doi: 10.1111/j.1365-2222.2010.03667.x
Clinical & Experimental Allergy, **41**, 378–388

ORIGINAL ARTICLE **Basic Mechanisms in Allergic Disease**

Characterization of syk expression in human lung mast cells: relationship with function

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Clinical & Experimental Allergy

Summary

Background Previous studies indicate that the protein tyrosine kinase, syk, is critical in transducing FcεRI-mediated signals. In human basophils, 'releasability' has been linked to the extent of syk expression. Human lung mast cells, like basophils, are also found to be variably responsive to IgE-dependent activation.

Objective The aim of the present study was to determine whether the wide variability in human lung mast cell responses, following IgE-dependent activation, has a relationship with syk expression.

Methods Mast cells were isolated from human lung tissue and 'releasability' was determined by activating the cells with a maximal releasing concentration of anti-IgE. Syk levels in mast cells were determined by immunoblotting and flow cytometry.

Results Histamine release from mast cells, challenged with a maximal releasing concentration of anti-IgE, ranged from 0% to 69% (mean±SEM, 24±2%, $n = 53$). A proportion of these preparations (nine out of 53) released very low levels of histamine ($\leqslant 5\%$) in response to anti-IgE. Flow cytometry of a subset of preparations indicated that a weak response to anti-IgE was not related to a lack of surface IgE. Immunoblotting and flow cytometry studies demonstrated that, compared with mononuclear cells, human lung mast cells express low and variable levels of syk. However, there was no correlation between syk expression and mast cell releasability. Nonetheless, a number of putative inhibitors of syk including NVP-QAB205 (EC_{50}, 0.2 μM) effectively attenuated the IgE-dependent release of histamine from mast cells.

Conclusion and clinical relevance These studies indicate that although syk may play an important role in mediating degranulation, the relative level of syk expression does not govern human lung mast cell releasability. Identification of the mechanisms that govern IgE-dependent activation of human lung mast cells is likely to be of wider clinical significance, given the central role that mast cells play in the development of allergic asthma.

Keywords basophil, FcεRI, histamine, human lung mast cell, IgE, lyn, syk
Submitted 6 August 2010; revised 1 October 2010; accepted 1 November 2010.

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Cite this as: S. Havard, A.-M. Scola, L. J. Kay, S. S. Ishmael, D. W. MacGlashan Jr and P. T. Peachell, *Clinical & Experimental Allergy*, 2011 (41) 378–388.

Introduction

The human lung mast cell has long been recognized as central to the mediation of asthma, especially asthma that has an allergic basis [1]. This is because the exposure of sensitized mast cells to precipitating allergens leads to the IgE-dependent generation of mediators that cause bronchoconstriction and promote inflammation [2, 3]. Moreover, the recent realization that mast cells contribute to airway hyperreactivity has underscored the prominent role that these cells play in asthma [4–6].

Despite the prominence of the mast cell as an important inflammatory cell in asthma, very little is known concerning the intracellular signalling pathways that lead to human lung mast cell activation. However, a significant body of work using rodent cell systems has shown that cross-linking of high-affinity IgE receptors (FcεRI) by antigen initiates the rapid recruitment of the protein tyrosine kinases, lyn and syk, to the FcεRI [7–9]. This recruitment of lyn and syk to the receptor activates the kinases and activated syk may especially play a prominent role in furnishing signals downstream [10]. A critical role

for syk in mast cell signalling has been amply reaffirmed in a number of studies not only in rodent systems [11] but also for human mast cells derived from peripheral blood [12] and for human basophils [13–16].

In human basophils, a particularly interesting association between syk and 'releasability' has been reported [13–16]. It has been known for some time that basophils show highly variable responses to IgE-dependent activation, with a substantial proportion of the population (one in seven people) being unresponsive to anti-IgE. These individuals who are refractory to activation are often referred to as 'non-releasers' and the concept of 'releasability' has been in existence for some time [17]. More recently, it has been shown that releasability is associated with syk expression such that non-releasers do not express syk, whereas releasers do [13–16]. In a screen of the general population (20% atopic), the expression of syk was found to be the best predictor of IgE-mediated histamine release [16]. Moreover, non-releaser basophils can be made to release in response to anti-IgE after a prolonged treatment with cytokines and the releaser phenotype is accompanied by the emergence of syk expression [15]. Taken together, these data suggest that syk plays a critical role in governing basophil releasability. As mast cells and basophils share some common attributes, it is possible that mast cells, like basophils, are similarly influenced by syk.

While heterogeneity in the responses of human lung mast cells to anti-IgE has been reported [18], the concept of mast cell releasability has not been developed meaningfully. In the present study, we provide evidence that different human lung mast cell preparations respond with substantial variability to IgE-dependent activation. Whether syk expression correlates with this releasability was explored.

Materials and methods

Buffers

Phosphate-buffered saline (PBS) was used in these studies. PBS contained (mM): NaCl 137; $Na_2HPO_4 \cdot 12H_2O$ 8; KCl 2.7; KH_2PO_4 1.5. PBS-fetal bovine serum (FBS) was PBS, which additionally contained: $CaCl_2 \cdot 2H_2O$ 1 mM; $MgCl_2 \cdot 6H_2O$ 1 mM; glucose 5.6 mM; FBS 2%; and DNase 15 μg/mL. PBS-human serum albumin (HSA) was PBS additionally supplemented with: $CaCl_2 \cdot 2H_2O$ 1 mM; $MgCl_2 \cdot 6H_2O$ 1 mM; glucose 5.6 mM; and HSA 30 μg/mL. The pH of all PBS buffers was titrated to 7.3.

