

Monomeric IgE Stimulates Signaling Pathways in Mast Cells that Lead to Cytokine Production and Cell Survival

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Summary

Although IgE binding to mast cells is thought to be a passive presensitization step, we demonstrate herein that monomeric IgE (mIgE) in the absence of antigen (Ag) stimulates multiple phosphorylation events in normal murine bone marrow-derived mast cells (BMMCs). While mIgE does not induce degranulation or leukotriene synthesis, it leads to a more potent production of cytokines than IgE + Ag. Moreover, mIgE prevents the apoptosis of cytokine-deprived BMMCs, likely by maintaining Bcl-X_L levels and producing autocrine-acting cytokines. The addition of Ag does not increase this IgE-induced survival. Since IgE concentrations as low as 0.1 µg/ml enhance BMMC survival, elevated plasma IgE levels in humans with atopic disorders may contribute to the elevated mast cell numbers seen in these individuals.

Introduction

IgE-induced mast cell activation is responsible for both allergic disorders, such as hay fever and asthma, and for host resistance to parasites via the secretion of proinflammatory mediators (Schwartz, 1994; Yamaguchi et al., 1999; Galli, 2000). The three major classes of proinflammatory mediators released by these cells are preformed granule-associated chemical mediators, newly synthesized arachidonic acid metabolites, and cytokines (Schwartz, 1994; Mekori and Metcalfe, 2000). IgE initiates these processes by binding to mast cells via the high-affinity receptor FcεRI, which belongs to the immune receptor superfamily and exists as a tetramer

of one α, one β, and two identical disulfide-linked γ chains. The α subunit binds the Fc portion of a single IgE molecule, while the β and γ chains are critical for mediating signal transduction events via their immunoreceptor tyrosine-based activation motifs (ITAMs), which, upon phosphorylation by members of the Src kinase family, attract Src-homology-2 (SH2)-containing signaling proteins (Turner and Kinet, 1999). IgE binding to the FcεRI is referred to as a “passive presensitization” step in the current mast cell signaling paradigm. FcεRI activation and signaling is thought to occur only when receptor-bound IgE is crosslinked via multivalent antigens (Ags) (Beaven and Metzger, 1993; Kinet, 1999).

We recently demonstrated that IgE binding, in the absence of Ag, triggered a number of signaling pathways in normal murine bone marrow-derived mast cells (BMMCs) (Huber et al., 1998a). We also showed that an important function of the SH2-containing inositol phosphatase SHIP in these cells was to prevent this signaling from progressing to degranulation, by hydrolyzing the IgE-induced generation of phosphatidylinositol (PI)-3,4,5-P₃. In the present study, we have further elucidated the signaling events triggered by IgE alone and the biological ramifications of these signaling events.

Results

IgE Alone Stimulates the Phosphorylation of the Erks, p38, JNK, and PKB in Normal Murine BMMCs

We recently reported that a 3 min exposure to 5 µg/ml IgE in the absence of Ag triggered the phosphorylation of the FcεRI β and γ subunits, Shc, and SHIP in normal murine BMMCs and that the presence of SHIP prevented IgE-induced signaling from progressing to degranulation (Huber et al., 1998a). To further explore the signaling potential of IgE with normal BMMCs, we carried out time course studies with IgE alone (monoclonal anti-DNP clone SPE-7 IgE, Sigma) and examined the phosphorylation of the mitogen-activated protein kinases, Erk 1/2, p38 (a.k.a. HogK), and JNK (a.k.a. SAPK). As can be seen in Figure 1A, 5 µg/ml of IgE alone stimulated near maximal phosphorylation of the Erks within 5 min, and this phosphorylation was still at maximal levels at 60 min of stimulation. The stress-activated kinases, p38 and JNK, were also phosphorylated in response to IgE alone, with p38 again being maximally phosphorylated within 5 min of stimulation and remaining at maximal levels following 60 min of exposure. Interestingly, JNK phosphorylation was relatively delayed, not peaking until 15 min poststimulation. In addition, as shown in Figure 1B, IgE alone stimulated the phosphorylation of the survival-enhancing kinase PKB at both Ser473 and Thr308. This dual phosphorylation, which is required for full activation of PKB (Alessi et al., 1997), indicated that IgE alone was also capable of recruiting PI-3-kinase to the plasma membrane, since PI-3-kinase is upstream of PKB phosphorylation and generates PI-3,4,5-P₃ at the plasma membrane to attract the PH-containing kinases, PDK-1 and PKB (Alessi et al., 1997). A reprobing of the

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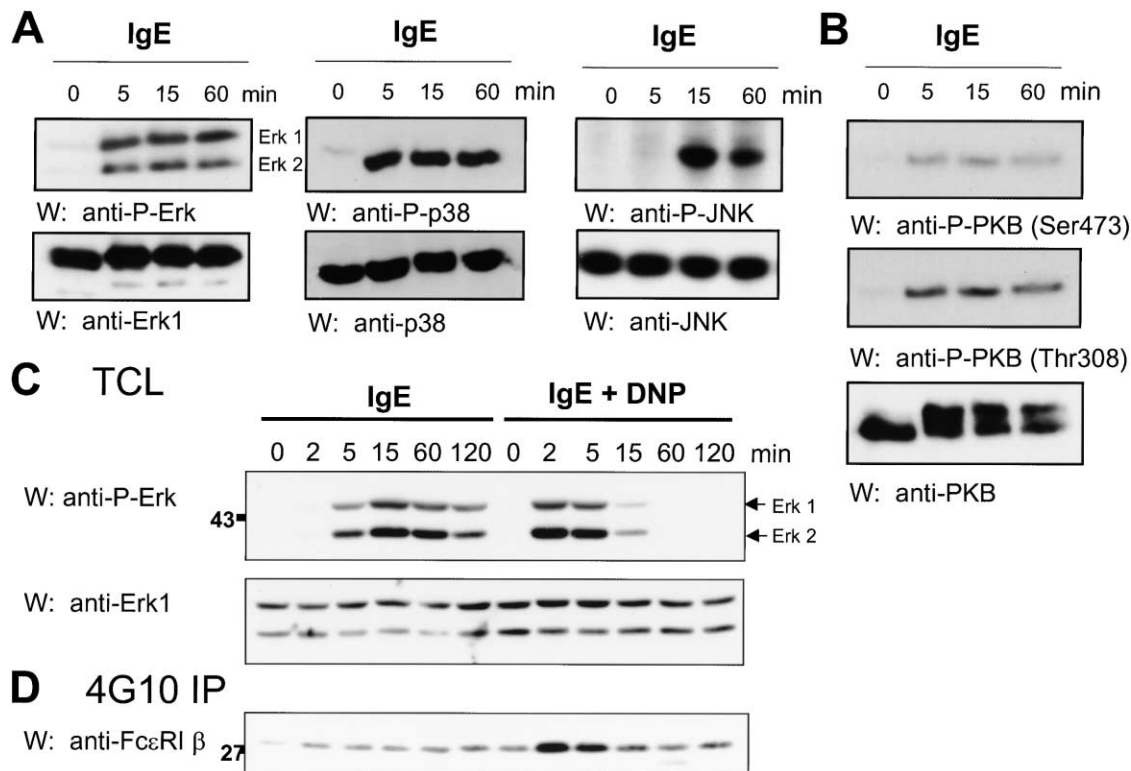


Figure 1. IgE in the Absence of Ag Stimulates Multiple Phosphorylation Events

BMMCs were treated with 5 μ g/ml IgE alone for the indicated times, and the SDS-solubilized total cell lysates were subjected to (A) Western analysis with phospho-specific antibodies to Erk 1/2 (left panel), p38 (middle panel), or JNK (right panel). The blots were then reprobed with antibodies to Erk 1, p38, and JNK, respectively, to confirm equal loading. (B) Western analysis was performed with anti-phospho-Ser⁴⁷³-specific PKB (top panel) and reprobed with anti-phospho-Thr³⁰⁸-specific PKB (middle panel) and anti-PKB (to confirm equal loading; bottom panel). (C) BMMCs, treated with 5 μ g/ml IgE alone for the indicated times or 5 μ g/ml IgE for 4 hr and then 20 ng/ml DNP-HSA for the indicated times, were SDS solubilized, and total cell lysates were subjected to Western analysis with anti-phospho-specific Erk 1/2 (upper panel) and then anti-Erk 1 antibodies (lower panel). Results are representative of six separate experiments. (D) BMMCs, treated as in (C), were lysed with TX-100 and subjected to immunoprecipitation with anti-phosphotyrosine (4G10) beads and Western analysis with anti-FcεRI β chain antibodies. Results are representative of three separate experiments.

blots shown in Figures 1A and 1B with anti-Erk1, anti-p38, anti-JNK, and anti-PKB, respectively, established equal loading.

