Immunologic Mechanisms in Heavily Irradiated Mice Treated with Bone Marrow

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HEAVILY IRRADIATED MICE treated with bone marrow from a different mouse strain (homologous marrow) recover from the immediate effects of irradiation, but often develop a peculiar fatal illness that comes on as radiation recovery progresses. This late illness is seen in other irradiated animal species treated with antigenically nonidentical marrow cells, and has been termed the "foreign bone marrow reaction." Irradiation with marrow donation from an identical strain (isologous marrow) causes only minimal and temporary illness.

The basic nature of the "foreign bone marrow reaction" is not known, but has been assumed to be related to immune processes since it appears as immune mechanisms return, and morphologically is manifested by severe atrophy of lymphatic tissue.

If these late deaths are due to histoincompatibility, is death due to host tissue reaction against the bone marrow graft, or graft against the host? Evidence is conflicting. Uphoff and Trentin have both demonstrated that reactions occur with donor marrow from parent strains into irradiated F1 hybrid mice, a situation in which the only incompatible antigens are in the host and any reaction, then, should be against host tissues. Similarly, Trentin has demonstrated skin graft compatibility when skin and marrow were from the same donor. Both Uphoff and Trentin conclude from their studies that the active immunologic processes are from the donor marrow tissue. There are a few inconsistencies in some of these experiments, however, that may possibly allow for other interpretation of the findings.

Morphologically, leukocytes, platelets and erythrocytes have all been demonstrated to arise from the donor marrow after irradiation. Recently, two observations have indicated that the lymphocytes, too, were derived from donor. Gengozian and Makinodan have reported that thymus lymphocytes in mice treated with rat bone marrow are immunologically identifiable as rat type. Similarly, Uphoff has reported that spontaneous lymphatic system neoplasms arising in animals previously treated with homologous mouse marrow usually are accepted in transplantation in the marrow donor strain rather than in the host strain.
All of these observations present strong arguments for the hypothesis that marrow infusion in irradiated animals transplants not only hematopoietic cells but also immunologically active tissue. Makinodan, however, has reviewed a number of observations that support the concept of humoral antibody production remaining a function of the host. He has noted that antibodies persist for prolonged periods after irradiation and marrow protection. Of major importance is the fact that the gamma globulin, by sensitive agar diffusion methods, remains of host and not donor type.

With these conflicting observations, all apparently technically correct, we undertook further studies of the immunologic mechanisms of the irradiated, marrow-treated mouse.

**Materials and Methods**

The studies were performed in irradiated C3Hx101 or C57 Black x 101 hybrid mice treated with isologous (strain specific) bone marrow or spleen cell suspension unless otherwise stated. Animals of both sexes 10 to 14 weeks of age were used in the experiments.

The irradiation technic has been described in detail elsewhere, but a total dose of 700 r by our calculations was used. This dosage was in the supralethal range, as evidenced by the fact that 142 mice treated as controls in various experiments all died before the 13th day postirradiation.

The donor marrow cell suspensions were prepared by injecting suspending medium (Hank's solution or saline) through a No. 23 gage needle and syringe attached to one end of the cut femoral shaft. The plugs of expressed marrow were suspended by agitation in and out of the syringe, and cell counts made by conventional white blood cell counting technics. Splenic cell suspensions were prepared by expressing minced spleen through a garlic press and suspending the cells by agitation to and fro in a tuberculin syringe. Standard irradiation protection was achieved by the cells of one femoral bone marrow or one-half a spleen.

Two methods of studying the state of immunity in mice were used. Both rely on a functional end point, rather than measurement of serum antibody, but are expressions of humoral antibody production capacity. We had previously found that chromium-labeled rat erythrocytes injected into normal mice were destroyed after 3 to 5 days. Mice previously injected with rat erythrocytes developed immunity, as evidenced by the observation that a second injection of cells was eliminated from the circulation within a few hours. In mice irradiated before rat erythrocytes had been given, no significant acceleration of cell destruction occurred. These findings are illustrated graphically in figure 1. In mice challenged with intravenous suspensions of labeled rat erythrocytes, the degree of immunity was established by the percentage of the injected radioactivity remaining in the circulation 24 hours after injection of the test cells. In normal animals given a single immunizing dose of rat cells 1 to 4 weeks before challenge, less than 20 per cent of the challenging labeled rat cells remained in the 24 hour blood sample. In control nonimmunized animals, almost 100 per cent of rat cells were retained for 24 hours.

