

Blood basophil numbers in chronic ordinary urticaria and healthy controls: diurnal variation, influence of loratadine and prednisolone and relationship to disease activity

C. E. H. Grattan, G. Dawn, S. Gibbs and D. M. Francis*

Department of Dermatology, Norfolk & Norwich University Hospital NHS Trust, Norwich and *St John's Institute of Dermatology, Guy's, King's and St Thomas' School of Medicine, St Thomas' Hospital, London, UK

Summary

Background The basopenia of chronic urticaria relates to histamine releasing autoantibodies in the serum of patients with autoimmune urticaria. This reduction in circulating basophils may be due to active recruitment into weals. If so, it might be expected that numbers in blood would be reduced when urticaria is active and increased after treatment. The primary aim of this study was to look at diurnal variation of basophil numbers in patients with chronic ordinary urticaria (not physical or vasculitic) in relation to disease activity and the effect of treatment with antihistamines and corticosteroids, and to compare the results with healthy controls. A secondary aim was to compare a standard manual counting method with automated basophil counts and to look at numbers of other circulating leucocytes that might be relevant to urticaria pathogenesis.

Methods Manual basophil counts using a toluidine blue stain and automated 5-part differentials (Coulter® Gen. S™) were performed at 4-hourly intervals from 08.00 to 20.00 in 10 healthy controls (six women, age 24 to 63 years) and seven chronic urticaria patients (five women, 24 to 50 years). All chronic urticaria patients had severe daily or almost daily urticaria. Only one of six chronic urticaria sera showed *in vitro* basophil histamine releasing activity. Counts were performed without treatment, after a week of taking loratadine 10 mg daily and after 3 days of adding prednisolone at 0.6 mg/kg/day (maximum 40 mg). Daily urticarial activity scores (UAS) were derived from weal numbers and itch, maximum 7.

Results There was no significant overall diurnal variation of basophil numbers in healthy controls or chronic urticaria patients. Mean (SE) manually counted basophil were higher in healthy controls than chronic urticaria (43.4/μL (2.1) vs. 4.4 (0.8), $P < 0.001$). Basophil counts were reduced in healthy controls on steroids (19.2 (1.9), $P < 0.001$) but increased in chronic urticaria (8.9 (1.9), $P < 0.001$). Loratadine did not influence them. UAS fell on treatment (3.3 (0.4) baseline, 1.4 (0.5) on loratadine and 0.5 (0.2) on prednisolone with loratadine, $P < 0.001$). There was a negative linear correlation between basophil numbers and UAS in untreated chronic urticaria patients ($P = 0.001$, Spearman rank correlation). Manual and automated basophil counts showed poor agreement. Lymphocyte numbers were lower in chronic urticaria than healthy controls. Neutrophils increased whereas lymphocytes and eosinophils decreased in all subjects on prednisolone. They were unaffected by loratadine.

Conclusion The results are consistent with the hypothesis that circulating basophils may be recruited from blood into urticarial weals during disease activity. Automated counts are not suitable for assessing basophil numbers in chronic urticaria. The relevance of reduced lymphocyte numbers in chronic urticaria needs to be explored.

Keywords antihistamines, automated differential, basophil, chronic urticaria, eosinophils, lymphocytes, manual counting, neutrophils, steroids

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Introduction

Basophils represent only about 1% of circulating leucocytes in health. Basophilia has been associated with chronic inflammation, endocrine disorders and infections, iron deficiency and some types of neoplasia but basopenia has only been linked with anaphylaxis and urticaria [1]. Rorsman first described a reduction of peripheral blood basophil in patients with chronic