Since it had been reported previously that IgE + Ag triggers the phosphorylation of these same signaling intermediates (Lu-Kuo et al., 2000), we compared the intensity and kinetics of phosphorylation of the Erks and PKB in response to IgE alone versus IgE + Ag (DNP-HSA). As shown with Erk 1/2 in Figure 1C, we found that phosphorylation events were slightly delayed but far more prolonged in response to IgE alone. Specifically, 5 μ g/ml of IgE for 4 hr, followed by an optimal concentration of Ag (20 ng/ml) for the indicated times, triggered a maximal phosphorylation of the Erks by 2 min, but this phosphorylation was barely detectable at 15 min and was back to baseline levels by 1 hr. With 5 μ g/ml IgE alone, on the other hand, Erk 1/2 phosphorylation was barely detectable at 2 min, peaked at 15 min, remained clearly visible at 2 hr, and was back to baseline levels at 4 hr (the zero time for IgE + Ag). Similar results were obtained with PKB (data not shown). Worthy of note is that the level of Erk 1/2 phosphorylation stimulated by 5 μ g/ml IgE alone was similar to that attained with 5 μ g/ml IgE + Ag. Similar results were obtained

when the cells were preloaded overnight, ruling out that the similar intensities were due simply to a transient desensitization by IgE (data not shown).

Having established that signaling events are initiated by IgE alone and with a similar intensity to that elicited with IgE + Ag when 5 μ g/ml IgE is used, we compared the tyrosine phosphorylation of the FcεRI itself. BMMCs were stimulated as above and the cell lysates subjected to immunoprecipitation with anti-phosphotyrosine beads and Western analysis with anti-FcεRI β subunit antibodies (Figure 1D). Interestingly, phosphorylation of the FcεRI β chain by IgE alone was less intense than that observed in response to IgE + Ag, but phosphorylation levels remained clearly detectable at 4 hr (i.e., the zero time for IgE + Ag). With IgE + Ag, on the other hand, phosphorylation of the FcεRI β chain was clearly faster and more intense, peaking at 2 min but returning to its baseline levels by 1 hr.

IgE Alone Likely Acts through the FcεRI and Lipid Rafts

We then carried out IgE dose response studies $\pm$ Ag, using Erk phosphorylation as an endpoint. As can be seen in Figure 2A, 2–5 μ g/ml of IgE (for 60 min) was

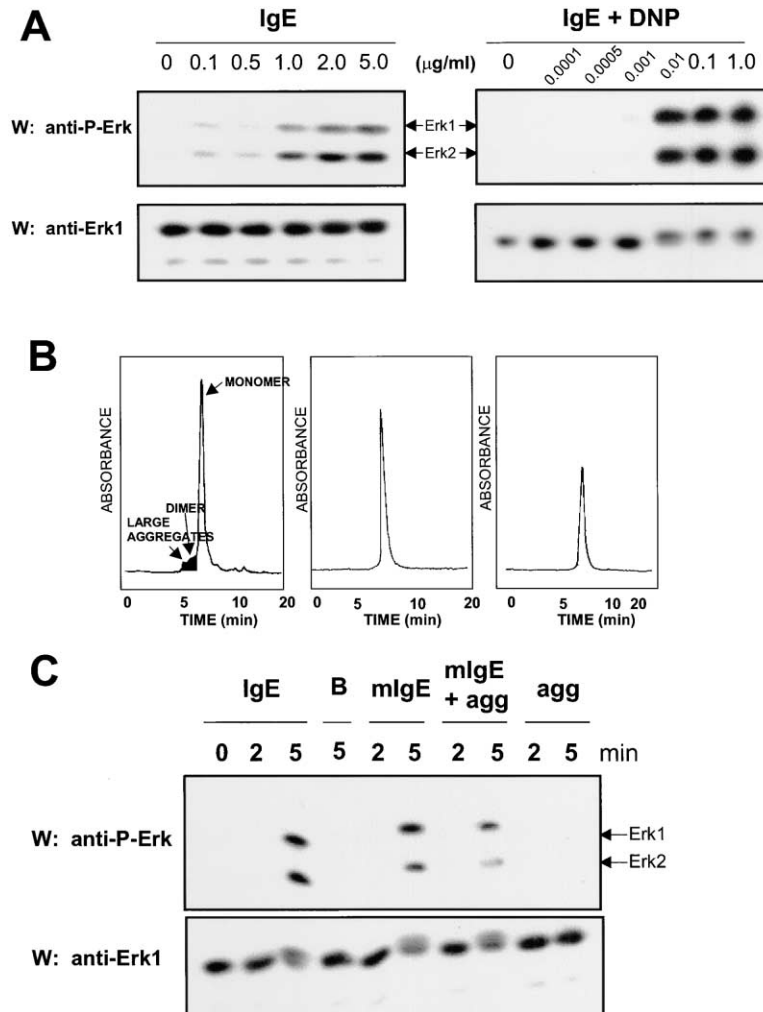


Figure 2. Monomeric IgE Stimulates Erk Phosphorylation

(A) BMMCs, treated with the indicated concentrations of IgE alone for 60 min or the indicated concentrations of IgE for 4 hr and then 20 ng/ml Ag for 2 min, were SDS solubilized, and total cell lysates were subjected to Western analysis with anti-phospho-specific Erk 1/2 (upper panel) and then anti-Erk 1 (lower panel). Results are representative of two separate experiments.

(B) Clone SPE-7 anti-DNP IgE (Sigma) was fractionated using a BioSep SEC S3000 column (left panel) and the monomer peak either rechromatographed immediately (middle panel) or after incubation for 7 days at 37°C in Tyrode's buffer (right panel). Results are representative of ten separate experiments.

(C) BMMCs were treated with 5 μ g/ml unfractionated IgE, HPLC buffer eluate (B), 5 μ g/ml mIgE, 5 μ g/ml mIgE + 0.5 μ g/ml aggregates (agg), or 0.5 μ g/ml aggregates alone for the indicated times, SDS solubilized, and subjected to Western analysis with anti-phospho Erk 1/2. The blot was reprobed with anti-Erk 1 to demonstrate equal loading. Results are representative of two separate experiments.