To obtain test cells, rat blood was obtained by heart puncture using a syringe lightly rinsed in heparin solution and was incubated with radioactive sodium chromate (approximately 1 microcurie/ml. of blood). The cells were washed twice and resuspended in 0.85 per cent NaCl to obtain 30 to 50 per cent erythrocytes. One-half milliliter of labeled cells was injected intravenously, and at the appropriate times the animals were sacrificed by decapitation and the radioactivity of a measured volume of blood detected in a well-type scintillation counter. Calculation of the rat erythrocytes remaining in circulation was based on the percentage of radioactivity retained, presuming an original 2 ml. blood volume in these 20 to 30 Gm. mice. Data from at least two mice, and usually more, were averaged for each determination.

Growth of the mouse leukosis E.L. 4 in C3Hx101 recipients was used as a further...
Fig. 1.—The disappearance of chromium\textsuperscript{51}-labeled rat erythrocytes from the circulation of normal, irradiated and previously immunized mice.

measure of antibody production capacity. Amos and Day\textsuperscript{23} reported that this tumor, indigenous in strain C57 Black mice, regularly regressed in C3H\times101 mice as humoral antibodies were produced. In the experiments, tumor cells from the ascitic phase of growth in a C57 Black mouse were injected subcutaneously in the experimental mouse. Total tumor cells implanted varied from 1.3 million to 3.0 million. The diameters of the subcutaneous tumors were measured at appropriate times postinjection, and data expressed as square millimeters of body surface involved with the tumor.

RESULTS

Studies of the immunologic response in the nonimmunized mouse after irradiation and isologous (strain-specific) marrow treatment.—Labeled rat erythrocytes were injected intravenously into irradiated, isologous marrow-treated mice at various times in the postirradiation period. Immediately
after irradiation, the rat erythrocytes exhibited a near normal life-span in the irradiated mouse. As noted previously, without active production of anti-rat erythrocyte antibodies the mouse exhibits no basic incompatibility toward these cells.\textsuperscript{12} Destruction of rat erythrocytes injected 10 days after irradiation was accelerated, but was not as rapid as in the normal mouse. Mice 30 days postirradiation and marrow treatment destroyed rat erythrocytes essentially as did normal animals. These observations are depicted in figure 2.

Similarly, the E.L. 4 tumor grew in the C3Hx101 recipients immediately after irradiation, but at two weeks immune mechanisms were beginning to reappear. After four weeks antibody production seemed essentially normal, as evidenced by the rapid regression of the implanted histoincompatible tumor (fig. 3).

Further evidence of the regeneration of antibody-production potential in these animals was obtained by later challenges of mice studied with rat erythrocytes in the postirradiation period. Thus, eight mice were given rat erythrocytes intravenously immediately postirradiation (as in figure 2) and were challenged 4 to 8 weeks later with Cr\textsuperscript{51}-labeled rat erythrocytes. Three of the eight mice reacted to the second challenge as an immunized mouse and destroyed the rat erythrocytes within 24 hours. All mice given rat erythrocytes two weeks after irradiation and marrow protection exhibited an immune response on challenge 4 to 8 weeks later.

Study of mice immunized before irradiation and treated with isologous
Fig. 3.—Regression rate of E.L. 4 tumor in histoincompatible mice (C3Hx101) at intervals following radiation and isologous marrow.

Marrow or spleen.—Mice immunized with a single intravenous injection of rat erythrocytes were irradiated one week later and treated with isologous bone marrow. These animals were then injected at later intervals with labeled rat cells, and the amount of cell destruction at 24 hours ascertained. During the first six weeks, at times when the previously described studies had indicated good return of responsiveness to antigen by the irradiated, marrow-treated mice, the animals remained solidly immune (fig. 4).

Gradually, during the later periods of observation, immunity was lost more rapidly in the animals treated with irradiation plus isologous bone marrow than in the nonirradiated control group (fig. 4). This loss of immunity by the irradiated, marrow-protected group as a late effect is highly significant. Statistical analysis using the ranking test of White was applied to the observations (fig. 4) beyond six weeks postirradiation, and the changes were found significant at the 1 per cent level.

