THE ROLE OF THYMUS AND BONE MARROW CELLS IN DELAYED HYPERSENSITIVITY*

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Delayed hypersensitivity (DH)[1] lesions have been adoptively transferred with immune cells from lymph nodes, spleen peritoneal exudates, peripheral blood, and thoracic duct lymph (1–5). Isotope labeling showed that these cells constituted a very small proportion of the cells in the lesion (4), and indirect evidence suggested that the sensitized cells were thymus dependent (6). Bone marrow cells constituted the majority of the mononuclear cells in a DH lesion and played a supporting or passive, although essential, role (3, 7, 8).

Experiments were conducted to evaluate the ability of the thymus to provide the sensitized cells capable of initiating a DH response and to determine whether the bone marrow cells have a specific immunologic function as well as a suporting function.

Materials and Methods

Antigen.—Bovine gamma globulin was obtained from Reheis Co., Inc., Chicago, Ill. The immunoglobulin G (BGG) fraction was isolated by elution from diethylaminoethyl (DEAE) columns with 0.01 M phosphate buffer. Deaggregated BGG was obtained from the upper one-half of the tube after centrifugation at 100,000 *g* for 2 hr. BGG-^{131}I was prepared by the method of McConahey and Dixon (9).

Animals.—Adult Lewis (Le) rats (Simonsen Laboratories, Gilroy, Calif.) were used as donors and recipients of cells. Recipients ranged from 130 to 150 g in weight.

Induction of Tolerance.—Tolerance was induced by injecting intravenously 10 mg of deaggregated BGG. To prove unresponsiveness the animals were challenged 1 wk after injection with 0.5 ml of complete Freund's adjuvant (CFA) containing 1 mg tubercle bacillus (H37Ra, Difco Laboratories, Detroit, Mich.) and 1 mg BGG/ml. The injections were given into the footpads and nuchal area. 7 days after challenge and 2 days after beginning oral potassium iodide the animals were given intraperitoneally 30 μg of BGG-^{131}I containing approximately 1 μCi of ^{131}I and were counted daily in a whole body gamma counter. The slope of disappearance of isotope reflected the immunologic state of the donors. A slope similar to those obtained in nonimmune rats indicated a state of tolerance (Fig. 1).

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[1] *Abbreviations used in this paper:* BGG, bovine immunoglobulin G; BSS, balanced salt solution; CFA, complete Freund's adjuvant; CFA-BGG, CFA containing BGG; DH, delayed hypersensitivity; PBS, phosphate-buffered saline.

Immunization.—Le rats were immunized with CFA containing BGG (CFA-BGG) as described above.

Skin Testing.—Skin testing was performed with 25 μg of BGG in 0.05 ml of 0.15 M phosphate-buffered saline (PBS) injected intradermally into the shaved flank. Injection sites were measured for erythema and induration at 6 and 24 hr, and the skin test sites were biopsied at 24 hr. Biopsies were fixed in 10% formalin and stained with hematoxylin and eosin. All immune and tolerant donors were skin tested 3 days before sacrifice. Donors designated as immune had gross lesions of greater than 14 mm at 24 hr while tolerant donors had lesions of less than 2 mm which on random biopsies were histologically negative. Biopsies were evaluated histologically by two investigators independently and were considered negative when mono-

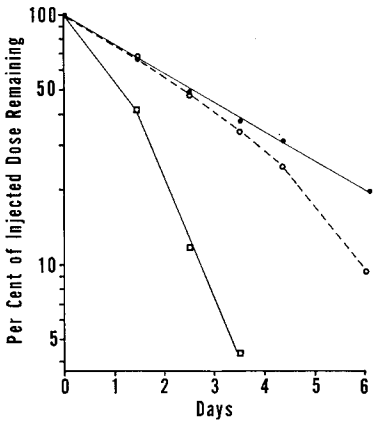

FIG. 1. Group 1 (tolerant, ●—●) rats injected i.v. with deaggregated BGG on day 14 and intradermally with CFA-BGG on day 7 (mean of eight animals). Group 2 (normal, ○ - - - ○) rats injected intradermally with CFA on day 7 (mean of three animals). Group 3 (immune, □—□) rats injected intradermally with CFA-BGG on day 7 (mean of nine animals). BGG-^{131}I given intraperitoneally on day 0.

nuclear cell infiltrates were absent. Positive biopsies were graded on a 1+–4+ basis, depending on the intensity of the mononuclear cell infiltrate and the microscopic extent of the lesion.