required for maximal Erk phosphorylation, whereas only 0.01 μ g/ml of IgE was required for maximal phosphorylation when a 4 hr pretreatment with IgE was followed with 2 min of 20 ng/ml Ag. Densitometric analyses of these two blots suggested an EC₅₀ of 1 μ g/ml for IgE alone and 0.005 μ g/ml for IgE + Ag. Similar results were obtained with RBL-2H3 cells (data not shown). This 200-fold difference in potency raised the possibility that IgE alone was stimulating these cells because of a small number of aggregates in the commercial IgE anti-DNP preparation. To test this, we fractionated this Sigma IgE via BioSep SEC S3000 gel filtration HPLC and found, depending on the lot number, that these anti-DNP IgE preparations contained from 5% to 20% aggregates (dimers plus higher molecular weight aggregates) (Figure 2B, left panel). The purified monomers were rechromatographed immediately following purification (Figure 2B, middle panel), 7 days after incubation at 37°C in Tyrode's buffer (Figure 2B, right panel) or 1 year after storage in PBS at 4°C (data not shown) and in all cases remained in the monomeric state (i.e., contained less than 0.5% aggregates, our lower limit of detection). To determine if the IgE-induced Erk phosphorylation was due to the presence of these aggregates, freshly prepared monomeric IgE (mIgE) (at 5 μ g/ml, as assessed

by an IgE ELISA), aggregates alone (at the level present in 5 μ g/ml of unfractionated IgE, i.e., 0.5 μ g/ml), or aggregates plus 5 μ g/ml mIgE were tested. As can be seen in Figure 2C, 5 μ g/ml mIgE could still robustly stimulate the phosphorylation of Erk 1/2. While this stimulation was slightly reduced compared to unfractionated IgE, the aggregates were very poor stimulants, and the addition of aggregates to mIgE did not increase the response over that seen with mIgE alone (Figure 2C). These results suggest that the aggregates in these commercial preparations of IgE are for the most part in inactive conformations. Of note, column eluate alone did not stimulate Erk phosphorylation (Figure 2C). We also assessed the levels of endotoxin in both our unfractionated and HPLC purified IgE preparations, since endotoxin has been shown to stimulate mast cells (Leal-Berumen et al., 1994). Using the highly sensitive LAL reagent clotting assay (Sullivan et al., 1983), we found the level of endotoxin was below the limit of detection (1.25 pg/ml). Concentrations up to 10⁶-fold higher than this had no effect on Erk phosphorylation.

The 200-fold difference in potency between IgE alone and IgE + Ag also raised the possibility that IgE alone was acting through a low-affinity IgE receptor. Of the known low-affinity IgE receptors on BMMCs, the most

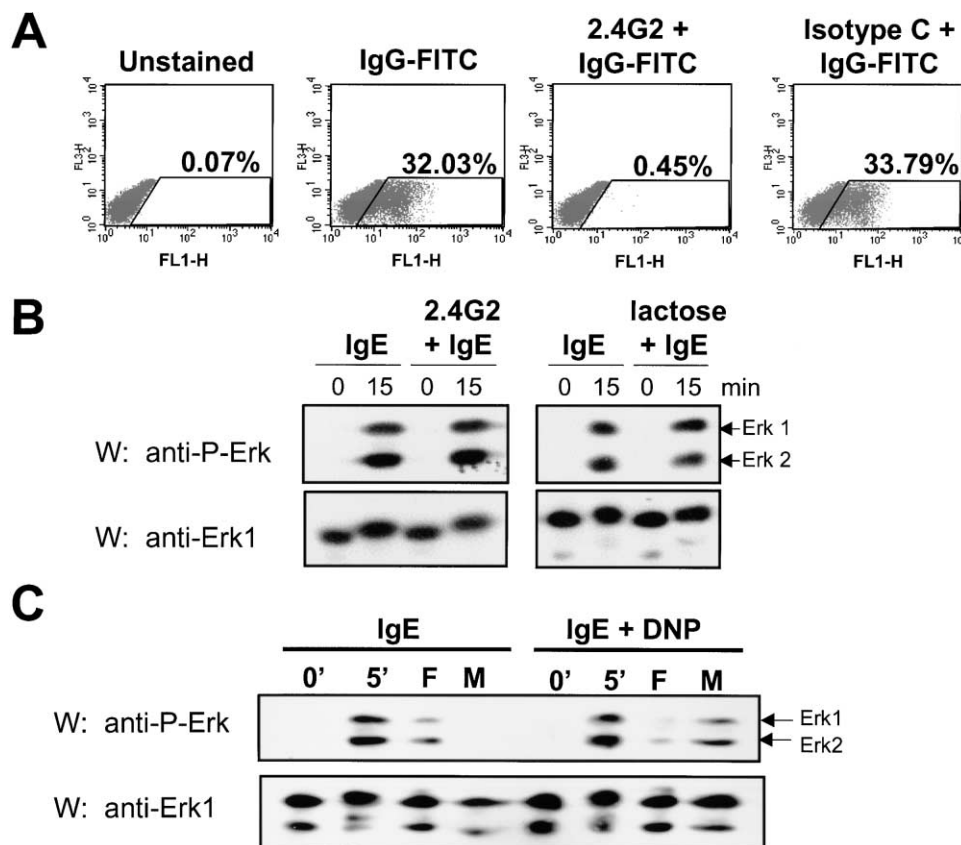


Figure 3. IgE Likely Acts through the FcεRI and Lipid Rafts

(A) BMMCs were pretreated $\pm$ 10 μ g/ml of 2.4G2 or isotype control (rat IgG2b anti-Mac1 α) antibody for 30 min at 4°C and then either left unstained or exposed to 30 μ g/ml heat-aggregated (63°C, 30 min [pH 8]; Daeron et al., 1980) FITC-mouse IgG for 30 min at 4°C and subjected to FACS analysis. The first panel shows the unstained control, the second panel shows the nonpretreated FITC-IgG sample, the third panel shows the 2.4G2 pretreated sample, and the fourth panel shows the isotype control pretreated sample. (B) BMMCs were either pretreated $\pm$ 10 μ g/ml 2.4G2 for 30 min at 4°C and then treated $\pm$ 5 μ g/ml IgE for 15 min (left panel) or pretreated $\pm$ 25 mM lactose for 10 min at 37°C and then treated $\pm$ 5 μ g/ml IgE for 15 min (right panel). (C) BMMCs were pretreated for 15 min $\pm$ 1.6 μ g/ml filipin III (F) or 7 mM methyl- β -cyclodextrin (M) and then treated $\pm$ 5 μ g/ml IgE or 20 ng/ml DNP-HSA for 5 min. Results are representative of two separate experiments.

likely candidate, given that IgE alone stimulates the phosphorylation of the β subunit of FcεRI, was FcγRIII. Although FcγRIII is expressed at relatively low levels at the surface of BMMCs (Kurosaki and Lobell, 1995), it also utilizes this β subunit (Kurosaki et al., 1992) and has been shown to be a low-affinity receptor for both IgG and IgE (Takizawa et al., 1992). To test this, we preincubated BMMCs with the FcγRIII/FcγRII blocking antibody 2.4G2 (Unkeless, 1979) under conditions which completely blocked binding to these receptors (Figure 3A) and found that it had no effect on subsequent IgE-induced Erk phosphorylation (Figure 3B, left panel). Another low-affinity IgE receptor that has been shown to be present on the rat basophilic cell line, RBL-2H3 (Frigeri and Liu, 1992), and thus may be present on BMMCs is the lectin Mac-2 (a.k.a. ϵ BP). This receptor binds to β -galactoside on the cell surface and thus can be efficiently eluted from cells with 25 mM lactose (Frigeri and Liu, 1992). However, this treatment did not diminish IgE-induced Erk phosphorylation (Figure 3B, right panel). These results suggest that IgE alone triggers Erk phosphorylation through the FcεRI.

To gain some insight into how IgE alone triggers these signaling events, we examined the effects of the lipid

raft disrupters filipin III (Aman and Ravichandran, 2000) and methyl- β -cyclodextrin (Vereb et al., 2000) on IgE-induced Erk phosphorylation. As can be seen in Figure 3C, both markedly inhibited IgE-induced Erk phosphorylation. This suggests that signaling induced by IgE alone, like IgE + Ag (Holowka et al., 2000), involves a critical detergent-resistant membrane (DRM) step.

