PURIFICATION AND CHARACTERIZATION OF THE MAJOR ALLERGEN FROM THE HOUSE DUST MITE (*Dermatophagoides pteronyssinus*) - ANTIGEN P_1.

by

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ABSTRACT

The house dust mite, Dermatophagoides pteronyssinus, produces potent allergens which are the most important cause of perennial rhinitis and extrinsic asthma in this country. The aim of this study was to purify the allergenic activity of D.pteronyssinus in order to have a better understanding of properties of allergens and also as a pre-requisite for studies on the antibody (ab) response to mites.

A partially purified allergen, F_4P_1, was obtained from D.pteronyssinus culture by gel filtration and block electrophoresis. This preparation (MW 15-25,000 daltons) had very good skin test reactivity and could be used in an antigen binding radioimmunoassay to measure IgG, IgA and IgE antibody (ab) to D.pteronyssinus. The F_4P_1 allergen was further purified by preparative isoelectricfocusing (IEF). The antigenic activity eluted from IEF fractions was predominantly distributed in two peaks, fraction 5 (Fr.5) (pI 4.7-5.4) and fraction 14 (Fr.14) (pI 6.6-7.1) which showed complete immunological cross-reactivity. The amino-acid composition of the fractions was almost identical and each fraction migrated as a single homogeneous band on gel electrophoresis (MW 24,000 daltons). The protein present in Fr.5 and Fr.14, which bears the common antigenic determinant P_1, was shown to be the major allergen of D.pteronyssinus. Allergic individuals had very similar skin test responses to purified preparations of P_1 and to crude mite extract. In addition, serum IgE ab to P_1 accounted for up to three-quarters of the IgE ab to the crude extract. Antigen P_1 is present on a heat-sensitive, soluble glycoprotein which is abundant in extracts of mite culture. The physical properties (molecular size and solubility) and quantities of antigen P_1 appear to be important in determining it's

allergenicity. These properties are in keeping with those of other major allergens derived from pollens.

The antigen binding assay was used to investigate the antibodies to D.pteronyssinus in serum from patients with perennial rhinitis, asthma and atopic dermatitis. It was also used to investigate the role of IgG 'blocking' ab in patients treated with injections of D.pteronyssinus extract (hyposensitization). In two studies treated patients were clinically improved following hyposensitization, but this did not appear to be related either to changes in IgG ab levels or to suppression of IgE ab responses.

In the past the use of heterogeneous extracts of house dust and mite culture as a source of mite allergens has given unsatisfactory results. Using the purified allergen it will be possible to make quantitative studies of allergen exposure and to investigate the cellular basis of the immune response to inhaled allergens.

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ABBREVIATIONS

ab	-	antibody
BA	-	binding activity
BBS	-	borate buffered saline, pH 8.0
BSA	-	bovine serum albumin
CIE	-	crossed immunoelectrophoresis
CRIE	-	crossed radioimmunoelectrophoresis
ExI	-	a non-dialyzable, aqueous extract of whole *D.pteronyssinus* culture
HSA	-	human serum albumin
IEF	-	isoelectric focusing
P-K	-	Prausnitz-Küstner
RAST	-	radioallergosorbent technique
Rye I	-	the group I protein from rye grass pollen
SDS-PAGE	-	polyacrylamide gel electrophoresis in sodium dodecyl sulphate

GENERAL

INTRODUCTION

1. Historical Background

The term "allergy", meaning "changed reactivity", was introduced by von Pirquet (1906) to describe the abnormal response of a host to a foreign antigen (the allergen). Allergic reactions were considered to represent a "supersensitive" (hypersensitive) form of immunity which resulted in local or systemic tissue damage. At that time the most well documented allergic reactions were anaphylaxis, serum sickness and "hypersensitivity of infection" (reactions to bacteria and fungi). All these reactions were specific for a given allergen and only occurred in previously sensitized hosts. They were distinguished on the basis of the time course of the response (immediate or delayed), the nature of the allergen and the route of sensitization. Immediate reactions were demonstrated using the cutaneous skin test. A drop of allergen solution was applied to the skin which was then "pricked" with a fine needle to allow the solution to penetrate the epidermis. The development of a wheal and flare at the test site within 15-20 minutes indicated sensitivity. This test was used to diagnose allergic diseases such as asthma, hay fever and food intolerance which were known to be acquired through natural exposure to environmental allergens and in which symptoms developed immediately following allergen challenge. Coca and Cooke (1923), in their classification of allergic reactions, proposed the term "atopy" (from the Greek "atopia", meaning strange disease) to describe these conditions which were also linked to heredity (Cooke and Vander Veer, 1916). The term atopy indicates immediate sensitivity to one or more common environmental allergens.

In 1921 Prausnitz and Küstner injected serum from atopic patients into the skin of non-atopic individuals and showed that the sensitized

sites would give a positive skin test on challenge with the specific allergen. These experiments demonstrated that atopic sensitivity was mediated by a serum factor. The technique of Prausnitz and Küstner, the P-K test, was widely used to investigate atopy and to make semi-quantitative assessments of patients sensitivity to allergen. Coca and Grove (1925) investigated the properties of the transferable serum factor which they termed "atopic reagin". The factor was found in serum from all atopic subjects and remained fixed in normal human skin for several weeks but could not sensitize most animal tissue. It did not fix complement and was partially destroyed by heating at 56°C for 30 minutes. Little more became known about the nature of atopic reagin until the advent of high resolution protein separation techniques about 30 years later.

Fractionation studies, using serum from atopic patients, initially associated reaginic activity with fast moving γ-globulin (Kuhns, 1954; Humphrey and Porter, 1957; Stanworth, 1959) and later with the newly discovered immunoglobulin, IgA (Heremans and Vaerman, 1962; Fireman, Vannier and Goodman, 1963). In the early 1960's most immunologists believed that IgA was the reaginic antibody (ab). Contrary to this view, some preparations of specific IgA ab were not active in P-K tests (Ishizaka, Ishizaka and Lee, 1966) and serum from allergic individuals with IgA deficiency contained reagin (see Loveless, 1966). The situation was finally resolved by the classic experiments of Ishizaka and colleagues. They showed that the activity of antiserum raised against reagin-rich fractions of serum could not be absorbed by IgG, IgM, IgA or IgD, or other myeloma proteins. Following successive absorptions the antiserum

still showed a line on immunoelectrophoresis against the reagin-rich fraction which was shown to be allergen specific by autoradiography (Ishizaka, Ishizaka and Hornbrook, 1966). The carrier of reaginic activity was ascribed to a unique class of immunoglobulin, γE or IgE. Subsequently, it was shown that the P-K titre of serum from atopic patients correlated with the concentration of IgE ab, measured by antigen binding assay, but not with other classes (Ishizaka, Ishizaka and Hornbrook, 1967).

Further studies on the properties of IgE were facilitated by the independant discovery of two IgE myeloma proteins; IgE (ND) in Sweden (Johansson and Bennich, 1967) and IgE (PS) in the United States (Ogawa et al, 1969). These proteins had the characteristic structure of immunoglobulins, being composed of two heavy chains and two light chains. Physicochemically, IgE differed from other monomeric immunoglobulins in having a slightly higher molecular weight (190,000 daltons) and S_{20w} (8.0) and a higher carbohydrate content (12%). The most important distinguishing feature of IgE was that the Fc portion had a very high affinity for surface receptors on mast cells. The cross-linking of mast cell bound IgE by allergen resulted in degranulation and the release of potent pharmacological agents, e.g. histamine and serotonin which mediated the symptoms of atopic disease. This mechanism is the Type I reaction of Gell and Coombs (1968). It is distinct from antibody dependant cell-mediated hypersensitivity (Type II), complex mediated hypersensitivity (Type III) and delayed hypersensitivity (Type IV).

With the availability of myeloma proteins a variety of methods were developed for measuring IgE including radioactive radial immunodiffusion (Rowe, 1969), solid phase radioimmunosorbent assays (Johansson, Bennich and Wide, 1968; Wide, 1971) and double antibody inhibition radioimmunoassays (Ishizaka, Tomioka and Ishizaka, 1970; Gleich, Averbeck and Swedlund, 1971). The levels of IgE in serum were shown to be several orders of magnitude lower than for other immunoglobulins, ranging from 2-200 International Units IgE/ml in serum from non-atopic individuals (equivalent to 5-500 ng IgE/ml). Elevated levels of serum IgE were found in patients with hay fever, asthma, eczema and the Wiscott-Aldrich syndrome (Bennich and Johansson, 1971). The highest levels (up to 200,000 units/ml) were found in patients infected with parasitic worms (helminths), which have an adjuvant effect on IgE production (Jarrett and Stewart, 1972; Strejan, 1975). The role of IgE in some of these conditions, e.g. eczema and helminth infection remains to be established. However, much progress has been made in our understanding of atopy by the development of in vitro methods of assessing ab to allergens, e.g. the histamine release assay (Lichtenstein and Osler, 1964), the radioallergosorbent test (Wide, Bennich and Johansson, 1967) and antigen binding assays (Ishizaka et al, 1967; Yunginger and Gleich, 1973; Platts-Mills et al, 1976, 1978).

