CHAPTER V

Immunological Studies with Radiation Chimaeras

Introduction

In stable radiation chimaeras the cells which are responsible for the immunological reactivity have been replaced by derivatives of the graft, and the immunological reactivity of chimaeras is therefore at least qualitatively similar to that of the donor animals.

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

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

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

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

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

Reactivity of radiation chimaeras

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

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

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

HOMOGRAFT REACTIVITY

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

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

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

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

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

They found that DBA/2 mice which had received BALB/c marrow, while accepting BALB/c skin, rejected C57BL skin grafts, thus demonstrating the presence of a competent anti-third party reactivity in their radiation chimaeras. Interestingly, it was found that (C57BL/HeN × A/HeNF)F_1 hybrid mice which had been restored with BALB/c marrow following irradiation accepted DBA/2 skin in 9 out of 29 cases. When restoration was accomplished with DBA/2 marrow, 12 out of 16 animals were found to accept BALB/c skin grafts. The BALB/c and DBA/2 strains share the most important histocompatibility antigens (at the so-called H-2 locus). The acceptance of skin grafts from an inbred strain antigenically slightly different from the bone marrow donor was tentatively ascribed to residual radiation effects.

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

In isologous chimaeras the ability to reject foreign skin grafts is

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

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

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

GRAFT VERSUS HOST REACTIVITY

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

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

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

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

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

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

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

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

More direct evidence of graft versus host immunological activity in radiation chimaeras was provided by Koller and Doak[203] who observed

