

KINETICS AND DISTRIBUTION OF ANTIGEN-SPECIFIC IgE-
SECRETING CELLS DURING THE PRIMARY ANTIBODY
RESPONSE IN THE RAT*

By J. D. SEDGWICK[‡] and P. G. HOLT[§]

From the Clinical Immunology Research Unit, Princess Margaret Hospital, Subiaco, 6008, Western Australia, and the Department of Microbiology, University of Western Australia

Since the identification of the IgE antibody class as a major source of reaginic activity (1), the mechanisms that regulate the IgE system have attracted increasing attention from immunologists, in view of the potential role of this antibody in allergic disease(s). While the low levels of IgE encountered in biological fluids have hampered the development of modern immunoassay techniques for the quantitation of this antibody isotype, the passive cutaneous anaphylaxis (PCA)¹ bioassay (2) has provided a means to illustrate the basic features of IgE immunoregulation.

Using this technique to monitor the development of IgE responses in experimental animals via measurement of serum antibody titers, it has been possible to show that the IgE system is tightly regulated by T lymphocytes (3, 4), which function in an apparently class-specific fashion (5, 6, reviewed in 7).

Despite the success of these studies on T-B cell interaction in the IgE response, relatively little direct information exists on the biology of the B cells committed to expression of the epsilon heavy chain. These cells have been thought to be derived from a single lineage committed to IgE production (8, 9). However, recent studies on precursor frequency suggest that the major influence attributable to the B cells themselves, with regard to isotype commitment, stems from their maturational state, with the predominant form of regulation occurring at the T cell level (10). Studies on the frequency of mature IgE immunoglobulin-secreting cells (ISC) during the course of primary and secondary antibody responses have not been technically feasible, and are clearly necessary for further elucidation of the immunoregulatory mechanisms that control expression of this isotype.

This paper describes a novel method for enumeration of IgE ISC based upon enzyme-linked immunosorbent assay (ELISA) methodology, which is used in the study below on the organ distribution and kinetics of development of IgE ISC during the course of the primary and secondary antibody response in the rat. The results of this study implicate the draining lymph node as the major site of IgE and IgG synthesis during the primary response, with the spleen contributing significantly only to the IgM class.

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[‡] Supported by a Commonwealth Postgraduate Research Award.

[§] To whom correspondence should be addressed at the Clinical Immunology Research Unit, Princess Margaret Hospital, Subiaco 6008 Western Australia.

¹ *Abbreviations used in this paper:* OVA, ovalbumin; PCA, passive cutaneous anaphylaxis; ELISA, enzyme-linked immunosorbent assay; ISC, immunoglobulin secreting cells; AP, alkaline phosphatase; Rb, rabbit; Sh, sheep; NRS, normal rat serum.

Materials and Methods

Animals and Immunizations. Male Brown Norway (BN) rats aged 12–16 wk were used in experiments that examined primary and secondary antibody responses. The animals were immunized intraperitoneally with 100 μ g ovalbumin (OVA) plus 10 mg aluminium hydroxide and rechallenged (where indicated) 14 d later with the same antigen-adjuvant dose. Male WAG rats were used as PCA recipients, and SPF Lou/M animals inoculated with *Nippostrongylus brasiliensis* larvae were used in studies on the enumeration of total numbers of IgE-ISC.

ELISA Reagents. The specificity of Rb-anti-rat IgG (γ -chain specific) (Cappel Laboratories, Cochranville, PA) and rabbit (Rb)-anti-rat IgM (μ -chain specific) (Nordic Immunology, The Netherlands) were confirmed in preliminary tests, and these reagents were used without further purification. Sheep (Sh)-anti-Rb IgG (a gift from Dr. G. Stewart of these laboratories) was repeatedly passed through an immunoabsorbent column comprising a 40% saturated ammonium sulfate precipitate of normal rat serum (NRS), to remove anti-rat IgG and IgM activity. Rb-anti-rat IgE (a gift from Associate Professor K. J. Turner of these laboratories) raised against the IR 162 IgE myeloma was initially immunopurified using IR 331 rat IgE myeloma protein, and subsequently absorbed against 50 and 40% saturated ammonium sulfate precipitates of NRS as above. Sh-anti-rat IgE (ϵ -chain specific) (Miles Laboratories Inc., Elkhart, IN) was similarly immunopurified, followed by absorptions against glutaraldehyde-cross-linked NRS, a 40% saturated ammonium sulfate precipitate of NRS and protein A-purified IgG from NRS. These reagents were monitored for cross-reactivity with purified IgM and IgG by conventional ELISA.

