"SECONDARY DISEASE" AMONG LETHALLY IRRADIATED MICE RESTORED WITH HAEMATOPOIETIC TISSUES FROM NORMAL OR ISO-IMMUNIZED FOREIGN MICE

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MICE given lethal doses of X-rays and then restored with bone marrow cells from normal allogeneic* mouse donors recover from the acute effects of radiation, but later often develop a wasting disease which may be fatal. This "secondary disease" is believed by most workers to result from an immune reaction by the injected cells against the host. This belief is strengthened by the observation that if foreign lymphoid cells from spleen, lymph-node or thymus are added to the bone marrow inoculum, the disease develops more rapidly and runs a more acute course (Ilbery, Koller and Loutit, 1958; Vos, de Vries, Collenteur and Van Bekkum, 1959); this effect varies according to the number of lymphoid cells injected.

If a reaction by the graft against the host is indeed responsible for secondary disease, it might be anticipiated that iso-immunization of the prospective bone marrow or spleen donors against antigens of the host strain would result in a somewhat earlier onset of the disease. While the present work was in progress, experiments testing this hypothesis were reported by Cosgrove, Upton, Schwartz, Congdon and Makinodan (1959) and by Chin and Silverman (1960). The former found that in one strain combination secondary mortality was increased by pre-immunization of the donor, while in another mortality was heavy whether or not the donor was pre-immunized. Chin and Silverman on the other hand, using rat donors and mouse hosts, found that pre-immunization of the donor did not affect mortality (except when as many as 5×10^7 rat spleen cells were injected in addition to bone marrow), and concluded that a graft-versus-host reaction was not responsible for delayed mortality in their animals.

Our experiments reported here show that the results of pre-immunizing the donors against host antigens and of injecting spleen in addition to bone marrow differ radically according to the genetic relationship of host and donor, and we believe that these differences provide suggestive new evidence concerning the pathogenesis of secondary disease.

MATERIALS AND METHODS

Male and female mice of the strains CBA, A and C3H maintained at Harwell by strict sib-mating, were used. Hosts 3–5 months old, were distributed into boxes of 5 and irradiated

* The terms "syngeneic" (= of the same highly inbred strain), "allogeneic" (= of the same species but not genetically identical) and "xenogeneic" (= of a different species) are now preferred to the trio "isologous", "homologous" and "heterologous", which have caused justified confusion to many whose primary concern is not transplantation immunity. (See Gorer, Loutit and Micklem, 1961.)

with 1007 rads (±3 per cent) of 250 kV X-rays by the standard technique of this laboratory (Corp, 1957).

In most experiments immunization of donor mice was carried out by 3 injections of foreign spleen suspension in physiological saline (each consisting of about 25×10^6 nucleated cells) at intervals of a fortnight—the first intravenous, the second intraperitoneal, the third subcutaneous. The mice were sacrificed and used as donors of haematopoietic tissues 10–14 days after the last injection. This method of immunization was used for Expts. I–II and IV–V. It has been found to engender a high level of serum antibody as judged by an *in vivo* cytotoxicity test (Loutit and Micklem, 1961). For Experiment III A-strain mice were immunized with a single intraperitoneal dose of CBA spleen cells (240×10^6 or 24×10^6 cells/mouse) and used as donors 7 days later.

Bone marrow and spleen suspensions for post-radiation treatment were prepared and administered intravenously as described by Bridges, Loutit and Micklem (1960). The bone marrow from the femora of one donor was divided among 5 recipients. When spleen was given, one donor also provided the material for 5 recipients (except in Expt. 6 : see below). The dose of nucleated bone marrow cells was thus approximately 5×10^6, and of spleen 25×10^6 per mouse.

Four experiments of essentially similar design were performed. Each comprised 5 groups of lethally irradiated mice which were treated as follows :

Group 1 : bone marrow from normal allogeneic donor.
Group 2 : bone marrow from iso-immune (against antigens of the prospective host) allogeneic donor.
Group 3 : bone marrow and spleen from normal allogeneic donor.
Group 4 : bone marrow and spleen from iso-immune allogeneic donor.
Group 5 : Saline only.

All the groups in a given experiment were treated within a span of 8 days.

The 3rd experiment comprised only Groups 1 and 2.

The strain combinations used were : Expt. I : A ⟶ CBA, Expt. II : CBA ⟶ A, Expt. III : A ⟶ CBA, Expts. IV and V : C3H ⟶ CBA.