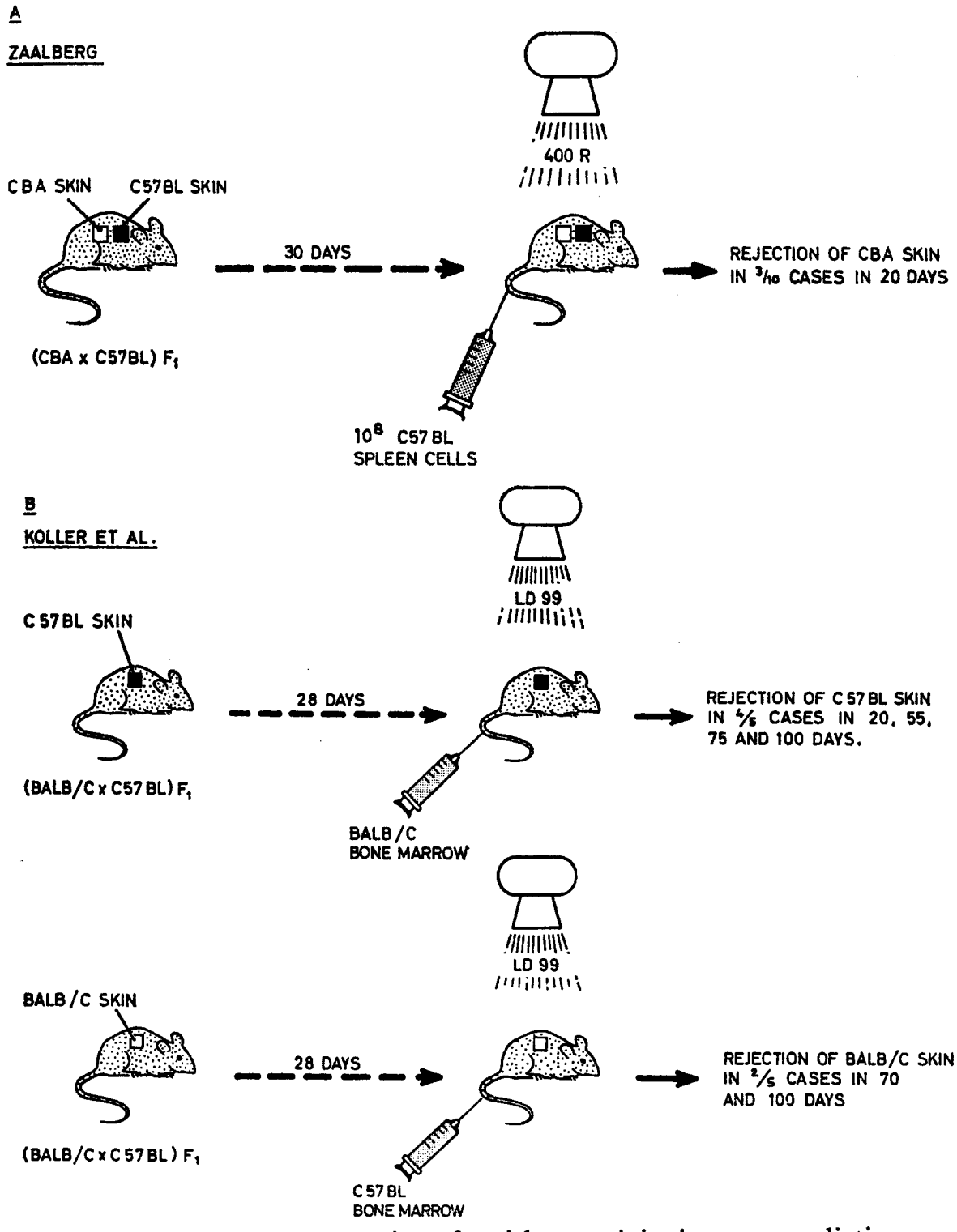

Figure V[1]. Demonstration of anti-host activity in mouse radiation chimaeras with skin grafting. Schematic representation of experiments by

(A) Zaalberg (1959)[467]
(B) Koller *et al.* (1961)[205]

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

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

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

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

REACTIVITY AGAINST OTHER ANTIGENS

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

case in isologous chimaeras, but the immunological recovery is severely impaired when graft versus host disease develops as is the case in incompatible host–donor combinations. As would be expected, an early recovery can be promoted in compatible combinations by the administration of large numbers of lymphatic cells.

