

ORIGINAL PAPER

Effect of stress on basophil function in chronic idiopathic urticaria

S. M. Dyke, B. S. Carey and E. R. Kaminski

Department of Immunology, Derriford Combined Laboratory, Derriford Hospital, Plymouth, UK

Clinical and
Experimental
Allergy

Summary

Background Chronic idiopathic urticaria (CIU) is a distressing skin condition involving recurrent itchy hives lasting 6 weeks or longer. The mechanism involves mast cell and basophil degranulation, which releases inflammatory mediators including histamine. In our clinical practice, we have observed that the onset of CIU is often preceded by a major life event.

Objective To investigate the role of the hormones of the hypothalamic–pituitary–adrenal (HPA) axis in the link between psychological stress and CIU.

Methods Thirty people with CIU and 30 normal controls were recruited. A flow cytometric CD63 expression assay was used to quantify basophil activation, and serum cortisol concentrations were measured as an indication of stress.

Results Both corticotrophin releasing factor (CRF) and adrenocorticotrophic hormone (ACTH) were shown to activate basophils. There was no significant difference between numbers of CIU patients and normal controls responding to CRF, ACTH or cortisol. However, the responses in the CIU patients were stronger than those in normal controls. There was also a trend towards higher serum cortisol concentrations in CIU patients. The basophil response to CRF and ACTH correlated with the serum cortisol concentration in normal controls, but not in CIU patients.

Conclusions Although our data have not supported the hypothesis that stress makes a major contribution to CIU, the heightened basophil response to CRF and ACTH and higher levels of serum cortisol do suggest a derangement of the HPA axis in CIU.

Keywords ACTH, adrenocorticotrophic hormone, basophils, chronic idiopathic urticaria, corticotrophin-releasing factor, cortisol, stress

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Correspondence:

Dr E. R. Kaminski, Department of Immunology, Derriford Combined Laboratory, Level 7, Derriford Hospital, Plymouth PL6 8DH, UK.

E-mail:

edward.kaminski@phnt.swest.nhs.uk

Introduction

Chronic urticaria is a skin condition characterized by a raised, itching weal-and-flare rash that appears with varying frequency for at least 6 weeks and can occur for many years [1–3]. It affects 0.5–1% of individuals at some time in their lives [3]. The same mechanism is shared by chronic urticaria and allergic reactions, namely the degranulation of mast cells and basophils, which releases mediators of inflammation, such as histamine. However, unlike allergies, degranulation in chronic urticaria is not initiated by an allergen interacting with IgE bound to cell surface receptors. A proportion of cases of chronic urticaria are predominantly due to physical factors such as delayed pressure, cold, heat, etc directly triggering mast cell degranulation, and these are classified as physical

urticaria [3]. In another proportion of cases of chronic urticaria, autoantibodies to the high-affinity IgE receptor have been demonstrated [4] and these are now classified as autoimmune urticaria [1]. Finally, the remaining patients with chronic urticaria for which no cause can be identified are classified as chronic idiopathic urticaria (CIU) [3]. It is recognized that physical urticarias often co-exist with CIU [1]. It has been suggested that autoimmune urticaria accounts for 30–50% of cases of chronic urticaria, including many patients previously diagnosed as CIU [5].

In our clinical allergy practice, we have made the observation that a relatively large proportion of patients with CIU give a history of a significant life event in the period leading up to the commencement of the symptoms and this has been confirmed by a recent study

(C. M. Chung, C. Symons, J. Gilliam and E. R. Kaminski, unpublished data). This is paralleled by the observation that in our own practice, only a relatively small minority (~5–10%) of patients with chronic urticaria have the autoimmune variety, as diagnosed by the autologous skin prick test or the presence of thyroid antibodies. A number of previous studies have proposed a role for stress in CIU [6–8].

