

In chronic idiopathic urticaria autoantibodies against FcεRII/CD23 induce histamine release via eosinophil activation

A. Puccetti^{†1}, C. Bason^{†1}, S. Simeoni[‡], E. Millo[‡], E. Tinazzi[‡], R. Beri[‡], D. Peterlana[‡], G. Zanoni[§], G. Senna[¶], R. Corrocher[‡] and C. Lunardi[‡]

**Department of Experimental Medicine, University of Genova, Genova, Italy, †Institute Giannina Gaslini, Largo Gerolamo Gaslini, Genova, Italy, ‡Department of Clinical and Experimental Medicine, University of Verona, Verona, Italy, §Department of Pathology, Section of Immunology, University of Verona, Verona, Italy and ¶Allergy Unit, Verona General Hospital, Verona, Italy*

Summary

Background Chronic idiopathic urticaria is a common skin disorder characterized by recurrent, transitory, itchy weals for more than 6 weeks. An autoimmune origin has been suggested based on the findings of auto-antibodies (Abs) directed against either the α subunit of the high-affinity IgE receptor or the IgE molecule in nearly half of the patients.

Objective To identify other autoantigen targets in patients with chronic idiopathic urticaria.

Methods We used pooled IgG derived from 133 patients with chronic idiopathic urticaria to screen a random peptide library to identify disease-relevant autoantigen peptides. Among the identified peptides, one was recognized by the vast majority of patients' sera. Abs against this peptide were affinity purified from the patients' sera and assayed for their ability to induce histamine release from basophils.

Results We identified a peptide that showed similarity with the low-affinity IgE receptor (FcεRII/CD23) expressed on lymphomonocytes and eosinophils. Anti-peptide IgG Abs purified from the patients' sera bound cell surface CD23 and were able to induce histamine release from basophils. This effect appeared to be mediated by the release of major basic protein from eosinophils upon engagement of CD23. The same effects were obtained with the sera from mice immunized with the CD23 peptide.

Conclusion Our results indicate that patients with chronic idiopathic urticaria have Abs against CD23 and that eosinophils, which infiltrate the skin of these patients, play a crucial role in maintaining the disease through the release of major basic protein upon engagement of the low-affinity IgE receptor by such auto-Abs.

Keywords anti-CD23 antibody, chronic idiopathic urticaria, low-affinity IgE receptor (FcεRII/CD23), major basic protein (MBP), monocyte chemoattractant protein-1 (MCP-1), random peptide library

Submitted 22 April 2005; revised 10 August 2005; accepted 20 September 2005

Introduction

Chronic urticaria is a common skin disorder characterized by recurrent and transitory itchy weals [1] occurring on a regular basis for more than 6 weeks [2]. In the majority of the patients, the cause remains unknown and in this case chronic urticaria is defined as chronic idiopathic urticaria (CIU) [2].

CIU appears to be of autoimmune origin in a substantial percentage of cases [2] since approximately 35–40% of patients have circulating IgG antibodies (Abs) directed against the α -subunit of the high-affinity IgE receptor [2, 3], and an

additional 5–10% have Abs against the IgE molecule [2, 3]. These autoantibodies induce basophils and mast cells to release histamine [4, 5].

CIU may be associated with autoimmune thyroiditis, mainly Hashimoto's thyroiditis [6]: indeed, 27% of patients with CIU have anti-thyroglobulin Ab, anti-microsomal Ab or both, and 19% of them have abnormal thyroid function. There is no evidence that these antithyroid Abs may be pathogenic for the development of the cutaneous manifestations; thyroiditis appears to be a parallel abnormality and may reflect the presence of an underlying autoimmune process [2].

The autoimmune origin of CIU is suspected when the skin test with autologous serum is positive. This simple test consists in the appearance of a typical weal with pruritus after the subcutaneous injection of 0.1 mL of autologous serum [7]. The presence in the serum of autoantibodies directed either against the alpha chain of the high-affinity IgE receptor or

¹These authors contributed equally to this work.

Correspondence: Claudio Lunardi, Department of Clinical and Experimental Medicine, University of Verona Policlinico, G.B. Rossi, P.le L.A. Scuro, 37134 Verona, Italy.

E-mail: claudio.lunardi@univr.it

against IgE Abs or against unknown autoantigens may explain such reactivity [8].

An alternative pathway for allergen-independent histamine release by basophils and mast cells is through the major basic protein (MBP) released by eosinophils [9, 10]. Indeed, incubation of human basophils and mast cells with MBP results in a non-cytotoxic, dose-dependent histamine release from target cells within 30–45 min. Such a mechanism contributes to the perpetuation of the symptoms in the "late-phase" of an allergic reaction [10] and may be relevant in CIU since eosinophils infiltrate the skin of these patients [4]. Moreover, MBP itself can cause a cutaneous weal and flare reaction when injected into human skin [11, 12].

IgE binding to the low-affinity IgE receptor (FcεRII/CD23) on eosinophils of atopic subjects results in the activation of these cells and in the release of MBP, suggesting that FcεRII/CD23 plays a pivotal role in eosinophil activation. Indeed, in atopic and nonatopic subjects, binding to FcεRII/CD23 on eosinophils induces increased eosinophil migration and expression of adhesion molecules [13].

