

C5a-induced in vitro basophil activation in patients with chronic urticaria: a pilot study

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Summary. *Background:* Complement component 5a (C5a) might be involved in the formation of wheals in patients with chronic urticaria (CU). We sought to compare the in vitro responsiveness of basophils to C5a in patients with CU and in a control group.

Methods: Basophil surface expression of activation marker CD63 induced by C5a, anti-FcεRI mAb or anti-IgE pAb was measured using flow cytometry in 17 patients with CU and in 10 healthy controls.

Results: Patients with CU showed significantly greater basophil CD63 surface expression induced by C5a (median [interquartile range]; 16.4% [13–25.1]; $P = 0.011$) than the group of healthy controls (10.7% [7.2–16.8]). In contrast, basophil CD63 response to anti-IgE and anti-FcεRI was lower in the CU group (12.3% [6–36.3]; 25.9% [12.5–60.5]) than in the control group (51.7% [6.7–84.3]; 62.1% [9.7–89.2]), although not statistically significant.

Conclusion: Results of this pilot study suggest that patients with CU might have an enhanced basophil response to stimulation with C5a, indicating that further studies in CU basophil responsiveness are needed.

Key words: Chronic urticaria, basophils, C5a, CD63.

Introduction

Chronic urticaria (CU) is defined as the daily or almost daily occurrence of wheals for at least six weeks, physical urticaria and urticarial vasculitis having been excluded. The pathogenesis of CU is not thoroughly understood, although mast cell degranulation and histamine release are believed to be of a central importance [1]. A subpopulation of patients have an autoimmune disorder associated with autoantibodies against the high-affinity IgE receptor FcεRI [2] and/or IgE [3] or with circulating antibodies against various thyroid antigens [4]. Other triggers such as chronic *Helicobacter pylori* gastritis could also be involved [5]. Anti-IgE receptor autoantibodies are thought to cause mast cell and basophil degranulation by cross-linking of the α-chain of

FcεRI [2, 3]. However, recent data suggest that this activation is further augmented by complement activation [6, 7] and release of complement component 5a (C5a) [8]. C5a acts directly through the G-protein-coupled C5a receptor on basophils or dermal mast cells and can induce both rapid and long-lasting cellular responses [9]. C5a is also a chemotactic factor for neutrophils, basophils and eosinophils, which are present in CU lesions [10]. Although in vitro C5a-mediated release of basophils and mast cells may be altered in several allergic and non-allergic conditions [11], there is a lack of data on C5a-induced basophil or mast cell response in patients with CU. However, in patients with CU it has been shown that basophils have reduced capacity for releasing mediators when activated through IgE receptors [12–13]. We wished to establish, by measuring surface expression of CD63 (glycoprotein 53) as a marker of basophil activation [14], whether basophils from patients with CU show an altered response to C5a-induced in vitro stimulation in comparison with healthy control persons.

Materials and methods

Study participants

We recruited 17 CU patients with recurrent whealing occurring at least four times a week for an average of 45 months (range 6–132) without clinical evidence of physically induced urticaria or of urticarial vasculitis. The average age of the patients was 51 years (range 34–70) and 12 were women. Ten healthy persons (average age 38 years; age range 28–49, 6 of whom were women) served as the control group. Controls were matched for sex but were younger than the patients. The clinical and laboratory characterization of the CU patients is summarized in Table 1. Antihistamine treatment was stopped at least four days before blood samples were collected and none of the patients was taking glucocorticoids, immunosuppressive drugs or tricyclic antidepressants at the time of venipuncture or in the past month. The study was approved by the National Ethics Committee and written informed consent was obtained from the participants.

Materials

Recombinant human C5a was obtained from Sigma Chemicals (St Louis, MO), mouse anti-FcεRI mAb from Bühlmann Laboratories (Allschwil, Switzerland) and goat anti-IgE pAb from Caltag Laboratories (Burlingame, CA). C5a was dissolved

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in dimethyl sulfoxide and stored at -80°C . For stimulation of basophils, C5a was diluted 1/1000 in cell incubation medium. The final concentration of dimethyl sulfoxide (0.1% vol/vol) had no effect on cellular function (data not shown).

