

## LYMPHOID CELL DEPENDENCE OF EOSINOPHIL RESPONSE TO ANTIGEN\*, ‡

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(Received for publication 10 May 1971)

The response to the presence of foreign material in immunologically competent animals is an elaborate, well integrated series of reactions and interactions involving multitudes of cells that arise predominantly in the myeloid and lymphoid tissues. Physiological activities in the inflammation include chemotaxis, ameboid movement, phagocytosis, intracellular and extracellular digestion, increases in protein and nucleic acid synthesis, and a release of effector molecules by activated cells. Cellular necrosis is also a conspicuous feature, with compensatory proliferation occurring both at the inflammatory site and in the central hemopoietic tissues (1-4).

Supralethal doses of irradiation deplete hemopoietic activity and decrease the capacity for an inflammatory response to foreign material (5). Transplantation of normal isogenic hemopoietic cells, specifically stem cells, effects a reconstitution of bone marrow and lymphatic tissue. In such animals hemopoietic differentiation may be studied morphologically, by determining the types of hemopoietic colonies which develop as discrete foci in the marrow and spleens (6, 7), and functionally, by determining the capacity of such animals to respond to an inflammatory stimulus (5). An indication of the rate of maturation of specific cell types can be obtained by measuring the relative numbers of cells responding with reference to time.

This report documents our findings concerning the inflammatory response of hemopoietically reconstituted irradiated animals to intraperitoneal injections of tetanus toxoid and its potentiation by transfer of cells from animals previously primed with the antigen. Special emphasis has been given to the eosinophil granulocyte and to serum antitoxin titers since these responses were most affected by the source and immunological capacity of the transferred cells.

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\* This investigation was supported by U.S. Public Health Service grants CA 03367, T1 CA 05021, and K6 CA 14,219.

‡ A preliminary report of this work appeared in 1969, *Anat. Rec.* **163**:269.

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*Materials and Methods*

*Experimental Procedure.*—The mice used were 3–5-month old (C57 × A)<sub>F</sub><sub>1</sub> hybrids maintained in a specific pathogen-free (SPF)<sup>1</sup> environment. Marrow cells were obtained by flushing the long bones with Gey's balanced salt solution. Spleen and thymus cells were obtained by expressing the tissues through 50-mesh screens into Gey's solution. Suspensions were aspirated to break up larger clumps of cells, flushed through a 200-mesh screen in a Swinny adapter (Becton-Dickinson and Company, Rutherford, N. J.), counted for viable cells, and brought to the desired concentration. Donor animals were primed 28–45 days before use by a single subcutaneous injection of 0.2 ml alum-precipitated tetanus toxoid (Eli Lilly & Co., Indian-

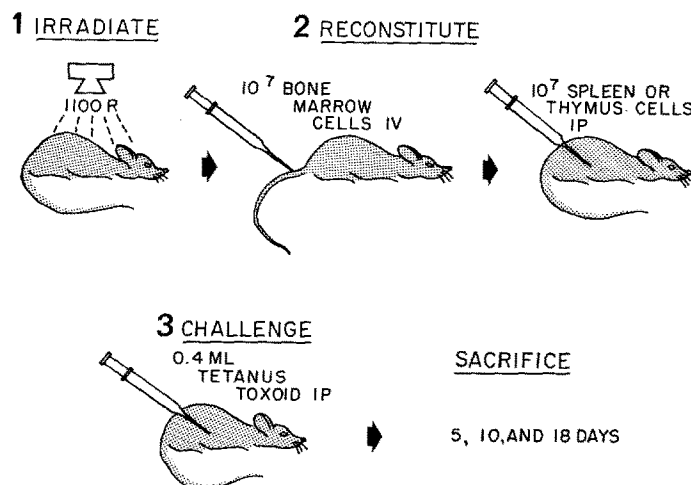


FIG. 1. Schematic drawing illustrating the experimental procedure.

apolis, Ind.) combined with 0.2 ml pertussis vaccine (Parke, Davis & Co., Detroit, Mich.) diluted with saline (1:10). Recipient animals were exposed to 1000 R or 1100 R from a <sup>137</sup>Cs source. Immediately after irradiation 10<sup>7</sup> bone marrow cells were injected in a volume of 0.5 ml Gey's solution in the tail vein and/or 10<sup>7</sup> splenic or thymic cells injected intraperitoneally in a volume of 1 ml. ½ hr later, an intraperitoneal injection of 0.4 ml alum-precipitated tetanus toxoid was given. Autopsies were performed at 5, 10, and 18 days postirradiation (see Fig. 1). The data presented in the tables and figures represent averages of six mice per group except where noted.