Preparation of inhibitors and stimuli

Piceatannol (10 mM stock), syk inhibitor I (10 mM), BAY 61-3606 (10 mM) and NVP-QAB205 (1 mM) were all prepared as stocks in dimethyl sulphoxide and stored frozen. Ionomycin was prepared as a stock solution (10 mM) in dimethyl sulphoxide and stored frozen in appropriate aliquots. Lyophilized polyclonal goat anti-human IgE antibody was reconstituted in distilled water and stored at 4 °C. The drugs were diluted to the desired concentration in buffer just before use. Preliminary experiments indicated that the vehicles used to prepare the drugs had no effect on mediator release assays.

Lung tissue

Human lung tissue was obtained from surgical resections of patients following surgery with the approval of the Local Research Ethics Committee. Most of the patients were undergoing surgery for carcinoma. The majority of the patients were Caucasian (90%), and there was an approximately 1 : 1 split of males and females.

Cell isolation

Mast cells were isolated from human lung tissue using a modification of the method described by Ali and Pearce [19]. Macroscopically normal tissue from lung resections was chopped vigorously for 15 min using scissors in a small volume of PBS buffer. The chopped tissue was washed over a nylon mesh (100 μm pore size; Incamesh, Warrington, UK) with 0.5–1 l of PBS buffer to remove lung macrophages. The tissue was reconstituted in PBS-FBS (10 mL/g of tissue) containing collagenase Ia (350 U/mL of PBS-FBS) and agitated using a water-driven magnetic stirrer immersed in a water bath set at 37 °C. The supernatant (containing some mast cells) was separated from the tissue by filtration over a nylon mesh. The collagenase-treated tissue was then reconstituted in a small volume of PBS-FBS buffer and disrupted mechanically using a syringe. The disrupted tissue was then washed over nylon gauze with PBS-FBS (300–600 mL). The pooled filtrates were sedimented (480×g, room temperature, 10 min), the supernatant was discarded and the pellets were reconstituted in PBS-FBS (100 mL). The pellet was washed a further two times. Mast cells were visualized by microscopy using an alcian blue stain [20]. Cell viabilities, as assessed by erythrosin B exclusion, were ⩾92%. Of the total cells, 3–13% were mast cells. This method generated approximately 6×10^5 mast cells/g of tissue. Mast cells prepared in this manner were incubated overnight in RPMI 1640 buffer supplemented with penicillin (10 U/mL), streptomycin (10 μg/mL), gentamicin (50 μg/mL) and FBS (2%). Mediator release experiments were performed the day after tissue digestion and cell isolation.

Mast cells of enhanced purity (10–35% purity) were generated by flotation over discontinuous Percoll gradients [21]. Mast cells were further purified using a MACS magnetic cell sorting system (Miltenyi Biotec, Surrey, UK) according to the manufacturer's instructions. In brief, following flotation over Percoll, mast cells (3 to 12×10^6 cells) were incubated with magnetic microbeads conjugated

to monoclonal antibody (clone AC126) recognizing human CD117 (also known as the c-kit) in the presence of blocking buffer (human IgG) for 30 min on ice. Cell separations were performed over an LS column held within a magnetic field (midiMACS separator, Miltenyi Biotech, Surrey, UK). Mast cell yields were usually >70% of the starting population and the purities ranged from 88% to 98%. Moreover, the antibody (clone AC126)-recognizing c-kit sees an epitope distinct from the binding site of the c-kit ligand [also known as the stem cell factor (SCF)]. Preliminary studies indicated that mast cell responses to SCF (and anti-IgE) were unaffected by the presence of the antibody.

Mediator release

Mediator release experiments were performed the day after the cells had been isolated. Experiments were performed in PBS-HSA buffer. Mast cells (20 000 mast cells/sample) were challenged with a stimulus, in a reaction volume of 300 μL, for 25 min at 37 °C for histamine release. When inhibitors were used, cells were incubated with or without an inhibitor for 15 min before challenge. After challenge, the cells were pelleted by centrifugation (480 g, room temperature, 4 min). Histamine released into the supernatant was determined using the modified [22] automated fluorometric method of Siraganian [23]. The total histamine content was determined by lysing aliquots of the cells with perchloric acid at a final concentration of 1.6%. Cells incubated in buffer alone served as a measure of spontaneous histamine release, which ranged from 2% to 8% of the total histamine content. Histamine release was thus expressed as a percentage of the total histamine content after subtracting the spontaneous histamine release. Previous studies of ours indicate that the spontaneous histamine release and responses to anti-IgE remain constant over 3 days, indicating that mast cell releasability does not change with time [24, 25]. All experiments were performed in duplicate.

In most experiments, mediator release was initiated with a goat anti-human IgE. Alternatively, mast cells were activated with antigen. Cells were passively sensitized (20 h, 37 °C) with a chimeric IgE (human heavy chain, murine lambda light chain), JW8-IgE (1 : 250; ~0.2 μg/mL), directed against the hapten 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) [26]. Preliminary studies indicated that these conditions for passive sensitization were optimal to ensure maximal responses to the antigen, NIP_{20}-HSA. After sensitization, cells were washed twice and activated with antigen (0.0001–100 ng/mL). In some experiments, mast cells were activated non-immunologically with ionomycin.

