

A role for C5a in augmenting IgG-dependent histamine release from basophils in chronic urticaria

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Background: Histamine release in chronic urticaria is initiated by cross-linking of the α subunit of Fc ϵ RI by means of IgG antibody, followed by complement activation.

Objective: We sought to further elucidate the mechanism by which complement augments histamine release and to assess the role of C5a.

Methods: We first quantitated the ability of purified C5a to initiate basophil histamine release and to be inhibited by antibody directed to the C5a receptor. Using this antibody, we quantitated its ability to inhibit histamine release induced by sera from patients with chronic urticaria. We also compared the ability of normal serum, C5-depleted serum, and C5-depleted serum after reconstitution with C5 to augment histamine release by IgG isolated from patients with chronic urticaria.

Results: As the concentration of C5a was increased up to 50 ng/mL, the percentage of histamine release increased and reached a plateau of 40% to 50%; this was inhibited by antibody to the C5a receptor. Preincubation of basophils with antibody to the C5a receptor inhibited basophil histamine release from 15 sera tested, with a range of 4% to 39%. Histamine release caused by patient IgG was augmented when normal serum was added but not when C5-depleted serum was substituted for normal serum. Augmentation of histamine release by patient IgG was again obtained when C5-depleted serum was reconstituted with C5.

Conclusion: Our conclusion is that pathogenic IgG cross-links the IgE receptor directly to cause histamine release, and activation is augmented by complement. C5a is the complement agonist that is responsible for the augmented histamine release. (*J Allergy Clin Immunol* 2002;109:114-8.)

Key words: Chronic urticaria, basophils, histamine release, complement, IgE receptor, α subunit

Approximately 30% to 40% of patients with chronic urticaria have a circulating IgG antibody directed to the α subunit of the IgE receptor,¹⁻⁷ which releases histamine from basophils^{1,2,4-6} or cutaneous mast cells.^{3,7} An additional 5% to 10% of patients have IgG anti-IgE antibody,^{8,9} and an unknown percentage can have both. How-

Abbreviation used

HSA: Human serum albumin

ever, the magnitude of histamine release is increased when whole serum is compared with purified IgG, and the difference appears to be due to complement activation.¹⁰

A role for complement is supported by absence of serum enhancement of pathogenic IgG if the serum is deficient in the second or fifth components of complement.⁷ Because C5-depleted serum should activate C4 and C3 normally and also release C4a or C3a anaphylatoxins, the presumption has been that C5a is the peptide responsible for the augmentation by complement. When we previously used peptides purported to interfere with the interaction of C5a with its receptors, they had significant agonist activity so that the results were indeterminate and did not allow confirmation of earlier observations.⁶ In this article we use an inhibitory antibody to the C5a receptor and reconstitute C5-depleted serum to assess the contribution of C5a to complement augmentation of IgG-dependent histamine release.

METHODS

Patients and control subjects

We selected sera (from 1997-2000) from patients with recurrent hives of greater than 12 weeks' duration, occurring at least 4 times a week, with individual lesions lasting over 4 hours. Patients with physically induced urticaria or clinical evidence of urticarial vasculitis were excluded. Antihistamine treatment was stopped at least 48 hours before serum samples were collected, and none of the patients were taking tricyclic antidepressants, corticosteroids, or immunosuppressive drugs at the time of venipuncture or in the recent past. Sera from normal subjects served as controls.

Leukocyte isolation

Fifteen milliliters of whole blood was mixed with 1.5 mL of 0.2 mol/L 10% EDTA and with 3.0 mL of 3% dextran-3% glucose-0.15 mol/L NaCl saline solution. It was sedimented for 90 minutes, the supernatant was removed, and the cells were placed into 50-mL conical tubes that were washed 3 times with HBSS-human serum albumin (HSA) containing HBSS (Gibco BRL), 4 mmol/L HEPES, and 0.3% HSA (Sigma). The cells were then resuspended in HBSS-HSA containing 2 mmol/L CaCl₂ and 1 mmol/L MgCl₂, and the leukocyte concentration was adjusted back to the original concentration of blood.

