

Lack of role for mononuclear cell-derived histamine releasing factors in occupational asthma due to western red cedar

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Summary

Occupational asthma due to Western Red Cedar (WRCA) is attributed to sensitization to plicatic acid (PA), but does not appear to be dependent on PA-specific IgE antibodies. Exposure to PA induces histamine release *in vivo* and *in vitro*, so if IgE is not important, other mechanisms of histamine release must presumably operate in WRCA. To explore the possible role of histamine-releasing factors in WRCA, peripheral blood mononuclear cells were obtained and cultured with PA, PA-albumin conjugate plicatic acid-human serum albumin (PA-HSA), grass pollen or Concanavalin A using a standard histamine releasing factor (HRF) generation protocol. Supernatants were dialysed to remove endogenous histamine and then assayed for histamine releasing activity using human basophils as targets and a Con A-induced bulk supernatant as an internal HRF standard. In contrast to some previous reports, spontaneous HRF release from the peripheral blood mononuclear cells (PBMC) of WRCA patients ($n=9$) and atopic asthmatic subjects ($n=5$) was not elevated compared with the non-asthmatic controls ($n=11$; five atopic and six non-atopic). Both PA and PA-HSA induced the production of small amounts of HRF by PBMC of WRCA patients, but a similar degree of HRF generation was also observed in PBMC from the atopic asthmatic, atopic non-asthmatic, and non-atopic subjects. In contrast, grass pollen induced the production of HRF by PBMC from the subjects with positive skin tests to grass pollen but not by PBMC of non-atopic subjects, confirming that our methods and assay were capable of detecting antigen-specific HRF production. Since neither PA nor PA-HSA induced significantly elevated HRF production from PBMC of WRCA patients, it seems unlikely that PA-induced HRFs play a substantial role in the pathogenesis of WRCA.

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Introduction

Western Red Cedar Asthma (WRCA) has been shown to be caused by sensitization to plicatic acid (PA), a low molecular weight organic compound in cedar dust [1]. In sensitive subjects, inhalation of PA induces bronchoconstriction and elicits both immediate and late-phase responses, analogous to those observed in other forms of allergic asthma [2,3]. Mast cells and their chemical mediators have been implicated in WRCA, since increased amounts of histamine and leukotrienes can be

detected in bronchoalveolar lavage fluid following inhalation of PA [4]. Similarly, peripheral blood basophils and bronchial mast cells from WRCA patients release histamine when exposed *in vitro* to PA [1,5]. Despite their clearcut sensitivity to inhaled PA, only 20% of WRCA patients have detectable amounts of PA-specific IgE [3,5], raising some questions as to the exact mechanism by which mast cell mediators are released in WRCA.

IgE-dependent degranulation is the best understood mechanism for histamine release from mast cells and basophils [6], but several other stimuli, including neuropeptides [7], platelet products [8,9] and various cytokines [10] can also induce histamine release from basophils and

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mast cells. In the context of asthma, particular attention has been paid to mononuclear cell-derived 'histamine releasing factors' (HRFs) [11], which have been reported to be generated by mononuclear cells from asthmatic patients, both in spontaneous cultures [12] and in response to appropriate allergens [13]. Moreover, spontaneous HRF generation has been found to be related to bronchial responsiveness in the peripheral blood mononuclear cells (PBMC) donor, suggesting that HRF generation may be relevant to the pathogenesis of bronchial inflammation [14]. We have therefore examined the possibility that HRFs may be involved in the pathogenesis of WRCA.

Materials and methods

Chemicals

Unless otherwise stated, all chemicals were purchased from Fisher Scientific Ltd, Vancouver, B.C., Canada. Calcium ionophore A23187, human serum albumin, ficoll and antibiotics were purchased from Sigma Chemical Co, St Louis, U.S.A. Plicatic acid (PA) was prepared from western red cedar dust by Forintek, Vancouver, B.C., Canada. Plicatic Acid-Human Serum Albumin conjugate (PA-HSA) was prepared as previously described [3] with approximately 25 PA molecules per albumin molecule (PA:Albumin ratio 13% w/w). Tyrode's buffer without calcium [5] was prepared fresh and used for all washing steps. For cell cultures, a culture buffer was prepared, which consisted of Tyrode's buffer with added calcium (0.2 g/l), human serum albumin 0.1%, penicillin 100 U/ml, streptomycin 100 µg/ml and amphotericin 0.25 µg/ml. Mixed grass pollen extract (grass pollen mixture No 5, containing pollens of Kentucky blue, orchard, red top, timothy and sweet vernal grasses) was purchased from Hollister-Stier Laboratories (Elkhart, Indiana, U.S.A.).

