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The β Subunit of the Type I Fcε Receptor Is a Target for Peptides Inhibiting IgE-Mediated Secretory Response of Mast Cells[1]

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Peptides originally derived from complement component C3a were earlier shown to inhibit the type I FcεR (FcεRI)-mediated degranulation of mucosal type mast cells. In the present study, we show that C3a7, a peptide with a natural sequence, and its modified derivative, C3a9, are powerful inhibitors of the above response of both serosal and mucosal type mastocytes. We demonstrate that these peptides inhibit FcεRI-induced membrane proximal events, suppress phosphorylation of the FcεRI β subunit, the protein tyrosine kinase Lyn, as well as the transient rise in free cytosolic Ca^{2+} level. The late phase of cellular response was also inhibited, as demonstrated by the reduced TNF-α secretion. Experiments using two independent methods provided evidence that the interaction site of complement-derived peptides is the FcεRI β-chain. This was further supported by fluorescence confocal microscopic colocalization and resonance energy transfer measurements. Taken together, these results suggest the presence of distinct "activating" and "inhibitory" motifs in the C3a sequence. Response to both is in balance under physiologic conditions. Furthermore, present data predict that such inhibitory peptides may serve as potent agents for future therapeutic intervention. *The Journal of Immunology,* 2005, 175: 2801–2806.

Mast cells play a central role in the pathogenesis of allergic diseases. Clustering of type I FcεR (FcεRI)[4] present in their plasma membrane initiates a coupling cascade culminating in the secretion of inflammatory mediators, including histamine, serotonin, proteases, leukotrienes, and several cytokines (1). Lyn, a src family protein tyrosine kinase (PTK) interacts with the β subunit of the receptor complex and undergoes phosphorylation and activation as a result of FcεRI clustering. Recruitment of Lyn to the ITAM motif-phosphorylated receptor subunits results in activation of Syk PTK, which in turn causes phospholipase C-γ activation, hydrolysis of phosphatidyl-inositide-4,5-bisphosphate, and a transient rise in free cytosolic $[Ca^{2+}]_i$. This in turn induces activation of protein kinase C culminating eventually in mediator secretion (2–4).

Mast cell progenitors represent a single lineage, giving rise upon migration into different tissues to two distinct phenotypes: the so-called serosal (connective tissue type) mastocytes residing in serosal cavities, in the skin, and respiratory tract; and the mucosal type mast cells found mainly in the gastrointestinal mucosal surfaces (5). Nevertheless, mast cell tissue-dependent differentiation is reversible; fibroblast-derived factors change mucosal type mast cells into serosal ones, whereas IL-3 favors the mucosal phenotype (1, 6). Besides tissue distribution, life span and mediator content of their intracellular granules are also different (5). Both types express FcεRI on their cell membrane, clustering of which provokes the secretory response.

In contrast to the FcεRI-mediated triggering of mastocytes, the peptidergic pathway of mast cell activation is unrelated to the former and only occurs in serosal mast cells, which are experimentally modeled by rat peritoneal or human skin mast cells. The peptidergic stimulus is triggered by exposure to polyamines or cationic peptides, such as substance P, or the complement activation products C3a and C5a (7). These complement-derived anaphylatoxins are among the most potent peptidergic activators, each reacting with their own specific receptors (C3aR and C5aR) expressed on the serosal type mastocytes. In contrast, mucosal mast cells, such as the rat basophilic leukemia cell line (RBL-2H3), do not appear to express these receptors and do not respond to such cationic peptides. Our lab had demonstrated earlier that C3a and some of its derivatives inhibit the IgE-mediated degranulation of RBL-2H3 cells, whereas C5a has no effect on this process (8, 9). We identified the inhibitory sequence motifs upstream of the C-terminal activatory stretch of C3a, ranging from positions 56–77 and also by a shorter sequence ranging from positions 56–64.

In the present study, we show results of experiments designed to study the capacity of C3a and its related peptides to inhibit different steps of the FcεRI-coupled signaling cascade to resolve the mechanism of its inhibitory action. Along these lines, we now demonstrate that C3a7, a peptide with the natural sequence of CC NYITELR, and its modified derivative C3a9 (DCCNYITR) are powerful inhibitors of the IgE-mediated response of both the mucosal and the serosal type mast cells. Earlier results showing that

FcεRI clustering-mediated intracellular protein tyrosine phosphorylation and $[Ca^{2+}]$ mobilization are suppressed by C3a (8) are now confirmed by the similar effects of the above peptides. Specifically, we show that tyrosine phosphorylation of the FcεRI β subunit as well as of Lyn, both playing a crucial role in FcεRI-IgE-mediated signaling, is reduced. In addition to these FcεRI-proximal events, later stimulus-response coupling processes are also inhibited, as judged from the decreased TNF-α secretion. Significantly, C3a7 and C3a9 also inhibited the FcεRI-mediated secretion of rat peritoneal (i.e., serosal type) mast cells, an effect consistent with their sequence lacking the C-terminal stretch of C3a, which confers its anaphylatoxic activity. Using independent experimental methods, evidence is presented that the site of C3a interaction leading to the inhibitory action is the FcεRI β-chain, a subunit that has been proposed to serve as an amplifier of this receptor's clustering-induced signaling (10, 11).