A potentially important technical consideration was whether the processes of tissue dispersion, mast cell isolation and subsequent purification alter cell functionality. In an attempt to address these issues, we compared the release of histamine induced by anti-IgE (1 : 300; ~3 μg/mL) from mast cells that are loosely associated with lung tissue and that can be dissociated by coarse chopping and simple washing of tissue with mast cells dissociated from the tissue following enzymatic and physical dispersion and found only modest differences in the extent of release (23±3% and 19±3%, respectively, $n = 27$). Enhancing mast cell purities further by flotation over Percoll gradients had no effect on histamine release while further purification of mast cells by immunomagnetic bead separations exerted a modest effect on release (24±4% and 18±3%, respectively, $n = 16$). These findings show that the processes of mast cell isolation and purification do not alter the functional responses appreciably. This suggests that cell integrity, including the signalling machinery, is unaffected by mast cell processing.

Immunoblotting

Cell extracts were prepared for use in immunoblotting using a lysis buffer (Tris 50 mM; NaCl 150 mM; PMSF 50 μg/mL; SBTI 50 μg/mL; leupeptin 5 μg/mL; aprotinin 5 μg/mL; Triton X-100 0.5%; sodium orthovanadate 1 mM; sodium iodoacetate 1 mM; pH 8.0) according to the methods described elsewhere [27]. Purified cells were incubated (20 min on ice) in lysis buffer (10 μL per 1×10^6 cells) with intermittent vortexing and then the lysates were clarified by centrifugation (13 000 g, 3 min). Cell lysates were either used immediately or snap frozen in liquid nitrogen and stored at −80 °C before use. Lysates were prepared for electrophoresis by resuspending in a concentrated sample buffer (final concentrations: Tris 60 mM; SDS 69 mM; bromophenol blue 0.5 mg/mL; glycerol 10% v/v; dithiothreitol 100 mM; pH 6.8) and heating to 96 °C for 10 min in a heating block. The sample was then centrifuged briefly (13 000 g for 5 s) before being applied to the gel. Samples were subjected to SDS-PAGE (Mini-Sub Cell GT System; Bio-Rad, Hemel Hempstead, UK) using 7.5% gels and then the separated proteins were transferred electrophoretically to nitrocellulose membranes. In each blot, an extract of a mononuclear cell preparation, isolated from a single donor, was also prepared [21] and run alongside mast cell lysates. This mononuclear cell preparation served as an internal control that was used to cross-reference and normalize densitometry data among blots.

Membranes were probed with mouse monoclonal antibody to syk (4D10; 1 : 200), followed by goat anti-mouse IgG secondary antibody (1 : 1000). The membrane was stripped (0.2 M NaOH, 15 min) and probed with rabbit polyclonal antibody to lyn (44; 1 : 600), followed by donkey anti-rabbit IgG secondary antibody (1 : 2000). The membrane was stripped again and probed with a rabbit polyclonal for actin (I19; 1 : 600), followed by donkey anti-rabbit IgG secondary antibody (1 : 2000). Protein bands were visualized by the addition of enhanced chemiluminescence (ECL) reagents and signals detected

on an ECL film. Band densities were assessed using densitometry (Kodak Digital Science 1D, version 3.0.1).

Flow cytometry

In flow cytometry experiments, mast cells were identified as the c-kit (CD117)-positive population using a monoclonal (IgG_1) anti-c-kit-PE (A3C6E2). Flow cytometry was performed on mast cells of enhanced purity (10–30%) following flotation over Percoll. In some experiments, mast cells of higher purities (80–95%) were also studied for comparative and confirmatory reasons.

In order to assess IgE expression by the c-kit positive population, cells were incubated with a goat anti-IgE-FITC or the corresponding isotype control (goat IgG-FITC). All incubations were performed in a PBS buffer containing BSA (0.1%), sodium azide (0.2%) and human IgG (0.02%). Cells were incubated with conjugated antibodies (1 : 20) for 30 min, after which the cells were washed three times in the supplemented PBS buffer and then fixed (30 min) with Medium A (Fix & Perm kit, Caltag, Carlsbad, CA, USA). After fixing, the cells were washed twice in PBS buffer containing FBS (5%) and stored in the same buffer at 4 °C. In some experiments, after fixing with Medium A, the cells were permeabilized with Medium B (Caltag, Fix & Perm kit) and stained for 20 min with the anti-syk mAb 4D10 (2 μg/mL) or the $mIgG_{2a}$ isotype control (2 μg/mL) in order to assess the intracellular syk content according to the methods that have been described [28]. Cells were then washed once with PBS containing FBS (5%) and incubated overnight with an Alexa 647-conjugated anti-mouse IgG_{2a} secondary antibody. The expression of syk was assessed in mast cells based on gating of c-kit-positive cells. This assay has now been used and validated by comparison with Western blotting in several different settings [28–31]. To ensure that the comparisons with basophils and other leucocytes were equivalent, the mast cell samples were analysed in parallel with basophil standard samples. In addition to calibrating the syk measurements, the measurements of cell surface IgE density relied on extensive flow cytometric calibration of basophil IgE densities [31–33] and the mast cell samples were also run in parallel with the calibrated basophil samples.

The IgE levels were determined in the United Kingdom using a FACSort flow cytometer (BD Biosciences, Oxford, UK), whereas the IgE and syk levels were determined in the United States using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The IgE data were analysed using Flow Jo (Tree Star Inc., Edition 7.2.4). The syk data were analysed using CellQuest (BD Biosciences, Version 3.2.1f1). The c-kit-positive cells were gated and the amount of fluorescence was quantified as the net mean fluorescence index (MFI), which was calculated as the geometric mean of the specific antibody signal subtracted by the geometric mean of the isotype control signal.

