

Reduction of increased serum neutrophil chemotactic activity following effective hyposensitization in house dust mite allergy

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Summary

Changes in the level of serum neutrophil chemotactic activity (S-NCA) were investigated in 20 subjects with allergic rhinitis, with or without asthma, undergoing clinically effective hyposensitization to house dust mite with Pharmedin® *Dermatophagoides pteronyssinus*. Two control groups were studied: 28 subjects with allergic rhinitis, with or without asthma, receiving placebo injections for 1 yr in a double-blind controlled trial with Pharmedin® *D. pteronyssinus* (from whom the actively treated group in this study were recruited), and eight non-atopic asymptomatic controls. S-NCA and serum IgE specific to *D. pteronyssinus* were measured in the subjects before, during (3-6 months) and 12 months after treatment, and once in the non-atopic controls. The mean S-NCA was significantly higher ($0.01 > P > 0.001$) in subjects before treatment (mean $\pm$ s.e. = 63.8 ± 3.6 arbitrary units of migration (AUM)) compared with the non-atopic controls (48.5 ± 3.7 AUM), but had fallen to normal levels after 6 months (46.8 ± 4.0 AUM) and 12 months treatment (45.2 ± 3.8 AUM). The levels of S-NCA in the placebo treated group were significantly higher than normal at the start of treatment (69.2 ± 4.1) and remained raised throughout the 12 months treatment. In the actively treated group, the level of S-NCA had fallen in 18 out of 19 subjects after 12 months immunotherapy, and was unaltered in one. Mean levels of *D. pteronyssinus* IgE rose during the first 6 months and declined to initial levels by the end of the treatment. We suggest that the elevated S-NCA may play a role in the maintenance of the allergic state through the induction of the late-phase reaction; that the abnormal S-NCA production is reduced by effective hyposensitization, and that measurement of S-NCA may be useful to monitor hyposensitization therapy. Complete characterization of S-NCA and the mechanisms of its control remain to be investigated.

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Introduction

There are now several recognized mediators, with polymorphonuclear leucocyte chemotactic activity, released during immediate hypersensitivity reactions, including histamine [1], leukotriene B₄ [2], eosinophil chemotactic factor [3], and both high and low molecular weight neutrophil chemotactic factors or activities (NCF or NCA) [4-6].

Serum neutrophil chemotactic activity may result from the combined effects of a number of factors, and must be distinguished from mast cell NCF (usually designated NCF or NCA), unless the source of the chemotactic activity has been defined. To emphasize this, we have designated serum neutrophil chemotactic activity as S-NCA in this paper. S-NCA has been shown to increase rapidly during the early phase of the allergic reaction in subjects challenged with specific antigen [7]. This is followed by a fall in S-NCA before a subsequent rise during the late-phase reaction [8,9].

The late-phase reaction, which can be demonstrated in

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the skin [10], nose [11], and lung [12], is now recognized to be important in allergic reactions. It is probably one of the best models for the study of allergic disease since it is thought to approximate more closely to clinical disease than the immediate response to antigen. The late reaction in the lung is followed by increased non-specific bronchial reactivity, and S-NCA seems to be important in the genesis of the late reaction. The influx of inflammatory cells, particularly neutrophils and eosinophils, is probably essential for the development of the late reaction which, in experimental models, can be abrogated by depletion of polymorphs [13]. Modification of the late reaction would be expected to be followed by reduced non-specific bronchial reactivity [14] and, indeed, in a study of hyposensitization in children, clinical benefit seemed to be associated with loss of the late reaction following antigen bronchoprovocation [15].

The mechanism of hyposensitization remains unclear, although for many years it has been said to be due to the generation of IgG 'blocking' antibody. Whilst this seems to be true in insect sting allergy [16] it remains to be established in inhalant allergies where the correlation between IgG titres and clinical efficacy following hyposensitization is often poor. However, lack of sufficiently sensitive assays for IgG antibodies to relevant allergens, together with the variable degree of clinical benefit following hyposensitization, make interpretation of findings from earlier studies difficult.