Materials and Methods

Reagents

Tissue culture media and supplements were purchased from Invitrogen Life Technologies. Triton X-100, p-nitrophenyl-N-acetyl-β-D-glucosamine, and anti-phosphotyrosine Ab PT-66 were purchased from Sigma-Aldrich. 2,4-Dinitrobenzene sulfonic acid-conjugated BSA (DNP_{11}-BSA), DNP-coated beads, and murine DNP-specific monoclonal A2 IgE were kindly donated by A. Licht (Rehovot, Israel). HRP-conjugated anti-mouse IgG and HRP-labeled anti-rabbit IgG were purchased from Sigma-Aldrich, and anti-Lyn Ab was purchased from BD Transduction Laboratories. ECL reagent was purchased from Amersham Biosciences, and materials used for SDS-gel electrophoresis were obtained from Bio-Rad. The Fluo-3 AM dye was obtained from Calbiochem. C3a and C5a were isolated as described previously (8).

Synthetic peptides and protein labeling

Peptide synthesis was conducted by solid phase technique using "Boc chemistry" (12) on 1 para-methyl-benzhydryl-amine-resin. The following sequences were synthesized: C3a7, CCNYITELR; C3a9, DCCNYITR; and a control peptide, DVSNYITR. Peptides were purified and characterized by reversed phase HPLC and mass spectrometry. Peptide C3a9 was labeled with Cy5 and A_2IgE with Cy3 as given by the instructions (labeling protocol for 0.1 M $NaHCO_3$) provided by Amersham Biosciences.

Cells

Bone marrow-derived mast cell (BMMC) were prepared from BALB/c mice as described by Nagao et al. (13). After 3 wk, an ~95% pure mast cell population was obtained, showing high expression of FcεRI and stem cell factor receptor (c-kit), as measured by flow cytometry.

RBL-2H3 cell line, obtained from Dr. R. Siraganian (National Institutes of Health, Bethesda MD), was maintained in DMEM supplemented by 5% FCS, 2 mM glutamine, and antibiotics in a humidified atmosphere with 5% CO_2 at 37°C. For the experiments, cells were harvested following detachment by 15 min incubation with 10 mM EDTA in DMEM.

Rat peritoneal mast cell (RPMC) were isolated as described previously (14). Mast cell preparations were ~95% pure, as evaluated by flow cytometric monitoring of FcεRI surface expression. Compound 48/80, a specific activator of serosal type mast cells (15), elicited ~50% release of the total β-hexosaminidase content of the isolated RPMC.

Secretory response of mast cells

Mediator secretion by mast cells in response to stimulation by FcεRI clustering was monitored by measuring activity of the secreted granular enzyme β-hexosaminidase, as described earlier (8). To study the effect of C3a and its derivatives on Ag-induced response, mast cells sensitized with saturating concentrations of DNP-specific A2 IgE were preincubated with a concentration range of the various peptides for 5 min at room temperature before exposure to suboptimal Ag concentrations (5 ng/ml).

Measurement of TNF-α by ELISA

TNF-α secretion by RBL-2H3 in response to FcεRI clustering in the absence and presence of the peptides was determined with a rat TNF-α ELISA kit (R&D Systems).

Immunoprecipitation and Western blot analysis

Mast cells were seeded in 10-cm petri dishes (7×10^6 cells/dish) and saturated with A_2IgE at 37°C in DMEM overnight. After washing, they were treated with peptides for 5 min, and FcεRI was clustered by incubating with 5 ng/ml DNP_{11}-BSA at 37°C for 2 min. The reaction was terminated by 500 μl of lysis buffer (1% Triton X-100, 50 mM HEPES, 100 mM NaF, 10 mM EDTA, 2 mM sodium orthovanadate, 10% glycerol, 10 mM sodium pyrophosphate, and protease inhibitor mixture 1:200 (pH 7.4)) per dish. The cells were scraped, and the protein content of the post nuclear supernatants was adjusted to equal values using the Bradford assay before precipitation by PT-66 phosphotyrosine-specific Ab-coated beads (10 μl of beads/sample). The proteins were eluted by sample buffer containing 2-ME and were separated by SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and developed using the indicated Abs and detected by ECL.