Materials

The following were purchased from the sources indicated: spleen tyrosine kinase inhibitor I (3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2, 3-dihydro-1H-indole-5-sulphonamide) (Calbiochem, Nottingham, UK); ionomycin, piceatannol (Tocris, Bristol, UK); EDTA, $CaCl_2.2H_2O$, $MgCl_2.6H_2O$ (BDH, Poole, UK); acrylamide, Tris, SDS (Bio-Rad); antibodies to syk, lyn, actin and secondary antibodies for blotting (Santa Cruz, Autogen Bioclear, Calne, UK); ECL reagents, ECL film, nitrocellulose membranes (Amersham, Little Chalfont, UK); monoclonal (IgG_1) anti-c-kit-PE (Miltenyi Biotec); goat anti-human IgE-FITC, mouse IgG_1-PE, goat IgG-FITC (Caltag/Invitrogen, Paisley, UK); human IgG (Cappel/MP Biomedicals, Solon, OH); mouse IgG_{2a} (Caltag/Invitrogen, Carlsbad, CA); and anti-mouse IgG_{2a} Alexa 647 secondary antibody (Molecular Probes/Invitrogen, Carlsbad, CA). All other compounds and reagents (including BAY 61-3606, anti-IgE, Percoll) were from Sigma (Poole, UK).

Data analysis

Maximal responses (E_{max}) and potencies (EC_{50}) were determined using non-linear regression analysis (GraphPad Prism, version 3.0a). The coefficient of variation (CV) was calculated as standard deviation ÷ mean.

Results

Mast cell releasability

The response of human lung mast cell preparations to anti-IgE was found to be highly variable. Some mast cell preparations released large amounts of histamine in response to anti-IgE, whereas others released very little (representative examples are shown in Fig. 1a). Out of a total of 53 mast cell preparations, nine released $\leqslant$5% of their total histamine content in response to a maximal releasing concentration (1 : 300) of anti-IgE (Fig. 1b).

Whereas the responses of some mast cell preparations to anti-IgE were relatively weak, we wondered whether this reflected a global unresponsiveness to activation. To test this, mast cells were activated not only with anti-IgE but also with the calcium ionophore, ionomycin (Fig. 1b). These studies showed that preparations that were weakly responsive to anti-IgE (3±1% histamine release, $n = 9$) and those that responded more effectively to anti-IgE (28±2%, $n = 44$) were activated to similar extents by ionomycin (53±3% and 59±2%, respectively).

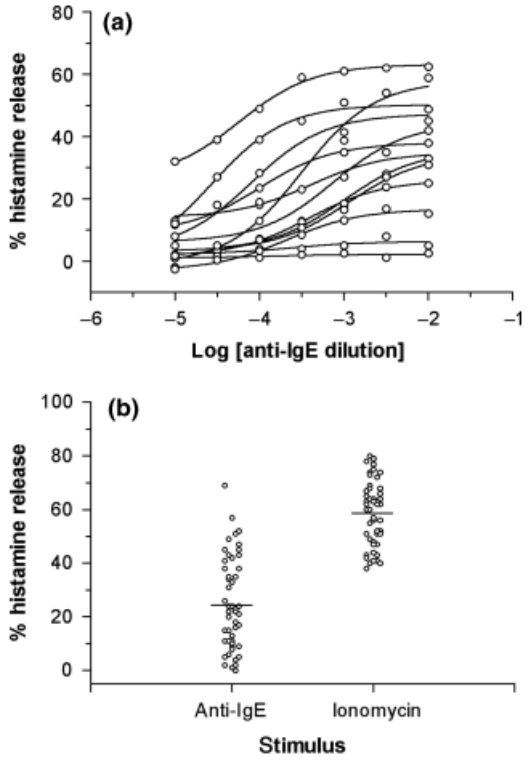

Fig. 1. Mast cell releasability. (a) Mast cells were activated for 25 min with increasing concentrations of anti-IgE. Concentration–response data for 13 human lung mast cell preparations are shown. (b) Mast cells were activated with maximally effective concentrations of either anti-IgE (1 : 300) or ionomycin (0.5 μM), in parallel, to induce histamine release. Each symbol represents data from a separate mast cell preparation and horizontal bars represent the mean histamine releases, which were 24±2% and 58±2% for anti-IgE and ionomycin, respectively ($n = 53$).

IgE density and releasability

In order to examine whether the IgE levels might influence release from mast cells, IgE density in a subset ($n = 13$) of mast cell preparations was determined by flow cytometry (Table 1). The IgE levels in the mast cells ranged widely, although the distribution was quite similar to that observed previously [18, 34]. The IgE distribution was dominated by a sub-population of mast cells with a mean density of around 10 000 IgE/cell and a rightward tail for about 20% of the preparations (> 40 000 IgE/cell).

Previous studies in basophils have not found a relationship between histamine release and cell surface IgE density [35]. However, in basophils, surface IgE is in vast excess of what is needed for stimulation; hence, a correlation would not be expected. By contrast, in these mast cell preparations, many express IgE densities that are near the thresholds for maximal release [35]; hence, a correlation could exist between IgE density and histamine release. However, no correlation was observed ($r^2 = 0.04$; $P = 0.53$; $n = 13$). These data indicate that IgE density does not explain the differences in histamine release among preparations.

