

***In vitro* investigations in asthmatic children undergoing hyposensitization with tyrosine-adsorbed *Dermatophagoides pteronyssinus* antigen**

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Summary

Successful hyposensitization to *Dermatophagoides pteronyssinus* in perennial childhood asthma was associated with a slight mean rise of serum IgG antibody to the mite antigen in contrast to a placebo group in whom this antibody fell slightly. No relationship was detected between the effect on asthma and the magnitude of this change. Nor was there any consistent effect on IgE or IgA antibody.

There was a wide range of total serum IgE and IgE, IgG and IgA antibody to *D. pteronyssinus* before treatment; this level did not predict the effect of treatment. Some patients lacked IgA antibody.

IgE antibody to timothy grass pollen was raised in some but not others. These levels did not change systematically during the study and improvement occurred in those who had this antibody as well as IgE antibody to *D. pteronyssinus*.

IgG and IgE antibodies to *D. pteronyssinus* were significantly correlated in the pre-treatment samples.

Lymphocyte thymidine uptake was lower after 8 weeks of treatment than in the control group, not only after stimulus with *D. pteronyssinus* antigen, *in vitro*, but also with antigen from *Candida albicans*. This was not a serum effect.

The late bronchial provocation response was lost only in those with serum IgA less than the log mean for age.

Introduction

Several controlled clinical trials of hyposensitization treatment for pollen allergy have confirmed the long-standing impression of benefit, though we have no evidence how it works. Various forms of extract and regimen have been used. Though *Dermatophagoides pteronyssinus* is thought to be the main cause of asthma, especially in children,

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reports of hyposensitization for housedust-induced asthma and rhinitis have been contradictory and controversial. Limited benefit has been reported using aqueous extracts by Taylor, Sanders and Norman, (1974) and D'Souza *et al.* (1973) in both children and adults though, again, no mechanism has been established. In contrast Gaddie, Skinner and Palmer, (1976) found no evidence of sustained benefit.

A study of Chinese patients with perennial rhinitis showed consistent benefit during maintenance with an aqueous extract of *D. pteronyssinus* antigen (Gabriel *et al.*, 1977). In these patients levels of IgG antibody to the P₁ antigen of *D. pteronyssinus* rose (Chapman *et al.*, 1980) but this did not correlate with the changes in symptom scores.

The present paper describes *in vitro* investigations made over 12 months in children with bronchial asthma who were in a double-blind controlled trial of hyposensitization with tyrosine-adsorbed *D. pteronyssinus* antigen. The beneficial *in vivo* results of this trial have already been briefly reported (Warner *et al.*, 1978) and are presented in detail in the accompanying paper (Price *et al.*, 1984).

Materials and methods

The trial and the fifty-one patients (twenty-seven active group; twenty-four placebo group) are described in the accompanying paper (Price *et al.*, 1984).

Serum IgG, IgM and IgA were measured by the single radial diffusion method (Fahey & McKelvey, 1965) using antisera supplied by Wellcome Reagents Ltd, and Seward Immunodiagnostics Ltd. Results were expressed in iu/ml.

Serum IgE was measured by a modification of the double antibody procedure (Nye *et al.*, 1975) using rabbit anti-human IgE (Hoechst Pharmaceuticals, England) in the first stage and donkey anti-rabbit serum (Wellcome Reagents Ltd) in the second stage. Results were expressed in iu/ml.

IgE antibodies were measured to *D. pteronyssinus* and timothy grass pollen using a modification of the RAST technique described by Ceska, Eriksson and Varga, (1972). Allergen extracts were kindly supplied by Bencard Ltd, and coupled to cellulose paper discs. In the final stage of the test an ¹²⁵I-labelled specific rabbit anti-human IgE (Fc_ε-immunosorbent purified) was added and bound radioactivity determined. Results were expressed as a percentage of the total radioactive counts which became bound. Appropriate positive and negative (cord serum) controls were included with each assay. Binding levels greater than 1.6 times the mean cord serum value for each allergen were regarded as positive.

IgG, IgA and IgE antibodies to the P₁ antigen of *D. pteronyssinus* were measured in selected sera by a double antibody radioimmunoassay, essentially as described by Chapman and Platts-Mills (1978). Measurements were made on duplicate or triplicate dilutions and the results expressed in units of binding activity by reference to a control curve obtained using a standard serum.

