

Effects of calcineurin inhibitors on an *in vitro* assay for chronic urticaria

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Summary

Background Chronic urticaria is a common skin disorder, which causes considerable morbidity. In approximately 40% of cases, patients have an autoimmune disorder in which functional antibodies cause degranulation of mast cells and basophils, and C5a complement augments this in varying amounts from patient to patient. Since the calcineurin inhibitor ciclosporin has been used in chronic autoimmune urticaria, we examined the effect of ciclosporin and other drugs on the release of histamine from basophils when stimulated by sera from patients with chronic autoimmune urticaria.

Methods Leucocytes from healthy donors were isolated and incubated in varying concentrations of ciclosporin, ascomycin, methotrexate, diphenhydramine or hydroxyzine for 30 min prior to stimulation with serum from urticaria patients known to have functional immunoglobulin (Ig)G antibodies directed against the alpha subunit of the IgE receptor. Histamine release was then measured.

Results Pre-incubating cells with ciclosporin and ascomycin produced dose-dependent inhibition of histamine release when cells were stimulated by sera of urticaria patients, by purified IgG from these sera, but not by C5a. Inhibition was not prevented by C5a receptor blocking antibodies. No inhibition was seen with methotrexate, diphenhydramine or hydroxyzine.

Conclusions This is the first demonstration of inhibition of histamine release by calcineurin inhibitors employing sera of patients with chronic autoimmune urticaria. These drugs may work by interfering with intracellular signalling in cells following cross-linking of the IgE receptor, but not following stimulation of the C5a receptor.

Keywords ascomycin, calcineurin, ciclosporin, immunosuppression, methotrexate, pimecrolimus, urticaria

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Introduction

Chronic urticaria is a common skin disorder characterized by widespread transient pruritic weals that recur from day to day for 6 weeks or more, producing considerable morbidity [1, 2]. Greaves and co-workers [3] demonstrated it to be an autoimmune disorder in at least 40% of patients, who possess functional autoantibodies that cause cross-linking of immunoglobulin (Ig)E receptors on basophils and mast cells to release inflammatory mediators and cytokines. Furthermore, it has been shown that these chronic autoimmune urticaria patients have more severe disease [4].

Treatment of chronic urticaria is a challenge and oral antihistamines (H_1 receptor antagonists), even in high doses, may not be effective [1]. Other additional therapies may include H_2 receptor antagonists, leukotriene antagonists [5, 6], UVB phototherapy [7], hydroxychloroquine, dapson,

methotrexate, plasmapheresis [8] or intravenous γ globulin [9]. However, none of these are reliably effective or are prohibitive based on cost/availability considerations. For severe cases, low-dose and alternate day systemic corticosteroids [1] have been recommended, and ciclosporin has also been shown to be particularly effective in randomized controlled trials involving the autoimmune subgroup. The mode of action of calcineurin inhibitors, such as ciclosporin, is, however, not clear [10].

In order to further examine mechanisms of action of some of these drugs in chronic autoimmune urticaria, we have assessed the effects of ciclosporin and ascomycin on basophil histamine release [11] as stimulated with sera or isolated IgG of patients.

Materials and methods

Leucocyte isolation

Leucocyte isolation was carried out as described previously [11]: 15 mL of whole blood from a healthy donor was mixed with 1.5 mL of 0.2 M EDTA and 3.0 mL of 3% dextran–3% glucose–0.15 M NaCl saline solution. After the mixture had

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sedimented for 90 min, the supernatant was transferred to conical tubes and washed three times with HBSS-HSA containing HBSS (Gibco BRL, Grand Island, NY, USA), 0.3% human serum albumin and 4 mM HEPES. Cells were resuspended in the histamine release buffer from the histamine ELISA kit (Immunotech International) at the original leucocyte concentration.