The late reaction in both the skin and the lung appears to be IgE-dependent [17,18], and blocking of mediator release in the immediate reaction may result in a decreased inflammatory late reaction. The mechanism of the loss of the late-phase reaction, however, has not been defined and there is no information on the effect of hyposensitization on S-NCA, or on the basal levels of S-NCA in house dust mite-sensitive subjects. In this study we investigated the effect of hyposensitization with an extract of *Dermatophagoides pteronyssinus* on S-NCA in subjects with allergic rhinitis, with or without asthma, over 1 yr.

Subjects and methods

Subjects treated with active immunotherapy

Twenty subjects, 11 male and nine female, with an age range of 22–41 yr, who had rhinitis due to allergy to house dust mite (and no other perennial allergy) were studied. Seventeen had asthma (which was mild in most) and none had eczema. The diagnosis was made on the basis of clinical history, and was confirmed by positive skin-prick and nasal challenge tests to *D. pteronyssinus*. All gave informed consent to hyposensitization with Pharmalgen® *D. pteronyssinus* (ALK, Denmark) in a study approved by

the hospital Ethics Committee. This was an open study (study 2), to which the placebo group, from a double-blind controlled study (study 1) with Pharmalgen® *D. pteronyssinus*, were recruited at the end of a 1 yr placebo treatment, and given a second year's active treatment. Pharmalgen® *D. pteronyssinus* had been shown to be effective against rhinitis in the double-blind controlled trial (decreased symptoms and decreased sensitivity to allergen on nasal challenge and skin testing after both 3 and 12 months treatment) [19,20, and a paper in preparation]. Subjects entered the study over a year, to minimize any seasonal influence.

Hyposensitization regime

Pharmalgen® *D. pteronyssinus* is a potent partially purified extract, which is biologically standardized and supplied freeze dried. It contains about 15% dry weight of the major allergen *Der pI*; 100 000 biological units (BU) are equivalent to approximately 260 000 IU of the international standard of *D. pteronyssinus* when assessed by RAST inhibition (manufacturer's data).

Injections were given subcutaneously using depot diluent throughout. The initial dose was 70 BU, with increments at 1–2 weekly intervals, up to a maximum of 30 000 BU or to a lower maximum tolerated dose. The regime was modified if a reaction to an injection occurred, or in the light of a history of recent or current infection, worsening of asthma or intercurrent illness, or if a fall in peak expiratory flow rate had occurred. When the top dose or maximum tolerated dose was reached, maintenance injections at this level were given monthly. The total treatment period was 1 yr. Concomitant drug therapy was restricted to beclomethasone nasal spray, cromoglycate nasal spray or the short acting oral antihistamines (terfenadine or chlorpheniramine) for rhinitis, and inhaled or oral salbutamol, beclomethasone inhaler, cromoglycate inhaler and/or oral theophylline for asthma. Most subjects took little or no drug therapy. Allergy avoidance measures were neither advised nor knowingly taken by any subject.

Placebo-treated subjects

The placebo-treated subjects from a separate double-blind controlled trial of hyposensitization with Pharmalgen® *D. pteronyssinus* (described above—study 1) were also studied. This group comprised 28 subjects, 17 male and 11 female, age range 19–52 yr (mean = $28.3 \pm \text{s.d. } 9.2$ yr). All had rhinitis due to *D. pteronyssinus* and had no other perennial allergy, and 21 also had asthma, which was mild in most cases. The same criteria for entry to this study were used as described above. Placebo injections of

histamine chloride were given at the same intervals as Pharmedin® *D. pteronyssinus*, starting at 0.7 µg (equivalent to '70 BU') to a maximum of 30 µg, for 1 yr. Concomitant medication was the same as that for the actively treated group.

Non-atopic controls

Ten asymptomatic healthy subjects, five males and five females, age range 18–45 yr, were also studied. Two of these were found to have positive *D. pteronyssinus* IgE antibody, as measured by the radio-allergosorbent test (RAST, Phadebas RAST®, Pharmacia, Sweden) and were subsequently excluded from the control group, but their S-NCA levels are reported. The remaining eight non-atopic subjects formed the second control group.

