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A kinetic analysis of the *in vitro* sensitization of murine peritoneal mast cells with monoclonal IgE anti-DNP antibody

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SUMMARY

Incubation of murine peritoneal cells with monoclonal IgE anti-DNP antibody *in vitro* led to sensitization of mast cells, measured as release of 5-HT upon challenge with DNP-HSA antigen. Sensitization was maximal at 0·3–3·0 μg/ml of IgE anti-DNP and declined above and below this concentration range. In kinetic studies, the time–course of sensitization was clearly divisible into an early slow phase of approximately 4 hr, followed by a more rapid linear phase from 4 to 48 hr. The early slow phase was more pronounced at lower concentrations of IgE anti-DNP (within the range 0·05–5·0 μg/ml). The degree of sensitization obtained after incubation of peritoneal cells with IgE anti-DNP for fixed periods (2, 4 and 8 hr) was markedly increased when the cells were washed and recultured in IgE-free medium, thus demonstrating that sensitization proceeds subsequent to an early stage of binding of IgE to receptors. Sensitization with IgE anti-DNP was blocked by addition of excess rat myeloma IgE, but only to a marked extent (> 50%) when the blocking immunoglobulin was added during the first 2 hr, thus providing further evidence that the major part of binding of the IgE antibody took place during this early stage, that is, prior to the phase of greatest sensitization. These findings indicate a period of delay between binding of IgE to receptors and functional sensitization, measured as mediator release in response to antigen.

INTRODUCTION

The activation of mast cells and basophils by antigen, to release the chemical mediators of immediate allergic reactions, proceeds via two distinct ligand–receptor interactions at the cell surface. Initially, at the stage of cell sensitization, IgE antibody binds to Fcε receptors in the cell membrane. Subsequently, the bound antibody acts as receptor for specific antigen; cross-linking of adjacent IgE molecules by divalent or multivalent antigen activates the secretory response of the cell (Ishizaka & Ishizaka, 1978; Metzger, 1978).

The dynamics of the interaction of IgE with its receptor on rodent mast cells, rat basophilic tumour cells, and human mast cells and basophils have been well characterized. These studies, which utilized radiolabelled or fluorescently labelled IgE, established that each IgE molecule binds to a single mobile receptor, of which there are approximately 10^5 per cell, and that binding is reversible, of high affinity, and follows simple saturation kinetics (Ishizaka, Soto & Ishizaka, 1973; Kulczycki & Metzger, 1974; Conrad *et al.*, 1975; Schlessinger *et al.*, 1976; Mendoza & Metzger, 1976; Coleman & Godfrey, 1981b; Pruzansky & Patterson, 1986). However, functional studies of the dynamics of IgE-dependent sensitization of mast cells and basophils have not progressed in parallel with binding studies, probably in part because of problems associated with the standardization and supply of antisera containing sufficient IgE of a given antigen specificity. Functional studies are further complicated since the presence of IgE antibodies of different specificities in sera leads to competition for receptors (Coleman & Godfrey, 1981a) and, furthermore, the problem of polyclonality is not fully resolved by affinity purification.

In order to overcome these difficulties, monoclonal IgE antibody directed against the haptenic dinitrophenyl (DNP) determinant was utilized in the present study for a detailed investigation of the magnitude, concentration-dependence and kinetics of sensitization of murine mast cells by IgE of a single specificity. The results reveal several points of interest that could not have been predicted from simple binding studies.

Abbreviations: DNP, dinitrophenyl; DNP-HSA, dinitrophenyl-human serum albumin; 5-HT, 5-hydroxytryptamine.

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MATERIALS AND METHODS

Sensitization of mast cells

Cells were obtained by lavage of the peritoneal cavity of female CBA/Ca mice (Bantin & Kingman, Hull) with 5 ml of 0·15 M phosphate-buffered saline (PBS), pH 7·2. The cells were pooled, sedimented by centrifugation (300 *g*, 5 min) and washed twice in PBS, before resuspension in complete RPMI-1640 medium (Gibco, Uxbridge, Middlx) supplemented with 2 mM L-gluta-

mine, 40 μg/ml gentamicin and 5% fetal calf serum (Sera-Lab, Crawley Down, Sussex) at a concentration of 1–2 × 10^6 cells/ml. Mast cells were identified by metachromasia in 0·01% aqueous toluidine blue, and comprised 3–5% of total cells. All preparations were free of erythrocytes. The cells were divided into 0·5 ml aliquots and incubated at 37° in 5% CO_2 in conical plastic cell culture tubes (Sterilin, Teddington, Middlesex). Monoclonal IgE anti-DNP antibody (ICN Biomedicals, High Wycombe, Bucks) was added at appropriate concentrations in a minimal volume (≤20 μl) of RPMI-1640 medium.