Patients

Sera were selected from patients who had recurrent hives occurring at least four times a week for more than 12 weeks, with individual lesions lasting over 4 h. None of the sera used was from patients who had a physical urticaria or who had urticarial vasculitis. None of the patients had recently taken immunosuppressants, corticosteroids or tricyclic antidepressants, and patients had been off all medication, including antihistamines for at least 4 days prior to venipuncture. The patients chosen were clearly positive for chronic autoimmune urticaria as assessed by histamine release from basophils. Autologous skin tests were not employed. We did not distinguish those with anti-IgE from those with anti-IgE receptor antibody. This study was approved by the Investigational Review Board (IRB) of the Medical University of South Carolina and informed consent was obtained from each participant.

Purification of immunoglobulin G

Whole IgG was isolated from patient sera using protein G (Amersham Biosciences, Uppsala, Sweden) affinity chromatography according to the manufacturer's instructions.

Histamine release from basophils and histamine assay

The same blood donor, who has a history of mild seasonal allergic rhinitis but is otherwise well, was used as his cells are known to be reactive to the stimuli of anti-IgE, functional anti-FcεRIα, and complement C5a without initial IL-3 priming. 75 µL of serum, buffer, purified IgG or complement C5a was incubated with 75 µL of whole leucocyte suspension for 40 min at 37 °C, as described previously [11]. Following incubation, supernatants were separated by centrifugation at 1200 g at 4 °C for 5 min. Aliquots of cells in two dilutions were boiled to determine the total histamine content of basophils in the leucocyte preparations.

Histamine release was measured with an enzyme immunoassay kit (Immunotech International, Marseille, France), according to the instructions of the manufacturer. The average release from duplicate samples was calculated and expressed as a percentage of total histamine content, after subtracting the spontaneous release from both, which was less than 5% of total histamine. The inhibition by pre-incubation with various drugs was calculated by expressing the proportion of percentage release with drugs.

Drugs and chemicals

Ciclosporin, ascomycin, methotrexate, diphenhydramine and hydroxyzine were purchased from Sigma pharmaceuticals. Stock solutions of 0.01 M of all these were made in methanol with the exception of ascomycin, which was dissolved in

ethanol. The reagents were bought at the highest grade available. Drugs were further diluted by the histamine release buffer used in the assay and 16.7 µL of drug solution was added to the leucocyte suspension for 30 min at room temperature prior to incubation.

Statistics

Statistical analysis was done employing Student's *t*-test. A *P*-value of <0.05 was considered significant.

Results

Calcineurin antagonists inhibit histamine secretion stimulated by serum from chronic urticaria or isolated immunoglobulin G but not by C5a

In order to study the effects of ciclosporin on histamine release from normal human basophils, we first stimulated cells with anti-IgE, as has been reported previously [12]. As shown in Fig. 1, there is a dose-dependent inhibition of histamine release between 100 (10^{-8} M) and 10 000 nM (10^{-6} M) ciclosporin. A maximum of over 70% inhibition was noted. We then examined whether a similar result would be obtained if chronic urticaria sera containing anti-FcεRIα antibody were used as the agonist. Figure 2 is representative of one such serum in which a dose-dependent inhibition is observed between 100 and 10 000 nM ciclosporin. We next surveyed a series of patients at 1000 nM (10^{-7} M) ciclosporin and found that all sera tested were inhibited (Fig. 3), with an average inhibition of 53.8%. Since we know that the pathogenic antibody is of the IgG class, we isolated IgG from several patients to remove complement; thus, the effects of IgG-induced stimulation alone could be observed. A dose-dependent inhibition of histamine release is demonstrated in Fig. 4 employing one representative serum; the results were similar in four of four such patients studied. By