Table 1. Variability in endogenous IgE densities among mast cell preparations

Mast cell prep	IgE (MFI)	IgE (molecules per cell)	Histamine release (%)
1	6	1050	15
2	9	1575	28
3*	18	3150	61
4	23	4025	0
5	28	4900	41
6	33	5775	56
7	36	6300	29
8	58	10 150	67
9	135	23 625	66
10	172	30 100	12
11	218	38 150	0
12	492	86 100	24
13	1136	1 98 800	24

IgE density in mast cells ($CD117^+$ population) is presented as either MFI units or molecules per cell. Histamine release induced by a maximal releasing concentration of anti-IgE is also shown. Preliminary cross-calibration studies indicated that 100 MFI units = 17 500 molecules per cell. While all the data in the table were generated using mast cell-enriched preparations (8–30% purity), IgE density in a purified mast cell (> 90%) preparation was also studied for preparation 3 (*), for which a net MFI value of 22 was obtained. The median IgE density was 36 MFI units and the mean of the log of the IgE data was 59 MFI units.
MFI, mean fluorescence index.

Antigen and anti-IgE as stimuli

Although surface IgE density was not a factor that determined the response to anti-IgE, a potential consideration was whether the nature of the IgE-dependent stimulus influenced the outcome. Anti-IgE has been used throughout as a way of monitoring mast cell responses but does it parallel the response that might be seen with antigen? The type and extent of aggregates induced by the anti-IgE antibody may differ from those induced by antigen [36, 37] or, alternatively, the interaction of the anti-IgE antibody (a goat polyclonal IgG) with low-affinity IgG receptors may modify the extent of release [38–41].

To address these potential issues, mast cells were incubated (20 h, 37 °C) with or without anti-NIP-specific IgE (JW8-IgE), after which the cells were then tested for responses both to the cognate antigen, NIP-HSA, and to anti-IgE. Representative data are shown in which a good, moderate and poorly releasing mast cell preparation to anti-IgE (Fig. 2a) were incubated with or without JW8-IgE before challenge with antigen (Fig. 2d) or anti-IgE (Fig. 2c). The data show that while good- and moderate-releasing preparations respond effectively to NIP-HSA, the poorly releasing preparation failed to respond effectively to either antigen or anti-IgE following passive sensitization with JW8-IgE. Indeed, there was an excellent correlation ($r^2 = 0.90$; $P < 0.0001$; $n = 26$) between the response of mast cells to

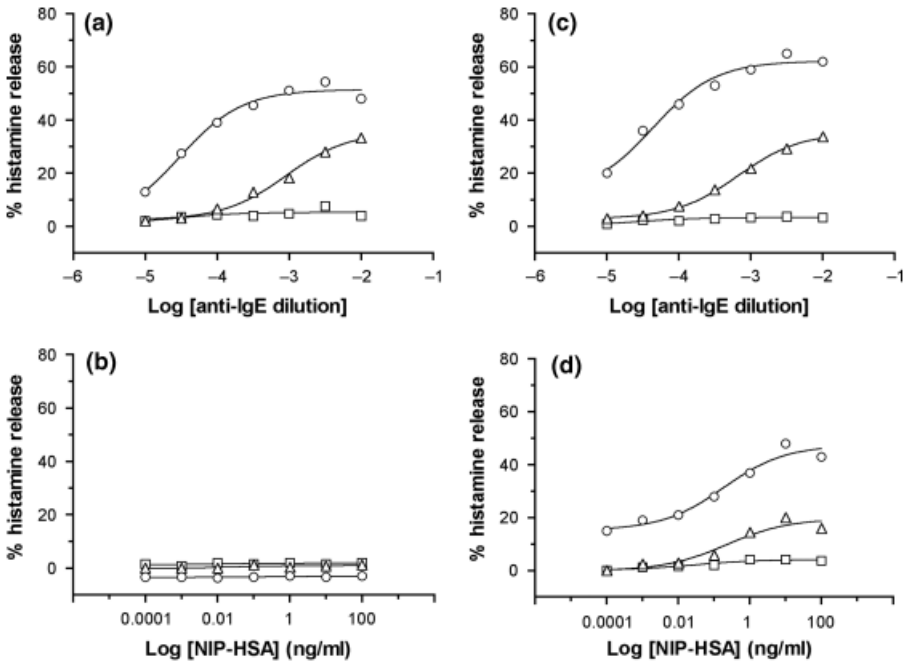

Fig. 2. Mast cell sensitization with antigen-specific IgE and responses to antigen. Three mast cell preparations known to respond well (circles), moderately (triangles) and very little (squares) to anti-IgE were incubated without (a, b) or with (c, d) JW8-IgE (1 : 250) for 20 h. After washing, the cells were challenged with either anti-IgE (a, c) or NIP-HSA (b, d) for 25 min in order to induce histamine release. NIP, 5-iodo-4-hydroxy-3-nitrophenacetyl; HSA, human serum albumin.

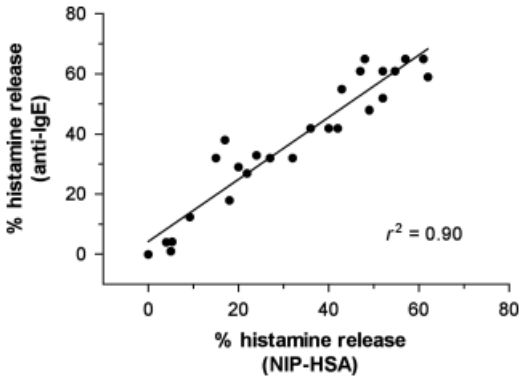

Fig. 3. Correlation between mast cell responses to anti-IgE and antigen. Mast cells were passively sensitized with antigen-specific IgE (JW8-IgE; 1 : 250) for 20 h. After washing, the cells were challenged with maximal releasing concentrations of either anti-IgE (1 : 300) or NIP-HSA (10 ng/mL) for 25 min in order to induce histamine release. There was an excellent correlation ($r^2 = 0.90$; $P < 0.0001$) between the response of mast cells to anti-IgE and to NIP-HSA following passive sensitization. The correlation is based on data from 26 mast cell preparations. NIP, 5-iodo-4-hydroxy-3-nitrophenacetyl; HSA, human serum albumin.

anti-IgE and the extent to which mast cells responded to NIP-HSA following passive sensitization (Fig. 3).

