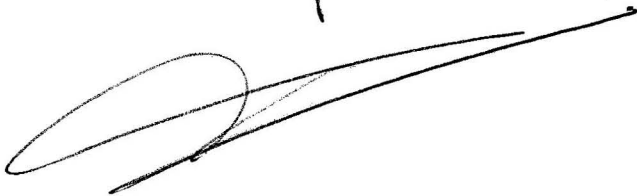# Radiation Chimaeras

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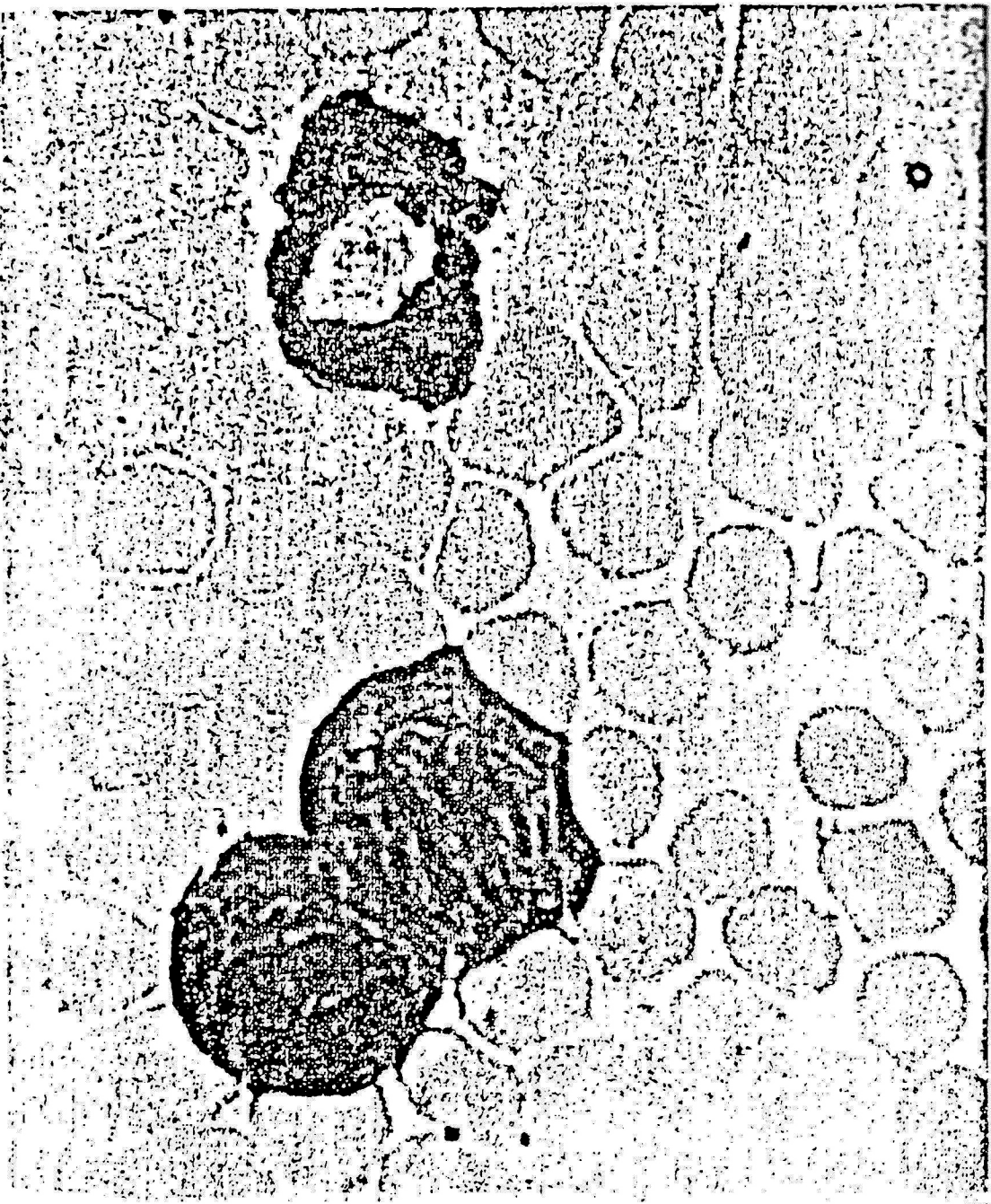
With compliments of the author

Blood smear from rat → mouse chimaera 12 days after irradiation
and rat bone marrow transplantation. The two (rat) granulocytes
at left show a positive alkaline phosphatase reaction, the (mouse)
granulocyte at right is negative (See pages 12 and 13)
Calcium-cobalt method for alkaline phosphatase, counter-stained
with May–Grünwald Giemsa. Magnification × 1200

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VI CLINICAL APPLICATIONS OF BONE MARROW TRANSPLANTATION AND RELATED EXPERIMENTS

Preface

The discovery of haematopoietic chimaerism, resulting from the intravenous administration of bone marrow cells into a lethally irradiated animal, has opened new ways to investigate numerous problems in the fields of immunology, haematology and tissue transplantation. In fact, radiation chimaeras have become such accepted tools for studies in these areas that there is now a tendency to neglect the original object of bone marrow transplantation as a cure for lethal exposure to ionising radiation. In addition, the outcome of clinical trials involving bone marrow transplantation in the treatment of disorders of the haematopoietic system, mainly leukaemia, has been disappointing; also insurmountable difficulties have been encountered in attempts to facilitate organ transplantations by inducing haematopoietic chimaerism in human patients. These factors have caused many investigators to abandon the idea that bone marrow transplantation can ever become a valuable asset to clinical medicine. It is our opinion that these failures have occurred mainly because the clinical applications were undertaken too soon, most of them before even the minimum of basic knowledge required to bridge the gap between mouse and patient had been obtained.

A particularly unexpected complication has arisen because of the immunological reaction of the lymphoid cells present in the transplanted marrow against the new host. This has confronted investigators with the formidable problem of identifying a completely new syndrome (generally called secondary disease) as well as with the task of unravelling its pathogenesis and devising methods for its prevention and treatment.

Many errors in extrapolation from the laboratory experiment to the patient have been made and much time was lost before it became evident that the graft versus host reaction in primates, including man, is incomparably more violent than in rodents. One of the main objects of this monograph is to present an exhaustive review of the comparative pathology of the immunological complications which occur after transplantation of foreign bone marrow, and to analyse the causes of the clinical failures in the light of the available experimental data.

This work has been greatly facilitated by our long-standing co-operation with the group led by George Mathé in Paris. Apart from being one of the pioneers in bone marrow transplantation, he has been the only clinician to conduct careful clinical trials whenever new experimental results seemed to require them. Because his clinical approach has always taken into full account the data obtained from experiments by his own group and by others, his accumulated clinical material represents by far the most important source of information on this aspect.

It as yet impossible to predict what therapeutic advantages will eventually be gained from the vast amount of research that has been

invested in radiation chimaeras. Quantitative evaluation of the current methods for storage of bone marrow, and the development of more appropriate freezing techniques, certainly warrant new clinical trials of autologous bone marrow transplantation. Recent advances in the control of secondary disease by treatment with cytotoxic and antimetabolic drugs, the new prospects offered by the introduction of anti-lymphocyte serum and the steady progress that is being made towards the identification of transplantation antigens in leucocytes as a method for the selection of compatible donors, all seem to provide grounds for a more optimistic outlook concerning the future of homologous bone marrow transplantation. Whatever the chances are, the stakes are so high that a continuation of the investigation of homologous bone marrow transplantation, both experimental and clinical, appears to be more than justified.

This monograph has been written for specialists and workers in related fields. We have made no special attempt to prepare a complete review of the literature on radiation chimaeras, but have preferred to discuss trends and ideas emerging from various lines of research, from our personal point of view.

Throughout the book we have employed the original terminology of transplantation, mainly because we did not consider it practical to change these terms so soon after their derivatives became established in the scientific language of the various European countries, including our own, where transplantation immunology is a relatively new addition to medical research. For those readers who have already forgotten the "old" terminology, we should say that isologous, homologous and heterologous are used here in place of syngeneic, allogeneic and xenogeneic respectively.

Acknowledgments are given on page 265.

The critical readers for whom this monograph is obviously intended are referred to four recent monographs which deal in different ways with the same subject: *Transplantatsionnyi immunitet i radiatsionnye khimery* (*Transplantation Immunity and Radiation Chimaeras*) by R. Petrov and Yu. Zaretskaya, Atomizdat, Moscow (1965); *L'Aplasie myelo-lymphoïde de l'irradiation totale* by G. Mathé, J. L. Amiel and L. Schwarzenberg, Gauthier-Villars, Paris (1965); *Tissue Grafting and Radiation* by H. S. Micklem and J. F. Loutit, Academic Press, New York and London (1966); *Bone Marrow Transplantation* by D. E. Pegg, Lloyd-Luke, London (1966).

We feel confident that these volumes contain all the information that we have omitted or neglected in the present one.

Rijswijk   D. W. VAN BEKKUM
October 1966   M. J. DE VRIES

CHAPTER I

History of the Radiation Chimaera

The term "radiation chimaera" was introduced by Ford et al.[143] (1956), to designate an animal which carries a foreign haemopoietic system, as a result of whole body irradiation followed by transplantation of haemopoietic cells derived from another animal.

It is impossible to state exactly when the first radiation chimaeras appeared on the scene in experimental biology, because this amazing product of radiation research was not recognised as such until about 1955.

The line of investigation which led to its discovery was initiated some time before 1949. In that year Jacobson et al.[193] published the results of a study on the protection of mice given an otherwise lethal dose by shielding of the spleen during the irradiation. This procedure caused an impressive reduction in mortality, and moreover the spleen appeared to be specific in this respect since shielding of other organs was much less effective, the only notable exception being the shielding of a whole leg.

In the mouse the spleen is essentially a haemopoietic organ and these results underlined the significance of the damage to the haemopoietic system for the development of that form of radiation sickness which is now generally known as "the bone marrow syndrome".*

In the experiments described above the spleen was exteriorised in order to permit effective and selective shielding and the investigators made every attempt to maintain the normal blood supply to the organ. In some cases, however, these attempts failed and the spleen was found to be completely infarcted after the completion of the irradiation. It was returned, nevertheless, to the abdominal cavity and subsequent observation of these animals showed them to be as equally well protected as the other animals in which the whole procedure had been technically successful.

Jacobson and his co-workers, with remarkable insight, drew the

* A description of radiation sickness and the bone marrow syndrome is given in Chapter II.

TABLE I: 1. Therapeutic effects of bone marow transplantation in irradiated animals
Data up to 1955

Recipient	Donor	Amount administered*	Survival at 21–30 days per cent	References
Mouse (CFI no. $_1$)	Isologous	0·1– 0·9 ⎫ 1 – 5 ⎪ cells 5 –10 ⎬ (× 10⁶) i.v. 10 –15 ⎪ 15 –20 ⎭	12 33 55 43 40	Jacobson et al. (1955)[190]
Mouse (4 strains)	Isologous	1·5 mg i.v. 1·5 mg i.p.	68–100 10–75	Congdon et al. (1952)[98]
Mouse (C3Hb)	Homologous (LAF₁)	1·5 mg i.v.	39	Congdon et al. (1952)[98]
Mouse (LAF₁)	Guinea pig	100 mg i.p. or i.v.	40–60	Lorenz et al. (1951)[231] Lorenz et al. (1952)[230] Congdon et al. (1952)[98]
Mouse LAF₁ and A	Rat (3 strains)	20–30 mg i.v.	0–72	Congdon and Lorenz (1954)[93]

Mouse	Dog	Not stated	None	Congdon and Lorenz (1954)[93]
Mouse	Rabbit	Not stated	None	Barnes and Loutit (1954)[28] Congdon and Lorenz (1954)[93]
Rat (Sprague-Dawley)	Isologous	50–100 mg i.v.	70	Fishler et al. (1954)[140]
Rat	Rat	Not stated	Increased	Chamberlain (1952)[73]
Rat	Rat	Not stated	Insignificant effect	Talbot and Gerstner (1951)[394] Lorenz and Congdon (1952)[227]
Guinea-pig (family 2, NIH)	Isologous	10 mg i.v.	85	Congdon et al. (1952)[98]
Guinea-pig	Guinea-pig	Not stated	Increased	Barnes and Loutit (1954)[28]
Guinea-pig	Rabbit	Not stated	None	Congdon and Lorenz (1954)[93]
Rabbit	Rabbit	2 femurs	Increased	Hilfinger et al. (1953)[177]
Hamster	Hamster	4 long bones and spleen of a 2 week old donor	Increased	Smith et al. (1955)[383]
Dog	Dog	Not stated	Equivocal effect	Rekers et al. (1950)[346]

* i.v., intravenous   i.p., intraperitoneal

conclusion that the infarcted spleens were probably equivalent to a spleen autograft and soon afterwards they reported that the implantation of un-irradiated autologous and isologous spleen after the irradiation had a similar therapeutic effect[195].

In addition, they were able to show that the intraperitoneal injection of isologous spleen cell suspensions was therapeutically effective[189]. In the same year Lorenz *et al.*[231] showed that lethally irradiated mice and guinea pigs can be protected by the parenteral administration of isologous bone marrow after the irradiation. In subsequent papers[229, 230], these investigators reported the therapeutic efficacy of homologous and heterologous bone marrow cell suspensions and "bone brei" as measured by 20 or 30 day survival.

The application of haemopoietic cell suspensions to the treatment of irradiated animals was greatly extended in succeeding years by the groups of both Jacobson and Lorenz as well as in a number of other laboratories. Much of this work was of an exploratory nature and consisted of the testing of various recipient–donor combinations. Few investigators were concerned with the quantitative aspects of the problem, as can be seen in Tables I: 1 and I: 2 which summarise the results published up to the end of 1955.

By that time the beneficial effect of haemopoietic cells in the prevention of "bone marrow death" had been firmly established and the interests of several leading groups of investigators in this field became centred on the mechanism of this therapeutic action.

The nature of the therapeutic action of haemopoietic cells

From his early experiments Jacobson had postulated that the mouse spleen contained a *humoral factor* capable of stimulating the regeneration of blood-forming tissues. This hypothesis was undoubtedly founded on the results of the histological observations on animals with shielded spleens[192, 196]. These had revealed that an initial massive destruction of the blood-forming cells occurred to the same extent in shielded as in non-shielded mice during the first few days. Beginning on the fourth day, however, an intensive proliferation of haemopoietic cells was observed in the bone marrow of the shielded mice, followed somewhat later by a regeneration of the lymphoid tissues. Complete restoration of the bone marrow was reached on the 8th day following irradiation and in the thymus between the 12th and 15th day.

The non-shielded mice could be studied until the time of death

TABLE I: 2. Therapeutic effects of spleen cells in irradiated animals

Recipient	Donor	Amount administered*	Survival at 30 days (per cent)	Remarks	References
Mouse (LAF₁)	Isologous	"Homogenate" of 1–2 spleens injected i.p.	53–79	60–70% of the cells were disrupted	Cole et al. (1952)[87]
		"Homogenate" of 28–160 mg i.p.	57–100	Irradiation dose not lethal to all control groups	Cole and Ellis (1953)[81]
Mouse (CBA)	Isologous	Ground infant spleens 1/20–4 per recipient i.v.	20–100		Barnes and Loutit (1953)[27]
		4 spleens i.p.	30		Barnes and Loutit (1954)[28]
Mouse (LAF₁)	Isologous	"Homogenate" of 1 spleen i.v.	No data	Accelerated recovery (following LD₇₅)	Smith et al. (1954)[382]
Mouse (CF₁ no. 1)	Isologous	0·5–11 × 10⁶ cells i.v.	50–72	Donors 2–5 days old	Jacobson et al. (1955)[190]
Mouse (LAF)	Homologous mouse LAF₂	"Homogenate" of 25–50 mg i.p.	Little or no protection		Cole and Ellis (1953)[81]
Mouse (LAF₁)	Homologous mouse A	1 spleen equivalent i.v.	56	High secondary mortality	Barnes and Loutit (1954)[28]
Rats (Sprague-Dawley)	Homologous (Sprague-Dawley)	"Homogenate" of 40–400 mg i.p. or i.v.	11–20		Cole and Ellis (1953)[81]

* i.v., intravenous   i.p., intraperitoneal

around the 10th day. They showed no haemopoietic regeneration at any time or at best a few isolated groups of erythropoietic cells. Since the astonishing migratory activity of haemopoietic cells had not been recognised at that time, the humoral factor concept was the logical outcome of these observations. The majority of the investigators were inclined to explain the beneficial effects of bone marrow injections and of post-irradiation parabiosis in a similar way, because it was found that these procedures resulted in an accelerated haemopoietic recovery wholly comparable to that seen after spleen shielding or spleen implantation.

As an alternative to the humoral hypothesis it was suggested—in particular by the Harwell group led by Loutit[27, 28]—that the active principle in spleen and bone marrow suspensions might be living cells, which would act at least for some time as a tissue graft. This cellular hypothesis received little support, particularly during the years immediately following Jacobson's and Lorenz's discoveries. In 1954 Lorenz and Congdon[229] wrote in a paper describing therapeutic effects obtained by the administration of homologous and heterologous bone fragments: "Therefore, if we assume the existence of humoral factors in irradiation protection by spleen shielding and bone marrow injection as postulated first by Jacobson, osseous tissues should also contain such a factor. This factor may be the same as in bone marrow protection. The experiments in which it was shown that rat bone marrow also affords protection against irradiation injury give additional proof that bone may contain such a factor. The histological evidence shows that rat bone transplanted into irradiated mice does not form bone marrow. Yet it protects irradiated mice."

Obviously it was of extreme importance to reach a clear decision about the mechanism involved. In the case of a humoral factor its isolation, identification and perhaps even its chemical synthesis appeared to be within reach of modern biochemical technology. The availability of a large supply of this factor in a readily usable form seemed attractive in view of the menace of atomic warfare, which was at that time even more of a reality than it is today.

HUMORAL HYPOTHESIS

The humoral hypothesis received what seemed to be strong direct support from the work of Cole's group at San Francisco[82, 83, 85, 196]. These workers found that cell-free homogenates of spleen and bone marrow were therapeutically effective and they claimed that this

could not be explained by the presence of intact cells in their preparations. On the basis of studies involving the fractionation of spleen "homogenates" and the "specific" destruction of cell nucleii in the "homogenates", Cole et al. postulated:

(a) "That the cell nuclei of normal mouse spleen contain a specific macromolecular nucleoprotein complex which is required for the cell growth and division, regeneration or maturation of critical haemopoietic tissue"; and

(b) "that the biological activity of this nucleoprotein is inhibited by ionizing radiations"[85].

Other workers were, however, unable to confirm these results[21, 190] and it must now be concluded that the findings of Cole et al. were due to the fact that these "homogenates" contained large numbers of intact viable cells. It should be mentioned that the same group was among the first to publish experimental proof for the cellular mechanism in 1956.

Indirect evidence which seemed to support the humoral hypothesis came from two sets of observations by Jacobson and his co-workers.

(1) Survival following irradiation and spleen implantation was not always accompanied by the persistence of the spleen graft[187]. However, this observation contradicted earlier reports by the same author and it was not subsequently confirmed by others. When one of the present authors reinvestigated this important point in 1954[47], a strong correlation was found between the survival of the irradiated mice and a take of the (isologous) spleen grafts.

(2) The degree of protection afforded by spleen shielding was not modified when the shielded spleen was removed completely 1 hour after the end of the irradiation[186]. Even when the splenectomy was performed five minutes after the irradiation some significant protection remained. These results are now explained by the fact that repopulation of the destroyed haemopoietic tissues can be accomplished by the seeding of an amazingly small number of (isologous or autologous) haemopoietic cells, but at that time the results seemed to fit the humoral hypothesis far better.

Perhaps the most forceful argument against the cellular mechanism was provided by the therapeutic successes which Lorenz et al.[98, 230] obtained with homologous and heterologous tissues. At that time, the

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idea of an heterologous transplant becoming established was com-
pletely at variance with the current biological dogmas. Evidently, it
was not sufficiently recognised that the immunological defence
reactions are very severely inhibited following lethal doses of radia-
tion, so as to permit the establishment of a foreign graft.

Even those investigators who favoured the cellular hypothesis
were initially prepared to accept the findings of Lorenz *et al.* as a
seemingly insurmountable obstacle. To quote from a paper by
Barnes and Loutit[28] in 1954: "One cannot presume that hetero-
specific (i.e. guinea-pig to mouse) cellular material would survive in
the host. . . . Therefore this indirect approach (of Lorenz) suggested
that cells that were doomed to die, were potent, presumably in
virtue of their supplying a chemical not a vital factor."*

In order to test such a possibility an attempt was made to supply
this chemical factor regularly by the daily administration of large
amounts of proven *cell free* spleen extract to irradiated mice for the
period of a week following the irradiation. This treatment failed to
reduce the mortality[118].

CELLULAR HYPOTHESIS

Barnes and Loutit, among others, were unable to confirm the
therapeutic effectiveness of heterologous bone marrow as described
by Lorenz *et al.* In addition, they and other workers made a variety
of observations which appeared to be far better explained by the
cellular than by the humoral mechanism. The observations in them-
selves constituted, however, no more than circumstantial evidence.

These results may be summarised as follows:

(1) The intravenous administration of haemopoietic cell suspen-
sions proved to be far superior to an intraperitoneal injection.

(2) Any procedure tested which would tend to decrease the via-
bility of living cells (e.g. heat, ionising radiation, freezing) was
found to decrease the effectiveness of the preparation. Conversely,
storage of the factor for appreciable periods in the frozen state
was found to be possible only when methods were used that were
known to favour the preservation of living cells[30].

(3) Mice treated with homologous spleen, although able to sur-
vive the 30th day after irradiation, had all died by day 100, in
contrast to mice receiving isologous spleen, which lived for 600

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* In the same paper these authors concluded that "the chemical hypothesis has
not been proved by the complete exclusion of the cellular hypothesis".

days and longer[29]. Such a delayed difference between the action of homologous and isologous material seemed more in accordance with a tissue grafting mechanism than with the effects to be expected from a humoral factor.

This striking difference between the action of isologous and homologous spleen had escaped the attention of Lorenz *et al.* because they employed a relatively short observation period of 20–30 days.

(4) When recipient mice were immunised against the donor strain prior to the irradiation, the administration of homologous spleen no longer resulted in a prolongation of survival time[28]. This finding could be explained by assuming that the humoral factor possessed antigenic properties, but again the cellular mechanism seemed to offer a more likely explanation.

(5) Finally, certain observations of Main and Prehn[239] very strongly suggested the replacement of host haemopoiesis by the donor type cells. These authors transplanted BALB skin to DBA mice which had been treated with (BALB $\times$ DBA)F_1 bone marrow following irradiation. The skin grafts took in the majority of the cases (see Fig. I[1]).

Shortly afterwards, Lindsley *et al.*[222] supplied direct evidence that transplantation and proliferation of haemopoietic cells can be achieved in irradiated rats under certain conditions. By the use of a serological method, they were able to demonstrate the presence of donor type erythrocytes several months after the irradiation and the bone marrow grafting.

In a limited number of cases an almost complete replacement of host erythrocytes had occurred but in others the majority of the erythrocytes were of host type. Although these results were highly significant they failed to prove satisfactorily the validity of the cellular mechanism; firstly, because the results failed to show a strong correlation between survival of the irradiated rats and the occurrence of replacement of host type by donor type erythropoiesis, and secondly, because the donor and recipient strains were genetically quite closely related (as evidenced by the subsequent observation that 20–25 per cent of permanent bone marrow transplants could be achieved following sublethal doses of X-rays)[295]. The occurrence of donor type erythropoiesis could, therefore, have been independent of the curative effect of the bone marrow.

From the nature of the various experiments described above it

can be deduced that by the end of 1955 there was a general tendency to abandon the humoral factor hypothesis in favour of the cellular mechanism. In fact a number of investigators had already set out to devise ways and means to settle this question.

DBA · 350R

DBA

DBA

4 WEEKS

BALB SKIN

NORMAL REJECTION (32 DAYS)

DBA · 750-800 R

(BALB x DBA) F₁

DBA

4 WEEKS

BALB SKIN

SLOWER REJECTION IN 29 PERMANENT TAKES IN 2

750-800 R

DBA

4 WEEKS

BALB SKIN

PERMANENT TAKES IN 33 REJECTION IN 3
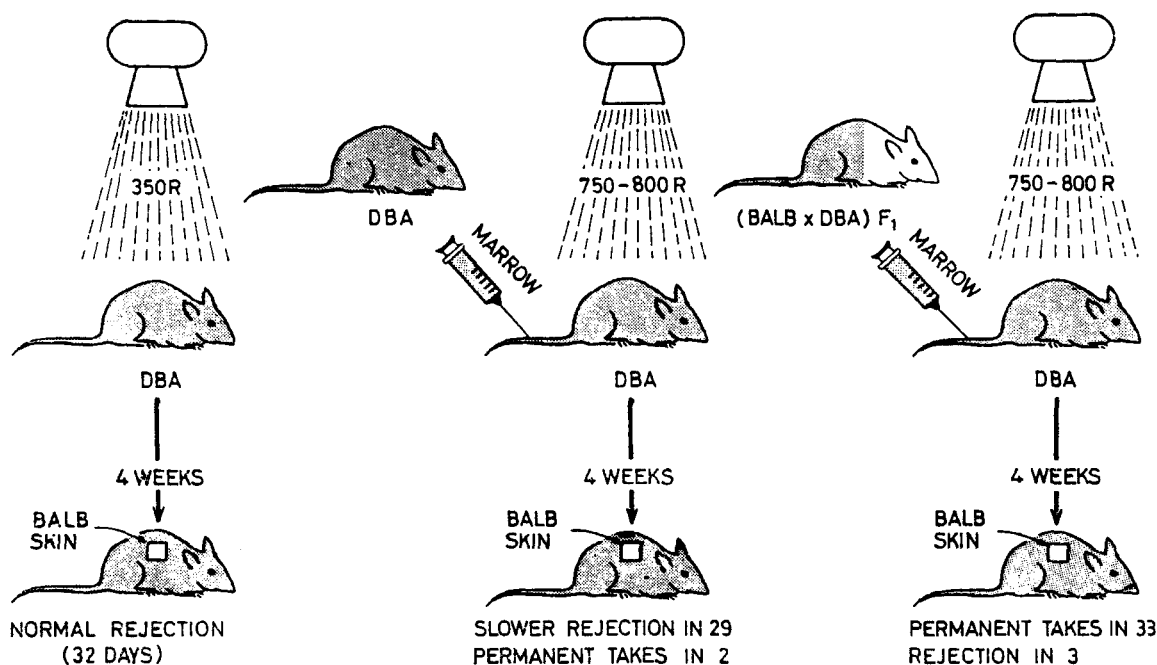
Figure I[1].   Schematic representation of the results obtained by Main and Prehn (1955)[239]

From left to right

Sublethally irradiated DBA mice reject a BALB skin graft normally. Lethally irradiated DBA mice treated with isologous bone marrow show delayed rejection of BALB skin grafts.
Lethally irradiated DBA mice treated with (BALB × DBA)F₁ bone marrow accept BALB skin grafts in the majority of cases.

IDENTIFICATION OF GRAFTED CELLS

In 1956 three research teams independently succeeded in supplying irrefutable proof of the cellular mechanism. That the three papers were published simultaneously—in March—in three different journals must be interpreted as pure coincidence, considering the procedures involved nowadays in publishing scientific papers. Surprisingly, there was little duplication in the methods employed in the three laboratories.

A very elegant technique for the identification of donor type cells in the mice treated with bone marrow was introduced by Ford *et al.*[143]. These workers were able to prepare chromosomal preparations of the haemopoietic cells in metaphase. Rat cells contain 42 pairs of chromo-
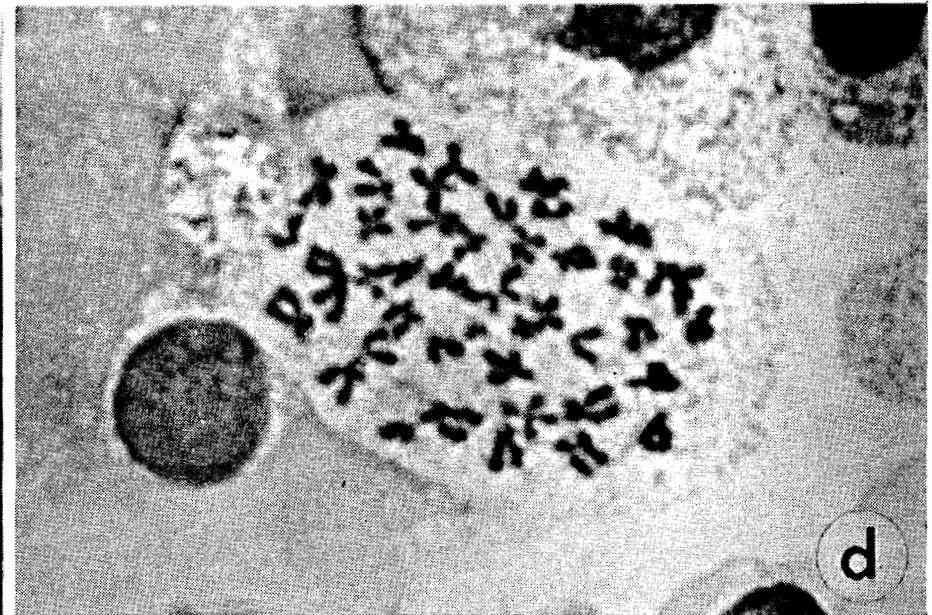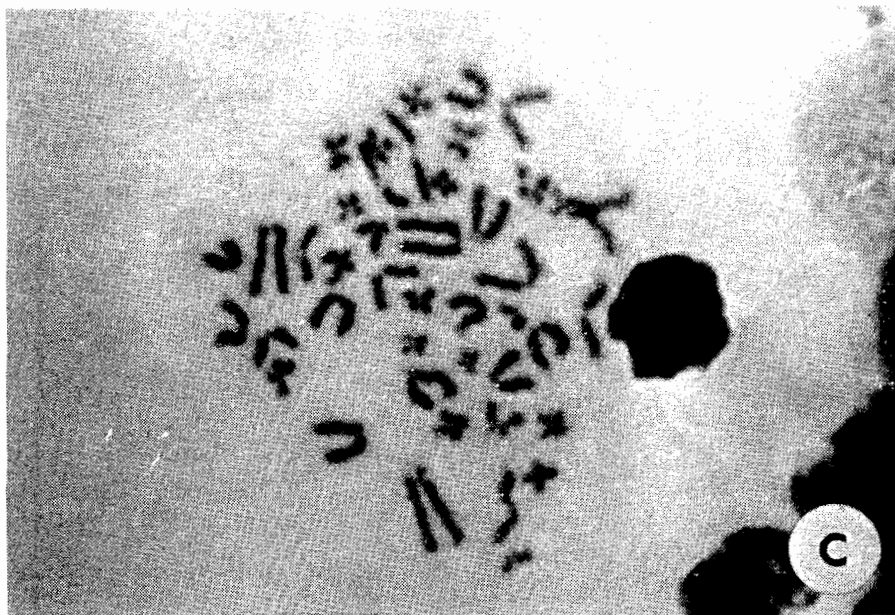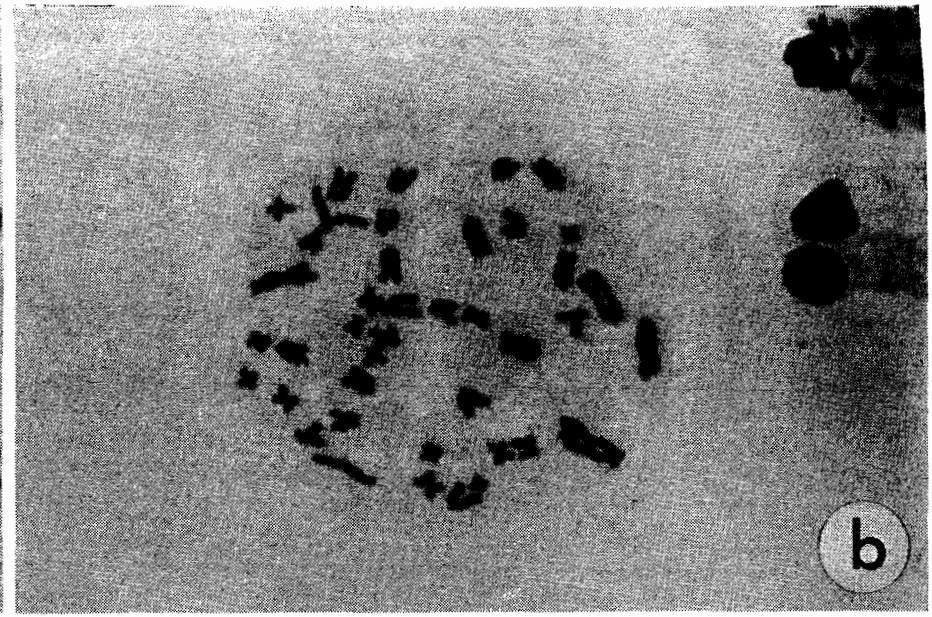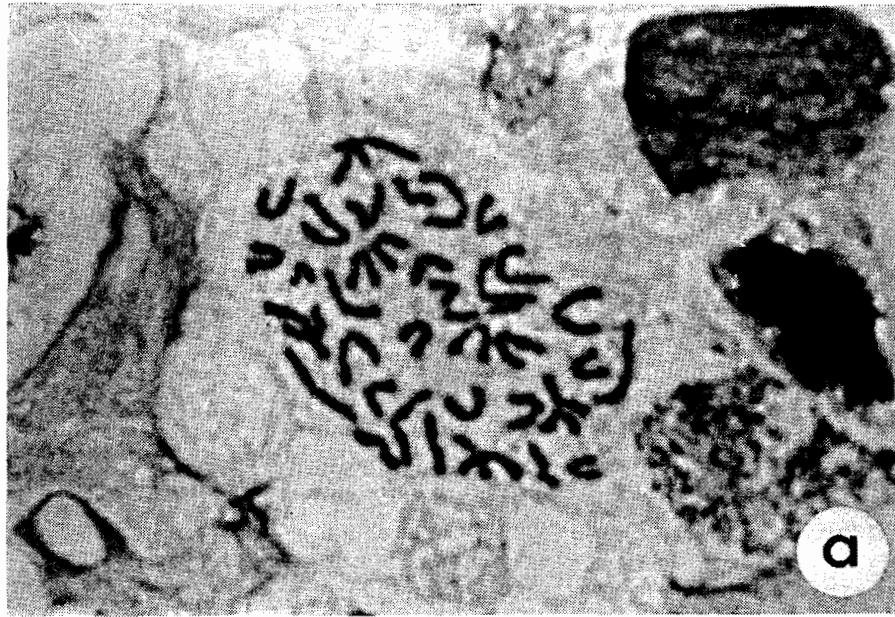
PLATE I:1. Squash preparations of cells from the bone marrow showing the chromosomes in metaphase

(a) Mouse   (b) Rat   (c) Mouse irradiated and treated with rat bone marrow 21 days previously
(d) Mouse irradiated and treated with bone marrow from a Syrian hamster 79 days previously
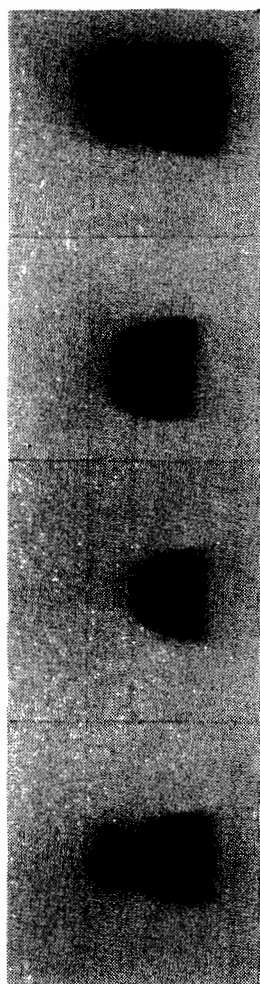
CBA MOUSE

C57BL MOUSE

CHIMAERA: CBA + C57BL BONE MARROW

CHIMAERA: C57BL + CBA BONE MARROW

a
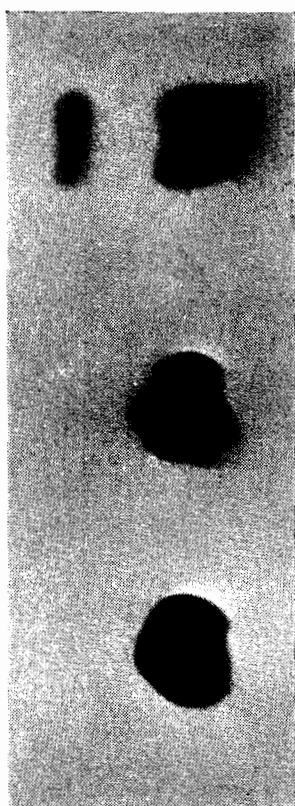
(RF × CBA)F$_1$ MOUSE

SYRIAN HAMSTER

CHIMAERA: (RF × CBA)F$_1$ MOUSE + HAMSTER BONE MARROW

b

(*Courtesy Dr. H. M. Klouwen, Rijswijk*)

PLATE I:2(a) and (b). Agar electrophoretograms of haemoglobins of various mouse strains, Syrian hamsters and mouse radiation chimaeras

(a) chimaeras 5 and 9 months after bone marrow transplantation
(b) chimaera 2 months after bone marrow transplantation

somes and a number of them have a characteristic cruciform (meta-centric) appearance in metaphase. The mouse cell has 40 chromosome pairs all with terminal centromeres. It is easy to distinguish, therefore, rat and mouse cells (Plate I: 1) in metaphase, and when this method was applied to haemopoietic cell preparations of mice treated with rat bone marrow, the majority of the cells was found to contain the rat-type chromosomes.

In addition, the group at Harwell employed donor mice which carried a clearly recognisable translocation on one of the chromosomes (T_6 mice) and they were able to show the presence of the marker chromosome in metaphase haemopoietic cells of the treated recipients. The authors correctly concluded that it is inconceivable that the host cells had accepted whole chromosomes from the donor cells, rejected some of their own to maintain a normal complement and still retained a balanced set for division. A transformation of host cell chromosomes through the influence of the injected foreign material was equally inconceivable, and these findings provided, therefore, indisputable proof of a cellular repopulation mechanism.

Donor type cells were identified with this technique not only in the bone marrow but also in the spleen, the lymph nodes and the thymus. Elegant and conclusive as this method is, it also possesses definite disadvantages for routine studies of this kind. The applicability is limited because the animal has to be either sacrificed or operated upon. Furthermore, only a small proportion of the cells in the tissues to be examined presents its chromosomes in the squash preparation in such a way that identification becomes possible. Finally, a cytological classification of the haemopoietic cells in metaphase is very difficult indeed.

The Rijswijk group provided proof of the cellular hypothesis in three different ways[438]. Two methods were at that time used for the rat–mouse chimaera. The first consisted of a serological identification of the erythrocytes with the use of specific agglutinating antisera. A slightly positive reaction with anti-rat serum was obtained as early as 10 days following rat bone marrow transplantation and the complete replacement of mouse erythrocytes by rat erythrocytes had occurred at about 60 days.

The mere demonstration of erythrocytes which agglutinated in the presence of anti-rat serum did not constitute a wholly satisfactory proof of the cellular mechanism since the possibility that a number of mouse erythrocytes had collected rat proteins on their outer surface

could not be excluded. The observation, however, that the proportion of cells reacting with anti-rat serum increased in the course of about 2 months following transplantation to reach 100 per cent, while simultaneously the proportion of cells reacting with anti-mouse sera decreased, was less subject to such criticism.

The method described requires only a few drops of blood from the tail and a single animal can be tested repeatedly for any period of time. The identification is limited, of course, to the erythropoietic system.

The second technique which was used permitted typing of the myelopoietic system only. It was a histochemical assay based on the observation by Wachstein[452] that rat granulocytes yield a strongly positive alkaline phosphatase reaction, while mouse granulocytes are essentially negative in this respect. After treatment with rat bone marrow, positive cells appeared in the blood of irradiated mice within a few days and after about 8 days the majority of the cells showed a positive reaction (see colour frontispiece). It could be argued, of course, that the injection of rat material might have induced alkaline phosphatase activity in mouse granulocytes but this type of reasoning is extremely far-fetched. However, some caution in an interpretation of these results was clearly necessary, as the authors pointed out: "Although the histo-chemical evidence as such cannot be accepted as unequivocal proof of the cellular hypothesis, it constitutes a substantial support for the two other arguments presented in this paper."

The same method was employed at San Francisco by Nowell et al.[293] who also found a replacement of alkaline phosphatase negative granulocytes by positive cells following the transplantation of rat bone marrow into irradiated mice. In addition, they observed a tremendous proliferation of positive cells in the bone marrow in the first weeks following transplantation.*

This method of distinguishing rat and mouse granulocytes has proved to be extremely suitable for the repeated typing of individual animals. The test can be performed with a small drop of tail blood and the differentiation of hundreds of cells can be carried out both

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* Recent observations from the authors' laboratory indicate that the percentage of alkaline phosphatase positive cells may vary somewhat between different rat strains. However, in all the strains so far investigated the majority of the granulocytes have been positive. Since in germfree and mono-contaminated gnotobiotic rats the granulocytes were found to be alkaline positive, there seems to be no relationship between the presence of infections and the alkaline phosphatase of the granulocytes in this species, in contrast to the findings in humans reported by Wachstein.

rapidly and reproducibly. Since control rat blood always yields more than 95 % positive granulocytes, for all practical purposes the finding of more than 5 % negative cells can be interpreted as evidence of the presence of mouse granulocytes.

The third procedure which was employed by Davids *et al.* to investigate the cellular hypothesis can best be described as a typing of the whole population of haemopoietic cells in the bone marrow. It was based on the observation by the same authors that the number of homologous (and heterologous) bone marrow cells required to prevent mortality in lethally irradiated mice is about 20 times larger than the number of isologous cells necessary to achieve a similar therapeutic effect.

The identity of the bone marrow of mice which survive the irradiation as a result of homologous bone marrow injection could be established, therefore, by the injection of graded numbers of these bone marrow cells into two strains of irradiated mice, one identical to the recipient and the other identical to the original bone marrow donor. This assay is depicted in Fig. I[2]. The results left no doubt that the majority of the bone marrow cells in the surviving recipients were of donor origin.

Thus, the publications which appeared in March 1956 provided independent evidence in favour of the cellular mechanism by four different procedures. These findings fully justified the acceptance of the earlier observation by Lindsley *et al.* as having established a similar state of events in their rats treated with homologous bone marrow.

A great number of other publications containing confirmatory, as well as additional evidence have subsequently appeared. Methods have been successfully worked out to identify all types of cells which have their origin in the haemopoietic system and a number of other host donor combinations have been investigated with similar results.

It can now be concluded with certainty, therefore, that under suitable conditions the host's haemopoietic system may be replaced by isologous, homologous and heterologous cells according to the type of bone marrow donor.

A list of the identification methods which are presently available is presented in Table I: 3. Many can be made to yield quantitative or semi-quantitative results of the proportion of host and donor type cells in the test sample. This holds in general for the serological assays and for the histochemical test. The electrophoretic method

for the identification of haemoglobins in suitable host donor combinations produces at best semi-quantitative results (Plate I: 2) and the drum stick counts in female–male combinations can give qualitative information only (Plate I: 3)[237].

C 57BL 30 DAY SURVIVAL %	NO.OF CELLS INJECTED (×10⁵)	CBA 30 DAY SURVIVAL %
0	1	0
0	2	0
70	8	0
100	16	0
100	32	0
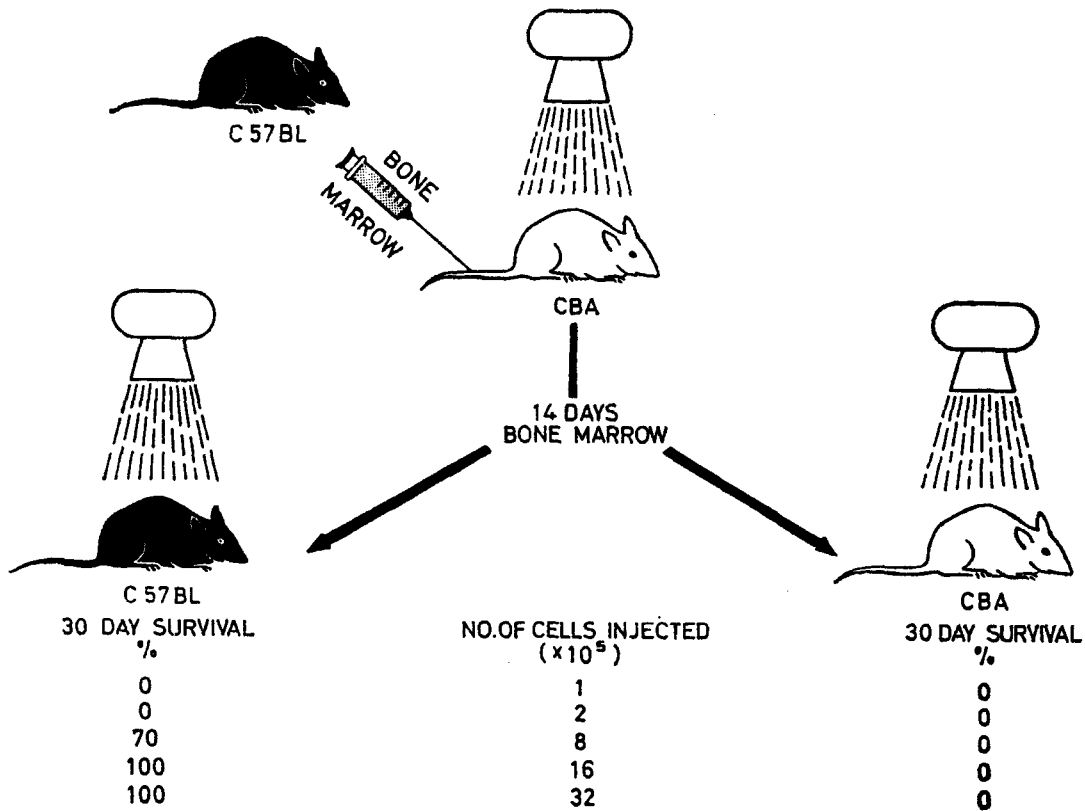
Figure I². Transplantation of bone marrow from a radiation chimaera as a method of identification of the cells

The bone marrow cells of lethally irradiated CBA mice which were treated with C57BL bone marrow were able to protect lethally irradiated C57BL mice but not lethally irradiated CBA mice, when injected in moderate numbers. Since protection with such small amounts of bone marrow is only possible in isologous host-donor combinations, this is proof that the original CBA recipient carried C57BL cells in its bone marrow at the time of the identification experiment. (van Bekkum et al., 1956)[51]

When bone marrow transplantation has been performed in human patients, it has usually been possible to employ differences in one or more minor blood groups between the donor and the recipient, so that a convenient differentiation of erythrocytes can be made. Differences in the leucocyte antigens between the donor and the recipient can be employed in principle, but as yet the application of this technique is limited to primates[117, 472] and dogs[473], for which the iso-

antisera available allow the identification of the "antigenic profile" of a leucocyte sample. To trace the myelopoietic cells of the donor in human patients, it would also be useful if the donor could be selected from the opposite sex as the recipient, in order that the drum stick count of the granulocytes could be followed.*

Summarising, the identification methods fall into four classes: cytological, serological, biological (serial transplantation) and biochemical (haemoglobin electrophoresis).

Immunological specificity of chimaeras

Attempts at identification based on the capacity to produce specific antibodies have not been listed in Table I: 3 because the evidence obtained by this approach is usually rather indirect and not sufficient in itself. Nevertheless, useful information in favour of the cellular hypothesis has been obtained by such experiments. The first attempt in this direction was made by Mitchison[283] who showed that irradiated mice treated with spleen cells of mice immunised with *Salmonella typhi* (H) antigen, showed a greater production of antibody than normal mice receiving the same treatment.

In irradiated CBA mice, Mitchison[283] further demonstrated the increase of A isoantigens and their persistence for at least 51 days following A spleen cell transplantation. The chimaeric tissues to be tested were injected into normal CBA mice which were challenged subsequently with an A tumour. Growth inhibition of the tumour was considered as evidence of the presence of A antigens in the tissue sample. A comparable method, which employed the disintegration of a non-vascularised Harderian gland homograft in pre-immunised mice, was reported by Merwin and Congdon[275].

The results of these two investigations were similar, in that evidence of the presence of donor type antigens was found in a number of haemopoietic tissues of the treated mice, including the lymph nodes and the thymus. It was later confirmed with more direct methods (see Table I: 3) that complete repopulation of these organs with donor type cells might occur; this means that in established radiation chimaeras the host's lymphatic tissue has been replaced by a population of donor derived lymphoid cells. Since the lymphoid system is the site of immunological reactivity, it follows that the immunological

* In the case of a male recipient this procedure theoretically carries an increased risk of graft versus host reactions, because the presence of sex-linked histocompatability genes has not been excluded in humans.

TABLE I: 3. Methods of differentiation between host and donor derived cells in radiation chimaeras

Cell type	Host-Donor combination	Method	Authors
Haemopoietic cells in general	Mouse–mouse Mouse–rat Mouse–hamster	} Chromosome identification	{ Ford et al. (1956)[143] Ford et al. (1956)[143] van Bekkum (1964)[44]
Bone marrow	Mouse–mouse	Serial transplantation	Vos et al. (1956)[438]
Erythrocytes	Rat–rat	Agglutination with specific antisera	Lindsley et al. (1955)[222]
	Mouse–rat	Agglutination with specific antisera	Vos et al. (1956)[438] Makinodan (1956)[241]
	Mouse–hamster	Agglutination with specific antisera	van Bekkum (1964)[44]
	Pigeon–pigeon	Agglutination with specific antisera	Shaw and Vermund (1959)[366]
	Pigeon–pigeon	Agglutination with *Phaseolus limensis* extracts	Shaw and Vermund (1959)[366]
	Pigeon–dove	Agglutination with specific antisera	Shaw and Vermund (1959)[366]
	Monkey–monkey	Modified Coomb's reaction	Owen (1961)[301]
	Mouse–mouse	Electrophoresis of haemoglobin	Rosa et al. (1958)[353] Welling and van Bekkum (1958)[453] Popp et al. (1958)[319]
	Monkey–monkey (Rhesus) (Cynomolgus)	Solubility of haemoglobin	Overman (1959)[298]
	Mouse–mouse	Solubility of haemoglobin	Popp and Cosgrove (1959)[318]

Granulocytes	Mouse–rat	Alkaline phosphatase	Nowell et al. (1956)[293] Vos et al. (1956)[438]
	Mouse–hamster	Alkaline phosphatase	van Bekkum (1964)[44]
	Rabbit–rabbit	Pelger anomaly in donors	Czerski et al. (1960)[115]
	Male–female rabbits	Drumstick appendages	Porter (1957)[323]
	Male–female dogs	Drumstick appendages	Porter and Couch (1959)[330] Mannick et al. (1959)[248]
	Male–female monkeys	Drumstick appendages	Magliulo et al. (1963)[237]
Thrombocytes	Mouse–rat	Agglutination with specific antisera	Smith et al. (1957)[380]
Thymus cells	Mouse–rat	Agglutination with specific antisera	Urso et al. (1957)[431]
	Mouse–rat Mouse– mouse (T 6)	Chromosome identification	Ford et al. (1956)[143]
	Mouse–mouse	Graft versus host test	Popp (1961)[317]
Lymph node cells	Mouse–rat Mouse–mouse (T 6)	Chromosome identification	Ford et al. (1956)[143]
	Mouse–rat	Cytotoxic test with antisera	Zaalberg and van Bekkum (1959)[469]
	Mouse–rat	Tissue globulin electrophoresis	Popp and Smith (1959)[321]
	Mouse–mouse	Cytotoxic test with antisera	Vos et al. (1960)[439]
Macrophages (peritoneal)	Mouse–mouse	Cytotoxic test with antisera	Balner (1963)[13]

reactivity of radiation chimaeras should have all the characteristics of that of the donor.

The skin transplantation experiments of Main and Prehn, which have been mentioned earlier, were the first to focus attention on this possibility. These findings were confirmed and extended soon afterwards by Trentin[414] and by Zaalberg et al.[470]. The latter workers demonstrated for the first time that rat skin can be made to grow on a mouse when the recipient is pretreated with total body irradiation and rat bone marrow transplantation.

Spontaneous blood chimaerism, a comparable but not identical condition, occurs naturally in some dizygotic twins of certain species (cattle[299], sheep[389], marmoset[55] and man[351, 465]) and is caused by exchange of haemopoietic cells in foetal life between the twin partners through vascular anastomoses. This results in the establishment of a mixed population of haemopoietic cells which persist in one or both partners into adult life. Anderson et al.[6] introduced the term genetical chimaera for this condition. It has been experimentally produced in chickens by artificial union of the chorioallantoic membranes of two embryos by Hasek[169]. Some authors have used the term blood or marrow mosaicism, but recently chimaerism seems to be the more generally accepted term.

It is already evident that the implications of these discoveries reach far beyond the treatment of radiation sickness. The homologous and even the heterologous transplantation of an extremely complex tissue, with localisations throughout the entire host organism has been shown to be possible by the simple procedure of an intravenous injection of a limited number of cells. In many cases this complex transplant remains permanently established with preservation of its various specialised functions, while the analogous cells of host origin do not return. Recently, it has even become apparent that macrophages as they occur in the peritoneal cavity, will be replaced by donor type cells in radiation chimaeras[13], but the fate of the so-called fixed macrophages still has to be ascertained.

Investigators in the fields of transplantation biology, immunology and haematology have been quick to recognise the unique possibilities of the radiation chimaera as an experimental tool.

In the following chapters many aspects of radiation chimaeras will be discussed. A wealth of experimental data is now available and many of the essential questions concerned with chimaerism have been answered. In dealing with these problems the authors have referred
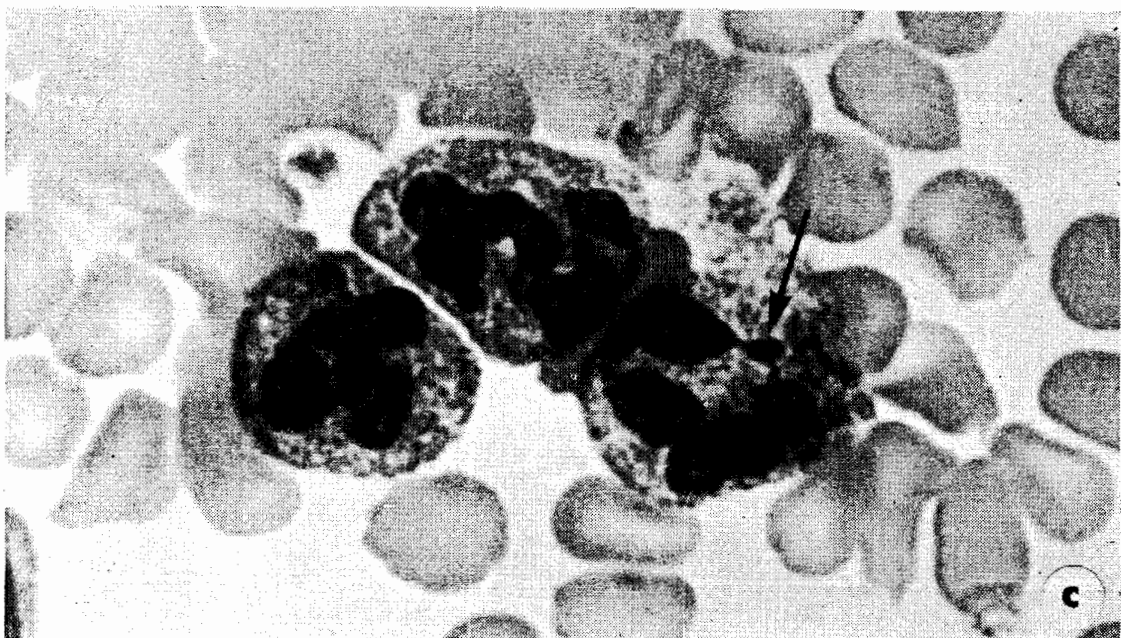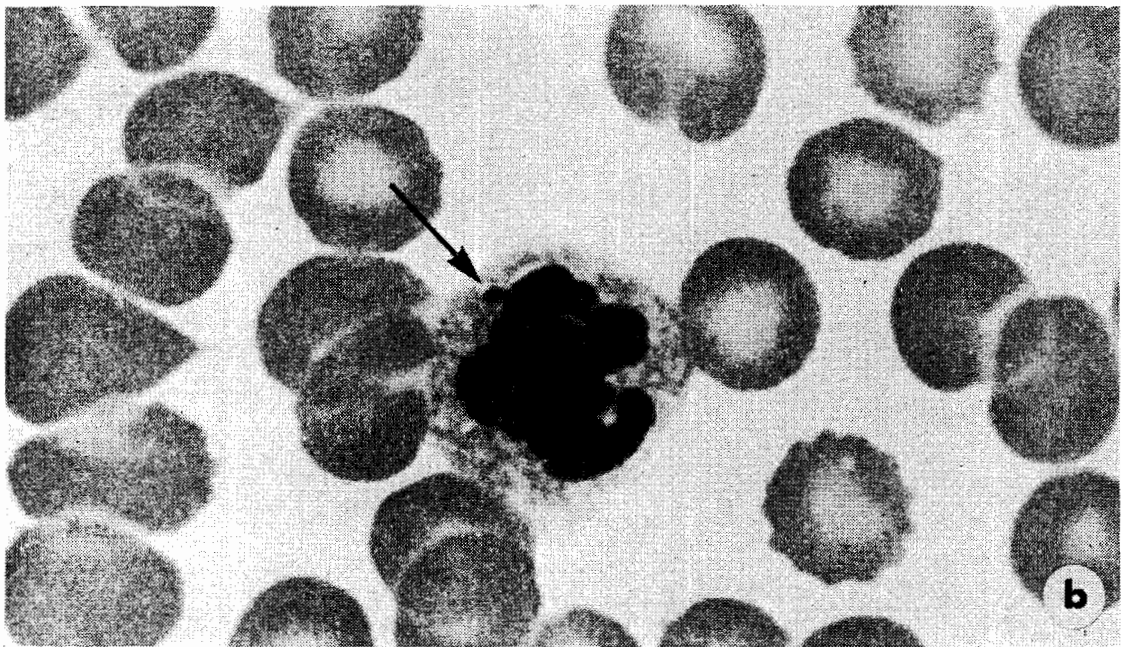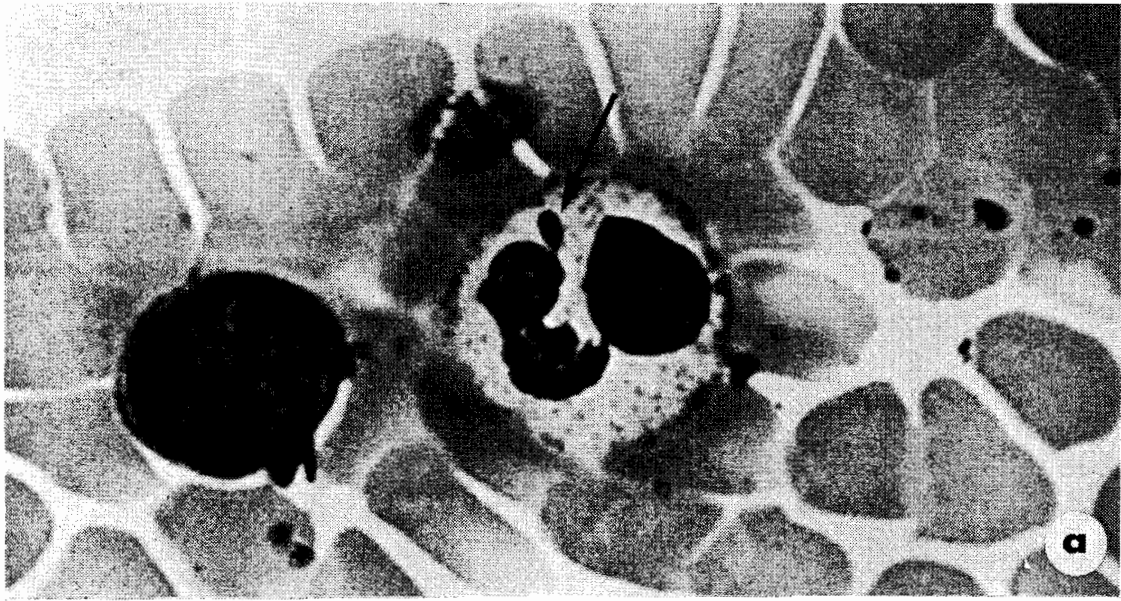
PLATE I: 3. Characteristic drumstick appendages in neutrophil granulocytes

(a) human female   (b) rhesus monkey female   (c) rabbit female

primarily to those papers which provide clearly interpretable results. The merit of such experiments can be evaluated properly only if information is available to prove the chimaeric state of the animals. It is a mistake to assume that the injection of an arbitrary number of bone marrow cells into an animal irradiated with a supposedly lethal dose of X-rays will always produce permanent haemopoietic chimaerism. These factors can be and have to be controlled. Since 1956 and 1957 a sufficiently large number of comparatively simple methods has been available to obtain exact information on the state of chimaerism of the experimental animals at any time. Studies in which this condition is not fulfilled have been intentionally neglected in this review in favour of those which meet this criterion even if the latter have been published at a later date.

CHAPTER II

The Production of Radiation Chimaeras and the Stability of the Chimaeric State

Among the various factors which determine the continued proliferation of haemopoietic cells in a new host, two conditions seem to be absolutely necessary. The first is that the immunological reactivity of the recipients towards the injected material must be minimal, otherwise the graft will be rejected by a mechanism comparable to the well known homograft reaction against other tissues, e.g. skin. The second important requirement seems to be that there must be a stimulus for the injected cells to proliferate. Obvious as this statement may be to the transplantation biologist, there is as yet very little information on the nature of this stimulus. Jacobson et al.[188] have shown that a certain level of erythropoietin is required to allow proliferation of the erythropoietic cells of a bone marrow graft. In their polycythaemic irradiated mice which received rat bone marrow, rat erythrocytes appeared in the circulation only after the haematocrit had decreased and about 2 weeks after rat granulocytes could be first detected. The results of such an elegant experiment are shown in Fig. II[1].

It seems likely that the natural environment of the haemopoietic cells—in other words the haemopoietic tissues—also provides a stimulus to proliferate. This possibility is based on the observation that, following the parenteral administration of the cells, proliferation is ultimately found predominantly in areas where haemopoiesis normally occurs, a phenomenon which has been quite adequately described by the term "homing". Furthermore, there are indications that a much more rapid proliferation occurs when the transplanted cells arrive in depleted or acellular haemopoietic tissues.

In the lethally irradiated animal the conditions for proliferation seem to be ideally fulfilled. The haemopoietic cells of the recipients have been lethally damaged, or in any case prevented from multiplying, so that the haemopoietic tissues become rapidly depleted or

even completely acellular. The capacity to react immunologically is severely inhibited, not only with respect to soluble antigens but also to transplanted tissues.
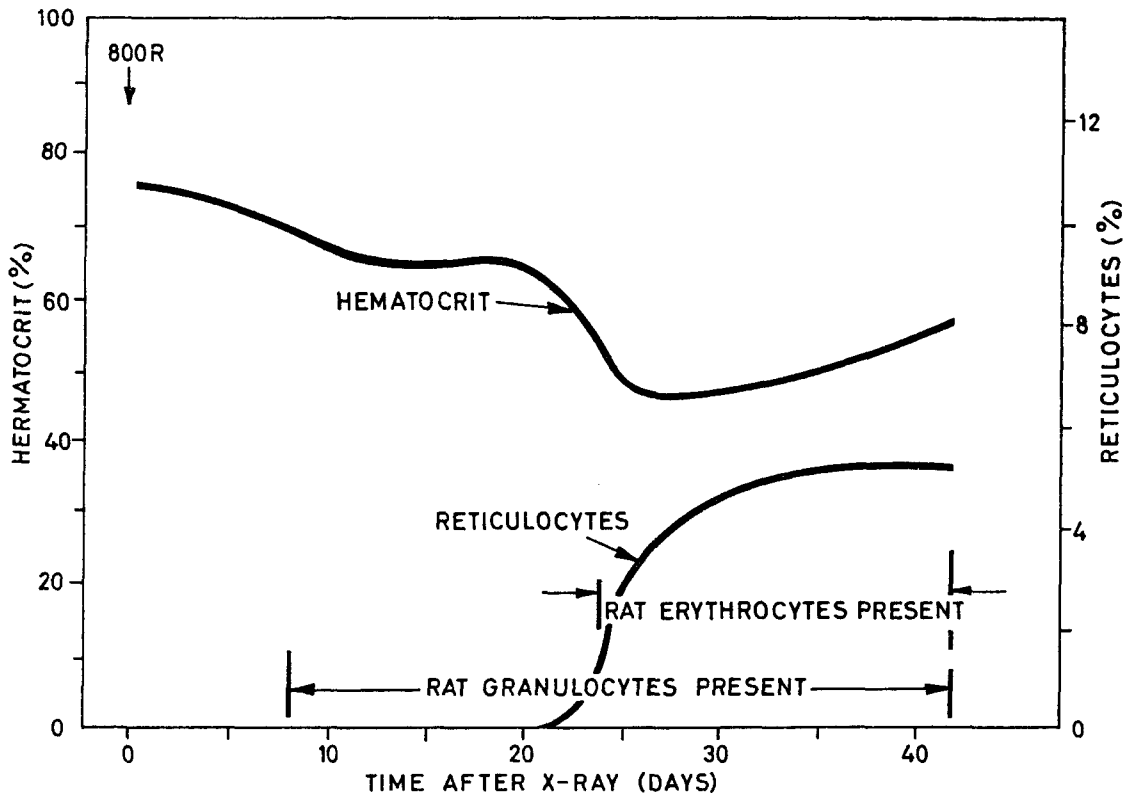
Figure II[1]. Response of a polycythemic X-irradiated mouse to rat bone marrow transplantation (Data from Jacobson et al. (1960)[188])

The haematocrit was maintained above 65 per cent for 18 days by mouse red cell transfusions, which were started 7 days before the irradiation. With the fall in haematocrit, the number of reticulocytes rose and a few days afterwards rat erythrocytes could be demonstrated. Granulopoiesis of rat origin (alkaline phosphatase positive cells) had already started by 8 days after the bone marrow transplantation. In controls not receiving red cell transfusions after the irradiation reticulocytes began to rise around the 10th day

Nevertheless, the number of host-donor combinations in which chimaerism has been successfully established and proved by identification of donor type haemopoiesis, is still rather limited: it includes homologous chimaerism in pigeons, mice, rats, rabbits, dogs, monkeys and in a limited number of human patients and heterologous chimaerism in mice (rat bone marrow and Syrian hamster bone marrow) and pigeons (dove bone marrow).

An attempt will be made in the following pages to discuss whether

the failure to obtain chimaerism with other host-donor combinations can be explained in terms of an inadequate suppression of the immunological reactivity of the host or by the absence of a stimulus to proliferate.

Antigenic differences between the host and the donor

The significance of this factor can be illustrated most clearly by referring to the numbers of cells which are required in various host-donor combinations in order to obtain a 30-day survival following an LD_{100} of whole body X-irradiation. These quantitative studies have been carried out most extensively with mouse recipients, in which the 30-day survival rate is a good indication of the take and continued proliferation of the haemopoietic graft.

In the case of homologous bone marrow roughly 80 times as many cells were required for optimal recovery as with isologous bone marrow and in the two successful mammalian heterologous combinations even more cells were needed (Fig. II^2).
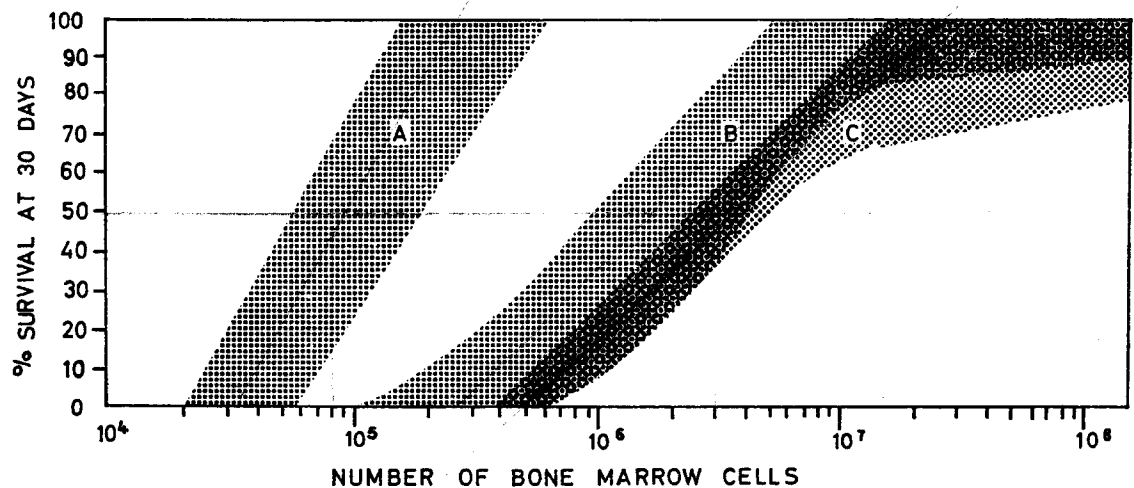
Figure II^2.   Number of bone marrow cells required in various host-donor combinations for restoration following lethal whole body irradiation

A   Isologous and parent → F_1 hybrid
B   Homologous and F_1 hybrid → parent strain
C   Heterologous (rat and hamster → mouse) combinations

Very little quantitative information has been reported in other species, but the few data available suggest a difference of a factor of 5–15 between autologous and homologous donor material in non-inbred experimental animals. The number of cells which have been used so far in man have always aimed at obtaining a maximal response.

TABLE II[1]: 1. Numbers of isologous, autologous and homologous cells required to provide 100% 30-day survival or maximal achievable protection following lethal whole body irradiation in various species

Species (see reference below)	Body weight (kg)	Nucleated cells (kg)		Proportion (Homol./Isol.)
		Isologous or autologous	Homologous	
(1) Mouse	0·02	5×10^6	4×10^8	80
(2) Rat	0·2	5×10^7	5×10^8	10
(3) Guinea pig	0·6	5×10^7	5×10^8	10
(4) Rabbit	2·5		1×10^8*	
(5) Monkey	3	4×10^7	2×10^8	5
(6) Dog	6	5×10^7	3×10^8	6
(7) Calf	50	3×10^7	$>8 \times 10^8$	>25
(8) Human (children and adults)		2×10^8	$2–10 \times 10^8$	1–5

(1) van Bekkum and Vos (1957)[49] (isologous and homologous)
Lewis and Trobaugh (1964)[221] ⎫
van Putten (1964)[338] ⎬ (isologous)
Vos et al (1961)[437] ⎭
(2) van Bekkum and Vos (1957)[49]
Balner et al. (1964)[18]
(3) van Bekkum (unpublished observations)[46]
(4) Porter (1957)[323]
(5) Crouch et al. (1961)[108]
Magliulo, van Putten and van Bekkum (unpublished observations)[238].
(Criterion is not 30-day survival but take in case of homologous bone marrow)
(6) Sullivan et al. (1959)[391]
Hager et al. (1961)[166]
(7) Mizuno et al. (1960)[285]
(8) Thomas et al. (1959)[406], (1961)[404]. (isologous)
Robins and Noyes (1961)[348] ⎫ (isologous bone marrow in case of
Mills et al. (1964)[282] ⎬ drug induced or idiopatic bone
Thomas et al. (1964)[408] ⎭ marrow failure)
Kurnick (1961)[209] (autologous, his data suggest that $10^7–10^8$ cells/kg might cause effective repopulation)
Mathe et al. (1959)[265,262] ⎫ (homologous)
Miller and Diamond (1961)[279] ⎭

* Maximal degree of recovery obtainable was not 100% due to complicating factors.

C

It is impossible to decide, therefore, upon the minimum effective number* of cells, and the data presented in Table II: 1 represent no more than rough guesses on the basis of meagre information.

The required cell numbers per body weight unit show surprisingly little variation between the species, namely 10^7–10^8 cells/kg for isologous and autologous transplantations and 10^8–10^9 cells/kg for homologous transfers. A notable exception is the value of 5×10^6 cells/kg for the isologous bone marrow transplantation in the mouse. The mouse data seem to be, however, by far the most reliable in view of the large number of experiments on which these are based.

IMMUNOLOGICAL REACTIVITY OF THE HOST

Whatever the cause may be of the exceptional position of the mouse with respect to isologous bone marrow transplantation, it is clear in all host-donor combinations that the required number of homologous cells is much larger than the number of isologous or autologous cells. The explanation for this difference has been assumed to be the *incomplete* destruction of the host's immunological reactivity. This assumption is based on the following evidence.

(1) The number of parent strain cells required to protect lethally irradiated F_1 hybrid mice is equal to the number of isologous cells. In the reverse combination many more cells are needed[49], as is the case with homologous and heterologous bone marrow cells. These results are markedly similar to those observed in tissue transplantability between F_1 hybrids and their parental strains. Normal F_1 hybrids uniformly accept grafted parental skin, while in the reverse combination the transplants are rejected.

Recently one exception to this general pattern of parent strain bone marrow efficacy has been discovered. When C57BL marrow is used to restore F_1 hybrids, up to 10 times as many cells are required as in the case of marrow from the other parent strain[269,316, 320]. The basis of this difference has not been elucidated, but it does not seem to be due to immunological factors.

(2) Following midlethal† or sublethal X-ray doses, transplantation of parental cells into F_1 hybrids has been successful, while the

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* The minimum number of cells required to provide 100 per cent 30-day survival or the highest protection achieved following lethal whole body irradiation. This value is preferred here to the number of cells required to protect 50 per cent of the animals, because the latter value has usually not been determined.

† Midlethal: in the dose range LD_{10}–LD_{90}. The reader is referred to page 28 for a description of dose-survival relationships in irradiated animals.

reverse combination meets with various degrees of failure. Similarly, homologous and heterologous chimaeras are much more easily produced by employing supralethal irradiation of the recipients than by lower radiation doses.

(3) Preimmunisation of the host against the bone marrow donors has been found to result in failure of the bone marrow to take following lethal irradiation[28]. This observation is in accordance with the discovery that the secondary immune response is much less inhibited by whole body irradiation than the primary reaction[170].

There are thus many reasons to support the belief that even a high dose of total body irradiation does not in itself completely suppress the capacity of the recipient to react against foreign antigens. However, there also appears to be some evidence that this active rejection is not the only factor involved in the failure of small numbers of foreign bone marrow cells to repopulate the host's tissues. It was observed by Vos et al.[437] that a 24-hour delay of the bone marrow transplantation improved the results in terms of 30-day survival rate, in particular when the recipients were subjected to a median lethal dose of X-rays. The latter experimental condition favours the preservation of a part of the immunological host versus graft reaction. Furthermore, the results in general were in agreement with the observation of both Taliaferro et al.[395] and Gengozian and Makinodan[151] on the time course of the inhibition of the primary immune response towards other antigens following whole body irradiation.*

Much to our surprise, however, when a lethal dose of X-rays was given, the number of homologous and heterologous cells required for effective recovery was found to be no less following delayed administration than with immediate injection[437]. What then might be the additional factor which determines the size of an effective haemopoietic graft?

A reasonably good speculation seems to be that naturally occurring "antibodies" inactivate a proportion of the injected foreign cells. For instance, Terasaki et al.[397] have shown that the lymph node cells of various species rapidly become eosin permeable when incubated with heterologous sera.

* Taliaferro et al.[395] showed that in rabbits the lowest peak titres following injection of sheep red blood cells occurred when the antigen was administered more than 12 hours after irradiation. Gengozian and Makinodan[151], found the immune reaction which develops in mice following the injection of the same antigen to be minimal when the interval was 1 day.

In a number of different laboratories it was demonstrated that immune antibodies in circulation are capable of preventing the proliferation of bone marrow cells (Loutit and Micklem, 1961[234]; Gorer and Boyse, 1959[161]; Santos et al., 1959[358]; Garver and Cole, 1961[148]; Balner et al., 1962[17]). This evidence is mostly derived from the fact that the immunity to injected marrow suspensions could be passively transferred by means of specific antisera, in some cases even in very small amounts.

Of course, it cannot be excluded that in some homologous and heterologous host-donor combinations a biochemical incompatibility in contrast to an immunological one, may exist as well, which might explain the large number of cells required for transplantation. In the personal opinion of the present authors the odds are very much against such an explanation at the moment.

It has been proposed by Wooles and DiLuzio[466] that the phagocytic activity of RES cells could be a factor in the removal of foreign bone marrow cells. They observed a decreased carbon removal rate (evidence of a decreased phagocytic activity) after 72 hours but not after 6 hours post irradiation in normal mice. A decreased survival of mice following irradiation and treatment with foreign bone marrow cells was observed when the RES of the recipients had been stimulated before the irradiation. The latter observation has not, however, been confirmed by Vos[436].

HETEROLOGOUS CHIMAERAS

Proof of a successful transplantation, in terms of long-lasting proliferation of the donor bone marrow, has been obtained until recently in only one heterologous mammalian combination, namely irradiated mice treated with rat bone marrow. Surprisingly, the reverse combinations have failed consistently. Therapeutic effects have been reported in a few other heterologous combinations. An increased survival time for irradiated mice was obtained following treatment with guinea-pig bone marrow[49, 230]; Syrian hamster bone marrow had a similar effect. The identity of the haemopoietic cells in the surviving animals was not determined. Recently, a significant number of 30-day survivals was obtained in supralethally irradiated mice by treatment with a much larger number (40×10^6) of Syrian hamster bone marrow cells. In the survivors a complete replacement of alkaline phosphatase negative mouse polymorph nuclear granulocytes by alkaline phosphatase positive (hamster) cells was observed[44]. After 60

days an almost complete replacement of mouse erythrocytes by erythrocytes agglutinating with mouse anti-hamster serum was observed. These results and the demonstration of cells containing characteristic hamster chromosomes in the bone marrow meant the establishment of a second type of stable heterologous radiation chimaera with the mouse as the host animal.

Irradiated rabbits have been protected by suspensions of foetal mouse liver and spleen[191], but no evidence of the presence of mouse haemopoietic cells was obtained in the survivors. This therapeutic effect of foetal mouse liver was not subsequently confirmed by Porter and Moseley[331].

In birds, Shaw and Vermund[366] reported the successful transplantation of dove bone marrow into irradiated pigeons. However, the survival time of these birds was slightly less than that of the saline treated irradiated controls, which seemed partially due to an unusually severe graft versus host reaction.

The results of homologous bone marrow transplantation between individuals of randomly bred populations have in general closely paralleled those obtained with transplantation between inbred strains of mice. Convincing evidence of bone marrow takes has been supplied in dogs and monkeys, but in both species long-term survivals have been extremely rare due to the intervention of graft versus host reactions.

The number of bone marrow cells required for homologous transplantation in humans is not known. The few cases in which a bone marrow take has been registered received between 2 and 10×10^8 cells/kg body weight. Values for the critical number of autologous or isologous cells are not available for obvious reasons. Extrapolation from the animal data presented in Table II: 1 suggests values between 5 and 8×10^7/kg body weight as compared to 10^7–10^8/kg suggested by the results of clinical studies by Kurnick. However, the limited number of patients who were successfully treated with isologous bone marrow following whole body irradiation all received 2×10^8 cells/kg or more.

The radiation dose

For those readers who are not familiar with dose-survival relationships in whole body irradiated animals a few introductory remarks may be useful.

RADIATION SYNDROMES

The nature of the clinical syndromes as well as the survival time following whole body irradiation are determined by the radiation dose. Three different radiation syndromes can be distinguished in mammals (see Fig. II[3]). Following doses exceeding about 12,000 r the animals develop symptoms characteristic of damage to the central nervous system and death ensues within hours or at the most 1 or 2 days. The survival time has been found to decrease with increasing dose. This form of radiation sickness has been termed the cerebral syndrome; it does not develop when the head is shielded during the irradiation.

In the dose region between 1,200 and 12,000 r mortality is caused by irreversible damage to the intestinal tract. The animals develop anorexia and excessive watery diarrhoea and die between the 4th and 5th day from protein loss and a disorganization of their water and mineral metabolism. This syndrome can be prevented to some extent by shielding the intestines during the exposure. It is of interest that this protective effect can even be obtained by shielding a small proportion of the ileum[392]. Apparently a relatively small piece of functional intestine is sufficient to correct the disturbances of resorption in the rest of the intestinal tract. A group of workers at Brookhaven, U.S.A.[90] has succeeded in preventing the intestinal death in a significant number of dogs by the continuous replacement of fluid and minerals combined with the administration of antibiotics.

Animals that have been exposed to lower dosages in the lethal range die from failure of the haemopoiesis. This form of radiation death is called bone marrow death and the clinical picture is known as the bone marrow syndrome. The underlying cause of the symptoms is the severe inhibition of cellular proliferation in the haemopoietic tissues which results in a depletion of the various cellular products of the tissues. In the mouse this is reflected in the peripheral blood by a granulocytopenia which reaches lowest values after a few days, a thrombocytopenia with minimum values between 8 and 12 days and anaemia which develops during the second and third week. As a result of interphase death* of the radiosensitive lymphocytes these cells disappear within 24 hours.

As a consequence of leucopenia the animals' resistance against bacterial infections decreases rapidly, which explains the common

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* Interphase death occurs in lymphatic cells within a few hours after irradiation and independently of mitosis. In most other cell types radiation death is associated with mitosis or attempted mitosis.

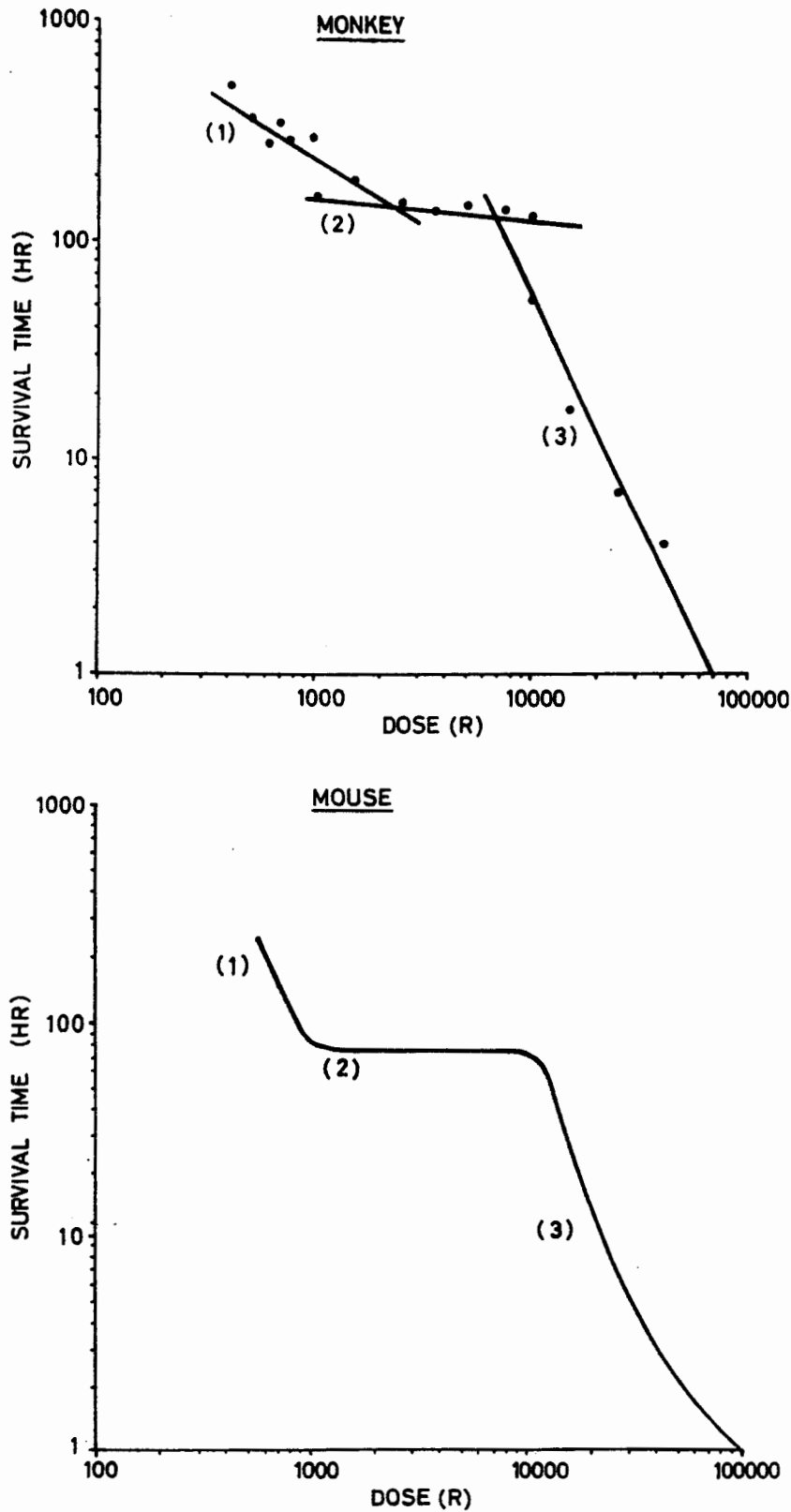Figure II[3].   Survival time following lethal whole body exposure
in monkeys and mice showing the three radiation syndromes.
Graphs are based on data from Quastler *et al.* (1951)[341], Cronkite
(1951)[105] and Rajewsky *et al.* (1953)[343] for the mouse and from
Pickering *et al.* (1959)[312] for the monkey

(1) Region of bone marrow syndrome
(2) Plateau of intestinal syndrome
(3) Region of cerebral syndrome

occurrence of sepsis and bacteriaemia in many animal species. The lack of thrombocytes results in a haemorrhagic diathesis with the possibility of diffuse small or localised massive haemorrhages (Plates II: 1(a) and (b)). Even if the latter do not develop, the loss of erythrocytes in the numerous microhaemorrhages may cause all possible degrees of anaemia which are not adequately corrected by the production of new red blood cells. A number of years ago the question of whether the haemorrhagic state is due solely to thrombocytopenia was very much disputed. Direct radiation damage to the vascular walls as well as the occurrence of a haemorrhage producing factor in the blood were postulated as additional causative factors. This problem has now been settled: the haemorrhages can be completely prevented by an adequate supply of fresh platelets either by way of thrombocyte transfusions or by injections of fresh blood.

INFECTIONS

Infections and septicaemia can be prevented in many cases by the administration of appropriate antibiotics. This does not necessarily prevent mortality because the animals die from haemorrhages instead.

In certain animal species, e.g. the mouse, bacteraemia is always present at the time of death; in other species, e.g. the rat, this is not the case. From 50 to 80 per cent of lethally irradiated rats die with sterile blood from multiple haemorrhages and severe anaemia. In our own series of irradiated monkeys, septicaemia was occasionally observed, but it should be noted that these animals received a rather intensive antibiotic treatment.

There is still a widespread misunderstanding concerning the origin of the micro-organisms which invade the blood stream of animals—in particular mice—suffering from the bone marrow syndrome, with ensuing mortality at about the 10th day. It is generally believed that these bacteria always originate from the intestinal flora by entering the blood stream by way of radiation induced lesions of the intestinal epithelium[280]. This concept was difficult to reconcile with the histological findings in the intestinal tract at the time of the highest incidence of bacteriaemia, since the epithelium is found to be completely regenerated and no longer shows lesions which could be considered as likely sites of entry for micro-organisms. In addition, Wensinck and Renaud[456] have identified micro-organisms found in the blood of lethally irradiated CBA mice as belonging to the normal

bacterial flora of the respiratory tract and their observation that infection of the cervical lymph nodes occurs prior to that of the mesenteric lymph nodes strongly support the idea that the invasion takes place in the oro-pharyngeal or respiratory regions.

On the other hand micro-organisms belonging to the normal intestinal flora have also been identified in cases of bacteriaemia accompanying radiation sickness by various investigators.

This apparent discrepancy has recently been explained by van der Waay et al. (D. van der Waay, personal communication) who showed that at least in irradiated mice the species of micro-organisms that invade the blood stream vary with the dose of whole body irradiation administered. In the lower dose region causing the bone marrow syndrome, intestinal bacteria are usually not predominant, but upon increasing the radiation dose above the LD_{100} minimum these species become almost exclusive invaders of the blood stream. In these cases the pathway of entrance is via the mesenteric lymph nodes and this does not necessarily have to occur by way of mechanical defects in the epithelial lining of the gut, since there is some evidence that under normal conditions bacteria penetrate the epithelium regularly to be inactivated in the lymphatic tissues of the intestine. It is highly conceivable that this defence mechanism remains partly effective following the lower lethal doses of irradiation, to break down completely following the higher doses, which would account for the differences in the types of bacteriaemia which develop.