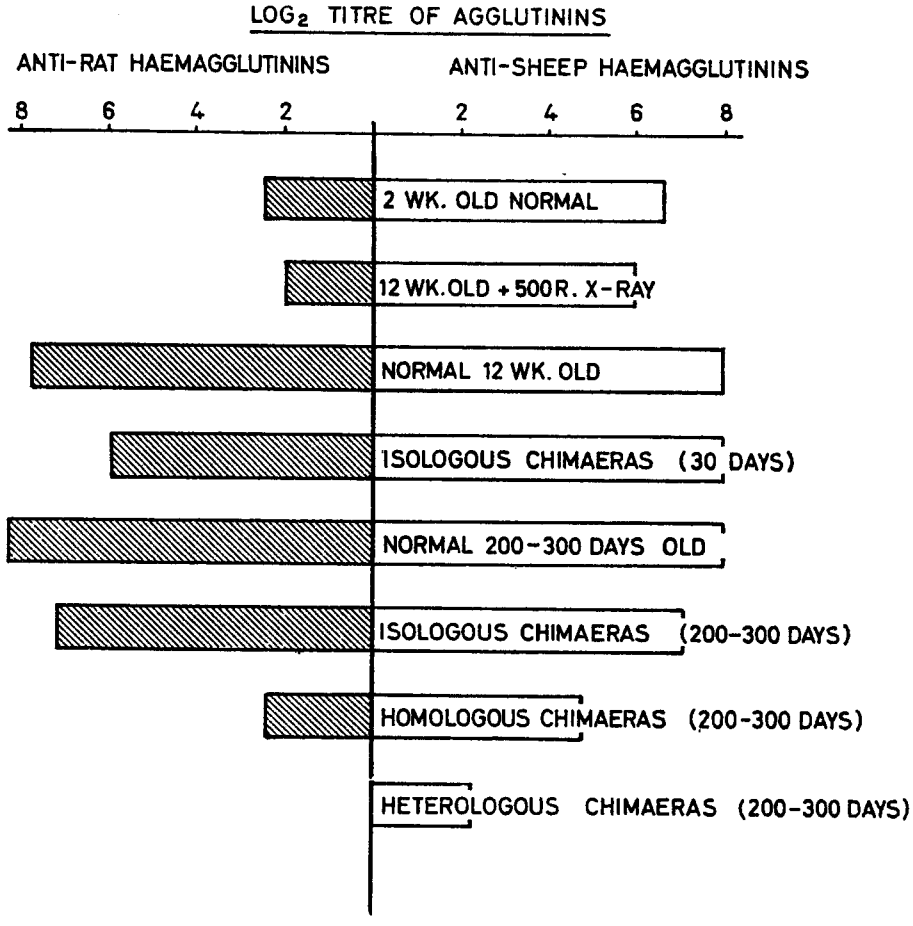

Figure V². Antibody formation in various mouse radiation chimaeras and their controls. Data from Gengozian *et al.* (1958)[152] and Makinodan *et al.* (1956)[245]

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

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

Many of their results were reviewed by Makinodan and Gengozian in 1960[244] and their main findings with chimaeras are summarised in Fig. V².

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

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

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

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

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

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

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

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

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

conditions of antigenic stimulation used by these investigators, rabbits will respond very actively to such antigens. Both species respond to STV. Surprisingly, rats treated with rabbit cells produced significant anti-BSA titres, while controls treated with rat embryo liver gave no response at all to BSA. Both types of "chimaeras" responded equally well to the STV vaccine. It has never been established whether the surviving rats in the study were indeed chimaeras. The authors state in their paper that "rabbit cells have not been demonstrated in the rats tissues in our experiments", and since rabbit eosinophylic granulocytes can readily be distinguished from similar rat cells, it must be assumed that chimaerism was not involved. Although these results still remain unexplained it may be that a temporary proliferation of rabbit cells had caused the production of a limited amount of anti-BSA antibody.

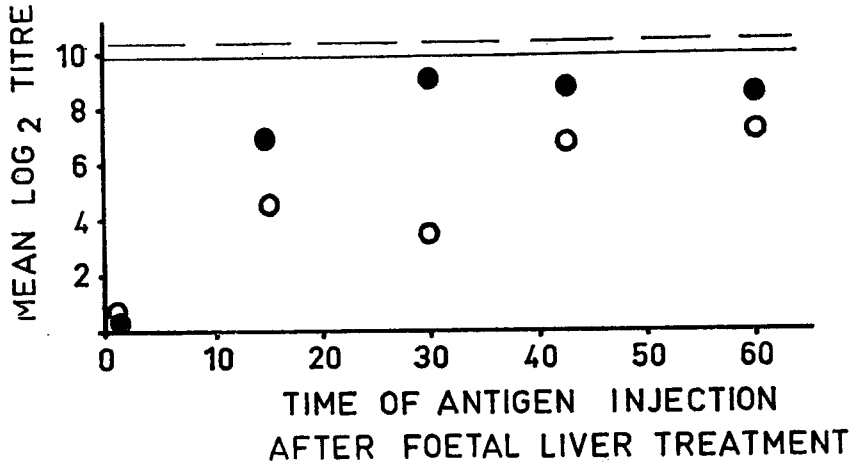

Figure V[3]. Agglutinin response 14 days after injection of sheep red blood cells (RBC) (●) and rat red blood cells (RBC) (o) in isologous chimaeras produced by injection of 40 × 10^6 nucleated foetal liver cells following whole body irradiation with a dose of 950 r. Data from Doria and Congdon (1962)[128]

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

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

Transfer experiments involving radiation chimaeras

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

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

TRANSFER OF IMMUNITY

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

TRANSFER OF IMMUNOLOGICAL TOLERANCE

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

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

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

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

In this notation the antigens with which the C57BL donor cells were confronted have been underlined.

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

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

* A close correlation had been found previously between the genotype of the erythropoietic cells and that of the lymphatic cells in radiation chimaeras.