Monitoring free cytosolic Ca^{2+} ion concentrations

A total of 5×10^6 IgE-sensitized cells in 0.5 ml RPMI 1640 medium was loaded with 5 μM fluo-3/AM indicator and 30 μg/ml Pluronic F-127 for 30 min at 37°C. After washing, the cells (at 5×10^5 cells/ml) were incubated with C3a7 or C3a9 peptides at 200 μM for 5 min at 37°C. Twenty seconds after initiation of flow cytometric recording, 5 ng/ml Ag (DNP_{11}-BSA) were added to the cells, and the changes in free calcium ion concentration were followed in the time-resolved mode of a BD Biosciences FACSCalibur flow cytometer. Data acquisition and analysis were performed with the CellQuest software (BD Biosciences).

Laser scanning confocal microscopy

RBL-2H3 cells were harvested and incubated either with 5 μM of the Cy3-conjugated IgE or simultaneously also with 200 μM Cy5-conjugated C3a9 peptide for 25 min at 4°C. After washing, the cells were fixed with 2% paraformaldehyde on ice for 20 min and then mounted on a coverslip precoated with 0.1% poly-L-lysine. The fluorescence signals from the Cy3-labeled IgE and the Cy5-peptide were analyzed in the green (excitation by 543 nm He-Ne laser) and red (excitation by 632 nm He-Ne laser) optical channels of a Zeiss LSM5 laser scanning confocal microscope. The cells were optically sliced to 512×512 pixel sections with 0.5-μm thickness. Estimates of the cross-correlation coefficients between fluorescence intensities as a measure of colocalization were conducted as described earlier (16).

Flow cytometric fluorescence resonance energy transfer (FRET) measurements

FRET between FITC-labeled C3a9 peptide (donor) and Cy3-labeled IgE (acceptor) both bound to the surface of RBL-2H3 cells was measured and evaluated using BD Biosciences FACSStar Plus flow cytometer, as described earlier (17, 18). RBL-2H3 cells were labeled with saturating concentrations of Cy3-IgE while the FITC-C3a9 peptide was at 150 μM, i.e., at doses similar to those used in the functional studies.

Covalent cross-linking of C3a to BMMC

BMMC were washed and incubated with C3a (50 μg/ml per $15–20 \times 10^6$ cells/sample, in PBS) for 10 min at room temperature. After another incubation of 20 min at room temperature with 5 mM of the cross-linking reagent bis-(sulfosuccinimidyl)-suberate (BS^3; Pierce), cells were washed and lysed. Polyclonal rabbit Abs specific to C3a (Behringwerke) were used for immunoprecipitation. Western blotting was conducted by a FcεRI β-chain specific Ab kindly provided by J. P. Kinet (Harvard University). As secondary Ab, HRP-conjugated goat anti-mouse IgG (DakoCytomation) was used. Detection was performed by ECL.

Surface plasmon resonance (SPR) measurements

SPR measurements were performed using a BIAcore instrument Model 2000 (Pharmacia). Peptides with the following sequences were synthesized and used: 1) the first extracellular loop of the rat and human FcεRI β-chain: STLQTSDFDDEVLLLYRAGYPF and SVLDISHIEGDIFSSFKAGY; and 2) the second extracellular loop of the rat and human FcεRI β-chain: NNSAYMNYCKDITEDDGCFVTS and KSLAYIHIHSCQKFFETKCF MAS, respectively.

All peptides were biotinylated at their N-terminal amino group and bound to the streptavidin-coated sensor chips (BIAcore) at low densities, ranging between 30 and 60 resonance units. C3a and C5a solutions in HEPES-buffered saline at five different concentrations, ranging from 10.5 to 656 nM, were injected at a flow rate of 20 μl/min. The surface of the chips was regenerated between measurements by 0.1 M HCl. Data were analyzed with BIAevaluation 3.0 software. The observed association rate

constants (k_{obs}) were plotted as the function of the C3a concentration and the slope of each plot was taken as specific rate constant of association (k_{on}), while the y-intercept was taken as the dissociation rate constant (k_{off}), then K_d was calculated as k_{off}/k_{on}.