Purification of IgG

IgG was isolated with protein G (Pharmacia Biotech) affinity chromatography according to the manufacturer's instructions. The flow through was also collected. Eluates containing IgG were run

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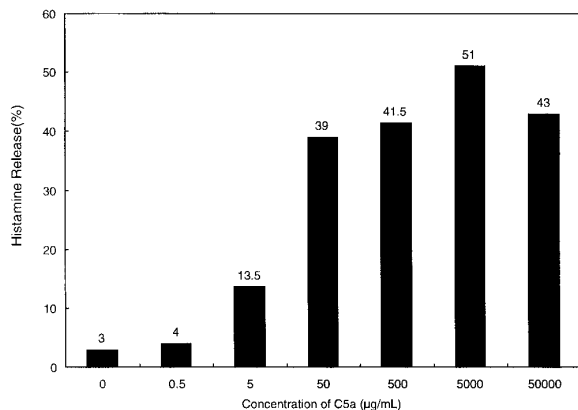


FIG 1. Histamine release (percentage) from donor basophils stimulated with increasing concentrations of C5a from 0 to 5000 µg/mL.

through an anti-IgE affinity column to deplete IgE. Immunoaffinity-purified, polyclonal goat anti-human IgE (Chemicon International, Inc) was immobilized on Sepharose (UltraLink, Biosupport Medium, Pierce), according to the manufacturer's guidelines. The purified IgG fractions were concentrated back to their original serum volume with a centrifugal filter device (Centricon and Centrprep, Millipore). The purity of IgG in the final solution was confirmed by separating the proteins on SDS-PAGE and staining the gel with silver stain. No other protein was detected. A Western blot was done to confirm that the final solution does not contain IgM. The IgG concentrations in whole serum and IgG fractions were measured by means of the Bradford method.

Activation of C5-depleted sera reconstituted with C5

C5-depleted sera (Quidel) were reconstituted with C5 (Quidel) to a final concentration of 70 µg/mL and to 19.6 mmol/L with CaCl₂. Incubation of serum with heat-aggregated gamma globulin was used to activate complement and test the ability of C5 to reconstitute C5-depleted serum. Heat-aggregated gamma globulin was prepared by dissolving 20 mg/mL gamma globulin (Sigma) in veronal-buffered saline containing 0.1% gelatin, 1.5×10^{-4} mol/L CaCl₂, and 1.0×10^{-3} mol/L MgCl₂ and heated at 63°C for 30 minutes. Reaction mixtures routinely consisted of 30% heat-aggregated gamma globulin and 70% C5-depleted serum before and after reconstitution with C5. Mixtures were incubated for 60 minutes at 37°C with frequent stirring and then centrifuged for 15 minutes at 20°C at 3000 rpm. Then the serum was carefully aspirated and immediately used in the histamine release assay. Patient IgG was then incubated with C5-depleted serum that was reconstituted with C5, and these mixtures were incubated with leukocytes and assayed for histamine release.

Histamine-release reaction

One healthy blood donor whose cells were reactive with anti-IgE antibody and responsive to many of the patient sera was chosen for the studies reported here. This donor was atopic, with a serum IgE level of 25 IU/mL.

Seventy-five microliters of activated serum, buffer, or C5a (Sigma) was incubated with 75 µL of leukocyte suspension for 40 minutes at 37°C. After incubation, the supernatants were separated by means of centrifugation at 3000 rpm for 5 minutes at 4°C, and histamine release was determined. Two replicate aliquots of cells were boiled to determine total basophil histamine content.

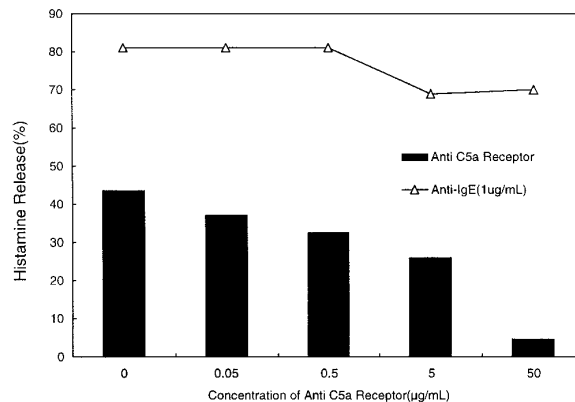


FIG 2. Basophils were preincubated with a 10-fold increasing concentration of anti-C5a receptor/CD88 from 0 to 50 µg/mL and stimulated either with C5a at 1000 µg/mL or anti-IgE at 1 µg/mL. Histamine release (percentage) was then measured.