In the uncomplicated bone marrow syndrome following supralethal doses of X-rays, the peak of mortality is between 10 and 14 days in mice, rats, rabbits, dogs and monkeys. Following lower radiation doses death may occur somewhat later, but mortality after the 30th day is rare. In a number of laboratories, contamination of the mouse colony with Pseudomonas aeruginosa has caused the occurrence of so-called early mortality, which means that the highest death rate occurs between the 5th and 7th days following irradiation. This contamination seriously interferes with the experiments, particularly because various prophylactic and therapeutic procedures have been found to be much less effective under these conditions. The significance of this complication is best demonstrated by the fact that in 1961 a symposium was devoted entirely to the subject of pseudomonas contamination in radiobiological laboratories[142]. The prevention of pseudomonas contamination of mouse colonies requires constant bac-

teriological supervision and the irradiated mice which are apparently susceptible to only a few *pseudomonas* bacteria have to be protected from infection by daily change and sterilisation of water bottles[451, 455]. In irradiated rats from a particular colony, the incidence of *pseudomonas* bacteraemia at the time of death following whole body irradiation was found to be over 50 per cent. Following the introduction of a number of sanitary precautions into the rat colony, amongst which the frequent sterilisation of drinking bottles was prominent, *pseudomonas* bacteraemia was no longer observed following irradiation of rats bred in the colony. More than half the irradiated animals showed sterile heart blood at death. In addition the mortality before the 10th day was drastically reduced.

Another micro-organism which may influence the survival time of lethally irradiated mice—although to a less extent than *pseudomonas*—is *proteus*. The peak of mortality for *proteus*-infected mice is around the 7th day. A standard method for the eradication of *proteus* from mouse colonies is not known at present. Recently, the difficulty has been overcome by the introduction of Enterobacteriaceae free mice (so-called Specific Germ Free mice) by D. van der Waay. These animals can be kept free of Enterobacteriaceae—including *proteus*—by employing an incomplete nursing barrier.

BONE MARROW THERAPY

Treatment with isologous bone marrow and spleen is generally successful in preventing mortality at all X-ray dose levels up to those which are followed by intestinal syndrome interference (death at the 4th or 5th day following irradiation). In contrast, the majority of successful transplantations of *foreign* bone marrow have been performed in animals subjected to irradiation with a dose exceeding the LD_{100} and several investigators[49, 150, 413] have reported the existence of a lower limit to the irradiation dose following which transplantation of foreign bone marrow and subsequent recovery can be obtained. They observed, moreover, that in the midlethal dose region the injection of homologous and heterologous bone marrow is ineffective and in some cases even harmful.

MIDLETHAL RADIATION DOSE

This unexpected phenomenon has been named the midlethal dose effect (MLD-effect). It is proposed to use this term only to designate the fact that the administration of foreign bone marrow causes an in-

creased mortality when compared with non-treated irradiated controls, rather than apply it to all cases where foreign bone marrow is merely ineffective (see Fig. II[4]). In the latter situation the bone marrow administration has *no* effect at all and therefore this can be more correctly described as the MLD-phenomenon[43].
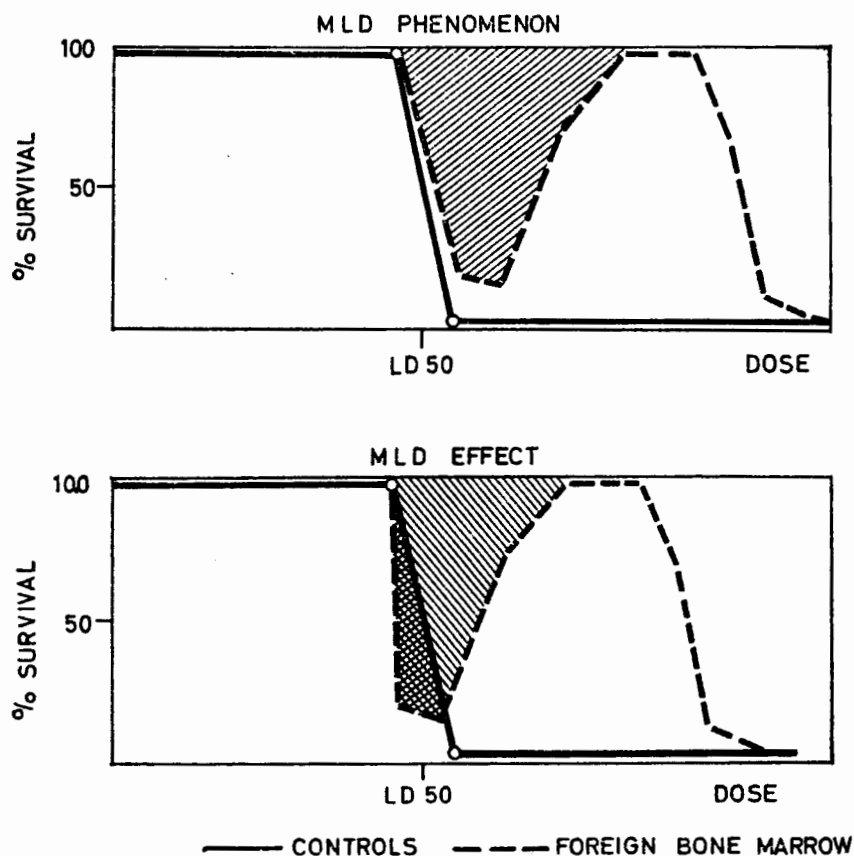
Figure II[4]. Theoretical survival curves for irradiated mice illustrating the concepts of the MLD phenomenon and the MLD effect. The crossed area in the lower graph represents the additional mortality caused by the administration of foreign bone marrow

On the basis of peripheral blood counts, Trentin[413] suggested in 1956 that the greater mortality at 550 r (than at 770 r) in mice treated with foreign bone marrow might be related to a less adequate haemopoietic recovery. Soon afterwards it was convincingly demonstrated that the deficient haemopoietic recovery indicated by Trentin is the result of a rejection of the transplanted cells. As shown by Congdon *et al.*[94] the usual intense proliferation of donor cells does occur initially, but is followed by a sudden disintegration of the haemopoietic graft about the 7th day after transplantation. This reaction has been referred to as the "acute rejection of the graft"[52]. It is obviously

caused by an immunological reaction on the part of the host. A certain proportion of these mice die because their own blood-forming tissues have not recovered sufficiently to prevent the development of pancytopenia.

As described above, the incidence of acute rejection of the graft is dependent on the dose of irradiation, since it can only occur if recovery of the host's immunological defence system is sufficiently fast. This interpretation is consistent with the results of immuno-genetic experiments. (CBA × C57BL)F_1 hybrids are completely protected by parent strain bone marrow following median lethal X-ray dosages; however, under the same conditions of irradiation, parent strain mice are incompletely protected by F_1 hybrid bone marrow. It is only in the latter combination that the host is potentially reactive against the graft. Although a satisfactory explanation has thus been obtained for the MLD-phenomenon, this does not automatically apply to the MLD-effect. There are two sets of observations which seem to provide some insight into this problem.

(1) Gengozian et al.[154] have shown that the detrimental effect of foreign bone marrow cells is more severe the greater the number of cells. In sublethally irradiated mice (640 r) the injection of 100 × 10^6 rat bone marrow cells caused 50 per cent 30-day mortality, while 240 × 10^6 cells resulted in 100 per cent mortality.

(2) The antigenic difference between donor and host determines the magnitude of the MDL-effect as well as the MLD-phenomenon. When CBA mice were treated with C57BL cells following a midlethal dose of irradiation, the mortality was 100 per cent. Treatment with (CBA × C57BL)F_1 bone marrow caused much less mortality[437].

It seems therefore that the enhanced mortality which characterises the MLD-effect can be evoked by increasing the dose of foreign cellular antigens as well as by increasing the antigenic difference between the host and donor cells. Both changes would result in a more intense immunological reaction on the part of the surviving host cells which might decrease the chances of survival of the host, for instance as a result of non-specific stress.

The immunogenetic factors involved have recently been extensively studied by Uphoff[426]. On the basis of a large number of homologous host-donor combinations this author has distinguished four classes of results of bone marrow transplantation following midlethal irradiation.

(1) An early lethal effect (equivalent to the MLD-effect).

(2) No beneficial or deleterious effect (conforming to the concept of the MLD-phenomenon).

(3) Initial protection followed by late deaths (probably secondary disease, see Chapter III).

(4) Lasting protection.

One extremely interesting conclusion of her investigation is that the occurrence of the MLD-phenomenon and the MLD-effect are determined by the immunogenetic properties of the *host only* and are limited to a small number of mouse strains.

Other observations confirm that the MLD-phenomenon is not always pronounced in incompatible host-donor combinations. Santos et al.[359] reported that LAF_1 mice treated with rat bone marrow and penicillin following low lethal dosages of X-rays in the range between LD_{10} and LD_{100} suffered only 14–20 per cent 30-day mortality and an MLD-effect was completely absent. Similarly, one of the present authors[43] found no evidence of an MLD-phenomenon in (CBA × C57BL)F_1 mice which were grafted with rat bone marrow, although in both parent strains considerable mortality occurs under the same conditions. On the other hand a pronounced MLD-effect was observed by Gengozian and Makinodan[150] in (C3H × 101)F_1 mice treated with rat bone marrow.

This means that at least in mice the deleterious effects of homologous bone marrow transplantation following midlethal irradiation are probably the exception rather than the rule, which would make the outlook for eventual clinical application of homologous bone marrow transplantation somewhat less pessimistic. The discovery of the MLD-effect has led to considerable caution in the recommendation of bone marrow transplantation as a treatment for accidental whole body irradiation, because the exact exposure dose is usually not known in the days immediately following the irradiation. Results of experiments with other animal species and in particular with primates have to be awaited, however, before a more reliable prediction of human reaction patterns can be made.

The MLD-phenomenon as well as the MLD-effect in mice can be partially or completely avoided by employing a 24 hour interval between the irradiation and the administration of foreign bone marrow[359]. This is also in agreement with the interpretation of the MLD-effect as presented here, since the primary antibody response in irradiated animals has been found to be weaker when the interval

between irradiation and the injection of the antigens has been 12–24 hours rather than shorter intervals[151, 395].

The conclusion from these quite extensive data is that failure to survive median lethal doses of irradiation and foreign bone marrow treatment is due to a secondary loss of the haemopoietic graft (MLD-phenomenon). This acute rejection of the graft may be so violent as to cause additional mortality of the irradiated host animals (MLD-effect). Apparently the graft rejections are manifestations of a residual immunological activity of the host, although admittedly the number of immunologically competent host cells that survive X-ray doses of the magnitude described would be small. In order to obtain an idea of the number of those cells involved in the anti-graft reactions, graded numbers of *isologous* lymph node cells have been administered to supralethally irradiated mice, in addition to a number of rat bone marrow cells which were known to afford maximal protection[49]. It was found that as few as $4-8 \times 10^5$ isologous lymph node cells could abolish the therapeutic effect of the rat bone marrow transplant and a similar effect was observed with at least 8×10^5 isologous thymus cells.

SURVIVING FRACTION OF THE IMMUNE SYSTEM

If the number of immunologically active cells surviving the LD_{100} of whole body irradiation (800 r) is S, it follows that $S + 4 \times 10^5$ lymph node cells are sufficient to reject a rat bone marrow graft. Rejection also takes place in a large proportion of the recipients following irradiation with an LD_{50} (650 r), so that at this dose level the number of surviving cells will probably be around $S + 4 \times 10^5$. By extrapolation of the dose survival curve for mouse lymph node cells irradiated *in vitro* as published by Smith and Vos[381] (Fig. II[5]) the surviving fraction at 800 r is 6×10^{-5}, and at 650 r 4×10^{-4}. This survival curve was based on measurements of the killing effect of homologous lymph node cells when injected into lethally irradiated F_1 hybrid mice, which capacity is no doubt also related to the capacity of the same cells to reject a foreign bone marrow graft.

The values derived above permit the following calculation of the population size of the lymphatic system of the mouse (n)

$$4 \times 10^{-4}n = S + 4 \times 10^5,$$
$$6 \times 10^{-5}n = S,$$
$$34 \times 10^{-5}n = 4 \times 10^5,$$
$$n = 12 \times 10^8,$$

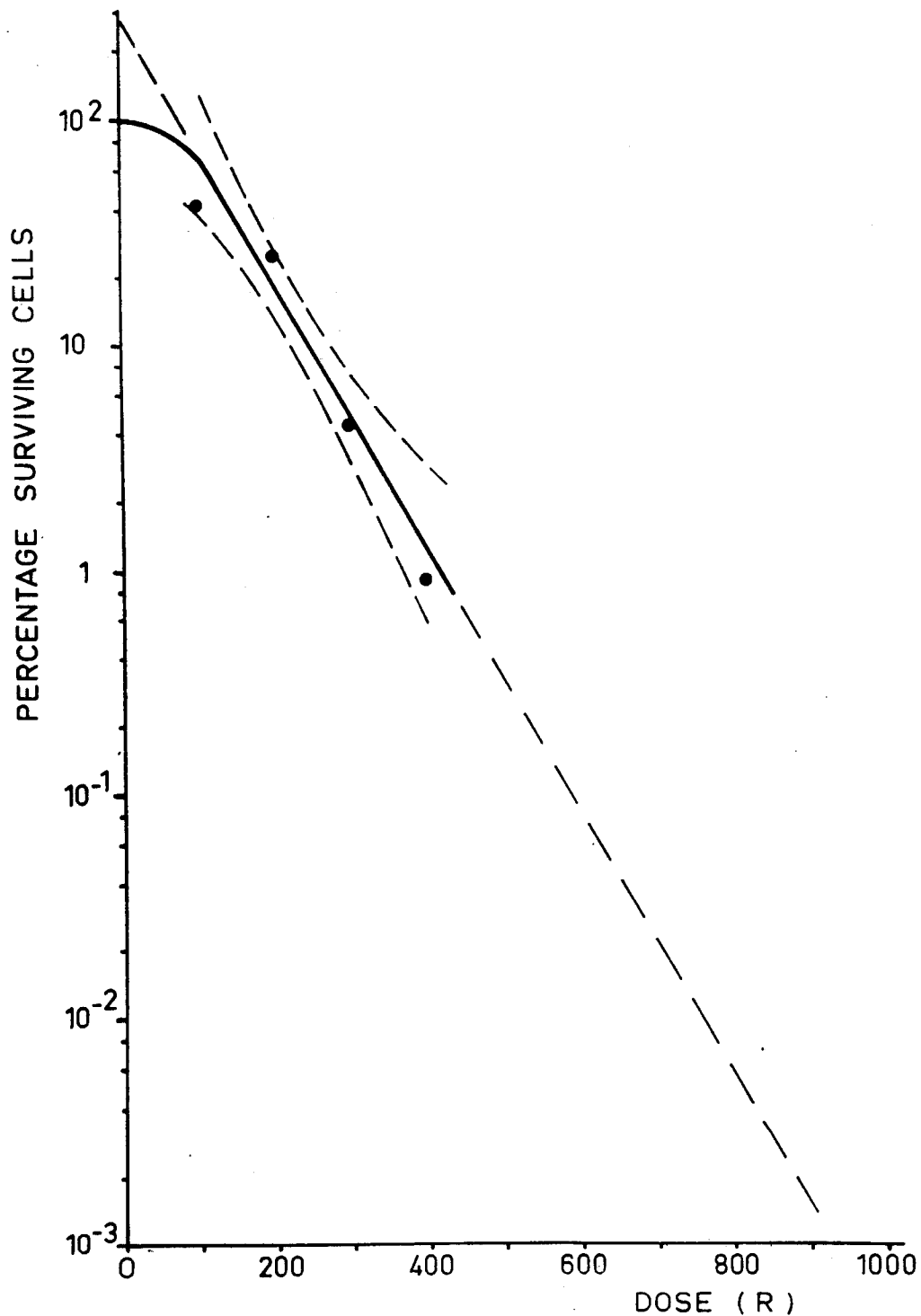Figure II[5]. Survival curve of mouse lymph node cells following *in vitro* irradiation. The curve is extrapolated from the data published by Smith and Vos (1963)[381]. The extrapolated portion is represented by the broken part of the line. The extrapolation was done by simple extension of the line drawn by Smith and Vos. Any other extrapolation methods seemed to involve too many additional uncertainties

A rough estimate based on routine cell counts yields $3 \cdot 5 \times 10^8$ as the total number of lymphatic cells in the adult mouse (thymus 5×10^7, spleen 10×10^7, lymph nodes 10×10^7, other lymphatic tissues and bone marrow approximately calculated as 10×10^7). Considering the number and magnitude of the errors involved in this type of calculation and the degree of uncertainty in the direct evaluation of the size of the lymphatic cell population, the agreement between the two values seems fair.*

The form of adoptive immunity resulting in the rejection of foreign bone marrow grafts which was described above, has been reinvestigated with isologous thymus cells by Congdon and Duda[92], who also confirmed histologically the acute rejection of the rat marrow graft. In addition they showed that this adoptive immunity can be effected with isologous spleen cells, white blood cells and also with bone marrow cells, but in all their tests very large numbers of lymphatic cells—10^7 and more—were employed. Jacobson et al.[194] have shown that shielding of a piece of intestine containing Peyer's patches during the irradiation prevents the take of foreign bone marrow in mice. On increasing the irradiation dose the chances of acute rejection of the graft become less, presumably because the host's immunological system is more effectively destroyed. Accordingly, after supralethal doses of irradiation this type of rejection no longer occurs.

So far, the discussion has been concerned with homologous and rat into mouse bone marrow transplantations. When more distantly related species are employed as bone marrow donors, permanent takes may not be achieved even when the irradiation dose is further raised to doses which cause death in about 4 days as a result of the intestinal

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* The same calculation based on survival curves produced by McCulloch and Till[267] for mouse bone marrow irradiated in vitro (using mouse protection as an index for the number of viable cells) yields 10^8 cells for n. The surviving fractions at 800 r (0 .001) and 650 r (0 .005) have also to be obtained by extrapolation. Using the survival curves published by Barendsen et al.[19] for human kidney cells grown in tissue culture the value for n becomes 8×10^6. The discrepancy between the latter values and the much higher value obtained by using the lymph node cell survival curves of Smith and Vos is most readily explained by the fact that lymphocytes suffer from a direct mechanism of radiation death, not involving mitosis (interphase death) and that this renders them much more radio-sensitive than kidney or bone marrow cells. It should furthermore be noted that all the survival curves used here were obtained by in vitro irradiation which may not necessarily yield identical survival factors as in vivo exposure. Finally, the curves by Barendsen refer exclusively to the capacity of cells for unlimited proliferation, while in the lymph node cell and bone marrow experiments other properties of the cells are most likely involved.

syndrome. Shekarchi and Makinodan[368] have studied the persistence of rat, hamster, guinea pig and rabbit bone marrow in mice irradiated with doses from 300 to 2,000 r by sacrificing the recipients at daily intervals and estimating the concentration of alkaline phosphatase positive (donor) cells in blood, bone marrow and spleen preparations. Their results with rat and hamster bone marrow are shown in Fig. II[6];
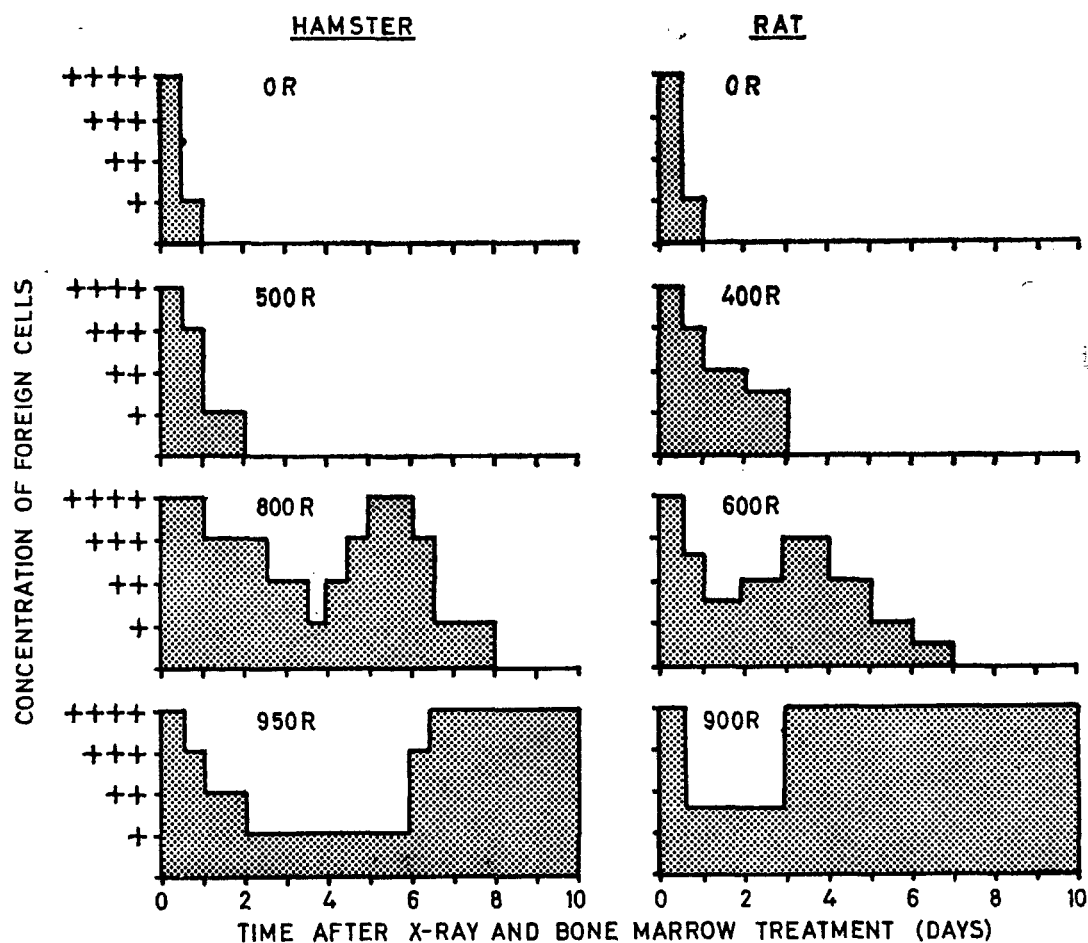
Figure II[6]. Donor type cells in spleen cell preparations (imprints) of irradiated mice following treatment with hamster and rat bone marrow. Figure from Shekarchi and Makinodan (1959)[368]. The mice received ~140 × 10^{6} nucleated cells i.v. and the donor cells were identified by their positive alkaline phosphatase reaction

guinea pig and rabbit cells persisted for less than 1 day following 930 r or lower X-ray doses. Following an irradiation dose of 1300 r, guinea pig cells persisted for 4 days and rabbit cells for 1 day. The latter persisted until the death of the recipients on the 4th day following a dose of 2,000 r. As it seems impossible that an immunological rejection becomes operative within 1 day after antigenic stimulation,

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the prolonged persistence of the foreign cells upon increasing the dose of irradiation to the recipient must remain unexplained.

Stability of the chimaeric state

Even when conditions prevail which are favourable to an initial proliferation of the grafted cells, the host may—after a longer interval —regain its capacity to react against the haemopoietic graft. This then leads to a more gradual replacement of donor type cells by host type cells which in turn allows a continued survival of the host animal. This recovery of the host's haemopoiesis has been described as a *reversion* and the animals undergoing this change have been called *reversals*. The process has to be distinguished from a *delayed rejection* of the bone marrow graft which is a subacute process leading to death from pancytopenia and which may occur any time between the 7th and 30th day following median lethal and low lethal doses of irradiation and foreign bone marrow transplantation.

Animals that eventually regain their own haemopoietic system completely, and can therefore, no longer be classified as chimaeras, are called *total reversals*. In our experience this recovery process, if it occurs, is usually under way or completed before the end of the 3rd month following transplantation. In exceptional cases the reversion remains incomplete for a very long time, during which time the animals maintain a *mixed population* of host and donor type blood cells. These cases were classified as *partial* reversals, which included those animals that at no time had exclusively donor type cells, while the term *true chimaeras** was used to designate animals that showed a complete absence of host type haemopoietic cells.

The permanency of the chimaeric state is determined by the same two factors as the initial take of the bone marrow graft, namely, the dose of irradiation to which the host animals are subjected and the degree of immunological incompatibility between the host and the donor.

RADIATION DOSE

As a logical consequence of the dose dependency of the acute rejection of the graft,'it has been found that the frequency of reversions diminishes when the dose of radiation is increased. The data that support this statement are mainly derived from studies of rat bone

* *Complete chimaeras* now seems to be a more suitable term.

marrow transplantations into mice. Gengozian and Makinodan[150] typed the erythrocytes of mice surviving 150 days following rat bone marrow transplantation after various doses of X-radiation. Following 400, 500 and 600 r, rat erythrocytes were found not to persist. A pronounced MLD-effect was observed with a dose of 710 r so that at this dose level no survivors remained available for typing. Following 800 r, only one out of seven 150-day survivors showed rat erythrocytes, but all the survivors (17 mice) following 950, 1150 and 1300 r were found to have rat erythrocytes. Santos et al.[359] determined the
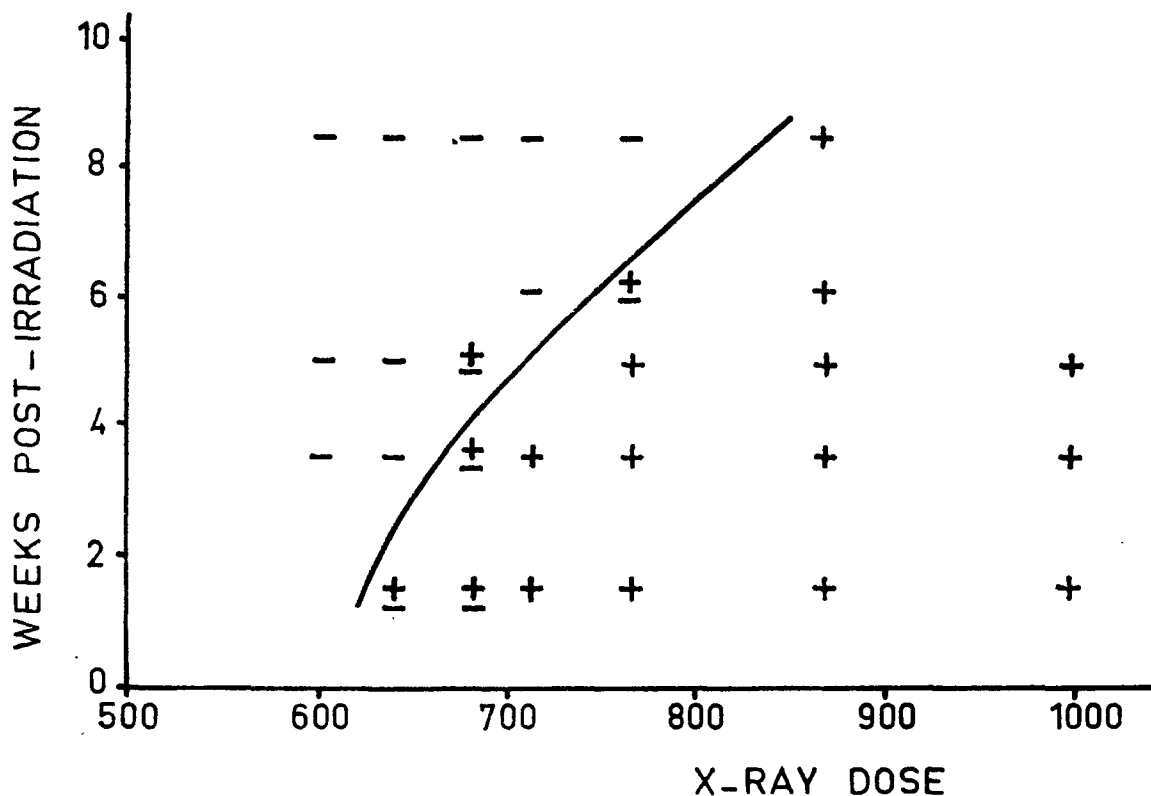
Figure II[7]. Incidence of chimaerism in relation to radiation dose in rat bone marrow treated mice. Data derived from Santos et al. (1958)[359], Table 2, who determined the incidence of alkaline phosphatase positive granulocytes in the peripheral blood at various times after transplantation of rat bone marrow
+ predominantly or exclusively donor type granulocytes
— host type granulocytes

persistence of alkaline phosphatase positive cells in mice surviving X-ray doses from 600 to 1000 r and rat bone marrow treatment. No MLD-effect was observed in those experiments in which animals were tested randomly up to 63 days after the irradiation. Their results (Fig. II[7]) also show a decreasing incidence of reversals with increase

of the X-ray dose. In a study involving two X-ray doses and more than 750 mice transplanted with rat bone marrow, Welling *et al.*[454] determined the frequency of true chimaeras, partial reversals and total reversals at 100 days (153 survivors), 200 days (94 survivors) and 300 days (49 survivors) following transplantation. Their identification was based on typing of the erythrocytes and the granulocytes in a sample of the peripheral blood. In the higher X-ray dose group only 2 partial reversals were observed, while the majority of the mice in the low X-ray dose group were total or partial reversals (Fig. II[8]).
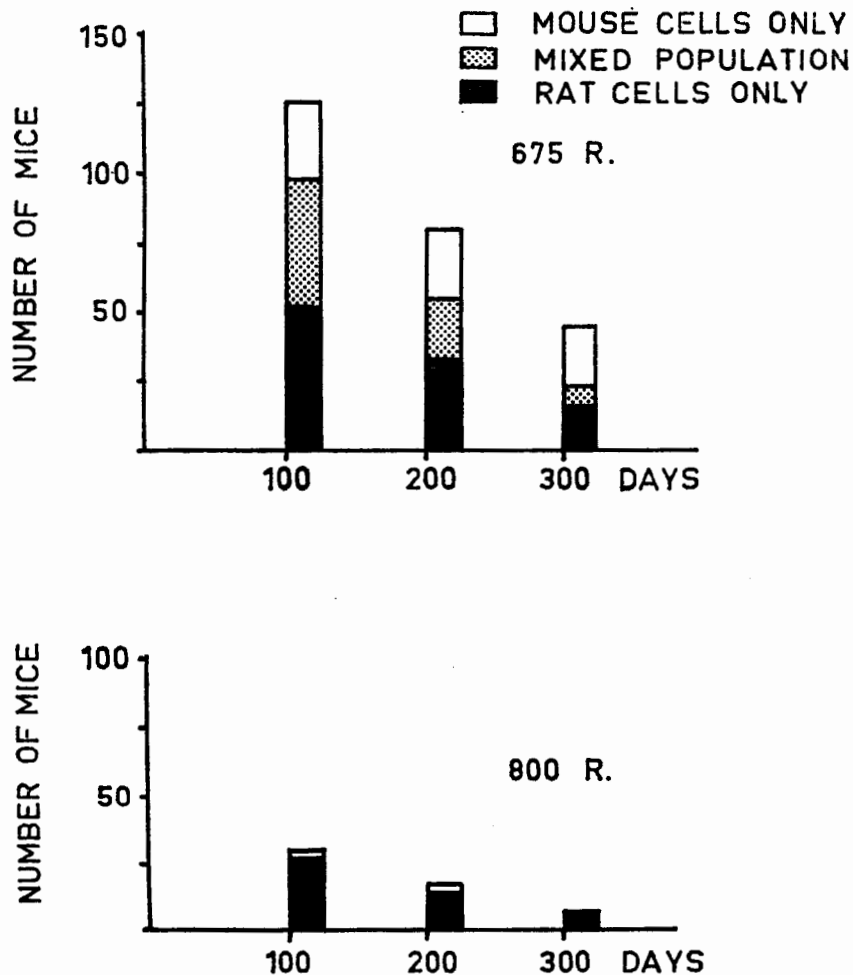
Figure II[8]. Incidence of reversals in mice surviving two dose levels of whole body irradiation followed by rat bone marrow transplantation. Data derived from Welling *et al.* (1959)[454]

The changes with time of the proportion of true chimaeras versus partial and total reversals are mainly due to a selective elimination by mortality of true chimaeras and partial reversals. In other words these data do not support the view as suggested by Ford *et al.*[144] that older

rat–mouse chimaeras have a greater tendency to reverse. These authors performed cytological studies on a number of mice treated with rat bone marrows up to 337 days following transplantation. In the older chimaeras relatively few donor type cells were observed, but this may merely reflect the better chances for long term survival of the reversals.

In rats it has also been found[18] that the incidence of reversals decreases with higher radiation doses. Below 950 r a high incidence of reversals was noted after homologous bone marrow transplantation, while none were seen after higher doses of X-irradiation.

HOST-DONOR INCOMPATIBILITY

The conviction that a high degree of incompatibility between host and donor favours the reversion process is based on a comparison between rat–mouse chimaeras and homologous mouse chimaeras. Ford et al[144] observed only very few reversions, and other investigators none at all, when homologous bone marrow was transplanted. More relevantly, Welling et al.[454] pointed out that when rat bone marrow was used for treatment following the same lethal X-ray dose, a high incidence of reversals was found.

Owen[300], using a haemolytic test system to identify the erythrocytes of homologous mouse radiation chimaeras, noted a great variety in the distribution pattern of host and donor type and also described the persistence of mixed populations for periods up to 100 days after irradiation. He did not attempt to relate his findings specifically to radiation dose, cell number injected, or other parameters, but stressed the uncertainties involved in applying information obtained with one host–donor combination to other cases.

Following the higher lethal doses of radiation, reversion seems to be promoted by decreasing the number of foreign cells administered. This was recently described by Balner[14] who studied homologous rat combinations and whose observations are in accordance with impressions derived earlier from experiments with other host–donor combinations.

MECHANISM OF REVERSION

It is not altogether clear at present whether the reversion process is primarily the result of an *immunological rejection* of the foreign bone marrow graft by the slowly recovering host or the consequence of a

recovery of the host's haemopoietic cells with a gradual, not immunologically determined, *replacement* of the donor type cells.

The latter mechanism was favoured in particular by the Harwell group. These investigators studied induced reversion in homologous mouse chimaeras by the administration of isologous spleen cells 14 days after homologous (bone marrow) transplantation. This artificial reversion was termed "induced transpopulation" and could not be produced by the administration of isologous lymph node cells. Furthermore, the spleen cells from mice that had been sensitised against the donor strain were no more effective than spleen cells from normal mice. This led them to conclude that both *spontaneous reversion* and *induced transpopulation* are due to the "superior physiological competence of the finally predominating cell line rather than to mastery through a reaction of immunity"[23].

On the other hand, it was pointed out by Welling et al.[454] that it seems unlikely that disappearance of the graft is merely due to (non-immunological) adverse milieu conditions, since the incidence of reversions in the rat-mouse chimaeras was much smaller after a dose of 800 r than after lower doses of X-rays. According to Barnes et al.[23] this difference has to be explained in terms of a decreased competitive power of the host cells following the higher dose of irradiation.

The failure of Barnes et al. to induce reversions by the injection of isologous lymph node cells forms an important argument in favour of the mechanism of reversion, as outlined by these authors. It is necessary to point out, however, that their findings are difficult to reconcile with the observations on adoptive immunisation described by Billingham et al.[65]. In CBA mice that were made tolerant to A skin by treatment with A spleen cells at birth, the injection of lymph node cells from normal CBA mice as well as from CBA mice which had been immunised against A tissues resulted in a rejection of the skin grafts. Leucocytes from sensitised mice were also effective in transferring immunity to tolerant mice, as long as 200 days after sensitisation[67]. It has recently become increasingly probable that the condition of actively acquired tolerance present in these CBA mice before the lymph node cell injections was a consequence of the fact that these mice were (at least partially) immunological and haematological chimaeras[419]. The breakdown of the tolerance as brought about by the isologous lymphoid cells thus seems to be the reflection of a forced reversion to the host type immunological system.

Another piece of information which seems relevant to the problem

of reversion has recently been provided by Balner[14]. In the course of a study on homologous rat bone marrow chimaeras, a number of reversals (which initially had a proved functional donor type bone marrow graft) were tested with donor type skin grafts. About 50 per cent of these animals were found to reject the skin graft in accordance with the expected pattern, but of the remaining animals some showed partial and others absolute tolerance towards the previous donor's skin. In a few animals of the latter category this tolerance was found to persist indefinitely as shown by several regraftings. These observations would suggest a non-immunological disappearance of the donor type haemopoietic cells during reversion.

In view of the evidence reviewed here, it appears difficult to arrive at a conclusion as to which of the two likely mechanisms of reversion, immunological or non-immunological has to be favoured at present. It should be noted that this distinction is rather academic since the complete return of host type haemopoiesis is, in the majority of cases, accompanied by the full return of host type immunological reactivity. As will be discussed later, it is now known that in certain not too distant host-donor combinations the grafted immune system becomes completely and specifically tolerant towards the host tissue antigens. Since this is generally not followed by reversion, it appears that the remnants of the host system are either incapable of proliferation or have become immunologically tolerant towards the graft. Mutual tolerance between host and donor systems also seems to underlie the cases of persistent partial chimaerism described earlier. Under conditions of mutual tolerance, which thus far seem to form the exception rather than the rule and which are limited to the more compatible host–donor combinations, a non-immunological mechanism of reversion seems very likely. However, it has to be kept in mind that the term tolerance, as used here, applies to tissue antigens, that is to a mixture of antigens of unknown composition and variable strength. If a variable susceptibility of different cell types to immunological attack and a tissue-specific antigen distribution are taken into account, it can be postulated that the host's tolerance will, in fact, be a complicated form of split tolerance, with preservation of sufficient reactivity towards part of the antigens to bring about a slow rejection of a population of haemopoietic cells and sufficient tolerance towards other components to allow the persistence of a skin graft. However, this would be at variance with the observations by Billingham et al.[64, 66] that at a "low degree" of tolerance haemopoietic cells

may survive long after skin grafts of identical genetic make up are rejected.

As to the exact nature of a non-immunological reversion, only speculations can be offered. The gradual replacement of the donor type cells by host type cells *could* be visualized as due to inherent characteristics of host and donor cells or to a difference in susceptibility to "milieu" factors—either stimulating or inhibiting.

REVERSION AND THEORIES OF HAEMOPOIESIS

The pattern of reversion was studied in great detail at Rijswijk[49,454] in rat → mouse chimaeras, using the peripheral blood erythrocytes and granulocytes as markers. The two main patterns of graft behaviour have already been discussed, namely, permanent true chimaerism and total reversal, the latter being usually completed within 100 to 150 days. A third category of mice presented mixed populations of host and donor cells for long periods of time, sometimes even for their complete life span. A similar prolonged coexistence of host and donor erythropoietic systems had previously been described by Odell and co-workers[296] in the course of a study of homologous rat chimaeras, where a low genetic disparity existed between host and donor. Comparable fluctuating mixtures of host and donor type platelets have been described in a series of rat → mouse chimaeras by Repplinger *et al.*[347] some of which were studied between 100 and 200 days following transplantation. All the partial reversals among the rat → mouse chimaeras described by Welling *et al.*[454] were found to have mouse erythrocytes, some of them exclusively so; the others had variable proportions of mouse and rat erythrocytes. Rat type granulo-

TABLE II: 2. Distribution of various types of partial reversals in mice as identified at about 200 days after irradiation and rat bone marrow transplantation*

Erythrocytes	Granulocytes		
	Rat	Mouse and rat	Mouse
Mouse	4	11	Total reversal
Mouse and rat	2	6	0
Rat	True chimaera	0	0

* Data derived from Welling *et al.*[454]

cytes were always present in these chimaeras, whether or not accompanied by mouse type granulocytes (Table II: 2). Interestingly enough, no partial reversals were encountered which exclusively carried either erythrocytes of rat origin or granulocytes of mouse origin.
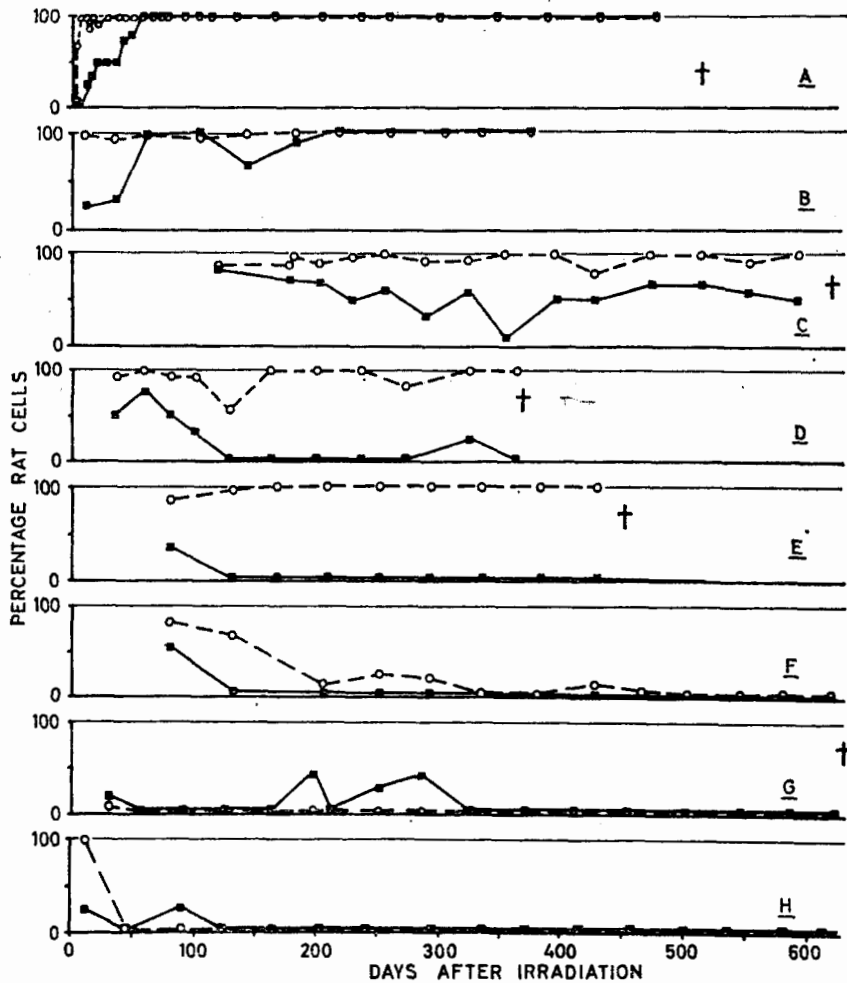

Figure II⁹. Various patterns of replacement of peripheral blood host cells by donor type cells as encountered in irradiated mice treated with rat bone marrow by Welling *et al.* (1959)[454]

O----O	Percentage of granulocytes showing positive alkaline phosphatase reaction (rat granulocytes)
■———■	Proportion of erythrocytes agglutinating with anti-rat serum
†	Death of the mouse
A,B	True chimaeras
C—G	Partial reversals, varying from near complete chimaerism (C) to near total reversal (F and G) of interest is the case of "split" chimaerism represented by (E)
H	Total reversal

Figure II[9] shows that some of these partial reversals showed considerable fluctuations in the relative numbers of host and donor type cells, but in others quite stable populations of mouse erythropoietic cells and rat myelopoietic cells seemed to be present for several hundred days. It should be pointed out that these are exceptional cases indeed, but their very nature seems to have an interesting bearing on the current theories concerning the origin of differentiated blood cells. It appears that, at least under the conditions prevailing in these chimaeras, the myelopoietic and erythropoietic systems proliferate quite independently and that very little differentiation of primitive cells from one system into stem cells of the other groups occurs (polyphyletism). This phenomenon provides one argument—admittedly an indirect one—against the stem cell hypothesis, which has become quite popular for explaining the repopulation of the haemopoietic tissues by donor cells following transplantation in irradiated animals.

The behaviour of the lymphoid cell series was studied by Zaalberg and van Bekkum[469] in rat → mouse chimaeras. The identification of lymph node cells was carried out by a cytotoxic test method employing specific antisera. The results obtained with a limited number of true chimaeras, partial reversals and total reversals showed that the identity of the lymphoid cells was closely linked to that of the other haemopoietic cells. Since the typing of the lymphoid cells required the sacrifice of the animals, follow-up studies on single animals could not be performed.

The necessity for caution in the interpretation of peripheral blood cell identifications is stressed by the results of Popp[316] who studied the fate of the graft in F_1 hybrid mice which were restored with parental bone marrow. A number of survivors were subsequently found to undergo a reversion of their erythrocyte type from donor type to host type, according to haemoglobin solubility characteristics. The bone marrow of these mice was found to contain, however, sufficient donor type stem cells to repopulate lethally irradiated, secondary recipients with erythrocytes of the primary donor's characteristics. This suggests that tests for the presence of latent donor stem cells should be performed in those situations where complete absence of donor tissue is crucial for the interpretation of the experiments.

Another development which throws light on the problem of reversions is concerned with the origin of the host cell population which replaces the grafted cells. From detailed chromosome studies on the

tissues of host-donor combinations which carried suitable chromosome markers, Barnes et al.[23] were able to show quite clearly that in cases of spontaneous reversion the regenerating host cells may stem from a very few cell clones, which could be identified cytologically by the presence of characteristic chromosomal rearrangements in mitotic cells. These chromosomal rearrangements obviously occurred as a result of the irradiation of the host.

Since in some reversals the majority of the cells in the bone marrow, the spleen, the thymus and the lymph nodes exhibited the same chromosome rearrangement, these findings in turn lend strong support to the monophyletic theory of haemopoiesis.

Variations of the irradiation regime

In the great majority of the experiments involving bone marrow transplantation the penetrating X- or γ-radiation has been administered to the recipient as a single dose delivered in a period of less than one hour. The influence of the dose of radiation has already been discussed and it has been concluded that supralethal doses within the limits indicated on pages 41 and 42 generally favour a take of the graft and result in a stable chimaera.

Fractionation of the dose and variations of the dose rate have so far received little attention.

FRACTIONATION

In rabbits it has been found advantageous to deliver the dose in two fractions divided by a 24 hour interval. This procedure was introduced because a relatively large proportion of rabbits develop irreversible shock following a single lethal dose of irradiation[333]. The effect of this fractionation on the transplantability of foreign bone marrow and on the fate of the chimaeras has not been investigated systematically.

The survival of rats following three fractions of whole body irradiation and treatment with autologous marrow was studied by Strelin and Shmidt[390]. The fractions of 350 r each were delivered at weekly intervals and, in the animals to be treated with autologous marrow, one leg was shielded during the irradiation. Immediately after the last exposure, between 17 and 20×10^6 bone marrow cells were extracted from the shielded femur and reinjected intravenously. In this group 60 per cent survived for 30 days against 20 per cent in the group in which leg shielding only was performed. Although these
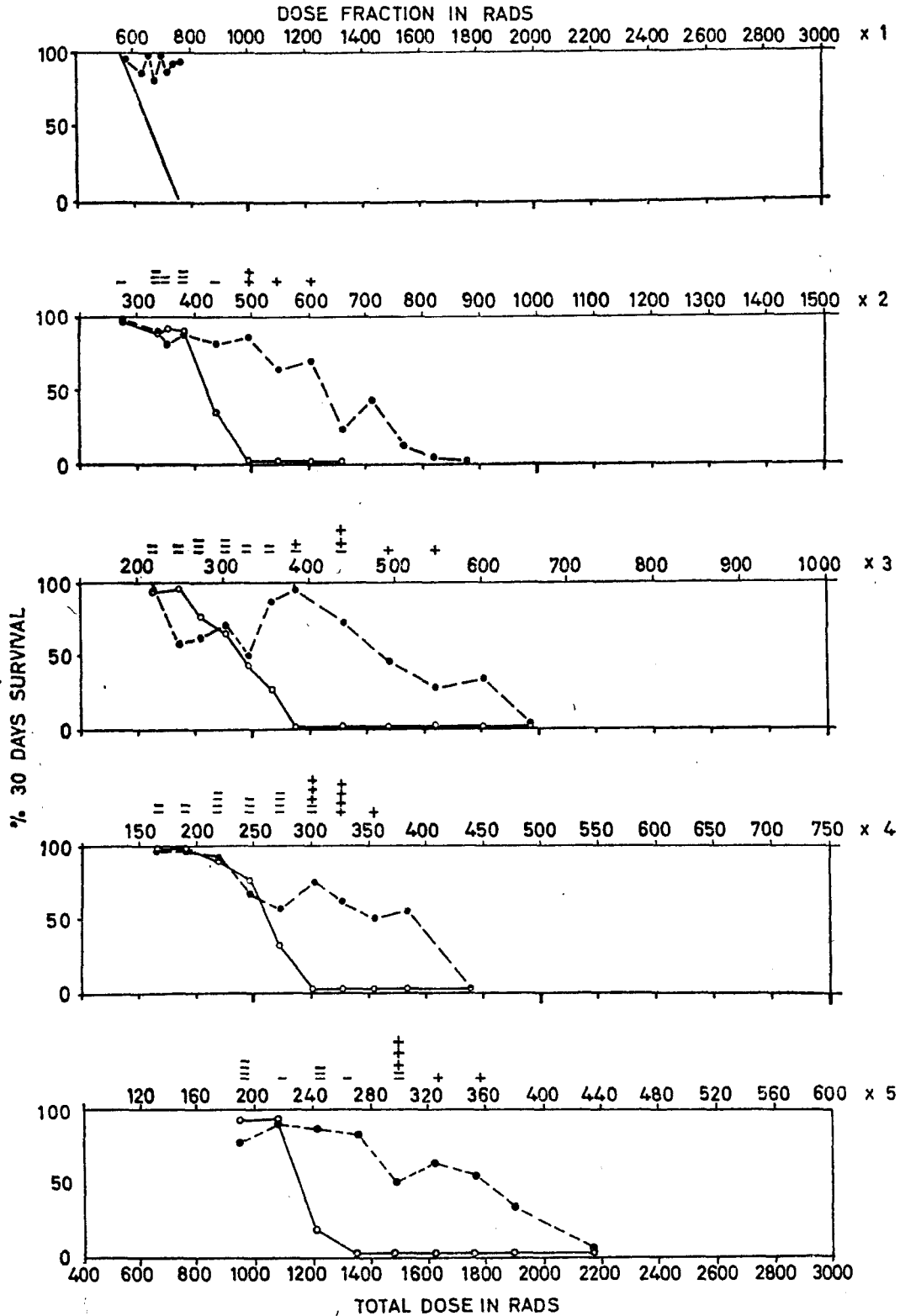
Figure II[10]. Therapeutic effect of rat bone marrow transplantation in mice following various doses of single and fractionated whole body irradiation. Figures are derived from van Bekkum (1964)[43]. The interval between the fractions was 24 hours, and the bone marrow was injected 2–6 hours after the last irradiation. Minus (reversals) and plus (chimaeras) signs at the top of each graph refer
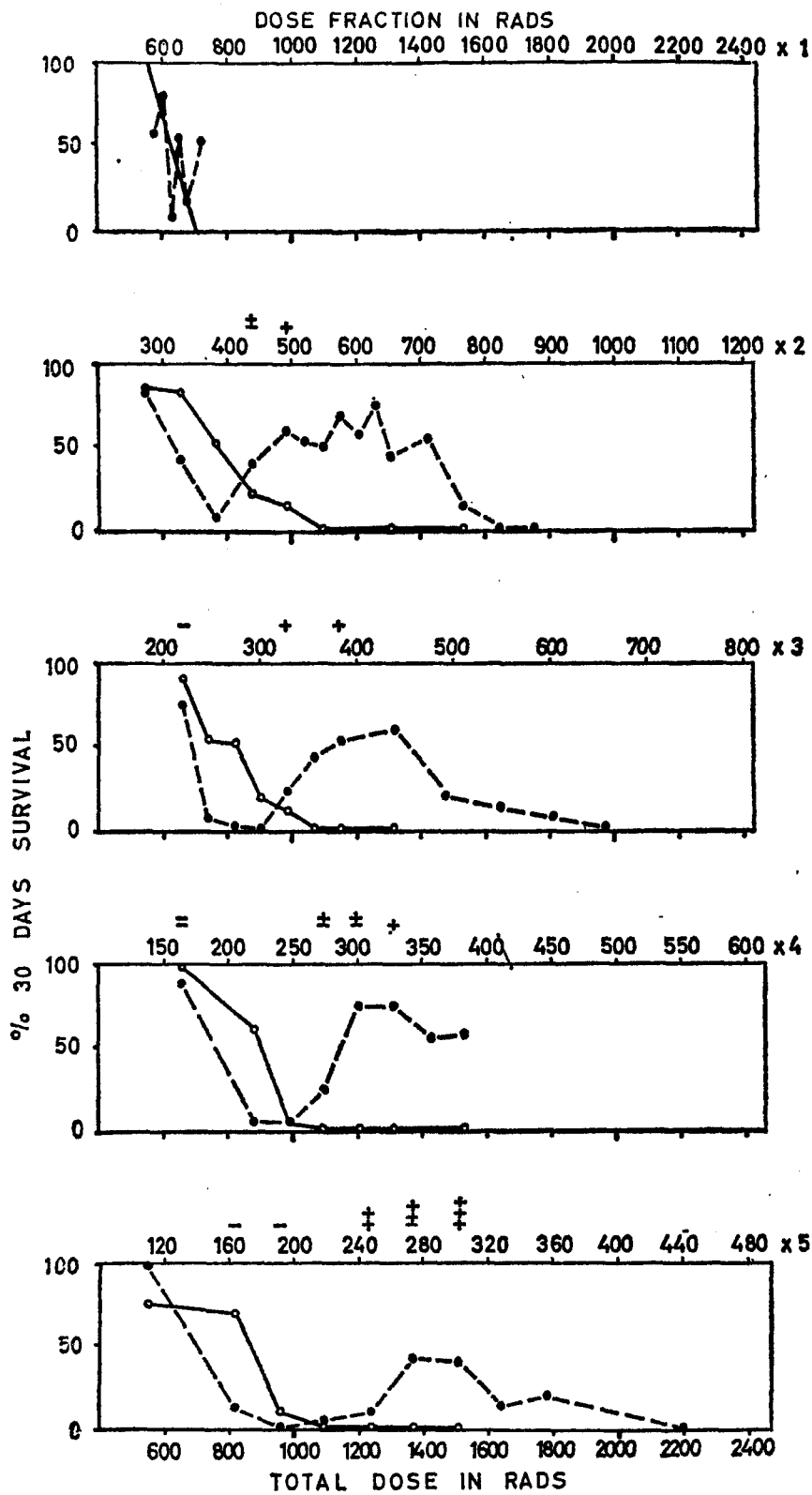
to the typing of erythrocytes and granulocytes, which was performed in groups of survivors 2–4 months after transplantation

(a) (CBA × C57BL)F₁ hybrid mice

(b) CBA mice

o————o Controls

● - - - - ● Rat bone marrow

experiments do not contribute much to the problem of fractionation they are of interest in showing that injected cells seem to be much more effective than cells remaining *in situ*.

In the present authors' laboratory an intensive study of rat bone marrow transplantation in mice subjected to fractionated irradiation has been performed during the past few years[43]. The total dose was delivered in 2-5 fractions separated by 24 hour intervals. The marrow was administered between 4 and 6 hours following the last irradiation. In the (CBA × C57BL)F_1 hybrid mice and C57BL mice—both strains which show very little of an MLD-phenomenon following a single radiation dose—a gradual decrease of effectivity of the bone marrow was found to occur at higher total doses, with no distinct interruption of the therapeutical effect (Fig. II[10(a)]). In contrast, a definite MLD-effect was observed in CBA mice with all the fractionation schedules (Fig. II[10(b)]) as well as following a single X-ray dose.

As with the single irradiation experiments chimaerism was induced only with total doses exceeding the LD_{100} minimum. The majority of these chimaeras died from severe secondary disease in the 2nd and 3rd month following transplantation.

Only one study has been published describing bone marrow transplantation following continuous irradiation over a long period[179]. The experimental animals (guinea pigs) were treated with isologous bone marrow at the completion of a 3 month period of whole body γ-irradiation. The bone marrow seemed to have a favourable influence on haemopoietic recovery and on survival, but the incidence of bone marrow takes could not be evaluated. Barnes *et al.*[20] have observed the beneficial effect of both isologous and homologous bone marrow in mice that received 1500 rads of whole body γ-irradiation over a period of 25 hours. These experiments were performed with mice which had received an inoculum of leukaemic cells before the irradiation and the number of animals employed was quite small.

INTERNAL RADIATION

Lorenz and Congdon[227] have reported the beneficial effect of isologous bone marrow transplantation in mice following the intravenous administration of a lethal dose of radon. Only a few attempts at bone marrow therapy following large doses of internally administered radioactive isotopes have appeared in the literature so far. Garvan *et al.*[147] treated rabbits with homologous bone marrow follow-

ing the administration of radioactive gold but the identification of donor cells was not performed in the survivors. Mathé *et al.*[264] found isologous as well as homologous bone marrow to be ineffective in the treatment of mice which were given lethal doses of radioactive gold intravenously.

A special technique of recycling internally administered Yttrium 90 chelated with diethylenetriamine pentacetic acid was developed by Winchell[462] to achieve selective irradiation of the tissues responsible for the homograft rejection, e.g. the lymphatic tissues. Dogs which were lethally irradiated by this method survived after the administration of autologous marrow[463] and successful homologous bone marrow transplantation between unrelated beagles was claimed. The evidence, however, does not fully support the thesis that the survival of these animals was due to a proliferation of the grafted cells. The author suggests that his method of irradiation is superior to external X- or γ-irradiation in the treatment of malignancies of the lymphatic tissues and in the preparation of large animals and possibly man for the transplantation of homologous tissues. Further experimental confirmation of this view-point is clearly needed.

IRRADIATION WITH NEUTRONS

Some information is available on the effect of bone marrow transplantation in animals subjected to neutron irradiation. Fission neutrons ($\sim$ energy 1 MeV) were employed by Vogel and Jordan[434], 2 MeV and 8 MeV cyclotron neutrons by Cole and Ellis[84] and 14 MeV neutrons produced by the ^{3}H (d, n) ^{4}He reaction by Randolph *et al.*[344]. In all cases isologous bone marrow or isologous infant spleen cells were injected after the irradiation. The results show such treatment to be less effective in reducing mortality than in animals subjected to X- or γ-irradiation. This is generally ascribed to the relative predominance of gastro-intestinal damage as compared with the destructive effects on the haemopoietic system following neutron exposure. In other words, the neutron irradiation seems to cause a relative shift of the gastro-intestinal syndrome towards lower radiation doses resulting in partial overlapping with the bone marrow syndrome, at least in mice. In neutron irradiated dogs no such enhancement of the gastro-intestinal component of the radiation sickness has been observed and in this species autologous bone marrow was found to be as effective as when used after γ-irradiation[2]. Nothing has been reported thus far on the foreign bone marrow treatment of

neutron irradiated animals, but in view of the relative preponderance of the intestinal damage, it is to be expected that the establishment of chimaeras will be more difficult to achieve than with X- or γ-radiation. An evaluation of the efficacy of foreign bone marrow in the treatment of animals exposed to neutron irradiation is obviously needed because in radiation accidents a mixed neutron and γ-irradiation is usually involved. However, information of use in the treatment of human patients will probably have to be obtained from experiments with large animals because of the important differences in distribution patterns of absorbed energy which occur in animals of different sizes, when radiation by neutrons or other high energy particles is employed.

Interval between irradiation and transplantation

The advantage of a 24 hour interval versus an interval of a few hours in the transplantation of foreign bone marrow has been sufficiently discussed in relation to the MLD-phenomenon and the MLD-effect.

As to the maximum interval after which marrow transplantation can modify radiation mortality, most of the information is based on experiments with isologous bone marrow or spleen (Table II: 3).

From the experiments of Unsgaard[422] it has to be concluded that for isologous cells the optimal interval is from o to 24 hours and that some therapeutic effect seems possible after as long as 8 days. The number of cells administered by Unsgaard is, however, exceptionally large when compared with the minimal number which permits about 100% survival (estimated at $1-5 \times 10^5$) when administered immediately after irradiation. Furthermore, the radiation dose employed in this study allowed 26 per cent of the control animals to survive without treatment. Both of these factors make it necessary to employ caution in accepting the above conclusion.

Rogacheva[350] compared the effect of the intravenous administration of 5×10^7 bone marrow cells at 2, 24 and 48 hours and at 3, 6 and 12 days following the irradiation of rats with an LD_{89} (1000 r). Optimal survival (88 per cent) was observed in the 24 hours group. The group treated after two hours showed 63 per cent survivors, the 48 hours group 52 per cent survivors and the 3 days group 27 per cent survivors. The survival of the rats which were treated at 3 or 6 days was not different from that of untreated rats. Although the marrow was termed isologous, the paper contains indications that the

PLATE II: 1(a). Extensive haemorrhages in the skin of a monkey which succumbed 15 days following whole body irradiation with a dose of 800 r

PLATE II: 1(b). Haemorrhages in the skin, the subcutaneous tissues and the wall of the intestines of a rat 12 days after whole body irradiation with a dose of 900 rads
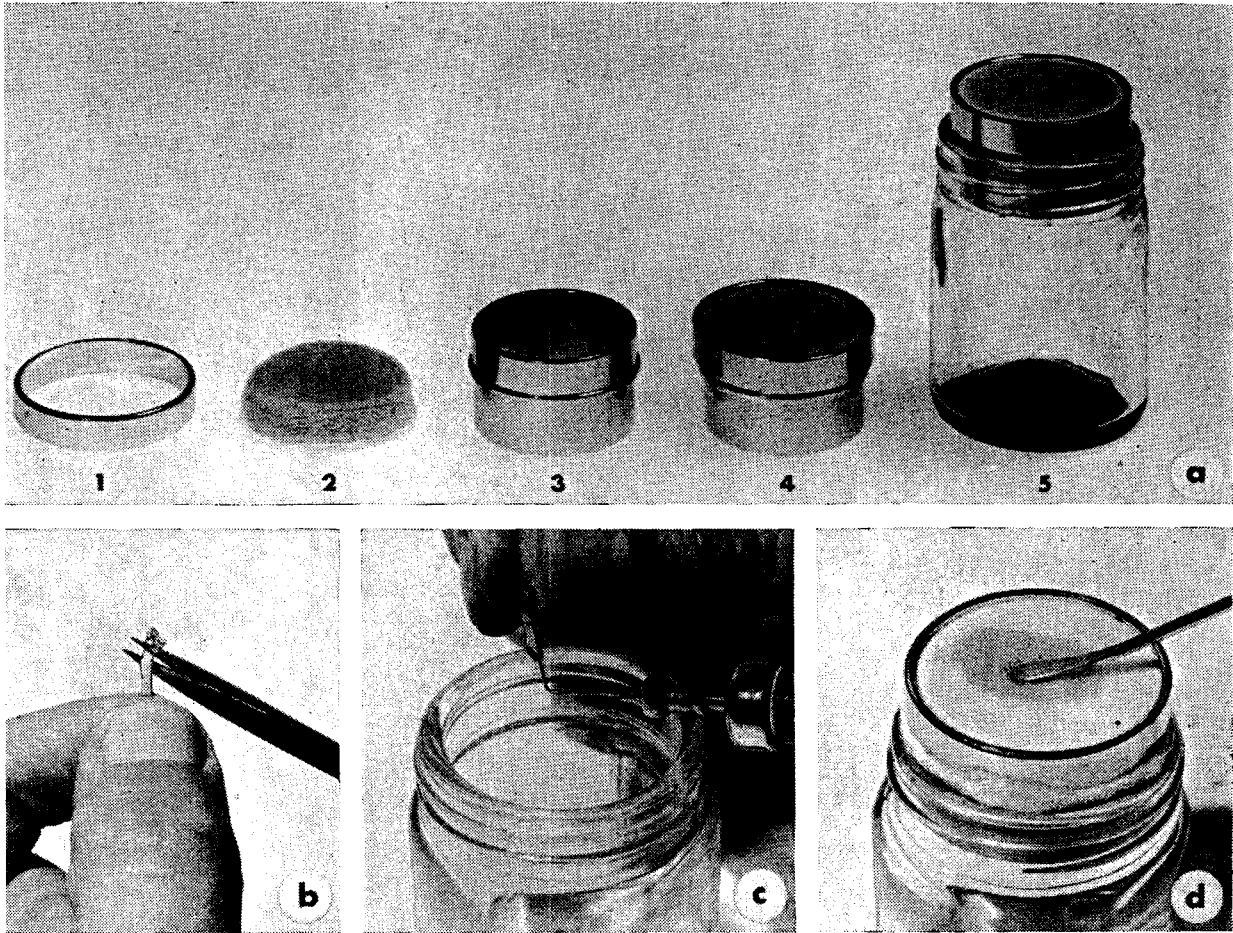
PLATE II: 2. The preparation of bone marrow suspensions

a) Bone marrow sieve: 6 layers of nylon gauze (2) are arranged over a metal cylinder (3). The nylon is stretched and held tightly in place by slipping the metal ring (1) over the cylinder (4); the completed sieve is then placed on a glass bottle (5)

b) The proximal end of the femur is cut with scissors

c) A bent needle no. 12 is moved lightly up and down and side wards inside the shaft to break up the small bone spiculae. Thereafter a small amount of Tyrode's solution (about $0 \cdot 5$ ml.) is forcibly injected when the needle tip is in the distal end of the femur. This expels the bone marrow which is collected in a glass vessel

d) The collected (pooled) bone marrow is suspended by moving it several times in and out of a wide tipped pipette and filtered through the nylon. Clumps are rubbed into the filter with a spatula and flushed through the sieve with medium

The volume of the suspension is measured and the number of cells are counted in Türk's solution (total number). The number of eosin resistant cells is estimated in solution of $2\%_0$ eosin in Tyrode's solution using a haemocytometer. The cells which take up eosin within one or two minutes are considered to be "non-viable". After adjusting the volume to the number of "viable" cells required, the suspension is injected into the tail vein of the recipients, $0 \cdot 5$ ml. in the mouse, $1-2$ ml. in the rat

Table II: 3. Effects of delayed bone marrow transplantation. Summary of results

Authors	Graft (host)	30 day survival (%) Immediate treatment	30 day survival (%) Delayed treatment interval
Jacobson (1954)[187]	Isologous spleen implants (mice)	38	20 (2 days)
Cole et al. (1952)[87]	Isologous spleen homogenate (mice)	90	85 (2 days)
Jacobson et al. (1954)[190]	Isologous b.m. (mice)	—	"some beneficial effect" (5 days)
Congdon et al. (1952)[98]	Isologous b.m. (mice)	68–100	"effective" (3 days)
Schwarz et al. (1957)[364]	Isologous b.m. 10^7 cells (mice)	not done	80 (3 days)
Unsgaard (1961)[422]	Isologous b.m. 2×10^7 cells (mice)	90 { radiation dose LD_{74} }	92 (1 day), 84 (3 days), 60 (5 days), 50 (8 days)
Vos et al. (1961)[437]	Rat b.m. 25×10^6 cells (mice)	74	85 (1 day), 65 (2 days), 50 (3 days), 25 (4 days)

		average survival time (days)	average survival time (days)
Shaw and Vermund (1961)[367]	Homologous b.m. (pigeons)	24	47 (5–7 days)
	Heterologous b.m. (pigeons)	9	27 (5–7 days)

E

strain was nòt strictly inbred. If indeed this were the case their results would be in agreement with those of Vos *et al.*[437] who also found 24 hours to be the optimal interval for heterologous bone marrow transplantation in mice.

It seems possible that the occurrence of the temporary takes of homologous bone marrow as observed by Mathé *et al.*[265] in some of the victims of the Vinca accident was related to the fact that these patients received the bone marrow graft as late as 3–4 weeks following the exposure.

The results obtained by Shaw and Vermund[367] with pigeons are in apparent contrast to the general tendency of the results obtained with mammals.

A delay of 5–7 days compared with immediate transplantation caused an average increase of the survival time of approximately 20 days for both homologous and heterologous bone marrow. The improved survival is attributed to the larger proportion of early reversions which were observed. This must have decreased the incidence and severity of graft versus host reactions, which caused early death when the marrow was transplanted immediately.

The majority of the observations on the maximum possible time lapse compatible with survival in bone marrow transplantations have apparently been made as incidental side-observations in the course of experiments performed for other purposes. The only systematic approach to the problem is the one published by Unsgaard[422]. Obviously, the answers to be expected will depend largely on the number of bone marrow cells injected, in the sense that larger time lapses will be possible with higher cell numbers. After all, the transplanted cells have to multiply to produce a sufficient number of peripheral blood cells and this production has to reach a certain rate before the effects of pancytopenia have led to irreversible lesions of the host's tissues. The 20×10^6 supposedly isologous bone marrow cells employed by Unsgaard represent a tremendous excess and therefore his results are of limited practical value. In fact studies of this kind may be much more interesting when aimed at obtaining an insight into the dynamics of repopulation of the irradiated host by the donor cells. For this purpose variations in both the number of cells and in the length of the interval have to be investigated simultaneously.

Grafting techniques and the nature of the graft

Nearly all the possible procedures of transplantation have been attempted by one investigator or another, a notable and obvious exception being the oral route. Again most of the available information is at best semi-quantitative and limited to casual experiments. Whole spleen transplantation has always been performed into the peritoneal cavity. The great majority of investigators have used cell suspensions which were injected intraperitoneally, intracardially and, almost exclusively in recent years, intravenously. The statement has been made that the intra-muscular and subcutaneous route are ineffective, while some weak therapeutic action was observed following intrathoracic administration[228]. This only means, however, that a certain amount of bone marrow which was highly effective on intraperitoneal or intravenous injection failed to induce an increased survival when the other methods of administration were employed. Rigidly controlled experimental conditions with a sufficiently large number of animals and a range of cell doses are required to assess the efficacy of any transplantation technique.

COLLECTION AND PREPARATION OF CELL SUSPENSIONS

Not only should the cell number be adequately estimated but it is also necessary to control the method of preparation of the suspension by counting the proportion of viable cells. Relatively crude ways of preparing the suspension, e.g. homogenisation of the tissue in a Potter Elvehjem apparatus or a Waring blender, and even the popular technique of forcing the tissues from a syringe through a thin needle, are bound to produce a larger percentage of non-viable cells than the more gentle methods of preparation. It is of interest that the highest incidence of takes in the field of human bone marrow transplantation has been obtained with a minimum of manipulation of the bone marrow cells: the method used by Mathé and collaborators[265] which consists of injecting the aspirated cells from the donor immediately into a vein of the recipient. This method, however, involves the risk of pulmonary emboli of fat, bone marrow and pieces of bone.

The method employed in the present authors' laboratory for bone marrow transplantation in mice and rats is illustrated in Plate II: 2.

Spleen and lymph node suspensions are prepared in a similar way from the minced tissues. It has been found that with lymph node cell suspensions it is usually necessary that the fat droplets be removed in order to prevent fatalities upon injection. The fat can be conveniently

removed by centrifugation. Sometimes this is also necessary when marrow from adult rats is to be administered to irradiated mice.

The acute toxicity of highly concentrated spleen cell suspensions can be effectively reduced by adding heparin in a concentration of 1:400. The toxicity is probably due to clumping but this seems an inadequate explanation for the apparent greater acute toxicity of homologous spleen suspensions.

Monkey bone marrow has been obtained by extrusion of the cells from the pelvic bones and the vertebral column in a tissue press. Considerable numbers of cells (up to 4×10^9 from immature animals) can also be collected from the living anaesthetised donor by puncture of the femur (Plate II: 3). In the latter case contamination with peripheral blood is of course unavoidable.

Human bone marrow has been obtained from living donors under general anaesthesia by multiple aspirations from the sternum, the ileum, the acromion, the ribs and the vertebral spines. The number of punctures may exceed 50 and between 300 and 400 ml. of a blood-bone marrow mixture may be obtained yielding 30–40 million nucleated cells per ml., according to Mathé and Amiel[253]. This amounts to a yield of $9–16 \times 10^9$ nucleated cells per donor and if corrected for the presence of peripheral blood leucocytes $8–14 \times 10^9$ bone marrow cells. The amount obtained by Pegg and Kemp[304] from a series of 50 patients was somewhat lower, $5–10 \times 10^9$ marrow cells as an average, with a maximum yield of 27×10^9 nucleated cells.

Excised bones from either surgical patients or from cadavers have been explored as a source of human bone marrow. It is interesting to note that in one clinic ribs have been surgically removed from volunteers for purposes of bone marrow transplantation. Most of the donors submitted to a two-rib resection. Almost complete regeneration of these ribs took place within approximately one year[410]. Where possible, the marrow cells are scooped from the bones which are then cut into small fragments and shaken with a buffered medium so that the cells are leached out. Another method is to obtain the cells by compression of the bones[345]. Ribs yield on the average $1–2 \cdot 5 \times 10^9$ cells and vertebral bodies between $3 \cdot 5$ and 10×10^9 cells according to various authors. For detailed descriptions of the procedure the reader is referred to the articles by Tocantins[410], Ferrebee et al.[138], Ray et al.[345], Schwartz et al.[363], and Pegg and Kemp[304].

With regard to the use of cadaver marrow it should be noted that information is lacking on the persistence of proliferative capacity after

death. Porteous[322] showed that motility of the leucocytes of the bone marrow may persist for at least 20 hours after death and Perry et al.[305] observed motile cells as long as 50 hours after death. The significance of this function in relation to the proliferative capacity is, however, unknown. Studies with larger animals on this important practical problem have thus far not been published.

ROUTES OF ADMINISTRATION

One systematic comparison has been made between the efficacy of the intravenous, intraperitoneal and intrasplenic route of injection of bone marrow in mice[51]. Graded numbers of isologous bone marrow cells were administered to lethally irradiated mice and the 30-day survival was taken as a criterion of successful proliferation of the grafted cells. The results showed that the intravenous and intrasplenic routes were equally effective, while roughly 70 times as many cells had to be given intraperitoneally to obtain the same percentage of survivals. Using the former methods of administration 10^5 isologous nucleated eosin-resistant cells were sufficient to cause approximately 100 per cent protection. The intramedullary injection of homologous bone marrow was found to be equally effective as intravenous administration, both resulting in about 60 per cent 30-day survivors under the conditions employed by Lebedev[216].

The injection of 5×10^5 cells into the testis or the brain was completely ineffective, although histological examination showed a limited degree of haemopoietic proliferation in some of the testicles. These peculiar transplantation sites were investigated because of the reported lack of reaction to homografts placed in those tissues.

It follows from the above experiments that the intravenous route of administration is the method of choice, especially so in the case of homologous or heterologous transfers. By any other route (except the intrasplenic and possibly also the intramedullary injection) the number of foreign cells needed would make the method completely impracticable.

LOCALIZATION OF INJECTED CELLS

Immediately following the intravenous administration of rat bone marrow cells to irradiated mice Nowell et al.[294] observed considerable numbers of alkaline phosphatase positive cells in the lungs, but after 24 to 48 hours the lungs were free of those cells, which were then to be found in the spleen and the bone marrow. Comparable results have

been obtained with ^{51}Cr labelled bone marrow cells in irradiated rats[165]. The donor cells were labelled *in vitro* and the label was followed for 24 hours in the tissues of the host animals (Fig. II[11]). Fifteen minutes after the injection the highest concentration was found in the lungs but this was a transient phenomenon and at 24 hours by far the greatest amount of relative activity was contained in the spleen and the bone marrow.

Balner *et al.*[17] studied the distribution of ^{3}H thymidine labelled donor cells in irradiated homologous mice. They observed an initial
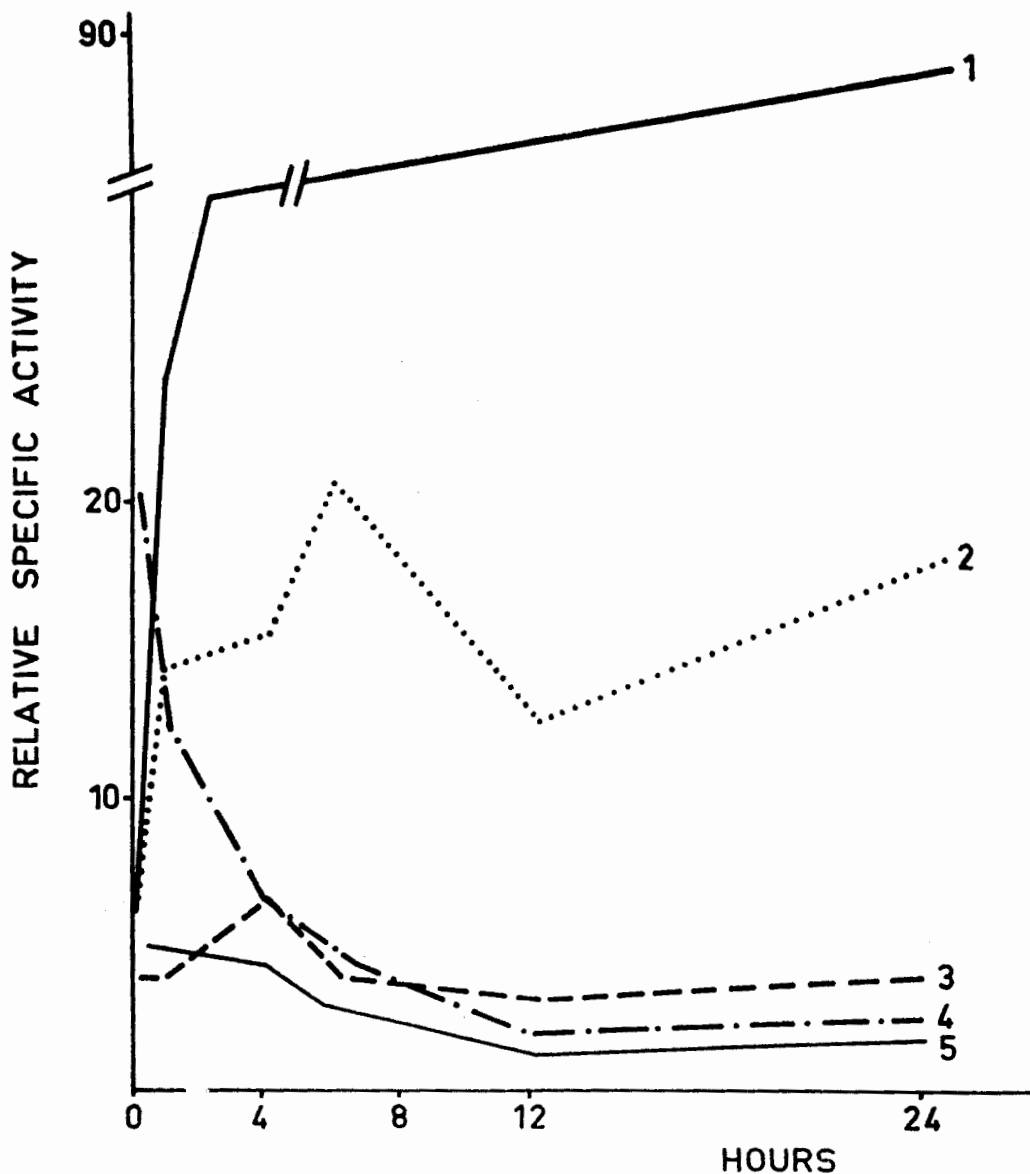
Figure II[11]. Concentration of ^{51}Cr label in various organs after intravenous administration of labelled marrow cells to lethally irradiated rats. Figure derived from Gregušová and Hupka (1961)[165]

1, Spleen; 2, Bone marrow; 3, Lungs; 4, Liver; and 5, Blood

accumulation of labelled cells in the lungs and the liver, which had disappeared after 24 hours. In the spleen and bone marrow, labelled cells were found from 3 hours following injection throughout the observation period of 8 days (Fig. II[12]).
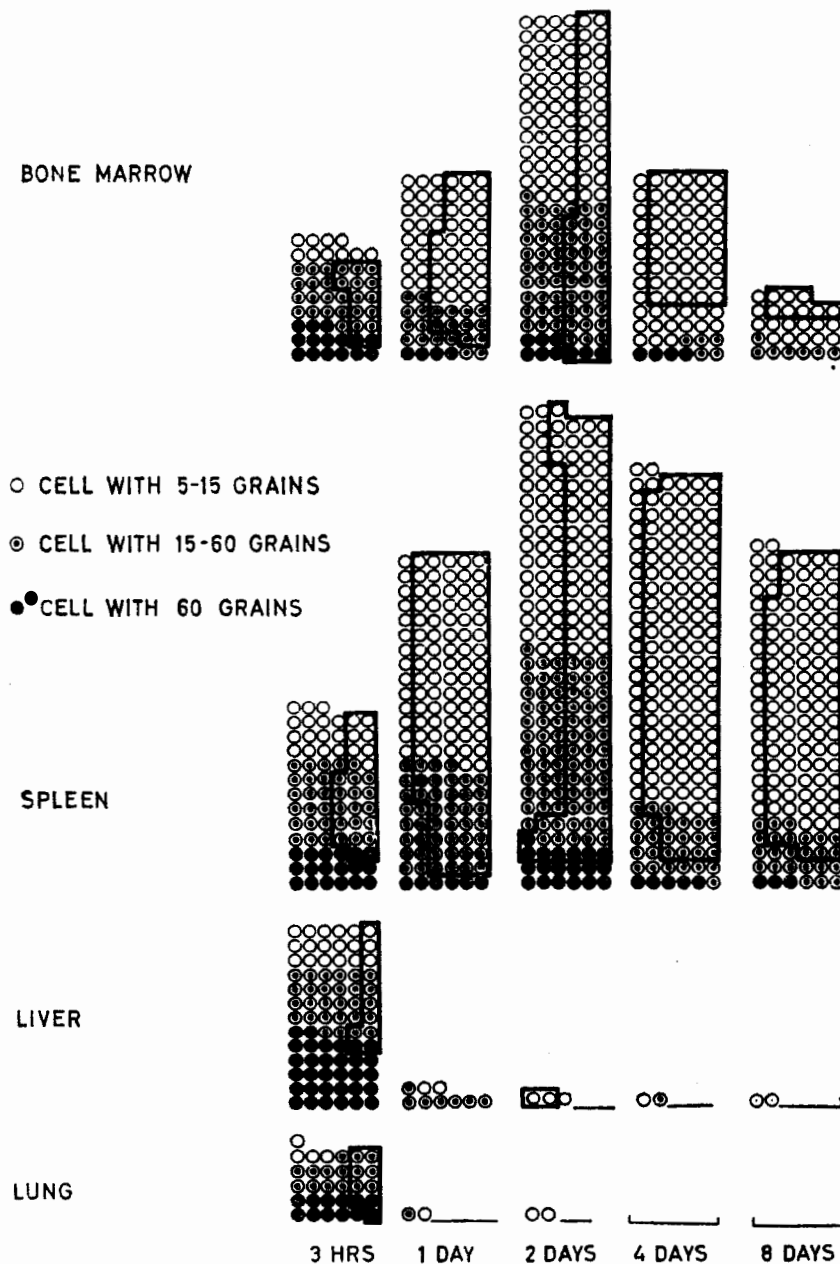
Figure II[12]. Relative numbers of labelled donor cells and intensity of label in organs of irradiated mice at various intervals following bone marrow transplantation. Data from Balner *et al.* (1962)[17]

The bone marrow was derived from mice of the same non-inbred stock as the recipients (Swiss mice) and was labelled *in vivo* with tritiated thymidine. Outlined areas indicate the number of cells found in either pairs or groups

In lethally irradiated rats the distribution of ^{3}H thymidine labelled bone marrow cells has been reported by Fliedner[141]. Approximately 10^8 bone marrow cells, with 50 per cent of the immature myelopoietic cells marked, were injected within an hour after irradiation. At 36 hours the largest number of labelled cells was found in the bone marrow. Up to 1 per cent of the cells in the bone marrow contained the label at that time, a lower number were found in the spleen (about $0 \cdot 1$ per cent) and labelled cells were sporadically observed in the lymph node smears. Some labelled segmented neutrophils were found in the peripheral blood which indicated the proliferation and maturation of the transferred precursor cells. The author concluded that the small number of labelled cells per total number of marrow cells in the recipient suggested that the proliferative potential of the transferred bone marrow cells must not be the most important factor in the recovery of haemopoiesis seen after marrow cell transfusion. This tendency to reintroduce a humoral mechanism seems not to be justified, since it is known from other studies that the number of precursor cells needed, for repopulation may be very small indeed.

Specific information on the repopulation pattern of the lymphatic organs has recently been provided by Ford and Micklem[145], who employed an ingenious method for identifying descendant cells of a bone marrow and a lymphoid graft by means of chromosome markers. The donor cell suspensions were derived from a co-isogenic line which for practical purposes could be considered as isologous with the recipient strain. The irradiated mice received 10^5 bone marrow cells $+10^7$ lymph node or thymus cells. The results provide strong evidence that precursors for thymic regeneration came from the injected bone marrow. Both the bone marrow and the lymphoid inoculum provided cells for the recolonisation of the lymph nodes, but the bone marrow seemed to provide the more permanent population of cells. These observations offer a cytological basis for the rapid loss of memory for immunological reactivity and specific immunological tolerance following transfer of haemopoietic cells into irradiated recipients[42]. This will be described in more detail in Chapter V.

EFFECTIVE CELL TYPE

The identification of the cell type responsible for the repopulation of the host's haemopoietic tissues has received a great deal of attention for two main reasons. One is based on the idea that a relatively small

number of these cells constitute the active component of the bone marrow. In this case a concentration of this cell type would increase the therapeutic activity many times. The other reason is based on interest in the more fundamental aspects of haemopoiesis. To mention only one aspect, the identification of such a common stem cell would be an extremely elegant way of proving the monophyletic hypothesis of blood cell formation.

Direct approaches to this problem—such as attempts to separate the effective cells from the ineffective ones—have so far failed to contribute much, except perhaps negative information. Most investigators agree for instance that lymph node and thymus cell suspensions are ineffectual in repopulating the bone marrow of irradiated animals[7, 49].

It has been claimed by Delorme[121] that thoracic duct cells can promote recovery of irradiated rats but the author did *not* satisfactorily exclude the possibility that this effect was due to the presence of non-lymphoid cells in the lymph, nor was it proved that recovery was accompanied by a proliferation of haemopoietic cells of donor genotype. Recently, the restorative capacity of lymphocytes has been extensively reinvestigated by Gesner and Gowans[156]. These workers succeeded in obtaining large numbers of lymphocytes from the thoracic duct of mice and found no evidence whatsoever of a therapeutic effect when these cells were administered to irradiated isologous recipients. This represents by far the most convincing study of its kind.

Spleen cells are much less effective in protecting lethally irradiated animals than bone marrow cells. In the case of isologous transplantation, roughly 20 times as many of them are needed as compared to bone marrow cells[49]. If all this serves to exclude the lymphoid cell as the effective cell type in the restoration of irradiated animals, it could be asked whether the effective cells reside predominantly in the erythropoietic or in the myelopoietic series. This problem has been approached in a similarly indirect way by Vos[435] and by Cole et al.[86], who selectively stimulated the erythropoiesis or the myelopoiesis of donor animals prior to the transplantation. The bone marrow and spleen suspensions from these mice were compared on the basis of effective cell numbers with suspensions obtained from normal donors. Phenylhydrazine pretreatment served to stimulate erythropoietic activity and myelopoietic stimulation was obtained by transplanting a mammary carcinoma to the donor mice. The results reported by the

two groups of investigators were similar, in that any deviation from normality of the cell population injected caused a decrease of the restorative capacity.

In the course of repeated transfers of isologous bone marrow and spleen cells in irradiated recipients van Bekkum and Weyzen[53] observed the appearance of an increasing proportion of immature cells—predominantly of the myelopoietic series—upon successive transfers. The de-differentiation was accompanied by a decrease of the restorative capacity of the cells which eventually led to loss of the transfer lines. These results suggest that the ability of haemopoietic cells to restore lethally irradiated mice does not depend so much on the most primitive cell types, but that the more mature types are also required to prevent the development of a fatal leucopenia and thrombocytopenia. There can be no doubt that differentiation needs time and it seems highly questionable whether a graft consisting exclusively of "stem" cells—if it were available—would be able to produce a sufficient number of mature leucocytes and thrombocytes within the 10 days or so before mortality from infection and haemorrhage occurs.

Another interpretation is that all the induced changes in the relative composition of the bone marrow described above were accompanied by a decrease in the number of stem cells and that these stem cells alone determine the rate of regeneration of the bone marrow and survival of the animals.

Leukemoid blood from tumour bearing mice has been shown to promote the recovery of isologous lethally irradiated mice[97], but failed to protect adequately in several homologous combinations. Excessive numbers of nucleated cells of the order of 10^8 were required to obtain 100% protection and the data published by Smith and Congdon[379] put the calculated minimal number of cells needed to show some effect in homologous mice at approximately 400×10^6. However, in the homologous experiments, the authors injected only 132×10^6 cells, which failed to influence survival.