More definite evidence of prolonged persistence of immunity was obtained in a group of mice subjected to repeated irradiation and protection with either isologous bone marrow or isologous spleen. These mice had first been hyperimmunized by three injections of rat erythrocytes. Three supralethal
irradiations with protection by bone marrow or spleen were performed at intervals of one month. After the three treatments all animals remained solidly immune to challenge. Indeed, for four and one half months all animals remained immune except for two that had received the largest injection of spleen cells.

Attempts to obtain passive transfer of immunity.—Passive immunization of normal mice by 0.3 ml. of hyperimmune mouse antirat erythrocyte serum produced an immune response to challenge with labeled rat erythrocytes that persisted for only two or three weeks. From this observation, it seemed evi-
dent that sudden cessation of active antibody production would result in loss of immunity by our testing method in a very brief period.

Repeated attempts failed to transfer antirat erythrocyte immunity by means of marrow donation from immune animals into irradiated recipients. In one experiment, irradiated mice were treated with either normal or hyperimmune donor marrow. No immediate immunity was detected by this transfer, and one month later both groups were given labeled rat erythrocytes intravenously. Both groups exhibited a destruction rate of these cells identical to that of normal mice (shown in figure 1). There was no evident secondary antibody response in the mice previously treated with rat-cell immune donor marrow.

With spleen suspensions from rat-cell immune mouse donors, it was possible to demonstrate transient immune response (lasting two to three weeks) to challenge with labeled rat erythrocytes.

Studies in animals treated with homologous (not strain specific) marrow.—Homologous marrow was injected into irradiated mice that had been previously immunized to rat erythrocytes. Swiss mouse marrow was used in immunized C3Hx101 mice. Since lymphatic tissue atrophy is regularly seen in these homologous marrow-treated animals, it was reasoned that previous immune processes might cease. Although there is no way to be certain of the “take” of a homologous marrow, we had been able to achieve “takes” of rat marrow in mice similarly treated, as evidenced by the circulation of immunologically identifiable rat erythrocytes. These homologous marrow-treated mice had frequent late deaths (the foreign bone marrow reaction) which also suggested that the marrow was of the donor strain. Because of the high death rate due to the foreign bone marrow reaction, only 28 mice out of several experiments were available for challenge four weeks or longer following irradiation and homologous marrow. Thirteen of these 28 animals retained their immunity to rat erythrocytes.

Some homologous-treatment animals of C3Hx101 strain had been treated with C57 Black x 101 marrow. E.L. 4 tumor, which is histocompatible with the donor only in this circumstance, was injected subcutaneously and followed for the occurrence of immune rejection.

One month after irradiation and isologous marrow treatment, we had found that antibody production was sufficient to induce regression of the tumor. In two experiments, 23 C3Hx101 mice given marrow from C57 Black x 101 donors survived for six to eight weeks. On challenge with E.L. 4, 13 mice rejected the tumor, but more slowly than normal C3Hx101 mice. Ten animals died, but some died with relatively small tumors and were thought to have had foreign bone marrow reaction as a cause of death.

In another experiment, C3Hx101 mice were given marrow from C57 Black x 101 mice, and C57 Black x 101 mice were given C3Hx101 marrow. Although only six animals from each group of homologous marrow-treated animals survived four weeks, these animals died at identical rates of progressively growing E.L. 4 tumor.

Although these experiments were not conclusive, it seemed that the most likely explanation of the observed failures of the tumor to regress in histo-
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incompatible recipients was due not to specific replacement with histocompatible marrow, but to the nonspecific inanition of animals suffering from the foreign bone marrow disease.

DISCUSSION

Our results, working primarily with a system of immunity based on characteristic patterns of in vivo lysis of rat erythrocytes by normal and immune mice, indicate that treatment of severe irradiation with bone marrow cells does not supplant the humoral antibody production mechanism of the host with those of the donor. Thus, ability to respond to antigen is temporarily impaired after irradiation and marrow protection, in a manner very similar to that of the sublethally irradiated mouse. Treatment of the immunized mouse with isologous marrow, isologous spleen or homologous marrow did not abruptly cause cessation of antibody production, although irradiation plus isologous marrow treatment shortens the general duration of immunity. Finally, only temporary transfer of immunity by donor spleen was achieved, and that immunity disappeared at the general time of recovery of the active immunologic processes of the host.

Makinodan in a series of observations has also concluded that humoral antibody production of the host is not displaced by tissues of the donor. On the other hand, there is the impressive array of observations that implicate the donor tissues as being of major importance. Particularly, the genetic studies of Trentin and Uphoff very strongly suggest that immune tissues from the donor regenerate and overwhelm the host. Certainly, the observations of Gengozian and Makinodan and of Uphoff indicate that donor lymphatic tissue is permanently transferred to the irradiated mouse.

