

## ORIGIN AND FATE OF IgE-BEARING LYMPHOCYTES

### II. Gut-Associated Lymphoid Tissue as Sites of First Appearance of IgE-Bearing B Lymphocytes and Hapten-Specific IgE Antibody-Forming Cells in Mice Immunized with Benzylpenicilloyl-Keyhole Limpet Hemocyanin by Various Routes: Relation to Asialo GM1 Ganglioside<sup>+</sup> Cells and IgE/CD23 Immune Complexes<sup>1</sup>

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The organs in which B cells bearing membrane-bound IgE (sIgE<sup>+</sup>) and benzylpenicilloyl (BPO)-specific IgE antibody-forming cells (AFC) first appeared were determined in BALB/c mice given BPO-keyhole limpet hemocyanin (10 µg) in aluminum hydroxide by various routes (i.p., gavage, s.c., i.v., or i.m.). In mice immunized by the i.p. route, the numbers and location of sIgE<sup>+</sup> B cells and asialo GM1 ganglioside (AsGm1<sup>+</sup>) cells, the location of IgE/CD23 immune complexes, and the numbers of BPO-specific IgE AFC in lymphoid organs were determined. With all routes of immunization, no sIgE<sup>+</sup> B cells or BPO-specific IgE AFC were ever detected in any organ before day 8. On day 8, with the s.c., i.v., or i.m. routes, sIgE<sup>+</sup> B cells and IgE AFC appeared exclusively in Peyer's patches (PP); with the i.p. or gavage routes, sIgE<sup>+</sup> B cells simultaneously appeared in both PP and mesenteric lymph nodes, whereas IgE AFC appeared only in PP. In mice immunized by the i.p. route, IgE/CD23 immune complexes and strikingly increased numbers of AsGm1<sup>+</sup> cells transiently appeared only in PP after the appearance and preceding the "disappearance" of the sIgE<sup>+</sup> B cells and IgE AFC. The data suggest that specific IgE responses originate in gut-associated lymphoid tissue and appear later in spleen. The data also associate the appearance of IgE/CD23 immune complexes and AsGm1<sup>+</sup> cells with the "disappearance" of sIgE<sup>+</sup> B cells and IgE AFC from PP.

sIgE<sup>+</sup> B cells, unlike B cells bearing other Ig isotypes, are not found in BM (1) or lymphoid organs of unsensitized animals (2), or of healthy (3) or allergic humans (4). sIgE<sup>+</sup> B cells have been detected in PP, but not in other lymphoid organs, of germfree rats (2), and in MLN and spleen of parasite-infested rats (5) and mice (6). IgE<sup>+</sup> B

cells are present in peripheral blood (4), and in MLN and spleen, but not in other organs (3), of patients with hyper-IgE syndrome, and in peripheral blood of patients with atopic dermatitis and AIDS (4). However, little is known regarding the sites of origin, migration pathways, and fate of sIgE<sup>+</sup> B cells, or of their presumed progeny.

We studied sIgE<sup>+</sup> B cells and hapten-specific IgE AFC<sup>3</sup> generated during an immune response to BPO-KLH in mice immunized by various routes (i.p., gavage, s.c., i.v., or i.m.). With the s.c., i.v., or i.m. routes, PP were the sites of first appearance of both sIgE<sup>+</sup> B cells and BPO-specific IgE AFC. With the i.p. or gavage routes, sIgE<sup>+</sup> B cells simultaneously appeared in PP and MLN; however, IgE AFC appeared later in MLN and spleen. Also, in mice immunized by the i.p. route, PP appeared to be the only sites in which both IgE/CD23 immune complexes and strikingly increased numbers of AsGm1<sup>+</sup> cells were simultaneously detected.

The data suggest that specific IgE responses originate in GALT and appear later in spleen. The data also associate the appearance of IgE/CD23 immune complexes and AsGm1<sup>+</sup> cells with the "disappearance" of sIgE<sup>+</sup> B cells and IgE AFC from PP.

#### MATERIALS AND METHODS

##### *Animals, Immunization, and Protocol*

BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were randomly bred for up to three generations in the animal quarters of the Pathology Department, State University of New York, Health Science Center, Brooklyn, NY. Only males (6 to 8 wk old) were used in these studies.

Mice were given BPO-KLH (10 µg) in alum or alum alone (0.2 ml) by various routes (gavage, i.p., i.m., i.v., or s.c.) on day 0. Mice were killed on days 0 to 18, and the numbers of sIgE<sup>+</sup> and BPO-specific IgE AFC in BM, thymus, PP, draining and OLN, including but not limited to MLN, cervical, axial, perigastric, inguinal, popliteal, and iliac LN, and spleen were determined by flow microfluorimetry and ELISPOT assay. For all routes of immunization, levels of homocytotropic antibody in serum were determined on days 0 to 18 by PCA.

In other studies, mice were injected i.p. with BPO-KLH (10 µg) in

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<sup>3</sup> Abbreviations used in this paper: AFC, antibody-forming cells; alum, aluminum hydroxide gel; AsGm1, asialo GM1 ganglioside; BM, bone marrow; BPO, benzylpenicilloyl; BPO-KLH, BPO-keyhole limpet hemocyanin; ELISPOT, enzyme linked immunosorbent spot assay; GALT, gut-associated lymphoid tissue; LN, lymph node; MLN, mesenteric lymph node; OLN, other lymph node; PP, Peyer's patches; sIgE<sup>+</sup> B lymphocytes bearing membrane-bound IgE; PCA, passive cutaneous anaphylaxis; D-PBS, Dulbecco's PBS; D-PBS-Az, D-PBS containing sodium azide (0.13%).

alum on day 0, days 0 and 21, or on days 0, 21, and 42. These mice were killed on days 0 to 72, and the numbers of  $\text{slgE}^+$  B cells,  $\text{AsGm1}^+$  cells, and BPO-specific IgE AFC in the various lymphoid organs were determined. In addition, the location of  $\text{slgE}^+$  B cells,  $\text{AsGm1}^+$  cells, and IgE/CD23 immune complexes in lymphoid organs was determined (peroxidase labeling of tissue sections). Furthermore, levels of homocytotropic antibody in serum were detected by PCA.

In each series of experiments, mice (6 to 12/group) were immunized and boosted with the same lot of Ag in alum.

#### Cell Suspensions

Cell suspensions were prepared in RPMI 1640 (GIBCO, Grand Island, NY), filtered through a nylon screen, and washed once in RPMI 1640. Erythrocytes were lysed using lysing reagent (Ortho Diagnostic Systems, Raritan, NJ), after which the cells were washed twice with RPMI 1640 and then resuspended in either PBS (GIBCO) or complete medium (RPMI 1640 containing heat-inactivated FCS (10%) (GIBCO), L-glutamine (2 mM) (GIBCO), 2-ME ( $5 \times 10^{-5}$  M) (Sigma Chemical Co., St. Louis, MO), and streptomycin (20  $\mu\text{g}/\text{ml}$ ) (Eli Lilly and Co., Indianapolis, IN)).

