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.

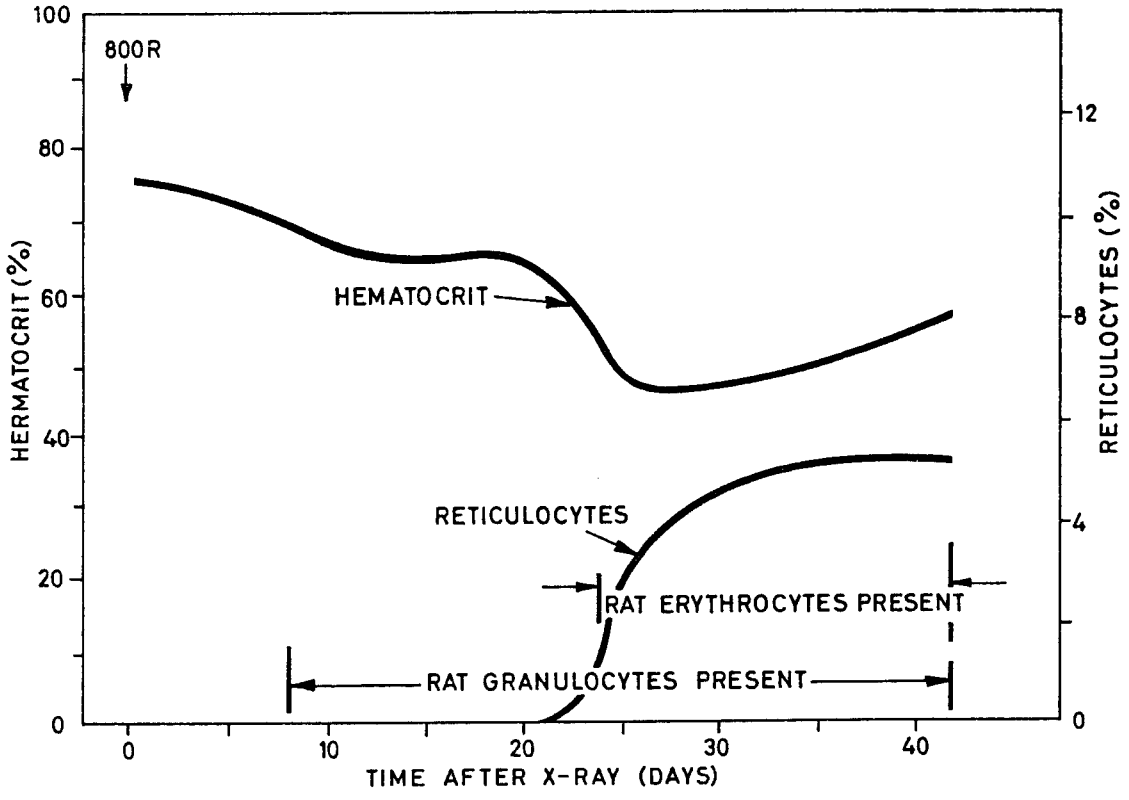


Figure II[1]. Response of a polycythemic X-irradiated mouse to rat bone marrow transplantation (Data from Jacobson *et al.* (1960)[188])

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]).

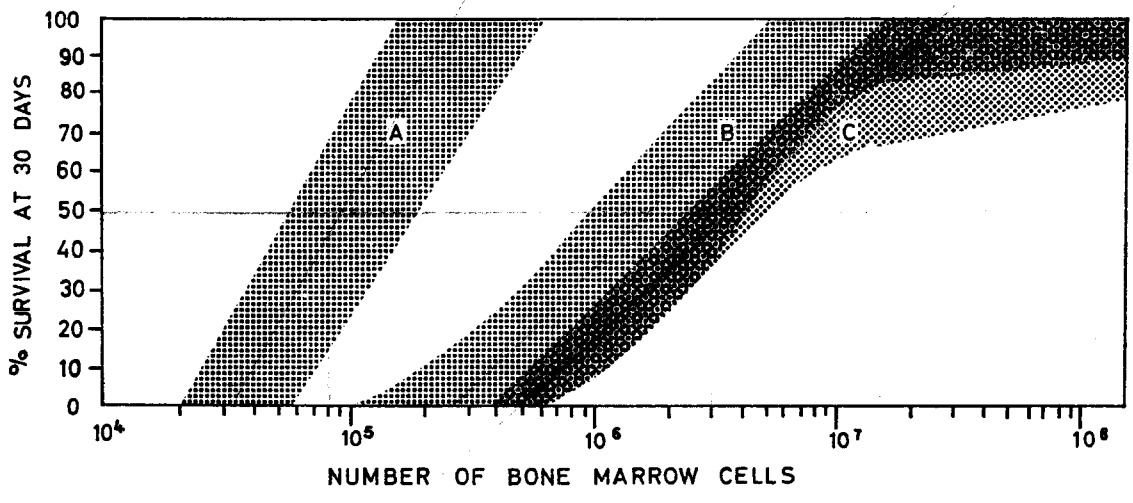


Figure II[2]. Number of bone marrow cells required in various host-donor combinations for restoration following lethal whole body irradiation

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]
Vos *et al* (1961)[437] } (isologous)

(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]
Mills *et al.* (1964)[282]
Thomas *et al.* (1964)[408] } (isologous bone marrow in case of drug induced or idiopatic bone 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]
Miller and Diamond (1961)[279] } (homologous)

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

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

* 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

* 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.

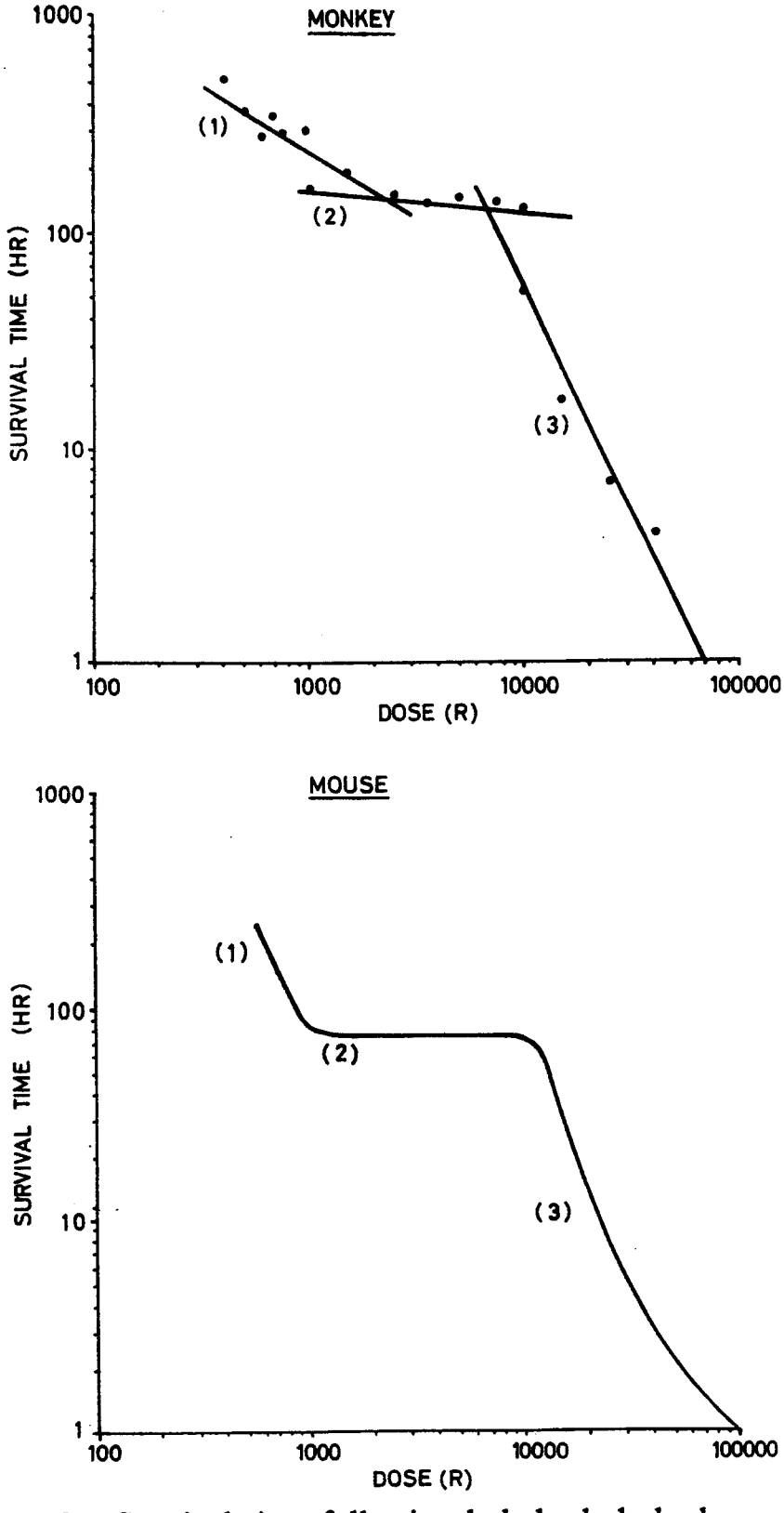


Figure II[3]. Survival time following lethal whole body exposure in monkeys and mice showing the three radiation syndromes. Graphs are based on data from Quastler *et al.* (1951)[341], Cronkite (1951)[105] and Rajewsky *et al.* (1953)[343] for the mouse and from Pickering *et al.* (1959)[312] for the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