The AP substrate used in the ELISA-ISC assay was 5-bromo-4-chloro-3-indolyl phosphate (5-BCIP) (Sigma Chemical Co., St. Louis, MO). Preparation of the substrate and its use in an agarose overlay are detailed elsewhere (11). Conventional ELISA assays used *p*-nitrophenyl phosphate (Sigma Chemical Co.) as the AP substrate.

Serum Antibody Determinations. IgM and IgG-anti-OVA levels were determined by conventional ELISA, using the method of Voller et al. (12). Serum IgE titers were measured via the PCA system (2).

Heterologous Adoptive Cutaneous Anaphylaxis (HACA) Assays. The method described in (13) was used, whereby single-cell suspensions of splenic and lymph node lymphocytes were injected intradermally into the shaved backs of WAG rats. The diameter of blueing after a subsequent challenge with OVA and Evans Blue was a measure of the IgE produced by the lymphoid cells in situ.

Enumeration of OVA-specific ISC by the ELISA Plaque Assay. A method recently developed in this laboratory for enumeration of IgM and IgG ISC was used in the present study, with some modification. Briefly, 24-well Nunc tissue-culture plates were used, precoated with OVA as described (11). 1.0 ml of a suspension of putative ISC (10^4 – 10^7 cells/ml) in Dulbecco's PBS plus 15% fetal calf serum was added to the washed wells, which were incubated for 2 h at 37°C (for IgG and IgM) or 4 h at 32°C (for IgE assays); these conditions were adopted after preliminary experiments, and represent optimal conditions for the respective isotypes.

After the initial incubation with cells and washing, appropriate developing antisera (Rb-anti-rat IgM, IgG, or IgE) were added to the wells and the plates were incubated for 2 h at 37 or 4°C overnight. Localized zones of complexed Rb-anti-rat Ig were subsequently developed via the application of a Sh-anti-Rb IgG-AP conjugate, followed by the AP substrate, 5-BCIP in 36°C gelling agarose (11). Macroscopic blue spots or "plaques" became visible within 15–30 min at room temperature, and were routinely counted after 1 h. 3 M NaOH was then added to the plates to stop the AP reaction if storage was desired.

Enumeration of Total IgE Secreting Cells. These experiments used mesenteric lymph nodes and spleen cells from Lou/M rats, prepared 14 d after the subcutaneous inoculation of 2,500 *N. brasiliensis* larvae. The assay system was as detailed above, except that precoating of plates was performed with Sh-anti-rat IgE instead of OVA. The antiserum was applied at 1 μ g/ml in coating buffer on the basis of preliminary dose-response experiments.

Results

ELISA-Plaque Production by IgE-ISC. The microscopic appearance of IgE-ELISA plaques is shown in Fig. 1, and the pattern is not discernibly different from that produced by cells secreting other antibody isotypes. These plaques were developed

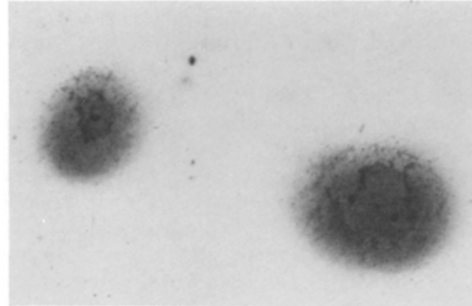


FIG. 1. Microscopic appearance of ELISA plaques produced by IgE ISC. Magnification, $\times 135$.

after initial incubation of parathymic lymph node cells from OVA-immunized rats on a plate coated with OVA, followed immediately by exposure of the plate to affinity-purified Rb-anti-rat IgE. The granular appearance of the plaques is typical; the isolated small dots exterior to the large plaques are artifacts, which appear to result either from the adsorption of diffused AP product onto particulate impurities within the gel or onto adhering cells.