*Determination of the Response.*—At the time of sacrifice of the recipient animals, brush smears were made of the peritoneal exudate and of the bone marrow of one femur from each animal for May-Grünwald-Giemsa staining and differential counting (8). The peritoneal cavity and the other femur were then flushed of cells with a known volume of Carpentier's fixative containing 0.2% eosin, thereby allowing the direct enumeration of eosinophilic granulocytes and total cells in the preparations. The numbers of mononuclear cells and neutrophilic and eosinophilic granulocytes were also determined as the product of the total cells counted

<sup>1</sup> Abbreviation used in this paper: SPF, specific pathogen-free.

TABLE I  
Differential Per Cent ( $\pm$  SEM) of the Predominant Cell Types Found in the Peritoneal Exudate 5, 10, and 18 Days After Irradiation, Cell Transfer, and Antigenic Stimulation

Group	Donor cells*		Mononuclear cells†			Neutrophilic granulocytes†			Eosinophilic granulocytes†		
	Spln	BM	5 days	10 days	18 days	5 days	10 days	18 days	5 days	10 days	18 days
1	N	—	64.6 $\pm$ 4.9	57.0 $\pm$ 4.0	78.3 $\pm$ 6.9	3.4 $\pm$ 0.4	17.0 $\pm$ 6.2	15.7 $\pm$ 4.3	31 $\pm$ 5.0	26.0 $\pm$ 3.8	5.7 $\pm$ 5.2
2	P	—	64.5 $\pm$ 14.8	89.5 $\pm$ 3.2	68.5 $\pm$ 11.6	5.5 $\pm$ 1.1	3.5 $\pm$ 1.8	25.2 $\pm$ 9.7	27.5 $\pm$ 13.9	1.5 $\pm$ 1.4	5.2 $\pm$ 3.1
3	—	N	59	72.3 $\pm$ 1.6	64.4 $\pm$ 4.1	0	14.3 $\pm$ 5.0	5.0 $\pm$ 1.1	39	13.0 $\pm$ 3.8	18.2 $\pm$ 4.4
4	—	P	76.5 $\pm$ 9.2	70.0 $\pm$ 12.5	57.6 $\pm$ 4.7	14 $\pm$ 7.0	7.2 $\pm$ 4.2	4.1 $\pm$ 1.6	9.2 $\pm$ 2.1	22.8 $\pm$ 14.3	32.4 $\pm$ 5.1
5	N	N	51.7 $\pm$ 7.3	88.2 $\pm$ 3.5	63.6 $\pm$ 3.5	5.3 $\pm$ 3.0	1.5 $\pm$ 1.1	8.2 $\pm$ 1.4	42.7 $\pm$ 9.5	8.0 $\pm$ 1.4	17.5 $\pm$ 3.2
6	N	P	65.7 $\pm$ 0.9	86.8 $\pm$ 3.0	60.3 $\pm$ 3.6	17.3 $\pm$ 4.3	5.5 $\pm$ 2.5	3.3 $\pm$ 0.8	17.0 $\pm$ 4.4	7.8 $\pm$ 3.8	31.6 $\pm$ 3.8
7	P	N	55.8 $\pm$ 14.0	83.7 $\pm$ 2.8	39.0 $\pm$ 2.8	10.8 $\pm$ 4.1	5.0 $\pm$ 1.2	1.7 $\pm$ 0.8	32.8 $\pm$ 17.8	11.0 $\pm$ 3.1	56.2 $\pm$ 3.1
8	P	P	55.7 $\pm$ 4.6	85.7 $\pm$ 5.4	35.3 $\pm$ 2.0	28.7 $\pm$ 8.9	10.7 $\pm$ 5.4	4.3 $\pm$ 3.1	15.7 $\pm$ 5.7	3.0 $\pm$ 1.0	58.0 $\pm$ 3.2

\* P = primed, N = nonprimed. Spl, spleen; BM, bone marrow.