Yeast opsonization was measured as previously described (Soothill & Harvey, 1976); results were expressed as an opsonization index (mean number of yeasts/poly-morph).

Functional C2 activity was measured as previously described (Turner *et al.*, 1978).

To measure lymphocyte responses to antigens lymphocytes were isolated from defibrinated blood by density centrifugation on Ficoll-Triosil. The cell concentration was adjusted to 1.5×10^6 /ml in RPMI medium containing either autologous serum or heterologous serum from healthy adult donors. Cells were cultured in the presence of *D. pteronyssinus* extract (batch No. R173, Beecham Pharmaceuticals), *Candida*

albicans extract (batch No. CAD6, Beecham Pharmaceuticals) in triplicate and PHA-6 (Burroughs Wellcome) (in duplicate). Plates were incubated at 37°C for 3 days (PHA) or 5 days (*D. pteronyssinus* and *C. albicans*), 10 µl of tritiated thymidine ([³H]TdR) (0.2 µCi; Radiochemical Centre, Amersham) were added to the cultures either 4 (PHA) or 24 hr (*D. pteronyssinus* and *C. albicans*) before harvesting and counting. A stimulation index was calculated for each. Preliminary experiments with cells from healthy subjects established the following optimal concentrations of the stimulants which were then used throughout: *D. pteronyssinus*, 10 and 40 µg/ml; *C. albicans*, 1, 10 and 100 µg/ml; PHA, 1, 2 and 4 µg/ml. The peak values of stimulation index were used in subsequent analysis of the data.

T-tests (paired or group) were performed on logged data, since most parameters appeared to have a log normal distribution. Wilcoxon's signed rank test, Fisher's exact test and Spearman rank correlation tests were also used as appropriate.

Results

There was no significant difference in the levels of IgG, IgA or IgM between the treatment and placebo groups at any time. As shown in Table 1 IgG and IgA levels increased significantly during the study in both groups, but IgM did not. Eleven of the fifty-one children had a serum IgA level below the department's age matched normal ranges (mean—2 s.d.) and one of these also had low IgM. The mean IgE level of the active group, which was initially non-significantly lower than that of the placebo group, increased significantly ($P=0.007$) by 1 year, although it was still lower than that for the placebo group.

Neither the IgE antibody levels to *D. pteronyssinus* (measured by either of the two methods) nor those to timothy grass differed significantly between the groups, and there was no significant change in either patient group during the study (see Fig. 1). The two assays used to measure IgE antibodies to *D. pteronyssinus* correlated well (see Fig. 2) ($r=0.68$, $P<0.001$).

There was no significant difference between the two patient groups in the initial IgG antibody levels to the P₁ mite antigen (Fig. 3) and these levels correlated significantly

Table 1. Antilog of log mean (± 2 s.d.) of serum immunoglobulin concentrations (iu/ml) for children with bronchial asthma at commencement of trial and after 12 months of therapy (0 and 12)

	IgG		IgA		IgM		IgE	
	0	12	0	12	0	12	0	12
Active	98	123	74	94	141	158	399	495
	(86–112)	(102–147)	(58–94)	(78–113)	(115–173)	(129–193)	(235–678)	(289–848)
	$*t=3.29$		$t=3.17$		$t=1.01$		$t=2.98$	
	$P=0.004$		$P=0.005$		NS		$P=0.007$	
Placebo	97	113	72	91	151	160	593	568
	(88–108)	(101–126)	(57–90)	(71–116)	(119–190)	(129–199)	(354–994)	(325–991)
	$t=2.56$		$t=4.01$		$t=0.77$		$t=0.13$	
	$P=0.018$		$P=0.0005$		NS		NS	

NS = not significant.

* Paired *t*-test.

