

The Biological Activity of Serum IgE Changes over the Course of a Primary Response

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Mast-cell degranulation is triggered by the bridging of Fc receptor-bound antigen-specific immunoglobulin IgE on the cell surface. *In vitro* experiments suggest that antibody affinity and nonspecific IgE may affect the mast-cell function, however, their importance *in vivo* is unclear. Investigations of the effects of these parameters on mast-cell sensitization were therefore carried out in a rat immunization model in which the IgE response is transient and peaks on days 10–15. Between these two timepoints, significant changes in the level of specific IgE were not observed, but the avidity of specific IgE increased ($P < 0.05$). Total serum IgE peaked on day 10 and slowly declined, with the relative proportion of specific to total IgE increasing from day 10–15 ($P < 0.05$). Despite similar levels of antigen-specific IgE, increasing avidity and an increased proportion of specific IgE between days 10 and 15, the biological activity of IgE in the serum peaks on day 10 and declines rapidly, dropping around seven-fold by day 15 ($P < 0.001$). Mechanisms that could explain this finding, such as differential expression of IgE isoforms and changes in the fine specificity of the IgE response, are discussed.

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INTRODUCTION

As early as 1960 the binding of 'sensitizing' antibodies to the mast cell was known to play a role in anaphylactic reactions [1] and in 1966, Ishizaka identified these antibodies as IgE [2]. It is now clear that cross-linking of FcεRI-bound IgE causes mast-cell degranulation [3]. This involves the binding of two or more cell-bound IgE antibodies to one molecule of antigen. Although antigen/antibody binding is well studied, many of the lessons learned about such interactions in solution may not apply at the surface of the mast cell [4]. Moreover, the binding kinetics of the IgE/antigen interaction appears to strongly affect intracellular signalling processes, and may therefore dictate the level of activation of the mast cell [5].

Many studies of the allergic response imply that the absolute amount of allergen-specific IgE produced is most critical to the sensitization process. Clinically, serum allergen-specific IgE is routinely quantified as an indirect measure of antigen-specific sensitization of mast cells, and consequently of the allergic status of the individual. While this has proven to be clinically useful, the predictive potential of serum IgE concentrations is far from perfect [6]. Other influences such as nonspecific IgE, which is a feature of allergic disease [7] and of helminth infection [8], may also be important in determining mast-cell reactivity [9, 10]. In contrast, high affinity may be relatively unimportant for IgE function [11].

Previously, using an *in vitro* system, we have investigated the effects of different parameters involved in the IgE–antigen interaction on mast-cell degranulation [11, 12]. These studies have suggested that four factors are most important to the IgE-mediated mast-cell function: the absolute concentration of antigen-specific IgE; the affinity of IgE for

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antigen; the presence of nonspecific IgE; and the valency of the antibody–antigen interaction. It has been unclear which of these might be important *in vivo*, but it is possible that variations of some or all of these parameters, either between individuals or over time in an individual, could dictate the level of antigen reactivity of mast cells armed with IgE.

In this study we have used a well-described rat model of IgE sensitization in order to investigate how the serum concentration and affinity of antigen-specific IgE, as well as the concentration of total IgE change over time during a primary immune response. Changes in these parameters are then compared with the ability of serum to confer antigen-specific reactivity to mast cells. In particular we have focused on the changes occurring at the peak of the IgE response. Both antigen-specific and total IgE were measured by ELISA and compared with the antibody-forming cell (AFC) response in the draining lymph nodes subsequent to antigen challenge. Changes in the avidity of IgE were also investigated. Results from these ELISA-based assays are contrasted with the functional activity of IgE measured by sensitization of the RBL-2H3 mast-cell line. While these results suggest that the concentration of antigen-specific IgE is important in determining the biological activity of serum IgE, none of the changes in the investigated parameters are able to fully account for the pattern of biological activity seen in this model.

MATERIALS AND METHODS

Animal procedures. Female, specific pathogen free, Brown Norway rats were purchased from the Animal Resources Centre (Perth, Western Australia). They were housed in clean conventional conditions in the University of New South Wales, School of Microbiology and Immunology animal house and were 10–12 weeks of age at the commencement of the experiments. Animals were immunized intraperitoneally with 50 µg/ml alum-precipitated OVA (OVA, Sigma, St Louis, MO, USA) in 1 ml phosphate-buffered saline (PBS). Groups of animals were euthanized at 0, 5, 10, 15, 20, 30 and 50 days post immunization. Blood was collected by cardiac puncture under ketamine/xylazine anaesthesia, and animals were killed by cervical dislocation. Serum was collected and stored in aliquots at -70°C . One animal from the day 15 group was omitted from the analysis, as all parameters indicated that it did not respond to immunization. For determination of IgE-secreting cell numbers, animals were immunized as above and groups of 4–5 animals were euthanized on days 0, 5, 10, 15 and 20. All animal procedures were approved by the UNSW animal ethics committee.