In a subsequent publication Merwin[274] reported the survival of lethally irradiated (BALB/cxA)F_1 mice after treatment with 25–100 $\times 10^6$ nucleated cells from the leukemoid blood of (BALB/c $\times$ C3H)F_1 donors. In a limited number of the survivors evidence for the presence of donor cells in the bone marrow and lymph nodes was obtained by a test method based on the detection of tissue antigens.

Compared with bone marrow cell suspensions the leukemoid blood cells are less effective by a factor of 100–1000. According to

Congdon et al.[97] nucleated cells from the leukemoid blood consist of 75–90% granulocytes and 9–25% lymphocytes. Immature forms were classified as metamyelocytes, myelocytes and monocytes which together constitutes between 0·3 and 0·9 per cent of the total number of nucleated cells. If it is assumed that these immature forms include all the types which are necessary for repopulation, 5×10^5 of these cells afforded complete protection. This has to be compared with the value of 10^5 found by van Bekkum and Vos[49] for isologous bone marrow cells which also includes, of course, a certain percentage of mature cells.

So far, attempts to separate the effective cell type from marrow suspensions by way of centrifugation have failed.[159] This may also be taken as evidence contrary to the idea that one type of stem cell is needed for the protection of irradiated animals.

FOETAL CELLS

Several investigations have been carried out with haemopoietic cells derived from embryos. The data obtained are summarised in Table II: 4. The emphasis of most of this work has been on the avoidance of secondary disease; the incidence of immunological complications (see Chapter III) has been definitely less than that observed after the transplantation of adult foreign bone marrow of the same genotype. In certain host-donor combinations, e.g. the experiments reported by Uphoff[425] who used parent strain mice as donor and the related F_1 hybrids as recipients, secondary mortality is virtually absent when foetal liver suspensions are transplanted, while adult marrow causes a high percentage of late mortality. In other combinations—homologous as well as heterologous[107]—the severity of secondary disease is merely diminished but not completely abolished. This, the main and so far the only obvious advantage gained by the use of embryonic tissues, is ascribed to the immature condition of the immunological system in the very young individual.

Crouch[107] has drawn attention to one major disadvantage of foetal liver suspensions. On the basis of a large number of quantitative experiments he found foetal cells to be considerably less effective in restoring the irradiated recipients than adult bone marrow cells. In isologous combinations 8–16 times as many foetal liver cells were required to obtain optimal protection, while in the homologous combinations tested, this factor was between 2 and 10. With 10^8 rat foetal liver cells 80% survival was obtained, which is 10 times the number

Authors	Recipients	Donor cells	Survival (%)	
Jacobsen *et al.* (1954)[190]	CF$_1$ mice	Isologous foetal liver suspensions;		
		16–20 days	*Day 30*	
		1–8 $\times$ 10^6 cells	50–75	
		0·1–1 $\times$ 10^6 cells .	25	
		5 $\times$ 10^4 cells	5	
Duplan (1956)[134]	Strain XVII mice	Isologous foetal liver (12–19 days)	*Day 30*	
		1–16 $\times$ 10^6 cells	28 (at most)	
Wolf and Duplan (1960)[464]	Strain XVII mice	Foetal liver and spleen	*Day 30*	
		(15 days) 5 $\times$ 10^6 cells	71	
		(18 days) 5 $\times$ 10^6 cells	68	
Congdon and Urso (1957)[99]	LAF$_1$ mice	Homologous "foetal blood-forming tissues"	No specific data: "may cause less delayed reactions than adult homologous tissues"	
Uphoff (1958)[425]	(C57BL $\times$ DBA/2)F$_1$ mice	DBA/2 and C57BL liver (14–16 days)	*Day 30*	*Day 90*
		3·7–7·2 $\times$ 10^6 cells (19–20 days)	100	100
		16 $\times$ 10^6 cells	100	70
Barnes *et al.* (1958)[26]	CBA mice	C57BL embryo spleen and liver	Decreased secondary mortality	
Lengerova (1959)[217]	Non-inbred H mice	Homologous foetal liver	*Day 30*	*Day 90*
		2 $\times$ 10^7 cells	65	65
		Rat foetal liver	No 10 day survivors	
		(18–19 days) 2 $\times$ 10^7 cells		
Porter (1959)[324]	Rabbits	20 days foetal liver	*Day 30*	*Day 90*
		350 $\times$ 10^6 cells	45	45
		670 $\times$ 10^6 cells	55	55
		27 days foetal liver	Not as effective	

	(C57L × A)F₁ mice	(101 × C3H)F₁ foetal liver (12–19 days) 38–60 × 10⁶ cells	95	90
		(12–19 days) 50–65 × 10⁶ cells	94	70
	(101 × C3H)F₁ mice	Rat foetal liver (12–21 days) 80–180 × 10⁶ cells	42	12
		Rat foetal spleen (12–21 days) 8–50 × 10⁶ cells	20	0
Crouch (1960)[107]	CBA and C57BL mice	Isologous foetal liver (14–16 days) 8–16 × 10⁵ cells	*Day 30*	*Day 60*
	CBA mice	C57BL foetal liver (14–16 days) 50–135 × 10⁶ cells	95	—
	C57BL mice	CBA foetal liver (14–16 days) 12–50 × 10⁶ cells	80–100	20
	(CBA × C57BL)F₁ mice	Rat foetal liver (14–16 days) 50–100 × 10⁶ cells	60–100	50–100
Tschetter et al. (1961)[420]	Webster strain mice (not inbred)	Homologous foetal liver 20 × 10⁶ cells	75–80 *Day 30*	20–56 *Day 90*
		12–14 days	66	50
		15–19 days	63	40
		Newborn–5 days	54	33
Thomas et al. (1963)[402]	Dogs	Liver, spleen and marrow from foetuses at or near term	Incidence of graft rejections, "takes" and secondary disease similar to that following treatment with adult marrow	

of adult bone marrow cells required for the same percentage of sur-
vival. Even if the values are corrected for the presence of about 50 per
cent presumedly inactive hepatocytes in the suspensions, it remains
likely that foetal haemopoietic cells are therapeutically less potent
than adult marrow cells by a factor of at least 2. This will not be a
great disadvantage when isologous cells are employed, but it may be a
serious one in the case of homologous transplantations, especially so
in man, where the available number of cells from a single embryonic
donor is indeed limited. It seems doubtful even, whether a sufficient
number of cells can be obtained from a suitably young human foetus
to permit the effective repopulation of an adult recipient. According
to van Putten[336] Rhesus monkey foetuses of an estimated age of 100
days (gestation period is $5\frac{1}{2}$ months) yield at best only slightly more
than the minimum number of adult bone marrow cells (8×10^8 for a
3 kg recipient) required to effect a take in a homologous recipient.
Consequently, foetal transplants of this size showed no restorative
effect in lethally irradiated monkeys.

Another possible disadvantage of foetal liver cells in the restora-
tion of haemopoiesis after lethal irradiation has been indicated by
Barnes et al.[35]. Using an isologous combination (CBA mice) they
observed a considerable incidence of late disease and mortality which
was attributed to an inadequate regeneration of the lymphatic tissues.
The addition of 5×10^6 isologous adult lymph node cells to the foetal
liver inoculum ($3-12 \times 10^6$ cells) prevented the development of this
secondary disease. It was suggested that foetal liver cell suspensions
may be deficient in lymphoid cell precursors and that secondary dis-
ease can be the result of an inadequate regeneration of the lymphoid
system. Most authors view this deficiency as the probable reason for
the apparent ease with which the foetal transplant becomes immuno-
logically tolerant towards its host. The results of Barnes et al. present
us with an apparently paradoxical situation with respect to the
applicability of foetal haemopoietic cells. On the one hand foetal
tissue is to be recommended because of the relative absence of im-
munologically active cells which would prevent the development of a
graft versus host reaction and, therefore, of secondary disease. On
the other hand the very absence of these cells would seem also to
promote the appearance of a seemingly identical complication. Con-
firmation of these results in other animal species, in particular in
primates, is obviously essential. The reader is referred to Chapter III
for an exhaustive discussion of this problem.

In conclusion, the results obtained with isologous foetal material again underline the fact that normal bone marrow seems to possess the optimal composition with respect to the therapeutically effective cell types and that the precursors of all haemopoietic cell series may be required for the optimal protection of irradiated animals. This seems compatible with the polyphyletic theory of haemopoiesis which postulates that after the very early embryonic stage each type of blood cell has its own particular "stem" cell, from which a rapid production and differentiation of derivative cells can occur. It is conceivable that foetal liver contains relatively less of these *specific* precursors than adult bone marrow. If on the other hand a multipotent mesenchymal stem cell is the sole determinator of repopulation, one is forced to conclude that foetal liver is a less abundant source of these cells than adult bone marrow.

CULTURE OF HAEMOPOIETIC CELLS

The *in vitro* culture of haemopoietic cells to provide the material for transplantation in irradiated recipients offers a whole range of possibilities for the applied as well as the more fundamental areas of our field of investigation. Theoretically, a successful method would yield unlimited numbers of cells and open the way to the *in vitro* production of haemopoietic cells of the antigenic composition most suitable for any particular recipient. Furthermore, it might become possible to investigate the exact stage of development of the various cell types most effective therapeutically. Finally, the large scale *in vitro* multiplication of the immunologically active components of the cell population could lead to the development of methods for the *in vitro* induction of tolerance towards the future host, for instance by the addition of appropriate antigens to the culture medium.

Such utopian ideas must have been in the minds of many investigators concerned with bone marrow transplantation. Unfortunately, only a few actual attempts at *in vitro* cultivation of haemopoietic cells have been described so far, and because of the difficulties involved these have contributed little to solve the problems at hand.

The most thorough series of investigations so far have been reported by Billen. In 1957 he succeeded in maintaining therapeutically active cells *in vitro* for 4 days[59] and in another paper this period was reported to be 24 days[58]. However, under the conditions of these experiments cell proliferation was not obtained in the cultures so that the procedure merely proved that a proportion of the cells survived.

Miller[278] (1956) has reported the maintenance of the therapeutic activity of embryonic liver cells in culture for 4 days, but his studies have apparently not been continued nor confirmed by others.

Billen subsequently investigated a well type culture method for bone marrow[60]. By the 4th day of cultivation the restorative capacity of the cells had decreased and by the 9th day the explanted cells had lost this capacity completely, although several stem cell types were found to persist in these inactive cultures.

By the use of tantalum wire as an overlay for the explants and foetal calf serum additions to the culture medium, Billen and Debrunner[61] succeeded in obtaining the continuous proliferation of several cell lines derived from mouse bone marrow. All the tests for restorative capacity of these cells in lethally irradiated mice, using from $0 \cdot 2 - 3 \cdot 2 \times 10^6$ cells per mouse, were negative.

The cultivation of mouse bone marrow in Algire-diffusion chambers has been the subject of investigations by Berman and Kaplan[56, 57]. These authors were able to cultivate bone marrow cells for periods up to 215 days in intraperitoneally implanted diffusion chambers. A significant change in the relative distribution of the various cell types occurred, however, between 20 and 30 days, resulting in a predominance of differentiated myeloid elements and histiocyte-like cells[56]. The capacity of the cells grown in diffusion chambers to restore lethally irradiated mice was investigated in the isologous combination. With $2 \cdot 3 \times 10^6$ cells cultured for 21 days, 30% survival was obtained compared with 100% survival with 3×10^6 fresh bone marrow cells. After the third week the protective ability of the cultivated cells decreased rapidly[57]. No further studies along these lines have appeared in the literature.

Methods of preservation

The need for adequate methods of storage for haemopoietic cells arose as soon as the clinical application of bone marrow transplantation was contemplated. The availability of autologous marrow permits the administration of considerably larger doses of radiation or cytotoxic drugs in selected cases, but this approach usually requires storage of a sufficient amount of the patient's own marrow collected before the start of the therapy. In the case of homologous donors, storage without the loss of the capacity to proliferate would permit the collection of bone marrow from such sources as cadavers and ribs etc. removed at operation, and it might also facilitate the establishment of bone

PLATE II: 3.   Bone marrow puncture in an anaesthetised monkey. The needle is driven through the knee-joint and the distal end of the femur into the narrow cavity with a small hammer. As soon as the point enters the cavity the mandrin is removed and marrow is aspirated with a syringe. The needle is gradually moved further into the cavity after each sampling

marrow banks. As early as 1955, Barnes and Loutit[30] were able to store mouse spleen cells at −70° C for periods from 2 to 83 days with the preservation of their therapeutic activity for isologous irradiated hosts. The method they used was the one devised by Smith[378] for the preservation of living cells with the aid of glycerol. Their results were correctly interpreted as supporting the concept of a transplantation mechanism to explain the therapeutic effect of the haemopoietic cell suspension.

Several investigators have extended and perfected this technique of freezing and storage and several modifications are in current use for the preservation of human haemopoietic tissue.

The efficacy of preservation methods can only be evaluated by testing the capacity of the stored cells to restore lethally irradiated recipients and this is obviously only feasible with animal material.

A number of so-called viability tests have been introduced to evaluate the condition of frozen human cells, among them the incorporation of labelled phosphate and tritiated thymidine into DNA, the exclusion of dyes like eosin and trypan-blue and the mitotic index of the cellular preparations following stimulation (the Stathmokinetic Index)[10, 178]. For none of these tests has it been proved convincingly that the results directly reflect the capacity of the cells for unlimited proliferation.

This situation is particularly unsatisfactory because in many instances of attempted bone marrow transplantation in man, the number of cells that can be obtained from a single donor barely approaches the number theoretically needed for a successful take.

A loss for instance of 50 per cent as a result of storage may well make the difference between survival and death of the patient. As for the studies with animal bone marrow, very few experiments have included the estimation of sufficiently detailed cell dose versus survival curves *in vivo* to permit an accurate evaluation of cell survival (Table II: 5). At best, a cell dose was selected which produced suboptimal protection with fresh material so that any decrease of viability after freezing and storage would be detectable. However, such a one point estimate is usually not very accurate because of the variability of the results obtained with fresh marrow. These data indicate that a reasonable degree of preservation can be obtained with the standard method of slow freezing (1° per minute) to −20° C followed by rapid cooling to −70° C or −79° C in 15% glycerol for spleen[30] and marrow[364, 307] of mice, when the *in vivo* tests were performed with

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TABLE II: 5. Preservation of haemopoietic cells—animal experiments

Authors	Material	Recipients	Technique	Evaluation Survival
Barnes and Loutit (1955)[30]	Spleen	Isologous mice	Glycerol —70° C 2–83 days	*In vivo* test (no cell dose titrations)
Schwartz *et al.* (1957)[364]	Marrow	Isologous mice	Glycerol —70° C 7–8 days	*In vivo* test (no cell dose titrations)
Porter and Murray (1958)[332]	Marrow	Homologous rabbit	Glycerol —70° C 7 days	*In vivo* test 1200 × 10⁶ cells/ rabbit Similar results as with fresh marrow
Phan and Bender (1960)[307,308,309]	Marrow	Isologous mice	Comparison of various pentoses, inorganic compounds and poly-alcohols with glycerol —70° C, 1 hour	*In vivo* test (no cell dose titrations)
Mannick *et al.* (1960)[249]	Marrow	Autologous dogs	Hanks solution or glycerol —79° C, 3–130 hours	Re-infusion 1–4·5 × 10⁹ cells 14/17 dogs survived
Lengerova and Abraham (1960)[218]	Foetal liver Adult spleen	Randomly bred mice	Glycerol —70° C, up to 6 months	*In vivo* test: Simonsen assay (not quantitative)
Githens *et al.* (1961)[157]	Foetal liver	Randomly bred mice	Various methods —50° and —80° C, 2 weeks	*In vivo* test (no cell dose titrations)

Reference	Tissue	Treatment and storage	Assay / Result
Ashwood-Smith (1961)[9]	Marrow	Dimethyl sulfoxide −79° C, 1 month	In vivo test: 5 × 10⁶ cells (no cell dose titrations)
Thomas and Ferrebee (1962)[403]	Marrow	Glycerol −79° C, 14 months	Re-infusion (no cell dose titrations)
Persidsky and Richards (1964)[306]	Marrow	Polyvinyl-pyrrolidone (PVP) 10%, −79° C / glycerol 15%, −79° C	In vivo test (two point assay) / 80% preservation / 90% preservation
van Putten (1964)[338]	Marrow (Parent strain)	Glycerol 15%, −196° C 1 week–2 months / 10% PVP	In vivo test (5-point assay) / 72% preservation / 70% preservation
van Putten (1965)[339]	Marrow	10% PVP and 10% PVP + 10% glycerol	90–100% preservation
	Foetal liver	10% PVP + 10% glycerol	40% preservation
Lewis and Trobaugh (1964)[221]	Marrow	Glycerol 15% −196° C, 1 week	In vivo tests 74–90% preservation (5-point recovery assay and 6-point spleen colony assay)

isologous recipients. Comparable results were obtained with auto-
logous dog marrow[249, 403], homologous rabbit marrow[332] and foetal
liver from non-inbred mice[157, 218] (Table II: 5). Phan *et al.*[311]
compared the storage of bone marrow cells frozen in glycerol at
different temperatures: $-30°$ C, $-79°$ C and $-190°$ C. They found
remarkable differences and by far the best preservation was obtained
at the lowest temperature (Fig. II[13]).
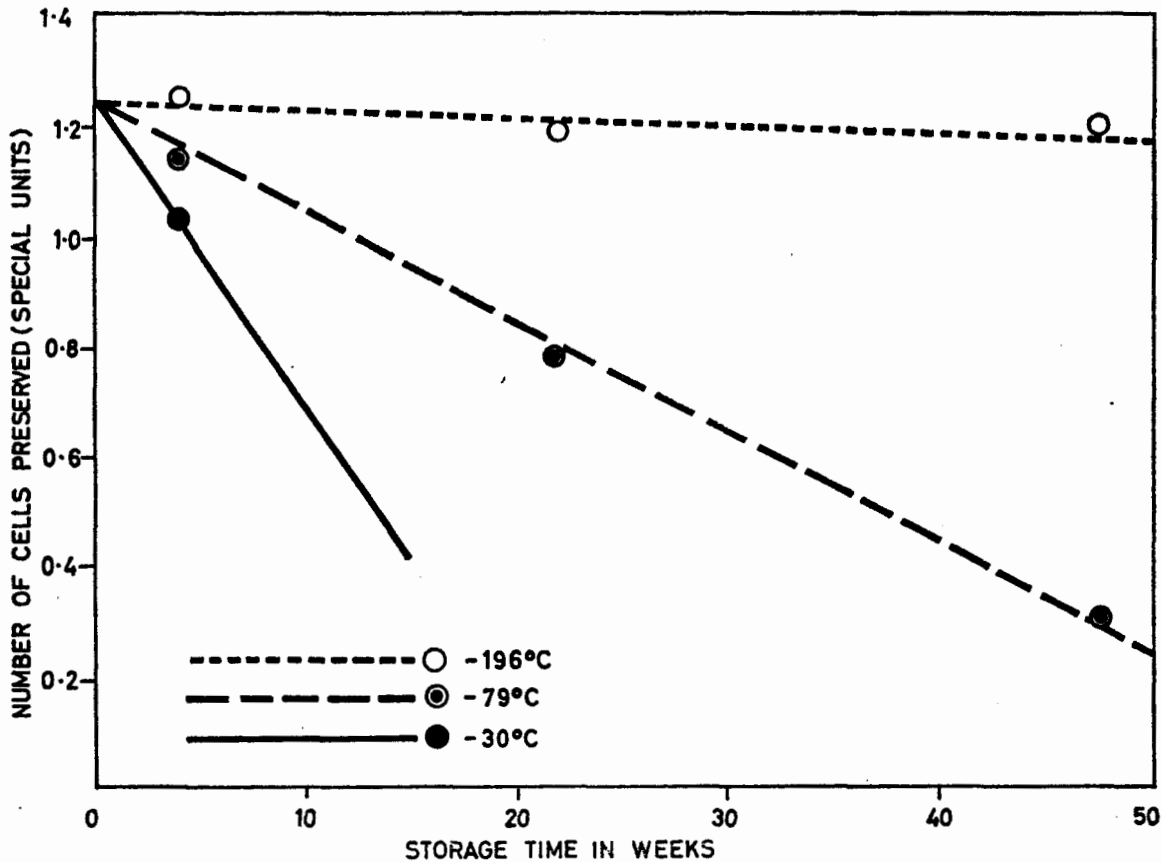
Figure II[13].   Degree of preservation of mouse bone marrow cells
upon storage at three different temperatures. Figure from Phan
et al. (1963)[311]. Even after a period of nearly 5 years, the cells stored
at $-196°$ C did not show a significant loss of protective capacity
(L. Smith, personal communication, 1964)

Dimethyl sulphoxide was stated to give superior results compared
to glycerol by Ashwood Smith[9] but this was not proved in the mouse
survival test. In these experiments isologous mouse marrow was
investigated.

Phan and Bender have tested the ability of various concentrations
of a number of compounds to protect mouse bone marrow cells from
the effects of freezing and thawing. These included polyalcohols,
mono- and disaccharides, amino acids and inorganic salts[308]. Among

the polyalcohols, iso-erythritol, D-ribitol, D-mannitol, D-sorbitol and i-inositol were effective. Among the sugar derivatives of polyalcohols tested, D-ribose was found to give very good protection.

Of the 15 amino acids that were investigated, all but L-cysteine, L-asparagine, L-lysine and L-arginine showed some protective ability but none were effective enough to warrant their application in the preservation of human bone marrow[310].

These authors also tested several inorganic salts and found some protection with sodium iodine, sodium bromide, sodium nitrate, sodium sulphate, sodium thiocyanate and sodium thiosulphate. Even the most effective among these were inferior, however, to the more effective representatives of the organic compounds[309]. Most of the results published by Phan *et al.* seem to require confirmation, since these authors added 3·5% polyvinylpyrrolidone (PVP) to their test media. They found PVP itself to be non-protective[54] (concentration 7%) but recent reports (Persidsky and Richards[306], van Putten[339]) show that PVP is an effective protective substance if added in a 10% concentration.

The Simonsen assay method, using splenomegaly as an indicator, was employed by Lengerova and Abraham[218] to demonstrate the preservation of immunological reactivity in spleen cells frozen in glycerol and preserved at $-70°$ C.

Recently van Putten has reported the results of a 5 point cell dose assay in lethally irradiated recipients, in which the recovery of the protective capacity of mouse bone marrow cells frozen in glycerol was found to be on the average 60 per cent[338]. After thawing, the suspensions were slowly diluted with Tyrode's solution according to a method described by Drašil[129]. Van Putten also studied a number of other freezing techniques as well as the influence of erythrocyte admixtures on the preservation of bone marrow cell viability. Freezing in dimethyl sulphoxide (DMS) proved to be slightly inferior to glycerol when Drašil's method of dilution was used after thawing. When the efficacy of undiluted suspensions was compared, DMS yielded much better results than glycerol (34% against 14% preservation of protective capacity). With mixtures of glycerol and PVP or DMS and PVP as well as with PVP alone, optimal storage efficiencies ranging from 70 to 100 per cent were obtained[339].

Very similar results were reported by Lewis and Trobaugh[221] who found 90% preservation of bone marrow cells frozen in 15% glycerol as determined in a 5 point mouse protection assay and 74%

in a 6 point spleen colony test. DMS appeared to be less efficient than glycerol.

Several of the storage techniques that were found to be most effective for mouse bone marrow were tested for their usefulness in the preservation of monkey bone marrow [339]. After freezing and storage the cells were reinfused into the original donors after these had been subjected to a standard lethal dose of whole body irradiation. By using graded numbers of cells in series of animals and comparison with the minimal number of fresh autologous bone marrow cells needed for protection, the storage efficiency could be evaluated. These experiments revealed marked differences between the species with respect to the efficacy of bone marrow storage methods. Some of the best methods for mouse bone marrow were found to result in poor cell survival when applied to monkey bone marrow. The best results were obtained with a mixture of PVP and glycerol but the storage efficiency did not exceed 50 per cent. Methods employing glycerol or DMS which have been widely used for the storage of human bone marrow were found to be completely ineffective with monkey bone marrow.

The methods that are currently in use for the preservation of human bone marrow and foetal cells are summarised in Table II: 6. Each group of investigators has administered the stored cells to human patients and found indirect evidence in favour of at least a temporary proliferation of the infused cells in some of the patients. However, in general the therapeutic effect of reinfusion of preserved autologous bone marrow has been disappointing, which has led several groups to abandon this form of treatment. These failures can now be attributed to the use of inadequate freezing media, in view of the data provided by van Putten for monkey bone marrow. The mixtures employed in the freezing of human bone marrow cells were all shown to cause excessive losses of viable cells when tested with monkey bone marrow. As pointed out earlier, it has not been possible to evaluate, in a really dependable way, the number of viable cells administered. In view of the differences for each species described above it cannot be predicted, of course, whether extrapolation to man of the results obtained with bone marrow storage in experimental animals will be of any value. However, quantitative *in vivo* testing of storage methods in large animals and particularly in primates seems to be the best approach to the solution of this practical problem. At present it seems logical to recommend the use of the 10% PVP + 10%

TABLE II: 6. Preservation methods in use for human haemopoietic cells

	Humble (1962)[70]	Kay (1962)[70]	Kurnick (1962)[70,211]	Loeb (1962)[70]	Lochte et al. (1959)[223] Ferrebee et al. (1959)[138]	Ferrebee et al. (1959)[139]
Cell type	Marrow	Foetal liver and spleen	Marrow	Marrow	Marrow	Foetal liver and spleen
Diluent	TC 199	TC 199	TC 199 or Osgood's solution	Hanks solution + 5% AB serum		Hanks solution + albumin 5% or Hanks solution + autologous serum
Heparin U/ml	10	10–20	2–5	None: 0·002% EDTA		—
Additive for freezing	15% G*	12·5% DMS	15% G	15% G		15% G
Rate of freezing at 1°/min down to	−15°	−15°	−40°	†		−15°
at higher speed to	2–3°/min–63°	5–10°/min −60°	Immediately in refrigerator			2°/min to −70°
Storage temperature	−79°	−79°	−80° to −95°	−79° or −190°		−70°
Thawing rapidly at 37°	+	+	−	+		+
slowly at 0–2°	−	−	+	−		−
Dilution with	—	—	½ volume 35% glucose	½ volume 50% glucose + (10 min. later) 2 volumes saline		½ volume 35% glucose + 2 volumes saline + 2 volumes saline

* G=glycerol   † Polge, Smith and Parkes technique (1949)[315]

glycerol mixture for human bone marrow. These recent developments also seem to justify a resumption of clinical trials with the transplantation of preserved autologous bone marrow.

As was first described by Urso and Congdon[429], the storage of untreated suspensions for short periods of time in the refrigerator and even at room temperature is possible with a surprisingly good preservation of the protective capacity. The dosage of cells was expressed as femur equivalents. If it is assumed that one femur yields about 10^7 cells and that optimal protection under the experimental conditions of these workers could be obtained with 10^6 cells (isologous combination) the data indicate more than a 90% loss in 3 days upon storage in the refrigerator and in 2 days at room temperature.

Refrigerator storage of haemopoietic cell suspensions has also been studied quantitatively in relation to the possibility of a selective killing of lymphoid cells[42]. Among other things, it was noted that the concentration of the suspension is a determinant factor in cell survival. For practical purposes it shoud be noted that bone marrow can be kept for a few hours at room temperature and for at least a day at 4° C without an appreciable loss of the protective efficacy.

The effects of storage on the capacity of homologous cell suspensions to initiate a graft versus host reaction will be discussed fully in the following chapter.
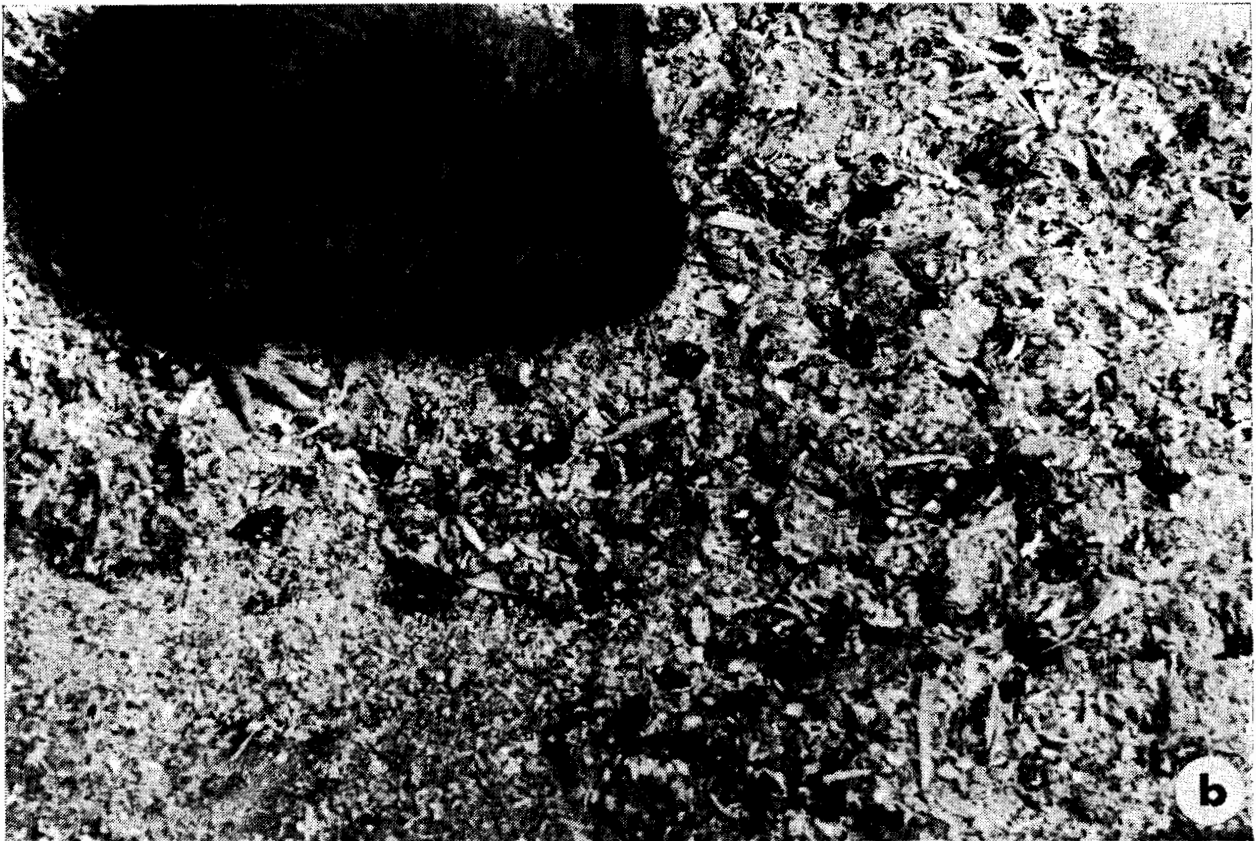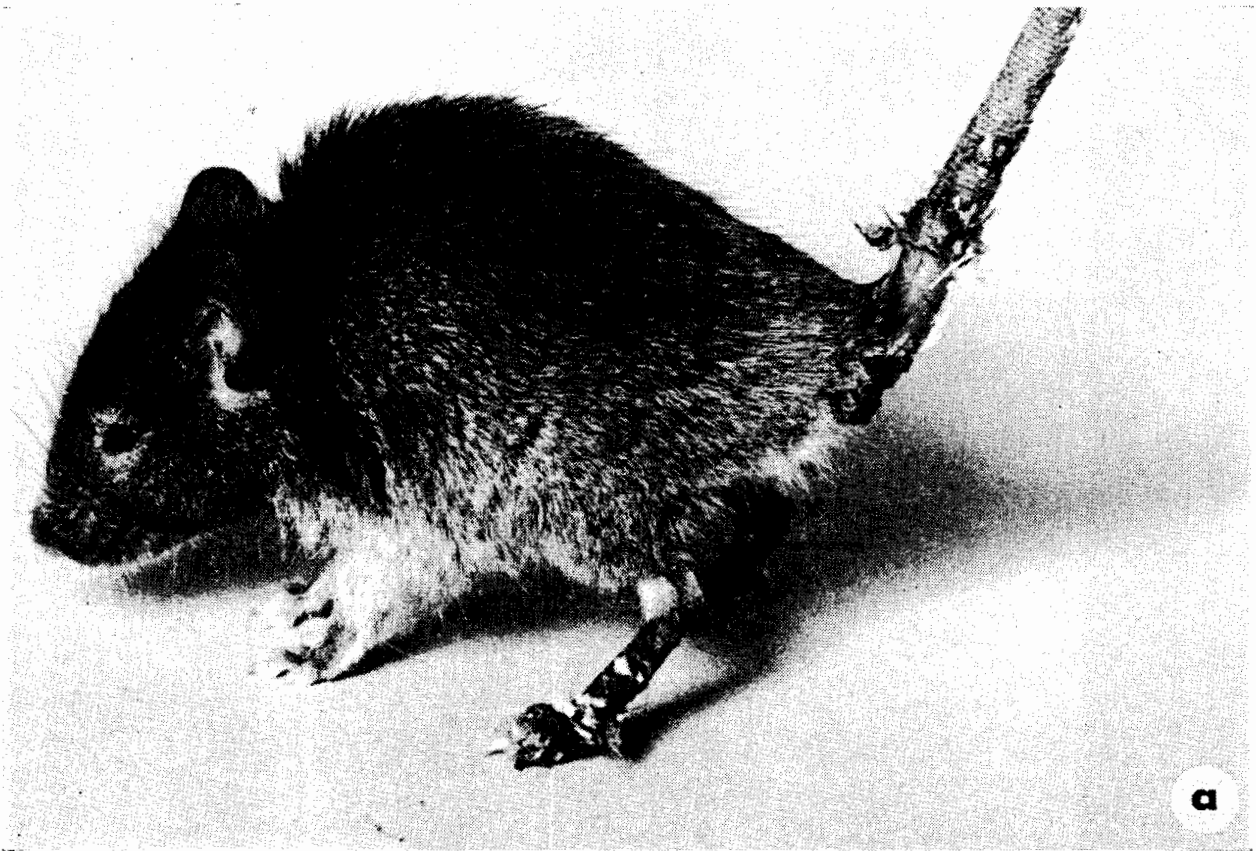
PLATE III: 1. "Diarrhoea" of secondary disease

(a) Sticky faeces adhering to the base of the tail as well as to the hind legs, causing severe desquamation of the skin in the anal region
(b) Characteristic appearance of the bedding of a cage with mice suffering from "diarrhoea". The bedding sticks to the wet faecal pellets
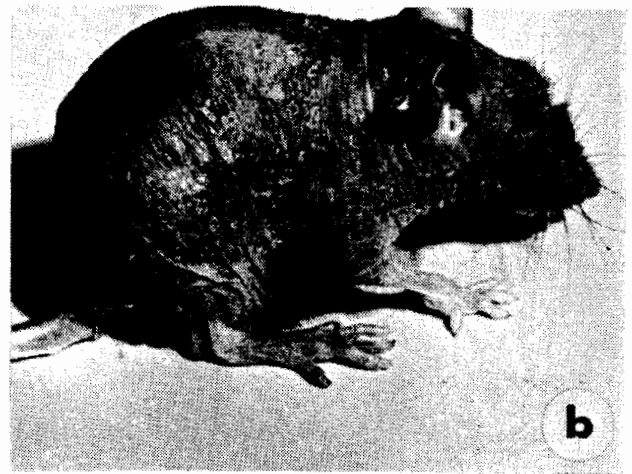
PLATE III: 2.　Various forms of skin lesions in mice suffering from secondary disease following foreign bone marrow transplantation

(a) Albino mouse with erythema showing at the snout, ears, feet and around the eyes. (b) Extreme alopecia, some scaling and characteristic folding due to thickening of the skin.　(c) and (d) Alopecia and crust formation. Note the partial loss of the ears as a result of desquamation and ulceration.　(e) and (f) Patchy alopecia, crusting and desquamation. Note the lesions of the skin of the hind feet.
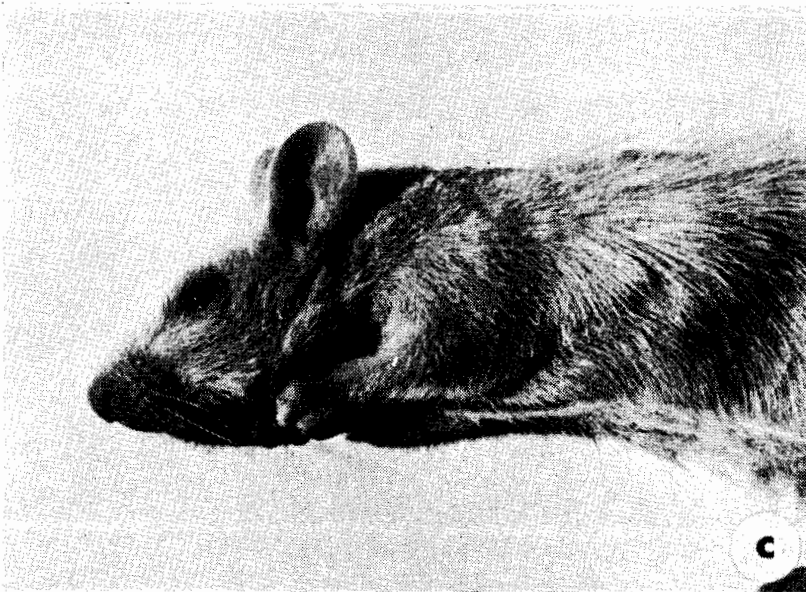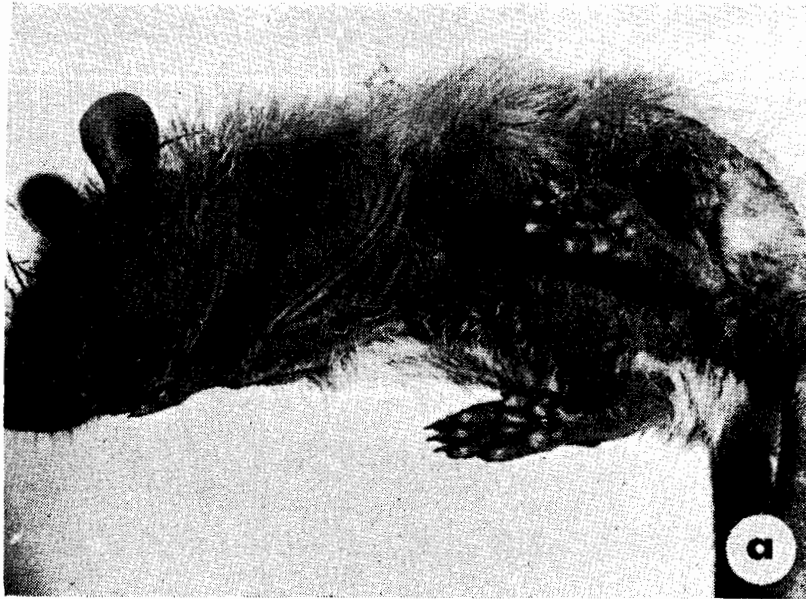
PLATE III: 3. Skin lesions of rats following irradiation and homologous bone marrow transplantation. Data from Balner *et al.* (1964)[18]

(a) and (b) Lesions at the height of the disease during the 5th week after transplantation. Note loss of hair, scaling and thickening of the skin on the legs.

(c) and (d) Advanced recovery of the skin with regrowth of hair, 2 weeks later
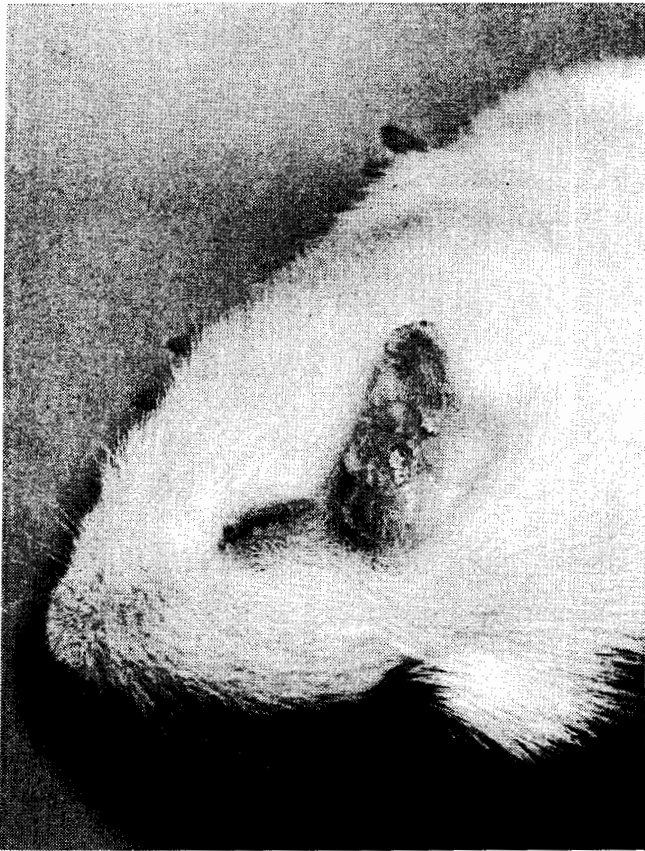
PLATE III: 4. Guinea-pig showing characteristic skin lesions 20 days after irradiation and homologous bone marrow transplantation. Lesions in guinea-pigs are most pronounced on the ears, around the eyes and in the skin of the feet
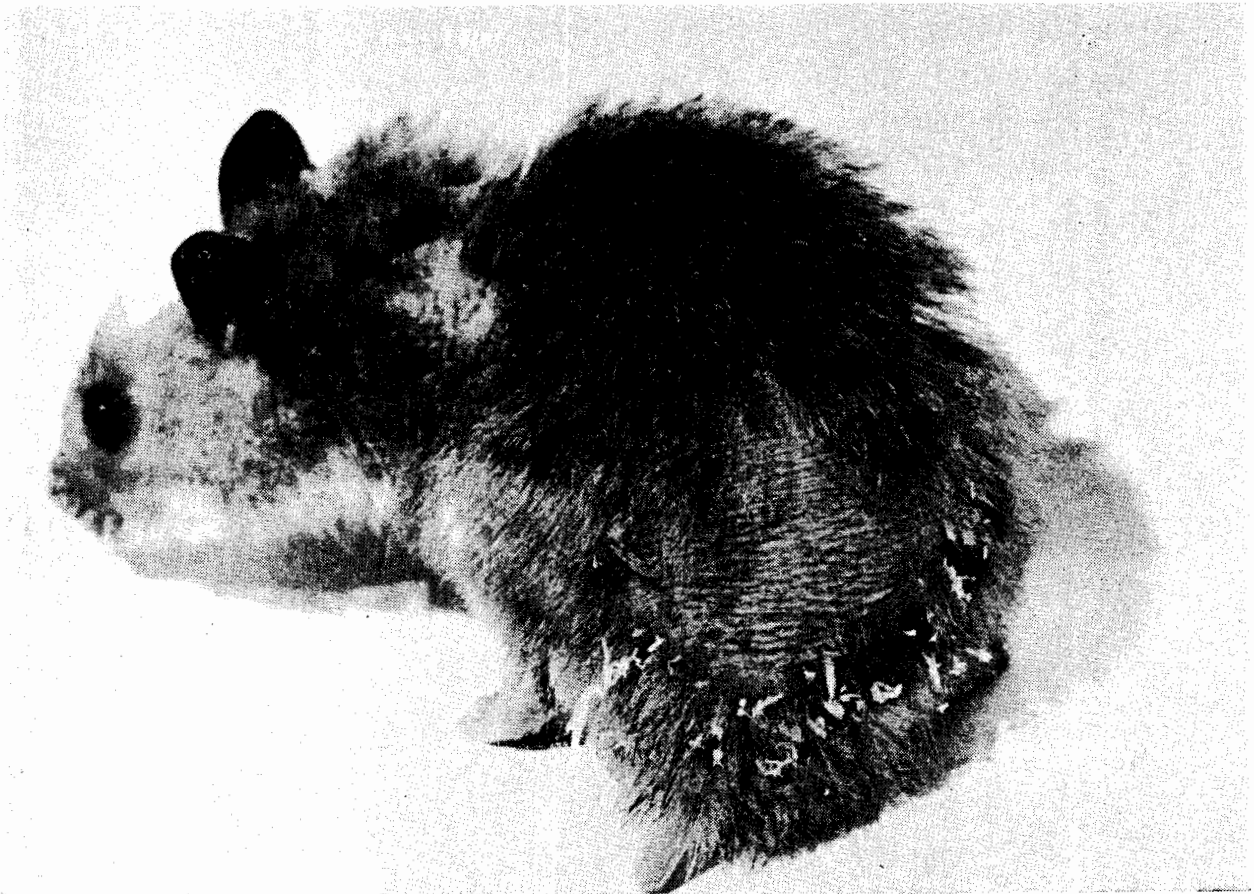
PLATE III: 5. Syrian hamster with cutaneous manifestations of secondary disease following irradiation and homologous bone marrow transplantation. Characteristic patchy alopecia and scaling

Immunological Studies with Radiation Chimaeras

Introduction

In stable radiation chimaeras the cells which are responsible for the immunological reactivity have been replaced by derivatives of the graft, and the immunological reactivity of chimaeras is therefore at least qualitatively similar to that of the donor animals.

The successful transplantation of haemopoietic cells in lethally irradiated recipients is usually followed by a variable period of immunological unresponsiveness or non-specific non-reactivity, while regeneration of lymphatic tissues is under way. This inert period is also observed in isologous chimaeras. Its duration is dependent upon the nature of the grafted cells, the longer periods being observed with foetal liver cells and bone marrow and very short ones with spleen cells or when lymph node cells are added to bone marrow. The larger the number of cells injected the shorter will be the inert period.

In homologous and heterologous chimaeras the immunological system has, at least initially, the inherent capacity to react against the host. Progressive anti-host reactivity results in secondary disease which may be fatal, or else anti-host reactivity may gradually change into a state of partial or complete specific immunological tolerance towards host tissue antigens.

In cases of secondary disease the reactions against other antigens (third party antigens) are usually subnormal, which is consistent with extreme atrophy of the lymphatic tissues. During periods of severe anti-host reactivity the reactions against third party antigens may be virtually absent; the less severe the secondary disease, the more nearly normal is the immunological reactivity.

The evidence which has provided the basis for these generalisations has been presented in previous chapters. In the first part of this chapter the actual immunological responses of radiation chimaeras to a variety of antigens will be briefly discussed. The subject has been

dealt with in several excellent reviews (Hasek and Lengerova (1960)[170]; Makindoan and Gengozian (1960)[244] and Koller *et al.* (1961)[202]). In the second part, transfer studies of immunologically competent cells involving radiation chimaeras will be analysed from the point of view of general immunological interest.

Reactivity of radiation chimaeras

In several of the investigations of the immunological activity of radiation chimaeras, the chimaeric state of the animals has not been confirmed, which greatly limits the value of these studies.

In many of these experiments donor type skin transplantations have been employed to prove the chimaeric state. If performed with the appropriate controls this method usually seems to be adequate. The persistence of tolerance towards donor skin after seemingly total reversion in rats,[14] as well as the mutual tolerance between host and donor cells described in a few partial (mouse) chimaeras[119], indicates that some caution is nevertheless necessary, but this situation appears to be the exception rather than the rule.

Transplantation immunity studies in radiation chimaeras have been performed generally with the purpose of obtaining information on the immunological interaction between donor and host immunological systems. In addition, a great number of investigations has been undertaken, using red blood cells or soluble materials as antigens, to evaluate the capacity of the transplanted immunological system of the chimaera to react against antigens in general. Some of these experiments were, however, also aimed at the elucidation of the identity of the antibody forming cells of the chimaeras.

HOMOGRAFT REACTIVITY

In the study of homograft reactivity of chimaeras, a number of workers have used homologous tumour transplants but the bulk of the information has come from skin grafting experiments.

The tumour transplantation approach is not a very attractive one because of the outcome of the assay is always determined by two opposing forces: the growth rate of the tumour cells and the immune reaction that has been induced. Nevertheless, the results reported by Barnes *et al.*[24], Ilbery *et al.*[184] and Feldman and Yaffe[136] are in agreement with the generalisations which have been presented above, in respect of the immunological behaviour of chimaeras.

Koller and Doak[203] found no significant difference in the time

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required for the restoration of the immune response against homologous tumours between mice receiving isologous adult bone marrow and those that were restored with foetal liver cells. The number of haemopoietic cells injected was quite high in these studies and it is quite possible that with lower cell numbers a difference would have been apparent.

Skin transplantation was introduced as a method of investigating radiation chimaeras by Main and Prehn[239] when the concept of chimaerism was not yet fully recognised, and their results contributed significantly to the acceptance of the phenomenon, as was pointed out earlier (Chapter I). Main and Prehn[240] extended their investigations in 1957 with a number of different host–donor combinations and also studied the effects on homograft reactivity of variations of the number of bone marrow cells and of the radiation dose.

They found that DBA/2 mice which had received BALB/c marrow, while accepting BALB/c skin, rejected C57BL skin grafts, thus demonstrating the presence of a competent anti-third party reactivity in their radiation chimaeras. Interestingly, it was found that (C57BL/HeN × A/HeNF)F$_1$ hybrid mice which had been restored with BALB/c marrow following irradiation accepted DBA/2 skin in 9 out of 29 cases. When restoration was accomplished with DBA/2 marrow, 12 out of 16 animals were found to accept BALB/c skin grafts. The BALB/c and DBA/2 strains share the most important histocompatibility antigens (at the so-called H-2 locus). The acceptance of skin grafts from an inbred strain antigenically slightly different from the bone marrow donor was tentatively ascribed to residual radiation effects.

The latter argument indicates that, at that time, the authors were not fully aware of the consequences of radiation chimaerism, since the donor system, which was *not irradiated*, determines the immunological responses of the chimaera. At present this phenomenon has to be ascribed rather to a decreased competence of the (donor) immunological system, as is generally encountered in homologous chimaeras, probably as a result of graft versus host activity and the ensuing lymphatic atrophy. Since typing of the cell population in the lymphatic tissues and bone marrow was not performed, an interpretation of their results with variations of cell number and X-ray dose is difficult.

In isologous chimaeras the ability to reject foreign skin grafts is

usually restored within one or two months,[14, 414], but in exceptional cases it may remain impaired for a long time. Tyan and Cole[421] for instance found delayed rejection of homologous skin grafts 350 days after lethal irradiation of $(C3H \times DBA/2)F_1$ mice followed by injection of 4×10^6 isologous bone marrow cells. Under the same conditions rat skin was rejected normally.

Skin transplants were employed extensively by Trentin in early studies[414] concerned with isologous and homologous bone marrow transplantation in lethally irradiated mice and in homologous combinations following sublethal irradiation. In the former experiments with homologous marrow the results suggested the production of chimaeras, since donor type skin or skin normally accepted by the donor strain was in general accepted by the test animals. Typing of haemopoietic tissues was not carried out. After sublethal irradiation and homologous bone marrow transplantation Trentin observed the rejection of donor type skin which pointed to a recovery of the recipient's own immunological system[413].

In conclusion, it seems that homograft reactivity is more quickly restored in isologous chimaeras and complete recovery is usually obtained after 30–50 days. Homologous chimaeras frequently exhibit impaired homograft reactivity, in particular when incompatible host–donor chimaeric combinations are studied.

GRAFT VERSUS HOST REACTIVITY

Soon after the discovery of radiation chimaerism attempts were made to solve the problem of the causes of secondary disease by studying the fate of skin grafts. In 1957 Zaalberg et al.[470] published their observations on rat mouse radiation chimaeras that were grafted with skin from various sources.

Rat skin derived from the same inbred strain as the bone marrow was grafted at 24–151 days after irradiation. In 10 out of 35 mice the skin graft remained in excellent condition for more than 52 days. It should be noted that this finding represented the first case of a successful heterologous skin transplantation in a mammalian species. The erythrocytes and the granulocytes of these mice were exclusively of the rat type, indicating a state of complete chimaerism.

In 9 other animals the rat skin slowly deteriorated and these individuals were found to possess a mixture of mouse and rat erythrocytes and granulocytes. Normal sloughing of the grafts occurred in three other cases which were found to have no rat cells in the

peripheral blood (total reversals). In 19 CBA mice treated with rat bone marrow, homologous mouse skin (C57BL) was transplanted simultaneously with the rat skin to investigate whether the rat donor cells would be capable of reacting against mouse antigens. In four of these the homologous mouse skin was rejected between 10 and 30 days after the transplantation while normal takes of the rat skin occurred.

It is not possible to conclude from these experiments whether the C57BL skin was rejected by the donor immunological system or by remnants of the host lymphatic tissues; the latter possibility seems, however, unlikely.

Very similar results were described later by Barnes et al.[22] in mice treated with rat bone marrow.

In 1959 Zaalberg[467] reported additional evidence suggestive of a reaction against host-type antigens by donor cells in radiation chimaeras (Fig. $V^{1(A)}$). Male (CBA × C57BL)F_1 hybrids were grafted with CBA and with C57BL skin. One month later when the grafts were well established these animals were sublethally irradiated (400 r) and injected intraperitoneally with 10^8 C57BL spleen cells which caused a prolonged graft versus host disease. After 20 days, three animals sloughed the CBA skin, while the C57BL skin remained normal. Six animals, including the three mentioned above, died within 41 days after the administration of the spleen cells with signs of diarrhoea and wasting. The rejection of the CBA skin grafts showed that the injected spleen cells were able to react against host type antigens, which was assumed to result in the subsequent death of the animals. The fact that in other mice with graft versus host disease the CBA skin was retained can be explained by assuming that the host tissues represent such an excess of antigen that the available antibodies—either "cellular" or humoral—failed to reach the skin graft in sufficient amounts.

Comparable experiments were reported by Koller et al.[205] using (BALB/c × C57BL)F_1 hybrid mice. Some hybrids were grafted with BALB/c skin and others with C57BL skin and 28 days later they were irradiated and received bone marrow from the *other* parental strain. Six out of 10 of these chimaeras subsequently rejected the well-established graft of the parent strain which was not identical to the bone marrow donor, the rejection times varying between 20 and 100 days (Fig. $V^{1(B)}$).

More direct evidence of graft versus host immunological activity in radiation chimaeras was provided by Koller and Doak[203] who observed
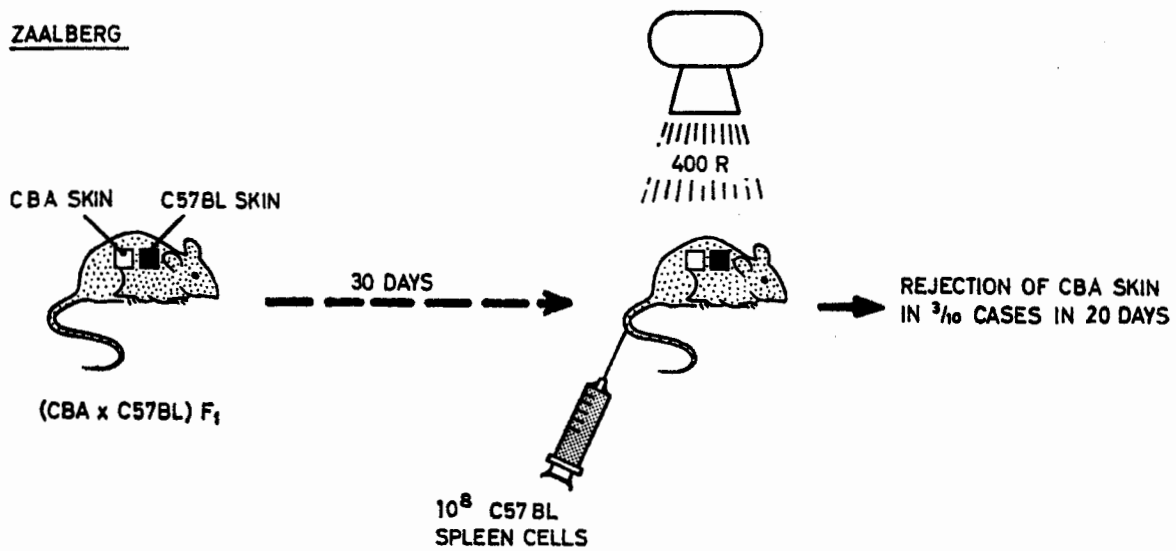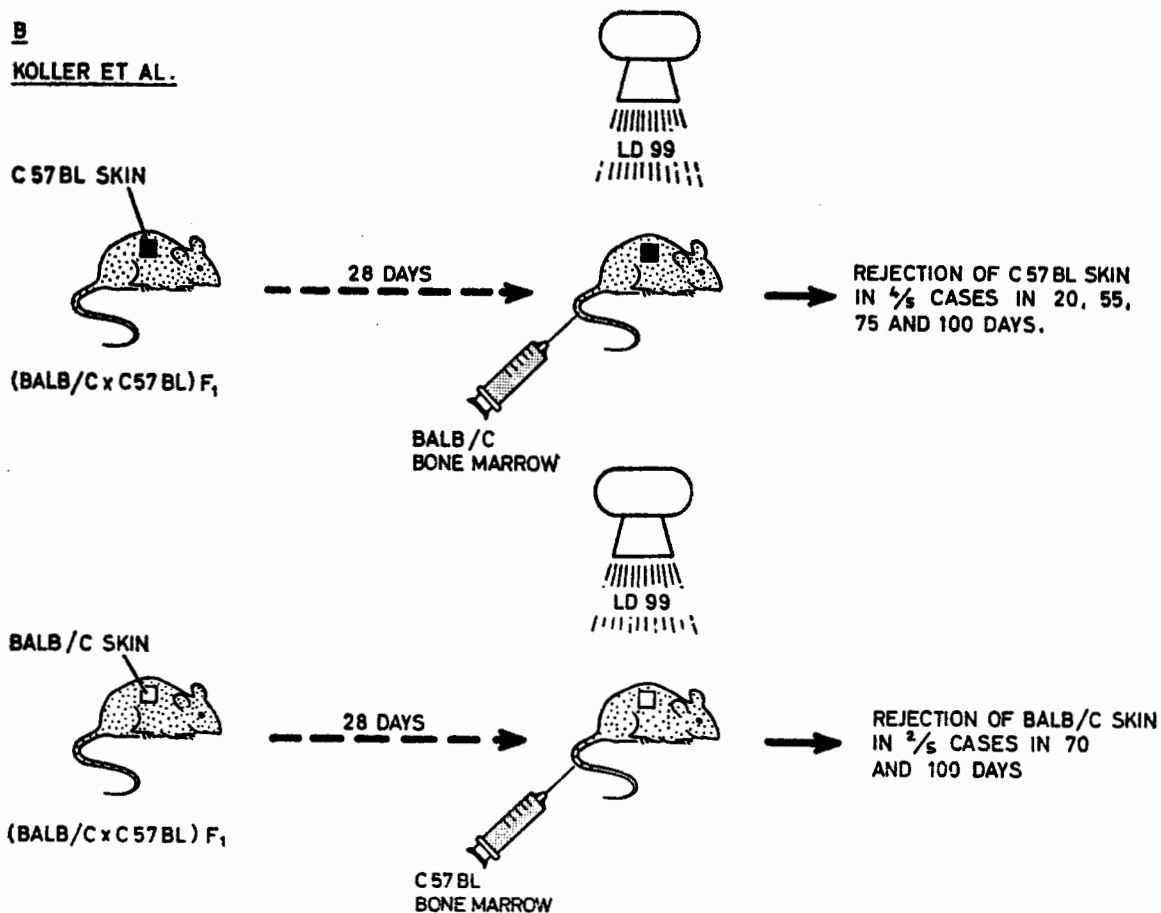
Figure V[1]. Demonstration of anti-host activity in mouse radiation chimaeras with skin grafting. Schematic representation of experiments by

(A) Zaalberg (1959)[467]

(B) Koller *et al.* (1961)[205]

In Koller's experiments parent strain skin grafts were established in F_1 hybrid mice. Thereafter, the recipients were exposed to X-irradiation and received bone marrow from mice of the other parent strain. A proportion of the skin grafts was subsequently rejected. In Zaalberg's experiment the skin grafts which were isologous to the bone marrow donors were retained, but two out of five of the grafts from the other parent strain were rejected

the rejection of CBA skin grafts in a number of BALB/c → CBA mouse chimaeras, as has already been mentioned in Chapter III. The CBA skin (host type) was grafted between 3 and 7 days following irradiation and was sloughed after 10-25 days. A large proportion of autografts (5 out of 7) were also rejected by homologous chimaeras, at least when the grafting was done within 24 hours after the bone marrow transplantation[124]. It is also of considerable interest that a similar rejection of isografts was seen in irradiated mice which were treated with homologous foetal liver cells instead of with homologous bone marrow, although foetal liver cells are generally believed to induce a less severe graft versus host reaction.

Doak and Koller drew attention to the strange phenomenon that the grafted CBA skin was rejected while the host CBA skin remained unaffected. This was tentatively interpreted as due to a local concentration of reactive cells of donor origin in the graft bed. If the authors had performed an histological examination of the *host* skin, they would probably have found evidence of a graft versus host reaction. The rejection of host type skin grafts under similar experimental conditions has not been found by other workers, so that either the transplantation method or the specific host–donor combination employed by Doak and Koller must have been particularly favourable for this type of response.

As discussed in Chapter III, page 87, Stastny *et al.*[385] have reported the rejection of autologous skin grafts in non-irradiated rats suffering from homologous disease as a result of the injection of massive numbers of foreign spleen and lymph node cells. These rats showed, however, a very severe dermatitis over the whole body surface of the characteristic graft versus host type. A similar rejection of isografts was observed by Balner[15] in lethally irradiated rats treated with homologous *spleen* cells, but never in bone marrow chimaeras of the same host–donor composition.

REACTIVITY AGAINST OTHER ANTIGENS

When considering the immunological responses of radiation chimaeras it should be kept in mind that in the period immediately following the lethal irradiation and bone marrow transplantation, the chimaeras are very similar to untreated irradiated animals in being either incapable, or having a very low ability, to react to primary antigenic stimulation. As the lymphatic system is regenerated from the donor cell precursors, reactivity may reappear. This is always the

case in isologous chimaeras, but the immunological recovery is severely impaired when graft versus host disease develops as is the case in incompatible host–donor combinations. As would be expected, an early recovery can be promoted in compatible combinations by the administration of large numbers of lymphatic cells.
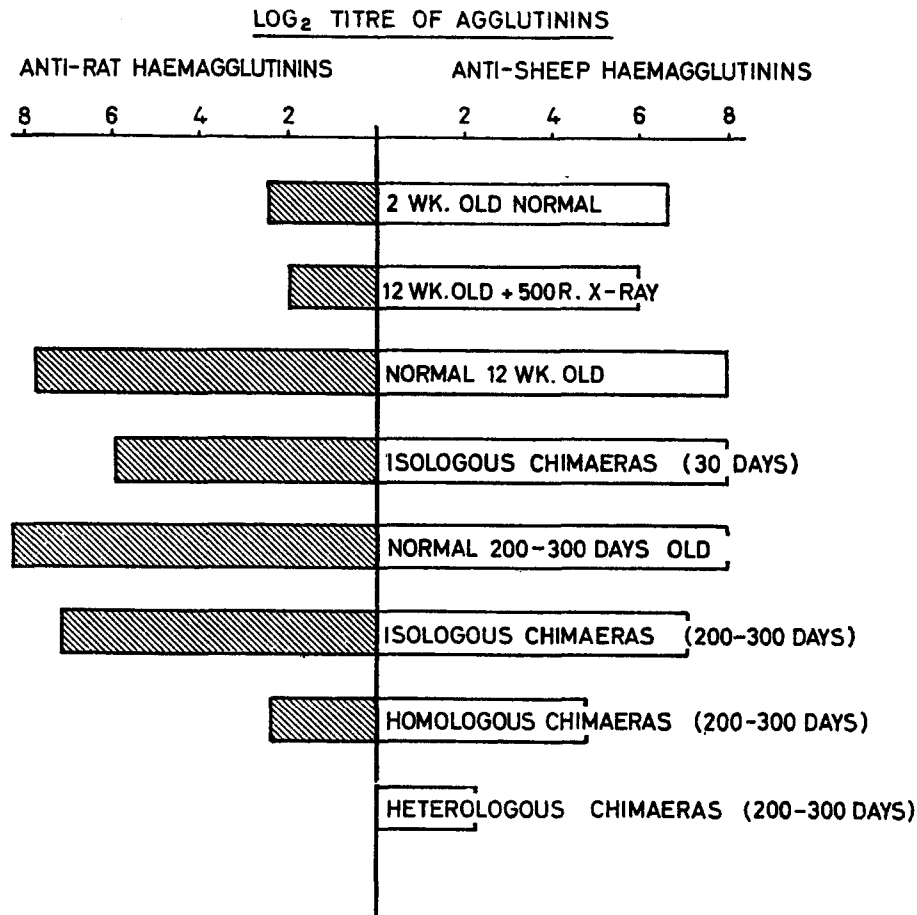
Figure V[2]. Antibody formation in various mouse radiation chimaeras and their controls. Data from Gengozian *et al.* (1958)[152] and Makinodan *et al.* (1956)[245]

Mouse marrow dose: 12×10^6 cells/recipient
Rat marrow dose : 140×10^6 cells/recipient

The most extensive quantitative research on this subject has been carried out at Oak Ridge by Makinodan and his collaborators. The antigens employed were predominantly erythrocytes of different animal species unrelated to either the host or the donor; the formation of haemagglutinins was taken as an index of immunological reactivity.

Many of their results were reviewed by Makinodan and Gengozian in 1960[244] and their main findings with chimaeras are summarised in Fig. V[2].

The responses of isologous chimaeras both at 30 and 300 days after transplantation were found to be near normal.*

A similar conclusion was reached by Garver et al.[149] who tested isologous radiation chimaeras with sheep and human erythrocytes and observed a return to normal reactivity up to 55 days following the transplantation.

In contrast, the primary response of homologous mouse chimaeras remained below normal, the response of mice treated with bone marrow, being greatly decreased according to Makinodan's group, and absent according to Garver et al.

Figure V[2] shows that in general the deficient animals' response as well as the response of young animals to rat erythrocytes is consistently lower than to sheep erythrocytes. A similar response was observed in mice recovering from various sublethal doses of whole body irradiation[243]. The fact that normal mice react to both these antigens with the production of roughly equal amounts of antibody led Makinodan to formulate his *recognition hypothesis*. According to this, maturation of the capacity of the antibody froming cell or of the antibody forming population of cells to recognise more closely related antigens is a function of age. X-irradiation, by reversing the immune mechanism of an adult animal to a less mature state, would lead to a loss of recognition capacity. The degree of destruction of immune reactivity by irradiation would decrease with increasing "foreignness" of the antigen. This hypothesis actually postulates the existence of different types of antibody producing cells, the one capable of reaction against less closely related antigens being more resistant to irradiation than the cells which are able to react against closely related antigens. Alternatively, one cell could possess different mechanisms of reaction against various antigens, the mechanism responsible for the reaction against less closely related antigens being again the most radiation-resistant. Such an interpretation, however, seems to be unrealistic, since it ignores the factor of the antigenic strength of the antigen, which, together with the dose and the mode of administration of the antigen, determines the strength of the stimulus to the immune system. It is well known that under otherwise comparable conditions,

* In a later study by Makinodan's group[155] values markedly below normal were obtained 160 days after *isologous* bone marrow transplantation in four different mouse strains. These lower mean values were accompanied by extreme variations in the responses, some mice showing normal reactivity, whilst others showed a very low response. An explanation for this discrepancy between the results of the two groups of experiments could not be found.

the antigenic stimulus decreases with increasing similarity of the antigens. A much simpler and therefore more attractive explanation of the different responses to rat and sheep erythrocytes encountered in young animals, in irradiated animals and in chimaeras, is that the partial deficiency of their immunological response was not evident against the sheep antigens because the antigenic stimulus provided by the latter is relatively strong. That roughly equal titres of antibodies to both antigens were found in normal animals may be due to the use of an excess of antigen in the case of sheep erythrocytes. This argument emphasises the necessity of performing comparative measurements with different antigenic stimuli by using in each case a standard fraction of the minimal dose of antigen which produces a maximal response.

Gengozian et al.[155] showed further that homologous foetal liver cells caused the same degree of restoration of immunological reactivity as isologous bone marrow. Gross morphological and histological examination showed that mice treated with foetal liver recovered as completely as the isologous chimaeras. This was in contrast to a group of mice treated with adult bone marrow of the same donor strain as the foetal liver group, which showed changes characteristic of secondary disease.

A more detailed study of the recovery of the agglutinin production in isologous foetal liver chimaeras was reported by Doria and Congdon[128]. They found a return to normality after about 30 days when sheep red blood cells were used as an antigen, while the response to rat erythrocytes was still slightly below normal at 50 days (Fig. V[3]). In view of the large number of foetal cells injected it seems likely that the number of lymphatic cell precursors in foetal liver is much smaller than in bone marrow. It is unfortunate that the authors provided no information on the histological appearance of the lymphatic tissues of their chimaeras.

An interesting technique was employed by La Via et al. in 1958[215] in a study of antibody formation in X-irradiated rats treated with rat or rabbit haemopoietic cells. Liver cells from rabbit embryos (10^8 per recipient) had been found to protect rats irradiated with a dose of 750 r. In order to establish whether donor or host type cells were subsequently producing antibody, the surviving animals were injected 7–14 days later with bovine serum albumin (BSA) and Salmonella typhimurium vaccine (STV). Although rats do not form precipitating antibodies to soluble antigens, e.g. BSA, under the

conditions of antigenic stimulation used by these investigators, rabbits will respond very actively to such antigens. Both species respond to STV. Surprisingly, rats treated with rabbit cells produced significant anti-BSA titres, while controls treated with rat embryo liver gave no response at all to BSA. Both types of "chimaeras" responded equally well to the STV vaccine. It has never been established whether the surviving rats in the study were indeed chimaeras. The authors state in their paper that "rabbit cells have not been demonstrated in the rats tissues in our experiments", and since rabbit eosinophylic granulocytes can readily be distinguished from similar rat cells, it must be assumed that chimaerism was not involved. Although these results still remain unexplained it may be that a temporary proliferation of rabbit cells had caused the production of a limited amount of anti-BSA antibody.
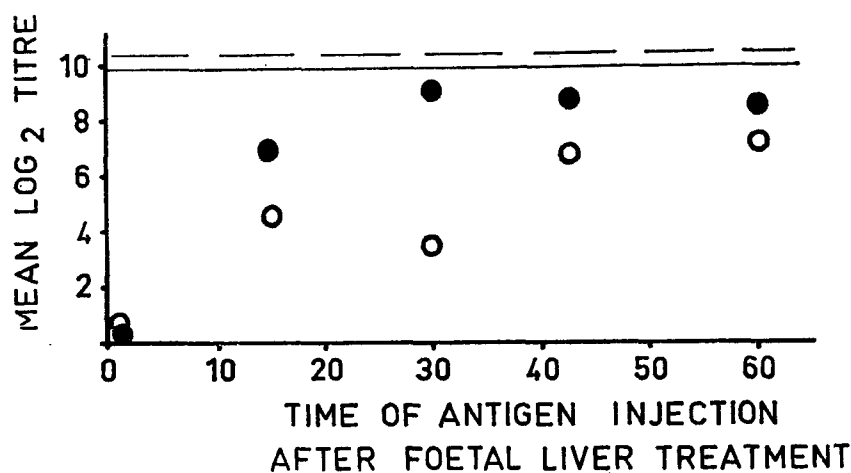
Figure V[3]. Agglutinin response 14 days after injection of sheep red blood cells (RBC) (●) and rat red blood cells (RBC) (o) in isologous chimaeras produced by injection of 40×10^6 nucleated foetal liver cells following whole body irradiation with a dose of 950 r. Data from Doria and Congdon (1962)[128]

——————— Anti-sheep RBC titre in normal mouse

— — — — Anti-rat RBC titre in normal mouse

Summarising, it may be said that the recovery of the immune response occurs after varying intervals of time following the transplantation. The length of the time interval and the completeness of the recovery closely parallel the recovery of the lymphatic system. In isologous combinations this is promoted by the addition of lymphoid cells to the bone marrow graft. When recovery is incomplete a strong antigenic stimulus provokes a larger response than a weak one.

Transfer experiments involving radiation chimaeras

The transfer of immunologically competent cells to irradiated animals has certain advantages for the student of immunological mechanisms. The fact that the irradiated animal cannot usually reject the transferred cells, makes it possible to measure their activity, unhampered by the host's own immunological reactions or by interfering processes which may be present in the donor of the transferred cells. To exclude completely any homograft reaction against the transferred inoculum, a supra-lethal dose of whole-body irradiation is recommended. In addition the degree of histo-incompatibility between the donor and the recipient should not be too great. In the case of isologous transfers, irradiation of the recipient serves to allow maximal proliferation of the injected cells and to permit measurements of the activity of the transferred cells without the occurrence of similar reactions on the part of the host. According to several authors, the host animal serves as a "living test tube" in these cases; this term implies the immunological inertness of the host in this sytem. In other experimental situations antigenic differences between host and donor are specifically chosen to study interactions between the immunologically competent cells of the graft and excess of (transplantation) antigens provided by the host.

In order to keep the recipients alive, these have to be treated with a sufficiently large number of haemopoietic cells which are sometimes present in the transfer inoculum. If not, bone marrow of the same source has to be administered as well.

TRANSFER OF IMMUNITY

In 1954 Harris et al.[167] demonstrated the transfer of bacterial agglutinin production into sublethally irradiated rabbits by the injection of spleen or lymph node cells of immunised rabbit donors. The production of agglutinins in the recipient was studied for a limited period only (10 days). Agglutinin production also occurred when cells were transferred from non-immunised donors, provided these cells were incubated in vitro with the antigen before injection. Since the rabbits were genetically not homogenous it is unlikely that a state of chimaerism was produced by the transfer of these lymphatic cells, but rather that a temporary persistence of the injected cells was responsible for the antibody production, as is the case in adaptive immunity.

Mitchison[283] was the first to realise that radiation chimaeras

might acquire the immunological reaction patterns of their donors. He transferred large numbers of spleen cells (10^8 intraperitoneally) from mice that had been immunised against *Salmonella typhi* (H) antigen to lethally irradiated isologous and homologous recipients and obtained a significant antibody titre against H antigens within 10 days following the transplantation. The studies were not extended for more than 20 days.

In non-irradiated recipients the titres obtained were *lower*, especially when the donors had been immunised only once, and this disparity served to discount the possibility that the titres in the irradiated recipients were caused by transfer of antigen with the spleen cells.

Recent studies on adoptive immunity to BSA (bovine serum albumin) by Mark and Dixon[250] have confirmed Mitchison's observation that upon the transfer of immune cells to irradiated recipients an increased antibody formation occurs compared with a similar transfer to non-irradiated isologous mice. Whether this effect is caused by a non-specific stimulation of the proliferation of all lymphoid cells in the irradiated animal or by a selective proliferation of the transferred antibody-producing cells has not been elucidated.

Makinodan *et al.*[246] have shown that in lethally irradiated mice, antibody could be produced in response to an intraperitoneal injection of antigen (rat erythrocytes) administered simultaneously with an intravenous injection of large numbers of isologous spleen cells. Bone marrow does not seem to contain sufficient immunologically competent cells to initiate a similar response. However, isologous bone marrow (12×10^6 cells) from presensitised donors did confer the capacity to respond to a dose of antigen administered immediately after the irradiation upon lethally irradiated recipients[153]. These results seem to confirm that in rodents bone marrow contains a much lower percentage of immunologically active cells than spleen and lymph nodes. It is logical to assume that the antibody production in the recipients will be a function of the number of immunologically active cells that have been transferred and this has in fact been demonstrated quite convincingly by Makinodan and co-workers[247] for the transfer of both primary and secondary responses.

These authors have also employed this relationship to measure the homograft reaction quantitatively by injecting a standard dose of antibody forming cells (A) and in addition graded numbers of cells

(B) that could react against the latter, into the same lethally irradiated recipients. The amount of antibody produced was thus inversely related to the homograft activity of the B-cells. They favour the term "*in vivo* tissue cultures of antibody forming cells" to designate this experimental set up. In a previous chapter, experiments by Doria were mentioned, in which this technique was ingeniously applied to obtain evidence of graft versus host reactivity in homologous chimaeras[126, 127] (page 91).

Radiation chimaeras have lately also been employed to collect information on one of the most intriguing properties of the immunological system: its *memory* to respond to specific antigens. This so-called anamnestic response has always been one of the keystones in the various theories of immunity. Any theory of immunity has to account for the long-lasting memory of previous antigenic stimulation, resulting in an increased and accelerated response upon secondary antigenic challenge. This property is the basis of the well-recognised state of immunity towards certain commonly employed antigens such as tetanus toxin, vaccinia virus, etc. It is on this immunity that vaccination against many infectious diseases depends.

For an explanation of specific anamnestic responses it is essential to decide whether part of the initially administered antigen remains involved in the processes accounting for the memory; the so-called direct antigen template theories according to Talmage and Cann[396] are based on this concept. If this is not so it must then be decided whether the antigen induces characteristic and stable changes in the immune cells, i.e. if they no longer require the persistence of the antigen for the maintenance through generations of cells of the property to react with a secondary response when stimulation with the specific antigen is renewed. The latter concept is incorporated in the so-called indirect antigen template theories as well as in the selective theories. The latter postulate either the existence of a great many natural templates or the existence of a large diversity of cell clones, each capable of producing one distinct type of antibody. The transfer of immunologically "committed" cells to lethally irradiated recipients, i.e. the use of radiation chimaeras, seems to offer a unique opportunity on the one hand to separate the *stimulated cells* from the stimulus (the initially administered antigen) and on the other hand to obtain an intense proliferation of the antigenically stimulated cells which allows an evaluation of the stability of the anamnestic response (i.e. the "memory") through a number of cell generations. This can be done

by challenging the chimaeras at various intervals after the transplantation and by a determination of their antibody production.

The transfer of hyper-immune cells to *non*-irradiated (isologous) recipients has been found to lead to a gradual loss of anamnestic response in about 3 weeks[250]. This might indicate that in the non-irradiated animal conditions are not favourable for the proliferation of injected cells, even in the absence of histo-incompatibility.

Obviously, one of the most critical points in this type of experiment is that the persistence and the continued proliferation of the transferred cells in the recipients at the time of the secondary challenge should be proved beyond any reasonable doubt. This requirement was not met in the experiments reported by Dixon and co-workers[123], who measured the antibody response to BSA injection several days after the transfer of pre-immunised lymph node cells to irradiated rabbits. They observed a successive loss in the ability to evoke an immune response upon delaying the injection of the antigen into the recipients of the lymph node cells. Since the transfer took place between non-inbred animals, it is quite likely that a homograft rejection (admittedly a weakened one because of the irradiation) was responsible for the disappearance of the response. Makinodan has emphasised the importance of employing genetically identical animals for such transfers, but it would be even more elegant to include a cell marker in the system to allow identification of the transferred cells and their descendants, in particular when longer intervals between the transfer and the immunity tests are being studied.

So far, the number of investigations of the type outlined above have been limited and the length of the interval between cell transfer and antigenic challenge has been rather short. For this reason, the question of the persistence of the antigen in the maintenance of immunological memory has not yet been answered.

Chin and Silverman[74] studied the isologous transfer of cells from donors immunised against *Salmonella typhii* into lethally irradiated mice. Following the transfer of 10^7 spleen cells from hyper-immune donors, a recall injection of antigen 22 days after the transfer evoked a typical booster response, not only in the irradiated recipients but also in the non-irradiated controls. Large amounts of isologous bone marrow could not, however, transfer the ability to respond anamnestically.

They also studied the "memory" of rat bone marrow transferred in sufficient amounts to repopulate the lethally irradiated mouse

recipients. Booster responses could not be induced 28 days after the transplantation and, furthermore, an increase in the number of bone marrow cells and the addition of spleen cells failed to induce secondary response when challenged some time after the transplantation. These negative results undoubtedly reflect the relatively poor recovery of the lymphatic system in these chimaeras and are probably also related to the occurrence of secondary disease.

Stoner and Bond[388] measured the levels of anti-tetanus toxin levels in sublethally irradiated isologous recipients of cells from immunised donors. An antigenic stimulus of toxoid was given 3 days after the transfer. Significantly higher titres were obtained following this booster in animals receiving bone marrow, spleen, thymus and lymph node cells when compared to those obtained from animals which had received no booster. By far the highest booster response was observed in the animals that received spleen cells. In the non-stimulated recipients detectable levels of antibody were found which indicated either a continued production by the transferred cells or a passive transfer of antibody with the cell suspension. This is in agreement with the earlier observations by Stoloff[387] that bone marrow cells from mice hyperimmunised with tetanus toxoid are capable of continuing anti-toxin production for more than 25 days after transfer to irradiated isologous hosts. The experiments by Silverman and Chin as well as those by Stoner and Bond suggest that spleen cells are far superior to bone marrow cells in the transfer of the anamnestic response. The duration of the anamnesis demonstrated in this way is not known since the last observation was 28 days after transplantation; furthermore, it seems that the anamnestic response is much more difficult to transfer in non-isologous combinations. In this context it may be added that Nossal and Larkin[291] failed to transfer immunity against mouse erythrocytes when bone marrow and spleen cells from immunised rats were injected into homologous irradiated (1000 r) recipients.

Experiments that are in some aspects comparable to cell transfer studies in irradiated animals were performed by Claman[75] who studied anti-BSA production in rabbits that were sublethally irradiated (400 r) 30 days and 60 days after antigenic stimulation. 50 to 60 days after the irradiation the response to a booster injection was greatly decreased compared to the response of non-irradiated control rabbits. In the latter cases no cells had been transferred, but the immunised lymphatic cell population was severely reduced by the irradiation

and subsequently allowed to regenerate before the anamnesis was assayed. These results suggest that a *proliferating* population is apt to "forget" its immunological experience which seems incompatible with a mechanism for anamnastic response in which a stable inheritable change in the cells capable of synthesising the antibody is postulated. It is also unlikely that the memory-carrying cells were destroyed by the irradiation, since other workers have shown that a quite normal response can be induced in immunised animals, when a challenge is made within a week or so of the irradiation.

The transfer of the anaphylactic reaction in guinea pigs has been reported by Stanković and Vlanovik[384]. Guinea-pigs were subjected to varying doses of whole body irradiation and received intravenously about 10^8 of either bone marrow or spleen cells and in some cases both, taken from donors which had been sensitised 6 days previously with horse serum. The surviving recipients were challenged 9 days and 16 days after cell transfer by the intravenous injection of horse serum. Significant anaphylactic reactions were observed in all three groups, while the non-irradiated controls either reacted weakly or not at all.

Some attempts to transfer transplantation immunity in lethally irradiated mice have been published by one of the authors[42]. The test system employed was the Simonsen assay: the increase of liver and spleen weight relative to body weight in mice following the injection at birth, of spleen cells capable of reacting against the tissues of the mouse strain. Spleen cells of donors pre-immunised against the strain of the newborn test animals evoke a stronger reaction than spleen cells from normal mice so that this system is suitable for the quantitative measurement of transplantation immunity. The isologous transfer of anti-CBA immunity to irradiated C57BL mice was only successful when massive numbers (10^8) of immune spleen cells were transferred and in that case an increased reactivity in the Simonsen assay was detectable 3, 6 and 10 days after transfer but no longer after 15 days. The transfer of such numbers of spleen cells is, however, likely to carry over a significant number of immune active cells into the newborn test mice.

Following the transfer of smaller numbers of spleen cells (2×10^7) or bone marrow (5×10^6) plus lymph node cells (5×10^6) evidence of increased reactivity against CBA antigens was absent in spleen cells of the recipients at 1–4 weeks following the transfer. These attempts to transfer an anamnestic reaction to transplantation

antigens have thus been completely negative. It would be of interest if these experiments were repeated using skin graft rejection as the assay system.

The conclusion at present seems to be that it is much more difficult to transfer immunity of any kind by way of cells to lethally irradiated animals than would be expected from the persistence of immunity in normal animals and man. This is particularly so when bone marrow is transferred and when there is proof that the specific antigen was lacking in the new host. Far more detailed information—preferably of a quantitative nature—is required before further interpretations can be made. The available data merely serve to underline the suitability of the radiation chimaera for fundamental studies of immunity. They suggest, furthermore, that the extensive proliferation of a small cellular inoculum in a lethally irradaited animal, together with a dilution of the particular antigen that is carried over in the inoculum, might represent a pronounced acceleration in the change of the "immune" cell population. In *normal* animals this change would occur only in the course of several years following a single contact with antigen.

TRANSFER OF IMMUNOLOGICAL TOLERANCE

On finding that specific immunological tolerance of the donor-type lymphatic cells towards host-type tissue antigens occurred in certain radiation chimaeras[41] an interesting possibility for the study of a number of problems related to immunological tolerance was opened up. As has been mentioned before, the immunological activity of the chimaeras was measured by the Simonsen assay. This test involves the injection of spleen cells—in radiation chimaeras these cells are of donor origin—into newborn mice of suitable antigenic composition. The magnitude of the graft versus host reaction which ensues is measured by the increase of liver and spleen weights of the baby mice. The question of whether or not this type of tolerance is dependent on the presence of excess antigen was the first one to receive attention. Excess of antigen has been shown in a variety of systems to be essential for the development of tolerance. In the case of radiation chimaeras (the specific tolerance of a chimaera's haemopoietic and lymphoid cell population towards host type antigens), the excess of antigen is represented by the whole of the recipient. The behaviour of a small sample of such a tolerant donor cell population in the *absence* of the specific antigen can be studied conveniently by the

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transfer of bone marrow or spleen cells from these chimaeras to a second irradiated host having a different immunogenetic composition. The excess of antigen represented by the tissues of the first host can be removed, for example, by the use of an animal which is isologous to the original bone marrow donor as the second host. The excess of antigen can also be replaced by an excess of antigen of a different type using another strain or a F_1 hybrid as the second recipient (see Fig. V[4]). If the development of tolerance were due to a clonal selection process with elimination of the cells that were capable of reacting, a tolerant population would have difficulty in regaining its reactivity when the excess of these antigens is removed.

After transfer of 5×10^6 bone marrow cells from C57BL → (CBA × C57BL)F_1 chimaeras to irradiated (RF × C57BL)F_1 hybrid mice, reactivity against the first host (i.e. against CBA antigens) was found to recur after about 30 days, reaching normal values after two months. In contrast, reactivity against the antigens of the new-born of the second host type (i.e. against RF antigens) remained absent so that a new and specific tolerance had developed upon the second transfer of cells[41]. These experiments were extended by a third transfer[42] so that the C57BL cells were finally passed through three different recipients as follows:

C57BL → (<u>CBA</u> × C57BL)F_1 → (<u>RF</u> × C57BL)F_1 →
(<u>CBA</u> × C57BL)F_1
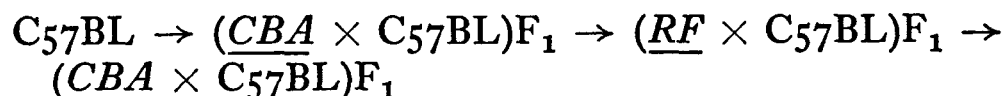
In this notation the antigens with which the C57BL donor cells were confronted have been underlined.

Before each transfer the condition of total chimaerism was verified by the serological typing of the erythrocytes, and reversals—if present—were excluded from the group which was sacrificed to provide bone marrow for the next transfer*. In the third hosts the C57BL cells again rapidly lost their tolerance towards the (RF × C57BL)F_1 tissues (the RF antigens) and again developed tolerance towards the CBA antigens of their actual host. Occasionally, instead of tolerance some anti-host reactivity was exhibited by the spleen cells, but these reactions were usually weak. The intervals between the subsequent transfers varied from between 100 to 200 days.