Among the chemokines with IgE-independent histamine-releasing activity, the monocyte chemoattractant protein (MCP-1) is the most potent [14]. MCP-1 may have a role in the development of allergic responses [15] and an increased MCP-1 level is present in bronchoalveolar lavage fluid in allergic asthmatic patients [16]. Monocytes express surface FcεRII/CD23 and therefore engagement of this receptor may induce MCP-1 release.

Since more than half of the patients with CIU do not show evidence of auto-Abs either against the high-affinity IgE receptor or against IgE Abs, we aimed at identifying novel pathogenetically relevant autoantigens by using the random peptide library approach, which we have used to define disease-fit peptide epitopes in other autoimmune diseases [17, 18]. In this study, we have identified a pathogenetically relevant autoantigen peptide-sharing homology with FcεRII/CD23 and we demonstrate histamine release through the production of MBP by eosinophils upon engagement of FcεRII/CD23 by specific autoantibodies.

Methods

Patients

We enrolled patients with CIU between January 1999 and December 2002. Diagnosis of the disease was based on the presence of itchy weals for more than 6 weeks and exclusion of any known cause of chronic urticaria such as food allergy or intolerance, drugs, urticarial vasculitis, physical urticaria, infectious agents, parasites, or autoimmune diseases [19]. We obtained sera from 133 patients (88 women and 45 men), with a mean age of 41.5 years (range 16–70), affected by CIU, before starting steroids or immune-suppressive therapy with cyclosporin-A. At the time of venopuncture, patients were free from antihistamines. Controls consisted of 90 people matched for age and sex, with a mean age of 37 years (range 20–68).

We took blood samples from patients and controls after obtaining oral or written informed consent.

Antithyroglobulin Abs and antimicrosomal Abs were measured using a commercially available ELISA kits (Diamedix, Miami, FL, USA) according to the manufacturer's instructions.

Soluble CD23 was measured by an ELISA assay (Bender Medsystems, Vienna, Austria). IgE levels were detected by the nephelometric technique.

Screening of a random peptide library with pooled sera immunoglobulin G obtained from patients with chronic idiopathic urticaria

To define disease-specific peptide epitopes, we purchased the FliTrx random dodecamer peptide library, which displays peptides on the surface of *Escherichia coli* using the major bacterial flagellar protein (FliC) and thioredoxin (Lu), from Invitrogen (Carlsbad, CA, USA). We screened the library with pooled IgG isolated through chromatography from the sera of 133 patients with CIU according to the manufacturer's instructions (FliTrx Panning Kit, Invitrogen) [17, 18]. After five sequential rounds of biopanning experiments, each preceded by a prepanning step with pooled IgG obtained from normal donors to remove bacteria that unspecifically bind Igs outside the antigen-binding site, the enriched library was grown and single colonies were expanded and induced to express the fusion peptides with tryptophan. *E. coli* bacteria were lysed in sample buffer. Lysates were separated in SDS-PAGE, transferred onto nitrocellulose and tested in Western blot to identify clones expressing a fusion peptide recognized by the pooled IgG fraction obtained from patients with CIU. Binding of pooled IgG to the fusion peptide was detected using a phosphatase-labelled anti-human IgG Ab (Sigma, St. Louis, MO, USA). DNA was extracted from positive clones and sequenced with an Automated Sequencing Apparatus (Perkin Elmer, Norwalk, CT, USA). A set of 15 peptides, out of the 25 peptides obtained from the last biopanning round, was synthesized and used in an ELISA assay to test individual patients' sera as well as control IgG immunoglobulins.

Peptide synthesis

We obtained synthetic peptides by solid-phase synthesis with the 9-fluorenylmethoxycarbonyl strategy [20]. We also synthesized an irrelevant control peptide, chosen on the basis of its biochemical properties, and its lack of similarity with all the peptides used in the study and all known human autoantigens, as verified with basic local alignment search tool program (BLAST) via the National Center Biotechnology Information (NCBI) BLAST network service.

Enzyme-linked immunosorbent assay

The direct and competitive ELISA assay for Ab binding to the synthetic peptides has been described elsewhere [21]. Briefly, synthetic peptides were used at a concentration of 20 µg/L in phosphate-buffered saline (PBS) to coat polystyrene plates (Immulon II, Dynax, Ashford, UK). After blocking for 1 h with 3% bovine serum albumin (BSA) in PBS, the Abs, diluted in 1% BSA, were added and incubated overnight at 4 °C. The plates were then washed once with 1% Tween in PBS and twice with PBS.

An alkaline phosphatase-conjugated anti-human IgG anti-serum (Sigma) in diluting buffer was then added and incubated for 3 h at room temperature. After washing, the bound enzymatic activity was measured with *p*-nitrophenyl-

phosphate (Sigma). Ninety sera samples diluted 1 : 200 from normal subjects were used as the control group. Optical density values higher than the mean + 3 SD of each serum dilution sample of the control group were considered positive. For the competitive assays, the amount of Ab that gave 50% of the maximum binding to the peptide was pre-incubated with different amounts of competitors or buffer for 1 h at 37 °C and then transferred to the peptide-coated plates. The assay was then carried on as the direct binding assay.

