Polyclonal IgE Induces Mast Cell Survival and Cytokine Production

Jun-ichi Kashiwakura1, Yuko Kawakami1, Keisuke Yuki2, Dirk M Zajonc1, Shunji Hasegawa1,6, Yoshiaki Tomimori1, Benjamin Caplan3, Hirohisa Saito4, Masutaka Furue5, Hans C Oettgen3, Yoshimichi Okayama2 and Toshiaki Kawakami1

ABSTRACT

Background: Ag-dependent activation of IgE-bearing mast cells is a critical first step in immediate hypersensitivity and other allergic responses. Recent studies have revealed Ag-independent effects of monoclonal mouse IgE molecules on mast cell survival and activation. However, no studies have been performed on the effects of polyclonal IgE molecules. Here, we tested whether polyclonal mouse and human IgE molecules affect survival and cytokine production in mast cells.

Methods: Mast cells were cultured in the presence of polyclonal mouse and human IgE molecules, and cell survival and cytokine production were analyzed.

Results: Polyclonal mouse IgE molecules in sera from mice with atopic dermatitis-like allergic skin inflammation, enhanced survival and cytokine production in mast cell cultures. Similar to the effects of monoclonal IgE, the polyclonal IgE effects were mediated by the high-affinity IgE receptor, FcεRI. Human polyclonal IgE molecules present in sera from atopic dermatitis patients were also capable of activating mast cells, and inducing IL-8 production in human cord blood-derived mast cells.

Conclusions: These results imply that polyclonal IgE in atopic dermatitis and other atopic conditions might modulate mast cell number and function, thus amplifying the allergic response.

KEY WORDS

allergy, antibodies, cell activation, human, mast cells/basophils

INTRODUCTION

Mast cells play a pivotal role in allergic inflammatory reactions and in defense against certain bacteria and parasites. In addition to these well-appreciated areas of mast cell function, recent studies have revealed the roles of mast cells in innate and adaptive immunity and implicated them in the pathogenesis of autoimmune diseases, chronic heart failure, cancer, and tolerance. Aggregation of FcεRI on mast cells by interaction of receptor-bound IgE with multivalent Ag induces their activation. Activated mast cells release a variety of preformed and de novo synthesized proinflammatory and immunomodulatory mediators, such as histamine, proteases, leukotrienes, prostaglandins, and various cytokines and chemokines. In addition to this classical IgE and Ag-dependent mechanism of mast cell activation, we and others have observed that survival and some activation events can also be induced in mouse mast cells by monomeric IgE in the absence of specific Ag. We also found that mouse monoclonal IgE molecules are quite heterogeneous with respect to their ability to induce survival and activation events in mouse mast cells: at one end of the spectrum, highly cytokinergic (HC) IgEs induce enhanced survival, degranulation, adhesion, migration, and expression of cytokines such as IL-6 and TNF-α; at the other extreme, poorly cytokinergic
(PC) IgEs mediate these effector functions inefficiently. Human monoclonal IgE molecules also affect human mast cells. Gilchrest et al. reported that the chemokine 1-309 mRNA and protein levels are upregulated by IgE alone in the presence of IL-4. Cruse et al. observed that human monomeric IgE stimulates cultured lung mast cells to release histamine, leukotriene C4 and IL-8. In addition, it was reported by Matsuda et al. that human monomeric IgE enhances chemokine production in cultured human mast cells, and this response was augmented by pre-incubation of the cells in IL-4.

The effects of monomeric IgE have mostly been demonstrated by in vitro culture experiments, although animal experiments have revealed that mouse IgE molecules can also promote survival and migration of mouse mast cells in vivo as well as the splenic mastocytosis induced by infestation with the parasite Trichinella spiralis. In this study, we set out to determine whether polyclonal IgE molecules in the absence of a specific Ag can affect mast cell biology.

**METHODS**

**MOUSE BONE MARROW-DERIVED MAST CELLS (BMMCs)**

Bone marrow cells from NC/Nga, C57BL/6, and FceRIα−/− mice were cultured in IL-3-containing medium for 4 to 6 weeks to generate mast cells (BMMCs) with more than 95% purity (c-Kit+ FceRI+ by flow cytometry). Animal experiments were approved by the Animal Care Committee of the La Jolla Institute for Allergy and Immunology (LIAI).