The association with stress implicates the neuroendocrine system in the disease aetiology. Psychological stress activates the hypothalamic–pituitary–adrenal (HPA) axis. This hormone cascade includes corticotrophin-releasing factor (CRF), adrenocorticotrophic hormone (ACTH), and cortisol. CRF has been associated with mast cell degranulation in rats [9], and the expression of the CRF receptor has been detected in human skin [10], with higher levels found in CIU patients [6].

The activation and degranulation of basophils can be assessed using flow cytometric detection of surface CD63 expression. This flow cytometric assay has been used to study the response of donor basophils to serum from CIU patients to detect autoantibodies [11–13]. However, recent work by Luquin *et al.* [14] suggests an intrinsic basophil abnormality in CIU. In this study, the CD63 expression assay was used to investigate whether the degranulation of basophils in CIU results from an abnormal response to the HPA axis hormones.

Patients and methods

Study subjects

Thirty consecutive patients diagnosed with CIU were recruited from the routine allergy clinic at Derriford Hospital, Plymouth. Four of these patients were on anti-histamine treatment. Thirty normal volunteer controls from the hospital laboratory staff were also recruited. These volunteers had never suffered from any form of urticaria, were not taking antihistamines and did not have allergies requiring ongoing treatment or avoidance of specific allergen triggers. All subjects gave written informed consent to take part in the study. All subjects were aged 18–65 years to avoid any confounding effects caused by the declining function of the immune system in old age. All data were anonymized before analysis. This study was approved by the South West Devon Research Ethics Committee (reference: 06/Q2103/20).

Sample collection

Peripheral blood samples were taken by a trained phlebotomist. All samples were taken during the time of the morning clinic (within 2.5 h) to minimize the effects of the diurnal variation in the cortisol level.

Basophil activation test

The basophil activation test is based on flow cytometric detection of CD63, which appears on the surface of basophils during degranulation. Basophils are identified by the presence of IgE bound to the cell surface via the high-affinity IgE receptor. The basophil activation test measures cell function, and hence all samples were analysed within 24 h of collection to minimize cell death and degradation. The BASOTEST kit manufactured by Orpegen (Heidelberg, Germany) was purchased from Becton Dickinson (Oxford, UK). All reagents are kit components unless stated otherwise. Preparations of CRF (human/rat), ACTH (human) and cortisol (corticosterone) were purchased from Sigma-Aldrich (Poole, UK). The peptide hormones, CRF and ACTH, were dissolved in BASOTEST wash buffer at 5 and 10 µg/mL, respectively. Cortisol was dissolved at 50 µg/mL in 0.5% ethanol in wash buffer due to its hydrophobic character. These hormone concentrations are orders of magnitude higher than physiological levels, but are based on the work of Theoharides *et al.* [9] on the response of mast cells in rat skin.

The manufacturer's instructions for the BASOTEST were altered to assess the *in vitro* response of basophils to the HPA-axis hormones. One hundred microlitres of heparinized whole blood was pre-incubated with 20 µL of IL-3-containing buffer at 37 °C for 10 min. The stimulation stage involved addition of 100 µL of test solution (hormone solution, wash buffer as negative control or 0.5% ethanol in wash buffer) and incubation for 2 h at 37 °C. Alternatively, 100 µL of f-Met-Leu-Phe (fMLP; 2 µM) was added as a positive control stimulation and incubated for 20 min at 37 °C. Degranulation was stopped by chilling on ice for at least 5 min. The cells were then labelled by incubating for 20 min with a two-colour staining reagent containing two monoclonal antibodies: phycoerythrin-conjugated anti-IgE and anti-CD63 conjugated to fluorescein isothiocyanate. This was carried out on ice to avoid the anti-IgE activating the basophils by crosslinking IgE bound to receptors on the cell surface. The erythrocytes were lysed and the leucocytes were simultaneously fixed by the addition of lysing solution and incubation at room temperature for 10 min. Finally, the cells were washed and resuspended in ice-cold wash solution. They were kept on ice and analysed within 2 h. Data on approximately 1000 surface IgE-positive cells were acquired on a FACS calibur flow cytometer (Becton Dickinson), and the percentage expressing surface CD63 was calculated.

