IgE- and IgE/Antigen-Mediated Mast Cell Migration

in an Autocrine/Paracrine Fashion

Running Title: IgE- and IgE/Antigen-Mediated Mast Cell Migration

By

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Abstract

Mast cells are the major effector cells for immediate hypersensitivity and chronic allergic reactions. These cells accumulate in mucosal tissues of allergic reactions, where IgE is produced locally. Here we provide evidence that, in addition to antigen that can attract IgE-bound mast cells, the type of IgE molecules that can efficiently activate mast cells can promote the migration of mast cells in the absence of antigen. IgE- and IgE/antigen-mediated migration involves an autocrine/paracrine secretion of soluble factors including adenosine, leukotriene B4, and several chemokines. Their secretion depends on two tyrosine kinases, Lyn and Syk, and they are agonists of G-protein coupled receptors and signal through phosphatidylinositol 3-kinase γ, leading to mast cell migration. In mouse experiments, naïve mast cells are attracted to IgE and IgE-sensitized mast cells are attracted to antigen. Therefore, IgE and antigen are implicated in mast cell accumulation at allergic tissue sites with local high IgE levels.
Introduction

Mast cells are the major effector cell type in IgE-mediated immediate hypersensitivity, chronic allergic diseases and the defense against certain parasites and bacteria. Traditionally, it is thought that mast cells bound to antigen-specific IgE via the high-affinity receptor (FcεRI) encounter multivalent antigen (the stimulation mode hereafter termed IgE+Ag), and then IgE-bound receptors are aggregated, leading to cellular activation.

Activated mast cells secrete preformed and newly synthesized proinflammatory mediators, such as histamine, proteases, lipids, cytokines and chemokines.

The mouse FcεRI consists of an IgE-binding α subunit, a β subunit and two molecules of signal-generating γ subunit. FcεRI aggregation leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the β and γ subunits by Lyn, and recruitment of Syk to the tyrosine-phosphorylated ITAMs of the γ subunit results in the activation of this protein-tyrosine kinase (PTK).

Activated Lyn and Syk as well as Fyn eventually lead to the activation of multiple signaling pathways including phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ/Ca^{2+}/protein kinase C (PKC), and mitogen-activated protein (MAP) kinases.

Cell-extracellular matrix interactions mediated by integrins play a critical role in multiple cellular functions including cell adhesion and migration. Activation of mast cells by FcεRI aggregation and stem cell factor (SCF) induces adhesion to fibronectin (FN) predominantly via integrin α5β1. Upon FcεRI aggregation and SCF stimulation, FN-adherent cells exhibit stronger effector functions such as histamine release and cytokine production than non-adherent cells.
We and others recently demonstrated that monomeric IgE can promote mast cell survival \(^9,10\). This observation, together with earlier studies showing that IgE in the absence of antigen can increase the surface expression of FcεRI \(^11-13\), has transformed the traditional view of IgE-mast cell binding as a “sensitization” step prior to receptor aggregation with antigen or other crosslinking reagents into a new one that monomeric IgE can induce survival and “activation” of mast cells \(^14\). We also found that IgE molecules display heterogeneity in that different IgE molecules induce varied levels of activation; at one extreme end of spectrum, some IgE molecules, termed highly cytokinergic (HC) IgEs, induce the production and secretion of various cytokines and other activation events including degranulation, whereas other IgE molecules, termed poorly cytokinergic (PC), do so very inefficiently \(^15\).

Mast cells accumulate at local inflammatory mucosal tissues, as seen in allergic rhinitis and asthma. Interestingly, class switch recombination and somatic hypermutation of the immunoglobulin gene and eventually IgE synthesis and secretion occur at such inflammatory mucosae \(^16-21\). In an allergic individual, local IgE production persists for a long period in the absence of allergen \(^20\). A variety of biological agents, including growth factors (e.g., SCF), chemokines (MCP-1/CCL2, MIP-1\(\alpha\)/CCL3, RANTES/CCL5, eotaxin/CCL11, SDF-1\(\alpha\)/CXCL12, etc. refer to \(^22\) for the nomenclature of chemokines), and adenosine nucleotides, are known to attract rodent mast cells \(^23-27\). Consistent with effects of the chemokines on mast cell migration, mast cells express appropriate receptors including CCR1-5, CXCR1-2 and CXCR4 \(^28-32\). Antigen can also cause the migration of IgE-sensitized mast cells, which can be suppressed by inhibitors of Rho-kinase/ROCK and p38 \(^33\). In this study, we have found that, in addition to IgE+Ag, HC IgEs can attract
mast cells. HC IgE- and IgE+Ag-induced mast cell migration involves autocrine/paracrine secretion of soluble factors. The initial phase of migration leading to the release of such factors depends on Lyn and Syk, and the following phase downstream of G-protein coupled receptor (GPCR) stimulation by these soluble factors requires PI3Kγ. In vivo mouse experiments also suggest that mast cells can be attracted to HC IgEs as well as antigen. Therefore, this study provides a novel mechanism for mast cell accumulation to allergic inflammatory sites.