IgE Alone Is More Effective than IgE + Ag at Increasing the Levels of Multiple Cytokines

Since stimulating normal BMMCs with IgE + Ag has been shown to trigger mast cell degranulation, the generation of arachidonic acid metabolites, and the synthesis of various cytokines (Mekori and Metcalfe, 2000), we compared the ability of IgE alone with IgE + Ag at eliciting these three biological responses. Specifically, we compared β -hexosaminidase release to monitor degranulation, LTC₄ production (using enzyme immunoassays [EIAs]) to monitor arachidonic acid metabolite production, and cytokine mRNA levels via RNase protection assays (RPAs). As expected, IgE + Ag stimulated all three responses (Figure 4). Surprisingly, however, while IgE alone was incapable of triggering degranulation or significant LTC₄ release (Figure 4A), it dramatically in-

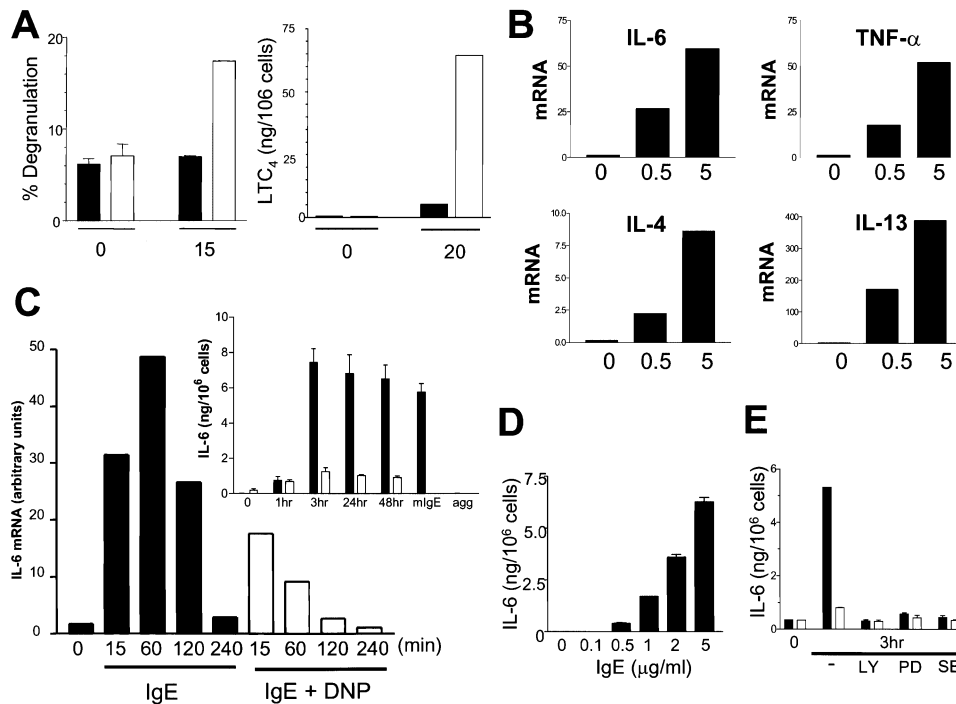


Figure 4. IgE Increases Multiple Cytokine mRNA and Protein Levels More than IgE + Ag

BMMCs, treated $\pm$ 10 μ g/ml IgE (filled boxes) for the indicated times or 10 μ g/ml IgE for 4 hr and then 20 ng/ml DNP-HSA (empty boxes) for the indicated times, were analyzed for (A) degranulation (left panel) and LTC₄ production (right panel). The values shown in the left panel are the means $\pm$ SEM of duplicate determinations, and both panels are representative of three separate experiments. (B) BMMCs, treated with IgE for 15 min at the indicated concentrations (in μ g/ml) were subjected to RNase protection assays (Riboquant), and relative band intensities of IL-6, TNF α , IL-4, and IL-13 were quantitated using a phosphorimager and standardized using levels of the housekeeping gene *GAPDH*. Results are representative of four separate experiments. (C) BMMCs, treated with 5 μ g/ml IgE for the indicated times or 5 μ g/ml IgE for 4 hr and then washed three times and exposed to 20 ng/ml DNP-HSA for the indicated times, were subjected to RNase protection assays as in (B). Results are representative of four separate experiments. The inset shows the levels of IL-6 protein (assessed by IL-6 ELISA) in medium conditioned by BMMCs treated as in (C). The last two lanes show the level of IL-6 secreted by 3 hr in response to 5 μ g/ml mIgE or 0.5 μ g/ml aggregates. Values are the mean $\pm$ SEM of eight determinations (duplicates of four separate experiments). (D) BMMCs were treated with the indicated concentrations of IgE for 24 hr, and the levels of secreted IL-6 were determined. (E) LY294002 (LY) at 50 μ M, 50 μ M PD98059 (PD), and 10 μ M SB203580 (SB) were added to BMMCs 30 min before the addition of 5 μ g/ml IgE or 20 ng/ml DNP-HSA, and the production of IL-6 was assessed after 3 hr of incubation. Values for (D) and (E) are the mean $\pm$ SEM of duplicate determinations and are representative of two separate experiments.

creased the mRNA levels of multiple cytokines (e.g., IL-6, TNF α , IL-4, and IL-13) in a dose-dependent manner (Figure 4B). In fact, IgE alone was substantially more potent than IgE + Ag at increasing the mRNA levels of these cytokines. A typical result, shown with IL-6 mRNA, is depicted in Figure 4C. Reminiscent of our protein phosphorylation data (Figure 1C), cytokine mRNA levels remained elevated longer in response to IgE alone than IgE + Ag (Figure 4C). However, this was not true for all cytokines examined. IL-5 mRNA levels, for example, were substantially higher following IgE + Ag than IgE alone (data not shown).

To determine if cytokine levels secreted by BMMCs in response to IgE alone versus IgE + Ag paralleled their mRNA levels, we compared IL-6 protein levels, by ELISA, in medium conditioned by BMMCs. As shown in the insert in Figure 4C, this was indeed the case, with secreted IL-6 levels being 5- to 10-fold higher following stimulation with IgE alone than with IgE + Ag. Similar results were obtained with IL-13 and TNF α (data not shown). Of note, identical results were obtained when a protease inhibitor cocktail was added to the medium (Sigma, P-8340), suggesting that this difference is not

due to a differential secretion of proteases or protease inhibitors. Also of note, mIgE was only slightly less potent than unfractionated IgE, while IgE aggregates were very poor stimulators of cytokine secretion (inset to Figure 4C). To ensure that our HPLC purification did not inactivate the IgE aggregates, we repeated our IL-6 secretion studies with monomeric and aggregate-enriched preparations obtained via a 60 min airfuge procedure. Bearing in mind the caveat that this procedure only removes larger aggregates, we found, once again, that all the IgE-induced production of IL-6 could be attributed to mIgE (data not shown).

Interestingly, while IgE-induced IL-6 mRNA levels peaked at 60 min, IL-6 protein levels did not reach plateau levels until 3 hr, and this discrepancy could suggest, as has been shown with TNF α production from RBL-2H3 cells (Baumgartner et al., 1994), that secretion from BMMCs is actively regulated via a calcium- and PKC-mediated pathway and that this pathway may be more active in response to IgE alone. An IgE dose response study was then carried out and revealed a similar EC₅₀ for IL-6 (and IL-13; data not shown) secretion (Figure 4D) to that seen for Erk phosphorylation.