#### Antibodies

Rat mAbs directed against mouse IgM (B-7-6; C-2-23), IgG1 (H143-225-8.1), IgE (641-46-48), and IgA (L22-8.1) were prepared at CIBA-Geigy, Basel, Switzerland, and assayed for specificity as described previously (7). These antibodies were selected for absence of isotype cross-reactivity using a panel of hybridoma cells which secrete anti-phosphorylcholine murine mAb of the various isotypes. In addition, rat anti-mouse mAb anti-IgE was purchased from PharMingen, San Diego, CA. Rat anti-mouse CD23 (B3B4) was generously provided by Dr. Daniel Conrad, Medical College of Virginia, Richmond, VA. Rat anti-mouse Thy-1.2 was purchased from Becton Dickinson, San Jose, CA. Rabbit anti-mouse  $\text{AsGm1}$  was purchased from Wako Chemicals, Richmond, VA. Fluorescein-conjugated mouse anti-rat IgG (H and L chains) in  $\text{F(ab')}_2$  form was purchased from Pel-Freeze Biological, Rogers, AR. Fluorescein-conjugated goat anti-rabbit IgG (H and L chains) was purchased from Cappel (Durham, NC). Horseradish peroxidase-conjugated sheep anti-rat Ig was purchased from Amersham (Arlington Heights, IL) and was selected for its lack of species cross-reactivity. All antibodies were titrated before use to determine the concentration at which optimal staining efficiency was obtained. Where required, antisera were centrifuged at 50,000 rpm for 30 min at  $4^\circ\text{C}$  to eliminate aggregates, after which they were stored at  $4^\circ\text{C}$ .

#### Morphologic Studies

**Immunofluorescence studies.** Suspensions of washed living cells ( $3 \times 10^6$  cells/ml; 0.1 ml/tube) were incubated with primary antibody (0.1 ml) appropriately diluted in D-PBS (GIBCO) containing sodium azide (Fisher Scientific, Springfield, NJ) (0.13%) (D-PBS-Az) for 30 min at  $4^\circ\text{C}$ . Cells were washed three times in D-PBS-Az, and then incubated with the appropriate fluorescein-conjugated antibody (0.1 ml) for 30 min at  $4^\circ\text{C}$ . Cells were washed three times in D-PBS-Az and counted in a FACS (Cytofluorograf IIs, Ortho Diagnostic Systems, Westwood, MA). Fluorescent cells were identified by simultaneous multiparameter analysis of low forward angle and right angle light scatter. Fluorescence histograms were obtained by plotting the relative fluorescence intensity of 10,000 cells on a linear scale of 1 to 1000 of increasing brightness. The gain on the photomultiplier tube delineating fluorescence intensity was adjusted so that  $>99\%$  of cells with background fluorescence (conjugated antibody of relevant isotype, but irrelevant Id) were scored in channels 1 to 50. Specific fluorescence is reported as the percentage of cells with relative fluorescence intensity scored in channels 51 to 1000. Data are expressed as the percentage of total cells.

In some experiments, cell surface Ig was removed by treatment of PP, MLN, or spleen cells with pronase, according to a modification of the method of Jones et al. (8) used in our previous studies (2). Briefly, cells were resuspended in Eagle's MEM (GIBCO) to which an equal volume of 0.5% pronase (*Streptomyces griseus* pronase type IV; Sigma) was added. They were incubated for 5 to 15 min at  $37^\circ\text{C}$ , placed on ice, and a large volume of RPMI 1640 was added to the culture vessel. The cells were washed twice with RPMI 1640 and their viability ( $>98\%$ ) determined by trypan blue dye exclusion. Aliquots of the enzyme-treated cells were examined for the presence of IgE, IgM, IgG, and IgA to ensure that they had been removed from the cell surfaces. The remaining cells were cultured for 12 h in serum-free RPMI 1640 supplemented with 5% BSA (Miles Laboratories, Elkhart, IN)  $\pm$  cycloheximide (50  $\mu\text{g}/\text{ml}$ ) (Aldrich Chemical Co., Milwaukee, WI). The cells were then reassessed to determine

the percentage of total cells that had regained surface IgE. At least 50% of the cells were recovered.

Cells were routinely treated with acetate buffer (0.05 M, pH 4.0) containing NaCl (0.085 M), KCl (0.005 M), and heat-inactivated FCS (1%) (GIBCO) for 1 min at  $4^\circ\text{C}$ . Tris buffer (0.1 M, pH 7.4) with 1% FCS was added to the suspension to neutralize the acid to the range of 7.3 to 7.4. Cells were then centrifuged at 1000 rpm for 10 min and washed twice with PBS. Viability as judged by trypan blue dye exclusion was  $>98\%$ . Katona et al. have reported that this procedure removes Ig cytophilically bound to cell surfaces (6).

The fluorescence-conjugated antibodies did not stain cells of any organ when used alone. They also did not stain cells if the specific antibodies were replaced by normal (goat, rabbit, or mouse) serum, an irrelevant antibody, or with PBS. MLN cells from *Nippostrongyls brasiliensis*-infested mice, pretreated with weak acid solution to remove cytophilic Ig (6), served as IgE $^+$  controls whereas thymocytes served as IgE-negative controls.

**Immunohistochemistry studies.** The location of IgE $^+$ , CD23 $^+$ ,  $\text{AsGm1}^+$ , and Thy-1.2 $^+$  cells in lymphoid organs was determined by indirect immunoperoxidase labeling of frozen serial sections. The location of IgE/CD23 immune complexes was determined using frozen and Carnoy's fixed paraffin serial sections, according to the method of Chalet and Wolf (10). For frozen sections, tissues were cut into 2-mm pieces, rinsed with ice-cold PBS, mounted in BSA (35%) (Sigma) and allowed to freeze for 20 min at  $-18^\circ\text{C}$ . Sections (7  $\mu$ ) were cut using a Cryocut microtome (American Optical Corp., Buffalo, NY), placed on glass slides, and used immediately. Alternatively, for paraffin-fixed sections, tissue samples were fixed in cold Carnoy's solution (11) and then embedded in paraffin (Paraplast, Fisher Scientific) until use. Tissue pieces embedded in paraffin were cut (4  $\mu$ ) using a microtome (Minot Rotary International Equipment Corp., Boston, MA) and mounted on glass microscope slides. Peroxidase-labeled cells and IgE/CD23 immune complexes were detected by light microscopy.

No IgE $^+$  cells or  $\text{AsGm1}^+$  cells were detected in tissue sections when peroxidase-conjugated antibodies were used alone or when the primary antibody was replaced with normal sera, an irrelevant antibody, or PBS. IgE/CD23 immune complexes were not detected in tissue sections when peroxidase-conjugated antibodies were used alone or when the primary antibodies were replaced with normal sera, an irrelevant antibody, or PBS. IgE $^+$  mast cells and plasma cells were detected in tissue sections prepared and stained according to the method of Mayrhofer et al. (12).