ELISAs. For the OVA-specific IgE ELISA, 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl of 10 µg/ml OVA in carbonate-bicarbonate buffer pH 9.6 (CBB). Plates were washed three times with washing buffer (PBS containing 0.05% tween-20), followed by blocking with 1% skim milk powder (SMP-Savings, Tooronga, Australia) in CBB for 1 h at room temperature (RT). After washing three times, two-fold falling serial

serum dilutions, from an initial dilution of 1 in 5, in PBS containing 0.05% tween-20 and 1% SMP (PBS-T-SMP) were added in duplicate to the plate and incubated at RT for 1 h. The plates were washed three times before a biotinylated monoclonal antirat IgE (Clone MARE-1, Zymed #03-9740, CA), in PBS-T-SMP, was added and incubated at RT for 1 h. After washing three times, avidin conjugated alkaline phosphatase (Zymed #43-4422), in PBS-T-SMP was added and again incubated for 1 h at RT. After three washes in washing buffer and three in distilled water, p-nitrophenyl phosphate substrate in 0.1M diethanolamine buffer was added and the plate developed for 6 h at 37°C . Absorbance was read at 405 nm and the titer of each serum was determined as the highest dilution giving an absorbance larger than twice that of a pooled negative reference serum. The OVA-specific IgE thiocyanate affinity assay took the form of a modified OVA-specific IgE ELISA, and was conducted as described by Pullen *et al.* [13], with some modifications. Sera were assayed at dilutions that had been determined to give a final absorbance of approximately 0.3. After serum incubation and washing, 100 µl of ammonium isothiocyanate (Sigma) (0.1, 0.2, 0.5, 1 and 2M) in 0.2M phosphate buffer was added and incubated for 15 min at RT. Wells containing 0.2M phosphate buffer alone served as a control, and each concentration of sodium isothiocyanate was assayed in triplicate. Following the thiocyanate elution step, wells were washed three times with washing buffer and the assay was completed as previously described for the OVA-specific IgE ELISA. The $\text{IC}_{50}\%$ was defined as the concentration of thiocyanate required to reduce the absorbance of a sample to 50% of the noninhibited value. This was determined by interpolation of the inhibition curves assuming linearity around the 50% point. In the total IgE ELISA, plates were coated with 100 µl of 5 µg/ml avidin (Sigma) in CBB at 4°C overnight. Plates were then washed three times with washing buffer and blocked at RT for 1 h with CBB–1%SMP. After washing three times, biotinylated monoclonal anti-IgE (Zymed) in PBS-T-SMP was added and incubated for 1 h at RT. Plates were washed and sera diluted in PBS-T-SMP were added and incubated for 1 h at RT. After serum incubation, plates were washed and polyclonal goat antirat IgE (Nordic Immunological Laboratories #GARa/IgE(Fc)/7s – Tilburg, the Netherlands) in PBS-T-SMP was added. Plates were then incubated at RT for 1 h. After washing, alkaline phosphatase conjugated affinity-purified rabbit antgoat IgG (Sigma #A4187) in PBS-T-SMP was added and incubated at RT for 1 h. Plates were then washed and developed as described for the OVA-specific ELISA above, with the exception that substrate was incubated for 4 h at RT. Sera were quantified against dilutions of purified LoDNP monoclonal rat IgE (a kind gift from Dr D. Conrad, Medical College of Virginia) at known concentrations.

Enzyme-linked immunospot (ELISpot) assays. The OVA-specific IgE ELISpot assay was performed essentially as described by Sedgwick and Holt [14]. Twenty-four well tissue culture plates (Greiner, Labortechnik, Frickenhausen, Germany) were coated overnight with 0.4 ml of 1 mg/ml aggregated OVA in CBB and washed three times with washing buffer. Wells were blocked with 1% bovine serum albumin (BSA, Sigma) in CBB for 1 h at RT. After washing three times with washing buffer and three times with PBS, serial dilutions of single-cell suspensions from parathymic lymph nodes (PTLN) were added, in duplicate, in PBS containing 10% fetal calf serum (FCS) (Trace Scientific, Noble Park, Australia). Plates were incubated in a vibration-free environment at 32°C for 3 h, and then

the cells were dislodged by washing vigorously four times with cold PBS-T. Two hundred microlitres of goat antirat IgE (Nordic) in PBS containing 0.05% tween-20 and 1% BSA (PBS-T-BSA) was added and incubated overnight at 4°C. The plates were then washed three times and incubated with 0.2 ml of alkaline phosphatase (AP)-conjugated antigoat IgG (Sigma) in PBS-T-BSA for 1 h at 37°C. After washing three times with washing buffer and three times with distilled water, plaques were developed by addition of 1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Bohringer, Manneheim, Germany) in 2-amino-2-methyl-1-propanol buffer, pH 10.25 containing 0.6% agarose [14]. Plates were incubated for approximately 15 h at 37°C and the development was stopped by the addition of 3M NaOH. Plates were stored at 4°C in the dark until examination, which was performed in a blinded manner. In the total IgE ELISpot assay, monolayers of RBL-2H3, which express the high affinity IgE receptor were used to capture IgE secreted from cells. RBL cells were plated at 7.5×10^5 /ml in Dulbeccos's modified Eagle's medium (DMEM) supplemented with 10% FCS (DMEM-10) in 24 well tissue culture plates and cultured for 18–24 h. Supernatant was discarded and monolayers fixed by the addition of 0.5% formaldehyde in PBS for 2 h. Plates were washed three times with washing buffer and blocked for 1 h at RT with PBS–1%BSA. Prior to the addition of cell suspensions, wells were washed three times with washing buffer and once with PBS. Single-cell suspensions of PTLN were added and incubated for 3 h at 32°C. The assay was then completed essentially as for the OVA-specific IgE assay described above, however, as RBL monolayers were sensitive to NaOH, plaques were counted directly after development.