† Based upon counts of 300-2600 cells except groups 3 and 4 in which only 100 and 200 cells were counted.

directly and the per cent cell type derived from differential counts. There was good agreement between the proportion of eosinophils noted in the hemocytometer chamber and the per cent of eosinophils counted on the smears.

Specific antibody titers were determined for blood samples obtained before autopsy at 18

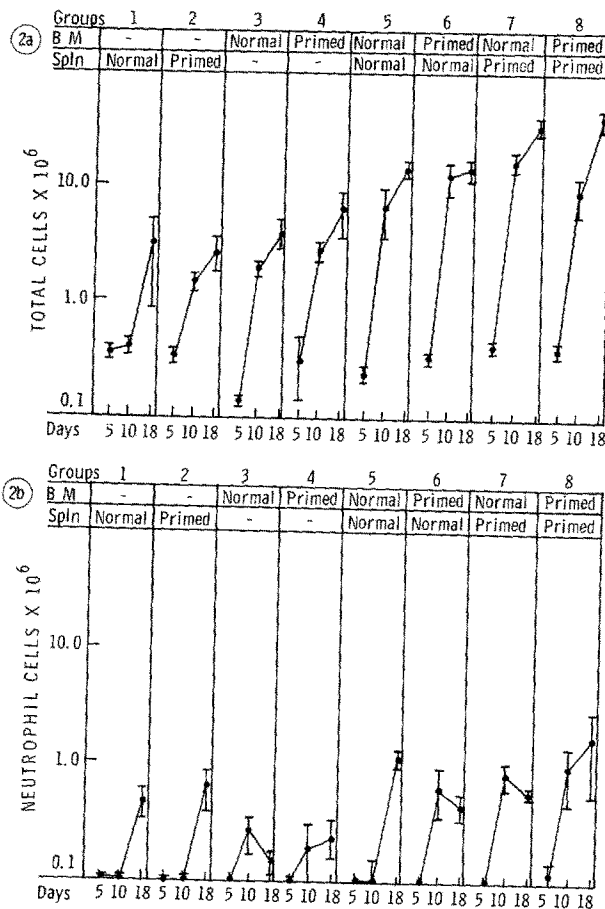


FIG. 2. Cells recovered from peritoneal cavity of mice on days 5, 10, and 18 after irradiation and reconstitution with normal or primed bone marrow (BM) and/or spleen (Spln) cells and intraperitoneal injection of tetanus toxoid on day 0. (a) Total number of cells in peritoneal exudate. (b) Number of neutrophils in peritoneal exudate.

days by measuring the capacity to neutralize tetanus toxin, using a modification of the bioassay method of Ipsen (9).

RESULTS

Fig. 2 illustrates the total number of cells and the number of each of the predominant types of cells which could be washed out of the peritoneal cavity.

The number of these cells represents the capacity of the reconstituted animals to mount an inflammatory response to the tetanus toxoid. It may be seen that on day 5 the total cellularity in each group was less than 1 million cells and was therefore less than the number of splenic cells injected earlier. Furthermore, on