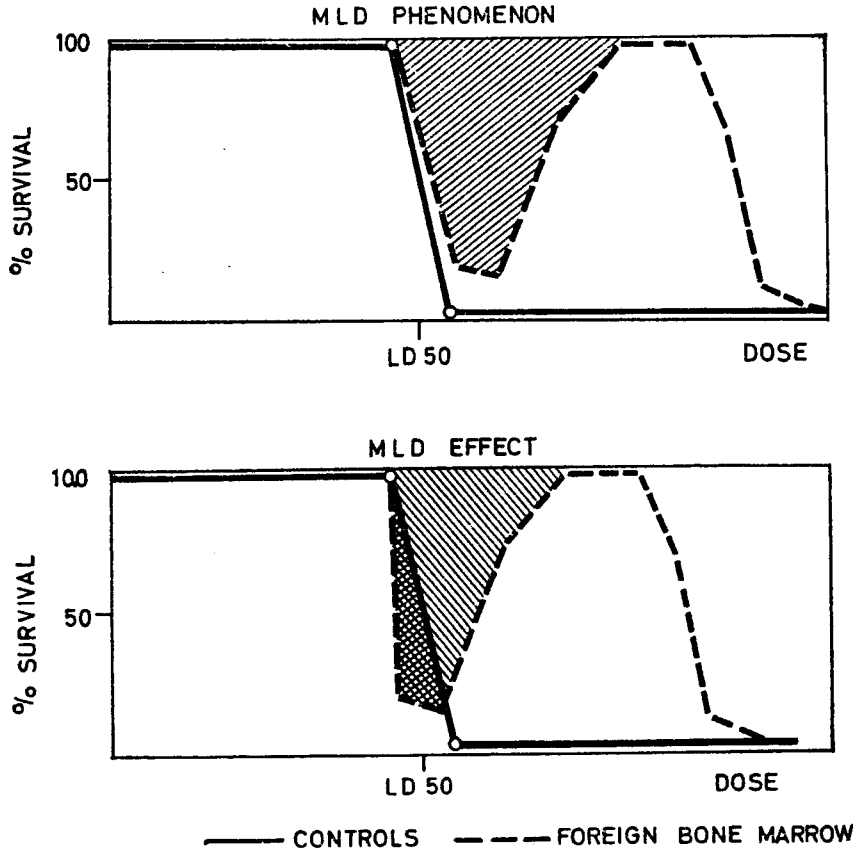


Figure II[4]. Theoretical survival curves for irradiated mice illustrating the concepts of the MLD phenomenon and the MLD effect. The crossed area in the lower graph represents the additional mortality caused by the administration of foreign bone marrow

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 immunogenetic 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 (C_3H × 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 $\times$ 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)

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

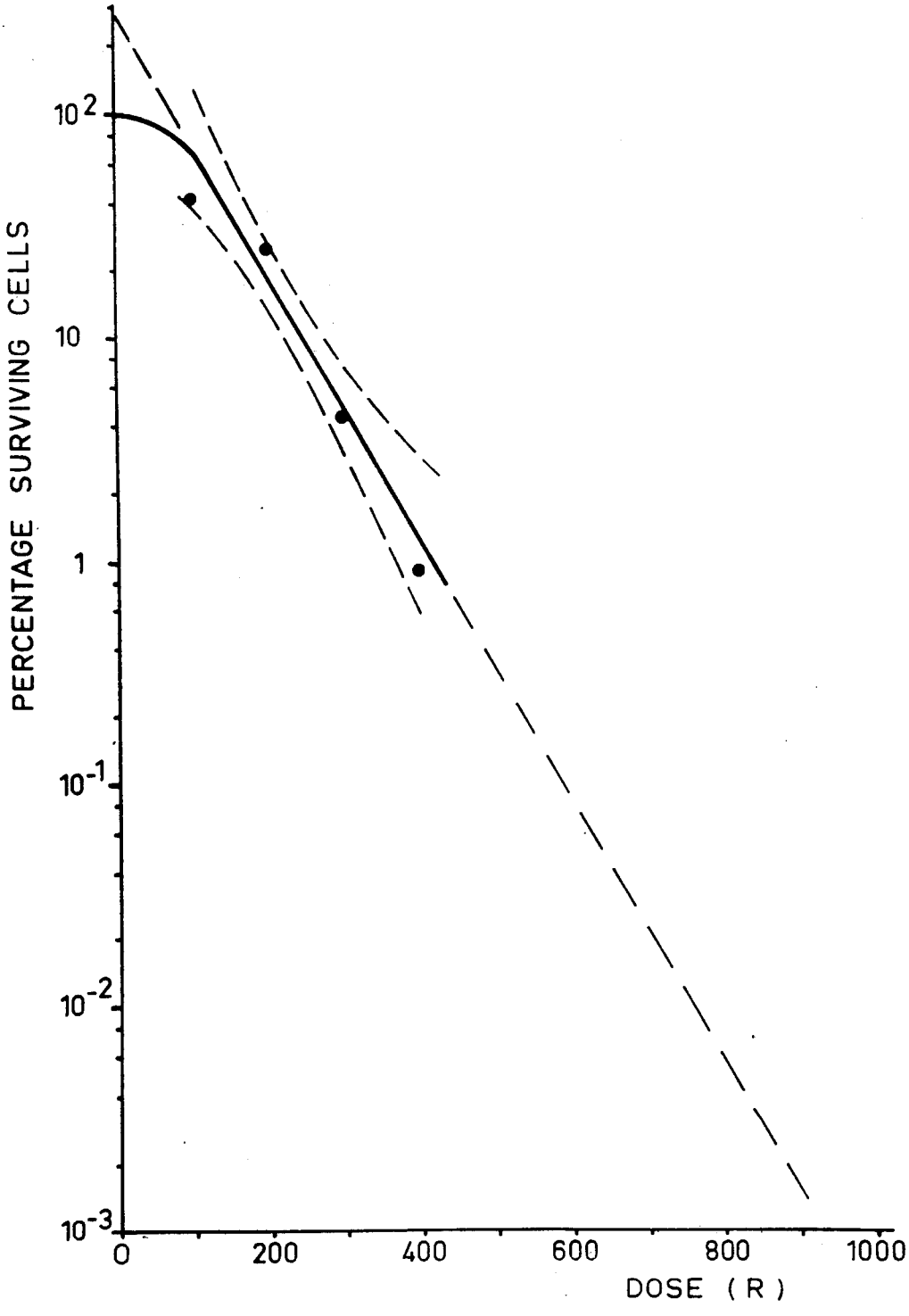


Figure II[5]. Survival curve of mouse lymph node cells following *in vitro* irradiation. The curve is extrapolated from the data published by Smith and Vos (1963)[381]. The extrapolated portion is represented by the broken part of the line. The extrapolation was done by simple extension of the line drawn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

* 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];

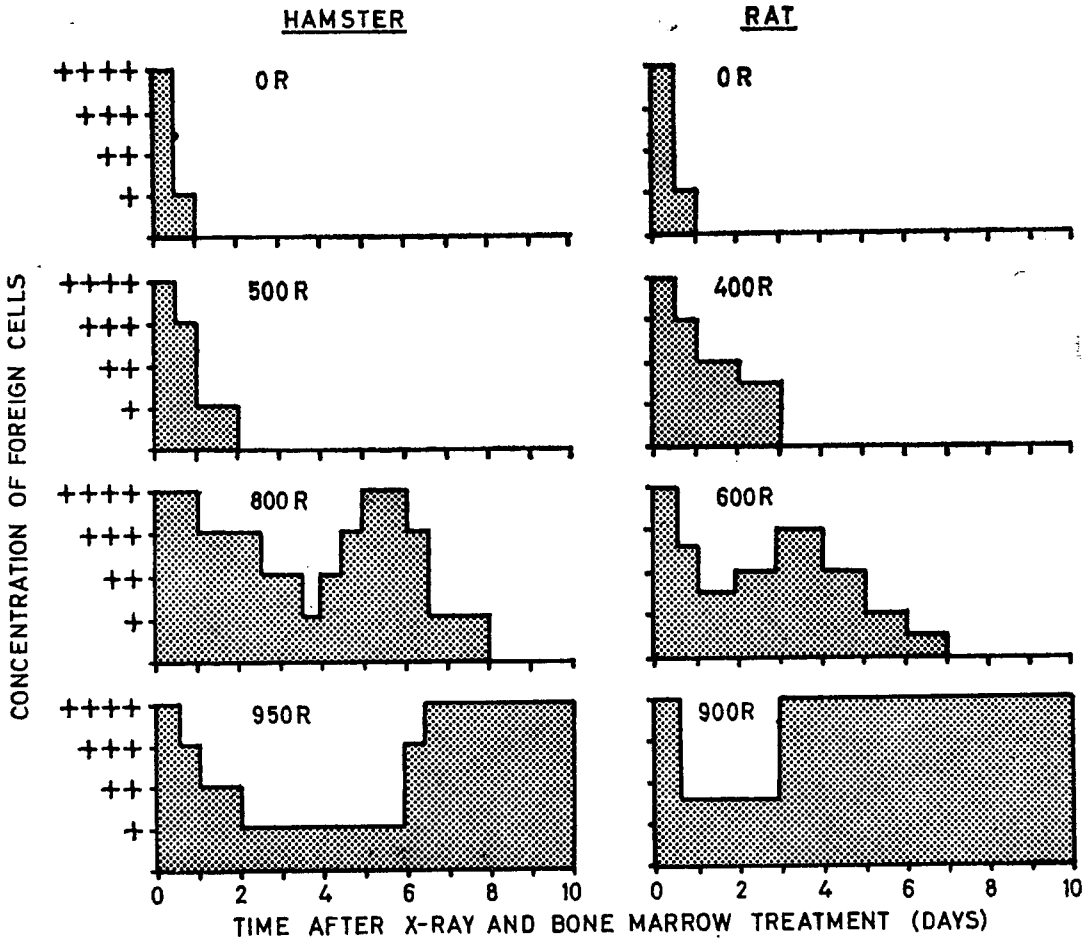


Figure II[6]. Donor type cells in spleen cell preparations (imprints) of irradiated mice following treatment with hamster and rat bone marrow. Figure from Shekarchi and Makinodan (1959)[368]. The mice received ~140 × 10^6 nucleated cells i.v. and the donor cells were identified by their positive alkaline 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,