RBL bioassay. In the standard RBL assay, 100 µl of RBL-2H3 cells at a concentration of 5×10^5 /ml in DMEM-10 containing 1.5 µl of tritiated serotonin (NEN, Boston, MA, USA) per ml, were dispensed into 96 well tissue culture plates (Greiner). Plates were incubated for 22–24 h at 37°C and then the medium was removed. Serial dilutions of serum in DMEM-10 were added in duplicate and incubated for 3 h at 37°C. Following serum incubation, wells were washed three times with DMEM-10 and a challenge solution of 1 µg/ml OVA in DMEM-10 was added and incubated for 30 min at 37°C. The percentage of released serotonin was determined as described previously [11], and titers of individual sera were defined as the highest dilution giving a serotonin release greater than twice that of a pooled negative reference serum. The potential effect of soluble serum factors upon RBL degranulation was examined in a modified RBL assay in which RBL cells were first incubated with serum from different timepoints, and were then activated in a non-antigen-specific manner. RBL plates were prepared as described above. Prior to the addition to assay plates, serum samples were prepared at a 1:10 dilution, and monoclonal LoDNP IgE was added to each sample to a final concentration of 10 µg/ml. This concentration of LoDNP was previously determined to saturate FcεRI on RBL cells (data not shown). Following serum incubation, plates were washed three times with DMEM-10%FCS and challenged with a 1:5000 dilution of goat polyclonal antirat IgE (Nordic), a concentration that had previously been determined to cause submaximal release under these conditions (data not shown). The assay was then completed as described for the standard RBL assay, above. The effect of total serum IgE levels on RBL reactivity was examined in a modified RBL assay. Five day 10 and seven day 15 sera were available for this assay. Sera were assayed at a dilution

of 1:10. Two aliquots of each diluted serum were prepared. One of these aliquots served as a control, while the second was spiked with exogenous monoclonal rat IgE (LoDNP). A different amount of LoDNP was added to each of these spiked samples, the amount having been calculated from measurements of the total IgE levels in that serum. Final IgE concentrations (endogenous IgE plus exogenous monoclonal IgE) in each spiked sample were adjusted to 25 µg/ml. The RBL bioassay was otherwise conducted as described for the standard assay above.

Statistical analysis. Serum titers were transformed to $\log_2$, and ELISpot results to $\log_{10}$. Statistical analysis was performed on these transformed values. Mean values were calculated for these results as geometric means. Other data were analyzed without transformation. For all experiments, a *post hoc* analysis was performed using Tukey's test for comparisons between multiple groups, with the exception of the results of the thiocyanate affinity and IgE index, where a *t*-test was used. Significance was accepted at the 0.05 level.

RESULTS

Biological activity of serum IgE by RBL bioassay

Mast cell arming activity was investigated with an *in vitro* assay using RBL-2H3 cells. This rat cell line has been shown to be homologous to mucosal mast cells [15], and is extensively used both as a rat mast-cell model, and as a general tool for investigation of receptor-mediated signalling. A similar bioassay has also been used previously by other investigators in order to measure the IgE activity of serum, and has been shown to correlate well with the passive cutaneous anaphylaxis (PCA) reaction [16].

The timecourse of biological activity of serum IgE is shown in Fig. 1. Biological activity peaked at day 10 (mean titer = 388), remaining detectable at low levels up to day 30. Between days 10 and 15, there was an approximately seven-fold drop in mean titer ($P < 0.001$).

Antigen-specific and total IgE concentrations

Antigen-specific IgE was measured in sera by ELISA, and these results are shown in Fig. 2. By this assay, IgE peaks between days 10 and 15 postimmunization, with a sharp rise from day 5–10 and a rapid decline after day 15. Although the mean titer of IgE rose slightly from day 10 (mean = 70) to day 15 (mean = 80), this increase was not significant ($P > 0.05$).

The kinetics of the antigen-specific IgE response contrasts with that of total IgE, which are shown in Fig. 3. IgE was detectable at low levels in several nonimmunized animals, and rose from day 5 postimmunization to reach peak levels (mean = 181 µg/ml) 10 days after immunization ($P < 0.01$ compared with all timepoints). Total IgE levels then declined steadily over the course of the experiment. However,

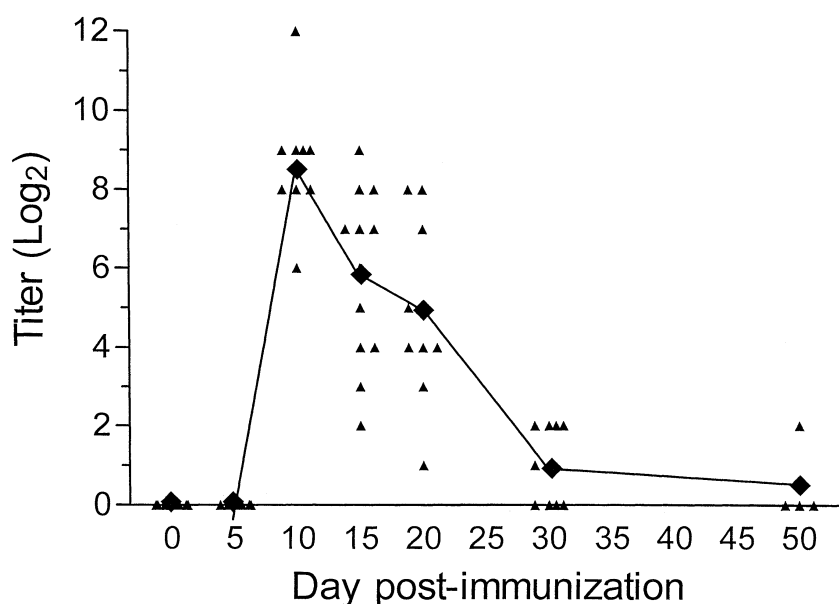


Fig. 1. Timecourse of mast cell arming activity in serum following immunization. Animals were immunized on day 0, and groups of animals euthanized on days 0 (nonimmunized) and 5, 10, 15, 20, 30 and 50 postimmunization. The ability of serum from each animal to confer antigen-specific reactivity to mast cells was determined by RBL bioassay. Sera were serially diluted from an initial dilution of 1:2 and results are expressed as endpoint titers. Symbols represent the titers of individual animals and the geometric mean of groups at each timepoint. At the peak of the IgE response, between days 10 and 15, the biological activity of antigen-specific serum IgE fell significantly ($P < 0.001$).

comparatively high levels (mean = 25 µg/ml) were still present 50 days after immunization.