**HUMAN CORD BLOOD-DERIVED MAST CELLS (CBMCs)**

Human CBMCs were generated as described. Briefly, CD34+ cells were purified from umbilical cord blood mononuclear cells and cultured in serum-free Iscove’s methylcellulose medium containing 200 ng/ml SCF, 50 ng/ml IL-6, 1 ng/ml IL-3, 1% insulin-transferrin-selenium (Invitrogen, Carlsbad, CA, USA), 50 μM 2-mercaptoethanol, 1% penicillin-streptomycin (Invitrogen), and 0.1% bovine serum albumin. On day 42 of culture, methylcellulose was dissolved in PBS and the cells were then suspended and cultured in Iscove’s modified Dulbecco’s medium supplemented with 100 ng/ml SCF, 50 ng/ml IL-6, and 5% fetal calf serum. After 15 weeks, the purity of the resulting CBMCs was ~98% (c-Kit+ FceRI+ by flow cytometry).

**SERUM PREPARATIONS AND PURIFICATION OF IgE**

Mouse sera were obtained from NC/Nga mice before and after atopic dermatitis (AD)–like lesion induction as described previously. Briefly, NC/Nga mice were immunized by two rounds of epicutaneous treatment with Dermatophagoides farinae extract (Der f, Greer Laboratories, Lenoir, NC, USA) and Staphylococcal enterotoxin B (SEB, Sigma-Aldrich, St. Louis, MO, USA). Sera with high IgE levels were obtained from mice with AD-like skin lesions with clinical skin scores of ≥8. In some experiments, mouse sera were obtained from wild-type and IgE−/− BALB/c mice epicutaneously immunized with ovalbumin (OVA) as described previously. IgE concentrations were determined by ELISA (BD Biosciences Pharmingen, San Diego, CA, USA).

Human sera were obtained from AD patients and normal subjects. Written informed consent was obtained from all AD and normal subjects, and approved by the Institutional Review Board of LIAI. Human IgE levels were determined by latex agglutination-turbidimetric immunoassay.

For IgG or IgE depletion, sera were incubated overnight at 4°C with agarose beads chemically conjugated with either protein A (Amersham Biosciences, Buckinghamshire, UK), anti-mouse IgE mAb (Pharmingen) or anti-human IgE Ab (provided by Dr. Ishizaka in LIAI). Sera were spun by microcentrifuge. Cleared sera were analyzed by SDS-PAGE followed by immunoblotting with anti-mouse IgG Ab (Amersham), anti-mouse IgE Ab (SouthernBiotech, Birmingham, AL, USA) or anti-human IgE Ab (EMD Biosciences Inc., La Jolla, CA, USA).

For purification of polyclonal IgE molecules from sera, sera were pooled from AD-induced NC/Nga mice and incubated with anti-mouse IgE-conjugated agarose beads for overnight at 4°C. After incubation, the beads were washed with PBS and eluted with 0.1 M glycine-HCl (pH 2.7). The eluted IgE molecules were further purified by incubation with protein A beads to exclude contaminated IgG molecules, and followed by gel filtration chromatography using Superdex S-200 column (Amersham) on an AKTApurifier (GE Healthcare, Piscataway, NJ, USA) to purify monomeric IgE (Fig. 3A). After purification, IgE preparation was dialyzed against PBS and concentrated by Microcon 10 (Millipore, Bedford, MA, USA).

**STIMULATION OF MAST CELLS WITH SERA**

Mouse BMMCs (1 × 10⁵ per 100 μl) in culture medium containing 10% FCS were stimulated with sera (10%) with high or low levels of IgE (from NC/Nga mice untreated or treated with Der f and SEB; alternatively from BALB/c or IgE−/− mice epicutaneously immunized with OVA) for 24 hours for cytokine measurement or 72 hours for cell growth measurement. When purified polyclonal IgE was used, BMMCs were resuspended in culture medium and stimulated by the polyclonal IgE (5 μg/ml) for 72 hours for the measurement of survival and cytokine production. As controls, aliquots of BMMCs were stimulated with 5 μg/ml of SPE-7 IgE (Sigma-Aldrich Corp., St. Louis, MO, USA). For human CBMC experiments, CBMCs (1 × 10⁵) were first incubated...
Effects of Polyclonal IgE on Mast Cells

Fig. 1 Effects of sera containing high IgE levels on mouse mast cells. BMMCs were incubated with 10% sera from AD-induced (AD) or normal-skin (non-AD) NC/Nga mice for 72 hours (A) or 24 hours (B). Some cells were also incubated with 5 μg/ml of SPE-7 IgE or medium alone (-). (A) Total live cell numbers were counted after trypan blue staining. (B) IL-6 and IL-13 secretions were measured by ELISA.