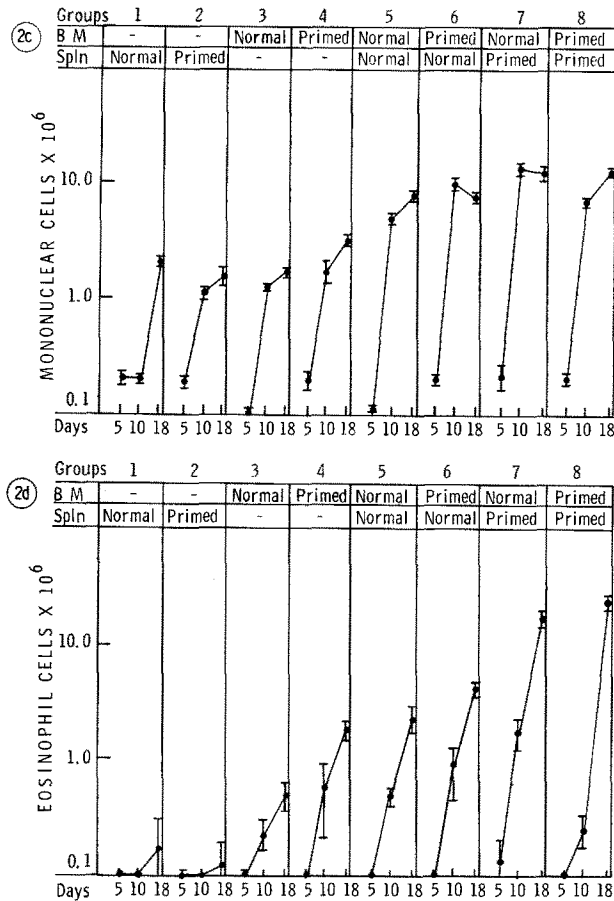


FIG. 2. See Figs. 2 a and b. (c) Number of mononuclear cells in peritoneal exudate. (d) Number of eosinophils in peritoneal exudate.

day 5 there was no significant difference between the two groups of animals not injected with splenic cells (groups 3 and 4) and the other groups.

Between days 5 and 18 the cellularity of the peritoneal cavity increased in all groups (Fig. 2 a). The extent and composition of this increase varied between groups and reflected the source of cells administered after irradiation and their prior experience with the specific antigen. The cellular responses of animals

receiving either spleen or marrow cells alone (groups 1-4) were consistently lower than responses obtained with combinations of marrow- and spleen-derived cells (groups 5-8), especially if the spleen cells were from antigen-primed ani-

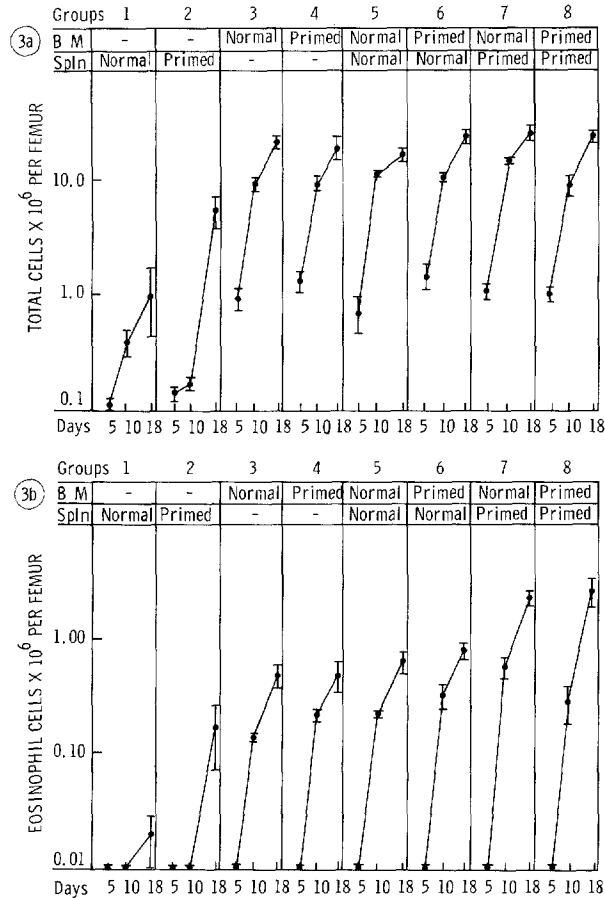


FIG. 3. Marrow cells recovered from one femur of mice on days 5, 10, and 18 after irradiation and reconstitution with normal or primed bone marrow and/or spleen cells, and intraperitoneal injection of tetanus toxoid on day 0. (a) Total marrow cellularity of one femur. (b) Number of eosinophils in one femur.

mals. Figs. 2 b, c, d, and Table I represent the quantitative determinations and per cent respectively of each cell type in the peritoneal exudate.

