A CLONAL ANALYSIS OF THE IgE RESPONSE AND ITS IMPLICATIONS WITH REGARD TO ISOTOPE COMMITMENT*

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Since its identification as a class of distinct antibody molecules (1), the IgE antibody system has been of great interest to immunologists because of its role in IgE-mediated allergic diseases. Until recently, however, the IgE response has been difficult to study because, as compared with other immunoglobulin (Ig) classes, it is synthesized and secreted into serum in very small quantities. Consequently, development of suitable reagents and adequate technology for quantitating IgE by modern immunoassay techniques, especially in experimental animals, has been hampered. Nevertheless, the ability to measure IgE antibody responses in a biological manner by the passive cutaneous anaphylaxis (PCA)[1] technique (2) made it possible to investigate many important parameters concerning regulation of the IgE response.

Considerable experimental evidence has been collected during the past 10 years which indicates that the IgE antibody response is tightly regulated, even more so than antibody responses of other Ig classes. Independent experiments by Okumura and Tada (3), Hamaoka et al. (4, 5) and Ishizaka and Okudaira (6) demonstrated the requisite participation of T lymphocytes in IgE responses. Moreover, very early in such investigations it became clear that a suppressor T cell mechanism plays a dominant role in minimizing the IgE response (7). Recent studies of various types performed in several different laboratories have identified several biologically-active soluble factors, many of which appear to be T cell products that selectively enhance or suppress IgE antibody responses with no detectable effects on antibody responses of other Ig classes (8–16; reviewed in 17).

Although the bulk of experimental work on the IgE system, such as those cited above, has been highly informative in terms of the complex regulatory mechanisms governing IgE responses, relatively little direct information has been gathered about the biology of the B lymphocytes committed to express the ϵ heavy chain isotype. Previous work clearly indicated that IgE B cells differed from IgG B cells in their relative sensitivities to regulatory T cell activities (18) and to the biological effects of ionizing irradiation (19). Such findings raised the question of whether IgE B cells

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[1] *Abbreviations used in this paper:* ASC, *Ascaris suum* extract; BSA, bovine serum albumin; DNP, 2,4-dinitrophenyl; KLH, keyhole limpet hemocyanin; MLN, mesenteric lymph node; PC, phosphorylcholine; PCA, passive cutaneous anaphylaxis; RIA, radioimmunoassay.

existed as a distinct lineage, unlike B cells committed to expression of other Ig isotypes. Indeed, certain recent studies have been interpreted as evidence for a separate IgE B cell lineage (20).

Because of the fundamental importance of this issue, we felt it essential to put to a direct experimental test the postulate that there is a separate subset of IgE-committed B cells. The only way to do this unambiguously is to study the IgE response at the clonal level. The recent development of IgE-secreting murine hybridomas made available the necessary reagents to develop a suitable solid-phase radioimmunoassay (RIA) system specific for murine IgE (21). This, in turn, made it possible to use the T cell-dependent splenic focus assay (22, 23) to analyze primary and secondary IgE B cells at the clonal level. The results of such studies, presented herein, provide information on the biology of IgE B lymphocytes including their frequency and tissue distributions in mouse strains of both the high and low IgE responder phenotypes (24) and the extent to which IgE-secreting B cells are committed to synthesis of IgE molecules. Collectively, the observations obtained in this system demonstrate that B lymphocytes capable of expressing IgE do not exist as a direct lineage of B cells, but rather, B lymphocytes display multipotentiality with regard to Ig heavy chain isotype expression, including IgE.

Materials and Methods

Hapten-Protein Conjugates. The preparation of 2,4-dinitrophenyl (DNP)-keyhole limpet hemocyanin (DNP_{10}-KLH), DNP-bovine serum albumin (DNP_{10}-BSA) and DNP_2-*Ascaris suum* extract (DNP_2-ASC) have been previously described (4, 25, 26). The subscripts indicate the number of moles of DNP per 100,000 mol wt of KLH, moles of DNP per mole of BSA, and moles $\times 10^{-7}$ of DNP/mg of ASC, respectively. ASC proteins were extracted from *Ascaris suum* (27) and kindly provided by Dr. Kurt Bloch, Massachusetts General Hospital, Boston, Mass.