Results

The peptides' inhibitory capacity on the secretory response of mucosal type mast cells

In earlier experiments, we have identified the C3a sequence motif responsible for inhibiting the IgE-mediated stimulation (9) of RBL-2H3 cells. Our results have clearly demonstrated that the C-terminal sequence of C3a (residues 65–77)—known to be of major importance in exerting anaphylatoxic and chemotactic activity of the complement-peptide—is not involved in that inhibition. However, upstream sequences, comprising residues 56–64 (CCNYITELR, designated C3a7) exert an inhibiting effect (9). Several analogues of this sequence were now synthesized, out of which an octapeptide, DCCNYITR, designated C3a9, is shown to be effective in inhibiting FcεRI-mediated secretion of mucosal type mast cells of the RBL-2H3 line (Fig. 1). IgE-sensitized cells were incubated with the peptides for 5 min before the stimulation with a suboptimal (5 ng/ml) Ag dose, followed by measuring activity of the secreted granular enzyme, β-hexosaminidase. Fig. 1A shows the dose-dependent inhibition exerted by these peptides on these cells' response.
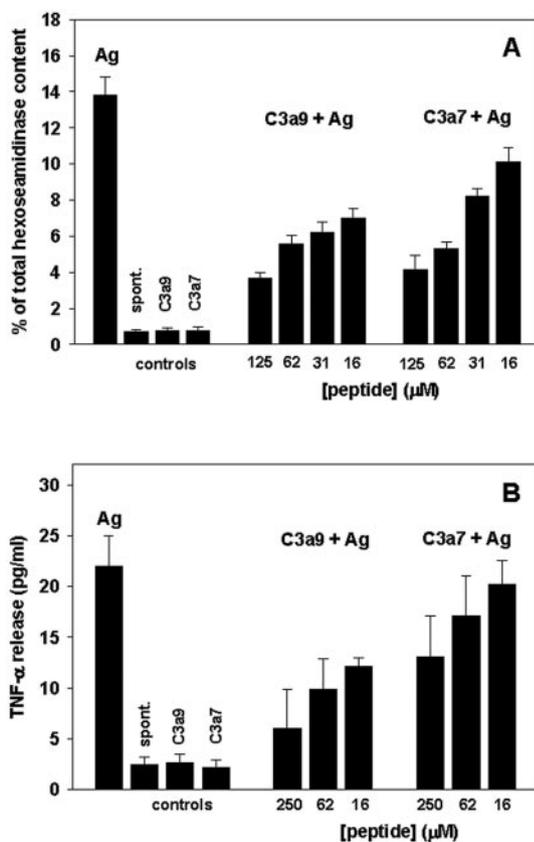

FIGURE 1. The inhibitory capacity of synthetic peptides C3a7 and C3a9 on the secretory response of RBL-2H3 cells. RBL-2H3 cells were saturated with the DNP-specific monoclonal A2 IgE and preincubated with the indicated concentrations of synthetic peptides C3a7 and C3a9 for 5 min at room temperature, followed by a suboptimal antigenic challenge (5 ng/ml). A, Release of the granular enzyme, β-hexosaminidase. Data are presented as net percentages of the cells' total enzyme activity. B, Secretion of TNF-α cytokine by RBL-2H3 cells cultured for 24 h in the presence of the peptides. (One representative experiment of five is shown. The error bars represent SD of triplicate samples.)

Similar experiments were conducted using BMMC. C3a was found to inhibit the IgE-mediated secretory response of these mucosal type mast cells as well (Table I), while C5a had no effect (data not shown), confirming earlier results obtained with RBL-2H3 cells (8). As shown in Table I, the inhibitory effect of the peptides C3a7 and C3a9 on a BMMC's secretory response is comparable to that exerted on RBL-2H3 cells. The control peptide had no effect on either system.

The peptides' inhibitory capacity on the secretory response of serosal type mast cells

Although mucosal type mast cells are nonresponsive to peptidergic stimuli, the serosal type ones have been known for a long time to be stimulated by cationic secretagogues (7). The anaphylatoxic peptides C3a and C5a initiate the cells' secretory response by binding to their respective receptors expressed by serosal type mast cells. To investigate the effect of the peptides on this mast cell type, RPMC were used. As expected, these cells did respond to both C5a and C3a (Table I). However, degranulation was inhibited when peptides C3a7 (a sequence upstream to the C3aR-binding stretch) or C3a9 (nonrelated to C3a) were added to the IgE-sensitized RPMC and stimulated 5 min later with a suboptimal Ag dose (Table I).

Peptides C3a7 or C3a9 suppress the FcεRI-clustering induced TNF-α secretion

The effect of the peptides on mast cells' late phase response was determined by measuring TNF-α secretion. To this end, supernatants were taken 24 h after stimulation of RBL-2H3 cells in the absence or in the presence of peptides C3a7 or C3a9, and the secreted cytokine concentration was determined by ELISA. As shown in Fig. 1B, both peptides dose dependently inhibited the release of this inflammatory cytokine. When added alone, the peptides had no effect.