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

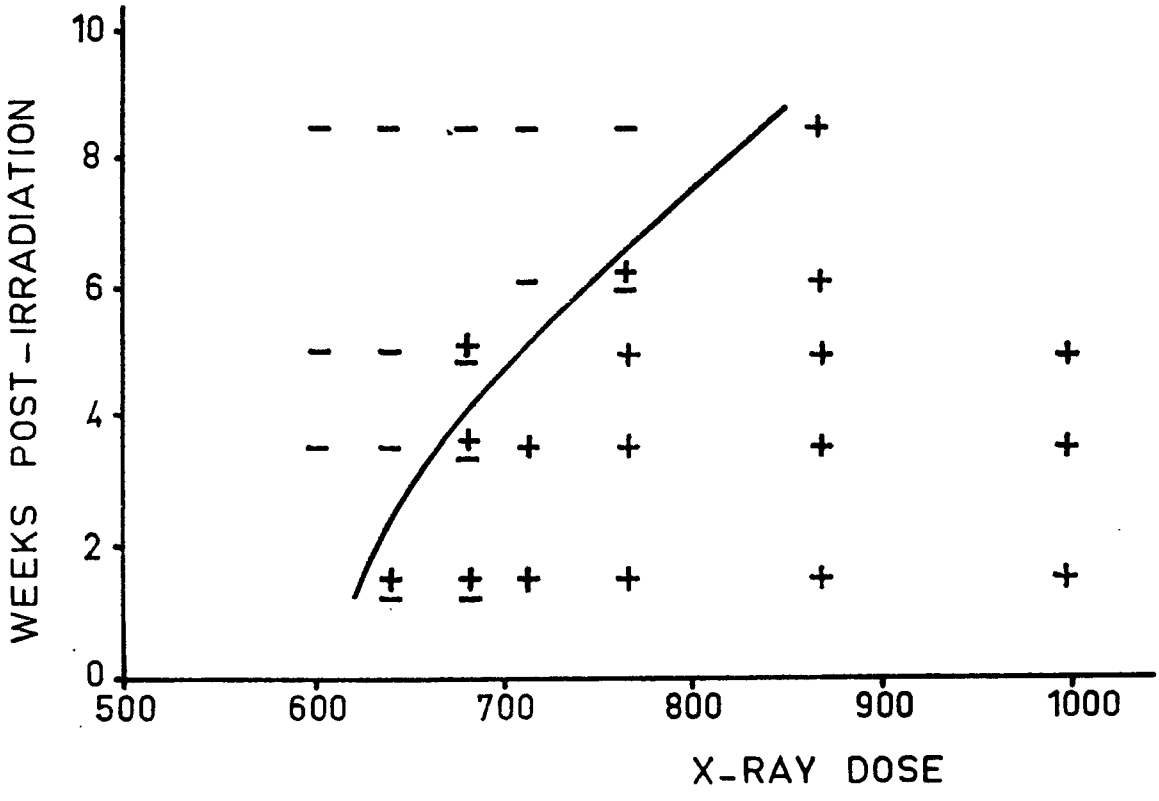


Figure II[7]. Incidence of chimaerism in relation to radiation dose in rat bone marrow treated mice. Data derived from Santos *et al.* (1958)[359], Table 2, who determined the incidence of alkaline phosphatase positive granulocytes in the peripheral blood at various times after transplantation of rat 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]).

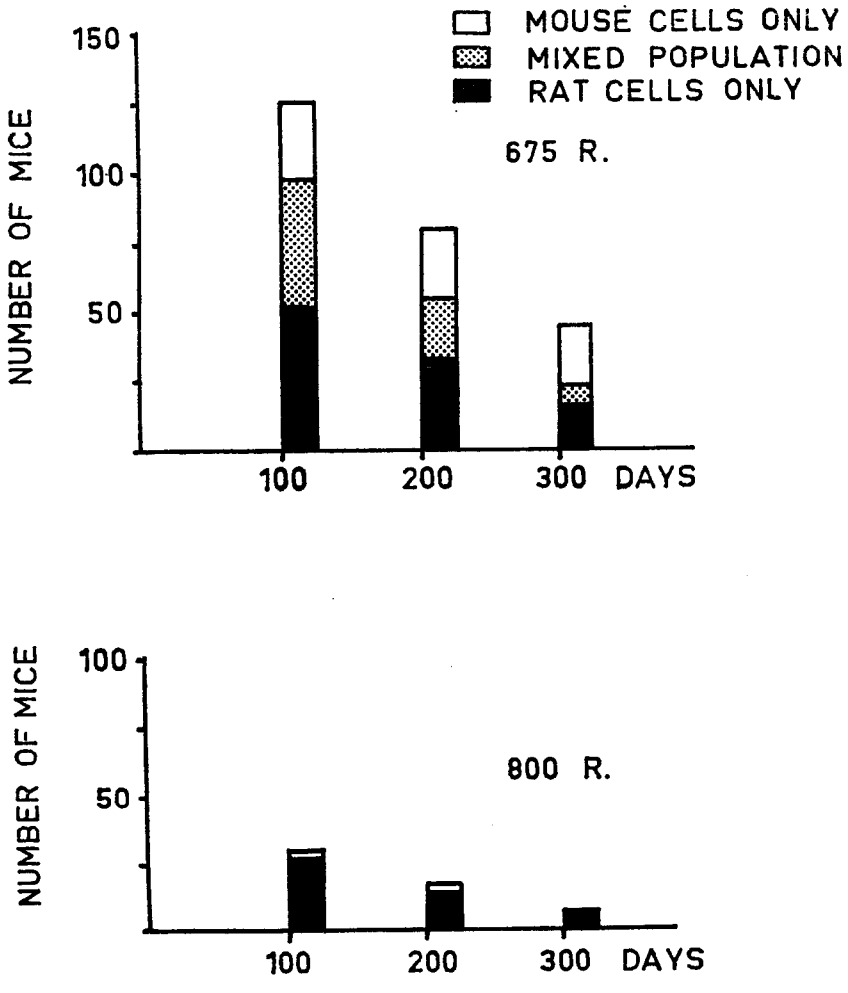


Figure II[8]. Incidence of reversals in mice surviving two dose levels of whole body irradiation followed by rat bone marrow transplantation. Data derived from Welling *et al.* (1959)[454]

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 Replinger *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.

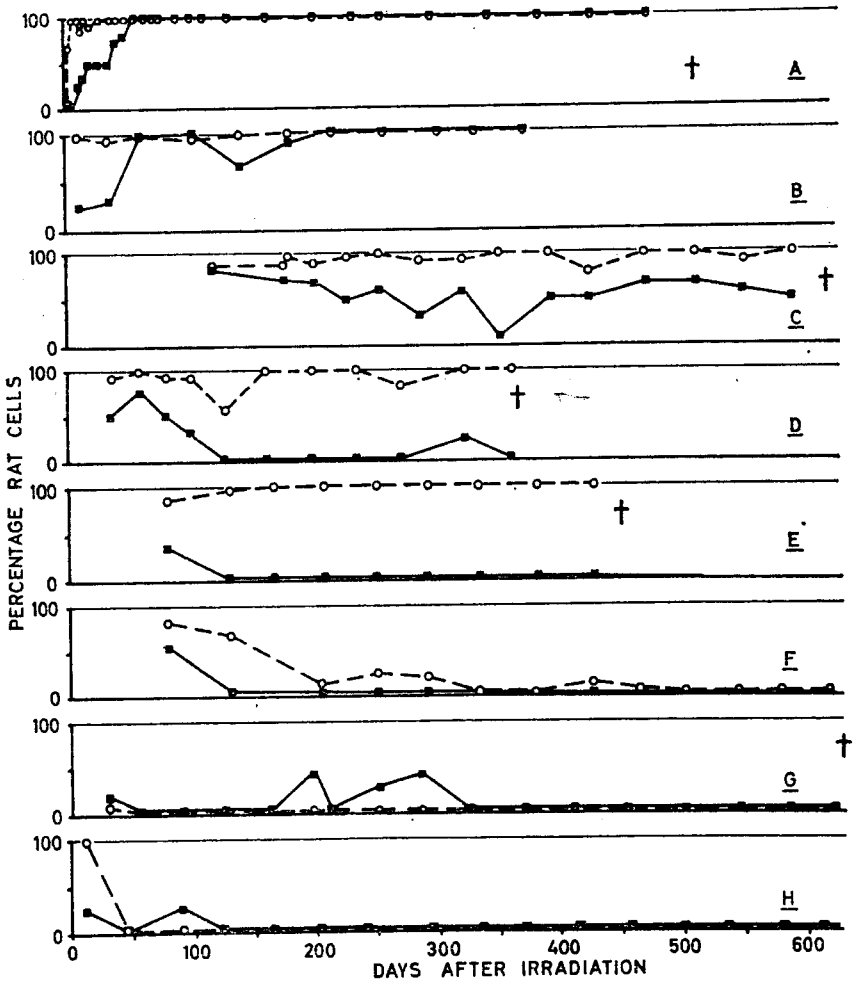


Figure II[9]. Various patterns of replacement of peripheral blood host cells by donor type cells as encountered in irradiated mice treated with rat bone marrow by Welling *et al.* (1959)[454]