Isolation of peptide-specific antibodies

To affinity purify the anti-peptides Abs, we coupled the synthetic peptides (5 mg peptide/g of dried Sepharose powder) to Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Serum samples diluted in PBS were applied to the columns. The columns were washed with PBS. Bound Ig were eluted with 0.1 M glycine pH 2.5 and dialysed against PBS.

Animals

Male Balb/c mice at 6–8 weeks of age were purchased from Charles River Laboratories (Lecce, Italy) and maintained in our own animal facilities. All experimental protocols were approved by the Ethical Committee for Animal Research of the University of Genova. To prepare a mouse antiserum directed against the CD23 peptide sequence, Balb/c mice were immunized subcutaneously with CD23 peptide coupled to the carrier protein keyhole limpet haemocyanin (KLH) (Sigma). After the fourth injection the animals were bled and the serum was collected.

Isolation of eosinophils

To prepare human eosinophils, granulocyte-enriched blood fractions were obtained from a buffy coat by Percoll gradient and purified using CD16 microbeads (Miltenyi Biotech, Bergish Gladbach, Germany). The resulting cell population was highly enriched with eosinophils (80–85% eosinophils).

Fluorescence-activated cell sorting analysis

Fluorescence-activated cell sorting (FACS) analysis was performed to detect the binding of affinity-purified antipeptide Abs to the human B cell line 22-1, known to express CD23, or to human eosinophils. Cells were incubated with commercially available anti-CD23 monoclonal Ab (Becton Dickinson, Mountain View, CA, USA), with mouse polyclonal anti-CD23 peptide antiserum, with affinity-purified anti-peptide C and anti-peptide CD23 Abs isolated from the sera of patients with CIU. After washing, cells were stained with R-phycoerythrin goat anti-mouse γ 1 (RPE) or with RPE-conjugated Fab2 fragment goat anti-mouse IgG (Jackson Laboratories, Bar Harbor, MN, USA), or with RPE-conjugated goat anti-human IgG and analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA.).

Immunoprecipitation of the CD23 molecule with anti-peptides antibodies

For detection of the CD23 molecule, the human B cell line 22-1, expressing CD23, was lysed and immunoprecipitated

with human affinity-purified Abs against the peptides C and CD23 and with the mouse antiserum directed against the CD23 peptide. Immunoprecipitates were resolved by SDS-PAGE and transferred onto Immobilon P membrane (Millipore, Bedford, MA, USA). Blots were incubated with the mouse antiserum raised against CD23. The Renaissance Chemiluminescence Kit (NEN, Boston, MA, USA) was used for detection.

Detection of histamine release in the peripheral blood of normal donors

We used a commercially available histamine immunoassay kit (Immunotech, Marseille, France). The enzyme assay for histamine is based on the competition between the histamine to be assayed in the sample and its enzyme conjugate, histamine-alkaline phosphatase, used as a tracer for binding to the Ab, coated onto microwells. The histamine in the sample to be tested and the histamine-alkaline phosphatase conjugate, when added to the microtitre well, compete for the binding to the Ab coated on the well. After incubation, the wells are rinsed to remove non-bound components. The bound enzymatic activity is measured by adding a chromogenic substrate. The intensity of the colour depends on the concentration of histamine in the sample. The concentration of histamine is calculated on the basis of a standard curve. Blood (diluted 1/7 in a diluting buffer) of two healthy individuals was incubated with 10 μ g of human-purified anti-CD23 peptide Abs, 10 μ g of human-purified anti-peptide C Abs and 10 μ L of antiserum obtained from mice immunized with the CD23 peptide or with 10 μ g of human Abs affinity-purified against an irrelevant peptide. Total cell lysate, which indicates the total amount of histamine present in the blood sample, was used as a positive control (total histamine release) and the blood sample incubated without Abs was the negative control (spontaneous histamine release). The samples were incubated for 30 min at 37 °C and then centrifuged, and the supernatants were collected and tested in the assay to detect histamine.

For the inhibition experiments of histamine release, the Abs were pre-incubated with 50 μ g of the corresponding peptide. For neutralization of MBP, 10 μ g of anti-MBP monoclonal Ab (Chemicon International, Temecula, CA, USA) was added to the sample together with the human anti-CD23-purified Ab. The remainder of the assay was then carried out as described above.

Detection of major basic protein and monocyte chemoattractant protein-1 in the supernatants used for the histamine release assay

To detect the levels of eosinophil MBP, we used the same supernatants as the ones used for the histamine release assay. At the end of the 30 min incubation with the Abs, the samples were centrifuged and the supernatants were collected. MBP was detected using Western blot analysis.

For Western blot, the supernatant was run in an SDS-PAGE and blotted onto a nitrocellulose paper. The blot was then incubated with a commercially available monoclonal Ab against MBP.

Alternatively, 50 μ L of the supernatant was spotted onto a nitrocellulose paper using a Bio-Dot apparatus (Biorad, Hercules, CA, USA).

After colour developments, the bands and the spots were analysed with the Versa Doc apparatus (Biorad), and the amount of MBP was quantified by densitometric analysis of the spots.