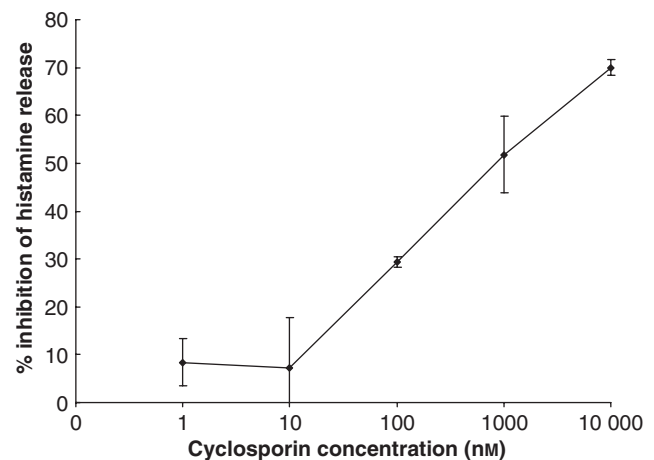


Fig. 1. Ciclosporin causes a dose-dependent inhibition of histamine release from basophils stimulated with anti-IgE. Leucocytes were isolated from a healthy donor and preincubated in varying concentrations of ciclosporin or reaction buffer alone for 30 min prior to stimulation with goat anti-human anti-IgE (100 µM). In the absence of ciclosporin 16.4 pmol histamine was released from 75 µL of leucocyte suspension containing approximately 2×10^5 cells/mL. Histamine release is expressed as percent inhibition by ciclosporin for each of the duplicates. Error bars express SEM. A dose-dependent inhibition of histamine release is observed with a IC_{50} occurring between 100 and 1000 nM ciclosporin.

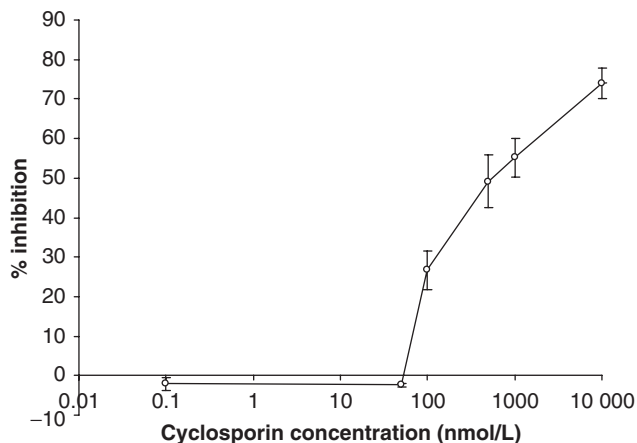


Fig. 2. Dose-dependent inhibition of histamine release from urticaria serum with ciclosporin. Leucocytes were isolated from a healthy donor and preincubated in varying concentrations of ciclosporin or reaction buffer alone for 30 min prior to stimulation with serum from a patient with chronic autoimmune urticaria. In the absence of ciclosporin 26.5 pmol histamine was released from 75 μ L of leucocyte suspension containing approximately 3×10^5 cells/mL. Histamine release is expressed as percent inhibition by ciclosporin for each of the duplicates. Error bars express SEM. A dose-dependent inhibition of histamine release is observed with a IC_{50} occurring between 100 and 1000 nm ciclosporin.