The main conclusion from this series of experiments was that immunological tolerance of lymphoid cells disappears rapidly, if not

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* A close correlation had been found previously between the genotype of the erythropoietic cells and that of the lymphatic cells in radiation chimaeras.
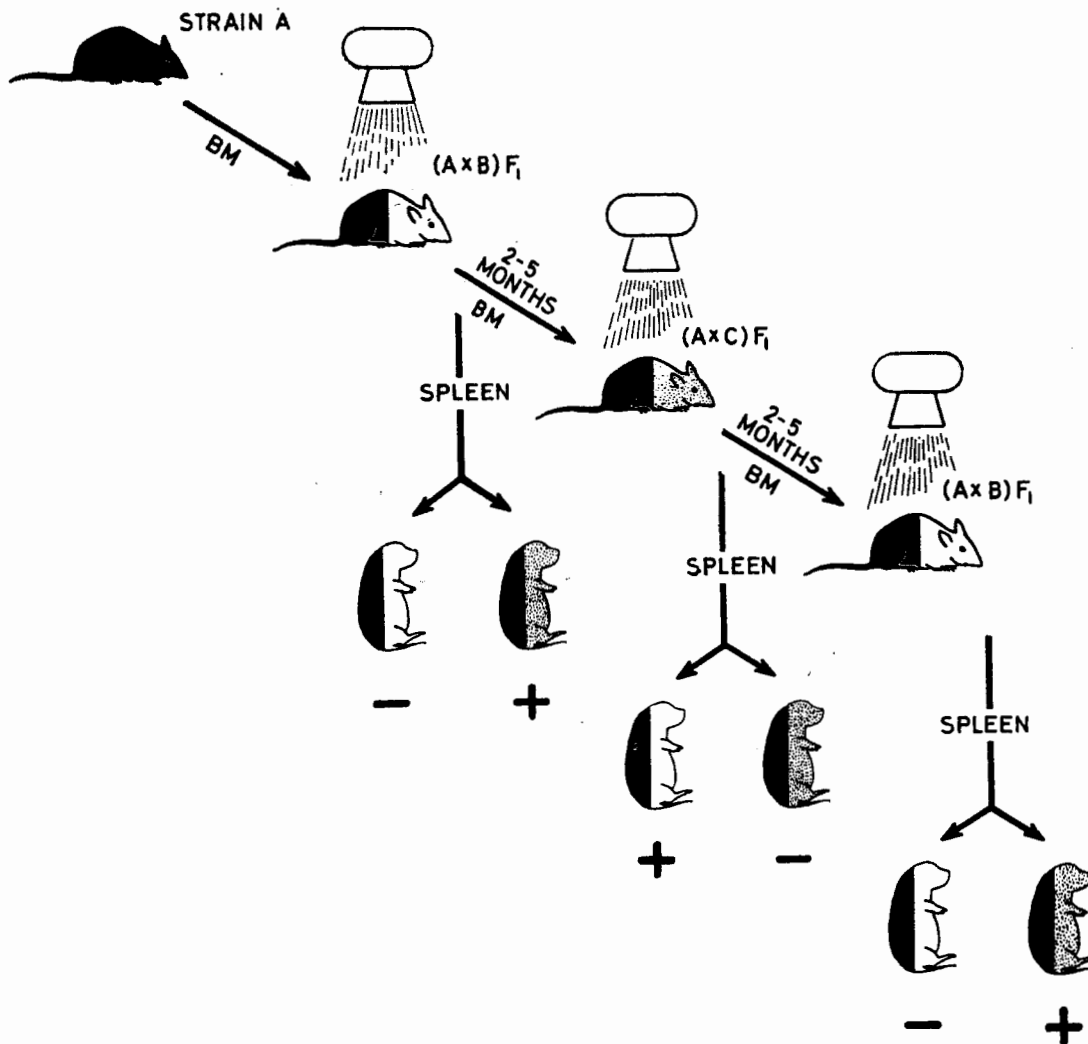
Figure V⁴. "Change of host" by transfer of bone marrow. The reactivity of the donor (C57BL) spleen cells is shown schematically by + and − signs beneath the symbols for the newborns. These experiments show the loss of specific tolerance of the donor type cells (A) towards the host (AxB)F_1, following transfer to a second host which lacks the antigens (B) which elicited the tolerance in the first host. After some time tolerance towards the new host (AxC)F_1 has developed. Following transfer to a third host (AxB)F_1 the situation is again reversed: loss of tolerance towards C type antigens and development of tolerance towards B type antigens

immediately, after the removal of the specific antigens and that a new type of tolerance, specific to the new host, may develop instead. Moreover, this finding has been confirmed several times with a different host donor combination, namely:

CBA → (CBA × *C57BL*)F_1 → (*RF* × CBA)F_1 → (CBA × *C57BL*)F_1
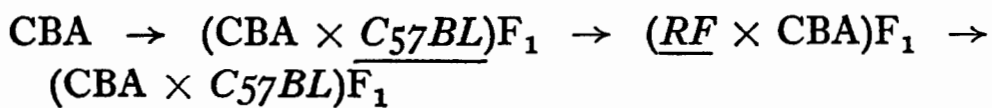
In order to test the possibility that the loss of specific tolerance

following transfer to a new host lacking these specific antigens, is related or due to the establishment of tolerance towards the second host, transfer of tolerant donor-type bone marrow cells of a chimaera to the original host strain was studied:

$$C57BL \rightarrow (\underline{CBA} \times C57BL)F_1 \rightarrow C57BL$$

It was found that under these conditions the tolerance to CBA antigens is also rapidly lost in the second host. This showed that the disappearance of the tolerance is independent of the development of a new tolerance, and suggests that tolerance depends on the persistence of an excess of specific antigen.

In view of observations discussed earlier, that populations of spleen cells or lymph node cells retain their immunological reactivity more readily than bone marrow cells upon transfer to an irradiated host, the above transfer system of tolerant cells from a chimaera back to the original donor type mice was employed with lymph node cells (5×10^6) or with spleen cells (2×10^7) in addition to the bone marrow. However, the addition of these lymphoid cells failed even to delay the reappearance of immunological reactivity.

In contrast, Zaalberg et al.[471] using the CBA $\rightarrow$ (CBA $\times$ C57BL)F$_1$ $\rightarrow$ CBA transfer combination and employing skin grafting to test for tolerance, found a more prolonged persistence of the tolerant state when 12×10^6 lymph node cells were transferred in addition to the standard number of bone marrow cells (3×10^6). One of the present authors (D. W. van Bekkum, L. E. J. Holt-Mour, and H. Balner, Transplantation, 3, 340–351, 1965) performed Simonsen assays of a similar transfer involving lymph node cells using the spleen cells of the second host. A return of weak but significant anti-C57BL reactivity was eventually observed, even in animals with intact C57BL skin grafts. The latter, however, showed evidence of a slight homograft reaction upon microscopic examination. These results indicate that the sensitivity of the Simonsen assay is equal to the most careful histological examination of skin grafts and that the macroscopic evaluation of skin grafts provides much less dependable information on the state of immunological reactivity of the host than the two former methods. It can be concluded that in the absence of the specific antigen, tolerance is maintained much longer when tolerant lymphoid cells are transferred instead of bone marrow cells only, at least under the conditions of this particular transfer experiment.

Since only two inbred strains have been studied in this way, it cannot yet be decided which one shows the more commonly occurring reaction pattern. Whether the return of a subnormal degree of reactivity should be designated as a loss of tolerance or as a persistence of partial tolerance appears entirely a matter of personal preference.

It is of interest to recall that tolerance was not maintained upon transfer of spleen and bone marrow from rats made tolerant to mouse erythrocytes to lethally irradiated homologous recipients[291].

The results described above have given rise to a number of speculations. It was pointed out previously[42] that populations of lymph node cells appear to differ in two important respects from bone marrow cells with respect to the induction and maintenance of immunological tolerance. The former cells are much less able to adapt to newly encountered antigens by developing a state of specific tolerance *and* they seem to contain a larger proportion of cells with a memory for tolerance. Both of these properties could belong to the same cell type, which would have to be sought among the more mature cells of the lymphoid series. Since transfer of tolerance was not successful when smaller numbers of lymph node cells were transferred, it was concluded that the manifestation of immunological memory is dependent on the size of the grafted population. The loss of tolerance described above was observed when intense proliferation of the transferred population of cells occurred, so that the properties of the original inoculum became rapidly "diluted". Even if the lymphoid cells which were transferred had a rather long life span, their presence would not have influenced the eventual reactivity of the resultant population of descendant lymphoid cells to any significant extent. For all practical purposes immunological memory should be considered as a property of a cell population rather than one of individual cells, since only the former has been evaluated directly. Zaalberg et al.[471] postulated that mature lymphoid cells cannot become tolerant following mere contact with excess of antigen. However, once tolerant, they can pass the tolerant state on to their descendants, even in the absence of the specific antigen. Bone marrow on the other hand contains lymphoid precursors which can develop into tolerant mature forms in the presence of excess of antigen. In the absence of antigen the precursors would develop into normally reactive lymphoid cells. Both gradual or rapid loss of tolerance upon transfer to a host lacking the specific antigen would—according to this hypothesis—be due to

a replacement of the tolerant lymphoid population by reactive cells derived from bone marrow precursors.

Quite recently evidence has been obtained that the presence of the thymus or factors derived from the thymus is required for the differentiation of immature precursor cells into mature immunologically active cells. Using thymectomised second recipients in transfer experiments which were otherwise similar to those described before, Zaalberg[468] found a further prolongation of specific tolerance upon transfer of lymphoid cells to recipients which did not contain the specific antigens. This would support the view that certain lymphoid cells could transmit specific tolerance to their daughter cells in the absence of the antigen, supposing that replacement by bone marrow derived cells was inhibited. It cannot be excluded, however, that the proliferation of lymphoid cells derived from lymph nodes was decreased in the absence of the thymus, so that the prolonged maintenance of tolerance was merely an expression of a prolonged persistence of the original lymphoid cell population.

Whilst the number of strain combinations investigated remains so limited, any interpretation of the results must be treated with caution. It will obviously be of great interest to know whether all these intricate theories are of general significance or whether they will be found to be related only to the mouse, the one species studied so far.

OTHER DATA FROM TRANSFER STUDIES

Serial transfer of C$^-$ bone marrow in irradiated C57BL hosts was studied by Koller and Doak[204] in an attempt to detect changes in the immunological behaviour of both donor and host cells during their coexistence in the chimaeras. The ability of the chimaeric marrow to cause survival of the recipients for more than 50 days was used as the criterion, but since the authors failed to confirm the chimaeric state of their animals the incidence of reversals remains unknown and makes an interpretation of their results impossible.

Barnes et al.[25] have maintained several "lines" of serially transferred CBA haemopoietic cells which contained the T6 marker chromosome in lethally irradiated CBA mice. They reported on 6 different lines which were each passed at intervals of roughly 12 months, for a period of more than 3 years. In some lines the T6 marker was identified up to the third transfer and these results suggest that they have succeeded in maintaining the donor cells in the foreign hosts for 40 months at least. The authors suggested that this approach

could be useful for the study of the ageing process at the level of cell populations and secondly that more information on the mechanism of leukaemogenesis might be gained from such experimental designs. So far no reports appeared to show that these exceedingly interesting suggestions have been followed up.

It is significant that the clinical observations of the recipient animals after the various transfers indicated no adaptation of the CBA/T6 cell line to its CBA environment. On the contrary, with an increasing number of transfers and also with an increase in the frequency of transfers, the ability of the cells to restore lethally irradiated CBA mice seemed to decline.

The term *adaptation* has been employed by several groups of investigators who carried out serial transfers of haemopoietic tissue in homologous irradiated host animals. The term has been used in a general sense in the earlier studies. No clear distinction was made between a modification of the antigenic properties of the serially transferred cells, rendering them more easily transplantable into homologous animals, and an adaptation of their immunological reactivity against the homologous environment (immunological tolerance towards the new host) causing less secondary disease in the recipients.

Transfer experiments by Urso et al.[432] showed a decreased incidence of secondary mortality in the second homologous transfer, which was tentatively ascribed to the transfer of a significant number of host haemopoetic cells. Cell typing was not performed, however, in these experiments. A secondary passage of rats → mouse chimaeric marrow led to a high incidence of reversals in the second hosts. Ilbery[183] working on the retransplantation of bone marrow from radiation chimaeras produced with homologous foetal liver cells reported a decreased incidence of homologous disease. As the donor cells were not identified and the number of mice employed was very small, these results are of limited significance. In a subsequent paper Ilbery and Winn[185] described the transfer of an homologous line of cells carrying a chromosome marker through three transfers, but their data failed to reveal a decrease of secondary disease in the recipients who received secondarily or tertiarily transferred cells. It is not clear on what grounds the authors have concluded that "adaptation" of their cell line to the homologous host did occur.

A specific immunological tolerance of the donor cells towards the host tissues was demonstrated unequivocally in the 4th passage of a

C57BL → (CBA × C57BL)F_1 hybrid transfer line in 1961 by van Bekkum and Weyzen[53]. The development of tolerance was initially thought to be promoted by the continuous transfer, but it was later found that tolerance had already developed after the first transfer. In the same study, the quantitative aspects of the transfer procedure were investigated in more detail[53], and an attempt was made to establish optimal conditions for the continuous transfer of haemopoietic cells both in isologous recipients and in a combination consisting of parent strain cells and F_1 hybrid recipients.

Particular attention was paid to the number of cells and the duration of the intervals required to maintain the transfer line. In the isologous transfers, when bone marrow dosages up to 8×10^6 cell per transfer were used, intervals of one or two weeks resulted in the loss of the transfer line due to the death of the recipient animals within the first four transfers (Fig. V^5). Bone marrow cells from the
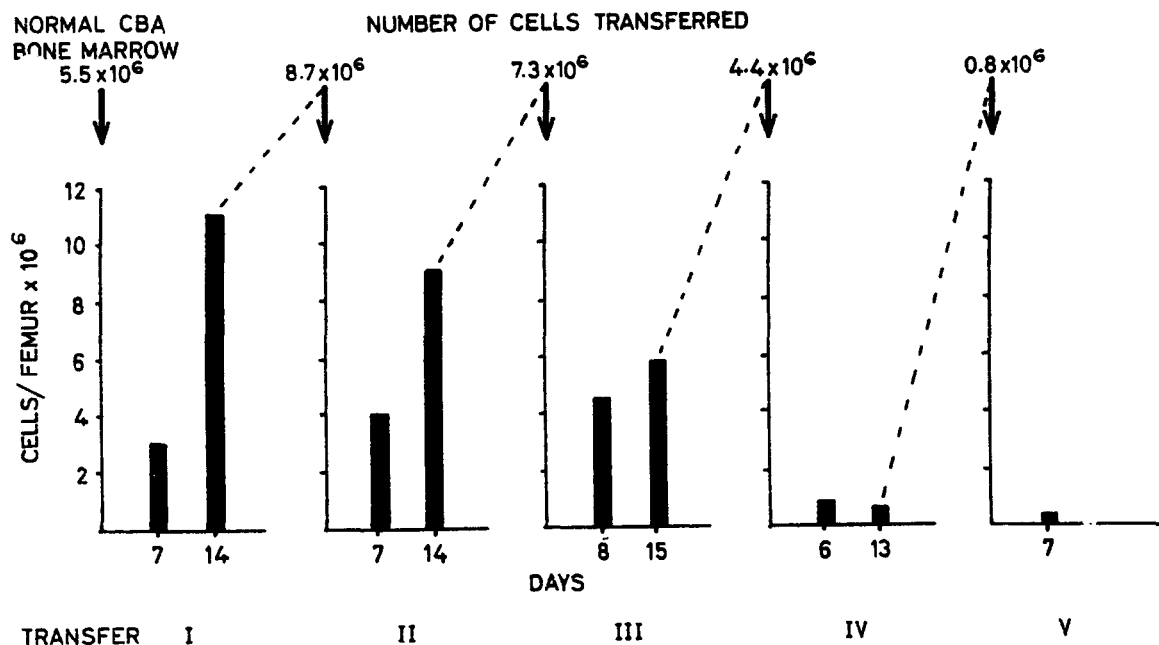
Figure V^5. Yield of bone marrow cells upon serial transfer in lethally irradiated isologous (CBA) hosts. Data from van Bekkum and Weyzen (1961)[53]

Normal value for CBA mice: 10^7 cells/femur
Intervals between transfers: 13–15 days as indicated by arrows

first transfer were tested on the 7th day following transplantation and were found to have a reduced ability to protect lethally irradiated isologous mice. It was thought that one of the factors responsible for

the diminished restorative efficacy of the transferred cells might be a relative decrease in the number of lymphoid cells in the bone marrow upon repeated transfer. Therefore, a repeated transfer of spleen cells plus bone marrow cells was compared with a transfer line in which an equivalent number of bone marrow cells was transplanted[53]. The spleen cell transfer failed in its 7th transfer but it was found that after the first few transfers the lymphoid elements in the spleen became largely replaced by myelopoietic and erythropoietic cells. Thus very few, if any, lymphoid cells were actually present in the subsequent transfers. Cudkowicz et al.[113] studied the addition of normal isologous lymphoid cells (10^7) to bone marrow from tertiary recipients of a bone marrow transfer series (5×10^5 cells at intervals of 35 days). Without additions such bone marrow proved inadequate in the protection of quarternary recipients. The addition of the lymph node cells enhanced the survival of the recipients but failed to increase the "lymphocyte" levels in the marrow. The latter were found to decrease to very low values upon successive transfers of bone marrow, and the capacity to take up ^{131}I labelled 5-iodo-2' deoxyuridine in the spleen also decreased. This uptake was taken as a reflection upon the capacity of the transferred cells to proliferate and the authors concluded from this and other studies[111, 114] that the marrow "lymphocyte" and *not* the lymph node lymphocyte is a primitive precursor of other types of haemopoietic cells. It was postulated, therefore, that depletion of bone marrow "lymphocytes" is the main cause of the eventual failure of transfer lines.

The present authors have not been able to maintain short interval bone marrow transfer lines by the addition of normal isologous lymph node cells at each transfer[46]. This seems to be in accordance with the observations of Micklem and Ford[277] that lymph node cells "homed" almost exclusively to the lymphoid tissues after intravenous administration to lethally irradiated mice. These studies were performed with chromosome markers.

It seems, therefore, that the decreased restorative potential of serially transferred haemopoietic cells has to be ascribed to a gradually decreasing content of so-called stem cells, this term being used to describe the primitive haemopoietic precursors which are required for the repopulation of the host's haemopoietic tissues. Indeed it has been shown by Cudkowicz et al.[114] as well as by Siminovitch et al.[369] that retransplanted bone marrow contains a decreased percentage of cells which are able to form colonies in the spleen of an irradiated

animal. This deficiency is particularly evident during the first two weeks following the initial bone marrow transplantation.

Furthermore, there are strong indications that colony-forming potency and the ability to protect lethally irradiated mice are closely related properties of bone marrow. Although the exhaustion of "stem" cells seems at present the most likely explanation for the failure to maintain serial bone marrow transfers made over a short interval of time, a depletion of certain more differentiated cell types, e.g. mega-karyocytes, has not been excluded as an (additional) causal factor.

A disease resembling secondary disease in several aspects was observed by Barnes et al.[34] in irradiated mice after restoration with isologous marrow which had been transferred several times. This disease could be prevented by the administration of normal compatible lymph node cells. The authors ascribe this beneficial action of the lymphoid cells to a trophic or metabolic function of the lymphocytes.

Serial transfers are facilitated by employing larger intervals of time and larger numbers of cells. Using a mean interval of 34 days and a mixture of bone marrow and spleen cells totalling 10–18×10^6 cells/mouse, an isologous CBA line was kept for 18 transfers until an outbreak of Tyzzer's disease among these mice terminated the experiments[53]. Since no chromosome markers were used, there was no proof that the original cell population persisted throughout the transfer line or, in other words, that cells derived from any of the successive hosts had not contaminated or even replaced the original line.

The possibility of distinguishing the transferred cells from the recipient cells was introduced by serially transferring C57BL cells in (CBA $\times$ C57BL)F_1 mice. One of those lines was maintained for 8 transfers with intervals of about 1 month between each tranfers. When the erythrocytes of survivors of the 8th transfer were typed at 65 days following transplantation, only 1 out of the 8 mice was found to be a complete chimaera. Apparently, a gradual replacement of the transferred C57BL population with F_1 hybrid cells had occurred, which suggests that even the heavily irradiated host cells were, over a period of time, in a more advantageous position than the continuously proliferating cells of the C57BL line.

Clearly, the possibilities of this technique have not been fully explored. In particular with the introduction of chromosomal cell markers the serial transfer system seems rather attractive for the study of a variety of problems, e.g. proliferation kinetics, ageing and leukaemogenesis.

Secondary Disease Following Bone Marrow Transplantation

Recognition of a secondary syndrome

In the first few years of experimental bone marrow grafting the ultimate fate of 30-day survivors among the treated animals seems to have received little attention. The reasons for this lack of interest are presumably to be sought in the emphasis that was placed on the elucidation of the nature of the protective factor in haemopoietic cells. In addition, a large proportion of the experiments were performed with isologous host donor combinations, which—as will be described—do not normally develop secondary disease.

The first report of secondary mortality was given in 1954 at the Liège Radiobiology Symposium by Barnes and Loutit[29] at a time when most other workers in the field favoured the hypothesis that the recovery factor was a chemical agent or hormone. The authors described that 9 out of 16 lethally irradiated CBA mice which received spleen cells of strain A mice survived 30 days, but that deaths occurred soon afterwards and that all mice had died before the 100th post-irradiation day. In contrast, treatment with isologous spleen cells afforded long-lasting protection.

In a later paper the same authors[31] were still unable to interpret the previously reported difference in long term survival rate between animals treated with isologous and with homologous spleen cells. They did mention for the first time, however, the possibility that the delayed death following homologous spleen transplantation could be the result of an immunological reaction. To quote them: "However if this . . . death is due to delayed production of histotoxic antibodies by the host against the graft or by the graft against the host . . . ". Thus, even before the cellular nature of the recovery factor had been formally established, they were able to outline the problem which was to keep the whole field of experimental bone marrow transplantation occupied for a number of years, namely: Is secondary mortality

caused by a host versus graft or by a graft versus host immunological reaction?

Soon afterwards others[38] confirmed the observations by the Harwell workers on delayed mortality following homologous bone marrow transplantation. In both a homologous and heterologous (rat to mouse) combination over 50 per cent of the 30-day survivors died before the 100th post-irradiation day.

At about the same time (1956) the Rijswijk group[77] drew attention to the severe diarrhoea which occurs in the animals during the period of delayed mortality. In addition, the animals showed various other symptoms, e.g. a severe weight loss (wasting) and skin lesions. The entire syndrome has been termed secondary disease. Secondary disease has remained over the years the term of preference since it provides a proper distinction from the *primary* disease (radiation sickness or the bone marrow syndrome) without reference to its, as yet, incompletely elucidated cause. Others have used the terms homologous disease and foreign bone marrow disease, thereby neglecting the reported observations of a similar syndrome following isologous bone marrow transplantation. As early as 1956[77] it was pointed out that delayed mortality can be observed in CBA mice treated with *isologous* bone marrow, when the dose of whole body irradiation is increased above the LD_{100} minimum. This suggested that factors other than immunological ones were involved in the pathogenesis of secondary disease.

Trentin[414] also published in 1956 some interesting details of delayed mortality in mice which had received foreign bone marrow following a standard LD_{100} of whole body X-irradiation (770 r). Each mouse received roughly 25×10^6 cells which is comparable to the number employed by the investigators at Rijswijk. In contrast to the long-lasting protection seen with isologous marrow, the transplantation of foreign marrow cells resulted in considerable mortality between days 21 and 100 in all 5 combinations tested. Marrow from F_1 hybrids injected into irradiated parent strain mice was on the average intermediate in protective activity between isologous and foreign strain marrow. In only 2 out of the 4 combinations tried, was the mortality appreciable after day 21.

Trentin's studies included the transplantation of host and donor type skin on the chimaeras; the results tended to confirm the earlier findings of Main and Prehn that skin types compatible with the host as well as those compatible with the donor were accepted. The author

discussed the suggetion made by Main and Prehn that the tolerance to skin of the marrow donor type might be similar to the acquired immunological tolerance produced by Medawar and his co-workers in newborn mice by the injection of large numbers of foreign spleen cells. Trentin formulated the issue with admirable clarity as follows: "After protection with homologous or heterologous marrow against an otherwise lethal dose of X-irradiation, does the host's antibody producing tissue survive in a functional sense, or is it completely replaced by the comparable system of the marrow donor? Only in the first case must one postulate an altered immunological specificity of the surviving host system."

Exactly the same question has been repeatedly raised in discussing the acquired tolerance produced by the injection of cells into newborn animals. Interestingly enough, the first convincing results suggesting at least partial replacement of the host's lymphatic system by donor type cells in such tolerant animals were provided by Trentin and Session in 1962[419].

Turning again to the problem of secondary disease it should be recalled that Trentin drew attention to the fact that his affected mice had essentially normal blood counts, which argued against bone marrow graft rejection as the underlying mechanism. Furthermore, he suggested the possibility of an immunological reaction of the graft-derived immunological system of the chimaera directed against the host.

Identification of secondary disease as a graft versus host disease

In 1957 the dispute over the pathogenesis of secondary disease became fully developed, as was reflected in the papers and discussions on this subject at the Gatlinburg symposium of that year. At this time the parties involved took more sharply defined positions.

The Harwell workers[24] were clearly in favour of a graft versus host immunological mechanism and extended this hypothesis by pointing out that exhaustion of the donor immunological system could occur as a result of the overwhelming and continuous presence of host type antigens. Such immunological exhaustion would fit in with the lymphoid atrophy described first by Congdon and Urso[99] and later by many others in chimaeras suffering from secondary disease. This condition could conceivably bring about death from a reduced immunological defence against infections.

Diametrically opposed to this graft versus host concept were the

ideas of the Oak Ridge group headed by Makinodan, who used the term "delayed foreign bone marrow reaction" and attributed it to host versus graft immunological reactivity[242]. Their hypothesis was based on studies of the influence of the X-ray dose on the occurrence of secondary mortality and their inability to detect rat globulins in the serum of rat → mouse radiation chimaeras. The second argument was a rather inadequate one since Makinodan's negative findings were contradicted by the clearcut positive results obtained by Weyzen and Vos[460], whose findings were reported at the same meeting[459]. These investigators were consistently able to demonstrate the presence of rat proteins in the globulin fraction of the serum of mice approximately 100 days after irradiation and rat bone marrow transplantation. The chimaeric state of these mice had been confirmed by typing of the erythrocytes and the granulocytes. Soon afterwards these observations were confirmed by Grabar et al.[163, 164] in rat → mouse chimaeras produced at Harwell.

THE GENETIC APPROACH

As was pointed out by Koller[201], the dilemma of which system reacts against other, thereby causing secondary disease, could theoretically be solved with ease by a study of host-donor combinations of the appropriate genetic constitution. According to the basic rules
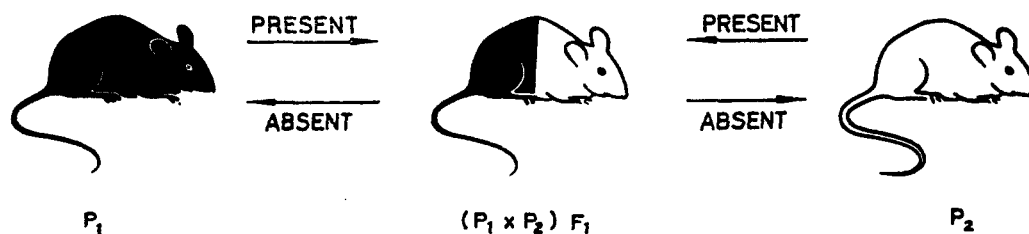
Figure III[1]. Theoretical occurrence of secondary disease in bone marrow transfers between mice of an inbred strain and their F_1 hybrids. Arrows indicate direction of bone marrow transfer

of tissue transplantation, F_1 hybrid mice cannot react against parental tissues while parent strain mice reject tissues of the F_1 hybrid. If a graft versus host reaction were involved, delayed mortality would not occur in F_1 → parent strain chimaeras, while being prominent in the reverse combination (Fig. III[1]).

The data initially available on this point were not conclusive. Koller's results—admittedly meagre ones—with $(A \times C57BL)F_1$ hybrids suggested that the F_1 was better as a donor than as a host.

Van Bekkum and Vos[49], using $(CBA \times C57BL)F_1$ hybrids found virtually no secondary mortality in either combination, and as mentioned before Trentin[414] found F_1 hybrid marrow to be intermediate between isologous and homologous marrow in causing secondary mortality. In a subsequent paper Trentin[416] attributed the late mortality of the mice treated with F_1 hybrid marrow to the use of very young recipients, suggesting that his results should be interpreted as affirming the graft versus host mechanism of secondary disease as pointed out above.

A few months afterwards Uphoff drew attention to what she called the "F_1 hybrid effect". When F_1 hybrid mice were exposed to a lethal dose of X-radiation and treated with bone marrow, the F_1 (isologous) marrow afforded better "protection" than marrow of either of the parent strains. Protection was used in this context as the equivalent of long term survival[423]. This Uphoff interpreted as support for a graft versus host mechanism in secondary disease. In the same paper preliminary experiments were announced which showed better "protection" being afforded by the F_1 hybrid marrow in a parental strain than by bone marrow of the parental strain in the hybrid. Details of these experiments, which were carried out with a number of inbred strains and their F_1 hybrids, appeared in 1958[427]. In every combination, except one, secondary disease developed in the F_1 hybrids which received parental marrow, while the mice which received F_1 hybrid bone marrow showed little or no secondary mortality. The one exception (no secondary disease following treatment with parent strain marrow) was provided by an F_1 hybrid whose parental strains (NALB/c and DBA/2) were identical at the H-2 locus, both being $H-2^d$. The H-2 locus is considered to be the most important histocompatibility locus in these strains. Some delayed mortality was observed in genetic combinations which did not allow for any graft versus host reaction, but these mice died from amyloidosis of the kidney or from pneumonia and failed to show the characteristic symptoms of secondary disease.

Thus, the genetic approach to the problem failed to provide uniform results in different laboratories. Furthermore, late radiation effects tended to complicate the picture as was to be expected from the observation of secondary mortality in certain experiments with isologous bone marrow transplantation. This confusion was increased by the variations observed in the time of onset of the symptoms of secondary disease and the inability of a number of investigators to

distinguish between delayed mortality due to a rejection of the bone marrow graft and secondary mortality in mice which carried adequately functioning foreign haemopoietic tissue. This distinction was first pointed out by de Vries and Vos[449] on the basis of an elaborate histological study.

ANALOGOUS CONDITIONS

Additional indirect evidence in favour of a graft versus host mechanism was derived from the similarity between secondary disease and the runting syndrome which results from the transplantation of homologous spleen cells into newborn mice, as described by Billingham and Brent in 1957[63].

Runt disease. The main symptoms of "runt disease" are greatly retarded growth and development, diarrhoea, varying degrees of hypoplasia of the lymphatic system, skin lesions and focal necrosis of liver cells. Every one of these symptoms has been encountered in mice suffering from secondary disease following foreign bone marrow transplantation. The hypothesis of a graft versus host immunological reaction stimulated a number of investigators to explore the effects of an injection of lymphoid cells in addition to bone marrow into irradiated recipients[88, 96, 184, 246, 362, 440]. The common trend which emerged from these various studies was in accordance with an immunological reaction of the injected cells directed against the host: higher numbers of lymphatic cells caused early mortality (within 6–10 days)*; with lower cell numbers secondary disease was obtained which was more pronounced than when following transplantation of bone marrow alone. Perhaps one of the most convincing pieces of evidence which support the graft versus host mechanism in marrow chimaeras has been the discovery that on increasing the number of lymphoid cells injected together with the bone marrow, the severity of secondary disease could be increased and the delay between grafting and the appearance of symptoms could be reduced.

Homologous disease. A syndrome called homologous disease has been induced in adult non-irradiated F_1 mice by the intravenous administration of large numbers (more than 10^7) of spleen or lymph node cells[292, 440]. Not all of the animals so treated died during the observation period, but the clinical and morphological findings in the

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* Also termed the "acute killing effect".

mice that succumbed, showed a striking resemblance to those found in mice dying with secondary disease.

Parabiosis. Finally Trentin[418] and van Bekkum *et al.*[39, 52] independently identified the disease which develops in the F_1 hybrid partner following parabiotic union with a mouse of one of the parent strains, as analogous to the diseases described above. The F_1 hybrid partner usually showed a progressive loss of weight, resulting in a characteristic wasted appearance. In five F_1 hybrid partners of such parabiotic twins as have been described, typical skin lesions developed

TABLE III: 1. Various forms of graft versus host reactions in rodents

Disease	Donor cells	Recipients	Survival Time (Days)
Secondary disease (foreign bone marrow reaction)	Foreign bone marrow	Lethally irradiated	20–100
"Acute killing" effect or early secondary disease	Foreign lymphoid cells	Lethally or sub-lethally irradiated	6–14
Homologous disease	Massive doses of parent strain lymphoid cells	Non-irradiated F_1 hybrids	20–60
Runting	Homologous lymphoid cells	Newborn or foetal	10–60
Complications of parabiosis	Union between F_1 hybrid and a partner of one of the parent strains		17–97

but the diarrhoea characteristic of secondary disease in mice was not observed. Liver necrosis and lymphoid atrophy were found microscopically in the F_1 partners.

All these findings lent substantial support to the concept of a graft versus host mechanism in secondary disease but still constituted no more than strong indirect evidence. Table III: 1 shows a summary of the different graft versus host syndromes which have just been described.

DIRECT EVIDENCE OF ANTI-HOST ACTIVITY

Many attempts have been made to obtain more direct proof of the immunological activity of the proliferating donor system directed

against host antigens. Evidently, one of the first things to look for would be the presence of humoral antibodies against host type erythrocytes in the chimaera's serum, the more so because haemolytic anaemia and jaundice have been observed in several species during the period of severe secondary disease. In homologous rabbit chimaeras both Porter[328] and Piomelli and Brooke[314] found a decreased half life for the recipient's erythrocytes labelled with ^{51}Cr and normal life spans for the donor erythrocytes. In addition, a positive direct antiglobulin reaction (Coomb's test) was obtained with the erythrocytes of the chimaeras. Moreover, when stored erythrocytes of the recipient were exposed to the serum of the chimaeric animal these erythrocytes could be agglutinated with antiserum to rabbit gamma globulin, indicating the presence of incomplete antibodies against the host in the serum of the chimaera. In a few cases such positive tests were obtained with donor erythrocytes, but these animals were presumedly in a stage of reversion to host type. Shaw and Vermund[366] have observed an extraordinarily strong agglutinating activity directed against host type erythrocytes in the serum of heterologous chimaeras produced by the transplantation of bone marrow from the ring dove into lethally irradiated pigeons. The haemagglutinins appeared as early as 4 days after bone marrow transplantation and caused *in vivo* agglutination of the erythrocytes; this may have contributed to the high incidence of early mortality in these pigeons.

In mouse and rat radiation chimaeras such unequivocal results have not been obtained. Although Uphoff mentioned in 1957 that Amos, using haemagglutination techniques, had detected antibodies against the host in her experimental F_1 hybrid mice[423], which had received parental marrow following irradiation, this finding was not subsequently confirmed.

Goodman and Smith[160] studied the life span of erythrocytes in radiation chimaeras and found quite variable values. In some early homologous chimaeras, host-type red cells disappeared abnormally fast, but at a later stage the rate of disappearance was normal. The authors believed the early rapid decrease of host cells to be the consequence of the radiation-induced haemorrhagic syndrome instead of the result of a graft versus host immunological reaction.

Conflicting data were obtained by Harriss *et al.*[168] who studied the rate of disappearance of ^{51}Cr labelled erythrocytes in sublethally irradiated F_1 hybrid mice which had received large numbers of lymph node or spleen cells from a parental strain. These animals

developed wasting and severe anaemia, which was accompanied by an accelerated loss of both host and donor type erythrocytes. Serum haemagglutinins directed against both the donor and recipient type erythrocytes were detected by Uyeki[433] in rat → mouse radiation chimaeras between 3 and 10 weeks following transplantation. Unfortunately, no tests were performed to establish in detail the chimaeric state of the experimental animals so that these results remain unexplained. It should be mentioned that Weyzen[458] has failed to demonstrate, directly or indirectly, antibodies against mouse erythrocytes in rat → mouse chimaeras at any time following transplantation.

A quite sensational development was provided by the reports of Koller and co-workers[124, 205] who showed that, in homologous mouse radiation chimaeras, host skin grafts were rejected in a large proportion of test animals if the skin was transplanted soon after the transplantation of the bone marrow. Other workers studying the behaviour of host and donor type skin in such chimaeras failed to observe this phenomenon and Koller's findings in bone marrow chimaeras have thus far not been confirmed.

However, Stastny et al.[385] have recently described rejection of autografts in rats that were suffering from severe homologous disease as a result of repeated injections with large numbers of lymphatic cells. These animals had been made tolerant to the homologous lymphoid cell graft by an injection after birth with spleen and lymph node cells. Balner[15] has similarly observed a rejection of skin autografts in irradiated rats, which had received large numbers of homologous spleen cells in addition to homologous bone marrow. The possibility of autograft rejection under conditions of graft versus host reactivity seems, therefore, to have been established. As will be discussed in Chapter IV, the histological changes in the skin of radiation chimaeras strongly suggest a similar reaction against the host's own skin and are not easily explained by any other mechanism. This lends support to the concept that the skin lesions which occur in secondary disease in a variety of species are caused by an immunological attack of the donor type lymphatic cells or their products against dermal and epidermal tissue. This concept was initially proposed on the basis of histological changes in the affected portions of the skin, reminiscent of the lesions noted in skin homografts in the course of their rejection.

G

TRANSFER EXPERIMENTS

A different approach was used by Feldman and Yaffe[135] who tried to demonstrate the production of anti-host antibodies by the bone marrow graft in a series of ingenious experiments. They assumed that circulating antibodies against the host tissues, if produced by the donor immunological system, would not be detectable because of their complete absorption by the isoantigens of the host's tissue. In an attempt to avoid this complication they transplanted the spleen and lymph nodes of the chimaeras into normal animals of the same inbred strain as the original donors, according to the following schedule:

C57BL   C3H   12 days
———————→   ————————————→   normal C57BL
spleen   900 r   spleen +
                                  lymph nodes
                                  of 3 mice
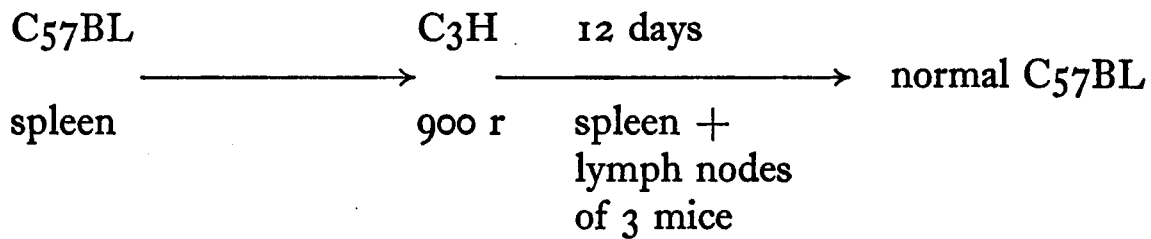
The serum from the second recipients showed a titre of 1 : 124 for agglutinins against C3H erythrocytes on the 5th day following transplantation. Suitable control animals showed no agglutinins. This finding indicated that the lymphoid cells from the chimaeras were stimulated to produce antibodies (agglutinins) against the host.

In another series of experiments the same investigators used a transfer of bone marrow cells to demonstrate a "second set" effect of graft versus host activity, as shown in Table III: 2.

They interpreted the shorter survival time of the *secondary* C3H recipients (II) of the C57BL bone marrow as the possible manifesta-

TABLE III: 2. Data provided by Feldman and Yaffe[135] to demonstrate anti-host sensitization of donor cells in mouse radiation chimaeras

Bone marrow from	Recipients (800r)	Mean survival time (days)
I C3H———→C3H Isologous chimaeras (12 d. following transplantation)	C3H mice	>60
II C57BL———→C3H Homologous chimaeras (12 d.)	C3H mice	7
III Normal C57BL mice	C3H mice	29
IV No bone marrow	C3H mice	8–9

tion of a secondary immunological response. Unfortunately, the number of cells administered in the successive transfers has not been reported and their study was incomplete because there were no histological data to permit a distinction between early death from inadequate haemopoietic graft prolliferation and death from a graft versus host killing effect. In view of the data published later by van Bekkum and Weyzen[53] on the serial transfer of haemopoietic cells in irradiated mice, it now appears unlikely that the number of cells which were transferred in Feldman and Yaffe's experiments were sufficient to protect the secondary host from haemopoietic failure.

Still another method has been introduced to study directly the extent of the anti-host immunological activity of chimaeric cells[41]. Spleen cells of chimaeras, which according to current identification tests had donor type haemopoiesis, were injected into newborn mice of the appropriate genetic constitution (see Fig. III²) according to the graft versus host assay devised by Simonsen and Jensen[374]. With this technique direct evidence of the anti-host immunological activity of the chimaeric cells has been obtained: spleen cells from chimaeras which are known to develop severe secondary disease induced all the signs and symptoms of a graft versus host reaction in the newborn mice. One could reason that this merely demonstrated that the donor system in the chimaeras retained the capacity to react against foreign antigens, but on the other hand it is illogical to suppose that this capacity would not become manifest in the chimaera. That the results of the Simonsen assay reflect the actual reactions of the donor cells in the chimaera was substantiated by the discovery that the magnitude of the graft versus host reaction in newborns injected with a standard number of spleen cells from radiation chimaeras paralleled the severity of the clinical and histological signs of secondary disease in the chimaeras. Furthermore, cells from chimaeras which had recovered completely from secondary disease were found to be specifically non-reactive to host type antigens in the Simonsen assay. This approach has proved to be extremely useful in the study of immunological tolerance (of the graft towards host type antigens) as it develops in certain host–donor combinations.

By using a modification of the Simonsen assay it has also been possible to demonstrate graft versus host activity in rat → mouse radiation chimaeras.

More recently Doria[126, 127] has provided substantial evidence in favour of graft versus host activity in radiation chimaeras. The intri-

A

MOUSE   RAT

BONE MARROW   BONE MARROW

B

MOUSE   MOUSE

VARIOUS INTERVALS

SPLEEN CELLS   SPLEEN CELLS

SIMONSEN ASSAY   MODIFIED SIMONSEN ASSAY

NEWBORNS   SUBLETHALLY IRRADIATED
$(A \times B)F_1$ NEWBORN MICE

MEASUREMENT OF   MEASUREMENT OF
SPLEEN AND LIVER   SPLEEN AND THYMUS
INDEX AFTER 10 DAYS   INDEX AFTER 10 DAYS
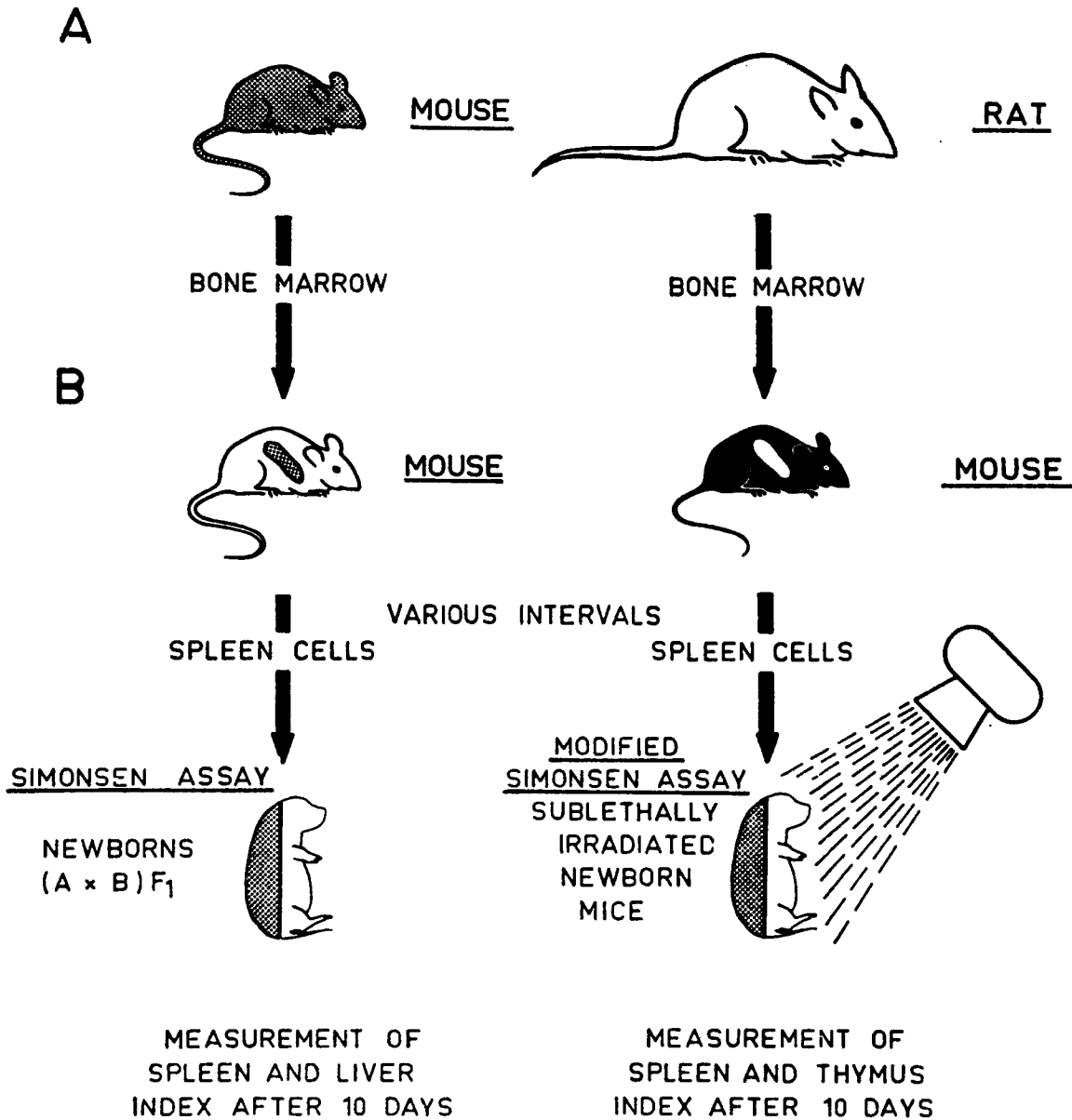
Figure III[2]. Schematic representation of graft versus host assay with spleen cells of radiation chimaeras to demonstrate anti-host activity

Left   : Homologous chimaeras
Right : Rat → mouse chimaeras
(A)   : donor
(B)   : radiation chimaera

cate test systems that were devised for this purpose are shown in Fig. III[3].

In the first test system the spleen cells of chimaera B (parent bone marrow into F_1) reacted in much the same way as the spleen cells from parental mice which had been sensitised against F_1 hybrid cells and this was considered as indirect evidence for the presence of anti-host activity in the chimaera.

(A) INDIRECT ASSAY
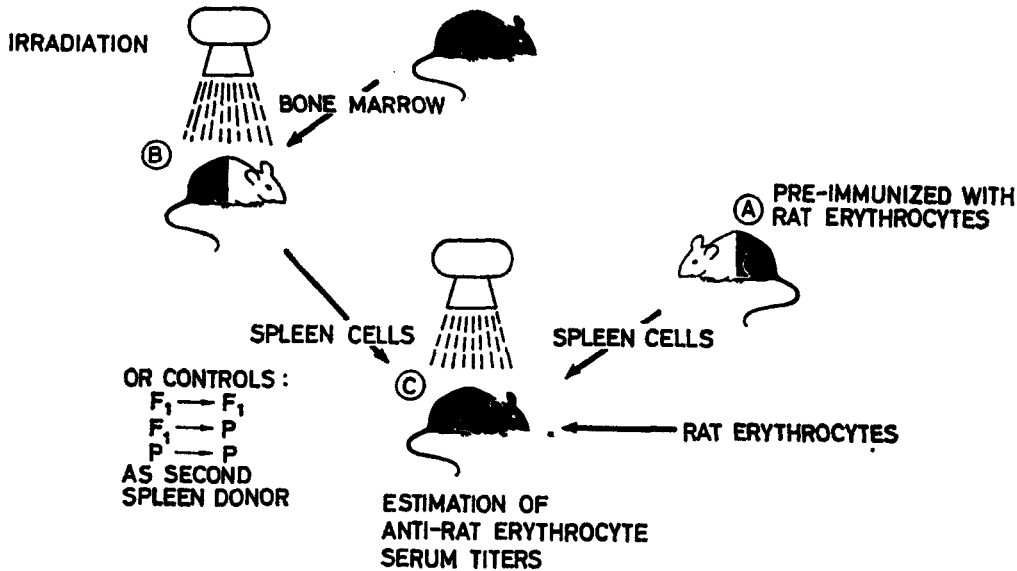
(B) DIRECT ASSAY
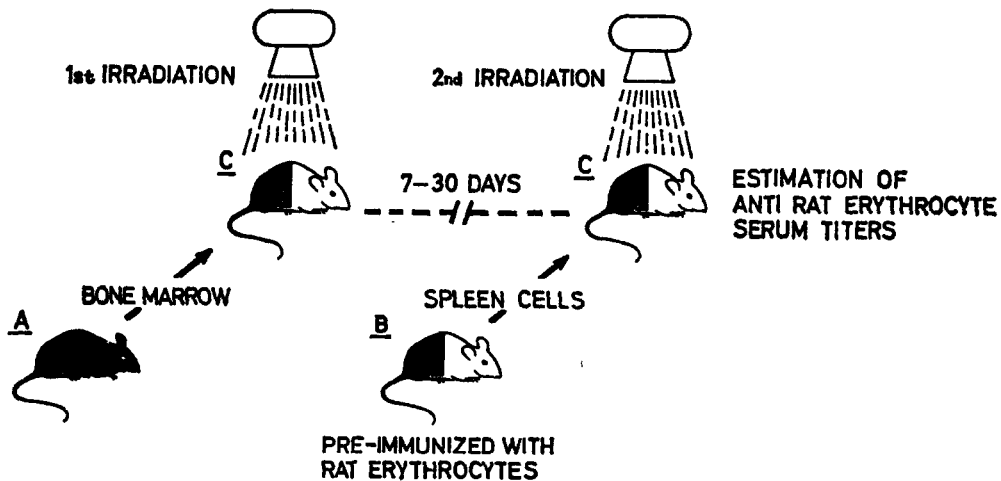
Figure III[3]. Transfer techniques employed by Doria[126, 127] in mice to demonstrate anti-host immunological activity in radiation chimaeras

(a) Indirect assay: In the case of graft versus host activity existing in chimaeric spleen donor B (P→F₁), these spleen cells will reject A donor cells when both B and A spleen cells are transferred to lethally irradiated recipient C. This results in decreased levels of anti-rat blood cells agglutinins in C, since such agglutinins can only be produced by A cells under the conditions of the experiment.

(b) Direct assay: In the case of graft versus host activity existing in the chimaera C (P→F₁) the spleen cells of B will be rejected and the production of anti-rat blood cell agglutinins by B cells will be diminished.

In Doria's second experimental set-up, evidence for a decreased functional activity of host type (F_1) cells that were transferred to $P \rightarrow F_1$ chimaeras was obtained and this was attributed to the presence of a graft versus host immunological reactivity. The results obtained with both experimental set-ups were exceptionally clear-cut.

THE MORPHOLOGICAL EVIDENCE

Although this subject will be dealt with in detail in Chapter IV it must be mentioned here that histological examination of the tissues of animals suffering from secondary disease has provided strong arguments in favour of a graft versus host pathogenesis. In particular the study of cases of severe secondary disease as they occur in monkeys after the administration of homologous bone marrow and in mice which have received large doses of lymphoid cells has been very illuminating. Under these conditions, which result in early death, destructive changes in host tissues notably the skin and the intestinal mucosa have been found to be accompanied by the infiltration of these tissues with lymphoid cells presumedly of donor origin. Admittedly, a causal relationship between these phenomena was not proved by these observations, but they made it very likely indeed.

If the experimental evidence described in the preceding paragraphs is taken all together it seems that there can be no doubt that the principal underlying cause of secondary disease is a graft versus host immunological reaction. The most convincing arguments in favour of such a mechanism have come from the use of certain genetical host-donor relationships, the demonstration that transferred spleen cells of the chimaera exhibit anti-host reactivity, significant morphological findings and finally from the observation that secondary disease can be provoked and intensified by the injection of homologous lymphoid cells. The manner in which the graft versus host reactions produce the variety of lesions and clinical symptoms as well as the actual cause of secondary death remain, however, the subject of continuing research and discussion.

Description of secondary disease and related syndromes

PATTERNS OF SECONDARY DISEASE AND MORTALITY

The severity and the course of secondary disease varies among different species (Fig. III[4]) and also within a species, depending on the source, nature and number of the grafted cells. The most severe
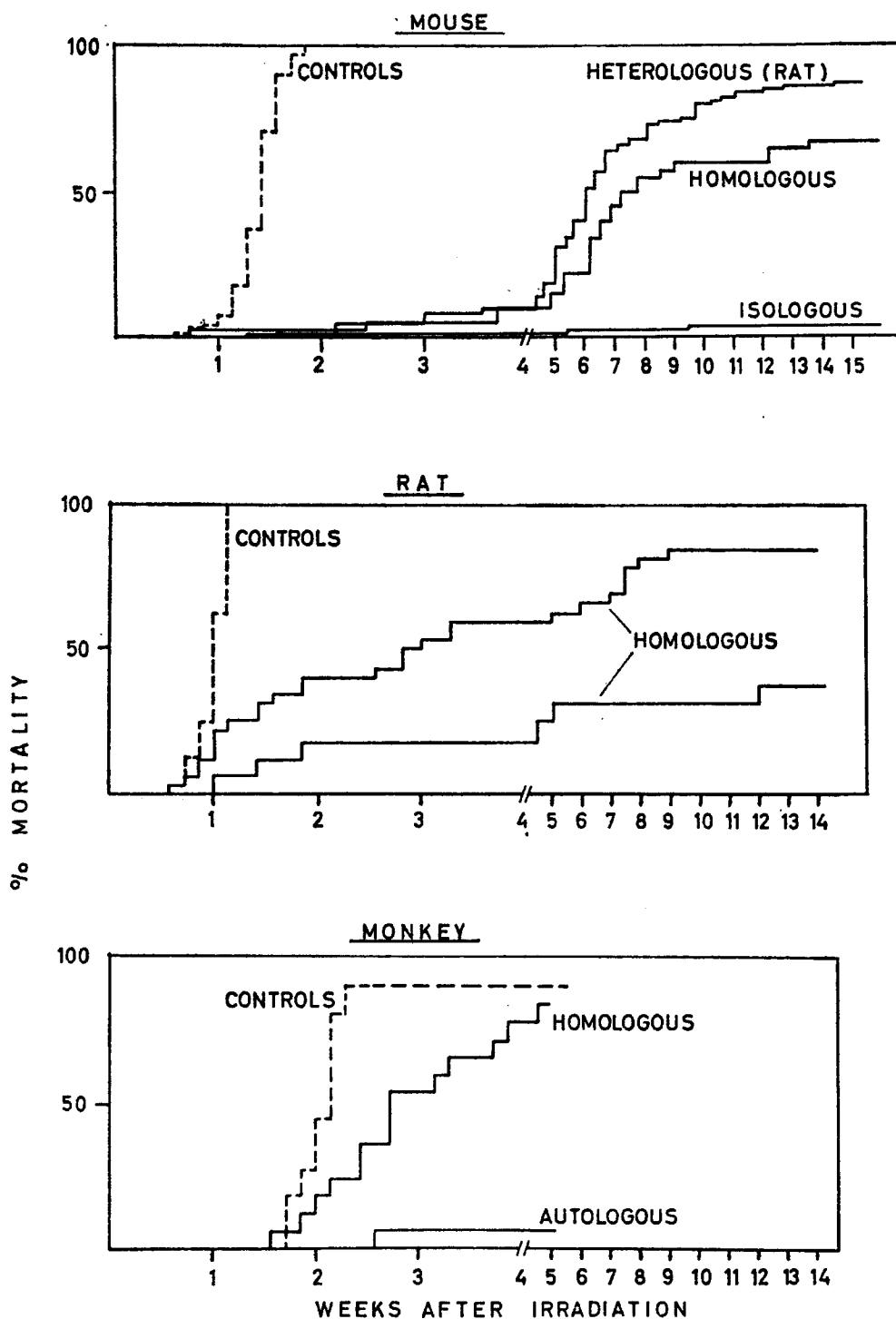
Figure III[4].   Time-pattern of secondary mortality in three animal species. Data from van Bekkum and Vos (1959)[49] for the mouse, Balner (1964)[18] for the rat, Crouch et al. (1960)[108] and van Putten[340] for the monkey.

The mortality within the first two weeks in rats following homologous bone marrow transplantation is due to graft failure and infection.

The curve for monkeys treated with homologous bone marrow is based exclusively on monkeys in which a take of the graft was evident from the presence of donor granulocytes. Other monkeys have been observed to die as early as 7 days after transplantation with characteristic signs of graft versus host disease and before regeneration of the bone marrow had even started

form following bone marrow transplantation has been encountered in monkeys and the limited experience with human patients has suggested a similarly severe course. Mortality in the monkey may occur so early that it falls within the period in which death from bone marrow failure occurs in untreated controls. The two causes of death can only be differentiated by a microscopic examination of certain affected tissues, notably the blood forming ones. In rodent chimaeras secondary disease usually begins after the 3rd week and secondary mortality can therefore be clearly distinguished from primary radiation death.

Much confusion has been created by the failure of some investigators to define secondary disease correctly. They failed to distinguish it from a delayed rejection of the bone marrow graft by means of post-mortem examination of the critical tissues and by an objective proof of the chimaeric state of the animals. A delayed rejection of the graft may be the cause of death in mice during the second half of the first month, when the radiation dose to which the recipients were subjected has been below a certain level, when the number of foreign bone marrow cells has been too small, or in cases where the immunogenetic difference between host and donor has been too large. Obviously, this condition can be easily recognised by the presence of bone marrow aplasia and pancytopenia and its effects (e.g. haemorrhage), which do not occur in typical secondary disease.

Following transplantation of homologous or heterologous bone marrow from certain species, irradiated mice recover rapidly from radiation sickness and begin to gain weight during the second week. However, between 20 and 30 days after the transplantation the faeces become abnormal and the animals start to lose weight again, which may give rise to varying degrees of wasting. In a proportion of the animals characteristic skin lesions appear as early as the beginning of the second month. These lesions may either subside, or persist for many months.

The peak of mortality from secondary disease falls in the second and third month and the animals which survive for more than 100 days thereafter show a death rate which is usually very low. At the end of the third month, and in some groups of mice even earlier, the diarrhoea and the wasting gradually disappear and it seems that at least partial recovery is taking place; this will be described in Chapter IV.

When the antigenic difference between host and donor is rela-

tively large, e.g. in the case of rat → mouse chimaeras, the recovery after the third month may be less striking, but a certain decrease in the severity of the symptoms is nearly always apparent.

The course of secondary disease in homologous rat chimaeras resembled that seen in mice, except that skin lesions and wasting were sometimes more prominent in rats. In the surviving rats the lesions had a tendency to disappear at the end of the third month and almost complete recovery occurred in the majority of the animals[14].

Porter[325, 327] has described a sequence of secondary disease symptoms and pathological changes in homologous rabbit chimaeras which resemble, in many aspects, those of the mouse. As a symptom of recovery from radiation sickness, the weight started to increase from about the 10th day after irradiation. In those animals which subsequently developed secondary disease, the body weight started to decrease between the 16th and the 40th day and this was accompanied by diarrhoea. Immune haemolysis of host cells was frequently found. The symptoms progressed until the animal died in an emaciated condition. In many animals the immediate cause of death was due to infection, e.g. pneumonia caused by Pseudomonas pyocyaneus. A sharp decrease of the mortality rate occurred at the end of the 3rd month.

A similarly rapid and fatal course of secondary disease, as is seen in monkeys, can be induced in mice by the injection of homologous lymphoid cells in addition to homologous bone marrow. The animals may die as early as the 6th day following transplantation without having developed a clearly defined clinical syndrome. This kind of death can be distinguished, nevertheless, from early radiation death on the basis of histological changes.

Early severe graft versus host reaction induces a number of characteristic microscopical lesions which will be described in detail in Chapter IV. The time of death alone is usually insufficient for a correct assessment of the cause of death. With graded numbers of immunologically competent cells, all degrees of secondary disease can be induced in the irradiated recipients, as is shown in Table III: 3. In these experiments the number of haemopoietic cells injected was in all cases sufficient to prevent death from bone marrow failure. Following the administration of larger amounts of bone marrow, the increase in absolute numbers of lymphoid cells in the graft induced progressively more severe anti-host reactions and an earlier death caused by this reaction consequently occurred.

TABLE III: 3. Mortality of irradiated (700r) (CBA × C57BL) F1 hybrid mice following treatment with spleen cells from C57BL mice†

Number of spleen cells	Days after irradiation*									
	0	10	20	30	40	50	60	70	80	90
20 × 10⁶	10	10	9	2	1	0	—			
10 × 10⁶	10	10	10	10	1	0	—			
5 × 10⁶	10	10	10	10	6	4	4			
1 × 10⁶	10	10	9	9	8	8	8			
0	10	10	0	—	—	—	—			

Mortality of irradiated (800r) CBA mice following treatment with bone marrow cells from C57 BL mice‡

Number of bone marrow cells	Days after irradiation									
	0	10	20	30	40	50	60	70	80	90
6 × 10⁷	20	20	12	1	0					
2 × 10⁷	20	17	14	9	3	1	1	1	0	
1 × 10⁷	30	30	30	24	23	16	11	10	9	8

* Figures in the body of the table represent number of mice surviving
† Data from van Bekkum (1959)[40]
‡ Data from van Bekkum et al. (1959)[88]

RADIATION CHIMAERAS

TABLE III: 4. Symptoms and pathology of secondary disease

	Mouse	Rat	Rabbit	Dog	Monkey	Man
Diarrhoea and colitis	+	—	+	+	+	+
Wasting	+	+	+	+	+	n.i.
Skin lesions	+	+	n.i.	+	+	+
Lymphoid atrophy	+	+	+	+	+	+
Infections	+	+	+	+	+	+
Liver necrosis	+	+	+	+	+	+
Immune haemolysis	—	—	+	n.i.	+?	n.i.
Appearance of secondary disease, days after transplantation	20–30	20–30	20–30	(30)	7–10	5–10

* n.i. = no conclusive information available

SYMPTOMS: DIARRHOEA AND WASTING

These two symptoms and the characteristic skin lesions constitute the main external signs of secondary disease following foreign bone marrow transplantation (Table III: 4). Many authors have described the typical hunched appearance* of the animals and also ruffling of the coat but since these symptoms are generally encountered in animals that are in the terminal state of disease, they cannot be considered characteristic.

Diarrhoea is the term generally employed to describe the condition in mice suffering from secondary disease, which produce abnormal faeces. The stools are bulky and soft and stick to the bedding material as well as to the sides of the cage. In severe cases faecal material adheres to the anal region, forming crusts. Even a mild degree of "diarrhoea" can easily be recognised by inspection of the cage contents, because normal mouse faeces do not stick to the bedding (Plate III: 1).
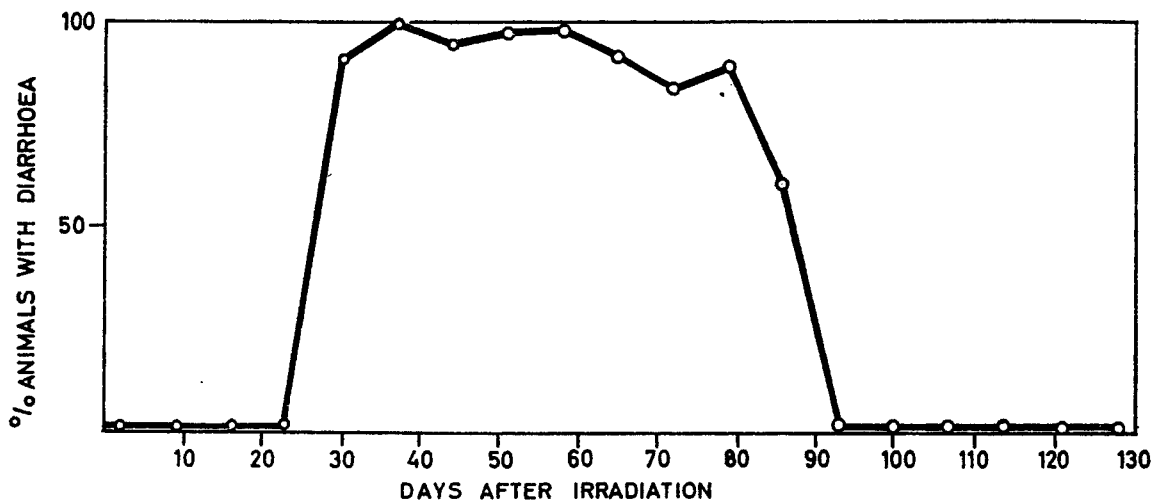
Figure III[5]. Incidence of diarrhoea in C57BL→CBA homologous radiation chimaeras. Data from de Vries and Vos (1959)[449]

It is very likely that wasting is at least partially attributable to the diarrhoea, but it has yet to be proved beyond doubt that the two are causally related. Although there is a very high incidence of diarrhoea in mice suffering from secondary disease (Fig. III[5]), an occasional mouse may be observed, which develops severe wasting without obvious diarrhoea. Furthermore, in other animal species wasting

* Chimaeras with severe widespread skin lesions sometimes develop a peculiar wobbly high-stepping gait, which has also been described in animals suffering from sever homologous disease[1].

has been described in the absence of diarrhoea following foreign bone marrow transplantation.

Diarrhoea has also been found to accompany "homologous" disease in F_1 hybrid mice injected with large numbers of parent strain lymphoid cells, but in these animals it seems not to be as marked as in bone marrow chimaeras after lethal irradiation. In both experimental situations as well as in sublethally irradiated F_1 hybrids treated with parent spleen cells, McRae[272] observed an abnormal handling of the food by the wasting animals. When given access to standard mouse pellets the animals chewed an increased amount of food per day but the amount actually swallowed was decreased. Therefore, the apparent food intake as estimated in the usual way by measuring the decrease of food pellets has to be corrected by the amount of food wasted. Normal mice spoil very little food in this manner (Fig. III[6](A) and (B)).

In one experiment the decrease in food intake largely explained the weight loss as shown in Fig. III[6](C). Lack of salivation seemed not to be the cause of the abnormal eating habit since a wet slurry of food was dealt with in a similar fashion. McRae also observed that this type of food was thrown out of the food dish to a greater extent than is normal by control mice.

McRae's careful observations are in disagreement with a statement by Kretchmar and Congdon[208] that the secondary body weight loss occurs in spite of a nearly normal food intake. The latter statement was not substantiated, however, by experimental data.

It has been shown that the dietary regimen may influence not only the diarrhoea but also the course of secondary disease. The addition of mixed cereals to the normal pelleted diet decreased the incidence and severity of the diarrhoea of radiation chimaeras and, furthermore, had a favourable effect on survival[50]. As will be shown in the next chapter, the diarrhoea can be completely explained by the colitis which is found in these animals. It is not clear, however, in which way the dietary regimen influences the degree of colitis.

In parabiotic F_1 hybrid mice suffering from a graft versus host reaction, diarrhoea was *not* apparent[52]. Chronic diarrhoea seems, however, to be a common feature in mice suffering from the "runting" syndrome following the injection of homologous spleen cells at birth[64]. No information is available on the role which diarrhoea plays in the development of wasting in these runts.

In the homologous rat bone marrow chimaeras which showed

distinct secondary disease and also a high incidence of mortality, diarrhoea was *absent* and intestinal lesions were always mild if present[14]. On the other hand diarrhoea was described as a characteristic symptom of "parabiotic disease" in rats by Nakić *et al.*[288]. Diarrhoea was also reported by Nisbet and Heslop[290] and by Krěn *et al.*[206] to accompany the runting syndrome in rats injected immediately after birth with homologous spleen cells. Billingham *et al.*[66], however, in an extensive study of runting in the rat, considered that diarrhoea was not a typical feature. The latter authors failed to find pathological changes in the intestinal tract of the runted animals and reported that the animals developed diarrhoea in the terminal stages
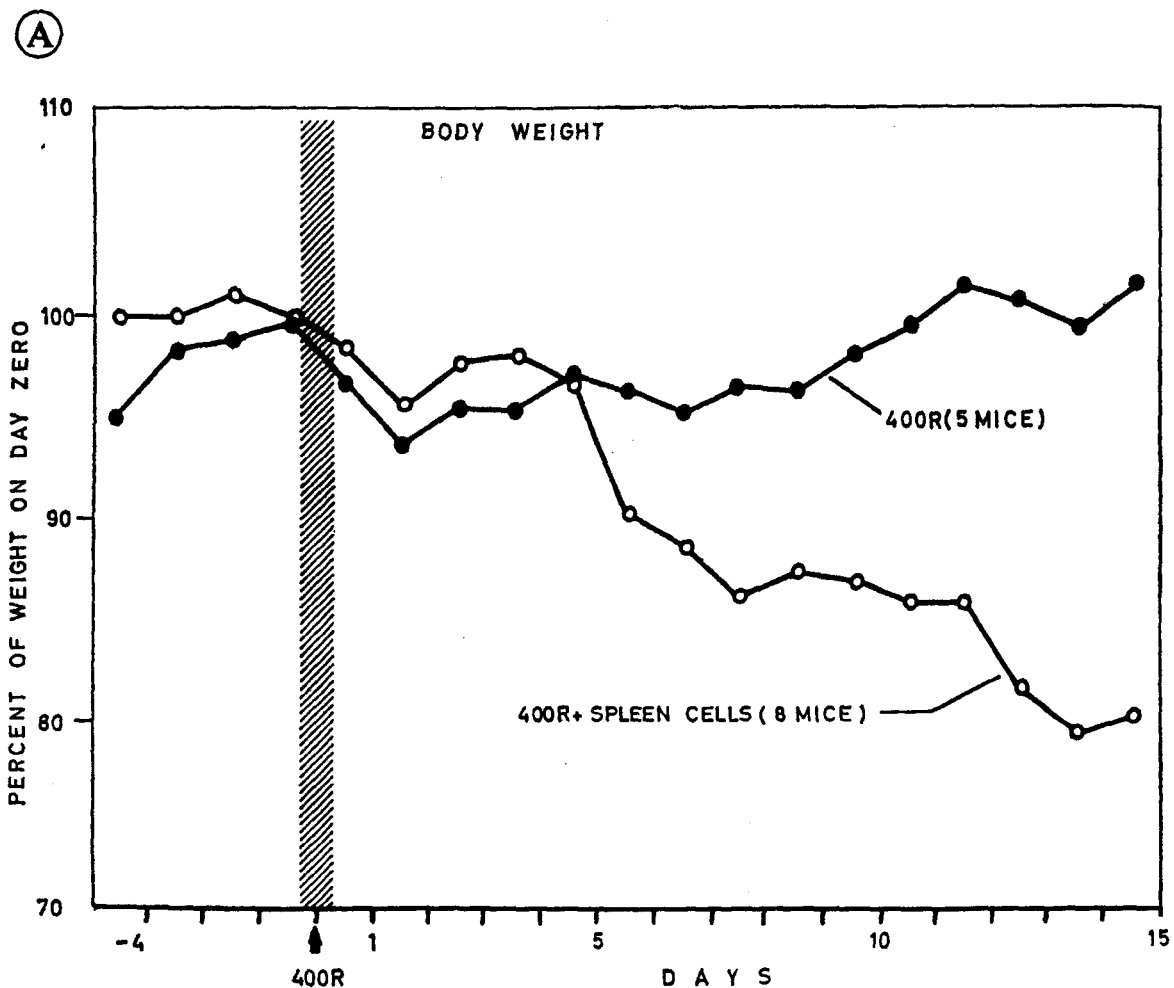
Figure III[6].   Body weight changes, food intake and food wastage in mice suffering from homologous disease. Figures from McRae (1960)[272]

(A) Body weight curves
(B) Apparent food intake curves and daily wastage of food
(C) Food intake obtained by subtracting food wasted from apparent food intake
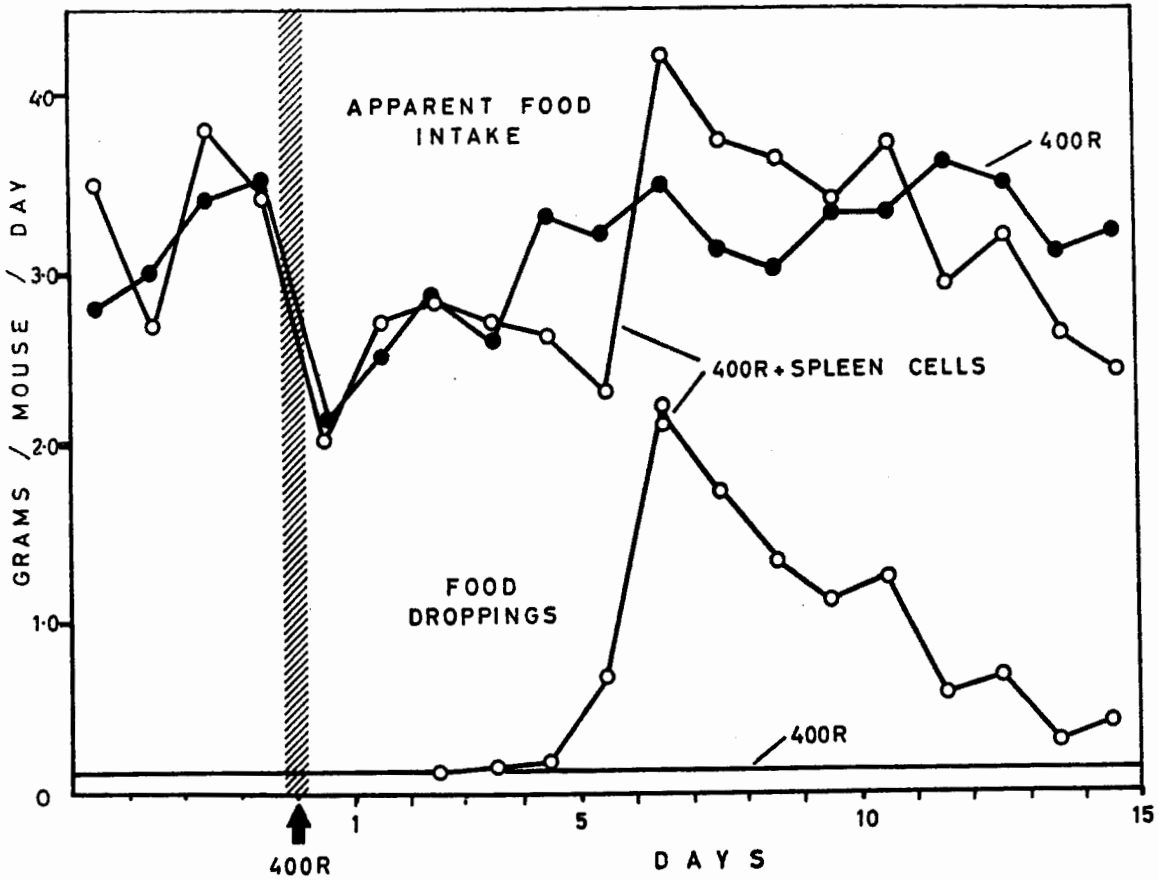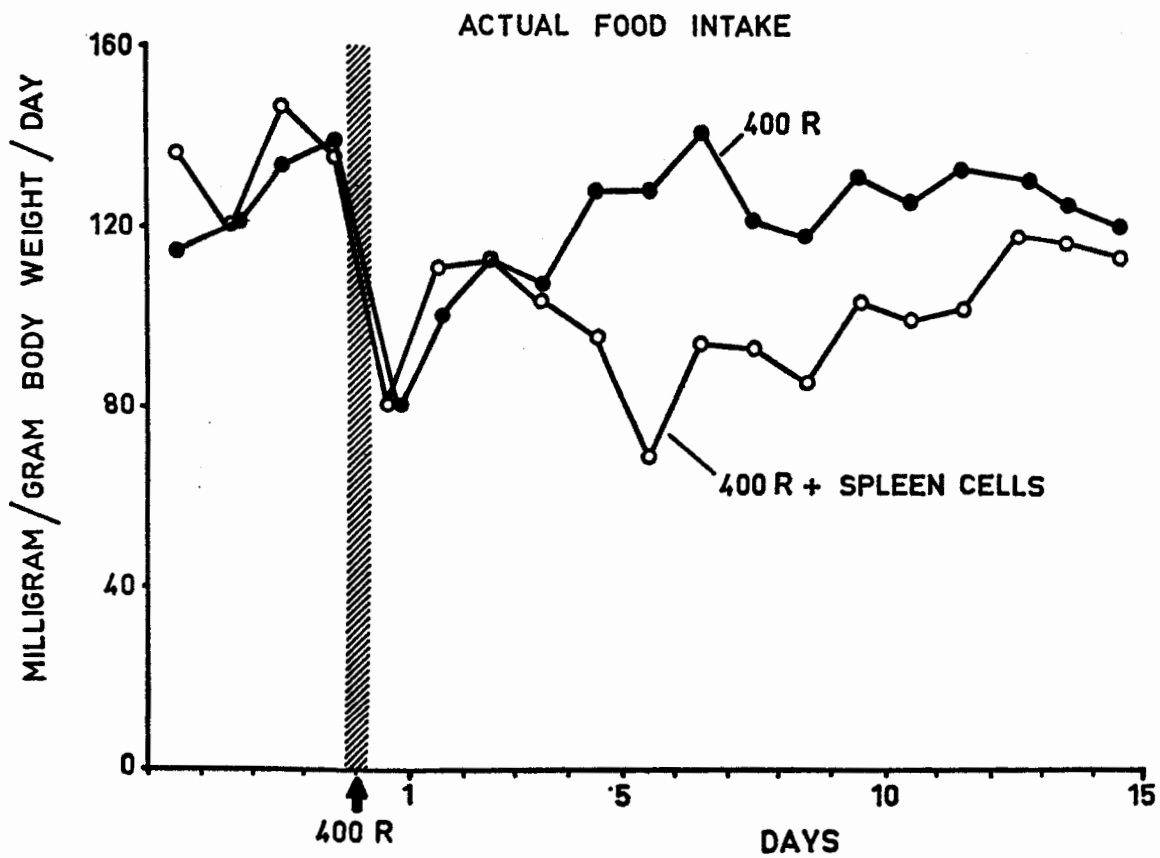
of the disease. They reported, furthermore, that the food intake of these runts was somewhat "limited" because of neglect by their mothers and their own inability to move easily as a result of serious skin lesions. The description by Porter and Cooper[329] of runt disease induced in rats by the injection of newborns with thoracic duct lymphocytes mentions diarrhoea as being "sometimes" present.

The absence of diarrhoea in rats suffering from secondary disease appears somewhat puzzling in view of the pronounced radio-sensitivity of the rat's intestinal tract. One possible explanation may lie in the use of specific pathogen free (SPF) rats in these experiments. The occurrence of colitis associated with diarrhoea might be dependent upon the composition of the microflora in the intestinal tract. The SPF rats used probably had a modified bacterial flora and were certainly free of intestinal parasites and protozoa. The fact that considerable wasting did occur in these animals would suggest that this condition is *not* caused by diarrhoea. It is of interest that the same SPF rats developed severe lesions of the crypts in the colon when given homologous spleen cells following a lethal dose of radiation, but that diarrhoea did not occur to any significant extent in these animals[18].

In guinea-pigs which showed characteristic skin lesions following a lethal dose of radiation and homologous bone marrow transplantation, diarrhoea was not observed. The secondary disease in these animals was not, however, severe, and only of a transitory nature.[46]

In Porter's exhaustive studies of secondary disease in homologous rabbit radiation chimaeras and of "runt disease" in young rabbits, diarrhoea and wasting were found to be characteristic symptoms of both diseases.

In the dog, watery stools have been noted in one or two cases of secondary disease but it is not clear whether it constitutes a major symptom since it was only reported as an incidental observation[78, 166]. Lesions of the intestinal tract similar to those which occur in rodents suffering from secondary disease have not been described so far.

In monkeys[108] as well as in human patients[263] severe diarrhoea is one of the outstanding characteristics of secondary disease. Anorexia is nearly always present in the monkeys and contributes considerably to the wasting. Weight loss was found to occur in all irradiated animals, including controls and monkeys treated with autologous bone marrow during the first 14 days. In the chimaeras the mean rate of
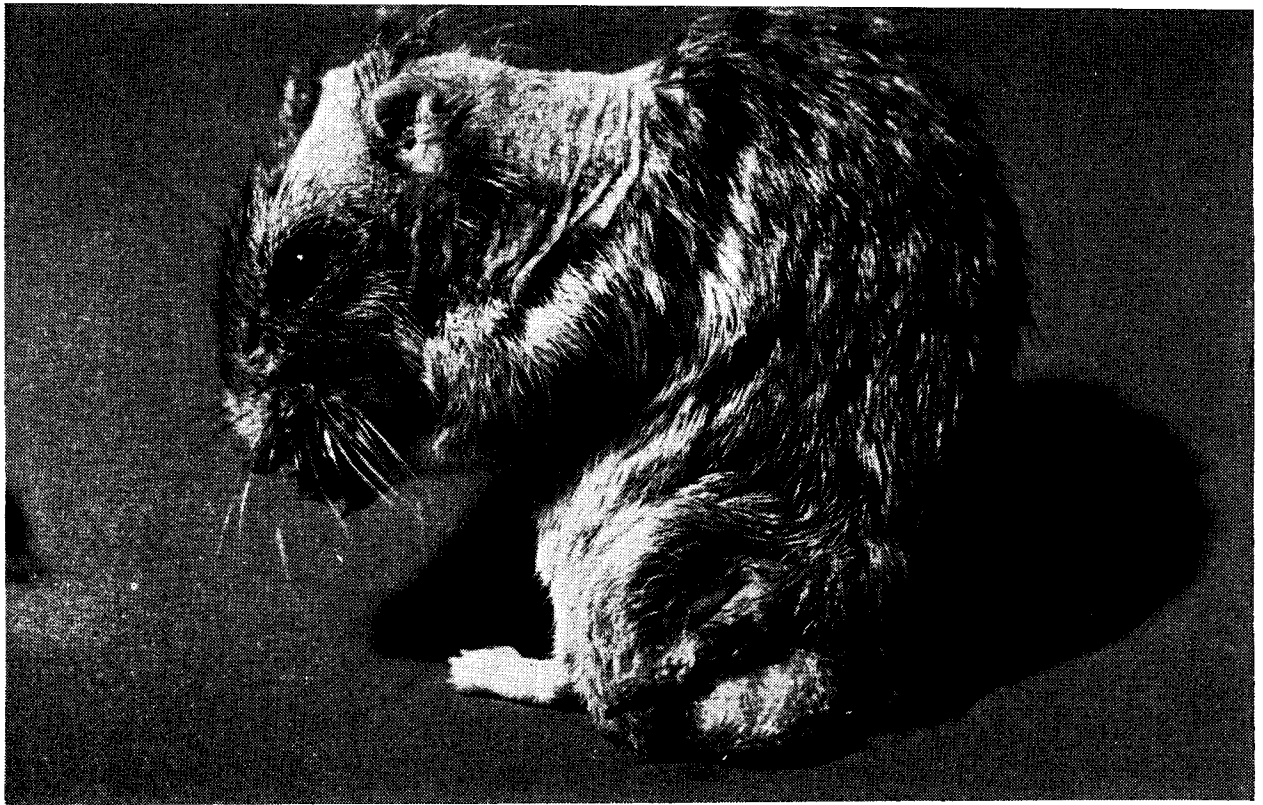
PLATE III: 6. Chinese hamster showing alopecia and scaling of the skin of the upper part of the body. Five weeks after irradiation and treatment with homologous bone marrow
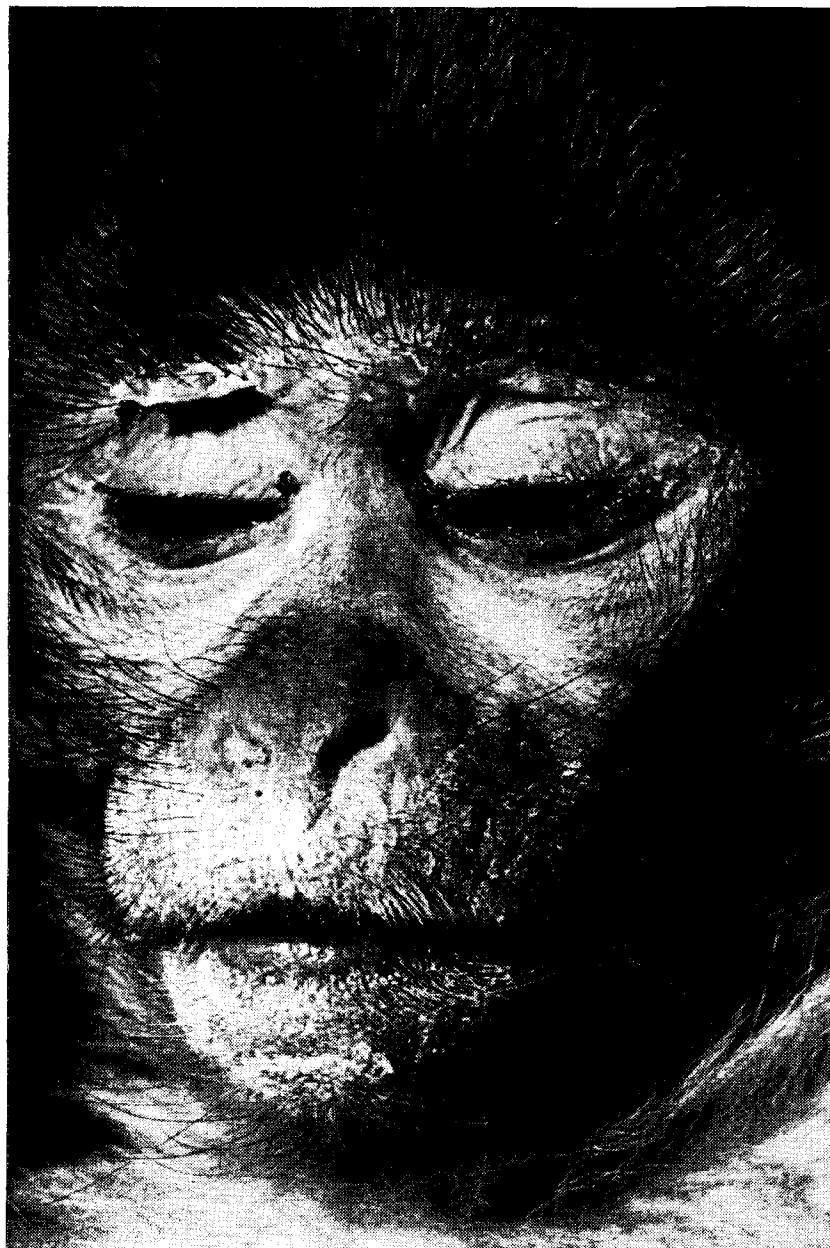
PLATE III: 7. Rhesus monkey suffering from secondary disease 17 days after irradiation and homologous bone marrow transplantation. Note scaling of the skin and crust formation predominantly in hairless areas of the face

PLATE III: 8.   Extensive desquamation of the facial skin in a patient with second-
ary disease. Photograph from Mathé *et al.* (1960)[263]

The picture was taken at 17 days after the transplantation of homologous bone
marrow and 29 days after the irradiation. The skin reaction had started as a
scarlatiniform eruption 3 days before the picture was taken

weight loss was 0·93 per cent per day compared to 0·72 per cent in the autologous group. The latter then began to recover as shown by a mean weight increase of 0·34 per cent per day, but the homologous chimaeras showed a marked loss of a further 1·23 per cent per day. The total weight loss of these animals was often as much as 25–30 per cent of the original body weight and this was obviously an important factor in determining the fatal outcome of the disease.

In the monkeys studied so far the diarrhoea and the anorexia start comparatively early, usually between 7 and 14 days after the bone marrow transplantation. The diarrhoea following homologous bone marrow transplantation can be distinguished from the radiation induced diarrhoea both by histological examination of the intestines (see Chapter IV) and also because the former is much more severe. In most cases the diarrhoea could not be controlled and tests for specific pathogenic organisms in the faeces were usually negative.

SKIN LESIONS

Secondary disease is accompanied by macroscopic skin lesions in all animal species so far investigated, except possibly in the rabbit. In rabbit chimaeras suffering from secondary disease the fur was described as "dull, dirty and being shed easily"[325] but no specific lesions either macroscopic or microscopic have been reported.

In the mouse and the rat the skin lesions which occur during secondary disease show a striking resemblance to the changes of the skin which develop in the course of graft versus host reactions not involving irradiation, such as the runting syndrome in young animals, parabiosis disease and homologous disease. In mice the incidence and the severity of the skin disturbances vary a great deal. Usually not more than 20 per cent of the animals develop the grosser signs of skin abnormalities which consist of partial or complete alopecia, erythema, scaling, crust formation and sometimes desquamation of large areas of the epidermis. Preferential sites are the snout, ears, the paws and the tail, these localisations being particularly obvious in albino mice. Some characteristic lesions are shown in Plate III: 2. When crust formation and ulceration are extensive, the movements of the animals are impeded and they acquire a peculiar gait, characterised by walking with straight legs and a hunched back.

In many cases the changes are reversible and recovery takes place, but severe skin lesions may persist for 6 months or longer. Although diarrhoea and wasting are generally not manifest beyond the 4th

H

month, skin lesions have been present in a minority of cases for much longer periods of time.

Another characteristic feature in coloured mouse strains is the delay of the radiation induced depigmentation of the hair. The delay of "greying" in radiation chimaeras is a reflection of the inhibition of the growth of a new fur of depigmented hairs. Possibly this inhibition is in turn caused by the catabolic effects of the graft versus host reaction (clinical and subclinical wasting).