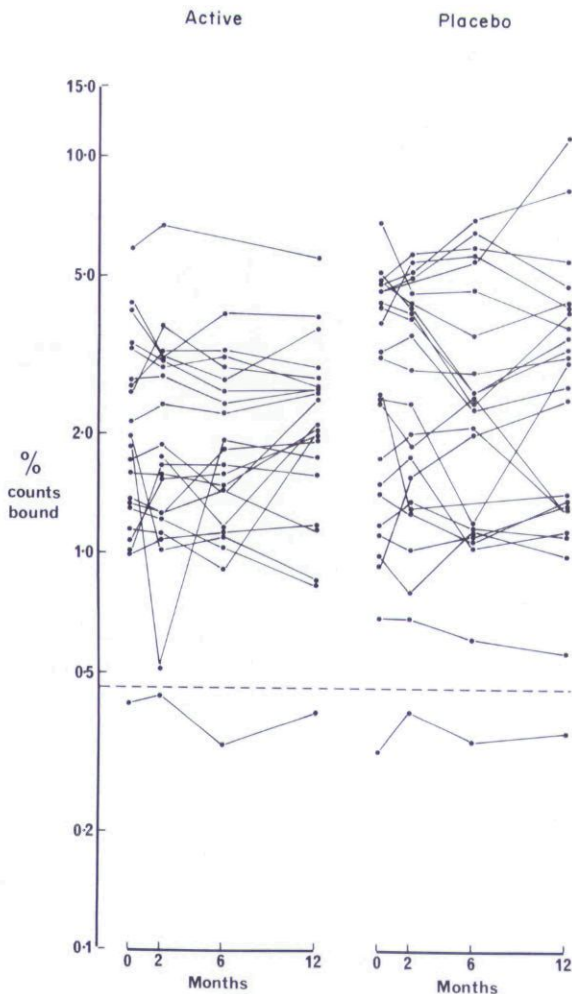


Fig. 1. IgE antibodies to *D. pteronyssinus* measured by RAST in the sera of children with bronchial asthma after random allocation to active hyposensitization with tyrosine-adsorbed mite antigen or placebo. The horizontal dashed line indicates the value for cord serum $\times 1.6$, a commonly used threshold of significant detection.

with the IgE antibody levels ($r=0.77$, $P<0.001$) (Fig. 4). The IgG anti- P_1 antibody levels fell in nineteen of the twenty-three children in the placebo group; this was significantly more frequent than in the active group (nine of the twenty) ($P=0.0114$, Fisher's exact test). IgG, IgA and IgE antibody to the P_1 antigen were measured by the double antibody method in sera from nineteen individuals (ten active, nine placebo) in whom pre-treatment G:E ratios were $<8:1$. This selection was made in order to reduce the possibility of interference by excess IgG antibody in the assay (see Fig. 5). No IgA antibody was detected in ten of these individuals at the beginning of the study. These ten did not have lower serum IgA concentrations than those who made IgA antibody. No patient in the active group who lacked IgA antibody at the start acquired it during the study, but two in the placebo group did. There were no consistent changes in IgA antibody in those who received the antigen. The slight rise of IgG antibody levels in this

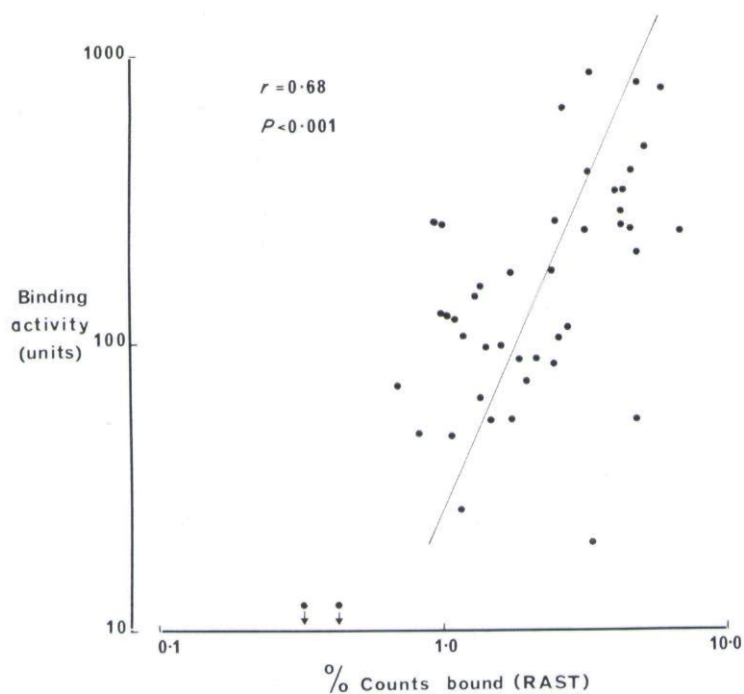


Fig. 2. Correlation between pretreatment serum IgE antibody levels to *D. pteronyssinus* measured by RAST using whole mite extract and by the double antibody antigen-binding assay using P₁ mite antigen.