ELISA
Human IL-8 and mouse IL-6 were measured by ELISA kits purchased from Biosource (Camarillo, CA, USA) and Pharamingen, respectively.

IMMUNOBLOTTING
Immunoblotting was performed as described previously.20 Densitometry of phosphorylated and total protein bands was performed using Quality One® version 4.6.2 (Bio-Rad Laboratories, Hercules, CA, USA).

STATISTICAL ANALYSIS
Differences between the 2 groups were analyzed by paired or unpaired Student’s t-tests. A difference in p value less than 0.05 was taken to indicate a statistically significant difference.

RESULTS
MOUSE POLYCLONAL IgE IN SERA CAN INDUCE SURVIVAL PROMOTION AND CYTOKINE PRODUCTION IN MOUSE MAST CELLS
We previously showed that a mixture of several monoclonal IgE antibodies can promote survival and induce cytokine production in mouse mast cells.21 However, no studies have been reported on the effect of polyclonal IgE on mast cell biology. In order to address this issue, we first tested whether sera containing high levels of IgE affect mast cell survival and cytokine production. Sera with 43.3–58.5 μg/ml of IgE derived from NC/Nga mice that had been treated with Der f and SEB (AD sera) were added to BMMC cultures at 10%. Cultures were kept without IL-3. Sera with low levels (<0.5 μg/ml) of IgE from non-treated NC/Nga mice (non-AD sera) served as a control. Live mast cells decreased from 1 × 10^5 cells to 4.5 × 10^4 cells after 72 hours in the absence of sera. Non-AD sera failed to affect the number of live cells. However, live mast cells were up to 80% more abundant in BMMC cultures containing AD sera than in cultures supplemented with non-AD sera (Fig. 1A). The effect of AD sera on the production of IL-6 and IL-13 was similarly greater than that of non-AD sera (Fig. 1B).

To explore the specific role of IgE in these sera in inducing mast cell survival and cytokine production, we depleted IgE using rat anti-IgE beads and repeated the BMMC culture experiments. Nearly 100% depletion of IgE was confirmed by immunoblotting (Fig. 2A). As controls, sera pretreated with rat IgG beads (negative control for IgE depletion) or protein A (for IgG depletion) were tested. No detectable differences in the effect on mast cell survival were seen between non-treated sera and sera pretreated with rat IgG beads (unpublished data). However, IgE-depleted sera showed reduced effects on both mast cell accumulation and cytokine production (Fig. 2B, C): IgE depletion reduced the effect on mast accumulation by 15–25% while it reduced the effect of IL-6 and IL-13 production by 22–30% and >90%, respectively. IgG-depleted sera showed a tendency toward reduction: the reduction in mast cell survival by IgG-depleted sera did not reach statistical significance (Fig. 2B), but IgG depletion induced a drastic reduction in IL-13 production (Fig. 2C). We observed the positive effects of high concentrations of IgG on mast
cell survival and cytokine production (unpublished data), suggesting that the IgG effects stem from stimulation of mast cells via the Fcγ receptors, probably FcγRIII and/or FcγRIV.

Sera containing high levels of IgE were obtained from another (BALB/c) mouse model of AD induced by epicutaneous sensitization with OVA.\textsuperscript{17} Sera were also obtained in identically immunized IgE\textsuperscript{-/-} mice. Sera from both WT and IgE\textsuperscript{-/-} mice induced IL-6 production (Fig. 2D). However, the effect of sera from IgE\textsuperscript{-/-} mice on IL-6 production to be at least 40% less. These experiments, together with IgE depletion experiments, demonstrate that IgE in sera can promote mast cell survival and cytokine production in vitro.