2. The properties of allergens

Von Pirquet (1906) originally used the term allergen to describe any substance which could induce hypersensitivity. Later, the agents

responsible for immediate reactions were described more specifically as "atopic allergens" (Coca and Cooke, 1923). For the purposes of this thesis the term allergen will be restricted to those substances which stimulate IgE-mediated allergic reactions. Even so, the term still has some subtle differences in meaning. Thus while an allergen may be the source of the basic material, e.g. grass pollen, house dust, it may also be an aqueous extract or a highly purified protein derived from that source. There are a wide variety of common allergens which may be characterized by their routes of exposure. Individuals may be exposed by inhalation, e.g. pollens, dust; ingestion, e.g. milk, eggs, fish; or injection, e.g. insect venoms, penicillin. Inhalant allergens are further distinguished by the very small doses (sub µg quantities) that are encountered during natural exposure.

Studies on the immune response in atopic individuals have been greatly enhanced by the use of highly purified allergens (Marsh, 1975; King, 1976). The first allergen to be obtained in a purified form was antigen E of ragweed pollen; an acidic glycoprotein molecular weight 38,000 daltons (King and Norman, 1962). Subsequently, purified allergens were obtained from rye grass pollen (Johnson and Marsh, 1965a), cod-fish (Elsayed and Aas, 1970) and vespid venoms (Shipolini et al, 1971). As a result of these studies it became apparent that allergens could be categorized as being either "major" or "minor" depending on their relative activities in a group of sensitive patients. Major allergens are defined by their ability to induce a ++ positive skin test at high dilution ($<10^{-3}$ µg/ml) in >90% of allergic individuals (Marsh, 1975). The incidence of positive skin tests to minor allergens is much lower

than this, though a small proportion of patients may be more sensitive to the minor allergen. Ragweed antigen E and the Group I protein of rye grass pollen (Rye I) are examples of major allergens. Minor allergens such as ragweed Ra3, Ra4 (BPA-R), and Ra5 and rye grass group II and group III proteins have also been highly purified (Johnson and Marsh, 1965a; Underdown and Goodfriend, 1969; Griffiths and Brunet, 1971; Lapkoff and Goodfriend, 1974). Most major allergens, and some minor allergens, exist as "isoallergens" (analagous to isoenzymes) (Johnson and Marsh, 1965b). These are closely related molecular species which are antigenically identical but have slightly different physiochemical characteristics, e.g. molecular size and isoelectric point.

Although it has been known for many years that house dust is a potent allergen and an important cause of rhinitis and asthma the purification of house dust allergens has met with little success. Positive immediate skin reactions to extracts of house dust were first reported over 50 years ago (Kern, 1921; Cooke, 1922). Early work on the isolation of dust allergens involved the extraction of large quantities of dust in ammonia solution followed by precipitation with organic solvents (Sutherland, 1942). The preparations obtained were largely carbohydrate in nature and polydisperse on electrophoresis (Rimington, Stillwell and Maunsell, 1947). The skin test reactivity of fractions separated by electrophoresis was widely distributed in both highly coloured and colourless solutions (Rimington et al, 1947; Vannier and Campbell, 1961). Berrens pursued these

rather crude fractionation techniques and reported the purification of a house dust allergen (allergen E), which was a brown coloured, polydisperse glycoprotein, molecular weight 20-25,000 daltons (Berrens and Young, 1961). While the physicochemical properties of this allergen have been studied in great detail (Berrens, 1970, 1974) it's allergenicity has not been fully established. Recent work has shown that comparatively high concentrations of allergen E (0.4-2.0 µg/ml) are required to elicit a positive skin test (Weemaes, Stoop and Berrens, 1977) and at present most workers do not consider this fraction to be a highly purified allergen (Marsh, 1975; King, 1976). The purification of specific allergens from crude dust extracts is extremely difficult because house dust, unlike most other allergens, is a heterogeneous material and some components, e.g. animal danders and fungal spores are themselves potent allergens. Most recent studies have been aimed at comparing the allergenicity of individual dust components obtained from a homogeneous source with that of crude dust extract (Voorhorst et al, 1967; Aalberse, 1974). Amongst these components mites, animal danders and fungi appear to be most important.

3. The relationship between house dust and mite allergens

Mites belong to an order of the class Arachnida, the Acari. Phylogenetically, they are more closely related to spiders, scorpions and ticks than to insects, being non-segmented and having eight, rather

than six, legs in the adult form. The sub-order Acaridae contains the two families which are most important in allergy, the Pyroglyphidae and the Tyroglyphidae (for classification see Appendix A.1.). Prior to 1960 there had been several reports that mites were involved in allergic and respiratory disease. Ancona (1923) reported "epidemic asthma" in 23 workers handling grains infested with Pyemotes ventricosus. Later, occupational asthma and pulmonary acariasis due to foodstuffs infested with other "storage" mites, including Acarus siro, Glycyphagus, Tyrophagus and Carpoglyphus sp, was also described (Storm van Leewen, 1925; Soysa and Jayawandena, 1945; Carter and D'Abrera, 1946; Taboada, 1954). Mites were also found in house dust (Dekker, 1928), but their importance was not fully realised until 1964 when Voorhorst, Spieksma-Boezema and Spieksma suggested that the pyroglyphid mite, Dermatophagoides pteronyssinus, was the major house dust allergen. Several lines of evidence were put forward to support this view. Large numbers of D.pteronyssinus were found in dust samples from Holland and other European countries (up to 500/g dust). There was a very good correlation between skin tests to D.pteronyssinus and house dust and the allergenicity of dust was proportional to the content of live mites. Furthermore, patients presented with the symptoms of dust allergy (rhinitis and asthma) more frequently during the mites breeding season when, presumably, there was more allergen in the environment.

The observations of the Dutch workers aroused great interest amongst biologists and acarologists, as well as allergists, because D.pteronyssinus (the house dust mite) was a virtually unknown mite

species. It was probably first isolated as D.scheremetewsky by Bogdanov during an outbreak of scabies in Moscow in 1854. The modern nomenclature is based on a synonymous species, Mealia pteronyssinus (Troussart, 1897). The life-cycle proceeds from the egg to a six-legged larva and then passes through several eight-legged, nymphal stages until the mature adult finally emerges after 20-30 days. Adult mites live for 8-10 weeks. They range from 250-400μ in length and have a finely striated cuticle which bears hair-like projections termed "setae" (Fig.I.1). The family Pyroglyphidae (Cunliffe, 1958) has been extensively described by Fain (1965, 1966) according to the size and shape of the body (idiosoma) and the leg segments, and the position and distribution of the setae.

The close association of D.pteronyssinus with Man derives from the food requirements and environmental factors which influence mite growth. The mites natural food is desquamated human skin scales which are abundant on household floors, in mattresses and padded furniture, and on clothes. On average the human sheds ~5g of skin scales per week (Kligman, 1964). Much smaller amounts (~ 200 mg) will support a growing colony of D.pteronyssinus for up to six months; by which time the population may have reached several thousands (Spieksma, 1967). The optimal conditions for mite growth in the laboratory, on cultures of human "whiskers" supplemented with powdered yeast, are a temperature of 25°C and 75-80% relative humidity. Cultures of D.pteronyssinus can be maintained at temperatures of 15-30°C but the population stops growing if the relative humidity falls below 70% (Spieksma, 1970). Conversely, at relative humidities of >85% the