A measure of the relative proportion of OVA-specific IgE in relation to the total IgE was calculated by dividing the antigen-specific IgE titer by the total IgE concentration (in µg/ml) for each animal. This data is presented in Fig. 4, and shows that the proportion of antigen-specific IgE in relation to total IgE increased significantly from day 10 to day 15 ($P < 0.05$).

Total and antigen-specific IgE secreting cells

In order to further characterize the IgE response, the kinetics of IgE production at a cellular level was examined. In this model, the primary IgE response to intraperitoneal antigen has been shown to occur almost exclusively in the parathyroid lymph nodes [17]. Therefore, using ELISpot assays, we investigated the presence of both antigen-specific and total IgE-secreting cells in the PTLN postimmunization. Both the OVA-specific and total IgE-forming cell responses

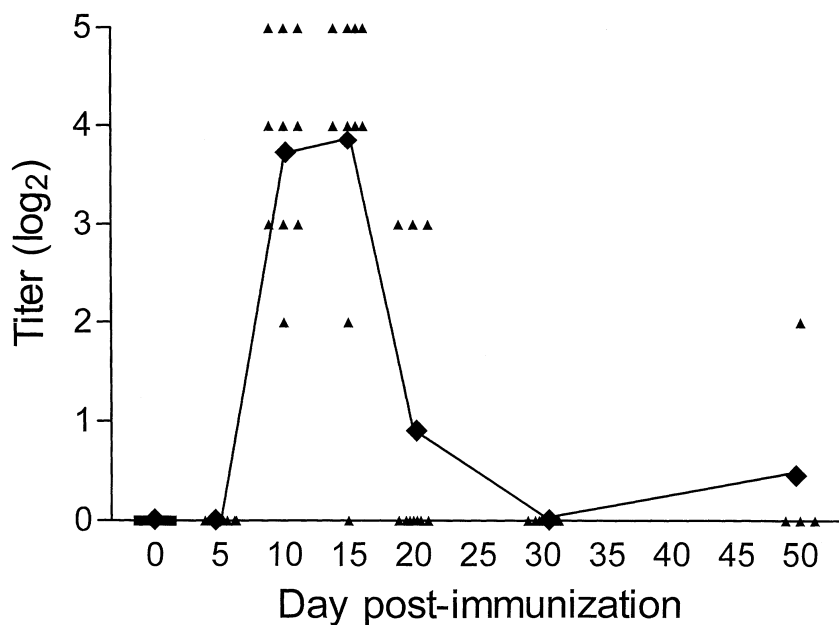


Fig. 2. Timecourse of ovalbumin (OVA)-specific IgE in serum by ELISA following immunization. Antigen-specific IgE was quantified in the sera of immunised animals by ELISA. Symbols represent the titers of individual animals and the geometric mean of groups at each timepoint. Between days 10 and 15 postimmunization, there was no significant change in the mean titer of OVA-specific IgE ($P > 0.05$).

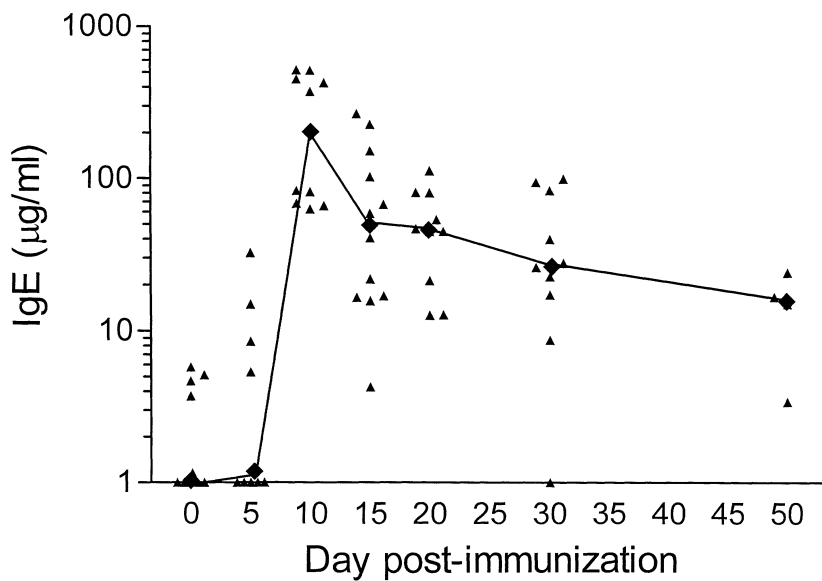


Fig. 3. Timecourse of total IgE in serum following immunization. Total IgE was quantified in the sera of immunized animals by ELISA assay. Symbols represent individual animals and the mean of groups at each timepoint.

in the PTLN were broadly similar to the serum responses as measured by ELISA. Numbers of OVA-specific IgE-secreting cells peaked around days 10–15 (Fig. 5). The increase from day 10 (mean = 382 cells) to day 15 (mean = 695 cells) was not significant ($P > 0.05$). IgE-forming cells (Fig. 6) were present at low numbers in PTLN from nonimmunized rats (mean = 60), but rose by day 5 ($P < 0.001$), and peaked on days 10–15 (day 10 mean = 1.45×10^4 cells; day 15: mean = 1.56×10^4 cells). There was no significant difference between days 10 and 15.