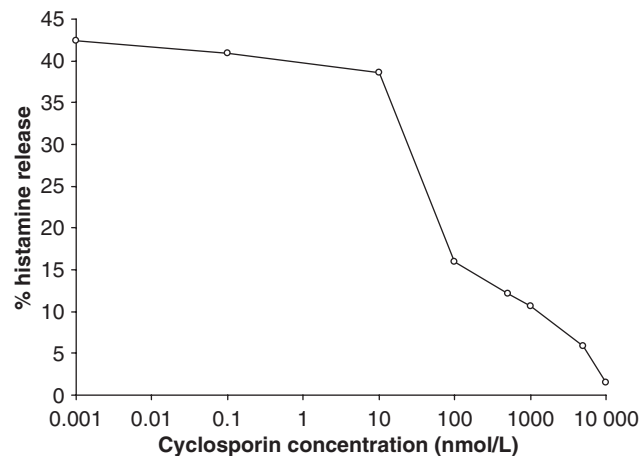


Fig. 4. Dose-dependent inhibition of histamine release by ciclosporin with immunoglobulin (Ig)G from urticaria serum. Leucocytes were isolated from a healthy donor and preincubated in varying concentrations of ciclosporin or reaction buffer for 30 min prior to stimulation with IgG isolated by protein G chromatography from the serum of a patient with chronic autoimmune urticaria. 26.5 pmol histamine was released from 75 μ L of leucocyte suspension containing approximately 2×10^5 cells/mL when boiled. Histamine release is expressed as percent of total for each sample after subtraction of spontaneous release. A dose-dependent inhibition of histamine release is observed with a IC_{50} occurring between 100 and 1000 nm ciclosporin.

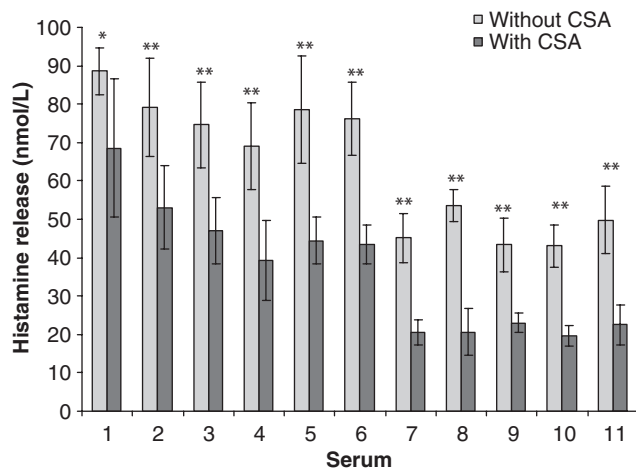


Fig. 3. Inhibition of histamine release with ciclosporin with various autoimmune urticaria sera. Leucocytes were isolated from a healthy donor and preincubated with 10 μ M ciclosporin or reaction buffer for 30 min prior to stimulation with serum from different patients with chronic autoimmune urticaria. Histamine release is expressed as percent of total histamine release when the same volume (75 μ L) of leucocyte suspension was boiled. The small spontaneous release has been subtracted for each of the duplicates. Total histamine release from 75 μ L leucocyte suspension (approximately 3×10^5 basophils/mL) was 16.9 pmol. Samples were assayed in sextuplet. Histamine release was inhibited in varying amounts for all sera tested. Error bars show standard deviation from the mean * $P < 0.05$ ** $P < 0.01$.

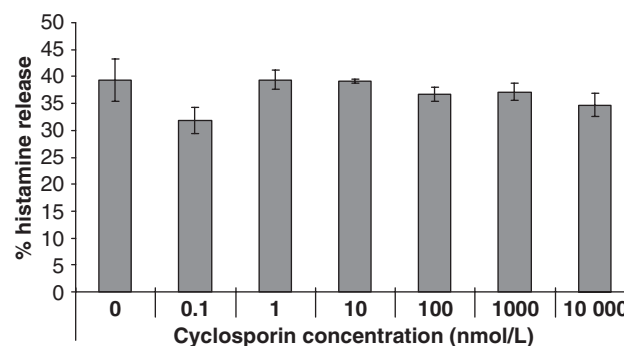


Fig. 5. Ciclosporin does not inhibit histamine release when cells are stimulated with C5a. Leucocytes were isolated from a healthy donor and preincubated in varying concentrations of ciclosporin or reaction buffer alone for 30 min prior to stimulation with 75 μ L of 20 μ M C5a. 43.9 pmol histamine was released from 75 μ L of leucocyte suspension containing approximately 3×10^5 cells/mL when boiled. Histamine release is expressed as percent inhibition by ciclosporin for each of the duplicates. Error bars represent SEM. Even at high doses of ciclosporin, no inhibition of histamine release is observed when C5a is the agonist.