In the 6th experiment, 3 groups of 10 CBA ♂ mice were irradiated and treated with 5×10^6 bone marrow cells plus 1×10^7 cells from, respectively, spleen, thymus and lymph nodes (mesenteric and subcutaneous) of C3H donors. Autopsy was performed on all mice found dead or moribund and, where the state of preservation warranted, tissues were fixed in buffered formalin for histological examination.

RESULTS

Survival

Experiment I : A → CBA.—Cumulative mortality data are shown in Fig. 1. The survival of CBA mice given normal A-strain bone marrow was comparable with that of mice similarly treated in this laboratory in the past (Barnes, Loutit and Westgarth, 1959). There were few deaths within 20 days of irradiation, but between 20 and 90 days all the animals became ill with secondary disease. Median survival was 57 days after irradiation. When the bone marrow donors were iso-immune to host antigens, median survival was reduced to 39 days, and the pattern of mortality presented a somewhat different appearance ; but the significance of this is doubtful. The addition of approximately 25×10^6 adult spleen cells to the restorative inoculum greatly accelerated the onset and course of secondary disease. Median survival times were 16 and 15 days after injection of normal and iso-immune cells respectively. However, survival was still better than among the saline-injected controls.

Experiment II : CBA → A.—Cumulative mortality data are shown in Fig. 2. Secondary mortality was less rapid than in the reciprocal strain combination (Expt. I). Median survival in animals given normal bone marrow was 161 days. This was reduced to 110 days where the bone marrow donors were iso-immune.

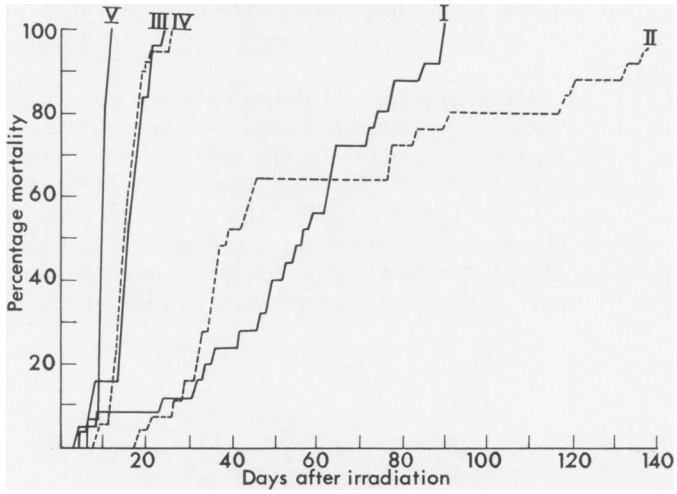

FIG. 1.—Cumulative mortality of CBA mice lethally irradiated and injected i.v. with bone marrow or bone marrow + adult spleen from normal or iso-immune A-strain donors. Number of mice per group shown in brackets.

I A b.m. (25).
II Immune A b.m. (25).
III A b.m. + adult spleen (25).
IV Immune A b.m. + adult spleen (25).
V Saline only (25).

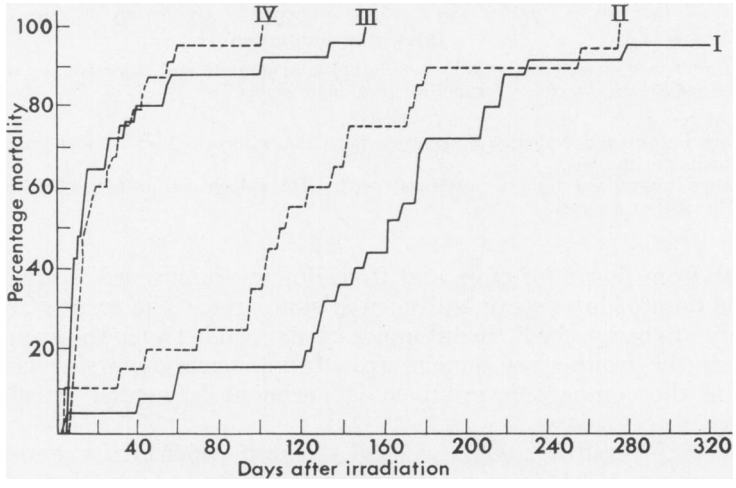

FIG. 2.—Cumulative mortality of A-strain mice lethally irradiated and injected i.v. with bone marrow or bone marrow + adult spleen from normal or iso-immune CBA donors. Number of mice per group shown in brackets. Five controls, injected with saline only, died within 12 days.