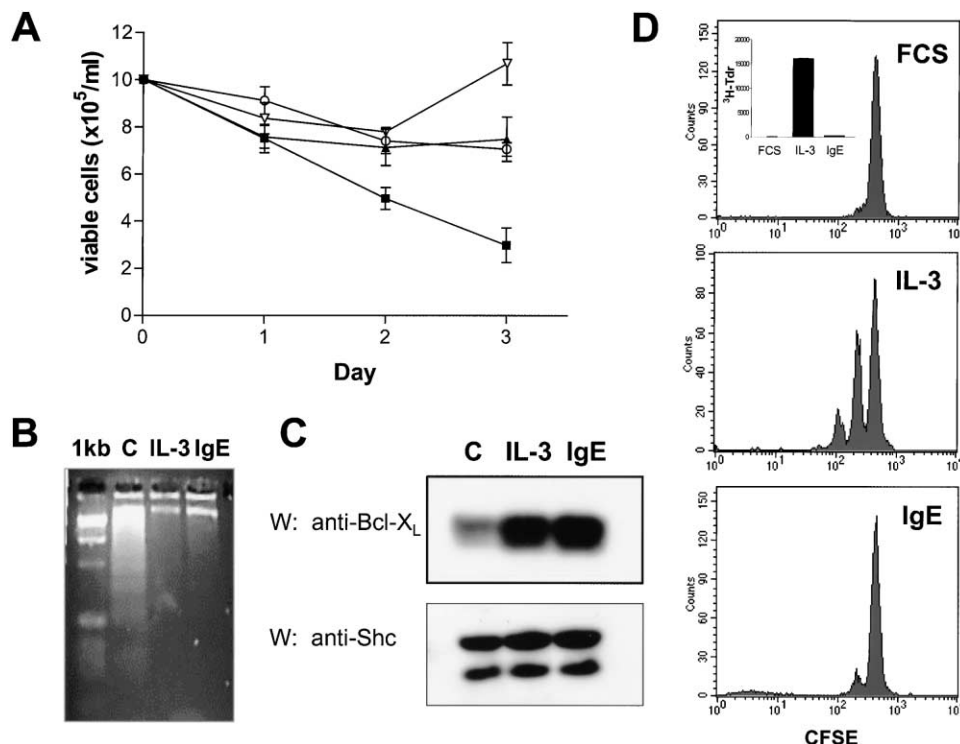


Figure 5. IgE Alone Maintains Bcl-X_L Levels and Prevents Apoptosis of BMMCs

(A) BMMCs derived from four separate mice were incubated separately for 72 hr with 10% FCS alone (closed squares) or plus 5 μ g/ml IgE (closed triangles), 40 ng/ml SF (open circles), or 30 ng/ml IL-3 (open triangles) and viable cells counted. Values are the mean $\pm$ SEM of eight determinations (duplicates for the four BMMC preparations). (B) BMMCs were incubated for 48 hr with 10% FCS alone (C) or plus 30 ng/ml IL-3 or 5 μ g/ml IgE, and the DNA was then extracted and subjected to agarose gel electrophoresis. 1 kb = 1 kb DNA ladder (GIBCO-BRL). Results are representative of two separate experiments. (C) BMMCs were cultured for 24 hr with 10% FCS alone (C) or plus 30 ng/ml IL-3 or 10 μ g/ml IgE, and total cell lysates were subjected to Western analysis with anti-Bcl-X_L and reprobing with anti-Shc antibodies to show equal loading. Results are representative of three separate experiments. (D) Representative FACS profiles of CFSE-stained BMMCs after 3 days of culture in 10% FCS alone (top panel), 30 ng/ml IL-3 (middle panel), or 10 μ g/ml IgE (bottom panel). The inset in the top panel shows [³H]thymidine incorporation after a 6 hr labeling on day 3, with 1 μ Ci/50,000 cell sample (2 Ci/mmol), and each point is the mean $\pm$ SEM of three determinations.

To gain some insight into the pathways involved in eliciting this robust production of inflammatory cytokines, BMMCs were stimulated with IgE alone or IgE + Ag for 3 hr $\pm$ the PI-3-kinase inhibitor LY294002, the MEK inhibitor PD98059, or the p38 inhibitor SB203580. As can be seen in Figure 4E, all three inhibitors blocked IL-6 production, suggesting that the PI-3-kinase, Erk, and p38 pathways all play a role in mediating IgE- as well as IgE + Ag-induced cytokine production/secretion. Of note, these inhibitors did not affect cell viability at the concentrations used, even after 24 hr of incubation with IgE-stimulated BMMCs (data not shown).

IgE Enhances Mast Cell Survival and Does So by Preventing Apoptosis

Since PKB, the Erks, and the cytokines IL-4, IL-6, and IL-13 have all been shown to enhance survival in some cell systems (Downward, 1998; Deng et al., 2000; Yanagida et al., 1995; Lai and Mosmann, 1999), we asked if IgE alone might be a prosurvival factor for BMMCs. As can be seen in Figure 5A, a 48 hr incubation of these cells with 5 μ g/ml IgE alone was as effective at preventing cell death as maximal levels of IL-3 or SF, two well-documented mast cell survival factors (Gommerman

and Berger, 1998). Similar results were obtained with a hybridoma supernatant from a different IgE (i.e., anti-erythropoietin IgE-26), at a final concentration of 10 μ g/ml (StemCell Technologies Inc., Vancouver, BC), but not from an equal volume of an IgG₁-producing hybridoma supernatant produced in the same medium (i.e., anti-glycophorin A at 20 μ g/ml; StemCell Technologies Inc.) (data not shown), ruling out the possibility that the purification procedure used in producing the commercial anti-DNP IgE altered its properties. Moreover, we observed equally potent IgE-induced survival of BMMCs from both C57B6 and Balb/C mice, indicating that our results are not mouse strain specific.

To determine if IgE was enhancing survival by preventing apoptosis, DNA was isolated from BMMCs following 48 hr of incubation $\pm$ 5 μ g/ml IgE or 30 ng/ml IL-3. As can be seen in Figure 5B, IgE alone was as effective as IL-3 at preventing DNA fragmentation. To gain some insight into the mechanism(s) underlying IgE's ability to prevent apoptosis, we compared the levels of the antiapoptotic protein Bcl-X_L in BMMCs, following 24 hr of incubation $\pm$ IgE or IL-3. As shown in Figure 5C, IgE, like IL-3, prevented Bcl-X_L levels from falling to that seen in starved cells. For this study, a 24 hr time

point was chosen to ensure that all the cells in the starved culture were still viable. An anti-Shc reprobing of the blot confirmed equal loading of the gel. The maintenance of this prosurvival Bcl-2 family member suggests at least one pathway by which IgE may be preventing BMMC death.

To determine if IgE was acting primarily as a survival factor or a mitogen, we carried out both [^{3} H]thymidine incorporation and CFSE vital dye (Glimm and Eaves, 1999) studies. As can be seen in the inset to Figure 5D, a 6 hr labeling of BMMCs, following 3 days with IgE, IL-3, or control medium, revealed that IL-3-containing cultures incorporated substantial levels of thymidine, while IgE-containing cultures did not. At this time, there were 4.0 (IgE), 1.8 (control), and 6.25 (IL-3) $\times 10^5$ viable BMMCs/ml. BMMCs were also stained with 5 μ M CFSE, flow cytometry sorted to obtain a homogeneous population of stained cells, and incubated for 3 days $\pm$ IL-3 or IgE. Similar to our [^{3} H]thymidine results, the CFSE results suggested that IgE is primarily a survival factor (Figure 5D).

Freshly prepared mIgE was also tested for its effect on BMMC survival. Dose response studies revealed that concentrations as low as 0.1 μ g/ml were capable of significantly enhancing survival (Figure 6A). IgE aggregates, on the other hand, at 0.5 μ g/ml, were found to be relatively poor enhancers of survival (i.e., approximately equal to 0.1 μ g/ml monomeric IgE; Figure 6A). This strongly suggested that, if there were low levels of contaminating IgE aggregates in our mIgE preparations, they were not playing a significant role in enhancing BMMC survival. Also of interest was our finding that mIgE did not have to synergize with factors in FCS to enhance survival, since similar survival enhancement was observed with medium containing 0.1% BSA instead of 10% FCS (data not shown). Interestingly, no enhancement in BMMC survival was observed when Ag was added to 5 μ g/ml mIgE (Figure 6B). However, it was conceivable that we were already at the maximal levels of survival attainable and so carried out mIgE dose response studies $\pm$ Ag and again found no enhancement of survival (Figure 6B).