Serum

Ten millilitres of blood were taken from subjects before, during (3 or 6 months) and after (12 months) treatment. Blood samples were always taken before any immunotherapy injection if this was given on the same day. The blood was allowed to clot and the serum separated and stored at -20°C in 0.5–1 ml aliquots. Serum was also collected from the controls and stored in the same way. The only differences between the placebo and active groups were, (a) the second blood sample was taken at 3 months not 6 months, and (b) the serum was stored for 1 yr longer.

Leucocyte chemotaxis assay for S-NCA

Leucocytes were separated from whole blood by the method of Boyum [21]. Briefly, 20 ml of heparinized venous blood from a normal donor were layered onto Lymphoprep® solution (Nycomed A.S. Oslo, Norway) (density 1.077 g/ml) and centrifuged at 400 × g for 20 min. The leucocyte-rich buffy coat was depleted of red blood cells by dextran sedimentation (Dextran 110, Fisons, Loughborough, U.K.) followed by osmotic shock. This procedure resulted in a predominantly neutrophilic cell population (93–95% neutrophils), with greater than 95% viability as assessed by trypan blue dye exclusion. The cell concentration was adjusted to 10⁶/ml in RPMI 1640 (Flow Laboratories, Rickmansworth, U.K.) and supplemented with 2 mM L-glutamine (Flow Laboratories) and 0.5% heat-inactivated new-born calf serum (Gibco Ltd, U.K.) as a protein source. A single neutrophil donor was used throughout.

Neutrophil chemotaxis was measured by a modified Boyden chamber technique, described below. Serum was thawed (without heat treatment) immediately before use

and vortexed. One hundred and fifty microlitres of each test sample were placed on a 13 mm antibiotic assay disc (Whatman, Maidstone, U.K.) in the lower chamber (24 well, flat bottom, plastic tissue-culture plate; Celltec, U.K.). This was overlaid with a 3.0 µm pore-size filter (Millipore, Molsheim, France) and 250 µl of the neutrophil cell suspension applied above in a closed upper chamber (inverted LP3 tissue culture tube cap; Falcon, U.K.). Serum was used undiluted in this assay. After 45 min incubation at 37°C in a humidified atmosphere the filters were removed and fixed in propan-2-ol, rehydrated (2 min), stained in Harris' haematoxylin (2 min), washed in water (2 min), dehydrated in propan-2-ol (2 min), cleared in xylene (overnight) and mounted on microscope slides. The distance of migration from the surface of the filters to the leading cells was measured in 10 random high-power fields for each sample, by the leading-front method [22], and the results were expressed in arbitrary units of migration (AUM). These units were read directly from the micrometer scale on the microscope at ×40 magnification, and were standardized between separate experiments by reference to positive, negative and quality controls. Variation between results from two separate experiments was low (coefficient of variation ~8%). All samples were run in duplicate and the result for each was calculated as the mean of the 20 individual fields read. Subjects' sera from all three time points were assessed in the same experiment, together with the normal control sera, RPMI negative controls (*n* = 5) and 2% casein (w/v) in RPMI as positive controls of chemotaxis (*n* = 5). Chemokinesis for the positive standard was assessed by applying equal concentrations of 2% casein on either side of the filter (*n* = 5), but was not tested for each serum sample. Samples were coded, randomized and counted by a single operator.

Characterization of S-NCA

Preliminary characterization of the S-NCA was carried out on a positive serum pool by fractionation using ammonium sulphate ((NH₄)₂SO₄) precipitation. Two point five millilitres of serum were precipitated with 10 ml 80% (NH₄)₂SO₄, centrifuged for 15 min at 3000 × g, and the supernatant collected. The precipitate was washed with a further 5 ml 80% ammonium sulphate, centrifuged and the supernatant pooled with the first supernatant to give an 80% fraction. To the residual precipitate, a series of concentrations of ammonium sulphate (70%, 60%, 50%, 40%, 30% and 20%) were added, the supernatant being collected each time, thus providing a total of seven fractions (80% to 20%). These were dialysed against polyethylene glycol, and then against distilled water.

