CHAPTER III

Secondary Disease Following Bone Marrow Transplantation

Recognition of a secondary syndrome

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

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

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

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

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

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

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

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

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

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

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

Identification of secondary disease as a graft versus host disease

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

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

Diametrically opposed to this graft versus host concept were the

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

THE GENETIC APPROACH

As was pointed out by Koller[201], the dilemma of which system reacts against other, thereby causing secondary disease, could theoretically be solved with ease by a study of host-donor combinations of the appropriate genetic constitution. According to the basic rules

Figure III[1]. Theoretical occurrence of secondary disease in bone marrow transfers between mice of an inbred strain and their F_1 hybrids. Arrows indicate direction of bone marrow transfer

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

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

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

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

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

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

ANALOGOUS CONDITIONS

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

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

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

* Also termed the "acute killing effect".

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

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

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

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

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

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

DIRECT EVIDENCE OF ANTI-HOST ACTIVITY

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

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

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

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

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

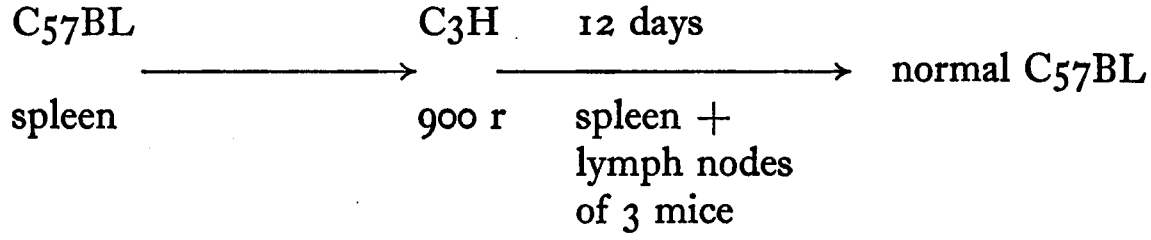
developed wasting and severe anaemia, which was accompanied by an accelerated loss of both host and donor type erythrocytes. Serum haemagglutinins directed against both the donor and recipient type erythrocytes were detected by Uyeki[433] in rat → mouse radiation chimaeras between 3 and 10 weeks following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

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

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

TRANSFER EXPERIMENTS

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



The serum from the second recipients showed a titre of 1:124 for agglutinins against C3H erythrocytes on the 5th day following transplantation. Suitable control animals showed no agglutinins. This finding indicated that the lymphoid cells from the chimaeras were stimulated to produce antibodies (agglutinins) against the host.

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

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

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

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

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

Still another method has been introduced to study directly the extent of the anti-host immunological activity of chimaeric cells[41]. Spleen cells of chimaeras, which according to current identification tests had donor type haemopoiesis, were injected into newborn mice of the appropriate genetic constitution (see Fig. III[2]) according to the graft versus host assay devised by Simonsen and Jensen[374]. With this technique direct evidence of the anti-host immunological activity of the chimaeric cells has been obtained: spleen cells from chimaeras which are known to develop severe secondary disease induced all the signs and symptoms of a graft versus host reaction in the newborn mice. One could reason that this merely demonstrated that the donor system in the chimaeras retained the capacity to react against foreign antigens, but on the other hand it is illogical to suppose that this capacity would not become manifest in the chimaera. That the results of the Simonsen assay reflect the actual reactions of the donor cells in the chimaera was substantiated by the discovery that the magnitude of the graft versus host reaction in newborns injected with a standard number of spleen cells from radiation chimaeras paralleled the severity of the clinical and histological signs of secondary disease in the chimaeras. Furthermore, cells from chimaeras which had recovered completely from secondary disease were found to be specifically non-reactive to host type antigens in the Simonsen assay. This approach has proved to be extremely useful in the study of immunological tolerance (of the graft towards host type antigens) as it develops in certain host–donor combinations.

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

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

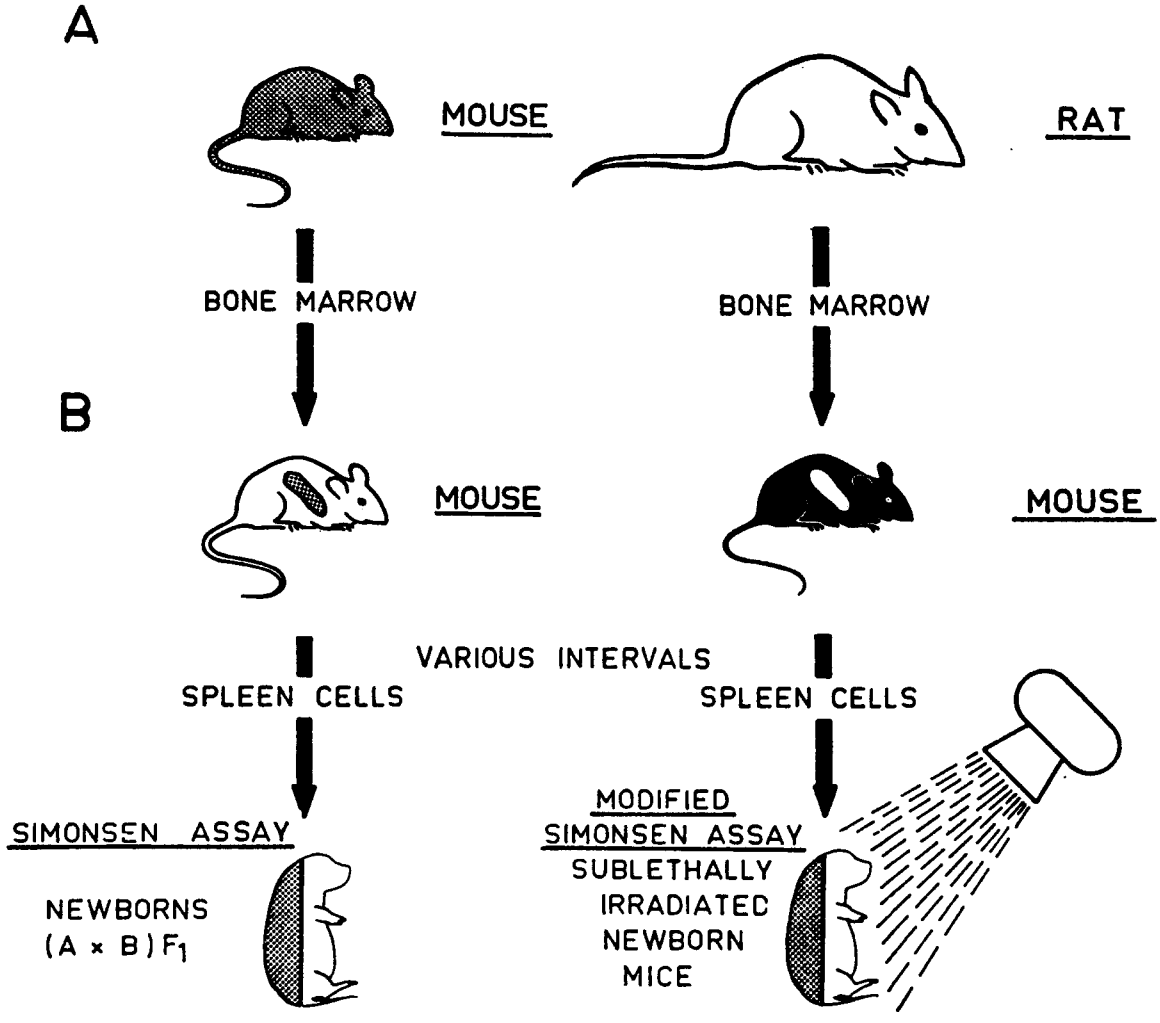


Figure III[2]. Schematic representation of graft versus host assay with spleen cells of radiation chimaeras to demonstrate anti-host activity

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

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

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

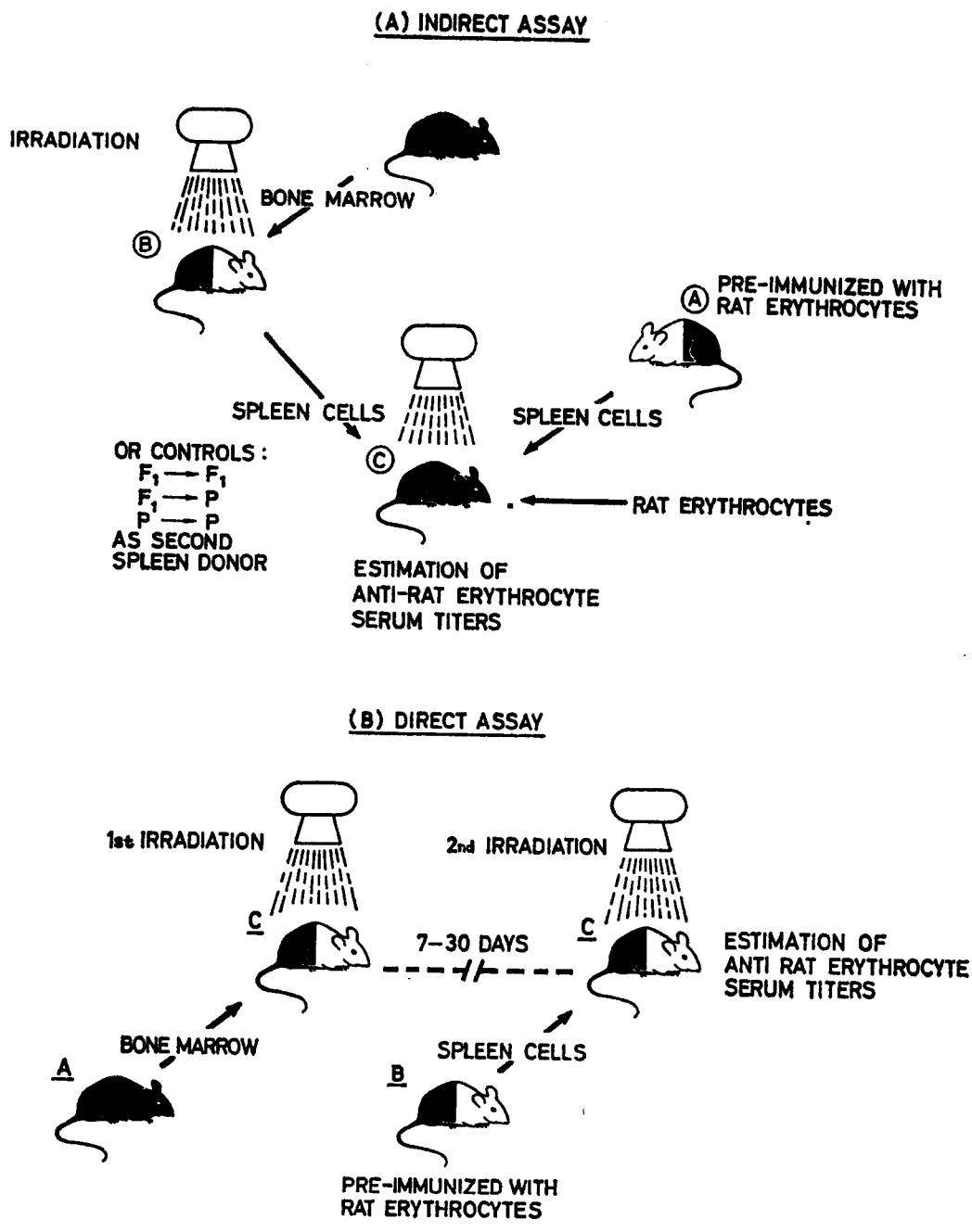


Figure III[3]. Transfer techniques employed by Doria[126, 127] in mice to demonstrate anti-host immunological activity in radiation chimaeras

(a) Indirect assay: In the case of graft versus host activity existing in chimaeric spleen donor B ($P \rightarrow F_1$), these spleen cells will reject A donor cells when both B and A spleen cells are transferred to lethally irradiated recipient C. This results in decreased levels of anti-*rat* blood cells agglutinins in C, since such agglutinins can only be produced by A cells under the conditions of the experiment.