Correspondence: C. Grattan, Department of Dermatology, Norfolk & Norwich University Hospital, Colney, Norfolk NR4 7UZ, UK.
E-mail: clive.grattan@nnuh.nhs.uk

urticaria, using a metachromatic granule stain [2]. His observation has been confirmed in more recent studies using manual counting [3, 4] and flow cytometry [4]. Absence of blood basophils relates closely to the presence of basophil histamine releasing activity in autologous serum [4] and therefore appears to be a useful marker for autoimmune urticaria. Autoimmune urticaria is now classified as a subset of ordinary urticaria [5]. The term 'ordinary' embraces all urticarias that cannot be attributed to a physical, vasculitic or contact cause and includes those cases with 'idiopathic' disease where the aetiology remains unknown despite full evaluation. Loss of granules as a reason for the apparent basopenia seems unlikely as basophil numbers are similar whether counted manually using a granule stain or flow cytometrically using dual staining with monoclonal antibodies against IgE and FcεRI on the cell surface [4].

It is possible that the peripheral blood basopenia is due to active recruitment of basophils into weals. Sustained histamine release from tissue basophils might explain why urticarial weals are more prolonged than after histamine injection in healthy subjects [6]. Preliminary data using the basophil-specific monoclonal BB1 indicate that basophils are increased in chronic urticaria weals of at least 12 h duration [7] but definitive evidence of migration into skin is still lacking. A relationship between diurnal urticarial activity and an influence of treatment with antihistamines and steroids on blood basophil numbers might be expected if this hypothesis is correct.

Manual counting of basophils is time-consuming and subject to errors due to the relatively small numbers of cells in the counting chamber. We have therefore compared it with automated counting using the basophil channel of a Coulter® Gen. S™ system, which uses a triple system to identify cells based on light scatter, impedance and the effect of high frequency signals. Size and granularity are some of the factors that influence the results. Accurate automated basophil counts could offer a simple clinical test to support a diagnosis of autoimmune urticaria and facilitate monitoring of disease activity. We have also looked for fluctuations in the number of other circulating leucocytes in healthy controls and chronic urticaria patients as neutrophils, eosinophils and lymphocytes are typically found in urticaria biopsies [8] and may be involved in its pathogenesis.

Methods

Subjects

Seven patients with daily or almost daily chronic urticaria (age range 24 to 50 years, median 35 years; five women) lasting more than 6 weeks (range 3 months to 26 years, median 12 months), referred to the Dermatology Department of the Norfolk and Norwich University Hospital by primary care practitioners, were recruited. Those with predominantly physical or vasculitic urticaria were excluded by a careful clinical assessment. Ten healthy controls (age range 24 to 63 years, median 39 years; six women), were recruited from technical, medical and nursing staff. Exclusions were C1 esterase inhibitor deficiency, children less than 16 years, pregnant or breast-feeding mothers, patients with known systemic lupus erythematosus, thyroid disease, Cushing's disease, active inflammatory bowel disease, dyspepsia, acute infection within 3 weeks, hypereosinophilia, hyperlipidaemia, and known immunosuppression. None of the

participants were on the long-acting antihistamine astemizole within 6 weeks, immunosuppressive medications, systemic steroids, non-steroidal anti-inflammatory drugs, doxepin, oestrogens or progestagens within 4 weeks or had had previous adverse reactions to loratadine or prednisolone.

Trial medication

All urticaria patients were asked to stop antihistamines at least 7 days prior to the study. Calamine lotion, crotamiton cream or 1% menthol in aqueous cream were allowed for symptomatic relief of pruritus. All participants started loratadine 10 mg mane on day 2 until the close of study (day 14). Prednisolone was introduced at 0.6 mg/kg body weight (maximum 40 mg) once a day after breakfast for the last 3 days of the study (days 12 to 14).

Blood samples

Venous blood was taken into K₃EDTA tubes (Vacutainer®, Becton Dickinson, New Jersey, USA) for manual and automated basophil counts at 08.00, 12.00, 16.00 and 20.00 on day 1 (baseline counts off treatment), after 1 week on loratadine and on the third day of taking prednisolone with loratadine (the 08.00 sample being taken just before the third and final dose of prednisolone). Venous blood was clotted for at least 30 min in plain Vacutainer® tubes before centrifugation at 450 g for skin testing. Residual serum was stored at -70 °C for histamine release assays.