#### Functional Studies

**ELISPOT assay.** ELISPOT assays, which quantify BPO-specific IgE AFC, were performed as previously described (13). Briefly, BM, thymus, PP, MLN, OLN, or spleen cells ( $4 \times 10^7$ ) from mice injected with BPO-KLH in alum, or with alum alone, or from unsensitized mice, were washed twice in RPMI 1640, resuspended in complete medium (2 ml), and then added to BPO-BSA-coated, 48-well tissue culture plates (0.5 ml/well, triplicate wells). The plates were incubated for 6 h at  $37^\circ\text{C}$  in a humidified atmosphere consisting of 7%  $\text{CO}_2$  in air. The wells were then washed three times with cold PBS-Tween (0.1%) (Tween 20, Aldrich) to ensure that all cells were removed. Rat mAb specific for murine IgE (2  $\mu\text{g}/\text{ml}$ , 0.25 ml/well) was added to appropriate wells, and the plates were incubated overnight at  $4^\circ\text{C}$ . The wells were then washed three times with PBS-Tween, after which horseradish peroxidase-conjugated sheep anti-rat Ig (1/2000, 0.25 ml) was added, and the plates were incubated for 2 h at room temperature. The wells were then washed three times with PBS-Tween and covered with substrate solution (0.5 ml). Substrate solution consisted of diaminobenzidine hydrochloride (400  $\mu\text{g}/\text{ml}$ ) (Sigma) in D-PBS, to which 1% aqueous cobalt chloride (30  $\mu\text{l}/\text{ml}$ ) and urea hydrogen peroxide (400  $\mu\text{g}/\text{ml}$ ) were added.

ELISPOTs, representing the sites of antibody secretion by individual AFC, appeared on plates by 2 h after addition of substrate solution and were fully darkened at 3 to 4 h. The plates were then washed twice with distilled  $\text{H}_2\text{O}$  and air dried. The individual ELISPOTs were counted using a dissection microscope (16 $\times$ ) (Wild, Heerbrugg, Switzerland). Data, representing the mean number of AFC from triplicate wells, are expressed as AFC/ $10^7$  cells.

In agreement with our earlier studies (13) peak numbers of ELISPOTs, representing BPO-specific IgE AFC, were observed in a 5- to 6-h ELISPOT assay at  $37^\circ\text{C}$ , using BPO-BSA-coated plates. No hapten-specific IgE AFC ( $<1$  AFC) were detected if a) plates were coated with BSA rather than BPO-BSA, b) assays were done at  $4^\circ\text{C}$ , rather than at  $37^\circ\text{C}$ , c) rat anti-mouse IgE antibody was omitted from the assay, and d) cycloheximide (50  $\mu\text{g}/\text{ml}$ ) was included in the ELISPOT assay. Furthermore, no hapten-specific IgE AFC responses were obtained from any organ (BM, thymus, mesenteric or other LN, spleen) of unsensitized mice ( $<1$  AFC).

**Passive cutaneous anaphylaxis.** To test for levels of homocytotropic antibody in sera of unsensitized and sensitized mice, the PCA assay of Ovary and colleagues was performed (14). Briefly, serum (0.2 ml from 4 to 6 mice, pooled) was injected into the tail vein of ether-anesthetized, syngeneic, unsensitized mice (three mice/group). At 72 h, BPO-BSA (1 mg/ml) (0.05 ml) was injected s.c. into the pinna of the left ear. Saline (0.05 ml) was injected s.c. into the right pinna (control). After 2 h, swelling in the left and right pinnae was measured using a micrometer caliper. Data represent mean values obtained in three experiments and are expressed as percent absolute increased swelling. Even though PCA is a semi-quantitative assay and not as sensitive as ELISA, PCA was performed instead of ELISA because we wished to test for the appearance of functional BPO-specific antibody in sera (i.e., able to bind to FcR on mast cells).

## RESULTS

### *slgE<sup>+</sup> B Cells and BPO-Specific IgE AFC in Lymphoid Organs of Unimmunized and Immunized Mice and the Effect of Boosting*

**Unimmunized mice.** No slgE<sup>+</sup> B cells or IgE AFC were detected in any organ (BM, thymus, PP, MLN, OLN, or spleen) of unimmunized mice, or of alum-treated mice, on days 0 to 70  $\pm$  boosting (Tables I and II) (data for alum-treated mice not shown).

**Immunized mice.** With all routes of immunization (i.p., gavage, s.c., i.v., or i.m.) no slgE<sup>+</sup> B cells (<1%) or BPO-specific IgE AFC (<1) were detected in any lymphoid organ of immunized mice until day 8 (Tables I and II). When mice were immunized by the s.c., i.v., or i.m. routes, slgE<sup>+</sup> B cells and IgE AFC were exclusively located in PP on day 8 (Table II). With the i.p. or gavage routes, slgE<sup>+</sup> B cells were detected in both PP and MLN on day 8 (Table I), whereas IgE AFC were found only in PP. IgE AFC were not found in MLN until day 10. With all routes of immunization, slgE<sup>+</sup> B cells and BPO-specific IgE AFC did not appear in the spleen until day 12 (Tables I and II). slgE<sup>+</sup> B cells were never detected in BM or in other lymphoid organs (Tables I and II; Fig. 1). However, IgE AFC appeared in BM, but not in OLN, on day 18 (Tables I and II).

With all routes of immunization, the appearance of slgE<sup>+</sup> B cells in PP and MLN was transient in that these cells were no longer detected in these organs by day 14 (Table I and II), but still were detected in spleen through day 18; with the i.p. route, slgE<sup>+</sup> B cells were detected in spleen through day 70 (Fig. 1).

**Effect of boosting.** Boosting either on day 21 or on days

21 and 42 (only i.p. route studied) did not result in either the reappearance of slgE<sup>+</sup> B cells in PP or MLN, the appearance of these cells in OLN or BM, or in an increase in their numbers in spleen (Fig. 1). However, boosting did induce the reappearance of BPO-specific IgE AFC in MLN and increased their numbers in spleen and BM (Fig. 2). Interestingly, boosting did not induce a reappearance of BPO-specific IgE AFC in PP.

**Identification of IgE<sup>+</sup> cells as slgE<sup>+</sup> B cells.** The slgE<sup>+</sup> cells in PP, MLN, and spleen were defined as B cells because they were Thy-1.2<sup>+</sup> typical small (7  $\mu$ m) lymphocytes that resynthesized in culture the IgE observed on their surface membranes after removal by pronase. No IgE<sup>+</sup> cells were detected when pronase-treated cells were cultured with the protein synthesis inhibitor cycloheximide (data for i.p. route given in Table III; data for other routes not shown). Throughout these studies, attempts were made to remove any possible cytophilic Ig from cell surfaces with weak acid treatment. However, similar numbers of slgE<sup>+</sup> cells were detected when IgE<sup>+</sup> cells obtained from PP, MLN, or spleen were examined  $\pm$  such treatment. Weak acid also had no effect on the numbers of slgE<sup>+</sup> B cells detected when pronase-treated cells, which had resynthesized surface IgE after 12 h of culture, were subjected to this treatment.