These fractions were run on gradient SDS-PAGE (8–25%). The fractions were stored at -20°C , then analysed for S-NCA activity in the bioassay described above.

Estimation of specific IgE

Specific IgE was measured in subjects and controls, in duplicate, using a Phadebas RAST[®] with *D. pteronyssinus* antigen coated discs. Results are expressed as Phadebas RAST units/ml (PRU/ml).

Results

Serum neutrophil chemotactic activity

In both groups of allergic subjects the pre-treatment S-NCA level was elevated compared with non-atopic subjects (mean $\pm$ s.e.m. S-NCA = 63.8 ± 3.6 AUM in Pharmalgen-treated subjects; 69.2 ± 4.0 in placebo-treated subjects; 48.5 ± 3.7 in non-atopic subjects) (Fig. 1, Table 1). In the actively-treated subjects S-NCA levels had fallen to normal at 6 months and remained normal at 12 months (Fig. 1). Comparison of means at 0 and 12 months showed that this fall was significant ($t=3.50$; 38 d.f.; $0.001 < P < 0.01$; Student's *t*-test). In contrast, there was no change in the mean S-NCA level in the placebo-treated group over 12 months (Fig. 1). Figure 2 shows the changes in S-NCA in individual subjects in the actively-treated group during treatment. S-NCA levels fell over the 12 months treatment period in every subject

Table 1. Statistical analysis (ANOVA) of changes in S-NCA expressed in arbitrary units of migration (AUM) during 12 months hyposensitization with Pharmalgen[®] *D. pteronyssinus* or placebo

	Mean S-NCA $\pm$ s.e. (AUM)		
	0 months	6 months	12 months
Pharmalgen treated (n)	$63.8 \pm 3.6^*$ 19	$46.8 \pm 4.0^\dagger$ (18)	$45.2 \pm 3.8^\dagger$ (19)
Placebo treated (n)	$69.2 \pm 4.0^*$ (28)	$65.0 \pm 6.5^{*\ddagger}$ (28)	$70.3 \pm 7.4^*$ (25)
Normal controls (n)	48.5 ± 3.7 (8)		

* Significantly different from normal controls ($P < 0.01$).

† Significantly different from placebo ($P < 0.01$).

‡ Measurement made at 3 months.

NB: *n* values are not always equal to the sample sizes stated in the text as some results were unreadable.

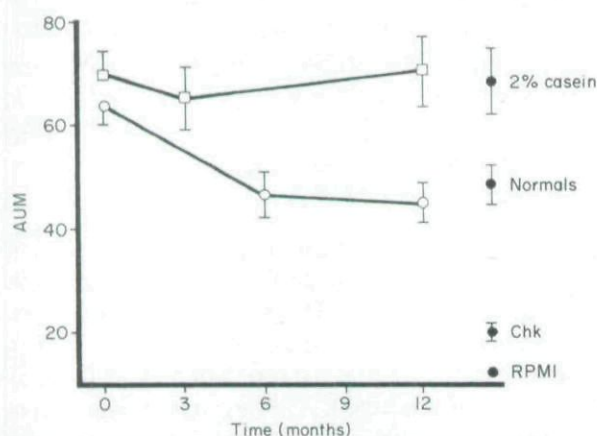


Fig. 1. Neutrophil chemotactic activity of serum (mean $\pm$ s.e.), expressed in arbitrary units of migration (AUM), from subjects with allergic rhinitis, treated with Pharmalgen[®] *D. pteronyssinus* ($\circ$, $n=20$) or placebo injections ($\square$, $n=28$) during 12 months hyposensitization, and from asymptomatic non-atopic subjects (normals, $n=8$). 2% casein = positive control of chemotaxis ($n=5$); RPMI = negative control of chemotaxis ($n=5$); Chk = chemokinesis of positive control ($n=5$).