The Autocrine Production of Cytokines Contributes to IgE-Induced BMMC Survival

To determine if cells cultured in the presence of mIgE were producing cytokines or other factors that contributed to the enhancement of BMMC survival, medium conditioned by a 3 day incubation of BMMCs with mIgE (10 μ g/ml) was treated with DNP-agarose beads to remove the IgE. This purified conditioned medium, which retained $\sim$ 0.5 μ g/ml of IgE (sample 3 in inset to Figure 7A), was then added to naive BMMCs and survival monitored. As can be seen in Figure 7A, this conditioned medium enhanced survival to the same degree as medium containing 10 μ g/ml IgE. Thus, IgE-stimulated cells were producing autocrine-acting factors that were sufficient to mediate the IgE-induced cell survival. This was also suggested by our finding that IgE-induced survival was cell density dependent (data not shown). Interestingly, if BMMCs were treated with 10 μ g/ml IgE for only 4 hr and the cell medium treated with or without DNP-agarose and added back to the cells, the survival was

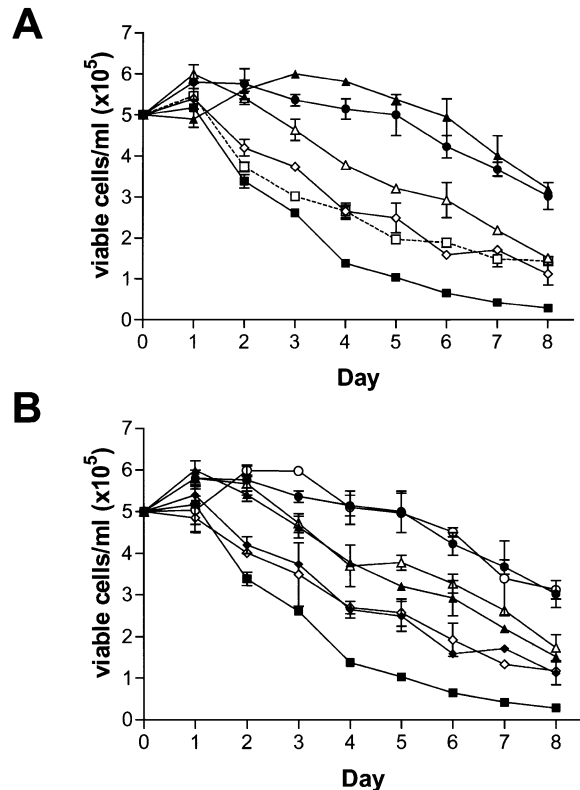


Figure 6. Monomeric IgE Enhances Mast Cell Survival

(A) BMMCs were cultured with 10% FCS alone (closed squares) or plus 0.1 (open diamonds), 1 (open triangles), 5 (closed circles), or 10 (closed triangles) μ g/ml mIgE or plus 0.5 μ g/ml IgE aggregates (open squares), and viable cells were counted on the days indicated. (B) BMMCs were cultured with 10% FCS alone (closed squares) or plus 0.1, 1, or 5 μ g/ml mIgE in the presence (open diamonds, open triangles, open circles) and absence (closed diamonds, closed triangles, closed circles) of 20 ng/ml DNP-HSA. Viable cell counts were determined on the days indicated. For (A) and (B), the values shown are the mean $\pm$ SEM of duplicate determinations, and similar results were obtained in two separate experiments.

similar (data not shown). This suggests that exposure to IgE for 4 hr is sufficient to enhance survival.

Finally, to see if the autocrine-acting factors could be the cytokines produced in response to IgE, we added a combination of cytokines (i.e., IL-2, IL-3, IL-4, IL-6, IL-13, and TNF α) to BMMCs cultured in the absence (Figure 7B) or presence (Figure 7C) of 0.5 μ g/ml IgE. The cytokine concentrations used were based either on levels in conditioned media from 3 day IgE-stimulated BMMCs cultures (for IL-3, IL-6, and TNF α , based on ELISAs) or from extrapolating RPA results (for IL-2, IL-4, and IL-13). As can be seen in Figure 7B, these cytokines significantly enhanced BMMC survival, although to a lesser degree than that achieved with 10 μ g/ml IgE (survival in the presence of 0.5 μ g/ml IgE alone is shown as a dashed line to compare to Figure 7C). However, as shown in Figure 7C, the combination of these cytokines with 0.5 μ g/ml IgE yielded a survival equal to that obtained with 10 μ g/ml IgE, at least for the first 3 days of culture. The subsequent drop in survival could be due to the omission of either one or more secreted cytokines or certain noncytokine secreted factors.

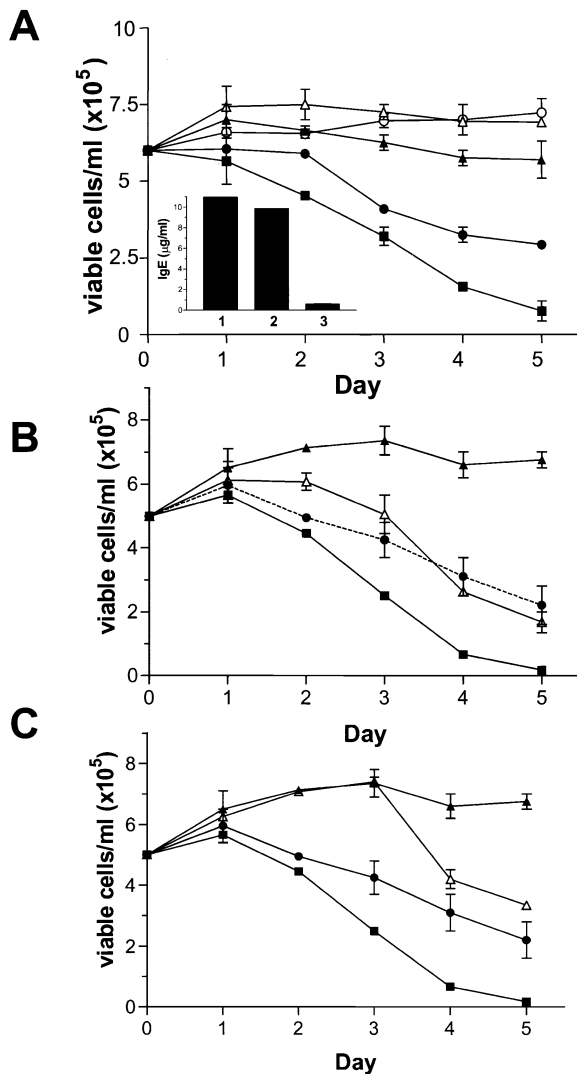


Figure 7. IgE-Induced Cytokines Act in an Autocrine Manner to Enhance BMMC Survival

(A) BMMCs were cultured with 10% FCS alone (closed squares) or plus 0.5 μg/ml mIgE (closed circles), 10 μg/ml mIgE (closed triangles), 3 day conditioned medium (open triangles), or 3 day conditioned medium treated with DNP-agarose to reduce IgE levels to 0.5 μg/ml (open circles), and viable cells were counted on the days indicated. The inset in (A) shows IgE levels in fresh medium containing 10 μg/ml IgE (1) and in this same medium following 3 days of incubation with BMMCs before (2) and after (3) treatment with DNP-agarose. (B) BMMCs were cultured with 10% FCS alone (closed squares) or plus 0.5 μg/ml mIgE (closed circles), 10 μg/ml mIgE (closed triangles), or a combination of 500 pg/ml IL-2, 10 pg/ml IL-3, 500 pg/ml IL-4, 5 ng/ml IL-6, 5 ng/ml IL-13, and 2.5 ng/ml TNFα (open triangles), and viable cells were counted on the days indicated. (C) BMMCs were cultured with 10% FCS alone (closed squares) or plus 0.5 μg/ml mIgE (closed circles), 10 μg/ml mIgE (closed triangles), or a combination of the same cytokines as in (C) plus 0.5 μg/ml mIgE (open triangles), and viable cells were counted on the days indicated. Values for (A), (B), and (C) are the mean ± SEM of duplicate determinations, and similar results were obtained in two separate experiments.

