

Generation of Heat-Labile Chemotactic Activity in Blood after Inhalation Challenge and its Relationship to Neutrophil and Monocyte/Macrophage Turnover and Activity

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The chemotactic activity of serum has been monitored in eight patients with bronchial asthma after inhalation of an allergen. Lactoferrin and lysozyme were measured in serum simultaneously as markers of neutrophil and monocyte/macrophage activity and turnover. The heat-labile chemotactic activity of serum showed a two-phase response with an initial reduction in activity (median = 37.5 min after challenge) ($P < 0.05$) followed by an increased activity (median = 60 min after challenge) ($P < 0.01$). The consistency of this pattern was highly significant ($P < 0.0005$). Heat-stable chemotactic activity was significantly increased ($P < 0.01$) 15 min after challenge.

The initial reduction in heat-labile chemotactic activity was observed only in those patients who developed a late asthmatic response and could suggest the involvement of immune complex-mediated reactions since a similar reduction in heat-labile chemotactic activity is produced by *in vitro* activation of serum with aggregated IgG.

The increased heat-labile chemotactic activity showed a close relationship ($P < 0.01$) to the extent of the immediate reduction in PEF-rate. No relationship was discernible between the chemotactic activity and the eventually occurring rise in PMN blood count. In contrast the activity and turnover of PMNs after challenge as reflected by serum-lactoferrin levels were significantly reduced ($P < 0.01$). A close correlation ($P < 0.001$) was found between the heat-labile chemotactic activity in serum and the serum-levels of lysozyme, suggesting the involvement of macrophages/monocytes in the asthmatic reaction and in the generation of the heat-labile chemotactic activity found in serum after inhalation challenge.

Key words: asthma; chemotaxis; granule-proteins; immune-complex; mast-cell.

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Participation of neutrophil granulocytes in allergic reactions has recently been suggested. The idea was formed by the demonstration of neutrophil chemotactic activity in patients with cold urticaria (29) and after allergen challenge of asthmatics (2). The modes by which neutrophils may affect the allergic reaction are manifold. By virtue of their high content of proteolytic enzymes both humoral aspects such as complement, coagulation and fibrinolysis, and cellular aspects such as basophils and platelets may be affected (21). Also the production of

oxygen-free radicals by activated neutrophils could take part in the tissue inflammation (16) often seen as a consequence of the allergic process.

The response to allergen challenge may be an immediate reaction only, or an immediate reaction followed by a late reaction several hours after challenge and generally with an interdeposited symptom-free period. The mechanisms involved in the immediate reaction are probably of mast cell/basophil origin including the liberation of vasoactive substance

(5). The nature of the late reaction is far less known but immunocomplex-activatable reactions have been suggested (13, 23). Recently, patients with dual asthmatic responses were demonstrated to activate FXII (Hageman-factor) during challenge, which could point to the involvement of coagulation mechanisms as well as activation of kallikrein-kinin and fibrinolytic mechanisms (8).

In the present investigation we have studied the production of chemotactic activity in eight asthmatic patients with both single and dual responses to allergen challenge. The activities were correlated to the symptoms of the patients in order to test the hypothesis that the degree of recruitment of neutrophils to the challenged organ may be responsible for the symptoms. Further substantiation of the possible neutrophil involvement was made by the simultaneous measurements of markers in the blood of neutrophil turnover and activity such as lactoferrin, a constituent of the secondary granules of neutrophils (25), and lysozyme, a constituent of both neutrophil and monocyte/macrophage granules (22, 25). The comparison of the levels of these two markers might also give an indication of the possible involvement of macrophages in the asthmatic reaction – information which was of some interest in the light of recent data which suggested that the alveolar macrophage is an active participant in various inflammatory reactions taking place in the lung (17).

PATIENTS AND METHODS

Eight patients with allergic bronchial asthma were studied. There were four women and four men with an age range from 17 to 39 years (mean = 28 years). They had positive skin prick tests and IgE-RAST tests to more than two common allergens. Inhalation challenge test was positive when performed with the allergen used in the present study to provoke bronchial obstruction by inhalation challenge. None of the patients had received corticosteroids within the past 3 months and all medication was stopped 24 h before and during the test period.