Preparation of peripheral blood leukocytes

For the basophil activation assay, 9 ml of whole blood collected in EDTA tubes (Vacutainer, BD, Plymouth, UK) was centrifuged at $550 \times g$ for 10 min at 4°C ; the buffy coat was collected (0.5 ml) and centrifuged again. The supernatant was discarded and the cell pellet resuspended in 450 μl of HEPES calcium stimulation buffer containing 2 ng/ml of interleukin 3 (IL-3) for 5 min.

Cell stimulation procedure

Cells were stimulated at 37°C in a shaking water bath for 45 min in final concentrations of $1.2 \times 10^{-8}\text{M}$ for C5a [8], 0.55 $\mu\text{g}/\text{ml}$ for anti-Fc ϵ RI (α chain of high-affinity IgE receptor) mAb and 0.5 $\mu\text{g}/\text{ml}$ for anti-IgE pAb. Basal surface expression of CD63 was determined in the same conditions by adding an equal volume of corresponding IL-3 containing stimulation buffer alone.

Basophil activation assay

Surface expression of the basophil activation marker CD63 was measured using the Flow-CAST test (Bühlmann Laboratories, Allschwil, Switzerland). Briefly, the stimulation was stopped by adding blocking buffer (ice-cold HEPES buffer without calcium and magnesium but with EDTA 0.27 mM) followed by incubation with a mixture of anti-human CD63 mAb labelled with phycoerythrin (anti-CD63 PE) and anti-human IgE labelled with fluorescent isothiocyanate (anti-IgE FITC). After incubation for 30 min on ice, lysing reagent was added

for 4 min at room temperature. Cell lysis was stopped with blocking buffer and after centrifugation the cells were resuspended in blocking buffer and analyzed within 2 hours on a FACSCalibur flow cytometer with CELLQuest software (BD, San Jose, CA). The initial cell gate was defined on a histogram by forward and side scatter characteristics and the second gate was defined around cells showing bright IgE expression identifying them as basophils. IgE-carrying monocytes and dendritic cells were excluded from the analysis by their different forward and side scatter characteristics and dim expression of IgE. CD63 surface expression was determined by analyzing 150–500 basophils in each probe. The percentage of activation was calculated as the number of cells positive for anti-IgE and anti-CD63 compared with the total number of anti-IgE positive cells. Other parameters were given simultaneously by FACS as the percentage of the anti-IgE positive population.

Statistical analysis

The distribution of data was recalculated using the Shapiro–Wilk normality test. The data were not normally distributed, therefore a Mann–Whitney U test was used to study differences between the groups. The strength of association between variables was obtained with the Spearman rank-order method. Probability values of $P < 0.05$ were accepted as significant. Unless otherwise stated, results are given as medians and interquartile ranges (IQR). GraphPad Prism 5 software was used for the analyses.

Results

The percentage of bright anti-IgE positive cells (basophils) in patients with CU (median [IQR]; 0.7% [0.4–1.2]) was not significantly different from the healthy control group (1.1% [0.7–1.7]; $P = 0.23$). There were no significant

Table 1. Clinical and laboratory characteristics of 17 patients with chronic urticaria

Sex	Duration of CU (months)	Recurrent angioedema	Positive autologous serum skin test	Elevated levels of Ab to thyroid ^a	Elevated levels of IgG Ab to <i>H. pylori</i> ^b	ASA intolerance ^c
F	48	Yes	No	No	No	Yes
F	40	No	Yes	Yes	Yes	No
M	56	No	No	No	No	No
M	38	Yes	No	No	No	No
M	60	Yes	No	No	No	Yes
F	58	Yes	No	No	No	No
F	32	Yes	No	No	No	No
F	38	Yes	No	No	No	No
M	46	Yes	No	No	No	No
F	43	Yes	No	No	No	Yes
F	36	Yes	No	No	Yes	No
F	40	No	No	Yes	Yes	No
F	12	Yes	Yes	Yes	Yes	No
F	24	Yes	No	No	Yes	Yes
F	6	Yes	No	Yes	Yes	No
M	132	No	ND	ND	Yes	No
F	60	Yes	Yes	No	Yes	Yes