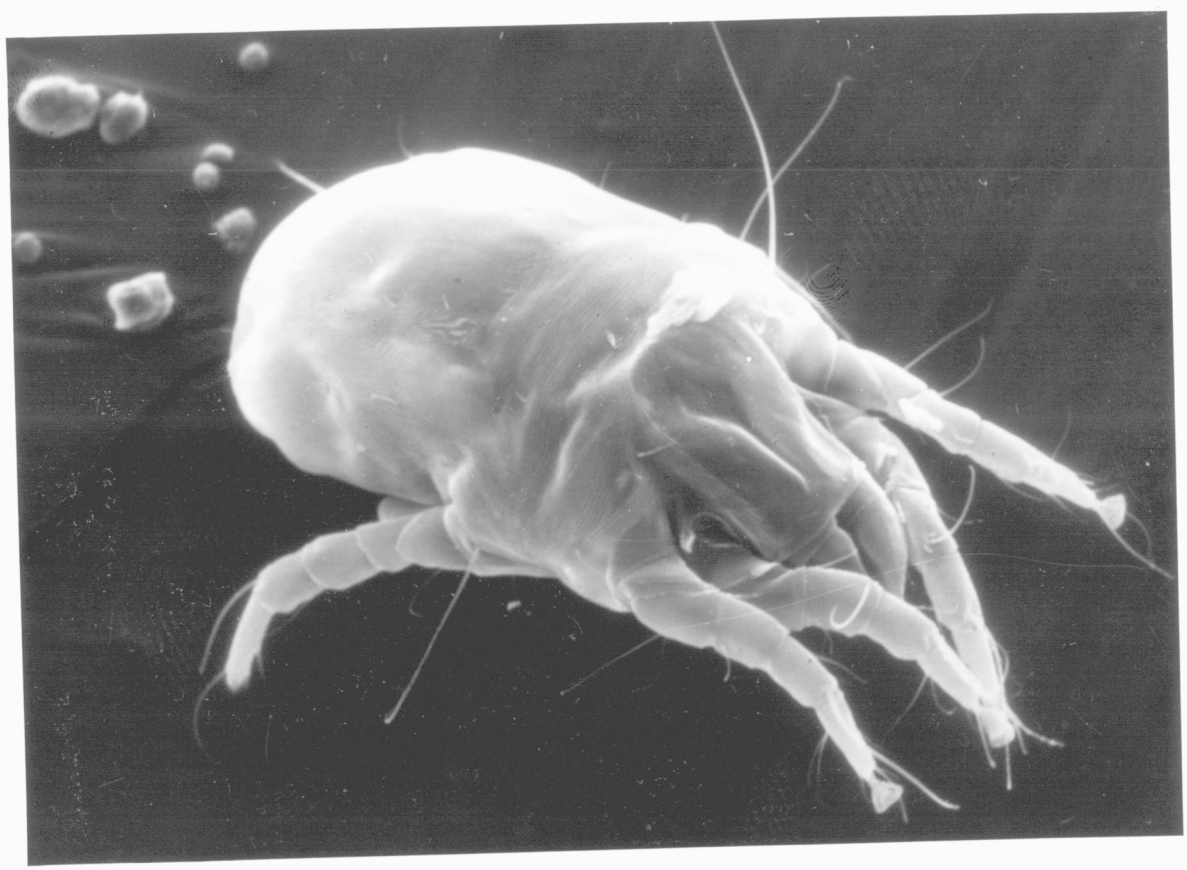


Figure I.1

Scanning electron micrograph of Dermatophagoides pteronyssinus. The particles (top left) are mite faeces. I am very grateful to Mr. E.R. Tovey and Mr. J. Clark for providing this figure.

cultures usually become overgrown by moulds. Humidity is one of the most important factors which influence mite growth. Under natural conditions mites obtain water directly with their food, or as a product of metabolism, or by both passive and active absorption from the surrounding air. The latter mechanism appears to be most important in enabling mites to survive large fluctuations in humidity (Arlian, 1977). For example, the relative humidity in a bed, when occupied, may be much higher than the minimum necessary for mite growth. The absorption of water at such times enables mites to survive when conditions are less favourable, i.e. when the bed is unoccupied (Wharton, 1976). Mites also develop quiescent protonymphs which survive prolonged periods of low humidity.

The family Pyroglyphidae contains two other species of allergenic importance, Dermatophagoides farinae and Euroglyphus maynei, which are also widely distributed in house dust samples. Many workers prefer to work with D.farinae because it is easily grown on cultures of dogmeal and shows some cross-allergenicity with D.pteronyssinus (Pepys, Chan and Hargreave, 1968). Attempts to culture E.maynei in the laboratory have not been successful and little is known of the allergenicity of this species. A good correlation between skin tests to all three species has been reported (Voorhorst, Spieksma and Varekamp, 1969). Studies on the distribution and allergenicity of Dermatophagoides sp. in relation to house dust allergy have, in most cases, confirmed the original observations of Voorhorst et al (1964). The genus has a worldwide distribution in areas where climatic conditions are compatible with mite growth (van Bronswijk and Sinha, 1971). Thus

D.pteronyssinus was found in dust samples from Europe, Asia and Africa (Oshima, 1967; Maunsell, Wraith and Cunnington, 1968; Miyamoto et al, 1968; Voorhorst et al, 1969; Anderson and Cunnington, 1974; Buchanan and Jones, 1974) and could account for 70% of the mites in dust (Spieksma and Spieksma-Boezeman, 1967). In North America D.farinae was the most prevalent species (Wharton, 1970, 1976). The ubiquitous occurrence of mites in dust samples from many countries appeared to explain the "universal" nature of the house dust allergen.

A great deal of evidence accumulated which supported the concept of "allergenic identity" between dust and mite allergens. There was a very good correlation between skin test responses to Dermatophagoides sp. and to house dust extract (Pepys et al, 1968; Spieksma and Voorhorst, 1969; Mijamoto, Oshima and Ishizaki, 1969; Biliotti et al, 1972). Furthermore, Voorhorst et al (1967) analysed 100 dust samples and showed that the skin test reactivity of each extract correlated with the number of mites it contained. Similar findings were also reported in England (Maunsell et al, 1968) and Japan (Miyamoto et al, 1970). Following the introduction of the radioallergosorbent test (RAST) to measure IgE ab to allergens a very good correlation was found between IgE ab to Dermatophagoides sp. and IgE ab to dust in serum (Morita et al, 1975; Virchow, Roth and Moller, 1976). It was also shown that mite extracts could inhibit the house dust RAST (Aalberse, 1974, 1978) and that rabbit anti-mite antibody could absorb out house dust allergens (Ricci, Biliotti and Romagnani, 1975). These results were confirmed in vivo. There was good agreement between P-K tests performed with extracts of house dust and of mites and P-K reactions to dust could be neutralized by Dermatophagoides allergens (Miyamoto et al, 1968; Kabasawa et al, 1976).

Several workers argued against the view that mites were the most important source of dust allergens. Berrens (1970, 1974) maintained that the conditions which favoured the growth of mites were also ideal for the generation of glycoprotein allergens, such as allergen E, by the non-specific chemical degradation of animal and plant material. In a detailed study of 20 highly dust allergic individuals Kawai et al (1972) did not find any correlation between leucocyte sensitivity to extracts of D.pteronyssinus and house dust using a quantitative histamine release assay. Over half their subjects were 10-100 fold more sensitive to the dust extract. Moreover, rabbit anti-mite antibody failed to block dust-induced histamine release in the majority (19/20) of patients. In retrospect, these findings probably reflect the low incidence of D.pteronyssinus on the east coast of the United States. On the west coast a good correlation was found between sensitivity to mites and dust using histamine release assays and eosinophil migration tests (Bullock and Frick, 1972).

4. The clinical significance of house dust mites

Unlike some mites, e.g. the scabies mite, house dust mites are not infectious agents. They have been mainly implicated in diseases mediated by immediate hypersensitivity, particularly perennial rhinitis and asthma, which are commonly associated with house dust allergy (Kern, 1921; Cooke, 1922). In Europe exposure to mites is almost unavoidable, consequently sensitization occurs at an early age and most allergic reactions to dust are seen in children (Sarsfield, 1974). Mite sensitivity generally declines with age and children often show 'spontaneous cure' by the time of puberty. Unfortunately, highly

atopic individuals may have become sensitized to other environmental allergens by then and they continue to suffer from allergic disease.