*Neutrophilic granulocytes* (Fig. 2 b) were a minor component of the inflammatory site at all periods studied. The maximum count, obtained in group 8, was less than 2 million, and represented 4.3% of the total cell content.

*Mononuclear cells* (Fig. 2 *c*), including small lymphocytes, monocytes, and large macrophages, increased greatly in number between the 5th and 10th days, with only slight changes occurring between days 10 and 18. There were significantly more mononuclear cells in those animals receiving both bone marrow and spleen cells.

*Eosinophilic granulocytes* (Fig. 2 *d*) constituted the other major component of the inflammatory exudate. In animals reconstituted only with spleen cells (groups 1 and 2) few eosinophils were found during the 18-day period. Animals reconstituted with normal bone marrow cells (group 3) had significantly greater numbers of eosinophils at days 10 and 18. An even greater eosinophil response occurred if the donor bone marrow cells were taken from animals which had

TABLE II  
*Percentage of Eosinophils in Femur Bone Marrow (Determined by Hemocytometer Counts)*

Group	Spln	BM	5 days	10 days	18 days
				%	%
1	N	—	*	*	2 (3)‡
2	P	—	*	*	3 (5)
3	—	N	*	2 (6)	2 (12)
4	—	P	*	2 (6)	2 (12)
5	N	N	*	2 (6)	4 (16)
6	N	P	*	3 (6)	3 (18)
7	P	N	*	4 (6)	9 (16)
8	P	P	*	3 (6)	11 (17)

\* Total cells were too few in number for accurate determination of percentage of eosinophils.

‡ Numbers in parentheses indicate number of animals in group.

been primed with the antigen (group 4). When normal spleen cells were given with the normal or primed bone marrow there was a slight increase in the capacity to mount an eosinophil response. (Compare group 3 with group 5 and group 4 with group 6.) However, a very marked increase occurred in the eosinophil response of animals reconstituted with immune spleen cells as well as bone marrow cells (groups 7 and 8). In these last two groups more than 15 million eosinophils were present in the peritoneal exudate, constituting more than 50% of the cells present on day 18.

*Bone Marrow Cellularity.*—The data in Fig. 3 illustrate the average number of cells (total and eosinophilic) in the marrow cavity of a single femur taken 5, 10, or 18 days after irradiation and cellular reconstitution. In animals which had been reconstituted with spleen cells alone (groups 1 and 2) there was a significant retardation in the recovery of marrow cellularity. In animals given  $10^7$  bone marrow cells, cellularity comparable to nonirradiated animals was

obtained between 10 and 18 days (groups 3-8). The addition of spleen cells did not significantly change the total number of bone marrow cells in the recipient. The massive peritoneal eosinophilia observed in animals given both bone marrow and primed spleen cells (groups 7 and 8, Fig. 2) was reflected in an increased number and proportion of eosinophilic granulocytes in the marrow (Fig. 3 *b* and Table II). It is apparent that proliferation and differentiation of eosinophils had been greatly stimulated in the bone marrow of these animals.

*Serum Antitoxin Titers.*—Blood samples for assay of tetanus antitoxin titers

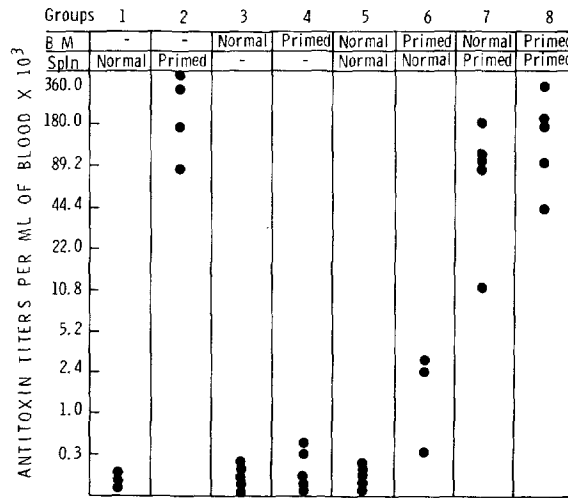


FIG. 4. Serum antitoxin titers of mice on day 18 after irradiation and reconstitution with normal or primed bone marrow and/or spleen cells, and intraperitoneal injection of tetanus toxoid on day 0.

were taken before autopsy on the 18th day. It may be seen in Fig. 4 that animals reconstituted with normal spleen and/or normal bone marrow cells (groups 1, 3, and 5) had no measurable antitoxin titers. Animals reconstituted either with immune marrow cells alone or together with normal spleen (groups 4 and 6) had low but detectable titers in five of nine animals, while all animals reconstituted with immune spleen cells (groups 2, 7, and 8) had very high antitoxin titers.