IgE Response Following Intraperitoneal Challenge. The main lymphatic drainage from the peritoneal cavity in the rat is to the parathymic lymph nodes (14), and these appear to represent the major site of IgE synthesis during the primary response. Fig. 2 demonstrates that by day 9 these nodes contained $\sim 12,000$ OVA-specific IgE-ISC; in contrast, 34,000 IgM and $\sim 117,000$ specific IgG-ISC were observed at this time. The primary IgE response was rapidly terminated after day 9, and by day 14 only 200 IgE-ISC were found in these lymph nodes. The splenic contribution to the primary response was restricted to the IgM isotype. Restimulation of these animals at day 14 failed to elicit significant changes in OVA-specific IgE or IgM-ISC numbers, whereas IgG responses displayed significant boosting. The relative frequencies of OVA-specific cells secreting the IgE and IgG isotypes during the primary response is in general agreement with the results of a recent clonal analysis of the mouse IgE response (10).

Serum anti-OVA PCA titers in these animals during the response mirrored figures for IgE ISC numbers in the draining node, which suggests that these lymph nodes represented the major site of IgE synthesis. ISC numbers in the tissues were clearly dissociated from serum titers for the other isotypes, indicating that significant IgM and IgG synthesis was occurring in organs that were not examined in this study.

Specificity of IgE-ELISA Plaques. Rat IgE of sufficient purity for direct blocking experiments was not available to us as the time of this study; however, a variety of indirect evidence attests to the specificity of the IgE-ELISA plaques in this system (see also reference 11): (a) precoating with irrelevant antigen(s) or inclusion of soluble OVA at the cell incubation step abrogated plaque development, and the latter was not seen in cells from nonimmune rats; (b) IgE ISC numbers in organs varied independently of IgM and IgG during the response (Fig. 2); and (c) splenocytes from hyperimmune rats yielded large numbers of IgG ISC without discernible IgE ISC (data not shown).

A number of experiments have also been performed with the parallel use of the HACA assay for IgE ISC. While direct comparisons between these systems are difficult, due to the semiquantitative nature of the HACA, it can be stated that samples that were negative for IgE ISC by the ELISA assay were consistently negative

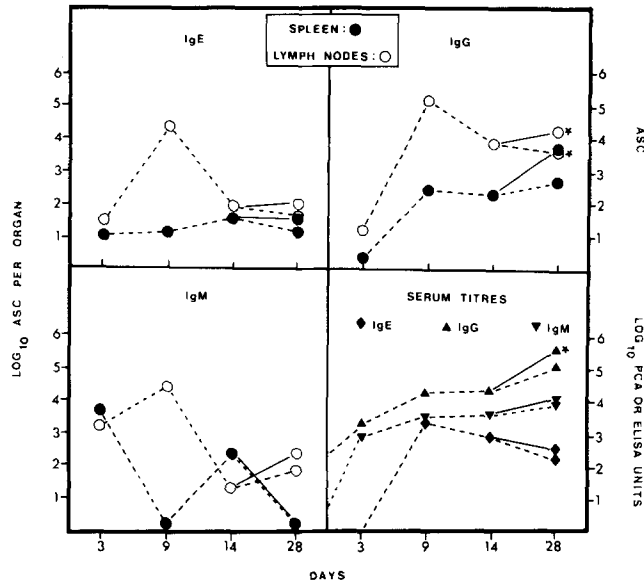


FIG. 2. Primary and secondary IgE response of BN rats. Animals were immunized on day 0 with OVA plus aluminium hydroxide, and their primary anti-OVA antibody responses were followed up to day 28 (----). One group was given a secondary challenge on day (—). Stars at day 28 represent significant secondary responses, comparing challenged with nonchallenged figures.