Methods

Antibodies and Other Reagents
IgE antibodies used were described previously. Anti-TNP IgG and antibodies against integrins were purchased from BD Biosciences Pharmingen; MIP-1α, MCP-1, and RANTES from R&D Systems; fibronectin, vitronectin, laminin, collagen type IV, DNP-lysine, adenosine, histamine, serotonin, and MRS 1523 from Sigma; PP2, piceatannol, wortmannin, LY294002, PD98059, SP600125, Gö-6976, PKI, KT5720, Y-27632, cytochalasin D, EGTA and leukotriene B4 (LTB4) from EMD Biosciences; N-oleoyl dopamine, U-75302, and LY2552833 from Cayman Chemical. SCF and ER-27319 were gifts from Kirin Brewery Co. (Tokyo, Japan) and Eisai (Tokyo, Japan), respectively. Terreic acid was described previously.

Cells and Stimulation
Bone marrow cells from wild-type and mutant mice were cultured in IL-3-containing medium for 4-6 weeks to generate mast cells (BMMC) with >95% purity (c-Kit+ FcεRI+ by flow cytometry). The following mutant mice were used: FcεRIα−/−, lyn−/−, fyn−/−, hck−/−, syk−/−, btk−/−, PKCβ−/−, PKCδ−/−, PKCθ−/−, and PI3Kγ−/−. For IgE+Ag stimulation, BMMC were sensitized by overnight incubation
with 0.5 μg/ml of H1-ε-DNP-206 (206) IgE. BMMC washed twice with buffer were used for migration assays. Animal studies were approved by the IRB of La Jolla Institute for Allergy and Immunology.

**In Vitro Migration Assay**

BMMC migration for 8 h was assayed using 24-well Transwell chambers (Corning) separated by 5-μm polycarbonate filters, the lower surface of which had been coated overnight with 20 μg/ml fibronectin and followed by blocking with 4% BSA in PBS at 37°C for 1 h, unless otherwise described. Under standard conditions, upper wells contained 10^6 cells/well in 0.2 ml medium consisting of RPMI/1% BSA/20 mM HEPES (pH 7.4) and lower wells contain 0.6 ml medium. Cells migrated into lower wells were counted using a hemocytometer. For inhibition with antibodies, BMMC were first preincubated with neutralizing antibodies (20-50 μg/ml) for 30 min, and then placed in upper wells. Both upper and lower wells contained neutralizing antibodies.

In some experiments, BMMC were prestained with 10 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probe) in 0.1% BSA/PBS at 37°C for 10 min before loading onto upper wells. Cells migrated into lower wells were quantified using flow cytometry.

**In Vivo Mast Cell Accumulation**

Gauze strips (~7 layers, 10 x 15 mm²) spotted with 50 μl of 10 μg/ml of SPE-7 IgE or PBS were applied to the shaved back skin of naïve NC/Nga mice for 24 h. This portion of the back skin was occluded with Tegaderm™ Transparent Dressing (3M) and BAND-AID®. Mice were sacrificed and skin regions were prepared to stain mast cells with toluidine blue. Mast cell numbers were counted under a microscope. In another type of in vivo experiment, mice were first treated similarly with 100 μl of 10 μg/ml anti-DNP (206) IgE or PBS into the back skin, and
one day later the same area was applied with 50 µl of 100 ng/ml DNP-HSA or PBS for 24 h. For mast cell quantification, dorsal skin samples were fixed in 10% formaldehyde, paraffin embedded and cut into 6 µm sections. Deparaffinized sections were stained with toluidine blue (pH 4.0) and analyzed by light microscopy. Cells between epithelium and panniculus carnosus were counted at a magnification of x400.

Data Supplements

Four figures are provided as supplemental materials: effects of IgE/antigen and IgG/antigen complexes on mast cell migration (Fig. S1), IgE- and IgE+Ag-induced migration of mouse mast cell lines (Fig. S2), requirements of fibronectin, vitronectin or laminin (Fig. S3), and autocrine/paracrine mechanism of mast cell migration (Fig. S4).

