ORIGIN AND FATE OF IgE-BEARING LYMPHOCYTES

I. Peyer's Patches as Differentiation Site of Cells Simultaneously Bearing IgA and IgE*

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There is considerable circumstantial evidence that gut- and bronchus-associated lymphoid tissues (GALT and BALT)[1] supply precursor cells for systemic and/or local IgE responses and that B lymphocytes of the IgA- and IgE-forming systems may have some similarities in their life histories (1). At the same time, direct investigations with the use of immunofluorescence and tissue culture techniques have led to conflicting observations and a strong emphasis on bone marrow and mesenteric lymph node (MLN) as the probable source for IgE-bearing B cells (1).

We report here a comparison of distribution profiles of B lymphocytes bearing different immunoglobulin isotypes in germ-free (GF) and conventionally reared (C) adult rats. A preliminary study (2, 3) suggested that in GF animals, the low level of stimulation with exogenous antigens and such bacterial components as endotoxic lipopolysaccharide might leave the cells at their lowest rates of proliferation and movement and permit a more precise definition of the source of IgE-bearing cells and their relation to cells of the IgA system.

Materials and Methods

Animals. Sprague-Dawley rats (males, 8–10 wk old) were used in all experiments. C and GF rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Immunofluorescence studies involving GF rats (and in some experiments, C rats) were done on the day of delivery to our laboratory. These animals were not fed or given water in our laboratory.

Cell Suspensions. Cell suspensions were prepared from bone marrow, thymus, spleen, MLN, popliteal, inguinal, and cervical LN (other LN), and Peyer's patches (4). Peripheral blood lymphocytes were separated on Ficoll-Hypaque gradients (density, 1.10 g/ml) (5).

Antisera. Monospecific antisera against rat immunoglobulin isotypes were prepared as described previously (6, 7). They included goat anti-epsilon chain (from IR2 immunocytoma), goat anti-mu chain (from IR202 immunocytoma), and rabbit anti-alpha chain (IR22 immu-

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[1] *Abbreviations used in this paper:* BALT, bronchus-associated lymphoid tissue; BM, bone marrow; BSA, bovine serum albumin; C rat, conventionally raised adult rat; GF rat, germfree adult rat; D-PBS-Az, Dulbecco's phosphate-buffered saline containing sodium azide; GALT, gut-associated lymphoid tissue; LN, lymph nodes; MLN, mesenteric lymph nodes; other LN, popliteal, inguinal, and cervical LN; PP, Peyer's patches; sIg, surface immunoglobulin.

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nocytoma). Rabbit anti-gamma chain was prepared by Dr. C. T. Ladoulis. The monospecific antisera were made specific by solid-phase absorptions. Fluorescein-conjugated IgG fraction of rabbit anti-goat IgG (heavy and light chain) and rhodamine-conjugated IgG fraction of goat anti-rabbit IgG (heavy and light chains) were purchased from N. L. Cappel Laboratories Inc., Cochranville, Pa.

All antisera and conjugates were routinely ultracentrifuged at 50,000 mol wt for 30 min at 4°C to eliminate aggregates, after which they were refrigerated. The fluorescein and rhodamine conjugates were usually adsorbed twice with spleen cells. However, conjugates used in conjunction with monospecific antisera to evaluate isotypes present on bone marrow cells were adsorbed five times with normal rat bone marrow cells and then twice with spleen cells. After ultracentrifugation and adsorption, virtually no nonspecific fluorescence was noted when cells of the various organs were examined.

Specificity of Staining. All antisera were titrated before use to determine the concentration at which optimum staining efficiency was obtained. The fluorescein- or rhodamine-conjugated antisera did not stain cells of any organ when used alone. They also did not stain rat cells if the specific antisera were replaced by normal rabbit or goat sera or by phosphate-buffered saline. Thymocytes served as Ig-negative controls. Thymocytes added to each sample in ratios of 3:1, 1:1, and 1:3 reduced the numbers of cells with membrane-bound fluorescence in the expected fashion.

The specificity of staining by the anti-epsilon antibody was internally controlled in each experiment. Cells that appeared to bear IgE were found in high concentrations in Peyer's patches and in lesser numbers in spleen of GF rats, whereas bone marrow, thymus, and MLN of these animals, and all lymphoid organs (excluding MLN and spleen) of C rats had virtually no IgE^+ cells. Anti-epsilon antibody is bound by mast cells (8). However, there were virtually no mast cells in Peyer's patches of GF rats, whereas mast cells were present in all LN of C rats, including those where no IgE-bearing cells were detected.