Animals and Immunizations. Inbred BALB/c, C57BL/6, SJL, and A/J mice used in these experiments were obtained from the Scripps Clinic and Research Foundation breeding colony. Adult mice 6–8 wk of age were injected i.p. with either 2 μg of KLH or 10 μg of ASC adsorbed on $Al(OH_3)$ gel (alum, 4 mg), and challenged i.p. with the same doses in saline 3–5 wk afterwards. These mice were irradiated 4–6 wk later (1,300 rad total-body irradiation from a ^{137}cesium source) and used as recipients for adoptive transfer of adult nonimmune or immune spleen, mesenteric lymph node (MLN), or bone marrow cells.

Mice used as donor sources of immune cell populations were injected i.p. with 2 μg of DNP_{10}-KLH adsorbed on 4 mg of alum followed by 2 μg of DNP_{10}-KLH in saline 3–5 wk later. These mice were rested 2–3 mo after the last injection before using.

Cell Transfers and Splenic Focus Assay. Spleen, MLN, or bone marrow cells taken from the appropriate strains of mice were injected into the carrier-primed, lethally irradiated recipients for analysis in the splenic focus assay as previously described (22, 23). Approximately 0.1×10^6–1×10^6 cells from the immune cell populations and 5×10^6–15×10^6 cells from the nonimmune populations were transferred per recipient. Fragment cultures derived from recipient spleens were incubated either in the presence of DNP-ASC at 1 μg/ml or DNP-KLH at 10^{-7} M DNP for 3–4 d. Culture supernates were collected on days 7, 10, 14, 17, and 21 and analyzed for the presence of anti-DNP antibodies.

RIA. Culture supernates collected at various days after antigenic stimulation were assayed for the presence of anti-DNP antibody using a polyvalent rabbit anti-mouse Ig in a solid-phase RIA as previously described (21, 28). At the same time, the supernates were also tested for anti-DNP antibodies of the IgE isotype using the same RIA except that rabbit anti-mouse IgE was used in place of the polyvalent anti-mouse Ig. The rabbit anti-IgE was raised against mouse IgE purified from an IgE-secreting hybridoma as described elsewhere (21); the resulting antiserum was rendered monospecific for IgE as described (21).

Supernates found to be positive for anti-DNP antibodies were subsequently tested for all Ig

isotypes through the use of the following anti-mouse class-specific antibodies and anti-light chain-specific antibodies (Litton Bionetics, Kensington, Md.): (*a*) anti-IgM, (*b*) anti-IgG1, (*c*) anti-IgA, (*d*) anti-IgG2a, (*e*) anti-IgG2b, (*f*) anti-IgG3, (*g*) anti-kappa, and (*i*) anti-lambda. These were made highly specific by adsorption on Sepharose that had been coupled with antibodies of known heavy chain class isolated from anti-DNP-secreting hybridomas (21). Data documenting their specificity have been provided elsewhere ([21]; and J. M. Teale, D. La Frenze, N. R. Klinman, and S. Strober. Immunoglobulin class commitment exhibited by B lymphocytes separated according to surface isotype. Manuscript in preparation.).

Results

Comparison of the Frequency of IgE-secreting Clones in Low and High IgE Responder Strains. To determine the frequency of B cells that can produce IgE anti-DNP antibodies after antigenic stimulation, donor spleen cells from various strains of mice were injected into the appropriate KLH-primed, lethally-irradiated recipients and analyzed in the splenic focus assay (22, 23) after stimulation with DNP-KLH. Because previous experiments have shown the importance of antigen dose in the IgE response (24, 29), doses of DNP-KLH from 10^{-6} M to 10^{-12} M DNP were initially tested. However, little detectable difference was observed in the IgE response among the various doses tested except when the antigen was diluted to $<10^{-9}$ M DNP. In this case the frequency of anti-DNP-secreting clones was lower and variable. Consequently, an antigen dose of 10^{-7} M DNP was used in subsequent experiments.