Assessing baseline basophil activation

One hundred microlitres of whole blood was diluted with 100 µL of ice-cold BASOTEST wash buffer to obtain the same dilution of the sample as that used for the basophil

stimulation test. Twenty microlitres of BASOTEST two-colour staining reagent was added and incubated on ice for 20 min to label the baseline CD63 without prior exposure to IL-3 buffer or incubation at 37 °C. The rest of the procedure was identical to that described for the basophil activation test.

Quantitation of serum cortisol

Serum cortisol concentration was measured by the Clinical Chemistry department using the routine immunoassay method on a DPC Immulite 2000 analyser.

Statistical analysis

The differences between percentages were tested by calculating the 95% confidence intervals (CIs) of proportion. Any overlap of the 95% CIs indicates that the percentages are not significantly different. Using the negative control to model on, the baseline basophil activation was tested using Student's *t*-test based on the mean and standard deviation of the CD63 fluorescence of the IgE-positive cells as given by the flow cytometer. Other analyses were carried out using non-parametric tests: Mann-Whitney *U*-test for hypothesis testing and Spearman's ranked correlation coefficient.

Results

Assay precision

To assess the variability of the basophil activation test performance, the negative and positive controls were tested in duplicate on three volunteer control samples. The 95% CIs for the percentage of basophils expressing CD63 overlapped in each pair, except the fMLP-positive controls for volunteer 2 (Fig. 1). The overlap indicated that there was no statistically significant difference between the two results. The difference between the two results for the positive control of volunteer 2 was statistically significant despite being the outcome of identical test conditions. The gap between the 95% CIs was small: only 0.4%. This variability was considered when interpreting the results of the basophil activation tests. The mean coefficient of variance for the negative controls was 16.17% and 5.67% for the positive controls.

Using the negative control to model baseline basophil activation

Baseline basophil activation, as indicated by CD63 expression, was assessed directly on eight samples: five CIU patients and three volunteer controls. Comparison between the baseline activation and the negative control showed no significant difference for six of the eight

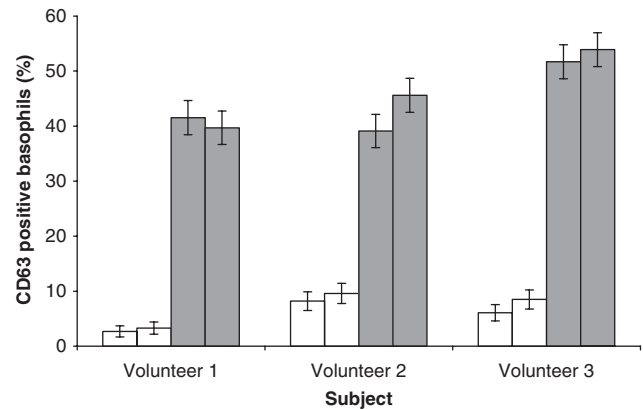


Fig. 1. Assessing basophil activation test assay precision. Controls were carried out in duplicate on blood samples from three volunteers. Open bars represent negative controls stimulated with BASOTEST wash buffer. Shaded bars represent positive controls stimulated with f-Met-Leu-Phe. Error bars represent 95% confidence intervals of proportion, calculated with n = number of surface IgE-positive cells.

samples (Table 1). One sample had a difference of only 1.32 fluorescence units, which was significant due to a small standard deviation, but not clinically significant. This left only one discrepant sample out of eight (12.5%; 95% CI = -10.4 to 35.4%). The negative control is therefore an acceptable model of baseline basophil activation.