Virtually all of the IgE<sup>+</sup> B cells detected in sections of PP, MLN and spleen were exclusively located in classical B cell areas (primary follicles), i.e., they were not found in thymus-dependent or other areas of these organs (data for i.p. route given in Table IV; data for other routes not shown); in addition, the slgE<sup>+</sup> B cells in PP, MLN, and spleen did not appear to be activated because they were small lymphocytes (7  $\mu$ m), which were not found in germinal centers of B cell follicles, and did not contain mitotic figures.

The IgE<sup>+</sup> cells were not mast cells because no mast cells were ever detected in either smears of cell suspensions or in primary B cell follicles of lymphoid organs, the sites in which virtually all IgE<sup>+</sup> cells were found. Although rare IgE<sup>+</sup> mast cells were detected in thymus-dependent and other areas of lymphoid tissue, and in gut lamina propria, no IgE<sup>+</sup> mast cells were detected in any site before day 8. No IgE<sup>+</sup> plasma cells were detected in B cell follicles. Rare IgE<sup>+</sup> plasma cells were detected in other sites of MLN and spleen, and most IgE<sup>+</sup> plasma cells were

TABLE I

Appearance of IgE-bearing B lymphocytes in lymphoid organs of mice given BPO-KLH in alum by the oral or i.p. routes: relation to IgE AFC<sup>a</sup>

| Route | Days after Sensitization | Fluorescent Cells (%) |    |     |     |     | IgE AFC/10 <sup>7</sup> Cells |     |     |     |     |
|-------|--------------------------|-----------------------|----|-----|-----|-----|-------------------------------|-----|-----|-----|-----|
|       |                          | BM                    | PP | MLN | SPN | OLN | BM                            | PP  | MLN | SPN | OLN |
| i.p.  | *0-7                     | <1                    | <1 | <1  | <1  | <1  | <1                            | <1  | <1  | <1  | <1  |
|       | 8                        | <1                    | 6  | 5   | <1  | <1  | <1                            | 18  | <1  | <1  | <1  |
|       | 10                       | <1                    | 9  | 7   | <1  | <1  | <1                            | 25  | 4   | <1  | <1  |
|       | 12                       | <1                    | 2  | 2   | 2   | <1  | <1                            | 30  | 11  | 6   | <1  |
|       | 14                       | <1                    | <1 | <1  | 4   | <1  | <1                            | 197 | 32  | 13  | <1  |
|       | 16                       | <1                    | <1 | <1  | 3   | <1  | <1                            | 58  | 79  | 32  | <1  |
|       | 18                       | <1                    | <1 | <1  | 5   | <1  | 52                            | 29  | 168 | 41  | <1  |
| Oral  | *0-7                     | <1                    | <1 | <1  | <1  | <1  | <1                            | <1  | <1  | <1  | <1  |
|       | 8                        | <1                    | 7  | 9   | <1  | <1  | <1                            | 22  | <1  | <1  | <1  |
|       | 10                       | <1                    | 12 | 17  | <1  | <1  | <1                            | 31  | 45  | <1  | <1  |
|       | 12                       | <1                    | <1 | <1  | 4   | <1  | <1                            | 107 | 60  | 3   | <1  |
|       | 14                       | <1                    | <1 | <1  | 5   | <1  | <1                            | 199 | 69  | 8   | <1  |
|       | 16                       | <1                    | <1 | <1  | 5   | <1  | <1                            | 48  | 92  | 12  | <1  |
|       | 18                       | <1                    | <1 | <1  | 7   | <1  | 61                            | 24  | 183 | 59  | <1  |

<sup>a</sup> BALB/c mice were fed (gavage) or injected i.p. with BPO-KLH (10  $\mu$ g) in alum on day 0. On days 0 to 18, the numbers of slgE<sup>+</sup> B cells and BPO-specific IgE AFC in BM, PP, MLN, and OLN, and spleen (SPN) were determined (flow microfluorimetry, ELISPOT assay). Data are from a representative experiment, one of three with similar results, and are expressed as percent total cells or as AFC/10<sup>7</sup> cells. \*Day 0, unsensitized mice.

TABLE II

Appearance of IgE bearing B lymphocytes in lymphoid organs of mice given BPO-KLH in alum by s.c., i.m., or i.v. routes: relation to IgE AFC<sup>a</sup>

| Route | Days after Sensitization | Fluorescent Cells (%) |    |     |     |     | IgE AFC/10 <sup>7</sup> Cells |    |     |     |     |
|-------|--------------------------|-----------------------|----|-----|-----|-----|-------------------------------|----|-----|-----|-----|
|       |                          | BM                    | PP | MLN | SPN | OLN | BM                            | PP | MLN | SPN | OLN |
| s.c.  | *0-7                     | <1                    | <1 | <1  | <1  | <1  | <1                            | <1 | <1  | <1  | <1  |
|       | 8                        | <1                    | 8  | <1  | <1  | <1  | <1                            | 14 | <1  | <1  | <1  |
|       | 10                       | <1                    | 9  | 3   | <1  | <1  | <1                            | 22 | 4   | <1  | <1  |
|       | 12                       | <1                    | <1 | <1  | 2   | <1  | <1                            | 35 | 11  | <1  | <1  |
|       | 14                       | <1                    | <1 | <1  | 3   | <1  | <1                            | 42 | 28  | 5   | <1  |
|       | 16                       | <1                    | <1 | <1  | 5   | <1  | <1                            | 34 | 31  | 16  | <1  |
|       | 18                       | <1                    | <1 | <1  | 6   | <1  | 43                            | 12 | 37  | 47  | <1  |
| i.m.  | *0-7                     | <1                    | <1 | <1  | <1  | <1  | <1                            | <1 | <1  | <1  | <1  |
|       | 8                        | <1                    | 2  | <1  | <1  | <1  | <1                            | 6  | <1  | <1  | <1  |
|       | 10                       | <1                    | 2  | 2   | <1  | <1  | <1                            | 12 | 3   | <1  | <1  |
|       | 12                       | <1                    | <1 | <1  | 1   | <1  | <1                            | 25 | 16  | <1  | <1  |
|       | 14                       | <1                    | <1 | <1  | 2   | <1  | <1                            | 61 | 20  | 9   | <1  |
|       | 16                       | <1                    | <1 | <1  | 5   | <1  | <1                            | 18 | 23  | 11  | <1  |
|       | 18                       | <1                    | <1 | <1  | 7   | <1  | 10                            | 3  | 27  | 14  | <1  |
| i.v.  | *0-7                     | <1                    | <1 | <1  | <1  | <1  | <1                            | <1 | <1  | <1  | <1  |
|       | 8                        | <1                    | 8  | <1  | <1  | <1  | <1                            | 11 | <1  | <1  | <1  |
|       | 10                       | <1                    | 8  | 3   | <1  | <1  | <1                            | 31 | 8   | <1  | <1  |
|       | 12                       | <1                    | <1 | <1  | 4   | <1  | <1                            | 40 | 13  | <1  | <1  |
|       | 14                       | <1                    | <1 | <1  | 4   | <1  | <1                            | 66 | 26  | 5   | <1  |
|       | 16                       | <1                    | <1 | <1  | 5   | <1  | <1                            | 30 | 38  | 21  | <1  |
|       | 18                       | <1                    | <1 | <1  | 6   | <1  | 44                            | 17 | 41  | 56  | <1  |