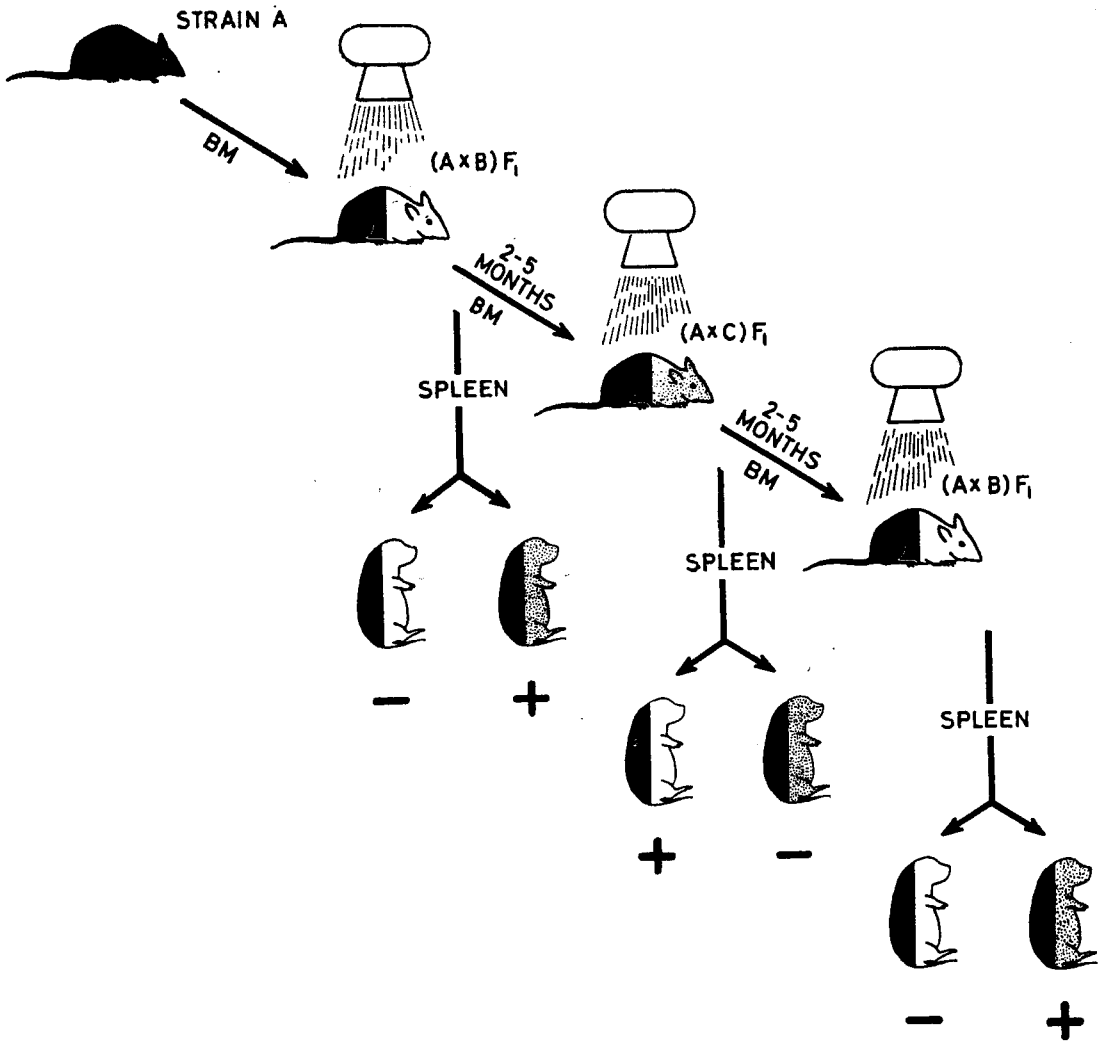

Figure V[4]. "Change of host" by transfer of bone marrow. The reactivity of the donor (C57BL) spleen cells is shown schematically by + and − signs beneath the symbols for the newborns. These experiments show the loss of specific tolerance of the donor type cells (A) towards the host (AxB)F_1, following transfer to a second host which lacks the antigens (B) which elicited the tolerance in the first host. After some time tolerance towards the new host (AxC)F_1 has developed. Following transfer to a third host (AxB)F_1 the situation is again reversed: loss of tolerance towards C type antigens and development of tolerance towards B type antigens