In rat chimaeras of a certain strain combination the skin lesions have been described by Balner et al.[18] as being more severe than is generally seen in mice, but in those animals which survive the period of severe wasting, a striking recovery of the skin lesions is usually seen (Plate III: 3). In contrast to the condition in mouse chimaeras, macroscopic skin lesions rarely persist beyond the 3rd month following transplantation. There is no reason to suppose that this is due to a qualitative difference between the two species. It is much more likely that in the homologous rat chimaera of Balner's host–donor combination, the graft develops an immunological tolerance towards the host tissues more regularly and more completely than in most mouse chimaeras.

Skin lesions very similar to those just described have been observed in guinea-pigs (Plate III: 4) and in Syrian hamsters (Plate III: 5) as well as in Chinese hamsters (Plate III: 6) following lethal irradiation and homologous bone marrow transplantation. In the first two species the lesions were of a transitory nature and seemed to be relatively mild. The course of the lesions in the latter species is not known because the studies have not been completed as yet.

In monkeys carrying a homologous bone marrow transplant the skin lesions are usually mild but severe dermatitis with appreciable loss of hair has occasionally been encountered (Plate III: 7). The skin lesions developed in the majority of the monkeys in which evidence of a take of homologous bone marrow was obtained[108]. Usually, in the second week following irradiation a patchy erythema appeared over the face and the anterior part of the chest. In a few days it had progressed into a uniform erythrodermia that gradually decreased in intensity. In the 3rd week after irradiation the affected skin became covered with scales, showed abnormal prominence of the orifices of the hair follicles and the disappearance of redness. The lesions could be easily distinguished from radiation erythema which appears on the 2nd or 3rd day after irradiation but which subsides after a few

days. The latter erythema was also seen in some control monkeys which had received no bone marrow and in monkeys receiving autologous marrow.

A serious erythematous and desquamative dermatosis involving virtually the whole body surface occurred in the few human patients in whom the take of a homologous bone marrow graft was established (Plate III: 8). Complete recovery from these lesions was observed in the only patient described until now in whom proliferation of foreign bone marrow could be followed for a prolonged period[258].

INFECTIOUS COMPLICATIONS

Autopsy of animals dying after the 1st month following foreign bone marrow transplantation frequently reveals multiple localised infections. This was extensively described by de Vries and Vos[449] in their study of the pathology of mouse radiation chimaeras. Their material showed a high incidence of pneumonia, chronic colitis and in addition a number of other infectious foci. On the other hand septicaemia was not a characteristic finding of secondary mortality, but instead more specific for those mice that died from acute or delayed bone marrow failure. It has already been mentioned that pneumonia caused by *Pseudomonas pyocyaneus* was a frequent cause of secondary death in rabbits. Infection was not a characteristic complication of secondary disease in the rats described by Balner *et al.*[18]; moreover, in this series the mortality was rather low and colitis as a consequence of the graft versus host reaction was notably absent. The incidence of infectious complications in monkeys treated with foreign bone marrow was much lower than in mice, but this may have been due to the acute course of secondary disease in the monkey and also because intensive antibiotic treatment was given to all the animals. Yeast infections, possibly produced by the prolonged antibiotic treatment, and lesions suggestive of virus infections have been described in monkeys as well as in the few human cases of secondary disease[263, 446].

The infectious complications of secondary disease have been related to the atrophic condition of the lymphatic tissues. Both conditions are more generally associated with the late form of secondary disease than with the severe early form.

INTENSITY OF GRAFT VERSUS HOST REACTION AND SECONDARY DISEASE

The relationship between the clinical course of the disease and the graft versus host activity in mouse chimaeras was established in a

series of investigations involving three different host-donor combinations: one homologous combination showing minimal secondary disease, one homologous combination showing severe secondary disease and the heterologous rat mouse combination which also shows severe secondary disease[48]. In all groups, animals were killed at intervals both for histological examination of the tissues and also in order to obtain spleen or lymph node cells to be used for the quantita-
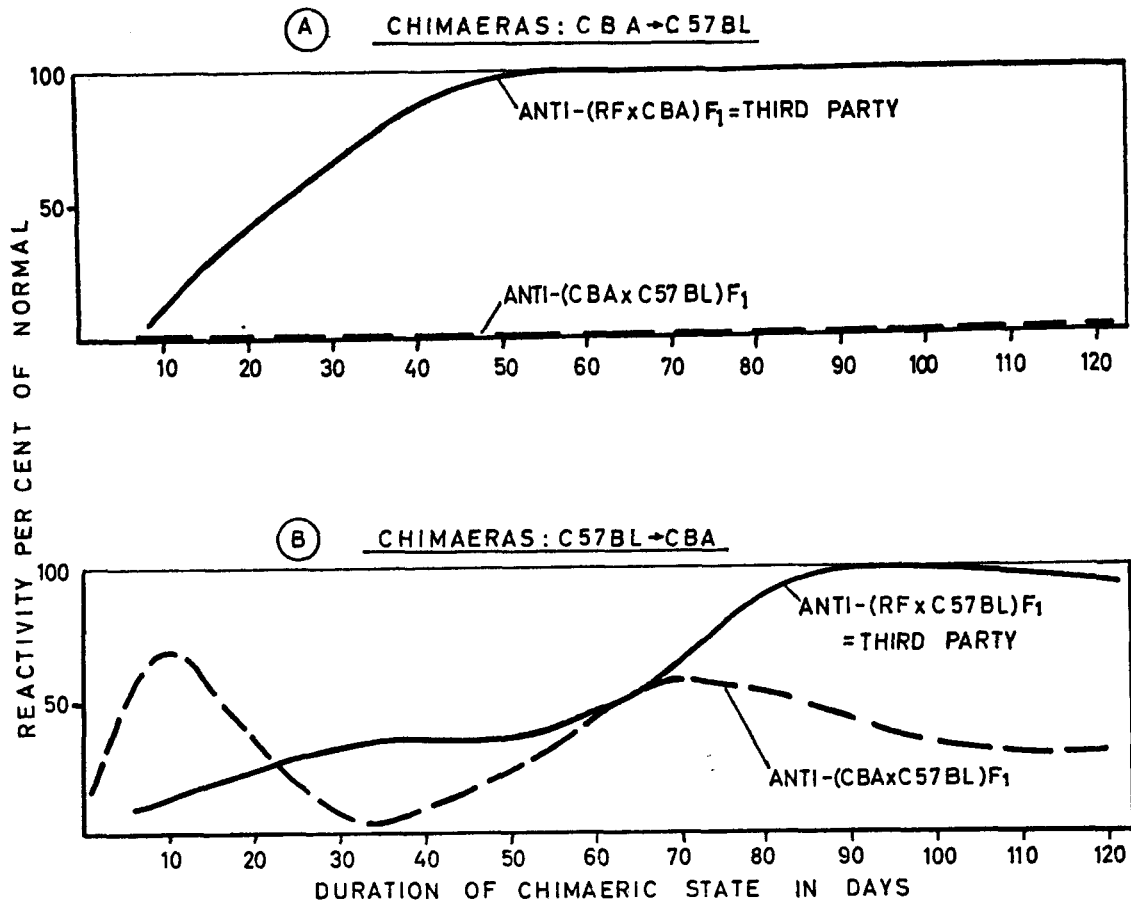
Figure III[7].   Schematic representation of results of anti-host and anti-third party homograft activities in two homologous chimaeras. Data from van Bekkum et al., 1962[48]

A: a host donor combination with little secondary disease
B: a combination which shows severe secondary disease

tive estimation of anti-host activity and anti-third party activity (immunological reactivity against tissue antigens not related to either host or donor) according to the method described by Simonsen and Jensen (see page 105).

In the first combination (Fig. III[7](A)) an initial period of non-specific non-reactivity—which also occurs after isologous bone marrow transplantation—was followed by a recovery of the reactivity

against third party antigens and this reactivity was completely restored near the end of the 2nd month. Anti-host reactivity remained zero at all times despite the gradual normalisation of the histological appearance of the lymphatic tissues. It is significant that histological changes characteristic of secondary disease were virtually absent in this strain combination. Apparently, in these cases the graft developed a specific immunological tolerance towards tissue antigens of the host type. This state of specific tolerance has been demonstrated with a variety of other methods and by a number of different investigators[41, 53, 79, 334, 441].

In the least compatible host–donor combination also depicted in Fig. III[7](B), significant anti-host reactivity was found as early as 10 days after transplantation. During the second and third month this reactivity fluctuated considerably, while the anti-third party reactivity recovered at a much slower rate than in the previous combination. The highest incidence of histological changes also falls in this period and most of the mice are then clinically ill. The pronounced lymphoid atrophy in the majority of the animals forms the most likely explanation for the negative or low positive anti-host reactions encountered during this period. After the 100th day there is a tendency for the anti-third party tests to normalise and for the anti-host reactivity to decrease, and this coincides with the disappearance of the diarrhoea and other overt clinical signs of secondary disease.

In the rat → mouse chimaeras only the anti-host reactivity of the lymphoid cells could be estimated. During the whole period of testing (14–280 days after transplantation) a high incidence of positive reactions was scored in the Simonsen assays. It should be recalled that these chimaeras exhibit severe secondary disease and have less tendency to recover after the 100th day, although there is a sharp decrease of the mortality rate at that time.

The findings in the incompatible host–donor combinations have been interpreted as follows (see Fig. IV[1]). Proliferation and possibly differentiation of lymphoid cells starts soon after the first contact with the host antigens; these (donor type) lymphoid cells become sensitised so that after an interval of 3–4 days the production of antibody can be expected. In our investigations the early positive anti-host reactions were accompanied by negative anti-third party tests, which might indicate that the immunological potency of the lymphatic system is wholly directed against host antigens. Reactive lymphoid cells are liable to perish in the course of their reaction with the antigen

according to Gorer and Boyse[161], who described this phenomenon as "allergic death" of the lymphocyte. This secondary destruction of regenerating lymphoid cells was described originally in homologous chimaeras by Congdon and Urso[99] and subsequently by many other workers.

As soon as the donor lymphoid cells reach the reactive stage, therefore, the increase of cells due to proliferation becomes counter-balanced or even outweighed by destruction. In the case of a large inoculum of lymphoid cells, as for instance when spleen cell suspensions are used, massive proliferation of the donor cells in the period between injection and sensitisation will result in such large numbers of reactive cells, with presumably a high initial wave of antibody production, that the host may be killed. In the case of a bone marrow graft the early lymphoid cell proliferation is much less intense, so that the initial immunological attack on the host will develop more slowly and will not immediately become lethal. Possibly the continuous destruction of reactive cells will keep the anti-host activity at a stationary level thereafter and result in the secondary atrophy of the lymphatic tissues.

At the end of the third month a slow recovery of the lymphatic system occurs and at the same time some return of reactivity towards third party antigens has been found which might imply that a simultaneous recovery of the antimicrobial defences could occur. The diarrhoea diminishes at that time and in mice treated with antibiotics (which suppress the diarrhoea) the withdrawal of the drugs during the 4th month does *not* result in an increased mortality or a return of the severe diarrhoea[50].

The fact that lymphatic tissue recovery at this stage is not accompanied by an aggravation of the secondary disease, strongly suggests that at least a partial specific tolerance towards host antigens has developed.

SECONDARY MORTALITY IN THE ABSENCE OF A FOREIGN GRAFT

The question of whether secondary death—that is, death after the 20th day (and usually before the 100th day in mice)—must *always* be attributed to a graft versus host reaction, has already been answered in the negative. Secondary mortality has been observed in four situations which do not involve a foreign haemopoietic graft, as follows.

   (1) In mice irradiated with a high supralethal dose of irradiation and treated with isologous bone marrow[52, 77]. These findings have

been interpreted by Koller et al.[202] as indicative of genetic heterogeneity within the CBA strain used, but this possibility had been excluded beforehand by the results of skin transplantations between individuals of this strain. The clinical syndrome characteristic of secondary disease after homologous bone marrow transplantation has been observed occasionally by Barnes et al.[24] following isologous bone marrow transplantation and more frequently following the treatment of irradiated mice with isologous foetal liver[35].

(2) In mice subjected to whole body irradiation given once a day for 2 to 5 days and treated with isologous bone marrow. In the groups receiving the higher total doses of irradiation secondary disease and mortality have been observed[46].

(3) Mice that received two or more massive doses of whole body irradiation that were together just sublethal without further therapy, developed a syndrome resembling secondary disease which resulted in death between 40 and 100 days after the second irradiation[286]. The incidence of delayed deaths varied between 0 and 92 per cent and was in most groups about 20 per cent. In general the delayed mortality was higher when the interval between the two doses had been longer but it seems more likely that this was due to the fact that the larger intervals were also associated with the higher total doses of radiation.

After single doses of radiation, delayed deaths were less frequent, although a rather high incidence (24 per cent) was seen in older females. The delayed deaths were preceded by a secondary loss of weight and the passing of moist faecal pellets with an excess of mucus, frequently causing excoriation of the anus. Mice survived in this state for many weeks or would show an improvement followed by a relapse. The condition was usually fatal but a proportion of the affected animals recovered.

(4) Delayed illness was observed by Corp and Neal[101] in mice surviving a high lethal dose of radiation (causing 80 per cent 30-day mortality) administered at a high dose rate (68 rads/min). These 30-day survivors developed a protracted illness with the passage of bulky, yellowish and mucoid faeces as the predominant symptom. The sticky nature of the faeces again caused excoriation of the anus. The animals so affected were clinically ill and showed a marked loss of weight. Death from this syndrome occurred up to 210 days following the irradiation. In two parallel experiments

in which the animals were exposed to equivalent total doses at much lower dose rates, the secondary syndrome was not observed.

None of these studies provided a histological basis for the intestinal symptoms, nor were the other tissues examined for the presence of lesions resembling those found in secondary disease following foreign bone marrow transplantation. It is perhaps significant that the four experimental series described all involved CBA mice. This clearly necessitates the extension of these observations to other mouse strains and preferably to other species.*

Pathogenesis of secondary disease

There seems little doubt that the secondary disease which develops in established chimaeras after haemopoiesis has been restored is caused initially by an immunological attack of the grafted cells on the host. However, there exists a considerable difference of opinion over the manner in which this initial reaction leads to the development of progressive wasting, the characteristic lesions and finally death.

Two other factors besides graft versus host reactivity are known to influence the severity and incidence of the secondary disease: the dose of radiation to which the recipients are subjected, and the decreased resistance of radiation chimaeras to micro-organisms.

DECREASED IMMUNOLOGICAL DEFENCE

The decreased resistance is very likely the result of the extreme lymphatic atrophy, which is most severe in the later stages of the protracted disease that occurs in bone marrow treated mice. The microflora of the animals probably determines the type and severity of the infections that develop.

The relationship between graft versus host reactivity, the lym-

* Delayed mortality has been recently observed in another mouse strain namely C57BL, following supralethal irradiation and treatment with isologous bone marrow. These experiments could only be performed with *proteus* free mice (produced by decontamination by antibiotic treatment or by foster nursing by Enterobacteriaceae free mice), because normal C57BL mice die from *proteus* bacteriaemia when exposed to whole body irradiation exceeding about 800 rads even when treated with bone marrow.

In addition, pathological changes characteristic of secondary disease have been found in mice showing the delayed illness following isologous bone marrow transplantation. These pathological changes are tentatively attributed to a radiation induced deficiency of the *thymus* epithelium, which leads to *autoimmune disease* as will be discussed in the next paragraph. (D. W. van Bekkum and M. J. de Vries, Proceedings of the Third International Congress of Radiation Research, Cortina d'Ampezzo, 26 June–2 July 1966.)

phatic atrophy and the characteristic lesions of secondary disease has been visualised in two different ways. The first attributes the majority of the specific lesions (except infections) directly to the graft versus host reaction as follows[52]:

GvH reaction ⟨ specific lesions: e.g. skin, liver, spleen

allergic death of lymphocytes → lymphatic atrophy → infections
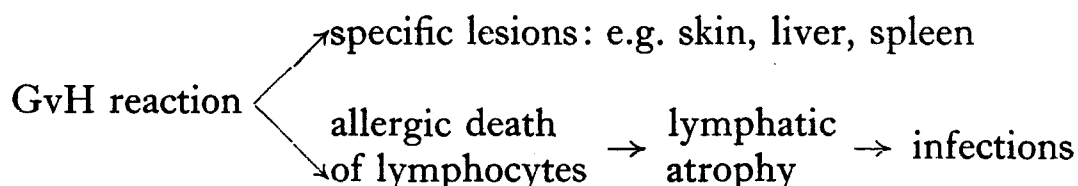
The second viewpoint, introduced by Loutit and Micklem[235], places more emphasis on the lymphatic atrophy, which has been considered to be the underlying cause of the specific lesions:

GvH reaction → allergic death of lymphocytes → lymphatic atrophy → specific lesions and infections
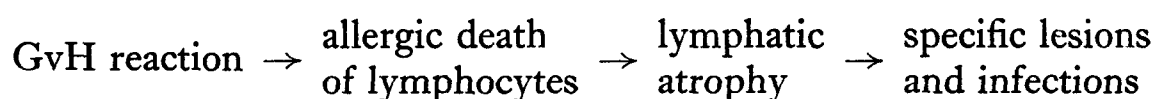
Some of the lesions were considered to result from infectious processes, whilst others were ascribed to the lack of lymphocytes, the latter being thus endowed with a "trophic" function[233], vital for many other cells. This hypothesis was based on a number of observations. A disease very similar to secondary disease was observed in irradiated CBA mice treated with the bone marrow of closely related C3H mice. This disease could be largely alleviated by the transplantation of C3H spleen cells in addition to the bone marrow[235] (in other, more distantly related, host–donor combinations transplantation of spleen cells accelerates and aggravates the secondary disease.) Loutit and Micklem[235] concluded from these results that lymphoid atrophy by itself might cause secondary disease. A decreased secondary mortality was also reported by Simmons et al.[371] when AKR lymphocytes were injected at 21 days into irradiated C3H mice treated with AKR bone marrow. However, these results need confirmation since the mortality in the group which received lymphocytes was already lower (20%) than in the control group (50%) before day 21.

Experiments along the same lines were reported by Barnes et al.[35] who observed a syndrome which resembled secondary disease in irradiated CBA mice after restoration with isologous foetal liver cells. The disease could be prevented by the addition of 5×10^6 isologous lymph node cells to the foetal cell inoculum. These authors noted, however, that the disease in the mice treated with foetal liver differed in *three* minor respects from the classical secondary disease in

homologous radiation chimaeras. Overt signs of infection were more common in the animals treated with foetal liver, they also tended to have slightly enlarged spleens and lymph nodes instead of the very small ones seen in mice treated with homologous bone marrow and finally the lymphoid follicles were not completely destroyed in animals treated with foetal liver.

The hypothesis favoured at Harwell that primary lymphatic atrophy can give rise to a syndrome comprising all the characteristics of secondary disease has always been extremely unattractive to the present authors. The main objection has been that many of the typical lesions have been encountered in the *acute* graft versus host reactions where *proliferation* instead of atrophy was the prevailing condition of the lymphatic system. Very recently, however, several authors have drawn attention to the similarity between the clinical symptoms and the pathology of the wasting syndrome which develops in mice after neonatal thymectomy and those of secondary disease in radiation chimaeras. This seemed to lend strong support to the Harwell hypothesis because primary lymphatic atrophy was thought to be the predominant feature of this disease. However, a subsequent pathological survey of the post-thymectomy wasting syndrome surprisingly revealed that the lymphatic atrophy in this condition seemed also of a secondary nature[447]. In addition to lesions that were strikingly similar to those seen in secondary disease, other sequelae were encountered which are highly specific for certain auto-immune diseases in humans. These findings have resulted in a completely different interpretation of the post-thymectomy wasting syndrome (as will be outlined in Chapter IV) and have invalidated the argument in favour of a causal role for lymphatic atrophy in producing the lesions of secondary disease.

RADIATION DOSE

A further factor which influences the severity of the secondary disease appears to be the dose of radiation to which the host was initially subjected. In a large series of comparative experiments, which involved various host–donor combinations, it was found that after higher doses of irradiation the incidence and severity of diarrhoea as well as the mortality were higher than after lower doses (Table III: 5). It was assumed that late radiation injury of the intestinal tract was a contributory factor in the determination of the degree of intestinal involvement in secondary disease[52]. This damage induced by radiation

TABLE III: 5. Influence of dose of whole-body irradiation on subsequent development of secondary disease and mortality in radiation chimaeras[52]

Recipient	Bone marrow donor	X-ray dose (r)	Percentage mortality at		Diarrhoea	Number of mice
			30-day	100-day		
CBA	CBA	675	0	0	Absent	30
CBA	CBA	800	26	31	Sporadic	19
CBA	CBA	950	22	71	Slight	89
F₁ hybrid	CBA	700	0	0	Absent	110
F₁ hybrid	CBA	800	0	0	Slight	60
F₁ hybrid	CBA	950	12	20	Severe	70

could render the intestines more susceptible to invading micro-organisms (colitis) or alternatively more liable to be the target of the sensitised donor lymphocytes. However, no convincing microscopic evidence to prove the existence of such late damage has so far been produced.

In this context it should be recalled that in the "secondary disease" which has been observed in the absence of a graft versus host reaction (which fits in with the recently developed hypothesis that thymic damage is the underlying cause of this form of "secondary disease"), diarrhoea and wasting were also more frequently observed in the groups which received the highest doses of radiation.

A summary of the relative importance of radiation dose and potential graft versus host activity is presented in Table III: 6.

TABLE III: 6. Factors involved in the production of secondary disease symptoms

Treatment	Mortality (secondary)	Skin lesions	Diarrhoea
Irradiation only	+	—	+
Foreign graft, *no* irradiation	+	+	+
Irradiation and foreign bone marrow	+ +	+	+ +
Irradiation, foreign bone marrow and foreign lymph-node cells	+ + +	+	+ +

It has been pointed out above that the transfer of foreign haemopoietic cells to lethally irradiated animals may be followed by a violent graft versus host reaction which brings about the early death of the host.* This is the case when irradiated mice or rats are given foreign lymphoid cells in addition to bone marrow, as well as in irradiated monkeys only treated with homologous bone marrow. There seems in fact to be no reason why this syndrome should be classified as a form of secondary disease, since the lethal graft versus host reaction frequently develops before recovery from the primary disease—radiation induced haemopoietic failure—has taken place. The condition may be termed alternatively "early or acute secondary

* "Acute killing" effect.

disease" in view of the similarity of its underlying cause with that of the classical secondary disease as it was originally described in mice. The exact nature of the reactions by which a strong graft versus host activity brings about death in the acute form of secondary disease remains largely obscure, although some speculations can be made on the basis of histological study of the tissues, as will be discussed in the next chapter.

Modification of secondary disease

PREVENTIVE MEASURES

If it is accepted that secondary disease is primarily a graft versus host disease, it follows that there are two main methods of prevention available. One is the use of compatible donors, that is donors with a minimum of immunogenetic disparity towards the host. The other is the use of haemopoietic grafts which contain a minimum of immunologically competent cells. The first approach seems highly favourable on both theoretical and experimental grounds. Many experiments with inbred mouse strains have shown that the severity of secondary disease is strongly related to the degree of histocompatibility difference between the host and the donor. Unfortunately, in clinical practice the selection of completely compatible host–donor combinations seems unobtainable except in the case of identical twins. However, a degree of compatibility may sometimes be obtained by choosing a close relative of the recipient. In addition there are indications that the matching of host and donors according to leucocyte antigens may be a profitable approach[116]. So far, the results of a limited number of skin and kidney transplantations, although by no means conclusive in proving the relevance of leucocyte antigens for histocompatibility, have been sufficiently encouraging as to stimulate an increased effort along those lines[16, 117, 352].

On theoretical grounds the second approach seems promising only with respect to the acute form of secondary disease as it appears in monkeys and humans where this seems to be a consequence of an initially high number of immunologically reactive cells. In these cases an elimination of the reactive cells might be profitable. It is not unlikely though that even if the acute form of secondary disease were prevented, the recipient might still be susceptible to the late form of secondary disease which is presumably caused by subsequent generations of reactive lymphoid cells. Theoretically, this pessimistic

attitude seems justified but it should be remembered that the late form of secondary disease is usually milder and more likely to be influenced favourably by conservative methods of treatment. Furthermore, the chances of the development of partial or even complete tolerance on the part of the donor system towards the recipient's antigens with concomitant regression of the secondary disease are, in general, much higher in the late form than in the early acute form of secondary disease.

Foetal haemopoietic cells seem to be the solution of our problem provided by nature, because of the relatively low proportion of lymphoid cells in foetal liver or spleen cell suspensions. Unfortunately, as has been concluded in the previous chapter, rodent experiments showed that secondary disease is diminished but not completely prevented following the administration of foetal liver suspensions. Furthermore, as has been mentioned before, Barnes et al.[35] found secondary complications following the use of isologous foetal liver cells which resembled secondary disease. Since these symptoms could be alleviated by the administration of isologous lymph node cells, they were attributed to a lack of lymphoid cell precursors. Whether or not this complication would arise after the use of foetal tissues for the treatment of human patients is not at present relevant. Far more important factors which prevent the clinical application of foetal liver cells are the difficulties in obtaining this material and also the large number of cells required for effective haemopoietic restoration, which would make pooling and therefore storage, necessary. The unavoidable losses which accompany storage of haemopoietic cells would increase even further the number of cells required.

A number of different attempts have been described to eliminate or mitigate the acute form of secondary disease by treatment of the donor cells either *in vivo* or *in vitro* before administration to the irradiated recipient.

Obviously, if the treatment is to be of any use, the immunologically active cells should be eliminated preferentially or selectively from the haemopoietic cells proper. The degree of selectivity should be quite high, otherwise the destruction of the haemopoietic cells will be difficult to compensate, since the number of cells obtainable from a single living donor is limited.

For the unequivocal proof of a selective elimination of immunologically competent cells from haemopoietic cell suspensions as distinct from a merely indiscriminate decrease of viable cells, elaborate

experiments are required[45]. It has been pointed out that in mice the severity and the incidence of secondary disease are functions of the number of homologous cells administered, whether it be spleen, lymph node or bone marrow cells, from which it follows that a decrease in the total number of viable cells can easily result in increased survival.
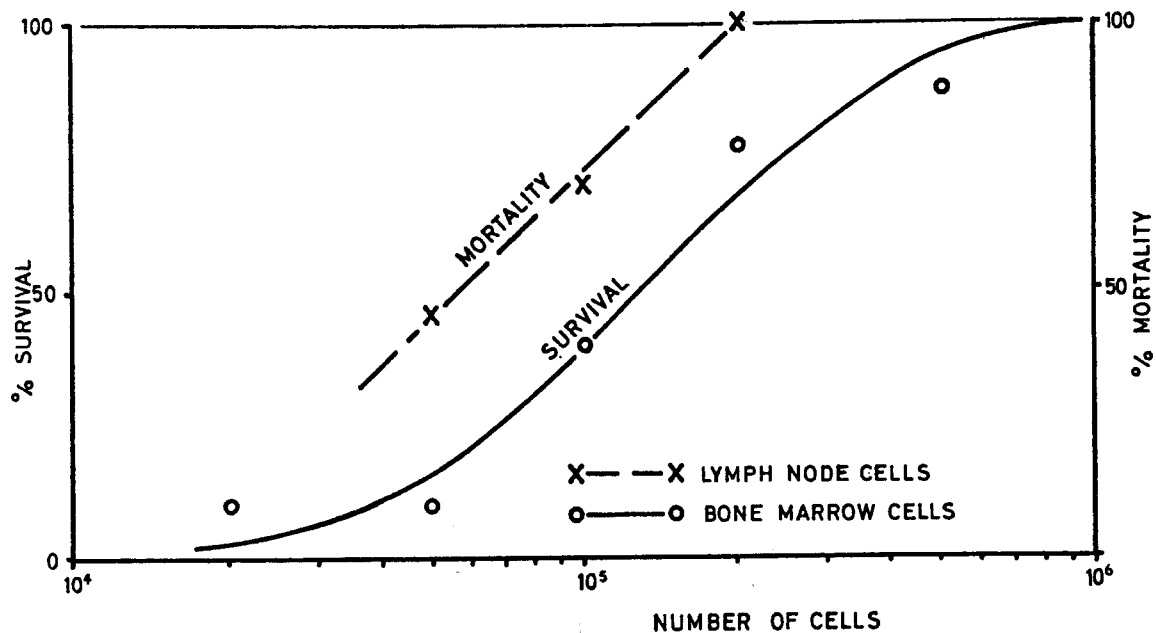
Figure III[8].   The relation between the number of CBA bone marrow cells and survival and between CBA lymph node cell dose and mortality in lethally irradiated (CBA × RF)F_1 hybrid mice. These relationships were used for the determination of the selective elimination of immunologically active cells from cell suspensions.

Data from van Bekkum (1964)[45]

The recipients were irradiated with a dose of 880 rads. The graph shows the mean values calculated from four experiments. The curve shown for the lethal effect of lymph node cells was obtained by injecting a standard number of bone marrow cells (10^6) and graded numbers of lymphoid cells into irradiated recipient mice. A fresh suspension of 10^6 bone marrow cells and $4 × 10^5$ lymph node cells was—in accordance with the graph—uniformly lethal. Pretreatment of this mixture—in the present case by storage at 4° C for one or two days—caused 90 and 85 per cent 30-day survival. This indicates a decrease of immunologically competent cells from $4 × 10^5$ to $2 × 10^4$ or less, that is by a factor of 20. The haemopoietic cells cannot have decreased further than from the initial 10^6 in the fresh suspension to $3 × 10^5$ which amounts to a factor of 3. The selectivity factor between inactivation of lymph node cells and haemopoietic cells is therefore 20/3 or about 6 in these experiments or 3 if extrapolation of the lymph node cell curve down to low cell numbers is presumed to be steeper downwards from the $5 × 10^4$ point

For such experiments it is thus imperative to record complete cell dose–survival curves for treatments with haemopoietic cell suspensions as well as with lymphoid cells (Fig. III[8]). These requirements have rarely been met, so that most of the evidence presented in the literature on the selective elimination of immunologically reactive cells can only be tentatively accepted.

PREIRRADIATION OF DONOR MARROW

Cudkowicz[109] has reported a decreased incidence of secondary disease when the donor mice were irradiated with a dose of 400–500 r of X-rays. He used a donor–recipient combination which showed a very high incidence of secondary mortality. It was calculated that the surviving fraction of the cells from the irradiated donors was in the order of 2·5 per cent. The standard dose of cells was 60×10^6 and since the mice treated with $1·5 \times 10^6$ fresh bone marrow cells showed the same incidence of secondary disease, it was concluded that a non-selective reduction of donor cells could not be the mechanism involved. However, preirradiation of the donor mice did reduce the ability of 60 million marrow cells to protect irradiated recipients against *early* radiation lethality, so that marginal numbers of viable cells were probably given when 60×10^6 cells from irradiated donors were injected. In fact, ten out of fifteen 90-day survivors were found to be reversals in one experiment. In other experiments the decreased incidence of secondary disease could not be ascribed to reversion but since the surviving fraction of irradiated cells was not actually determined, a non-selective decrease cannot be excluded.

In a recent study by Amiel et al.[5] the beneficial effect of preirradiation of the donor animals was not confirmed, but the number of animals employed was rather small. Cole and Davis[80] have reported the absence of secondary mortality in homologous radiation chimaeras following the use of donors which were sublethally irradiated 12 or 16 days prior to the transplantation. It is possible that changes in the bone marrow composition are responsible for this effect, since it is known that the regeneration of lymphoid cells proceeds more slowly than that of haemopoietic cells.

It has been reported by Cudkowicz[109] that the protection of irradiated donor mice with AET* did not increase the incidence of secondary disease in the recipients. If AET protected all cells to an

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* A well known radioprotective substance: S-(2-amino-ethyl)-isothiouronium dihydrobromide.
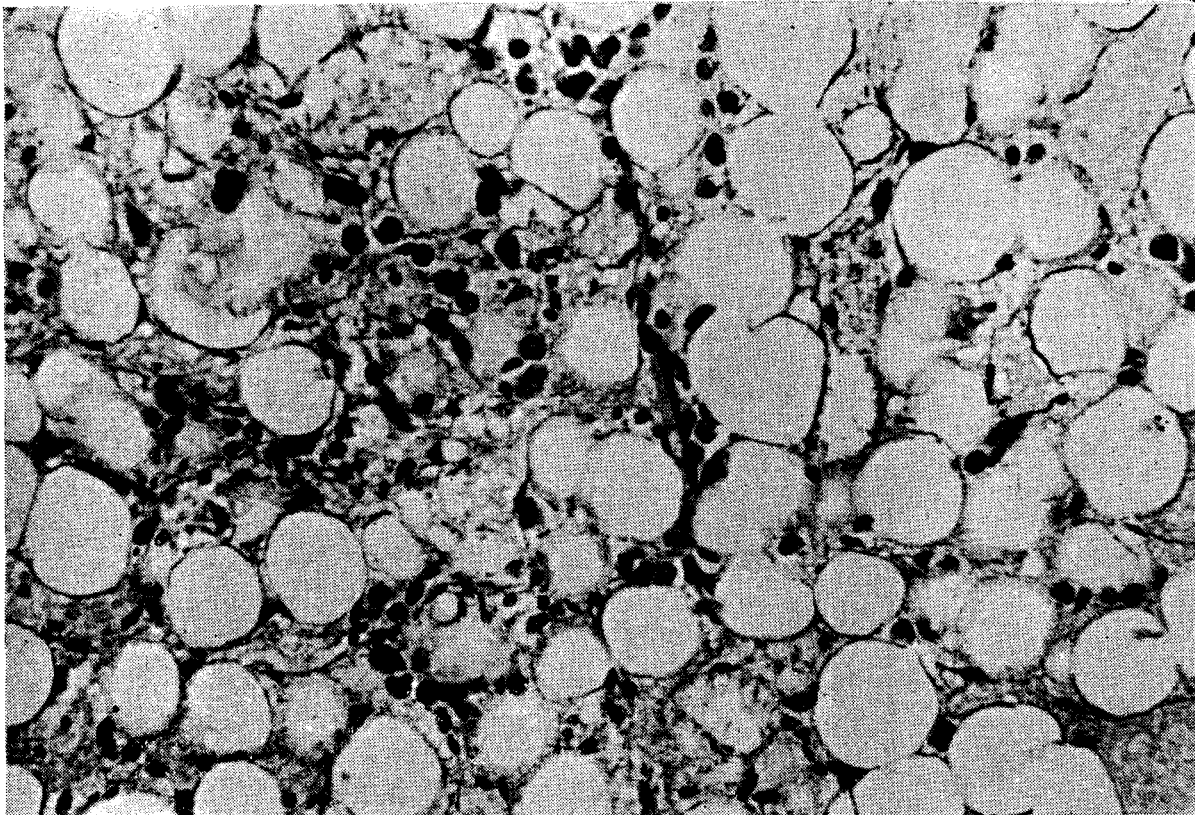
PLATE IV: 1. Aplasia of bone marrow in control monkey 15 days following lethal irradiation. The marrow space is occupied by fat cells and collections of plasma cells and histiocytes. Hematoxylin-eosin (HE). Magnification × 190
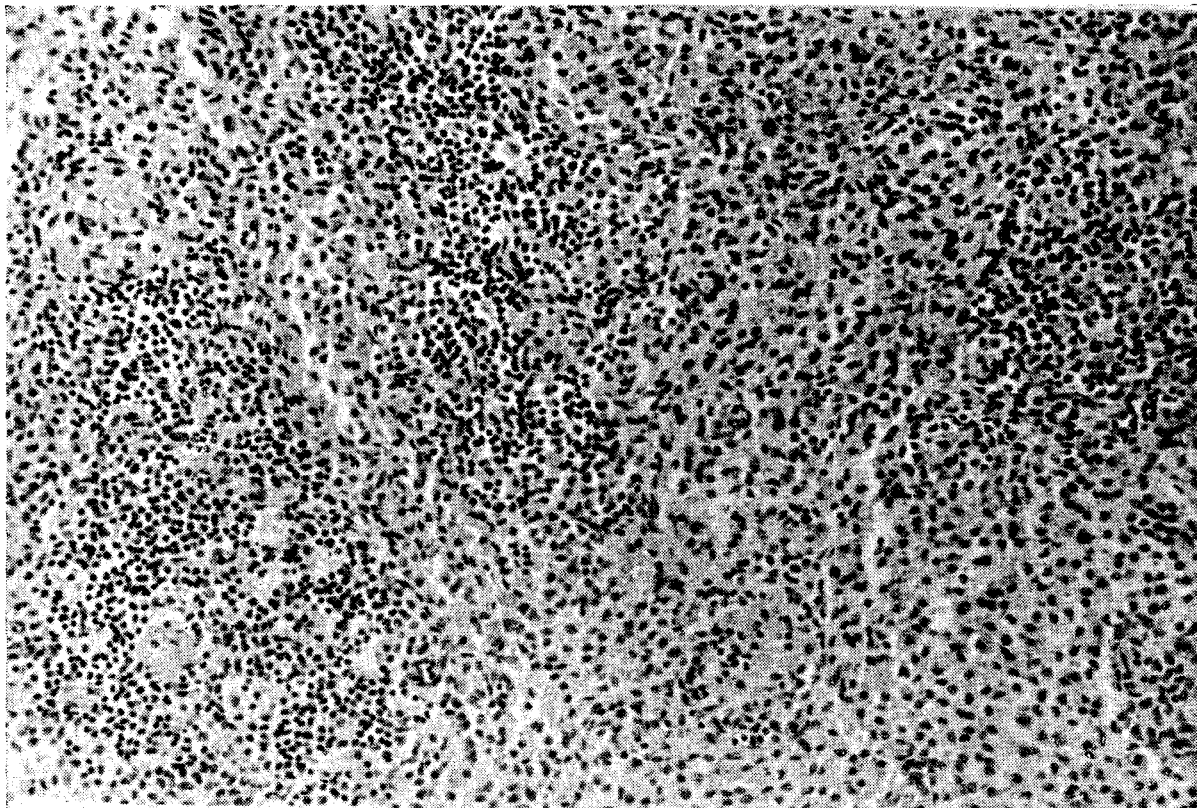
PLATE IV: 2. Atrophy of lymph node in control monkey 15 days following lethal irradiation. Note severe decrease of cellularity and absence of follicles. A few collections of lymphocytes are dispersed within the reticular stroma, however. (HE). Magnification × 30
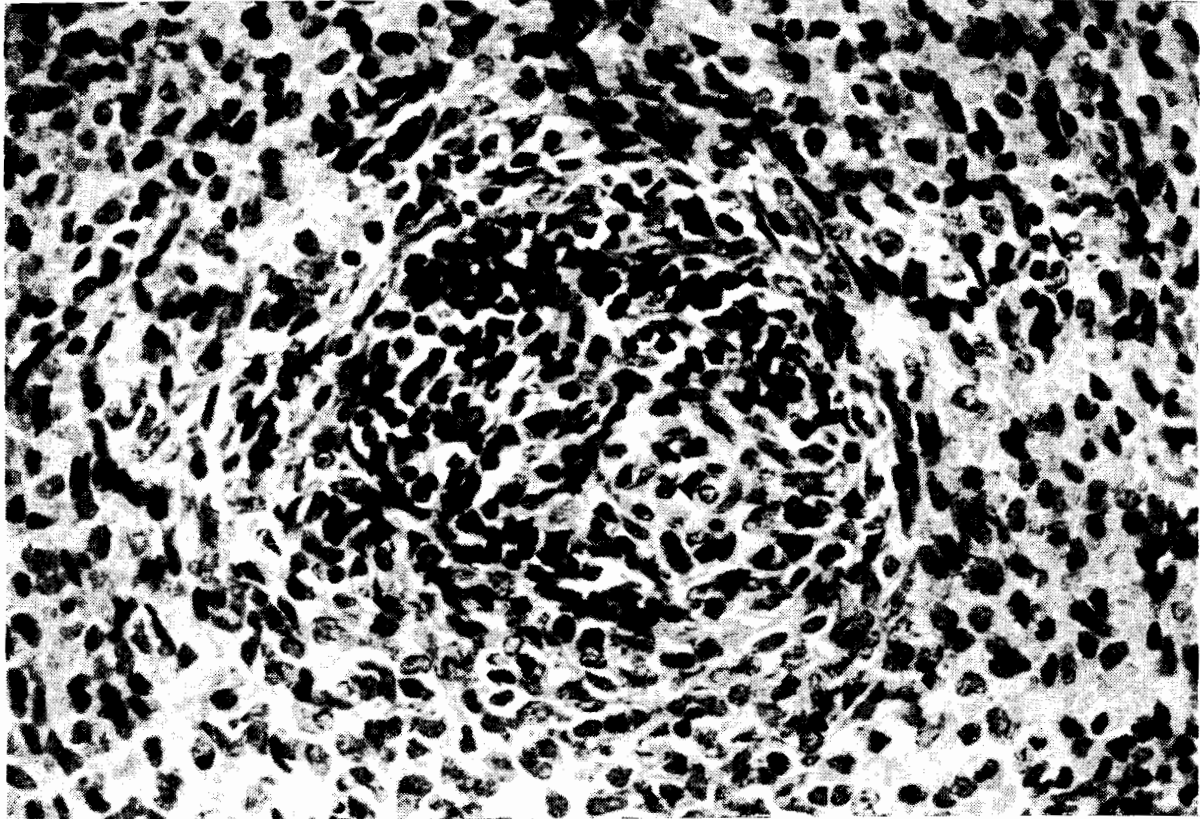
PLATE IV: 3. Atrophic lymphatic follicle in the spleen of a mouse, 4 days following lethal irradiation. A few lymphocytes remain in the stroma surrounding a splenic arteriole. (HE). Magnification × 300
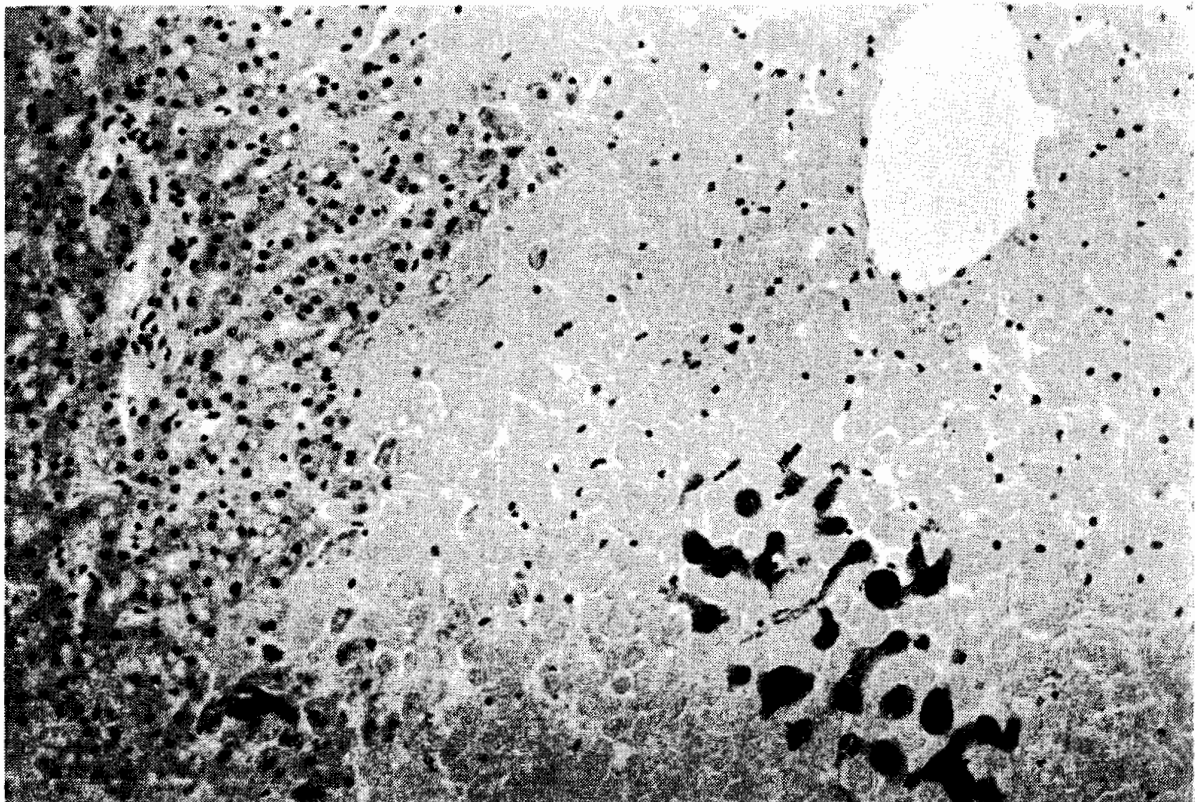
PLATE IV: 4. Septic liver necrosis in control monkey which died 15 days following lethal irradiation. Note darkly staining clumps of bacteria, total loss of liver cell structure in centre and right of the picture, and absence of any cellular inflammatory response. (HE). Magnification × 120

equal degree, the number of viable haemopoietic *and* lymphoid cells would have been larger in the suspensions derived from the AET protected mice and consequently a higher incidence of secondary disease would have been expected to occur in the recipients. These findings suggested, therefore, that AET selectively protects haemopoietic cells as compared to lymphoid cells. The observations were subsequently extended to *in vitro* irradiation and chemical protection of donor spleen cells, with similar results[110]. Other investigators were, however, unable to confirm this selective action of AET[91, 370].

INCUBATION OF DONOR MARROW

A different approach was described by Cosgrove and co-workers in a series of papers[102, 112, 120]. This entailed the pretreatment of the donor cells by incubation with recipient type liver tissue, a procedure which was intended to eliminate the immunologically reactive cells. In the test system, sublethally irradiated F_1 hybrid recipients were treated with parent strain spleen cells and incubation of the donor cells was performed at $10°$ C for 19 hours. When the pretreated cells were used, a marked reduction of mortality was observed compared with controls which received cells that had been similarly incubated with donor type liver tissue. Unfortunately, the evidence presented is not sufficient to exclude a non-specific reduction of the cell number. One difficulty was pointed out by the authors themselves[112], namely, that adult mouse liver contains viable immunologically active cells, able to induce a graft versus host reaction; this in fact prevents the design of proper control experiments.

Mathé *et al.*[257, 259] have tried to confirm these observations but have noted in control experiments that preincubation of homologous cells in Tyrode's solution alone caused a substantial decrease of secondary mortality in the recipients. Their procedure entailed the incubation of known numbers of bone marrow or lymph node cells at $37°$ C for one or two hours. As a result of the preincubation the subsequent mortality was reduced from nearly 100 per cent to about 60 per cent. The capacity of small numbers (10^6 and 10^5) of *isologous* bone marrow cells to restore lethally irradiated mice was not affected by the incubation and the authors concluded that a selective elimination of lymphoid cells was probably taking place. More recently Amiel and Mathé[4] have studied the effect of preincubation in a quantitative way and found that more than $97·5$ per cent of the lymph node cells were killed, while bone marrow cells seemed to be un-

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affected. However, the two types of cells were incubated separately, so that their results do not necessarily apply to mixtures of lymphoid and haemopoetic cells as are employed in clinical practice—normal human bone marrow being such a mixture.

With the test system described in Fig. III[8] it was found that storage of the cell suspension at 4° C for one or more days caused preferential inactivation of immunologically competent cells. The estimated selectivity factors* varied between 2·5 and 10, which seems insufficiently high to render the method clinically useful. A small number of attempts to free homologous monkey bone marrow from lymphoid cells by this method failed to give a significant decrease of secondary disease[336].

Experiments with homologous bone marrow transplantations into mice by Schwarzenberg et al.[365] have suggested that freezing and subsequent storage at low temperatures might affect the lymphoid cells more severely than the haemopoietic cells. If this were found to apply in a similar way to human bone marrow, new possibilities might present themselves for the use of stored cadaver marrow. The loss of viability upon storage might well be counter-balanced by the relatively large yield compared to the number of cells that can be obtained from living donors, together with the possible advantage of a decreased incidence of secondary disease. So far it has not been possible, however, to confirm these findings in mice, using a slightly modified form of Schwarzenberg's method for the preservation of bone marrow.

It cannot be expected that any procedure of selectivity will be perfect, in the sense that only immunologically reactive cells are eliminated while all haemopoietic cells remain viable. For methods to become clinically applicable, however, a high degree of selectivity seems to be required in view of the difficulties involved in obtaining sufficiently large amounts of homologous bone marrow.

POOLED DONOR MARROW

Mathé and his co-workers[255] have recently discovered another possibility for decreasing the severity of secondary disease. They mixed the bone marrow of donors of 4 inbred mouse strains and injected 2 × 10^{7} of these pooled cells into lethally irradiated recipients that were homologous to all 4 of the donor strains. Secondary mor-

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* The selectivity factor is the ratio between inactivation of immunologically active cells and inactivation of haemopoietic cells.

tality was much less than would have been expected from previous experience with any one of the homologous combinations. The behaviour of skin transplants in the surviving mice suggested that the cell lines which were most compatible with the recipients preferentially repopulated the hoemopoietic tissues of the host. This procedure brings to mind the experiments reported several years ago by Wilson et al.[461]. Pooled homologous bone marrow from 5 immunologically mature donors was administered to adult rabbits after lethal whole body irradiation ($1-2 \cdot 5 \times 10^9$ nucleated cells per rabbit). The survival rate beyond 4 weeks was lower than for animals who received marrow from a single donor. Of the 16 rabbits successfully grafted with pooled marrow and also grafted with skin from the various donors, 15 accepted skin from 2 or more donors of the marrow pool. This might indicate the development of some degree of mutual tolerance between the several donor cell lines and would not necessarily mean the survival of the one most compatible to the recipient.

Evidence for the development of graft versus graft tolerance in chimaeras receiving haemopoietic tissue from 2 donor strains was provided by Lengerova et al.[219] by the use of foetal liver cells from 20-day gestations in which mutual tolerance might be more easily induced.

It is of tremendous interest that so far the first human radiation chimaera to survive for 20 months was the result of a transplantation by Mathé's group[258] of the pooled bone marrow from 6 donors, who were all close relatives of the recipient. Evidence was obtained that only one donor cell line repopulated the patient's bone marrow, and this donor was identified by both a skin grafting test and by the study of leucocyte antigens to be the one most closely related to the patient. Attempts to prevent the severe secondary disease in monkeys following homologous bone marrow transplantation by the use of pooled bone marrow have so far, however, been unsuccessful.[340]

MISCELLANEOUS METHODS

A few other ways of modifying a graft versus host reaction have been reported. Adrenalectomy was found by Kaplan and Rosston[199] in 1959 to ameliorate the homologous disease which was induced by the injection of parent strain thymic cells into sublethally irradiated F_1 hybrid mice. One interpretation offered by the authors was that adrenalectomy stimulates lymphopoiesis in the hybrid hosts, thus enabling them to compensate more effectively for the massive dis-

integration of lymphocytes which occurs in the course of homologous disease.

A second modifying factor has been described recently by van Putten[337]. In mice that were thymectomised when 6 weeks old and subsequently irradiated, the secondary disease and mortality following treatment with rat bone marrow was significantly reduced compared with non-thymectomised controls. It was concluded that in the group treated with heterologous bone marrow the absence of the thymus delayed the recovery of the lymphatic tissue, thereby decreasing the anti-host immunological reactivity. The discovery that thymectomy *increased* the incidence of secondary mortality in isologous chimaeras was also attributed to deficient development of the immunological system. These latter observations seem to argue against the introduction of thymectomy into clinical practice as a method of preventing secondary disease. In addition, it is unlikely that thymectomy will ameliorate the early severe graft versus host reaction since it is believed to be caused by the action of immunologically competent cells which are already present in the bone marrow of primates.

Finally, the acute lethal graft versus host reaction caused by the injection of parent strain lymph node cells into irradiated F_1 hybrid recipients was shown to be prevented by the simultaneous administration of a 10 fold number of tolerant lymph node cells[335]. These latter cells were harvested from long standing parent strain $\rightarrow F_1$ chimaeras in which the donor system was known to have become specifically tolerant to host tissue antigens. The animals thus treated also received parent strain bone marrow and failed to show any delayed type secondary disease, although they were found to be chimaeras according to red cell blood typing. Obviously, this finding has only experimental interest at present.

In the case of human bone marrow the *in vivo* production of a cell population which has been made tolerant to the future host seems to be out of the question. The only alternative, namely, the *in vitro* production of those tolerant cells, appears to be equally impossible at present. However, the experiments of van Putten clearly show that should a successful method of growing haemopoietic cells *in vitro* be developed, several new approaches to tackling the problem of secondary disease would present themselves.

TREATMENT OF SECONDARY DISEASE

As early as 1957 Congdon and Urso[99] attempted to treat the "delayed homologous bone marrow reaction", the term employed by them for secondary disease at that time. They used gonadotrophin, hydrocortisone, oestrogen, diphenhydramine hydrochloride and streptomycin in mice, without any success.

Of more significance was Uphoff's discovery that treatment with the folic acid antagonist amethopterin (methotrexate) caused some decrease of secondary mortality in homologous mouse chimaeras[424]. The dosage employed—1·5 mg/kg body weight at 48-hour intervals, starting 14 days after irradiation—seemed to be rather toxic, causing some degree of mortality by itself, but Lochte and Thomas[226] found that this could be avoided by giving only 4 doses on days 1, 3, 5, and 7. This treatment resulted in the complete absence of secondary weight loss and mortality in a homologous mouse combination which otherwise showed 90% death from secondary disease. Amethopterin has been found to be quite effective in combating runt disease of mice following the intravenous injection at birth of homologous spleen cells[355].

In lethally irradiated dogs treated with homologous bone marrow the Cooperstown group[225, 399, 401] has repeatedly described the beneficial effect of post-transplantation administration of amethopterim. They also gained the impression that this drug decreased the incidence of graft rejection when the treatment was started before or on the day of the bone marrow transfusion[401, 405].

Recently Thomas and Epstein (*Cancer Research 25*, 1521–24, 1965) have reported the survival beyond 150 days of nearly 50 per cent of a series of 20 dogs following irradiation, homologous bone marrow transplantation and treatment with amethopterin. In the same series graft rejection occurred in 5 dogs. It is not clear from the published data whether secondary disease in dogs is of a similarly severe type to that seen in primates, although it seems that the time of appearance of the symptoms is much later in dogs. Pretreatment with 6-mercaptopurine or urethane prior to irradiation was found to promote the take of homologous bone marrow in dogs by Cole and Alpen[78], but the effect of post-transplantation treatment on the secondary disease is not yet known.

Another interesting approach was explored by Cole and Davis[80]. They treated mouse chimaeras with anti-donor isoimmune serum which was effective in the prevention of secondary mortality in a

small number of animals. One would expect that this would be due to the rejection of both lymphoid and haemopoietic donor cells and the subsequent discontinuation of the chimaeric state, however, the surviving animals were still found to accept host *and* donor type skin, while skin grafts of an unrelated third party were rejected.

An essentially symptomatic treatment of secondary disease has also been found to be effective in mice. By the addition of cereals as a supplement to the standard pellet diet diarrhoea and the weight loss of mice treated with rat bone marrow was influenced beneficially and delayed mortality was decreased[50]. The addition of the antibiotic aureomycin to the food causes an even more substantial amelioration of secondary disease[50]. When the antibiotic is administered to animals suffering from the disease there is usually, within a week, a striking decrease or even a complete disappearance of diarrhoea and an arrest of the wasting. When the therapy is withdrawn, the diarrhoea and wasting quickly return unless the animals have passed the 4th month following transplantation, when the secondary disease generally becomes spontaneously less severe or disappears completely.

Beneficial effects of a similar nature have been noted with a number of other antibiotics in mice, after *both* parenteral and oral administration: streptomycin, penicillin, terramycin and a mixture of neomycin and bacitracin (orally only). Furthermore, the feeding of sulfaguanidine has proved to be effective[46] (Table III: 7).

In the authors' laboratory, antibiotic treatment starting 30 days after transplantation has been routine practice in many experiments involving long term observations on radiation chimaeras in which secondary disease by itself was not the subject of the study.

So far, it has not been possible to explain the beneficial effects of the antibiotic treatment. It seems likely that a suppression of the secondary infection of intestinal lesions would cause the diarrhoea to disappear, but attempts to correlate the histological appearance of the intestines with the clinical symptoms and the composition of the bacterial flora have failed to provide proof for such a mechanism. However, the striking decrease of both mortality and the severity of intestinal symptoms does suggest that the latter play an important part in the outcome of the disease.

The relative ease with which late chronic secondary disease is counteracted by antibiotics stresses the importance of finding methods to prevent the actue lethal phase of secondary disease in primates.

Quite recently considerable progress has been made in this re-

TABLE III:7. The effect of antibiotics on secondary mortality of irradiated (900r) (CBA × C57BL) F_1 hybrid male mice treated with rat bone marrow

Experiment	Treatment	Number of survivals				
		0	→ 30	90	↔ 120	180 days
I	None	20	18	8	7	6
	Aureomycin*	20	20	18	18	17
	Neomycin–bacitracin†	20	18	16	16	16
	Pen–streptomycin‡	20	20	19	19	17
II	None	15	15	7	7	5
	Aureomycin§	20	13	9	9	8
	Sulfaguanidine‖	15	15	14	14	13

* Orally: 3 mg/g food
† Orally: Neomycin 0·5 mg/g food Bacitracin 25 u/g food
‡ Subcutaneously: Penicillin 2000 U, Streptomycin 1 mg daily
§ Orally: 1 mg/g food
‖ Orally: 5 mg/g food
→ = beginning and ↔ = end of treatment

spect (D. W. van Bekkum, *Oncologia* 26, Suppl. 60–72 (1966); N. C. Muller-Bérat, L. M. van Putten, D. W. van Bekkum, *Ann. N. Y. Acad. Sci.* (in press)). By using a model system consisting of lethally irradiated mice in which an acute type of secondary disease was induced by the administration of homologous spleen cells, it was discovered that the administration of amethopterin or cyclophosphamide within 4 days after transplantation is effective in preventing the fatal graft versus host reaction. A significant proportion of the treated mice survived as stable chimaeras, suggesting that treatment with the cytostatic agents acts selectively on the immunologically active cells of the graft, leaving the proliferation of the haemopoietic cells relatively undisturbed.

This principle was then applied to monkeys following lethal irradiation and homologous bone marrow transplantation, where it was found to be equally successful. Treatment with large doses of the drugs within 4 days after marrow transplantation resulted in a dramatic suppression of the acute secondary disease in the majority of the cases. Cyclophosphamide proved to be the more effective of the two drugs. Although the cytostatic agents were administered well before the first signs of haemopoietic recovery were apparent in the peripheral blood, this caused remarkably little delay of haemopoietic recovery. The monkeys which thus survived the acute phase following homologous bone marrow transplantation subsequently developed symptoms of secondary disease. These symptoms were in general, however, less severe and this later form of secondary disease seems to have much in common with the syndrome seen in rodents. Attempts to combat the secondary disease in its later stages by prolonged treatment with the same cytostatic agents and with anti-lymphocyte serum have yielded promising results in that survival beyond 60 days has been repeatedly obtained.

These results taken together with the impressive advances which are being made in the field of tissue typing for the selection of compatible donors, seem to provide a sound basis for the expectation that the hazards of homologous bone marrow transplantation may be decreased to an acceptable level in the not too remote future.
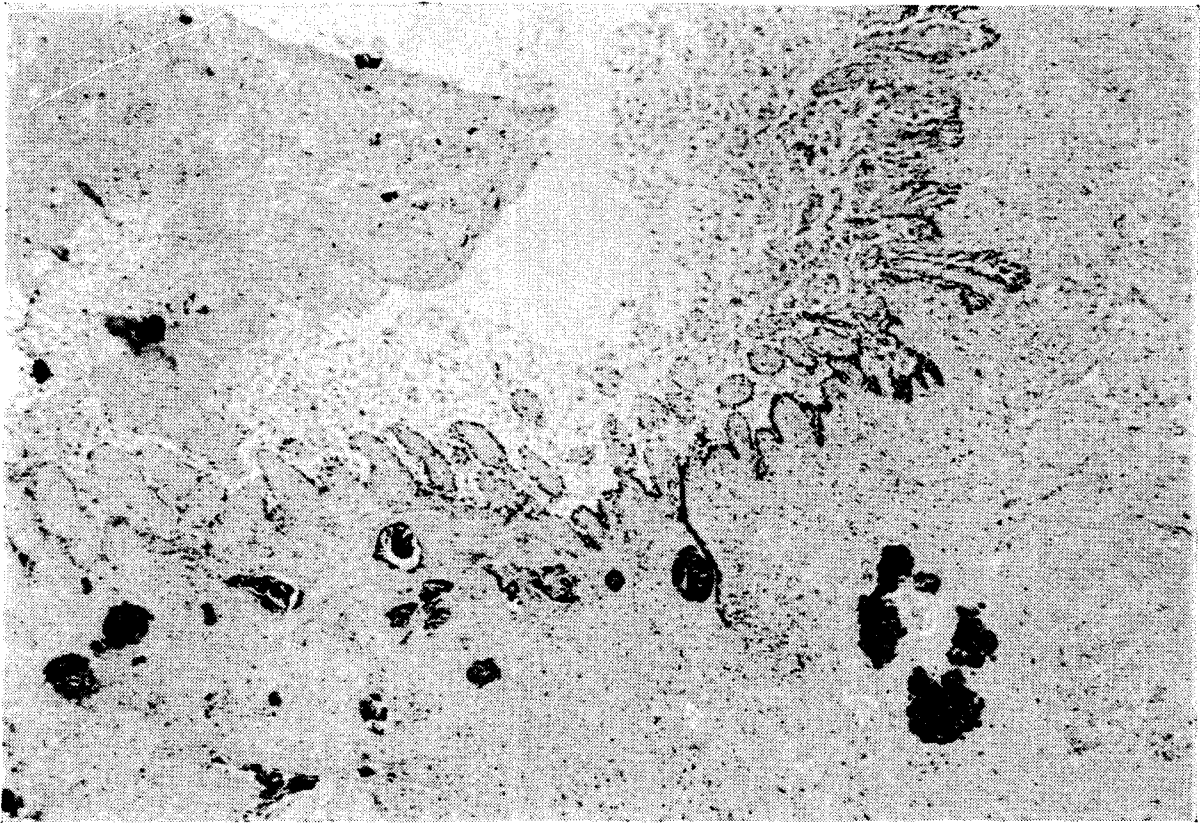
PLATE IV: 5.   Ulcer of oral mucosa in control monkey which died 15 days
following lethal irradiation. Desquamation of the epithelium with haemorrhage
and necrosis of submucosa. Note darkly staining accumulations of bacteria and
absence of cellular exudation. (HE). Magnification × 30

PLATE IV: 6.   Early regeneration of sternal bone marrow on the 4th day in
lethally irradiated mouse treated with rat bone marrow. Marrow still shows
poor cellularity but proliferated reticular cells and young stem cells (haemocy-
toblasts) can be seen between dilated sinusoids. (HE). Magnification × 190
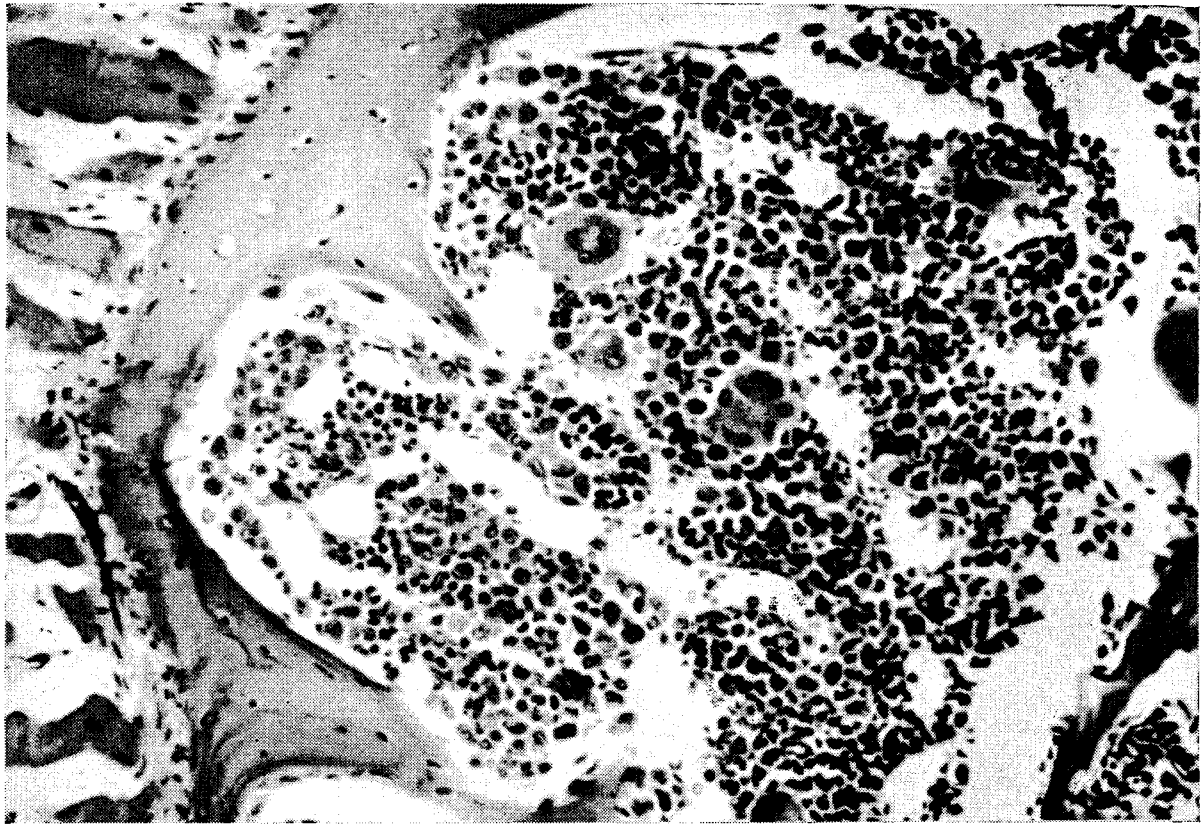
PLATE IV: 7.   Advanced regeneration of sternal bone marrow on day 5 in mouse treated with rat bone marrow. Highly cellular marrow with many immature cells. Mature cells—especially of the white series—are still lacking. (HE). Magnification × 190
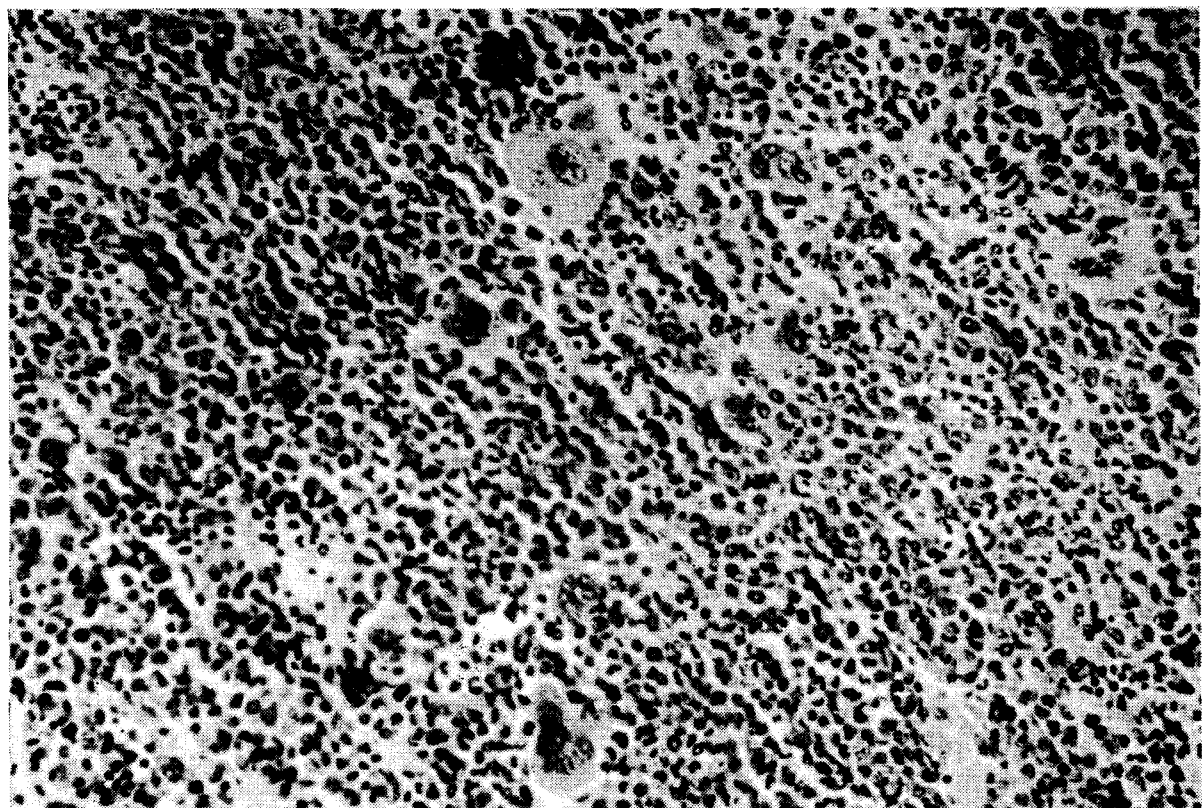
PLATE IV: 8.   Extensive extramedullary haemopoiesis in red pulp of spleen in homologous mouse chimaera on day 44. (HE). Magnification × 190
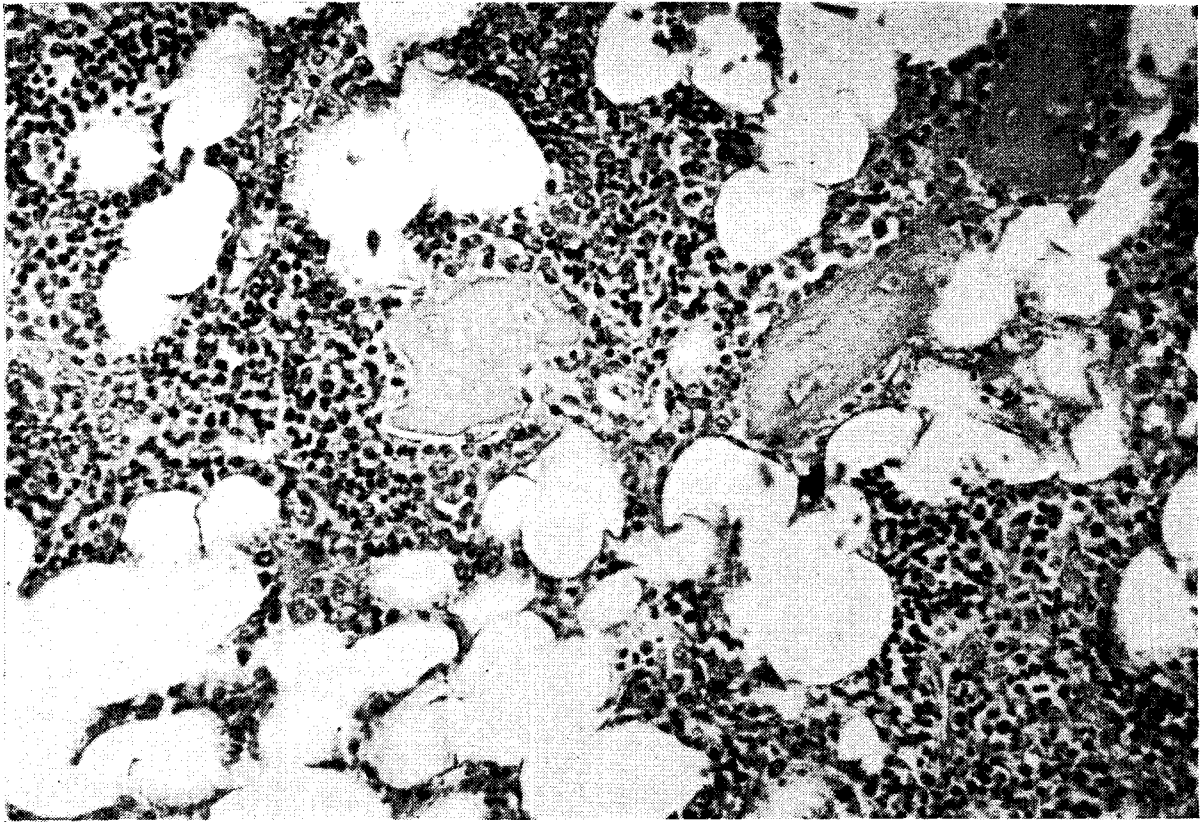
PLATE IV: 9. Advanced regeneration of bone marrow (rib) in a leukaemic child, 30 days after whole body irradiation with 900 r and treatment with homologous bone marrow. Fairly cellular marrow, all cell lines are represented. Photograph from Mathé *et al.* (1960)[263]. (HE). Magnification × 190
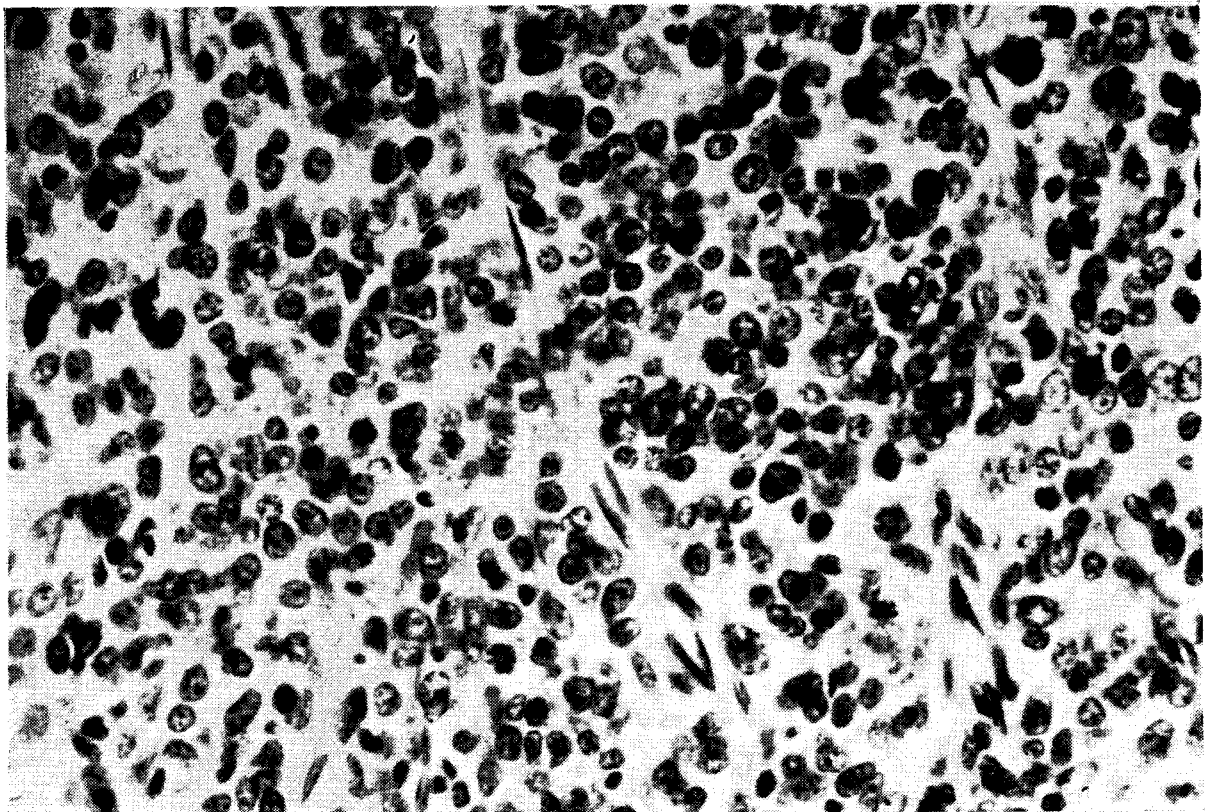
PLATE IV: 10. Early regeneration of thymus cortex on day 7 in lethally irradiated mouse treated with isologous bone marrow. Appearance of mitotic figures and small collections of lymphoblasts. (HE). Magnification × 480

PLATE IV: 11. Complete restoration of structure of splenic white pulp in a mouse 38 days after lethal irradiation and treatment with isologous bone marrow. Many mature lymphocytes and a lymphatic follicle are seen. (HE). Magnification × 120
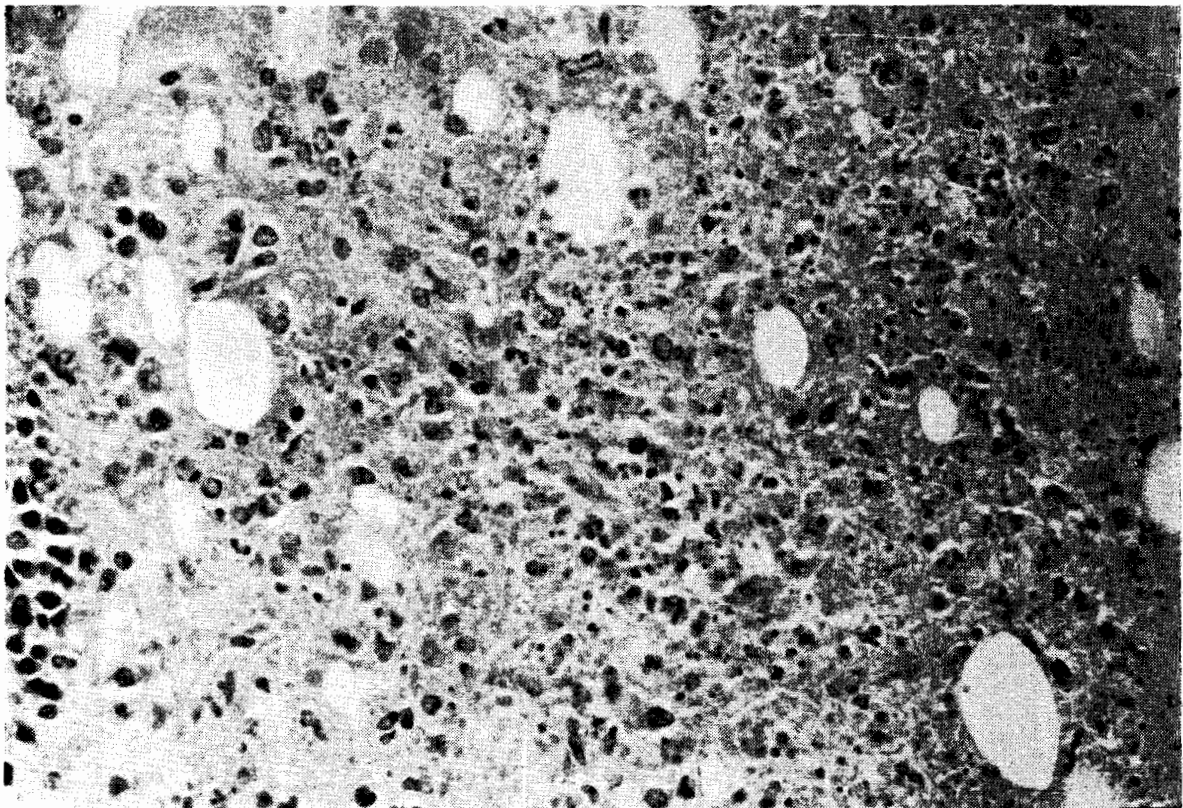
PLATE IV: 12. Rejection of bone marrow graft in a rat on the 12th day following low lethal irradiation (550 r) and treatment with homologous bone marrow. Sternal bone marrow, showing necrosis and many disintegrated cells. In lower left corner a number of intact erythroblasts and myeloid cells can be seen. (HE). Magnification × 300

CHAPTER IV

Pathology of the Radiation Chimaera

INTRODUCTION

The pathology of the radiation chimaera has been studied systematically in the mouse[99, 122, 184, 449], the rat[14, 18], the rabbit[325] and the rhesus monkey[446]. Some information has been published on other species including the dog[166, 330, 400] and man[263], but these observations are of an incidental nature. Moreover, the interpretation of the findings in the latter cases has usually been complicated by the fact that the identification of cells of the donor type has either not been performed or has yielded equivocal results so that it is not possible to be sure that the lesions encountered were characteristic of the chimaeric state.

The most important complication of an established graft of foreign bone marrow is secondary disease and since this condition is an entirely new pathological entity, emphasis is placed in this chapter on the pathological changes which have been found to accompany this disease. In order to identify the characteristic pathology of secondary disease it is obvious that the lesions have to be clearly differentiated from those due to whole body irradiation. This is the reason that the bone marrow syndrome will be described first in a separate section.

In several studies the conditions of the bone marrow transplantation were such that a delayed rejection of the graft took place in a proportion of the animals, while others remained stable chimaeras which developed secondary disease at about the same time. Because investigators were unaware of these two possibilities, particularly in early studies on the subject, some confusion arose concerning the nature of the lesions which were observed in such mixed groups of animals. The pathology which is considered typical for a delayed rejection of the bone marrow graft has therefore been treated more extensively in a later section.

The bone marrow syndrome

In the mouse, irradiation with lethal doses below 1200 r invariably leads to death from the bone marrow syndrome if no treatment is given. Since the pathology of the bone marrow syndrome is well known, those aspects necessary to differentiate this condition from secondary disease will be more fully discussed.

In most animal species bone marrow aplasia following lethal irradiation develops in about 2–3 days (Plate IV: 1). After 24 hours, mature granulocytes and megakaryocytes are the only haemopoietic cells left. After 48 hours an acellular marrow is found, which consists of reticular cells dispersed between dilated sinusoids. A few megakaryocytes may still be found. In men and monkeys infiltration with plasma cells and histiocytes—cells which are relatively radioresistant—is conspicuous. The state of aplasia persists till the death of the animal. Small groups of stem cells which consist mainly of erythroblasts may be noted during this interval and probably represent abortive attempts at regeneration.

Atrophy similarly develops in the lymphatic tissues of the lymph nodes (Plate IV: 2), spleen (Plate IV: 3), intestinal tract and thymus. After 24 hours depletion of lymphoid cells is pronounced and the lymphatic follicles have disappeared. However, even after *supra*lethal doses small numbers of mature lymphoid cells may be found dispersed in the reticular stroma and a further decrease in cellularity occurs in the following days. In a proportion of the animals large reticular cells and histiocytes with swollen nuclei and prominent nucleoli, sometimes displaying abnormal mitoses, are found in the follicular remnants. Frequently, the surrounding tissue and the medullary cords are heavily infiltrated with plasma cells.[95] It has been stated that this reticulo-histiocytic and plasmocellular reaction is a response to antigens from invading micro-organisms but this hypothesis seems to have been made less likely by the recent discovery of a similar cellular reaction in irradiated germfree animals[36]. Others have investigated this phenomenon and have ascribed it to an immunological reaction against the breakdown products of damaged and dead cells which are released in the first few days after irradiation.

The lymphatic sinusoids are dilated and filled at first with histiocytic cells, and after the first week with erythrocytes which are sometimes partly engulfed by macrophages. The latter finding is a reflection of the concurrent haemorrhagic diathesis. In a

minority of animals small groups of lymphoblasts may be found at the end of the first week, which probably represent abortive regeneration.

The aplasia of the blood-forming organs is reflected in the peripheral blood by pancytopenia. The rate of disappearance of the various mature cellular elements is mainly due to a complete inhibition of their production by the radiosensitive stem cells in the haemopoietic tissues and determined, therefore, by the normal life span of the mature radioresistant elements in the blood. An exception are lymphocytes which die in interphase soon after irradiation both in the lymphatic tissues as well as in the peripheral blood. In the case of the erythrocytes, extra-vascular losses after the first week greatly enhance the development of the anaemia.

In the mouse, as a consequence, maximum depression of lymphocytes, granulocytes, thrombocytes, reticulocytes and erythrocytes generally occurs within 24 hours, 72–96 hours, 9 days, 48 hours and 14 days respectively.

In some animal species—guinea-pig and man—temporary abortive recovery, in particular of the neutrophil granulocytes, has been described. Although normal values are not reached, abortive recovery of granulocytopoiesis in the host may interfere with a correct interpretation of the results of attempted therapy, e.g. bone marrow transplantation.

In the second week following the irradiation, depletion of thrombocytes and leucocytes leads to haemorrhages and septicaemia which terminate the life of the animal. Because of the absence of a cellular reaction against micro-organisms, the septicaemic lesions have a characteristic appearance. At autopsy various organs may be found to be studded with rounded yellowish white foci which on microscopic examination are found to consist of areas of bland necrosis of the parenchymal cells and stroma (Plate IV: 4). Although infiltration with inflammatory cells is absent, masses of bacteria may be seen in the centre of these lesions. In addition, capillaries plugged with bacterial emboli are also present.

Haemorrhages are found in the skin, mucosal and serosal surfaces and in a number of parenchymal organs. The distribution of the haemorrhages is often characteristic of the animal species. Rodents are characterised, for example, by haemorrhages in the pyloric part of the stomach, in the myocardium and in the testes. These are most likely the result of physiological vascular traumata occurring during the

intense muscular activity in these organs*, which are normally repaired by the adherence of thrombocytes at the site of the vascular defect and the subsequent deposition of fibrin. The haemorrhagic diathesis may also induce characteristic lesions in the stomach. Superficial haemorrhages in the mucosa may lead to widespread cystic dilation of glands by compression of the necks of these structures. Sometimes, similar changes may be noted in the large intestine.

Ulcers occur in the oral cavity and the intestinal tract; these may be caused by bacterial infection, haemorrhage or both. Massive haemorrhage may cause ischaemic necrosis by vascular compression. In the monkey large haemorrhages occur in the colon, which lead to mucosal ulceration, total necrosis of the intestinal wall and often perforation to the peritoneal cavity. Microscopically the lesions show desquamation of surface epithelium and necrosis, bacterial infiltration and some haemorrhage of the underlying tissue which is covered with membranes of fibrin. In monkeys, as well as in mice and rats, mucosal necrosis of the colon and caecum, apparently caused by bacterial invasion, is frequently observed (Plate IV: 5). In many cases it is impossible to determine whether the lesion is initiated by bacterial invasion or by haemorrhage. The lesions which have been described are very similar to the necrotic ulcers of the oral mucosa and gastrointestinal tract found in human cases of pure agranulocytosis in which platelet counts are normal. Here, bacterial invasion can be considered to be primarily responsible.

Radiation induced intestinal changes

Radiation doses which cause death from haemopoietic failure do not give rise to intestinal denudation. Significant radiation induced changes may be noted, however, in these cases in the first 4 days after irradiation. It is necessary to describe these lesions in some detail since they must be distinguished from the intestinal lesions due to secondary disease. The latter may be confused with the radiation induced changes in the case of an early severe graft versus host reaction.

Forty-eight hours after irradiation, the crypts in the small intestine are shallow and nuclear pyknosis and fragmentation of crypt cells is present. A small number of crypts are usually cystically dilated and show desquamation of degenerated crypt cells. The height of the in-

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* In the case of the testis the vascular traumata are believed to occur as a result of its frequent movements through the inguinal canal, which causes a slight deformation of the organ during the passage.

testinal villi is decreased, and the covering epithelial cells are flattened. Between the affected crypts, varying numbers of regenerated crypts are present, which are lined with a hyperchromatic pseudo-stratified epithelium showing many mitoses. Similar, but less severe changes are found on the following days and recovery is completed on day 6. The large intestine is much less affected, although in the first week some degeneration and an increase in the number of mucoid cells in the crypts may occur. As was mentioned earlier, ulcerations in the colon after the first week are in most cases secondary to haemorrhage and infection.

Recovery of haemopoiesis in bone marrow treated animals

The sequence of changes in the haemopoietic tissues after the intravenous injection of haemopoietic cells has only been studied extensively in irradiated mice. Less complete observations have been reported on the haemopoietic recovery in other species, e.g. rabbits, rats, dogs, monkeys, and human patients.

BONE MARROW

In mice the injection of very large amounts of isologous bone marrow may result in a visible recovery in the recipient's marrow on the 2nd and 3rd day[428]. Following the administration of the usual amount of isologous or homologous bone marrow, i.e. 10^6 and 10^7 cells per mouse respectively, recovery may be noted on the 4th day at the earliest[449]. Between the fat cells and the dilated sinusoids of the aplastic bone marrow, proliferation of reticular cells and large stem cells—haemocytoblasts—has occurred. (Plate IV: 6). On the 5th day cellular bone marrow is usually found, the haemopoietic elements still consisting mainly of stem cells of all cell lines (Plate IV: 7). During the following days, maturation of the marrow continues and the composition of the bone marrow appears to be normal on day 7. Hypercellularity of the bone marrow, caused by stem cell hyperplasia, is often seen during the first month following the recovery and may persist after that time in animals treated with homologous or heterologous bone marrow.

Simultaneously with the marrow regeneration, foci of extramedullary haemopoiesis appear in the spleen, liver, and lymph nodes. In the first month following treatment the spleen may be greatly enlarged due to extensive infiltration of the red pulp with haemopoietic cells (Plate IV: 8). After that time extramedullary haemopoiesis

diminishes and towards the 6th week, in mice treated with isologous bone marrow, it has returned to a normal level. Persistence of excessive extramedullary haemopoiesis is usually seen, however, in mice treated with homologous or heterologous bone marrow (Plate IV: 8).