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

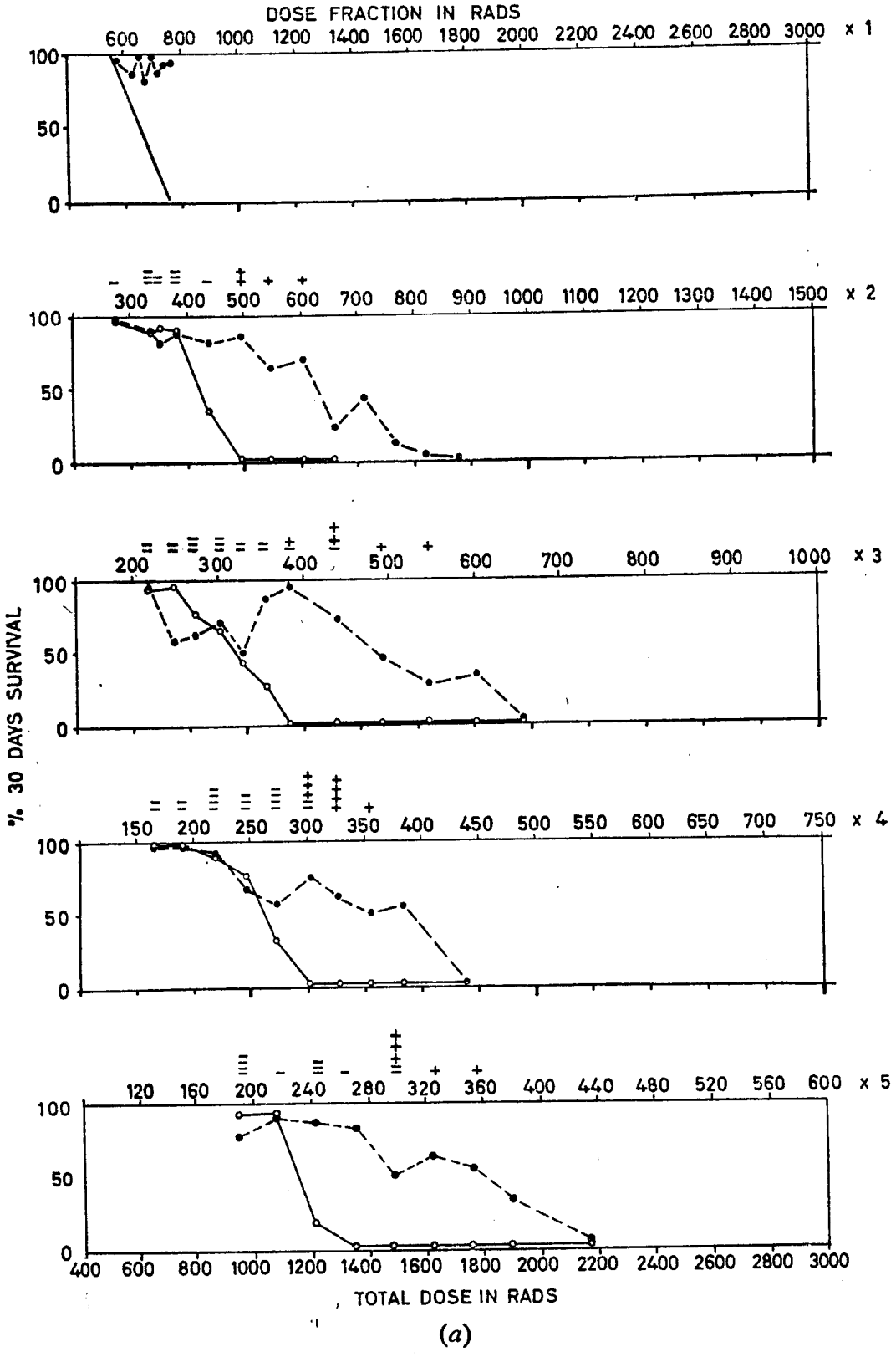
In the great majority of the experiments involving bone marrow transplantation the penetrating X- or γ-radiation has been administered to the recipient as a single dose delivered in a period of less than one hour. The influence of the dose of radiation has already been discussed and it has been concluded 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

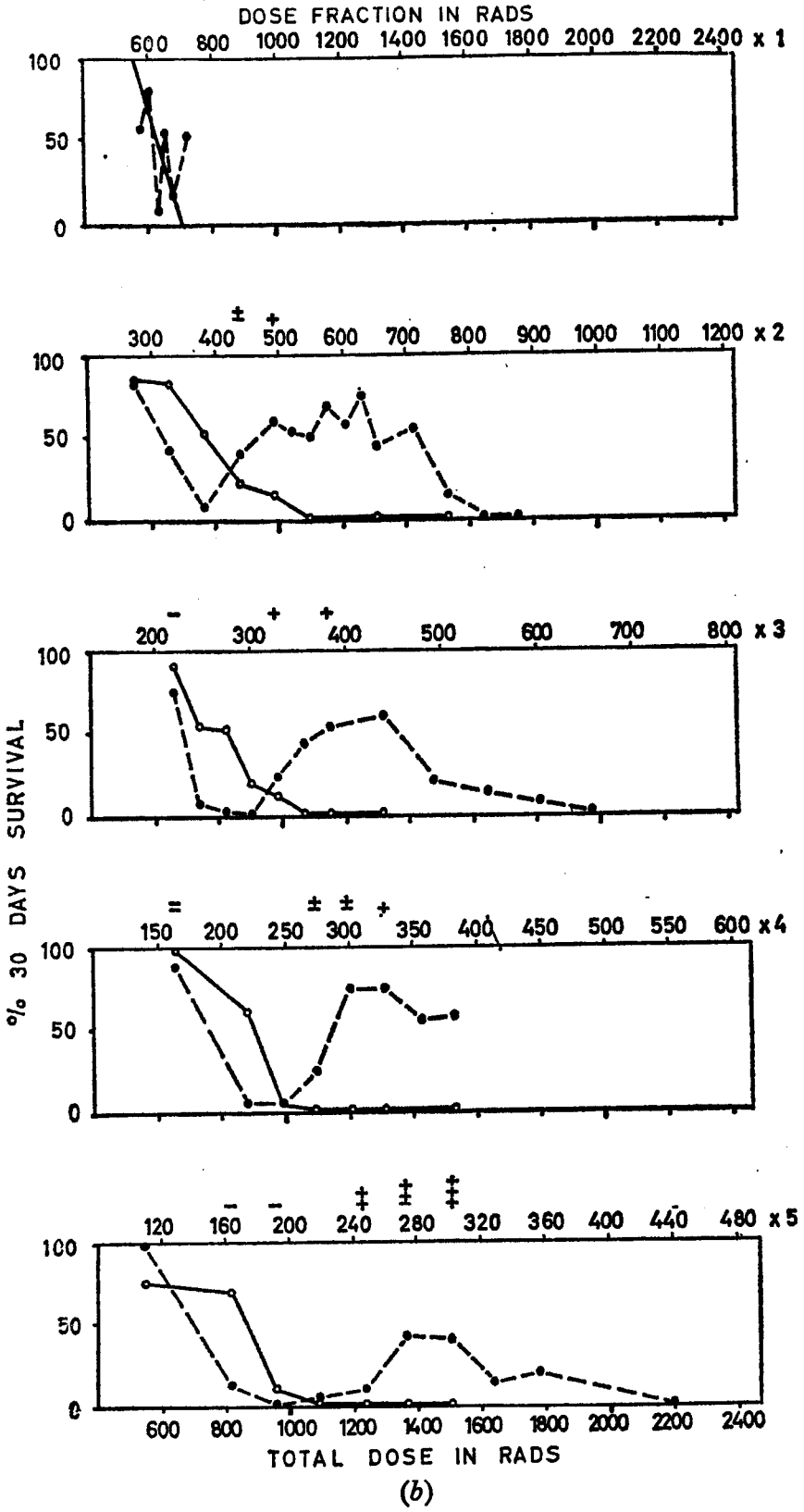
In rabbits it has been found advantageous to deliver the dose in two fractions divided by a 24 hour interval. This procedure was introduced because a relatively large proportion of rabbits develop irreversible shock following a single lethal dose of irradiation[333]. The effect of this fractionation 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 $\times$ 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



(*a*)

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



(*b*)

to the typing of erythrocytes and granulocytes, which was performed in groups of survivors 2–4 months after transplantation

(a) (CBA × C57BL)F_1 hybrid mice
(b) CBA mice
○———○ 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 0 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

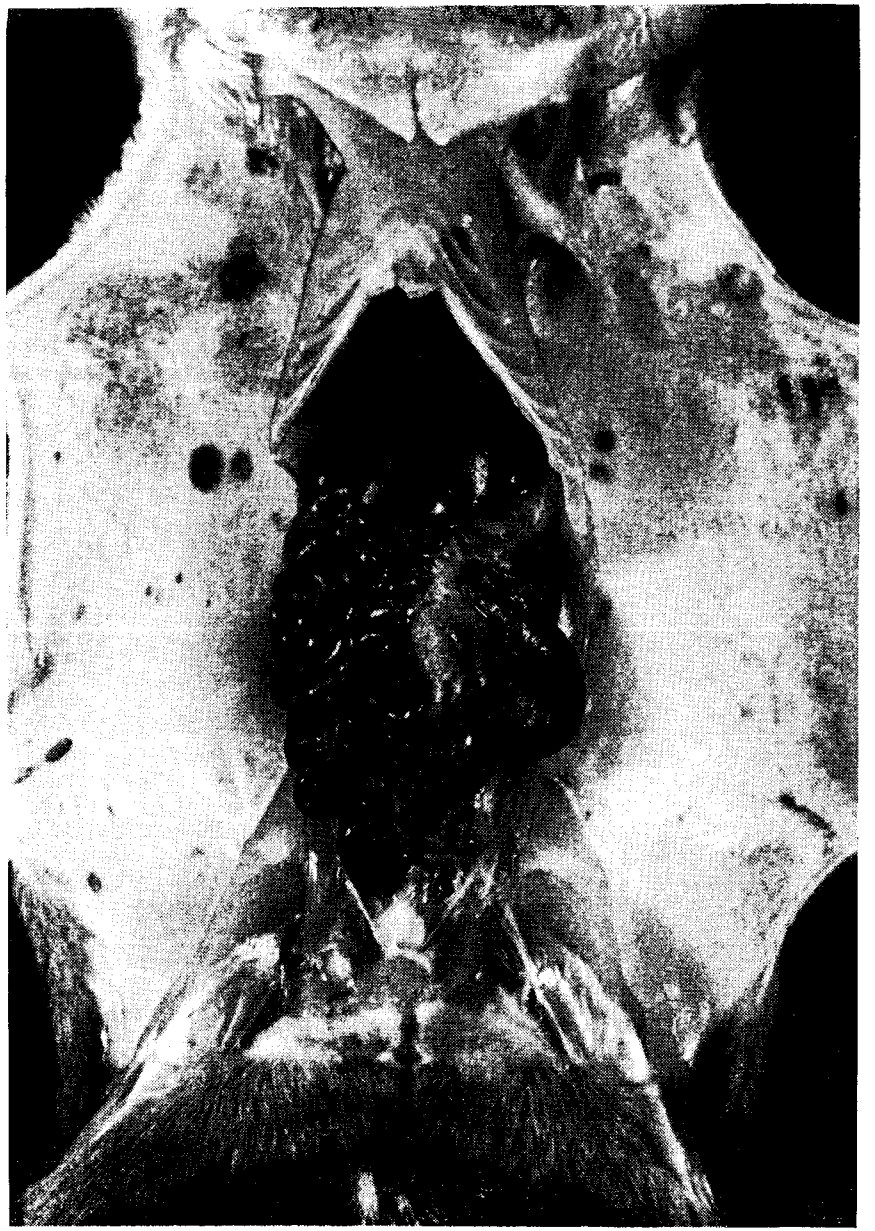


PLATE II: 1(b). Haemorrhages in the skin, the subcutaneous tissues and the wall of the intestines of a rat 12 days after whole body irradiation with a dose of 900 rads