Effects of antihistamine treatment on basophil response

Initially, CIU patients were recruited independently of whether or not they were taking antihistamine treatment for their condition. However, it was observed that basophils from patients on treatment had a different response profile to the stimulation conditions (Fig. 2). They had an elevated baseline activation as indicated by the negative control. The positive control response was the same irrespective of treatment. Only four patients on antihistamines were recruited, which accounts for the large standard errors of the means compared with the untreated group. Nonetheless, it can be seen that the mean result for those on antihistamines was high in response to all three hormones compared with the untreated group, but consistent with the negative control. In order to avoid this effect biasing the outcome of the study, all subsequent patients were recruited at initial diagnosis before commencing treatment with antihistamines. The results from the four patients taking antihistamines were excluded from the analyses of the basophil activation test results.

Response to f-Met-Leu-Phe as an indicator of successful assay performance

The validity of the basophil activation test was assessed by calculating the 95% CIs of the percentage of cells

Table 1. A Student's *t*-test to test the use of the basophil activation test negative control as a model for the baseline basophil activation

	Negative control (fluorescence intensity)		Baseline activation (fluorescence intensity)		<i>t</i> -test statistic	Significance
Subject	Mean	Standard deviation	Mean	Standard deviation		
CIU patients						
1	10.67	39.03	8.78	37.36	1.12	NS
2	20.57	49.83	9.87	28.95	5.13	Difference
3	13.64	65.92	10.35	25.64	1.47	NS
4	5.35	9.23	4.03	4.01	4.06	Difference
5	2.70	2.77	2.75	3.38	− 0.40	NS
Volunteer controls						
6	9.26	62.79	5.96	11.19	1.63	NS
7	6.17	17.77	5.73	14.36	0.69	NS
8	9.62	29.05	8.44	26.09	0.96	NS

The two-tailed test 95% critical value for significance of the *t*-test statistic is ± 1.96 . CIU, chronic idiopathic urticaria; NS, not significant.

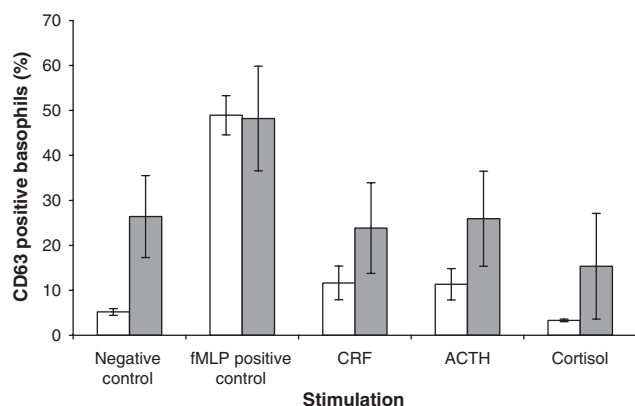


Fig. 2. Basophil responses from untreated chronic idiopathic urticaria (CIU) patients compared with those taking antihistamines. Open bars represent the mean of the untreated CIU patients [$n = 22$ for the negative control, f-Met-Leu-Phe (fMLP)-positive control and corticotrophin-releasing factor (CRF) stimulation; $n = 20$ for adrenocorticotrophic hormone (ACTH) stimulation and cortisol stimulation]. Shaded bars represent the mean of the CIU patients taking antihistamines ($n = 4$ for the negative and positive controls, CRF stimulation and ACTH stimulation; $n = 2$ for cortisol stimulation). Error bars represent the standard error of the mean.

expressing CD63 in the negative and positive controls for each sample. The positive control must be greater than the negative control and there must be no overlap in CIs in order for the test to be considered valid. This was the case in all the samples, except one, which was from a CIU patient. This sample was excluded from subsequent analyses of basophil activation test results because the test was deemed to have failed. While performing these analyses, it was observed that one volunteer control gave extreme responses to all stimulation conditions. It was decided to exclude these data as well, in order to avoid outlier effects on the statistical tests, although diagnosing their condition was beyond the scope of this study.