Immunofluorescence Assay. Suspensions of washed, living cells, 2.5×10^6, were incubated with 10 μl of the appropriate monospecific antiserum (diluted 1:100) in Dulbecco's phosphate-buffered saline containing 0.13% sodium azide (D-PBS-Az) for 45 min at 4°C. The cells were washed twice in D-PBS-Az and then 10 μl of the appropriate conjugated antiserum (1:20 dilution) was added. After another 45 min incubation at 4°C, the cells were washed three times, resuspended in 1 drop D-PBS-Az, and examined in a fluorescence microscope. In double-labeling studies, the two monospecific antisera were added to the cell suspension in tandem. The cells were incubated for 45 min at 4°C and then washed twice. The two conjugates were then added in tandem. The cells were incubated for 45 min at 4°C and then washed three times. The order in which the two monospecific antisera (and the two conjugated antisera) were added did not alter the results obtained.

Removal of Surface Immunoglobulins. In some experiments, cell surface Ig (sIg) was removed by treatment of Peyer's patches or spleen cells with pronase, according to the method of Jones et al. (9). Briefly, cells were resuspended in Eagle's minimal essential medium to which an equal volume of 0.5% pronase (*Streptomyces griseus*, pronase type IV; Sigma Chemical Co., St. Louis, Mo.) was added. They were incubated for 5–15 min at 37°C and were then placed on ice, and a large volume of medium was added to the culture vessel. The cells were carefully washed and their number and viability assessed (trypan blue dye exclusion test). Using the double-labeling techniques described above, untreated cells were examined for the proportions of cells bearing IgE and/or IgA surface markers. Aliquots of the enzyme-treated cells were examined for the presence of IgE and IgA to ensure that they had been removed from the cell surfaces. The remainder were cultured up to 12 h in serum-free RPMI 1640 supplemented with 5% bovine serum albumin (BSA; Miles Laboratories, Inc., Elkhart, Ind.). The cells were then reassayed to determine the percentages of total cells that had regained either IgE or IgA and of those with both markers. 50–70% of the cells were lost during this procedure.

Identification of Cells with Surface Immunoglobulin. Cells with surface fluorescein or rhodamine were identified and counted at a magnification × 400 using a Leitz Orthoplan fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.) with epi-illumination. The numbers of Ig^+ cells in 5–20 randomly selected fields were counted. At least 1,000 cells were counted per sample. Data are expressed as percent of total cells.

Histological Staining. Tissues to be examined for the presence of mast cells were fixed in cold Carnoy's fluid (10) and then stained with Alcian blue-Safranin (11).

Results

Peyers's Patches. Peyer's patches (PP) of adult C rats and GF rats of the same age differed strikingly in the distributions of cells bearing membrane-bound immunoglobulin of the different isotypes. High concentrations of cells with membrane-bound IgE (~20% of total cells) were found in PP of GF rats, whereas IgE^+ cells were absent from PP of C rats (Fig. 1; Tables I and II). The numbers of IgA^+ cells were also almost threefold higher in PP of GF rats when compared with C rats. In contrast, PP of GF rats had greatly reduced numbers of IgM-bearing cells (4%) when compared with C rats (23%). In some experiments, virtually no IgM-bearing cells were detected.

Of the IgE-bearing cells in PP of GF rats, approximately one-half were simultaneously positive for IgA (Table III). When these PP cells were treated with pronase to remove membrane-bound immunoglobulins and maintained in culture, both IgE and IgA reappeared within 12 h (Table IV). The proportion of doubly labeled cells was similar to that of the untreated population. Thus, PP cells of GF rats synthesize the IgE and IgA observed on their surface membranes.