contrast, when we stimulated basophils with C5a [13], we saw no inhibition of histamine release by pre-incubation with ciclosporin up to 1000 nm (Fig. 5). When we used anti-CD88 antibodies to block the C5a receptor and stimulated with the serum from one patient (Fig. 6) we saw about 47% inhibition. Pre-incubation with ciclosporin resulted in a further dose-dependent inhibition of histamine release presumably because of inhibition of the effect of anti-Fc ϵ RI α antibodies. We next examined the ability of ascomycin to inhibit histamine release

by sera from chronic urticaria patients. A dose-response inhibition to urticaria serum is demonstrated in Fig. 7. There is a strikingly similar dose-response inhibition noted in each between 100 nm and 10 μ M ascomycin, and a dose of 100 nm ascomycin significantly inhibited all 11 sera tested (Fig. 8). The average percent inhibition was 43.9, similar to that seen in Fig. 3 employing ciclosporine (41.2% inhibition). However, the concentration of ascomycin employed was 10-fold less. The antihistamines diphenhydramine and hydroxyzine, and the immunosuppressant methotrexate, have no effect on histamine secretion stimulated by serum from chronic urticaria patients. Figure 9 demonstrates that no inhibition of histamine was observed by pre-incubating cells with a wide concentration range (between 1 and 100 000 nm) of these drugs.

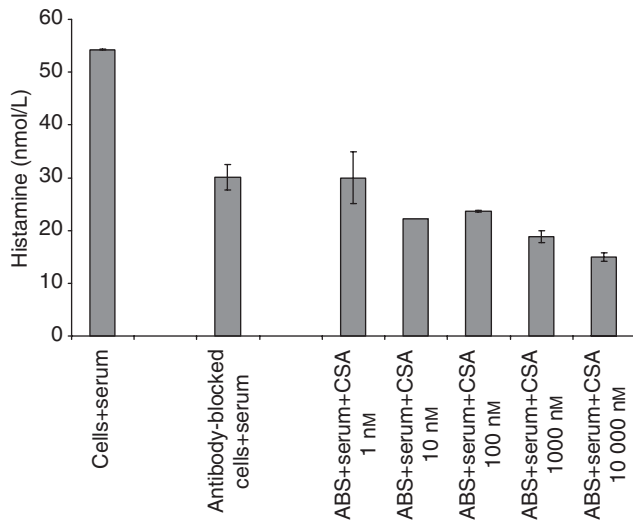


Fig. 6. Cyclosporin has a further dose-dependent inhibitory effect after blocking C5a receptors. Leucocytes were isolated from a healthy donor and preincubated with anti-CD88 antibodies and/or cyclosporin or reaction buffer alone for 30 min prior to stimulation with serum from a patient with chronic autoimmune urticaria. 21.4 pmol histamine was released from 75 μ L of leucocyte suspension when boiled. The results shown are the mean of duplicates, and the cells plus serum alone released a mean of 54 nmol/L. The anti-CD88 alone gave 47% inhibition. Further inhibition could be appreciated between 10 and 10 000 nm cyclosporin. ABS, antibody blocked cells; error bars represent SEM.

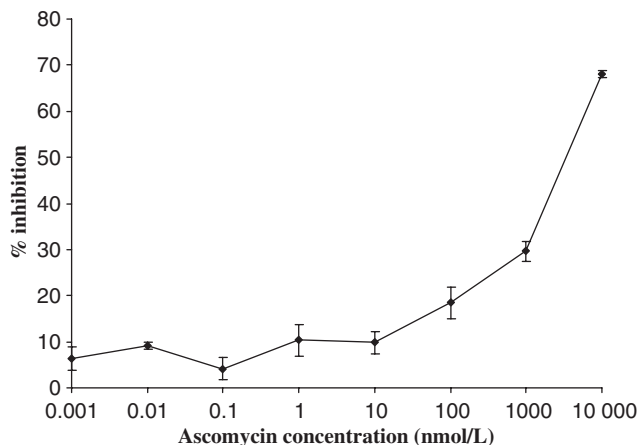


Fig. 7. Dose-dependent inhibition of histamine release by ascomycin with urticaria serum. Leucocytes were isolated from a healthy donor and preincubated in varying concentrations of ascomycin or reaction buffer alone for 30 min prior to stimulation with serum from a patient with chronic autoimmune urticaria. Without ascomycin 12.2 pmol histamine was released from 75 μ L of leucocyte suspension containing approximately 3×10^5 cells/mL. Histamine release is expressed as percent inhibition by ascomycin for an average of the quadruplicate samples. Error bars express SEM. A dose-dependent inhibition of histamine release is observed with a IC_{50} occurring between 1000 and 10 000 nm ascomycin.