I CBA b.m. (25).
II Immune CBA b.m. (20).
III CBA b.m. + adult spleen (25).
IV Immune CBA b.m. + adult spleen (26).

When normal or iso-immune CBA spleen cells were injected, most mice died very rapidly (median survival 11 and 13 days respectively); but a minority survived considerably longer.

Experiment III.—In Experiments I–II donor animals were hyper-immunized with 3 injections of host cells. Hyperimmunization, while producing large amounts of circulating humoral antibody, may not be the best way of producing sensitized cells such as have been shown by Billingham, Brent and Medawar (1954) to be involved in the reaction to foreign skin grafts, and which may also be of primary importance in the pathogenesis of " secondary disease ". A single large dose of spleen cells might possibly be more effective in producing cellular sensitization. In the present experiment, accordingly, survival of irradiated mice injected with

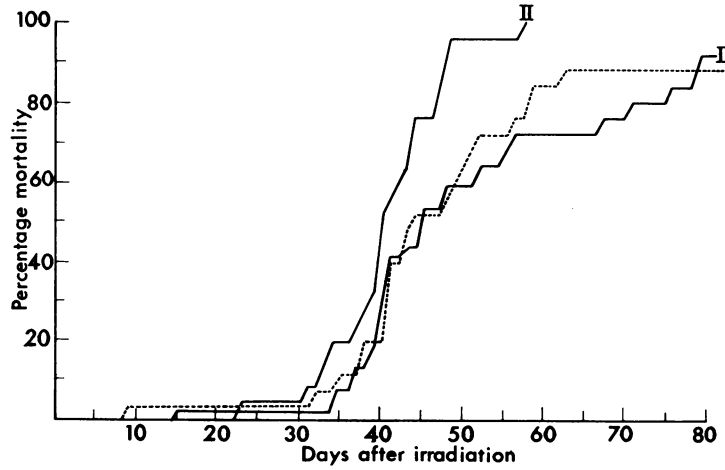

FIG. 3.—Cumulative mortality of CBA mice lethally irradiated and injected i.v. with bone marrow from A-strain donors. Fifteen mice in each group.

I Normal donors.

II Donors immunized 7 days previously with CBA spleen cell suspension injected i.p. (24×10^6 cells per mouse).

III Donors immunized 7 days previously with CBA spleen cell suspension injected i.p. (240×10^6 cells per mouse).

bone marrow from normal donors and from donors immunized 7 days previously with a single dose of host strain spleen was compared. The results are shown in Fig. 3. There appears to be little difference in survival between the groups. Secondary disease in the group given bone marrow from normal donors developed rather faster than in the comparable group in Experiment I; median survival was 45 days as opposed to 57 days.

Experiments IV and V: C3H → CBA.—These experiments were performed some months apart, but they were of similar design and yielded indistinguishable results. The mortality data have therefore been pooled and are presented in Fig. 4. The effects of adding spleen to the inoculum and of iso-immunizing the donors were in very sharp contrast with those seen in Experiments I and II. The injection of normal C3H spleen cells did not exacerbate secondary disease; on the contrary, it reduced it. Iso-immunization of the donors, on the other hand, greatly accelerated the disease even when bone marrow only was injected.

Experiment VI.—In Experiments IV and V injections of spleen from non-immune C3H donors did not induce secondary disease in the irradiated CBA host. This was in contrast with our results with A-strain donors and with the observed severity of the foreign spleen reaction in other laboratories (Makinodan, Gengozian and Shekarchi, 1958 ; Vos *et al.*, 1959). The present experiment showed that this apparent absence of reaction was also found after the injection of normal lymph-node or thymus cells. Three groups of irradiated CBA mice were treated with 2–5 $\times 10^6$ C3H bone marrow and 1 $\times 10^7$ C3H spleen or thymus or lymph-node