<sup>a</sup> BALB/c mice were injected s.c., i.m. or i.v. with BPO-KLH (10 µg) in alum on day 0. On days 0 to 18, the numbers of sIgE<sup>+</sup> B cells and BPO-specific IgE AFC in BM, PP, MLN, and OLN, and spleen (SPN) were determined (flow, microfluorimetry ELISPOT assay). Data are from a representative experiment, one of three with similar results, and are expressed as percent total cells or as AFC/10<sup>7</sup> cells. \* Day 0, unsensitized mice.

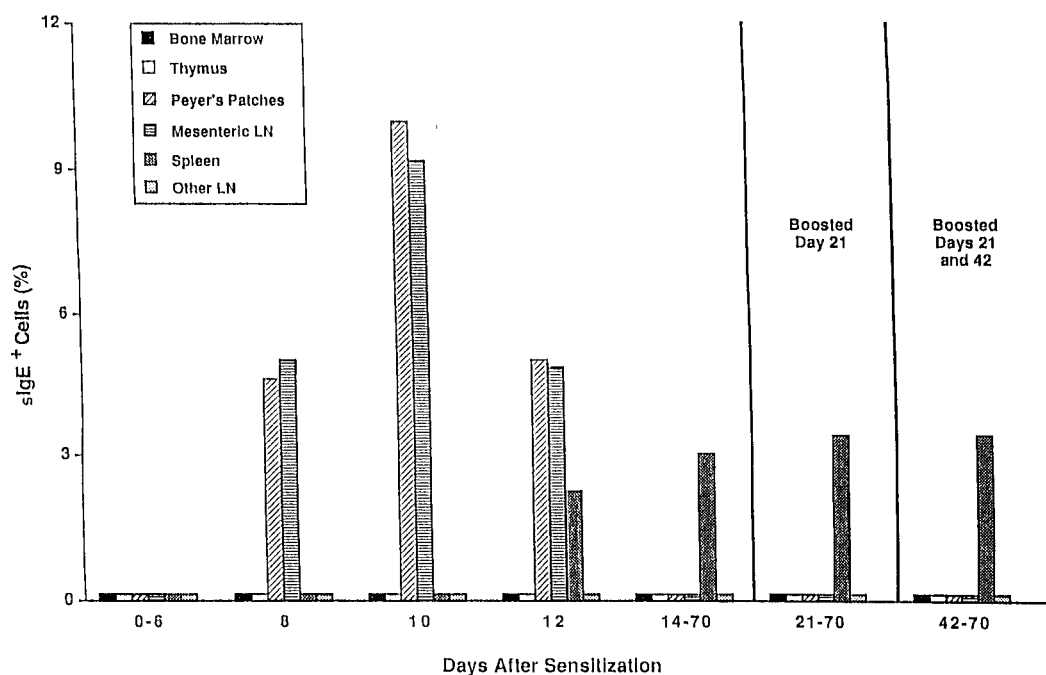


Figure 1. sIgE<sup>+</sup> B cells in BM and lymphoid organs of BPO-KLH sensitized mice. BALB/c mice were injected i.p. with BPO-KLH (10 µg) in alum on day 0, similarly treated (boosted) on day 21 or on days 21 and 42, and killed on days 0 to 70 (day 0, unsensitized). BM (two femurs per mouse), thymus, PP (four per mouse), mesenteric LN, spleen, and other (including axial and inguinal) LN cells from 4 to 6 mice were pooled and their surface markers determined using indirect immunofluorescence (flow microfluorimetry). Data represent mean values from two experiments and are expressed as percentage of total cells.

detected in gut lamina propria. However, no IgE<sup>+</sup> plasma cells were detected in any site before day 8.

#### IgE/CD23 Immune Complexes and AsGm1<sup>+</sup> Cells in PP, MLN, and Spleen of Mice Immunized by the i.p. Route

PP. When mice were immunized by the i.p. route (other routes not studied), IgE/CD23 immune complexes were detected in the "cap," areas of virtually all B cell follicles of PP on days 12 to 20 (Table V), the only sites in which sIgE<sup>+</sup> B cells also were observed (Table IV). The typical

appearance of these IgE/CD23 immune complexes is shown in Figure 3. Strikingly increased numbers of AsGm1<sup>+</sup> cells were detected in PP on days 10 to 22 (to fivefold) (Table VI). These cells were located adjacent to most B cell follicles of PP at this time (data for days 8 to 14 given in Table IV). The appearance of IgE/CD23 immune complexes in PP (Table V) and of increased numbers of AsGm1<sup>+</sup> cells in PP (Table VI) followed the appearance and preceded the disappearance of sIgE<sup>+</sup> B cells and BPO-specific IgE AFC in PP (compare Tables I and VI and Fig. 2).

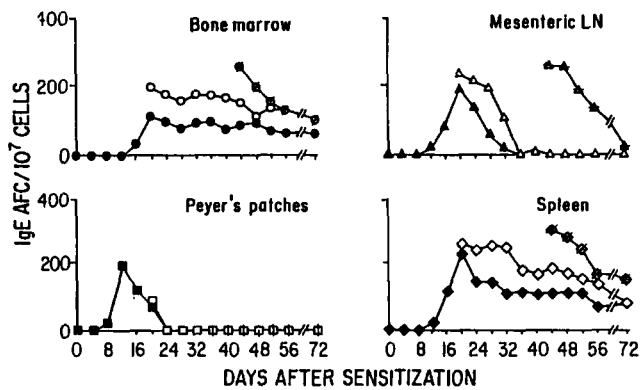


Figure 2. Effect of boosting on the numbers of BPO-specific IgE AFC in BM and lymphoid organs of BPO-KLH sensitized mice. BALB/c mice were injected with BPO-KLH (10  $\mu$ g) in alum on day 0 (black) and boosted on days 21 (white) or on days 21 and 42 (gray). BM (two femurs per mouse), thymus, PP (four per mouse), mesenteric LN, and spleen cells from four to six mice were pooled and the numbers of BPO-specific IgE AFC determined in the ELISPOT assay. Data represent mean values from two experiments and are expressed as AFC/ $10^7$  cells.