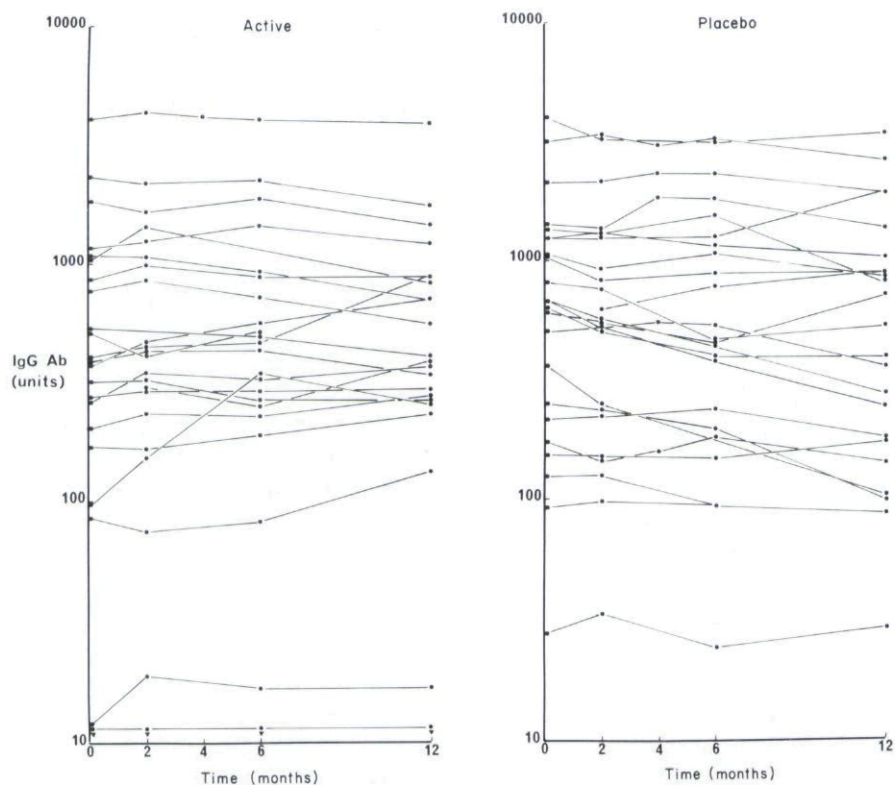


Fig. 3. IgG antibodies to P₁ antigen measured by the double antibody antigen-binding technique in asthmatic children receiving either active or placebo hyposensitization therapy. The lower limit of sensitivity in the assay was ten binding units.