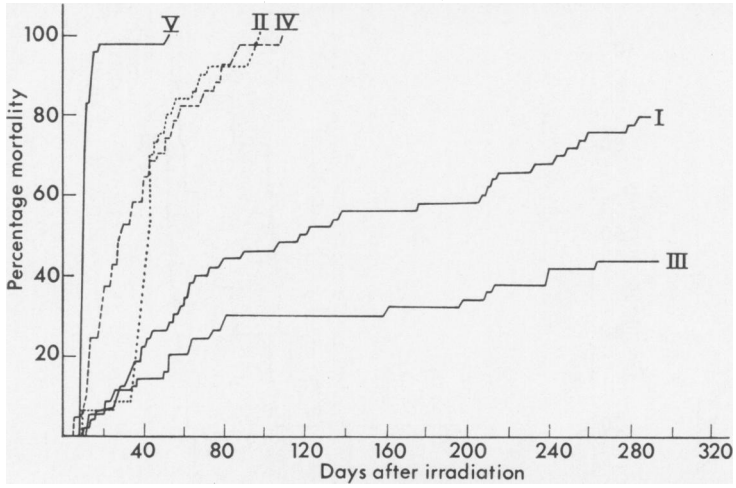

FIG. 4.—Cumulative mortality of CBA mice lethally irradiated and injected i.v. with bone marrow or bone marrow + adult spleen from normal or iso-immune C3H donors. Number of mice per group shown in brackets.

I C3H b.m. (50).
II Immune C3H b.m. (50).
III C3H b.m. + adult spleen (50).
IV Immune C3H b.m. + adult spleen (50).
V Saline only (50).

cells. Mortality is shown in Fig. 5. The long term survival of the spleen-treated mice was less good than in Expts. IV–V ; nearly all the deaths were attributable to lung infections. The survival of mice given thymus was better, and that of mice given lymph-node better still.

Clinical and pathological observations

All the groups (apart from the saline-injected controls) in all the experiments showed in some degree symptoms of what might broadly be termed " secondary disease ". But in detail there were important differences, probably related to the extent of antigenic diversity between donor and host and to the amount of viable lymphoid cells present in the inoculum given after irradiation. The different types of " secondary disease " are described below :—

Type 1 : *As found in* CBA *mice treated with one of the following :—normal* A *bone marrow ; iso-immune* A *bone marrow ; iso-immune* C3H *bone marrow ; iso-immune* C3H *bone marrow and spleen.*—Nearly all mice recovered from the immediate effects of radiation and began to regain weight. Beginning at the 3rd–

4th weeks after irradiation the weight began to fall again. Thenceforth the animals became increasingly emaciated and lethargic, and suffered from severe diarrhoea. They were hunched, huddled together more than normal mice, and were dull and ruffled of coat. Hair-growth was slight or absent. By death the body weight had sometimes sunk to as low as half the weight before irradiation. At autopsy the lymph-nodes and spleen were found to be small and most of the cells (especially the lymphoid elements) had been replaced by a palely staining eosinophilic hyaline substance. The liver and kidneys were small and the former often had areas of necrosis. Slight myocardial calcification was common. The thymus was completely

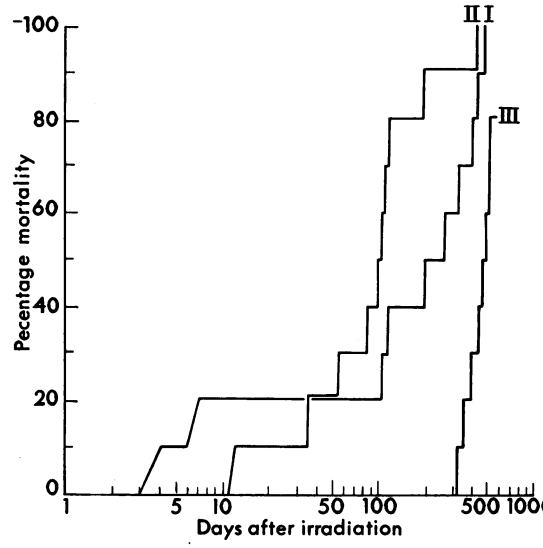

FIG. 5.—Cumulative mortality of CBA mice lethally irradiated and injected i.v. with bone marrow + thymus, spleen or lymph-node from normal C3H donors. Ten mice in each group.

I Bone marrow + thymus.
II Bone marrow + spleen.
III Bone marrow + lymph-node.

involuted. The lungs usually appeared normal. The bone marrow showed hyperplasia of granulopoietic elements. Haemorrhage and jaundice were absent. Overt signs of infection were rare. Dr. J. A. H. Brown of this laboratory has examined bacteriologically many CBA mice treated with A bone marrow and has never found bacteraemia except in the terminal stages of the disease (within about 24 hr. of death). A very similar secondary disease follows treatment of irradiated CBA mice with C57BL bone marrow.