Histamine assay

Histamine release was measured with enzyme immunoassay (Immunotech International), according to the manufacturer's instructions. All histamine-release experiments were done in duplicate, and the results are expressed as a percentage of total histamine content. Spontaneous histamine release from the cells was less than 8% of total histamine.

Statistical analysis

We used the Student *t* test for comparing paired means. A *P* value of less than .05 was considered statistically significant.

RESULTS

Histamine release from basophils stimulated with different concentrations of C5a

Basophils were stimulated with increasing concentrations of C5a from 0 to 5000 ng/mL, and histamine release (percentage) was quantitated (Fig 1). As the concentration of C5a was increased up to 50 ng/mL, the percentage of histamine release increased and then reached a plateau of 40% to 50%, as the C5a was further increased 1000-fold.

Inhibition of histamine release from sera of patients with chronic urticaria using anti-C5a receptor

We next assessed the ability of antibody directed to the C5a receptor (Accurate Chemical and Scientific Corp) to inhibit the complement-dependent increment in histamine release observed with patient sera. First, we determined the concentration of anti-C5a receptor that causes a significant diminution in basophil responsiveness to C5a. Basophils were preincubated with 10-fold increasing concentrations of anti-C5a receptor (between 0.05 µg/mL and 50 µg/mL) and challenged with C5a at 1000 µg/mL or with anti-IgE at 1 µg/mL. There was a dose-dependent decrease in C5a-dependent histamine release that approached zero at 50 µg/mL. This concentration was chosen for subsequent experiments (Fig 2). The anti-

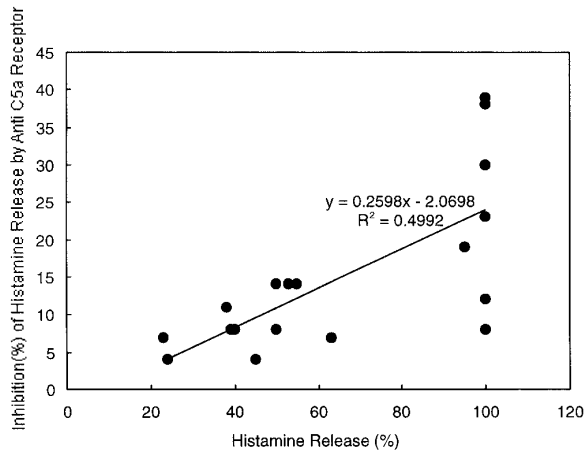


FIG 3. Fifteen patient sera whose histamine releases exceeded 20% were selected in this experiment. Basophils were preincubated with buffer or anti-C5a receptor (50 μ g/mL) and then were stimulated with patient sera. The X axis indicates the histamine release (percentage) with basophils preincubated with buffer and stimulated with patient sera, and the Y axis indicates the inhibition of histamine release (percentage) on preincubation of basophils with anti-C5a receptor.