Challenge of mast cells

Following sensitization the cells were diluted 10-fold in PBS, sedimented (300 *g*, 5 min) and washed twice in 5 ml PBS before resuspension at 2–4 × 10^5/ml in complete RPMI-1640 medium (as used for sensitization) supplemented with 20 mM HEPES to give pH 7·0 at 37°. Duplicate aliquots of cells (0·2 ml) were added to 0·2 ml of the following, prewarmed to 37° in a water bath: DNP-HSA (hapten density 13:1; prepared by the method of Park *et al.*, 1987) in complete HEPES-RPMI medium; HEPES-RPMI medium alone; and 0·05% Triton X-100 (Sigma, Poole, Dorset) to lyse the cells. Incubation was continued for 5 min at 37°. Supernatant fractions (0·15 ml) were separated from cells after centrifugation (300 *g*, 5 min). In some experiments goat anti-mouse IgE (ICN Biomedicals) was used in place of DNP-HSA antigen.

Release of 5-hydroxytryptamine (5-HT)

The secretory response of mast cells was assayed as release of 5-HT, which is selectively taken up by rodent mast cell granules and released in parallel with endogenous mediators such as histamine upon stimulation of exocytosis (Morrison *et al.*, 1974). Two microcuries of 5-hydroxy[G-^{3}H]tryptamine creatinine sulphate (specific radioactivity 17 Ci/mmol; Amersham International, Amersham, Bucks) were added to cell cultures (0·5 ml) 1 hr prior to challenge. The cells were washed free of unincorporated [^{3}H]5-HT before challenge with antigen, as described in the previous section. Release of incorporated [^{3}H]5-HT at challenge was measured as supernatant radioactivity by scintillation spectrometry, and net percentage release calculated as:

$$\left(\frac{a}{c} \times 100\right) - \left(\frac{b}{c} \times 100\right),$$

where a is the radioactivity (d.p.m.) recovered in the supernatant fraction from stimulated cells, b is the radioactivity (d.p.m.) recovered in the supernatant fraction from unstimulated cells, and c is the total radioactivity (d.p.m.) per reaction mixture. Each assay tube contained 1000–5000 mast cells and each mast cell incorporated approximately 100 d.p.m. of [^{3}H]5-HT. The difference between duplicate assays was consistently < 10% of the mean. Spontaneous release from unstimulated cells was 1–5% of total cellular [^{3}H]5-HT.

RESULTS

Concentration dependence of sensitization

Preliminary experiments had demonstrated release of [^{3}H]5-HT from sensitized cells at DNP-HSA antigen concentrations of 0·01–100 μg/ml, with optimal release above 1·0 μg/ml. Sensitized cells failed to respond to HSA, thus demonstrating specific sensitization to the DNP hapten. To study the dependence of sensitization on concentration of IgE antibody, cells were incubated with IgE anti-DNP within the concentration range 0·01–30 μg/ml for 18 hr at 37°, and challenged with either 1·0 or 100 μg/ml of DNP-HSA. The results showed a bell-shaped concentration–response curve with optimal sensitization at 0·3–3 μg/ml of IgE antibody (Fig. 1). Under these conditions 10–20% of cellular [^{3}H]5-HT was released. The shape of the concentration–response curve did not differ between the two concentrations of antigen used (Fig. 1).