Type 2: As found in CBA *mice treated with normal or iso-immune* A *bone marrow plus spleen.*—Secondary disease in these mice was similar to the above, but much earlier in its onset and more rapid in its course. Body weight was not regained after irradiation. The mortality overlapped that of the saline-injected controls, but histologically the cell-treated animals differed radically from the controls in that their bone marrow was exuberantly cellular, while that of the controls was aplastic. Myocardial calcification was much more frequent and severe than in Type 1 disease.

Type 3 : *As found in* CBA *mice treated with normal* C3H *bone marrow.*—Clinically the secondary disease of these mice closely resembled Type 1 for several weeks. However it was less lethal and about half the animals regained some weight, grew a healthy coat of grey fur and lived for up to a year after irradiation. In contrast to Type 1, infection appeared to be an important cause of both early and late deaths. Redness of peritoneum and mesentery, and consolidation of lungs were the commonest findings at autopsy. Lymph-nodes and spleen were often of near-normal size or even slightly enlarged.

Type 4 : *As found in* CBA *mice treated with normal* C3H *bone marrow plus spleen.*—Here such secondary disease as there was resembled Type 3. But mortality was lower and many of the mice were unaffected by the disease altogether. They grew healthy grey coats, had little or no diarrhoea, and enjoyed a life-span comparable with that of mice treated with syngeneic (CBA) bone marrow (Barnes, Loutit and Micklem, 1962).

Type 5 : *As found in* A *mice treated with normal or iso-immune* CBA *bone marrow.*—A mice rarely suffered from diarrhoea, but wasting and atrophy of lymphoid tissue were found. Pleural effusion, pulmonary consolidation, or both were generally found at autopsy.

Type 6 : *As found in* A *mice treated with normal or iso-immune* CBA *bone marrow plus spleen.*—Findings at autopsy resembled those in Type 2 secondary disease, but myocardial calcification was not seen.

DISCUSSION

The results of these experiments illustrate the diverse nature of what is commonly called " secondary disease " and the variability of its course and outcome. There is every reason to think that yet other manifestations of this protean disorder are found in other laboratories and different strains of mice.

Some of the mortality data confirm the observations of other workers that treatment with foreign spleen (with or without bone marrow) is followed by a more rapid demise of the irradiated hosts than treatment with bone marrow alone (Makinodan *et al.*, 1958 ; Vos *et al.*, 1959). On the other hand data from Experiments IV, V and VI (C3H → CBA) are in direct conflict with these observations ; there the addition of mature lymphoid tissue from spleen, lymph nodes or thymus to the inoculum did not shorten, but markedly lengthened, the survival of the irradiated hosts.

The different experiments also yielded contrasting data on the effect of immunizing the donors of bone marrow and spleen against host antigens. In two strain-combinations (CBA → A ; A → CBA) iso-immunization of donors had at most a small effect on the survival of the hosts. Yet in a third strain-combination (C3H → CBA) under similar conditions survival was dramatically abbreviated.

The contrasts provided by the experimental results are striking and interesting. It is useful to survey them in relation to the hypothesis that secondary disease in lethally irradiated mice is fundamentally due to a reaction by the grafted tissue against the host. This hypothesis is now supported by a considerable amount of evidence (Uphoff, 1957 ; Trentin, 1957 ; Barnes, Ford, Ilbery and Loutit, 1958 ; Uphoff and Law, 1958 ; Feldman and Yaffe, 1958), although some studies (Van Bekkum, Vos and Weyzen, 1959) suggest that other factors too may play a part. At first sight our present results may be divided into two portions, those which

support the hypothesis and those which do not. On the one hand we found that in certain strain combinations mortality from secondary disease was increased when iso-immune tissue donors were used or when spleen was injected in addition to bone marrow. These observations are paralleled in the literature (Makinodan *et al.*, 1958 ; Ilbery *et al.*, 1958 ; Vos *et al.*, 1959 ; Cosgrove *et al.*, 1959) and are most readily interpreted in terms of a graft-versus-host hypothesis. There is no reason to doubt that a graft-versus-host reaction is often at least an initiating factor in secondary disease. On the other hand, we found that in other strain combinations iso-immunization of the donors had at most a marginal effect on the survival of the hosts, and that the addition of spleen to the inoculum did not increase or accelerate secondary disease, but on the contrary almost prevented its occurrence. These two observations appear to be in conflict with the graft-versus-host hypothesis. The first of them does not really conflict seriously. Where there is strong incompatibility between donor and host (in this case a difference at the H-2 locus), it would not be surprising if the induction period for the immune response were so short that pre-immunization of the donor had little effect on the outcome of the disease. The second observation conflicts with the graft-versus-host hypothesis in a more important way and must be considered in detail.