The presence of MCP-1 in the supernatants used for the histamine release assay was investigated using the commercially available test MCP-1 Biotrak Elisa (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Statistical analysis

We analysed qualitative variables with the χ^2 test. We assessed quantitative data with the Mann–Whitney *U*-test for the variables that did not present a normal distribution. We considered $P < 0.05$ to be significant.

Results

Immunoglobulin E antibodies, sCD23 levels and prevalence of antithyroid antibodies in the sera of patients and controls

No significant difference was found in IgE Ab levels between patients and controls, median value of 68.5 IU/mL (IQR 32.5–141) and 54 IU/mL (IQR 20–109), respectively, $P = 0.07$, by the Mann–Whitney *U*-test.

The median values of sCD23 levels were 16.6 IU/mL (IQR 7.4–29.5) in the patients and 22.1 IU/mL (IQR 17.4–27.6) in the control group, $P = 0.065$ by the Mann–Whitney *U*-test.

The prevalence of antithyroid Abs was significantly higher in patients with CIU than in controls; in particular, antithyroglobulin Abs were present in 17 out of 133 patients (13%) and in two out of 90 controls (2.2%), $P < 0.011$ by χ^2 test and anti-microsome Abs were present in 26/133 patients (19.5%) and in 7/90 controls (7.5%), $P < 0.025$ by χ^2 test.

Peptides obtained with the random peptide library screening and recognized at a high frequency by patients' sera

By screening a dodecamer random peptide library with pooled IgG obtained from the patients affected by CIU, we identified three peptides (peptide A: AILLAISVRQH, peptide B: GLRLDADKLVGV and peptide C: GLRGLKLEGQQQL) that were specifically recognized by a large percentage of the individual patients' serum IgG by both direct and competitive ELISA. In particular, peptide A was recognized by 51.9% (69 positive patients out of 133) of patients' serum IgG, peptide B was recognized by 57.8% of patients (77 patients out of 133) and peptide C was recognized by 77.4% of patients (103 positive patients out of 133).

Reactivity against the identified peptides was not detected with the individual sera of 90 age- and sex-matched healthy controls. These results indicate that peptide C contains an epitope specifically recognized by the vast majority of sera from patients with CIU.

Sequence homology between the identified peptides and human proteins

We next compared the peptide amino-acid sequences with known sequences in a protein data bank, using the BLAST via the NCBI BLAST network service and found that the identified peptides share sequence similarity with different autoantigens (Fig. 1). In particular, peptide A shows homology with keratin type IV and peptide B with desmocollin-3. Most importantly, peptide C is homologous to FcεRII/CD23.

Since peptide C is recognized by a much higher percentage of sera as compared with the other two peptides and it shares homology with a cell membrane receptor that could be involved in the pathogenesis of CIU, we used this peptide for the following set of experiments.

Anti-peptide C antibodies recognize the homologous CD23 peptide and bind cell surface CD23 molecule

We isolated the IgG Ab component against the peptide C from the sera of five patients with chronic urticaria by affinity chromatography using a peptide-sepharose column. The Abs affinity purified against the peptide C bound the homologous peptide (GLRNLDLKGEFI) of the CD23 molecule (amino acids 222–233) in ELISA (Fig. 2a). Conversely, Abs affinity purified from the patients against the CD23-derived peptide cross-reacted with peptide C (Fig. 2b).

Moreover, the binding of Abs affinity purified against the CD23 peptide to the solid-phase peptide was cross-inhibited by peptide C, but not by an irrelevant control peptide (Fig. 2c).

To further assess the specificity of the cross-reactivity of these Abs, we used the CD23 peptide to test the panel of patients with CIU using ELISA. We were able to detect IgG Abs against the CD23 peptide in 87 out of 133 patients, whose sera recognized both the peptide C and the CD23 peptide (65.4%).

These data indicate that the sera of patients with CIU contain IgG Abs able to recognize a CD23-derived sequence.

As Abs against the peptide C recognized the CD23-derived peptide, we sought to determine whether Abs affinity purified against the peptide C were able to bind human cells expressing CD23. Indeed, affinity-purified Abs reacted with the CD23-positive human cell line 22-1 as shown by FACS analysis (Fig.

Peptide A	A I L L A I S V R Q H G
Keratin	V Q Q L Q I S V D Q H G
Peptide B	G L R L D A D K G V G V
Desmocollin	P S K L D A E K L V G —
Peptide C	G L R G L K L E G Q Q L
CD23	G L R N L D L K G E F I

Fig. 1. Sequence homology between the peptides obtained and known human proteins. Peptide sequence was compared with known protein sequences in a protein data bank, using the BLAST via the NCBI BLAST network service (I, identity; •, indicates conservative substitution).