Cell Transfers.—Donors were anesthetized with ether and exsanguinated by cardiac puncture 14 days after immunization or challenge. Lymph node cells were obtained by teasing the regional nodes apart in balanced salt solution (BSS). The thymus was freed of parathymic lymph nodes and cells were recovered by pressing the organ through a wire screen in BSS. Bone marrow cells were obtained by flushing BSS through the femoral and tibial bones and dispersing the cells by repeated aspiration through a 21 gauge needle.

All cell suspensions were washed once with BSS, counted in a standard hemacytometer, and adjusted to a concentration of 5×10^8 cells/ml before intravenous injection into the recipients. Recipients were irradiated within 12 hr of transfer with a Picker 200 kv dual beam source (Picker X-ray Corp., White Plains, N. Y.) which delivered 53 R/min. They received either 550 R, in the early experiments, or 700 R, total body, in later experiments, to insure that there was little or no escape from the effects of irradiation during a 15 day observation period. There were two major groups of recipients; one was stimulated by antigen in CFA at the time of transfer of cells derived from various organ sources; one was not stimulated by antigen after infusion of cells from thymus and/or bone marrow.

RESULTS

The first adoptive transfer experiments were designed to determine whether bone marrow played an active immunological role as a source of initiator or trigger cells or a passive part in the expression of the DH lesion. Irradiated recipients (550 R) were given immune or tolerant lymph node cells[2] (1×10^8) and immune or tolerant bone marrow cells (2.5×10^8) (Table I). All six animals receiving immune lymph node cells had positive gross and microscopic lesions on the 10th day after transfer whether the bone marrow was derived from

TABLE I

*Effect of the Immune Status and Number of Bone Marrow Cells on Adoptive Transfer of DH to BGG in Irradiated Recipients**

Bone marrow donor status	Lymph node cells, 1×10^8 donor status	Histologic evidence of DH to BGG in 24-hr biopsies‡ Day of skin test after transfer		
		4	5	10
Tolerant	Immune	+ (5.0)§	—	+ (2.5)
	Tolerant	—	—	—
Immune	Immune	+	—	+
	Tolerant	—	—	—
None	Immune (5×10^8)		—	—

* Recipients given 550 R just before cell transfers; three animals included in each group.

‡ The results are expressed only as positive or negative for each group; responses within groups were uniform.

§ The animals skin tested on day 4 received 5×10^8 bone marrow cells of the type indicated; those tested on days 5 and 10 received 2.5×10^8 bone marrow cells.

immune or tolerant animals. Conversely, if tolerant lymph node cells and immune bone marrow cells were infused, no lesion developed. The same experiment was repeated with an increased number of bone marrow cells (5×10^8). The results were similar except that the skin tests were now positive at 4 days (Table I). Thus the bone marrow from immune animals played a passive role in the unstimulated recipients and did not detectably enhance development of the DH lesion compared with marrow from tolerant animals. The necessity for the presence of bone marrow cells was evident from the lack of response in animals receiving only immune lymph node cells.

Immune bone marrow cells alone or combinations of bone marrow and thymus cells, from normal, tolerant, or immune donors, were infused into recipients that had received 700 R total body irradiation. These recipients

[2] Immune, normal, and tolerant cells mean cells removed from immune, normal, and tolerant donors, respectively.

were not stimulated by antigen in CFA and were skin tested on days 5, 8, and 11 after cell infusion. Occasional weak inflammatory foci were seen histologically (Table II), but in no group did more than two rats exhibit microscopic infiltrations of mononuclear cells. These responses were not considered positive skin reactions to specific antigen but rather were compatible with a small inflammatory response to the mechanical injury produced by injection.

TABLE II

Attempted Adoptive Transfer of DH by Infusions of Bone Marrow or Thymus–Bone Marrow Combinations into Irradiated (700 R) Rats

Bone marrow, 5×10^8 cells donor status	Thymus, 2.5×19^8 cells donor status	Histologic evidence of DH to BGG in 24 hr biopsy*		
		Day 5	Day 8	Day 11
Immune		0, 0, 0, 0, 0	0, 0, 0, 1+, 1+	0, 0, 0, 0, 0
Immune	Immune	0, 0, 0, 0, 1+	0, 0, 0, 0, 0	0, 0, 0, 0‡
Immune	Normal	0, 0, 0, 0, 0, 1+	0, 0, 0, 0, 1+, 1+	0, 0, 1+, 0, 0, 1+
Immune	Tolerant	0, 0, 0, 0, 0, 1+	0, 0, 0, 0, 1+, 1+	0, 0, 0, 0, 0, 1+
Normal	Immune	0, 0, 0, 0, 1+	0, 0, 0, 1+, 1+	0, 0, 0, 0, 0
Tolerant	Immune	0, 0, 0, 0, 0, 1+	0, 0, 0, 0, 0, 1+	0, 0, 0, 0, 0

* Histologic findings are given for each recipient and are graded on a 0–4+ basis, although no reactions greater than 1+ were found in this experiment.