*Thymus-Derived Cells.*—Other experiments were undertaken to determine if thymic cells acted in a manner similar to splenic cells in their capacity to augment the eosinophil response to antigen (groups 9-12 in Table III). Thymic cells from primed or normal mice were injected intraperitoneally into irradiated mice reconstituted with primed or normal bone marrow, and autopsies performed only on the 18th day. Although recovery of the bone marrow occurred



in all animals, the actual number of eosinophils in the inflammatory exudate was much greater in animals reconstituted with cells from primed animals. There were  $0.8 \times 10^6$  exudative eosinophils in animals receiving normal thymic and bone marrow cells. In animals receiving thymic cells from primed animals and normal bone marrow cells, the exudative eosinophils increased to  $3.7 \times 10^6$ . Animals receiving primed bone marrow cells plus normal thymic cells had  $3.2 \times 10^6$  eosinophils. Animals receiving both primed thymus cells and primed bone marrow cells had  $7.5 \times 10^6$  eosinophils in the inflammatory exudate. In the three groups receiving primed cells, the eosinophils comprised from 26 to 44% of the cells in the inflammatory exudate. These groups also synthesized humoral antitoxin. Only trace amounts were found in some of the animals in

TABLE III

*Cells in Peritoneal Exudate of Mice on Day 18 after Irradiation and Reconstitution with Normal or Primed Bone Marrow and Thymus Cells and an Intraperitoneal Injection of Tetanus Toxoid*

Groups:	9	10	11	12
Bone marrow	Normal	Normal	Primed	Primed
Thymus	Normal	Primed	Normal	Primed
Mononuclear cells $\times 10^6$	$3.43 \pm 0.62$	$7.29 \pm 0.81$	$4.88 \pm 1.21$	$7.94 \pm 0.75$
Eosinophils $\times 10^6$	$0.82 \pm 0.45$	$3.72 \pm 0.67$	$3.21 \pm 1.32$	$7.55 \pm 1.13$
Neutrophils $\times 10^6$	$0.21 \pm 0.03$	$0.25 \pm 0.07$	$0.42 \pm 0.11$	$0.23 \pm 0.03$
Humoral antitoxin: titers $\times 10^3$ per ml blood	<0.3	$\pm 0.3$	$\pm 1.0$	7.0
Number of mice	10	32	12	26

group 10 which received primed thymic cells and normal bone marrow cells. The highest titers were in group 12 in which both primed bone marrow and thymus cells were used for reconstitution.

## DISCUSSION

In all irradiated animals receiving bone marrow cells the marrow first became depleted and then completely repopulated between days 10 and 18. In those animals reconstituted only with bone marrow the inflammatory exudate consisted of less than  $6 \times 10^6$  cells. However when splenic cells were also injected the total number of inflammatory cells reached a level of  $36.8 \times 10^6$  cells (group 8). Thus the addition of splenic cells had only a negligible effect on bone marrow cellularity, but a marked effect on number of cells found in the inflammatory exudate.

It is therefore considered unlikely that the failure of marrow-reconstituted animals to show a normal inflammatory response in the peritoneal cavity is

due to a compromised hemopoietic cellularity. Rather it seems more likely to be due to the absence of peripheral chemotactic and/or specific hemopoietic factors normally provided by cells derived from the spleen or thymus.

