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Requirement for a Bone Marrow-Derived Component in the Expression of Cell-Mediated Antibacterial Immunity

H. HAHN[1]

Trudeau Institute, Inc., Saranac Lake, New York 12983

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Mice were X irradiated with 400 R and 1 week post-irradiation were found to be unable to develop antilisterial immunity after active or passive immunization with immunologically committed spleen lymphocytes from *Listeria*-immune donors. This consequence of irradiation disappeared spontaneously within 21 days of exposure to X rays. Mice irradiated with as much as 900 R could be passively protected by immunologically committed lymphoid cells from *Listeria*-immune donors 10 days after irradiation if they had been given normal bone marrow cells on the day of irradiation. It is concluded that, in addition to immunologically committed lymphocytes, a second cellular component is needed for the expression of antibacterial immunity. This second component is bone marrow derived.

Cell-mediated immunity to intracellular bacterial infections is commonly considered to rest upon a collaborative effort of immunologically committed lymphocytes (10) of the T cell class (5, 15) and mononuclear phagocytes acting nonspecifically (9). No direct evidence has yet been presented, however, proving the indispensability of the second cell component which is thought to express antibacterial immunity, nor has the tissue from which it is ultimately derived been identified.

In the experiments reported here, ionizing irradiation of mice was used to destroy the animal's capacity to muster an effective defense against *Listeria monocytogenes*. Cellular reconstitution of irradiated mice showed that, in addition to committed lymphocytes, a second cell type was needed to restore the animal's capacity to express cell-mediated antibacterial immunity. The cell concerned was present in the bone marrow of nonimmune subjects.

MATERIALS AND METHODS

Mice. Outbred, specific-pathogen-free mice of the CD-1 strain (Charles River Breeding Laboratories, Wilmington, Mass.) or mice of the inbred A/J strain (Jackson Laboratories) were used. Both were bred at the Trudeau Institute. Animals of both sexes were used, but not in the same experiments.

Microorganisms. A mouse-virulent strain of *L. monocytogenes* (strain EGD) was used. It was maintained by continuous passage in mice, the intravenous mean lethal dose being less than 5×10^3 organisms. For infection, a log-phase culture was counted in a Petroff-Hauser chamber and suitably diluted in saline to contain the desired number of bacteria in 0.2 ml. All inocula were injected intravenously, and the viable counts were confirmed by plate count using Trypticase soy agar.

Bacterial enumeration in liver and spleen. The procedures have been described previously (16).

Preparation of spleen cell suspensions and measurement of protective immunity. For passive protection experiments, cotton column-filtered spleen cell suspensions from immunized mice were prepared as described (8). Appropriate numbers of viable cells were injected intravenously into nonimmune recipient mice, immediately followed by the bacterial challenge inoculum. Protection against the challenge infection was measured in terms of the numbers of viable bacteria present in spleens and livers 24 and 48 h postinfection.

Bone marrow and cell transfer. Marrow cells were extruded from the femur or tibia of freshly killed A/J mice by flushing the marrow cavity with 0.5-ml volumes of Hanks balanced salt solution containing 1% fetal calf serum and 5 IU of heparin per ml. After breaking up the cellular plugs, the suspension was filtered through four layers of gauze. The cells were counted and suitably diluted in balanced salt solution for intravenous injection.

Irradiation. Irradiation was performed with a small-animal irradiator as previously described (18).

Histology. Samples of livers and spleens were cut from weighed organs so that bacterial counts could be performed on the same tissues and corrected for weight change. The tissues were fixed overnight in 10% formaldehyde, washed and dehydrated in ethanol, embedded in hydroxymethylacrylate, and sectioned to a thickness of 2 to 3 μm. Sections were stained with methyl green-pyronin (13).

[1] Present address: Department of Medical Microbiology, The University of Mainz, D 65 Mainz, Germany.

RESULTS

Growth of Listeria in irradiated mice. A group of 15 CD-1 mice was exposed to 400 R of gamma irradiation. After 1 week they were infected, along with unirradiated controls, by an intravenous injection of approximately 2×10^3 *L. monocytogenes*.

Figure 1 shows growth of *Listeria* in normal and irradiated mice. The former displayed evidence of developing resistance after 48 h of infection, but in the irradiated animals the organisms grew uninterruptedly throughout the period of observation. On the other hand, mice irradiated 24 h before infection proved resistant, and bacterial growth patterns did not differ measurably from those in control animals (results not shown).

Irradiation at 400 R represents a sublethal dose from which most adult mice eventually recover. It was of interest, therefore, to determine the rate at which mice regained their ability to develop an immune response against *L. monocytogenes* after 400 R of gamma irradiation.