Subjects

Nine patients with proven WRCA were recruited from the University of British Columbia Respiratory Clinic, Vancouver General Hospital, Canada. All had developed symptomatic asthma while working with western red cedar and had a positive bronchoprovocation test to plicatic acid (defined as 20% or greater fall in FEV₁ within 10 hr of challenge [2, 15]. The specificity of the PA-bronchoprovocation response was validated by the requirement for a stable FEV₁ during the sham challenge performed on the preceding day.) Four of these WRCA patients had isolated late-phase responses, three had early and late-phase asthmatic responses, while two had isolated immediate asthmatic responses. The atopic group consisted of

five atopic asthmatics and five atopic non-asthmatics. Atopic asthmatics were recruited from a local allergy clinic and by advertising for volunteers. The diagnosis of atopic asthma was confirmed by a compatible history, positive immediate skin test to grass pollen, and methacholine PC20 less than 4 mg/ml. Atopic, non-asthmatic volunteers were recruited from laboratory personnel and hospital staff: none had any symptoms of asthma, but all five had positive immediate skin tests to grass pollen (weal diameter ≥ 5 mm in each group). Non-atopic, non-asthmatic volunteers were recruited from laboratory personnel and hospital staff: none had any symptoms of asthma, methacholine PC20 was 20 mg/ml or greater and immediate skin tests were negative to 14 common airborne allergens. Further details of the subject groups are given in Table 1.

Mononuclear cell culture

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized peripheral venous blood by dextran sedimentation and density gradient separation over ficoll. The cells at the ficoll interface were aspirated, washed twice in Tyrode's buffer and adjusted to 10 million cells/ml. Aliquots of 10 million cells were placed in round-bottomed polypropylene tubes (Sarstedt, Numbrecht, Germany), centrifuged and then set up in culture in the presence of PA 100 µg/ml, PA-HSA 100 µg/ml, grass pollen extract 100 PNU/ml or Con A 50 µg/ml. Parallel cultures without antigen or mitogen were set up as negative controls. Following the methods of Alam *et al.* [12–14], PBMC were cultured for a total of 24 hr at 37°C in the presence of 5% CO₂. After the first 4 hr, the cells were centrifuged and washed twice to remove excess antigen and any mediators or cytokines that might have been released immediately after exposure to antigen. Cells were then resuspended in fresh culture buffer for the remaining 20 hr of culture. At the end of the culture period, the cells were centrifuged and the supernatants aspirated. Supernatants were dialysed overnight against Tyrode's buffer using a membrane with a 5000 D cut-off, in order to remove low molecular weight substances (e.g. histamine and products of metabolism). Dialysed supernatants were then stored at -80°C until assayed for histamine releasing factor.

Histamine releasing factor assay

Granulocyte preparations were obtained by dextran sedimentation of peripheral venous blood and density gradient separation over ficoll. The pelleted cells were washed twice in Tyrode's buffer and adjusted to 2×10^6 cells/ml. Aliquots of 2×10^6 cells were placed in round-

Table 1. Demographic characteristics, baseline spirometry, medication usage and skin-test results in the three principal study groups

Variable	WRCA (n=9) (mean)	Atopic asthma (n=5) (mean)	Atopic non-asthma (n=5) (mean)	Normal (n=6) (mean)
Age (yr)	40.6	29.2	27.2	32.1
FEV ₁ (% predicted)	85.8	98.7	100.2	106.2
FVC (% predicted)	99.1	95.1	95.5	101.5
Asthma	9	5	0	0
Sex (M:F)	9:0	1:4	5:0	4:2
Smoke (Never:Ex)	7:2	4:1	5:0	4:2
Grass pollen +ve	3	5	5	0
Skin-test -ve	6	0	0	6
Drugs				
β -2 agonist	6	4	—	—
Inhaled steroids	6	1	—	—
Theophylline	1	1	—	—