CU chronic urticaria; F female; M male; ND not determined. ^aTSH receptor antibody. ^bThe level of IgG Ab to *H. pylori* was measured on the day of basophil testing. ^cClinical history of intolerance to acetylsalicylic acid (ASA).

differences in the basal percentage of basophil CD63 surface expression between CU patients (5.5% [4–7.2]) and the control group (5.2% [3.7–6.2]; $P = 0.53$). However, after stimulation with C5a, the percentage of CD63-expressing basophils was significantly higher in CU patients (16.4% [13–5.1]; $P = 0.011$) than in healthy controls (10.7% [7.2–16.8]) (Fig. 1). In contrast, the basophil response to anti-IgE pAb and anti-FcεRI mAb was lower in patients with CU (12.4% [6–36.3]; 25.9% [12.5–60.5]) than in the control group (51.7% [6.7–84.3]; 62.1% [9.7–89.2]), although the difference did not reach statistical significance ($P = 0.18$; $P = 0.17$) (Fig. 2). There was a highly significant positive correlation between the basophil response to anti-IgE pAb and anti-FcεRI mAb stimulation ($R = 0.8$, $P = 0.0001$). There was no correlation between other variables.

Clinical and laboratory data in subgroups of CU patients were also compared. We could not find any significant differences in C5a, anti-IgE pAb or anti-FcεRI mAb basophil responsiveness between CU subgroups according to occurrence of angioedema ($P = 0.87$, 0.1 or 0.28), positive autologous serum skin-test results ($P = 0.11$, 0.42 or 0.59), elevated levels of Ab to thyroid ($P = 0.76$, 0.51 or 0.86) *H. pylori* ($P = 0.42$, 0.13 or 0.54) or clinical history of ASA intolerance ($P = 0.71$, 0.79 or 0.23).

Discussion

Previous studies showed that basophil IgE-dependent release of histamine and sulfidoleukotrienes is reduced in patients with CU in comparison with healthy persons [12, 13]. Our findings demonstrate a similar trend, as basophil activation induced by anti-FcεRI or anti-IgE stimuli tended to be lower in patients with CU. However, we showed that basophils from patients with CU displayed an increased response after activation with ana-

phylotoxin C5a. The contrasting effect of an anti-IgE receptor-triggered response and a C5a triggered response was demonstrated by surface up-regulation of basophil activation marker CD63 [14].

The serum of CU patients induces an inflammatory response and marked expression of CD63 on basophils [15]. However, in 2005 Luqin et al. [16] suggested that CU basophils may be hyporesponsive to C5a stimulation. Nevertheless, the results of their study may have been influenced by technical problems, as basophile histamine release after C5a stimulation was very low in the healthy controls, who released only around 4% of the histamine content after C5a stimulation. In contrast, an earlier study by Miura and MacGlashan [17], for example, and a recent study by Marsland et al. [18] have shown that basophils of normal donors release about 40% of their histamine content after C5a stimulation. Calcineurin inhibitors were able to inhibit the in vitro release of histamine when basophils were stimulated with urticaria sera but not when stimulated with C5a [18].

Many reports have suggested that complement could play an important role in the pathogenesis of CU [6–8]. IgG autoantibodies (anti-FcεRI or anti-IgE autoantibodies) from patients with CU can induce in vitro activation of complement [6–7] and liberation of C5a [8], which contribute to the higher magnitude of histamine release and may participate in enhanced dermal mast-cell degranulation in skin lesions of patients with CU. Therefore, C5a might also be a potential therapeutic target in CU. Ying et al. [10] found significant recruitment of basophils into the inflammatory cell infiltrate in CU lesions. Those basophils might significantly contribute to the enhanced in situ inflammatory response to C5a. In addition, Ying et al. [10] found that the inflammatory and cytokine profile in the skin lesions of patients with CU with and without circulating IgG au-

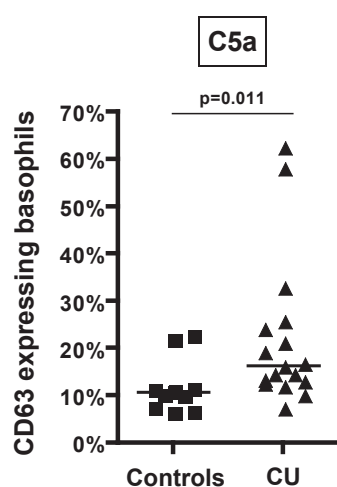


Fig. 1. Basophil response determined by the percentage of surface CD63-expressing cells, gated according to their bright surface IgE expression after stimulation with C5a from healthy control persons ($n = 10$) and patients with chronic urticaria ($n = 17$). The horizontal solid bars indicate the median value for each group