(b) Direct assay: In the case of graft versus host activity existing in the chimaera C ($P \rightarrow F_1$) the spleen cells of B will be rejected and the production of anti-rat blood cell agglutinins by B cells will be diminished.

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

THE MORPHOLOGICAL EVIDENCE

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

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

Description of secondary disease and related syndromes

PATTERNS OF SECONDARY DISEASE AND MORTALITY

The severity and the course of secondary disease varies among different species (Fig. III[4]) and also within a species, depending on the source, nature and number of the grafted cells. The most severe

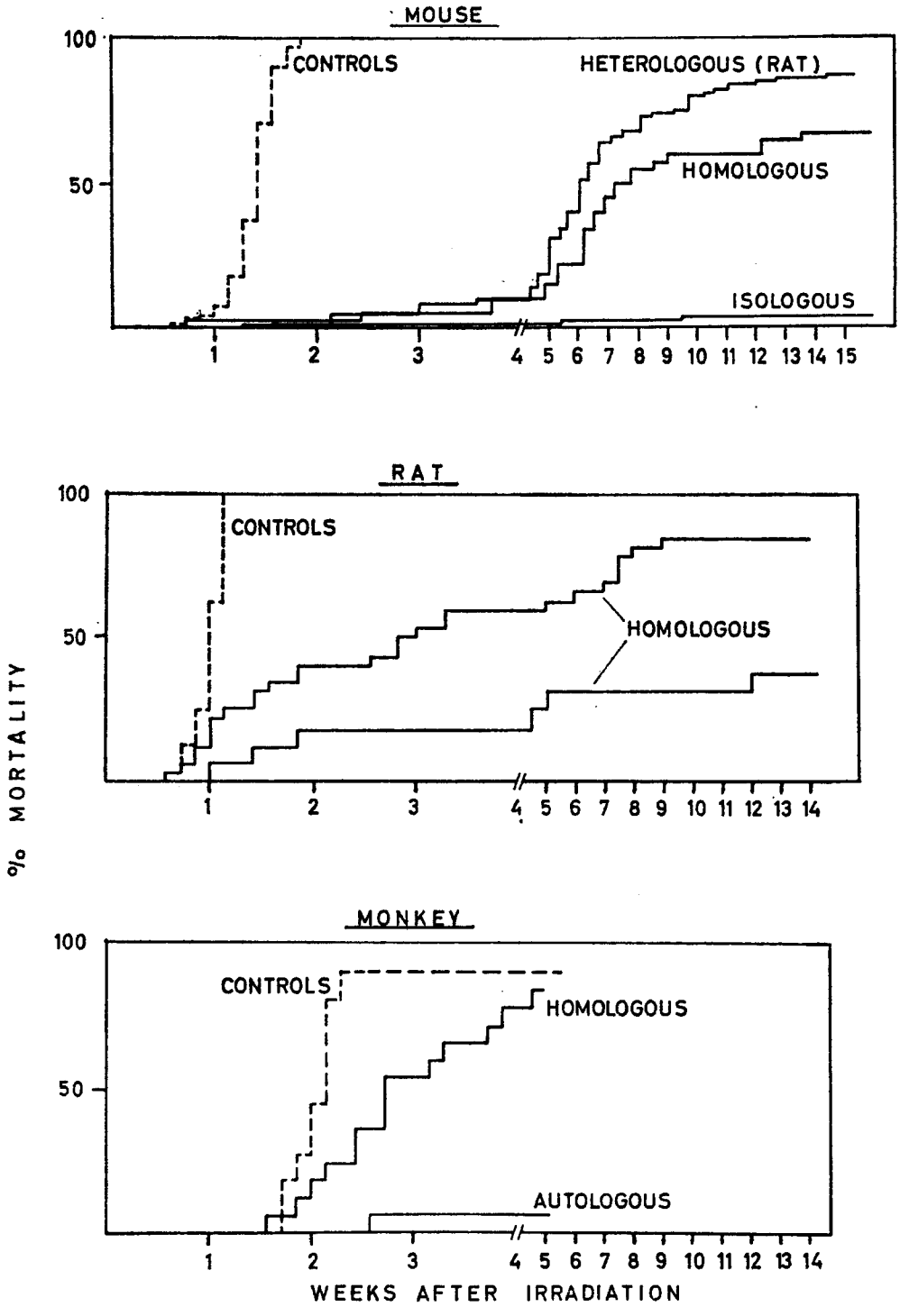


Figure III[4]. Time-pattern of secondary mortality in three animal species. Data from van Bekkum and Vos (1959)[49] for the mouse, Balner (1964)[18] for the rat, Crouch *et al.* (1960)[108] and van Putten[340] for the monkey.

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

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

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

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

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

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

When the antigenic difference between host and donor is rela-

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

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

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

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

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

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

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

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

Number of bone marrow cells	Days after irradiation									
	0	10	20	30	40	50	60	70	80	90
6×10^7	20	20	12	1	0					
2×10^7	20	17	14	9	3	1	1	1	0	
1×10^7	30	30	30	24	23	16	11	10	9	8

* Figures in the body of the table represent number of mice surviving
† Data from van Bekkum (1959)[40]
‡ Data from van Bekkum *et al.* (1959)[52]

TABLE III: 4. Symptoms and pathology of secondary disease

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

* n.i. = no conclusive information available

SYMPTOMS: DIARRHOEA AND WASTING

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

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

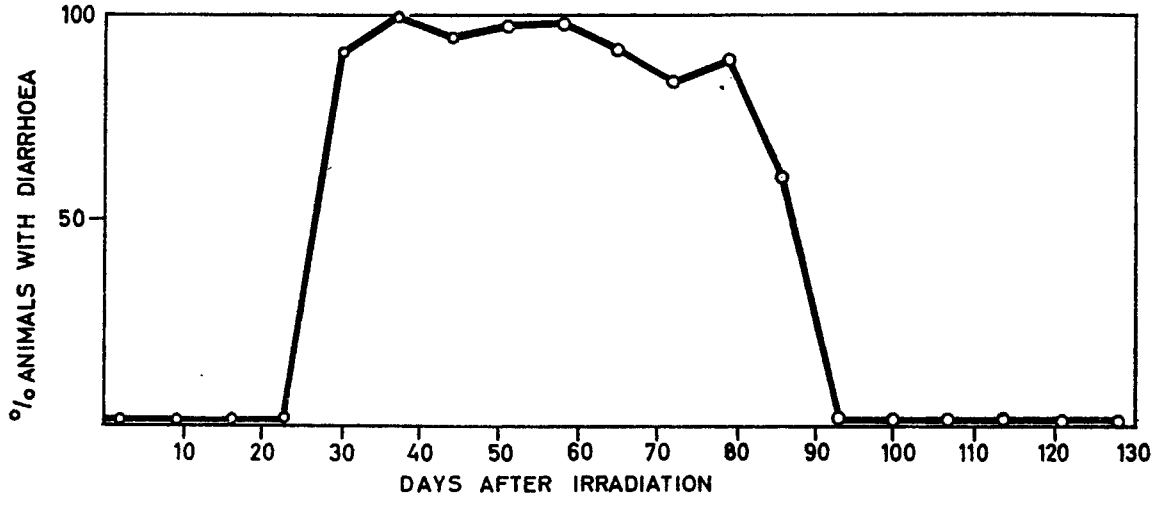


Figure III[5]. Incidence of diarrhoea in C57BL→CBA homologous radiation chimaeras. Data from de Vries and Vos (1959)[449]