TABLE III  
Regeneration of surface IgE on PP, MLN, and spleen cells after enzyme stripping<sup>a</sup>

| Days after Sensitization | Organ  | Hours after Pronase | Cycloheximide in Culture (50 $\mu$ g/ml) | Fluorescent Cells (%) |
|--------------------------|--------|---------------------|------------------------------------------|-----------------------|
| 8                        | PP     | No treatment        | —                                        | 6                     |
|                          |        | 0.5                 | —                                        | <1                    |
|                          |        | 12.0                | —                                        | 5                     |
|                          |        | 12.0                | +                                        | <1                    |
|                          | MLN    | No treatment        | —                                        | 5                     |
|                          |        | 0.5                 | —                                        | <1                    |
|                          |        | 12.0                | —                                        | 4                     |
|                          |        | 12.0                | +                                        | <1                    |
| 10                       | PP     | No treatment        | —                                        | 9                     |
|                          |        | 0.5                 | —                                        | <1                    |
|                          |        | 12.0                | —                                        | 6                     |
|                          |        | 12.0                | +                                        | <1                    |
|                          | MLN    | No treatment        | —                                        | 9                     |
|                          |        | 0.5                 | —                                        | <1                    |
|                          |        | 12.0                | —                                        | 5                     |
|                          |        | 12.0                | +                                        | <1                    |
| 12                       | PP     | No treatment        | —                                        | 9                     |
|                          |        | 0.5                 | —                                        | <1                    |
|                          |        | 12.0                | —                                        | 7                     |
|                          |        | 12.0                | +                                        | <1                    |
|                          | MLN    | No treatment        | —                                        | 4                     |
|                          |        | 0.5                 | —                                        | <1                    |
|                          |        | 12.0                | —                                        | 3                     |
|                          |        | 12.0                | +                                        | <1                    |
|                          | Spleen | No treatment        | —                                        | 3                     |
|                          |        | 0.5                 | —                                        | <1                    |
|                          |        | 12.0                | —                                        | 2                     |
|                          |        | 12.0                | +                                        | <1                    |
| 18                       | Spleen | No treatment        | —                                        | 3                     |
|                          |        | 0.5                 | —                                        | <1                    |
|                          |        | 12.0                | —                                        | 4                     |
|                          |        | 12.0                | +                                        | <1                    |

<sup>a</sup> BALB/c mice were injected i.p. with BPO-KLH (10  $\mu$ g) in alum on day 0. On days 8, 10, 12, and 18, the numbers of IgE<sup>+</sup> cells in PP, MLN, or spleen were determined before and after treatment with pronase (see *Materials and Methods*). Aliquots of the treated cells were cultured for 12 h  $\pm$  cycloheximide (50  $\mu$ g/ml), after which the numbers of IgE<sup>+</sup> cells were determined. Data are from a representative experiment, one of three with similar results, and are expressed as percent total cells.

As stated, boosting on day 21, or on days 21 and 42, did not induce the reappearance of either sIgE<sup>+</sup> B cells (Table I, Fig. 1) or BPO-specific IgE AFC (Fig. 2) in PP. In contrast, boosting on day 21 did induce the reappearance

TABLE IV  
Location of IgE<sup>+</sup> Cells in lymphoid organs on days 8 to 14 of an immune response to BPO-KLH: relation to AsGm1<sup>+</sup>, CD23<sup>+</sup>, and Thy-1<sup>+</sup> cells<sup>a</sup>

| Organ                                  | Peroxidase-Labeled Cells |                    |                    |                   |
|----------------------------------------|--------------------------|--------------------|--------------------|-------------------|
|                                        | IgE <sup>+</sup>         | AsGm1 <sup>+</sup> | Thy-1 <sup>+</sup> | CD23 <sup>+</sup> |
| <b>PP</b>                              |                          |                    |                    |                   |
| B cell areas                           |                          |                    |                    |                   |
| Primary follicles                      | ++                       | ++                 | ±                  | ++++              |
| Secondary follicles (germinal centers) | —                        | ++                 | —                  | ++                |
| Thymus-dependent areas (TDA)           | —                        | ±                  | +++++              | +                 |
| <b>MLN</b>                             |                          |                    |                    |                   |
| B cell areas                           |                          |                    |                    |                   |
| Primary follicles                      | ++                       | ++                 | ±                  | ++++              |
| Secondary follicles                    | —                        | +                  | —                  | ++                |
| Paracortex (TDA)                       | —                        | +                  | +++++              | +                 |
| Medulla                                | —                        | ±                  | +                  | +                 |
| <b>Spleen</b>                          |                          |                    |                    |                   |
| B cell areas                           |                          |                    |                    |                   |
| Primary follicles                      | +                        | ++                 | ±                  | ++++              |
| Secondary follicles                    | —                        | ++                 | ±                  | ++++              |
| Periarteriolar lymphoid sheath (TDA)   | —                        | ±                  | +++++              | +                 |
| Red pulp                               | —                        | ±                  | +                  | +                 |

<sup>a</sup> BALB/c mice were injected i.p. with BPO-KLH (10  $\mu$ g) in alum on day 0, killed on days 8 to 14, and their PP (4/mouse), MLN and spleen removed (6 to 12 mice/group). Peroxidase-labeled cells were enumerated in serial sections (5 to 10/organ, cut at 7  $\mu$ m). Data represent mean values from two experiments and are expressed as arbitrary units of + to +++++, in order of increasing magnitude.

TABLE V  
Location of IgE/CD23 immune complexes in PP<sup>a</sup>

| Site                                   | Day of Boost | Peroxidase-Labeled IgE/CD23 Complexes: Days after Immunization |       |       |       |       |
|----------------------------------------|--------------|----------------------------------------------------------------|-------|-------|-------|-------|
|                                        |              | 0-11                                                           | 12-20 | 21-24 | 25-30 | 31-70 |
| B cell areas                           |              |                                                                |       |       |       |       |
| Primary follicles                      |              | -                                                              | +     | -     | -     | -     |
| Cap (corona) areas                     |              | -                                                              | +     | -     | -     | -     |
| Other areas                            |              | -                                                              | -     | -     | -     | -     |
| Secondary follicles (germinal centers) |              | -                                                              | -     | -     | -     | -     |
| Thymus-dependent areas                 |              | -                                                              | -     | -     | -     | -     |
| B cell areas                           |              |                                                                |       |       |       |       |
| Primary follicles                      | 21           |                                                                |       | -     | +     | -     |
| Cap (corona) areas                     |              |                                                                |       | -     | +     | -     |
| Other areas                            |              |                                                                |       | -     | -     | -     |
| Secondary follicles                    |              |                                                                |       | -     | -     | -     |
| Thymus-dependent areas                 |              |                                                                |       |       |       |       |
|                                        |              |                                                                |       | -     | -     | -     |

<sup>a</sup> BALB/c mice were injected i.p. with BPO-KLH (10  $\mu$ g) in alum on day 0 and similarly injected (boosted) or not on day 21. Mice were killed on days 0 to 70, and serial sections (5 to 10/organ, cut at 7  $\mu$ m) evaluated for the presence of either IgE or CD23 containing immune complexes (peroxidase-labeling technique). Data are from two experiments and are expressed as either positive (+) or negative (—).

of IgE/CD23 immune complexes (Table V), and increased numbers of AsGm1<sup>+</sup> cells (Table VI) in PP. When mice were boosted on day 21, IgE/CD23 immune complexes reappeared in cap areas of B cell follicles of PP on day 25, where they persisted through day 30 (Table V). The numbers of AsGm1<sup>+</sup> cells again increased in PP within 24 h after boosting (28%), peaked on day 25 (37%), remained high through day 27 (15%), and returned to levels seen in PP of unsensitized mice after day 28 (8%) (Table VI). IgE/CD23 immune complexes and AsGm1<sup>+</sup> cells were not studied in mice boosted again on day 42.