**POLYCLONAL IgEs IN SERA ARE MOSTLY MONOMERIC AND NOT BOUND TO Ag**

The observed effects of polyclonal IgE contained in these sera could be mediated either by free IgE or by Ag-bound IgE antibodies. To evaluate these possibilities, we first examined whether sera derived from OVA-immunized mice contain the Ag, OVA. OVA in sera from WT mice was immunoprecipitated with anti-OVA polyclonal antibody, and immune complexes were precipitated with protein A-agarose (proA in Fig. 2E-1). Immunoblot analysis of unpurified supernatants (sup) and protein A precipitates showed that there are no detectable levels of OVA in sera (Fig. 2E-1). The size distribution of IgE molecules in these sera were then examined. Analysis by native PAGE and subsequent immunoblotting revealed that more than 90% of IgE molecules in the sera are present as a monomer (unpublished data). This amount of monomeric IgE in sera is slightly lower than those (>95%) of a typical highly cytokinergic IgE, SPE-7, and a typical poorly cytokinergic IgE, H1 DNP-ε-206, before these IgE preparations are ultracentrifuged. Ultracentrifugation prior to experimentation removes dimeric or oligomeric forms of IgE (unpublished data).

**Fig. 2** Effects of IgE in mouse sera on mast cells. (A) Immunoblotting of IgE- or IgG-depleted sera. (B, C) Effects of the sera were analyzed as in Figure 1. nd, non-depleted sera. (D) IL-6 production by sera from epicutaneously OVA-immunized mice. < d.l., less than detection limit. (E-1) Sera from OVA-sensitized mice were first immunoprecipitated with anti-OVA antibody. Immune complexes precipitated with protein A-agarose were divided into 9 parts and separately run on SDS gels together with supernatants (sera devoid of OVA), followed by immunoblotting with anti-OVA antibody. (E-2) Sera from OVA-sensitized mice were analyzed for IgE and IgG levels by immunoblotting. Arrowhead, IgG heavy chain. *p < 0.05. ns, not significantly different.
Effects of Polyclonal IgE on Mast Cells

**POLYCLONAL IgE TRANSDUCES ITS EFFECT VIA FcεRI**

The activating effects of monomeric IgE mAbs have shown transduction via FcεRI. To test whether the effect of polyclonal IgE in sera is also mediated by FcεRI, we tested AD sera on BMMC derived from FcεRIα−/− mice. As expected, no differences were seen in mast cell numbers and cytokine production between non-treated and IgE-depleted sera (Fig. 3A, Top). These results demonstrate that the effects of polyclonal IgE in AD sera on mast cell survival and cytokine production depend on FcεRI.

As would be expected for signaling via FcεRI, incubation of WT cells with 10% sera containing high IgE levels (‘AD sera’) induced tyrosine phosphorylation of cellular proteins, similar to that induced by SPE-7 IgE (Fig. 3B). Phosphorylation patterns of LAT (Y191), PLC-γ2 (Y1217), and p38 MAPK (T180/Y182) induced by ‘AD sera’ were generally comparable to those induced by SPE-7 IgE (Fig. 3C). Importantly, phosphoproteins induced by ‘AD sera’ were drastically reduced in FcεRIα−/− cells, suggesting that

---

**Fig. 3** Mouse polyclonal IgE can induce mast cell accumulation via FcεRI. (A) BMMCs from WT and FcεRIα−/− mice were incubated with the indicated sera. Live mast cells and cytokines were quantified. *p < 0.05. ns, not significantly different. (B, C) Immunoblot analysis of BMMCs stimulated with SPE-7 IgE or sera from Der f/SEB-treated NC/Nga mice with antiphosphotyrosine mAb 4G10 (B) or indicated antibodies (C). Ratio of phosphorylated to total protein intensities are shown. Results representing two independent experiments are shown.
these phosphorylation events were caused mainly by FceRI-bound IgE in 'AD sera'. However, phosphorylation patterns of Akt (S473) and ERK (T202/Y204) in WT were different between the two different stimuli (Fig. 3C). Moreover, 'AD sera'-induced phosphorylation patterns of Akt and ERK were similar in WT and FceRIα−/− cells. These observations are consistent with the idea that 'AD sera' contain a non-IgE factor(s) that can stimulate mast cells and induce phosphorylation of ERK and Akt. In addition, we found that a non-IgE factor(s) induced strong tyrosine phosphorylation of 3 proteins in the range of 100–130 kDa in FceRIα−/−, but not WT cells (Fig. 3B), suggesting that FceRα regulates the cellular susceptibility to such a factor(s). However, the identity of these FceRIα−/− cell-specific tyrosine-phosphorylated proteins remains to be determined.