Cell counting

Manual basophil counts were performed at a 1/5 dilution of a toluidine blue-based stain [9] using a modified Fuchs-Rosenthal (Manners) chamber, as described previously [10] and expressed as counts/μL of whole blood. Automated counts were made using the basophil channel of a Coulter® Gen. S™ system (Coulter Electronics Ltd, Luton, UK) within 6 h of venesection during routine full blood counts. The lower limit of automated counting was 10 basophils/μL; any counts below this were reported as zero. Lymphocyte, neutrophil, monocyte and eosinophil counts were taken from the same five-part automated differentials.

Basophil histamine release assays

Serum histamine releasing activity was tested on basophils from a healthy well-characterized donor with low serum IgE, as described previously [4]. Each serum was tested on basophils from which IgE had been eluted by lactic acid treatment [11] and, in a separate experiment, on basophils that had been passively sensitized with myeloma IgE (a gift from Dr J Hakimi, Hoffman La-Roche, Nutley, NJ, USA). The results were expressed as the percentage of total histamine content in each sample released from the cells into the supernatant after correction for background serum fluorescence and control spontaneous release. Histamine release > 5% was regarded as positive.

Urticarial activity assessments

Patients recorded the number and size of weals, severity of itching and episodes of angio-oedema at 4-hourly intervals between 08.00 and 20.00 during the 2-week study period. A scoring system of 0 to 4 points was used for weal severity (no weals, 0; 1 to 10 small weals, 1; 11 to 50 small weals or 1 to 10

large weals, 2; > 50 small weals or > 10 large weals, 3; almost covered, 4) and 0 to 3 for pruritus (none, 0; mild, 1; moderate, 2; severe, 3) as published previously [12]. Thus, the least urticarial activity score (UAS) for any time point was 0 and the greatest was 7.

Autologous serum skin tests (ASST)

Intradermal skin tests with 50 μ L of autologous serum were conducted on the volar forearm and any resulting weal response measured at 30 min, as described previously [13]. A pink weal response 1.5 mm or greater than an adjacent normal saline control skin test was regarded as evidence of endogenous histamine releasing activity due to functional autoantibodies or a mast cell specific factor [14].

Ethics approval

The study protocol was approved by the Norwich Health District Ethics Committee. All participants gave written informed consent.

Statistical analysis

Baseline basophil counts in healthy controls and chronic urticaria patients were compared with an unpaired *t*-test after checking for normal distribution of the data (normality test, Statworks™, Cricket Software Inc., Philadelphia, PA, USA). Differences between leucocyte counts at 4-hourly intervals over the day were compared with 1-way ANOVA. Differences in diurnal counts at baseline and on treatment were compared with 2-way ANOVA. UAS were compared on and off treatment with the Wilcoxon signed ranks test. Spearman rank correlation was used for examining linear association between baseline basophil counts and UAS. Comparison of manual and automated basophil counting methods was done by agreement of methods analysis [15]. All statistical tests were two-tailed and assumed a 5% level of significance.

Results

Healthy controls

There was no significant diurnal variation in basophil numbers counted manually without treatment. Mean (SE) counts/ μ L over the 12-h baseline period were 43.4 (2.1). There was no significant difference on loratadine (44.9; 1.8) but there was a significant reduction on prednisolone (19.2, 1.9; *F*-ratio 117.4, $P < 0.001$, two-way ANOVA). Basophil numbers fell rapidly after a morning dose of prednisolone (Fig. 1a) (*F*-ratio 85.0, $P < 0.001$, one-way ANOVA).

There was poor agreement between manual and automated basophil counts (Fig. 2). Automated counts two SD either side of the mean difference between methods might be as high as 33/ μ L above the equivalent manual count or 24 below it (range of the difference 57).