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

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

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

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

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

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

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

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

In the homologous rat bone marrow chimaeras which showed

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

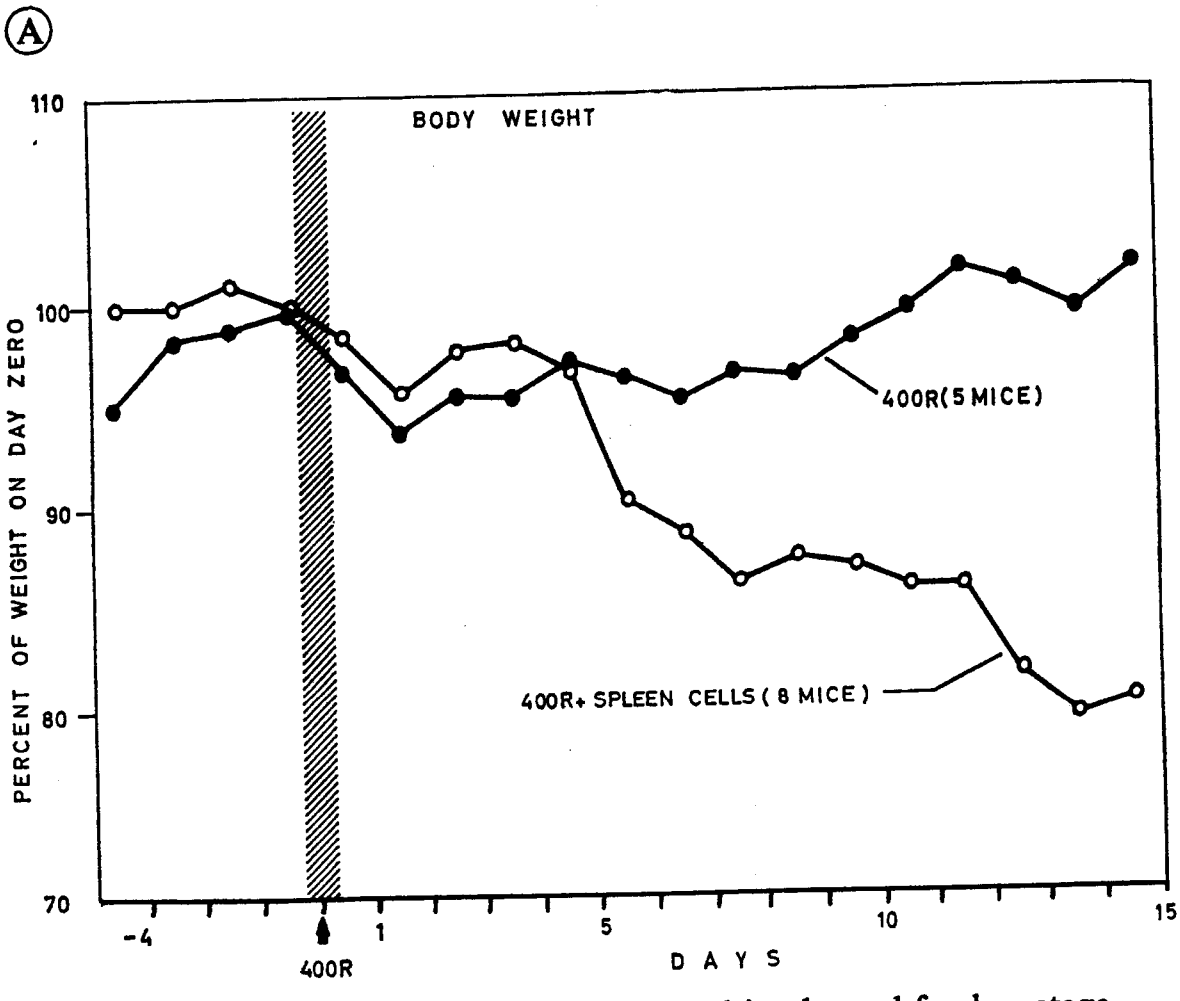
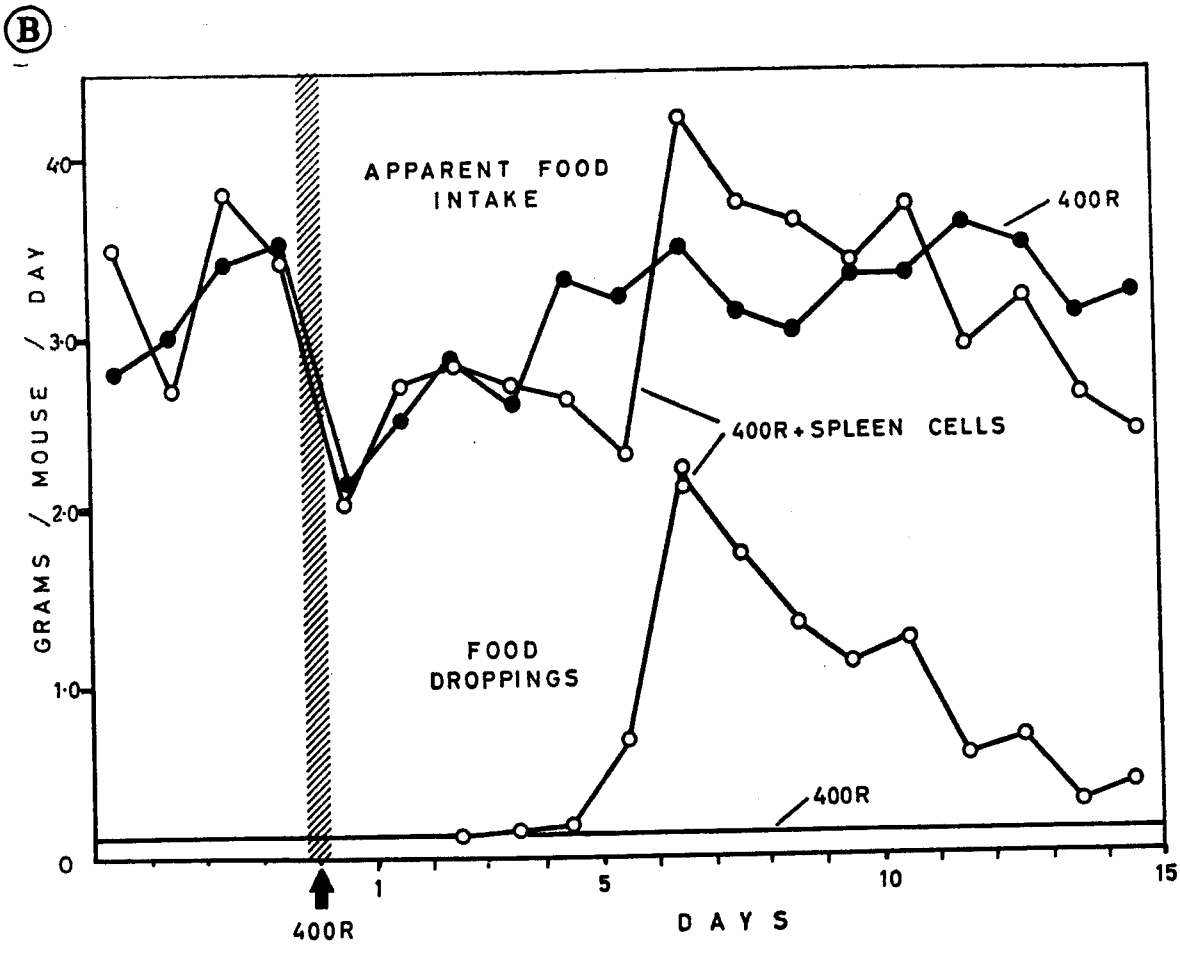
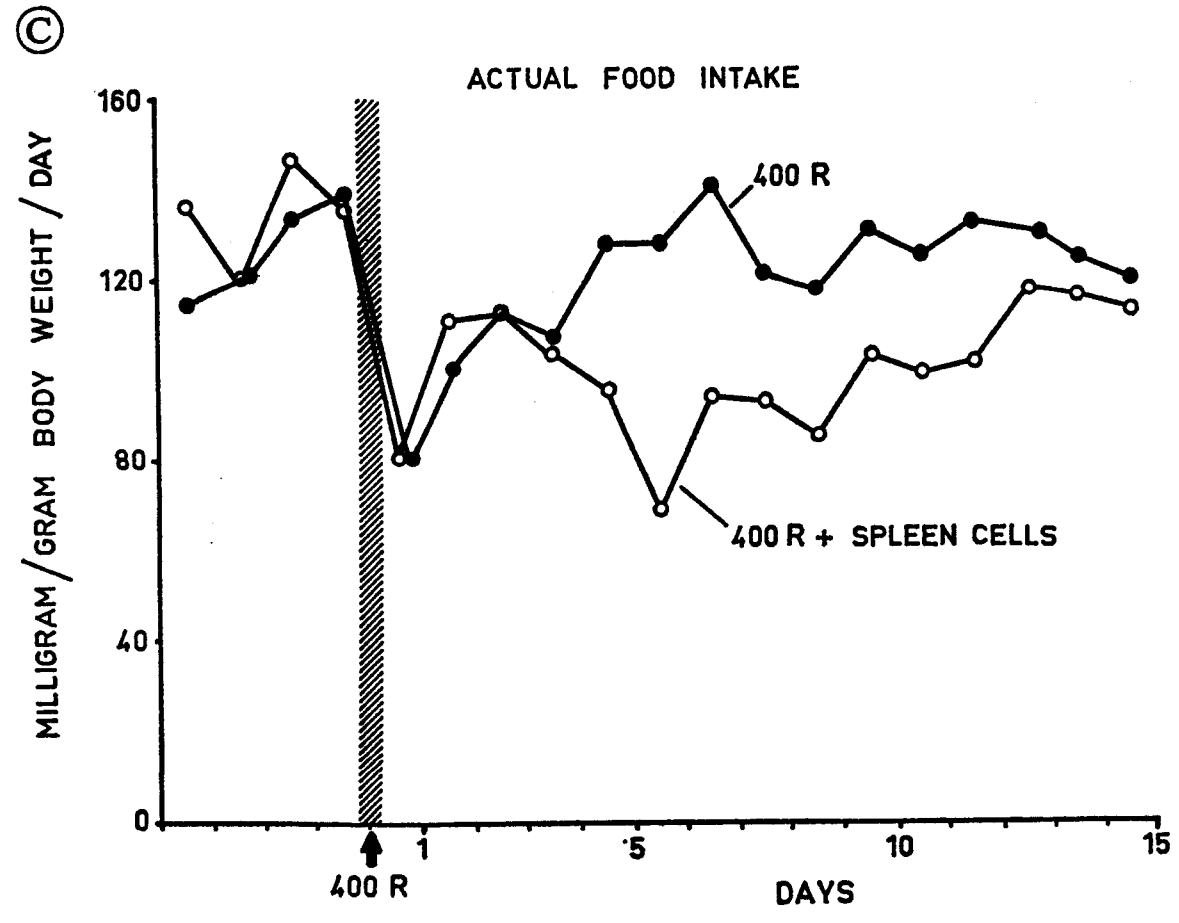


Figure III[6]. Body weight changes, food intake and food wastage in mice suffering from homologous disease. Figures from McRae (1960)[272]

(A) Body weight curves
(B) Apparent food intake curves and daily wastage of food
(C) Food intake obtained by subtracting food wasted from apparent food intake


B
APPARENT FOOD INTAKE
400R
400R+SPLEEN CELLS
FOOD DROPPINGS
400R
GRAMS / MOUSE / DAY
40
30
20
10
0
400R
1
5
10
15
DAYS


C
ACTUAL FOOD INTAKE
400 R
400 R + SPLEEN CELLS
MILLIGRAM/GRAM BODY WEIGHT / DAY
160
120
80
40
0
400 R
1
5
10
15
DAYS

of the disease. They reported, furthermore, that the food intake of these runts was somewhat "limited" because of neglect by their mothers and their own inability to move easily as a result of serious skin lesions. The description by Porter and Cooper[329] of runt disease induced in rats by the injection of newborns with thoracic duct lymphocytes mentions diarrhoea as being "sometimes" present.

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

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

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

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

In monkeys[108] as well as in human patients[263] severe diarrhoea is one of the outstanding characteristics of secondary disease. Anorexia is nearly always present in the monkeys and contributes considerably to the wasting. Weight loss was found to occur in all irradiated animals, including controls and monkeys treated with autologous bone marrow during the first 14 days. In the chimaeras the mean rate of

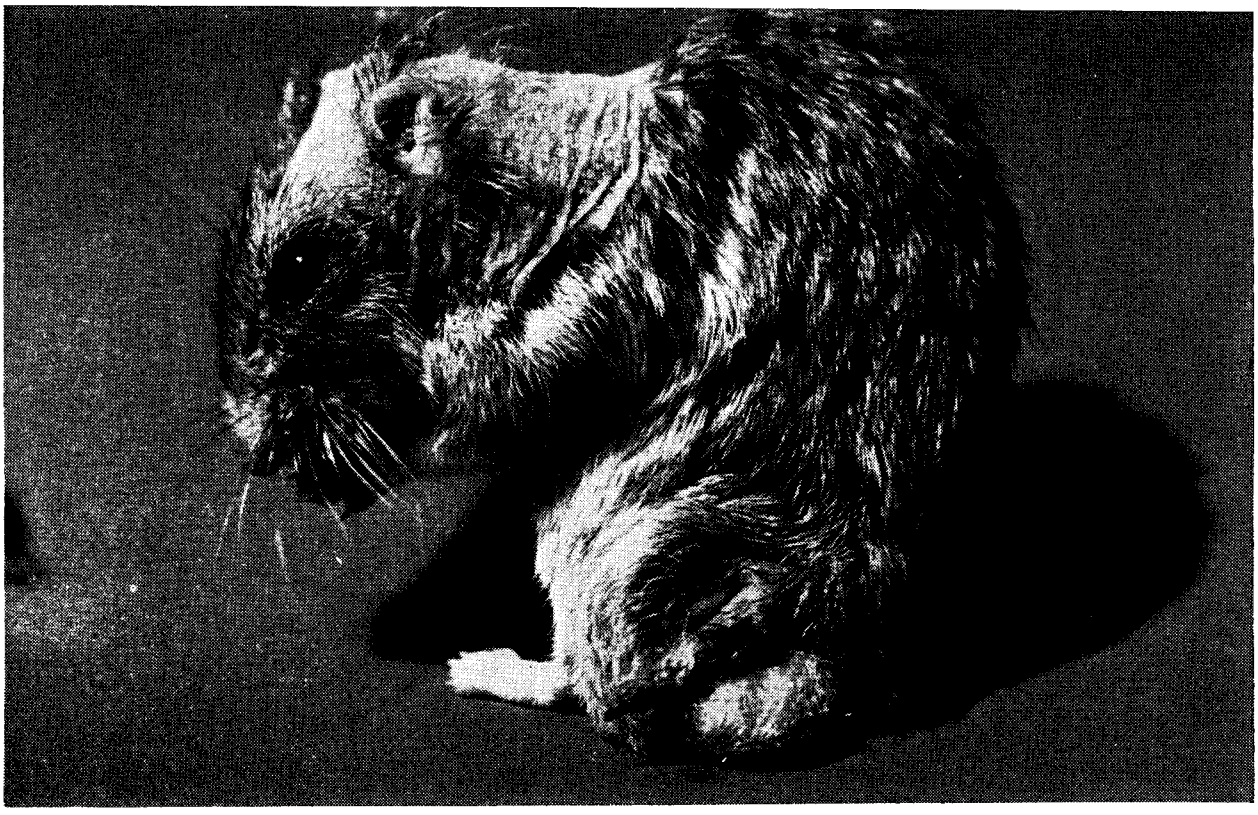


PLATE III: 6. Chinese hamster showing alopecia and scaling of the skin of the upper part of the body. Five weeks after irradiation and treatment with homologous bone marrow