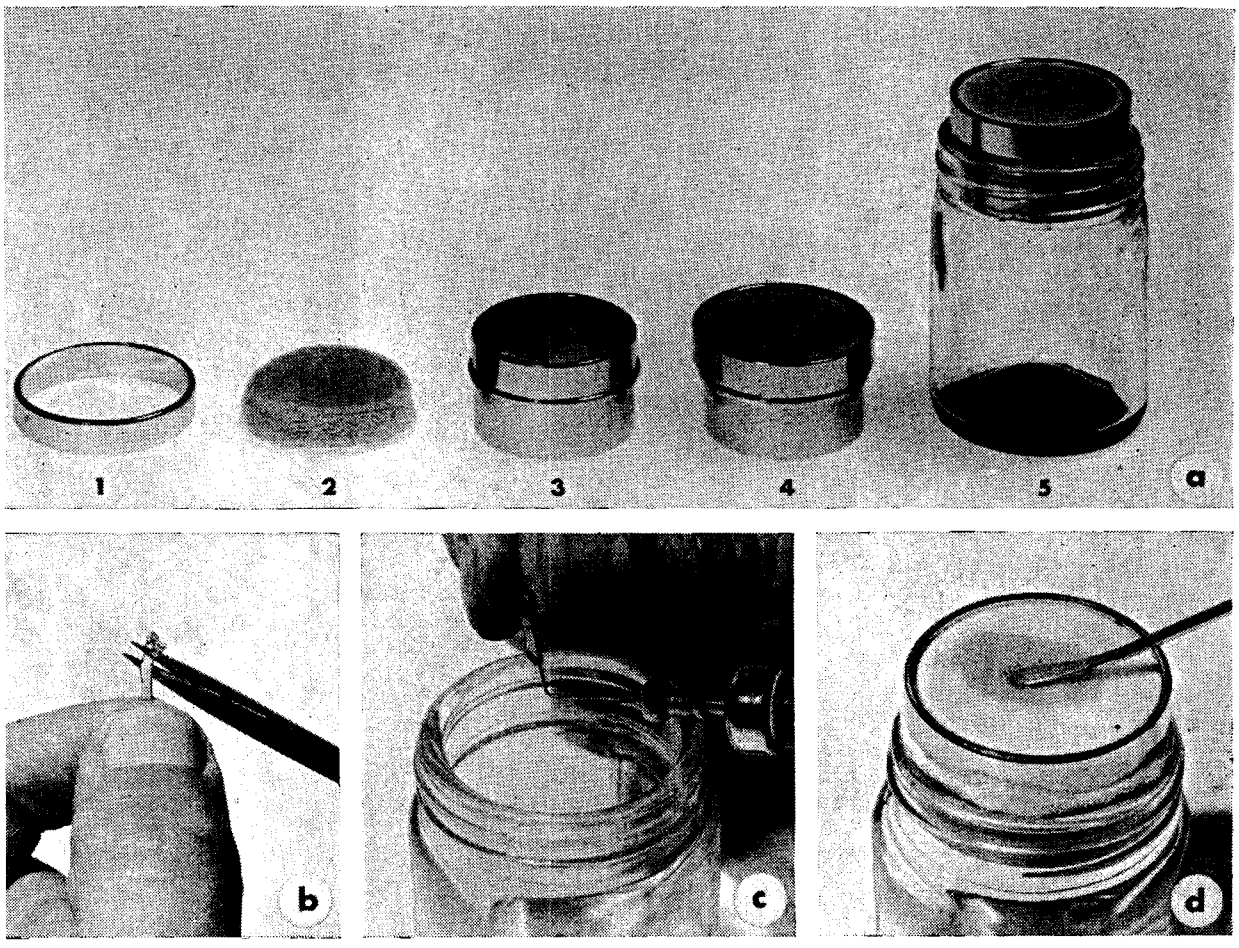


PLATE II: 2. The preparation of bone marrow suspensions

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 0·5 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 Turk's solution (total number). The number of eosin resistant cells is estimated in a solution of 2‰ 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, 0·5 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	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)		
Shaw and Vermund (1961)[367]	Homologous b.m. (pigeons)	24	47	(5–7 days)
	Heterologous b.m. (pigeons)	9	27	(5–7 days)

strain was not 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

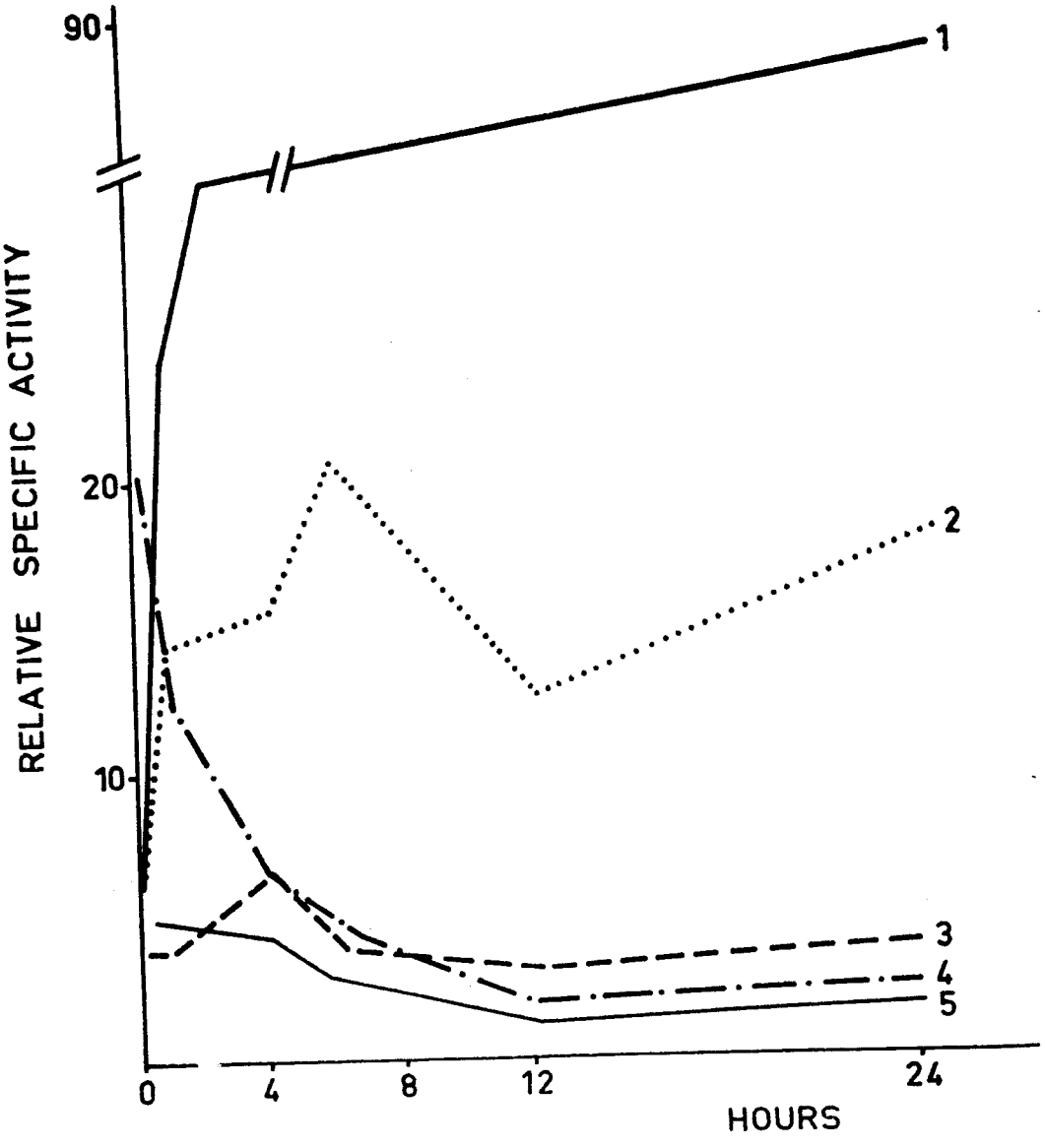


Figure II[11]. Concentration of ^{51}Cr label in various organs after intravenous administration of labelled marrow cells to lethally irradiated rats. Figure derived from Gregušová and Hupka (1961)[165]

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]).