Confirmatory flow cytometry experiments indicated that mast cells express significantly ($P < 0.05$) greater levels of surface IgE following passive sensitization. IgE MFI units were 49±18 (mean±SEM) and 67±21 following overnight incubation of mast cells in buffer or with JW8-IgE, respectively ($n = 6$). Increases in IgE density following passive sensitization were similar for mast cells that released well or poorly in response to anti-IgE.

Syk and releasability

The releasability of human basophils has been linked to syk expression [13–16]. In order to determine whether a similar situation might hold for human lung mast cells, the syk (and lyn) content was determined by immunoblotting. Visual inspection of immunoblots indicated that there was no apparent relationship between releasability and syk or lyn expression (Fig. 4). In a total of 24 preparations (Fig. 5a), densitometric analysis of immunoblots indicated that, relative to mononuclear cells and normalized for actin levels, there was a considerable variation in the expression of syk and lyn in mast cells (CV was 0.89 for syk and 0.60 for lyn). Moreover, mast cells express less syk (0.47±0.12) but more lyn (1.65±0.20) than mononuclear cells when the values are normalized for equivalent actin levels. If the syk and lyn content is calculated on a per cell basis, then mast cells express even less syk (~0.3) and about the same amount of lyn as mononuclear cells. However, there was no correlation between mast cell releasability and either syk (Fig. 5b) or lyn (Fig. 5c) expression.

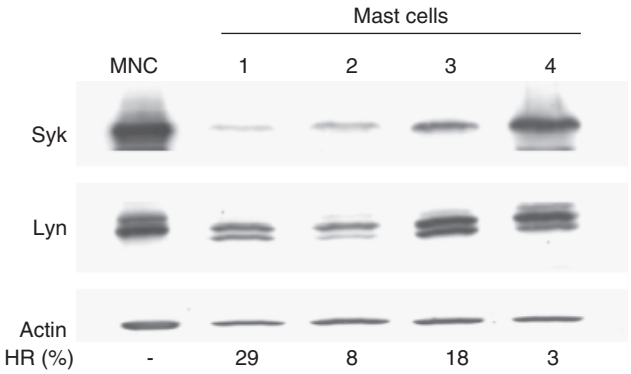

Fig. 4. Representative immunoblot for syk and lyn in mast cells. Four mast cell preparations (6 00 000 cell equivalents) at purities of 96–98% were solubilized and subjected to SDS-PAGE along with a mononuclear cell (MNC; 3 00 000 cell equivalents) preparation. Following electrophoretic transfer to a nitrocellulose membrane, the membrane was probed with an antibody to syk, the membrane was stripped and probed with antibody to lyn and the membrane was stripped again and probed with antibody to actin. Histamine releases (HR) of the mast cell preparations, in response to a maximal releasing concentration of anti-IgE (1 : 300), are provided.

In order to confirm these findings, the expression of syk in mast cells was also determined by flow cytometry. Assessment of syk using this method also indicated that there was considerable variability in syk expression in mast cells (CV = 0.65). Moreover, these studies reaffirmed that there was no correlation ($r^2 = 0.09$; $P = 0.43$) between syk expression and releasability (Fig. 6). For example, the expression of syk was essentially the same in three preparations that released 0%, 17% and 52% of the total histamine content in response to a maximal releasing concentration of anti-IgE (preparations 1, 4 and 8 in Fig. 6).

An attendant benefit of the flow cytometry studies was that a direct comparative assessment of syk expression in mast cells and basophils could be made. The average syk protein content (250±53 MFI, $n = 9$) in mast cells was more than double the average syk content in human basophils (110±16 MFI, $n = 16$) [30]. As 150 MFI units equate as 34 000 molecules of syk/cell [28–31], mast cells and basophils express, on average, 57 000 and 25 000 molecules of syk/cell, respectively.