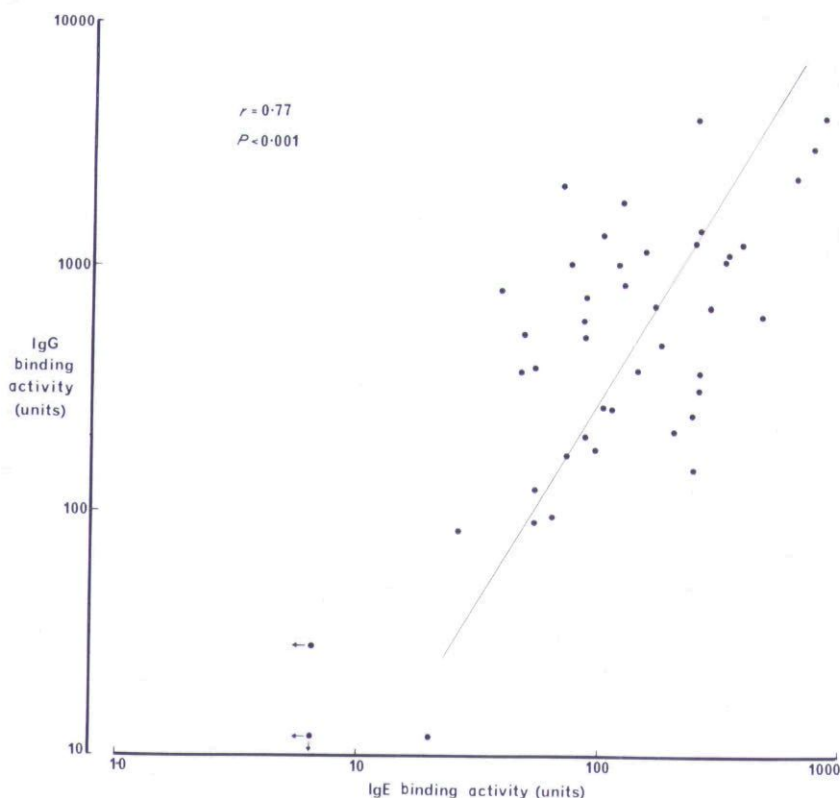


Fig. 4. Correlation between the levels of IgG and IgE antibody to the P_1 antigen in pre-treatment samples from asthmatic children measured by the double antibody antigen-binding assay.

group of treated patients, as in the treated series as a whole, was the only consistent change in the antibodies in either group.

Lymphocyte TdR uptake in the presence of *D. pteronyssinus* antigen was significantly lower at 8 weeks in the patients receiving active treatment than it was in the patients' own pre-treatment samples ($P < 0.05$, Wilcoxon signed rank test), whether studied in autologous or heterologous healthy adult serum (Table 2 and Fig. 6). The difference between the active and placebo groups 8 weeks after commencement of treatment was not significant. Surprisingly, a fall of response was also found after 8 weeks of treatment when cells were cultured with *Candida* antigen in autologous serum ($P < 0.01$, Wilcoxon signed rank test) though not in heterologous serum. The phytohaemagglutinin responses did not differ significantly (Table 2).

Relationship between in vivo and in vitro data

A reduction of drug score was associated with loss of the late reaction in the treated group of the series (Warner *et al.*, 1978; Price *et al.*, 1984). The initial IgG and IgE anti-mite antibody levels were not obviously related to the fall in drug score or loss of the late reaction. However, all those who lost their late reactions had serum IgA levels less than the population log mean for age; this was significantly more frequent than among those who did not do so. There was a similar non-significant association with a fall in drug score (Table 3).

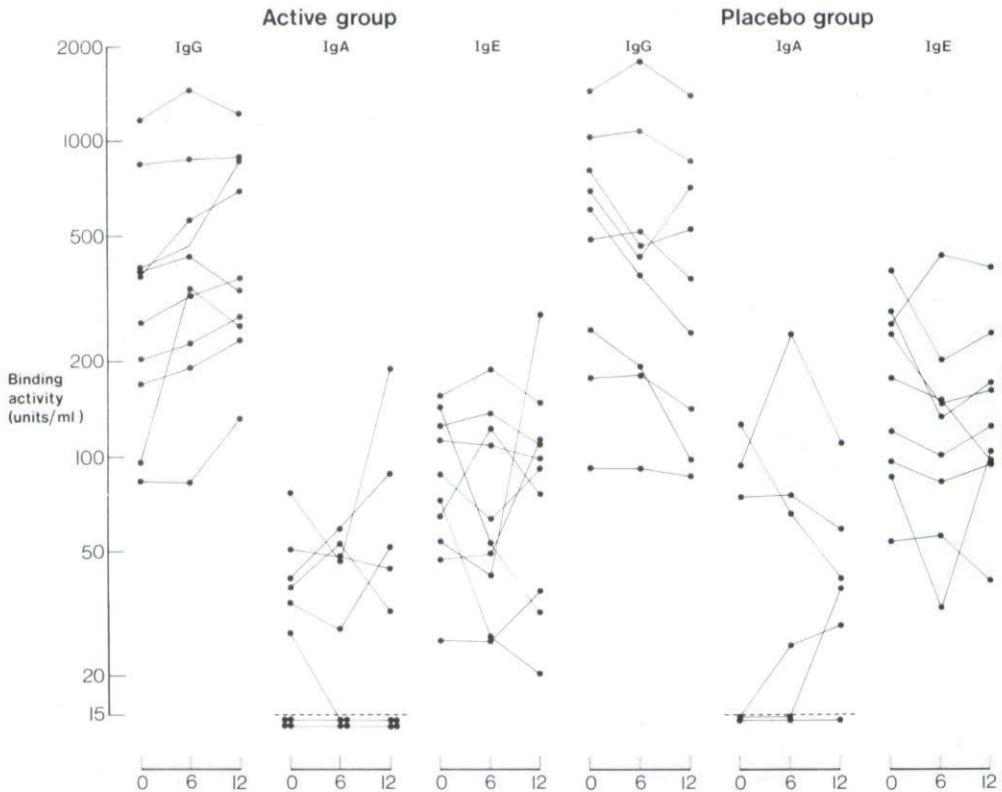


Fig. 5. IgG, IgA and IgE antibodies to P_1 antigen in nineteen sera selected from both the active and placebo groups. The lower limits of detection in the assays were six units for IgG and IgE and fifteen units for IgA.