The total frequency of anti-DNP-secreting clones was detected in a solid-phase RIA with a polyvalent rabbit anti-mouse Ig, and the proportion of these clones secreting the IgE isotype was determined by RIA with rabbit anti-mouse IgE. From Table I it is apparent that the frequency of nonimmune B cells that secrete IgE after antigenic stimulation is extremely low, representing only 3–4% of the total clonal response. Notably, there seemed to be little difference in the frequencies of IgE-secreting B cells among the various high and low IgE responder strains. That is, the SJL and C57BL/6 mice, which usually produce little or no IgE, show similar frequencies to the high responder A/J and BALB/c strains, indicating that the low responder strains

TABLE I

Clonal Analysis of IgE Response in Classical Low and High IgE Responder Strains

Strain*	Frequency of DNP-specific clones/10^6 spleen cells transferred‡		Percentage of clones secreting IgE
	Total	IgE	
BALB/c	1.5 ± 0.3§	0.05 ± 0.02	3.3
SJL	1.2 ± 0.07	0.04 ± 0.01	3.8
A/J	1.7	0.05	2.9
C57BL/6	1.1	0.04	3.6

* IgE responder phenotypes (24): BALB/c, A/J = high; SJL, C57BL/6 = low.

‡ Donor spleen cells were injected into KLH-primed recipients and after 16–24 h the recipient spleens were used to make fragment cultures, which were then incubated for 3 d in the presence of the antigen DNP-KLH. Supernates were collected starting at day 7 after stimulation and the presence of anti-DNP-secreting clones was detected in RIA.

§ Results of three experiments ± SE of the mean. Results represent the average of two experiments where the SEM is omitted.

TABLE II

Clonal Analysis of IgE Response Using ASC-primed Recipients

Source of donor cells (BALB/c)	Frequency of DNP-specific clones/ 10/6 spleen cells transferred*		Percentage of clones secreting IgE
	Total	IgE	
Spleen	1.13 ± 0.15‡	0.08 ± 0.01	7.1
Bone marrow	0.60	0	0
MLN	1.20	0.10	8.3

* Cell suspensions from the sources indicated were transferred into ASC-primed recipients. Fragment cultures were stimulated with the antigen DNP-ASC.

‡ Results presented as the mean of three experiments ± SEM. Results represent the average of two experiments where the SEM is omitted.

TABLE III

Clonal Analysis of IgE Response from Memory B Cells

Source of donor cells (BALB/c)	Frequency of DNP-specific clones/10^6 spleen cells transferred*		Percentage of clones secreting IgE
	Total	IgE	
Spleen	33.10 ± 4.6	2.50 ± 0.60	7.6
MLN	34.10	4.85	14.2

* See footnotes to Table I; the same procedure was followed except donor cell suspensions were obtained from mice previously primed with DNP-KLH.

are not deficient in precursor B cells capable of IgE production. The range of IgE antibodies secreted by individual clones was 8–32 ng/well.

Clonal Analysis of IgE Responses Using ASC-primed recipients. It has been well-documented that a number of parasites, including *Ascaris suum*, preferentially stimulate the IgE response (30, 31). Because the frequency of IgE-secreting clones was low in comparison to the total frequency of DNP specific clones, nonimmune BALB/c donor cells from spleen, bone marrow, and MLN were analyzed in the splenic focus assay using recipient mice primed with ASC and using DNP-ASC as the antigen in the culture system. The results, presented in Table II, show only a slight increase in the splenic frequency of DNP-specific IgE-specific clones using the ASC-primed recipients as compared with the KLH-primed recipients (Table I versus Table II). In addition, it was found that the frequency of IgE-secreting clones obtained from MLN was slightly higher than that obtained from spleen, but that no IgE-secreting clones were detected in the bone marrow cell population.

Clonal Analysis of the IgE Response from Immune Lymphocyte Populations. To determine the effect of prior antigen exposure on the frequency of anti-DNP IgE-secreting clones, donor cells were taken from mice primed 8–12 wk previously with DNP-KLH and analyzed in the splenic focus assay using KLH-primed recipients. The results shown in Table III indicate that previous exposure to antigen substantially increases the frequency of B cells capable of producing IgE, and the actual proportion of immune B cells that subsequently secrete IgE is approximately double the proportion found in primary B cell populations. It should be noted, however, that the fraction of immune B cells capable of IgE production remains relatively small, representing <15% of the total clonal response. Immune lymphocytes from the MLN seemed to be the most

TABLE IV

Combination of Isotypes Occuring with IgE-secreting Clones

Source of donor cells	Number of IgE-secreting clones analyzed	Percentage of clones secreting the following combinations of isotypes*				
		ϵ alone	$\epsilon + \mu$	$\epsilon + \alpha$	$\epsilon + \gamma$‡	$\epsilon \geq$ two other isotypes
Primary B cells						
Spleen	24	0	4.1	0	8.3	87.5
Memory B cells						
Spleen	31	0	0	0	16.1	83.8
MLN	10	0	0	0	0	100

* Clones secreting IgE anti-DNP were reanalyzed by RIA for the presence of other anti-DNP isotypes using the following anti-heavy chain-specific antibodies: (*a*) anti-IgM, (*b*) anti-IgG1, (*c*) anti-IgG2a, (*d*) anti-IgG2b, (*e*) anti-IgG3, and (*f*) anti-IgA.