Animals repopulated with  $10^7$  splenic cells (groups 1 and 2) had a diminished bone marrow cellularity during the whole 18-day period. They also had fewer cells taking part in the inflammatory response to the tetanus toxoid than did the groups receiving bone marrow cell injections. These animals had enormous spleens which contained large numbers of lymphoid cells indicating that cells were present which induced rapid regeneration and enlargement of the spleen. However, there were insufficient stem cells with bone marrow potential to permit the complete repopulation of femurs within the experimental period. This compromised hemopoietic potential of the marrow must have been a contributing factor to the diminished inflammatory response in the peritoneal cavity observed in these animals.

It was noted that reconstitution with normal bone marrow cells alone enabled the animals to mount a higher eosinophil response to antigen than did reconstitution with normal or primed splenic cells alone. Reconstitution with primed bone marrow, or normal bone marrow plus primed splenic or thymic cells augmented the eosinophil response even further. The primed lymphoid cells must have reacted in some manner with the bone marrow cells and augmented their capacity to mount a response, since splenic cells alone were not capable of inducing either eosinopoiesis or a local eosinophilia. However, primed bone marrow cells, either alone or in the presence of lymphoid cells, appeared to have much less capacity to induce an eosinophil response to antigen than did primed splenic cells in the presence of normal bone marrow. Primed bone marrow appeared to have about the same capacity to induce an eosinophilia as did primed thymic cells plus normal bone marrow.

It is generally assumed that the cellular composition of the inflammatory exudate is affected not only by increased permeability of the blood capillaries and venules, but also by the release of chemotactic mediators and other effector molecules specific for each of the different cell types involved (4, 10, 11). In addition, factors must act upon bone marrow stem cells to augment hemopoiesis and replace the specific cells consumed during the inflammatory response. The data obtained in these experiments suggest that thymic and splenic cells must participate in the production of specific chemotactic and hemopoietic stimuli for eosinophils. These factors induced a localization and accumulation of eosinophils at the site of injection of antigen and, in addition, they must also induce increased numbers of precursor stem cells to differentiate into eosinophil granulocytes.

Since the eosinophil response is mediated through or at least associated with antigen experience, the question is raised as to whether the eosinophilia is a direct consequence of toxin-antitoxin reactions. In our experiments the mech-

anisms necessary for the rapid initiation of antitoxin production resides almost exclusively in the presence of splenic cells that have been previously exposed to antigen (groups 2, 7, and 8). A much weaker antibody response was obtained in animals given bone marrow or thymic cells from primed donors (groups 4, 6, and 10). Animals reconstituted with normal marrow and spleen cells (group 5) did not produce detectable antitoxin titers, but they did mount a local eosinophil response on day 18. Thus eosinopoiesis and eosinophil accumulation did occur in the absence of measurable humoral antitoxin. In contrast, high antitoxin titers were present in animals with markedly reduced marrow cellularity and depressed eosinophil response in the inflammatory exudate (group 2). This would indicate that the capacity to produce high antitoxin titers by transfer of primed spleen cells must not be dependent upon an eosinophilia in the recipient mice.

A number of factors have been described which can induce a local accumulation of eosinophils. Experiments of Litt (12, 13) and Kay (14) indicate that eosinophils are chemotactically attracted to antigen-antibody complexes, and Ward (15) has suggested that complement may also be involved in these complexes. The experiments of Cohen et al. (16) indicate that large molecular aggregates per se may be the major stimulus. However, these experiments involve transient eosinophil responses during acute inflammation, or in vitro chamber migration experiments in which the eosinophil and neutrophil responses are identical. In chronic inflammation the neutrophil and eosinophil cells demonstrate entirely different patterns of response (3, 17). The experiments reported at this time with reconstituted animals are similar to those of the chronic type of response and the factors involved in chemotaxis and proliferation of eosinophils must be different.

The prolonged accumulation of eosinophils found in the inflammatory exudate after a challenging injection of antigen was shown in earlier experiments to be a consequence of ameboid motion combined with chemotaxis to specific mononuclear cells present in the exudate (18). The data presented in the present experiments suggest that the factors which cause eosinopoiesis, chemotaxis, and accumulation of mature eosinophils are dependent in large measure upon the influence of the thymus and/or spleen. The cells which stimulate eosinophils in the presence of antigen increase in number or in potential activity in the spleen and thymus after antigen priming. Parathymic lymph nodes have been shown to be embedded in or situated close to the mouse thymic capsule (19, 20). Although precautions were taken to dissect thymic tissue free of the associated lymph nodes, it is possible that the augmented eosinophil response induced by primed thymus could be due in part to the presence of small amounts of lymph node cells suspended along with the thymic cells. In any case, cells found in the lymphatic tissue are involved in the initiation of an eosinophil accumulation.