Patients were informed about the purpose and nature of the study and all gave their consent to participate.

The patients were hospitalized and under constant supervision during the test period. Only quiet sedentary activity was allowed. Each patient was given an inhalation challenge with an allergen dose sufficient to provoke a fall in peak expiratory flow (PEF) of more than 20%. The allergens used were *Dermatophagoides pteronyssinus* in five patients and cat dander in three patients. The allergens were produced and standardized by Allergologisk Laboratorium, Copenhagen.

All challenges started at 9 a.m. PEF was measured before the challenge and every 15 min for the first 2 h after challenge and subsequently every 30 min for an additional 12 h or until a late reaction was established.

An immediate reaction was present if PEF fell more than 20% within the first hour after challenge, and a late reaction was present if PEF values fell more than 20% from the highest value reached after reversal from the immediate reaction and the development of clinical signs of increasing bronchospasms. A dual response consisted of an immediate reaction followed by a late reaction.

Six developed a dual asthmatic response and two only an immediate asthmatic response.

Blood was taken via a catheter placed in an antecubital vein. Samples were drawn before the challenge, every 15 min for 1½ h after the challenge and 2, 3, 4 and 6 h after the challenge. Serum was collected and stored at -70°C until assayed. Blood leucocytes were counted in a Coulter counter and a differential count was done on a blood smear. Blood eosinophils were counted in Fuchs-Rosenthal chambers using Rud's modification (24) of Dunger's method (10).

PMN migration was measured by means of the leading-front method (31) in a modified Boyden-chamber (30) as described previously (14). Filter pore-size was 3 µm. PMN-concentration was $1.5 \times 10^9/l$ diluted in Gey's buffer, and incubation time 60 min. Results are expressed as means of three to five readings on duplicate filters. The coefficient of variation of

the method was 10%. The chemotactic activity of serum was measured in the following way: Normal PMNs were suspended in Gey's buffer and placed above the filter. 10% fresh or heated (56°C, 30 min) serum, also diluted in Gey's buffer, was placed below the filter. Migration was allowed for 60 min. As a control the PMNs were allowed to migrate towards 10% pooled normal fresh serum and the migration towards this pool was referred to as 100% migration. The chemokinetic activity of serum was measured as follows: Normal PMNs were suspended in 10% serum and incubated at room temperature for 10 min before the migration experiment. This PMN-suspension was placed above the filter and Gey's buffer was placed below the filter. Migration was allowed for 60 min. As a control the PMNs were incubated in 10% normal pooled serum and the migration in the presence of this pool was referred to as 100% migration.

Aggregated IgG was made by heat-treatment of an IgG-solution (20 g/l) (Kabi, Sweden) at 63°C for 30 min.

Radioimmunoassays of lactoferrin and lysozyme were performed as described earlier (20, 26).

Student's paired and un-paired t-tests were used for statistical evaluation of differences be-

tween groups. Friedman's method for randomized blocks was used for some purposes.

RESULTS

Fig. 1 shows the variation in chemotactic activity of fresh serum obtained from eight patients after inhalation challenge with an allergen. The mean chemotactic activity of the sera before challenge was $143\% \pm 39\%$ which is significantly higher ($P < 0.05$, Student's t-test) than that of a reference population ($100\% \pm 11\%$) of healthy individuals. After challenge the chemotactic activity was reduced with a significant reduction observed after 30 min ($P < 0.05$, paired t-test, as compared with 0 min activity). This reduction was followed by a significant increment ($P < 0.01$, paired t-test, 30 min versus 60 min activity) at 60 min followed again by a significant reduction ($P < 0.02$, paired t-test, 60 min versus 360 min activity) down to prechallenge levels. The minimum chemotactic activity occurred within 15–60 min (median 37.5 min) after challenge and the maximum chemotactic activity after 45–120 min (median 60 min). The consistency of the variations of the chemotactic activity after challenge in the eight patients was tested by means of the Friedman's