Many years before D.pteronyssinus was isolated clinicians observed that asthma was common in people who lived in damp houses built on poorly drained soils. They knew that symptoms improved if patients moved to drier areas in the mountains or beside the sea (Storm van Leewen, 1927). In Switzerland the incidence of asthma decreased proportionately at higher altitudes and correlated with the lower mite content of dust obtained from these areas (Voorhorst, Spieksma and Varekamp, 1969; Spieksma, 1970). All this circumstantial evidence supported the view that dust-induced asthma was attributable to mite allergy. The hypothesis was further supported by the demonstration of mite-specific IgE in the majority of symptomatic individuals. Positive immediate skin test responses to extracts of Dermatophagoides sp. were reported in over 80% of children with asthma (Maunsell et al, 1968; Morrison-Smith et al, 1969; Kabasawa et al, 1976) and in adult patients up to 60 years of age (McAllen, Maunsell and Assem, 1970; Kurimoto and Baba, 1978). Nasal and bronchial provocation tests with mite extracts were also demonstrated in a high proportion of patients (Sarsfield, 1974; Pauli et al, 1979). In one study more than half the patients gave positive bronchial responses to 1µg or less of D.pteronyssinus allergen: an amount which was considered to be less than the weight of a whole live mite (McAllen et al, 1970). Much larger quantities of allergen were required for provocation tests than for skin tests. However, positive reactions were generally obtained with smaller amounts of mite allergens than house dust allergens (Mijamoto et al, 1968; Pauli et al, 1979). Mite-specific

IgE was demonstrable in serum from most asthmatic patients using the RAST (Mijamoto et al, 1974; Turner, Baldo and Anderson, 1975; Kurimoto and Baba, 1978) or by passive sensitization of the skin (Mijamoto et al, 1968; Kabasawa et al, 1976) or normal human tissue (Assem and McAllen, 1970). These studies also showed a good correlation between the presence of serum ab and the results of challenge tests.

The precise mechanism by which allergens induce the symptoms of asthma is not known. Extrinsic asthma is frequently a chronic condition and symptoms may occur at times when there is no apparent exposure to allergens. It seems likely that the allergic reaction to mites and other allergens provides the initial stimulus for asthmatic attacks. Mites themselves are too large to penetrate the smaller airways. However, there is some evidence that the allergen is present in the secretions or excretions of mites; probably in the faecal particles which are 10-50μ in diameter (Voorhorst et al, 1964, 1967; Halmai and Alexander, 1971). The smaller particles could act as aeroallergens and enter the lungs in sufficient quantities to cause symptoms and possibly to cause bronchial hyper-reactivity.

5. Aims of the present studies

The correlation between sensitivity to mites and to house dust is by now well established. However, studies on the humoral and cellular response to D.pteronyssinus have been severely limited because purified allergens were not available. The aim of these studies was to purify an allergen from mite culture which could be radiolabelled and used in an antigen binding assay to measure class

specific ab to D.pteronyssinus. There was an urgent need for such an assay to compare the ab response to mites in atopic and non-atopic individuals, to assess the ab response following desensitization treatment and to investigate the site of ab production. In addition, it was hoped that more knowledge of the properties of mite allergens would add to our understanding of inhalant allergens in general.

This thesis is arranged in four chapters each with four sections; an Introduction, Methods, Results and Discussion. The Methods are given in outline only, but contain sufficient information for the interpretation of the Results. Full details of all experimental protocols are given in the Appendices. There is also a General Discussion.

C H A P T E R I

Partial purification of a *D.pteronyssinus* allergen

1.1 Introduction

The purification of mite allergens has been hampered by technical difficulties in growing mite cultures with a high allergen content. Previously, cultures of D.pteronyssinus were grown on human skin scales, animal danders and fishmeal (Spieksma, 1967, 1976; Miyamoto, Ishii and Sasa, 1975). Extracts of these cultures were extremely heterogeneous and often included culture medium components which were potentially allergenic, e.g. fish proteins. This type of extract was used in early gel filtration studies which established that mite allergens and house dust allergens eluted in the same molecular weight range (10-63,000 daltons) (Miyamoto, Oshima and Ishizaki, 1969; Romagnani et al, 1972). The quantities of crude mite allergen used in those studies (10-100mg) were generally too low for purification purposes. In the early 1970's Bencard Limited developed a successful method for the continuous culture of large numbers of D.pteronyssinus (several thousand mites per gram of culture) on non-ectodermal mammalian tissue in streams of humidified air. These cultures gave a much higher yield of mite allergens (1-2g extract/100g culture) and have been used throughout the present studies on allergen purification.

This chapter describes the preparation of a partially purified allergen from D.pteronyssinus culture by gel filtration and preparative electrophoresis. The allergen was radiolabelled and used in an antigen-binding assay to measure IgG, IgA and IgE ab to D.pteronyssinus. These assays can give quantitative measurements of the amount of ab in serum (Mulligan, Osler and Rodriguez, 1966) and have been applied to different ab classes (Osler, Mulligan and Rodriguez, 1966; Newcomb

and Ishizaka, 1967). Antigen binding techniques rely on the use of purified or at least partially purified allergens. They have previously been used to investigate the ab response in grass pollen and ragweed hay fever (Yunginger and Gleich, 1973; Platts-Mills et al, 1976, 1978) but have not been used in mite allergy because purified allergens were not available.

1.2 Methods

100g D.pteronyssinus whole culture (Bencard Limited, Betchworth, England) was extracted in 1L BBS by stirring overnight at 4°C. The extract was centrifuged (23,000g for 60 minutes) and the dark brown supernate was concentrated down to 50ml over an Amicon PM10 membrane filter (Amicon, Lexington, Mass. U.S.A.). This membrane concentrated molecules with a molecular weight of >10,000 daltons. Residual low molecular weight material was removed by extensive dialysis against three 2L changes of BBS over two days. The non-dialyzable material contained D.pteronyssinus allergens and soluble culture medium components with a molecular weight of >8,000 daltons. This preparation was termed Extract I (ExI). Fractionation of ExI by gel filtration and pevikon block electrophoresis yielded a partially purified allergen, F_4P_1. Fractions isolated during the purification procedure were assessed by skin testing, direct RAST and immunodiffusion. Quantitative intradermal skin tests were carried out using serial ten-fold dilutions of allergens from 10µg/ml down to 10^{-6}µg/ml in 0.05% human serum albumin in 0.9% phenol saline. The solutions were sterilized by passage through an 0.45µm Millipore filter. Skin tests were performed by successive intradermal injection of 0.05ml of increasing concentrations of allergen. The diameter

of the wheal and flare at the test site was recorded after 15-20 minutes. Intradermal skin testing was used to compare the allergenic potency of F_4P_1 and ExI. The antigens present in these fractions were also compared by Laurell electrophoresis using rabbit antiserum to Extract I (rabbit anti-ExI).

The F_4P_1 preparation was labelled with ^{125}I and used in an antigen binding assay to measure ab to D.pteronyssinus in serum from mite-allergic individuals and non-allergic controls. The fluid phase ab was allowed to combine with radiolabelled F_4P_1 and then all the ab of one class was precipitated with monospecific antiserum; anti-IgG, anti-IgA or anti-IgE (Fig. 1.1). The radioactivity bound by the washed precipitates was directly related to the quantity of class-specific anti-mite ab. The assay for IgE ab was compared with other methods of measuring IgE ab to D.pteronyssinus, i.e. P-K testing and the RAST. The P-K tests were performed using sera from five healthy, mite-allergic individuals (Australia antigen negative) which were fractionated with saturated ammonium sulphate to obtain IgE rich fractions (33-50% cut). These fractions were diluted from 1:10 to 1:1600 in human serum albumin in phenol saline and sterilized through an 0.45µm Millipore filter. Two non-allergic recipients (total serum IgE 31ng/ml and 175ng/ml) were injected intradermally with 0.05ml of suitable dilutions of the fractions and after 24 hours each site was challenged with 0.05ml of a 1µg/ml solution of ExI. The wheal and flare reactions were recorded after 15-20 minutes.

Further details of the methods used in this chapter are given in Appendix A2, A3 and A4.

0.1ml serum dilution

+

0.1ml ^{125}I F_4P_1 (~ 7ng)

+

(0.1ml IgE myeloma protein diluted 1:100)
Incubate for 4 hours at room temperature