C rats contain about twice as many PP as their GF counterparts (H. G. Durkin and B. H. Waksman, unpublished data). Individual PP from C rats are much larger in size and each contains about fourfold more cells than those of GF rats. To evaluate the possibility that IgE^+ cells might not be detectable in the larger PP of C rats because of a dilution effect, we examined T cell-depleted (thymectomized, lethally irradiated, bone marrow-reconstituted) rats (12). In these animals, values for IgM-

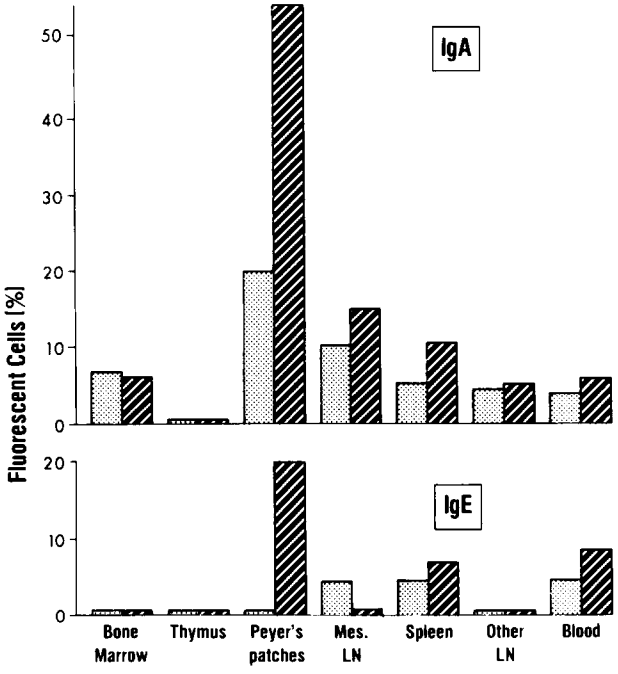

FIG. 1. Relative numbers of cells with membrane-bound IgA and IgE (indirect immunofluorescence) in organs of C (▩) and GF (▨) adult rats (data from Tables I and II). Mes LN, MLN.

TABLE I

Distribution of Cells Bearing Different Membrane-bound Immunoglobulin Isotypes in Adult GF Rats

Number of experiments*	Organ	Fluorescent cells‡			
		IgM	IgG	IgA	IgE
		%	%	%	%
3	Bone marrow	14 (11–20)	7 (7–8)	6 (3–8)	0
>30	Thymus	0	0	0	0
13	PP	4 (1–8)	2 (1–3)	56 (23–78)	20 (8–28)
6	MLN	10 (6–14)	1 (0–2)	14 (9–18)	0
10	Spleen	18 (12–23)	2 (2–3)	11 (9–13)	7 (6–8)
6	Other LN	19 (15–24)	1 (0–2)	5 (2–7)	0
4	Blood	8 (5–9)	1 (0–2)	6 (5–6)	7 (5–8)

* Two rats per experiment.
‡ Average percent of total cells in suspension (1,000 cells counted). Range in parentheses.

TABLE II

Distribution of Cells Bearing Different Membrane-bound Immunoglobulin Isotypes in Adult C Rats

Number of experiments*	Organ	Fluorescent cells*			
		IgM	IgG	IgA	IgE
		%	%	%	%
3	Bone marrow	21 (18–33)	6 (5–7)	7 (7–8)	1 (0–2)
>30	Thymus	0	0	0	0
11	PP	23 (18–29)	1 (0–2)	19 (16–28)	0
6	MLN	20 (16–22)	3 (2–4)	11 (9–14)	4 (3–5)
6	Spleen	26 (18–34)	4 (4–5)	6 (5–8)	3 (3–4)
6	Other LN	17 (12–22)	1 (0–2)	4 (3–8)	0
4	Blood	5 (5–6)	7 (6–8)	3 (3–4)	4 (3–4)

* Same as in Table I.

TABLE III

*Percent of Lymphocytes in PP and Spleen of GF Rats Simultaneously Bearing IgA and IgE on Their Membranes**

Organ	Fluorescent cells‡			Doubly labeled cells	
	IgA	IgE	IgA + IgE‡	Percent of total IgA cells	Percent of total IgE cells
	%				
PP	57 (45–76)	18 (9–24)	9 (3–12)	15	50
Spleen	11 (9–13)	7 (6–8)	4 (2–4)	36	57

* Data from four experiments, two rats per experiment.
‡ As in Table I.

TABLE IV

*Regeneration of Surface IgA and IgE in GF Rat PP Cells after Enzyme Stripping**

Hours after pronase	Fluorescent cells‡			Doubly labeled cells	
	IgA	IgE	IgA + IgE	Percent of total IgA cells	Percent of total IgE cells
		%			
No treatment	58 (55–63)	19 (15–23)	11 (10–12)	19	57
0.5	0	0	0	—	—
12.0§	32 (28–36)	12 (11–14)	6 (5–7)	20	58

* Data from three experiments, two rats per experiment.
‡ As in Table I.
§ 50–70% cell loss at 12 h.

and IgA-bearing cells in PP, as well as in bone marrow, spleen, and LN, increased two to three times over the values obtained in C rats. However, no IgE^+ cells could be detected in PP or bone marrow of any of these animals (data not shown).