**MLN and spleen.** IgE/CD23 immune complexes were never detected in MLN, or spleen  $\pm$  boosting. Increased numbers of AsGm1<sup>+</sup> cells transiently appeared in MLN (days 10 to 12) and spleen (days 14 to 18) after the initial appearance of sIgE<sup>+</sup> B cells in these organs (Table VI).

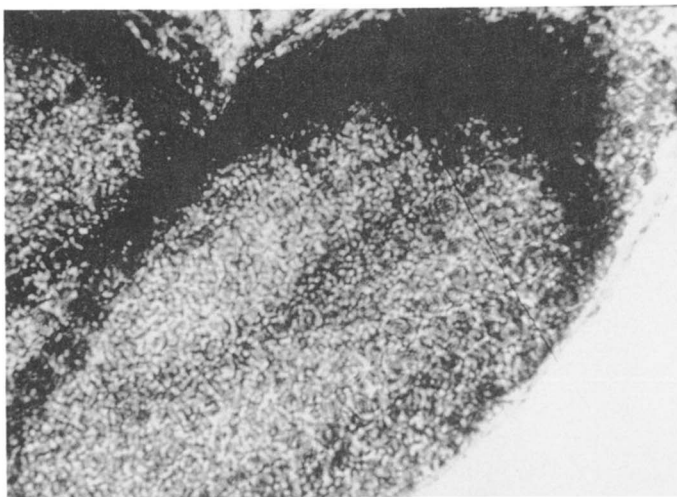


Figure 3. Typical appearance of immunoperoxidase-labeled IgE-containing immune complexes. Complexes are located in the "cap" (corona) of the follicular B cell areas of PP (dark staining areas) of BPO-KLH (10  $\mu$ g)-sensitized mice ( $\times 40$ ). Similar staining patterns were observed when PP were stained using anti-CD23 mAb.

However, in contrast to PP, boosting did not induce the reappearance of increased numbers of AsGm1<sup>+</sup> cells in either MLN or spleen.

#### Homocytotropic Antibody in Serum

Regardless of the route of immunization, homocytotropic antibody was never detected in serum before the appearance of IgE<sup>+</sup> B cells or IgE AFC in PP, or in PP and MLN (day 8). With all routes of immunization, homocytotropic antibody was always first detected in serum after day 8, persisted in serum through day 70, and was increased by boosting (data not shown).

#### DISCUSSION

Several findings stand out from the results reported here. Specific IgE responses appear to originate in GALT

and occur later in spleen. During these responses, PP were the only organs in which both IgE/CD23 immune complexes and strikingly increased numbers of AsGm1<sup>+</sup> cells were detected. Furthermore, the data associate the transient appearance of IgE/CD23 immune complexes and increased numbers of AsGm1<sup>+</sup> cells with "disappearance" of slgE<sup>+</sup> B cells and IgE AFC from PP.

The absence of slgE<sup>+</sup> cells from BM and other organs of unimmunized and alum-treated mice, and the first appearance on day 8 of slgE<sup>+</sup> cells in PP of mice immunized by the s.c., i.v., and i.m. routes, suggests that commitment of B cells to production of membrane bound IgE and to secretion of BPO-specific IgE takes place in PP. However, because slgE<sup>+</sup> B cells simultaneously appeared in PP and MLN when mice were immunized by the i.p. and gavage routes, it also appeared that slgE<sup>+</sup> B cells simultaneously arose in PP and MLN; or that slgE<sup>+</sup> B cells first arose in PP and then rapidly migrated to MLN. The exclusive presence of IgE AFC in PP on day 8, seen even with the i.p. and gavage routes, suggests that specific IgE responses arose in PP. Thus, it may be that a) Ag induced the expression of membrane-bound IgE on IgE-negative B lymphocytes in primary follicles of PP and that some of these cells differentiated into BPO-specific IgE AFC; and b) some slgE<sup>+</sup> B cells migrated from PP to MLN and later, via blood and lymph, to spleen, where some differentiated into BPO-specific IgE AFC.

The idea that IgE<sup>+</sup> B cells arise first in PP is consistent with our earlier studies of germfree rats, which also provided evidence that PP were the sites of first appearance of slgE<sup>+</sup> B cells (2). However, support for the idea that slgE<sup>+</sup> B cells can also arise in MLN can be inferred from our studies of the hyper IgE syndrome. It was deduced from these studies that because hyper IgE syndrome patients lacked PP, and because high numbers of IgE<sup>+</sup> cells were detected only in MLN, the source of the high numbers of slgE<sup>+</sup> B cells found in the peripheral blood was most likely the MLN (3). In this regard, studies

TABLE VI  
Appearance of slgE<sup>+</sup> B lymphocytes in PP, MLN, and spleen: relation to AsGm1<sup>+</sup> cells<sup>a</sup>

| Days after Sensitization | Day of Boost | Fluorescent Cells (%) |                    |                   |                    |                   |                    |
|--------------------------|--------------|-----------------------|--------------------|-------------------|--------------------|-------------------|--------------------|
|                          |              | PP                    |                    | MLN               |                    | Spleen            |                    |
|                          |              | slgE <sup>+</sup>     | AsGm1 <sup>+</sup> | slgE <sup>+</sup> | AsGm1 <sup>+</sup> | slgE <sup>+</sup> | AsGm1 <sup>+</sup> |
| 0 to 6                   |              | <1                    | 8                  | <1                | 2                  | <1                | 3                  |
| 8                        |              | 5                     | 7                  | 5                 | 3                  | <1                | 2                  |
| 10                       |              | 10                    | 18                 | 11                | 10                 | <1                | 3                  |
| 12                       |              | 5                     | 20                 | 4                 | 10                 | 3                 | 2                  |
| 14                       |              | <1                    | 40                 | <1                | 2                  | 5                 | 10                 |
| 16                       |              | <1                    | 24                 | <1                | 3                  | 4                 | 8                  |
| 18                       |              | <1                    | 20                 | <1                | 3                  | 5                 | 6                  |
| 20                       |              | <1                    | 16                 | <1                | 2                  | 6                 | 3                  |
| 22                       |              | <1                    | 14                 | <1                | 3                  | 4                 | 2                  |
| 24 to 70                 |              | <1                    | 8                  | <1                | 3                  | 5                 | 3                  |
| 22                       | 21           | <1                    | 28                 | <1                | 2                  | 4                 | 2                  |
| 23                       |              | <1                    | 39                 | <1                | 3                  | 5                 | 2                  |
| 24                       |              | <1                    | 35                 | <1                | 2                  | 5                 | 2                  |
| 25                       |              | <1                    | 37                 | <1                | 3                  | 4                 | 3                  |
| 26                       |              | <1                    | 20                 | <1                | 1                  | 6                 | 3                  |
| 27                       |              | <1                    | 15                 | <1                | 3                  | 4                 | 4                  |
| 28                       |              | <1                    | 8                  | <1                | 2                  | 5                 | 4                  |
| 29                       |              | <1                    | 8                  | <1                | 2                  | 4                 | 3                  |
| 30-70                    |              | <1                    | 8                  | <1                | 3                  | 5                 | 2                  |
| Unsensitized             |              | <1                    | 8                  | <1                | 2                  | <1                | 2                  |