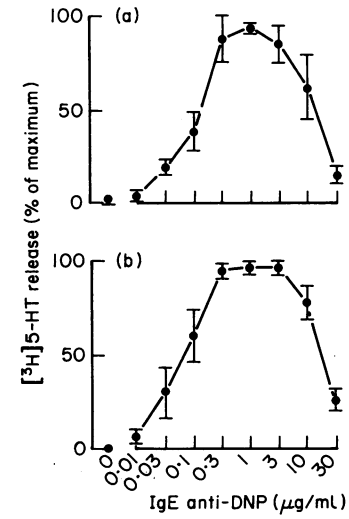

Figure 1. Dependence of [^{3}H]5-HT release on concentration of IgE anti-DNP antibody. (a) Cells challenged with 1·0 μg/ml DNP-HSA. (b) Cells challenged with 100 μg/ml DNP-HSA. Results are means ± SEM for three experiments. Each experiment was performed with cells pooled from three mice.

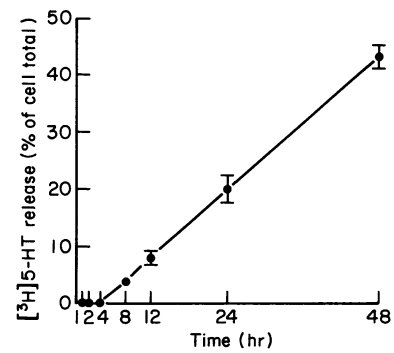

Figure 2. Kinetics of mast cell sensitization with 0·5 μg/ml of IgE anti-DNP. Results are means ± SEM for three experiments, each with cells pooled from three mice.

Kinetics of sensitization

Peritoneal cells were incubated with 0·5 μg/ml of IgE anti-DNP for various times and challenged with 1·0 μg/ml of DNP-HSA. Sensitization invariably proceeded according to the following time-course: relatively little sensitization was obtained during

Table 1. [^{3}H]5-HT release as percentage of 24 hr response

IgE anti-DNP	Time (hr) 2	4	8
0·05 μg/ml	0·7±1·5	0·7±0·4	6·0±0·7
0·5 μg/ml	1·7±1·4	4·1±0·2	15·7±1·0
5·0 μg/ml	5·4±1·2	8·1±1·9	17·1±0·8

Results are means±SEM for three experiments.

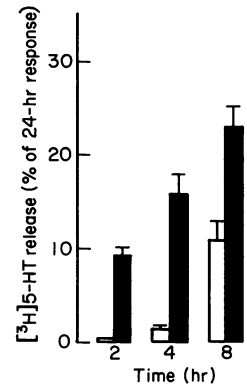

Figure 3. [^{3}H]5-HT release from mast cells incubated with 0·5 μg/ml of IgE anti-DNP for the times indicated either without (open columns) or with (filled columns) reculture in IgE-free medium to a total culture time of 24 hr. Release is expressed as percentage of release obtained after 24 hr incubation with the antibody. Results are means±SEM for five experiments, each with cells pooled from two mice.

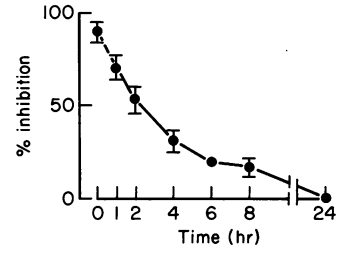

Figure 4. Inhibitory effect on sensitization of excess rat myeloma IgE added at various times after IgE anti-DNP. Results are means±SEM for three experiments, each with cells pooled from three mice.

the first 4 hr of incubation, whereas subsequent to 4 hr sensitization proceeded at a faster rate, which appeared to be constant between 4 and 48 hr (Fig. 2). After 48 hr of sensitization with the monoclonal antibody, challenge with antigen led to a mean net release of greater than 40% of total cellular [^{3}H]5-HT (Fig. 2).

Further kinetic experiments showed that the initial rate of sensitization was dependent on the concentration of IgE anti-DNP, and that the slow phase was more pronounced at low antibody concentrations (Table 1). At a low concentration of IgE anti-DNP (0·05 μg/ml), negligible sensitization was observed during the first 4 hr. At higher concentration (0·5 and 5·0 μg/ml) time-dependent and concentration-dependent sensitization was observed during the first 4 hr, but remained slow relative to sensitization subsequent to 4 hr (Table 1).

The characteristic time–course of sensitization of mast cells, as shown in Fig. 2, was not influenced by the concentration of fetal calf serum: identical kinetics were observed in the presence of 5%, 0·5% and 0% serum, although cell viability and stability were reduced at the lower levels of serum.