Results

HC IgEs Can Induce Mast Cell Migration

Given the ability of IgEs to induce mast cell activation, we examined whether IgEs can promote the migration of mouse bone marrow-derived mast cells (BMMC) in the absence of antigen. As shown in Fig. 1A, three HC IgEs in a lower well induced vigorous migration of BMMC from an FN-coated upper well after 8 h of incubation in a transwell assay, whereas typical PC IgEs such as H1-ε-DNP-206 (206) and IgE-3 did not. Dose-response experiments (Fig. 1B) indicated that the extent of migration induced by a typical HC IgE (SPE-7) increases in a dose-dependent manner up to 30 µg/ml. A significant migration was observed at 1 µg/ml IgE, a concentration reachable in a subset of atopic conditions. Kinetic experiments (Fig. 1C) showed that HC IgE-induced migration is smaller in magnitude than that induced by SCF and plateaus within 10-15 h. Consistent
HC IgE-Induced Migration Signals Through the FcεRI and Integrins

We next confirmed that IgE-induced migration is mediated through FcεRI. BMMC from \textit{FcεRIα}−/− mice \(^{38}\) failed to migrate in response to HC IgEs or IgE+Ag, whereas the mutant cells were attracted to SCF as vigorously as wild-type cells (\textbf{Fig. 1D}). Consistent with the notion that these IgE-dependent migrations are triggered by FcεRI aggregation, these migrations were inhibited by monovalent hapten, DNP-lysine (\textit{Table 1}). Further, BMMC migration was induced by incubation with PC IgE plus DNP-HSA, conditions which mimic acute allergic situations in that antigens are present around IgE-bound mast cells and are different from IgE+Ag in that unbound IgE molecules were removed (IgE+Ag) or not (PC IgE plus DNP-HSA) (Data Supplements, \textbf{Fig. S1A} online). By contrast, BMMC migration was not induced by IgG, IgM (unpublished data), or anti-TNP IgG plus TNP-BSA (\textbf{Fig. S1B} online). Efficient HC IgE-dependent migration was seen with BMMC and mouse mast cell lines (MC/9 and MCP-5) (\textbf{Fig. S2} online) incubated in upper wells coated with FN, vitronectin or laminin (\textbf{Fig. S3} online). However, collagen or BSA coating did not promote BMMC migration efficiently. These results implicated the involvement of integrins in HC IgE- and IgE+Ag-mediated mast cell migration. This was confirmed by the strong inhibition of migration with a neutralizing antibody against integrin β1. β2 and β7 integrins may play a minor, if any, role in IgE-dependent mast cell migration (\textbf{Fig. 1E}).
Comparison between HC IgE- and Chemokine-Induced Migration

Several chemokines are known to attract mast cells \(^{26,39,40}\). Remarkably, migration induced by 10 µg/ml SPE-7 IgE was more pronounced than that by mast cell-attracting chemokines, RANTES, MCP-1 and MIP-1\(\alpha\) (Fig. 2A). When used in combinations, HC IgEs did not synergize to induce mast cell migration with SCF or any of the tested chemokines, and HC IgEs determined the extent of migration (Fig. 2B & C), suggesting a hierarchically dominant role for HC IgEs in complex situations where various chemoattractants are present. Signaling hierarchy was well known among several chemoattractants of neutrophils \(^{41}\). An alternative possibility is that the mediators released by HC IgE-activated mast cells, e.g., proteases, inactivate SCF, chemokines or their receptors. Unlike SCF or the chemokines, which induce the movement of mast cells towards the increasing gradient of chemoattractant concentrations (chemotaxis), checkerboard analysis indicated that HC IgEs induce a mixture of directional and non-directional (chemokinesis) movements (Fig. 2 D & E).

Signaling Requirements for HC IgE- and IgE+Ag-Induced Migration

FcεRI stimulation by IgE+Ag triggers activation of non-receptor PTKs of Src, Syk and Tec families and several signaling pathways \(^{3,4,14}\). Pharmacological inhibition suggested that HC IgE- and IgE+Ag-induced migration requires Src and Syk kinases, but not Tec kinases (Table 1). Consistent with these data, IgE-dependent migration was abrogated in syk\(^{-/-}\) cells (Fig. 3B), but not affected by Btk deficiency (Fig. 3C). Among the tested Src PTKs, Lyn and Fyn played major and minor roles, respectively, in IgE- and IgE+Ag-
dependent migration whereas Hck played no part in this function (Fig. 3A). These pharmacological experiments (Table 1) and previous genetic and biochemical experiments\textsuperscript{10,15} indicate that signaling events induced by HC IgEs are almost the same as those by IgE+Ag. Downstream of these kinases, PI3K and some MAP kinases (p38 and JNK) appeared essential from pharmacological experiments. A general inhibitor (Ro31-8425) of PKC, an inhibitor (Gö-6976) of Ca\textsuperscript{2+}-dependent PKC isoforms and a Ca\textsuperscript{2+} chelator (EGTA) all inhibited IgE-dependent migration while inhibitors of protein kinase A or Rho-kinase did not. Consistent with these results, deficiency of PKC\textbeta, PKC\textepsilon, or PKC\texttheta modestly reduced IgE-dependent migration (Fig. 3D).