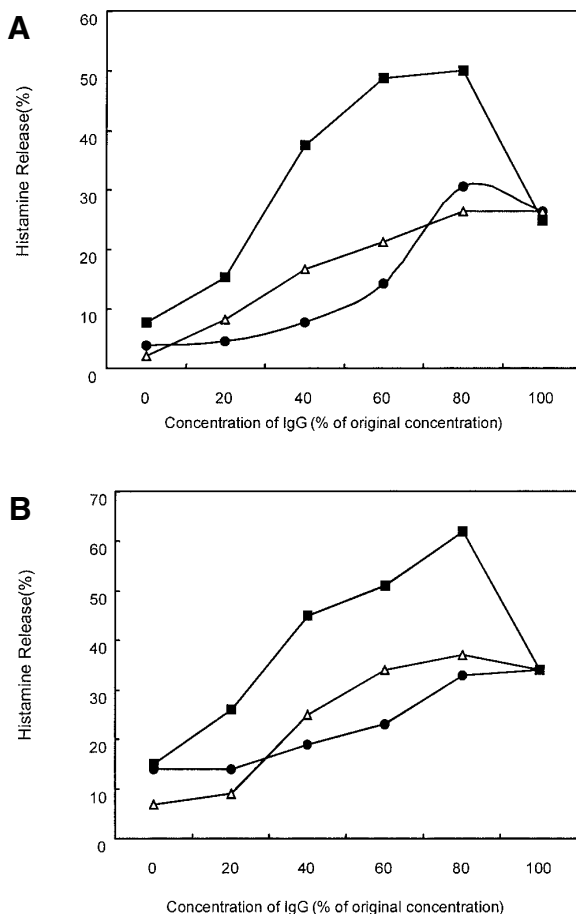


FIG 4. Histamine release (percentage) from human basophils stimulated with various concentrations of patient IgG plus buffer (open triangles), normal serum (filled squares), or C5-depleted serum (filled circles). **A** and **B** represent 2 different patients studied in this matter.

C5a receptor treatment did not inhibit histamine release by anti-IgE.

We preincubated 50 μ g/mL anti-C5a receptor/CD88 with basophils for 60 minutes. Then treated and untreated basophils were incubated with 15 sera from patients with chronic urticaria (Fig 3). All 15 sera demonstrated inhibition that varied between 4% and 39% because of anti-C5a receptor. There was good correlation between the absolute histamine release with sera from patients with chronic urticaria and the percentage of inhibition of histamine release ($R^2 = 0.5$) attributable to C5a.

Effect of complement on basophil histamine release with patient IgG and C5-depleted serum

Although pathogenic IgG can cross-link the IgE receptor directly to cause histamine release, the activation is augmented by complement.¹⁰ The presumption is that C5a is the complement agonist that is responsible for the augmented histamine release, and our studies have tried to further clarify this issue.

First, purified IgG from patients whose sera tend to release histamine well was concentrated to the serum IgG level of each patient serum. For patients 595 and 788, the IgG concentrations were 20 and 11 mg/mL, respectively. The IgG was added to normal serum, buffer, and C5-depleted serum to examine the effect on histamine release from basophils (Fig 4). In Fig 4 the middle line indicates the percentage of histamine release obtained with purified IgG when tested at various concentrations, the top line represents the percentage of histamine release when IgG was added to normal serum, and the bottom line represents the histamine release when IgG was added to C5-depleted serum.

Histamine release was augmented when normal serum was added but not when C5-depleted serum was added. Histamine release with the latter serum added was similar to that of the IgG alone.

Release of histamine by activated serum

Incubation of heat-aggregated gamma globulin with human serum generates histamine-releasing activity in the serum. We wished to assess the requirement for C5 by reconstituting C5-depleted serum with purified C5. We first tested this with aggregated IgG. A mixture of C5-depleted serum plus C5 was incubated with heat-aggregated gamma globulin, and basophil histamine release was quantitated. Optimal histamine release was noted when the reaction mixture consisted of 30% heat-aggregated gamma globulin and 70% C5-depleted serum reconstituted with C5.

Next, purified IgG fractions from 5 patients, buffer, and normal IgG were mixed with normal serum, C5-depleted serum, or C5-depleted serum reconstituted with C5. Then these mixtures were incubated with leukocyte suspensions with frequent mixing and centrifuged. The supernatant was aspirated and immediately assayed for histamine. The results are shown in Fig 5. The percent-

age of histamine release observed on incubation of buffer with C5-deficient serum was 17%, and this was unchanged on addition of C5 (histamine release, 16%). These values were subtracted from the histamine release percentage observed on incubation of patient IgG or normal IgG with C5-depleted serum or reconstituted C5-deficient serum, as shown in Fig 5. The histamine release observed on incubation of pathogenic IgG with reconstituted serum was statistically significantly greater ($P = .01$) for all 5 patients when compared with incubation of pathogenic IgG with C5-depleted serum, whereas control IgG had no histamine-releasing capability.