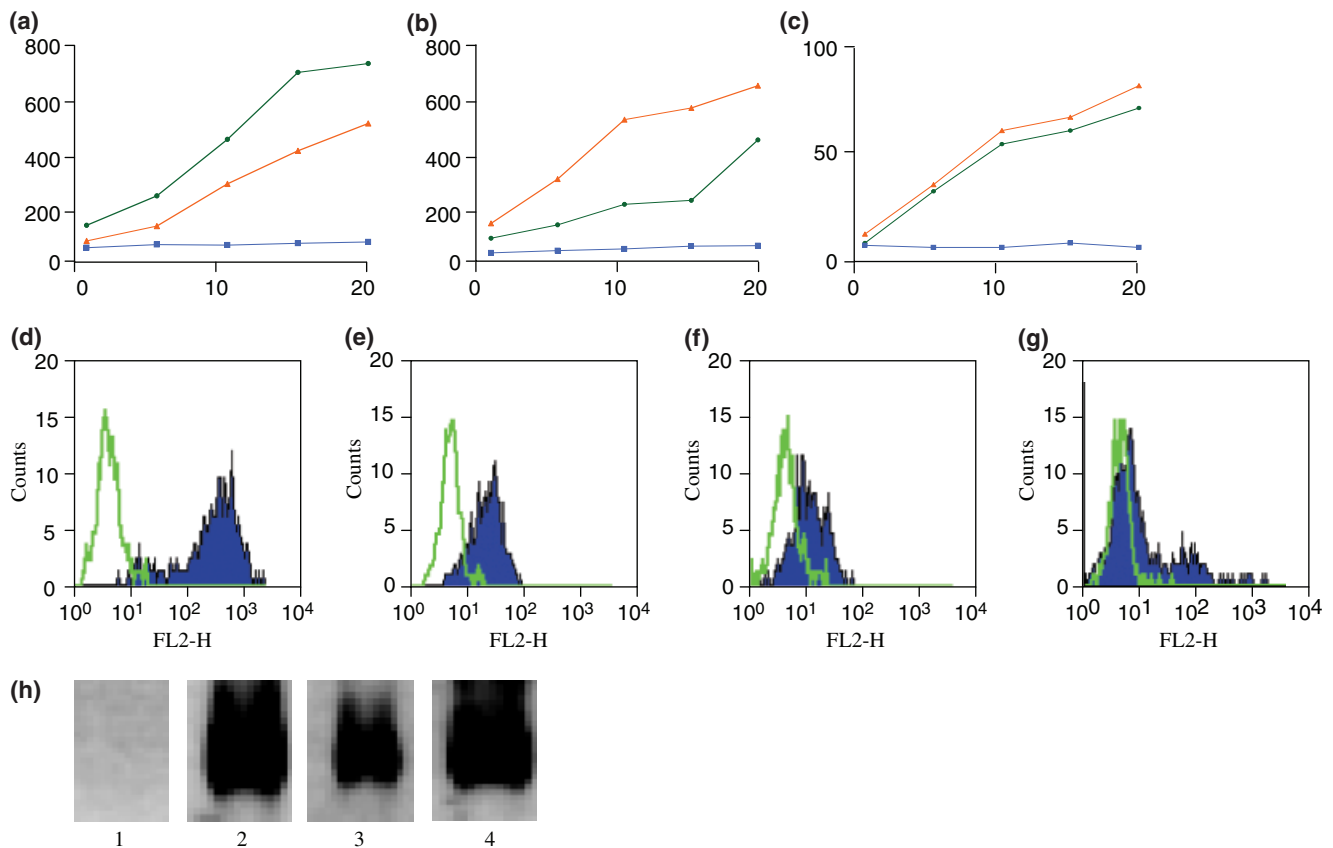


Fig. 2. Antibodies directed against peptide C and peptide CD23 bind the CD23 molecule. IgG antibodies affinity purified against the peptide C (▲) cross-react with peptide CD23. (●, antibodies affinity purified against the CD23 peptide; ■, antibodies affinity purified against an irrelevant control peptide). Data represent absorbance at 405 nm; antibody concentration (horizontal axis) (μg/mL). (b) IgG antibodies affinity purified against the peptide CD23 (●) cross-react with peptide C (▲), antibodies affinity purified against the peptide C; ■, antibodies affinity purified against an irrelevant control peptide). Data represent absorbance at 405 nm; antibody concentration (horizontal axis) (μg/mL). (c) Binding of affinity-purified antibodies against the CD23 peptide to the solid-phase peptide is specifically inhibited by the peptide CD23 (●), by the peptide C (▲) but not by an irrelevant control peptide (■). Data represent percent inhibition; inhibitor concentration (horizontal axis), μg/mL. (d–g) FACS analysis of the binding of antibodies to the human B cell line 22-1: commercially available monoclonal antibody directed against CD23 (d), mouse antiserum directed against peptide CD23 (e), antibodies affinity purified against peptide C (f) and antibodies affinity purified against peptide CD23 (g). Percentage of positive cells = 98.5% (d), 41.5% (e), 27.4% (f) and 19.8% (g). FL2-H = fluorescence intensity. (h) Lysates from 22-1 cells immunoprecipitated either with an antibody affinity purified against an irrelevant control peptide (lane 1), with a mouse antiserum directed against peptide CD23 (lane 2), with affinity-purified anti peptide C antibodies isolated from a patient with CIU (lane 3), and with affinity-purified anti-peptide CD23 antibodies isolated from a patient with CIU (lane 4). Immunoprecipitates were revealed with a mouse antiserum raised against peptide CD23. A band of 45 kDa was present in lanes 2–4.