**PURIFIED POLYCLONAL IgE CAN INDUCE SURVIVAL PROMOTION AND CYTOKINE PRODUCTION IN MOUSE MAST CELLS**

The role of IgE (e.g.15–25% for mast cell accumulation and 22–30% for IL-6 production) in the effect of sera on mast cell survival and cytokine production might initially appear to be modest. However, given the possibility that sera, particularly from animals with inflammation, contain many factors that might modulate mast cell survival and proliferation, it is possible that IgE effects would be obscured by the background. Indeed, NC/Nga mice treated with Der f and SEB had high mRNA levels of IL-4 (up to 40 fold over non-treated control) and IL-6 (up to 3 fold) in draining lymph nodes (T. Ando and T. K., unpublished data). Therefore, these and other cytokines as well as other protein and non-protein factors may be present in such sera. However, IL-4 was shown to induce mast cell apoptosis and therefore it was not surprising to find that the relative contribution of IgE in sera to alterations in mast cell biology is limited. Indeed, IL-13...
production was reduced by more than 90% through IgE depletion. To exclude the effects of the factors that might affect mast cell biology, we purified monomeric IgE to near homogeneity by combining affinity and gel filtration chromatographies from pooled sera taken from Der f/SEB-treated NC/Nga mice (Fig. 4A, B). As shown in Figure 4C, D, the added polyclonal IgE (at 5 μg/ml) in a monomeric form to mast cell cultures in the absence of IL-3 or SCF promoted mast cell survival and induced IL-6 production. The magnitude of these effects induced by polyclonal IgE was intermediate between them elicited by SPE-7 and 206 IgEs, and similar to that induced by a mixture of various HC and PC IgEs in a previous study.7

**POLYCLONAL IgE CAN INDUCE IL-8 PRODUCTION IN HUMAN MAST CELLS**

We and other investigators have reported that, similar to the effects of IgE on mouse mast cells, IgE antibodies also modulate cytokine and chemokine production in human mast cells.9-11 Therefore we evaluated polyclonal IgE effects on IL-8 production in human CBMCs. Sera from AD patients (n = 8) were used as a source of high-level polyclonal IgE (993–24,194 IU/ml), while sera from non-AD individuals (n = 2) were used as controls. Human CBMCs secreted higher amounts of IL-8 in the presence of 5% or 10% AD sera than non-AD sera, although the differences in IL-8 production between AD and non-AD sera varied considerably among CBMC preparations (Fig. 5B and unpublished data). Depletion of IgE from AD sera (Fig. 5A) reduced their effects on IL-8 production compared with that of non-depleted AD sera (Fig. 5B). We conclude that polyclonal IgE can affect chemokine production in human mast cells.

**DISCUSSION**

Ag-independent IgE effects on mast cell survival and activation, as well as the heterogeneity of IgE molecules in this regard, have been shown mainly using mouse IgE mAbs (reviewed in ref.8). To the best of our knowledge, this is the first study on the effects of polyclonal IgE on mast cell biology. Polyclonal IgE antibodies present in sera from mice with AD-like allergic skin inflammation and polyclonal IgE purified from the same sera can promote mouse mast cell survival and cytokine production, resembling mouse IgE mAbs. Similar to mouse IgE mAbs, polyclonal IgE also exerts its effect via the FcεRI. Our data underscores the role of IgE responses in mast cell survival.7,12,13 Similarly, AD sera containing high levels of IgE induced IL-8 production in human CBMCs. In this case, it is assumed that human FcεRI conveys the effect of human monoclonal and polyclonal IgE molecules in mast cells.