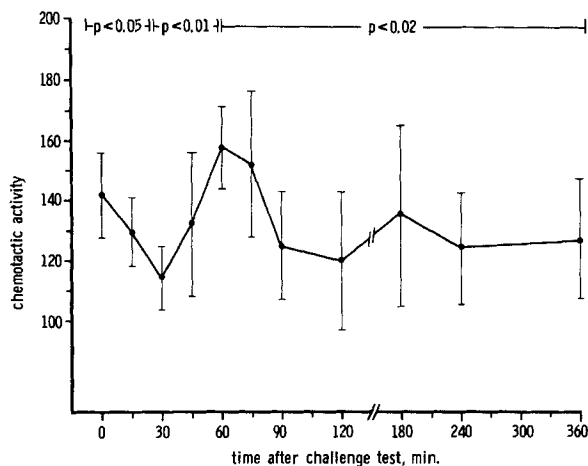


Fig. 1. Chemotactic activity of fresh serum from eight asthmatic patients after allergen challenge. Results are presented as means $\pm$ SEM. The significance of differences in chemotactic activities after various time periods after challenge was tested by means of Student's t-test. The following pairs were tested: 0–30 min, 30–60 min, 60–360 min. The statistical significances are indicated on the figure.

Table 1

Chemotactic activity of fresh and heated normal serum after activation of the fresh serum with aggregated IgG

	Fresh serum (10%)	Heated serum (10%)
Unactivated	100 $\pm$ 2	53 $\pm$ 2 <i>n</i> = 15
Activated:		
32 mgIgG/l	83 $\pm$ 3 <i>P</i> < 0.001 <i>n</i> = 9	57 $\pm$ 2 NS <i>n</i> = 6
64 mgIgG/l	77 $\pm$ 2 <i>P</i> < 0.001 <i>n</i> = 14	67 $\pm$ 2 <i>P</i> < 0.001 <i>n</i> = 14

100% fresh serum was incubated with aggregated IgG for 60 min at 37°C. After incubation the aggregated IgG was removed by ultracentrifugation and one part of the incubate was heated at 56°C for 30 min.

Results are means $\pm$ SEM and the statistical significance evaluated by means of Student's un-paired t-test as compared with the unactivated sera. NS = not significant.

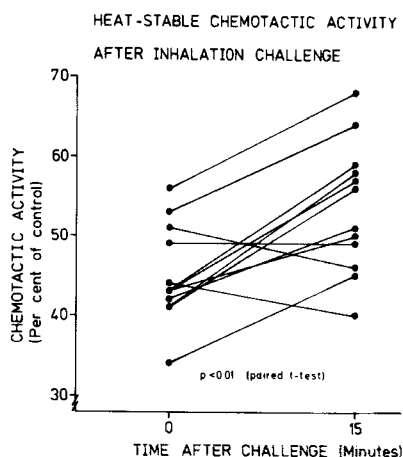


Fig. 2. Chemotactic activity of heat-treated sera (56°C, 30 min) from 12 asthmatic patients before and 15 min after challenge. The significance of the change in activity was tested by means of Student's paired t-test as indicated on the figure.

method for randomized blocks. The consistency of the pattern was highly significant ($P < 0.0005$).

The chemotactic activity of a normal serum is reduced by heat-treatment (56°C, 30 min) to approximately 50% of that of a fresh serum (Table 1). Heat-treatment of the patients' sera also reduced the chemotactic activity. The chemotactic activity in heated sera from 12 patients obtained 15 min after challenge showed a significant increase ($P < 0.01$, paired t-test) (Fig.

2). In two patients the heat-stable chemotactic activity was measured in all sera obtained after challenge. In one of these patients the maximum occurred after 90 min, and in the other patient after 120 min.