bottomed polypropylene tubes, centrifuged and then resuspended in 0.4 ml of dialysed culture supernatant, previously diluted with an equal volume of Tyrode's buffer containing HSA and antibiotics. Cells and supernatants were incubated for 30 min at 37°C in a water bath with continuous shaking, and then centrifuged (10 min 400 g). The supernatants were aspirated and stored at -80°C until assayed. Cell pellets were resuspended in 1 ml Tyrode's buffer, then frozen and thawed twice to release residual histamine. After the final thaw, tubes were centrifuged and supernatants were then aspirated and stored at -80°C until assayed. Histamine was detected by a single isotope radio-enzymatic assay as previously described [4]. The sensitivity of this assay is 20 pg histamine and the coefficient of variation of repeat measurement less than 2.5%. All histamine measurements were performed in duplicate. Results were expressed as the mean percentage of histamine released (i.e. amount of histamine in the supernatant divided by the sum of residual histamine in the cell pellet and the supernatant histamine). Net histamine release due to each supernatant was calculated by subtracting the percentage histamine release from control samples of target cells challenged with culture buffer alone. In preliminary studies, a bulk supernatant from Con A-stimulated PBMC was prepared and aliquotted for use as an internal HRF standard in subsequent assays. To allow comparison between samples tested on different days, HRF activity was standardized by expressing the percentage histamine released by each supernatant as a percentage of the activity of the internal HRF standard in the same assay.

Statistics

HRF generation to different stimuli was compared within groups by paired or unpaired *t*-tests as appropriate. HRF generation to the same stimulus was compared between groups by single factor analysis of variance. In each case, *P* values less than 0.05 were regarded as statistically significant.

Ethical approval

This study was approved by the Ethics Committee of the University of British Columbia, Canada.

Results

In preliminary dose-response studies, PBMC from several donors were stimulated with Con A, and the resulting supernatants were tested on the basophils of six different donors. This work confirmed that HRF activity was induced by Con A, in a dose-dependent manner, with a peak response at 25–50 µg/ml. PBMC viability was greater than 85% after the 24 hr cultures and there was no difference in the amount of HRF generated when RPMI-1640 was used instead of Tyrode's buffer in the culture medium. Although all six basophil donors responded to some extent to the Con A-induced supernatants, the magnitude of histamine release varied between basophil donors. A 72 hr time course study was also performed, which showed that maximal HRF activity was generated by 24 hr. As negative methodological controls, it was confirmed that no HRF was present in the supernatant of

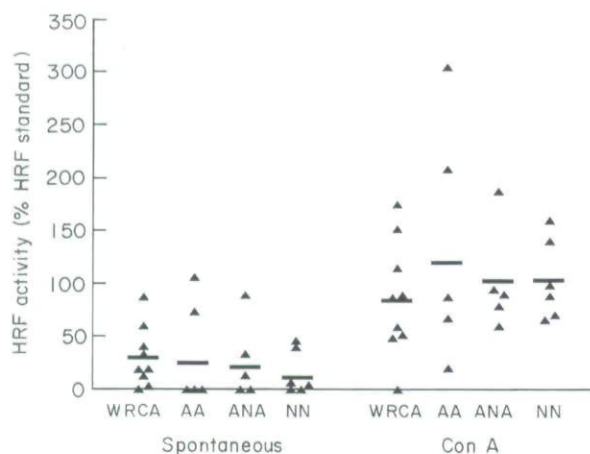


Fig. 1. Individual data for spontaneous and ConA-stimulated generation of HRF from cells of WRCA patients (WRCA; $n=9$), atopic asthmatics (AA; $n=5$), atopic non-asthmatics (ANA; $n=5$) and non-atopic non-asthmatics (NN; $n=6$). Results expressed as percentage of activity of HRF internal standard. Bars represent group means.

cells exposed for 1 hr to Con A or in buffer containing Con A cultured for 24 hr without cells and then dialysed as described above.

To standardize the assay as far as possible, all subsequent experiments were performed using target basophils obtained from a single atopic donor, whose cells had a low spontaneous release (mean $12.1 \pm 1.5\%$; $n=5$) and a

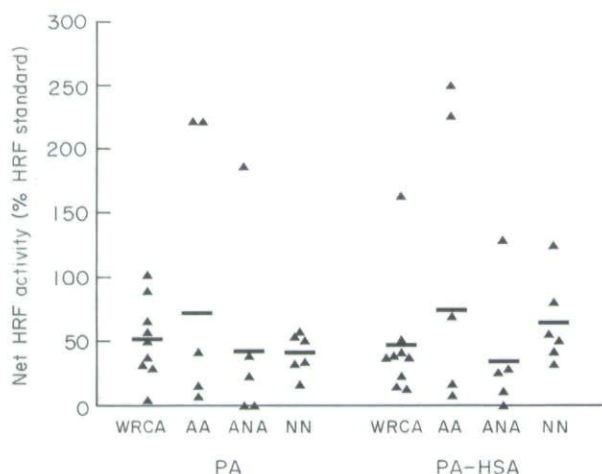


Fig. 2. Net generation of histamine releasing factor by PBMC cultured with plicatic acid (PA) 100 $\mu\text{g/ml}$, PA-HSA conjugate 100 $\mu\text{g/ml}$. Subject groups labelled as in Fig. 1. Results expressed as percentage of activity of HRF internal standard after subtracting HRF activity produced by cells stimulated with buffer in the same experiment. Bars represent group means.