2d–g). In order to verify whether the ligand on the 22-1 cell line was the CD23 molecule, cell lysates were immunoprecipitated with Abs affinity purified against peptide C. These Abs immunoprecipitated a band of 45 kDa that was identified as CD23 by binding of a mouse Ab directed against the CD23 peptide (Fig. 2h). The same results were obtained using human Abs affinity purified against the CD23 peptide (Fig. 2h).

These results indicate that Abs against the peptides C and CD23 isolated from the sera of patients affected by CIU recognize the cell surface receptor protein CD23.

Antibodies against peptides C and CD23 and mouse-derived anti-CD23 antibodies induce histamine release in peripheral blood from normal donors

We next wished to verify whether the Abs affinity purified against the peptides C and CD23 are functionally active and able to induce histamine release. Peripheral blood from two normal subjects was incubated for 30 min with affinity-purified anti-peptide Abs. The histamine release was approximately 50% of the total level of histamine present in the

samples after cell lysis and it was slightly higher in the samples incubated with the anti-CD23 peptide Abs. The level of histamine release obtained after incubation with mouse anti-CD23 peptide Abs was nearly similar to the total histamine present in the sample after cell lysis (Fig. 3).

Finally, the histamine release obtained after incubation with Abs affinity purified against an irrelevant peptide was similar to the level observed in the absence of Ab stimulation (spontaneous histamine release) (Fig. 3).

Histamine release is induced by major basic protein secreted by eosinophils upon engagement of the CD23 molecule

As the low-affinity receptor for IgE is expressed by macrophages and by eosinophils but not by basophils and as histamine release may be triggered by MCP-1 released by monocytes or by MBP released by eosinophils, we finally wished to clarify whether the histamine release is related to the secretion of MCP-1 or MBP. We did not detect MCP-1 in the supernatant of peripheral blood after a 30 min incubation

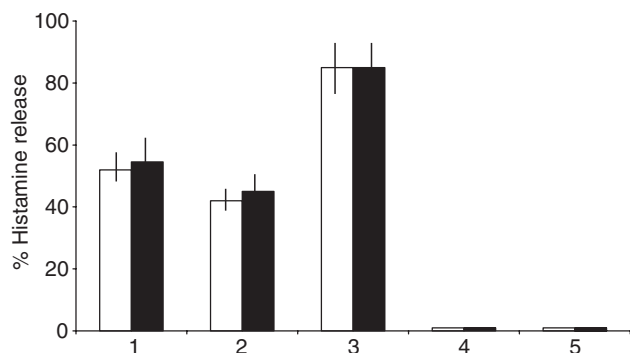


Fig. 3. Antibodies against peptides CD23 and C induce histamine release in the supernatant of peripheral blood. Percent of histamine release in the peripheral blood of two normal donors (empty and black bars) after incubation with human anti-CD23 peptide affinity-purified antibodies (1), with human anti-peptide C affinity-purified antibodies (2) and with mouse anti-CD23 peptide antibodies (3). Incubation with human antibodies purified against an irrelevant peptide (4) gave a result similar to spontaneous histamine release (5). The anti-CD23 peptide antibodies raised in mice gave the highest percentage of histamine release.



Fig. 4. Antibodies against peptide C and CD23 induce major basic protein (MBP) production by eosinophils. Immunoblot analysis of peripheral blood supernatants following incubation with: no antibody (lane 1), antibodies affinity purified against an irrelevant control peptide (lane 2), antibodies affinity purified against peptide C from a patient with chronic idiopathic urticaria (lane 3) and antibodies affinity purified against peptide CD23 from two different patients (lane 4 and 5). The blots were revealed with a commercially available anti-MBP antibody. A band of approximately 14 kDa is present in lanes 3–5.

with the Abs against the peptide C and CD23 (data not shown), whereas there was a significant release of MBP as detected by Western blot analysis (Fig. 4). MBP was not detected after incubation with no Abs or with Abs affinity purified against an irrelevant peptide (Fig. 4). Moreover, the pre-incubation of affinity-purified anti-peptide Abs with the relevant peptide inhibited the production of MBP (data not shown) and the histamine release (Fig. 5a). Finally, histamine release was strongly reduced by the neutralization of MBP with the specific monoclonal antibody (Fig. 5b).

The ability of anti-peptide Abs to bind CD23 on eosinophils was confirmed by FACS analysis of purified human eosinophils (Fig. 6a–c).

These results show that Abs directed against the peptide C and CD23 are able to induce histamine release in the peripheral blood of normal donors through the production of MBP by eosinophils after engagement of the CD23 molecule.

Discussion

We have found that the prevalence of anti-thyroid Abs in patients with CIU was significantly higher than in controls, as previously reported [6]. The levels of total IgE Abs as well as the levels of sCD23 were not statistically different in patients and controls; these findings have not been previously reported in CIU and are in accordance with the idea that atopy has no relevance in the pathogenesis of CIU.