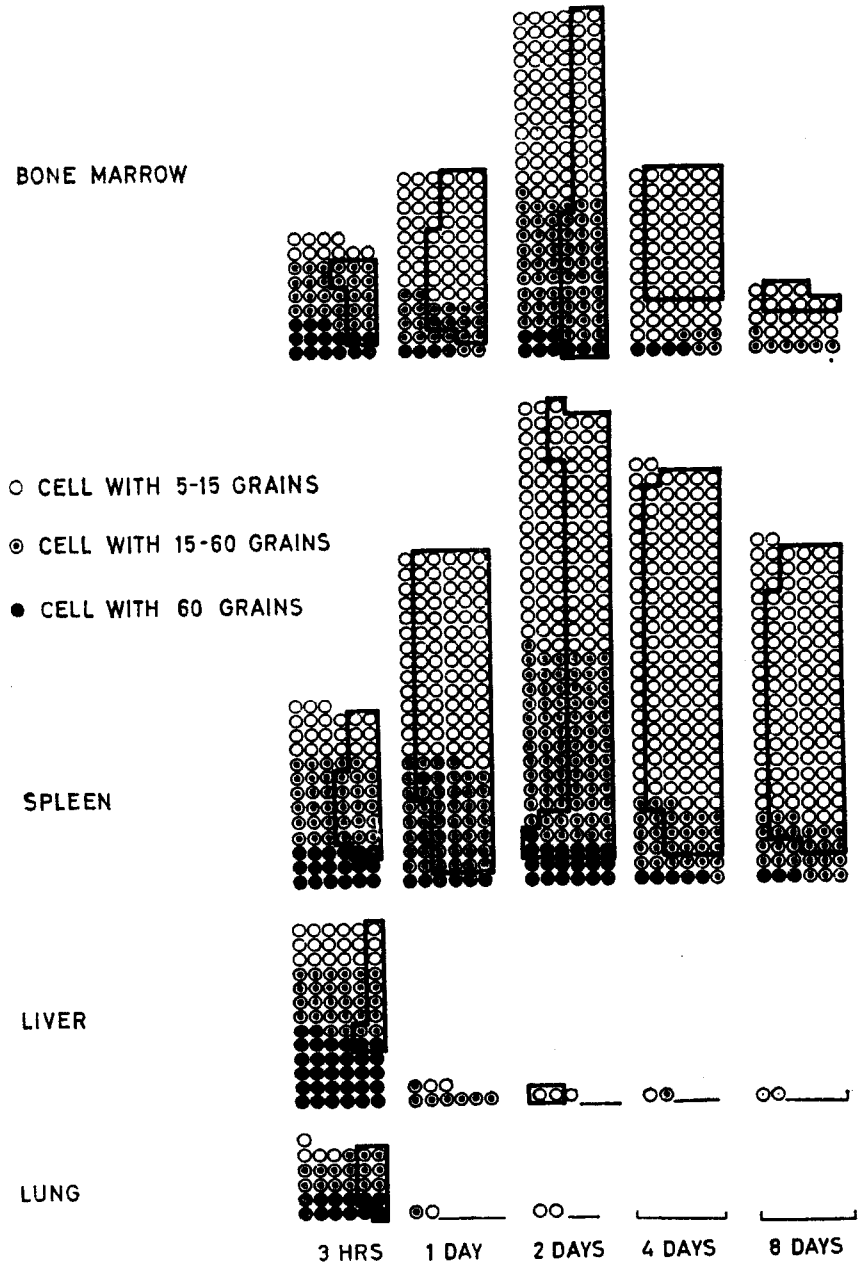


Figure II[12]. Relative numbers of labelled donor cells and intensity of label in organs of irradiated mice at various intervals following bone marrow transplantation. Data from Balner *et al.* (1962)[17]

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 0·1 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	
		$0{\cdot}1–1 \times 10^6$ cells	25	
		5×10^4 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) 5×10^6 cells	71	
		(18 days) 5×10^6 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*
		$3{\cdot}7–7{\cdot}2 \times 10^6$ cells	100	100
		(19–20 days)		
		16×10^6 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*
		2×10^7 cells	65	65
		Rat foetal liver (18–19 days) 2×10^7 cells	No 10 day survivors	
Porter (1959)[324]	Rabbits	20 days foetal liver	*Day 30*	*Day 90*
		350×10^6 cells	45	45
		670×10^6 cells	55	55
		27 days foetal liver	Not as effective	

		(12–19 days)		
		38–60 × 10^6 cells	95	90
	($C_{57}L$ × A)F_1 mice	(101 × C_3H)F_1 foetal liver		
		(12–19 days)		
		50–65 × 10^6 cells	94	70
	(101 × C_3H)F_1 mice	Rat foetal liver		
		(12–21 days)		
		80–180 × 10^6 cells	42	12
		Rat foetal spleen		
		(12–21 days)		
		8–50 × 10^6 cells	20	0
Crouch (1960)[107]	CBA and $C_{57}BL$ mice	Isologous foetal liver	*Day 30*	*Day 60*
		(14–16 days)		
		8–16 × 10^5 cells	95	—
	CBA mice	$C_{57}BL$ foetal liver		
		(14–16 days)		
		50–135 × 10^6 cells	80–100	20
	$C_{57}BL$ mice	CBA foetal liver		
		(14–16 days)		
		12–50 × 10^6 cells	60–100	50–100
	(CBA × $C_{57}BL$)F_1 mice	Rat foetal liver		
		(14–16 days)		
		50–100 × 10^6 cells	75–80	20–56
Tschetter *et al.* (1961)[420]	Webster strain mice (not inbred)	Homologous foetal liver	*Day 30*	*Day 90*
		20 × 10^6 cells		
		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 survival. 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 restoration 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 disease 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 immunologically 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 immunologically 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. Confirmation 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

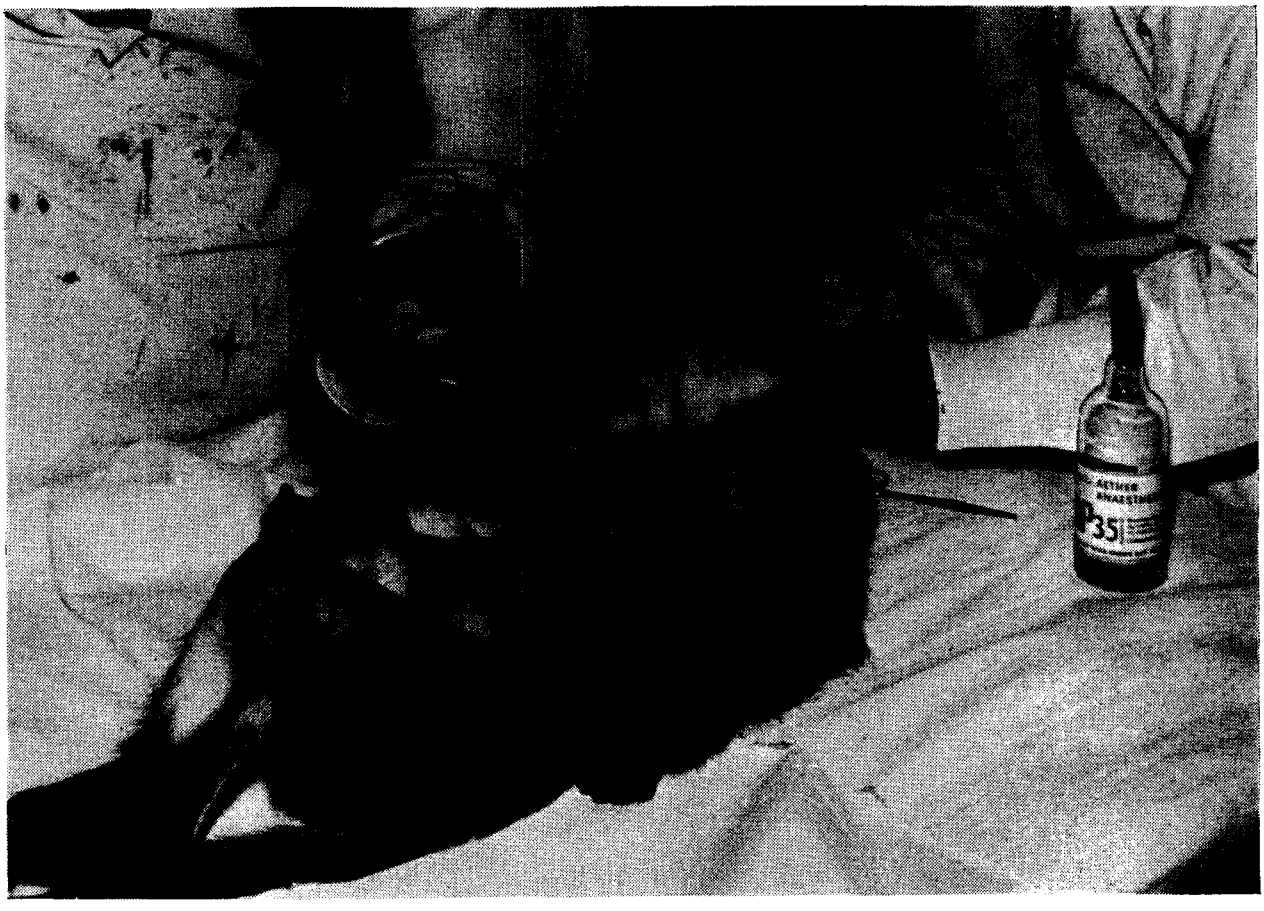


PLATE II: 3. Bone marrow puncture in an anaesthetised monkey. The needle is driven through the knee-joint and the distal end of the femur into the narrow cavity with a small hammer. As soon as the point enters the cavity the mandrin is removed and marrow is aspirated with a syringe. The needle is gradually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

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 × 10^6 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 polyalcohols 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 × 10^9 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)

Ashwood-Smith (1961)[9]	Marrow	Isologous mice	Dimethyl sulfoxide −79° C, 1 month	*In vivo* test: 5×10^6 cells (no cell dose titrations)
Thomas and Ferrebee (1962)[403]	Marrow	Autologous dogs	Glycerol −79° C, 14 months	Re-infusion (no cell dose titrations)
Persidsky and Richards (1964)[306]	Marrow	Isologous mice	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)	F_1 hybrid mice	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	Isologous mice	10% PVP and 10% PVP + 10% glycerol	90–100% preservation
	Foetal liver	Isologous mice	10% PVP + 10% glycerol	40% preservation
Lewis and Trobaugh (1964)[221]	Marrow	Isologous mice	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 autologous 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]).