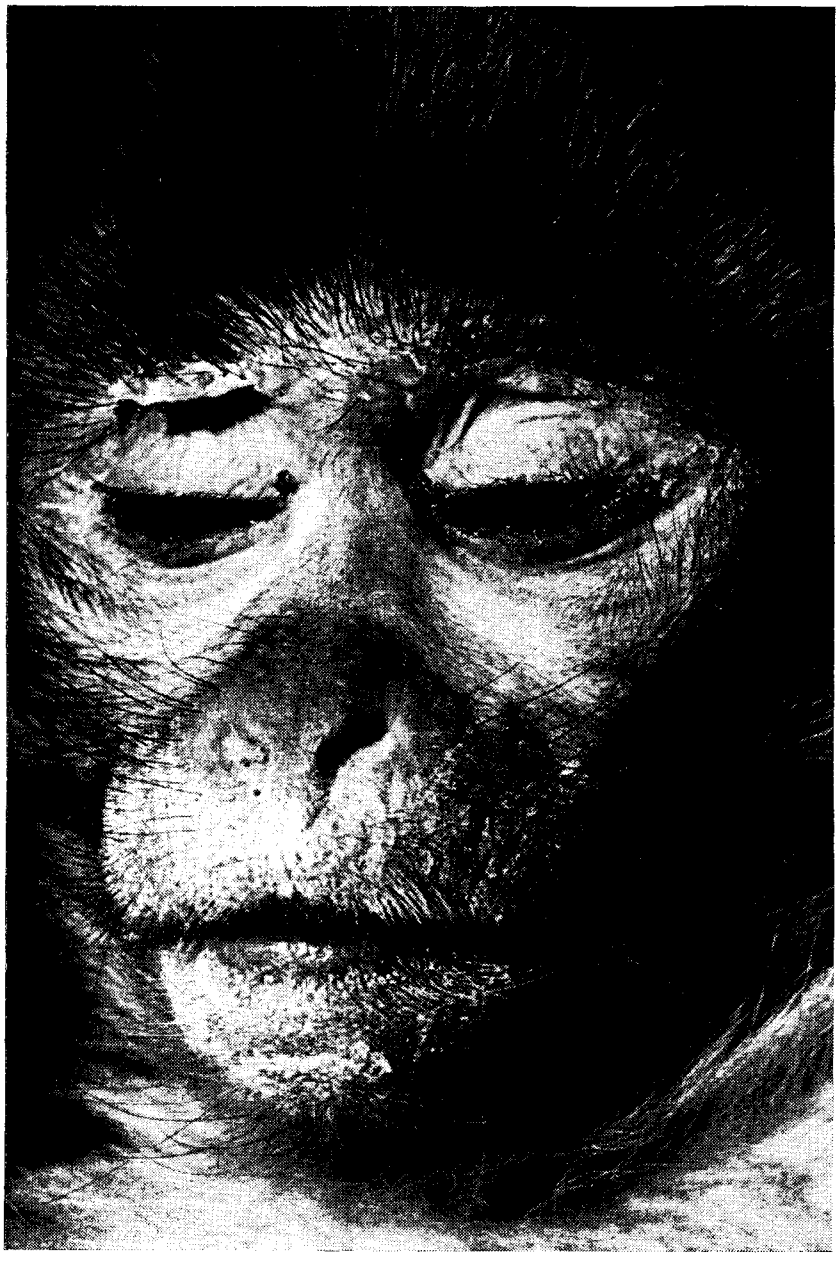
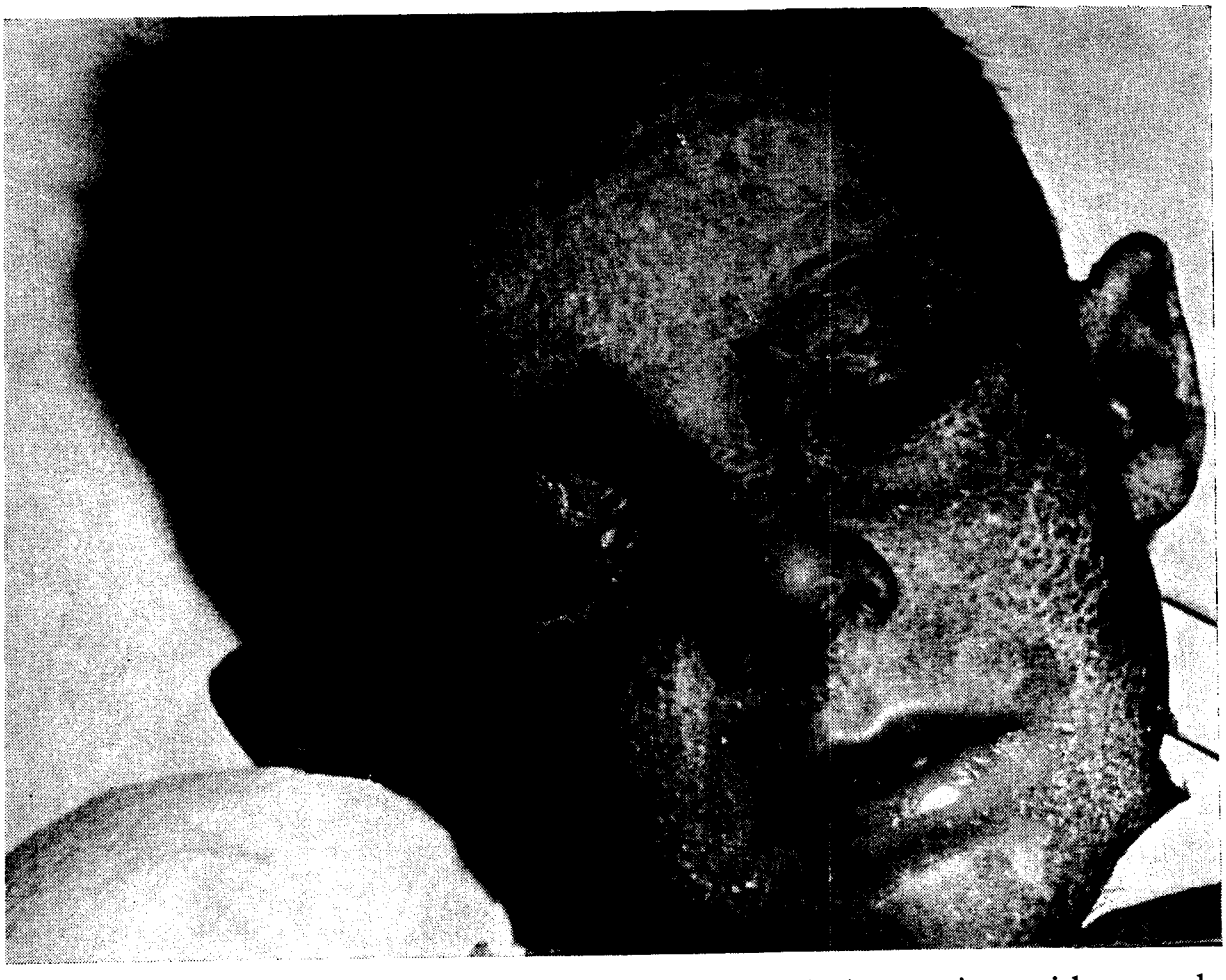


PLATE III: 7. Rhesus monkey suffering from secondary disease 17 days after irradiation and homologous bone marrow transplantation. Note scaling of the skin and crust formation predominantly in hairless areas of the face



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

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

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

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

SKIN LESIONS

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

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

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

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

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

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

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

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

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

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

INFECTIOUS COMPLICATIONS

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

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

INTENSITY OF GRAFT VERSUS HOST REACTION AND SECONDARY DISEASE

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

series of investigations involving three different host-donor combinations: one homologous combination showing minimal secondary disease, one homologous combination showing severe secondary disease and the heterologous rat mouse combination which also shows severe secondary disease[48]. In all groups, animals were killed at intervals both for histological examination of the tissues and also in order to obtain spleen or lymph node cells to be used for the quantita-

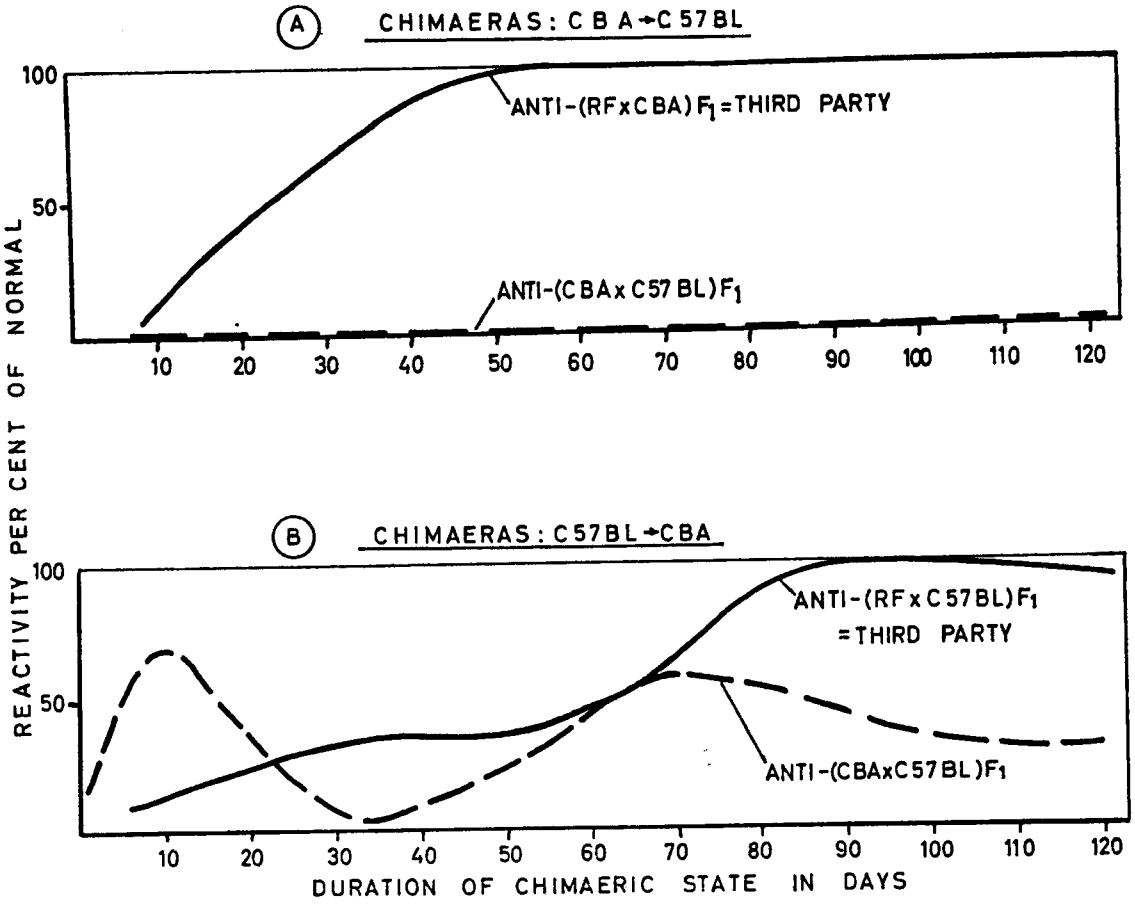


Figure III[7]. Schematic representation of results of anti-host and anti-third party homograft activities in two homologous chimaeras. Data from van Bekkum *et al.*, 1962[48]

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

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

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

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

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

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

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

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

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

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

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

SECONDARY MORTALITY IN THE ABSENCE OF A FOREIGN GRAFT

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

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

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

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

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

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

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

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

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

Pathogenesis of secondary disease

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

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

DECREASED IMMUNOLOGICAL DEFENCE

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

The relationship between graft versus host reactivity, the lym-

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

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

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

GvH reaction → specific lesions: e.g. skin, liver, spleen
GvH reaction → allergic death of lymphocytes → lymphatic atrophy → infections

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

GvH reaction → allergic death of lymphocytes → lymphatic atrophy → specific lesions and infections

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

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

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

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

RADIATION DOSE

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

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

Recipient	Bone marrow donor	X-ray dose (r)	Percentage mortality at		Diarrhoea	Number of mice
			30-day	100-day		
CBA	CBA	675	0	0	Absent	30
CBA	CBA	800	26	31	Sporadic	19
CBA	CBA	950	22	71	Slight	89
F_1 hybrid	CBA	700	0	0	Absent	110
F_1 hybrid	CBA	800	0	0	Slight	60
F_1 hybrid	CBA	950	12	20	Severe	70

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

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

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

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

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

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

* "Acute killing" effect.