Are these two sets of observations necessarily incompatible? Since all the data obtained appear sound, it seems likely to us that the general observations must be accepted as fact. Therefore, we must conclude that in animals treated with bone marrow some immune functions of the host are retained, while some factors are derived from the donor. One can easily postulate, for example, that antibody production continues in certain of the host cells at the same time that donor cell lymphocytes or lymphatic tissue (or possibly even granulocytes) are reacting in another, different way against the host tissues or against a skin graft.

It would also be possible to explain these divergent postulates on the basis of humoral antibodies being produced by the host tissue, with tissue antibodies coming only from the marrow graft. Certainly, no definitive evidence exists for the assumption that these two types of immune responses are basically different. However, such an explanation would seem to fit with the known facts.

Our studies confirm the repeated observations that the cells responsible for continued antibody production are not radiosensitive, and that they do not seem to be replaced by donor cells in these experiments. Indeed, almost all of our observations could be readily explained by the hypothesis that the cells responsible for sustained antibody productions are radioresistant and
nontransplantable, while the cells capable of the rapid subdivision that has been demonstrated to occur after antigen injection\(^\text{19}\) are both radiosensitive and transplantable from normal bone marrow. Such a simple cellular explanation of the complex observations in the immunology of marrow transplantation seems unlikely, however. More complete understanding of the immunologic problems posed by marrow transplantation seems likely to add basic understanding to the whole process of immune reactions.

**Summary**

1. Humoral antibody production has been studied in severely irradiated mice treated with isologous (same strain) or homologous (different strain) bone marrow.

2. The two methods of study involved functional end points of humoral antibody production as evidenced by in vivo lysis of rat erythrocytes or by regression of mouse leukemia E.L. 4 in histoincompatible mouse recipients.

3. Humoral antibody production was lost after irradiation and isologous marrow treatment, but recovered partially in two weeks and almost completely in four weeks.

4. Established immunity was not abruptly terminated after irradiation and treatment with either isologous or homologous marrow, although there was premature loss of immunity to rat erythrocytes by the irradiated, isologous marrow-treated mouse.

5. Permanent immunity could not be transferred by isologous marrow or spleen from immunized donors to irradiated recipients.

6. Treatment of mice histoincompatible to E.L. 4 leukemia with histocompatible donor bone marrow failed to establish rejection of the tumor.

7. These studies support the concept that humoral antibody production in irradiated, marrow-treated mice remains a function of the host rather than of the transplanted tissues.

8. These studies failed to clarify the conflicting evidence concerning the mechanism of the late illness that occurs after treatment of the irradiated mouse with bone marrow from a different strain or species.

**Summario in interlingua**

1. Le production de anticorpore humoral esseva studiate in severmente irradiate muses que esseva tractate con medulla ossee isologe (ab muses del mesma racia) o homologe (ab muses de altere racias).

2. Le duo methodos de recerca comportava functional punctos terminal del production de anticorpores humoral—evidentiate per le lyse in vivo de erythrocytos de rattos o per le regression de leucosis murin E.L.4 in recipientes histoincompatibile.

3. Le production de anticorpore humoral esseva perdite post irradiation e tractamento con medulla isologe, sed illo esseva recovrate partialmente intra duo septimanas e quasi completamente intra quatro septimanas.

4. Le immunitate, un vice establite, non esseva terminate abruptemente post le radiation e le tractamento con medulla isologe o con medulla homologe, sed il esseva notate un perdita prematur de immunitate contra erythrocytos de ratto in irradiate muses tractate con medulla isologe.
5. Immunitate permanente non poteva esser transferite per medulla isologe o splen isologe ab donatores immunisate a recipientes irradiate.
6. Le tractamento de muses histoincompatible a leucosis E.L.4 con medulla ossee ab histocompatibile donatores non resultava in le rejection del tumor.
7. Iste studios supporta le theses que le production de anticorpore humoral in irradiate muses tractate con medulla ossee remane un function del hospite e non del histos transplantate.
8. Le studios non succedeva a clarificar le observationes contradictori relative al mechanismo del morbo tardive que occurre post le tractamento del irradiate muses con medulla ossee ab un altere racia o un altere specie.

REFERENCES
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