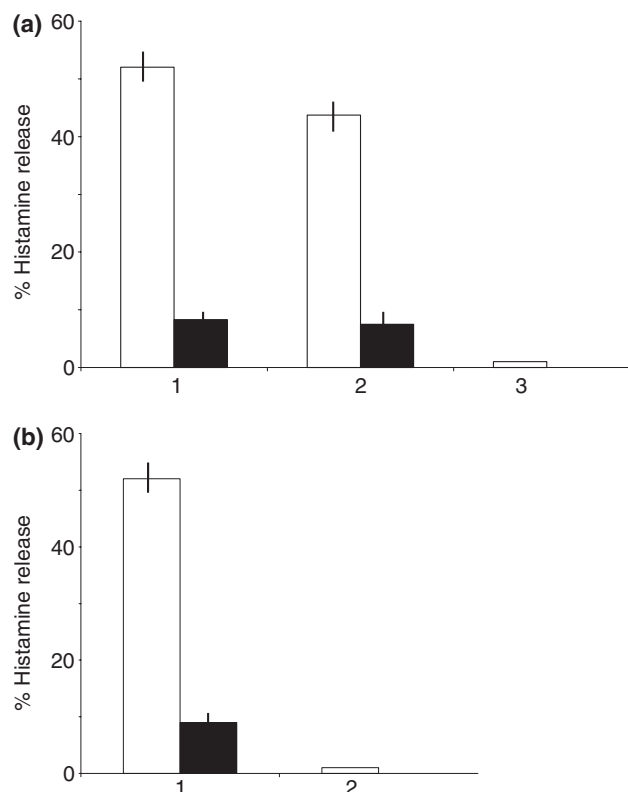


Fig. 5. Inhibition of histamine release in peripheral blood. (a). Histamine release by human anti-CD23 and anti-C peptides antibodies (empty bars) was inhibited by pre-incubation with the relevant peptides (black bars) (1 and 2, respectively); spontaneous histamine release (3). (b). Histamine release induced by human anti-CD23 peptide antibodies (white bar) was inhibited by neutralization of major basic protein (MBP) with an anti-MBP monoclonal antibody (black bar) (1); spontaneous histamine release (2).

This study aimed at identifying pathogenetically relevant autoantigen targets in CIU, a disorder for which an autoimmune origin has been shown in less than half of the cases. Indeed, autoantibodies directed against either the α subunit of the high-affinity IgE receptor or against IgE themselves and able to induce histamine release from basophils have been found in 40–50% of the sera of patients with CIU [1, 8]. It is unknown so far whether other autoantigen targets are present in these patients. To this aim, we used the random peptide library, a technique we have applied already to the study of other autoimmune disease conditions such as systemic sclerosis [17] and Cogan's syndrome [18]. Indeed, the random peptide library approach is a powerful molecular biology method for the identification of ligands for disease-specific Abs whether the antigen is known or not. This approach identifies linear and conformational epitopes and can be applied to analyse disease-specific antigen targets recognized by pooled serum Igs obtained from different patients affected by the same disease.

Using this approach, we have identified three peptides (A, B, C) that were recognized at various percentages by the individual sera of patients affected by CIU. The first two peptides share similarity with two proteins of the skin: peptide A with keratin type IV and peptide B with desmocollin-3. However, autoantibodies directed against keratin are frequently detected in early rheumatoid arthritis [22, 23], while Abs against desmocollin can be found in patients with