Avidity of IgE

The relative avidity of the OVA-specific IgE in sera on days 10 and 15 postimmunization was measured by thiocyanate

elution, and the results of this assay are shown in Fig. 7. The avidity of serum IgE increased significantly ($P < 0.01$) between days 10 and 15, with the mean $IC_{50}\%$ of thiocyanate concentration increasing from 0.26 ± 0.11 M on day 10 to 0.46 ± 0.16 M on day 15.

Non-specific effects on RBL degranulation

Soluble factors such as interleukin (IL)-3, IL-5 [18] and stem-cell factor [19] have been shown to influence the ability of mast cells to degranulate. Sera from different timepoints following immunization were therefore examined for the presence of nonspecific inhibitory or stimulatory activity (Fig. 8). RBL cells were incubated with sera and then activated in a nonantigen-specific manner via the FcεRI. Compared

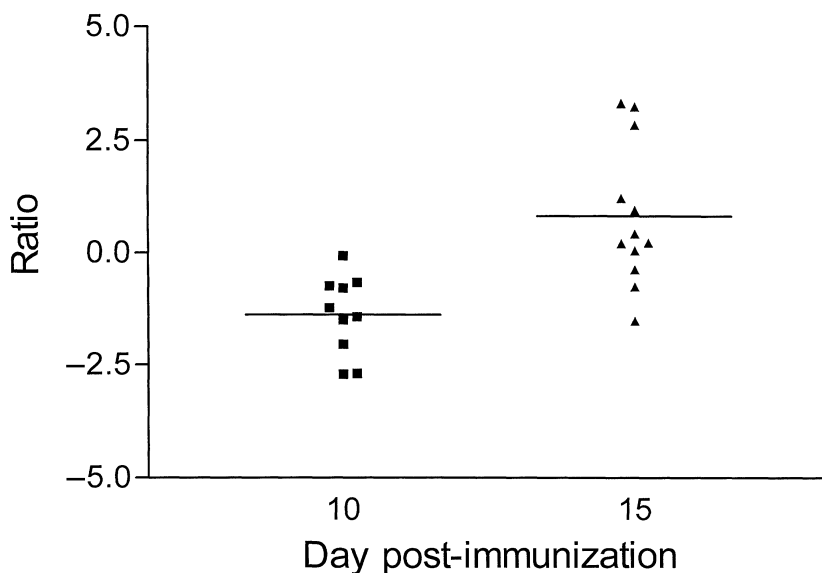


Fig. 4. Ratio of specific to total IgE on days 10 and 15 postimmunization. A measure of the relative amounts of antigen-specific to total IgE on days 10 and 15 for each animal was determined as the specific IgE index by dividing the anti-OVA IgE titer (Fig. 2) by the total IgE concentration (Fig. 3). Symbols represent individual animals and the mean of groups at each timepoint. The ratio of antigen-specific to total IgE increased significantly from day 10–15 postimmunization ($P < 0.05$).

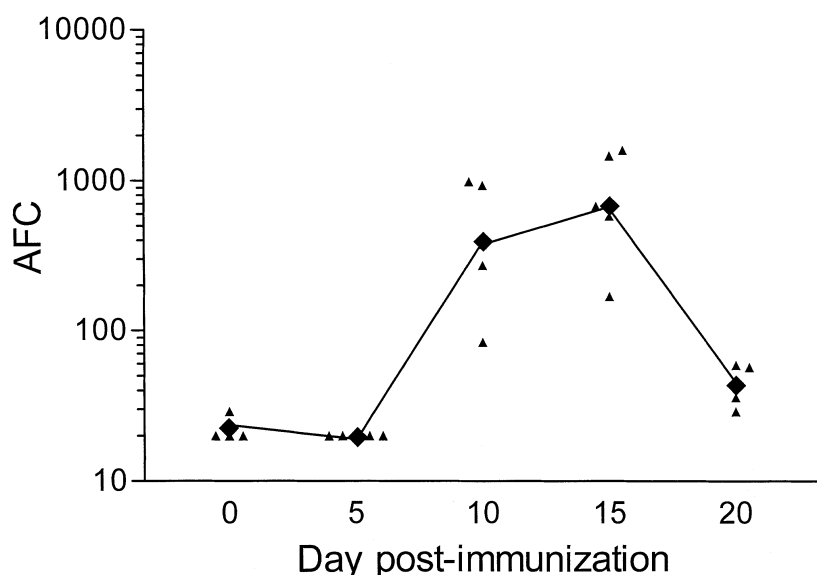


Fig. 5. Timecourse of OVA-specific IgE secreting cells in parathymic lymph nodes (PTLN) following immunization. Animals were immunized on day 0, and groups of animals euthanized on days 0 (nonimmunized) and 5, 10, 15 and 20 days postimmunization. Absolute numbers of OVA-specific IgE antibody-forming cells (AFC) were quantified at each timepoint in an ELISpot assay. Symbols represent the numbers of cells in individual animals and the geometric mean of groups at each timepoint.

with nonimmunized animals, neither potentiation nor inhibition of release was seen at any timepoint ($P > 0.05$ for all comparisons).

As serum from animals 10 days postimmunization had significantly higher levels of total IgE than other timepoints (Fig. 3), it was felt that increased levels of IgE could influence the function of RBL cells, possibly by causing increased expression of FcεRI [20]. To investigate this, sera from days 10 and 15 were tested in a modified RBL assay in which total IgE levels were equalized. In this assay, sera were used at a final dilution of 1:10. For all day 10 and 15 sera, this dilution saturates FcεRI in the RBL assay and leads to maximal degranulation. A separate, identical set of diluted sera were spiked with nonspecific IgE to bring the total concentration of IgE in each sample to a constant

amount. This procedure removed the influence of total IgE levels on the RBL reactivity. Sera from day 10 retained significantly more biological activity than did those from day 15 rats (Fig. 9). This occurred even though ELISA results showed that day 15 sera had similar concentrations of OVA-specific IgE to day 10 postimmunization animals (Fig. 2).