DISCUSSION

Approximately 30% to 40% of patients with chronic urticaria have demonstrable IgG autoantibodies directed to the α subunit of the IgE receptor,¹⁻⁷ and an additional 5% to 10% have anti-IgE antibodies.^{8,9} Initially, cross-linking of the α subunit was assumed to lead to cell activation and secretion, as demonstrated with a rat basophil leukemia cell,^{1,4} human basophils,^{1,2,4-6} and cutaneous mast cells.^{3,7} However, subsequent studies reported that purified IgG from patient sera were inactive or poorly reactive with cutaneous mast cells or human basophils and that a source of serum complement is needed for activation to occur.^{6,7} Thus an anaphylatoxic complement fragment, presumably C5a, was thought to mediate the direct activation of basophils and mast cells. The most recent data demonstrate that activation occurs with IgG alone, which is augmented by complement activation,¹⁰ and it is with low IgG input that activation appears largely complement dependent. The inability of C2-deficient serum to augment IgG activation suggests activation by the classical pathway.⁷ However, proof that C5a is liberated and is the cause of the histamine release is lacking. C3a or even C4a are alternatives, although C5a is the most potent on a molar basis¹¹ if vascular permeability in human skin is the criterion.

Our approach was to first compare histamine release with patient IgG at serum concentration with that obtained on addition of the IgG to normal serum or C5-depleted serum. Although it has been reported previously that C5-depleted serum does not support the augmentation of histamine release seen with normal serum,⁷ this serum is immunologically depleted and may not be identical to serum collected from patients with C5 deficiency. Unfortunately, C5-deficient patients are not currently available. Thus we believed it to be essential that the C5-depleted serum be reconstituted with C5 to demonstrate that it then behaves comparably with normal serum and to thereby ensure that other complement components have not been significantly altered by the procedure. Our data do indeed support a critical role for C5 in the process (Fig 5), and we can infer that C3a or C4a are contributing very little. We then used antibody to the C5a receptor to inhibit activation of basophils with sera of patients with chronic urticaria. An optimal dose was chosen (Fig 2), and the inhibition observed averaged 15%,

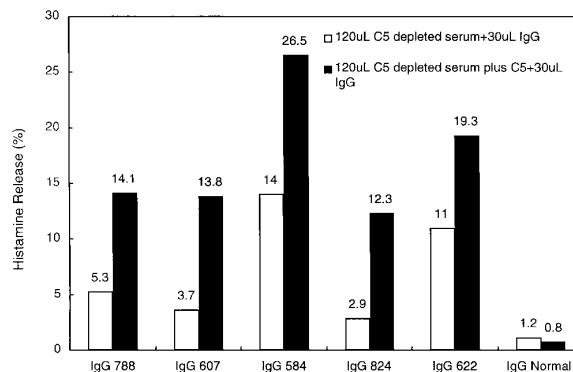


FIG 5. Histamine release (percentage) observed on incubation of 5 patients' IgG or normal IgG with C5-depleted serum (open bars) or C5-depleted serum reconstituted with C5 (black bar).

with a maximum at 39%. The percentage of inhibition appeared proportional to the percentage of histamine release so that sera with particularly high histamine release have a greater contribution by complement (Fig 3), although the IgG reactivity is also substantial. The data (Fig 4) also support that at lower IgG input (ie, less than normal serum concentration), maximal histamine release can still be observed with a greater percentage contribution by complement.

Our data reaffirm that cross-linkage of the IgE receptor α subunit by pathogenic IgG of patients with chronic urticaria causes histamine release, which is augmented by complement, and we now demonstrate that C5a is responsible for that augmentation. Although the cellular infiltrate seen in chronic urticaria is reminiscent of an allergic late-phase reaction,¹²⁻¹⁴ some of the differences observed may be attributable to the chemotactic activity of C5a. Patient variance in the contribution of IgG and complement to cell activation may contribute to the heterogeneity observed among patients.

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