Challenge of isolated peritoneal cells with anti-IgE antibody at concentrations up to 1/20 failed to induce release of [^{3}H]5-HT, thus suggesting little or no endogenous cell-bound IgE on the harvested mast cells. However, to investigate further the possibility that the presence of endogenous cell-bound IgE may have influenced the kinetics of sensitization, cells were pretreated with glycine-HCl buffer, pH 3·5, by the method shown by Ishizaka & Ishizaka (1974) and Kulczycki & Metzger (1974) to completely dissociate receptor-bound IgE. Following this treatment the time–course of sensitization was unchanged, thus demonstrating that in these kinetic experiments any endogenous IgE was of no influence.

In order to establish whether the initial slow phase of sensitization corresponded to a slow phase of IgE binding, or whether it represented a 'lag' phase in functional sensitization subsequent to IgE binding, peritoneal cells were incubated with 0·5 μg/ml of IgE anti-DNP for 2, 4 and 8 hr. At each time-point cells from one culture tube were washed and challenged with antigen while cells from a second culture tube were washed three times in PBS (dilution factor ⩾10,000), resuspended in IgE-free complete RPMI-1640 medium, and incubated again at 37°, to give a total culture time of 24 hr (in IgE-containing followed by IgE-free medium). After 24 hr, the cells were washed again and challenged with antigen. The results of these experiments (Fig. 3) show clearly that sensitization to the DNP antigen progressed after removal of IgE anti-DNP from the culture medium. The effect was most marked when cells were incubated with IgE anti-DNP for 2 or 4 hr: at these times negligible sensitization was obtained (0·1±0·4% and 1·4±0·4%, respectively, of the response obtained after 24 hr sensitization with the antibody), whereas reculture of the cells in IgE-free medium led to a marked increase in sensitization (9·3±0·8% and 15·9±2·1%, respectively of the response after 24 hr sensitization). Sensitization of cells increased by 93-fold following reculture subsequent to 2 hr in the presence of the antibody, and 11-fold following reculture subsequent to 4 hr in the presence of the antibody.

So far, these experiments demonstrated that sensitization of cultured murine mast cells with IgE anti-DNP antibody was characterized by an early slow phase and a later more rapid phase that did not appear to correspond to the rate of binding of IgE to receptors, since addition of antibody for 2 or 4 hr allowed good sensitization only when the cells were returned to culture for an additional period in IgE-free medium. To investigate further the kinetics of binding of the IgE antibody, the effects of an excess of blocking IgE were studied at various times after addition of the sensitizing antibody. Affinity-purified rat myeloma IgE (Burt, Hastings & Stanworth, 1986; kindly provided by Dr D. R. Stanworth) was added in 10-fold excess to peritoneal cell cultures at 0, 1, 2, 4, 6 and 8 hr after addition of 0·5 μg/ml of IgE anti-DNP. The results showed that sensitiza-

tion was inhibited by 90% when excess myeloma IgE was added simultaneously with the IgE-DNP, but as the myeloma IgE was added at later times the inhibitory effect progressively decreased such that after 6 hr no further blocking was observed (Fig. 4). It is evident that although relatively little sensitization of mast cells was obtained during the first 4–6 hr of incubation with 0·5 μg/ml of IgE anti-DNP, during this initial period the major proportion of binding of the antibody to the cells was complete.

DISCUSSION

Our understanding of the interaction between IgE and the Fcε receptor on mast cells and basophils has advanced a great deal as a result of direct binding studies using radiolabelled IgE. These studies have provided estimates of the association constant of the interaction, forward and backward rate constants, and number of Fcε receptors per cell (Ishizaka *et al.*, 1973; Kulczycki & Metzger, 1974; Conrad *et al.*, 1975; Coleman & Godfrey, 1981b; Pruzansky & Patterson, 1986). In the present study of the sensitization of murine peritoneal mast cells with monoclonal IgE anti-DNP, measured as release of 5-HT upon stimulation with DNP antigen, some fundamental and novel aspects of the sensitization process were revealed, that may not have been predicted from a simple dynamic model of IgE binding.