Tyrosine phosphorylation of Lyn, PI3K, and the β subunit of FcεRI is suppressed by the inhibitory peptides

Fig. 2A shows that the FcεRI clustering-induced enhancement of protein tyrosine phosphorylation of several intracellular proteins is markedly reduced upon exposure of the RBL-2H3 cells to 200 μM C3a7 (*lane 3*) or C3a9 (*lane 4*) before Ag stimulation. Based on this result and on our earlier findings (Fig. 1), we set out to investigate further details of these FcεRI-proximal events. These are known to include phosphorylation by the src family PTK Lyn of the ITAM of the FcεRI β and γ subunits (19). As shown in Fig. 2B, phosphorylation of Lyn is strongly reduced when the cells are stimulated by Ags in the presence of C3a7 or C3a9, while the control peptide had no effect. Phosphorylation of the FcεRI β-chain was also examined and found to be decreased compared with that of cells treated by the control peptide (Fig. 2B). Tyrosine phosphorylation of PI3K, the enzyme regulating phosphatidylinositides' phosphorylation levels and therefore being a key coupling element, was also found to be reduced upon exposure to

Table I. *Inhibitory effect (IC$_{50}$, μM) of C3a and C3a-derived peptides on the IgE-mediated degranulation of RBL-2H3, BMMC, and RPMC[a]*

	RBL-2H3	BMMC	RPMC
C3a[b]	2,4	0,2	Activation[b]
C3a7	52	69	123
C3a9	51	69	105
Control peptide	No effect		No effect

[a] For the degranulation assay, suboptimal Ag-dose was used.
[b] C3a, when added to nonsensitized cells, stimulated only the serosal type RPMC but not the mucosal type RBL-2H3 and BMMC.
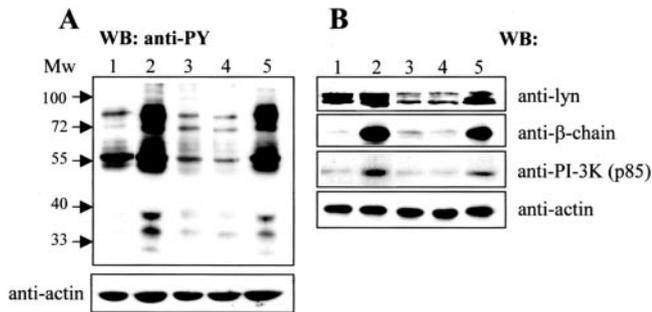
FIGURE 2. C3a7 and C3a9 inhibits tyrosine phosphorylation of Lyn, β subunit of FcϵRI, and PI3K. IgE-sensitized adherent RBL-2H3 cells were incubated for 5 min with C3a7, C3a9, or the control peptide before a suboptimal antigenic challenge. Cells were then lysed and immunoprecipitated with anti-phosphotyrosine Ab PT-66. Samples are as follows: *lane 1*, nonstimulated cells; *lane 2*, Ag-stimulated cells; *lane 3*, C3a7-pretreated cells; *lane 4*, C3a9-pretreated cells; *lane 5*, cells pretreated with control peptide. *A*, General phosphorylation pattern of RBL-2H3 cells. Western blot analysis was developed by PT-66 Ab. *B*, Phosphorylation of Lyn, β-chain of FcϵRI, and PI3K. Western blots were developed with Abs reacting with Lyn, the β-chain, and PI3K-specific Ab, respectively. Whole cell lysates were immunoblotted with anti-actin (*bottom rows*) to confirm that equal amounts were loaded. (One representative experiment of five is shown in each panel.)

peptides C3a7 and C3a9 (Fig. 2*B*). These peptides did not cause by themselves any change in the pattern of tyrosine phosphorylated proteins of RBL-2H3 cells (data not shown).

The peptides inhibit the Ag-induced transient elevation of $[Ca^{2+}]_i$

As the transient increase in $[Ca^{2+}]_i$ is one of the earliest events induced in Ag-activated cells, we examined the effect of C3a7 and C3a9 on this process in RBL-2H3 cells. As illustrated in Fig. 3, this process is markedly inhibited when peptides C3a7 or C3a9 were added to the cells 5 min before antigenic challenge. Both the initial rapid rise of $[Ca^{2+}]_i$, as well as the subsequent sustained elevated level assigned to the ion influx from the extracellular medium, were suppressed. In controls, where the peptides were added alone, no effect could be resolved.
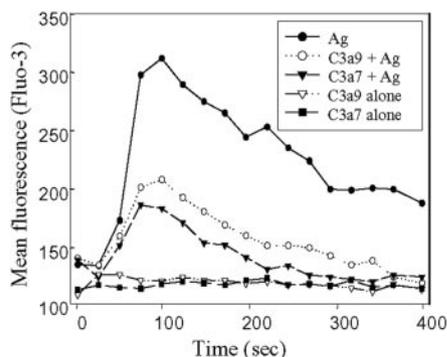

FIGURE 3. Inhibition of Ag-induced rise of cytosolic Ca^{2+} in mast cells by peptides C3a7 and C3a9. RBL-2H3 cells sensitized with Ag-specific IgE and loaded with fluo-3 acetoxymethyl ester were preincubated with 200 μM of peptides C3a7 and C3a9 for 5 min at room temperature. At 20 sec, Ag (5 ng/ml) was added to the cells, and the fluorescence intensity was monitored in time. In control samples, stimulation by the Ags in the absence of the peptides as well as the activity of peptides added alone was also tested. (One representative experiment of three is shown.)