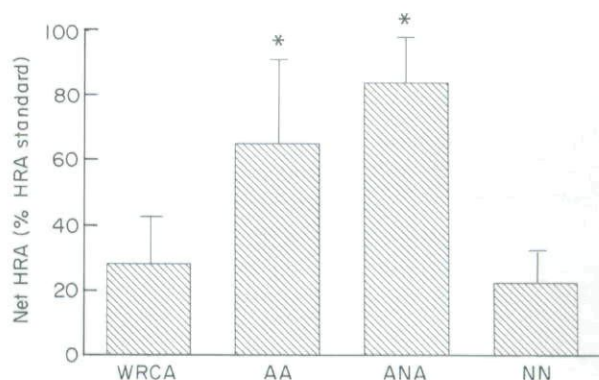


Fig. 3. Generation of grass pollen-induced histamine releasing factor by PBMC of WRCA patients. Subject groups labelled as in Fig. 1. Results expressed as percentage of activity of HRF internal standard after subtracting HRF activity produced by cells stimulated with buffer in the same experiment. Bars represent mean values. * = $P < 0.05$.

consistent response to Con A-stimulated PBMC supernatants in the pilot experiments. Using pooled supernatants from Con A-stimulated PBMC the HRF assay was demonstrated to be sensitive and to have a linear concentration response down to a dilution of 1:16. Slightly unexpectedly, it was found that for basophils from a given donor, under the conditions chosen, the amount of histamine released in the HRF assay was not affected by varying the number of target cells ($n=3$ data not shown). However, percentage release of total histamine (the usual parameter for this type of assay) was inversely related to target cell number, because total histamine is proportional to target cell number. These characteristics imply that the assay has target cell excess. These conditions are ideal for comparing differential HRF production, but have important implications for the way that results are expressed (see discussion). Optimum separation between Con A-stimulated supernatants and buffer controls was achieved when aliquots of 2 million leucocytes were challenged with supernatants which were diluted with an equal volume of Tyrode's buffer. These conditions were used for all subsequent assays. In addition, all HRF results were normalized to the activity of the internal standard to ensure that observed differences in HRF activity were due to differences in HRF and not to day-to-day variations in target cell number. The HRF internal standard was derived from a bulk culture of Con A-stimulated PBMC and in the four definitive HRF assays, it induced net histamine release of between 25 and 34% of total histamine (mean $\pm$ s.e.m. $29.3 \pm 1.8\%$).

Spontaneous HRF generation (from PBMC cultured without antigen or Con A) was similar in all subject groups (Fig. 1). In view of previous reports [12–14], the

atopic asthmatic group was compared separately with the atopic non-asthmatics and the non-atopic non-asthmatics but spontaneous HRF production was found to be similar in all groups (Fig. 1). Con A served as a positive control. Mean levels of HRF from Con A-stimulated PBMC were very close to the activity of the Con A standard. Cells from WRCA patients generated slightly less HRF in response to Con A than did PBMC from normal patients ($83 \pm 19\%$ vs $91 \pm 23\%$ of HRF standard; $P = \text{n.s.}$) while PBMC from atopic asthmatics showed increased responsiveness to Con A as compared with atopic non-asthmatics (mean $137 \pm 30\%$ vs $101 \pm 22\%$ of HRF standard respectively; $P = \text{n.s.}$)

PBMC cultured with PA yielded supernatants with increased HRF compared with spontaneous cultures. However, the mean increase in HRF production was comparable in all four subject groups (WRCA, Western Red Cedar Asthma; AA, Atopic Asthma; ANA, Atopic Non-Asthmatics; NN, Normal Non-Asthmatics), with the atopic asthmatic group showing the greatest increase (respective means WRCA: 51.8%, AA: 71.7%, ANA 42.1, NN: 40.5% of HRF standard; $P = \text{n.s.}$) (Fig. 2). PA-HSA also increased the amount of HRF generated by PBMC of all three groups, but with this antigen, PBMC from the atopic asthmatic and non-atopic groups produced rather more HRF than did PBMC from WRCA patients (WRCA: 46.3%, AA: 73.6%, ANA 34.4%, NN: 63.7% of HRF standard; $P = \text{n.s.}$) (Fig. 2).