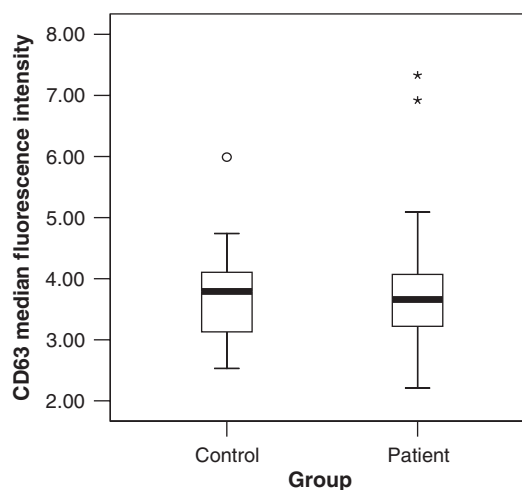


Fig. 3. Distribution of baseline basophil activation. CD63 median fluorescence intensity given in arbitrary fluorescence units. Subject group: control, volunteer control ($n = 27$); and patient, chronic idiopathic urticaria patient ($n = 22$). Open circles and asterisks represent outlying data and extreme outliers, respectively.

Baseline basophil activation and serum cortisol concentration

There was very little variation in the baseline basophil activation. No difference was found in the baseline expression of CD63 between the CIU patients and volunteer controls using a Mann–Whitney test to compare the median fluorescence intensity of the negative controls in each group ($P = 0.904$) (Fig. 3). There was a trend towards higher serum cortisol concentrations in the CIU patients compared with the volunteer controls (Fig. 4). However, using a Mann–Whitney test, this difference was not statistically significant ($P = 0.071$). There was no relationship between serum cortisol concentration and baseline basophil activation ($\rho = 0.008$, $P = 0.960$) and a wide variation in the serum cortisol concentrations, ranging from 111 to 897 nmol/L.

Basophil responses to stress hormones of the hypothalamic–pituitary–adrenal axis

The results of the basophil activation test using the hormones of the HPA axis as stimulation are summarized in Fig. 5. The data were analysed in the same way as for the assay precision study. The number of cells collected for each test was used to calculate the 95% CIs of the percentage of basophils expressing CD63. If the 95% CI for the response to a hormone did not overlap with the 95% CI for the negative control, the basophils from that individual were deemed to have responded to the hormone. Based on the assay precision study, any sample with a gap between the 95% CIs of $<0.4\%$ was not classified as a responder.