Table 2. Lymphocyte responses to three stimulants in asthmatic patients receiving active or placebo hyposensitization treatment

Peak stimulation index in autologous serum (median and observed range)						
	<i>D. pteronyssinus</i>		<i>Candida albicans</i>		PHA	
	A	B	A	B	A	B
Active	3.2 (0.5-27.0)	1.4 (0.3-43.0)	3.3 (0.2-7.5)	1.1 (0.05-6.0)	26.5 (1-86)	14 (0.9-81)
	$P < 0.05^*$		$P < 0.01^*$		NS	
Placebo	3.5 (0.2-28.5)	2.2 (0.4-28.8)	2.4 (0.2-5.0)	2.4 (0.1-6.4)	19.5 (1.5-204)	18 (2-110)
	NS		NS		NS	

A, sample obtained before treatment.

B, sample obtained 8 weeks after commencement of treatment.

* Wilcoxon's signed rank test.

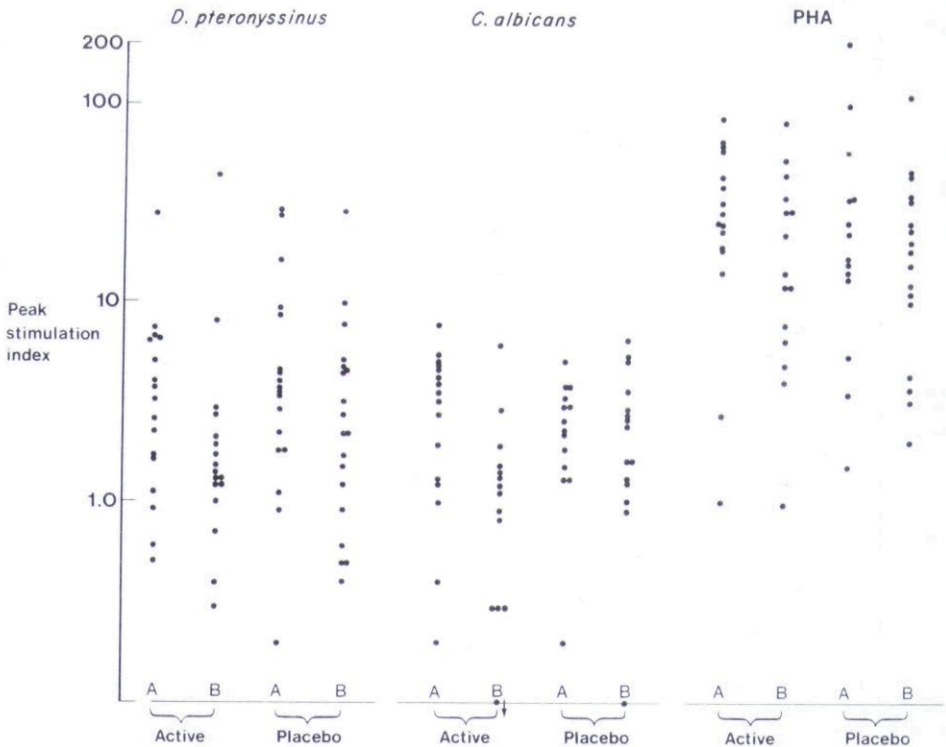


Fig. 6. Lymphocyte responses to three stimulants in asthmatic children receiving active hypsensitization or placebo (A) before treatment, and (B) 8 weeks after starting treatment. All tests were performed in autologous serum.

Table 3. The association of pre-treatment serum IgA less than log mean for age with loss of the late bronchial reaction and with fall in drug score to less than 50% of initial level

	Late reaction		Drug score	
	Lost	Persisted	Fall	No fall
IgA < mean	9	5	9	5
IgA > mean	0	6	2	5
P=0.024	NS		NS	

Yeast opsonization was defective (<2.5 yeasts/polymorph) in nine of the fifty-one patients studied. The asthma of three of the patients with defective yeast opsonization improved in the pre-trial period and so they were not included in the trial. Only two such patients were treated; one improved and one did not, similar to the group as a whole. Low levels of C2 (<47% of a normal human serum standard) were not observed in any of the patients in this series.