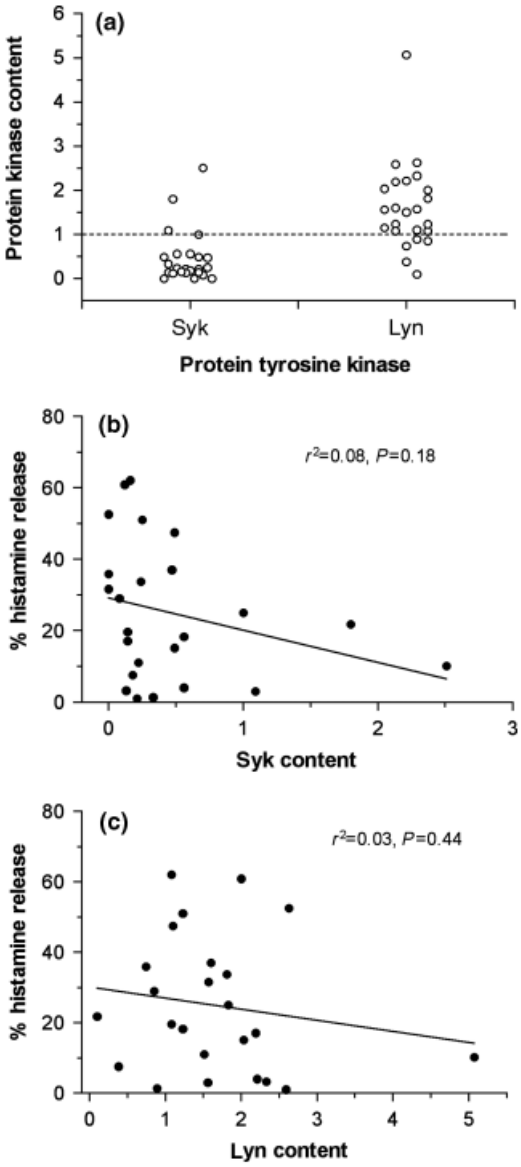

Fig. 5. Syk and lyn content in mast cells. (a) Syk and lyn content in mast cells was determined by densitometric analysis of immunoblots and is expressed relative to the content of these kinases in a mononuclear cell preparation that was used in all blots and normalized relative to cell actin content. Each point reflects the kinase content in a given mast cell preparation, and data from 24 mast cell preparations are shown. Correlations between (b) syk content and histamine release and (c) lyn content and histamine release are also shown.

Role for syk in release

Although no correlation was observed between releasability and syk expression, that syk might be involved in IgE-dependent activation of mast cells was suggested by studies in which putative inhibitors of syk attenuated histamine release from mast cells induced by anti-IgE (Fig. 7) but not that induced by ionomycin (data not shown). Piceatannol (EC_{50}, 32 μM), syk inhibitor I (0.5 μM), BAY 61-3606 (0.3 μM) and NVP-QAB205 (0.2 μM) all inhibited IgE-mediated histamine release in a concentration-dependent manner and with potencies (EC_{50} values) in the range expected for these inhibitors.

Discussion

It has been known for some time that human basophils are variably responsive to activation by anti-IgE [17]. A more recent report has intimated that human lung mast cells are, similarly, variably responsive to IgE-directed

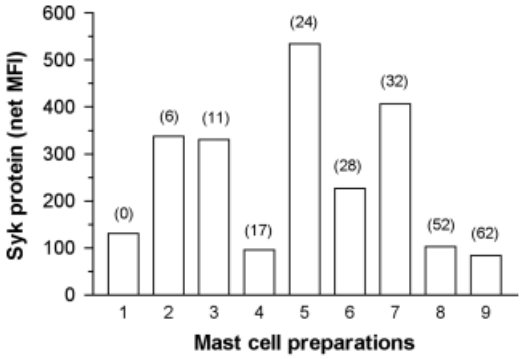

Fig. 6. Syk content in mast cells as assessed by flow cytometry. Fixed mast cells were permeabilized in order to determine the syk content in the $CD117^+$ population. Syk expression is given as a net mean fluorescence index (MFI), the difference between the sample labelled with an anti-syk Ab and a relevant isotype control. Values in brackets show the % histamine release of each of the mast cell preparations in response to a maximal releasing concentration of anti-IgE.

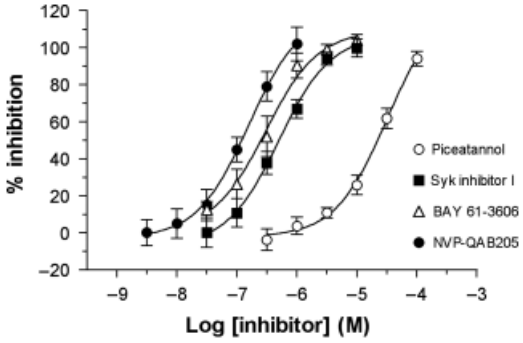

Fig. 7. Inhibition of mediator release by syk inhibitors. Cells were incubated with or without increasing concentrations of a given inhibitor for 15 min before challenge with anti-IgE (1 : 300) for a further 25 min for histamine release. Results are expressed as the % inhibition of the unblocked histamine release, which was 27±5%. Values are means ±SEM, $n = 8–10$.

activation [42]. In the present work, we provide evidence, in a large data set, that mast cells show markedly different levels of histamine release in response to IgE-directed activation. In this study, we have attempted to determine why this might be the case.

Some mast cell preparations fail to release any histamine following challenge with anti-IgE. One possible reason for this failure might relate to the density of IgE at the cell surface. However, our data demonstrate that 'non-releaser' mast cells express endogenous levels of IgE that are comparable to those seen in 'releaser' mast cells indicating that IgE expression does not determine releasability. These data are in accord with previous studies demonstrating that IgE density (determined by stripping mast cells of IgE and performing radioimmunosorbent tests) does not equate with mast cell releasability [18].

Attempts to make 'non-releaser' mast cells release histamine by passively sensitizing with antigen-specific IgE (JW8-IgE) and challenge with antigen (NIP-HSA) or anti-IgE failed to lead to a conversion in phenotype. Indeed, the demonstration that passive sensitization led to maximal levels of histamine release induced by antigen that were almost identical to those seen with anti-IgE strongly indicates that releasability is an intrinsic property of the cell. Each mast cell preparation, therefore, has a maximal ceiling of IgE-dependent histamine release, irrespective of whether this is driven by anti-IgE or antigen. These data indicate that while surface IgE expression is clearly necessary for release, surface IgE expression does not determine the extent of release this being governed by some property intrinsic to the cell.

One factor that could influence releasability is the expression of the protein tyrosine kinase, syk. Studies in human basophils indicate that syk expression is a predictor of histamine release [13–16]. Both basophils [16] and human lung mast cells (this study) show a large range in syk expression. However, in mast cells, unlike the situation for basophils, we were unable to demonstrate a correlation between syk expression and releasability when the syk content was determined by immunoblotting and densitometry.