Involvement of Autocrine/Paracrine Soluble Factors, GPCRs, and PI3K\gamma in HC

**IgE- and IgE+Ag-Induced Mast Cell Migration**

To test potential involvement of autocrine/paracrine secretion in migration, we examined whether \textit{Fc\varepsilon RI\alpha\textminus} mast cells that cannot directly respond to IgE can migrate in the presence of wild-type cells. A significant proportion of CFSE-labeled \textit{Fc\varepsilon RI\alpha\textminus} mast cells mixed at a ratio of 1:1 with non-labeled wild-type cells in upper wells migrated to lower wells in response to HC IgE and IgE+Ag (Fig. S4 online). Further, \textit{Fc\varepsilon RI\alpha\textminus} cells in upper wells were attracted to lower wells that contained wild-type cells in the presence of HC IgE or anti-DNP IgE-sensitized wild-type cells in the presence of DNP-HSA (Fig. 4A). More directly, supernatants of wild-type mast cells cultured with HC IgE or IgE+Ag for 0.5, 1, 2 or 6 h could attract \textit{Fc\varepsilon RI\alpha\textminus} mast cells with similar efficiency (Fig. 4B). These results strongly indicate that \textit{Fc\varepsilon RI\alpha\textminus} cells alone or in the mixed cultures
with wild-type cells migrated to lower wells that contained HC IgE- or IgE+Ag-induced soluble factors secreted from wild-type cells.

A variety of chemicals and peptides are released from IgE+Ag- and HC IgE-stimulated mast cells within 30 min and exert their functions on multiple cell types including mast cells themselves \(^{10,15,42}\). For example, histamine (and serotonin) mediates increased vascular permeability in passive cutaneous anaphylaxis, and LTB\(_4\) and sphingosine 1-phosphate induce the migration of various leukocytes \(^{43-46}\). Several chemokines such as MCP-1, MIP-1\(\alpha\), MIP-1\(\beta\)/CCL4, and MIP-2 are produced by BMMC stimulated weakly by IgE+Ag \(^{47}\), and MCP-1 secretion can be induced by fast dissociating antigens and characterized as an exception of the kinetic proofreading regimen \(^{48}\). Like mast cell-produced chemokines such as MCP-1 and MIP-1\(\alpha\) (Fig. 2), adenosine and LTB\(_4\) vigorously induced migration of BMMC while histamine, serotonin, or sphingosine 1-phosphate did not (Fig. 4C and unpublished data). Adenosine and LTB\(_4\) as well as chemokines are ligands of GPCRs. Consistent with this, migration of \(Fc\varepsilon R\alpha^-\) BMMC to supernatants of wild-type mast cells that had been cultured with HC IgE or IgE+Ag was blocked by \(\sim 60\%\) by pertussis toxin, indicating that some soluble factors utilize G\(\alpha\)-coupled receptors (Fig. 4D). The role of adenosine in the migration was confirmed by the inhibition with adenosine deaminase that converts adenosine to inosine and an adenosine A\(_3\) receptor inhibitor, MRS 1523 (Fig. 4D). The involvement of LTB\(_4\) was indicated by the inhibition with a 5-lipoxygenase inhibitor (N-oleoyl dopamine) and inhibitors of LTB\(_4\) receptors BLT\(_1\) (U-75302) and BLT\(_2\) (LY2552833) (Fig. 4E). Parenthetically, these inhibitors of LTB\(_4\) synthesis or receptors did not affect SCF-induced migration (unpublished data). Furthermore, roles of mast cell-produced
chemokines in the migration were shown by the inhibition by neutralizing antibodies. Antibodies to MIP-1α, MIP-1β, and RANTES individually inhibited weakly (10-25%) but in a combination inhibited HC IgE- and IgE+Ag-induced mast cell migration more strongly (~50%) (Fig. 4F). Adenosine, LTB₄ and chemokines are all agonists of GPCRs and signal through PI3Kγ⁴⁹. Strikingly, HC IgE- and IgE+Ag-induced mast cell migration was drastically reduced in PI3Kγ-deficient cells (Fig. 5A). Therefore, it appears that IgE- and IgE+Ag-dependent migration results from the signals originated from FcεRI aggregation that lead to a rapid release of multiple GPCR agonists including adenosine, LTB₄, and several chemokines. Thus, activation of the GPCRs and their downstream signaling molecule, PI3Kγ, is crucial for mast cell migration.