+

0.1ml goat anti-IgG, anti-IgA or anti-IgE
Incubate overnight at $4^{\circ}C$

Wash the precipitate 4 times and count the residual radioactivity in a γ-counter

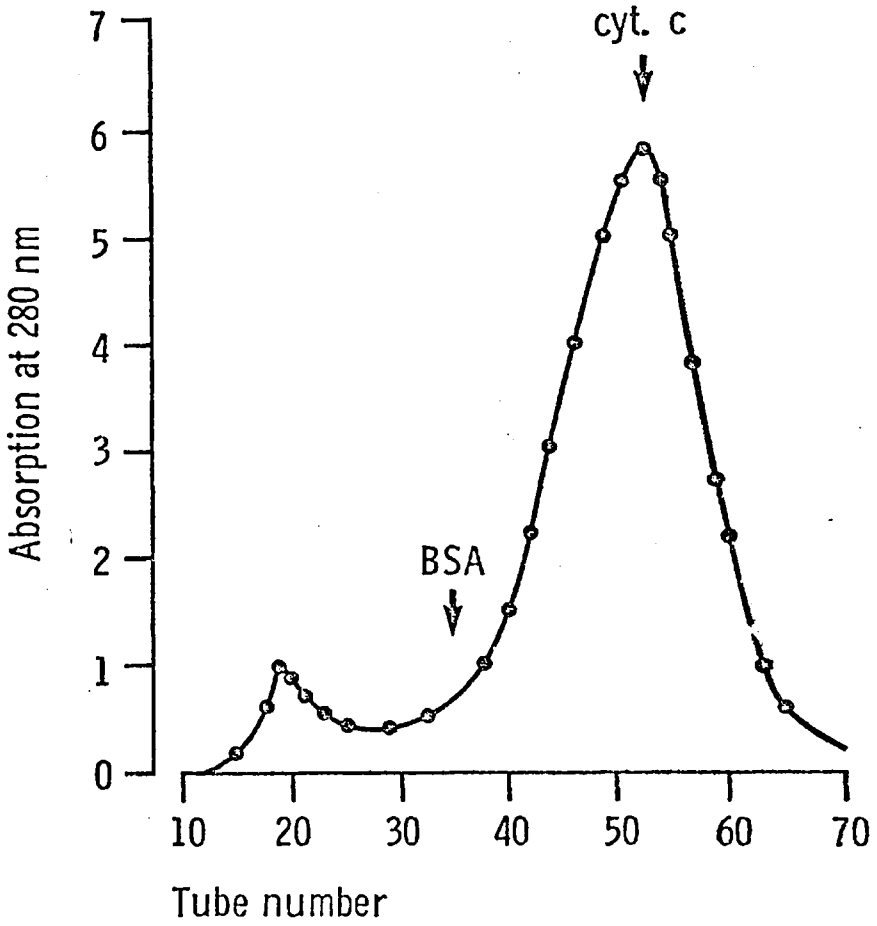
Figure 1.1

Assay for IgG, IgA and IgE antibody to D.pteronyssinus

1.3 Results

1.3.1 Preparation of the partially purified allergen F_4P_1

The components of D.pteronyssinus ExI were initially separated according to molecular size by gel filtration through Sephadex G-100. The eluted fractions showed two protein peaks: a small peak eluted with the void volume (MW >100,000 daltons) and a very broad peak eluted with MW 5-65,000 daltons as indicated by the positions of BSA (63,000 daltons) and cytochrome 'C' (12,500 daltons) markers (Fig. 1.2). The eluate was divided into six fractions (F1-6) which were tested in vivo by skin testing and in vitro by direct RAST to determine the distribution of D.pteronyssinus allergens. A semi-quantitative evaluation of the results is given in Fig. 1.2. The dilutions of F3 and F4 required to give a positive skin response (>6 x 6mm wheal) were 10-100 fold greater than for other fractions in the six mite-allergic subjects tested. Some highly sensitive individuals (4/6) gave positive skin responses to F3 and F4 at very high dilution, i.e. 10^{-4}-10^{-5}µg/ml. These two fractions appeared to contain potent D.pteronyssinus allergens with very similar skin test reactivity. Serum IgE ab to the six fractions was assessed by direct RAST using ten sera from mite-allergic individuals. The overall pattern of results was very similar to that obtained by skin testing (Fig. 1.2). Representative data for six sera are given in Table 1.1. In most cases there was significant binding to paper discs coupled to F2-5 when compared with control discs coupled to human serum albumin. All ten sera showed maximal binding to discs coupled to F4. There was no significant binding to any of the fractions by four control sera. The direct RAST results suggested that a higher proportion



Fraction no.	1	2	3	4	5	6
Skin reactivity	+	++	+++	+++	++	+
RAST	+	++	+++	+++	++	+

Figure 1.2

Fractionation of Dermatophagoides pteronyssinus Extract I on Sephadex G-100. Approximately 100mg ExI was applied to an upward flow, gravity fed Sephadex G-100 column (75 x 2.5cm) equilibrated in BBS at 4°C. Samples of 6.3ml were collected at a flow rate of 10ml/hr. Six fractions were pooled (1-6) and the skin test reactivity of each fraction was assessed by intradermal skin testing. Each fraction was also coupled to cellulose discs and used for RAST. Typical results are shown on a semi-quantitative basis (+ to ++++).

TABLE 1.1

Assessment of Sephadex G-100 fractions of D.pteronyssinus ExI by Direct RAST[a]

	Radioactivity (cpm) bound by paper discs coupled to:-							
	Sephadex G-100 fraction							Human Serum Albumin
Serum	1	2	3	4	5	6	ExI	
J.L.	1690	3443	3920	4743	1807	799	6224	650
S.J.	587	732	657	780	558	500	891	582
M.B.	624	1152	1507	1953	948	498	3083	460
M.K.	762	2362	3608	4844	1046	867	5776	570
A.B.	1611	3419	4067	6257	2002	879	7461	635
P.K.	564	964	1388	1734	796	523	2247	563
Controls[b]	490±79	529±79	466±53	506±90	581±104	484 ± 57	546 ± 102	480±40

(a) One microgram of each fraction (protein content determined by the method of Lowry et al, 1951) was coupled to a paper disc. Allergen-coupled discs were incubated with 0.1ml 1:12.5 dilution of serum followed by 2ng ^{125}I-labelled goat anti-IgE (total radioactivity added = 35,000 cpm).

(b) Values are the mean ± one standard deviation of four sera from skin test negative persons.

of the serum IgE ab was directed against allergens present in F4, therefore, this fraction was selected for further purification.

The proteins present in F4 were further analysed by pevikon block electrophoresis and the electrophoretic sub-fractions were assayed for protein content and tested on immunodiffusion against rabbit anti-ExI. The fraction was resolved into two protein peaks (Fig. 1.3). The larger peak, F_4P_2, migrated faster than albumin, was dark brown in colour and showed no reactivity on immunodiffusion. The smaller peak, F_4P_1, migrated with β-mobility, had a faint brown colour and was strongly reactive on immunodiffusion, showing one major and one minor precipitin line against the rabbit antiserum (data not shown). The F_4P_1 fraction was rechromatographed over Sephadex G-100 and it eluted as a single, apparently homogeneous, peak MW 15-25,000 daltons. This preparation was used for radio-labelling and for comparison with ExI and is hereafter referred to as F_4P_1.

The degree of purification was assessed by comparing the antigenic composition of F_4P_1 with that of ExI by Laurell electrophoresis. The analysis showed that at least 15 different components of D.pteronyssinus culture formed precipitin arcs against rabbit anti-ExI (Fig. 1.4a). In contrast, only one major precipitin arc and 2-3 smaller, more weakly staining arcs were detected in F_4P_1 using the same antiserum (Fig.1.4b). The predominant antigen in F_4P_1 appeared to correspond to the largest, most heavily stained precipitin arc in ExI. The Laurell analyses showed that a considerable degree of purification had been achieved and, even though the