Our experiments are in agreement with the results reported recently by Basten, et al. (21, 22) who demonstrated the role of large lymphocytes in the mediation of eosinophilia in response to *Trichinella* infection. In their experiments, irradiated rats exposed to live parasitic challenge did not develop an eosinophilia unless reconstituted with lymphocytes as well as bone marrow cells. When lymphocytes from sensitized donors were used together with normal marrow cells, a "secondary" type of eosinophil response was obtained. Transfer of the capacity to mount an early eosinophilia was obtained adoptively in normal rats by large thoracic duct lymphocytes collected 3-5 days after infection of the donors with *Trichinella*.

It has been shown that neonatal thymectomy significantly reduces the capacity of mice<sup>2</sup> and rats (22) to mount an eosinophil response to antigen. Collectively, these experiments indicate that thymic-derived or thymic-dependent cells, which are present in the lymphatic organs and in the circulating blood and lymphatic vessels, will induce a local accumulation of eosinophils and an increased eosinopoiesis in the presence of antigen. There is no evidence that thymic or splenic lymphoid cells per se give rise to eosinophils, but instead they serve to augment the capacity of hemopoietic stem cells to produce eosinophils. A similar augmentation or synergism of thymic-derived and bone marrow cells was demonstrated by Claman et al. (23) in the case of humoral immunity. Recently Barchilon and Gershon (24) and Hilgard (25) noted a synergism of thymus and marrow cell populations in the graft-vs.-host reaction. These similarities strongly suggest that the eosinophil response reflects in some manner either humoral or cellular immunity. Our experiments suggest that at least during the anamnestic response, the production of an eosinophil response and the synthesis of humoral antibody are not interdependent. Further work is necessary to obtain an understanding of the role of eosinophils in these reactions and of the manner in which antigen stimulates mononuclear cells to induce eosinophil chemotaxis and augment eosinophil differentiation from hemopoietic stem cells.

#### SUMMARY

Quantitative determinations were made of the capacity of isogenic bone marrow, spleen, and thymic cells from primed and/or nonprimed mice to repopulate the hemopoietic tissues and to mount an inflammatory and antibody response to specific antigen (tetanus toxoid) in heavily irradiated and reconstituted recipients.

Spleen cells from primed mice but not from normal mice had the capacity to adoptively transfer an anamnestic antitoxin titer in irradiated animals in the

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<sup>2</sup> Cohn, A., T. J. Athanassiades, and R. S. Speirs. 1971. Inhibition of mononuclear and eosinophil responses to antigen following neonatal thymectomy. Submitted for publication.

absence of transplanted bone marrow cells, and during retarded myeloid regeneration.

Spleen cells alone or bone marrow cells alone produced an insignificant and a moderate peritoneal eosinophil response, respectively, to antigen.

In the presence of bone marrow cells, normal spleen cells augment the capacity of recipient animals to mount an eosinophil response to antigen. A much greater augmentation occurs in animals reconstituted with splenic or thymic cells from primed animals.

The increase in antitoxin titers appears to be independent of the response of eosinophils since: (a) marked accumulation of eosinophils can occur in animals with no measurable humoral antitoxin, and (b) high antitoxin titers can occur in animals which do not have marked eosinophil responses.

It is suggested that a thymic-derived or thymic-dependent mononuclear cell population is necessary for optimal eosinophil response to antigen. The neutrophil and mononuclear cell responses to antigen are determined by different mechanisms from those which determine the eosinophil response. These studies together with earlier findings strongly indicate that the eosinophil granulocytes play a role in the immune response to antigen.

Suggestions and discussion of Mr. M. Gallagher and Mrs. Elizabeth Speirs, and technical assistance of Mrs. Elizabeth Priest are gratefully acknowledged.

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