C3a binds to the FcϵRI β-chain

Covalent cross-linking of C3a to BMMC. Because no C3aRs could be detected on mucosal type mast cells (7), we postulated that the peptides exert their inhibitory effect via interaction with a different cell membrane component, probably the FcϵRI. In earlier experiments, we observed no interference by C3a with the interaction between IgE and the FcϵRI (8); consequently, we assumed that it does not interact with the α-chain of the tetrameric FcϵRI complex. Because the ITAM-bearing β-chain had earlier been reported to act as an amplifier of the FcϵRI response (11), we set out to examine whether C3a interacts with this membrane protein on BMMC. Cells were incubated with C3a followed by adding the covalent cross-linking agent, BS[3] reagent. This was followed by cell lysis and immunoprecipitation with polyclonal C3a-specific Abs. Protein samples were separated by SDS-PAGE and analyzed by Western blotting, using FcϵRI β-chain-specific Abs. As shown in Fig. 4*A*, only a single protein was observed (*lane 1*), while none could be resolved in the control sample (*lane 2*). Because of the specific reactions of the covalently cross-linked complex to each component, i.e., for C3a on one hand and for the β-chain on the other, the only band appearing in *lane 1* with the mass of ~45 kDa is a covalent complex of C3a (9 kDa) and the β-chain (36 kDa). These results are in full agreement with our earlier ones obtained using RBL-2H3 cells (9).

SPR measurements of the interaction between C3a and the FcϵRI β-chain. To further investigate the interaction of C3a with the β-chain of FcϵRI, SPR measurements were conducted using immobilized oligo-peptides with sequences of the first and second extracellular loops of both the human and the rat tetraspan molecules. Human C3a and, as control, C5a were used as analytes. As shown in Fig. 4, *B* and *C*, C3a bound to the first extracellular loop of both the human and rat protein with K_d values of 250 and 520 nM, respectively. No interaction could be detected between C3a and the second extracellular loop of the cell membrane protein (data not shown). As expected from earlier results (8, 9), C5a did not react with any tested sequences of FcϵRI β-chain (data not shown).

C3a9 is colocalized with FcϵRI-bound IgE on intact RBL-2H3 cells

To visualize details on intact cells of the spatial relation between the C3a-derived peptides and the IgE bound to the FcϵRI, confocal laser scanning microscopic measurements were performed using a Cy3-conjugated IgE and a Cy5-conjugated C3a9 peptide on RBL-2H3 cells. A pixel-by-pixel analysis of the cross-correlation between Cy3 and Cy5 emission signals (cross-correlation coefficient: $\geq$0.54) indicated that the cell-bound peptide is highly colocalized with the IgE bound to the FcϵRI α subunit. This is in agreement with all the other findings and strongly supports that the peptide's interaction site is within the multisubunit FcϵRI receptor complex of mast cells (Fig. 4*D*). In addition, human monocytes lacking the β-chain had also been tested by confocal fluorescence microscopy and no detectable peptide binding was found (data not shown).

The above results were further supported by results of FRET measurements indicating proximity at the nanometer scale (18). A flow cytometric resonance energy transfer efficiency histogram (Fig. 4*E*), showing a mean FRET efficiency of 24%, clearly indicated a molecular proximity between FITC-C3a9 peptide and the Cy3-IgE bound to the surface of RBL-2H3 cells.

Discussion

Mast cells' secretion of inflammatory mediators is initiated either by FcϵRI-dependent or by independent agonists. In the case of