A significant initial reduction in heat-labile chemotactic activity was found in five of the six patients who developed a late asthmatic reaction but not in those who only developed an immediate reaction. The reduction could either be due to a reduction in the chemotactic activity or due to the production of an inhibitory principle. To test for these possibilities patient-sera with reduced activities were incubated with 50% normal serum. The chemotactic activity of all five sera was normalized by this measure, which indicated that the reduced activity was due to a consumption of the chemotactic activity. Since suggestions have been put forward that inhalation of an allergen gives rise to the formation of circulating immune complexes (13, 23), we also studied the effect of immune complexes on the chemotactic activity of fresh serum *in vitro*. In Table 1 the chemotactic activity of fresh serum is shown to be significantly reduced ($P < 0.001$) by the incubation of fresh serum with aggregated IgG. In parallel to this reduction in fresh serum the activity of heated serum was significantly increased ($P < 0.001$). The observed alterations in the chemotactic activities *in vivo* could thus be due to similar mechanisms.

Since the method of assaying chemotactic activity may be influenced by alterations in chemokinetic activity the chemokinetic activity of all sera was measured before and after heat-treatment (56°C, 30 min). The chemokinetic activity of fresh serum was, before challenge, on average $93.8 \pm 14\%$ ($\pm$ SD), which was not significantly different from the activity of normal serum. After challenge, the chemokinetic activity showed small but insignificant variations and in no case paralleled the variations in chemotactic activity. Heat-treatment of the sera did not change this pattern. These results indicated that the variations in chemotactic activity were real and not due to alterations in the chemokinetic activity of serum during challenge.

To investigate the possible relationship between the chemotactic activity formed after challenge and the asthmatic reaction, the peak chemotactic activity of fresh serum was correlated to the reduction in PEF during the immediate reaction. A close relationship ($P < 0.001$) was found between these two variables (Fig. 3) with the highest activities in those patients who had the heaviest reductions in the PEF. The reduction in PEF occurred in all cases within the first 45 min after challenge (median 30 min), which means that this reduction occurred well before the peak in chemotactic activity.

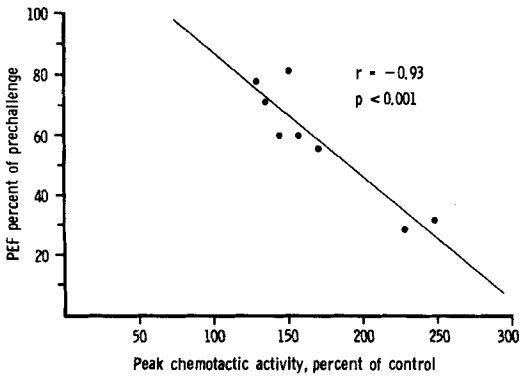


Fig. 3. Relationship between the maximal reduction in PEF-rate during the immediate hypersensitivity reaction and the peak chemotactic activity after challenge. The reduction in PEF-rate is expressed in percent of the prechallenge PEF-rate. Chemotactic activity is expressed as the percent activity of a pooled fresh normal serum (control).

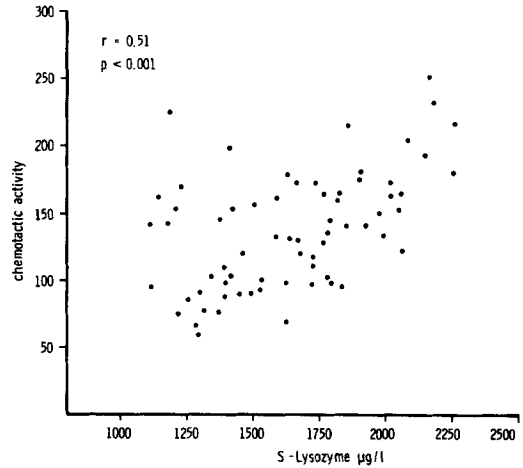


Fig. 5. The relationship between the chemotactic activity of fresh serum and S-lysozyme of all sera obtained just before and after allergen challenge of the eight asthmatic patients. The statistics of the correlation are given on the figure.