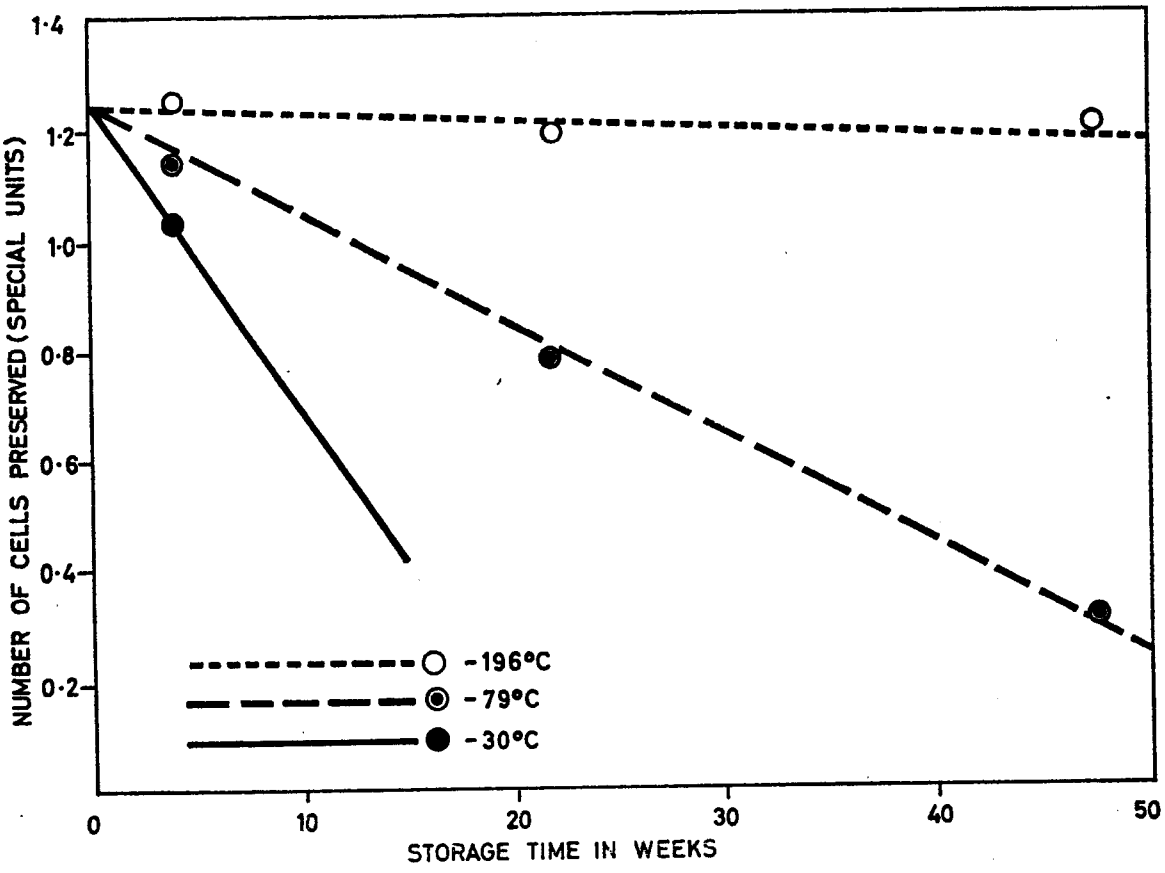


Figure II[13]. Degree of preservation of mouse bone marrow cells upon storage at three different temperatures. Figure from Phan *et al.* (1963)[311]. Even after a period of nearly 5 years, the cells stored at −196° C did not show a significant loss of protective capacity (L. Smith, personal communication, 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.

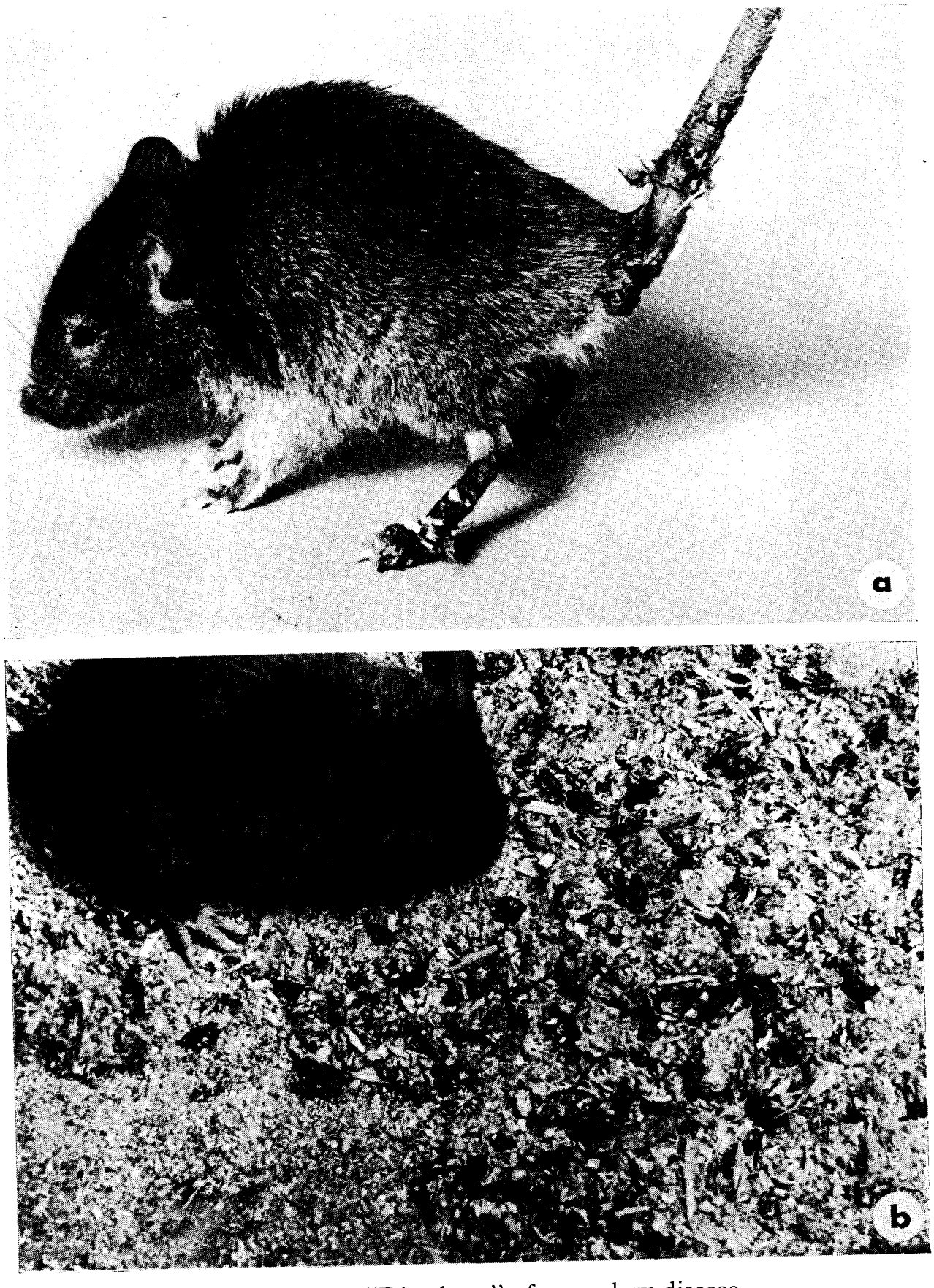


PLATE III: 1. "Diarrhoea" of secondary disease

(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

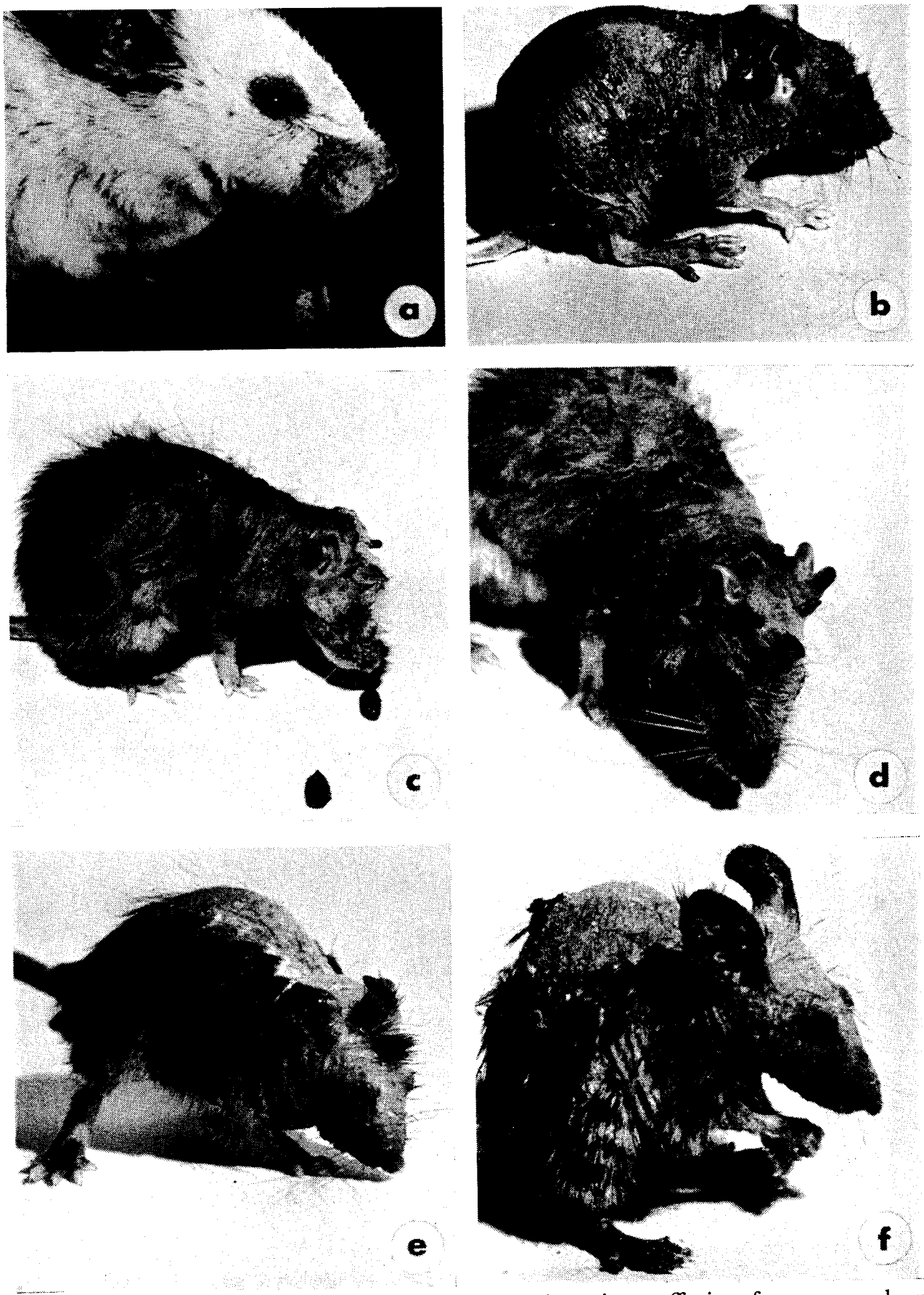


PLATE III: 2. Various forms of skin lesions in mice suffering from secondary disease following foreign bone marrow transplantation

(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.