Discussion

These *in vitro* studies of sera from asthmatic children undergoing successful hypsensitization with injections of tyrosine-adsorbed glutaraldehyde-modified *D.*

pteronysinus antigen detected no clear change which could be related to either the clinical improvement or the loss of late bronchial reactivity (Warner *et al.*, 1978; Price *et al.*, 1984b). In the treatment group we noted an absence of the fall of IgG antibody which occurred in the placebo series and this may be analogous to the selective rises in serum IgG antibodies observed in adults undergoing hyposensitization with aqueous extracts of ragweed (Yunginger & Gleich, 1973) and ryegrass (Platts-Mills, 1979). Previous studies have similarly failed to observe *in vitro* test results which correlated with clinical improvement in pollen allergy (Ahlstedt & Eriksson, 1977), and in mite allergy, in which IgG antibodies do rise (Chapman *et al.*, 1980). Despite our clear-cut therapeutic effect, the rise in IgG antibodies in the treatment group was much less than that reported by Chapman *et al.* (1980), but these authors used an aqueous extract and possibly different antigen doses. Similarly, a significant rise of IgG antibody was reported by Hanneuse, Pintens and Delespesse (1982) in forty-one adults with respiratory allergy after hyposensitization with either aqueous or tyrosine adsorbed *D. pteronyssinus* antigen. However, no clinical results were presented and it is not possible to compare data from distinctly different patient groups.

In a recent separate study of the late reaction in lung, skin and nose we also failed to find any association with the level of IgG antibodies (Price *et al.*, 1984a) so our data provide no support for the view that the late reaction is mediated by IgG, or that hyposensitization works in atopic allergy by inducing 'blocking' IgG antibody, as it may do in bee-sting allergy (Lesso, Sobotka & Lichtenstein, 1977). The lack of effect on IgA antibody suggests that, whatever the role of IgA deficiency in atopic allergy (Taylor *et al.*, 1973), recovery following hyposensitization does not depend on this. Unfortunately the treated group were too few to analyse different aspects in many subgroups (e.g. those with yeast opsonization deficiency) but those with relatively low IgA deficiency did better than those without. This needs further study, but perhaps provides some support for the view that the effect of hyposensitization may be analogous to the spontaneous recovery from much childhood atopic disease and the transience of low serum immunoglobulin deficiency in some. It is probable that the rise of serum IgG and IgA, observed in both treatment and placebo groups, represents normal maturation of immunoglobulin levels.

We have confirmed earlier reports (Platts-Mills, 1979; Chapman *et al.*, 1980; Hill *et al.*, 1981) of a strong positive correlation between IgE and IgG antibody levels to the inhalent allergens in pre-treatment samples. These levels are not related to the clinical state of the patients or their response to treatment other than hyposensitization (Hill *et al.*, 1981). However, in the latter study the spontaneous clinical improvement observed over a 5-year period was paralleled by a progressive decline in both IgG and IgE antibodies to *D. pteronyssinus*. The possibility remains that there is an antibody response in a single subclass which underlies the therapeutic effect, but there is no evidence for this.

The suppression of *in vitro* lymphocyte responses to *D. pteronyssinus* is similar to that observed with grass pollen antigen in patients receiving hyposensitization with this allergen (Romagnani, Biliotti & Ricci, 1975). Its relevance is doubtful, and the similar effect on lymphocyte responses to candida suggests that it is an irrelevant secondary antigen-non-specific effect, such as the binding of antigen-antibody complexes to cell surface receptors, despite the observations of a low response with *D. pteronyssinus* in donor serum.

There are many possible ways by which hyposensitization might work, and

possibly different forms of such treatment might work in different ways; these results with tyrosine-associated antigen may not apply to other systems. However, the choice of the dose was arbitrary and may well not be optimum.

We suspect that none of the factors we measured are directly relevant to the mechanism of either the therapeutic effect of hyposensitization or the late bronchial reaction. However, the clinical results in this study suggest that elucidation of the mechanism of the late reaction might well solve the problem of the mechanism of hyposensitization.

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