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

Modification of secondary disease

PREVENTIVE MEASURES

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

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

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

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

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

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

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

experiments are required[45]. It has been pointed out that in mice the severity and the incidence of secondary disease are functions of the number of homologous cells administered, whether it be spleen, lymph node or bone marrow cells, from which it follows that a decrease in the total number of viable cells can easily result in increased survival.

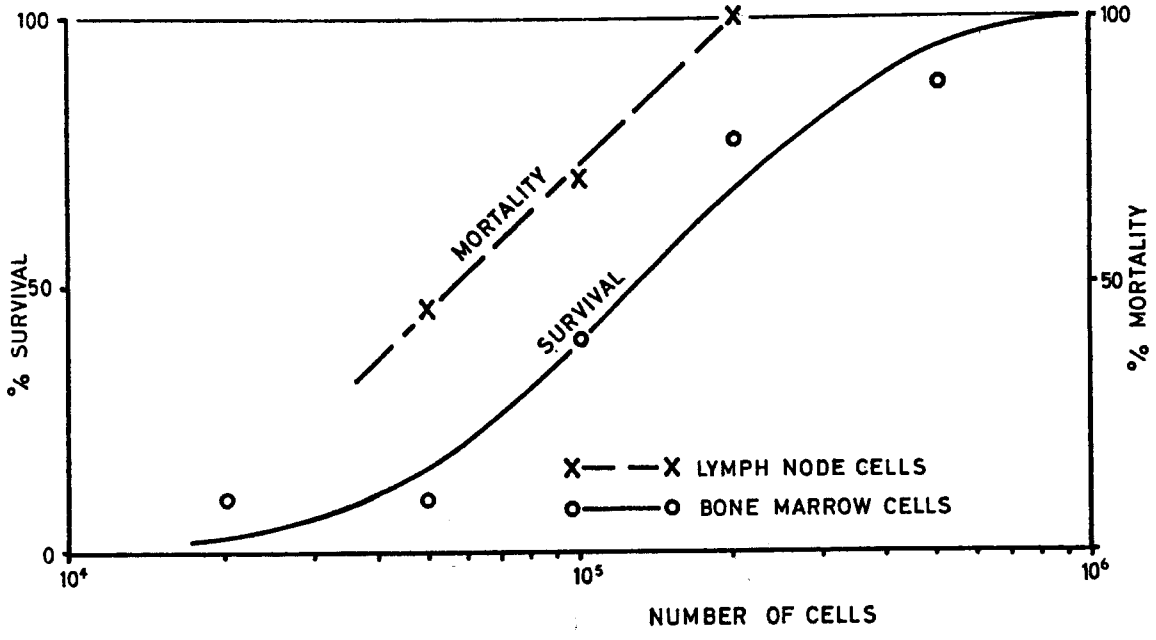


Figure III[8]. The relation between the number of CBA bone marrow cells and survival and between CBA lymph node cell dose and mortality in lethally irradiated (CBA × RF)F_1 hybrid mice. These relationships were used for the determination of the selective elimination of immunologically active cells from cell suspensions.

Data from van Bekkum (1964)[45]

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

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

PREIRRADIATION OF DONOR MARROW

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

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

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

* A well known radioprotective substance: S-(2-amino-ethyl)-isothiouronium dihydrobromide.

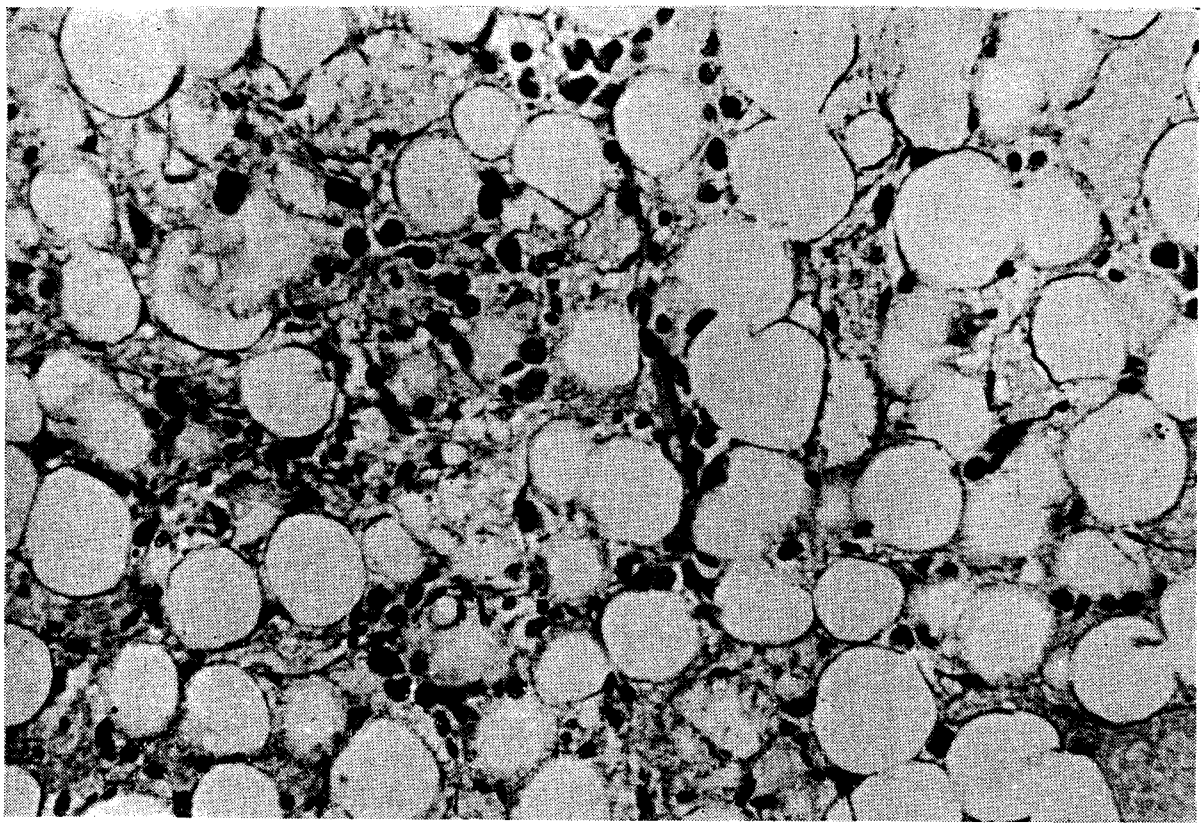


PLATE IV: 1. Aplasia of bone marrow in control monkey 15 days following lethal irradiation. The marrow space is occupied by fat cells and collections of plasma cells and histiocytes. Hematoxylin-eosin (HE). Magnification × 190

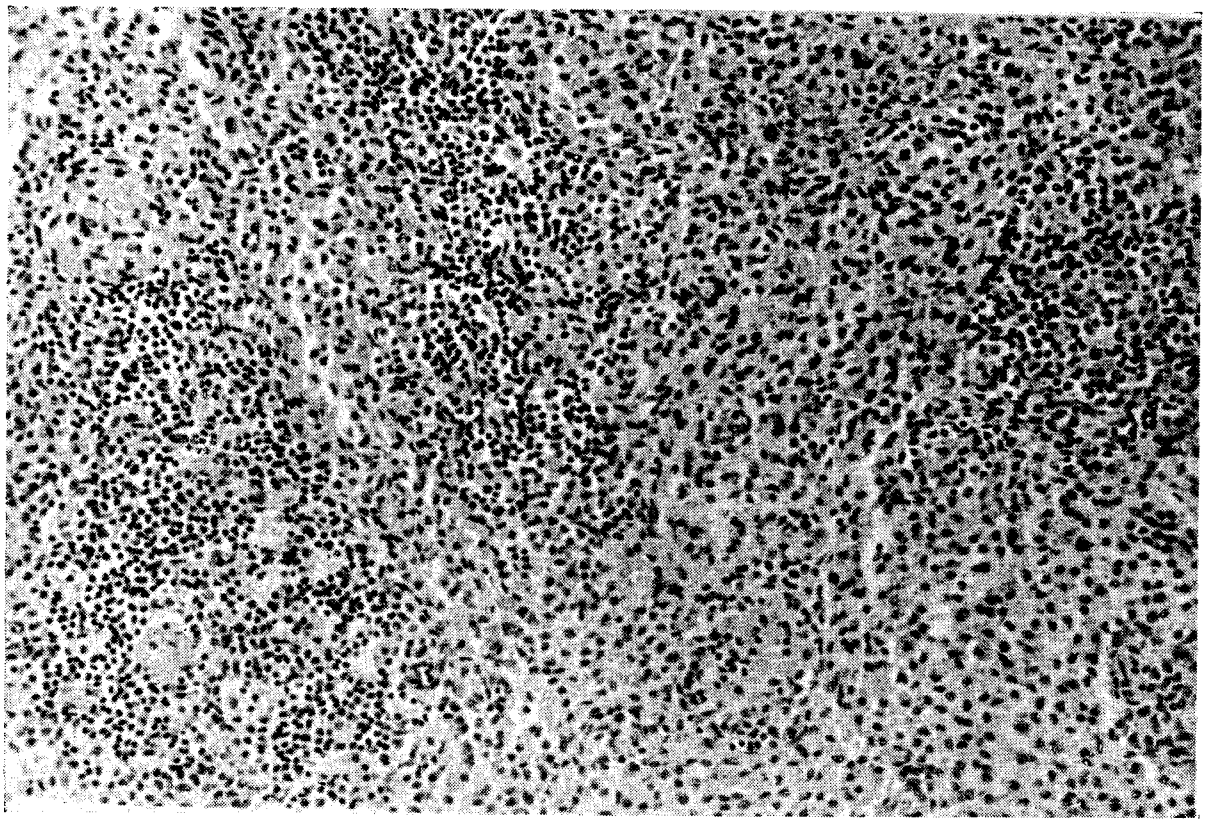


PLATE IV: 2. Atrophy of lymph node in control monkey 15 days following lethal irradiation. Note severe decrease of cellularity and absence of follicles. A few collections of lymphocytes are dispersed within the reticular stroma, however. (HE). Magnification × 30