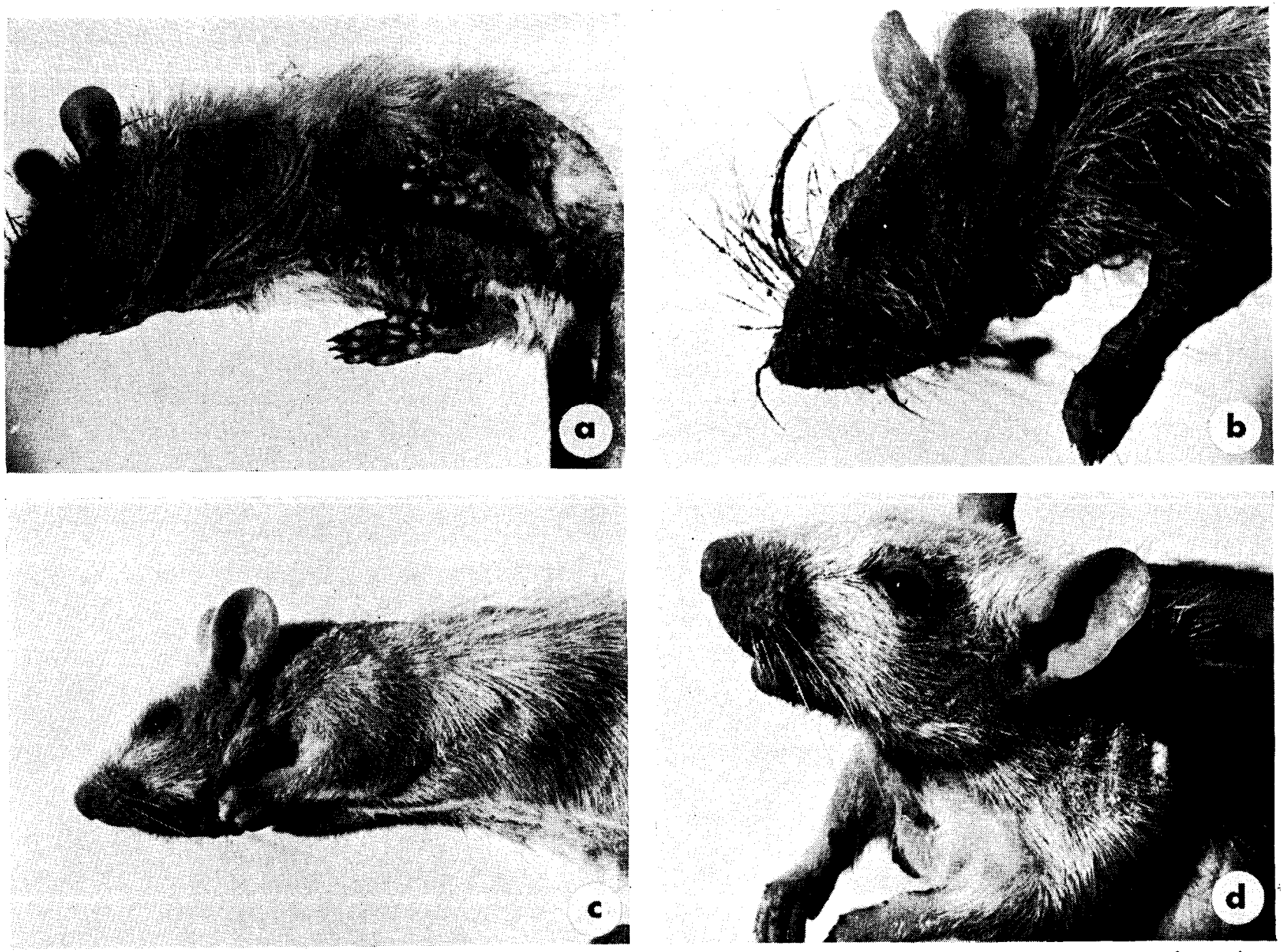


PLATE III: 3. Skin lesions of rats following irradiation and homologous bone marrow transplantation. Data from Balner *et al.* (1964)[18]

(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

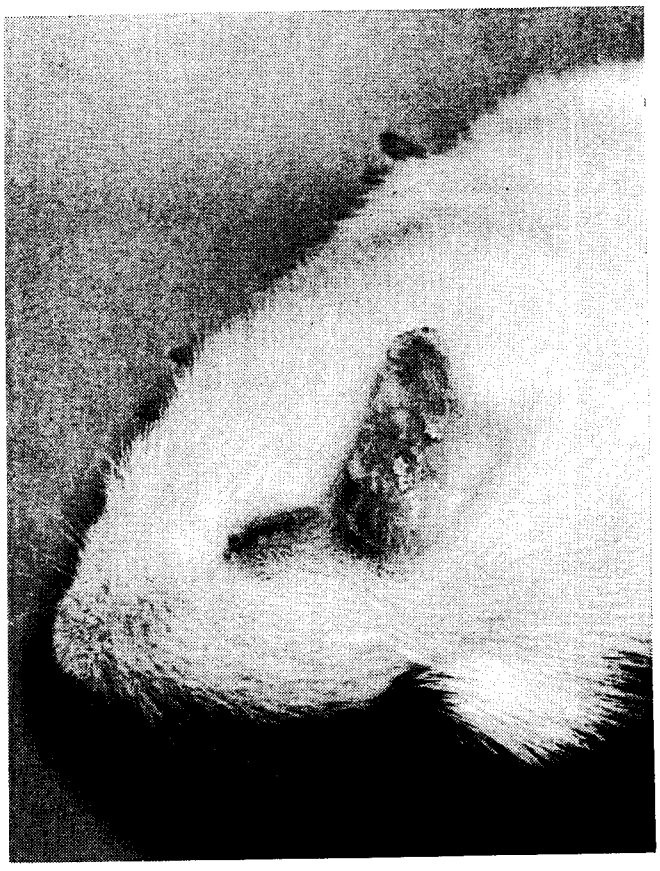


PLATE III: 4. Guinea-pig showing characteristic skin lesions 20 days after irradiation and homologous bone marrow transplantation. Lesions in guinea-pigs are most pronounced on the ears, around the eyes and in the skin of the feet

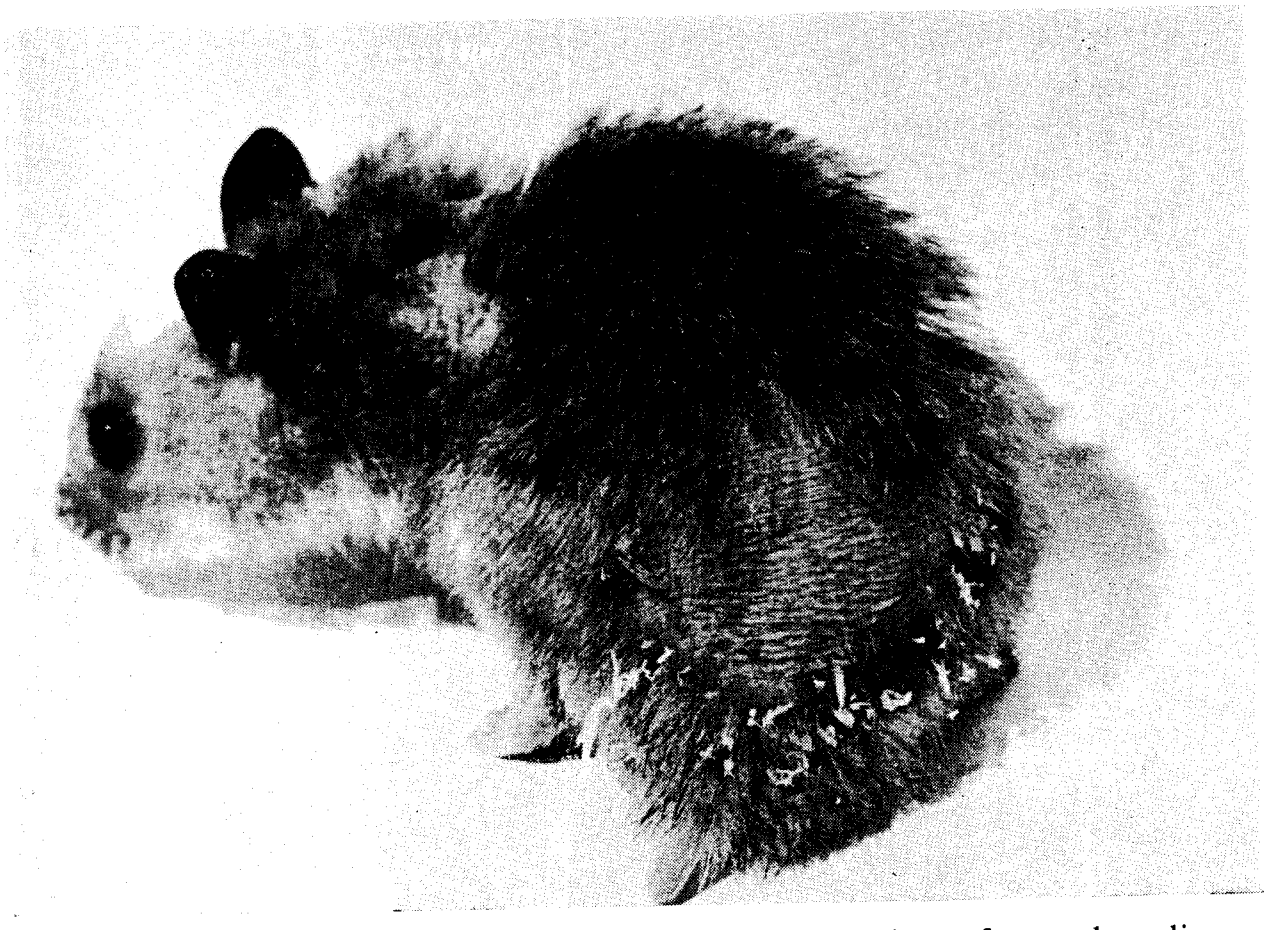


PLATE III: 5. Syrian hamster with cutaneous manifestations of secondary disease following irradiation and homologous bone marrow transplantation. Characteristic patchy alopecia and scaling