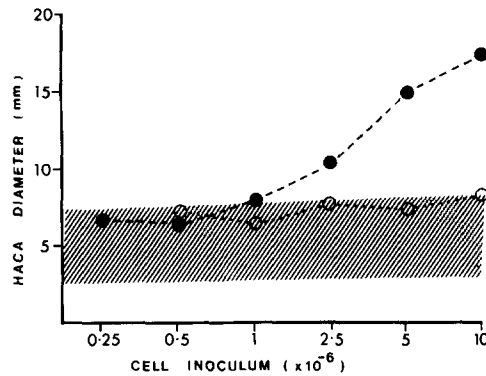


FIG. 3. HACA assay for IgE ISC. Data shown represent diameters of OVA-specific HACA reaction elicited by the day 9 lymph node (●) or spleen (○) used in the experiments in Fig. 2, which showed 97 and 0 IgE ISC/10⁶ cells, respectively, in the ELISA plaque assay. Hatched area represents the range of results obtained with cells from nonimmune animals.

by HACA. Fig. 3 illustrates the relative sensitivity of the two methods applied to cells from rats actively synthesizing IgE: the cells tested here were the day 9 sample from the lymph nodes in Fig 2. 2.5×10^6 cells represented the minimum requirement for a positive HACA response in this experiment, whereas the plaque assay detected 50 IgE ISC after addition of only 5×10^5 cells.

IgE-ISC in N. brasiliensis-infested Rats. Precoating of plates with Sh-anti-rat IgE before the addition of lymphocytes permits identification of IgE-secreting cells on the basis of isotype. Table I indicates that infestation of rats with a helminth parasite differentially stimulates the expansion of IgE-secreting clones in mesenteric lymph nodes, but not in central lymphoid organs such as the spleen. This observation is

TABLE I
Total Numbers of IgE ISC in Helminth-parasitized Rats

Animals	ISC/10 ⁶ lymphoid cells		ISC/organ		Cell yields × 10 ⁶	
	Mesenteric lymph node	Spleen	Mesenteric lymph node	Spleen	Mesenteric lymph node	Spleen
Controls	<5	<5	33	<10	33	112
Parasitized	2,652	<5	472,056	740	178	185

Male Lou/M rats were inoculated subcutaneously with 2,500 third-stage *Nippostrongylus brasiliensis* larvae. Single-cell suspensions were assayed for total IgE ISC as detailed in Materials and Methods. Results shown are means from three animals.

consistent with earlier studies using fluorescence techniques (15) and a reverse hemolytic plaque assay for IgE ISC (16).

Discussion

The results of this study illustrate the potential usefulness of the ELISA plaque assay in the study of cellular aspects of IgE immunoregulation. The impact of the Jerne hemolytic plaque assay on progress in the general area of humoral immunity is a matter of record, but the lack of a suitable assay for cells secreting the IgE isotype has meant that many of the assumptions concerning regulation of this antibody at the tissue level are inferential, or at best based upon indirect evidence. It is hardly surprising, therefore, that issues as basic as the nature of primary sites of IgE synthesis still remain controversial, with opinion polarized either for (17, 18) or against (15, 19) a mucosal location for cells secreting this antibody isotype. The importance of this issue in relation to allergy cannot be overstated, given that many of the proposed strategies for immunological intervention are conceptually based upon stimulating negative control mechanism(s) that operate at the level of the antibody-secreting cells.

The present study, although restricted to a single mode of antigenic challenge in a single species, provides the first line of direct evidence relevant to this question. The demonstration of antigen-specific IgE synthesis in the regional lymph node draining the site of primary immunization, and of major clonal expansion of IgE-secreting cells in the mesenteric lymph nodes after infestation with a gut-dwelling helminth parasite, supports the view that production of this antibody isotype is not primarily a mucosal phenomenon. The further application of this technique to a wider range of experimental models is required, however, before this issue can be resolved.

It must be stressed, in conclusion, that the precision of this technique depends absolutely upon the use of suitably purified and absorbed anti-Ig reagents. This is particularly the case when enumerating small numbers of IgE ISC against a 10-fold background of IgG ISC, as occurs during the primary immune response.

Summary

A new assay system is described for the enumeration of antigen-specific IgE immunoglobulin-secreting cells (ISC) based on an enzyme-linked immunoabsorbent assay. Using this technique to monitor the organ distribution of OVA-specific ISC after primary immunization of rats, ~12,000 specific IgE ISC were detected at the peak of the response in the draining lymph nodes compared with 117,000 IgG ISC; the splenic anti-OVA response was restricted to the IgM class. Using plates precoated

with anti-rat IgE instead of antigen, total IgE ISC were enumerated in normal and helminth-parasitized rats. The assay system detected up to 5×10^5 IgE ISC in mesenteric lymph nodes from parasitized animals compared with <50 in controls.

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