We next examined which step, FcεRI stimulation-induced release of GPCR agonists or downstream signaling from GPCRs, is dependent on Lyn and Syk. For this purpose, we tested whether CSFE-labeled FcεRIα⁻/⁻ BMMC in upper wells migrate to lower wells containing wild-type, lyn⁻/⁻ or syk⁻/⁻ BMMC in the presence of SPE-7 IgE or SCF. In the presence of SPE-7 IgE, wild-type BMMC attracted FcεRIα⁻/⁻ cells vigorously, but the migration of the latter cells was abrogated towards SPE-7 IgE-incubated lyn⁻/⁻ and syk⁻/⁻ cells, although FcεRIα⁻/⁻ cells migrated vigorously to SCF irrespective of the genotype of mast cells present in the lower wells (Fig. 5B). Similar results were obtained when CFSE-labeled FcεRIα⁻/⁻ cells in upper wells were cultured with lower wells containing 206 IgE-sensitized mutant cells in the presence of antigen or supernatants of 206 IgE-sensitized mutant cells that had been cultured in the presence of antigen (unpublished data). Therefore, these results indicate that Lyn and Syk are required for HC IgE- and IgE+Ag-induced release of soluble factors.
We next examined whether Lyn and Syk are required for signaling downstream of
GPCRs as well. For this purpose, we tested whether wild-type, lyn\(^{-/}\) and syk\(^{-/}\) BMMC in
upper wells are attracted to lower wells containing 206 IgE-sensitized wild-type cells in
the presence of antigen or supernatants of 206 IgE-sensitized wild-type cells cultured in
the presence of antigen. The mutant cells migrated as vigorously as wild-type cells under
these conditions (Fig. 5C and unpublished data). Furthermore, the mutant cells were also
attracted to adenosine, LTB\(_4\), MCP-1, and MIP-1\(\alpha\) as efficiently as wild-type cells
(unpublished data), consistent with our previous study showing that Syk is not required
for mast cell migratory responses to GPCR stimuli \(^{50}\). Therefore, we conclude that Lyn
and Syk are required for the initial phase of mast cell activation leading to the release of
GPCR agonists, but not for the late phase of GPCR activation and further downstream
events, in HC IgE- and IgE+Ag-induced migration.

**In Vivo Mouse Models for IgE- and IgE/Ag-Induced Mast Cell Accumulation**

To evaluate the pathophysiological relevance of our in vitro observations on IgE- and
IgE/Ag-induced mast cell migration, we performed in vivo mouse experiments. First, we
tested whether epicutaneous application of IgE on the back skin of naïve mice can attract
mast cells. Microscopic analysis of toluidine blue-stained samples showed that the local
density of mast cells in SPE-7 IgE-applied areas was significantly increased after 24 h
compared to that in PBS-applied areas while a modest increase was observed in 206 IgE-
treated areas (Fig. 6A & B). Most mast cells were localized in perivascular regions of
the dermis. SPE-7 IgE-applied areas contained \(\sim 10\%\) mast cells that had degranulated
while such degranulating mast cells were very rare in PBS- or 206 IgE-applied areas.
Another type of in vivo experiment was designed to test whether antigen can induce mast cell migration in vivo. Mice were epicutaneously treated with anti-DNP (206) IgE or PBS into the back skin, and one day later the same area was applied to with DNP-HSA or PBS. Microscopic analysis showed that mast cell numbers in IgE/antigen-applied areas are much higher after 24 h treatment than those in PBS/PBS, PBS/antigen- or IgE/PBS-applied areas (Fig. 6C). Similar to the effects of SPE-7 IgE on mast cell accumulation, antigen-treated areas had degranulating mast cells. Although these in vivo experiments do not reveal the mechanism of mast cell accumulation, the perivascular location of these cells suggest that mast cells or their precursors were recruited from the circulation rather than from nearby tissues. Alternatively, mast cells might have proliferated in situ, although this is less likely because of the short assay time and the low capacity of mast cells to proliferate in response to HC IgEs and IgE+Ag.

**Discussion**

This study provides evidence that HC IgEs in addition to IgE+Ag can promote the migration of mast cells. This migration is mediated mainly through integrin β1 and more potent than some chemokines, and involves two phases: an early phase of Lyn and Syk-dependent release of multiple soluble factors including adenosine, LTB₄, and chemokines, and a later phase of PI3Kγ-dependent signaling following the activation of GPCRs by these factors. These in vitro observations are consistent with in vivo mouse experiments in which mast cell accumulation was induced by epicutaneous application of HC IgEs and IgE+Ag.
The in vivo results suggest that HC IgEs and IgE+Ag can induce accumulation of mast cells without prior inflammation. However, it is conceivable that, in allergic individuals, some inflammatory reactions such as the infiltration of helper T cells have occurred when IgE synthesis in B cells takes places at mucosal sites in the nasal cavity and lung in response to antigen exposure \(^{16-21}\). Given the vast variety of proinflammatory mediators secreted from activated mast cells \(^{51}\), IgE- and IgE+Ag-induced mast cell accumulation would amplify inflammatory reactions by recruiting other cells such as T cells, eosinophils, monocytes, and neutrophils. For instance, histamine plays an important role in the pathogenesis of atopic asthma by enhancing the secretion of Th2 cytokines and inhibiting the production of Th1 cytokines \(^{52}\). LTB\(_4\) recruits T cells and myeloid cells \(^{43-45}\), and mast cell-produced cytokines and chemokines can recruit T cells, eosinophils, monocytes, and neutrophils. CC chemokine transcripts coding for I-309/CCL1, MIP-1\(\alpha\), MIP-1\(\beta\), and MCP-3/CCL7 are among the most dramatically enhanced ones in IgE+Ag-stimulated mast cells \(^{53}\). The ability of HC IgEs to attract mast cells suggests that this amplification of inflammation can last as long as local IgE synthesis continues even after the elimination of antigen. Overall, IgEs in the absence as well as presence of allergen are implicated in mast cell accumulation at allergic tissue sites with local high IgE levels.