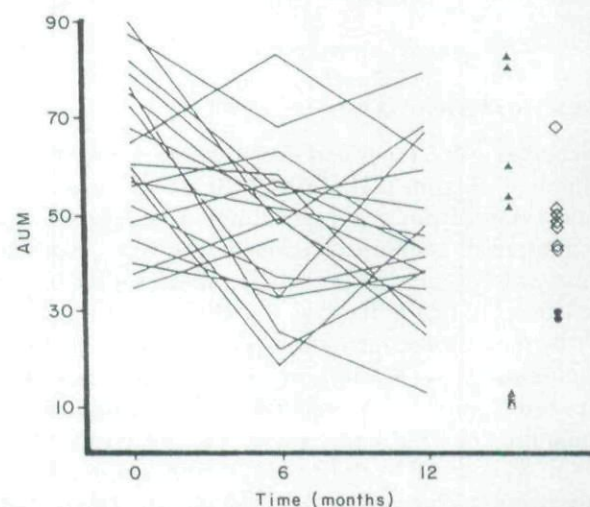


Fig. 2. Neutrophil chemotactic activity of serum (S-NCA), expressed as arbitrary units of migration (AUM), in Pharmalgen-treated subjects before and after 6 and 12 months hyposensitization. Lines join data for individual subjects. The controls shown are: (Δ) = RPMI (negative control of chemotaxis); ($\blacktriangle$) = 2% casein (positive control of chemotaxis); ($\diamond$) = serum from asymptomatic non-atopic subjects; ($\bullet$) = serum from asymptomatic subjects with *D. pteronyssinus* IgE antibody. One sample was unreadable at 12 months.

Table 2. Statistical analysis (ANOVA) of changes in *D. pteronyssinus*-specific IgE (PRU/ml, Phadebas RAST) during 12 months hyposensitization

	Mean specific IgE $\pm$ s.e. (PRU/ml)		
	0 months	6 months	12 months
Pharmalgen treated (<i>n</i>)	39.2 $\pm$ 4.4 (20)	43.4 $\pm$ 5.0 (20)	39.8 $\pm$ 5.0 (19)
Placebo treated (<i>n</i>)	34.5 $\pm$ 4.9 (28)	35.8 $\pm$ 4.9* (28)	35.6 $\pm$ 5.2 (25)
Normal controls (<i>n</i>)	2.0 $\pm$ 0.4 (8)		

* Measurement made at 3 months.

NB: *n* values are not always equal to the sample sizes stated in the text (20 subjects, 28 placebos and eight controls) as some sera were not available.

All means were significantly higher than control levels ($P < 0.01$) but did not show significant longitudinal differences.

except one, in whom it remained level. Mean values $\pm$ the standard error (s.e.) were compared by analysis of variance in Table 1.

It is of interest that the two asymptomatic controls with positive RAST values had low S-NCA levels, below those of the non-atopic controls (Fig. 2). These two were not, however, included in the mean values shown for non-atopic control subjects. (Fig. 1 and Table 1). No correlation was apparent between S-NCA levels and the severity of symptoms at the start of treatment, but this may have been due to the small sample size. The amount of migration due to chemokinesis was low in the positive control for chemotaxis (Fig. 2) and, although not tested, directional migration towards serum samples was assumed to be due to the presence of mainly forward pointing lamellipodia from most of the neutrophils present.

S-NCA characterization

Two peaks of neutrophil chemotactic activity were detected by bioassay of the fractions collected after $(\text{NH}_4)_2\text{SO}_4$ precipitation in the 60% and 80% fractions. The SDS-PAGE band-pattern in the 60% and 80% fractions indicated that the NCA probably falls into molecular weight ranges of (a) less than 30 kD and (b) between 45 and 80 kD. This is being investigated further. No activity was detected in fractions containing the higher molecular weight bands.