‡ One animal died on day 9.

TABLE III

*Capacity of Bone Marrow Cells to Confer DH in Irradiated, Antigen-Stimulated Recipients**

Bone marrow, 5×10^8 cells	Histologic evidence of DH to BGG in 24-hr biopsies on day 8‡
Immune	2+, 2+, 2+, 2+, 2+, 3+
Normal	0, 0, 0, 0, 0, 0, 1+, 1+

* Recipients were given 700 R just before cell transfer and CFA-BGG immediately after transfer.

‡ Histologic findings are graded on a 0–4+ basis and results are given for each recipient.

The contribution by marrow of specifically reactive cells was further examined in recipients given 700 R whole body irradiation and stimulated with CFA-BGG after cell infusions. Under these circumstances the group receiving normal bone marrow cells alone responded weakly in two of eight recipients, while those receiving immune bone marrow cells produced moderate to strong responses in all six recipients at 8 days, as shown in Table III. Thus, in the presence of continued antigenic stimulation, bone marrow from immune animals was capable of providing both specifically sensitized cells and nonspecific cells.

In this experiment two of eight recipients of normal bone marrow developed weak positive reactions on day 8. In other experiments also, there were some reactions which might have represented "escape" of the irradiated host, i.e., partial recovery of bone marrow. The dose of irradiation was chosen to permit survival of recipients of thymus cells alone; thus the host's bone marrow was severely damaged but not completely eliminated. This dose was also too low to destroy all host lymphoid tissue. Control animals which were irradiated

TABLE IV

*Development of DH to BGG with Normal Thymus and/or Bone Marrow Cells in Irradiated, Antigen-Stimulated Recipients**

Irradiation dose	Sources of cells ($\times 10^8$)		Histologic evidence of DH to BGG in 24-hr biopsies‡	
	Thymus	Bone marrow	Day 8	Day 11
700 R	2.5	0	0, 0, 0, 0 0, 0, 0, 0	0, 0, 0, 0 0, 0, 0§
	0	5.0	0, 0, 0, 0 0, 0, 1+, 1+	0, 0, 0, 0 0, 1+, 1+, 2+
	2.5	5.0	1+, 1+, 2+ 2+, 2+, 3+	3+, 3+, 3+ 4+, 4+, 4+
850 R	0	5.0	0, 0, 0 0, 0, 1+	0, 0, 0 0, 0, 1+
	2.5	5.0	0, 0, 0, 1+	0, 2+, 2+, 3+

* All recipients given irradiation just before cell transfer and CFA-BGG immediately after transfer.

‡ Histologic findings are graded on a 0–4+ basis and results are given for each animal.

§ One recipient died on day 9.

and stimulated with CFA-BBG but given no cells were included in each experiment (not shown in Tables). These animals were uniformly unresponsive during the period of observation reported.

Normal bone marrow and thymus cells were used alone or together in irradiated (700 R) CFA-BGG–stimulated recipients. Neither cell type alone was consistently effective in producing a lesion at day 8 or 11, yet the combination of both produced lesions at these times (Table IV). When normal bone marrow and normal thymus cells were given together to animals receiving 850 R irradiation, significant lesions were first evident on day 11. When normal bone marrow alone was infused into recipients of 850 R irradiation only one of six showed a weak positive lesion (Table IV). These

animals were considerably more ill than recipients of 700 R which may account for the delay in appearance of positive lesions.