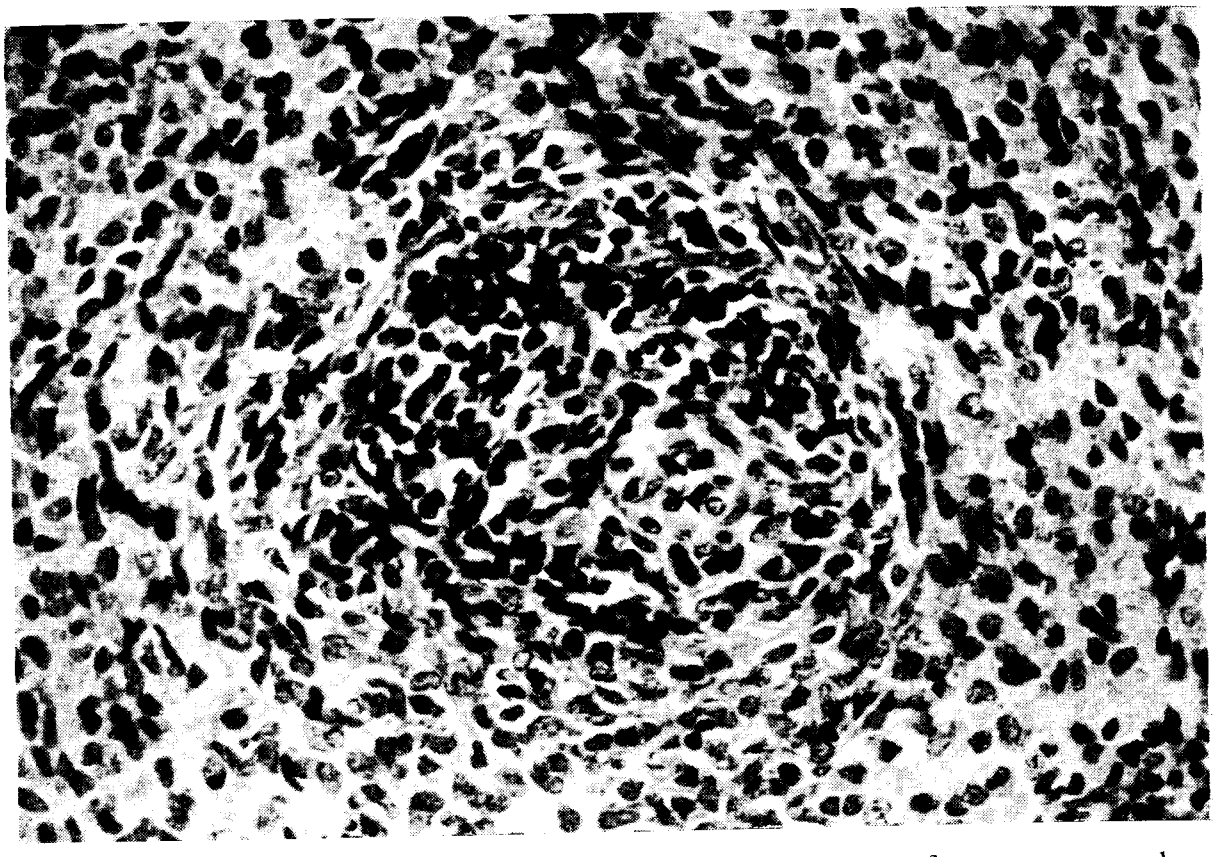


PLATE IV: 3. Atrophic lymphatic follicle in the spleen of a mouse, 4 days following lethal irradiation. A few lymphocytes remain in the stroma surrounding a splenic arteriole. (HE). Magnification × 300

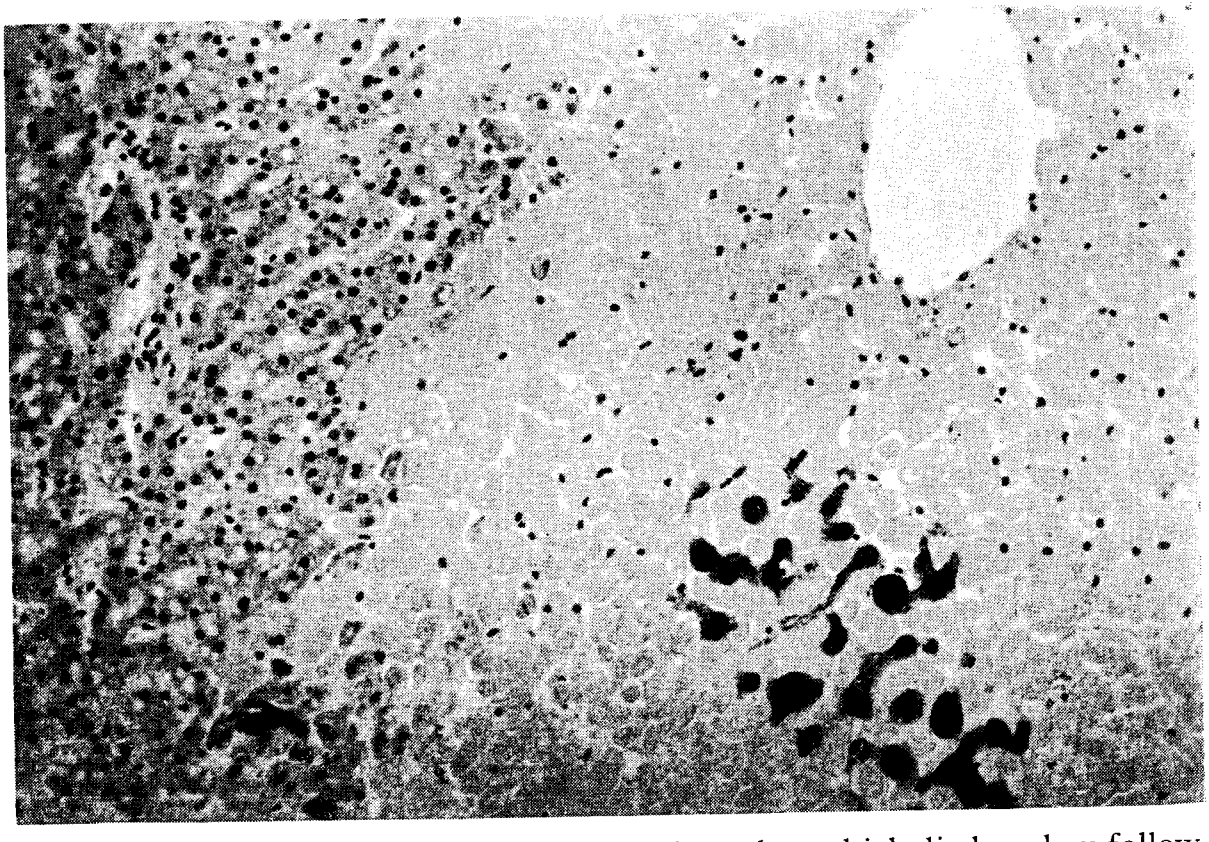


PLATE IV: 4. Septic liver necrosis in control monkey which died 15 days following lethal irradiation. Note darkly staining clumps of bacteria, total loss of liver cell structure in centre and right of the picture, and absence of any cellular inflammatory response. (HE). Magnification × 120

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

INCUBATION OF DONOR MARROW

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

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

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

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

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

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

POOLED DONOR MARROW

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

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

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

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

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

MISCELLANEOUS METHODS

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

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

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

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

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

TREATMENT OF SECONDARY DISEASE

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

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

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

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

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

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

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

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

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

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

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

Quite recently considerable progress has been made in this re-

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

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

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

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

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

These results taken together with the impressive advances which are being made in the field of tissue typing for the selection of compatible donors, seem to provide a sound basis for the expectation that the hazards of homologous bone marrow transplantation may be decreased to an acceptable level in the not too remote future.

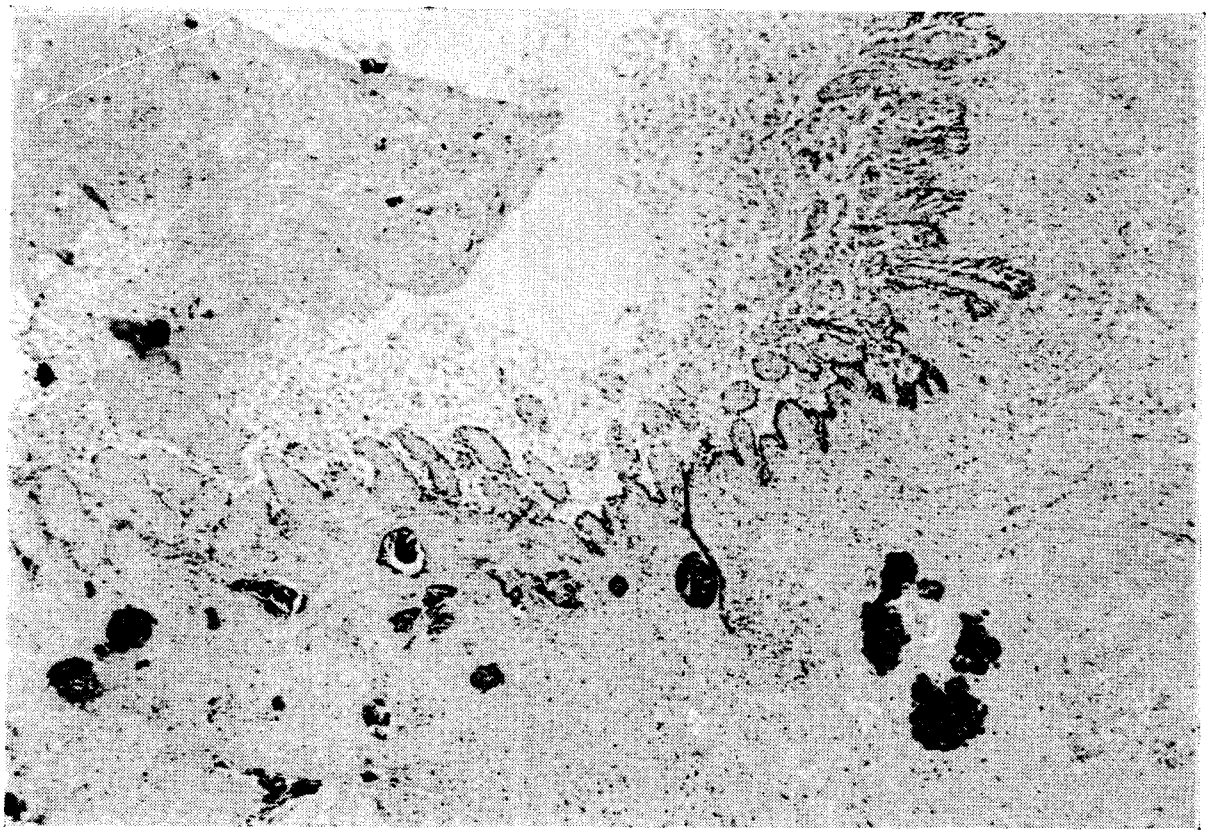


PLATE IV: 5. Ulcer of oral mucosa in control monkey which died 15 days following lethal irradiation. Desquamation of the epithelium with haemorrhage and necrosis of submucosa. Note darkly staining accumulations of bacteria and absence of cellular exudation. (HE). Magnification × 30