FIGURE 4. Interaction of C3a with the β-chain of the high-affinity IgE receptor. *A*, Detection of the covalent complex of C3a and β-chain of FcεRI on BMMC. C3a was cross-linked to BMMC by BS³ reagent and lysed. The cell lysate was immunoprecipitated with a C3a-specific rabbit Ab, run on 10% SDS-PAGE, and blotted to nitrocellulose sheet. Western blot analysis was developed by an Ab specific to the β-chain of FcεRI (*lane 1*). In the control sample (*lane 2*), no C3a was present. *B* and *C*, SPR measurements. Biotinylated peptides representing the first extracellular loop of the rat (*B*) and human (*C*) FcεRI β-chain were immobilized to sensor chips, and their interaction with C3a analyte was followed in real time. Calculation of binding parameters was done as described in *Materials and Methods*. (One representative experiment of two is shown.) *D*, Confocal microscopic images of RBL-2H3 cells labeled with Cy3-IgE and Cy5-C3a9. Equatorial slices (*upper row*) and a composite of three optical slices (512 × 512 pixels with 0.5-μm thickness) at the top of the cell (*lower row*) are shown for a representative cell. Cy3 fluorescence images (*left*), Cy5 fluorescence images (*middle*), and their overlay (*right*) are displayed. (One representative experiment of three is shown.) *E*, Histogram of FRET efficiency between FITC-C3a9 (donor) and Cy3-IgE (acceptor) on RBL-2H3 cells. The FRET efficiency was determined on a cell-by-cell basis, and the mean of the histogram (10⁴ cells) gave a value 24%, indicating a significant molecular proximity of the two labeled species.
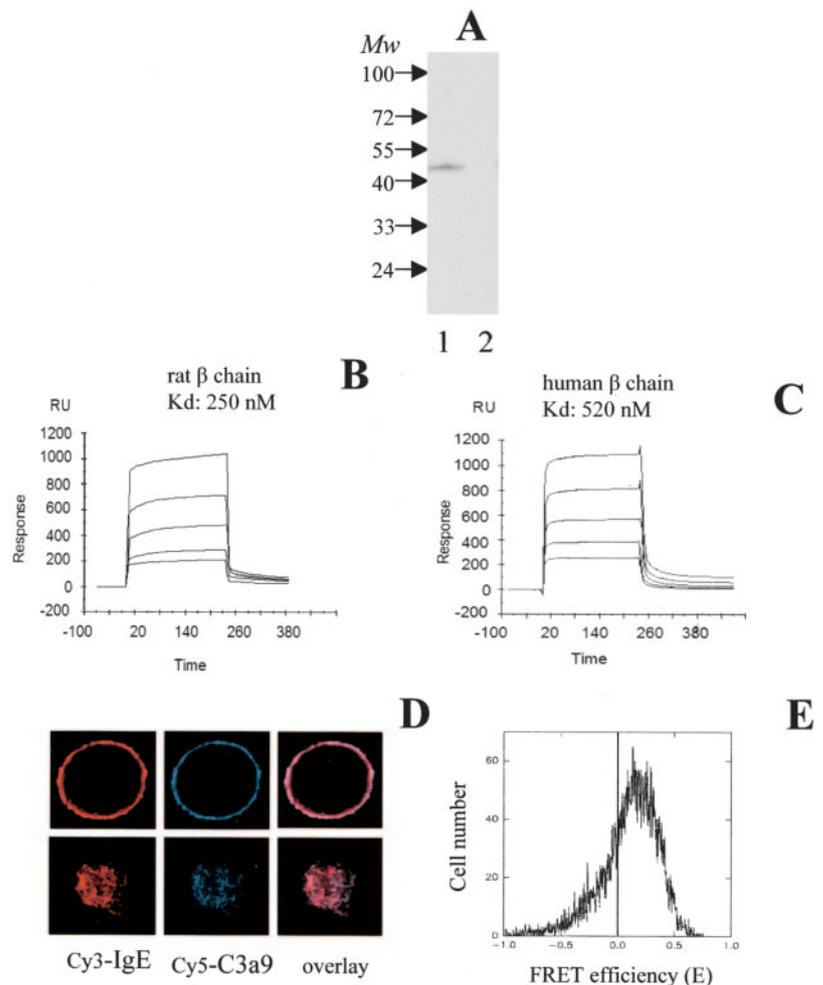
serosal type mast cells and basophils, micromolar concentration of the complement-derived anaphylatoxins, C3a and C5a, has been shown to provide a FcεRI-independent secretory stimulus, while mucosal type cells did not respond to these cationic peptides (7). Using mucosal type mast cells of the RBL-2H3 line, we had earlier demonstrated that C3a, but not C5a, inhibits the FcεRI clustering-induced response (8).

The FcεRI β-chain has been reported to act as an amplifier of mast cell response (10, 11). In addition, an association between atopy and variants of the β-chain had been revealed (20). Using BMMC, it has been shown recently that IL-10 and IL-4 reduces the expression of the β-chain, without affecting the α and γ subunits' expression (21). This down-regulation was found to be functionally significant.