Discussion

We demonstrate herein that IgE alone triggers multiple signaling pathways and a more robust secretion of cyto-

kines than IgE + Ag in primary murine BMMCs. Moreover, we show that IgE alone enhances the survival of these BMMCs and does so, at least in part, via the secretion of autocrine-acting cytokines. This challenges, to some extent, the current paradigm, which states that binding of IgE to FcεRI is a passive presensitization step that does not lead to intracellular signaling unless receptors are subsequently crosslinked (Beaven and Metzger, 1993). Our findings are not totally unprecedented, however, since earlier studies have shown that IgE alone increases FcεRI expression both in vitro and in vivo in mouse and human mast cells (Hsu and MacGlashan, 1996; MacGlashan et al., 1997; Yamaguchi et al., 1997, 1999), and this upregulation consists of an initial protection from internalization/degradation phase and a subsequent cyclohexamide-sensitive upregulation phase (Yamaguchi et al., 1997). Importantly, our results are not totally in conflict with the current paradigm, since they are still consistent with signaling being mediated via aggregation of FcεRI. Related to this, while our studies with 2.4G2 and lactose strongly suggest that mIgE is initiating signaling via FcεRI, future experiments with BMMCs from FcεRIα-deficient mice will be required to confirm this.

To address the major question of how IgE alone is stimulating BMMCs, it is highly unlikely that the aggregates in the commercial IgE anti-DNP preparations are responsible, since they are very poor stimulators of Erk phosphorylation, IL-6 production, and survival. Even if trace amounts of active aggregates are present in our mIgE preparations, more should be present in our aggregate preparations, and yet we always observe far less activity in them. Given the extensive evidence in the literature, we favor a model in which mIgE activates BMMCs by triggering FcεRI aggregation. The only question is whether mIgE does this by first aggregating in solution within the BMMC milieu, perhaps in response to cell-secreted factors, and then binding to the FcεRI or by binding to the FcεRI first and then triggering aggregation. Against the former, we do not see any detectable aggregation upon storage of mIgE in PBS or in medium containing 0.1% BSA (the medium in which we stimulate BMMCs with mIgE), and we do not see any increased activity when we preincubate mIgE with BMMCs (data not shown). We thus favor a model in which mIgE binds to the FcεRI and reduces, to a modest extent, given its EC₅₀ of 1 μg/ml, an inherent repulsion between neighboring receptors, allowing them to slowly form small clusters in lipid rafts and trigger a low but prolonged signaling of various intracellular pathways. Relevant to the concept of IgE reducing an inherent repulsion between neighboring FcεRI, we found in our survival studies with various IgE-producing hybridomas that the supernatants from three different IgE-anti-DNP hybridomas were more potent (based on IgE ELISAs) than those of five other IgE-producing hybridomas. On the other hand, one IgE to an as yet unknown epitope was even more potent than the IgEs against DNP. This suggests perhaps that the charge/conformation within the Fab hypervariable region of IgE influences its ability to aggregate FcεRI. Related to this, IgE-anti-Epo26 was significantly less potent than IgE-anti-DNP at inducing IL-6 secretion, and this correlated with a reduced ability to enhance survival. Interestingly, adding DNP-lysine to BMMCs 1 hr after the addition of mIgE-anti-DNP abruptly halted IL-6 se-

cretion. Addition of DNP-lysine to IgE-anti-Epo, on the other hand, had no effect, ruling out nonspecific inhibition. This is consistent with a model in which the binding of DNP-lysine to IgE abrogates the ability of IgE to reduce the inherent repulsion between neighboring IgE receptors and both prevents the formation of and disrupts IgE/FcεRI cell surface aggregates. This is both compatible with and sheds new light on early studies in which DNP-lysine was used to disrupt signaling initiated by DNP-albumin-induced aggregates (e.g., Kawakami et al., 1992). We also found that if we washed mIgE-treated BMMCs free of mIgE after 1 hr and then added DNP-lysine to the cells, it still abruptly halted IL-6 secretion. This suggests that DNP-lysine is disrupting aggregates at the cell surface rather than in solution, and this effect is consistent with IgE triggering signaling via receptor aggregation.

As to why IgE alone stimulates cytokine secretion to a much greater extent than IgE + Ag but is incapable of triggering degranulation or LTC₄ production, it is possible that intensity and kinetic differences in signaling are responsible. Thus, while we cannot rule out qualitative differences, it may not be necessary to invoke them. IgE + Ag triggers a much more rapid and robust tyrosine phosphorylation of the receptor β subunit and more rapid Erk phosphorylation, and this higher signaling intensity early on may allow several signals to converge with sufficient intensity to surpass the threshold for degranulation and leukotriene production. The weaker, longer lasting signal triggered by IgE alone, due perhaps to the relatively unsynchronized, slow on rate of IgE (Kulczycki and Metzger, 1974), the possible requirement for a sufficient saturation of receptors to facilitate IgE-IgE aggregation, and/or the far slower internalization rate of uncrosslinked IgE/FcεRI (Mao et al., 1993) may play a pivotal role in allowing cytokines to be actively secreted from BMMCs. Related to this, Kawakami et al. (2000) recently proposed that the enhanced secretion of TNFα and IL-2 they observe in IgE + Ag-stimulated Lyn-deficient BMMCs might be due to a prolonged activation of the Erks and JNK.

As to how the prolonged signals we observed with IgE alone might lead to maintenance of Bcl-X_L and more cytokine production, the Erks have been shown to play an antiapoptotic role in some cell types (e.g., Deng et al., 2000), and PKB is a well-established prosurvival kinase (Downward, 1998). PKB activation leads, among other things, to the activation of NFAT, which is a well-known activator of cytokine gene expression in mast cells (Kitaura et al., 2000), and NFκB (Ozes et al., 1999), which upregulates the synthesis of many cytokines, including IL-6 (Kitaura et al., 2000; Marquardt and Walker, 2000), and directly upregulates the antiapoptotic protein Bcl-X_L (Khoshnaw et al., 2000). As well, both p38 and JNK have been shown to enhance inflammatory cytokine synthesis (Kozawa et al., 1999), the latter via upregulating c-Jun (Kitaura et al., 2000). Related to this, our finding that IgE-induced IL-6 secretion is inhibited by LY294002, PD98059, and SB203580 implicates the PI-3-kinase/PKB, ERK, and p38 pathways in this process.

Cytokine production has also been shown to be regulated in mast cells, at the level of secretion, and the prolonged signaling induced by IgE may preferentially enhance this step as well. Specifically, Baumgartner et al. (1994) found there is an Ag-induced calcium- and

PKC-dependent secretion step, independent of protein synthesis, that determines the release of TNF from RBL-2H3 cells. It is thus possible that IgE alone, perhaps because of its prolonged signaling, stimulates the release of IL-6 for a longer period of time than IgE + Ag. Related to this, these authors found that the addition of DNP-lysine 1 hr after the addition of DNP-BSA, to disaggregate the residual cell surface complexes, immediately halted secretion (and promoted intracellular degradation of TNF). This suggests that a continuous Ag-induced signal is required to maintain secretion. As to the identity of the cytokine(s) that enhances IgE-induced BMMC survival, we assessed the level of SF in medium conditioned by 3 days with IgE, since de Paulis et al. (1999) showed that SF is released from human mast cells. However, it was below the limit of detection of the ELISA (less than 250 pg/ml), and levels of neutralizing anti-SF that could inhibit 30 ng/ml of SF from enhancing BMMC survival did not reduce the survival effects of IgE. Thus, SF does not appear to be an important player in IgE-induced BMMC survival.