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

CBA → (CBA × *C57BL*)F_1 → (*RF* × CBA)F_1 → (CBA × *C57BL*)F_1

In order to test the possibility that the loss of specific tolerance

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

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

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

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

In contrast, Zaalberg *et al.*[471] using the CBA → (CBA × *C57BL*)F_1 → CBA transfer combination and employing skin grafting to test for tolerance, found a more prolonged persistence of the tolerant state when 12×10^6 lymph node cells were transferred in addition to the standard number of bone marrow cells (3×10^6). One of the present authors (D. W. van Bekkum, L. E. J. Holt-Mour, and H. Balner, *Transplantation*, *3*, 340–351, 1965) performed Simonsen assays of a similar transfer involving lymph node cells using the spleen cells of the second host. A return of weak but significant anti-C57BL reactivity was eventually observed, even in animals with intact C57BL skin grafts. The latter, however, showed evidence of a slight homograft reaction upon microscopic examination. These results indicate that the sensitivity of the Simonsen assay is equal to the most careful histological examination of skin grafts and that the macroscopic evaluation of skin grafts provides much less dependable information on the state of immunological reactivity of the host than the two former methods. It can be concluded that in the absence of the specific antigen, tolerance is maintained much longer when tolerant lymphoid cells are transferred instead of bone marrow cells only, at least under the conditions of this particular transfer experiment.

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

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

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

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

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

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

OTHER DATA FROM TRANSFER STUDIES

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

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

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

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

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

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

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

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

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

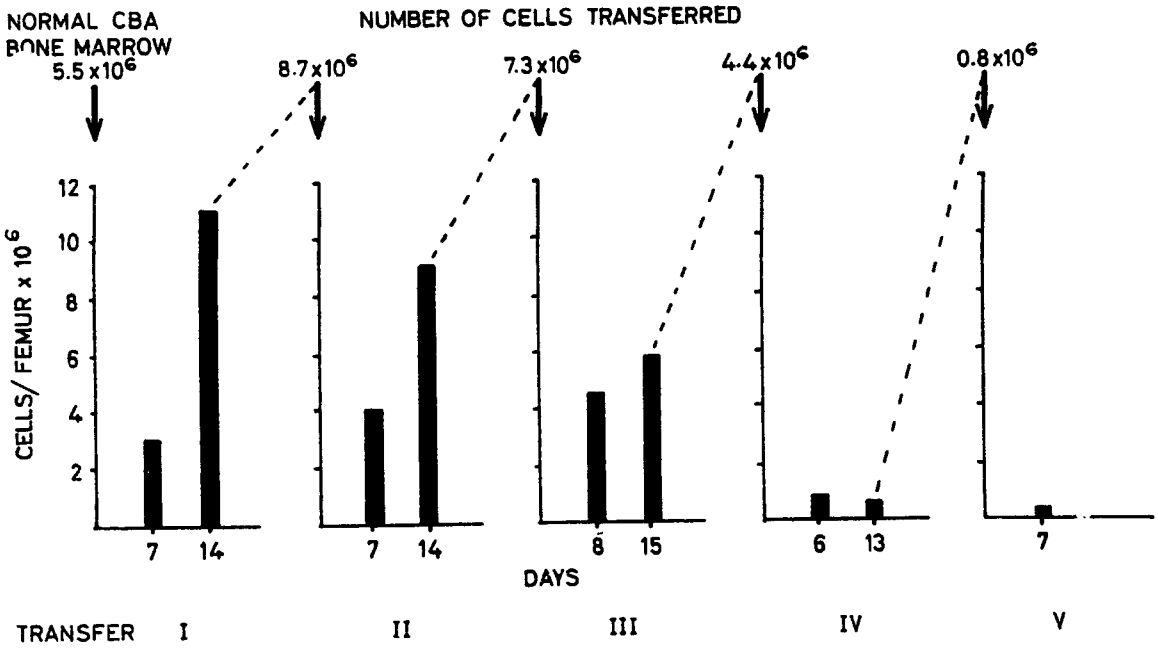

Figure V[5]. Yield of bone marrow cells upon serial transfer in lethally irradiated isologous (CBA) hosts. Data from van Bekkum and Weyzen (1961)[53]

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

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

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

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

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

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

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

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

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

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

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