Information on bone marrow histology is available for a number of other recipient species. In rabbits treated with homologous bone marrow, repopulation has been followed up and, in those animals which suffered from secondary disease, hyperplasia was found to be present[325]. In rats and guinea pigs examined approximately 14 days after isologous bone marrow transplantation the appearance of the bone marrow had returned to normal[445]. In rats treated with homologous bone marrow, bone marrow recovery started on day 6. The histological pattern of regeneration in the following days was similar to that in mice. However, in many rats relatively hypocellular or even acellular areas persisted in an otherwise normally maturated marrow[14, 18]. In monkeys[446] early repopulation of the bone marrow has been noted on day 6 and 7 following transplantation of autologous or homologous marrow. These investigations have suggested that, following cell doses of comparable effectiveness with respect to radiation protection, bone marrow regeneration in monkeys occurs later than in mice. Repopulation of the bone marrow has also been shown to occur in irradiated patients after treatment with homologous bone marrow[263], but the time of onset of repopulation and its completion after the bone marrow transfusion has not yet been exactly established (Plate IV: 9).

LYMPHATIC TISSUES

The time and the extent of the regeneration of the lymphatic tissues is dependent on both the number of lymphoid cells contained in the injected cell suspension and also the host donor combination.

Following the administration of bone marrow only, regeneration is first seen on the 6th day after autologous treatment (monkeys), isologous combinations (mice) and in homologous and heterologous combinations (mice and monkeys) (Plates IV: 10 and 17). Compared with mice, the repopulation of lymphatic tissue in monkeys is relatively early if the time of bone marrow recovery is taken into account. This could be one of the reasons for the more severe character of secondary disease in monkeys, as has been mentioned in Chapter III.

At the end of the first week collections of large immature lymphoid cells, presumably lymphoblasts, appear between the reticular cells of

the stromal tissue in the follicles of the spleen, the cortex of the lymph nodes and thymus and the remnants of the accumulations of the lymphatic tissue in the intestinal tract. Mitotic figures are seen in these areas of regeneration. In the days following, the number of lymphoid cells increases in both autologous and isologous combinations. Mature lymphocytes appear and follicle formation occurs. Advanced regeneration is found in the second week and in the course of the 3rd and the 4th week the lymphatic tissues attain a normal histological appearance (Plate IV: 11).

In a number of homologous or parent to F_1 combinations in mice, complete regeneration of the lymphatic tissues similarly occurs in about a month. In other homologous or heterologous combinations in mice and in homologous combinations in rats, rabbits, monkeys, and men, the initial regeneration is followed by secondary changes which will be discussed in the section dealing with secondary disease.

PERIPHERAL BLOOD

The peripheral blood changes which follow irradiation and bone marrow transplantation have been studied in mice, monkeys, and men.

The destruction of the host's bone marrow is reflected in the gradual disappearance of the mature elements in the blood, similar to that seen in non-treated irradiated animals.

The take and proliferation of the bone marrow graft is indicated by the reappearance of reticulocytes as early as the 5th day, followed by reticulocytosis in the 2nd and 3rd week. The neutrophil count increases after the 5th day, followed by a rise of the thrombocyte count on the 9th day and of the mononuclear cells at the end of the 2nd week. After treatment with isologous bone marrow the peripheral blood cell count is restored to normal by the end of the 4th week, the values of the mononuclear cells being depressed the longest[149].

In monkeys recovery of the peripheral blood elements to their normal levels occurred later than in mice and, moreover, the time of recovery was found to be more variable (1–3 weeks). This was probably due either to differences in the number of bone marrow cells injected in these particular experiments or to a slower proliferation rate of the grafted cells in monkeys.

In the human cases in which the irradiation factors were carefully controlled and bone marrow grafting proved to be successful, recovery of the peripheral blood count occurred after about 13–20 days[263].

In the recovery phase many immature cells appeared in the peripheral blood. Myelocytes, erythroblasts, large erythrocytes and large basophilic thrombocytes, were noted as transient features in the smears.

In comparison with animals treated with isologous bone marrow, those treated with homologous or heterologous bone marrow showed abnormalities of the peripheral blood count during or following the recovery phase; these abnormalities will be discussed in the next section.

Graft rejection in homologous and heterologous chimaeras

The cause of the delayed death of animals treated with foreign bone marrow, in contrast to the almost uniform survival of those treated with isologous or autologous marrow, has been an important issue for some time.

As has been mentioned earlier, two opposing explanations were postulated: a graft versus host or a host versus graft reaction. A follow-up study of lethally irradiated mice treated with homologous or heterologous bone marrow showed that particularly after irradiation in the lower lethal dose range, either of these immunological reactions might be responsible for delayed death of the chimaeras[449].

The proportion of animals suffering from a graft rejection, or failure of the graft to take, varies with both the radiation dose and the host donor combination, as discussed in Chapter III. Death from a delayed rejection of the marrow graft after an initial take usually occurs earlier (14–20) days than death from a graft versus host reaction (after the 20th day). The two modes of death may, however, overlap in time or, as has been found in monkeys, may even be present simultaneously.

THE "SPLENIC WHITE PULP REACTION"

It has to be assumed, therefore, that even after lethal radiation doses the immunological reactivity of the host is not completely suppressed. A histological counterpart of such immunological activity of the host was described by Congdon[95]. This reaction, designated as the splenic white pulp reaction, was found to occur in lethally irradiated mice. These histological changes will be described in some detail, since they may reflect the ability of the host to reject a foreign graft after irradiation in the lower lethal dose range.

The reaction was observed in the first 3 days following bone
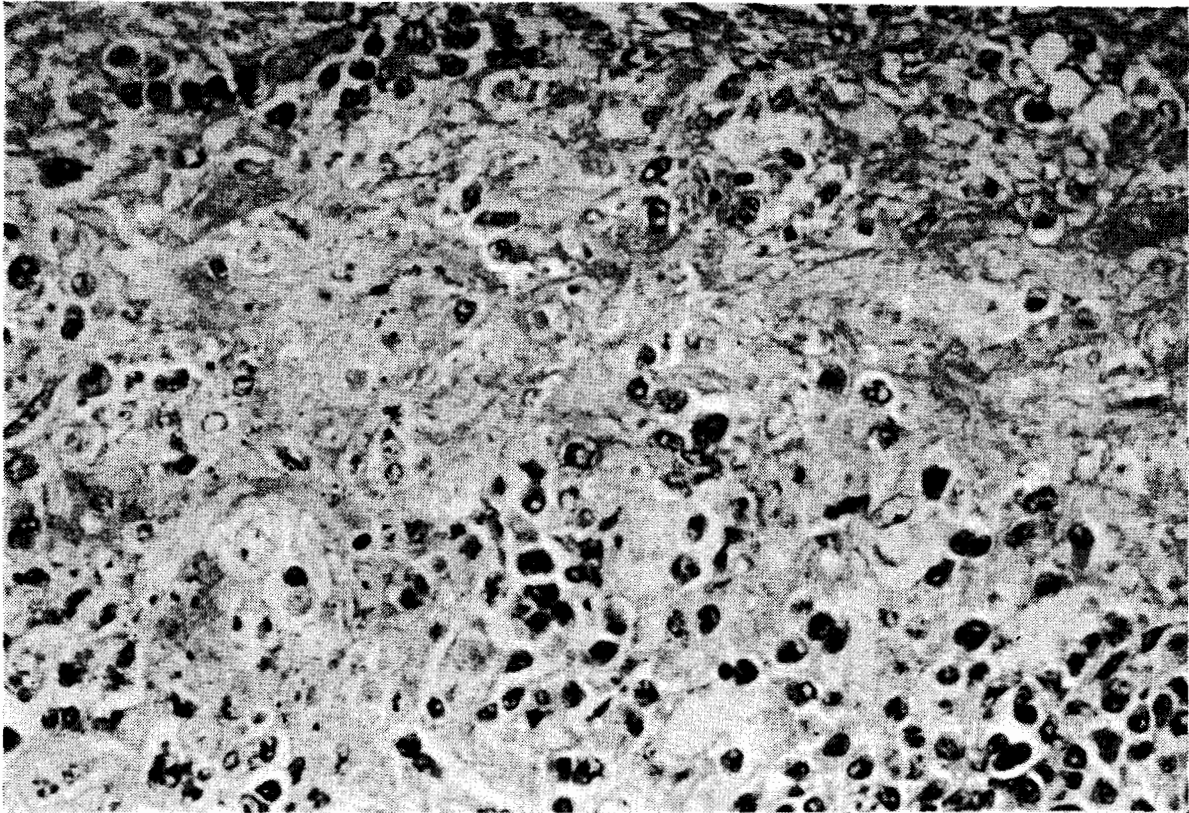
PLATE IV: 13. Fibrinoid necrosis in red pulp of spleen in an irradiated rat rejecting a homologous bone marrow graft at 9 days. Deposition of finely fibrillar fibrinoid substance and disintegrated cells in centre of picture. Note surrounding normal myeloid cells. (HE). Magnification × 300
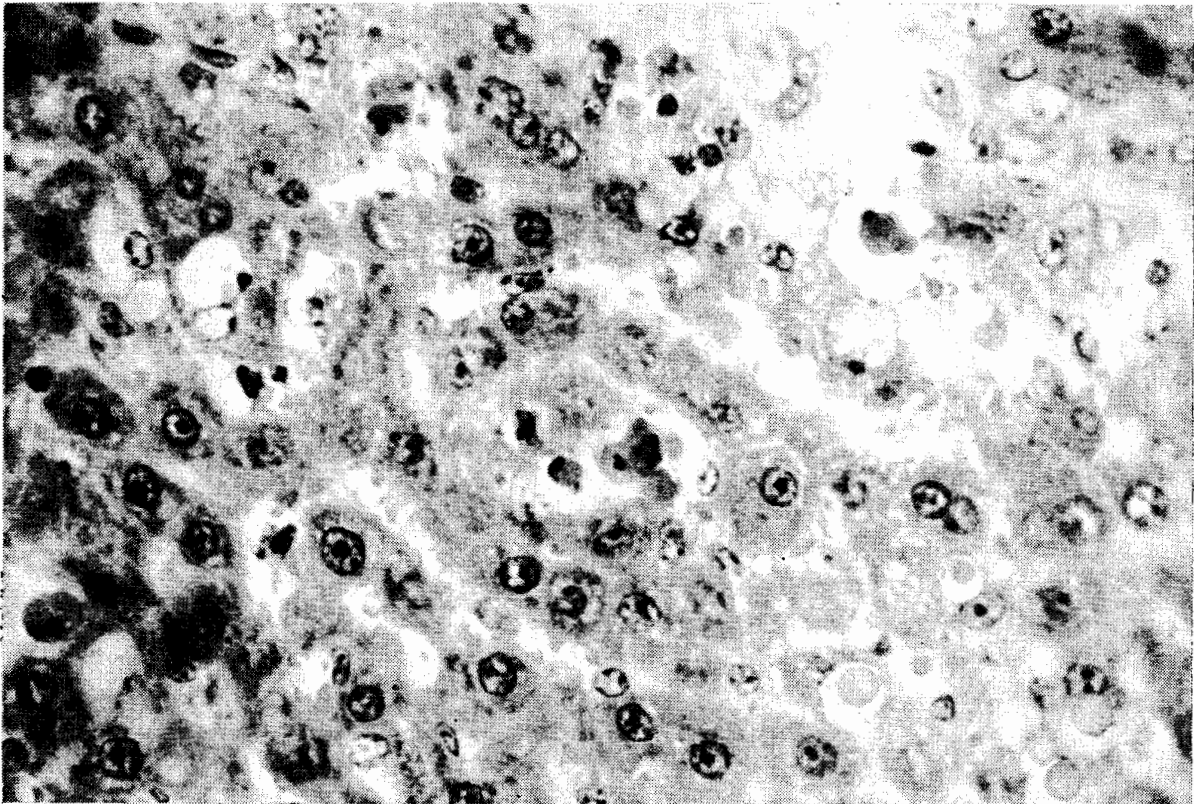
PLATE IV: 14. Isolated disintegration of liver cells in lethally irradiated rat dying 9 days after treatment with homologous spleen cells. (HE). Magnification × 300
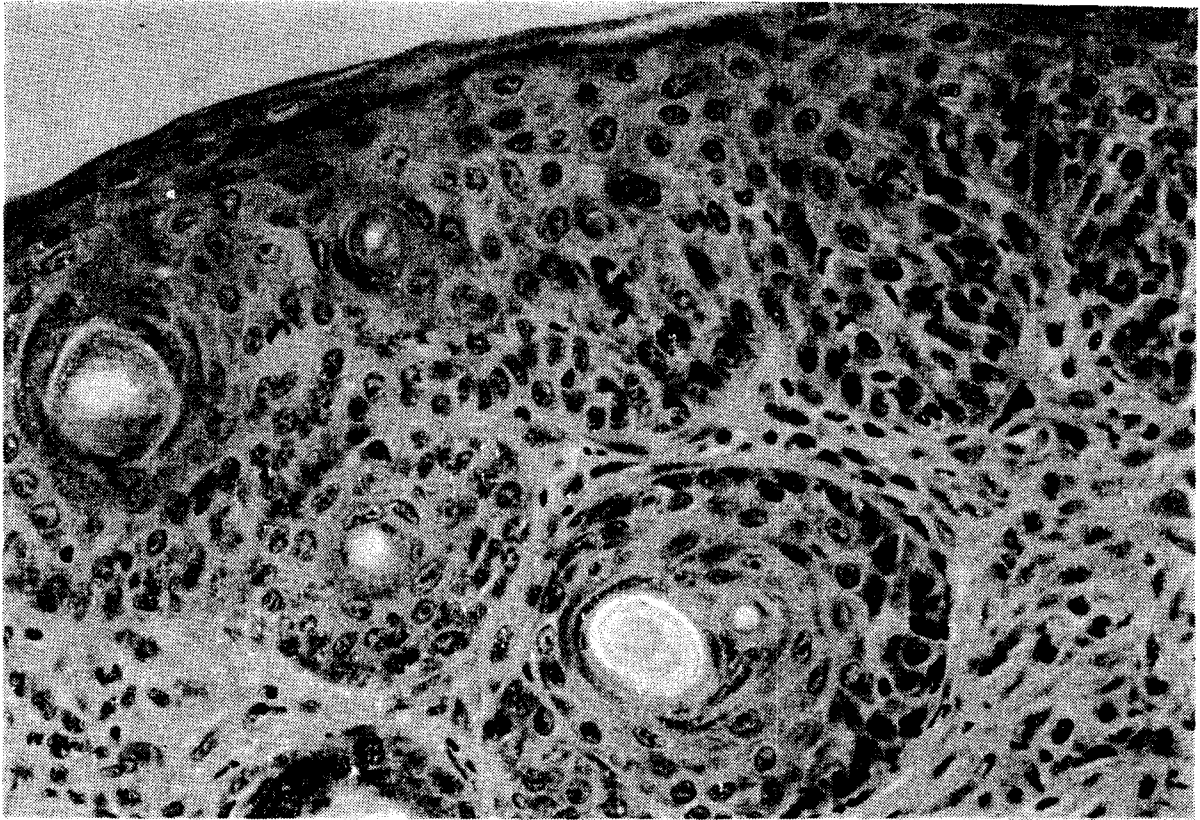
PLATE IV: 15. Hyperplastic change (acanthosis) of epidermis and hair follicles in 8-month old homologous mouse chimaera. Note slight infiltration of dermis with lymphoid cells and histiocytes. (HE). Magnification × 300
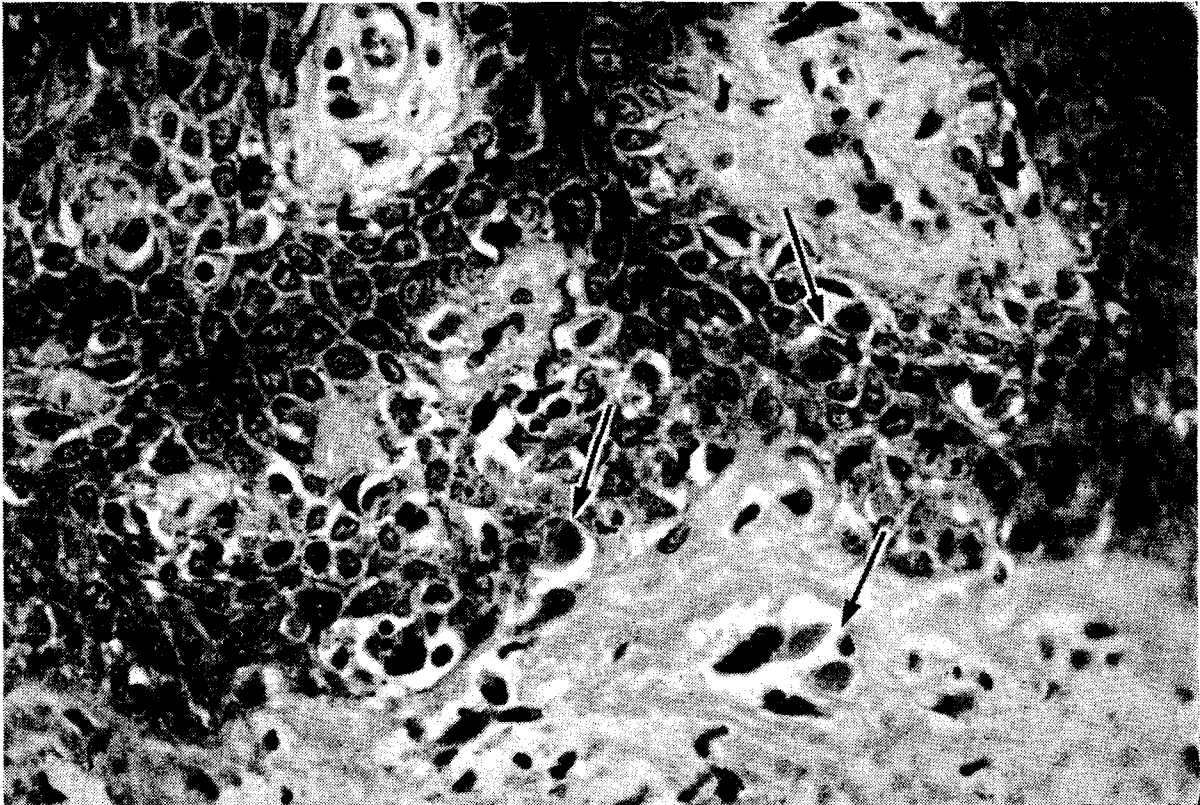
PLATE IV: 16. Skin biopsy of leukemia patient suffering from severe secondary disease after irradiation with 800 r and treatment with pooled bone marrow of 6 donors. The epidermis is infiltrated by lymphoid cells. In the immediate surrounding of these cells the epithelial cells display degenerative changes, most pronounced being a vacuolisation of the cytoplasm. Some cells are necrotic: rounded homogeneously eosinophilic cell remnants indicated by arrows (dyskeratosis). (HE). Magnification × 300
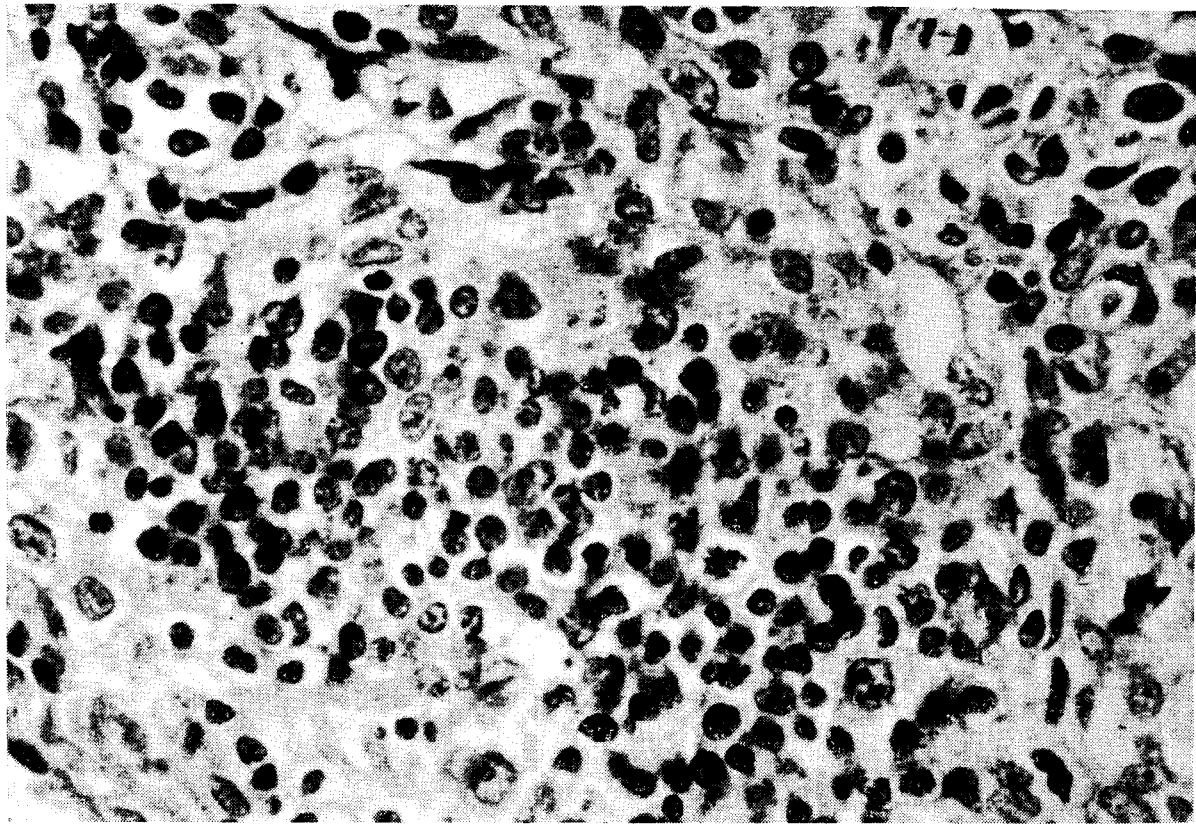
PLATE IV: 17. Early regeneration of lymphatic tissue in lymph node cortex of homologous mouse chimaera on day 7. Note focal collection of lymphoblasts and lymphocytes. Several mitoses are present. (HE). Magnification × 480
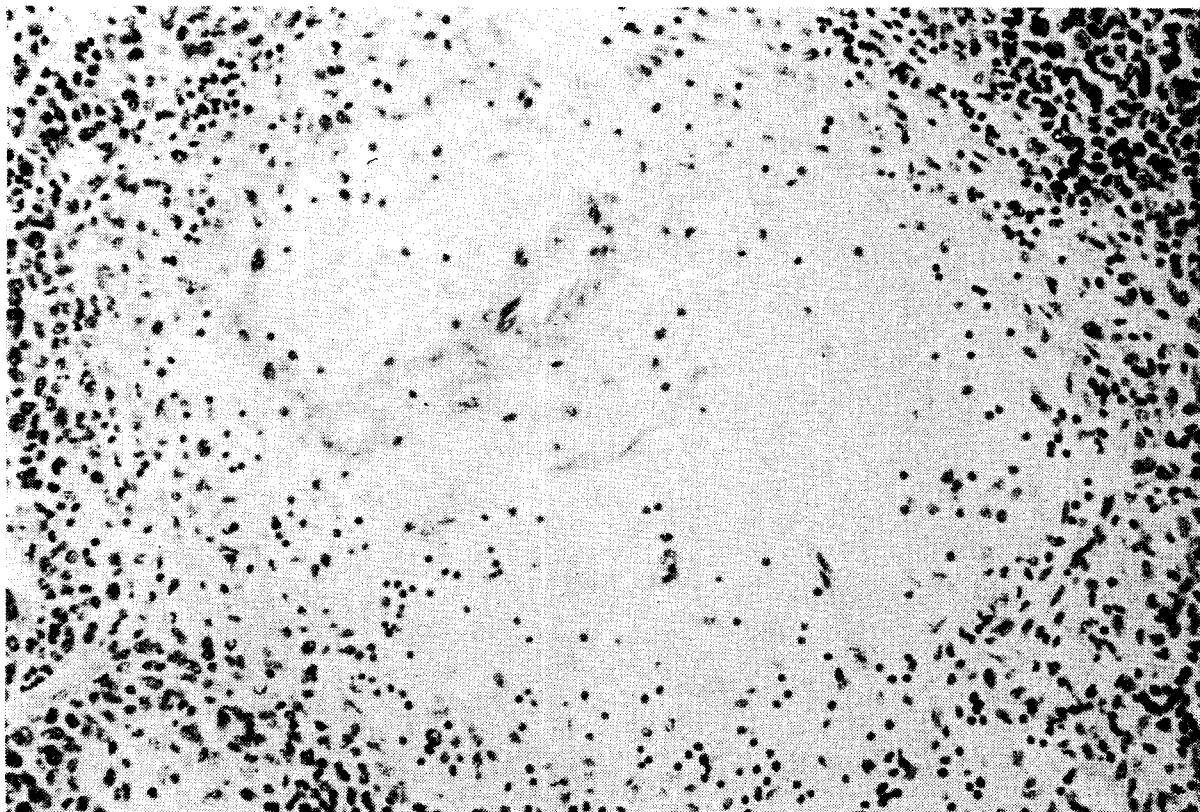
PLATE IV: 18. Complete atrophy of lymphatic tissue surrounding splenic arteriole in spleen of homologous mouse chimaera on day 44. Surrounding the empty follicle are many haemopoietic cells. (HE.) Magnification × 190
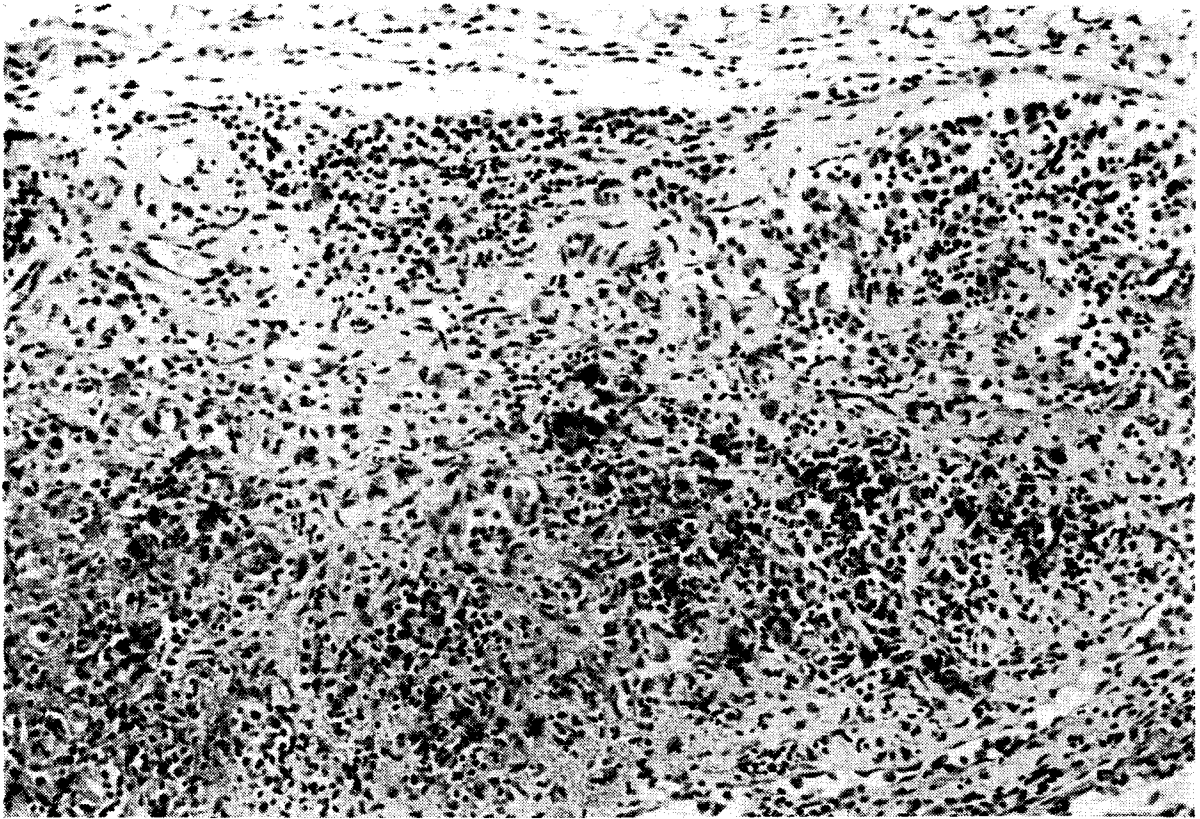
PLATE IV: 19.   Severe atrophy of lymph node of 6-week old heterologous (rat bone marrow treated) mouse chimaera. Note absence of lymphatic follicles, scarcity of lymphoid cells and fibrosis. (HE). Magnification × 120
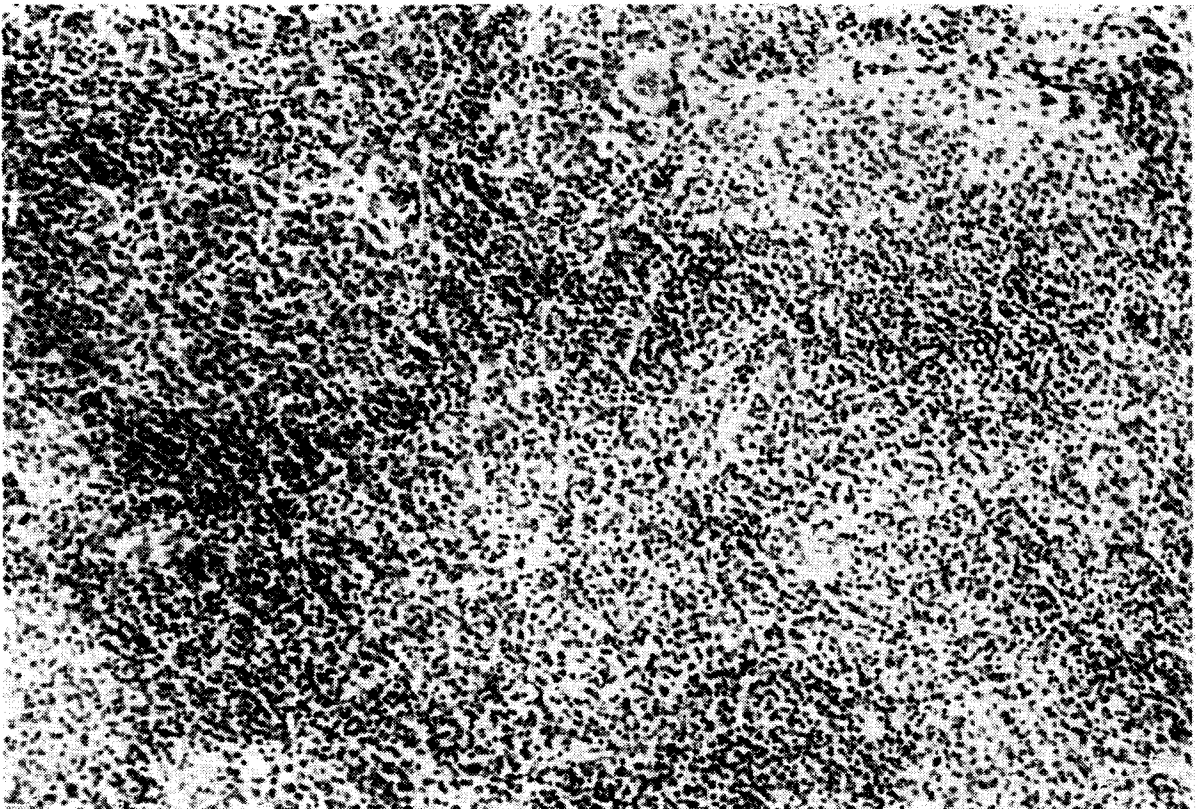
Plate IV: 20.   Complete regeneration of lymphatic tissue of spleen in 79-day old homologous mouse chimaera. The host–donor combination in this case generally produces severe secondary disease (CBA host, C57BL donor). A large lymphatic follicle is present in upper left corner. (HE). Magnification × 120
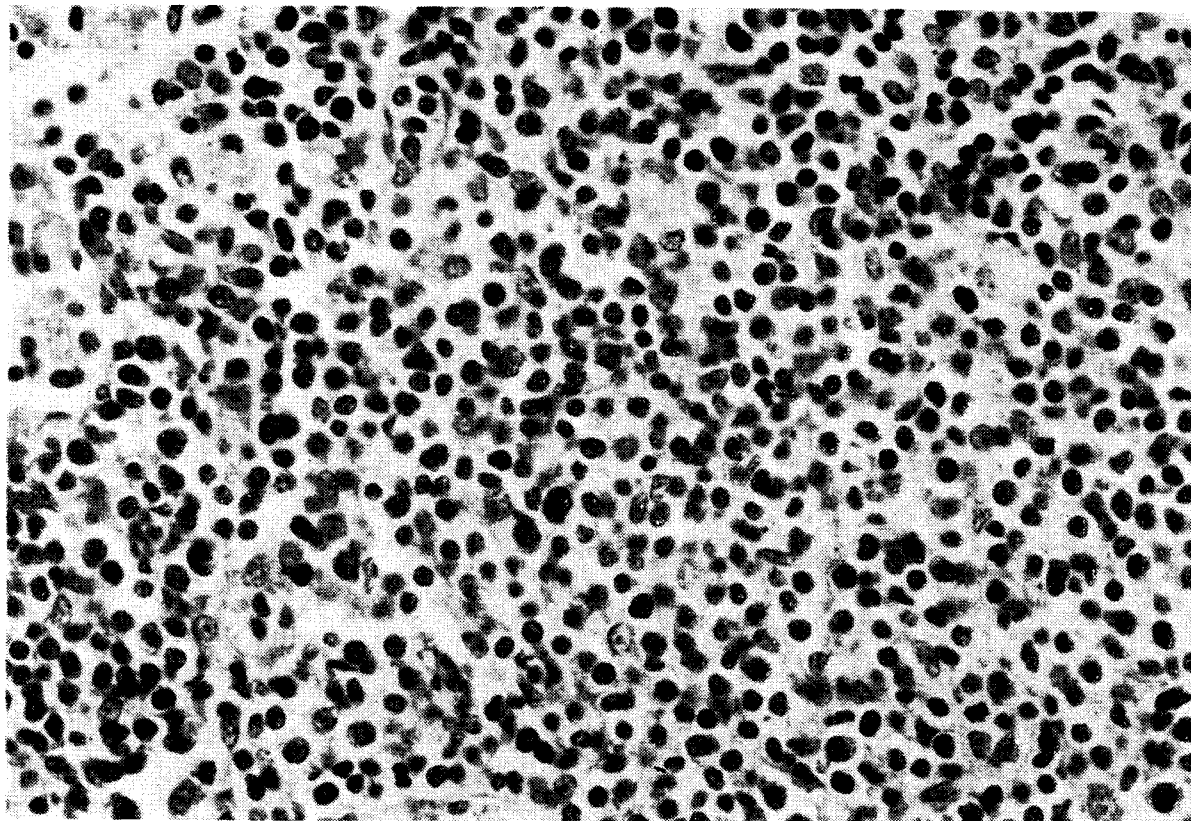
PLATE IV : 21.   Intense lymphopoietic activity in mesenteric lymph node of monkey that died on day 7 following treatment with homologous bone marrow. The animal suffered from severe secondary disease. Note many lymphoblasts, medium-sized and small lymphocytes and several mitoses. (HE). Magnification ⨯ 300
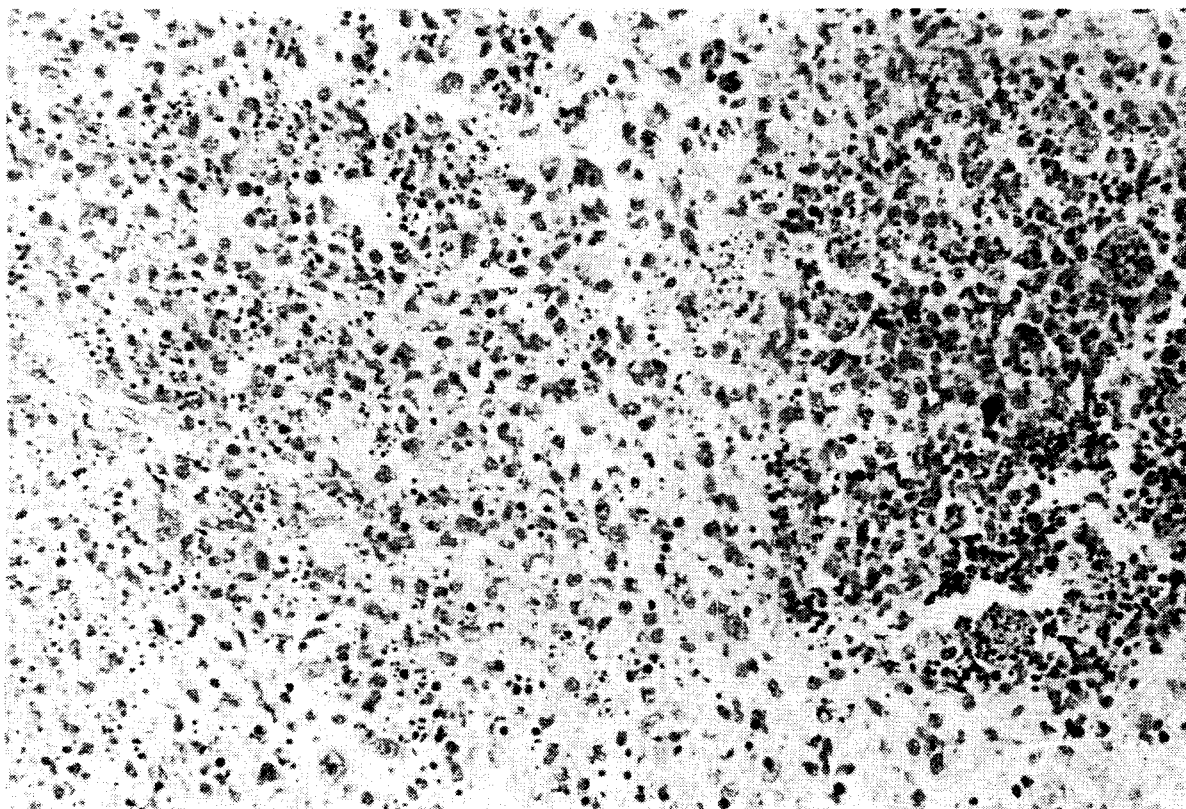
PLATE IV : 22.   Massive karyorrhexis of lymphoid cells in mesenteric lymph node of homologous monkey chimaera on day 14. Note follicular remnant on right side of picture. The stromal cells appear unaffected. (HE). Magnification ⨯ 190

PLATE IV: 23. Granulomatous reaction in lymph node of 24-day old homologous mouse chimaera. Note presence of many histiocytes (epitheloid cells) and several multinuclear giant cells of histiocytic origin (arrows). (HE). Magnification × 300

PLATE IV: 24. Intense lymphopoietic activity in mesenteric lymph node of CBA mouse on day 4 after irradiation with 800 r and treatment with 10^6 C57BL lymph node cells. Many lymphoblasts and medium-sized lymphocytes are seen, while a few small lymphocytes are also present. Note several mitoses. Compare with Plate IV: 21 of a homologous monkey chimaera, treated with bone marrow

PLATE IV: 25.   Interstitial pneumonitis of probable viral aetiology in homologous monkey chimaera (47 days). Large nuclear inclusion body in alveolar cell indicated by arrow. (HE). Magnification × 1200

PLATE IV: 26.   Ulcerating lesion in colonic mucosa of homologous monkey chimaera (27 days) showing many large cells with nuclear inclusion bodies. Two of these are indicated by arrows. (HE) Magnification × 480

PLATE IV: 23. Granulomatous reaction in lymph node of 24-day old homologous mouse chimaera. Note presence of many histiocytes (epitheloid cells) and several multinuclear giant cells of histiocytic origin (arrows). (HE). Magnification × 300

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Plate IV: 26. Ulcerating lesion in colonic mucosa of homologous monkey chimaera (27 days) showing many large cells with nuclear inclusion bodies. Two of these are indicated by arrows. (HE) Magnification × 480

PLATE IV: 27. Focal necrosis of adrenal cortex in leukaemic child, which died 43 days after irradiation with 890 r and treatment with bone marrow from his own mother. Several giant cells with prominent nuclear inclusion bodies are present; two of these are indicated by arrows. Photograph from Mathé *et al.* (1960)[263]. (HE) Magnification × 300
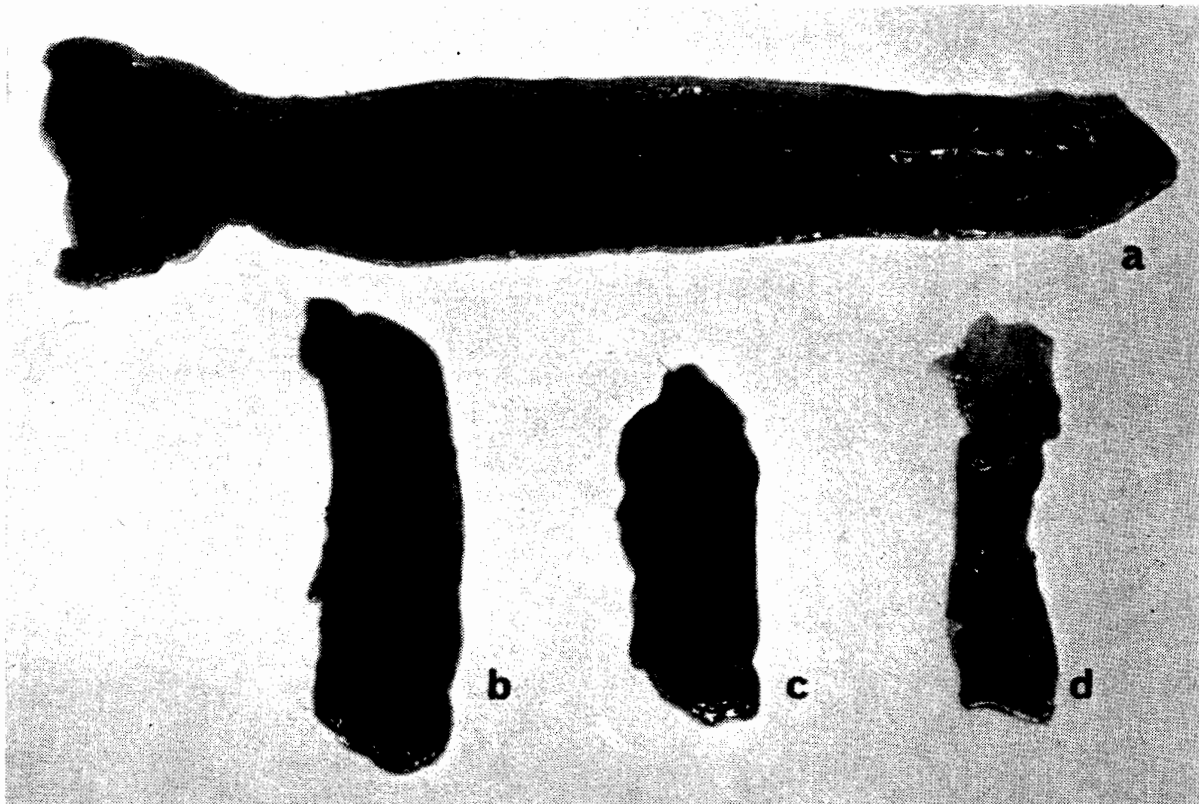
PLATE IV: 28. Shedding of the mucosa of colon (a), ileum (b) and jejunum (c) in homologous monkey chimaera (13 days) with severe secondary disease. Greyish-green membranes of sloughed mucosa project into the lumen. The duodenum (d) appears normal.

PLATE IV: 29. Atrophy of mucosa of cecum and ascending colon in homologous monkey chimaera (19 days). Note smooth glistening surface of mucosa and unusual prominence of lymphatic follicles, appearing as greyish-black dots.
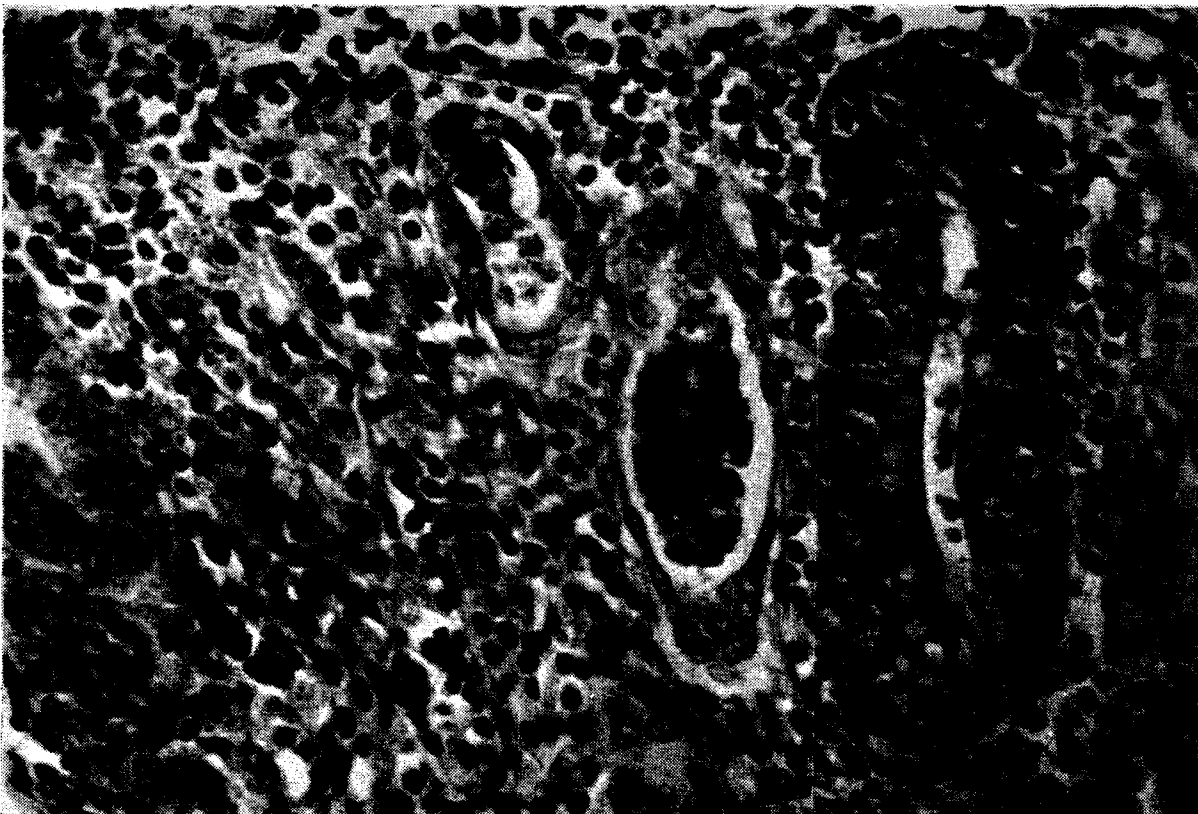
PLATE IV: 30. Acute disintegration of the crypts in colon of homologous monkey chimaera, that died on day 7. The mucosa is heavily infiltrated with lymphocytes which have also penetrated the crypts. Note severe karyorrhexis in the crypts and two crypts in centre distended with desquamated necrotic cells. (HE). Magnification × 300

PLATE IV: 31.   Cystic degeneration of crypts in colon of homologous monkey chimaera at day 26. The dilated atrophic crypts are lined with a single layer of flattened epithelium. Hyperplastic crypt in upper left corner. (HE). Magnification × 120
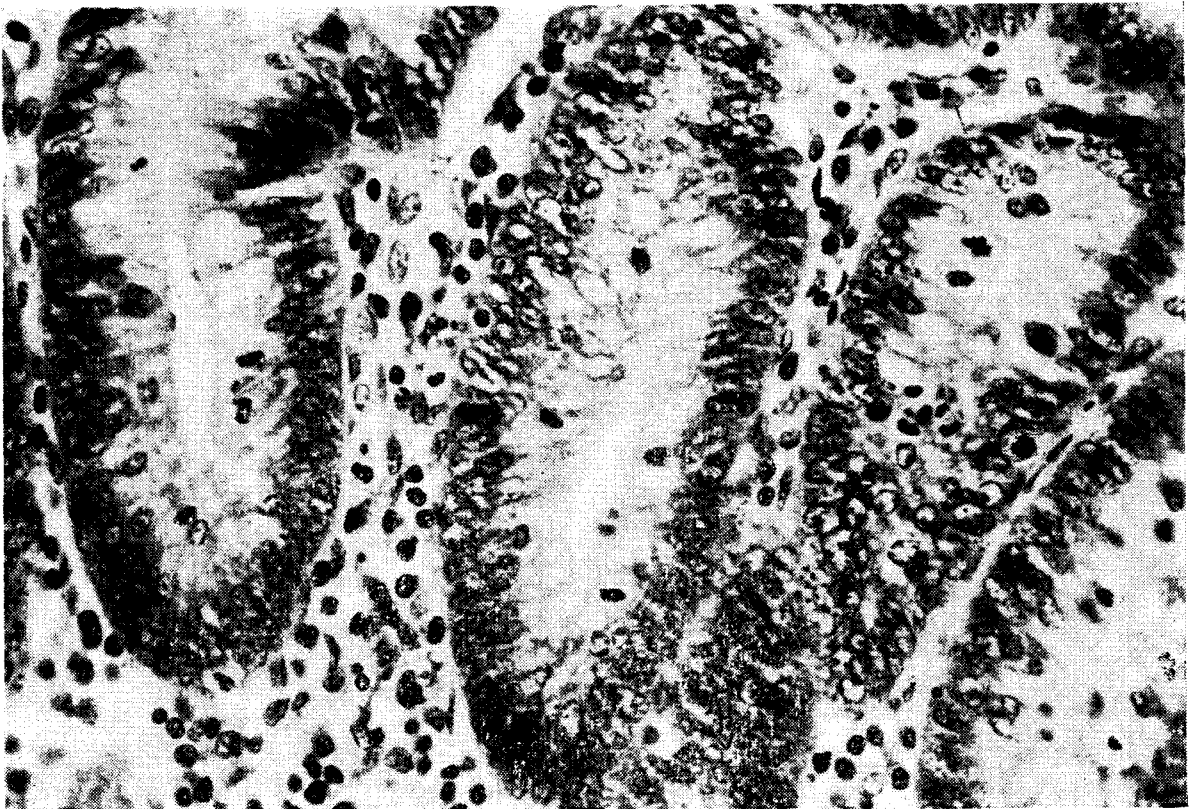
PLATE IV: 32. Hyperplastic crypts in colon of homologous monkey chimaera (same as in Plate IV: 30). The crypts are lined with a tall darkly staining epithelium showing many mitoses. (HE). Magnification × 300
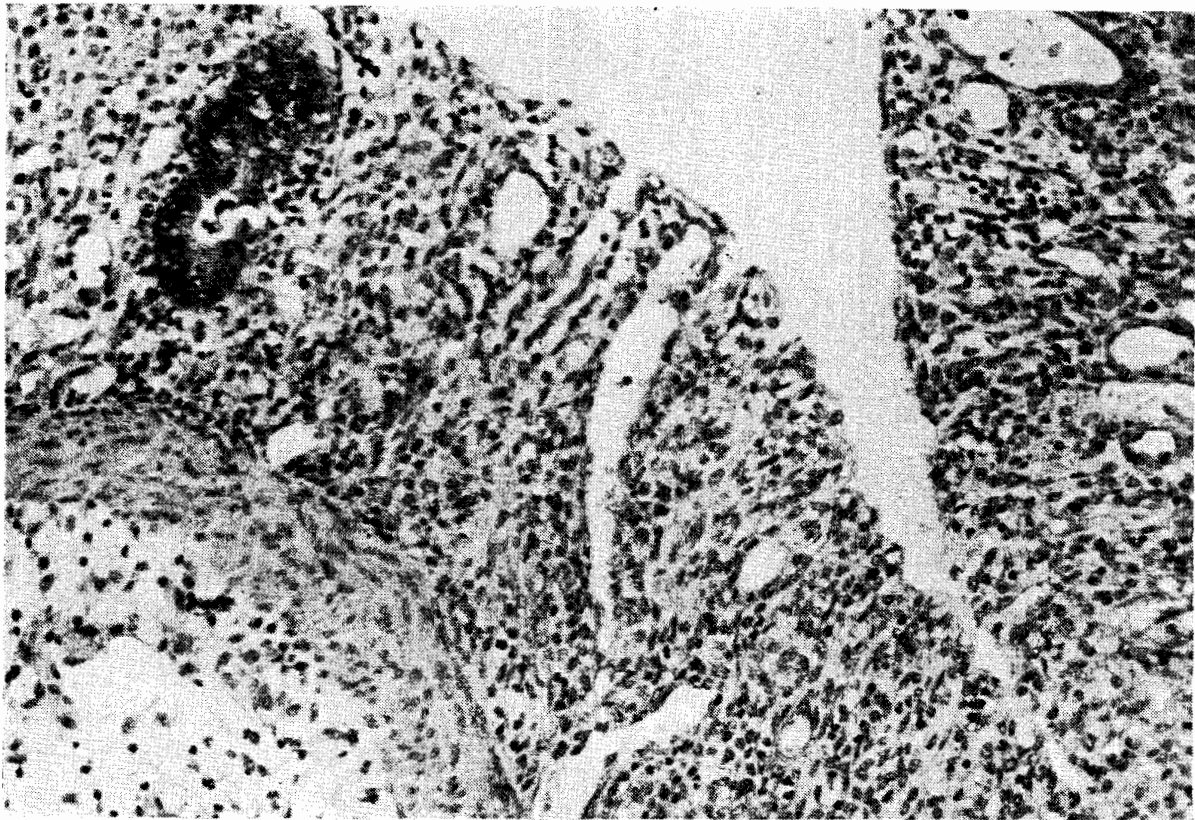
PLATE IV: 33. Denudation of colonic mucosa in homologous monkey chimaera. Note absence of surface epithelium and almost total loss of crypts. The stromal cells of the mucosa appear normal. (HE). Magnification × 120
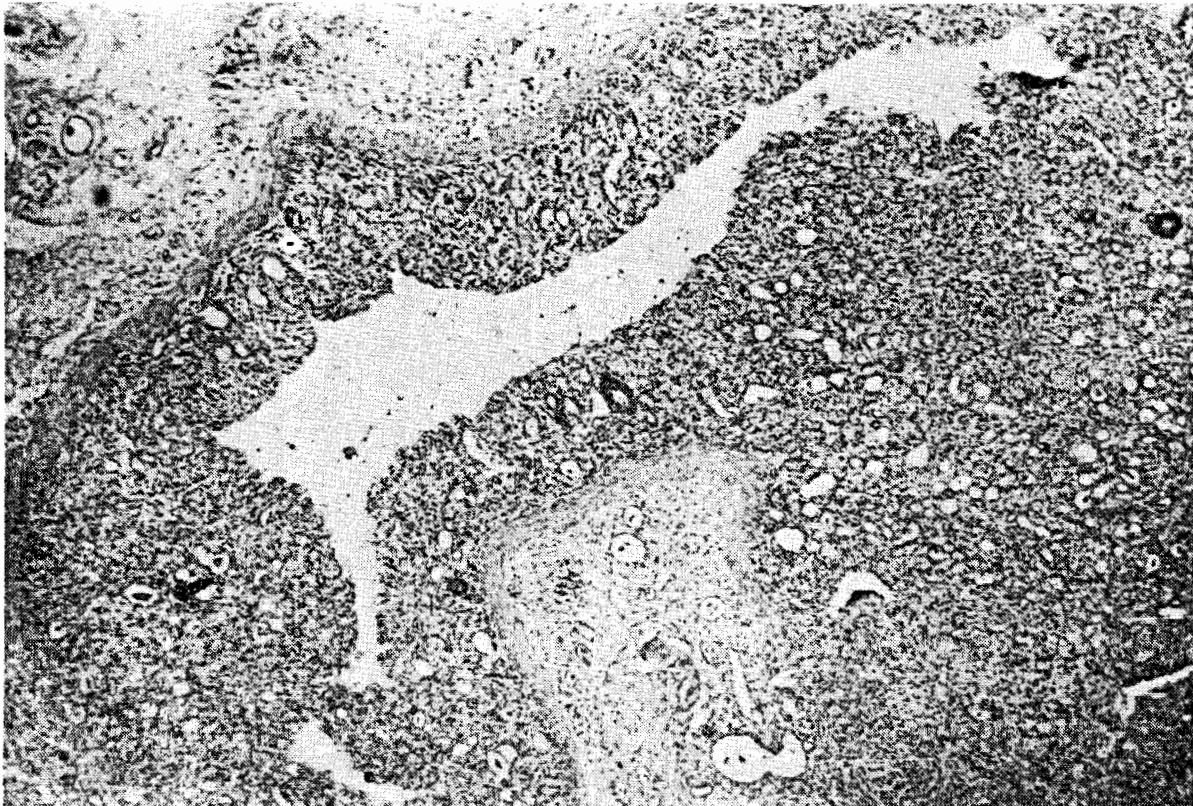
PLATE IV: 34. Near total loss of surface and crypt epithelium in ileum of homologous monkey chimaera (same animal as in Plate IV: 33). (HE). Magnification × 30
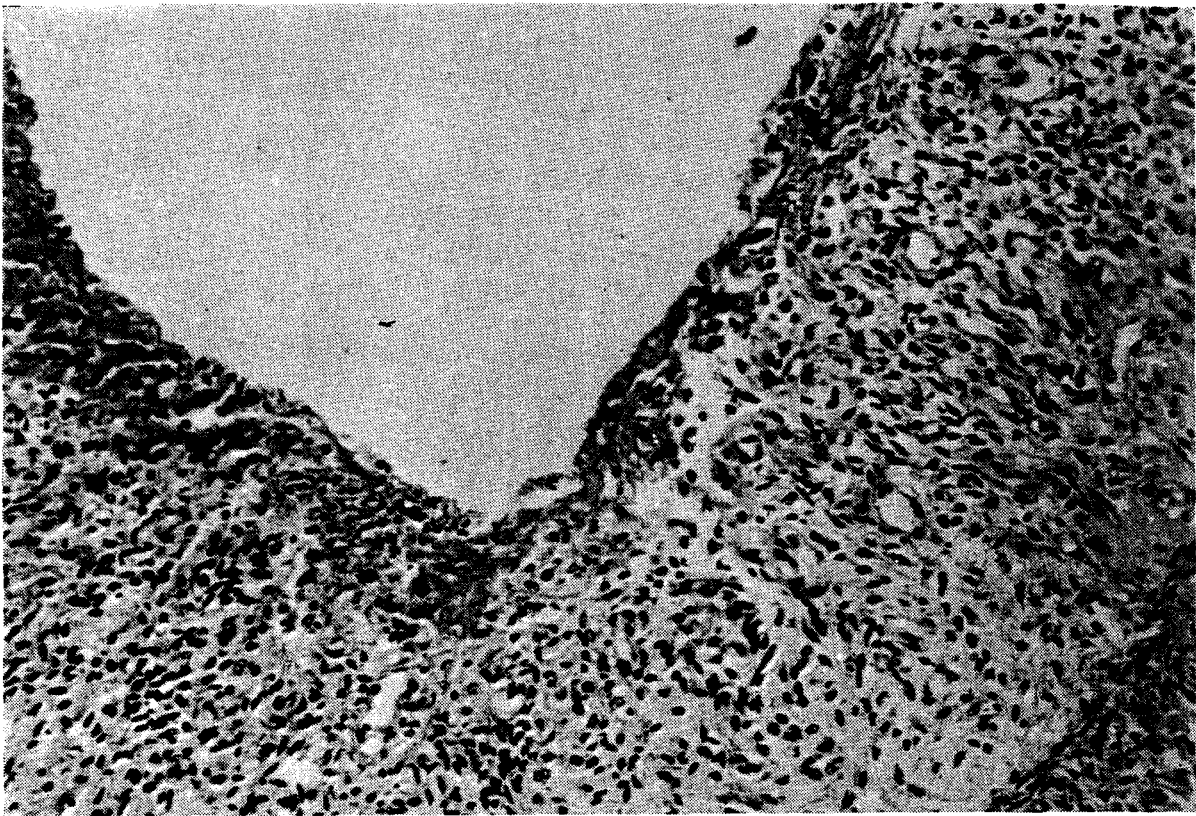
PLATE IV: 35. Total denudation of mucosa of ileum in a human radiation chimaera, transplanted with bone marrow of his mother, dying 43 days following the irradiation. Surface epithelium and crypts are absent. The substantia propria is covered with a thin layer of fibrin. (HE). Magnification × 120
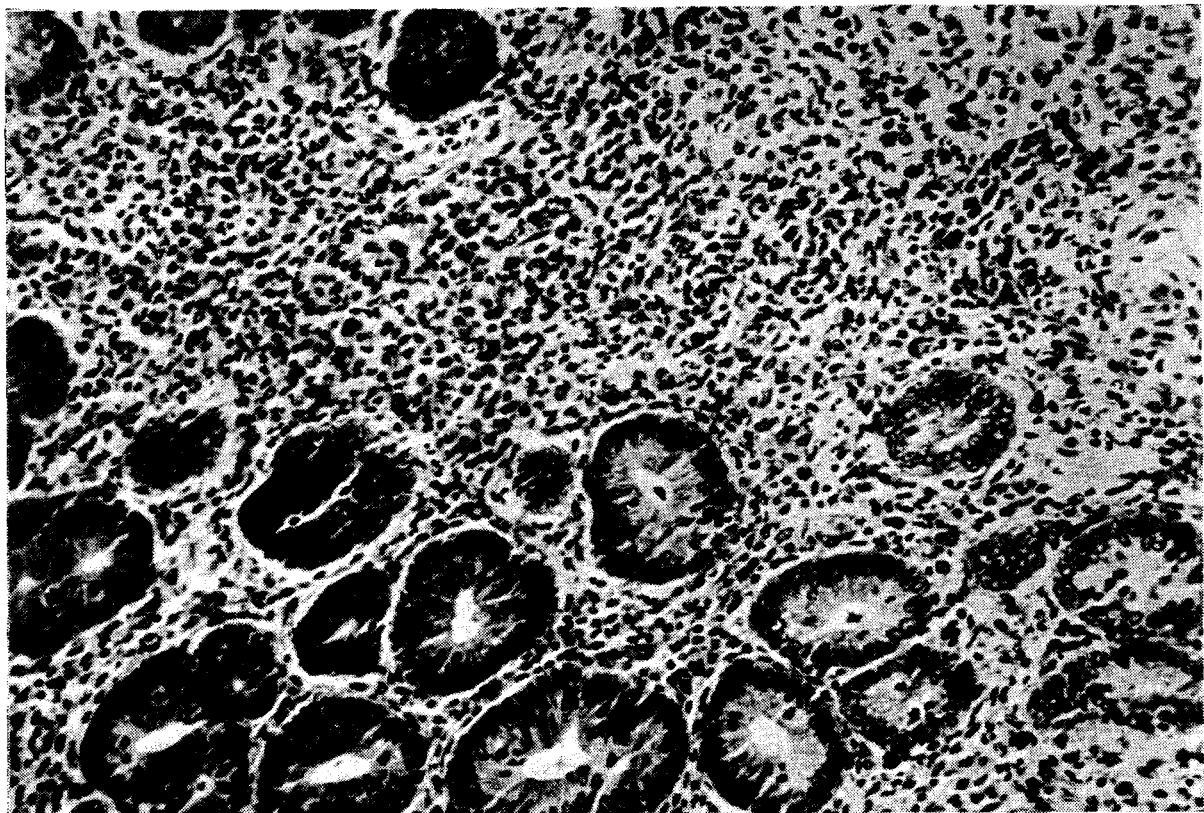
PLATE IV: 36. Chronic colitis in a rat → mouse chimaera 44 days after bone marrow transplantation. A dense infiltration of polynuclear leucocytes, plasma cells and lymphocytes separates the crypts, some of which are hyperplastic. (HE). Magnification × 190
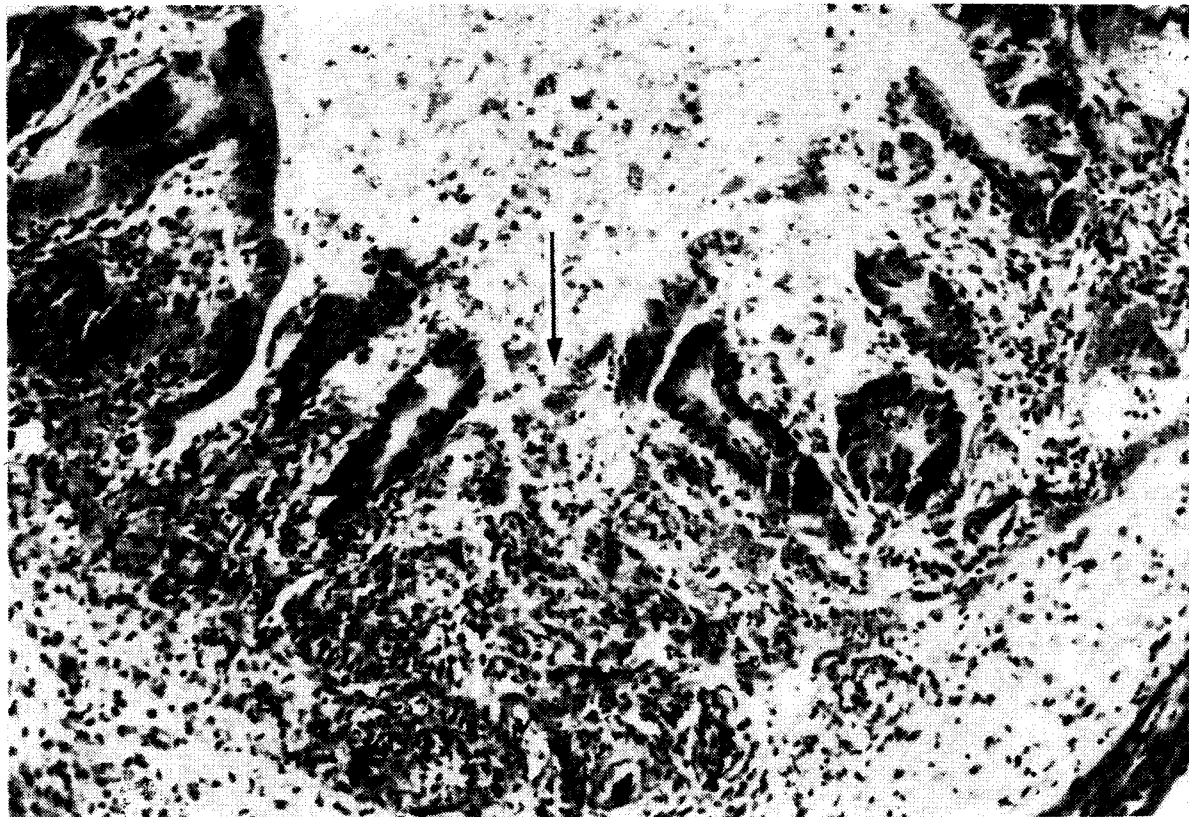
PLATE IV: 37. Chronic colitis in rat → mouse chimaera on day 35. The inflammatory infiltrate spreads through the submucosa into the muscular coat. There is pronounced oedema of the submucosa. Note destruction of crypts and small area of ulceration (arrow). (HE). Magnification × 120
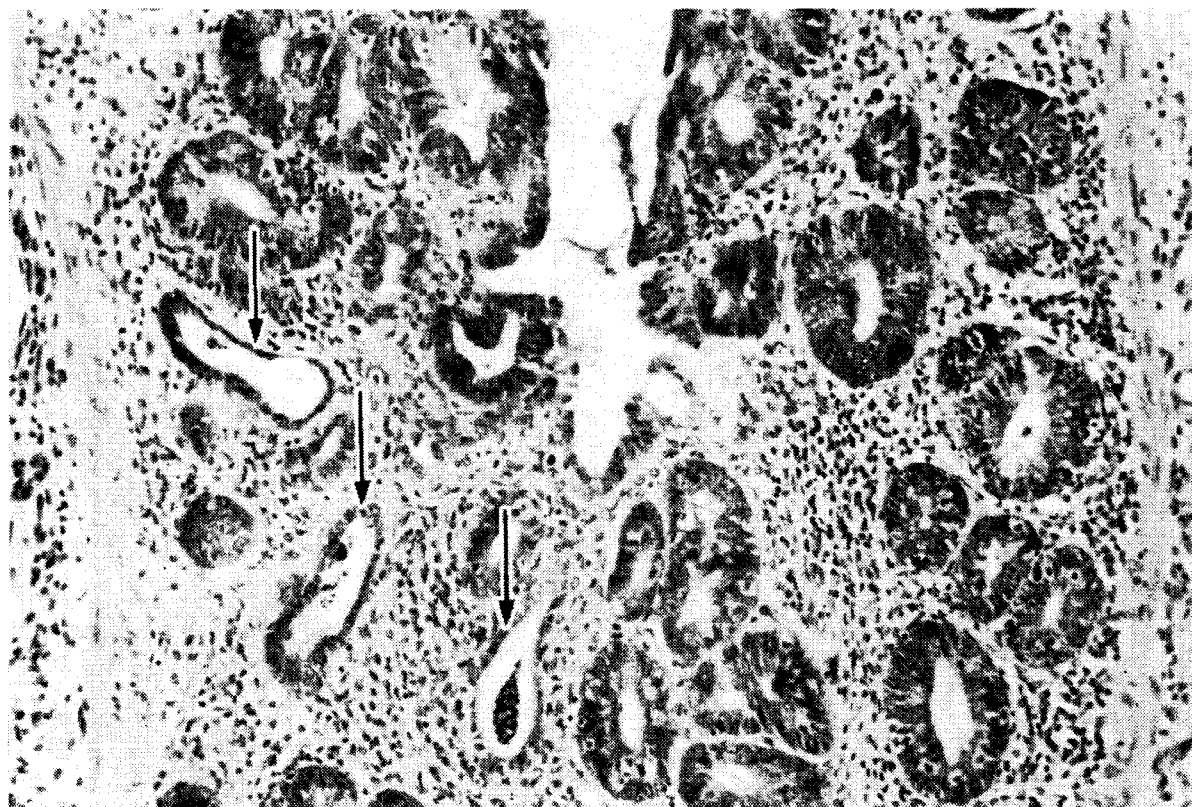
PLATE IV: 38. Chronic colitis in rat → mouse chimaera 9 months after transplantation. Several cystically dilated degenerating crypts are seen (arrows), lined with flattened atrophic epithelium. The crypt at the base is filled with polynuclear leukocytes (crypt abscess). The other crypts are hyperplastic. (HE). Magnification × 120
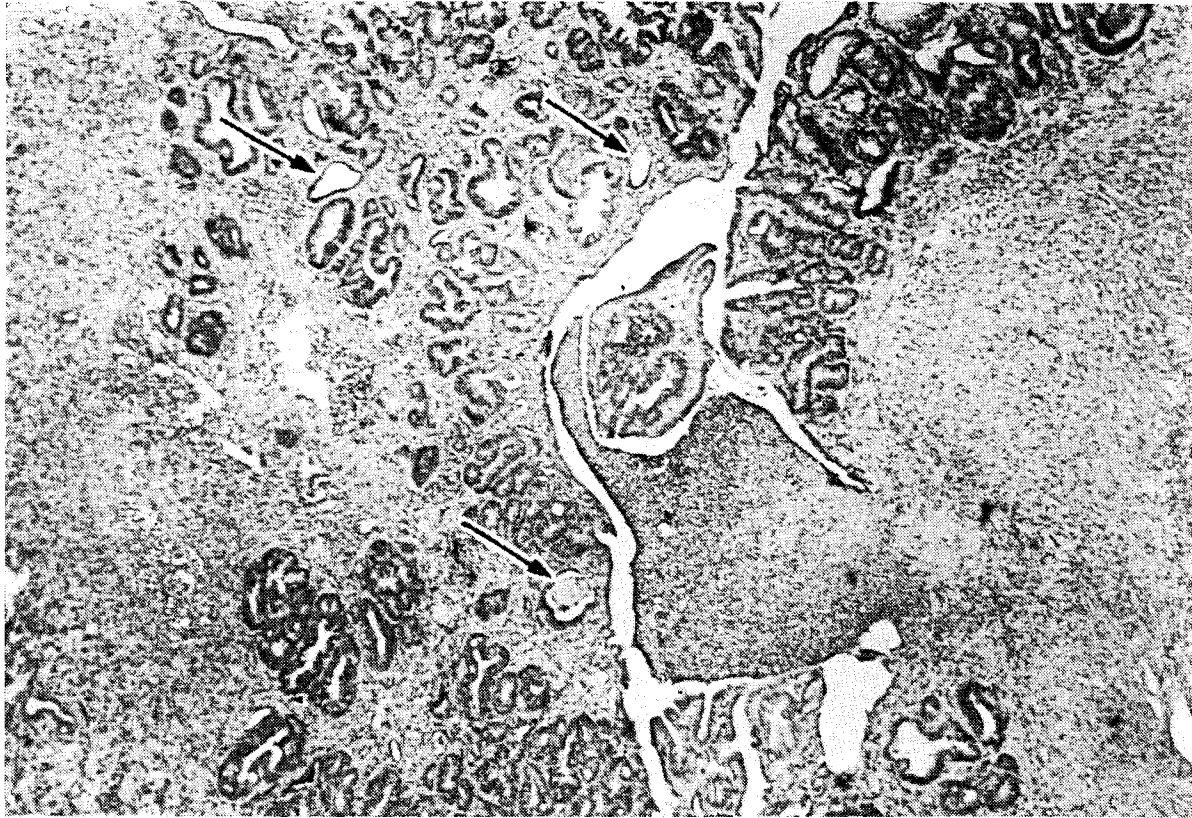
PLATE IV: 39(a). Chronic ulcerative colitis in rat → mouse chimaera at 6 weeks. A dense chronic inflammatory infiltrate spreads through all the layers of the colonic wall. Small funnel-shaped ulceration of mucosa at right. Several cystically dilated crypts showing a cystic change can be seen (arrows). (HE). Magnification × 30
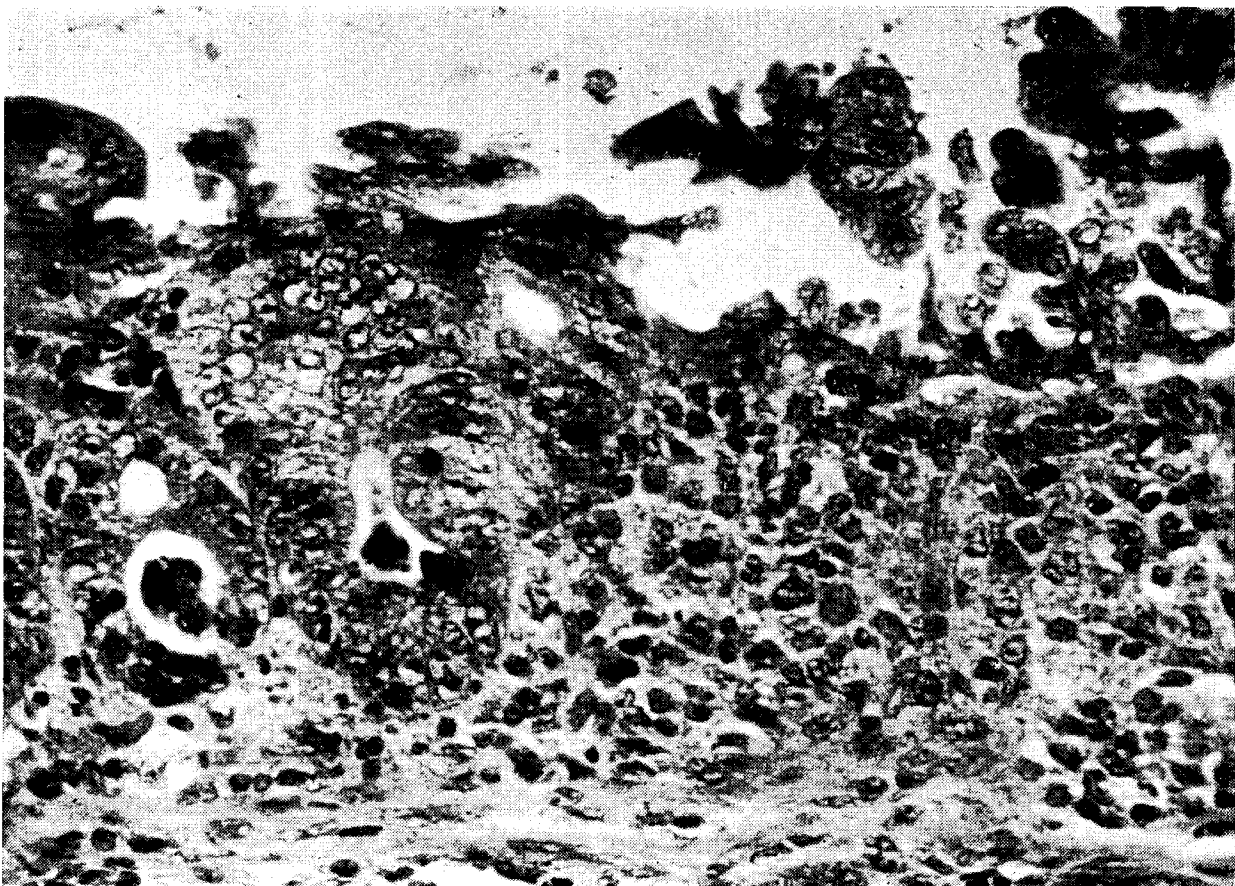
PLATE IV: 39(b). Crypt degeneration in colon of germ-free ND$_2$ mouse on day 17 following irradiation and treatment with rat bone marrow. The donor bone marrow was proved to be sterile. Note slight lymphoid cell infiltration; the mucosa at right side of picture is completely deprived of crypts. The lesion
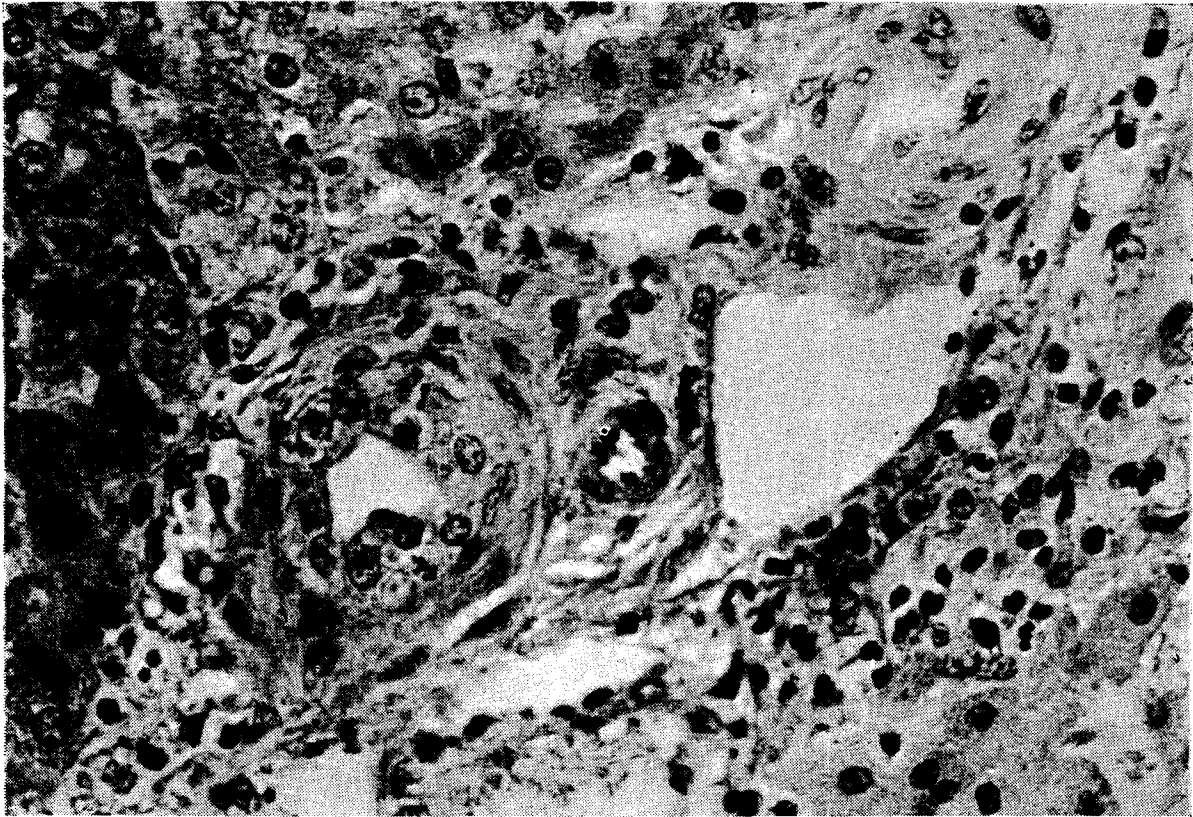
PLATE IV: 40. Degenerative changes in epithelium of small bile duct in homologous monkey chimaera on day 7. The epithelium is swollen and there is karyorrhexis of nuclei. Note slight infiltration of periportal space with lymphoid cells and histiocytes. (HE). Magnification × 300

PLATE IV: 41. Multiple periportal foci of liver necrosis in 14-day old homologous monkey chimaera at day 14. (HE). Magnification × 30
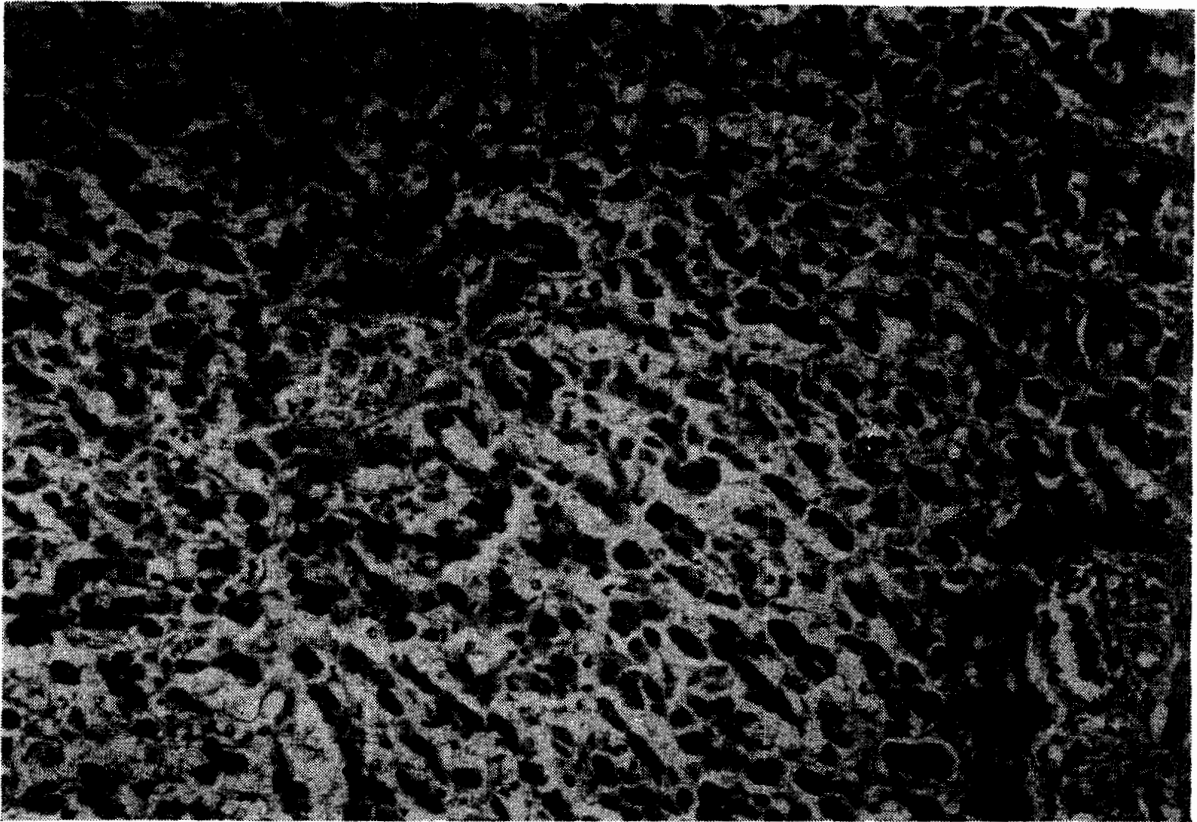
PLATE IV: 42. Massive dissociation and necrosis of liver parenchyma in homologous monkey chimaera at day 26. (HE). Magnification × 12

PLATE IV: 43. Pronounced histiocytic reaction in liver of homologous mouse chimaera (52 days). Histiocytes (epitheloid cells) have proliferated between liver cell cords. In upper left corner an infiltrate is protruding into the lumen of a vein. A focal necrosis is also present (arrow). (HE). Magnification × 190

PLATE IV: 44. Severe acanthosis, follicular hyperkeratosis and dyskeratosis (2 dyskeratotic cells are indicated by arrows) in homologous monkey chimaera (26 days). Note dermal infiltrate of lymphoid cells and histiocytes, surrounding the hair follicles, extending into the epithelium. Compare with normal monkey epidermis in Plate IV: 45. (HE). Magnification × 120