Results presented here and earlier (8, 9) suggest a modulator function for this FcεRI component, i.e., being also capable of suppressing the cellular response. This notion that the FcεRI β-chain has also a capacity of inhibiting the cellular response received rather compelling support from recent studies of Rivera and colleagues (22). In the present study, we provided evidence from two distinct experimental approaches that C3a binds specifically to the β subunit of the receptor complex: specific Ab detection in Western blot and SPR measurements. Using the latter technique, we further showed that the first extracellular loop of the tetraspan β-chain (both rat and human) is specifically responsible for binding the inhibitor. These data strongly suggest that the inhibitory peptides do not penetrate the cells but rather are exerting their action probably by inducing a conformational change in the β-chain, an alteration that may affect its interactions with downstream effector elements. This process then leads to the inhibition of the Ag-induced mast cell secretory response. An alternative mechanism for the C3a uncoupling the cascade may be due to potential interference with raft association of the receptor, inhibiting its clustering as well. A detailed study of this process is ongoing in our laboratories.

As a further step, we found that synthetic peptides such as C3a7—representing a stretch of the natural sequence—as well as its modified sequence, C3a9, also exert inhibition of the FcεRI-mediated secretory response of both RBL-2H3 and BMM cells where the latter represents more authentic physiological conditions.

Complement peptide interaction with its receptors on serosal type mast cells and the subsequent coupling to the secretory response were extensively studied earlier (23, 24). The question emerged whether C3a7 and C3a9 peptides can still bear the anaphylatoxic capacity of the intact C3a molecule on serosal type mast cells? The observation that C3a7 and C3a9 do inhibit the FcεRI-mediated activation of the serosal type RPMC as well, while the intact C3a molecule, containing the sequence responsible for the anaphylatoxic capacity was indeed an effective secretagogue of serosal type mast cells, leads us to propose the presence of distinct separate "activating" and "inhibitory" sequences in C3a (Fig. 5).

Several early steps of the FcεRI stimulus-response coupling network were now examined to resolve at which stage do the C3a-derived peptides cause their inhibition. The earliest biochemical process identified so far is the activation of Lyn PTK (19), which
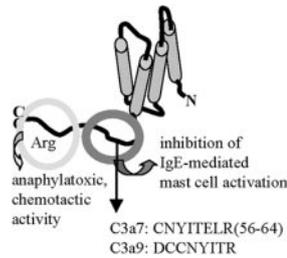
FIGURE 5. Schematic drawing of C3a structure indicating activatory and inhibitory motifs.

results in its own phosphorylation, activation, and FcεRI β- and γ-chain ITAM phosphorylation. Once the FcεRI subunits are phosphorylated, additional Lyn molecules are recruited to the FcεRIβ, whereas the phosphorylated FcεRIγ ITAM form distinct binding sites for the signal-amplifying PTK Syk (25). Hence, we investigated the effect of C3a-derived peptides on both the cells' general tyrosine phosphorylation pattern and that of specific proteins such as the FcεRI β subunit, Lyn, and PI3K in FcεRI-activated RBL-2H3 cells. These were all reduced substantially in the presence of C3a-derived peptides.

Downstream of Syk activation, the linker for activation of T cells plays an important role as a scaffold organizing additional signaling molecules that contribute to the rise of intracellular $[Ca^{2+}]$ level followed by degranulation and cytokine production (26). Mast cells have the feature of storing preformed cytokine TNF-α in their granules, which are immediately released, while secretion of de novo-synthesized TNF-α is sustained during several hours poststimulation in the late phase response (27, 28). In recent years, mast cell-derived TNF-α secretion was shown to play a crucial role in neutrophil recruitment, and effective immunity against enterobacterial infections (29) was extensively investigated. Our present findings show that C3a-derived peptides suppressed not only the FcεRI-induced degranulation but also the de novo TNF-α production and secretion.

The observation that all these FcεRI-proximal signal-coupling processes are suppressed suggests that the site of the peptides' action is indeed upstream to the Ca mobilization and PTK Syk activation, i.e., at the level of FcεRI complex. Indeed, both results of the peptides' binding obtained with SPR and the coprecipitation experiments, as well as those of the FRET measurements, all suggest that the peptide binding site is located on the β-chain of the FcεRI complex.

In conclusion, our data are consistent with a novel general regulatory mechanism of mast cells' response to the FcεRI stimulus because peptides C3a7 and C3a9 have been shown to effectively inhibit this process in mucosal as well as serosal type mast cells. Thus, these peptides are capable of modulating the response already at the very early coupling events, leading to inhibition of both early and late phase secretion. Our preliminary data suggest that the inhibitory peptides may also influence membrane compartmentation (raft association) of FcεRI thought to be critical in formation of the coupling platform. Future experiments aim at understanding the details of interaction between C3a7 and C3a9 peptides and the FcεRI complex, its consequence(s) at membrane level, and in vivo testing of the peptides in intact animal model systems for allergy.

Acknowledgments

Disclosures

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