This and previous studies \(^{10,15}\) have shown that intracellular signaling events induced by HC IgEs and IgE+Ag are very similar, if not identical: Src and Syk family PTKs are activated, intracellular Ca\(^{2+}\) concentrations are increased, and several serine/threonine kinases such as MAP kinases, PKCs, and Akt are also activated. These signaling events induced by Fc\(\varepsilon\)RI aggregation by either HC IgEs or IgE+Ag (Table 1
and 15) result in a variety of biological outcomes such as degranulation, histamine synthesis, leukotrienes release, receptor internalization, cytokine production, migration, and survival 9,10,15,54. Consistent with the similarities in signaling between HC IgE- and IgE+Ag-stimulated cells, migrations induced by these two modes of FcεRI stimulation require identical signaling molecules such as Lyn, Syk and PI3Kγ. Importantly, both migrations utilize soluble autocrine factors as a part of the migratory mechanism. Among numerous chemical and peptide agents rapidly secreted from activated mast cells, adenosine, LTB4 and several chemokines were identified as the mediators for mast cell migration. Unlike RBL-2H3 rat mast cells 55, sphingosine 1-phosphate did not substantially induce the migration of BMMC (Fig. 4C). As discussed above, these mediators influence an in vivo inflammatory process by recruiting not only mast cells but also other types of inflammatory cells. Assuming that PI3Kγ is involved only in signal transduction of GPCRs 49, this autocrine/paracrine mechanism seems essential for mast cell migration. Thus, only residual levels of migration were observed with PI3Kγ−/− mast cells (Fig. 5A). Notably, pertussis toxin inhibits HC IgE-, IgE+Ag-, and adenosine-induced migrations by ~60%. This indicates that these migrations are mediated mainly via Gαi. However, other Gα proteins or G protein-independent signals may also be involved; for example, integrins might supply additional signals for migration. In line with this possibility, Lyn is required for FN-mediated migration in RBL-2H3 cells 56. In any event, this study has extended the previous findings that soluble factors including adenosine are utilized to amplify Ca2+ and degranulation responses in IgE+Ag-stimulated mast cells 49,55. LTB4 or chemokines do not induce degranulation. It will be interesting to investigate whether other biological responses of mast cell activation depend on an
autocrine/paracrine mechanism and what molecules mediate such responses. Our knowledge on this mechanism will expand our choices of anti-inflammatory drugs beyond currently used anti-histamine and anti-leukotriene drugs that target this process.

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SUPPLEMENTAL MATERIAL IS AVAILABLE ONLINE AT THE TIME OF FINAL PUBLICATION ONLY.
References


31. Oliveira SH, Lukacs NW. Stem cell factor and IgE-stimulated murine mast cells produce chemokines (CCL2, CCL17, CCL22) and express chemokine receptors. Inflamm Res. 2001;50:168-174.


Table 1. Inhibition of BMMC migration induced by SPE-7 IgE, IgE+Ag or SCF by various pharmacological inhibitors

<table>
<thead>
<tr>
<th>Target</th>
<th>Inhibitor</th>
<th>SPE-7 IgE</th>
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<td>IgV</td>
<td>DNP-lysine</td>
<td>0.4 µM</td>
<td>0.4 µM</td>
<td>&gt;100 µM</td>
</tr>
</tbody>
</table>

*BMMC were preincubated with pharmacological reagents for 30 min before placed in upper wells at the time of addition of 10 µg/ml of SPE-7 IgE or 100 ng/ml of SCF to lower wells. For IgE+Ag stimulation, BMMC were sensitized by overnight incubation with 0.5 µg/ml of 206 IgE. BMMC washed with buffer were preincubated with inhibitors for 30 min before placed in upper wells at the time of addition of 10 ng/ml of DNP-HSA. Concentrations for 50% inhibition (IC₅₀) are indicated.