PLATE IV: 45. Skin of monkey irradiated 50 days previously and treated with autologous bone marrow. Completely normal appearance of the skin. Note that the epidermis has only 2–3 cells layers. (HE). Magnification × 120
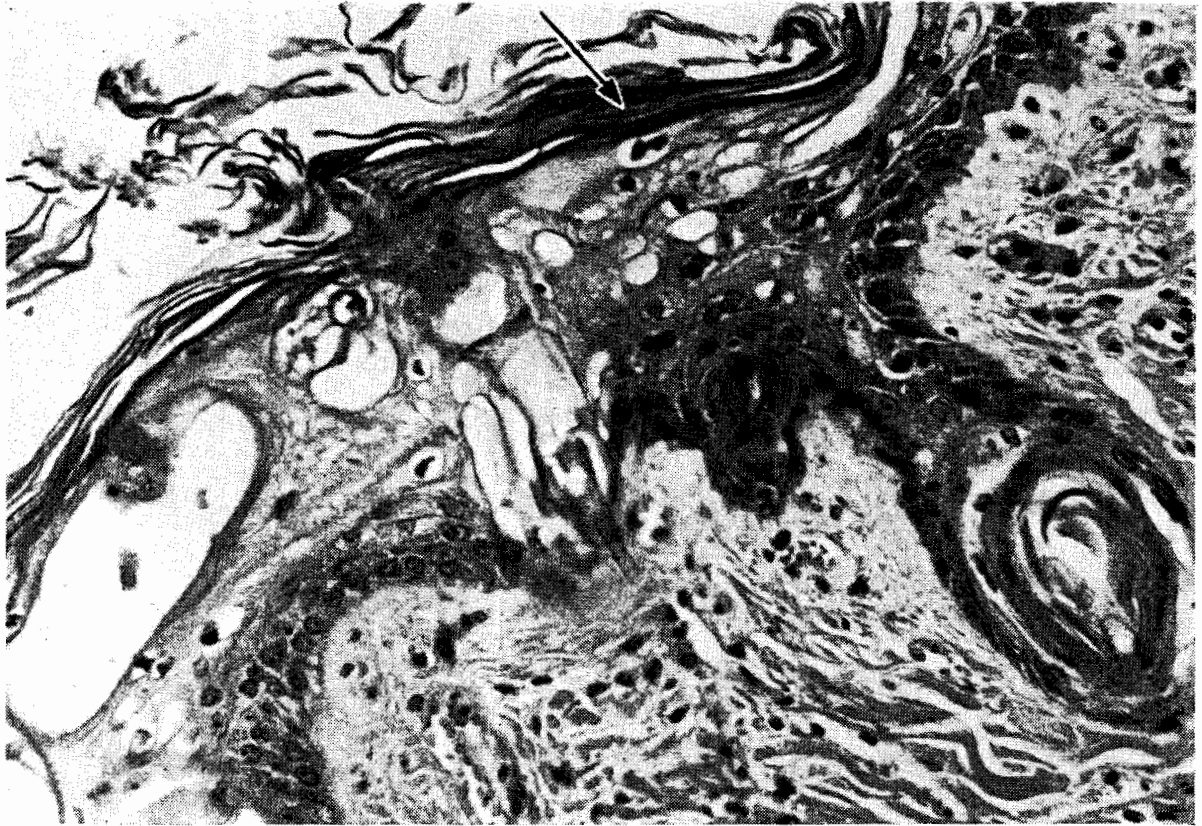
PLATE IV: 46. Vacuolar alteration of malpighian layer of epidermis, hyperkeratosis and parakeratosis (arrow) in homologous monkey chimaera (19 days). An atrophic hair follicle plugged with keratin is present. (HE). Magnification × 190
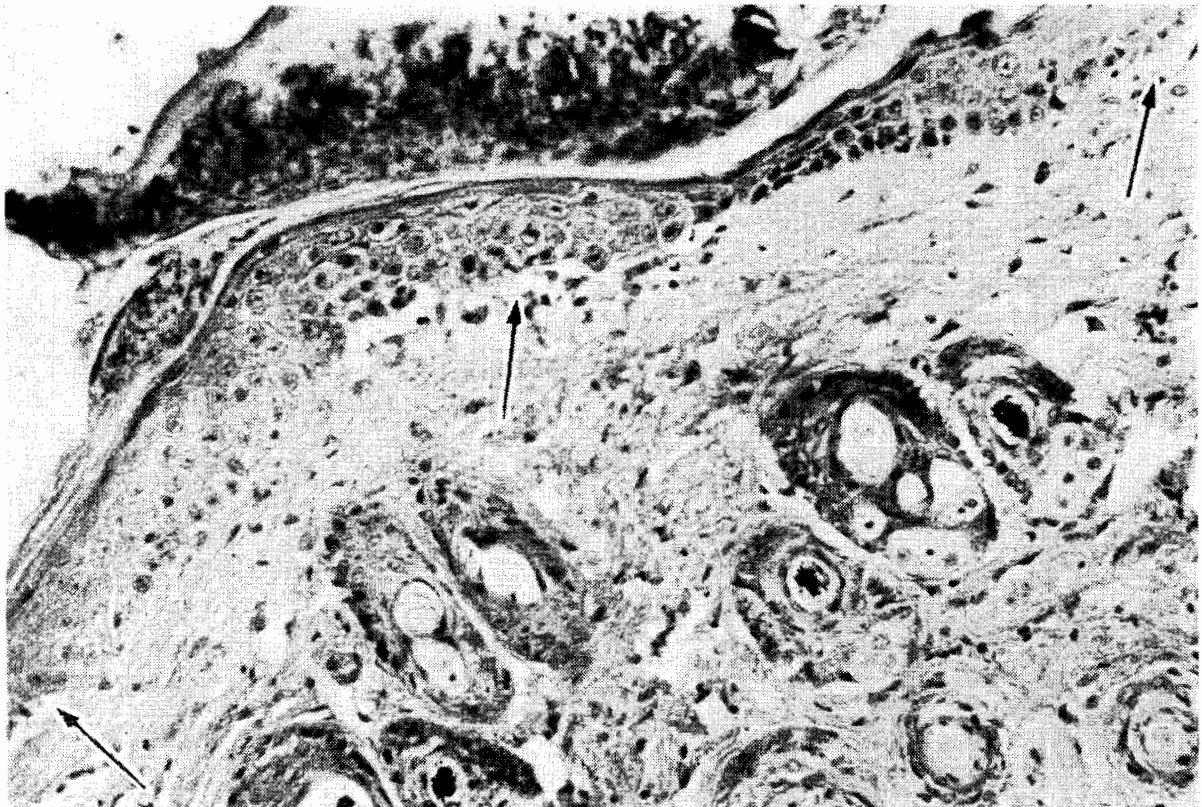
PLATE IV: 47. Basal cell vacuolisation (arrows) of epidermis in a homologous mouse chimaera at 2 months. In addition acanthosis and focal parakeratosis is shown. (HE). Magnification × 190
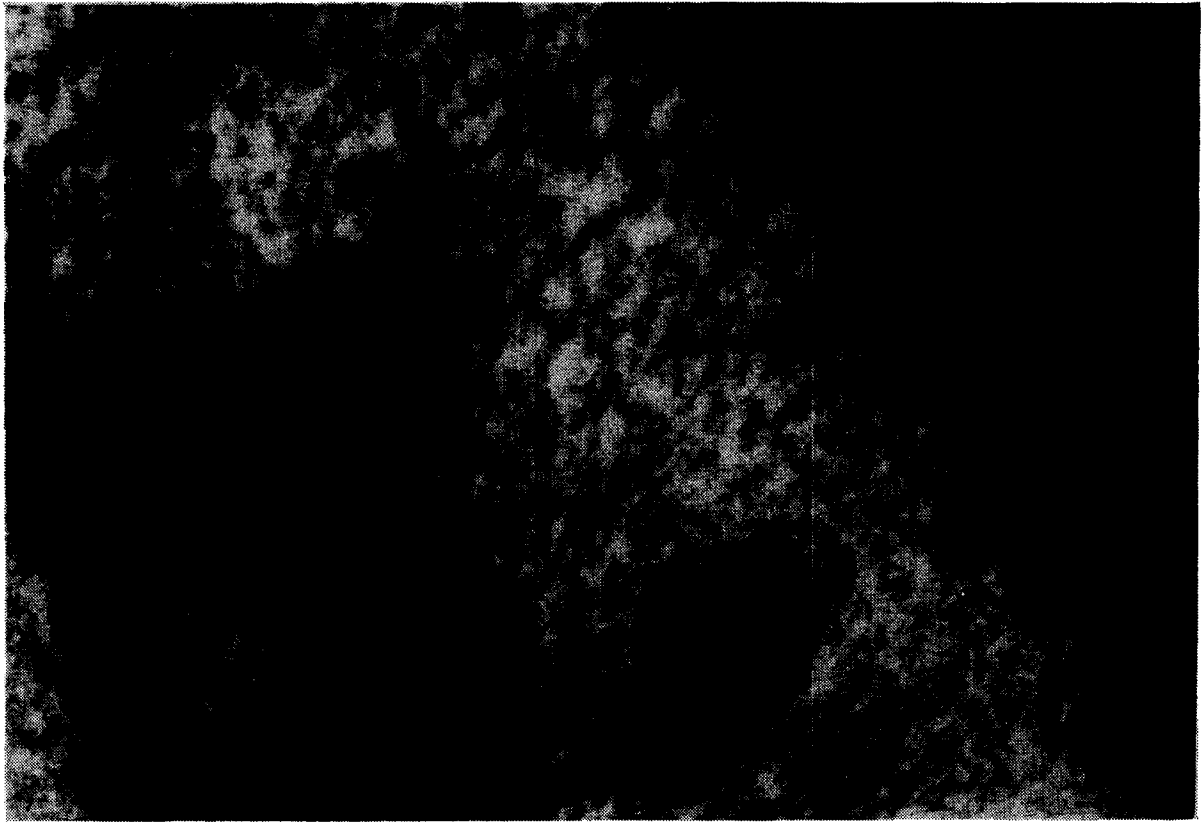
PLATE IV: 48. Erythematous desquamative dermatosis in lethally irradiated leukaemic girl 12 days after homologous bone marrow transplantation (23 days after irradiation). Note also extensive scaling of the skin of the face of the patient in Plate III: 8. Photograph from Mathé et al. (1960)[263]
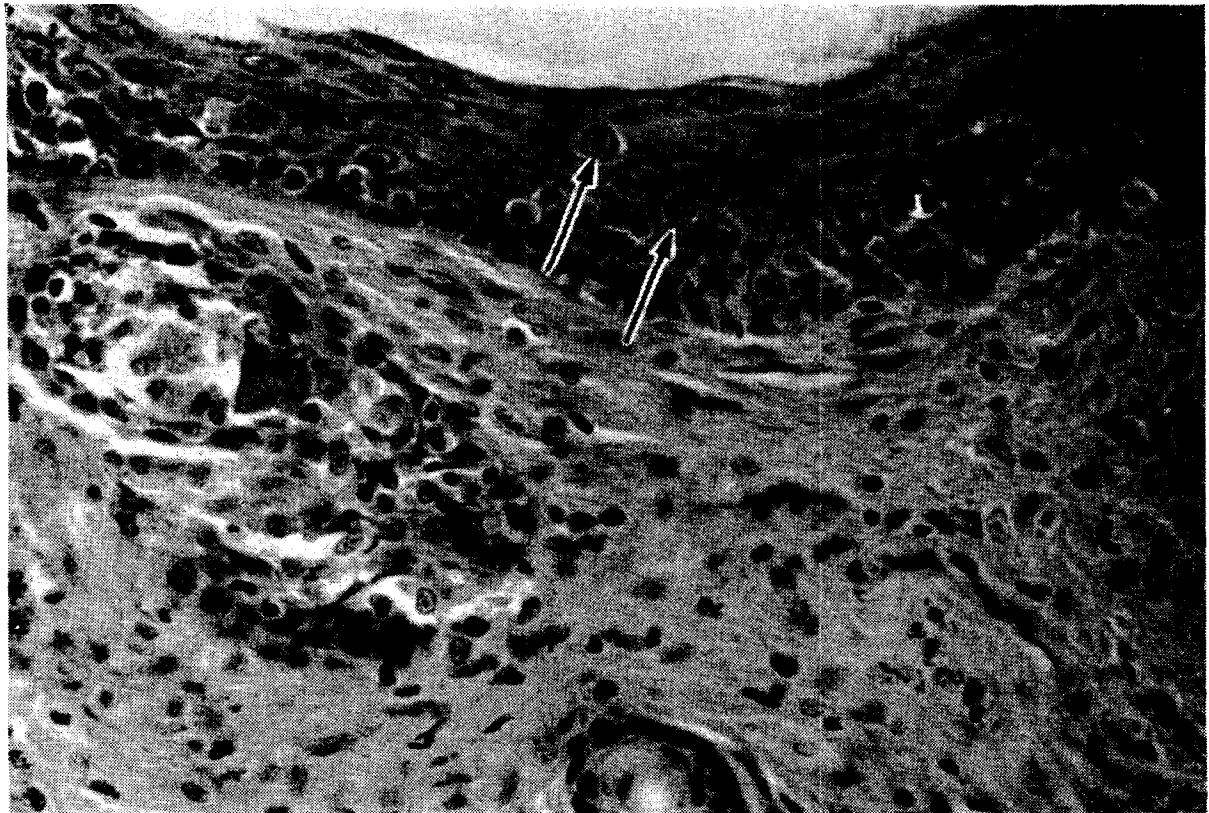
PLATE IV: 49. Acanthosis and dyskeratosis (arrows) of epidermis in mouse chimaera treated with rat bone marrow (5 weeks). Note lymphoid cells invading epidermis and partly destroyed hair follicle at left side. (HE). Magnification × 190
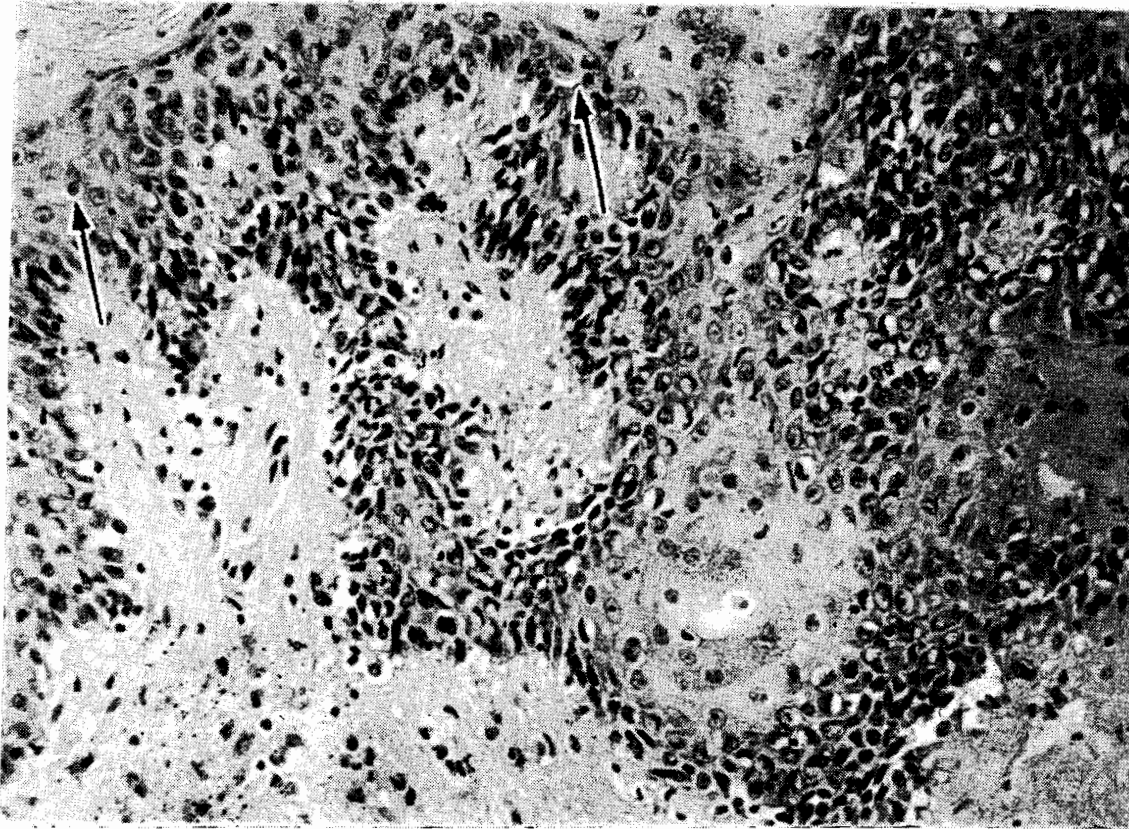
ᴛᴇ IV: 50. Infiltration by lymphoid cells accompanied by basal cell
ʟolisation of epidermis in lethally irradiated leukaemic child treated with
ʟologous bone marrow. The child died 30 days later with severe secondary
disease. Arrows indicate dyskeratotic cells. (HE). Magnification × 190
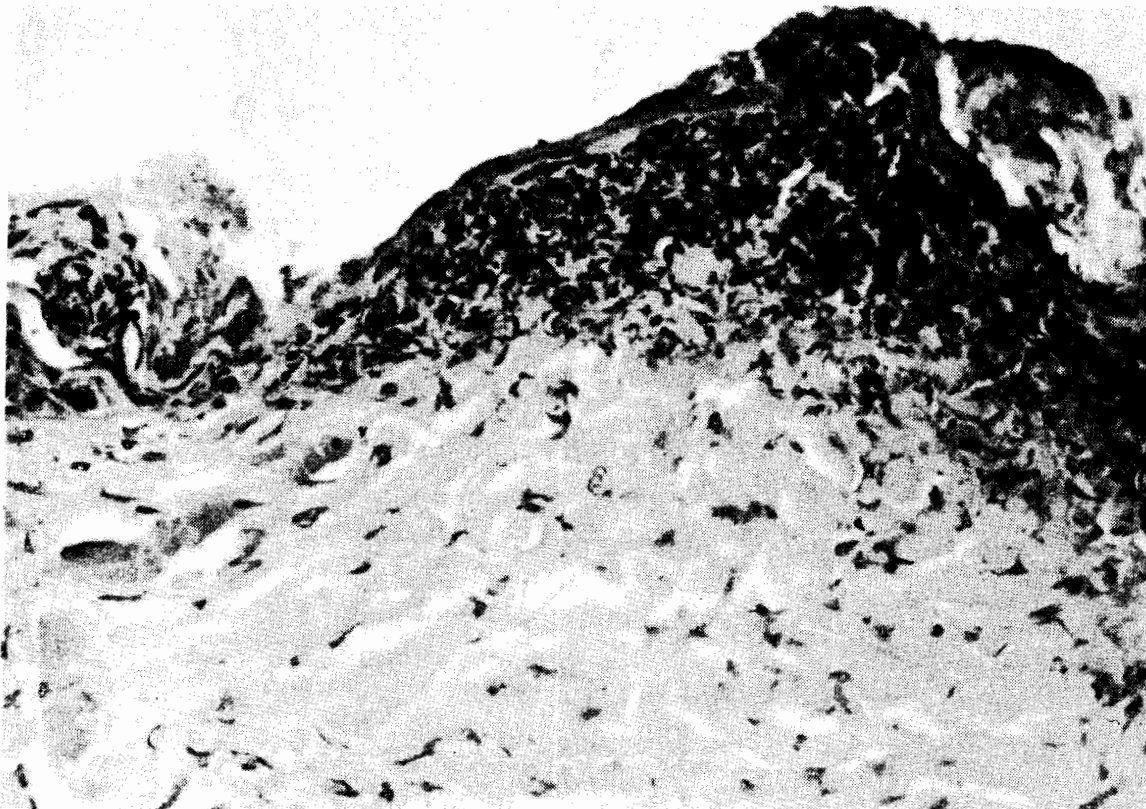
ᴀᴛᴇ IV: 51. Total necrosis of epidermis in rat chimaera treated with bone
.ʀʀow   and   spleen   cells.   Photograph   from   Balner   (1963)[14].   (HE).
Magnification × 300

marrow transplantation and afterwards subsided. Similar changes although to a lesser extent occurred in a proportion of irradiated non-treated mice. It was concluded, therefore, that in these cases the reaction was due to bacterial antigens.

The splenic white pulp reaction is first evident between 24 and 48 hours after the irradiation and transplantation of foreign haemopoietic tissue when an enlargement of the splenic follicles is seen. This enlargement is due to an accumulation of large pale cells with vesicular pleomorphic nuclei possessing prominent nucleoli. The eosinophilic cytoplasm of these cells may or may not have a peripheral basophilic rim. In Giemsa-stained smears, reticular cells, histiocytes and plasmo-blasts with irregularly shaped, lobulated or segmented nuclei and a few binucleated cells were noted. Among these cells mitoses are seen, a number of which show mitotic aberrations such as anaphase bridges, clumping of chromosomes, pyknosis and karyorrhexis at telophase.

Changes similarly indicative of the immunological reactivity of the host have also been observed in the cortex of the lymph nodes. From the 2nd day to the 6th day histiocytes, plasmoblasts and plasma cells appear in the red pulp of the spleen and the medullary cords of the lymph nodes. In smears these cells show nuclear abnormalities as described above.

Three arguments may be put forward in support of the view that the splenic white pulp reaction is a response on the part of the host and represents, therefore, a relatively radioresistant part of the host's immunological reactivity. Firstly, appearance of the cells taking part in the reaction precedes the proliferation of bone marrow cells in animals treated with bone marrow and that of lymphoblasts and lymphocytes in animals treated with lymph node or spleen suspen-sions. The reactive cells seem to disappear when proliferation of the grafted cells is evident histologically (4th day). Secondly, a similar reaction may occur in irradiated non-treated animals stimulated with non-cellular antigens. Thirdly, nuclear and mitotic aberrations sug-gest that these cells have been damaged by radiation. Furthermore, the reaction is even more prominent and persists till the 6th and 7th day in sublethally irradiated animals.

It may be assumed that the histiocytes, plasmoblasts and plasma cells which appear after the 2nd day are partially abnormal descend-ants of the relatively radioresistant immunologically reactive reticular cells. It might well be that these are the cells which are involved in an

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immunological reaction of the host against the graft which, in a proportion of cases, might result in graft rejection.

On the other hand, it is clear that this residual host reactivity can only persist temporarily and will decline rapidly. Since both the reticular cells and their descendants display morphological signs of radiation damage, it may be assumed that these cells have a restricted life span or, alternatively, that delayed radiation induced death will occur after one or more divisions. The splenic white pulp reaction subsides accordingly, after the 3rd day and this may well be related to the fact that graft rejection occurs in a variable proportion of animals following the minimum LD_{100} dose of radiation and only rarely after higher doses.

THE PATHOLOGY OF GRAFT REJECTION

In the animals in which the graft is rejected, persistent pancyto-paenia or development of reticulocytopenia, aplastic anaemia, neu-tropenia and thrombocytopenia occurs after initial recovery in the first month following lethal irradiation doses. The bone marrow in such animals shows arrest of maturation, necrosis of haemopoietic tissue or various degrees of hypocellularity (Plate IV: 12). Interest-ingly, massive karyorrhexis is also seen in the lymphatic follicles of the lymph nodes and spleen, indicating that lymphoid cells derived from the donor are destroyed concurrently with the haemopoietic cells. The cell fragmentation in the lymph nodes is especially marked around small blood vessels and lymphatic channels. In the red pulp of the spleen, at the site of extramedullary haemopoiesis, massive deposition of fibrinoid substance is a notable feature (Plate IV: 13).

A peculiar granulomatous reaction has been observed in the destroyed haemopoietic and lymphatic tissues of animals which reject their graft. Massive proliferation of reticuloendothelial cells of an epitheloid type occurs in the red pulp of the spleen, the lymph nodes and the bone marrow[94]. In addition, multinucleated cells of the foreign-body type are seen. It is not known whether the epitheloid cell reaction is primarily connected with the antibody-response against the graft, or whether it represents an aspecific histiocytic response to cellular disintegration. Following sublethal radiation doses the described changes occur between 6 and 8 days after the irradiation and grafting of foreign marrow.

The animals that reject their graft die with the usual changes of

the bone marrow syndrome which have been described previously, viz. septicaemia, haemorrhage and severe anaemia.

Secondary disease

In contrast to the graft rejection discussed in the previous paragraph an immunological reaction of the graft against the host is usually a far more important cause of delayed mortality in lethally irradiated homologous or heterologous radiation chimaeras. The animals show a complex of symptoms which is now preferably denoted as secondary disease. In broad outline the pathological changes characteristic of secondary disease have many similarities in common in a variety of animal species: mice, rabbits, rats, dogs, monkeys and man (see Table III: 4).

A number of factors may influence, however, the incidence, the extent and the type of the various lesions, e.g. the species of donor and recipient, the radiation dose, the number of lymphoid cells contained in the injected cell suspensions, the previous sensitisation of the host by donor antigens and the duration of the chimaeric state.

In particular, the occurrence of extensive early proliferation of donor lymphoid cells in the host may determine the prevalence of either the lesions found in the early form of secondary disease (degenerative changes in the intestines, the liver and the skin) or the lesions found in the more chronic forms of secondary disease (inflammatory changes due to infectious processes).

General pathology and pathogenesis of secondary disease

Secondary disease may be defined as a disease of radiation chimaeras which causes morbidity and mortality in the presence of a regenerating bone marrow graft and in which the characteristic lesions of the bone marrow syndrome are absent.

The fundamental change in secondary disease is infiltration with lymphoid cells and histiocytes associated with the degeneration and loss of cells in a great variety of organs other than the bone marrow. These changes may take the form of isolated cellular degeneration and in that case they are found only on detailed histological inspection (Plate IV: 14). Enlarged cells may be found disseminated throughout the tissues which show increased eosinophilia of the cytoplasm and nuclear pyknosis. In addition, an increased mitotic frequency is noted, which suggests a compensation for the loss of cells.

In a number of tissues more extensive damage is inflicted as is

revealed by necrosis and degenerative changes (pyknosis, karyorrhexis and fragmentation) of large numbers of cells. In addition to cellular degeneration, regenerative changes are apparent to a varying extent in most of the affected tissues, which suggests that the capacity for cell division is not primarily impaired.

The combination of degeneration and repair gives the lesions a rather complex appearance. In some tissues, e.g. the intestinal epithelium, the degenerative changes prevail, which results ultimately in mucosal denudation. In other organs, e.g. the epidermis, cell death as evidenced by dyskeratosis and vacuolar degeneration, is often of minor importance compared with the massive regeneration. The latter may lead to hyperplastic changes such as acanthosis (Plate IV: 15), which is partly responsible for the characteristic appearance of the skin in secondary disease. Rats, when treated with lymphoid cells in addition to bone marrow, are an exception in this respect, since extensive necrosis of the epidermis may occur in this species. In the surviving animals these lesions may eventually heal. In general, the recovery of the affected tissues may be either complete or fibrosis and atrophy may develop at the site of lesions inflicted earlier.

One other aspect of the primary lesions which appear in secondary disease seems to be particularly important in connection with the pathogenesis of the syndrome. In most tissues and especially in those which are severely affected—intestinal mucosa, skin and liver—slight to heavy infiltration with round cells, probably lymphoid cells and histiocytes, is characteristic. Lymphocytes appear to have penetrated into the epithelium of the hair follicles, the epidermis, the intestinal crypts and the periportal connective tissue of the liver. One often finds degenerating epithelial cells in the immediate vicinity of the invading cells (Plate IV: 16). It seems logical, therefore, to connect the round cell infiltration with the presence of dead cells. Evidence has been accumulated that the lymphoid cell population in the lymphatic tissues of the host is predominantly if not completely of the donor type. It is attractive to postulate, therefore, that immunologically competent cells migrate from these tissues to peripheral sites and that a cytotoxic effect on host cells in a number of target tissues is a direct consequence of the immunological activity of the donor cells.

Specific pathology

LYMPHATIC TISSUES

The changes in the lymphatic tissues are partly dependent on host species, host–donor combinations, radiation dose and the number of lymphoid cells injected.

As has been mentioned earlier, in some homologous and parent to F_1 combinations in mice, treatment with bone marrow only is followed by complete repopulation of the lymphatic tissues with lymphoid cells. Compared with isologous combinations in the same species, the recovery occurs at the same rate or is only slightly retarded[442]. Significantly, in these combinations, no symptoms, or only minor ones, of secondary disease are apparent.

In other homologous combinations and after treatment with rat bone marrow, regeneration occurs initially, as revealed by a few mitoses and the appearance of blast-cells and small collections of mature lymphoid cells, at the end of the first and in the course of the second week following treatment (Plate IV: 17). After the third week, when the animals suffer from severe secondary disease, it is common to find complete atrophy of the lymphatic tissues.

The splenic follicles of these mice are small and composed only of reticular cells (Plate IV: 18). The cortex of the lymph nodes is contracted, and a few lymphocytes are dispersed in an empty reticular stroma. The sinusoids are dilated and filled with histiocytes, which frequently contain phagocytosed erythrocytes or haemosiderin granules (Plate IV: 19). In the medullary cords varying numbers of plasma cells may be present. Similar atrophic changes are found in the thymic cortex. Frequently, deposition of collagen occurs in the lymphatic follicles of spleen and lymph nodes.

In mice surviving the period during which secondary disease is clinically observable, regeneration of the lymphatic tissues is eventually seen. After the second month complete restoration may be found in a varying proportion of animals (Plate IV: 20).

A similar atrophy of the lymphatic tissues, as was described for mice, has been observed in children who died following irradiation and treatment with homologous bone marrow. This characteristic biphasic response of the lymphatic tissues which occurs in incompatible host–donor combinations has been described most completely in lethally irradiated monkeys[446] and rabbits[325] treated with homo-logous bone marrow, and in irradiated F_1 hybrid mice treated with

parental lymph node cells in addition to bone marrow[252]. Initial proliferation of lymphoid cells in the first two weeks is followed by widespread necrosis of the partially regenerated lymphatic tissues and subsequent atrophy. In the early regenerative phase many lymphatic stem cells, transitional cells, plasma cells and histiocytic cells are seen (Plate IV: 21). In the degenerative phase massive karyorrhexis and fragmentation of lymphoid cell occurs, while the reticular supporting stroma remains intact (Plates IV: 18, 22). Deposition of fibrinoid substance in the centre of the lymphatic follicles may be a characteristic finding.

As was mentioned in the previous chapter, Gorer and Boyse[162] as well as Congdon and Urso[99] have related the destruction of lymphoid cells to an antigen-antibody reaction and have pointed to the similarity of this phenomenon with that observed in certain "allergic" reactions. When the lymphoid cells come into contact with host antigens, both the antibody-producing and antigen-carrying cells are destroyed in the reaction (so-called "allergic death" of cells) and the continuation of this mutually destructive process leads to exhaustion atrophy of the lymphatic tissues. Thereafter, the severity of the primary effects of the graft anti-host reaction diminishes, but secondary effects of the atrophy of the lymphatic tissues may cause other complications which will be discussed in the following section.

It might be thought that the atrophy of the lymphatic tissues is due to the debilitated condition of the animals. Generalised and complete destruction of the lymphatic tissues is, however, a rare phenomenon and only seen in very acute infections or as a transient feature after massive doses of cortisone. We may accept, therefore, that changes in the lymphatic tissue are a consequence of an immunological reaction as described above and are specific for graft versus host reactions in general and secondary disease in particular.

In homologous rabbit[325] and mouse chimaeras[99, 445] a striking granulomatous reaction has been described in lymph nodes and spleen, which is probably related to the destruction of lymphoid cells in the regressive phase following early regeneration. Many histiocytic cells of epitheloid morphology appear in addition to multinucleated cells of the foreign body type (Plate IV: 23). As discussed earlier, a similar granulomatous reaction has been found in the haemopoietic tissues during a graft rejection, i.e. as a consequence of a host versus graft reaction (see page 136). It is not known whether the histiocytic cells are immunologically active and involved in an immunological

reaction, either graft versus host or host versus graft, or whether the histiocytic response represents merely a secondary reaction to cellular degradation products.

It should be stressed at this point, that deposition of fibrinoid substance and plasma cell infiltration are both features which *cannot* be considered as specific for secondary disease or any other reaction to tissue antigens, since both are regularly seen in irradiated animals not treated with haemopoietic cells.

In lethally irradiated mice treated with large numbers of foreign lymphoid cells (1×10^6 cells and more) an apparently excessive regeneration occurs in the lymphatic tissues. The animals presumably die at an early stage before the secondary atrophy occurs. The histological manifestations of this acute killing effect are as follows: On the 4th day collections of large cells with pale nuclei and a varying amount of cytoplasmic basophilia appear in the centre of the splenic follicles and the cortex of the lymph nodes (Plate IV: 24). In smears these cells can be identified as lymphoblasts and young lymphocytes. Many mitoses are seen which usually show no aberrations. This suggests that these cells have not been irradiated and are, therefore, of donor origin. In the following 2 days the accumulations of immature lymphoid cells greatly enlarge, the entire lymphatic tissues being massively infiltrated and showing a resemblance to lymphosarcoma. In addition to lymphoblasts and lymphocytes many plasmoblasts and plasma cells are present. It seems likely that the enormous proliferation of what are presumably donor type lymphoid cells is responsible for the early mortality and the severe character of the disease in these animals. The lymphoid cells produced in these tissues may migrate to peripheral sites and inflict widespread cytotoxic damage, especially to epithelial cells.

As has been discussed in Chapter III, specific, complete or partial immunological tolerance of the graft towards the host may develop in a chimaera after a variable time, depending on the host-donor combination. In homologous or heterologous mouse chimaeras suffering from secondary disease, the development of tolerance seems to coincide with a diminishing severity or disappearance of symptoms of secondary disease and a recovery of the animal's immune reactivity against infective agents.

Histological examination of the lymphatic tissues of such mice has shown that following atrophy of the lymphatic tissue present during the period of secondary disease, gradual repopulation with lymphoid

cella parallels the development of tolerance. It seems very likely that this new population of donor lymphoid cells which has been demonstrated to be partly or completely non-reactive towards host antigens, reacts normally against both other tissue antigens and also bacterial and viral antigens. This would explain the improvement of the clinical condition of these animals. The diminished anti-host reactivity can, by itself, explain the termination of the period of continuous lymphoid cell depletion, because overwhelming "allergic death" of lymphoid cells caused by an excess of host antigens no longer occurs.

The relationships outlined above between the cellularity of the lymphatic tissues and the age, clinical condition and immunological reactivity of the radiation chimaera are schematically represented in Fig. IV[1].

I - PERIOD OF RADIATION ATROPHY
II - " " EARLY REGENERATION - ACUTE SECONDARY DISEASE
III - " " "ALLERGIC DEATH" OF LYMPHOID CELLS
IV - " " ATROPHY = CHRONIC SECONDARY DISEASE, INFECTIONS
V - " " LATE REGENERATION - SPECIFIC IMMUNOLOGICAL TOLERANCE,
          IMPROVEMENT OF CLINICAL CONDITIONS
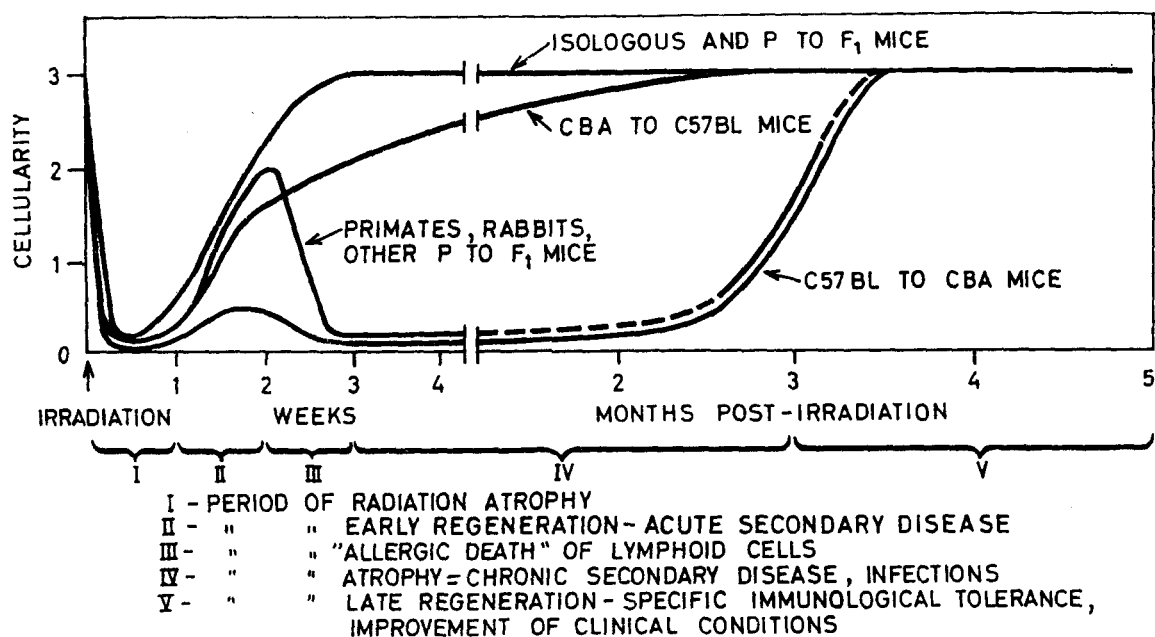
Figure IV[1]. Cellularity of lymphatic tissues, clinical condition and immunological reactivity of chimaeras. The cellularity of the lymphatic tissues has been arbitrarily estimated by recording the presence of follicles, lymphoblasts, mitotic activity and the number of mature lymphocytes.

o = total atrophy
1 = a few lymphoblasts, mitoses
2 = advanced regeneration, mature lymphocytes present, follicles
     of subnormal size and in subnormal numbers
3 = normal appearance

INFECTIOUS DISEASE

As has been discussed in the preceding section, severe atrophy of the lymphatic tissues may occur in chimaeras (mainly bone marrow

treated mice) surviving the first 2 weeks following treatment. This atrophy may explain the impairment of immunological defence against infective agents, which expresses itself as an increased susceptibility of these chimaeras to infections.

Other factors probably promoting infection are radiation induced lesions creating a *porte d'entrée* and lesions caused by the primary effects of the graft versus host reaction. Without a doubt the latter factors are of importance in the causation of the inflammatory lesions frequently found in the intestines of mouse chimaeras.

Among the most important infectious lesions found in mice, rabbits, dogs, and monkeys suffering from secondary disease are bacterial bronchitis and pneumonia. In some mouse experiments the incidence of pneumonia has been so high as to become the major cause of death. Miscellaneous lesions found in mice which are presumably caused by bacterial invasion are endocarditis, abscesses of the salivary glands and epididymitis. In dogs interstitial nephritis due to *Leptospira canicola* has been described. In monkeys pericarditis and cystitis was observed in a few cases.

Evidence has been accumulated that radiation chimaeras may be exceptionally sensitive to virus infections or, alternatively, widely prevalent latent viruses may become activated in the immunologically crippled animals. In a large proportion of dogs that died after treatment with homologous bone marrow, focal necrosis of the liver was found, evidence suggestive of contagious canine hepatitis[400]. In several of these cases, inclusion bodies characteristic of this disease were found in cells of the liver and other organs. In other dogs lesions suggestive of distemper occurred: liver necrosis, bronchitis, bronchiolitis, interstitial pneumonia, with metaplasia of the bronchial epithelium and epithelialisation of alveoli, and inclusion bodies in the epithelial cells of the urinary tract.

In monkey chimaeras several cases suspected of virus infection have been reported.[446] In one case interstitial pneumonitis was found with foci of desquamation of alveolar cells and acidophilic nuclear inclusion bodies in cells lining the alveoli and the terminal bronchioles (Plate IV: 25). Another case showed ulcerative colitis. In the mucous membrane many large cells were present with amphophilic nuclear inclusion bodies surrounded by a clear halo (Plate IV: 26). The inclusion bodies resembled those found in herpes infection and cytomegalic inclusion disease (salivary gland virus disease). Focal collections of histiocytic cells with similar inclusions were found in the

spleen of a third monkey. Hepatitis showing a morphology suggestive of a viral aetiology with many nuclear inclusion bodies in liver cells has been observed only once in a monkey chimaera. Comparable lesions were never encountered in irradiated control monkeys, monkeys treated with autologous marrow or non-irradiated monkeys of the colony.

In a 6-year-old boy—one of the leukaemic children successfully transplanted with bone marrow by Mathé et al.—adrenal lesions reminiscent of viral infection were observed which resembled those found in the intestines and spleen of the monkeys described in the preceding section[263]. The adrenal cortex showed areas of focal necrosis at the margin of which giant cells with amphophilic nuclear inclusion bodies were seen (Plate IV: 27).

Infection with mycelia or yeast-like organisms may complicate secondary disease in several species. In a dog treated with homologous bone marrow Candida was found in ulcers in the ileum[400]. In monkeys dermatitis and necrotising oesophagitis with abundant growth of yeast-like organisms have been reported[446]. In one of these cases the organism was cultivated and identified as Candida albicans. Necrotising bronchitis with the presence of mycelia in the bronchial lumen occurred in one of Mathé's clinical cases, a child suffering from secondary disease following homologous bone marrow transplantation. In a second case massive mycelial septicaemia explained the presence of meningitis and areas of cerebral colliquative necrosis. In the meningeal membrane, the cerebral vessels and the brain substance numerous mycelial threads were shown to be present[263].

Candida albicans is a widely distributed normally harmless inhabitant of the intestinal tract. It is presumed that invasiveness of this organism and ensuing systemic infection is promoted not only by the impairment of the immunological defence but also by the treatment with broad-spectrum antibiotics often used to prevent bacterial infection.

Finally, the list of infective agents which endanger the health of the radiation chimaera would not be complete without the mention of helminths. Rhesus monkeys are heavily infected with a nematode, Oesophagostomum apiostomum, a parasite allied to the human hookworm. The worm penetrates the colonic mucosa and may be buried deep in the intestinal wall. Secondary bacterial infection of the worm lesions is a major complication. Perforation and peritonitis have been cited as explanations for the death of a number of monkeys harbouring

this parasite, after irradiation and treatment with homologous bone marrow. It may thus be visualised that the intestinal worm lesions promote bacterial septicaemia in the immunologically crippled chimaera.

HAEMOPOIESIS

Hypercellularity of the bone marrow which continues to be present after the first month, is noted in mice which have been successfully treated with homologous or heterologous bone marrow. Usually, the cellularity is due to hyperplasia of the myeloid series with in addition a shift to the left, i.e. an increased number of immature cells. In some animals erythroblastosis is noted. An excessive stimulation of haematopoiesis is also suggested by the persistence of extramedullary haemopoiesis in the spleen of these chimaeras (Plate IV: 8). The myeloid hyperplasia could be easily explained by the high incidence of infection. On the other hand, the possibility cannot be excluded that the remaining immunological activity on the part of the host causes increased peripheral destruction of mature donor haemopoietic cells, thereby provoking bone marrow hyperplasia.

In the peripheral blood the outstanding feature is lymphocytopenia, which is the haematological counterpart of the atrophy of the lymphatic tissues discussed previously. Secondly, an absolute increase of the neutrophil count is frequently found. The neutrocytosis probably invalidates the second assumption given above, that the myeloid hyperplasia is due to increased peripheral destruction of neutrophils.

Lastly, mouse chimaeras often display an anaemia of slight or intermediate severity. This anaemia is usually accompanied by reticulocytosis, which according to one author[349] ceases to exist after the first month, but was found to persist in our experiments. One of the possibilities is that the reticulocytosis is caused by haemolysis. In rabbit chimaeras the existence of immune haemolysis of *host* erythrocytes has been demonstrated by the application of Coomb's tests and the injection of ^{51}Cr labelled erythrocytes[313, 326]. Haemolytic anaemia in the first two months, could be explained, therefore, by increased peripheral destruction of host erythrocytes, which the donor erythropoietic pool is temporarily unable to compensate. Haemolytic anaemia occurring after the second month[449] cannot be explained on this basis, however, since host erythrocytes are usually no longer present at that time.

The general debility of the mice suffering from secondary disease could also facilitate the development of a deficiency anaemia with reticulocytosis. This could explain the regular finding of macrocytosis in the chimaeras. It may be concluded that the question of the cause of the anaemia in certain radiation chimaeras has not yet been settled.

The thrombocyte counts in mouse chimaeras are rather variable. Slight thrombocytopenia is found in a number of animals. Especially after the transplantation of rat marrow when an unexplained thrombocytosis occurs in about one-third to one-half of the population from 30 to 130 days following transplantation.

GASTRO-INTESTINAL TRACT

Radiation induced changes of the intestinal mucosa may be seen in chimaeras shortly after irradiation. These changes are, however, of minor importance, since they never result in mucosal denudation and disappear after 5-6 days. On the other hand, important and highly characteristic lesions are found in homologous and heterologous chimaeras suffering from secondary disease. These intestinal lesions were first described in mice and subsequently discovered in human patients, monkeys, and rabbits. They have been very important in an understanding of the pathogenesis of secondary disease and the mortality of homologous or heterologous radiation chimaeras.

The lesions may take one of two forms. In the first, acute widespread crypt degeneration in the mucosa is the principal feature, while inflammatory changes are of little importance or completely absent. This form is seen in mice treated with homologous lymphoid cells which die early, 6-12 days following irradiation. Identical lesions are always found in monkeys treated with homologous or heterologous bone marrow, dying between 6 and 50 days following transplantation. Similar lesions have been described in two human patients treated with homologous bone marrow[263]. The presence of these severe lesions does not seem to be compatible with the survival of the individual.

The second form is a chronic inflammation of the colon and the terminal ileum and occurs in mice and rabbits treated with bone marrow. This form is probably not directly lethal—although it is the cause of persistent diarrhoea and emaciation—and it may be combated successfully in mice with antibiotics.

Acute degeneration of the mucosal crypts

The gross lesions are best described by taking as an example the appearance of the intestine of a monkey treated with homologous bone marrow.

The mucosal surface of the colon and ileum and sometimes of the entire intestinal tract is congested, moist and often covered with tightly adherent greenish-gray membranes (Plate IV: 28). Multiple small superficial erosions with a red base may be found scattered along the entire tract including the stomach.

More frequently the mucosa has a smooth surface with loss of the normal structure. The atrophy is accentuated by an unusual prominence of lymphatic follicles which appear as brownish-gray dots (Plate IV: 29). The wall of the intestine feels firm and oedematous.

Microscopically, the lesions may have a patchy distribution. In addition, the colon and ileum are usually most severely affected, although the lesions may extend sometimes to the stomach.

What appears to be the earliest phase of the lesion is widespread massive karyorrhexis and cell disintegration in the intestinal crypts (Plate IV: 30). The degenerated epithelial cells are desquamated and accumulate in the glandular lumina. This is followed by cystic dilation of the degenerating crypts, which become lined with a layer of flattened epithelium (Plate IV: 31). Between areas of crypt necrosis, crypt regeneration is usually apparent (Plate IV: 32). The hyperplastic crypts are lined with a pseudo-stratified cylindrical epithelium showing hyperchromatic nuclei and many mitoses. In the earliest and most severe lesions many crypts seem to have disappeared, until only few, widely dispersed crypts remain (Plate IV: 33). The intervening lamina propria is condensed and shows a heavy infiltration by lymphoid cells, a few plasma cells and probably some histiocytes (Plate IV: 30, 32). In the small intestine the villi are shortened or disappear completely.

Finally, the surface epithelium is lost from large stretches of mucosa, which are covered by a fibrinous membrane infiltrated by granulocytes (Plate IV: 34). In a number of cases denudation of almost the entire intestinal mucosa occurs. The submucosa shows severe oedema and is slightly infiltrated by lymphoid cells and plasma cells. Similar observations have been made in human radiation chimaeras (Plate IV: 35).

Certain aspects of the changes in the intestinal mucosa are reminiscent of radiation induced damage. Several observations are in-

compatible, however, with the assumption that radiation is the primary factor in the production of the intestinal lesions. At these dose levels irradiated control animals and animals treated with autologous or isologous bone marrow show only slight, if any, degeneration of the crypts during the first 4–6 days following the irradiation; mucosal denudation has never been observed. Moreover, in the homologous chimaeras no parallelism has been found between the extent of the lesions and the radiation dose, while the time interval between irradiation and death in these animals is appreciably longer than would have been expected if a radiation induced intestinal syndrome had been the cause of death. Finally, the preferential localisation of the lesions in the terminal part of the intestinal tract is not typically found in animals dying of the radiation induced intestinal syndrome. Experiments with mice have clearly shown that the occurrence of acute degeneration of the mucosal crypt is aetiologically related to the number of lymphoid cells injected and to the early massive proliferation of lymphoid cells in the lymphatic tissues of these mice[443].

These observations have shown convincingly that the severe intestinal lesions which accompany early mortality after the administration of homologous lymphoid cells in mice and of bone marrow in monkeys, as well as those seen in chronic secondary disease, are primarily caused by an immunological reaction. The evidence presented in Chapter III has led to the conclusion that this is a graft versus host reaction. In addition, the acute disintegration of crypt cells in the presence of lymphoid cell infiltration suggests, as discussed previously (page 147), that the damage is the result of a direct cytotoxic effect of antibody producing cells and not of mitotic inhibition of the germinal crypt cells as proposed by Cole and Rosen[89].

The possibility is not excluded, however, that the irradiation might be an additional factor in the production of the described lesion. Latent radiation damage of the crypt cells could manifest itself by cell death during or after mitosis and by a shortening of the life span of the daughter cells. The loss of cells due to the graft versus host reaction would not be adequately compensated then by the increase of the mitotic frequency. Ultimately mucosal denudation would be the outcome.

Chronic ileocolitis. Chronic inflammatory changes of the intestines are seen in mice treated with homologous or heterologous bone marrow, during the period of severe diarrhoea and weight loss, i.e.

1-3 months following the irradiation. Chronic colitis has also been reported to occur in homologous rabbit chimaeras. The lesions as they occur in mice will be described in detail.

On gross examination, the coecum and ascending colon show vascular congestion and oedema. The wall is stiff and appears thickened on cross section. Microscopically, the lesions are situated in the colon and terminal ileum and usually have a patchy distribution. The intestinal crypts are separated by a dense infiltration of granulocytes, lymphocytes and plasma cells occasionally mixed with eosinophils (Plate IV: 36). The submucosa is oedematous and the lymph vessels are dilated (Plate IV: 37). The chronic inflammatory infiltration often spreads through the submucous and muscular coats in the subserosal layer. Small blood vessels at these sites may become occluded with thrombi. Scattered through the mucous membrane are crypts with a cystic appearance due to regeneration or atrophy of the glandular epithelium. Individual crypt cells show disintegration and desquamation into the lumen of the crypts. Some of the affected crypts are lined by a single layer of flattened cells (Plate IV: 38). The wall and the lumen of the glands may be infiltrated with granulocytes, creating the appearance of crypt abscesses. In some parts of the intestine, groups of crypts have, apparently, disappeared; in other parts, hyperplastic regenerating crypts are prevalent. In severe cases multiple small funnel-shaped ulcerations are seen which are covered with a fibrinous cellular exudate and these ulcerations may penetrate the mucosa as far as the muscular coat (Plate IV: 39). The severity of the lesions may vary from slight cellular infiltration of the mucosa to extensive inflammation of the entire intestinal wall with accompanying peritonitis. The widespread loss of crypts and mucosal denudation as described in the preceding paragraph is not, however, seen.

In older chimaeras atrophy of the mucosa and replacement fibrosis with slight cellular infiltration of the lamina propria may represent the healing stage although degenerating crypts may still be found scattered throughout the mucosa.

The question of the pathogenesis of these lesions initially posed a number of difficulties but the discovery of the acute syndrome in monkeys and mice has been used to arrive at a satisfactory explanation. Parallel with the much more extensive degeneration in the acute syndrome, the loss of crypts could be considered as the direct result of a graft versus host reaction. The mild crypt degeneration in itself, however, cannot be considered to be lethal. The favourable effect of

antibiotic treatment on the diarrhoea and the mortality of mice treated with foreign bone marrow suggests that bacterial infection must be an important contributory factor in the aetiology of secondary disease which appears after the first month following treatment. It could be easily visualised that the minor epithelial defects caused by the graft versus host reaction are foci of increased susceptibility to bacterial invasion. In conjunction with the general impairment of immunological defences, caused by the atrophy of the lymphatic tissues at this stage of secondary disease, widespread secondary infection of the intestines can easily develop.

This view has been amply confirmed by recent studies of germ-free mice treated with rat-bone marrow (D. W. van Bekkum, D. van der Waay and M. J. de Vries. *J. Exp. Hematol.* (1965) **8**, 3-5). In these mice crypt lesions were clearly present while there was a complete lack of the inflammatory component which dominates the lesions in conventional chimaeras (Plate IV: 39(a)).

After about the third month the mortality and the incidence of diarrhoea diminish sharply. Examination of surviving chimaeras at this time has revealed that in certain relatively incompatible host-donor combinations minor lesions of the crypts persist, although the inflammatory changes are minimal. The lymphatic tissues of these animals are partly or completely regenerated. The latter fact points to the development of a partial immunological tolerance of the graft towards the host, as has been discussed in the previous chapter. With the advent of the regeneration of the lymphatic tissue the immunological defence against micro-organisms apparently recovers, enabling the animal to combat secondary infection of the intestinal lesions.

Incidentally, it may be noted that the chronic ileocolitis of secondary disease has some morphological features in common with chronic ulcerative colitis in man. One can only speculate about the possible aetiological relationship of these two diseases, especially since an auto-immune reaction has been postulated as the cause of the human disease.

LIVER

In the literature, necrosis of the liver is among the earliest and most frequently reported lesions in radiation chimaeras in all the species so far investigated. The characteristic lesions must be differentiated from septic necrosis, which can occur in irradiated non-treated animals and after the rejection of a foreign bone marrow graft.
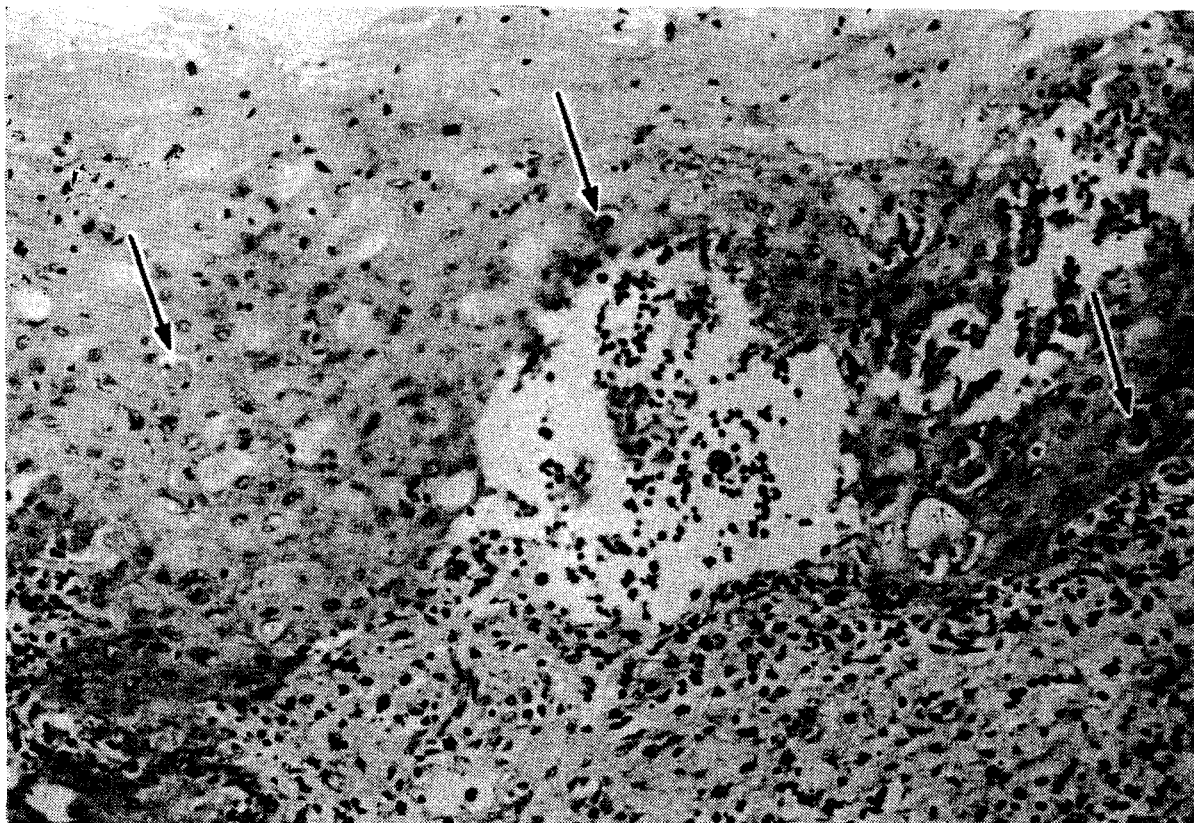
PLATE IV: 52.   Squamous epithelium of oesophagus of homologous monkey chimaera (7 days) showing changes similar to those of the epidermis in secondary disease: infiltration by lymphoid cells, vacuolar degeneration with bulla formation and scattered necrotic cells ("dyskeratosis", see arrows). (HE). Magnification × 220
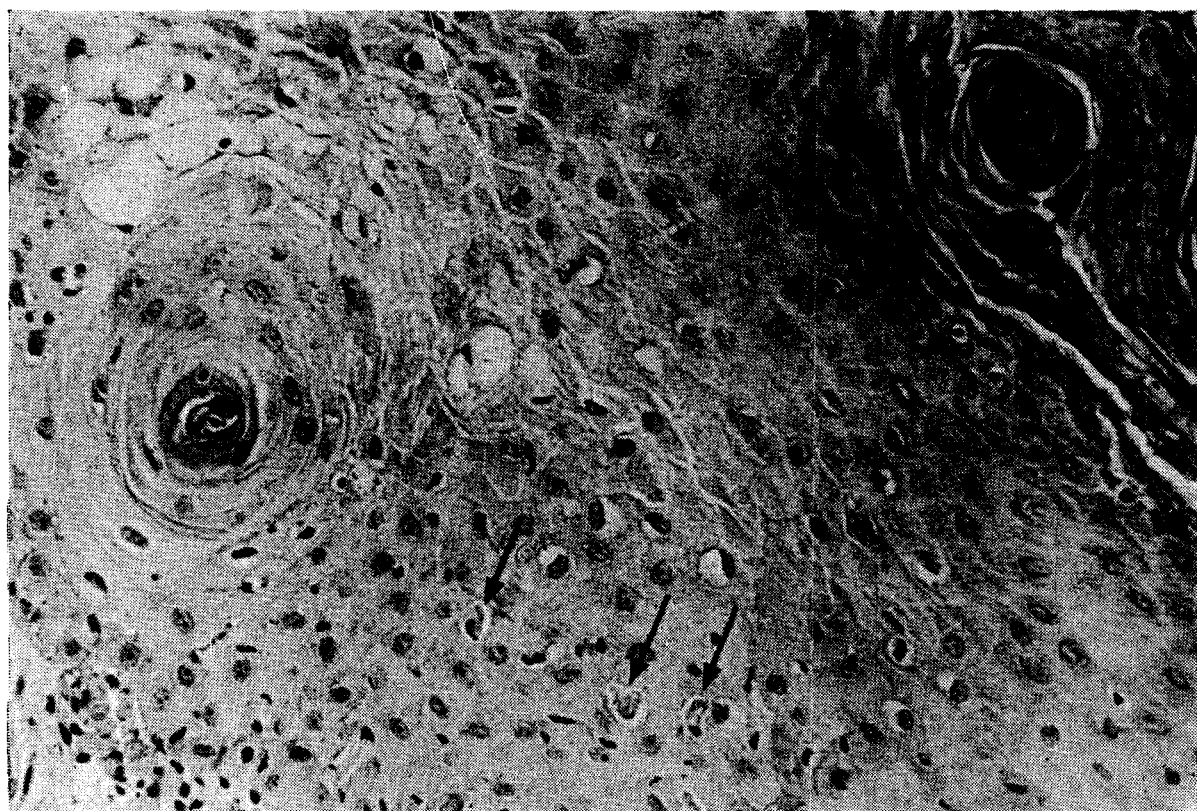
PLATE IV: 53.   Rejection of a human skin homotransplant, biopsy taken on day 8. Acanthosis, parakeratosis (upper right), dyskeratosis (arrows) and vacuolar degeneration in malpighian layer. The epidermis is sparsely infiltrated with lymphoid cells. (HE). Magnification × 190
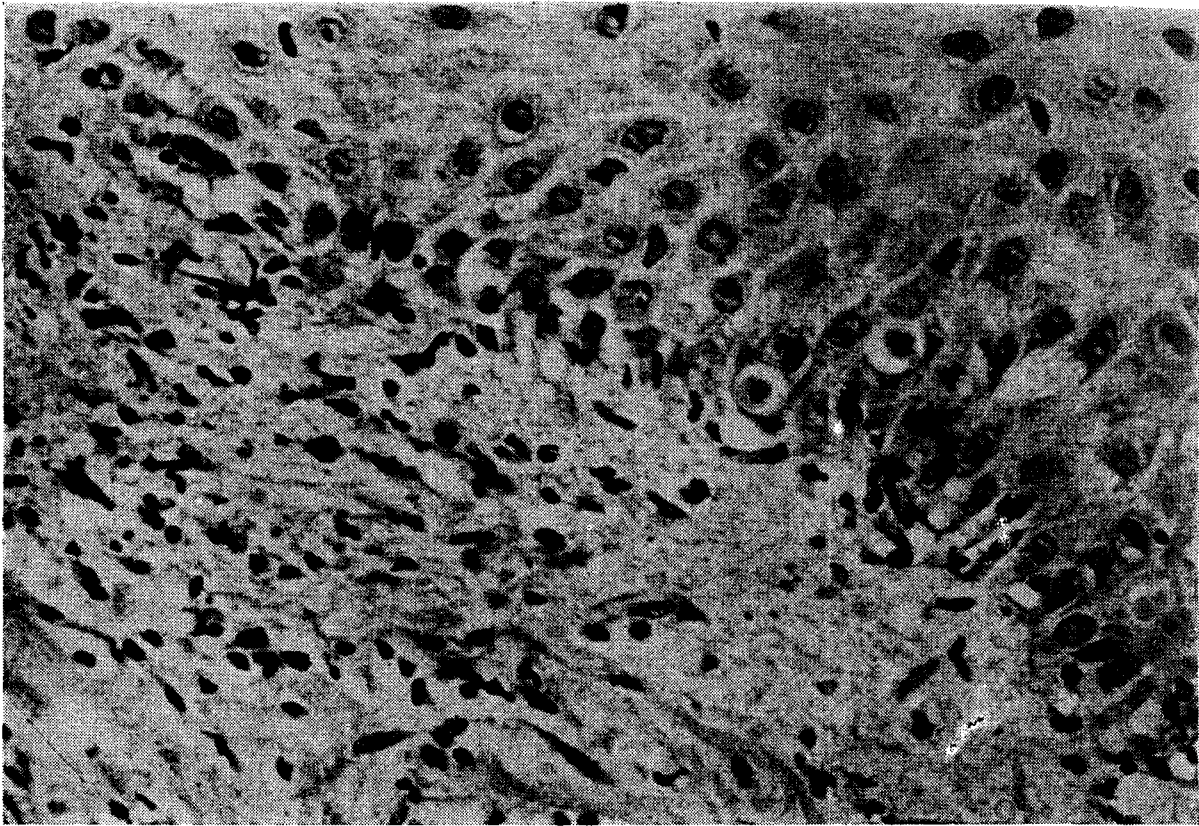
PLATE IV: 54. Rejection of a human skin homotransplant, biopsy taken on day 6. Lymphoid cell infiltration of corium and base of epidermis, basal cell vacuolisation. (HE). Magnification × 190
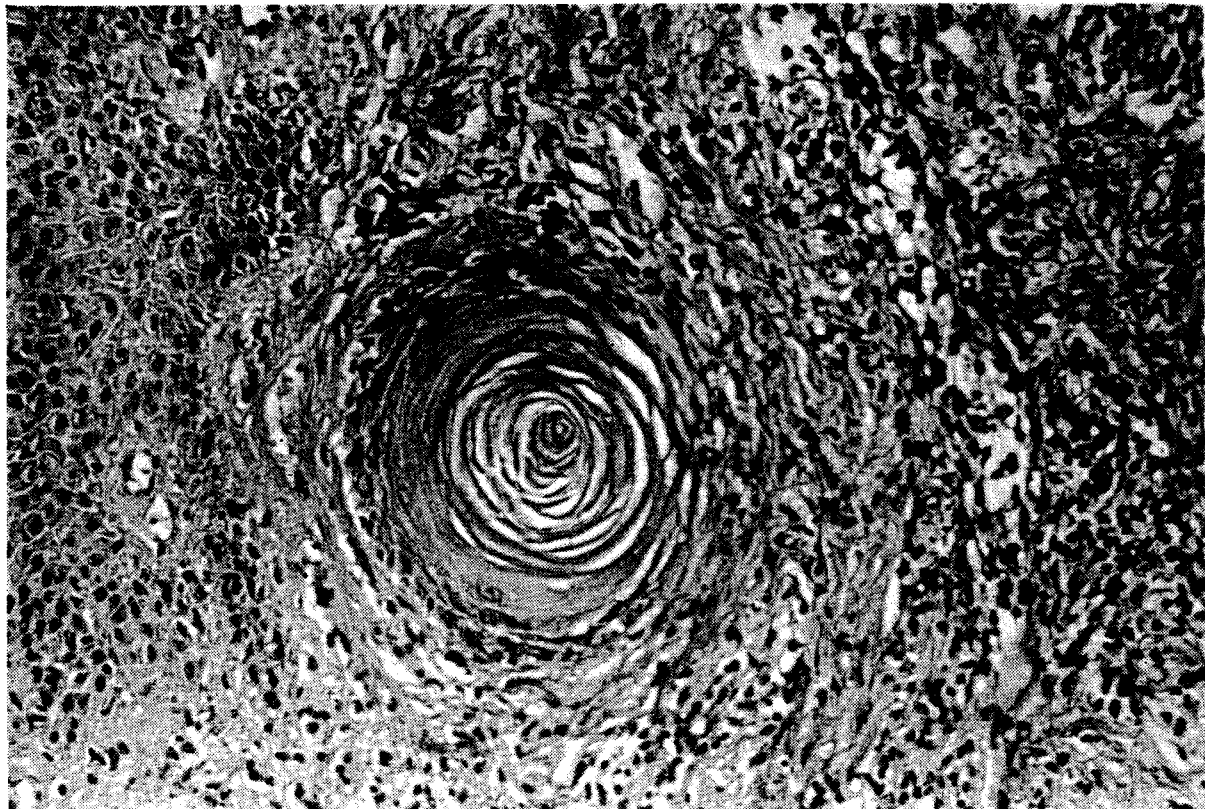
PLATE IV: 55. Rejection of a human skin homotransplant, biopsy taken on day 12. Hair follicle plugged with keratin (follicular hyperkeratosis). (HE). Magnification × 190
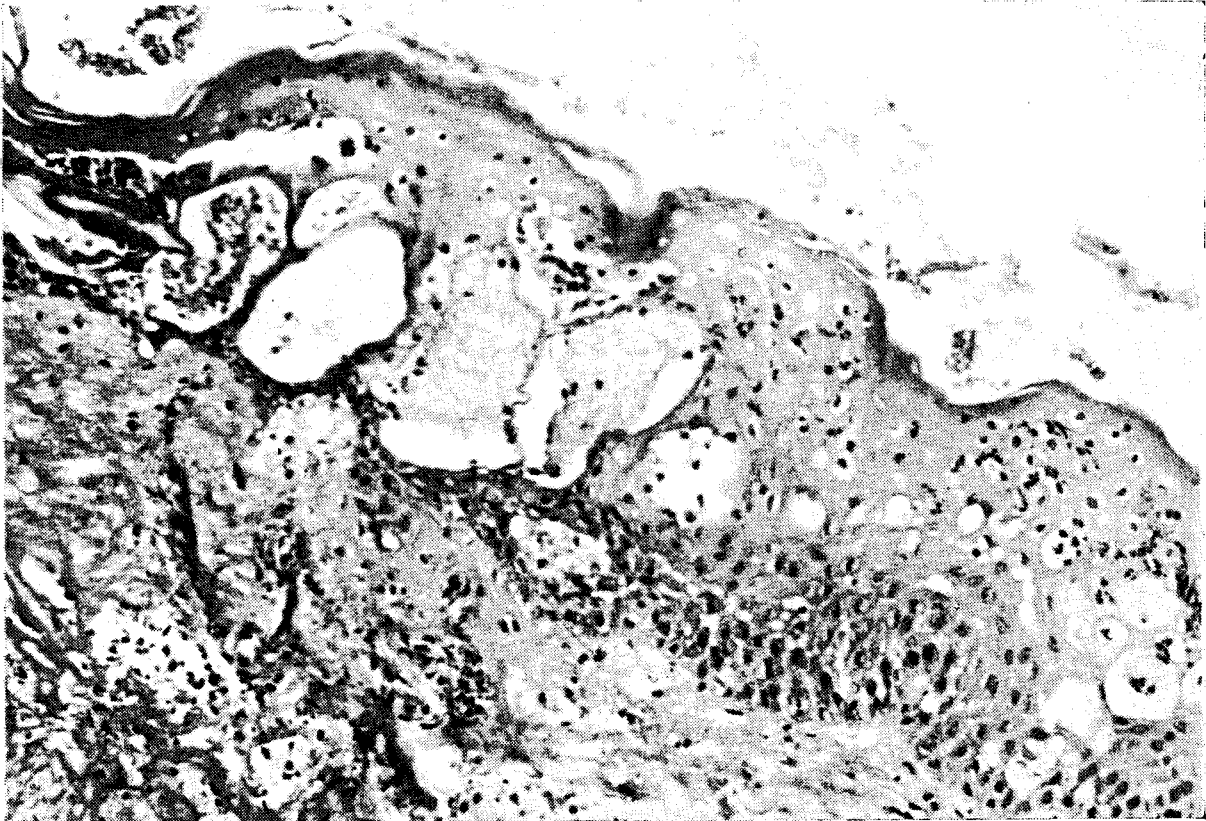
PLATE IV: 56.   Rejection of a human skin homotransplant, biopsy taken on day
9. Lymphoid cell infiltration, vacuolar degeneration with bulla formation,
parakeratosis (upper left). The superficial layers of the epidermis are necrotic.
(HE). Magnification × 120
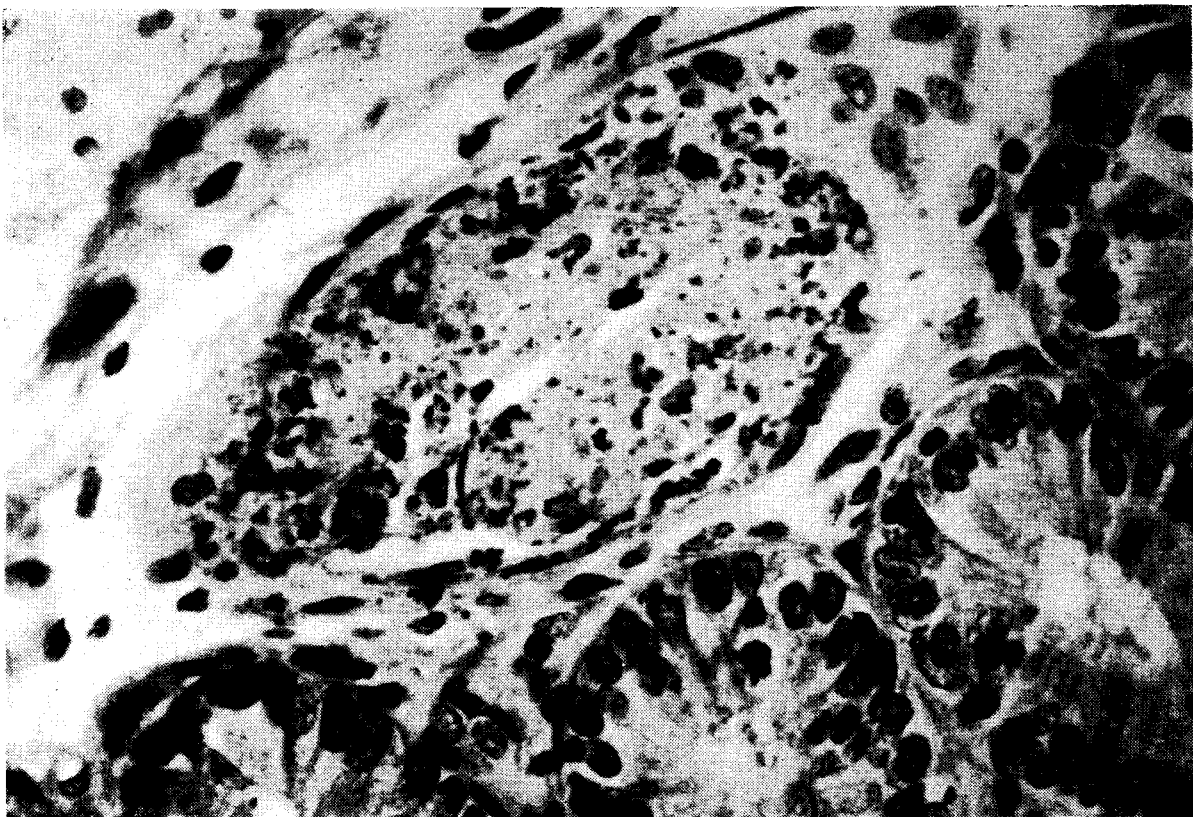
PLATE IV: 57.   Arteriolar necrosis in submucosa of colon of homologous mouse
chimaera (17 days). (HE). Magnification × 480
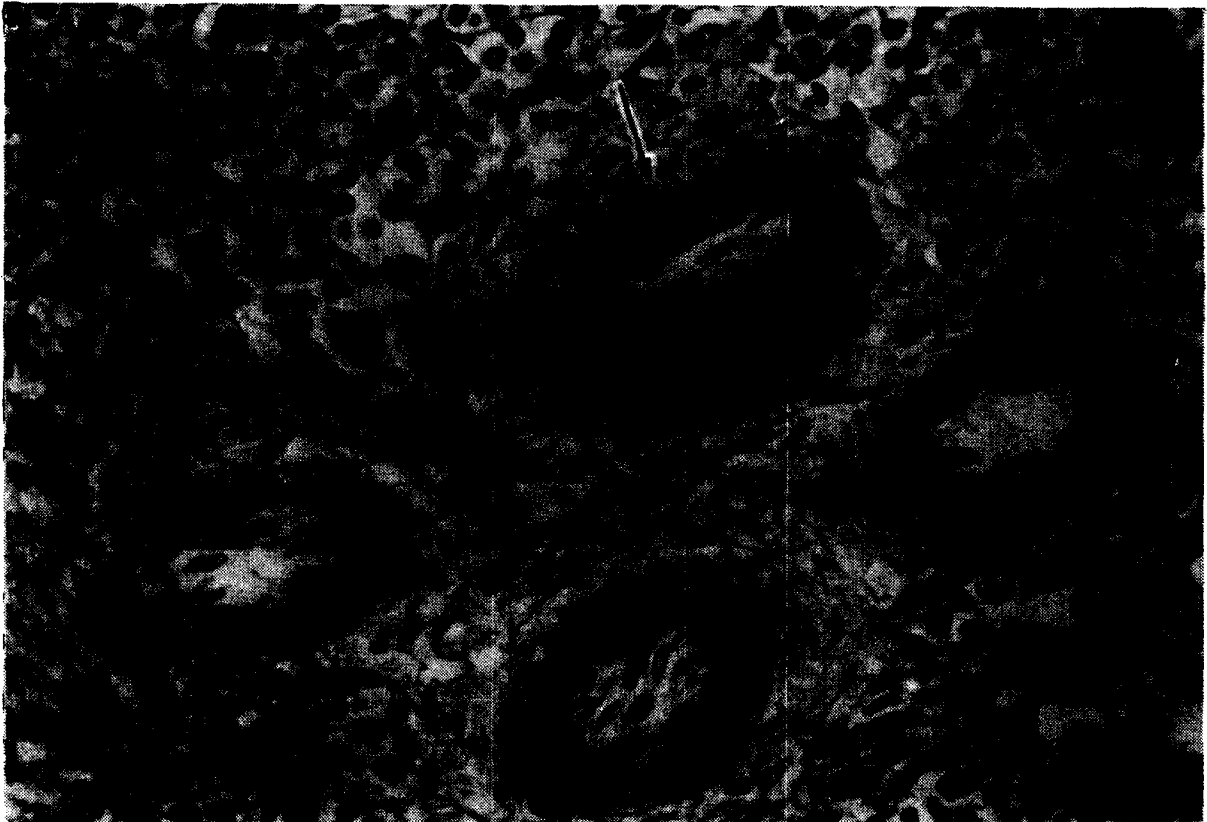
PLATE IV: 58.   Subendothelial deposition of PAS-positive fibrinoid material in splenic arteriole (arrow) of human radiation chimaera (lethally irradiated leukaemic child treated with homologous bone marrow). Photograph from Mathé *et al.* (1960)[236]. (Periodic acid Schiff.) Magnification × 120

In septic necrosis both the parenchymal cells as well as the stromal cells are affected (Plate IV: 4), and masses of bacteria are usually found in the centre of the lesions. Furthermore, there is a conspicuous lack of a leucocytic inflammatory response to the infection in lethally irradiated animals that have not been treated with bone marrow.

In secondary disease the lesions are restricted to the hepatic cells and the bile duct epithelium. In the less severe cases degenerated liver cells, which show cytoplasmic eosinophilia and nuclear pyknosis are sparsely disseminated throughout the liver lobules (Plate IV: 14). Eosinophilic globules resembling Councilman bodies represent cells which have undergone lysis of the nucleus. The epithelium of the smaller bile ducts may be swollen, thereby almost obliterating the lumen, whilst the epithelial cells of these ducts display cytoplasmic eosinophilia and pyknosis (Plate IV: 40).

Many mitotic figures of both the liver cells and the bile duct epithelium are found. Some of these show various mitotic aberrations probably due to irradiation damage.

In the more severely affected livers, the changes are readily visible. The surface and cross sections show multiple ill-defined pale-gray areas, with loss of lobular structure. The liver may be reduced in size and softer than normal. In mice, the liver surface may exhibit a peculiar variegated aspect, with an alternation of red coloured depressions and slightly elevated yellow-to-gray areas.

Microscopically, multiple foci of disruption of liver cell cords and necrosis are seen (Plate IV: 41). The parenchymal dissociation may involve whole liver lobules. Foci of overt necrosis are seen mainly periportally, but they may also have a pericentral or midzonal localisation. In a number of cases large areas of liver necrosis are present in which the supporting connective tissue is condensed and the hepatic sinusoids are dilated (Plate IV: 42). The periportal connective tissue is usually moderately infiltrated with lymphoid cells and histiocytes. In mice extensive myelopoiesis is frequently present. The proliferation of fibroblasts and bile duct structures is noticed in what appear to be the later stages of the lesion.

Lastly, the occurrence of a pronounced histiocytic reaction must be mentioned (Plate IV: 43). The significance of this reaction, which has been mainly observed in mice and rats, may be similar to the histiocytic infiltration of lymph nodes described earlier. The liver cell cords are separated by large numbers of cells with oval, indented or elongated nuclei and an eosinophilic cytoplasm. The infiltration

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often extends through the liver lobule into the wall of a central vein and seems to be the cause of atrophy of liver cell cords.

It is noteworthy that in all species the liver lesions have to be differentiated from natural occurring infections: viral hepatitis in man, monkey, dog and mouse, coccidial infection in rabbits and Tyzzer's disease in mice.

Since the lesions resemble those of hepatitis the possibility that an activated virus infection is an aetiological factor has been considered. Although inclusion bodies have been found in dogs, they have not been seen in mice and only exceptionally in homologous monkey chimaeras. In mice and monkeys, irradiation only, or treatment with autologous or isologous bone marrow, does not cause comparable liver lesions. The very rapid development of similar lesions in newborn mice injected at birth with homologous lymph node cells, or in monkeys treated with bone marrow—i.e. cases in which the anti-viral defences are presumably not jeopardised by lymphatic tissue atrophy —also suggests that a graft versus host reaction may be primarily responsible.

In mice, infection with *Bacillus piliformis* (Tyzzer's disease) has been considered as the cause of the hepatic lesions. During epidemics of the disease, the causative agent can be detected easily in the liver lesions of mice dying of this infection. However, in an extensive search for the organism in mouse chimaeras, using PAS-stained liver sections the characteristic intracellularly located slender rods could not be detected. Moreover, the presence of similar liver lesions in germ-free mice treated with rat-bone marrow, does not favour an infectious aetiology (D. W. van Bekkum, D. van der Waay and M. J. de Vries: *J. Exp. Hematol.* (1965) **8**, 3–5).

JAUNDICE

Jaundice is frequently found in homologous monkey chimareas. Since it has not been observed so far in irradiated non-treated monkeys, nor in monkeys treated with autologous bone marrow, the condition seems to be somehow related to the chimaeric state. The jaundice might be caused by hepatic cellular damage. One argument against hepatocellular jaundice is the fact that liver necrosis may be present without jaundice and *vice versa*.

Another possibility is that the described degeneration and swelling of epithelium of the bile ducts results in obstructive jaundice. Finally, haemolysis of either host- or donor-type erythrocytes as discussed in a

previous section must be considered. Which of the factors or combination of factors mentioned is in fact responsible remains to be settled.

SKIN

A generalised dermatosis is so regularly found in radiation chimaeras, that it may be considered as highly pathognomonic of secondary disease. In addition, the morphological alterations of the skin provide strong evidence in favour of the immunological nature of secondary disease since they are very similar to the lesions seen in the reverse condition, namely, the rejection of a skin homograft by a normal host in which irradiation is not involved as a complicating factor.

Each of the morphological features which will be discussed below have been described in conjunction with experimental and clinical skin grafting, especially when the rejection has been slow. This is the case when grafted isologous male skin is rejected by female mice of certain strains, when histo-incompatibility does not involve the strong H_2 antigen in mice, or when homologous skin is grafted in hamsters. This similarity leads to the attractive assumption that, although ultimate sloughing of the epidermis is rarely seen in chimaeras, except in rats treated with homologous lymphoid cells, the whole skin of the host is actually being slowly rejected by a functioning transplant of immunologically competent cells of donor origin.

The macroscopical appearance of the early lesion has been best observed in monkeys and man. A macular erythema appears on the skin of the face, at first surrounding the orbits and the mouth and spreading to the trunk and arms (Plate IV: 48). In the days following, coalescence of the individual maculae may lead to the appearance of a diffuse erythrodermia. The skin is warm, dry and appears infiltrated and oedematous. In human cases the development of bullae which may rupture and leave a denuded epidermis, has been described. Following the erythrodermic phase extensive dry scaling occurs which also begins on the face and ears (Plate III: 5). Characteristic is the widening and protrusion of the orifices of the hair follicles which are plugged with horny substance. Ulceration or fissuring of the skin is sometimes found and ultimately total desquamation of the epidermis may occur. This has been frequently observed in rats, especially when treated with homologous lymphoid cells.

In mice the fur has a characteristic ruffled appearance and epilation is often seen (Plate III: 2). In older mouse and rat chimaeras

the skin may be appreciably thickened and have a parchment-like consistency.

The microscopic appearance of the skin in the early erythematous phase has not been studied adequately, because the chimaera usually dies in the second and desquamative phase. The latter lesions are microscopically similar in mice, monkeys, and man.

Hyperplasia of the epidermis is the most conspicuous feature (Plates IV: 15 and IV: 44 should be compared with Plate IV: 45 of normal monkey). The acanthosis involves the hair follicles which may be surrounded by broad mantles of epithelium. Hyperkeratosis with distension of the follicles by accumulated horny substance— follicular hyperkeratosis—explains the extensive scaling of the skin. Localised persistence of nuclei sometimes in rounded masses— parakeratosis—may be found in the horny layer (Plates IV: 46 and IV: 47).

Dispersed between the epithelial cells of the epidermis and the hair follicles are numerous rounded cells with a homogeneously eosinophilic cytoplasm and a pyknotic or disintegrating nucleus (Plates IV: 16, IV: 44 and IV: 49). These dyskeratotic cells appear isolated or in small collections and may even be found between cells of the basal layer.

Also very characteristic, and frequently seen in chimaeras of all species, is liquefaction degeneration of cells of the basal layer of the epidermis. Rows of vacuoles are seen on the margin of the epidermis and corium, sometimes accompanied by separation of epidermis and corium (Plates IV: 47 and IV: 50). Other degenerative features are the total disappearance of the basal cell layer and a vacuolar degeneration of cells of the stratum spinosum (Plate IV: 46). In rats total necrosis of the epidermis and the underlying corium may be seen (Plate IV: 51).

In some cases, the hair follicles may show signs of atrophy instead of hyperplasia, the follicular remnants being covered with a few layers of pale, swollen epithelial cells (Plate IV: 46).

In the epidermis or superficially in the corium, globular structures consisting of concentrically layered, flattened epithelial cells may be seen in monkeys and in man. It is supposed that these represent a metaplastic change in the excretory ducts of the sweat glands.

The corium and subcutis show intercellular oedema and are infiltrated to a varying degree with lymphocytes, histiocytic cells, plasma cells and occasionally with granulocytes. The cellular infiltra-

tion often surrounds the hair follicles, sweat glands and small vessels and penetrates between the epithelial cells of the appendages and the epidermis (Plates IV: 16, IV: 44, IV: 46, IV: 49 and IV: 50). Sometimes a granulomatous reaction with the appearance of multi-nucleated giant cells is seen in the vicinity of remnants of disintegrated hair follicles. In later phases, the cellular infiltration diminishes and an increased number of fibroblasts in the corium is noted. In older mouse chimaeras the epidermis may be reduced to a few cell layers and the number of hair follicles appears to have decreased. Below the atrophic epidermis the corium shows some fibrosis.

The similarity of the skin lesions in secondary disease and the skin changes during a homotransplant rejection is clearly brought out by comparing the described lesions with Plates IV: 53 to IV: 56, taken from biopsies of human skin homografts.

It is noteworthy that epithelial changes resembling those of the skin have been observed in the oesophagus of a few monkeys (Plate IV: 52).

KIDNEYS

The kidney lesions found in chimaeras are of minor importance and are probably not specific. Albuminous degeneration of the convoluting tubules with albuminous casts in the collecting tubules is regularly observed in monkeys treated with homologous bone marrow. In a few cases a number of convoluted tubules scattered throughout the cortex showed distinct necrosis of the epithelium. Strongly eosinophilic hyaline and granular casts, partly surrounded by multinuclear cells, probably derived from desquamated tubular epithelium, have been found in the collecting tubules. Glomerular and vascular changes were not apparent.

The renal tubular changes have not been attributed to the direct effect of the graft versus host reaction and can be explained by secondary effects, such as shock, anoxia, haemolysis or hepatic damage.

CARDIOVASCULAR SYSTEM

There is little evidence that vascular changes are of great importance in secondary disease. This is of particular significance in a consideration of whether secondary disease is morphologically allied to the collagen diseases and the so-called allergic diseases in man (lupus erythematodes, periarteritis nodosa, allergic arteritis and others) as discussed in a later section.

In the CBA mouse strain, animals dying from secondary disease may display severe calcification of the cardiac musculature. This process is probably related to inanition or other sequelae of the severe debilitation of the mice, because it likewise occurs in irradiated non-treated mice but not in irradiated mice treated with isologous bone marrow, where comparable debilitation is absent.

A generalised necrotising arteritis, mainly affecting arterioles and capillaries, but sometimes also larger arteries, has been observed in a number of mice treated either with homologous or heterologous bone marrow after a minimum LD_{100} dose of radiation (Plate IV: 57). The affected vessels were strongly stained by the PAS method and showed nuclear fragmentation in the muscular coat. The endothelium was swollen and sometimes the lumen was occluded by a thrombus. The vascular wall and adventitia were often infiltrated with lymphoid cells, plasma cells and granulocytes.

The lesions occurred in the first month following the irradiation, during the period of maximum incidence of graft rejections and before the appearance of secondary disease. In a few mice septicaemia coincided with the vascular alterations. These observations suggest that the vascular lesions are either septic in origin or are related in some other way to the graft rejection. The latter possibility is supported by the observation that necrotic changes in small vessels, accompanied by thrombosis and deposition of fibrinoid substance, have been observed in mice at the end of the first week following irradiation with midlethal doses and injection of homologous bone marrow.

In monkeys vascular changes have not been observed. In two of Mathé's young patients, who both died following a take of a homologous bone marrow transplant, massive deposition of fibrinoid substance in the wall of small arterioles in the spleen was a striking feature (Plate IV: 58). The accumulations of fibrinoid material apparently caused the obliteration of the vascular lumina which in turn explained the development of multiple foci of haemorrhagic infarction. Vascular necrosis was not apparent, however, nor were vascular changes found in other sites.

OTHER ORGANS

Isolated degeneration of cells accompanied by an increase of mitotic frequency, as discussed in the section on general pathology, has been observed in the adrenal cortex, the pancreatic acini as well

as the islet tissue, the salivary glands, and the transitional epithelium of the renal pelvis.

Of the other endocrine glands, the thyroid has been examined in a few monkeys treated with homologous bone marrow. No apparent abnormalities could be detected.

The central nervous system was studied in a series of mice treated with homologous bone marrow and lymph node cells. Pathological changes were not apparent in sections routinely stained with haematoxylin and eosin.

In cross-striated muscle tissue of mice and monkeys sarcosporidiosis was sometimes noted. Lesions which could be attributed to secondary disease were not, however, found.

In the testes of mice treated with homologous or heterologous bone marrow, recovery of spermatogenesis was generally delayed in comparison to mice treated with isologous bone marrow. This might be attributed to the debilitated condition of the mice. Ovaries were examined in a few monkeys treated with homologous bone marrow. No differences were apparent between these monkeys and those irradiated and not treated, or monkeys irradiated and treated with autologous bone marrow.

The causes of death in secondary disease

As has been discussed in previous sections, secondary disease may manifest itself as an acute illness with early mortality, or as a chronic illness from which the animals die relatively late in the course of the disease. The direct causes of death may be different in both syndromes.

In the chronic protracted phase of secondary disease, which occurs in mice treated with bone marrow, the animals usually die showing signs of a generalised infectious disease. As for instance with pneumonia, this may be responsible for the death of many chimaeras. One of the most commonly found conditions in these animals is chronic ileocolitis. It is probably this infectious complication which is responsible for the progressive clinical deterioration and wasting of the chimaeras. Significant in this respect is the fact that both the mortality and the wasting can be largely prevented by treatment with antibiotics. One of the factors which could explain these effects of the chronic colitis might be a continuous loss of protein. This has been investigated by Friedberg[146] who injected human serum albumin labelled with ^{131}I into lethally irradiated mice treated with rat bone

marrow. He concluded that an increased fractional rate of loss of endogenous serum albumin occurred in these chimaeras, which he ascribed to increased catabolism (presumably due to infection, immunological processes, etc.) or leakage of the protein through the damaged intestinal mucosa.

Other factors, such as anorexia and the continuous immunological destruction of cells, may contribute to this state of uncompensated catabolism, leading to a general debilitation and death, even in those cases in which there is no complicating secondary infection.

It can be concluded that in the chronic late phase of secondary disease the graft versus host lesions in themselves are usually not of sufficient severity to explain the mortality and that death must have been caused by a number of partly still ill-defined complications.

In contrast, death of the chimaeras in the early, acute phase of secondary disease can much more easily be ascribed to the direct effects of the graft versus host reaction: intestinal denudation, hepato-necrosis and diffuse disintegration of cells in other vital organs. Acute denudation of large stretches of the intestinal mucosa may lead to shock from dehydration, disturbance of electrolyte metabolism and loss of protein. It also seems possible that shock could be induced by endogenous intoxication from the toxic products formed in the acute generalised destruction of cells.

In a comparatively small proportion of animals, death is readily explained by the extensive morphological damage of the liver. Whether haemolysis is a factor of importance in the production of mortality in the acute syndrome has not been adequately evaluated. As has been discussed in a preceding section, the jaundice which is frequently present in monkey chimaeras might have been caused by haemolysis. An argument against the interpretation of haemolysis as a lethal complication is that severe haemoglobinuric nephrosis is rarely seen in these animals. On the other hand, massive acute haemolysis could have caused death from shock before the development of such renal lesions.