Thymus cells alone in an irradiated host would not be expected to produce an early DH lesion since bone marrow cells are needed to provide the nonimmunologic component that is also essential. To evaluate the influence of previous antigenic experience on thymus cells, we combined thymus cells with bone marrow from tolerant animals. Thymus cells from normal, immune, or tolerant donors were injected into irradiated (700 R) recipients followed by challenge with CFA-BGG. As shown in Table V, immune thymus cells were more effective in the development of DH reactions and in initiating DH at an

TABLE V

*Effect of Immunologic Status of Thymus on the Development of DH in Reconstituted, Irradiated, Antigen-Stimulated Recipients**

Status of donors of thymus cells (2.5×10^8 cells)	Histologic evidence of DH to BGG in 24-hr biopsies‡	
	Day 8	Day 11
Immune	1+, 1+, 1+, 2+, 2+ 3+, 3+, 3+	1+, 2+, 2+, 2+, 3+ 3+, 3+, 3+
Normal	0, 0, 0, 0, 1+, 1+, 1+ 1+, 1+	0, 0, 1+, 1+, 1+, 2+ 2+, 2+, 2+
Tolerant	0, 0, 0, 0, 0, 1+, 1+ 1+, 1+	0, 0, 1+, 1+, 1+, 1+ 1+, 2+, 2+

* All recipients received 700 R whole body irradiation, 5×10^8 bone marrow cells from tolerant donors, and were challenged after cell transfer with CFA-BGG.

‡ Histologic findings are graded on a 0–4+ basis and results are given for each animal.

earlier time than normal or tolerant thymus cells. No differences were seen between groups receiving normal or tolerant thymus cells combined with tolerant bone marrow cells.

DISCUSSION

The results cited indicate that cells from the bone marrow can serve both actively as the source of specifically sensitized cells and passively as nonspecific responder cells. The nonimmunologic responder function of the bone marrow cells was shown by the response of unstimulated recipients which received immune lymph node cells and bone marrow from tolerant donors. In the absence of bone marrow cells no lesion was produced. The presence of either tolerant or immune marrow cells and immune lymph node cells allowed the lesion to develop and no difference in intensity or size of the lesion was evident between the two groups. We were unable to obtain positive skin lesions if the skin

testing was done immediately after cell transfer, but by increasing the number of bone marrow cells given we could decrease the time interval between cell transfer and development of a positive lesion. Thus a critical number of bone marrow cells appeared to be necessary before a DH lesion could develop in the presence of sensitized lymph node cells and either immune or tolerant bone marrow could provide those cells.

Specifically sensitized cells could, however, be demonstrated in immune bone marrow by continued antigenic stimulation. In irradiated recipients stimulated with CFA-BGG, bone marrow cells alone from immune donors produced significant DH skin lesions compared with those from bone marrow cells of normal donors. The marrow of immunized animals apparently contained specifically sensitized cells which under the influence of antigenic stimulation could initiate the DH reaction. This does not imply that the sensitized cells either originated or were sensitized in the marrow; they well might have migrated to marrow after sensitization elsewhere. Additional antigenic stimulation was probably required to expand the sensitized cell population by cell division and/or recruitment. These results agreed with those of Foerster et al. (10), who found bone marrow from immune "responder" guinea pigs effective in transferring responsiveness to nonresponder irradiated Hartley guinea pigs which were subsequently stimulated. Also, in additional experiments, with normal F_1 (Strain 2 $\times$ 13) marrow these investigators found a positive skin tests in only one of six Strain 13 guinea pigs that received marrow cells alone, whereas with F_1 spleen and lymph node cells plus nonresponder Strain 13 marrow cells skin tests were positive in 7 of 10 animals (10). Thus both rat and guinea pig immune marrow contain all of the elements needed for expression of DH, although the evidence suggests that these cells are present in small numbers. This concept may explain the success of Buckley et al. (11) who transplanted bone marrow to a patient with chronic mucocutaneous candidiasis. While the possible effect of transfer factor was not ruled out, the delayed development of a positive skin test in their patient was contrary to usual transfer factor conversions and was explicable by considering that a sensitized cell population expanded during continued antigenic stimulation.

The ability of normal bone marrow alone to provide all the elements for induction of DH under conditions of continued antigenic stimulation was equivocal. Two of eight irradiated recipients (700 R) and one of six irradiated recipients (850 R) developed positive lesions following infusion of normal bone marrow and stimulation by CFA-BGG. These responses may indicate that normal marrow was capable of complete reconstitution, or may reflect a contribution by the recipient of relatively radioresistant immunologically susceptible cells which enabled DH to develop. An alternative explanation of these results is that normal marrow stem cells repopulated the thymus and peripheral lymphoid organs, as has been shown histologically (12), although

the time required for this to occur compared with the time required to develop DH skin lesions in our animals makes this explanation unlikely. While the ability of normal bone marrow cells to provide trigger cells for DH was uncertain, a comparison of Tables III and IV indicated that during the induction of DH in an irradiated stimulated recipient the marrow played more than a passive role. Normal thymus cells combined with normal bone marrow cells gave more vigorous responses in a greater proportion of animals than did normal thymus cells and tolerant bone marrow cells.