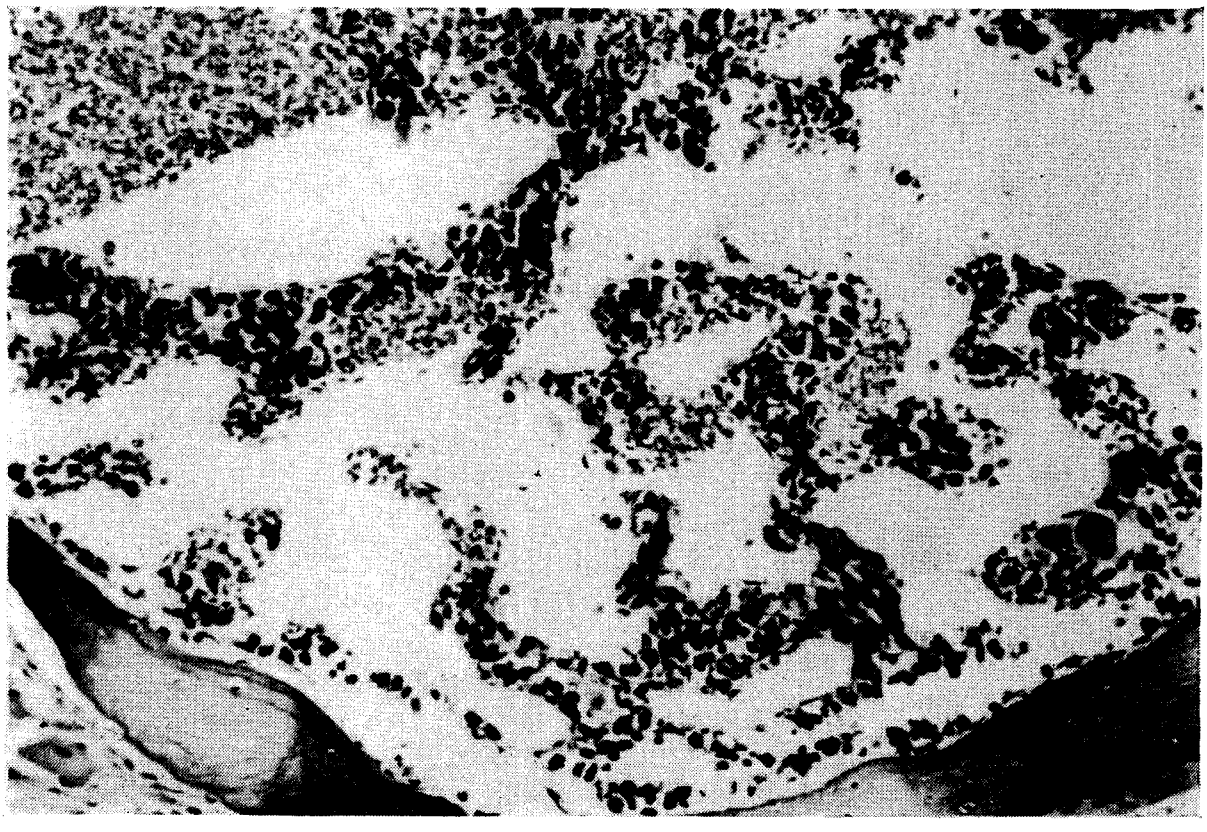


PLATE IV: 6. Early regeneration of sternal bone marrow on the 4th day in lethally irradiated mouse treated with rat bone marrow. Marrow still shows poor cellularity but proliferated reticular cells and young stem cells (haemocytoblasts) can be seen between dilated sinusoids. (HE). Magnification × 190

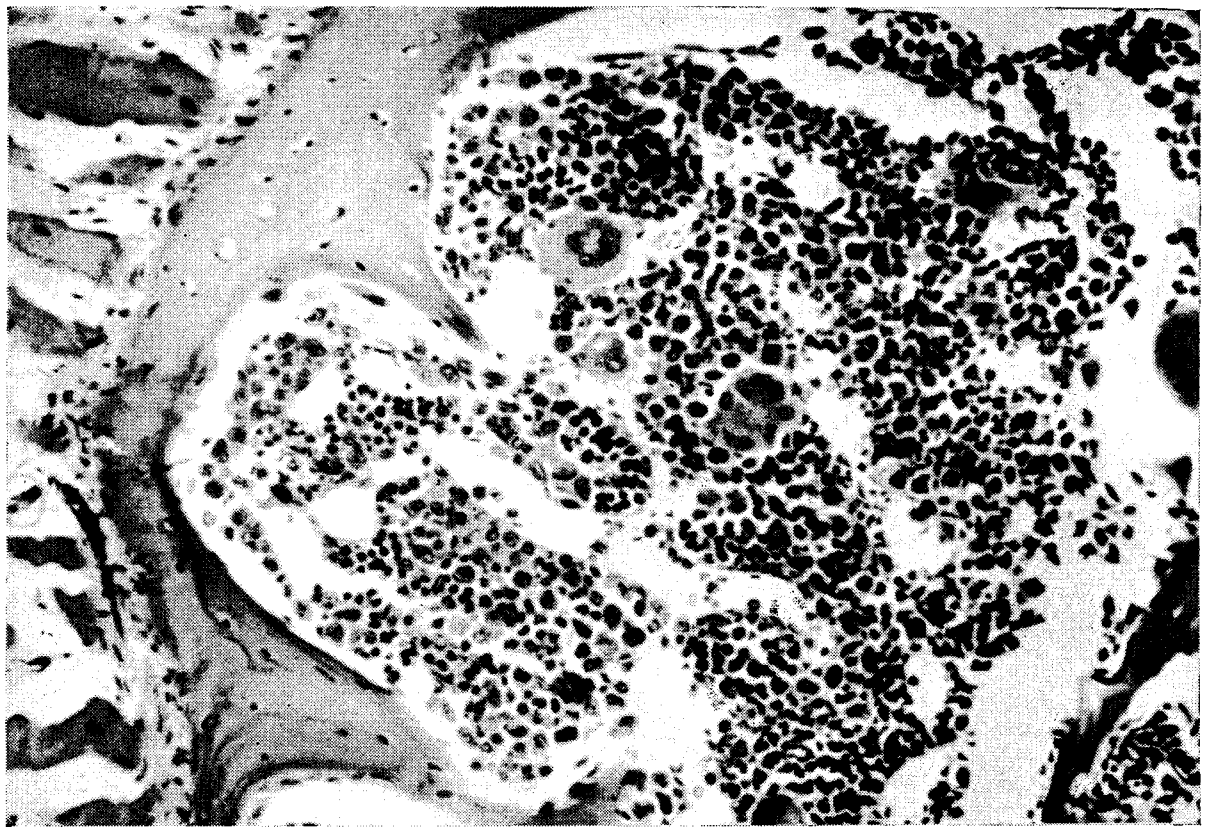


PLATE IV: 7. Advanced regeneration of sternal bone marrow on day 5 in mouse treated with rat bone marrow. Highly cellular marrow with many immature cells. Mature cells—especially of the white series—are still lacking. (HE). Magnification × 190

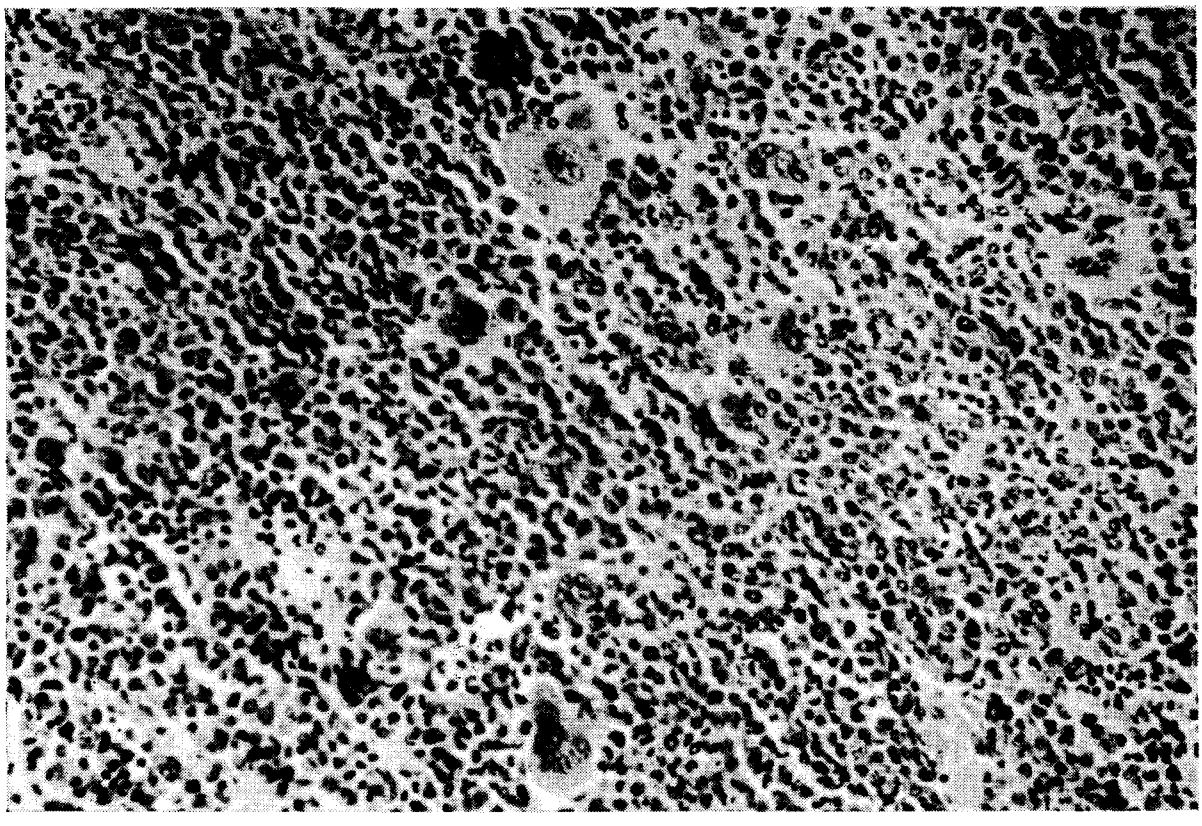


PLATE IV: 8. Extensive extramedullary haemopoiesis in red pulp of spleen in homologous mouse chimaera on day 44. (HE). Magnification × 190

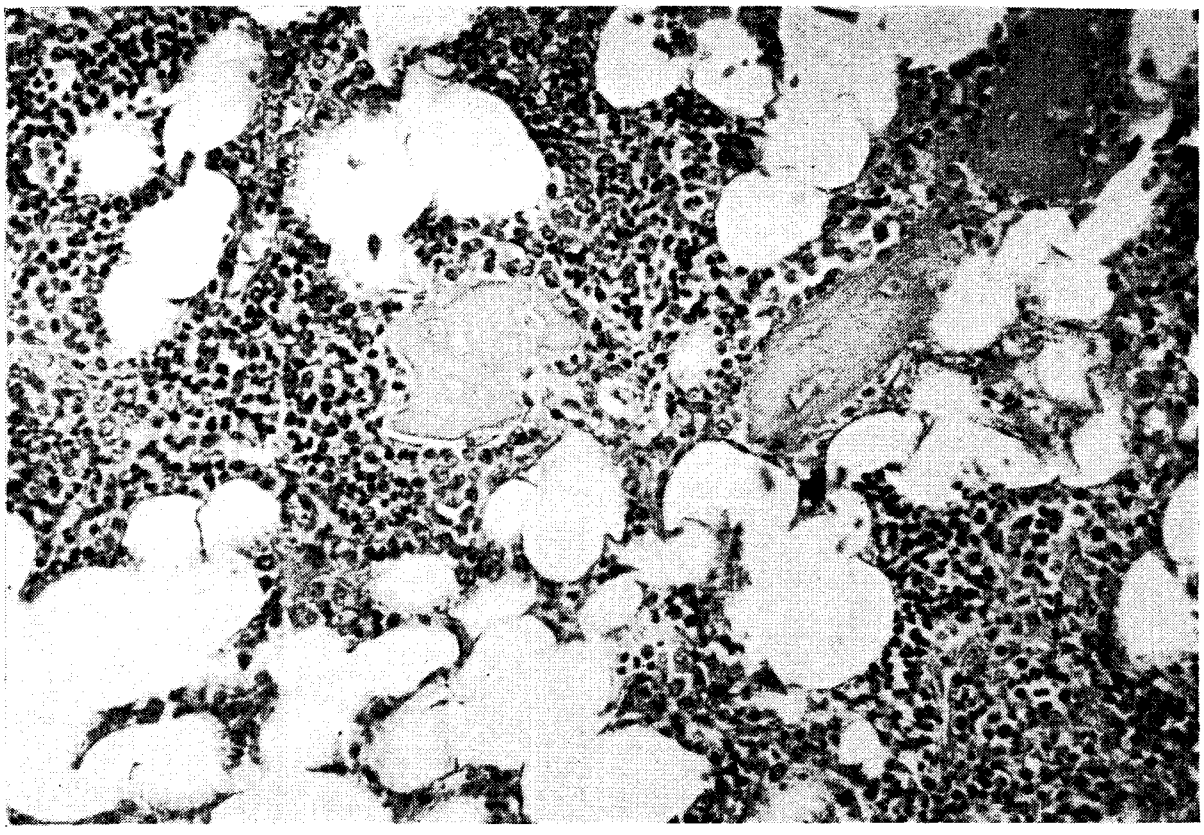


PLATE IV: 9. Advanced regeneration of bone marrow (rib) in a leukaemic child, 30 days after whole body irradiation with 900 r and treatment with homologous bone marrow. Fairly cellular marrow, all cell lines are represented. Photograph from Mathé *et al.* (1960)[263]. (HE). Magnification × 190