The possible effects of the chemotactic activity on PMN and monocyte turnover and activity was studied by monitoring the variations in cell counts after challenge and by following the serum levels of lactoferrin and lysozyme. The PMN blood cell count showed a significant increase ($151\% \pm 14.4\%$ ($\pm$ SEM)) of initial ($P < 0.02$, Student's test) after more than 120 min after challenge. No relationship between this PMN increase and the chemotactic activity after challenge was discernible. The serum-lactoferrin levels showed a slight but insignifi-

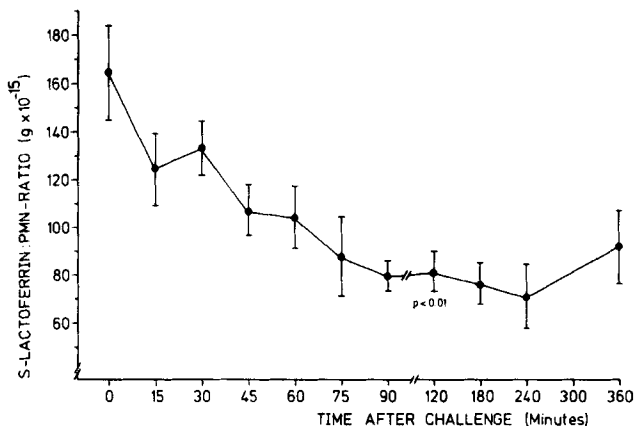


Fig. 4. S-lactoferrin: Blood PMN-ratio in eight asthmatic patients after allergen challenge. The results are expressed as means $\pm$ SEM. Statistical differences were evaluated by means of Student's paired t-test and indicated on the figure.

cant tendency towards a reduction after challenge. Since the only source of lactoferrin in the blood is the PMN, a very close relationship between the lactoferrin levels and the PMN turnover or activity was expected. These data on PMN blood cell count and serum-lactoferrin levels therefore suggested that the activity and turnover of the PMN after challenge were unaltered or even lower than normal. This latter suggestion was further strengthened by the significant reductions in the LF/PMN-ratio shown in Fig. 4. The reductions in the ratio appeared to start immediately after challenge and became significantly reduced after 90 min ($P < 0.05$, Student's *t*-test) with the lowest ratio 120 min ($P < 0.01$, Student's *t*-test) after challenge.

Pre-challenge levels of serum-lysozyme were $1514 \mu\text{g/l} \pm 321 \mu\text{g/l}$ ($\pm$ SD). In six of eight patients serum-lysozyme levels rose significantly after challenge, i.e. more than 10%, which is the intraindividual variation in healthy individuals (unpublished, Venge). Peak levels were observed after 60 min (median) with a range from 15–120 min. The variations in serum-lysozyme showed a significant co-variation with the chemotactic activity of fresh serum ($P < 0.001$) (Fig. 5). Serum-lysozyme levels did not co-vary with the levels of lactoferrin. These data suggested a relationship between the activity of monocytes/macrophages and the production of chemotactic activity after inhalation challenge.

DISCUSSION

The production of chemotactic activity after inhalation challenge (2) was confirmed by the present study. The predominant activity was heat-labile, occurred at a maximum 60 min after inhalation of the allergen and was normalized again after 90–120 min. The relevance of the heat-labile chemotactic activity to the allergic (bronchospastic) reaction was suggested by the very close relation to the reduction in lung function in the immediate reaction. This activity had no apparent relation to the late asthmatic reaction. Heat-stable activity was also formed after challenge and was detectable in samples obtained 15 min after challenge. Others have found significant heat-stable ac-

tivity already after 1 min with maximum activity after 10 min (3).

A consistent finding in our study was the initial reduction in chemotactic activity mostly within the first 15–30 min after challenge. *In vitro*, a reduction in the heat-labile chemotactic activity in serum is induced by immunecomplex (i.e. aggregated IgG)-activation of fresh serum (Table 1) (to be published, Håkansson & Venge). The phenomenon found *in vivo* in our patients could therefore be due to similar mechanisms. Interestingly, the five patients who had significant initial reductions in the heat-labile chemotactic activity all developed a late asthmatic reaction. Although the material is small this finding could lend support to the suggestion that the late allergic response is induced by immunecomplex-mediated mechanisms (13, 23).