Chronic urticaria patients

Six patients developed a pink weal response at least 1.5 mm greater than an adjacent normal saline control injection within 30 min of intradermal injection of 50 μ L of their own serum (mean diameter (SD) 3.1 (1.3) mm). Five of these sera were available for assay on basophils. Only one showed *in vitro*

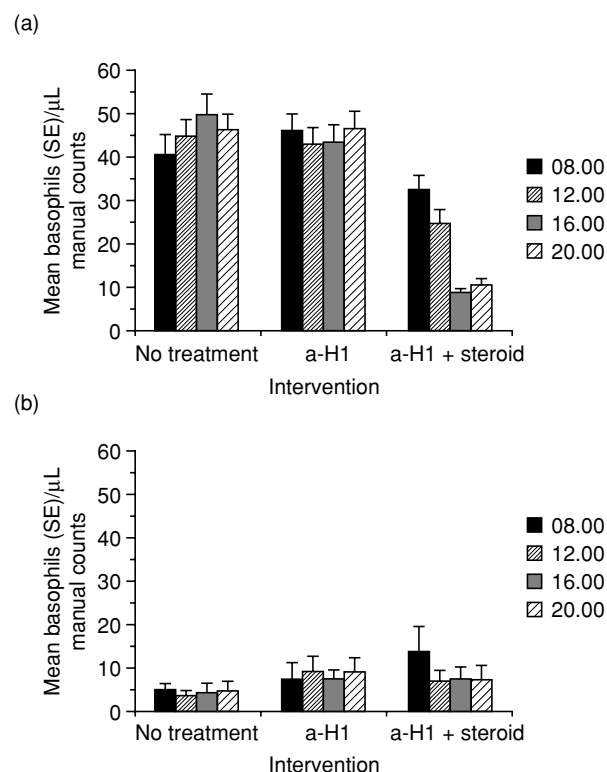


Fig. 1. (a) Basophil numbers in healthy controls during the day at baseline, on loratadine and after the addition of prednisolone, showing no consistent diurnal variation but a sharp fall after steroids. (b) The equivalent data for chronic urticaria patients, showing substantially lower basophil numbers at baseline but a slight overall rise on loratadine and on prednisolone. The counts were similar to healthy control values at the comparable 16.00 and 20.00 readings. a-H1 = loratadine; the number keys to the shaded columns are times of the day.

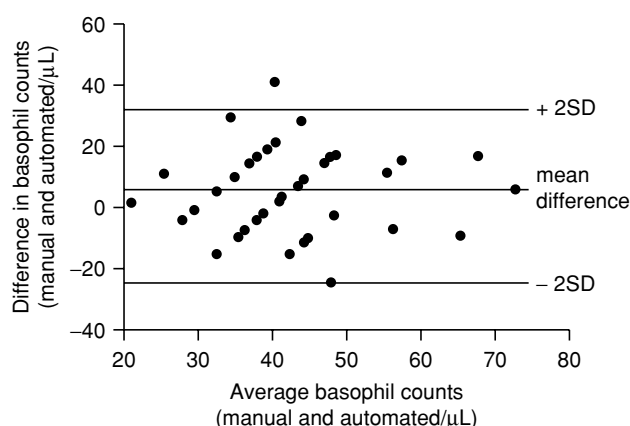


Fig. 2. Wide scatter of variation around the mean difference between manual and automated counting methods, illustrating the poor agreement between these two methods for counting blood basophil numbers in healthy controls.

histamine releasing activity (65% on IgE-stripped basophils, 19.4% on basophils passively sensitized with myeloma IgE), consistent with the presence of anti-Fc ϵ RI autoantibodies. The only skin test negative serum showed no *in vitro* activity.

In contrast to healthy controls, baseline basophils in chronic urticaria were reduced (4.4 (0.8), $t = 16.03$, $P < 0.001$).