Bone Marrow. No IgE^+ cells were found in bone marrow (BM) of C or GF rats at any time, in agreement with earlier studies of Ishizaka et al. (13). Approximately 20% of the BM cells carried other immunoglobulin isotypes, including 6–7% IgA cells (Fig. 1; Tables I and II).

Other Lymphoid Organs. The numbers of lymphocytes carrying IgA increased as much as twofold in the spleen, LN, and blood of GF rats, and the percentages of IgE^+ paralleled IgA^+ cells almost perfectly, with the single exception of LN (Fig. 1; Tables I and II). No IgE-bearing cells were present in mesenteric or other LN, whereas in MLN of C rats, IgE^+ cells consistently amounted to ~4% of the total cells.

Of the IgE^+ cells in spleen of GF rats, one-half were simultaneously positive for IgA (Table III). The low numbers of IgE^+ cells found in spleen and MLN of C rats precluded their use for a stripping experiment.

Discussion

Three findings stand out from the results reported here. First, bone marrow of GF and C rats did not contain any cells bearing membrane-bound IgE. Second, PP in GF rats contained high proportions of $sIgE^+$ lymphocytes—up to 28%. Spleen and blood contained far fewer IgE^+ cells, and mesenteric and other LN had none. Finally, a large proportion of PP cells in GF rats carried both IgA and IgE.

The absence of IgE-bearing cells from bone marrow of both GF and C rats, and the presence of high numbers of IgE^+ cells in PP but not in MLN of GF rats suggest that commitment of B cells to production of membrane-bound IgE may take place in PP (and presumably in other GALT and BALT elements). The stimulus to differentiation may be a structural element of PP, or it may be an antigenic, mitogenic (endotoxin), enzymatic, or hormonal stimulus from the intestinal lumen.

It is also probable that stimuli from the intestinal lumen cause proliferation of $sIgE^+$ cells in PP, followed by their rapid emigration to the periphery. Thus, in C rats, $sIgE^+$ cells were absent from PP, but were found in MLN and also in blood and spleen. The available evidence suggests that these cells migrate from PP to MLN and then, via the blood, to the spleen. As to their further fate, the presence of IgE^+ plasma

cells in intestinal lamina propria of *Nippostrongylus brasiliensis*-infested rats has been reported by Ishizaka et al. (1, 14, 15). In direct contrast, Mayrhofer et al. found IgE^+ mast cells but no IgE^+ plasma cells in this site, either in normal or *N. brasiliensis*-infested rats (16). In rats infested with *Dipetalonema vitae*, the majority of IgE-bearing plasma cells are found in sites close to the parasites and remote from the mucosae (Chassoux and Bazin, unpublished observations). In related studies, oral antigen (BSA or ovalbumin) given to rats or rabbits was shown to induce strong and persistent IgE responses (17–19). In monkeys and atopic humans, sIgE-bearing B lymphocytes are found in germinal centers of PP, mesenteric and bronchial LN, tonsils, and adenoids; and IgE-secreting plasma cells are found in gastrointestinal and respiratory mucosae (20). IgE is secreted into intestinal fluid (21) and into nasal washings of asthmatic patients (22).

With respect to the divergent findings of Ishizaka et al. (1, 14, 15) and Mayrhofer et al. (16) concerning the identity of the IgE^+ cells found in intestinal lamina propria (plasma cells [1] and mast cells [16]), it should be noted that Cebra et al. (23) have observed a strong tendency for PP cells to adsorb immunoglobulins passively, and there has been extensive research on mast cell receptors for IgE (1, 8, 14). The virtual absence of mast cells from PP of GF rats appears to rule out the possibility that such cells played a role in the findings reported here.

The relation of IgE to other immunoglobulin isotypes raises additional issues. Ishizaka et al. (13, 24–28) found $sIgM^+$, but no IgE^+ lymphocytes in bone marrow, spleen, or MLN of normal adult rats. However, after infestation with *N. brasiliensis*, IgE-bearing cells appeared in appreciable numbers in bone marrow and all lymphoid organs, excluding thymus. They were present in spleen at 24 h but did not appear in bone marrow until 4 d. B lymphocytes (virtually all $IgM^+D^+E^+$ cells) from MLN of these rats produced a factor that could convert IgM^+ cells in normal rat bone marrow to $sIgM^+E^+$ cells in vitro (27). In the studies of Tada and Ishizaka (20), IgE and IgA were always found in different cells.