DISCUSSION

The goals of this study were to describe the kinetics of a primary IgE immune response as determined by enzyme immunochemical techniques, and to compare this with the kinetics of functional IgE activity as determined by bioassay. The biological activity of serum IgE at the peak of the immune response was then compared with changes in the

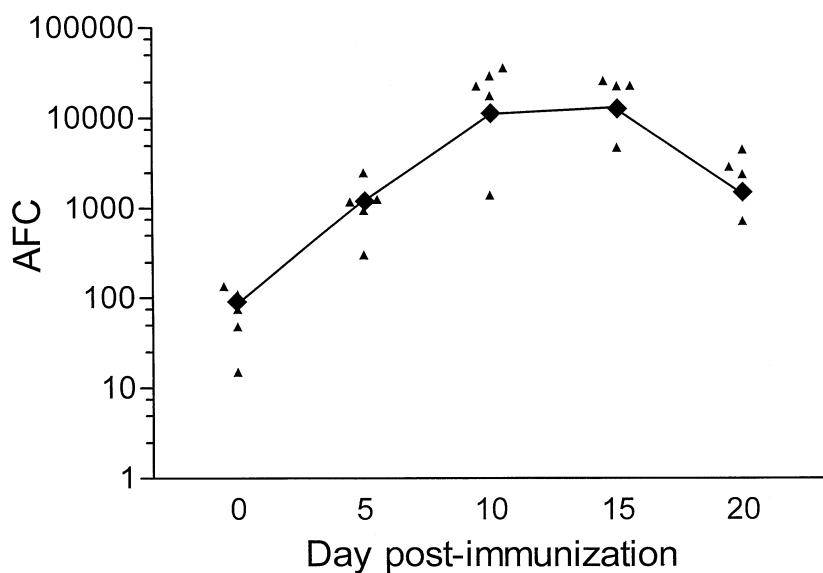


Fig. 6. Timecourse of total IgE-secreting cells in PTLN following immunization. Absolute numbers of IgE AFC of any specificity were quantified in an ELISpot assay. Symbols represent the numbers of cells in individual animals and the geometric mean of groups at each timepoint.

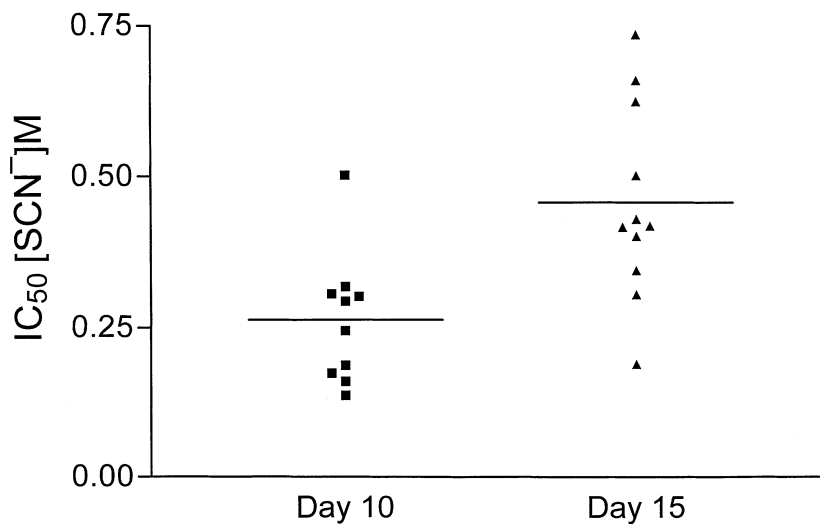


Fig. 7. Avidity of OVA-specific IgE on days 10 and 15 postimmunization. The avidity of OVA-specific IgE in serum was determined in animals on days 10 and 15 post immunization by thiocyanate elution. Data given are IC₅₀ values, defined as the concentration of thiocyanate ion required to inhibit the binding of specific IgE to OVA by 50%. Symbols represent individual animals and the mean of groups at each timepoint.

affinity of antigen-specific IgE, and the presence of potentially blocking nonspecific IgE.

The OVA-specific IgE response as measured by both RBL assay and ELISA arose early and was transient. This transience is likely to reflect the short life of plasma cells in the primary response [21] and rapid clearance of IgE from the blood [22]. However, comparison of the biological activity of antigen-specific serum IgE from different timepoints with the concentration of antigen-specific IgE showed two notable discrepancies. Firstly, the ELISA results suggested that the antigen-specific IgE response in serum is nearly complete by day 20; while biological activity was still detectable in the serum on day 30. This suggests that the RBL assay was more sensitive. More salient however, is that between days 10 and 15 (the peak of the antigen-specific IgE response) there was an approximately seven-fold drop ($P < 0.001$) in the biological antiova IgE activity. Over the same time period there was no change in the concentration as determined by ELISA. This finding led us to focus on these timepoints for further investigation of the influence of nonspecific IgE and of IgE affinity on biological activity.

While serum IgE responses have been investigated in many experimental animal systems, most of these studies examine either the biological activity of the IgE response (typically by PCA), or IgE levels by *in vitro* antibody-based methods such as ELISA or RAST. A measure of biological activity, such as the PCA test or *in vitro* degranulation assay, is probably the more appropriate approach in many cases, but there are surprisingly few direct comparisons between immunochemical and bioassay techniques. Positive correlations between ELISA and biological activity have been reported previously [23–25]. However, there is also conflicting evidence, most notably from Lindsey *et al.* [26] who found a significant negative correlation of ELISA with PCA using OVA as a test antigen. The possible mechanisms underlying this finding were not discussed.