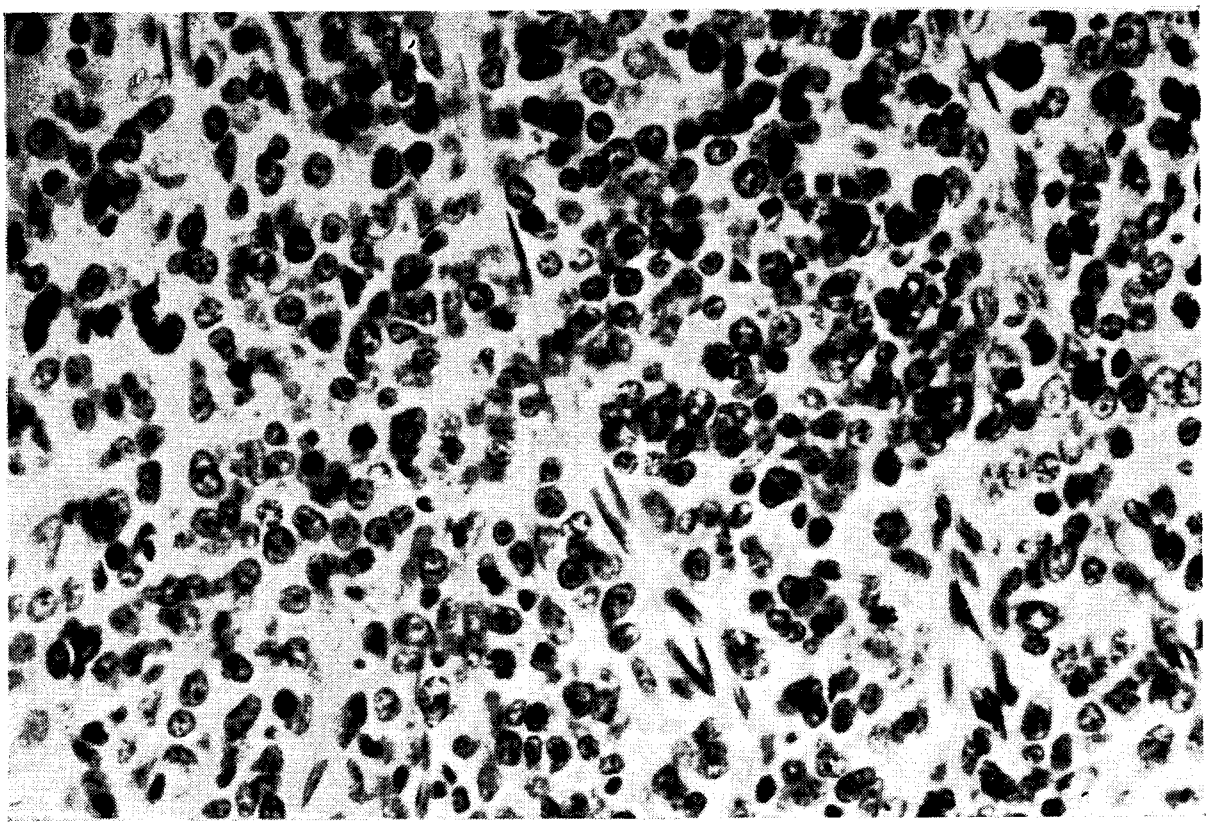


PLATE IV: 10. Early regeneration of thymus cortex on day 7 in lethally irradiated mouse treated with isologous bone marrow. Appearance of mitotic figures and small collections of lymphoblasts. (HE). Magnification × 480

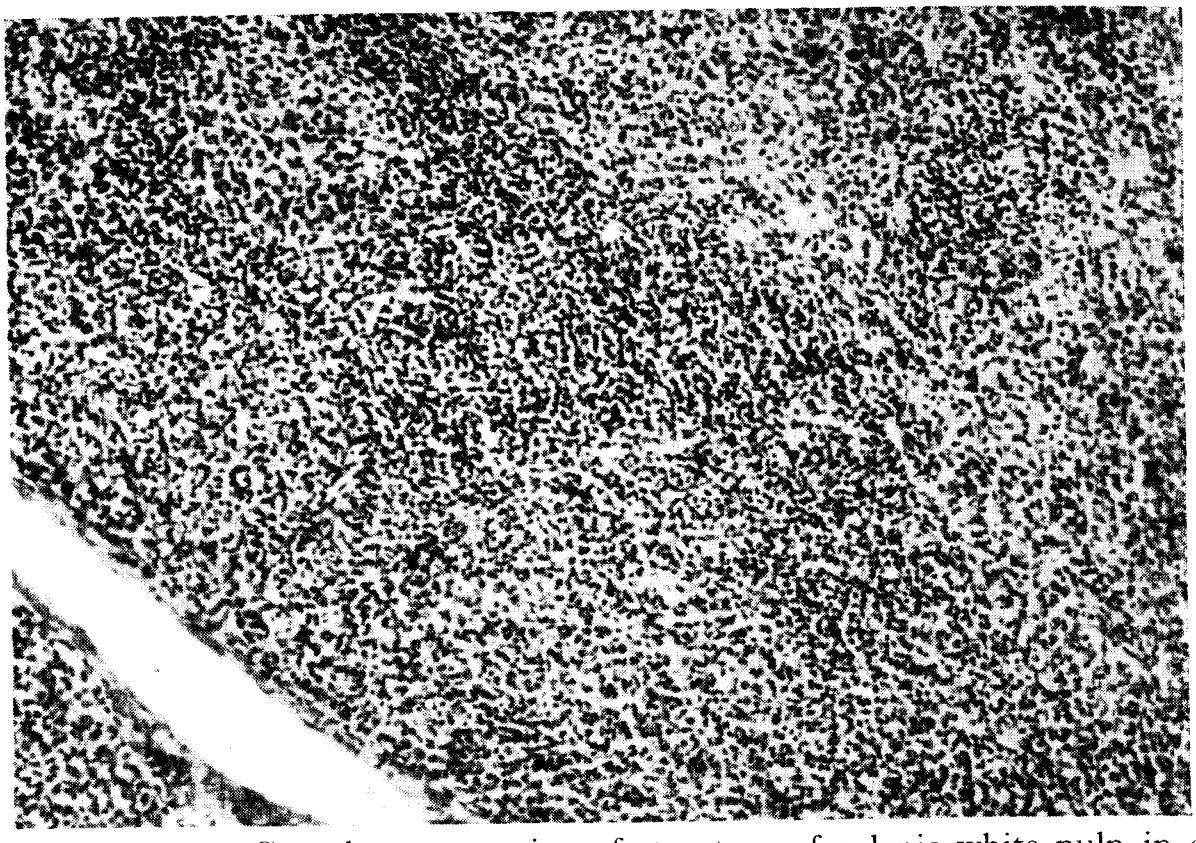


PLATE IV: 11. Complete restoration of structure of splenic white pulp in a mouse 38 days after lethal irradiation and treatment with isologous bone marrow. Many mature lymphocytes and a lymphatic follicle are seen. (HE). Magnification × 120

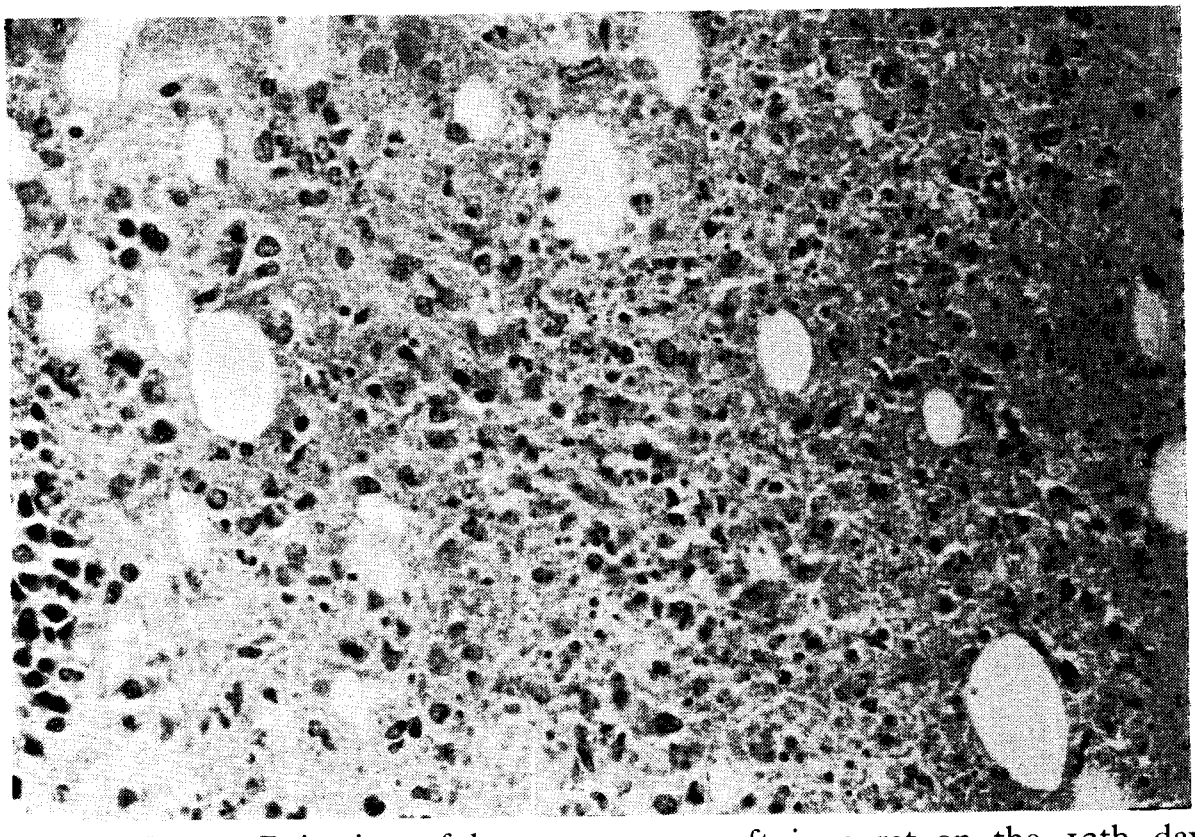


PLATE IV: 12. Rejection of bone marrow graft in a rat on the 12th day following low lethal irradiation (550 r) and treatment with homologous bone marrow. Sternal bone marrow, showing necrosis and many disintegrated cells. In lower left corner a number of intact erythroblasts and myeloid cells can be seen. (HE). Magnification × 300