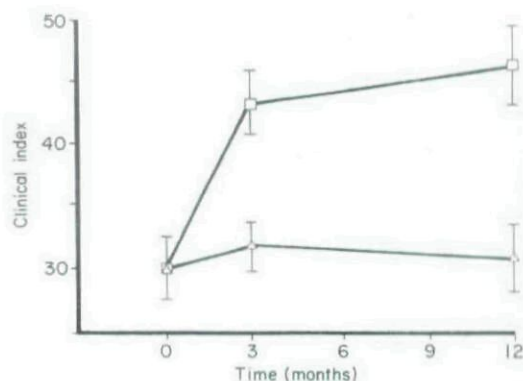


Fig. 3. Efficacy of Pharmalgen® *D. pteronyssinus* (□, *n* = 28) and placebo (△, *n* = 28) hyposensitization in the double-blind trial (study 1). The Clinical Index (CI), a compilation of 5 clinical measurements (see text), is expressed in arbitrary units. An increased CI indicates improvement.

Specific IgE

Serum IgE specific to *D. pteronyssinus* was measured at 0, 6 (3 months for the placebo group) and 12 months for each subject undergoing immunotherapy (Table 2). Results are expressed in PRU/ml. When the actively treated group was split according to RAST class (0–4), the 16 subjects with RAST scores ≥ 3 at the start of treatment showed a significant increase in mean specific IgE at 6 months (mean $\pm$ s.e. 47.8 $\pm$ 4.8 pre-treatment to 54.4 $\pm$ 4.1 at 6 months; $t = 2.06$; 30 d.f.; $0.02 < P < 0.05$), but this fell towards pre-treatment levels (49.6 $\pm$ 4.8) by the end of the treatment. This trend was not apparent when the 4 subjects with low RAST scores at the start of the treatment were included. The mean IgE level in the placebo group did not differ from that of the actively-treated subject before treatment, and remained relatively unchanged throughout the 1 yr treatment period (Table 2). There was an inverse correlation between the levels of specific IgE and S-NCA ($r = -0.313$; $t = -2.274$; 43 d.f.; $0.02 < P < 0.05$) when all the data from the actively-treated group were compared, but no correlation was evident at individual time points.

Clinical assessment

The clinical outcome of immunotherapy with Pharmalgen® *D. pteronyssinus* in this trial will be reported in detail separately. Data on the following parameters were collected to assess the effects on rhinitis: diary card symptom scores, drug scores, visual analogue scores, nasal challenge and skin-prick test. In a double-blind placebo-controlled trial (study 1) the same preparation was shown to be clinically effective at both 3 and 12 months [19,20]. The Clinical Index (CI), which is a compilation of the five

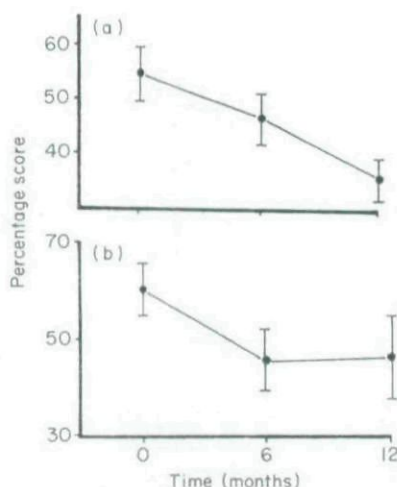


Fig. 4. Effect of hyposensitization with Pharmalgen® *D. pteronyssinus* on (a) skin-prick test and (b) visual analogue scores over 1 yr. Percentage score = maximum possible percentage (VA) or observed (SPT) value. SPT results were significantly lower after 12 months ($P < 0.025$, Mann-Whitney *U*-test), the fall in VA score after 6 months did not reach significance ($0.1 > P > 0.05$).

parameters above, is shown for study 1 before and after 3 and 12 months treatment (Fig. 3). The Pharmalgen-treated group was significantly better than the placebo-treated group at both 3 months ($P < 0.01$) and 12 months ($P < 0.01$, Mann-Whitney *U*-test). For the Pharmalgen-treated subjects in this open study (study 2) data for skin-prick test and rhinitis symptoms by visual analogue score are shown (Fig. 4).