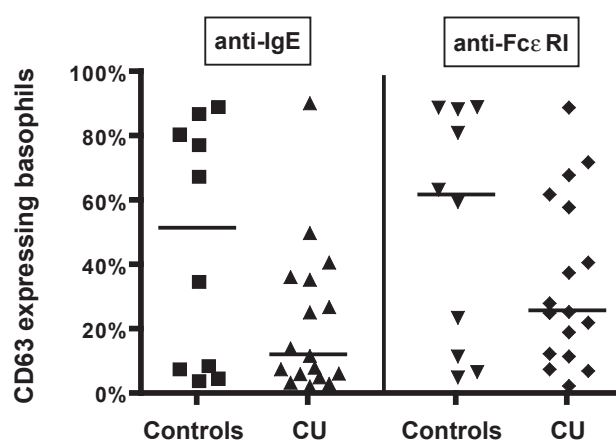


Fig. 2. Basophil response determined by the percentage of surface CD63-expressing cells after stimulation with anti-IgE pAb or anti-FcεRI mAb in healthy control persons ($n = 10$) and patients with chronic urticaria ($n = 17$). The horizontal solid bars indicate the median value for each group

toantibodies are the same and that the skin cell infiltration in both subgroups is likely to be the result of a C5a-mediated chemotactic response. C5a-mediated basophil activation via binding of the G-protein-coupled C5a receptor induces not only a rapid basophil degranulation response but also continuous generation of leukotriene C4, IL-4 and IL-13 for a period of 16–18 h [9, 19]. This long-lasting cellular response mediated by C5a can therefore differently promote certain basophil or dermal mast-cell functions.

Recent findings suggest an additional pathogenic mechanism in CU patients: activation of the coagulation cascade resulting in thrombin production [20]. Thrombin is a serine protease that may play a key role in urticaria, being able to induce edema through an increase in vascular permeability and mast-cell activation and degranulation, and to induce the production of C5a. Such mechanisms seem to be active in the majority of CU patients; however, their relationship with anti-FcεRI or anti-IgE autoantibodies is still a matter of research.

We also demonstrated that the percentage of basophils showing bright IgE fluorescence was slightly lower in patients with CU than in healthy control persons. Indeed, several previous studies have reported basopenia in CU [13, 21], although others have not [12]. An absolute basophil count was not included in our study.

The incidence of asthma, allergic rhinitis and atopic dermatitis in CU patients is comparable with the distribution of these disorders in the population and identification of an external allergen responsible for hive formation is very rare. However, basophils from patients with allergic disease frequently show an altered in vitro response to C5a [11], which could be in correlation with the severity of clinical symptoms. To eliminate the influence of atopy on the variability of the results we strictly excluded such persons from the study.

Several researchers [11, 22] suggest that C5a is able to enhance in vitro production of sulfidoleukotrienes in persons with acetylsalicylic acid intolerance. Hence, it is interesting that persons with intolerance to single or multiple non-steroid anti-inflammatory drugs (NSAIDs) showed a high prevalence (30% for single and >90% for multiple) of positive autologous serum skin tests [23]. Moreover, it was very recently demonstrated that NSAID intolerance often precedes the onset of CU by years, especially in individuals with acetylsalicylic acid intolerance [24]. Perhaps altered responsiveness to C5a is a possible link between NSAID intolerance and CU [25]. Nevertheless, in our study we were not able to find any differences in basophil response to C5a between CU individuals concerning their clinical history of intolerance to acetylsalicylic acid.

In conclusion, we have compared basophil surface expression of CD63 by various stimuli in patients with CU and in healthy control persons. The basophils from the CU patients showed significantly higher activation after C5a stimulation, indicating that CU basophils could be targeted very effectively by C5a, which further suggests their possible importance in the pathogenesis of CU.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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