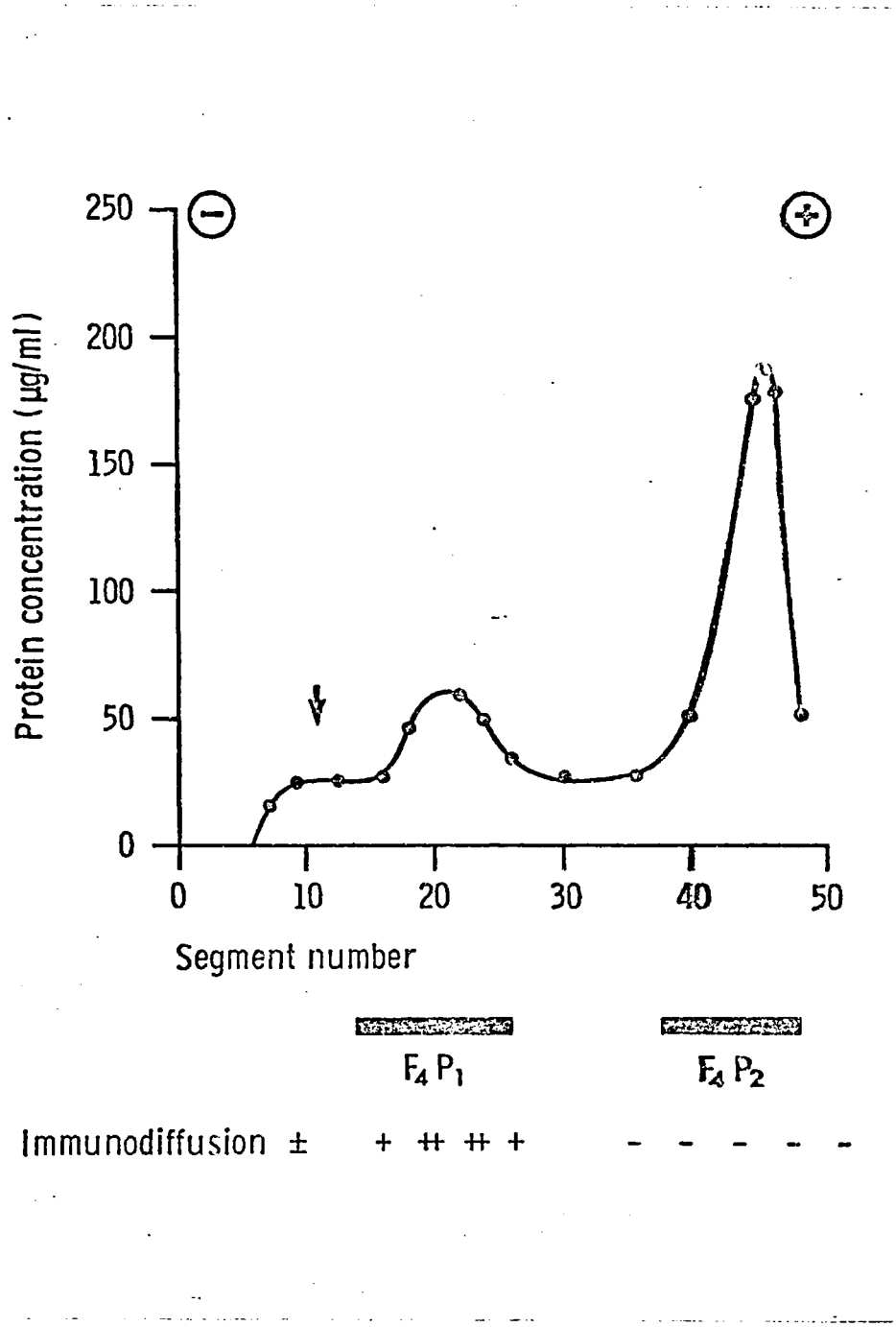


Figure 1.3

Pevikon block electrophoresis of Sephadex G-100 Fraction 4. Arrow indicates the position at which 30mg F_4 was applied to the block. The protein concentration was determined by the method of Lowry et al (1951).

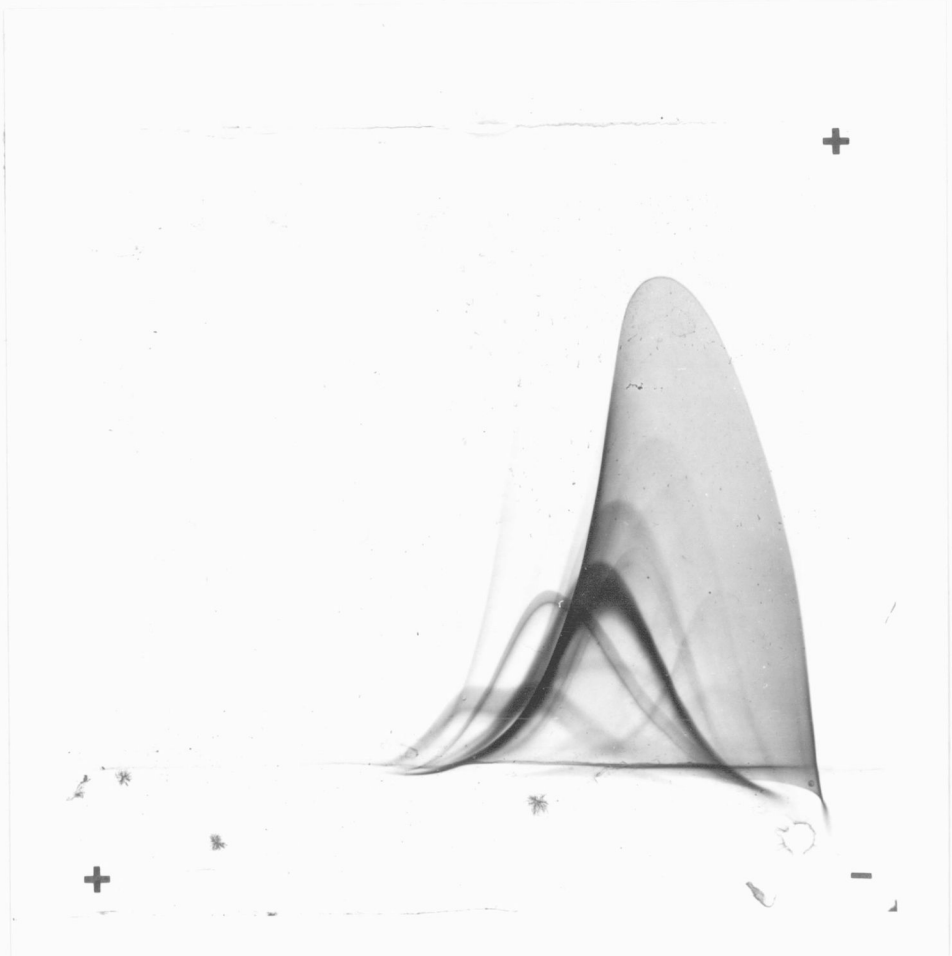


Figure 1.4(a)

Laurell electrophoresis of D.pteronyssinus Extract I.

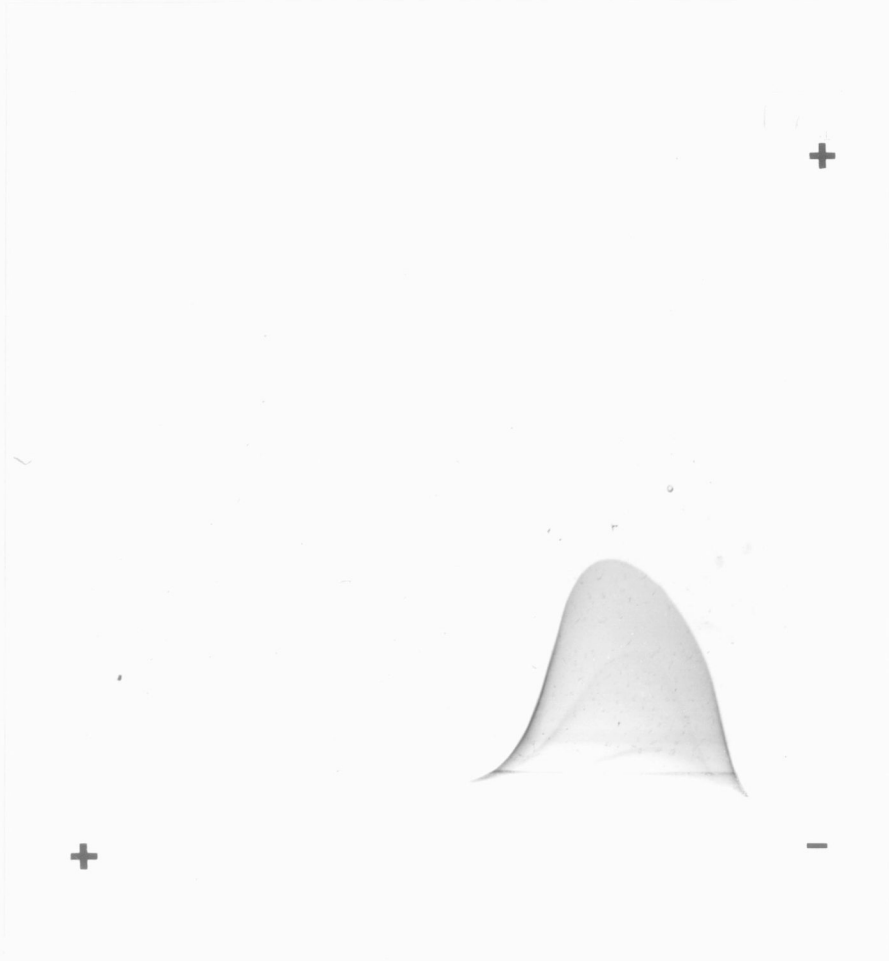


Figure 1.4(b)