One of the major obstacles to the investigation of the IgE response is that its measurement by ELISA can be problematic. In the present study, direct ELISA using endpoint titer was chosen as the most appropriate method of quantifying antigen-specific IgE. This assay design should be generally tolerant to competing antibodies [27–29]. Nevertheless, in order to confirm the ELISA results, the kinetics of the IgE antibody-forming cell response in the PTLN was determined by ELISpot. By their nature, ELISpot assays for minor isotypes are less prone to competition by other antibody classes, and as the serum half-life of IgE in the rat is in the order of 12 h [22], the AFC response should closely mirror that of serum IgE. This was indeed the case, with numbers of OVA-specific IgE secreting cells in the PTLN peaking on days 10 and 15, with low but still detectable numbers of IgE-secreting cells in the PTLN on day 20. This suggests that the OVA-specific IgE ELISA accurately reflects serum concentrations. Despite the concordance of these results, they are still inconsistent with the pattern of biological activity seen in sera from animals at days 10 and 15.

As seen in the OVA-specific response, levels of total IgE in the serum rise rapidly to a peak at day 10. But unlike the antigen-specific response, total IgE levels decline slowly, remaining elevated to at least day 50 postimmunization. The results of the ELISpot assays show that total IgE-secreting cells in the PTLN outnumber antigen-specific cells by around two logs at the peak of the response, with a sustained nonspecific IgE response continuing afterwards. Furthermore, significant numbers of total IgE-secreting cells were found within the spleen on day 10, while antigen-specific IgE AFC were not found in this organ at any time following immunization (data not shown). This suggests that most serum IgE in this model is in fact nonspecific. Similar patterns of IgE production have been reported previously in serum [30, 31] and at a cellular level [32, 33] in the BN rat. Non-specific IgE has been shown to block

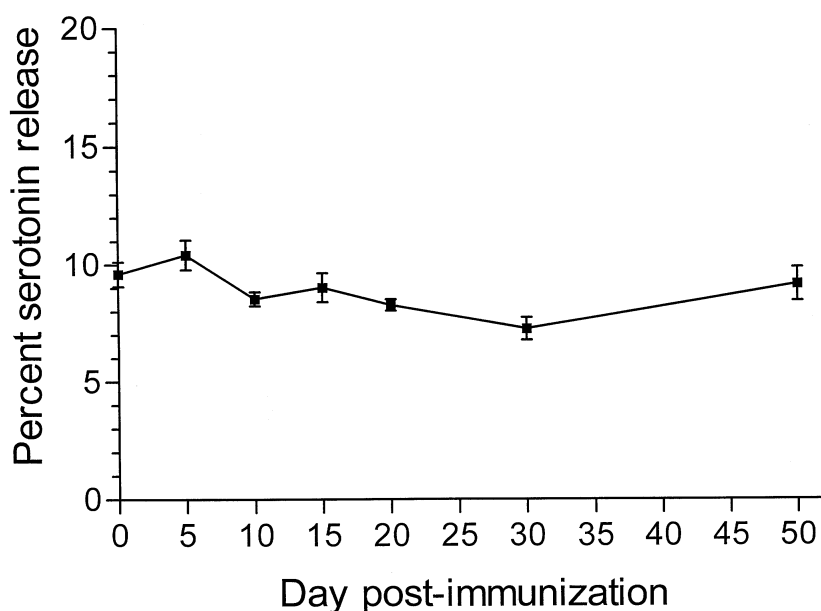


Fig. 8. Effect of soluble factors in sera from immunised animals on RBL degranulation. In order to determine if factors within the serum lead to either potentiation or inhibition of degranulation, sera were tested in a modified RBL assay. RBL cells were preincubated with serum from immunized animals that was diluted 1:10. Cells were then incubated with exogenous IgE at a concentration that saturated cell-surface FcεRI. They were subsequently challenged with a concentration of anti-IgE that caused suboptimal degranulation. The mean and standard deviation of serotonin release of groups of animals is shown.

the responsiveness of mast cells to specific antigen challenge [9, 10, 34]. In the present study however, despite the low ratio of OVA-specific to total IgE secreting cells, serum from days 10 and 15 was still able to efficiently arm RBL cells. In fact, the relative proportion of antigen-specific IgE in the pool of total serum IgE increases from day 10–15 (Fig. 4). This argues that mast cells can be effectively sensitized even when a relatively low proportion of cell-surface IgE is antigen-specific, and that the inhibition by nonspecific IgE is not a major factor influencing mast-cell function in this model.

High affinity for antigen has been argued to be important for antibody function [35] and affinity maturation is a fundamental feature of the antibody response. In this study, changes in the functional avidity of antigen-specific IgE at the peak of the immune response were measured by thiocyanate elution ELISA. The IC₅₀ values of sera from both days 10 and 15 (Fig. 7) are relatively low in comparison to previously published values for monoclonal IgE [11] or hyperimmunized IgG [13], suggesting that the IgE present at these times is of comparatively low avidity. The production of low avidity IgE is consistent with the early stage of the primary response, prior to extensive affinity maturation. Importantly, the avidity of serum antiOVA IgE increased significantly from day 10–15 postimmunization, which is in keeping with the general paradigm of increasing antibody affinity during an immune response. At these two timepoints, however, the change in avidity is again inconsistent with the changes in the observed biological activity of the immune serum.

As neither the concentration nor the affinity of OVA-specific IgE, nor changes in the proportion of nonspecific IgE seemed able to account for the pattern of biological activity as determined by the RBL assay, other influences

on mast-cell activation were investigated. Soluble factors such as interferon (IFN) α/β [36], stem-cell factor [19], IL-3 and IL-5 [18] have been shown to modulate mast-cell activation, and their presence in serum samples could affect the RBL cell degranulation. However, when sera were tested to determine if they could enhance or suppress degranulation, no influence on RBL degranulation was found (Fig. 8).