Basophils were only consistently absent in the one patient with *in vitro* evidence of autoantibodies. There was no significant diurnal variation. Numbers rose after treatment with loratadine (8.2; 0.1) and prednisolone (8.9, 1.8; *F*-ratio 14.3, $P < 0.001$, two-way ANOVA) (Fig. 1b).

UAS fell significantly from baseline (3.3; 0.4) on loratadine (1.4, 0.5; $P < 0.001$, Wilcoxon signed ranks test) and prednisolone (0.5, 0.2; $P < 0.001$, Wilcoxon signed ranks test). There was a significant negative correlation between baseline basophil numbers and UAS (Fig. 3; Spearman rank correlation 0.612, $P = 0.001$).

All automated basophil counts were zero at each time point.

Lymphocyte numbers/ μL whole blood (mean $\times 10^3$, SE) were consistently lower in untreated chronic urticaria than healthy controls (2.05 (0.12) vs. 2.66 (0.12), $t = -3.576$, $P = 0.001$) and on loratadine (2.11 (0.09) vs. 2.67 (0.12), $t = -3.474$, $P = 0.001$). They fell on steroids in both chronic urticaria and healthy controls, but by a smaller amount in chronic urticaria (1.76 (0.16) vs. 2.04 (0.20), $t = -1.017$, $P = \text{not significant}$) (Fig. 4).

Eosinophils/ μL whole blood (mean, SE) were lower in chronic urticaria than healthy controls (121 (13) vs. 198 (10),

$t = 4.701$, $P < 0.001$) but became undetectable after prednisolone in both. There was no diurnal variation in chronic urticaria.

Neutrophils/ μL whole blood (mean $\times 10^3$, SE) were slightly more numerous in untreated chronic urticaria than healthy controls (5.5 (0.3) vs. 4.5 (0.3), $t = 2.543$, $P = 0.013$). They increased after steroids (6.4 (0.5) vs. 7.5 (0.3), $P = \text{not significant}$).

Discussion

The findings of this study reaffirm the reduction of peripheral blood basophils in active chronic urticaria. Collectively, there was no significant diurnal variation in numbers in healthy controls or chronic urticaria patients. There was, however, an association between numbers and urticarial activity, which supports the hypothesis that basophils may be actively recruited from the circulation into weals by endothelial adhesion molecule expression secondary to chemokines, such as interleukin-8, released from dermal inflammatory cells. Improvement of urticaria on antihistamines and oral corticosteroids is well known. The mechanisms for this improvement may include inhibition of migration of basophil into skin by reducing markers of activation and vascular adhesion molecule expression. Anti-allergic properties have been ascribed to loratadine [16], which might contribute to its clinical benefits in urticaria in addition to its antihistaminic action. Enhanced apoptosis of basophils by steroids could explain the fall of basophil numbers in healthy controls, while the slight increase from baseline in chronic urticaria patients might result from a predominant inhibition of recruitment.

It may be relevant that the only patient with consistently absent stainable blood basophils at baseline also showed high levels of functional autoantibodies in her serum. This raises the possibility that functional autoantibodies enhance basopenia by binding to Fc ϵ RI, leading to expression of basophil surface activation markers such as CD63 and enhancing selective removal by the reticuloendothelial system. Inhibition of anti-IgE induced histamine release has been reported in chronic urticaria basophils [17], and up-regulation of histamine release by interleukin-3 [18], illustrating the functional changes that occur in addition to basopenia. Reduced (as opposed to absent) blood