In the C3H $\rightarrow$ CBA strain combination, treatment with normal adult bone marrow was followed by symptoms of secondary disease as enumerated above. If the syndrome was set in motion by a graft-versus-host reaction, one would expect it to be intensified (as it was in other strain combinations) by the addition of spleen to the inoculum. But this did not happen. On the contrary, the animals treated with spleen in addition to bone marrow fared much better than those treated with bone marrow only. A few died with secondary disease, but most avoided it altogether and became healthy, grey-coated mice without any sign of diarrhoea. Injection of normal thymus or lymph node cells in place of spleen resulted in even better survival. These results suggest that a graft-versus-host reaction in CBA mice treated with normal C3H bone marrow either does not occur or, if it does, is so slight as to lead to negligible damage. The symptoms of this secondary disease must have some other cause—a cause which is largely removed by the injection of lymphoid cells. The simplest explanation is that secondary disease in these animals is caused by an inadequate lymphoid system and that this is in turn caused by an inadequate number or failure to mature of lymphoid precursors in the therapeutic bone marrow inoculum. It is not certain in what ways lymphoid inadequacy could produce secondary disease symptoms, and our current ignorance concerning the functions of lymphocytes makes lengthy speculation pointless. Perhaps the most likely way is by making the chimaera susceptible to chronic infections. In any case it would follow that one of the prerequisites of a healthy chimaera is an adequate initial transplant of lymphoid precursors.

This brings us to another important point. In most strain-combinations the transplantation of normal allogeneic lymphoid tissue into lethally irradiated mice results in a rapid anti-host reaction. There is reason to think that, despite the similarity of their H-2 antigens, C3H cells can under suitable conditions mount quite a strong immune reaction against CBA antigens ; in the present experiments treatment with iso-immune C3H bone marrow and spleen resulted in heavy mortality of CBA recipients, presumably due to an anti-host reaction. For some reason, nevertheless, non-immune C3H cells do not react against the CBA host ; in other words they do not become sensitized. A-strain cells are evidently well

able to become sensitized against the CBA hosts in the body, and it is difficult to explain why C3H cells should not do likewise. However it is possible to advance a speculative explanation in terms of immunological tolerance.

It is now well-known that at least in some strain combinations, treatment of lethally irradiated mice with foetal allogeneic tissue (liver and spleen) is followed by far less secondary disease than treatment with adult bone marrow (Barnes, Ilbery and Loutit, 1958 ; Uphoff, 1958 ; Lengerová, 1958 ; Urso, Congdon and Owen, 1959). Our experience has been that the disease is often completely avoided. This contrast between foetal and adult cell therapy has been widely attributed to immunological tolerance shown to the host by the grafted foetal tissue. Such an explanation is theoretically acceptable, since animals are known to become tolerant of iso-antigens to which they are exposed in embryonic or neonatal life. Work in this laboratory (Lengerová, Micklem and Dent, 1961 ; and unpublished) has provided direct evidence in favour of the tolerance hypothesis by demonstrating that an embryonic graft does in fact show tolerance to another graft of cells injected shortly afterwards and may therefore by inference show it to the host. It is clear, then, that secondary disease may be largely or completely avoided under conditions where the graft may, and probably does, show tolerance to the host. Now it may be that the capacity to show tolerance is not the exclusive property of immature animals. It may also be present in adult animals, if at some early stage of their maturation cells respond to an antigenic stimulus by becoming tolerant ; or alternatively (following the clonal selection hypothesis of Burnet (1957, 1959) if potentially reactive immature cells are at some stage in their development destroyed by contact with antigen. The idea that tolerance may be produced in the adult was put forward by Loutit (1956) and by Lederberg (1958). If it is true that maintenance of tolerance depends on the persistent presence of antigen, then (as Medawar (1960) has argued) the clonal selection hypothesis almost necessarily involves the continuation into adult life of the capacity to develop tolerance, and such a continuation is by no means necessarily ruled out even if the clonal selection hypothesis is rejected. Supposing that the capacity to show tolerance does indeed continue into adult life, its persistence would normally be masked by the immune response given to an antigen by the mature reactive cells. But if only a few mature reactive cells were available to react, then the mask might not operate. There is reason to think that the stage at which animals pass from immaturity (*i.e.* tolerance-responsiveness) to maturity (*i.e.* immune-responsiveness) is not the same for all antigens. This rests on the observation of Billingham and Silvers (1960) that C57BL female mice may be made tolerant of C57BL male iso-antigen (governed by a locus on the Y-chromosome) as long as 17 days after birth, which is a far longer tolerance-responsive period than has been found with stronger iso-antigens (Billingham and Brent, 1959). In the ontogenesis of the whole animal, then, tolerance is accorded to some antigens for longer than to others. It seems reasonable to transpose this observation to the cellular level and suggest that the capacity to show tolerance to strong iso-antigens is lost at an earlier stage of cell maturation than when weak iso-antigens are concerned. In our experiments strains CBA and A have mutually strong iso-antigens, while in C3H and CBA the mutual antigens are probably much weaker (being identical at the important H-2 locus). It is tempting to think that the failure of normal C3H lymphoid cells to react against the CBA hosts, and their apparent ability to provide the chimaera with an adequate tolerant lymphoid tissue, is due to only