Discussion

The ability of drugs including calcineurin inhibitors to inhibit histamine release from basophils stimulated by anti-IgE has been studied previously [12, 14], but we are not aware of any studies examining their action specifically in chronic urticaria. Chronic autoimmune urticaria is dependent not only on the cross-linking of IgE receptors (by anti-Fc ϵ RI α or anti-IgE) but also on the activation of complement [13]. Cross-linking

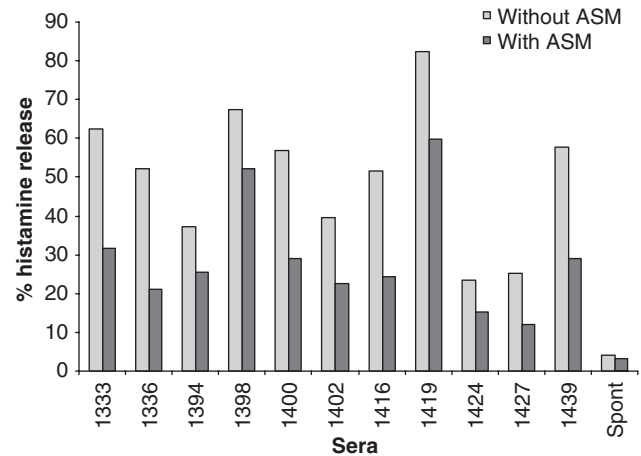


Fig. 8. Inhibition of histamine release with 100 nm ascomycin with various autoimmune urticaria sera. Leucocytes were isolated from a healthy donor and preincubated with 100 nm ascomycin or reaction buffer alone for 30 min prior to stimulation with serum from different patients with chronic autoimmune urticaria. Histamine release is expressed as percent of total histamine release when the same volume (75 μ L) of leucocyte suspension was boiled. The control (spontaneous release) is shown at the right-hand side of the figure. Total histamine release from 75 μ L leucocyte suspension (approximately 5×10^5 basophils/mL) was 46.4 pmol. Histamine release was inhibited in varying amounts for all sera tested.

of IgE receptors leads to histamine release via a calcineurin-dependent signal transduction pathway, whereas complement C5a receptors act through G-proteins. Histamine release by patient sera or isolated IgG can be inhibited by cyclosporin and ascomycin, but the contribution by complement is not. Kikuchi and Kaplan [11, 13] reported that on average about 30% of histamine release in the basophil release assay may be attributed to complement activation, although this is variable from patient to patient. Analysis of IgG subtypes by Soundararajan et al. [15] demonstrated that while most patients have functional IgG1 and/or IgG3 antibodies, some have IgG4, which we know does not fix complement.

Calcineurin inhibitors such as cyclosporin have previously been shown to inhibit histamine release from basophils and mast cells when stimulated with anti-IgE antibodies and pimecrolimus (ascomycin) seemed to be more potent than cyclosporin [14]. They also have inhibitory effects on other important components of the inflammatory/immune response, most notably T lymphocytes [16], and also eosinophils [17]. Basophils are dependent on T cell derived IL-3 for priming, although IL-3 levels of sera and supernatants tested were not determined. The randomized controlled trial of cyclosporin in urticaria by Grattan et al. [10] demonstrated that its *in vivo* efficacy might be because of inhibition of antibody production, rather than inhibition of basophil or cutaneous mast cell secretion. For example, the autologous serum test to stored serum was not reduced in responsive patients. Nevertheless, some patients respond to cyclosporin within 4–5 days, a time interval suggesting an effect on inflammation apart from antibody production. Although this might be because of effects on cells other than basophils or mast cells, our observations suggest that effects on these cells should be reconsidered. We demonstrate significant *in vitro* inhibition of the histamine releasing ability of the antireceptor antibody but no effect upon the complement augmentation. We have not tested cyclosporin inhibition of cutaneous mast