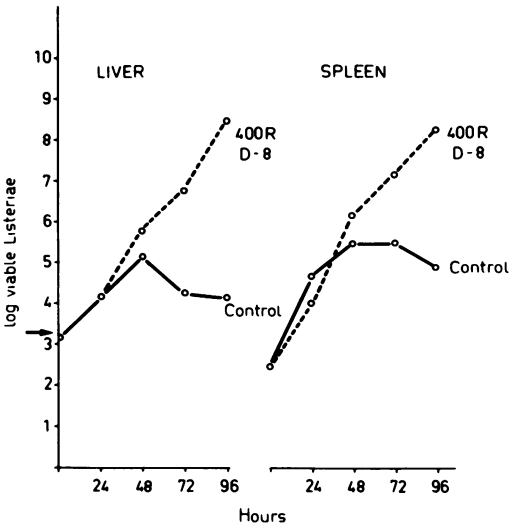

FIG. 1. *Growth of Listeria monocytogenes in livers and spleens of mice which have been irradiated with 400 R 1 week before infection. Arrow on ordinate denotes size of bacterial inoculum.*

Recovery of responsiveness to Listeria after whole-body irradiation. Groups of mice were drawn from a large pool of animals of comparable age. At varying times before a challenge infection, mice belonging to one group were irradiated (400 R). An excess of mice in each treatment group made it possible to choose 20 animals of comparable weight from each of the treatment groups. On the day of challenge, mice of all groups, including a control group of unirradiated animals, were injected intravenously with approximately 10^3 living *L. monocytogenes*. Bacterial counts were performed 24, 48, 72, and 96 h after infection.

On days 10 and 17 after irradiation, the animals were poorly equipped to defend themselves, but by day 24 they could again check the infection in liver and spleen and showed a marked splenomegaly (Fig. 2). This feature of the normal response to infection (14) was not seen in mice infected 10 or 17 days after irradiation. The spleens in these mice were almost acellular and teeming with bacteria when examined on the 4th day of infection. It is presumably because the spleens had become essentially necrotic that bacterial counts did not continue to increase beyond the 3rd day (Fig. 2b).

The paucity of phagocytic cells in the lesions of animals infected 10 or 17 days after irradiation could have been due to the unavailability of circulating monocytes because of damage sustained by bone marrow precursors. If true, this would mean that an infusion of lymphoid cells obtained from preimmunized donors does not suffice to protect irradiated mice, and that at least one more component is needed for passively transferred immunity to be expressed. This possibility was examined in the following experiment.

Effect of irradiation on passive protection with immune lymphoid cells. Previous observations had shown that the capacity to express delayed-type hypersensitivity reactions is lost in parallel with the disappearance of monocytes from the circulation and that this does not occur abruptly in animals exposed to 400 R. Instead, monocytes disappear gradually from the circulation when their source of supply from precursors in bone marrow is cut off by ionizing irradiation (18). Therefore, the possibility could be tested that the capacity to express cell-mediated antibacterial immunity after passive transfer of immune lymphoid cells and infection might be a function of the available supply of a radiosensitive component that disappears gradually in irradiated animals.

Groups of 10 mice of comparable weight were selected from pools of mice that had been exposed to 400 R of irradiation either 21, 10, or 1 day previously. The irradiated mice, together with appropriate controls, were injected intravenously with 10^8 spleen cells obtained from immune donors with a 6-day-old listerial infec-

tion. The irradiated mice, the unirradiated controls, and a group of animals that had received neither cells nor irradiation were then infected intravenously with 2×10^4 viable *Listeriae*, and bacterial growth was measured for the ensuing 48 h (Fig. 3).

The data indicate that whereas mice could be adoptively immunized 1 or 21 days after irradiation, no protection was conferred at all on mice irradiated 10 days previously. Thus, it was clear