In conclusion, the function of bone marrow cells in DH can be summarized on two operational levels. Firstly, in immune animals the bone marrow does contain trigger cells which can be readily demonstrated under conditions of continued antigenic stimulation. Secondly, in the presence of sensitized lymphocytes DH lesions are expressed only in the presence of bone marrow and at this level of DH responsiveness the bone marrow acts in a passive nonimmunologic role.

The role of cell cooperation in the development of DH was explored by using irradiated stimulated recipients which received thymus and/or bone marrow cells from normal donors. Animals receiving both cell types developed significant lesions on days 8 and 11, whereas rats infused with either cell type alone did not produce skin reactions. This evidence for bone marrow–thymus cell cooperation during the induction of DH is in accord with the observations of Claman et al. (13) and Miller and Mitchell (14) on the induction of antibody response to certain antigens. Similarly, in the area of cellular immunity, cooperation between cells from these two sources has been described for the induction of contact sensitivity in mice (15), the mouse footpad response to methylated human serum albumin (16), the homograft reaction (17), and both in vivo (18) and in vitro (19) graft *versus* host reactions. Our findings provide further evidence that bone marrow–thymus cell cooperation is important, if not essential, during induction of cellular immunity. Our experiments, however, in addition to those cited, required the presence of marrow cells to express the lesion and therefore from these experiments it was not possible to determine whether thymus cells alone in an irradiated, antigen-stimulated host might have produced "trigger" cells. It was clear that trigger cells were not consistently produced in the absence of thymus cells.

Our last set of experiments demonstrated that, when combined with tolerant bone marrow, cells found within the thymuses of immune animals were capable of producing a DH lesion in an irradiated, antigen-stimulated recipient, whereas those within the thymuses of normal or tolerant donors did not, or did so weakly. Thus, the evidence presented here indicated the ability of normal thymus cells to permit the recipient to develop DH and demonstrated the presence of immunocompetent cells in the thymus glands of immune animals. These results were in accord with those of Asherson and Ptak, who found

that normal thymus and bone marrow cells could partially restore contact sensitivity to mice rendered tolerant to picryl chloride and irradiated before cell transfer (20). They also reported that such restoration could not be accomplished in nonirradiated recipients. The recent report by Takada et al. provided evidence that survival of donor cells was better in irradiated hosts than in normal hosts and thus the irradiated recipient may be a much more sensitive animal to demonstrate donor cell function (21). The failure to transfer DH with immune thymus cells in normal guinea pigs (22) and the failure to restore DH in neonatally thymectomized rats with normal thymus (23) might have been because of poor survival of donor cells in a normal recipient. In addition, we have antigenically challenged our recipients to expand a small sensitized cell population and have based our results on histologic characteristics which permitted a more precise evaluation of the nature of a small lesion than does gross measurement. Whether the thymus cells in immune donors acquired their specific responsiveness within the thymus, or whether cells sensitized elsewhere migrate (back) to the thymus, was not established by our experiments. Under appropriate circumstances thymus cells from an immune animal could be shown, however, to contain specifically sensitized cells or at least cells committed to a particular antigen.

SUMMARY

Adoptive transfer experiments were performed to define the immunological role of thymus and bone marrow cells in the induction of delayed hypersensitivity (DH). The results indicated the following. (*a*) Bone marrow from immune donors contained cells capable of being stimulated by antigen to initiate the expression of DH. (*b*) Bone marrow from nonimmune or tolerant donors contained cells that were needed to complete the expression of DH after the infusion of immune lymph node cells. (*c*) Normal bone marrow and thymus cells cooperated in the irradiated recipient to induce the most vigorous skin reactions to specific antigen; these reactions were seen only when the recipients were stimulated by antigen. Either cell type alone was ineffective. (*d*) In the presence of tolerant bone marrow cells, thymus cells from immune donors gave a more vigorous response than did thymus cells from normal or tolerant donors. (*e*) There was suggestive evidence that thymus cells were the source of trigger elements that initiated DH. (*f*) Antigen in the irradiated recipient was necessary to induce DH after infusion of bone marrow cells alone, or bone marrow and thymus cells together.

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