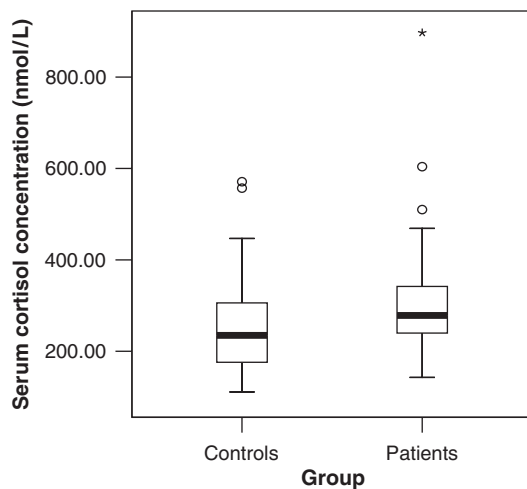


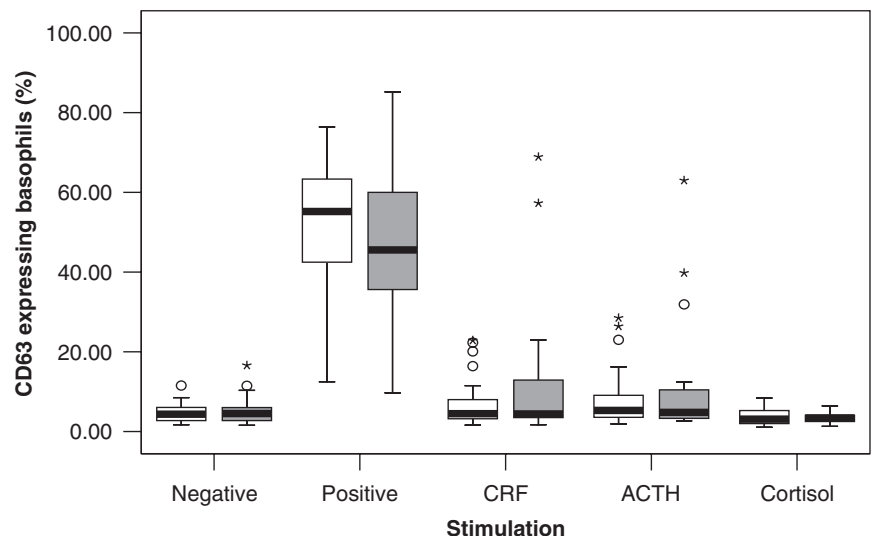
Fig. 4. Distribution of serum cortisol concentration. Subject group: controls, volunteer controls ($n = 30$); and patients, chronic idiopathic urticaria patients ($n = 22$). Open circles and asterisks represent outlying data and extreme outliers, respectively.

There were a few significant responses to CRF in both the CIU patient group and the volunteer control group. Four of the 22 CIU patients in the analysis (18.2%; 95% CI = 2.1–34.3%) responded, with a mean increase in the percentage of basophils expressing CD63 of 34.2% (95% CI = 31.3–37.1%). Six of the 27 volunteer controls (22.2%; 95% CI = 6.5–37.9%) responded, with a mean increase in basophils expressing CD63 of 11.1% (95% CI = 9.2–13.0%). There were five significant responses to ACTH in basophils from 20 CIU patients (25%; 95% CI = 6.0–44.0%) and nine from 27 volunteer controls (33.3%; 95% CI = 15.5–51.1%). The mean increase in the percentage of basophils expressing CD63 was 24.5% (95% CI = 21.8–27.2%) for the CIU patients and 10.8% (95% CI = 8.9–12.7%) for the volunteer controls. Two CIU patients responded to both CRF and ACTH. All six of the volunteer controls who responded to CRF also responded to ACTH.

In five of the 47 subjects (10.6%; 95% CI = 1.8–19.5%), the percentage of basophils expressing CD63 decreased following stimulation with cortisol. Three were volunteer controls and two were CIU patients. The mean reduction was 5.5% (95% CI = 4.1–6.9%). Three of these subjects also showed reductions in the percentage of basophils expressing CD63 in response to the 0.5% ethanol control. In total, six subjects had reduced basophil activation in response to the ethanol control. The ethanol solvent may therefore also have been a factor in the suppression of basophil activation in the tests stimulated with cortisol.

The basophil response to stimulation with the peptide hormones was calculated as the percentage of basophils expressing CD63 in response to CRF or ACTH minus the percentage in the negative control. The response to cortisol was calculated by subtracting from the negative control. These differences were then compared between CIU patients and volunteer controls using Mann–Whitney

Fig. 5. Summary of the basophil activation test using the hormones of the hypothalamic–pituitary–adrenal axis as stimulation. Open boxes represent the responses of basophils from volunteer controls ($n = 27$ for all stimulation groups). Shaded boxes represent the responses of chronic idiopathic urticaria patients [$n = 22$ for the negative control, f-Met-Leu-Phe positive control and corticotrophin-releasing factor (CRF) stimulation; $n = 20$ for adrenocorticotrophic hormone (ACTH) stimulation and cortisol stimulation]. Open circles and asterisks represent outlying data and extreme outliers, respectively.



tests. No statistically significant differences were found between the responses to CRF ($P=0.817$), ACTH ($P=0.540$) or cortisol ($P=0.606$).