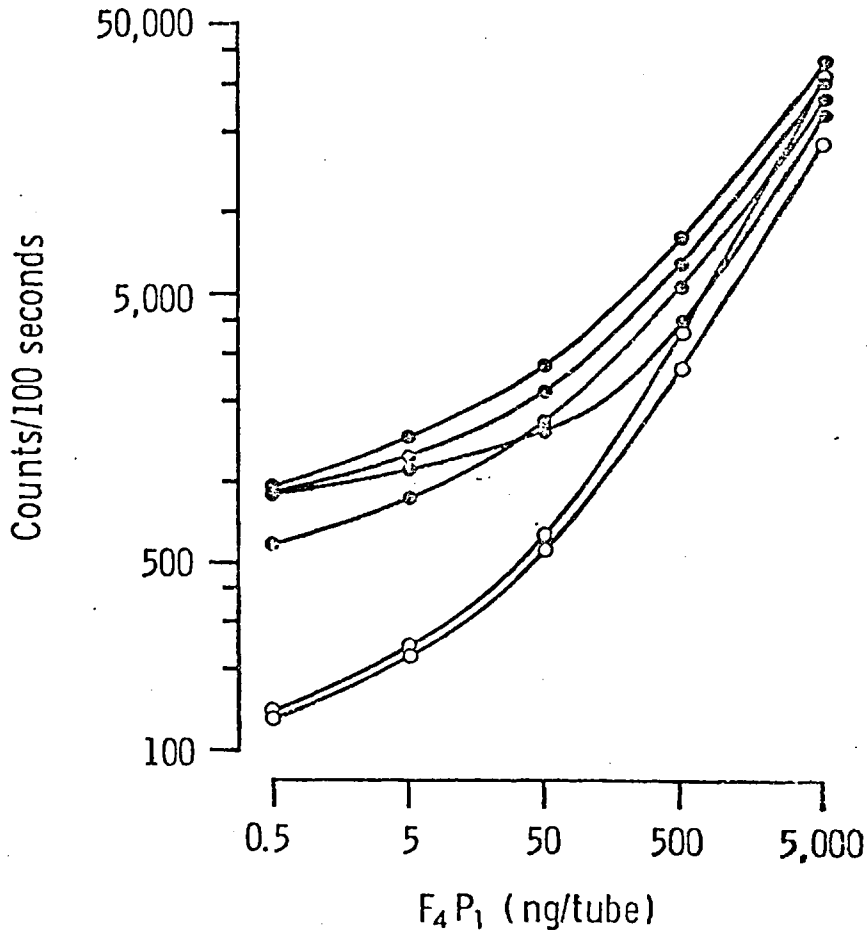
Laurell electrophoresis of the partially purified D.pteronyssinus allergen, F_4P_1.

F_4P_1 was not completely pure, the results justified further studies on the allergenicity of this preparation.

1.3.2. Use of F_4P_1 in an antigen-binding assay to measure ab to D.pteronyssinus

During the course of these studies several attempts had been made to establish antigen-binding assays using ^{125}I-labelled ExI or Sephadex G-100 fractions 3 and 4. These assays were not very sensitive because the labelled allergens had low specific activity (<20% ^{125}I was incorporated into protein) and gave high levels of non-specific binding. Some sera from mite-allergic individuals did show significant binding compared to control sera, but the differences were always less than ten-fold. The radiochemical properties of ^{125}I-labelled F_4P_1 were much better than those of the impure extracts (see Appendix A.2.5) and this preparation could be used in an antigen binding assay to measure IgG, IgA and IgE ab to D.pteronyssinus (Fig. 1.1, Appendix 4.1). Initially, the assay was set up using 7ng ^{125}I-labelled F_4P_1/tube; an amount of antigen similar to that used in antigen binding assays for pollen allergens (Platts-Mills et al, 1976, 1978). The effect of antigen concentration was investigated by varying the amount of F_4P_1 added over a 4 log range, from 0.5ng to 5µg F_4P_1/tube. The greatest differences in binding between sera from allergic and non-allergic individuals were achieved by adding 0.5-5ng F_4P_1/tube for both IgG and IgE ab (Figs. 1.5a and 1.5b). At higher antigen concentrations (over 50ng F_4P_1/tube) the absolute level of radioactivity bound by non-allergic sera (i.e. non-specific, non-antibody binding) increased and the sensitivity of the assay was progressively reduced.

(a)



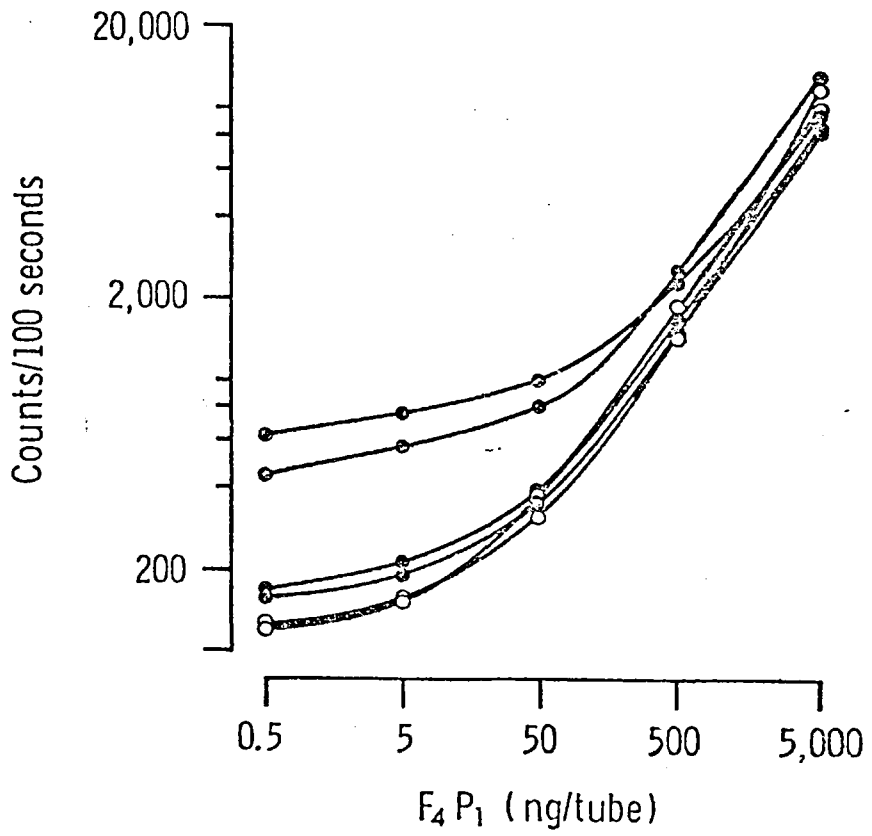
Percent radioactivity bound by IgG

Allergic	28.80	6.06	2.13	1.00	0.44
Non-allergic	3.74	1.01	0.43	0.34	0.23

Figure 1.5(a)

Effect of antigen dose on antigen binding by IgG ab. Six sera diluted 1:12.5 were incubated for 4 hours with 0.1ml of serial ten-fold dilutions of a solution containing 5µg ^{125}I F_4P_1 and 95µg cold F_4P_1 in 2ml (total F_4P_1 concentration = 50µg/ml). The radioactivity bound by serum from skin test positive (●——●) and skin test negative (O——O) individuals following precipitation with anti-IgG is shown. The percentage of radioactivity bound by an allergic serum (top line in Fig.) and a non-allergic serum (bottom line in Fig.) for each quantity of F_4P_1 added is also shown.

(b)



Percent radioactivity bound by IgE

Allergic	18.10	3.19	0.78	0.29	0.16
Non-allergic	3.65	0.66	0.26	0.18	0.16

Figure 1.5(b)

Effect of antigen dose on antigen binding by IgE ab. Legend as for Fig. 1.5(a). Sera from skin test positive (●——●) and skin test negative (O——O) individuals were precipitated with anti-IgE.

Similar increases in the negative background were observed using IgG and IgE myeloma sera which contained high levels of immunoglobulin without allergen specificity (data not shown). These data suggested that the increased binding by non-allergic sera was truly non-specific and not mediated by low-affinity ab. Although maximal differences in binding appeared to be achieved using 0.5ng F_4P_1/tube, the radioactivity (cpm) added at this level was so low that the non-specific binding could not be distinguished from the background 'noise' of the γ-counter. Therefore, all assays for each class of ab were routinely carried out using 7ng ^{125}I-labelled F_4P_1.

The antigen binding assay was used to measure ab to D.pteronyssinus in serum from 34 allergic persons who had positive prick tests to a commercially available D.pteronyssinus extract (Bencard Limited) and 31 non-allergic persons with negative prick tests. The majority of the sera from prick test positive persons contained detectable IgG (32/34), IgA (22/34) and IgE (27/34) binding activity (BA) for F_4P_1 (Fig. 1.6). In these sera the relationship between classes was usually IgG BA > IgE BA > IgA BA and IgE BA was never found in the absence of IgG BA. The allergic group included 28 persons who had perennial rhinitis and/or asthma related to house dust sensitivity, and six persons with rhinitis whose symptoms did not seem to be caused by house dust. Sera from these six, 'asymptomatic' persons lacked detectable IgE BA and IgA BA, but 4/6 contained low levels of IgG BA (<20 units BA/ml). Similarly, neither IgE BA nor IgA BA was detected in sera from prick test negative persons though 10 sera contained detectable IgG BA. The high incidence of BA for F_4P_1 in sera from allergic persons suggested that this preparation contained a major allergen from D.pteronyssinus.