An extensive literature shows that B cells bearing sIgA differentiate in PP, and IgA-bearing blasts migrate via the MLN and thoracic duct lymph and blood to mucosal lamina propria, where they differentiate into IgA-secreting plasma cells (1). The migration pattern proposed here for IgE-bearing cells appears to be identical for at least a major part of the pathway. Accordingly, we found that the distributions of $sIgA^+$ and $sIgE^+$ cells were roughly parallel in certain organs of GF and C rats. However, there were some discordances. The numbers of IgA-bearing cells in spleen of C and GF rats were twice those of IgE^+ cells, yet there were equal numbers of these cell types in blood. In bone marrow, IgA^+ cells amounted to 6–7%, whereas no IgE^+ cells were found. Likewise, in GF rats IgA^+ but not IgE^+ cells were found in mesenteric and other LN. These findings agree with human studies noting parallelism between the two immunoglobulins (1). Furthermore, deficiency of IgE synthesis was found in 44% of a group of patients with IgA deficiency and in 80% of subjects with ataxia telangiectasia (29). However, it is also possible that sIgE and sIgA are synthesized simultaneously. The inductive stimuli could involve the same or different mediators and could be simultaneous or sequential. Further study must resolve these questions. It is, however, of interest that a recent report describes an IgE myeloma that also produces IgA (30). The discordant findings cited above imply that cells bearing both IgA and IgE may have different migratory properties from cells bearing IgA or IgE

alone. However, some of these findings could be explained by sequential acquisition of IgA and IgE.

In PP of GF rats in some experiments, virtually no IgM-bearing cells were observed. This observation suggests either that IgM-bearing cells had lost sIgM before differentiating to sIgA$^+$ and to sIgE$^+$ cells or that the appearance of IgA and IgE surface markers on PP cells was independent of the presence of IgM. In mouse PP, IgD is the principal cell surface immunoglobulin (31, 32). 5–15% of Ig$^+$ cells carry IgM, and the remainder bear either IgM and IgD or IgD alone. The latter are precursors of IgE-secreting cells. In rabbit PP, >50% of the cells bear sIg, and this is mostly IgA (9, 33, 34). These cells were not examined for the presence of sIgE. In rabbit PP cell cultures with pokeweed mitogen, almost all IgA-secreting cells were derived from IgM-negative cells (35). Unless there is major difference among species, some PP cells must be positive for sIgD, A, E or even for sIgM, D, A and E. Others may bear IgD alone, IgD and IgA, or IgD and IgE.

It seems appropriate that production of IgE, which plays an important role in mucosal (and other) defenses against parasites, like production of IgA, which plays a similar role in mucosal defenses against viruses, should be initiated in special mucosal lymphoid organs as exemplified by PP.

Summary

Peyer's patches (PP) from adult conventionally raised (C) and germ-free (GF) rats of the same age differed strikingly in the distributions of cells bearing membrane-bound immunoglobulin of the different isotypes. High concentrations of cells with membrane-bound IgE (~20% of total cells as determined by indirect immunofluorescence) were found in PP of GF rats, whereas IgE$^+$ cells were absent from PP of C rats. The numbers of IgA$^+$ cells were also almost threefold higher in PP of GF rats when compared with C rats. In contrast, PP of GF rats had greatly reduced numbers of IgM-bearing cells (4%) when compared with C rats (23%); in some experiments virtually no IgM-bearing cells were detected. The levels of IgA- and IgE-bearing cells in spleen of GF rats also were increased (to 11% and 7%, respectively).

Of the IgE-bearing cells in PP and spleen of GF rats, approximately one-half were simultaneously positive for IgA. When these PP cells were treated with pronase to remove membrane bound immunoglobulins and maintained in culture, both IgE and IgA reappeared within 12 h. The proportion of doubly labeled cells was similar to that of the untreated population.

No IgE$^+$ cells were detected in bone marrow of C or GF rats at any time, in agreement with the findings of Ishizaka et al., although up to 20% of bone marrow cells bore other immunoglobulin isotypes, suggesting that IgE-bearing cells arise in the PP either *de novo* or by switching from precursors carrying IgM or IgA.

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