*Although BMMC were insensitive to Y-27632, MC/9 mouse mast cells were sensitive with IC₅₀s of 5-10 µM for SPE-7-, IgE+Ag- and SCF-induced migration.
Figure Legends

Figure 1. **HC IgEs and IgE+Ag induce the migration of BMMC through FceRI and β1 integrin.** (A) HC, but not PC, IgEs induce BMMC migration efficiently in an 8h transwell assay. BMMC, sensitized overnight with anti-DNP IgE (206) and washed, were also attracted to a lower well containing DNP-HSA. In comparison, SCF (100 ng/ml) was also tested in the same assay. Also shown are dose response (B) and time course (C) experiments using 10 µg/ml 206 IgE, 10 µg/ml SPE-7 IgE, 10 ng/ml DNP-HSA and 100 ng/ml SCF. (D) Migration assays were performed with FceRIα−/− and control BMMC. (E) Migration of wild-type BMMC was inhibited by neutralizing antibodies against integrins.

Figure 2. **Comparison between HC IgE- and chemokine-induced migration of BMMC and checkerboard analysis of HC IgE-induced mast cell migration.** (A) SPE-7 IgE-induced migration of BMMC was compared with those induced by mast cell-attracting chemokines, MIP-1α, MCP-1 and RANTES. (B & C) BMMC migration was induced by SPE-7 IgE in combination with chemokines or SCF. (D) BMMC in upper wells containing various concentrations of SPE-7 IgE were incubated with various concentrations of SPE-7 IgE in lower wells. (E) BMMC in upper wells containing various concentrations of SCF were incubated with various concentrations of SCF in lower wells.

Figure 3. **Effects of Src, Syk, Btk, and PKC deficiencies on IgE-induced migration.**
IgE- and IgE+Ag-induced migration assays were performed using Src family PTK-deficient (A), Syk-deficient (B), Btk-deficient (C), and PKC-deficient (D) BMMC.

**Figure 4. Involvement of adenosine, LTB₄, and chemokines in an autocrine/paracrine mechanism of IgE- and IgE+Ag-induced mast cell migration.**

(A) CFSE-labeled wild-type and FceRIα⁻ BMMC in upper wells were attracted to lower wells containing wild-type cells and SPE-7 or 206 IgE-sensitized wild-type cells and antigen. (B) Supernatants of wild-type BMMC incubated with SPE-7 for the indicated periods attracted FceRIα⁻ cells. Supernatants of PC IgE (206)-sensitized wild-type cells incubated with antigen for the indicated periods also attracted FceRIα⁻ cells. (C) Migration was induced by adenosine and LTB₄, but not sphingosine 1-phosphate (S1P) or histamine. (D) Pertussis toxin (PTX), adenosine deaminase (ADA), and adenosine A₃ receptor inhibitor MRS 1523 inhibit the migration of CFSE-labeled wild-type BMMC from upper wells to lower wells containing IgE-sensitized wild-type cells and antigen or adenosine. (E) 5-Lipoxygenase inhibitor (N-oleoyl dopamine, ODA), BLT₁ receptor inhibitor (U-75302), and BLT₂ inhibitor (LY2552833) inhibit the migration of CFSE-labeled wild-type BMMC from upper wells to lower wells containing IgE-sensitized wild-type cells and antigen. U-75302 and LY2552833 at 1 µM each were used in a combination (U+LY) as well. (F) Neutralizing antibodies to several chemokines inhibit the migration of CFSE-labeled wild-type BMMC from upper wells to lower wells containing IgE-sensitized wild-type cells and antigen. All the antibodies at the higher concentrations each were used in a combination (all) as well.
Figure 5. IgE- and IgE+Ag-induced mast cell migration can be divided into the early phase of Lyn/Syk-dependent release of soluble factors and the later PI3Kγ-dependent phase. (A) PI3Kγ−/− BMMC were defective in migration in responses to SPE-7 IgE, IgE+Ag or adenosine. (B) FceRIα−/− cells in upper wells were incubated with lower wells containing wild-type, lyn−/− or syk−/− cells in the presence of SPE-7 or SCF. (C) Wild-type, lyn−/− or syk−/− cells in upper wells were incubated with lower wells containing 206 IgE-sensitized wild-type cells in the presence of DNP-HSA or SCF.