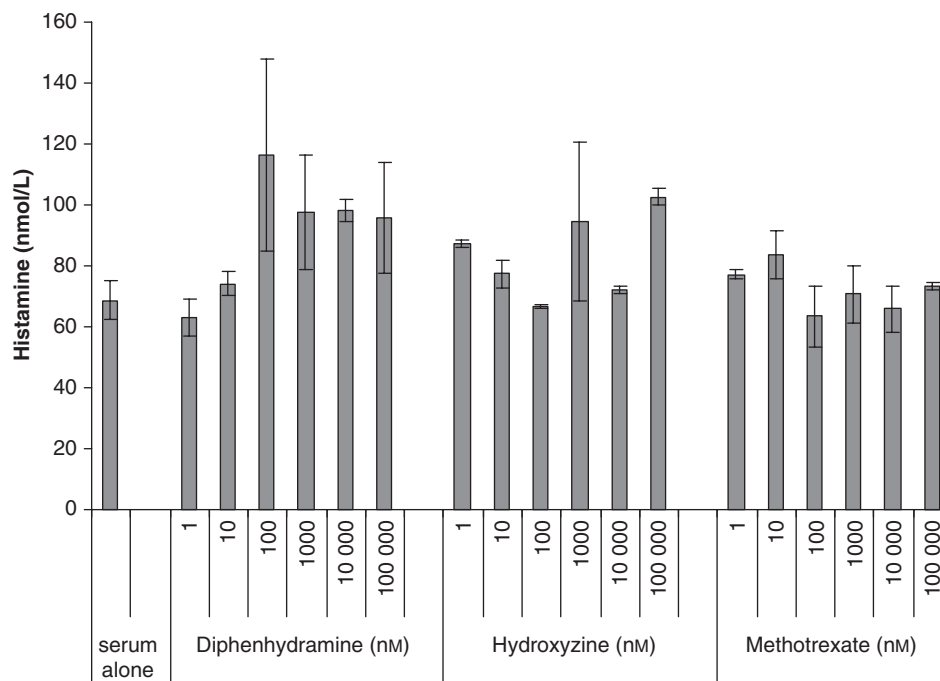


Fig. 9. Absence of dose response for serum with diphenhydramine, hydroxyzine and methotrexate. Healthy donor leucocytes were preincubated with varying concentrations of diphenhydramine, hydroxyzine or methotrexate or with buffer alone for 30 min prior to stimulation with serum from a patient with chronic autoimmune urticaria. 46.3 pmol was released from boiling 75 μ L leucocyte suspension. Error bars represent SD from the mean for duplicates. No inhibition is seen even at high doses of these drugs.

cells; however, basophils do infiltrate the skin of patients with chronic urticaria [18] and may contribute to the inflammatory response. Since calcineurin inhibitors such as ciclosporin and ascomycin inhibit histamine release from IgE receptor cross-linking but not C5a receptor stimulation, failure of these drugs to satisfactorily treat chronic autoimmune urticaria may relate to this, at least in part.

Ascomycin or pimecrolimus is currently available in topical form as Elidel[®] cream (Novartis Pharmaceuticals, East Hanover, NJ, USA) and reports of phase I/II trials for oral pimecrolimus in psoriasis and atopic dermatitis have shown that it is a safe and effective alternative to ciclosporin in these dermatoses and may have fewer side-effects [19–21]. Since it inhibits basophil histamine release at a concentration that is comparable with ciclosporin, we believe that oral pimecrolimus may potentially be an effective treatment for autoimmune urticaria.

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