Correlation of basophil activation with serum cortisol concentration

The responses to CRF and ACTH were calculated as the difference between the percentage of basophils expressing CD63 in the stimulated test and in the negative control. The relationship between these differences and the serum cortisol concentration was then explored using a Spearman's correlation coefficient. In the volunteer controls, the *in vitro* responses to both CRF and ACTH correlated with the serum cortisol concentration ($\rho=0.432$, $P=0.024$; and $\rho=0.432$, $P=0.025$, respectively). However, in the CIU patients this correlation was lost for both CRF ($\rho=0.010$, $P=0.968$) and ACTH ($\rho=0.270$, $P=0.295$).

Discussion

There is evidence that CIU may be associated with psychological stress [6–8]. This is proposed to be a two-way interaction, chronic skin diseases are known to heighten psychological distress [15], while chronic stress may be a trigger of CIU [3, 8]. In this study, a basophil activation test was used to look at whether an abnormal basophil response to the hormones of the HPA axis is involved in the mechanism of this link.

The comparison of baseline basophil activation between CIU patients and volunteer controls showed no difference in baseline CD63 expression. This is surprising, because basophils are thought to be involved in mediating the disease [16] and CD63 is a marker of basophil activation; it would therefore be expected that the basophils would be more active in patients with CIU. Therefore, the assumptions must be called into question. One possibility is that basophils are not involved in the disease process, which is entirely mediated by mast cells, or that basophils have to be recruited to the skin before they are activated as is the case for neutrophils in contact hypersensitivity [17]. Another is that CD63 expression may not be a good marker of activation for basophils in this disease. CD63 appears on the surface of basophils during degranulation and has previously been shown to be correlated with histamine release [18]. Perhaps the role of basophils in CIU is independent of their degranulation response. For example, human mast cells and basophils are a major source of cytokines, such as IL-4 and IL-5 [19], which could contribute to the inflammatory skin lesions.

Measuring serum cortisol as a surrogate marker of chronic stress found a trend towards higher serum cortisol concentrations in CIU patients. Measuring the cortisol concentration in a 24-h collection of urine would have given a more accurate indication of chronic stress and

may therefore have shown a more significant difference between the two groups. If CIU patients were found to have higher cortisol production than normal controls, this would be contrary to findings in atopic dermatitis where a blunting of the diurnal rhythm of cortisol production and a decreased cortisol response to acute stress are observed and implicated in the disease mechanism [20, 21].

The main purpose of the study was to investigate the response of basophils to the hormones of the HPA axis in order to determine their role in CIU. There were subjects in both the CIU patient group and the volunteer control group whose basophils responded to CRF and/or ACTH. Therefore, this response is not diagnostic of CIU. However, it does suggest that basophils are sensitive to activation by these peptide hormones. Roughly the same proportion of subjects responded to CRF or ACTH in the CIU patients and the volunteer control groups. It would be interesting to test whether the presence of autoantibodies affected the basophil responses. The responses were stronger in the CIU patients, resulting in a higher percentage of basophils expressing CD63. Therefore, basophils from CIU patients may be hyperresponsive rather than hypersensitive.

The final hormone of the HPA axis is cortisol. Corticosteroids are known to suppress cytotoxic T cell immunity and the results suggest a mild suppressive effect on basophil activation. When CIU patients are treated with corticosteroids, it may therefore not only reduce the inflammation but may also modulate the mechanism of the disease.

The working hypothesis of this study was that basophils from patients with CIU would have a lower threshold for activation by hormones of the HPA axis than basophils from normal controls. This was not shown to be the case, as no significant difference in basophil responses was found to any of the hormones tested. There is, however, a correlation between basophil responses to CRF or ACTH and serum cortisol concentration in volunteer controls that is lost in CIU patients. This derangement of basophil function is consistent with the basophil abnormality reported by Luquin *et al.* [14] in CIU patients. In conclusion, our data do not substantiate a major contribution of the hormones of the HPA axis to CIU.

Disclosure

There are no competing interests.

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