<sup>a</sup> BALB/c mice were injected with BPO-KLH (10  $\mu$ g) in alum on day 0, boosted or not on day 21, and killed on days 0 to 70. PP, MLN, and spleen cells from 6 to 12 mice were pooled and their surface markers determined after acid treatment (see *Materials and Methods*) using indirect immunofluorescence (flow microfluorimetry). Data represent mean values from two experiments and are expressed as percentage of total cells.

of Ishizaka et al. (5) and Katona et al. (6) in *N. brasiliensis*-infested rodents reported the presence of IgE<sup>+</sup> cells in MLN before they appeared in spleen or BM. Unfortunately, PP were not examined in either of those studies.

The identity and site of origin of the IgE-negative precursors of sIgE<sup>+</sup> B cells first seen in GALT in the present studies are unknown. Also unknown is the mechanism by which the B cells were induced to express IgE on their surfaces. However, it is well recognized that specific IgE responses are T cell mediated (reviewed in Reference 15). Thus, it is likely that T cells, other cells (Ag-processing cells? AsGm1<sup>+</sup> cells?) and also factors produced by these cells (e.g., IL-4, IFN- $\alpha$ ) are acting in concert to induce IgE-negative B cells to express surface IgE.

In the present studies, IgE responses disappeared from PP after the appearance and before the disappearance of IgE/CD23 immune complexes. It is possible that the IgE/CD23 immune complexes located exclusively in PP may have acted as down-regulators at these sites. It is significant that the IgE/CD23 immune complexes were localized to the cap areas of B cell follicles because it is known that B cells migrate into follicles via the cap areas (16, 17) and would come into intimate contact with these IgE/CD23 immune complexes. This suggests that a form of immune complex-mediated inhibition or down-regulation may be occurring at this site. Isotype-specific down-regulation of IgE responses in vitro by IgE-containing immune complexes has been clearly demonstrated by Saxon and Hassner (18). In this regard, recent studies by Maeda et al. have described IgE/CD23 immune complexes on follicular dendritic cells of murine MLN after infestation with *N. brasiliensis* (19). Thus, it appears likely that the IgE/CD23 immune complexes observed in the present studies are also bound to follicular dendritic cells.

In the present studies, IgE responses disappeared from PP after the appearance of IgE/CD23 immune complexes, and increased numbers of AsGm1<sup>+</sup> cells. Furthermore, after boosting, increased numbers of AsGm1<sup>+</sup> cells were again observed only in PP. At this time, PP, unlike MLN or spleen, contained no IgE<sup>+</sup> B cells or IgE AFC. AsGm1<sup>+</sup> cells may represent NK cells or natural suppressor cells (for review see Reference 20). Because these cells have cytolytic potential (20), are potent producers of IFN- $\gamma$  (21), and because IFN- $\gamma$  down-regulates IgE production (22-24), suppression of IgE responses by AsGm1<sup>+</sup> cells may affect B cells directly and/or act through other cells, by such diverse mechanisms as cytolysis and cytokine production.

The IgE<sup>+</sup> cells detected in PP, MLN, and spleen in the present studies were defined as B lymphocytes with membrane-bound IgE because they resynthesized the IgE detected on their surface membranes after pronase treatment. In addition, virtually all IgE<sup>+</sup> cells in lymphoid organs were located in classical B cell areas. In this regard, studies of Ishizaka et al. (5) and Katona et al. (6) have reported similar numbers of sIgE<sup>+</sup> B cells (2 to 5%) in MLN and spleen of *N. brasiliensis*-infested rats (5) and mice (6), where they persisted for longer periods.

It is of interest that widespread cytophilic binding of IgE to cell surfaces was not detected either in the present studies or in those of Ishizaka et al. (5). In contrast, Katona et al. reported that most IgE<sup>+</sup> cells in MLN of parasite-infested mice represented cells with IgE cytophilically bound to cell surfaces (6). The reason for these

differences is not clear; however, studies have demonstrated phenotypic and functional heterogeneity in CD23 among activated or resting B and T cells (25), monocytes (26), and perhaps NK cells (27). The increasing battery of mAb directed against IgE or CD23, which is presently being developed, should be of help in resolving these issues. Nevertheless, it appears that during a specific immune response in mice, and in parasite-infested rats, IgE<sup>+</sup> B cells, IgE AFC, and high serum IgE levels may coexist without widespread cytophilic binding of IgE to lymphocyte surfaces. Durkin et al. (3), studying patients with hyper-IgE syndrome detected a subpopulation of peripheral blood B cells that expressed both sIgE and CD23, and found that the CD23 molecules present on the surfaces of these cells were devoid of cytophilically bound IgE (4). Furthermore, studies of Kikutani et al. demonstrated that in hyper IgE syndrome, the majority of CD23<sup>+</sup> B cells were immature cells that did not express membrane-bound IgE and to which no IgE was cytophilically bound (28).

It seems unlikely that the sIgE<sup>+</sup> cells identified in the present studies were confused with mast cells bearing cytophilic IgE because no mast cells were ever detected in cell suspensions or in B cell follicles of PP, MLN, or spleen. Although a few IgE<sup>+</sup> mast cells were identified in thymus-dependent and other sites of these organs, IgE<sup>+</sup> mast cells were readily detected in gut lamina propria. Therefore, widespread degranulation of mast cells, resulting in "phantom" mast cells of the type described by Claman et al. during graft vs host reactions (29) seems not to have occurred. Nevertheless, the presence of a rare IgE<sup>+</sup> phantom mast cell cannot be strictly ruled out.

It appears from the present study that commitment of murine B cells to expression of membrane-bound IgE first occurs in GALT. It also appears that differentiation of sIgE<sup>+</sup> B cells into IgE AFC occurs first in PP and later in MLN, spleen, and BM. Furthermore, the data suggest that PP, as well as other elements of GALT, may provide, in addition to T cells and cytokines, other cellular and molecular regulators of IgE responses, perhaps in the form of IgE/CD23 immune complexes and increased numbers of AsGm1<sup>+</sup> cells.

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