Grass pollen was used as a specificity control. When cultured with grass pollen 100 PNU/ml, PBMC from atopic subjects generated HRF with a mean activity of $74.1 \pm 13.6\%$ of the internal HRF standard (AA & ANA vs NN: 74.1% vs 21.9%; $P = 0.02$) (Fig. 3).

Discussion

This study has used a carefully standardized assay to assess the possible role of histamine releasing factors in asthma due to western red cedar. A modest amount of HRF was generated spontaneously by PBMC from each of the four subject groups. Grass pollen-induced HRF production was evident in the atopic group but not in the WRCA or non-atopic subjects. Whether treated as raw data or standardized to the internal HRF standard, there was no evidence of WRCA-specific HRF generation in response to either PA or PA-HSA.

When attempting to establish a histamine releasing factor assay, review of the literature revealed wide variation between different authors' assay conditions. HRF has been assayed on human and animal basophils, in varying states of purity and with wide variation in the number of target cells, even in different studies from the same authors [12–19]. Our experiments showed that

the HRF assay is not necessarily linear with respect to target cell number and, as others have reported, there was wide variation between the responses of different basophil donors [20,21]. Most importantly, our assay had characteristics indicating target cell excess. Target cell excess means that the amount of histamine released depends on the supernatant activity, but does not depend directly on the amount of histamine present in the target cells. Such an assay can be used to demonstrate small differences in the activity of different supernatants, but care is needed in presenting the results. If results are expressed as percentage histamine release, then increasing target cell numbers will reduce the percentage release as the denominator (total histamine) is increased without a commensurate increase in the numerator (amount of histamine released). When using basophils from different donors or basophils obtained on different days from the same donor, it is thus advisable to use an internal HRF standard to circumvent this problem. In the present study, to maximize reproducibility, the definitive HRF assays were performed on a single basophil donor, who gave a consistent response to Con A-derived supernatants and a low level of spontaneous histamine release. Moreover, dose-response studies were performed to select assay conditions which gave a sensitive and linear response to dilutions of Con A-induced supernatants.

Some early studies of HRFs in asthma suggested that PBMC from asthmatic subjects spontaneously elaborated HRF in the absence of mitogen [12–14]. This has been partially confirmed by others [22]. In the present study, however, no significant difference was found between spontaneous HRF production between PBMC from the asthmatic and the non-asthmatic groups. Our results with Con A and grass pollen confirm that our HRF assay was both sensitive and specific: cells from virtually all subjects generated HRF in response to Con A, while grass pollen induced HRF production only by PBMC from donors with positive skin-test from grass pollen. The minor variation between groups in the magnitude of Con A-induced HRF is interesting but not statistically significant. In so far as there was a trend, PBMC from WRCA patients gave less Con A-induced HRF than non-atopic non-asthmatic controls, but PBMC from atopic asthmatics produced more Con A-induced HRF than atopic non-asthmatics.

It is important to note that grass pollen-induced HRF was not found in non-atopic subjects, indicating that the HRF in grass pollen-induced supernatants cannot be attributed to allergen carry-over. Similarly, the negative controls indicate that HRF activity is not due to inadvertent transfer of Con A.

PA and PA-HSA both induce histamine release from the basophils and mast cells of WRCA patients but

several lines of evidence suggest that this may not be via an IgE-dependent mechanism [5]. The present study indicates that PBMC-derived histamine releasing factors cannot account for the antigen-specific histamine release observed *in vitro* and *in vivo* in WRCA. This observation does not completely exclude the possibility that cytokines or soluble factors from PBMC may prime histamine-containing cells *in vivo* for greater release upon later challenge *in vitro* [22–25]. Such 'release-enhancing factors' have been described for neutrophils, eosinophils and basophils [22–26], so it is entirely plausible that release enhancing factors may also operate on mast cells. Similarly, the cytokine IL-8 has been shown to inhibit basophil histamine release induced by other cytokines [27]. Other physiological mechanisms by which mast cells and basophils can release mediators include complement activation, especially C5a [28], and platelet products [8,9]. Future studies will need to address the role that these mechanisms may play in WRCA.

In conclusion, this study has been unable to demonstrate a role for PBMC-derived histamine releasing factors in the pathogenesis of WRCA. The assay used was sufficiently sensitive to detect grass pollen-induced HRF generation by PBMC from atopic subjects, while PBMC from WRCA patients were able to generate HRF in response to the mitogen Con A, showing that they were capable of generating HRF if appropriately stimulated, but did not do so in response to PA or PA-HSA. It remains possible that PBMC-derived cytokines play a part in the pathogenesis of WRCA but evidence for this hypothesis will have to be sought by using alternative functional assays.

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