An important outstanding issue, of course, is whether IgE alone enhances mast cell survival in mice. In this regard, it has been known for some time that serum IgE levels are elevated in patients with allergic disorders or parasitic infections (Bennich and Johansson, 1970) and correlate with both disease severity and protective immunity to parasites (Corry and Kheradmand, 1999). This can be explained, at least in part, by the finding that mast cells undergo IgE-dependent FcεRI upregulation, and this enables the cells to bind more IgE and thus be activated to release mediators with lower concentrations of a given antigen (Galli, 2000; Kinet, 1999; Yamaguchi et al., 1997, 1999). Our results suggest that elevated levels of IgE may also be stimulating the release of inflammatory cytokines. While this cytokine release may not lead, in the absence of degranulation and leukotriene production, to an inflammatory response by itself *in vivo*, it may contribute to a subsequent, Ag-initiated inflammatory response. As well, since the concentration of IgE that promotes survival in our BMMC assay (0.1–10 μg/ml) is present in mouse and human sera exposed to parasites and allergens (Bennich and Johansson, 1970), it may be responsible, at least in part, for the elevated numbers of mast cells observed under these circumstances.

Experimental Procedures

Mast Cell Isolation

Bone marrow cells aspirated from 4- to 8-week-old C57B6 mice were derived as described previously (Huber et al., 1998a). By 8 weeks in culture, greater than 99% of the cells were c-kit and FcεRI positive, as assessed by fluorescein isothiocyanate (FITC)-labeled anti-c-kit antibodies (PharMingen, Mississauga, Canada) and FITC-labeled IgE (anti-erythropoietin 26), respectively (Huber et al., 1998a, 1998b).

BMMC Stimulation, Immunoprecipitation, and Western Blotting

To treat BMMCs ± IgE alone, the cells were starved (i.e., incubated without IL-3) for 4 hr at 37°C in IMDM + 10% FCS + 150 μM MTG and then washed three times with IMDM + 0.1% BSA and resuspended in IMDM + 0.1% BSA for 5 min at 37°C before adding 5–20 μg/ml anti-DNP IgE (i.e., monoclonal anti-dinitrophenyl [DNP] clone SPE-7 IgE [Sigma]) for the indicated times. To stimulate with IgE + Ag, BMMCs were preloaded with 5 μg/ml IgE for 4 hr in IMDM + 10% FCS + 150 μM MTG, washed three times to remove unbound

IgE, and then resuspended in IMDM + 0.1% BSA for 5 min at 37°C before adding 20 ng/ml DNP-HSA (30–40 moles DNP/mole human serum albumin; Sigma) for the indicated times. The cells were then washed with 4°C PBS and solubilized either by boiling for 2 min with SDS-sample buffer (using 5×10^5 BMMCs/sample for total cell lysates) or with 1% TX100 (using 10×10^6 BMMCs/sample) in 1 ml of 4°C phosphorylation solubilization buffer (Liu et al., 1994) and subjected to immunoprecipitation and Western blotting as described (Liu et al., 1994). Polyclonal antibodies against the phospho-S473 and phospho-T308 forms of PKB, PKB, the phospho-T180/Y182 p38, and the phospho-T202/Y204 Erk 1/2 were from New England BioLabs, Inc. (Mississauga, Canada). Polyclonal antibodies against Bcl-X_L and Shc were from Transduction Laboratories (Lexington, KY). The anti-Erk1 antibody (Erk-1-CT) was a gift from Dr. S. Pelech (University of British Columbia). 4G10 beads used for immunoprecipitation were from Upstate Biotechnology (UBI, Lake Placid, NY).

Degranulation and Leukotriene Assays

IgE- or IgE + Ag-induced degranulation was measured as described (Huber et al., 1998a). LTC₄ levels were analyzed with an enzyme immunoassay (EIA), according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

RNase Protection Assay and ELISAs

For mRNA analysis, BMMCs were stimulated as above and RNA isolated using TRIzol Reagent (GIBCO-BRL, Burlington, Canada). Cytokine mRNA levels were quantitated using a Riboquant™ Multi-Probe RNase Protection Assay (RPA) (PharMingen) according to the manufacturer's instructions, using [³²P]dUTP (NEG-307H from NEN Life Science Products Inc, Boston, MA). Quantitation of cytokine mRNA levels, using a phosphorimager (Storm 860, Molecular Dynamics), was standardized based on levels of the housekeeping gene *GAPDH*. Mouse IL-3, IL-6, TNF α , and IgE ELISAs (BD Pharmingen, San Diego, CA) and IL-13 and SF ELISAs (R&D Systems, Minneapolis, MN) were performed according to the manufacturer's instructions.

Preparation of Monomeric IgE

Commercial preparations of clone SPE-7 anti-DNP IgE (Sigma) were fractionated using a Waters HPLC system with a BioSep SEC S3000 gel filtration column (300 $\times$ 7.8 mm; Phenomenex, Torrance, CA) equilibrated and run with PBS (10 mM K phosphate, 0.14 M NaCl [pH 7.4]). Fractions (0.5 ml) were collected and monomer and aggregate peaks, based on OD280 readings, pooled separately. Freshly prepared mIgE and aggregates were used in all studies.

Survival Studies

BMMCs were washed with IMDM and incubated at 5×10^5 cells/ml in IMDM + 10% FCS or 0.1% BSA, $\pm$ IL-3, SF, or IgE, as indicated, in Falcon 3047 24-well flat-bottom plates (total volume 0.2 ml/well). Viability was assessed by trypan blue exclusion. The murine cytokines, IL-2, IL-4, and IL-13 were from R&D Systems; IL-3 and IL-6 were from StemCell Technologies; IL-4 was a generous gift from Dr. K. Humphries; and TNF α was from Peprotech (Rocky Hill, NJ).

CFSE Studies

BMMCs (5×10^6 /ml) were stained with 5 μ M CFSE for 10 min at 37°C, as described by Glimm and Eaves (1999), washed with 4°C HBSS, and resuspended at 5×10^5 BMMCs/ml in IMDM + 10% FCS for 5 hr at 37°C. A 20 channel gate from an FL-1 histogram was collected on a FACStar Plus cell sorter (Becton Dickinson, San Jose, CA) and the collected cells set up in survival assays and analyzed daily by FACS.

Apoptosis Assays

Apoptotic DNA fragments were visualized as described by Herrmann et al. (1994). In brief, 5×10^6 BMMCs were washed with IMDM and cultured for 48 hr in IMDM + 10% FCS $\pm$ IL-3 or IgE. Cells were lysed in 1% NP40 in 20 mM EDTA, 50 mM Tris-Cl (pH 7.4). Lysates were brought to 1% SDS and digested at 37°C for 3 hr with RNase A (5 μ g/ μ l), followed by 12 hr with Proteinase K (2.5 μ g/ μ l). Ammonium

acetate (10 M) precipitated DNA was separated by electrophoresis on a 1% agarose gel.

Removal of IgE from BMMC-Conditioned Medium

DNP-beads were made by coupling 2,4-dinitrobenzenesulfonic acid (Aldrich, Oakville, ON) to hydrazide CarboLink™ Coupling Gel (Pierce, Rockford, IL) as described in Inman and Dintzis (1969). In brief, 1 ml of gel was washed in saturated Na₂B₄O₇·10H₂O (pH 9.3) and incubated at 23°C for 1 hr with 2.5 ml of 20 mg/ml 2,4-dinitrobenzenesulfonic acid in Na₂B₄O₇·10H₂O. DNP-bead preparations were then washed consecutively with 0.1 N acetic acid, water, and 0.1 N NH₄OH, followed by storage in PBS + 0.02% azide (Truffa-Bachi and Wofsy, 1970). IgE was depleted from 3 day conditioned media by two consecutive 1 hr incubations at 23°C, with 100 μ l DNP-beads/ml conditioned media.

Acknowledgments

We would like to thank Christine Kelly for typing the manuscript. This work was supported by the National Cancer Institute of Canada (NCI-C) and the Medical Research Council of Canada (MRC-C), with core support from the British Columbia Cancer Foundation and the British Columbia Cancer Agency. J.K. holds a Natural Sciences and Engineering Research Council of Canada (NSERC) studentship. G.K. is a Terry Fox Cancer Research Scientist of the NCI-C, supported by funds from the Canadian Cancer Society and the Terry Fox Run.

Received October 13, 2000; revised May 16, 2001.

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