Comparison of secondary disease with runt disease and homologous disease

Haemopoietic chimaerism may be attained without the aid of irradiation by taking advantage of the immunological immaturity of newborn animals of certain species or the inability of the F_1 to react against parental antigens.

Mice and rats injected at, or shortly after birth, with adult homologous haemopoietic cells fail to grow at a normal rate and may show one or more of a variety of clinical symptoms: emaciation, stunting, a hunched appearance, ruffling of the fur, thickening of the skin and diarrhoea[62, 66, 375, 376]. Death may occur within 4 weeks. This condition is called "runt disease". A similar disease develops after hatching in chickens, the embryos of which were injected with adult homologous spleen cells[62] or again after parabiotic union with another embryo[372]. Adult F_1 mice injected with parental lymphoid cell suspensions likewise develop a runting syndrome, denoted as homologous disease[417].

As has been described earlier, these syndromes are the outcome of an immunological reaction of the foreign cells against the host. It is of considerable interest that the pathological changes, as described in animals suffering from runt disease and homologous disease[1, 66, 162, 385], are very similar to those found in secondary disease. A possible exception are the intestinal lesions, which have not been reported *in extenso* although diarrhoea is apparently often present. A study of mice suffering from runt disease by the present authors has shown that degeneration of crypts occurs in a proportion of cases, although it is less severe than that found in radiation chimaeras; denudation of the mucosa is absent. One of the explanations for this might be that, the other abnormalities seen in runts prove lethal before significant intestinal changes can develop. On the other hand, the radiation could be an important secondary factor in the production of these lesions in radiation chimaeras.

Profound haematological abnormalities are present in runts as well as in animals suffering from homologous disease[297, 372]. Haemolytic anaemia with high reticulocyte counts and a positive antiglobulin test occurs regularly. Acute haemolysis with jaundice has been observed occasionally. In rats the anaemia may be of an aplastic type[66]. Other abnormalities are neutrocytosis, lymphopenia and thrombopenia. These data point either to an increased destruction of mature peripheral blood cells or to destruction of the immature precursors in the bone marrow or to the two conditions together. Both result from the activity of the grafted immunologically competent donor cells.

In the lymphatic tissues atrophy of the lymphatic follicles, sometimes accompanied by necrosis, is an important feature of runt disease. Contrary to what is generally found in radiation chimaeras, and

in spite of the lymphoid atrophy, the animals show an enlargement of spleen and lymph nodes, which is caused by an intense proliferation of reticular cells, histiocytes and plasma cells. In the liver a similar cell infiltration is found in the portal triads and in the sinusoids between the liver cell cords. When this infiltration is pronounced, the liver cords show atrophy and dissociation. Foci of necrotic liver cells surrounded by histiocytes may be found. In addition, many mitoses of liver cells and an increase of haemopoiesis are seen. The question is, once again, whether the histiocytic cells are the cause of the cell destruction or merely scavenger cells engaged in the clearing away of dead cells. According to our own observations, the destruction of liver cells is found much more frequently in runts than in radiation chimaeras.

The skin changes have been examined extensively in runted rats by Billingham and his group[66] and in rats suffering from homologous disease by Stastny and co-workers[385] and are closely similar to those observed in secondary disease.

It may be concluded that the graft versus host reaction causes a number of highly characteristic lesions which are found both in runt disease and homologous disease, as well as in secondary disease in a variety of animal species.

Graft versus host diseases and auto-immune diseases

In a number of human diseases, among them the so-called collagen diseases, auto-antibodies have been demonstrated which are directed against a large variety of antigens contained in cells (erythrocytes, leucocytes, thrombocytes, cell nuclei) and tissues (brain, colon, kidney, liver, striated muscle) and against antigenic cell products (γ-globulin, thyroglobulin, lens proteins). Formation of such auto-antibodies may be accompanied by either an organ localised auto-immune disease with the lesions confined to the particular organ or tissue in which the antigen is present, e.g. auto-immune thyroiditis, or with a more generalised auto-immune disease with lesions spread throughout many organs and tissues.

Since the cellular antibodies in graft versus host diseases are also directed against host antigens, it is reasonable to speculate whether these diseases could represent an experimental model for human auto-immune disease. Oliner and co-workers[297] have postulated such a model for mice treated with parental spleen cells because of the occurrence of splenomegaly and certain haematological abnormalities:

haemolytic anaemia with a positive Coombs test, leucopenia and thrombocytopenia. Similarly, by an investigation of adult tolerant rats treated with homologous spleen cells, Stastny and co-workers arrived at the conclusion that the skin lesions presented by the animals mimicked those of human lupus erythematodes and sclerodermia and that a common pathogenesis could therefore be postulated.

On the other hand, a number of differences between a graft versus host disease and an auto-immune disease are apparent. The similarities and dissimilarities will therefore be summarised.

GENERAL FEATURES

Fibrinoid necrosis of collagen and of blood vessels is a universal feature of the generalised auto-immune diseases—e.g. lupus erythematodes, polyarteritis nodosa and rheumatoid arthritis—but is found only rarely in graft versus host disease. Although this change has been described by Stastny[385, 386], it should not be forgotten that his rats were treated with exceptionally large numbers of spleen cells (200–800 million).

Until now the LE-cell phenomenon and the occurrence of haematoxylin bodies in the tissues have not been reported in graft versus host diseases. Certain autoimmune diseases are frequently accompanied by hyperplasia of the thymus and the other lymphatic tissues with the formation of lymphatic nodules in the medulla of the thymus. In graft versus host disease, on the other hand, atrophy of the lymphatic tissues is frequently found whilst lymphatic nodule formation in the thymic medulla does not occur.

Among the similarities are the previously mentioned haematological phenomena, splenomegaly mainly caused by extramedullary haemopoiesis, and possibly hypoplasia or atrophy of the lymphatic tissues in the terminal stages of the disease. Interestingly, the lesions of graft versus host diseases, as well as those of auto-immune diseases, show a constant association of infiltration of the diseased tissues by lymphoid cells with desquamation of the cells of these tissues.

ORGAN- OR TISSUE-SPECIFIC FEATURES

In the generalised auto-immune diseases the blood vessels, the heart, the joints and the serous membranes are frequently affected. Necrotising arteritis, nephritis, myocarditis, endocarditis, arthritis and serositis are rare in graft versus host disease, although the occurrence of endocarditis, myocarditis and polyarthritis has been

described in rats suffering from homologous disease after treatment with very large numbers of homologous spleen cells[386].

Lesions of the thyroid, adrenal, striated muscle, ocular tissues, brain and testis, similar to those found in organ-localised auto-immune disease, have not been reported in graft versus host disease. In graft versus host disease the absence of such lesions might be explained by the existence of a blood-tissue barrier, which prevents the circulating donor lymphoid cells from gaining access to the particular organ-specific antigens. In organ-localised auto-immune disease it is assumed that this barrier is primarily disturbed, causing otherwise "shielded" antigens, to which the immunological apparatus is not tolerant, to appear in the general circulation.

One other human disease which is considered to be possibly of an auto-immunological nature, has to be mentioned here: colitis ulcerosa. The features of human ulcerative colitis are reminiscent in several respects of the chronic colitis of mice treated with foreign bone marrow: subacute to chronic inflammation of the mucous membrane, multiple superficial ulcerations, crypt destruction and oedema and lymph stasis in the submucosa. It is attractive to postulate that the pathogenesis of both forms of colitis is similar and that immune or auto-immune damage of crypt epithelium leads to small mucosal defects, which are secondarily infected by the intestinal flora.

As already mentioned, the skin changes in graft versus host disease have some characteristics in common with those in lupus erythematodes. Lupus erythematodes is characterised by either acanthosis or atrophy of the malpighian layer of the epidermis and hair follicles, hyperkeratosis with keratotic plugging of the hair follicles, liquefaction degeneration of the basal cells and a lymphocytic infiltrate in the dermis in the vicinity of the dermal appendages; these characteristics are nearly always present in graft versus host diseases. However, fibrinoid degeneration of the dermal collagen is frequently found in the acute and sub-acute forms of lupus erythematodes, but only rarely in graft versus host disease. On the other hand the dyskeratosis of epidermal cells which is a highly significant change in graft versus host disease is far less conspicuous in lupus erythematodes. It has been mentioned before that a number of visceral lesions characteristic of disseminated lupus erythematodes, i.e. lesions of the heart, serous membranes, arteries and the kidneys are only rarely present in graft versus host disease.

It may be concluded, therefore, that although graft versus host

disease and auto-immune disease have an immunological basis, care must be taken before an identical pathogenesis for the lesions is accepted.

Graft versus host diseases and the immunological deficiency syndromes

The decreased resistance of radiation chimaeras to infections, which is the main cause of mortality in mice treated with foreign bone marrow, has induced many authors to compare secondary disease with a number of clinical or experimentally produced "immunological deficiency syndromes".

In patients suffering from congenital or acquired agammaglobulin-aemia and in essential lymphopenia[409], either atrophy of the lymphatic tissues or a defect in the production of plasma cells and gamma-globulins is at the root of a generalised susceptibility to infections. The immunological defect in agammaglobulinaemics is further underlined by the fact that homotransplants of skin show prolonged or occasionally even indefinite survival in these patients[158].

It was mentioned in Chapter III that Loutit[233] has postulated that a primary defect in lymphocyte production is the common aetiological factor in all graft versus host diseases. Although lymphatic tissue atrophy is the main cause of death in some forms of graft versus host disease, it has been pointed out already in a preceding section of this chapter that this atrophy is a secondary complication of the graft versus host reaction. Furthermore, in other forms of graft versus host disease, infection plays a minor role. Symptoms, lesions and death are a direct consequence of the graft versus host reaction. Loutit's theory seems to have been supported, however, by the observation that an experimentally produced wasting disease, induced by neonatal thymectomy in rodents, closely resembled clinically the wasting caused by the graft versus host reaction, although no such graft is present in thymectomised animals.

The wasting in neonatally thymectomised rodents may be accompanied by diarrhoea and skin lesions. From the earlier investigations of Miller et al.[281] a primary defect in the development and maturation of the lymphatic tissues was at first held responsible for this wasting disease. Recent studies prove, however, that in the thymectomised animal initial development of the lymphatic tissues precedes a secondary disintegration and atrophy[447]. Apart from this finding a number of other lesions in these mice are very similar to those found in graft versus host reactions.

In the lymphatic tissues and the liver a pronounced histiocytic reaction is apparent. This reaction and an intense plasmocytosis explains the enlargement of the lymph nodes in certain stages of the wasting syndrome. Diffuse isolated degeneration of liver cells and sometimes extensive necrosis of the liver may be present. Focal disintegration of the crypts occurs in the small and large intestines and is reminiscent of the intestinal lesions of chronic secondary disease in mice. Acanthosis accompanied by focal parakeratosis and vacuolisation of basal cells has been observed in the skin although dyskeratosis is rarely seen. In some cases atrophy of the epidermis accompanied by fibrosis of the dermis has been noted.

These skin changes, apart from bearing a resemblance to those seen in secondary disease, are reminiscent of lupus erythematodes, a human auto-immune disease. Interestingly, other lesions found in the human disease were discovered in thymectomised mice. In the kidney, fibrinoid necrosis of glomerular capillaries as well as the wire-loop change, a characteristic pathological feature, were repeatedly found. Foci of fibrinoid necrosis in the myocardium and endocardium with deposition of thrombi on the endocardial surface have been observed and it may be recalled that these changes are common in the heart disease in human lupus. In L.E.-cell preparations of the blood of thymectomised mice, typical L.E. cells have not been found till now, but rosette-like configurations of leucocytes, containing inclusions reminiscent of agglutinated and changed thrombocytes, were found in a few cases. In one of the mouse strains tested, positive Coomb's tests associated with anaemia were observed in a significant number of the wasted mice.

On the basis of these findings it has been postulated that neonatal thymectomy in mice may induce an experimental auto-immune disease, possibly by a defect in the self-recognition mechanism, caused by the absence of the thymus[447]. Secondary atrophy of the lymphatic tissues ultimately occurs, presumably by a mechanism similar to that described for animals with graft versus host disease, the so-called "allergic death" of immunologically competent cells. The secondary depletion of lymphoid cells would then account for the extraordinary susceptibility of neonatally thymectomised mice to infections, especially to viral disease.

It appears, therefore, that the pathology of the post-thymectomy syndrome lies somewhere intermediate between that of graft versus host syndromes and human auto-immune diseases, sharing features

with both groups of diseases (Table IV: 1). From the findings in thymectomised mice it is tempting to speculate whether, in certain human immunological deficiency diseases, some primary process leads to lymphatic tissue depletion. This, as yet, unknown process might well be of an auto-immune nature.

Whatever may be the true relation between the experimental and the human diseases, the discovery of the graft versus host reaction offers many new experimental approaches to the study of auto-immunity in man.

TABLE IV: 1. Pathology of graft versus host reactions, thymectomy syndrome and human autoimmune disease

	Graft vs. host	Thymectomy	Lupus erhy-thematodes
Splenomegaly	+	+	+
Liver necrosis	+	+	Lupoid hepatitis?
Intestinal lesions	+	+	Colitis ulcerosa?
Skin			
Acanthosis, hyperkeratosis parakeratosis	+	+	+
Dyskeratosis	+	±	—
Basal cell vacuolation	+	+	+
Focal necrosis corium	+	+	+
Wire loop kidney	—	+	+
Endocarditis	—	+	+
Arteriolar changes	+*	—	+
Spleen			
Histiocytic and plasmocytic reaction	+	+	+
Lymphocyte disintegration	+	+	+
Anemia, neutropenia, Lymphopenia, Thrombopenia	+†	+	+
L.E.-cells	—	±‡	+

* Man
† Runt disease
‡ Rosettes with atypical inclusions

CHAPTER VI

Clinical Applications of Bone Marrow Transplantation and Related Experiments

The identification of haemopoietic chimaerism following the transplantation of bone marrow to lethally irradiated animals offered a variety of theoretical possibilities not only for the restoration of an atrophic haemopoietic system but also for the replacement of abnormal blood forming tissue. In addition, this development seemed to be the beginning of a real breakthrough in the field of organ transplantation because it had been demonstrated in experimental animals that the homograft rejection could be completely avoided by the replacement of the host's immunological system by cells of the future organ donor.

Interest has been very much concentrated on the application of bone marrow transplantation in the treatment of two conditions: haemopoietic failure—radiation-induced or from other causes—and leukaemia. In neither of these conditions, however, has any consistent success been achieved, although a considerable number of clinical trials have been made in the past 5 years. In addition, a few unsuccessful trials have been made with high doses of whole body irradiation and bone marrow replacement as a preliminary to kidney transplantation.

Apart from many disappointing experiences, it seems, however, that some limited but real progress had been made, which justifies a careful continuation of clinical work concerned with bone marrow transplantation. Many of the clinical trials were destined to fail from the outset, however, because some if not all conditions known from animal experiments to be necessary for graft acceptance were ignored. In other instances the results have made it quite clear that extrapolation from rodents to man is a very unreliable approach in transplantation biology.

One of the fundamental advances of recent years seems to be the realisation that the reactions of monkeys to both irradiation and bone

marrow transplantation resemble, in many respects, those of humans. It is reasonable to expect, therefore, that continued experimentation with monkeys will eventually contribute substantially to a more precise evaluation of the possibilities and the limitations of bone marrow transplantation in human patients. In one or two centres the same problem is being thoroughly studied in dogs but it is not certain, as yet, whether this species exhibits a graft versus host reaction of comparable severity and pathology following homologous bone marrow transplantation as is seen in primates.

The problems associated with graft rejection and with a graft versus host reaction are absent when isologous bone marrow is available. A number of leukaemic partners of identical twins have been treated with a high dose of whole body irradiation followed by transfusion of fresh isologous bone marrow. These trials will be discussed in more detail later: let it be sufficient to note here that it is now generally accepted that the leukaemia cannot be eradicated by doses of radiation after which bone marrow transplantation could be expected to be beneficial.

The only other application of isologous bone marrow which may be justified is its use for the restoration of haemopoietic function in cases of bone marrow failure. The opportunity for this kind of therapy arises, of course, only rarely, but the results so far reported are encouraging.

Autologous bone marrow, while having the same immunological advantages as isologous cells, is obviously not available for the treatment of spontaneous bone marrow failure. Its application in the treatment of malignant diseases in which the bone marrow is not affected would seem, however, to be a logical development. If a sufficient quantity of autologous bone marrow is collected and stored before the administration of large doses of radiation or of cytotoxic drugs, a subsequent haemopoietic failure can be treated effectively. In most cases this approach requires adequate freezing and storage facilities but, unfortunately, the effectiveness of storage methods cannot be evaluated accurately because of the absence of markers to demonstrate the proliferation of the infused cells.

It has been pointed out previously (Chapter II) that the best approach to the problem of preservation of bone marrow cells at low temperatures is provided by the experiments with monkey bone marrow. It seems worth while to consider the collection and storage of autologous bone marrow from patients who are undergoing kidney

transplantation followed by prolonged treatment with immunosuppressive agents. Bone marrow aplasia is a not infrequent complication in such cases and the reinfusion of stored autologous bone marrow might be tried to restore the patient, although this measure obviously entails the risk of a rejection of the kidney graft.

When autologous cells are not available the question arises, whether or not homologous bone marrow is of any use in the treatment of bone marrow aplasia caused by cytotoxic drugs. These problems have received extensive, but by no means exhaustive, study in experimental animals.

Treatment of haemopoietic failure following irradiation

HOMOLOGOUS BONE MARROW TRANSPLANTATION

The restoration of lethally irradiated subjects with bone marrow transplants has without any doubt a most impressive experimental basis. Much of this basic work with animals was initiated with the aim of finding a method to treat accidentally irradiated people and victims of nuclear warfare. Thus far, clinical bone marrow transplantation has been performed only once for this purpose, namely, in the treatment of the people involved in the now famous Vinca accident. Six laboratory workers, five men and a woman, were heavily irradiated with neutron and gamma rays in the course of a critical nuclear excursion of a Zero energy reactor at the Boris Kidrich Institute of Nuclear Sciences, Belgrade, Yugoslavia. The victims were flown to Paris and treated by a team of French and Yugoslav physicians headed by Jammet and Mathé[197, 198, 265].

The doses of radiation to which the victims were exposed have been estimated in a number of ways by several groups of investigators, who arrived at widely differing results (Table VI: 1). The clinical findings were in accord with the view that patient B. had received a sublethal dose of irradiation and that V. had probably been the most heavily exposed. Although the other patients had all been exposed to irradiation within the lethal dose range, it was not possible to decide whether in fact the irradiation would have proved lethal without bone-marrow transplantation. It has to be taken into account that an inhomogenous dose distribution is very likely to occur under the conditions of such accidents which tends to lower the biologically effective total dose.

The patients were nursed under conditions of strict aseptic isola-

Table VI: 1. Estimated neutron and gamma doses received by the persons involved in the Vinca accident

Patient	Jammet et al. (1959)[198]		Hurst and Ritchie (1959)[182]		Auxier (1961)[12]		
	neutron (rem)	gamma (rem)	neutron (rem)	gamma (rem)	charged particle dose (rad)	H(n, γ)D gamma dose (rad)	total radiation dose (rad)
V	210	630	320	320	89	133	214
M	214	642	290	290	87	130	209
G	230	690	300	300	90	135	189
D	256	768	250	250	91	136	192
H	174	522	210	210	66	99	158
B	102	306	175	175	45	67	95

tion to prevent the introduction of pathogenic micro-organisms (so-called barrier nursing), and treated with antibiotics, blood and platelet transfusions and other supportive measures.

Fourteen days after the exposure, patient V. received 4×10^9 spleen and liver cells from a 5 month old human foetus, which failed to result in any clinical or haematological improvement. During the 4th week the patient developed peritonitis and symptoms of intestinal invagination, followed by anuria and jaundice. On the 27th day this patient received $8 \cdot 5 \times 10^9$ homologous bone marrow cells, but died a few days later from a massive haemorrhage. The other 4 patients received between $8 \cdot 5$ and 14×10^9 nucleated homologous bone marrow cells between days 27–32 after the exposure. This was followed in some cases by a rapid and in others by a more gradual recovery of the peripheral blood elements. In 3 patients a slight increase of donor type erythrocytes was seen for a short time; however, the proportion of donor type erythrocytes decreased to insignificant values within a month or two. Satisfactory clinical and haematological recovery was obtained 4 months after the exposure and a follow-up study over 2 years revealed persistent low lymphocyte values but no other haematological abnormalities.

Although the clinical course and the treatment of these patients has been reported extensively, there is no general agreement over the contribution made by bone marrow transplantation to the recovery of the patients. Cronkite and Bond[106] in particular have favoured the possibility that the increase of the peripheral blood cell counts which occurred soon after the bone marrow transfusions may have been spontaneous, and that the presence of donor type red cells for a few weeks cannot be considered as convincing evidence for the take of the bone marrow graft.

The fate of these patients without bone marrow treatment is, of course, open to speculation but in view of the other radiation sequelae, for instance the prolonged azoospermia[342], it seems likely that in at least 2 cases, the bone marrow was life-saving. Even a limited and temporary proliferation of the graft could well be of great benefit, since it might carry the patient over an extremely dangerous period. Although the patients survived for as long as four weeks prior to bone marrow transplantation it does not follow that the dose of irradiation was, in each case, sublethal because extensive symptomatic treatment was given during that period.

Regeneration of the host's haemopoietic tissues with concomitant

disappearance of the grafted cells prevented the occurrence of secondary disease in all patients. The cause of this rather early reversal is not clear, but it may have been related to the type of radiation exposure which undoubtedly resulted in an inhomogeneous dose distribution. The experience with the Yugoslav patients also indicates that the risk of an MLD effect in man is probably less than would be expected from the experiments with certain mouse strains (see Chapter II).

The feasibility of homologous bone marrow transplantation was thoroughly discussed at a Symposium of the World Health Organization on the Diagnosis and Treatment of Radiation Injury, held in 1960. Loutit[232], in his appraisal of the reports, thought that the MLD effect could be largely discounted in man and that if this were so, marrow could be prescribed with much more confidence than was formerly justified. The only remaining concern would be the development of a graft versus host reaction after higher dose levels of irradiation, when a permanent take of the foreign bone marrow is more likely to occur.

It is evident that at these dose levels the alternative to the possibility of the development of secondary disease is the risk of the patient succumbing to bone marrow failure.

The opposing view-point has been expressed with great perspicity by Lajtha[213] who concluded that any dose of single whole-body radiation which would allow survival with marrow grafting, would also allow recovery of the remaining host marrow elements without the need for marrow grafting, if careful symptomatic therapy were applied. In his opinion "there appears to be no *prima facie* case for marrow grafting either following irradiation or following treatment with tumour chemotherapeutic agents". His thesis is based mainly on the use of dose-survival curves for proliferating cells of various types following *in vitro* and *in vivo* irradiation, to predict haemopoietic recovery in humans. According to this reasoning, the number of haemopoietic cells which survive an LD_{50} of whole body irradiation would be equal to the minimum number of haemopoietic cells required for successful autologous therapy after lethal irradiation. The former cell number (10^9 cells/kg body weight using the survival curve from Lajtha's paper and 500 rads as an LD_{50} for man) appears to be greater than those which have usually been employed in clinical trials (10^8 isologous cells/kg body weight). Pegg[302] has calculated that the number of bone marrow cells which would be

expected to survive a 100 per cent lethal dose of whole body irradiation in various species is 3–16 times higher than the number of isologous cells known to provide complete protection. One explanation of this discrepancy would be provided by the demonstration of the greater radiosensitivity of bone marrow cells *in vivo* than the hitherto accepted value of Do=140 r.* Recently, McCulloch and Till[268] have indeed demonstrated a Do of .95 rads for mouse bone marrow irradiated *in vivo*. This would leave less than 10^6 cells intact out of the total number of 10^9 bone marrow cells of a 25 gram mouse following 800 rads of whole body irradiation ($\sim LD_{100}$). For effective isologous marrow therapy in mice most authors have found 10^5–10^6 cells necessary.

These considerations tend to invalidate many of Lajtha's objections to marrow therapy. If the same radio-sensitivity applies to human bone marrow, a dose of 500 rads would leave 7×10^7 surviving cells per kg body weight which makes the therapeutic effects of 10^8 isologous cells/kg much more acceptable. Lajtha also refutes the idea of tiding the patient over the crucial days with a temporary *homograft* because daily platelet transfusions would be much more effective. Theoretically this may be true, but it is general clinical experience that the prolonged maintenance of adequate thrombocyte levels in thrombopenic patients is extremely difficult. In addition, a bone marrow graft which is functioning only temporarily may also produce granulocytes which may be more effective in the prevention of dangerous infections than any substitution therapy or antibiotic treatment. The difficulties in the collection of a sufficiently large number of cells, as pointed out by Lajtha, have been largely overcome, and Mathé's group has shown that even a single living donor can supply enough cells to repopulate an irradiated recipient to a significant extent.

* For an exponential survival curve, Do (mean lethal dose or inactivation dose) is defined as the dose required to reduce the surviving fraction of cells to 37 per cent of the original (approximately to e^{-1}). For other survival curves the Do may be described as a measure of the rate at which surviving cells are killed by a given increment in dose. Do instead of a LD_{50} has been used most often because of its convenience in the application of the target theory of the action of ionizing radiations on individual cells. Lajtha used a Do value of 160 rads for his calculations: after a whole body dose of 500 rads, 10 per cent of the bone marrow cells would survive; that is, if adult marrow contains 5×10^{11} bone marrow cells, 5×10^{10} cells are expected to survive. This value is to be compared with 10^8 isologous cells/kg body weight which was found to bring about regeneration in patients following even higher doses of whole body irradiation.

O

In conclusion, it seems fair to say that in Lajtha's categorical rejection of bone marrow transplantation, theoretical considerations have been allowed to outweigh an impressive body of experimental data obtained from experiments both with a variety of animal species and also the few proven takes of homologous bone marrow in leukaemic patients; the latter will be discussed in a later section.

AUTOLOGOUS BONE MARROW REINFUSION FOLLOWING IRRADIATION

The study of autologous bone marrow therapy is preferable in larger animals because of the difficulty in obtaining a sufficient quantity of material in smaller ones. Bone marrow can be stored at room temperature for several hours without appreciable loss of viability so that there is ample time for irradiation of the animal if fresh bone marrow has to be reinfused. In the treatment of human patients, whole body irradiation at lethal doses is usually spread over one to two days and in some cases over much longer periods of time so that the preservation of the marrow at low temperatures becomes imperative.

Alpen and Baum[2] have shown that dogs can be protected from a lethal dose of 600 r (midline dose in air) by a minimal number of $1\cdot5 \times 10^8$ fresh autologous marrow cells per kg body weight. The Cooperstown group has studied the therapeutic value of autologous marrow after storage at $4°$ C for periods of up to 96 hours and at $-79°$ C for 25–120 hours[249], and has reported on more than 30 dogs which survived lethal whole body irradiation as a result of this treatment[166]. The midline X- and γ-ray doses varied between 600 and 1800 r (dose rates as low as 2 r/min. were used in some cases). The number of bone marrow cells which were administered varied between 2×10^8 and 1×10^9 nucleated cells/kg but the minimal number required for protection was not reported.

Similarly in monkeys, fresh autologous bone marrow was effective following whole body X-ray exposures of 850–925 r[108], the minimum number of cells required for consistent recovery being about 10^8/kg[336].

In view of these data it would be expected that adequate treatment of adult human patients requires at least 5×10^9 autologous bone marrow cells or 10^8 cells per kg.

The use of frozen autologous bone marrow for the treatment of haemopoietic depression in patients with malignant tumours following partial body irradiation has been studied most extensively by

TABLE VI: 2. Amount of isologous bone marrow administered to irradiated leukaemic patients

Authors	Age	Midline dose of radiation	No. of isologous bone marrow cells ($\times$ 10^9)	Effect on haemopoiesis
Atkinson et al. (1959)[11]	1 y./6 m.	255 r	2·65	Regeneration
Thomas et al. (1959)[406]	2 y./11 m.	1000 r*	2·5	Regeneration
	4 y./9 m.	750 r	3·9	Late regeneration
Thomas et al. (1961)[404]	25 y.	840 r	17·0	Regeneration
	38 y.	1595 r	9·6	Defective regeneration

* This patient had received 200 r whole-body irradiation 3 months previously

Kurnick and his collaborators[209, 210, 212]. His data reveal no clear relationship between therapeutic success in terms of haemopoietic recovery and the number of reinfused cells; successes were recorded with as few as 4×10^8 cells and failures with 10 times as many cells. This may be related to the fact that many patients still had areas of active bone marrow although the total production of cells was inadequate. Kurnick considers autologous bone marrow of definite value in the treatment of radiation induced bone marrow aplasia even in cases of long duration. In his opinion, however, haemopoietic depression following chemotherapy does not generally require treatment with bone marrow because of the strong tendency for spontaneous recovery.

Following whole body irradiation of three children in the terminal stage of leukaemia with doses between 470 and 550 r, McGovern et al.[271], administered autologous bone marrow, which had been stored for 5 months at $-70°$ C. In the two patients who received $5 \cdot 4$ and $3 \cdot 0 \times 10^9$ nucleated cells no repopulation of the marrow was seen, while in the third patient recovery of haemopoiesis occurred following the administration of $2 \cdot 3 \times 10^9$ cells. The effectiveness of the preservation in all these studies with frozen autologous marrow remains completely unknown and, moreover, no proof has been obtained that a take of transplanted cells was responsible for the recovery of the blood forming system.

The number of cells used in the therapy of whole body irradiation with fresh isologous marrow is somewhat higher than 10^8/kg body weight (Table VI: 2), but it remains possible that an overdose of cells was administered to these patients. An evaluation of the results in terms of numbers of bone marrow cells administered is difficult, in view of the wide variations in radiation exposure and because the subjects were suffering to a varying degree from a variety of diseases.

Autologous bone marrow after chemotherapy

EXPERIMENTS WITH ANIMALS

Studies with experimental animals on the feasibility of bone marrow grafts as a way to combat the lethal effects of high doses of cytotoxic drugs have been in progress since 1957. As would be expected with those drugs that cause death from haemopoietic failure, the results leave no doubt that this method can be effective in the absence of immunogenetic differences between host and donor.

Inspection of Table VI: 3 reveals, however, that the available data leave many questions unanswered. The number of bone marrow cells administered has been uniformly rather high, so that no systematic information has emerged concerning the minimal quantity of bone marrow required for protection. Since in many experiments only partial protection was obtained, it must be concluded that bone marrow therapy is possibly less effective in these cases than with cases of whole body irradiation. At least in mice, this could be due to the relatively more pronounced damage to the intestinal epithelium produced by many of these drugs.

Since the majority of the experiments have been performed with a single dose of the toxic agent at a particular level, the toxic range in which bone marrow grafting may be useful still remains unknown. The work published to date does not represent much more than "screening" of the efficacy of bone marrow, and a more detailed and refined evaluation of the possibilities must involve far more time and larger numbers of animals.

It must also be realised that it is still uncertain whether the results thus obtained could be extrapolated with safety to humans.

CLINICAL TRIALS

Clinicians, nevertheless, have been quick to use this new addition to their therapeutic arsenal, particularly since reinfusion of autologous bone marrow entails no undue risk to the patient. As with its application in heavily irradiated patients, the availability of autologous bone marrow would seem to allow the administration of higher doses of chemotherapeutic agents, which might otherwise induce irreversible damage to the haemopoietic system. It must be pointed out at once that clinical experience with this technique is far too limited to allow an evaluation of its possibilities in the treatment of malignant tumours. So far it has been used predominantly in very advanced cases, in which the response to any form of treatment is difficult to assess.

The minimum conditions necessary for a sensible application of autologous bone marrow in combination with massive doses of cytotoxic drugs can, however, be listed.

(1) *A sufficiently large number of bone marrow cells should be available for reinfusion.* The procedure employed for instance by Black et al.[68] in cancer cases, in which 20 ml. of sternal marrow (by our estimate $0 \cdot 3-1 \times 10^9$ nucleated cells) were reinfused

Table VI: 3. Effects of treatment with isologous and autologous bone marrow after lethal doses of cytotoxic drugs: animal experiments

Chemotherapeutic agent	Animals	Bone marrow	Results	Authors
Thioguanine 2 doses of 85 mg/kg i.p.	C_3H mice carrying Ehrlich ascitis carcinoma	2×10^7 isologous cells i.p.	Prolongation of survival time	Sartorelli and Le Page (1957)[360]
T.E.M. 5 mg/kg (LD $_{100}$)	C_3H and $C_{57}BL$ mice	Over 10^7 isologous cells i.v.	7% 30-day survival	Rudivic et al. (1958)[357]
T.E.M. 0·3 mg/kg i.v. (LD$_{200}$)	Dogs	10–15 ml. autologous cells 18 hr later	5 out of 6 dogs survived	Costakel et al. (1960)[103]
N mustard two doses: 3 and 4 mg/kg (LD$_{90}$)	Swiss mice	10^7 isologous cells i.v.	30% survival	Tran BaLoc et al. (1958)[412]
Myleran 21 mg/kg per os (LD$_{100}$)	Inbred rats	32–128 $\times$ 10^6 isologous cells i.v.	40–60% 30-day survival	Dunjic and Maisin (1960)[132]
Dimethyl-Myleran (CB 2348) 7·5 mg/kg i.p. (LD$_{100}$)	Inbred August rats	3–7 $\times$ 10^7 isologous cells i.v. or i.p.	Up to 100% survival when injected within hours	Talbot and Elson (1958)[393]

Dimethyl-Myleran 16 mg/kg	CBA mice	3×10^7 isologous cells	Complete protection	Floersheim and Elson (1961)[141a]
6-mercaptopurine 100 mg/kg + Myleran 16 mg/kg	CBA mice	3×10^7 isologous cells	Complete protection	Floersheim and Elson (1961)[141a]
1, 6-bis-chloro-ethyl-amino-1,6 deoxy-D-mannitol 120 mg/kg s.c. (LD_{50}) and 140 mg/kg s.c. (LD_{200})	C57BL mice	5×10^6 isologous cells i.v.	Reduction of mortality at LD_{50} to 16%, at LD_{100} to 55%	Rudivic (1962)[356]
Thio-T.E.P.A. 28–40 mg/kg i.v. (LD_{20-100})	BDF1 mice	10^7 isologous cells i.v.	Slight decrease of mortality at lowest dose of drug	Lochte et al. (1963)[224]
5–6 mg/kg i.v.	Dogs	Stored autologous marrow i.v.	Decreased mortality, no effect at 8 mg/kg of drug	Lochte et al. (1963)[224]

i.p., intraperitoneal   i.v., intravenous

after treatment with 0·4–0·65 mg/kg nitrogen mustard, seems bound to result in complete failure because of the small number of cells used. Black et al.[68] were nevertheless of the opinion that the recovery of the haemopoietic system was more rapid than in comparable cases without marrow reinfusion. It is, however, always questionable whether small numbers of patients with advanced disease provide sufficient basis for comparison.

(2) *The effect of marrow reinfusion cannot be evaluated when doses of chemotherapeutic agents have been administered which themselves allow a rapid and spontaneous recovery of haemopoiesis.* A typical example of this is shown in the report of Smiley et al.[377]. These authors reinfused between 1·3 and 3·0 × 10⁹ autologous bone marrow cells into five patients after treatment with 0·4 mg/kg of nitrogen mustard. The rate of recovery of the haemopoietic systems of these patients was equalled by a further five patients who received only the nitrogen mustard; in all cases complete or nearly complete recovery had occurred by the 26th day after treatment.

The recent results of Meyer et al.[276] also seem to be in this category. Their treatment consisted of four daily doses of 0·2–0·3 mg of nitrogen mustard, after which spontaneous recovery of the haemopoietic system was seen in all seven patients. The rise in the blood counts started as early as 14 days after treatment. In 9 other patients bone marrow preserved by freezing was reinfused 6–12 days after the last injection of nitrogen mustard; this failed to result in a more rapid return of the haemopoietic system to normal.

(3) *The bone marrow should be administered at a time when the chemotherapeutic agent is no longer active.* Nitrogen mustard seems to be inactivated very rapidly so that reinfusion can be performed shortly after an injection or course of injections[68, 289]. For many other agents information on this point is inadequate. When the toxic action is expected to persist for more than 24 hours, low temperature preservation of the bone marrow becomes necessary, since storage at 4° C for more than 1 day may lead to an appreciable loss of viable cells.

(4) *When low temperature storage of the marrow is employed, a considerable loss of its restorative capacity may be expected.* Methods which allow very good preservation of mouse bone marrow have been found to be inadequate with monkey bone

marrow and, since direct information on this aspect of preservation cannot be obtained with human marrow, even the best methods should be regarded as leaving no more than 50 per cent of the cells viable. The absence of any beneficial effect from autologous marrow in the patients reported on by Meyer et al.[276] may also be related to the use of rather low numbers of frozen cells: $4-9 \times 10^9$ per patient. An efficiency of 50 per cent in the preservation (the best so far obtained with monkey bone marrow) would leave $2-4 \times 10^9$ cells available which is slightly below the estimated minimum number.

(5) *Autologous bone marrow can serve as an adjunct to "super-dosage" chemotherapy only when the use of larger doses of the drug is prohibited by its depressive action on the bone marrow.* The margin which separates marrow depression from lethal toxic effects on other tissues should be relatively large. In one of the patients described by McFarland et al.[270], extensive "maceration" of the mucosa of the oesophagus and the stomach was found at autopsy, 19 days after treatment with $1 \cdot 4$ mg/kg of nitrogen mustard. It should also be remembered that "super-dosage" of chemotherapeutics, even when supported by bone marrow, may not be tolerated by patients who are in a terminal stage of the disease.

(6) *"Super-dosage" chemotherapy is likely to cause a severe decrease of immunological defences so that "barrier nursing" as well as supportive therapy with antibiotics and blood or platelet transfusions are required.*

In most of the clinical work reported, the degree of effectiveness of autologous bone marrow reinfusion is limited to the general impression that favourable effects—in terms of a more rapid haemopoietic recovery—are obtained with many patients.[200, 209, 303]. The majority of these studies were carried out after treatment with nitrogen mustard. McFarland et al.[270] observed rapid recovery of the haemopoietic system in 3 patients who received almost 3 times the recommended dose of nitrogen mustard ($1 \cdot 1$ mg/kg versus $0 \cdot 4$ mg/kg) followed by autologous bone marrow. Clifford et al.[76] have investigated the use of large doses of nitrogen mustard administered over a period of 3 days for the palliation of patients in East Africa with advanced malignant tumours, where radiotherapy was not available. They report on 3 patients who survived a dose of 2 mg/kg with autologous bone marrow therapy, while 3 other patients not

treated with bone marrow died after the same dose of nitrogen mustard.

Pegg *et al.*[303] have reported on autologous bone marrow replants not only after administration with nitrogen mustard but also after treatment with mannomustine, phenylalanine mustard and cyclophosphamide. In the patients treated with the latter two drugs the authors failed to observe any benefit from the bone marrow treatment.

In 38 cases reported by Hill and Loeb[178], massive chemotherapy with a variety of agents (actinomycin D, vincaleucoblastine, amethopterin and 6-mercaptopurine) and therapy with radioactive isotopes were supported by autologous bone marrow reinfusion.[178] This was followed by a satisfactory response in 21 cases. In agreement with several other authors, they consider the use of autologous bone marrow of value, because it provides a degree of "insurance" against irreversible haemopoietic complications.

Haemotological responses similar to those in patients used as controls were seen in 3 patients by Kretchmar *et al.*[207] and in 4 patients by Dunnigan and Brown[133], after treatment with autologous marrow following large single doses of nitrogen mustard (0·6–1·1 mg/kg). Kretchmar *et al.* stress the fact that an early, quite rapid and *spontaneous* recovery of the haemopoietic system was seen in a number of patients who had received 1 mg/kg of nitrogen mustard in a single dose. Both groups reinfused the marrow shortly after its collection without recourse to freezing. Technical reasons, as Dunnigan and Brown have themselves pointed out, may have been responsible for the failure to demonstrate any effect of the autologous marrow.

Any discussion of the effects of autologous bone marrow would be incomplete without mention of the work of Conrad and Crosby[100]. In their studies 8 patients with advanced Hodgkin's disease were treated with massive single doses of nitrogen mustard (0·95–1·5 mg/kg) while orthopaedic tourniquets were applied to one or more extremities during the infusion in an attempt to protect the bone marrow. Radioactive iron uptake studies afterwards provided evidence of a more pronounced erythropoietic activity in the protected areas, but no proof was obtained that the aplastic marrow spaces were seeded with cells from the protected marrow.

Homologous bone marrow after chemotherapy

EXPERIMENTS WITH ANIMALS

In animal experiments, homologous bone marrow has been much less successful than autologous bone marrow in the treatment of chemotherapeutic drug toxicity as is shown in Table VI: 4. Several investigators have compared the effects of isologous and homologous bone marrow after the same drug dosage. However, in some cases the same number of homologous and isologous bone marrow cells were tested, which makes the comparison of limited value because it is known from radiation studies that many more homologous cells are required to obtain a prolonged proliferation of the donor cells. In very few cases has any attempt been made to identify donor type haemopoietic cells in the survivors. It seems that Cree[104] in his experiments with rabbits is the only author who has obtained proof of permanent takes following treatment with aminochlorambucil. The protective effects with homologous bone marrow in myleran treated rats by both Weston *et al.*[457] and by Dunjic[130, 131] are so impressive that it is likely that a temporary proliferation of donor bone marrow occurred. Dunjic found that survival was better following higher doses of myleran and the injection of larger numbers of cells; this points to a take of the graft. It is regrettable that Ambrus *et al.*[3] made no attempt to demonstrate the presence of donor cells in monkeys which had survived lethal amounts of nitrogen mustard as a result of bone marrow therapy. The absence of secondary disease in these animals suggests that the grafted cells did not persist.

It becomes evident from these data that the problem of foreign bone marrow transplantation following cytotoxic drugs has remained largely unexplored. Admittedly, the dangers of the development of secondary disease which result from a successful take of the bone marrow are discouraging, but this should be no reason to neglect a field which may still conceal interesting possibilities.

CLINICAL TRIALS

Haemopoietic failure caused by cytotoxic drugs has been treated with homologous bone marrow in a limited number of clinical trials. The results tend to confirm the findings obtained with experimental animals, that takes are temporary at best and that beneficial effects are in most cases doubtful or indeed absent altogether. Pegg *et al.*[303] presented data on 12 patients who had been treated with various

TABLE VI: 4. Effects of treatment with homologous bone marrow transplantation following chemotherapy: animal experiments

Chemotherapeutic agent	Animals	Bone marrow	Results	Authors
Thioguanine 2 doses of 85 mg/kg	C₃H mice carrying Ehrlich ascitis carcinoma	2×10^7 homologous cells i.p.	Prolongation of survival, no proof of "take"	Sartorelli and Le Page (1957)[360]
T.E.M. 5 mg/kg (LD₁₀₀)	C₃H and C57BL mice	Over 10^7 homologous cells i.v.	No protection	Rudivic et al. (1958)[357]
N mustard 2 doses 3 and 4 mg/kg (LD₉₀)	Mice: 3 inbred strains	10^7 homologous cells i.v.	No protection	Tran BaLoc et al. (1958)[412]
N mustard 5–10 mg/kg i.v. (LD₁₀₀: 8 mg)	Swiss mice	10^7 homologous cells i.v.	10–60% protection at LD₁₀₀ when injected 30 min after drug, no identification of donor cells in survivors	Ambrus et al. (1962)[3]
1–2 mg/kg i.v. (LD₁₀₀)	Rhesus monkeys	$1–10 \times 10^8$ homologous cells i.v.	5/6 monkeys survived, no proof of "take"	Ambrus et al. (1962)[3]

Aminochlorambucil 30–40 mg/kg s.c. (LD_{85})	Rabbits	Homologous bone marrow or foetal haemopoietic tissue	40–60% survival and evidence of graft "takes"	Cree (1962)[104]
Myleran 23–35 mg/kg per os (LD_{100})	Rats, non-inbred	$32–128 \times 10^6$ homologous cells i.v.	Nearly complete protection with the higher cell number and the larger dose of Myleran, no proof of "take"	Dunjic (1962)[130,131]
Myleran 20 mg/kg i.v. (LD_{100})	Rats, non-inbred	125×10^6 homologous cells i.v. 1 or 3 times	Complete protection, no proof of "takes"	Weston et al. (1957)[457]
Dimethyl-Myleran 16 mg/kg	CBA mice	3×10^7 homologous cells i.v.	No effect	Floersheim and Elson (1961)[141a]
6-mercaptopurine 100 mg/kg + Myleran 16 mg/kg	CBA mice	3×10^7 homologous cells i.v.	No effect	Floersheim and Elson (1961)[141a]

i.p., intraperitoneal   i.v., intravenous

chemotherapeutic agents, sometimes in addition to radiotherapy, and after which homologous bone marrow was infused. Only 4 patients received the minimal number of cells theoretically required for a take of the graft. In one of these, some evidence of a temporary proliferation of the donated cells was obtained by a rise, for a brief period, in donor type neutrophils. In none of the cases was the bone marrow reported to have had a beneficial clinical effect. Haurani et al.[171] described a smaller series of patients treated with homologous bone marrow after high doses of nitrogen mustard or 6-mercaptopurine and in which evidence of the proliferation of the grafted cells was absent.

Miller and Diamond[279] reported a temporary take of homologous bone marrow in a patient with Hodgkin's disease who developed pancytopenia after treatment with nitrogen mustard. The patient received 5×10^9 fresh bone marrow cells from a donor with the sickle cell trait. The sickle cell count in the recipient rose 6-fold during the first 3 weeks over the immediate post-infusion value, and then decreased to zero over the next 2 months.

Recently a temporary take of myeloid cells was observed in 3 children who received large numbers of peripheral granulocytes (a total of $2-9 \times 10^{11}$ cells) during intensive therapy with amethopterin for actue lymphoblastic leukaemia[220]. The cells were obtained by plasmophoresis from patients with chronic myeloid leukaemia. Persistent mitoses of the transfused cells containing the Philadelphia chromosome were detected as long as 52 days after transfusion. No symptoms of secondary disease were observed. Similar white blood cell transfusions involving $3 \cdot 10^{10}-10^{12}$ nucleated cells were performed by Mathé et al. (L. Schwarzenberg, G. Mathé, J. de Grouchy, C. de Nava, M. J. de Vries, J. L. Amiel, A. Cattan, M. Schneider and J. R. Schlumberger, Israel Journal of Medical Sciences 1, (1965) 925-56) in 33 patients with leukaemia, haematosarcoma or epithelioma during an agranulocytic stage of the disease. In 7 of those patients symptoms of secondary disease developed after the transfusion, which suggests a take of the lymphoid elements present in the myeloid leukaemic cell suspensions infused.

Evidence suggestive of temporary takes of homologous bone marrow of type O donors after radiotherapy or treatment with cytotoxic drugs was obtained in a number of patients by the observation of a rise of the level of O-positive erythrocytes for as long as 3 weeks after transplantation[284].

Three other partial or temporary takes of a homologous haemo-poietic graft have been reported in the literature. Beilby et al.[37] report the treatment of a patient with Hodgkin's disease who developed severe hypoplasia of the bone marrow after receiving amino-chlorambucil. Infusion of bone marrow from the patient's sister resulted in an increase of donor type (D positive) erythrocytes which amounted to 24 per cent 6 months after the transplantation. Curiously enough the donor's skin graft was rejected.

Another patient with Hodgkin's disease whose bone marrow became aplastic after treatment with TEM, was treated with pooled bone marrow from 4 donors. Donor type erythrocytes were present in large numbers (up to 50 per cent) 3 months after the grafting but 2 months later these cells had disappeared[172].

Proliferation of donor type cells was also observed[71] in a patient with pancytopenia who was treated with foetal liver cells following a course of thio-TEPA for the treatment of mammary carcinoma. Immediately after the infusion, the white cell and platelet counts rose sharply and donor type erythrocytes increased until a maximum of 9·5 per cent was reached on day 16. Thereafter, host cell regeneration started and the donor type erythrocytes eventually disappeared. The number of foetal liver cells administered was, unfortunately, not reported.

The total number of reported cases in which some evidence was present of a short period of proliferation of the infused homologous cells is certainly larger than would have been expected from a study of the results of experiments with animals. Perhaps this is related to the fact that in some of the patients suffering from Hodgkin's disease or leukaemia the immunological system is already severely depressed before chemotherapy is initiated. Another possibility seems to be that the immune system is sufficiently damaged by certain cytotoxic drugs as to prevent an immediate rejection of the transplanted bone marrow cells. Compared to the situation after whole body irradiation, however, recovery of the host's lymphatic system occurs quite rapidly and results in a rejection of the foreign cells.

It is of interest that both Thomas et al.[401] and Cole and Alpen[78] observed an improved acceptance of a homologous bone marrow graft in dogs treated with radiation plus amethopterin or 6-mercapto-purine. Whether the immune suppression by the addition of chemicals is greater than that provided by increasing the doses of radiation is one of the many questions that invite further experimentation.

Whole body irradiation and transplantation of haemopoietic cells in the experimental treatment of leukaemia

The treatment of leukaemia by irradiation is seriously hampered by the fact that the malignant cells are usually widely disseminated at the time of diagnosis. The local irradiation of a tumour site, e.g. a lymphomatous gland or a spleen, often has a limited effect because of the influx of non-irradiated tumour cells from outside the radiation field. This is illustrated by the early experiments of Hollcroft and co-workers[180] on the treatment of lymphomas in mice. A whole body dose as small as 50 r administered simultaneously with a local dose of 1000 r to a subcutaneously transplanted lymphoma, was found to be at least as effective as 6000 r administered to the tumour alone. When the local dose and the whole body dose were separated by a 5 minute interval a substantial decrease in the effectiveness of the treatment was already apparent.

The possibility of restoring lethally irradiated animals by bone marrow transplantation has greatly stimulated research on the treatment of leukaemia by whole body irradiation. It was hoped that the post-irradiation grafting of bone marrow would allow the use of much higher radiation doses than was formerly regarded as possible. In addition, the total dose could be given in one session, thus further enhancing the lethal effect on the tumour.

It was clear from the beginning, however, that the maximum dose would be limited to about 1000 r in view of the danger of intestinal radiation death, which cannot be prevented by bone marrow transplantation.

The first attempt was made by Hollcroft and co-workers[180] who used a transplantable leukaemia in inbred guinea-pigs. These authors found remissions, as evaluated from the peripheral lymphocyte count, the duration of which varied more or less linearly with the radiation dose. No permanent cure could, however, be obtained. These disappointing results were subsequently confirmed by other workers[32, 236, 254, 260, 361, 373, 448], and are in contrast with the initially promising experiments reported by Barnes *et al.*[20] These authors obtained a considerable number of cures in mice carrying a transplantable lymphosarcoma following irradiation with a dose of 1500 r given over 25 hours, and treatment with isologous bone marrow. They could not, however, repeat their earlier success[33], nor have others succeeded in obtaining an improved rate of cure by the use of protracted irradiation. Furthermore, the treatment of spontaneous tumours failed to

yield better results than had been obtained in the experiments with transplanted tumours[254, 260]. Quite satisfactory results have been published by Trentin[415] with the Gardner lymphosarcoma. Of eleven C3H mice, irradiated and treated with isologous bone marrow on the day of inoculation of the tumour, 10 were alive after 190 days. It might be questioned, however, whether in addition to the irradiation, antigenic differences between tumour and host may have contributed to the tumour regression. Such antigenic differences are not uncommon in tumours which have been frequently transferred over a number of years.

Significant tumour regression has been reported by the use of homologous or heterologous bone marrow and lymphoid cells in animals receiving whole body irradiation[20, 448]. The prevention of the recurrence of the tumour following radiation doses that by themselves were insufficient to eradicate it, has been attributed to a graft versus host reaction, the tumour being considered as part of the host. The simultaneous occurrence of severe secondary disease, has until recently, however, invalidated this mode of treatment.

In the following paragraphs the factors which determine tumour regression and the survival of experimental animals will be analysed. The experiences so far obtained with this form of treatment in human patients will also be reviewed.

THE EFFECT OF THE IRRADIATION

In clinical literature great emphasis has been placed on the radiosensitivity of tumours. Differences in the results of radiation therapy of different tumours as measured by decrease of tumour volume, remission time, symptom free interval and the rate of cure have been attributed to differences in the radiosensitivity of the cells which form the tumours.

Recent radiobiological research has indicated, however, that the differences in radiosensitivity between many types of cells are quite small when the irradiation is performed *in vitro* under standard conditions of oxidation and if the reproductive capacity of the cells is used as a criterion[214]. It is beyond the scope of this book to discuss all the factors which might possibly modify the radiation response of malignant cells with similar "intrinsic" radiosensitivity, when irradiated *in vivo*. For the present discussion it is relevant to mention only that the important study of Hewitt and Wilson[173, 174] has shown that the dose-effect relationship for the survival of mouse leukaemic

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cells when irradiated in the liver *in vivo* does not differ materially from that obtained with many lines tested *in vitro* (Fig. VI[1]).

Hewitt and Wilson reported limited observations on 4 other leukaemic cell lines which indicated that the radiosensitivity of these leukaemias did not differ radically from that found for the CBA leukaemia used in their earlier studies[176]. The only factor which significantly influenced the radiosensitivity of the leukaemic cells was the oxygen supply. Tumour cells irradiated under anoxic conditions
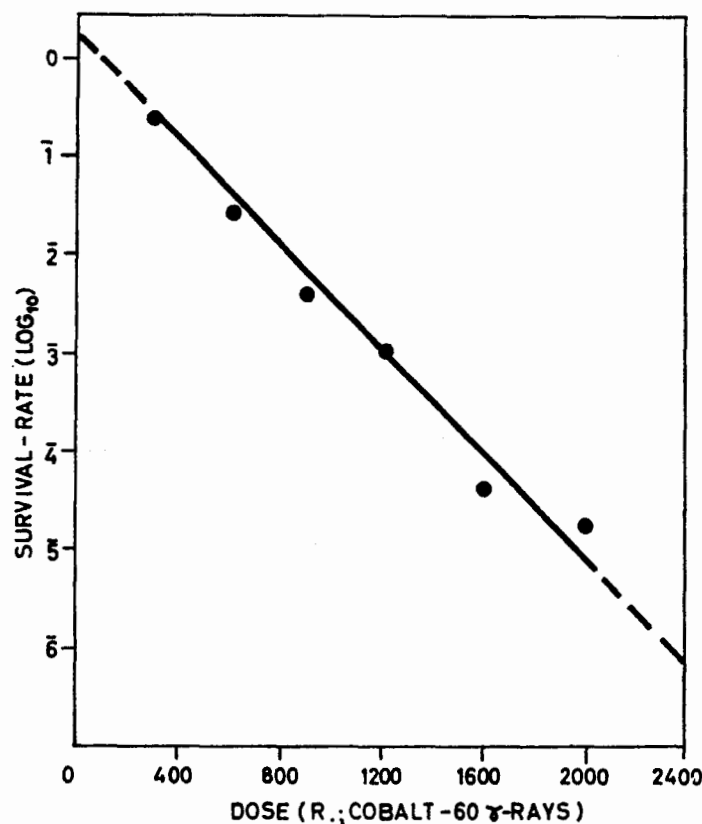
Figure VI[1].   Cell-survival curve, obtained by Hewitt and Wilson
(1959)[174] for mouse leukaemia cells irradiated *in vivo*

in recently killed mice appeared to be 2·3 times more radioresistant than cells irradiated in living mice[173].

In the authors' laboratory the assay is carried out with leukaemic spleen instead of liver (Fig. VI[2]). The Do values* obtained with 3 different strains of leukaemia (2 lymphosarcomas and 1 myeloid leukaemia) were found to be a factor of about 2 higher than those of Hewitt and Wilson[174]. This fits in very well with the theory that in contrast to leukaemic cells in the liver, those in the spleen are under anoxic conditions. Hewitt and Wilson also found that leukaemic cells

* The term Do has been explained on page 199.

in the peripheral blood behave as anoxic cells[176]. They explained this rather unexpected finding by postulating a rapid turnover of leukaemic cells, so that cells which were at anoxic sites during the irradiation were afterwards found present in the blood. We may extend this hypothesis and assume that these cells entered the circulation from the spleen.
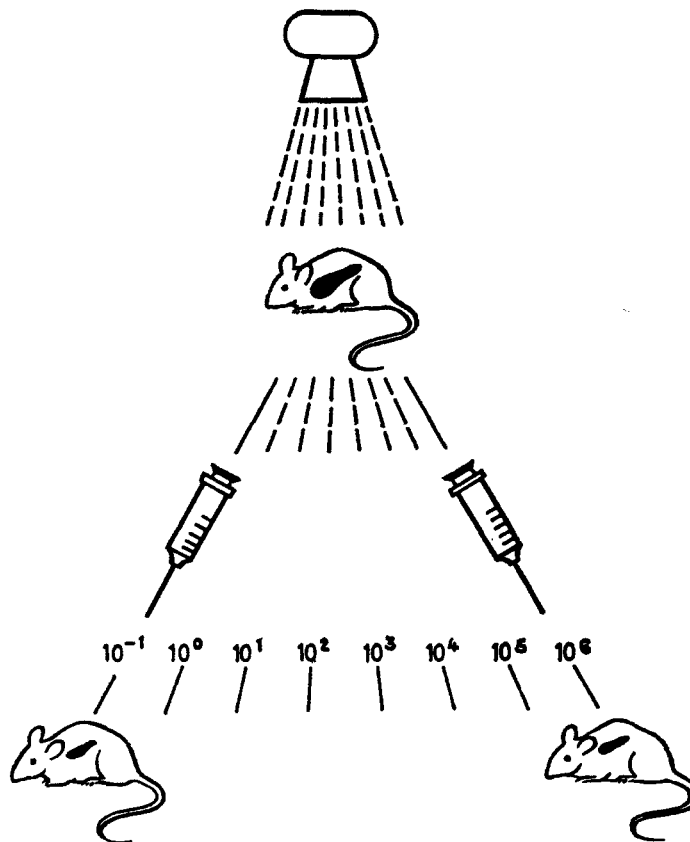
Figure VI². Schematic representation of the *in vivo* assay of the radiosensitivity of leukaemia cells. A tumour-bearing mouse is irradiated. Serial dilutions of a suspension of the leukaemic spleen are prepared after the irradiation and injected into normal mice. The number of cells required to produce leukaemia in 50% of the recipients ED_{50} (Effective Dose 50%) is compared with the ED_{50} for non-irradiated leukaemic cells

Until now, only a restricted number of different leukaemias have been tested, and it might be doubted whether the survival curves of other leukaemias would have a comparable shape. However, for the present discussion we shall assume that this is the case and, furthermore, that tumour cells from all sites are fully oxygenated.

With further reference to Fig. VI¹ it can be seen that a dose of 2000 r will cause a reduction of the tumour cell population by a factor of 10^5. This means that in a population of mice bearing tumours of a

size of 10⁴ cells, 90 per cent would be cured, assuming that each single surviving tumour cell would be able to grow out and cause a recurrence. It will be clear that in overt leukaemia, especially in humans,the population of malignant cells will be much larger than 10⁴. Since the dose of whole body irradiation cannot be increased above approximately 1000 r in view of the danger of death by intestinal damage, radiation alone cannot be expected to cure leukaemia. A logical consequence of these considerations is the recommendation that the irradiation of the patients should be carried out during a remission, when the number of malignant cells is at its lowest and the surviving fraction after a certain fixed dose of radiation is, therefore, as small as possible.
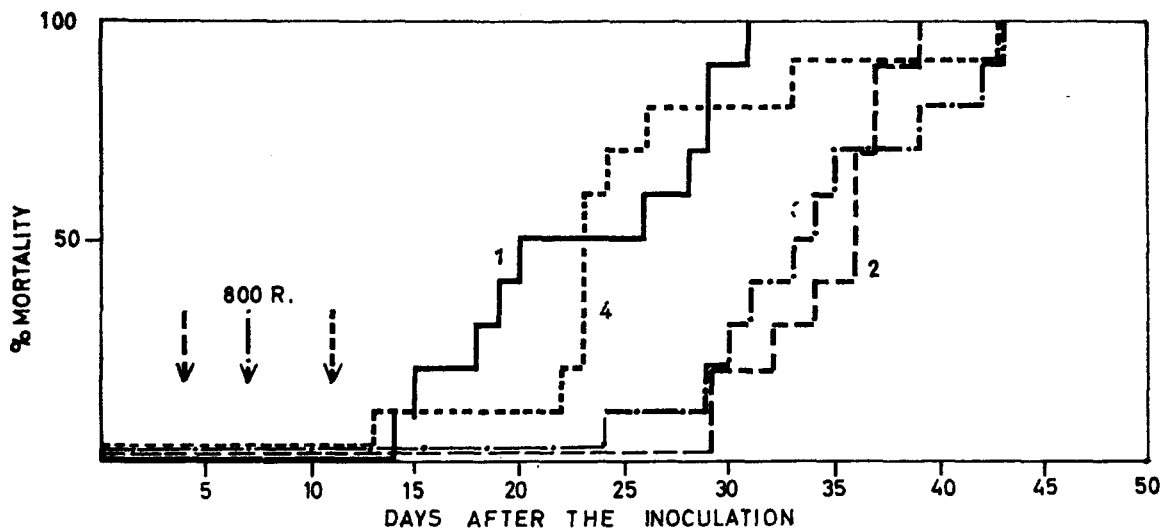
Figure VI³. Survival of C57BL mice, inoculated with o·5 × 10⁶ lymphosarcoma cells, irradiated with 800 r at 4, 7 or 11 days after the inoculation_respectively, followed by injection of 6 × 10⁶ isologous bone marrow cells

1 = Non-irradiated control mice
2 = Mice irradiated 4 days after tumour inoculation
3 = Mice irradiated 7 days after tumour inoculation
4 = Mice irradiated 11 days after tumour inoculation

These pessimistic predictions on the efficacy of a lethal dose of radiation as a cure for leukaemia are borne out by the experimental data obtained from mice. In Fig. VI³ the survival of mice treated with isologous bone marrow after irradiation at different intervals following tumour inoculation is shown. Although some prolongation of survival is obtained in mice irradiated within a week of inoculation, the results are not very impressive.

(A)   DAYS AFTER THE INOCULATION
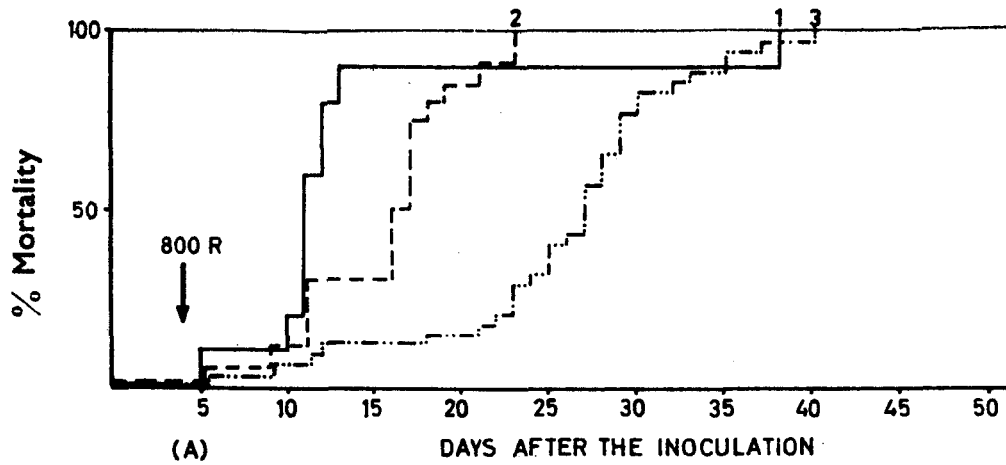
Figure VI⁴(a). Effect of irradiation (800 r) and injection or rat bone marrow and lymph node cells on the survival of mice inoculated with lymphosarcoma (o·8 × 10⁶ cells)

1 = Non-treated control mice

2 = Mice irradiated and treated with rat bone marrow (36 × 10⁶ cells)

3 = Mice irradiated and treated with rat bone marrow (36 × 10⁶ cells) and lymph node suspension (20 × 10⁶ cells)

(B)   DAYS AFTER THE INOCULATION
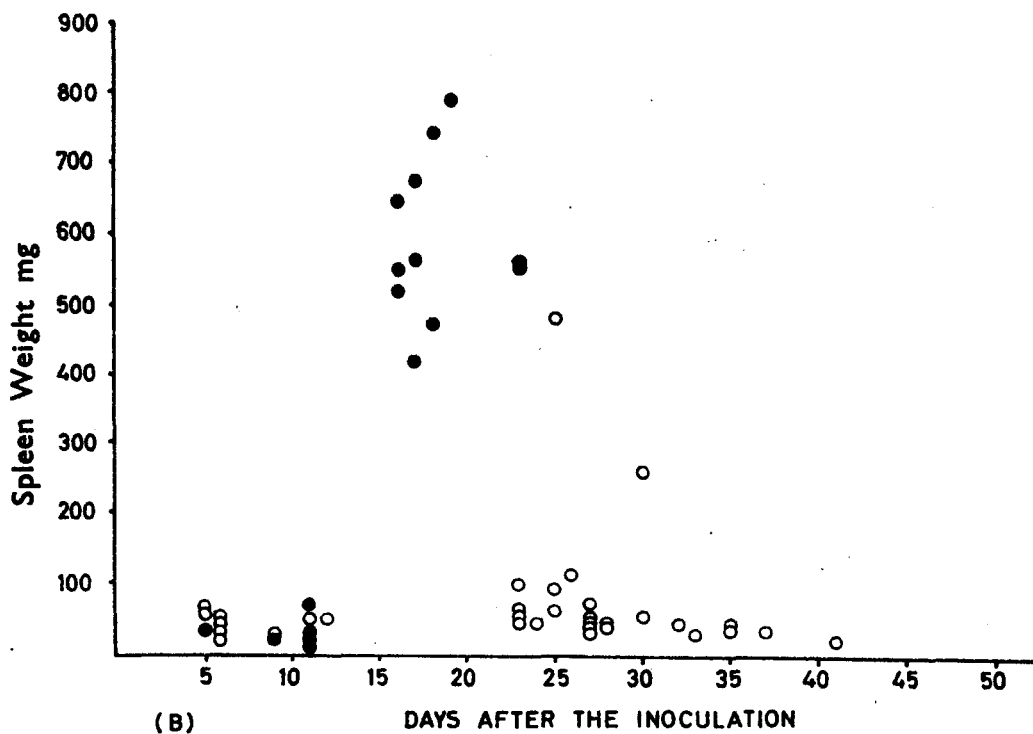
Figure VI⁴(b). Effect of irradiation and injection of rat bone marrow and lymph node cells on the growth of a C57BL lymphosarcoma

Spleen weights of mice of Fig. vi⁶(a)

● Heterologous bone marrow

○ Heterologous bone marrow and lymph node suspension

THE HOST-DONOR COMBINATION

If it is accepted that leukaemia cannot be cured by whole body irradiation alone, other means must be sought to supplement the effect of the irradiation. The use of bone marrow transplantation by itself seemed to provide the possibility of a further reduction in the number of malignant cells that survive irradiation. A foreign haemopoietic transplant is able to react immunologically against its host. Since a tumour, even if slightly antigenic, shares most antigens with its host, it might be expected that a graft versus host reaction would also affect the cells of the tumour.

It was indeed found by Barnes and Loutit[32], de Vries and Vos[448] and Mathé and Bernard[261] that the transplantation of foreign haemopoietic cells could result in a variable prolongation of survival time or even in a permanent cure of a small proportion of animals. As would be expected, the effect of tumour inhibition appeared to be most pronounced when, in addition to bone marrow, lymphoid cells were given to initiate an earlier and more severe graft versus host reactions.

In Plate VI: 1 the dramatic effect on tumour growth in one of these experiments is shown. The effect of tumour inhibition shown by the foreign haemopoietic cells seems not to be due to the wasting of the animals which accompanies the secondary disease, as was suggested by Barnes and Loutit[32]. This was shown by experiments with a C57BL lymphosarcoma which was inoculated into (CBA $\times$ C57BL)F_1 hybrids[448]. The growth of the tumour was inhibited following the irradiation and the administration of CBA bone marrow and CBA lymphoid cells, while similar cells of C57BL origin had no effect. Nevertheless, both treatments induced secondary disease and wasting in the F_1 hybrids to a similar extent.

Although the effect of tumour inhibition shown by foreign haemopoietic cells is quite spectacular in some host-donor combinations, the survival time of the animals is, at best, only slightly prolonged when compared to that of non-treated controls or mice treated with isologous marrow (Fig. VI[4] (a) and (b)). Only exceptionally was an animal found to survive permanently. It seems to be extremely difficult to induce that precise degree of graft versus host reactivity which will kill the leukaemic cells but which is at the same time mild enough to allow survival of the host (Table VI: 5).

ATTEMPTS AT CONTROLLING THE GRAFT VERSUS TUMOUR REACTION

Several attempts have been made to limit the graft versus host

TABLE VI: 5. Effects of the injection of varying numbers of homologous lymphoid cells on tumour recurrence after irradiation and on death from secondary disease*

Treatment	Mortality interval: days after treatment	Mean spleen weight at death (mg)	Main cause of death
Non-irradiated, non-treated	9–10	701	Lymphosarcoma
CBA bm	11–18	510	Lymphosarcoma
CBA bm + 0·8 × 10⁶ CBA Ly cells**	12–15	104	Secondary disease
CBA bm + 0·4 × 10⁶ CBA Ly cells	12–18	100	Secondary disease
CBA bm + 0·2 × 10⁶ CBA Ly cells	18–26	120	Secondary disease
CBA bm + 0·1 × 10⁶ CBA Ly cells	15–23	602	Lymphosarcoma

* C57BL mice inoculated with C57BL lymphosarcoma, irradiated 4 days after tumour inoculation with 800 r, treated with 10 × 10⁶ bone marrow cells and varying doses of lymph node cells—10 mice in each group[44].
** bm, bone marrow   Ly, lymph node

reaction in order to prevent death from secondary disease, while preserving the inhibiting effects on the tumour.

(1) *Induced reversion of the chimaeras to host type haemopoiesis shortly before the peak of secondary mortality.* This was accomplished by a second relatively low dose (200 r) of irradiation followed by the injection of isologous haemopoietic cells. The majority of the mice treated in this way died, however, from a recurrence of their lymphosarcoma. This finding indicates that a protracted graft versus host reaction is required to prevent regrowth of the malignant cells.[444]

(2) *Treatment with homologous lymph node cells which are tolerant towards the host.* This approach could only be expected to result in a cure if the tumour contained specific antigens of sufficient strength. Although up to 60 per cent of cures have been obtained in a suitable experimental set-up with this method, these results appear to be of academic interest only. Firstly, it is highly questionable whether human leukaemias possess tumour-specific antigens of sufficient strength to be susceptible to an immunological attack by host-tolerant cells. Secondly, the clinical application of this mode of treatment would require the *in vitro* induction of tolerance of the donor lymphoid cells to the prospective host, the possibilities for which seem to be remote at present.[444]

(3) *The grafting of large numbers of isologous lymphoid cells in addition to isologous bone marrow following lethal irradiation.* One of the present authors has reported a significant prolongation of life and even long term survival of C57BL and (CBA $\times$ C57BL)F$_1$ mice carrying a transplantable lymphosarcoma, following this form of treatment[448, 450]. Originally the possibility of a competition between the normal and the malignant lymphoid cells was favoured, but subsequent experiments have made it more likely that the beneficial effect of the treatment has to be ascribed to antigenicity of the tumour cells.

Although the practical significance of these experiments seems to be as remote as that of the preceding ones, treatment with large numbers of *isologous* lymphoid cells might be considered in those rare leukaemic patients who have an identical twin partner available as donor. In the case of tumour specific antigens being present, the likely possibility is that the transplanted cells would start an immunological reaction against the leukaemic cells.

COMPLICATIONS OF THE TREATMENT OTHER THAN SECONDARY DISEASE

Whole body irradiation of leukaemic mice may result in an appreciable rate of mortality in the first week following the irradiation. This is especially true in the cases where the animals have large leukaemic spleens at the time of irradiation. In cases involving such mice, autopsy showed large haemorrhagic areas in both lungs, which on microscopic examination proved to be infarctions, caused by massive blockage of the pulmonary arterioles by large numbers of disintegrated tumour cells and DNA (Plate VI: 2). Such thrombo-emboli have also been described in irradiated leukaemic AKR mice[181]. The fragments of tumour cells are probably removed from the spleen and the liver after the irradiation and carried to the lung by way of the portal circulation and the hepatic veins.

Another complication, due to massive radiation-induced destruction of tumour cells, is renal insufficiency caused by deposition and subsequent blockage of the renal tubules by urates. This latter complication is well known to occur in human cases of leukaemia following irradiation.

Theoretically, still another seemingly paradoxical complication may result from the irradiation of a tumour-bearing animal, namely, an increase in the rate of the proliferation of tumour cells which survive the irradiation. This may be the case when the tumour has antigenic properties, which impede its proliferation in a normal animal. The abrogation of the immunological defence by the irradiation would then result in enhanced growth of the tumour. This was actually found to occur in at least one specific host-transplantable tumour combination.

THE CLINICAL APPLICATION OF BONE MARROW TRANSPLANTATION IN THE TREATMENT OF LEUKAEMIA

Until now, about 60 patients suffering from leukaemia have been treated by total body irradiation and the infusion of isologous bone marrow from identical twins[11, 404, 406,] stored autologous bone marrow[271] or homologous bone marrow [8, 171, 258, 262, 263, 271, 404, 407].

The evaluation of the results, especially with respect to the effect of homologous bone marrow on the leukaemia, is exceedingly difficult, mainly because adequate non-treated control patients are never available, and because in most of the cases proof of a take of the marrow transplant is either lacking, or unconvincing. Careful analysis of the data reveals that evidence for a marrow take has been obtained in only

6 or 7 cases treated with homologous bone marrow. Two takes of homologous bone marrow grafts have been described by Mathé et al.[263] in 2 leukaemic children, both of whom died from secondary disease after about one month. In two other patients similarly treated a temporary existence of donor type erythropoiesis was accompanied by symptoms of secondary disease during the second and third month following transplantation[251, 262]. The symptoms disappeared at the same time as did the donor type erythrocytes, while simultaneously a rise in the lymphocyte count (presumably host type) occurred. These 4 patients were irradiated with a dose of 870 to 950 r and received 11-34 $\times$ 10^9 bone marrow cells from a single homologous donor each.

Quite recently, a successful take of a bone marrow homograft of more than one year duration was obtained in a 26-year old patient by Mathé and co-workers[258]. Following whole body irradiation with two divided doses of 400 rads each, this patient was given pooled bone marrow derived from 6 relatives. The bone marrow was administered 5 days after the second irradiation and the patient developed pronounced secondary disease beginning about 10 days after the transplantation and lasting two months. After 8 months the recipient's blood was completely repopulated with erythrocytes of the genotype of one of the donors (a brother). The patient produced γ-globulins of a type characteristic of the donors (a differentiation between the donors could not be made with this technique). A skin graft of the donor, whose erythrocytes were identified in the patient, was retained, while skin from the other donors—simultaneously grafted—was rejected.

It is of great interest that the graft which was retained came from the donor who had been classified as closest to the host according to leucocyte antigen determinations in spite of the fact that the sera used in these determinations were probably not monospecific. This donor was, together with one other donor, also closest to the recipient on the basis of the histocompatibility test* currently employed by the group in Paris[256].

The Cooperstown group has reported one temporary and incomplete take of homologous bone marrow in a heavily irradiated

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* A subject unrelated to the recipient of the bone marrow and the donor is grafted with the recipient's skin. After this graft has been rejected, the subject receives a skin graft from each of the prospective donors. A second set reaction in any of these grafts is interpreted as an indication that histocompatibility factors between that donor and the recipient are shared.

leukaemic patient. There were no clear-cut symptoms of secondary disease in this case although an active bone marrow was found at autopsy[398, 404, 407].

Kurnick[210] has reported a uniform lack of success in a limited number of homologous bone marrow transplantations. Similarly, disappointing findings were reported by Haurani et al.[171] in a series of 9 leukaemic patients. Following 300–500 r of whole body irradiation these patients received 4–29 × 10⁹ fresh homologous bone marrow cells from excised ribs. At no time could donor type cells be demonstrated in the recipient's blood, which is not surprising in view of the low dose of radiation. A single unsuccessful attempt to transplant homologous bone marrow was reported by Meighan and Bean[273] who transfused 4 × 10⁹ cells into a leukaemic patient after 700 r of whole body irradiation.

Andrews et al.[8] described a series of 7 leukaemic patients who had been given homologous bone marrow after whole body irradiation. Four cases received pooled marrow from several donors, in most cases closely related to the patients. The absence of any evidence of "takes" can be explained by the sublethal doses of whole body irradiation employed (270–620 r) in all but one patient. The latter received 6·8 × 10⁹ pooled cells from 5 donors in her "immediate family", following a radiation dose of 940 r. The patient died 17 days later with bone marrow aplasia.

A serious difficulty in an evaluation of the results of treatment is that in human leukaemia, remissions lasting for several months may be obtained after sublethal whole body irradiation without the administration of any bone marrow[8]. Remissions of long duration obtained by treatment with autologous bone marrow[271], isologous bone marrow[404], or homologous bone marrow[171, 407] after sublethal radiation doses, can also be ascribed to this unexplained effect of whole body irradiation on human leukaemia. Such remissions may even be obtained in patients unresponsive to treatment with cortisone or chemotherapy. In a case reported by Haurani and co-workers[171] whole body irradiation with a dose of only 50 r during a remission apparently sufficed to extend the leukaemia-free period of the patient to 7 months.

Taking all these considerations into account, it appears that the conclusions drawn from the experiments with mice are well borne out by the clinical observations. Radiation only, even with supralethal doses does not eliminate the leukaemia. This disappointing conclusion

is also applicable to the patients who were treated during a remission, when the total number of leukaemic cells would have been minimal. Remission times, obtained after lethal doses of whole body irradiation and treatment with isologous bone marrow (obtained from identical twins) have been two months at the most.

Evidence which suggests that the immunological activity of homologous haemopoietic cells against tumour cells has an additional effect by inhibiting the recurrence has been obtained by Mathé[258, 262]. Two children with acute lymphoblastic leukaemia were treated with homologous bone marrow. Apart from other evidence of a temporary take of the graft, they both suffered for a short period from secondary disease. Compared with the cases treated with isologous bone marrow, the remissions were of relatively long duration, 5 and 6 months respectively. These two cases, in which spontaneous reversal to host type haemopoiesis occurred, may be compared with the experiments in mice in which reversal was induced early in the period of secondary disease. They tend to confirm the view that in order to completely eradicate the leukaemia the graft versus host activity should not only be severe but also of a protracted nature.

In the patient whom Mathé[258] treated with pooled marrow from 6 donors, a remission was obtained, which lasted for 20 months until the time of his death from another cause. The patient suffered from severe secondary disease, recovered and then remained a chimaera. This case is the only one in which a long-lasting suppression of leukaemia in a human chimaera has occurred. The same reservations presented earlier with respect to the possible occurrence of remissions due to radiation only must, however, be applied to this case.

In 2 other patients definite evidence of chimaerism was provided[263]. Both died following the irradiation after 29 and 31 days respectively, and the autopsy findings pointed to the presence of severe secondary disease. Histological studies failed to reveal any evidence of leukaemia. Because of the relatively short period of survival of these children and the fact that they were treated during a remission, it is not possible to decide whether the homologous transplant had any additional inhibitory effect on the leukaemic process.

As with mice, several complications other than secondary disease have been observed in human patients. Acute death occurred within 48 hours in 2 patients due to massive destruction of leukaemic cells following the irradiation.[8] The death of these patients was probably

caused by pulmonary thrombo-emboli of disintegrated cell nuclei and DNA. Such emboli have actually been observed in a patient with a large leukaemic spleen who received irradiation.[181]

Acute renal failure, due to deposition of urates in the kidney, may occur in the first week following the irradiation[171]. It is accompanied by a high level of uric acid in both serum and urine. Lethal renal complications have not been reported however.

A complication, not observed in mice, is brought about by the bone marrow transfusion. It appears to occur mainly when haemopoietic cells from multiple sources (more than 1 donor or bone marrow combined with foetal tissues) are given simultaneously or within an interval of a few days. Its clinical manifestations are respiratory distress and sometimes the development of acute cor pulmonale. In the pulmonary arterioles of such patients multiple emboli of fat, bone and bone marrow have been found (Plate VI: 3), accompanied by infarctions and oedema of the surrounding pulmonary tissue[263, 271, 404].

Finally, the frequent occurrences of necrotising mycelial infections of the oesophagus and gastro-intestinal tract have to be mentioned[263, 404]. It might be assumed that leukaemic patients are especially prone to develop such infections. On the other hand it has been found, that monkeys treated with homologous bone marrow display a similar tendency to develop more or less generalised mycelial infections. The same applies to the frequent occurrence of viral diseases in irradiated monkeys and patients treated with homologous bone marrow.

It must be concluded that total body irradiation followed by bone marrow transplantation is an extremely hazardous treatment with, until now, only a few apparent successes. It is in particular the group of Mathé which has made outstanding contributions in this field and which has distinguished itself by a persistent exploration of the possibilities of this method in the treatment of leukaemia. So far, it has not been shown conclusively that such favourable effects as have been reported could not have been obtained with the use of sublethal total body irradiation alone.

Treatment of other blood diseases with bone marrow

Many attempts to treat various kinds of defective haemopoiesis with bone marrow from either animal or human sources can be found throughout the medical literature of the past half century. In a review by Congdon et al.[98] the first report is cited as far back as 1891.

With a few exceptions the bone marrow or bone marrow preparations were always given orally until the 1940's when the intramedullary or intravenous administration of bone marrow became more popular. Few clinicians were thinking in terms of a true replacement of the diseased haemopoietic tissue by proliferating donor cells; the current idea was, in fact, that stimulating factors present in normal bone marrow might induce a recovery of the host's blood-forming system. We shall not discuss the conflicting reports that have appeared on the usefulness of bone marrow in the treatment of pancytopenia and related diseases, but shall limit ourselves to the studies of recent years, when information on the fate of the transplanted cells has been sought.

The only animal experiments in this field were performed by Russell and her collaborators at Bar Harbor, who have published an impressive *oeuvre* on the experimental treatment of congenitally anaemic mice by implantation of normal bone marrow.

They succeeded in curing not only the mild form but also the lethal form of this macrocytic anaemia by the transplantation of marrow from normal isologous donors. This was done initially after the irradiation of the anaemic hosts, but later it was found that irradiation is not required for a successful replacement of the abnormal cells with normal erythropoietic tissue[354]. It seems that a small implant of normal blood forming tissue can replace the host's erythropoiesis because of the much higher rate of proliferation of the former. This has recently been confirmed by McCulloch et al.[266] who have shown that marrow from anaemic mice is less capable than bone marrow from normal mice by a factor of 200 in its ability to proliferate and to form visible colonies in the spleen of irradiated animals. It is thus possible to produce (isologous) erythropoietic chimaeras without whole body irradiation but it is as yet unknown whether the chimaeric state extends to the other haemopoietic cell types. In particular, it would be interesting to know if the lymphatic tissue of these animals had been repopulated by donor-type cells as well.

In view of the unpredictability of spontaneous recovery and the fluctuations which occur in human bone marrow hypoplasias, the only reliable criterion for an effective take of the graft in clinical cases is the identification of specific characteristics of the donor cells after haemopoietic regeneration has occurred. As there is usually no reason to expect a critical depression of the immune function in these diseases, it cannot be expected that a graft of homologous bone marrow

will take. In recent years two series of patients with aplastic anaemia or other bone marrow hypoplasias were treated with bone marrow[171, 303], and in another series the patients were treated with foetal haemopoietic cells[69]. A few cases responded favourably to the therapy but *in no case* was convincing proof of even a temporary take obtained. Some patients received corticosteroids which were intended to inhibit rejection of the foreign cells and one patient was conditioned with whole body irradiation (200 rads).

A further negative result was reported by Domz[125] who attempted to restore a case of acquired hypogammaglobinaemia with bone marrow from the patient's husband. Since the homograft reaction in these patients is not always significantly decreased, a take of the foreign cells was not *a priori* to be expected.

Without even entering into all the details such as the number of cells, the source of cells and other crucial factors, it is clear that recent clinical experiences have confirmed previous observations both on human patients and on experimental animals that a take of foreign haemopoietic cells cannot be achieved without a drastic suppression of immune reactivity of the recipient. Even in the anaemic mice used in Russell's experiments, *homologous* bone marrow was not permanently established unless the host animals were pretreated by irradiation. Such pretreatment would hardly seem to be justified even in the most severe cases of bone marrow aplasia in view of the present possibilities of symptomatic treatment.

It should be kept in mind that pancytopaenia can be due to toxic factors or unfavourable conditions of internal *milieu* which are not necessarily removed by the introduction of normal bone marrow. This may have been the cause of failure in the case reported by Fernbach and Trentin[137], who transplanted (isologous) bone marrow from an identical twin partner into a pancytopenic child (Table VI: 6). The anaemia subsided only temporarily and no improvement of the other blood cell types occurred. Four other attempts to treat marrow failure of unknown or toxic origin with bone marrow from an identical twin donor have been reported, three of which were successful (Table VI: 6). The lack of success in the case described by Tocantins and McKenna[411] may have been due to the rather small number of cells administered. Although there could obviously be no proof of a take in the patients who responded well to the treatment with isologous bone marrow, their rapid clinical improvement was sound evidence for the proliferation of the injected cells.

TABLE VI: 6. Cases of marrow failure treated with bone marrow from identical twins

Authors	Age (years)	Cause of marrow failure	No. of cells administered ($\times 10^9$)	Recovery after treatment
Fernbach and Trentin (1963)[137]	3	Chloramphenicol and/or sulfonamides	3·6 (2 months later 2·8)	No
Tocantins and McKenna[411] (cited by[408])	15	Unknown	2·0	No
Robins and Noyes (1961)[348]	7	Anticonvulsant drugs	5·5	Yes
Mills et al. (1964)[282]	9	Unknown	7·4	Yes
Thomas et al. (1964)[408]	9	Unknown	6·1	Yes

In view of these favourable results it seems justifiable to re-commend a resumption of therapeutic trials with homologous marrow as soon as criteria become established for the selection of comparatively histocompatible (or at least incompatible) bone marrow donors. Cautious pretreatment of the recipient with immune suppressive drugs may then even be considered.

Production of chimaerism as a preparation for organ transplantation

The discovery that the successful transplantation of foreign bone marrow allows the permanent survival of other tissue grafts from the same donor—or in the case of inbred animals from the donor strain—has immediately given rise to the hope that this principle would be applicable in clinical work. However, the severity of the secondary syndrome which has so far always complicated the continued proliferation of homologous bone marrow in human patients seems to prohibit this approach at present, even if the technical difficulties involved in obtaining sufficient bone marrow as well as a kidney from the same donor could be overcome. In dogs, where the donor material provides no problems, this approach has been largely unsuccessful. Only a single dog has been described[248] in which the kidney homograft remained intact until the time of death, 49 days after the transplantation. This animal was subjected to 1300 r of whole-body irradiation and received a fresh homologous bone marrow graft 8 days later. On the 24th day after irradiation, transplantation of a kidney from the same donor was performed. At that time the peripheral blood counts were increasing and donor type (female) leucocytes were present. The animal died from pneumonia on the 73rd day after irradiation. A similar experiment performed by Calne[72] failed, probably because the bone marrow graft was rejected. Following whole-body irradiation with a dose of 900 r, the recipient's own marrow recovered and the kidney graft showed a typical homograft reaction when explored 10 days after the transplantation (37 days after irradiation).

Murray et al.[287] subjected a patient to similar treatments prior to the transplantation of a homologous kidney. Following whole-body irradiation with a dose of 600 r the patient received 107 × 10^9 pooled marrow cells from 17 donors among which were siblings, unrelated adults and the kidney donor (an unrelated infant). Three days later the kidney was grafted. Death from pulmonary infection and haemorrhage occurred on the 32nd day after irradiation; there was

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no evidence of rejection of the kidney graft nor of a take of the bone marrow.

Because of the extreme dangers involved in lethal whole-body irradiation and in view of the consistent failures to induce haemo-poietic chimaerism in man, this method of pre-treating kidney recipients has not been pursued. Sublethal irradiations in combina-tion with prolonged treatment with so-called immunosuppressive drugs have been found to be more successful in preventing the homograft reaction. As yet the stability of the "tolerance" induced in this way towards the kidney homograft, once the drugs have been withdrawn, is uncertain. Equally uncertain is the question of whether this form of treatment will preclude further attempts to facilitate organ transplantation in human patients by changing them into radiation chimaeras. Obviously, this will depend on how much pro-gress is made in overcoming the difficulties and hazards involved in the production and maintenance of haemopoietic chimaerism in man.

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SUBJECT INDEX

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