‡ This grouping includes clones secreting ϵ + any one of the IgG subclasses.

TABLE V

Complete Isotype Analysis of IgE-secreting Clones

Source of donor cells	Number of clones analyzed	Percentage of clones secreting the following isotypes*								
		ϵ	μ	γ1	γ2a	α	γ3	γ2b	κ	λ
Primary B cells										
Spleen	24	100	41.6	62.5	12.5	50.0	4.2	50.0	70.8	29.1
Spleen	60	0	31.7	56.7	3.3	56.7	3.3	31.7	90.0	10.0
Memory B cells										
Spleen	31	100	22.0	87.0	29.0	51.6	32.3	61.3	87.0	12.9
MLN	10	100	20.0	100	30.0	100	20.0	40.0	70.0	30.0

* The same procedure was followed as in Table IV except 60 primary, splenic DNP-specific clones not secreting IgE were also analyzed for the presence of other Ig isotypes.

enriched for B cells capable of secreting IgE as compared with the spleen and bone marrow.

Complete Isotope Analysis of IgE-secreting Clones. An extremely important point in view of the possibility that the IgE response involves a separate subset of B cells committed to the production of IgE is the question of whether clones synthesizing IgE are synthesizing other Ig isotypes as well. To test this point, the IgE-secreting clones were examined for the presence of other Ig isotypes, and the results are shown in Table IV. The data are presented as the percentage of the IgE-secreting clones that are secreting the various combinations of isotypes. Most noteworthy is that no DNP-specific, IgE-secreting clones were found which secreted solely IgE from either primary or memory cell populations. Moreover, the vast majority of clones were secreting at least two other isotypes in addition to IgE.

In Table V, a complete isotype analysis of the IgE-secreting clones is presented. It is evident that IgE is found in combination with all of the other isotypes tested and mostly with two or more different isotypes. Also included in Table V is the complete isotype analysis of 60 primary clones from the splenic B cell population that are not

secreting IgE. Note that the distribution of non-IgE isotypes does not appear to differ substantially from the distribution of these isotypes present in the IgE-secreting clones. The results of the isotype analysis above as well as other studies (J. M. Teale, D. La Frenze, N. R. Klinman, and S. Strober. Immunoglobulin class commitment exhibited by B lymphocytes separated according to surface isotype. Manuscript in preparation.) indicate that the majority of B cells, including those that can secrete IgE, are not committed to a particular Ig class but rather are multipotential with regard to isotype production.

Discussion

The purpose of this study was to analyze the IgE response in a clonal manner so that the frequency and tissue distribution of IgE-secreting B cells and information pertaining to the commitment of isotype expression exhibited by these B cells could be ascertained. The results herein indicate that <3–4% of the splenic B cells stimulated by the antigen DNP-KLH secrete the IgE isotype. The same low frequency was observed in the four strains of mice tested including SJL and C57BL/6 which are known to be low IgE responder strains (24). This result demonstrates conclusively that the low IgE responder phenotype cannot be attributed to a lack of B cells capable of producing IgE. This is consistent with the observations and interpretations of Chiorazzi et al. (32, 33) and Watanabe et al. (8, 34), who were able to convert the low IgE responder phenotype to a high IgE responder phenotype by various manipulations such as low-dose irradiation and immunosuppressive drugs. The mechanism by which these manipulations enhanced an IgE response in the low IgE responder strain appeared to reflect a reduction or elimination of nonspecific suppressor T cells that normally minimize the IgE response (33, 34).