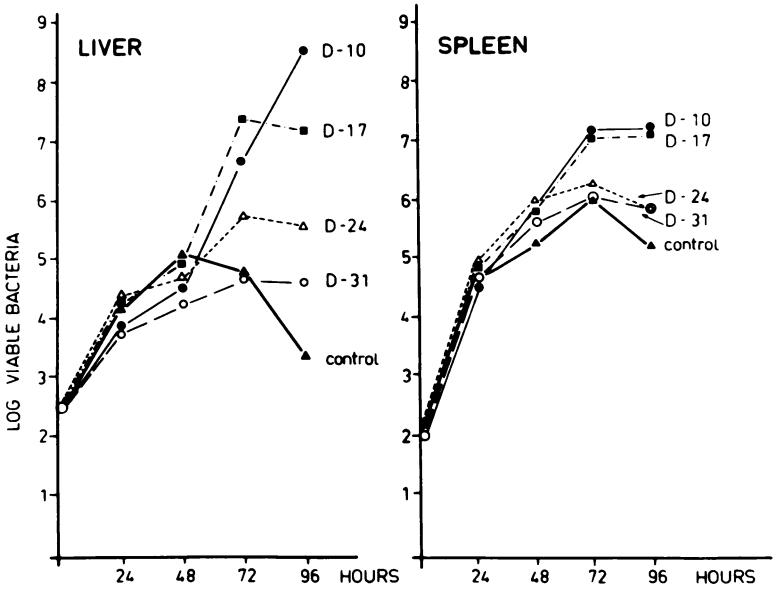

FIG. 2. *Growth of Listeria monocytogenes in livers (a) and spleens (b) of mice that have been irradiated with 400 R before infection on the days indicated. Bacterial counts in spleens did not increase beyond levels reached on day 3, presumably because at this time the spleens were essentially necrotic.*

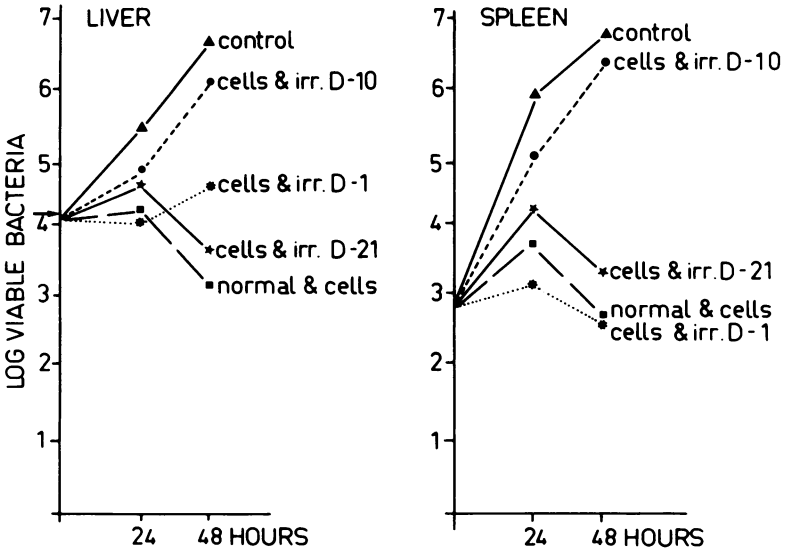

FIG. 3. *Growth of Listeria monocytogenes in livers and spleens of mice that have been adoptively immunized and irradiated with 400 R either on day 1, 10, or 21 before adoptive immunization and infection. Control: No cells, no irradiation; normal cells: immune lymphoid cells, no irradiation.*

that an immune spleen cell suspension lacked a component that is essential for host resistance. The missing component was evidently still present in the irradiated recipient 1 day after exposure and was restored spontaneously within 21 days. It was missing, however, around day 10 post-irradiation. This finding made it possible to obtain objective evidence that bone marrow is the source of an essential element in cell-mediated antibacterial immunity. Normal bone marrow was used in analogy with Lubaroff and Waksman's finding (6) that cells from this source can restore delayed-type hypersensitivity to adoptively immunized animals that have been irradiated before cell transfer.

Bone marrow as a source of cells needed for the expression of anti-Listeria immunity. A group of 10 A/J mice was irradiated with 900 R and on the same day received 10^8 isogeneic bone marrow cells intravenously. Ten days later these animals were adoptively immunized with spleen cells from immune donors, and immediately thereafter they were infected with *L. monocytogenes*. (Control experiments had shown that irradiated A/J mice did not differ conspicuously from CD-1 mice in their behavior toward infection with *L. monocytogenes*.)

Bone marrow restored to mice irradiated with as much as 900 R the capacity to benefit from the protective effects of immune spleen cells at a time (day 10) when animals given a much lower dose of irradiation (400 R; Fig. 3) were shown to be totally unprotected (Fig. 4). Since mice do not survive the larger dose of irradiation without marrow reconstitution, the status of unreconstituted mice could not be determined.

DISCUSSION

Immunity to certain intracellular bacterial infections results from a successful interaction between immunologically committed lymphocytes and phagocytic cells, both free and fixed (12). However, though the essential role of committed lymphocytes has been convincingly proven (10), the evidence for the participation of mononuclear phagocytes is largely circumstantial because it rests on indirect evidence showing that they divide (11), accumulate at sites of infection (11), and become functionally hyperactive during the course of infection with facultatively intracellular bacteria (7). These findings left open the question of whether the observed changes in the mononuclear phagocyte population at large were spurious or indicated an indispensable role in antimicrobial immunity. Irradiation was used to deplete mice of cells that are relevant to antibacterial defense. Reconstitution with cell populations of known origin was subsequently done to restore the capacity to express cell-mediated immunity.