Although the main conclusions of the study relate to the kinetics of sensitization, some other points may be worth noting. Firstly, the degree of sensitization was found to be dependent on the concentration of antibody according to a bell-shaped rather than a linear relationship: maximal sensitization was obtained at an antibody concentration of 0·3–3·0 μg/ml but declined at concentrations below and above this range. It is evident that at high concentrations of IgE antibody effector function is reduced, presumably, by analogy with the classical precipitin reaction, because of reduced cross-linking of receptor-bound antibody by antigen in conditions of antibody excess. Secondly, it is noteworthy that at an optimal concentration of IgE antibody, after 48 hr of sensitization, greater than 40% of [^{3}H]5-HT was released upon stimulation with specific antigen, thus demonstrating that a large proportion (>40%) of 5-HT, and presumably other granule-stored mediators, are functionally linked to the activation–secretion coupling mechanisms triggered by interaction between antigen and a single species of IgE antibody.

In kinetic studies, the time–course of sensitization with IgE antibody was clearly divisible into an initial slow phase of approximately 4 hr, and a later, more rapid phase, during which sensitization progressed at a constant rate up to at least 48 hr. The slow phase was most pronounced at lower concentrations of antibody. It is clear that sensitization does not proceed at the rate of binding of IgE, which is governed by the relationship: $v_0 = k_1 (IgE_0) (R_0)$, where v_0 is the initial rate of binding, k_1 is the forward rate constant, IgE_0 is the initial concentration of IgE, and R_0 is the initial concentration of free Fcε receptors (Kulczycki & Metzger, 1974). According to this relationship the rate of binding is highest initially but declines as the concentration of free Fcε receptors declines. Under the conditions used by Kulczycki & Metzger (1974), [^{125}I]IgE (1–4·5 μg/ml) bound rapidly to rat basophilic tumour cells (10^6/ml) during the first 15 min, but at a progressively declining rate, and reached a maximum after 90 min. Similar kinetics of binding of [^{125}I]IgE to human basophils were observed by Pruzansky & Patterson (1986) using comparable concentrations of ligand and cells. It is thus clear that the biphasic kinetics of sensitization observed in this study differed markedly from those that would be predicted by consideration of the dynamics of IgE binding.

In order to establish whether binding of DNP-specific IgE has indeed taken place during the early, slow phase of sensitization, experiments were performed in which mast cells were incubated with the antibody for various times, then washed to remove unbound antibody, resuspended, and re-incubated to give a total culture time of 24 hr. These experiments showed that further incubation of cells in IgE-free medium, after 2, 4 or 8 hr in contact with the antibody, led to increased sensitization to antigen that was most marked after 2 and 4 hr incubation with IgE. However, sensitization did not reach that obtained after 24 hr contact with the antibody.

In a further series of experiments the sensitization of mast cells with DNP-specific IgE could be blocked by addition of excess rat myeloma IgE, but only to a marked extent (≥50%) when the blocking immunoglobulin was added during the first 2 hr, thus providing evidence that the major part of binding of IgE antibody took place at an early stage, even though very little active functional sensitization of the cells took place during this time.

The slow kinetics of sensitization of murine mast cells with monoclonal IgE antibody are consistent with the observation that the IgE-dependent sensitization of human mast cells in chopped tissue and dispersed cell preparations requires long incubation times: a period of 18–20 hr incubation with atopic sera is used by most workers (Orange, Austen & Austen, 1971; Coleman & Godfrey, 1981b; Coleman *et al.*, 1985). The author has found that sensitization of dispersed human lung mast cells follows similar kinetics to those reported here for murine mast cells, that is, poor sensitization was obtained for incubation times of less than 8 hr (≤3% net histamine release) whereas good sensitization (≥12% histamine release) was obtained at 16 and 24 hr (J. W. Coleman, unpublished data).

In conclusion, this study has shown that the *in vitro* sensitization of murine mast cells with IgE anti-DNP appears not to be directly related to the kinetics of binding of the antibody and, furthermore, sensitization can proceed in the absence of extracellular IgE. Perhaps an association between the Fcε receptor and a second molecule subsequent to binding of IgE, or some other second event or signal is a prerequisite for sensitization, but any further comments must remain speculative at this stage.

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