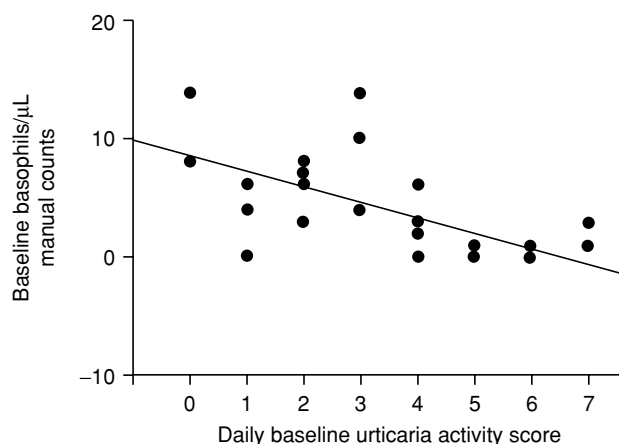


Fig. 3. Significant negative downward linear correlation between basophil numbers and urticarial activity in chronic urticaria patients (Spearman rank correlation, $r = 0.612$, $P < 0.001$).

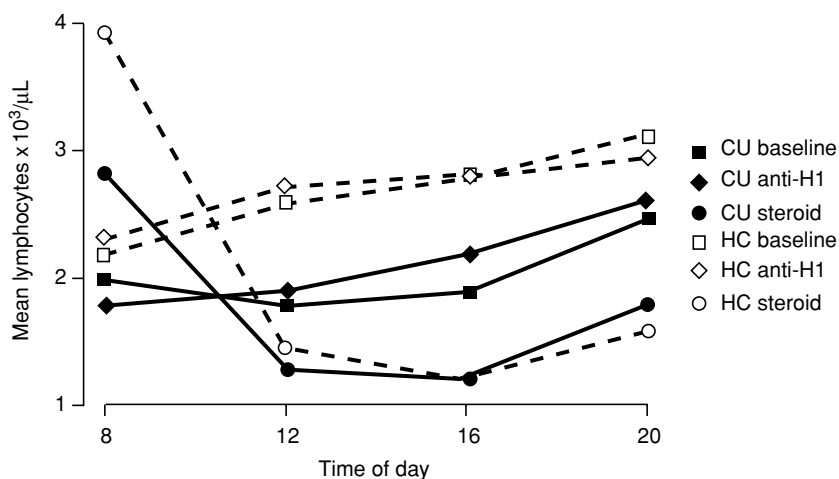


Fig. 4. Combined figure for lymphocyte counts at different times of day in healthy controls and chronic urticaria patients, showing consistently lower lymphocyte numbers in chronic urticaria with little effect of loratadine but a marked reduction on steroids, which was relatively greater for healthy controls than chronic urticaria.

basophil numbers have been shown for patients with 'non-autoantibody' chronic urticaria [3]. These may be the individuals who have a mast cell specific factor [14] that can elicit a positive ASST but will not show *in vitro* activity on basophils. The proposed recruitment of basophils into weals in response to adhesion molecule expression that results from mast cell degranulation could be expected to occur whether the activating stimulus is an autoantibody or some other histamine releasing agent.

There was poor agreement between manual counts based on a granule stain and automated basophil counts based on size and granularity in healthy controls, due partly to the low number of leucocytes counted in an individual sample (about 4000). Failure to discriminate between low ($< 10/\mu\text{L}$) and absent basophil numbers in chronic urticaria implies that the current methodology is unlikely to be of value in screening for chronic urticaria patients with functional autoantibodies. Programming automated differential counts to screen larger numbers of leucocytes (such as 40 000) might improve the level of agreement between these methods.

A reduction in total blood lymphocytes has not been reported previously to our knowledge although increased proliferation of activated B cells and increased expression of CD_{40L} on *in vitro* activated T cells has been reported in the blood of chronic urticaria patients [19], pointing to functional as well as numerical changes in lymphocyte populations.

The relative reduction of blood eosinophils in chronic urticaria compared with healthy controls is consistent with their presence in lesional skin biopsies [8] and may be analogous to the increased numbers of basophils and eosinophils found in the late cutaneous reaction of immediate hypersensitivity reactions [20]. The role of tissue eosinophils is unclear but it is possible that release of toxic eosinophil major basic and eosinophil cationic proteins further augments histamine release from mast cells in the late phase of urticarial weals.

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