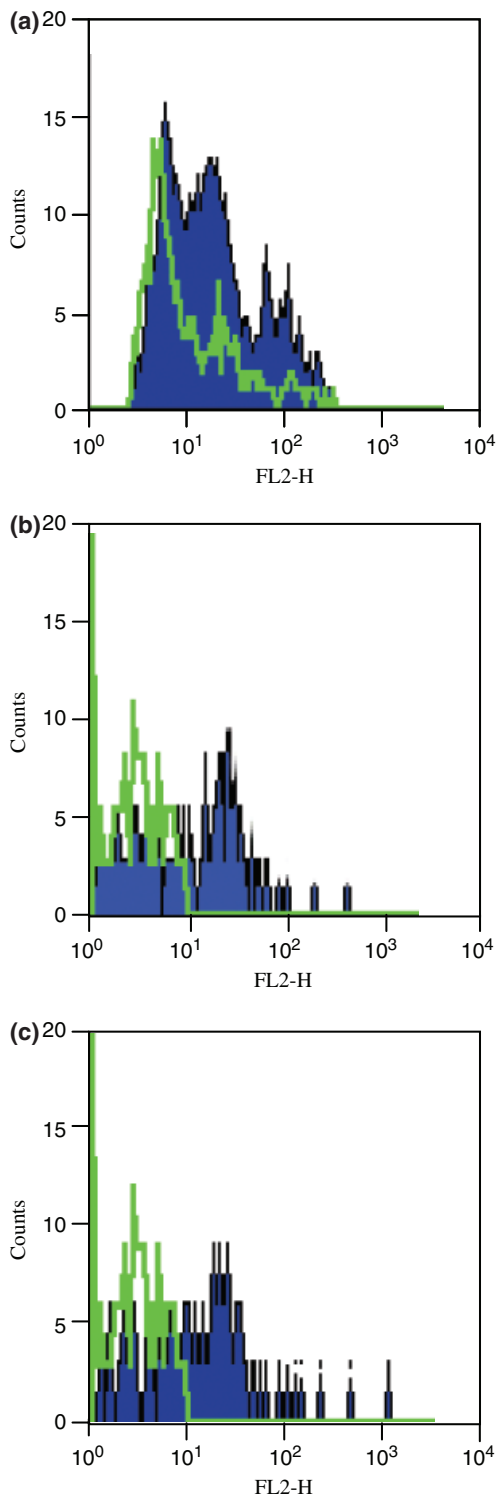


Fig. 6. Binding of anti-peptide antibodies to human eosinophils. FACS analysis of purified human eosinophils incubated with mouse antiserum directed against peptide CD23 (a), human antibodies affinity purified against peptide C (b) and human antibodies affinity purified against peptide CD23 (c). Percentage of positive cells = 39.5% (a), 63.7% (b), and 72.2% (c). FL2-H = fluorescence intensity.

pemphigus [24, 25]. Moreover, the CIU patients' sera reacted with these two peptides at a lower frequency when compared with peptide C. As this peptide shares homology with FcεRII/

CD23, which might have a role in the pathogenesis of CIU, and the vast majority of CIU patients' sera recognized the peptide in ELISA, we decided to focus our attention on peptide C.

FcεRII/CD23, a transmembrane glycoprotein of 45 kDa expressed on several cell types including B and T lymphocytes, monocytes, follicular dendritic cells, neutrophils, platelets and eosinophils [26], is thought to play a role in regulating IgE synthesis [27, 28] and its engagement on eosinophils results in the release of the granule content outside the cell [13].

We have demonstrated that Abs affinity purified from patients' sera against peptide C or its homologue on the CD23 molecule recognize CD23 expressed on the surface of the human B cell line 22-1, known to express CD23, and on human eosinophils.

Moreover, the same Abs immunoprecipitate a band of approximately 45 kDa, recognized as CD23 by binding of an antiserum raised against the CD23 peptide. Interestingly, these affinity purified Abs are able to induce histamine release, considered a pivotal mediator in the induction of the skin lesion typical of CIU. When blood from two normal donors is incubated in the presence of these Abs, we observe histamine release and the phenomenon is specific for the Abs studied as Abs directed against an irrelevant control peptide do not elicit any effect and histamine release induced by anti-peptide Abs can be blocked by pre-incubation of the Abs with the corresponding peptide.

As histamine is released by basophils and mast cells that do not express CD23, we aimed at clarifying the mechanism(s) underlying this process induced by the anti-CD23 Abs.

We observed that following stimulation of peripheral blood cells with the anti-peptide Abs for 30 min, eosinophils release high amounts of MBP. This protein is so named because it accounts for about 55% of the eosinophil granule proteins. MBP has several biological properties: it is a potent helminth toxin, has bactericidal properties, neutralizes heparin, increases bronchial reactivity and induces bronchospasm, causes histamine release from basophils and mast cells. Indeed, it has already been shown that incubation of MBP with basophils and mast cells induces histamine release within 30–45 min in a dose-dependent manner [9, 10]. As we detected MBP in the supernatant obtained from peripheral blood incubated with anti-CD23 peptide antibodies after a 30 min incubation, we believe that 30 min is a sufficient time to induce MBP and subsequent histamine release.

The secretion of MBP is specifically triggered by the interaction between the anti-peptide Abs and surface CD23 molecule, as the addition of the CD23 peptide, which competes for the binding of Abs to the CD23 molecule, almost abolishes the liberation of MBP.

It is worth noting that the same biological effect can be obtained using an antiserum raised against the CD23 peptide, further confirming the importance of this peptide sequence in the process.

Moreover, the neutralization of MBP with the specific monoclonal antibody reduces the histamine release from basophils, further confirming that MBP is indeed the relevant mediator for the histamine release after engagement of the CD23 molecule on eosinophils.

Another substance that can induce the release of histamine from basophils is MCP-1 [29], which belongs to the C-C subgroup of chemokines and has a potent basophil histamine release activity. It is produced by macrophages, monocytes and many other cells such as fibroblasts, endothelial and epithelial cells, and plays a pivotal role in the inflammatory response because of its ability to attract monocytes and T lymphocytes into the sites of inflammation. A normal basophils response to MCP-1 has been recently reported in patients with CIU [30]. However, under the experimental condition that we have used to detect histamine release, we did not observe an increased concentration of MCP-1 in the supernatant of peripheral blood. Indeed, we used a 30 min incubation for the histamine assay and this short time duration may explain why the levels of MCP-1 remain unchanged. Therefore, we cannot rule out that MCP-1 might be involved in histamine release at a later stage.

In conclusion, the identification of FcεRII/CD23 as a relevant autoantigen target in patients with CIU and the demonstration that anti-CD23 Abs present in these patients are able to bind the ligand expressed on eosinophils surface and to induce histamine release through a direct and non-cytotoxic effect by extracellular release of MBP confirm that CIU is of autoimmune origin.

Acknowledgements

This work was supported by grants from Italian Ministry for Scientific Research and Technology (MURST) (A. P. and C. L.), Regione Veneto Ricerca Finalizzata Venezia-Italia and Cariverona Foundation (C. L. and R. C.).

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