Mice exposed to 400 R of irradiation 1 week before infection were, in fact, unable to defend themselves against a listerial infection (Fig. 1). However, this capacity was not lost forever, but recovered spontaneously within 21 days of irradiation. The failure to muster an effective

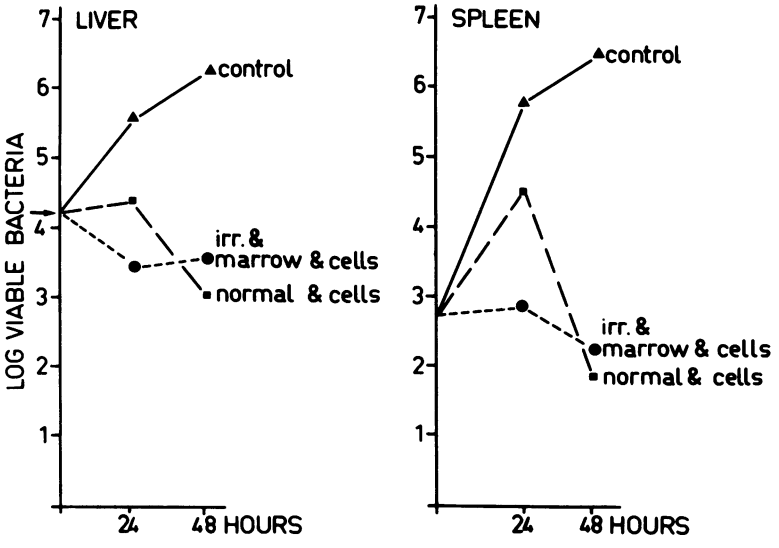

FIG. 4. *Mice irradiated with 900 R and infused with isogeneic bone marrow cells from nonimmune donors are able to express antibacterial immunity when infected and adoptively immunized with spleen cells from actively immunized donors.*

antibacterial defense was accompanied, histologically, by a conspicuous absence of mononuclear phagocytes from lesions. Recovery of the capacity to resist infection was accompanied by the reappearance of monocytic cells within lesions of liver and spleen. Similar effects of 400 R on the development of dermal tuberculous lesions in rabbits have been reported by Kambara et al. (3).

Grafting of irradiated mice with lymphoid cells from immune donors showed that such spleen cells provided protection when given on days 1 or 21 post-irradiation. Grafts performed on day 10 post-irradiation, however, left the animal totally unprotected (Fig. 3). Obviously, the spleen of immune donors does not contain all the cellular components needed to provide antibacterial immunity. Apparently the radiosensitive component that is also needed is functionally active 1 day after irradiation. It then disappears but is present again 21 days after sublethal irradiation.

Subsequently, clear evidence was obtained to show that bone marrow, in restoring a defense mechanism to the irradiated host, contributed essentially to the final expression of antibacterial immunity. This conclusion was based on the finding (Fig. 4) that a greater than normal protection was achieved (at the time when irradiated animals show their greatest susceptibility to challenge) if irradiated recipients had been given normal bone marrow cells before adoptive immunization with immune lymphoid cells. This finding can only mean that the immunity mediated by specifically committed lymphocytes is indispensably dependent upon a cell whose precursors are present in the marrow of nonimmune subjects.

This study does not establish the identity of the cells involved, but indirect evidence indicates that monocytes precursors are the essential elements supplied by a transfusion of normal bone marrow. This evidence has been accumulated in relation to the expression of delayed-type hypersensitivity rather than resistance to infection. Lubaroff and Waksman (6) showed that irradiated animals can be induced to respond to antigen if they are supplied with both sensitized lymphocytes from immune donors and monocyte precursors from the bone marrow of nonsensitized animals. Similar findings pointing to the indispensable role of macrophages in the expression of delayed-type hypersensitivity reactions have been reported by others (2, 19). These findings prove that specifically committed lymphocytes do not function alone to produce delayed-type immunological reactions. My observations show that the same is true of cell-mediated immunity to infection. It has been reported previously that irradiated mice (17) or cyclophosphamide-treated rats (4) cannot be protected by a massive infusion of *Listeria*-sensitive lymphocytes. This study confirms and extends these observations by demonstrating that the adoptively immunized, irradiated subject can efficiently protect itself against a *Listeria* challenge if it is also supplied with precursor cells present in bone marrow.

Using an experimental approach similar to the one used in this study, Campbell et al. (1) found a requirement for bone marrow-derived cells for the development of active immunity against *Listeria* infection in mice. However, the data reported allow no definite conclusion as to whether the bone marrow-derived cells are instrumental in the development of immunity to *Listeria* infection or whether they are needed in the expression phase, or both.

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