To validate the measurements of syk by immunoblotting, mast cell samples were also analysed by flow cytometry. This method has been used recently to assess syk levels in a range of leucocytes including basophils [28–31]. The flow cytometry studies in mast cells led to the conclusion reached using the immunoblotting approach that variability in syk expression does not readily explain the variability in IgE-mediated histamine release.

The flow cytometry studies also allowed some quantitative analysis of syk expression in mast cells, relative to human basophils and other leucocytes, as methods have been developed to calibrate the number of molecules of syk per cell in leucocytes [28–31]. With respect to syk expression, mast cells express about 57 000 molecules of syk/cell. This constitutes about twice the levels of syk expressed by basophils (25 000 molecules of syk/cell) but considerably lower than the syk content in monocytes and dendritic cells (7 50 000 molecules/cell) or neutrophils and eosinophils (2 75 000 molecules/cell). In addition, the range of syk expression in mast cells was similar to that seen in basophils [16]. The CV of syk expression in basophils is approximately 0.70, and in these human lung mast cells, somewhere between 0.70 and 0.90 depending on the method used. The CV (about 0.20–0.25) of syk expression in other leucocytes approaches the error one would expect from the methodology [30]. These findings indicate that the characteristics of syk expression in mast cells closely resemble the situation seen for basophils. As the characteristics of syk expression in human lung mast cells seem to follow those seen in basophils, an expectation might be that a similar relationship between syk and releasability might hold for mast cells. However, in the present study, no such relationship was found.

One explanation for this difference is that whereas syk is rate limiting for IgE/FcεRI-driven signal transduction in basophils, the same relationship does not hold in mast cells. Human basophils are known to express approximately 1 50 000 IgE/FcεRI/cell (mean for the full population; [33]) and 25 000 molecules of syk [30], leading to a ratio of 1 syk/6 IgE/FcεRI, on average, and as low as 1 syk/50 IgE/FcεRI in poor responders. This relationship of lower levels of syk relative to IgE/FcεRI limits full signal transduction in basophils. In the current study, mast cells were found to express about 10 000 IgE/FcεRI/cell, on average, which is consistent with previous studies [18, 34]. By contrast, syk expression averaged 57 000 molecules/cell, leading to an inverted ratio of 6 : 1 for syk to IgE/FcεRI. For mast cells, syk does not appear to be a rate-limiting signalling element during stimulation with anti-IgE as it does in basophils. These findings, together with the observation that the lowest level of syk expression found in mast cell preparations (measured by flow cytometry) is greater than the average for basophils, leads to the prediction that the level of syk expression in mast cells should be sufficient to drive adequate histamine release.

A previous anecdotal report [43] of a human lung mast cell preparation that failed to respond to anti-IgE and was found to lack syk (as assessed by Western blotting) is not inconsistent with our study in that a range of syk expression is possible. It is reasonable to surmise that if no syk is expressed a mast cell would be incapable of IgE-mediated signalling leading to histamine release. However, while this is a possible outcome in an isolated preparation, in general, the level of syk expression does not explain the variance in histamine release observed in our study.

Although our data indicate that the expression of syk does not govern releasability in human lung mast cells, that putative inhibitors of syk were found to inhibit histamine release induced by anti-IgE in a concentration-dependent fashion suggests an involvement of this kinase in degranulation. The rank order of the potency of these compounds of NVP-QAB205 > BAY 61-3606 > syk inhibitor I > piceatannol as inhibitors of histamine release is consistent with the potency of these compounds as inhibitors of syk [28, 44–46]. While the selectivity of piceatannol has been questioned [47], that the third-generation syk inhibitor, NVP-QAB205 [28], is an effective inhibitor of mast cells provides more reliable evidence that syk is involved in mast cell degranulation.

In summary, these studies have established that human lung mast cells express syk at levels that are considerably lower than most other types of leucocytes, making them more similar to basophils in this respect. In addition, the range of syk expression in mast cells, as in basophils, is broad. These similarities in the characteristics of syk expression in mast cells and basophils suggest that syk may play a similar role in regulating mast cell and basophil activity. And while syk appears to play an important role in mast cell degranulation, as selective syk inhibitors prevent histamine release, syk does not appear to govern mast cell releasability. This may be because, unlike the situation for human basophils, syk is not a rate-limiting enzyme in the context of IgE/FcεRI-directed signalling in human lung mast cells. As syk does not appear to determine mast cell releasability, it is possible that alternative signalling molecules may be involved. In this context, the inhibitory protein phosphatase, SHIP (SH2-containing inositol-5′-phosphatase), could play an influential role as it has been shown to influence secretion in basophils [16, 48].

These studies highlight the heterogeneity that exists between mast cells and basophils in the relative constitution of components of the same signalling pathway and how this impacts on the response.

Clinical relevance

Given the central role that mast cells play in the development of allergic asthma, identification of the mechanisms that govern IgE-dependent activation of human lung mast cells is likely to be of wider clinical significance. To this end, these studies confirm that inhibiting syk is a viable way to stabilize human lung mast cell responses.

Acknowledgements

We are grateful to Mr J. Edwards, Mr J. Rao, Mr T. Locke, Mr G. Cooper and Mr D. Hopkinson (Cardiothoracic Surgery), Dr S. K. Suvarna, Dr J. Hyde, Dr P. Kitsanta and Dr C. Layton (Histopathology) at the Northern General Hospital, Sheffield, for their invaluable help in providing lung tissue specimens. We thank Dr Sue Newton for help and advice with flow cytometry. We thank GlaxoSmith-Kline for the gift of NVP-QAB205 and we thank Dr Birgit Helm (Molecular Biology and Biotechnology, University of Sheffield) for the kind gift of JW8-IgE and NIP-HSA. This work was supported by Asthma UK.

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