In an attempt to increase the proportion of DNP-specific clones that could secrete the IgE isotype, nonimmune donor cells were injected into recipients primed with the parasitic ASC antigen. However, this experimental protocol produced only marginal increases in the frequencies of IgE-secreting B cells. In contrast, prior exposure of donor lymphocytes to antigen resulted in as much as 50-fold increases in the frequency of IgE-secreting B cells. This was particularly true of immune cells from MLN that enriched for IgE-secreting B cells. This finding is consistent with earlier studies concerning the localization of the IgE response after immunization or parasitic infection (35, 36).

Whether or not a separate lineage of B cells committed to IgE synthesis exists is fundamental to our understanding of how the IgE response is regulated. Because a variety of regulatory phenomena have been described that affect the IgE response preferentially and have little or no effect on other Ig classes (17), and because there have been differences observed in certain biological properties of IgE B cells (18, 19), it has been suggested that IgE B cells may exist as a separate subset. Inferential evidence for a separate lineage of IgE B cells was obtained by Ishizaka et al. (37) who demonstrated the appearance of surface-IgE-bearing B cells, the putative precursors of IgE-producing cells, in the neonatal rat. However, because of the extremely small percentages of IgE-bearing cells obtained and the inherent problems of the fluorescent staining technique itself, establishing a precursor-product relationship between the IgE-bearing cells and IgE-synthesizing cell would be essential for an accurate interpretation of these results. Indeed, a recent study by Teale et al. (J. M. Teale, D. La

Frenze, N. R. Klinman, and S. Strober. Immunoglobulin class commitment exhibited by B lymphocytes separated according to surface isotype. Manuscript in preparation.) suggests that IgG-bearing primary B cells may be an artifact of the fluorescent staining technique.

More direct evidence for a separate lineage of IgE committed B cells was obtained from studies by Kishimoto et al. (20) in which the immunologically defective CBA/N mice could produce IgE anti-phosphorylcholine (PC) antibodies but not IgG or IgM anti-PC antibodies in response to PC-KLH. However, studies by Clough et al. (38) as well as by R. Bargatze, H. Yamamoto, and D. H. Katz, who used a similar protocol (unpublished results), indicate that CBA/N mice are capable of producing all three isotypes (IgM, IgG, and IgE) in response to PC. The basis for the differences in results obtained by the latter investigators as contrasted to those of Kishimoto et al. (20) has yet to be ascertained. Thus, little concrete information has been heretofore available concerning the nature of the B cell capable of producing IgE and whether or not these B cells constitute a distinct subset of cells.

The results presented in this paper demonstrate an absence of a separate lineage of B cells committed to IgE synthesis. Indeed, the vast majority of IgE-secreting clones analyzed in this study were also secreting at least two other isotypes in addition to IgE. It is important to note that a number of IgE-secreting clones were analyzed from both primary and secondary B cells in which three or more classes were secreted, all of which bore the relatively rare lambda light chain. This, together with the well-documented fact that the splenic focus assay system illuminates individual B cell clones (22, 23) means that the possibility that they arose from more than one clone per well is virtually excluded. Consequently, from these and other experiments (J. M. Teale, D. La Frenze, N. R. Klinman, and S. Strober. Immunoglobulin class commitment exhibited by B lymphocytes separated according to surface isotype. Manuscript in preparation.) it appears that B cells are not committed to a particular isotype as previously suggested (39, 40) but rather exhibit potential for multiple class expression. The implication of these findings is that the aforementioned enhancing and suppressive phenomena which preferentially affect the IgE response must somehow influence the multipotential B cell in a way that either permits or prevents the synthesis of IgE without affecting the synthesis of other Ig isotypes. The mechanism by which this regulation occurs is presently being investigated.

Summary

In a clonal analysis of the IgE response, it was found that a small proportion of primary or nonimmune B cells in spleen and mesenteric lymph nodes can be stimulated by antigen to produce IgE-secreting clones. In addition, there appears to be no substantial difference in the frequency of such cells between the classical low and high IgE responder strains. An analysis of immune, or memory, B cells revealed substantial increases in the frequency of B cells secreting IgE as compared with primary B cells, although the actual proportion of B cells secreting IgE remained relatively low. When the IgE-secreting clones derived from either primary or secondary B cells were reanalyzed for the presence of other isotypes, it was found that all clones secreting IgE were secreting at least one other isotype, with the majority secreting two or three other isotypes in addition to IgE. This demonstrates that there is no distinct subpopulation of B cells committed to IgE expression per se.

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