The origin of the subsequent increase in chemotactic activity is unknown. Previous studies on heat-stable chemotactic activity have suggested the mast cell as the source of this activity (3). Demonstration *in vivo* of mast cell-derived chemotactic products from various species (28) has also recently been made and may support this notion. The production of the heat-labile chemotactic activity of our study was clearly related to the immediate reaction and seemed to be a consequence of the reaction. Other mast cell-derived products occur in the plasma within minutes after inhalation of the allergen (6) and the time-course of the appearance of the heat-labile chemotactic activity therefore makes the mast cell a less likely source. Our findings of a close relationship between the serum-lysozyme levels and the chemotactic activity was an interesting indication which could suggest the monocyte/macrophage as the source. The alveolar macrophages are most likely involved in the reactions taking place in the lungs (15) after inhalation of an allergen since monocytes/macrophages appear to have specific IgE-receptors on their surfaces (7, 19). Recently, alveolar macrophages have been shown (12, 17) to produce low molecular weight compounds with preferential chemotactic activity for PMNs, some of which may be analogous to the activity found in our patients.

In a recent study a relationship was demonstrated between the degree of neutrophilia after challenge and the production of heat-stable activity (4). These findings were interpreted to mean that the production of chemotactic activity after challenge was responsible for the mobilization of neutrophils and reflected the mobilization of neutrophils to the lung tissue. In our study we also observed a rise in blood PMNs which became significant for the whole group 120 min after inhalation of the allergen. We found, however, no relationship between this increment and the production of heat-labile or heat-stable chemotactic activity. Normally, there is a very close relationship between numbers of blood neutrophils and the serum-levels of lactoferrin (11). Raised levels of lactoferrin probably reflect an increased turnover or activity (18, 26, 27) of the neutrophil population in the circulation. Therefore, if the raised number of PMNs observed after challenge was a reflection of an increased turnover of the cells a corresponding rise in serum-lactoferrin levels would have been expected. This was not the case. On the contrary the serum-lactoferrin levels had a tendency to drop and the ratio between lactoferrin and PMN was significantly reduced after challenge. The simple explanation of these findings would be an unaltered or even lower than normal turnover of PMN after challenge, with a redistribution of the marginated blood PMNs to the blood stream: a phenomenon which is analogous to the leukocytosis produced by stress or corticosteroids (1). Other explanations which can not be excluded, but which probably are less likely, would be a reduced activity of the circulating PMN with a reduction in the secretion of lactoferrin and other granule-proteins to the plasma or a faster than normal elimination rate of lactoferrin from the plasma after challenge.

In conclusion we have confirmed the findings of others showing the generation of heat-stable chemotactic activity in blood in asthmatics after inhalation challenge (2). However, using fresh unheated serum we have demonstrated in this report that the net chemotactic activity early on after challenge is reduced and after approximately 60 min is followed by an

increase. These circumstances, and the fact that we were not able to find any indications of an increased turnover or activity of neutrophils after challenge, question the relevance of the described heat-stable activity to the mobilization of PMNs after challenge (4).

The findings of a relationship between the extent of the reduction in PEF during the immediate reaction and the formation of heat-labile chemotactic activity, as well as a relationship between the latter and serum-lysozyme levels, give rise to the following two hypotheses. One is that the alveolar macrophage is stimulated during the reaction to secrete both lysozyme and the heat-labile chemotactic factor and the stimulatory mechanism could either be an effect of the allergen directly on the macrophage or an effect mediated via the activation of the mast cell. The other would be that the mast cell is the producer of the chemotactic activity and simultaneously stimulates the alveolar macrophage to produce its various products. Whatever mechanism is responsible the important implications of the present results may be that the alveolar macrophage is an active participant in the asthmatic reaction.

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