Discussion

We have demonstrated that the mean S-NCA level was elevated in subjects with allergic rhinitis (not undergoing acute challenge), and that over the course of clinically effective hyposensitization with *D. pteronyssinus* the mean level of S-NCA fell ($P < 0.01$) to that of normal non-atopic controls. In the only subject in whom the S-NCA remained unaltered, the levels were within the control range at the start and throughout the treatment. In contrast, placebo treatment had no effect, with S-NCA levels remaining raised throughout the treatment. A somewhat analogous situation has recently been demonstrated for eosinophil chemotactic activity (ECA) in birch pollen allergy [23]: in untreated subjects a rise in ECA occurred during the pollen season, but this failed to occur in subjects who had received a pre-seasonal course of immunotherapy. In house dust mite allergy, where subjects are chronically exposed to low doses of allergen, mediator release is likely to be continuous, although not at the maximal level seen in antigen challenge studies. The

observed reduction in S-NCA levels is compatible with the concept that hyposensitization reduced mediator release.

The reason for the considerable variation in the change of S-NCA levels between 0 and 6 months is unclear but it might be related to acute, sub-acute or sub-clinical reactions induced by the allergen extract used in hyposensitization at the time the 6 month blood sample was taken. At this stage of therapy subjects were on variable doses and had a variable incidence of reactions to treatment. Measurements made after treatment was completed would be unlikely to be influenced by reactions, and these and the pre-treatment measurements might be expected to be more stable and more representative of the subjects' usual clinical status. In 18 out of 19 subjects S-NCA fell between 0 and 12 months.

In the 16 subjects with high RAST scores the mean level of specific IgE showed a significant increase at 6 months, but by 12 months had declined to a level similar to that seen at the start of treatment. This is consistent with the pattern observed in other hyposensitization trials [24]. In the placebo group mean IgE levels remained near pre-treatment levels throughout the year.

Although the inverse correlation noted between the levels of specific serum IgE and S-NCA was statistically significant ($0.05 > P > 0.02$), this may be of no biological significance.

Why subjects with specific IgE, exposed to allergen, do not all develop clinical allergy is not known. The very low S-NCA in our two control subjects with *D. pteronyssinus* IgE antibodies may simply reflect a lack of the conditions necessary for mast cell activation but, alternatively, there could be a factor suppressing S-NCA. Further studies are needed to explore these possibilities.

In order to investigate the mechanism(s) of the effect of hyposensitization on S-NCA it is clearly important to determine the specificity of the response and the source of the S-NCA that is released. The activity reported in this paper is not strictly comparable to that reported by others [4,6-8] since its heat stability and chemical characterization are, as yet, incomplete. The activity described in this paper was found in freshly frozen serum. It seems possible that some of the activities reported in the literature are of similar origins and perhaps modified according to the procedures used to isolate and characterize them. However, it does appear that several different mediators with chemotactic activity for neutrophils are released during allergic reactions. NCA is classically associated with mast cell activation and the surface bound IgE-antigen interaction that leads to degranulation and release of mediators [3,25]. If the S-NCA was mast cell-derived, the reduction in S-NCA levels would be consistent with blocking of mast cell activation. Recently, however, NCA has been

reported to be produced by alveolar macrophages [6] and lymphocytes [26], particularly T cells [27]. NCA release has been shown to be initiated by both immunologically specific (i.e. antigen-specific) and non-specific stimuli such as in physical urticarias [28,29] and in exercise-induced asthma [30,31]. Effects other than, or in addition to, blocking of mast cells might therefore be involved. We are currently investigating NCA from isolated and cultured CD4+ and CD8+ lymphocytes from *D. pteronyssinus*-sensitive and -normal subjects.

The rapid changes in the levels of NCA in the early and late phases of allergic anaphylaxis [5-7] suggest a role for NCA in its induction. Kay and Lee [9] suggested that S-NCA may be a useful means of monitoring the efficacy of treatment with drugs such as cromoglycate. Our findings suggest that measurements of S-NCA might also be helpful in monitoring hyposensitization therapy where, except in bee sting allergy in which specific IgG titres can be used, there is no useful laboratory test. It seems possible that S-NCA plays a role in the maintenance of the allergic condition, and that hyposensitization serves to reduce the levels of S-NCA by mechanisms which remain to be elucidated.

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