Figure 6. In vivo models for IgE- and IgE+Ag-induced mast cell accumulation. (A) Gauze strips spotted with 10 μg/ml of 206 or SPE-7 IgE or PBS were applied to the shaved back skin of naïve NC/Nga mice for 24 h. The mice were sacrificed and skin samples were prepared to stain mast cells. Representative photomicrographs are shown. (B) Total and degranulating mast cell numbers per high-power field were counted. (C) NC/Nga mice were first treated with anti-DNP (206) IgE or PBS on the back skin, and one day later the same area was applied to with gauze strips spotted with DNP-HSA or PBS for 24 h. Total mast cell numbers per high-power field were counted.
Kitaura, Fig. 1

A

PC IgEs

HC IgEs

Migrated cells (x10^4)

<table>
<thead>
<tr>
<th>PBS</th>
<th>206</th>
<th>27-74</th>
<th>C48-2</th>
<th>SPE-7</th>
<th>IgE-3</th>
<th>C38-2</th>
<th>SCF</th>
<th>0</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE (10 µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Migrated cells (x10^4)

<table>
<thead>
<tr>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>30</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>206 (µg/ml)</td>
<td>SPE-7 (µg/ml)</td>
<td>SCF (ng/ml)</td>
<td>DNP-HSA (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

Migrated cells (x10^4)

SPE-7

IgE+Ag

SCF

Incubation time (h)

0 | 5 | 10 | 15

D

Migrated cells (x10^4)

<table>
<thead>
<tr>
<th>0</th>
<th>3</th>
<th>10</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE-7 (µg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP-HSA (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E

% Migrated cells

SPE-7

IgE+Ag

SCF

control

anti-β1

anti-β2

anti-β7

anti-β1+anti-β7
Kitaura, Fig. 2

**A**

Migrated cells (x10^4)

<table>
<thead>
<tr>
<th>PBS</th>
<th>10</th>
<th>5</th>
<th>50</th>
<th>5</th>
<th>50</th>
<th>5</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE-7 MIP-1α (µg/ml) (ng/ml)</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>MCP-1 RANTES (ng/ml) (ng/ml)</td>
<td>5</td>
<td>50</td>
<td>5</td>
<td>50</td>
<td>5</td>
<td>50</td>
<td>5</td>
</tr>
</tbody>
</table>

**C**

Migrated cells (x10^4)

<table>
<thead>
<tr>
<th>SPE-7 (µg/ml): 0</th>
<th>10</th>
<th>0</th>
<th>10</th>
<th>0</th>
<th>10</th>
<th>0</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCF (ng/ml): 0</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D**

Migrated cells (x10^4)

<table>
<thead>
<tr>
<th>Upper well: 0</th>
<th>1</th>
<th>3</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPE-7 (µg/ml) 1</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

**E**

Migrated cells (x10^4)

<table>
<thead>
<tr>
<th>Upper well: 0</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCF (ng/ml) 1</td>
<td>0</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

**Lower well:**

- **A:** SPE-7 IgE µg/ml
- **C:** SPE-7 (µg/ml)
- **D:** SPE-7 (µg/ml)
- **E:** SCF (ng/ml)
Kitaura, Fig. 3

A

Migration cells (x10^4)

SPE-7 (µg/ml) 0 3 10

DNP-HSA (ng/ml) 0 1 10

B

Migration cells (x10^4)

SPE-7 (µg/ml) 0 10

DNP-HSA (ng/ml) 0 10

C

Migration cells (x10^4)

SPE-7 (µg/ml) 0 10

DNP-HSA (ng/ml) 0 10

D

Migration cells (x10^4)

SPE-7 (µg/ml) 0 3 10

DNP-HSA (ng/ml) 0 1 10

- wt
- fyn -/-
- lyn -/-
- hck -/-

- wt
- syk -/-

- wt
- syk -/-

- wt
- syk -/-

- wt
- PKCβ -/-
- PKCε -/-
- PKCθ -/-

- wt
- syk -/-

- wt
- syk -/-

- wt
- syk -/-

- wt
- syk -/-
Kitaura, Fig. 4
Kitaura, Fig. 5

A

![Graph showing the effect of Adenosine, DNP-HSA, and SPE-7 on migrated cells. The x-axis represents the concentration of each substance, and the y-axis represents the number of migrated cells.]

B

![Graph showing the effect of SCF and SPE-7 on migrated cells. The x-axis represents the concentration of each substance, and the y-axis represents the number of migrated cells.]

C

![Graph showing the effect of SCF and DNP-HSA on migrated cells. The x-axis represents the concentration of each substance, and the y-axis represents the number of migrated cells.]

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Kitaura, Fig. 6

A

PBS

206 IgE

SPE-7 IgE

B

Total MCs

Degran. MCs

Cells per HPF

30

20

10

0

PBS 206 SPE-7

C

Total MCs

Cells per HPF

20

10

0

Day 1: PBS IgE

Day 2: PBS PBSAg Ag
IgE- and IgE/antigen-mediated mast cell migration in an autocrine/paracrine fashion

Jiro Kitaura, Tatsuya Kinoshita, Masaaki Matsumoto, Shaun Chung, Yuko Kawakami, Michael Leitges, Dianqing Wu, Clifford A Lowell and Toshiaki Kawakami