Ag-independent IgE effects on mast cell biology have been shown to be mainly due to monomeric IgE, but not IgE aggregates. In the past, various groups have used FPLC or HPLC to independently purify monomeric IgE and IgE aggregates from commercial or laboratory preparations of IgE mAbs and the effects of monomeric versus aggregate IgEs on mast cell activation have been assessed.6,22,23 In the present study, we purified monomeric, polyclonal IgE using affinity and gel filtration chromatographies. Our findings and previous observations by others6,22,23 demonstrate that monomeric IgE exhibits mast cell stimulatory activity regardless of IgE being monoclonal or polyclonal. Although there is a remote concern that IgE might aggregate in culture medium, we believe this is unlikely, as demonstrated by biophysical simulations performed by Schweitzer-
Stenner and Pecht\textsuperscript{24}. Additional concerns have been raised that mast cells might be stimulated by IgE adherent to plastic plates. However, such dependence on plastic surfaces would be inconsistent with the observation that tyrosine phosphorylation and Ca\textsuperscript{2+} responses occur within seconds after IgE treatment,\textsuperscript{22,23} and it would also be incompatible with our findings concerning the heterogeneity of IgE molecules with respect to mast cell activation.\textsuperscript{7} ELISA experiments have confirmed that virtually no IgE is bound to culture plates in these cultures (unpublished data). Although the concept of ‘monomeric IgE effects’ might still be considered as heretical by some researchers, we have shown that monomeric IgE mAbs induces aggregation of FcεRI,\textsuperscript{7} in the same manner as IgE plus antigen, the conventionally accepted trigger of mast cell activation.

Our results also indicate that mouse sera containing high levels of IgE have a low degree of IgE aggregates, at least when stored at 4°C for a few weeks. Previous studies showed that IgE aggregates in preparations of IgE mAbs exhibit weak or strong mast cell stimulatory activity.\textsuperscript{5,22,23} These observations suggest the possibility that in vivo mast cells in inflammatory settings might be exposed to stimulatory effects not only of monomeric IgE, but also of IgE oligomers even when specific Ag is not present. Considering the fact that IgE is produced locally at sites of allergic inflammation,\textsuperscript{25} it is possible that the in vivo effects of IgE on mast cells may be significantly greater than the 15–30% increases in mast cell survival and cytokine production, which were observed in cultures containing only 10% sera. The accumulation of splenic mast cells during infestation with \textit{Trichinella spiralis}, which elicits a robust IgE response was enhanced more than two-fold in wild-type mice compared with \textit{IgE−/−} mice.\textsuperscript{13} It is believed that mast cells increased in the intestine of \textit{T. spiralis}-infected mice migrated to spleens then were eliminated during the recovery phase. As the number of jejunal mast cells was intact in \textit{IgE−/−} mice, it can be interpreted that intestinal mast cell expansion in response to \textit{T. spiralis}-infection is not affected by IgE. However, the number of splenic mast cells was reduced in \textit{IgE−/−} mice, probably due to lack of IgE-mediated protection from apoptosis. Furthermore, we have observed that IgE antibodies strongly promote the accumulation of mast cells in the airways of mice subjected to repeated inhalation of an aqueous extract of the allergen, \textit{Aspergillus fumigatus}.\textsuperscript{26}

Polycional IgE seems to be composed of HC and PC IgE molecules. Compiled data indicate that 7 out of 21 mouse IgE mAbs tested are HC IgEs, while the remaining 14 mAbs are PC (unpublished data). The potency of purified polyclonal IgE was intermediate between those of HC and PC IgE mAbs (Fig. 4C, D). These results are consistent with our previous findings that the survival effects of a mixture of 6 mouse mAbs were intermediate between HC and PC IgEs.\textsuperscript{7} Our findings on the effects of polyclonal IgE suggest that patients with chronically high levels of IgEs might have an expanded pool of mast cells that may be subject to activating stimuli including polyclonal IgE, with or without allergens. Mast cells in patients who preferentially produce HC IgEs would be more prone to activation than in patients who preferentially produce PC IgEs.\textsuperscript{8} Although patients with PC IgEs would probably benefit most by avoiding allergens, those with high HC IgEs would not respond effectively to such a strategy. Therefore, treating patients who produce HC IgE but not PC IgE with anti-IgE therapy would be a reasonable therapeutic approach.

ACKNOWLEDGEMENTS

This study was supported by grants from the National Institutes of Health AI50209 (T.K.) and AI054471 (H. C.O.) and Grants-in-Aid for Scientific Research (C) program of the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government (project No. 20591195 to Y.O.).

REFERENCES

12. Kitaura J, Kinoshita T, Matsumoto M et al. IgE− and IgE+ Ag-mediated mast cell migration in an autocrine/