Levels of total IgE, which may affect the responsiveness of mast cells to antigenic stimulation by increasing the expression of FcεRI [20], did not account for the differences in biological activity between days 10 and 15. Sera from day 10 and 15, which contained similar levels of antigen-specific IgE by ELISA, were spiked with exogenous IgE such that the total levels of IgE were equivalent in all samples. Under these conditions, day 10 postimmunization sera retained significantly more biological activity than those from day 15 (Fig. 9). The results of this assay also demonstrate that the presence of large amounts of exogenous nonspecific IgE are capable of blocking the biological activity of OVA-specific IgE in day 15 serum. This is consistent with previously published studies that demonstrated blocking of antigen-specific IgE activity by nonspecific IgE [9, 10]. Interestingly, serum from animals 10 days postimmunization were largely unaffected, which suggests that there are other influences on the biological activity of serum IgE that are stronger than that of nonspecific IgE.

It is possible that the differences in biological activity at days 10 and 15 are due to changes in either the primary protein structure or glycosylation of IgE present in the serum. Several IgE mRNA splice variants have been identified in humans [37, 38], and these may be differentially regulated over time in an individual [39, 40]. At least one

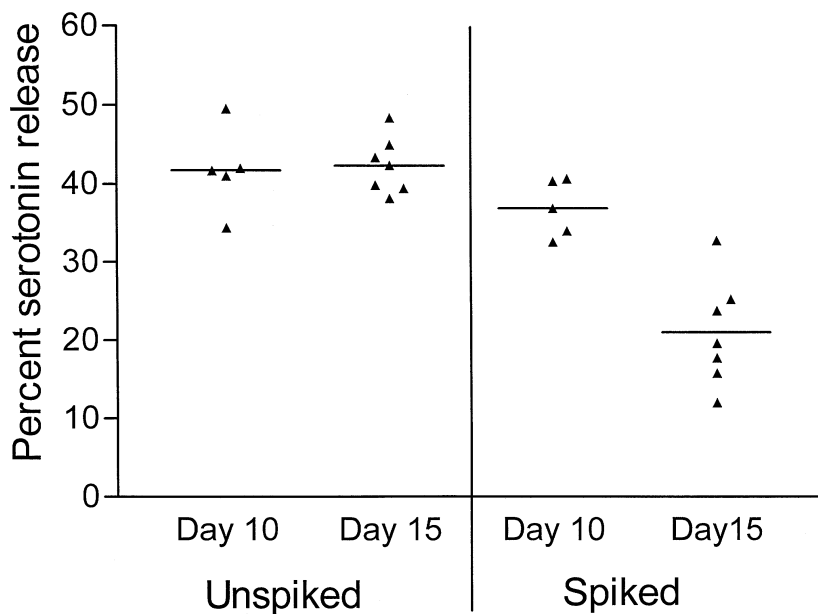


Fig. 9. Effect of total IgE concentration on RBL degranulation. Sera from animals immunised 10 or 15 days previously were tested in a modified RBL assay. Sera were assayed at a 1 : 10 dilution with or without the addition of exogenous monoclonal IgE. In 'spiked' samples, monoclonal IgE was added such that the total IgE concentration in each serum (endogenous plus exogenous monoclonal) was 25 µg/ml. Sensitized cells were challenged with 1 µg/ml OVA, and the percent of serotonin release measured. Symbols represent individual animals and the mean of each group. The serotonin release from the day 15 'spiked' group was significantly different from all other groups ($P < 0.05$). Significant differences were not seen between other groups.

of these alternative isoforms is able to bind to FcεRI to induce degranulation in mast cells when cross-linked by antigen, but with a reduced biological activity when compared to 'classic' secreted IgE [40]. Alternatively, differential IgE glycosylation has been described in sera from allergic patients [41]. This may be of significance, as glycosylation of IgE appears to be involved in its binding to the FcεRI [42, 43]. Neither multiple isoforms of IgE, nor differential glycosylation of IgE have been described in the rat to date. However, changes in these aspects of the response over time could potentially influence the biological activity of serum IgE.

In previous studies [11, 12] we have identified one further factor which may effect the biological activity of IgE; the valency of the antigen. Although divalent cross-linking has been shown to be sufficient to activate mast cells and basophils [3], the degree of aggregation of receptors may affect activation [11, 12, 44]. While most *in vitro* investigations of IgE-mediated mast-cell degranulation typically use either haptenated or aggregated antigen to cross-link monoclonal surface IgE, this may be unlike the situation *in vivo*, where monomeric protein antigen is likely to be the degranulating agent. Cross-linking almost certainly occurs here owing to the bridging of IgE antibodies recognizing different epitopes on an antigen molecule [45]. Consequently, changes in the number of epitopes recognized *in vivo* could potentially have modulating effects on mast-cell function. Changes in epitope patterns have been proposed to account for differences between RAST and skin-test results [6], and have been argued to influence the ability of IgG to activate effector mechanisms [46]. Although the present study was unable to address this question, there is some evidence that the number of epitopes to which the immune system responds can change during the course of a response [47, 48].

In conclusion, in this model we have shown that the affinity of antigen-specific IgE, as well as the presence of nonspecific IgE, both appear to be relatively unimportant in determining biological activity. Although the concentration of antigen-specific IgE is influential, other elements of the response appear to be important, and may change at the time of peak IgE production. Certainly the ability of serum to arm mast cells for antigen-specific reactivity changes dramatically early in the primary immune response. Studies investigating the molecular isoforms and fine specificity of the IgE response will be important in further clarifying this phenomenon.

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