the most mature cells in the inoculum being able to give an " adult " immune reaction to the weak host antigens. These mature cells might die in the normal course of events before the induction period of the primary immune response against the host could be completed ; where the donors were immunized against CBA antigens, and therefore no induction period in the irradiated hosts was necessary, there was apparently a pronounced anti-host reaction.

The above argument would receive further experimental support if it could be shown that in the CBA ← immune C3H combination irreversible tissue damage resulting in secondary disease, is done during the first few days after treatment. This concept of immediate, irreversible tissue damage is supported by the results of Siskind and Thomas (1959) using an analogous system (the injection of allogeneic spleen cells into newborn mice). They found that if syngeneic spleen cells were given to newborn mice, within 30 minutes after allogeneic spleen cells, the runting syndrome caused by the latter was prevented ; but if the injection of syngeneic cells was delayed for one day, runting was not prevented. Such an experimental approach could be employed to determine at what stage after irradiation and treatment with allogeneic tissue secondary disease becomes inevitable and irreversible. If the distinction suggested above between the secondary disease in CBA ← normal C3H bone marrow chimaeras (held to be due to lymphoid inadequacy) and the disease of some other allogeneic chimaeras (held to be due to lymphoid inadequacy superimposed and perhaps consequent upon initial tissue damage) is real, then it might be possible in the first case to cure the disease at any stage by an adequate transplant of myeloid and lymphoid tissue syngeneic with the host, while in the second case such treatment would at best provide some alleviation of the symptoms.

CONCLUSIONS

The arguments advanced above are largely speculative. However it appears that at least two causative factors may operate in secondary disease. These are : an inadequacy of the chimaera's lymphoid system and a graft-versus-host immune reaction. Under most conditions adjustment of the therapeutic inoculum to remedy the first cause (by including more lymphoid precursors) must be expected to exacerbate injury due to the second cause (by including of necessity more immunologically reactive cells). But in one set of conditions we have found that this drawback is not present. It may be that these conditions can be reproduced in other donor-host strain combinations where the antigenic differences are not too great. That would have encouraging implications for the treatment of irradiated humans with foreign adult cells.

SUMMARY

Mice lethally irradiated and treated with foreign bone marrow frequently suffer from " secondary disease ". The clinical appearance, time of onset and severity of the disease vary with the donor tissue and the host. In two donor-host strain combinations iso-immunization of the donor against antigens of the prospective host has little effect on the syndrome, while addition of spleen cells (normal or iso-immune) to the bone marrow inoculum markedly accelerates and aggravates it. In a third combination, on the other hand, where donor and host are closely related, iso-immunization of the donor accelerates and aggravates the syndrome,

while the addition of normal spleen to normal bone marrow actually reduces its incidence and results in better health and longer survival of the hosts.

A speculative explanation of these contrasting results in terms of immunological tolerance is put forward.

It appears that major causes of secondary disease in radiation chimaeras are lymphoid atrophy and a reaction by the graft against the host. Under certain conditions the former may be present and produce the disease even when the latter does not occur.

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