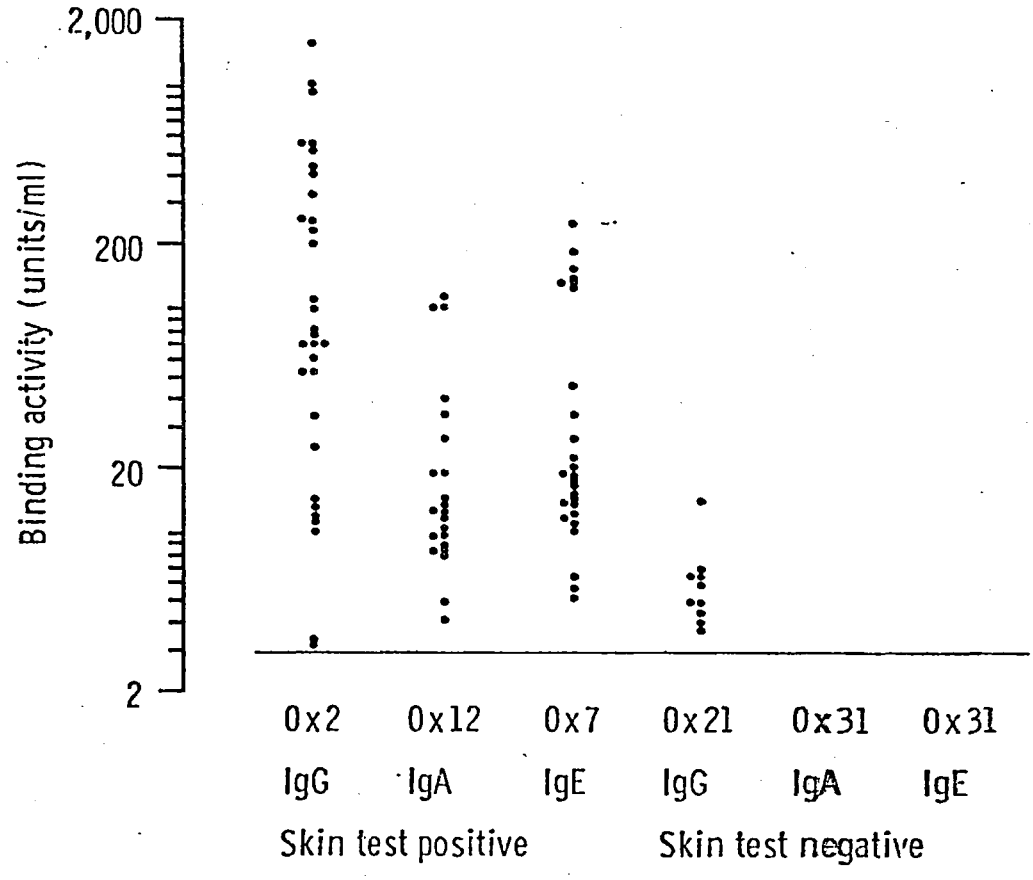


Figure 1.6

IgG, IgA and IgE BA for F_4P_1 in the serum of skin test positive and skin test negative persons measured by antigen binding radioimmunoassay. The minimum detectable level of BA (units/ml) is indicated by the solid line. The number of sera without detectable BA is shown below this line.

The clinical symptoms, intradermal skin test reactivity and serum ab levels of 15 prick test positive persons are shown in Table 1.2. Quantitative intradermal skin tests were used to compare the allergenic potency of F_4P_1 and ExI. All 14 persons tested showed positive reactions to both extracts. Although skin sensitivity amongst different subjects varied over a four log range, each individual responded to similar dilutions of the two allergens. The majority of the patients reacted to a higher dilution of F_4P_1 than ExI (Table 1.2) suggesting that the minimum quantity of F_4P_1 required to give a positive skin test was about one-fifth the quantity of ExI. To check the specificity of the skin tests five non-allergic persons were tested with 10µg F_4P_1: none gave a positive reaction. Two persons with grass pollen hay fever who were sensitive to 10^{-6}µg Rye I also failed to react to 10µg F_4P_1. In general, intradermal skin test reactivity did not correlate with measurements of serum IgE ab or total IgE (e.g. compare J.L. and S.S. in Table 1.2). However, there was a very good correlation between IgE BA for F_4P_1 and IgE ab to D.pteronyssinus measured either by P-K testing or by RAST. The IgE rich serum fractions from five prick test positive persons all gave positive P-K tests, whereas two sera from prick test negative persons and from the persons with grass pollen hay fever described above were completely negative. The P-K test was ~ 5-fold more sensitive than the other methods of measuring serum IgE ab. Thirty-three of the sera from skin test positive persons were assayed for IgE ab by antigen binding assay and RAST. The results (Fig. 1.7) showed an excellent quantitative correlation between the two methods by linear regression analysis ($r = 0.94$, $p < 0.001$). Analysis of the levels of BA in Fig. 1.6 showed a very good correlation between IgE BA

TABLE 1.2

Antibody levels in fifteen prick-test-positive subjects assessed by intradermal skin-testing, antigen-binding assay, RAST, and P.K. testing

Subject (Age)	Symptoms attributable* to house dust allergy*	Intradermal skin-test† Extract I 'μg/ml'	Intradermal skin-test† F_4P_1 'μg/ml'	RAST‡ Rast units/ml	BA for F_4P_1 IgG units/ml	BA for F_4P_1 IgE units/ml	BA for F_4P_1 IgA units/ml	Ratio IgG BA:IgE BA	P.K. titre§	Total IgE (ng/ml)
J.S. (17)	Rhinitis (grass, egg)	n.d.	n.d.	2125	563	184	27	3.1:1	1600 (666)	8850
J.L. (19)	Rhinitis, asthma	10^{-5}	$10^{-5\cdot5}$	775	1040	135	119	7·7:1	400 (136)	2059
M.B. (18)	Rhinitis, facial swelling	10^{-5}	$10^{-5\cdot5}$	91	330	28	< 4	11·8:1	200 (43)	1728
S.J. (39)	Rhinitis, asthma (grass)	$10^{-4\cdot5}$	10^{-5}	< 8	34	6	< 4	5·9:1	25 (17)	146
R.G. (30)	Asymptomatic (grass)	10^{-3}	10^{-3}	10	< 4	< 4	< 4	—	10 (< 4)	1620
M.K. (24)	Rhinitis	10^{-5}	10^{-6}	325	443	130	13	3·4:1		1824
S.B. (16)	Asthma	10^{-5}	10^{-6}	425	201	143	104	1·4:1		1162
S.M. (27)	Rhinitis	10^{-4}	10^{-5}	53	258	20	19	13·6:1		192
W.M. (50)	Rhinitic (grass)	10^{-5}	$10^{-5\cdot5}$	n.d.	81	21	14	3·85:1		1483
P.J. (19)	Mild rhinitis (grass)	10^{-4}	10^{-5}	66	113	14	< 4	8·1:1		2880
D.T.R.(45)	Rhinitis	10^{-4}	10^{-5}	25	72	14	8	5·1:1		643
K.B. (20)	Asymptomatic (grass)	$10^{-2\cdot5}$	10^{-3}	< 8	10	< 4	< 4	—		1248
P.M. (40)	Asymptomatic (cat)	10^{-3}	10^{-3}	< 8	< 4	< 4	< 4	—		1474
S.S. (39)	Rhinitis (grass)	10^{-5}	$10^{-5\cdot5}$	10	236	12	9·8	19·6:1		377
K.M. (26)	Rhinitis (grass)	10^{-6}	10^{-6}	144	55	47	11	1·2:1		655

* Other allergens giving clinical symptoms and positive skin-tests are indicated in parentheses. Grass = grass pollen.

† For both intradermal skin-testing and PK-testing a wheal of 6×6 mm was regarded as positive. Log dilutions of allergen solutions were used—intradermal skin-tests were assessed to the nearest ½ log to distinguish strongly positive results.

‡ RAST units were obtained by reference to a control curve.

§ PK titres are the mean values obtained from tests on two recipients. The IgE BA (units/ml) of the IgE-rich serum fractions used in PK-testing is given in parentheses.

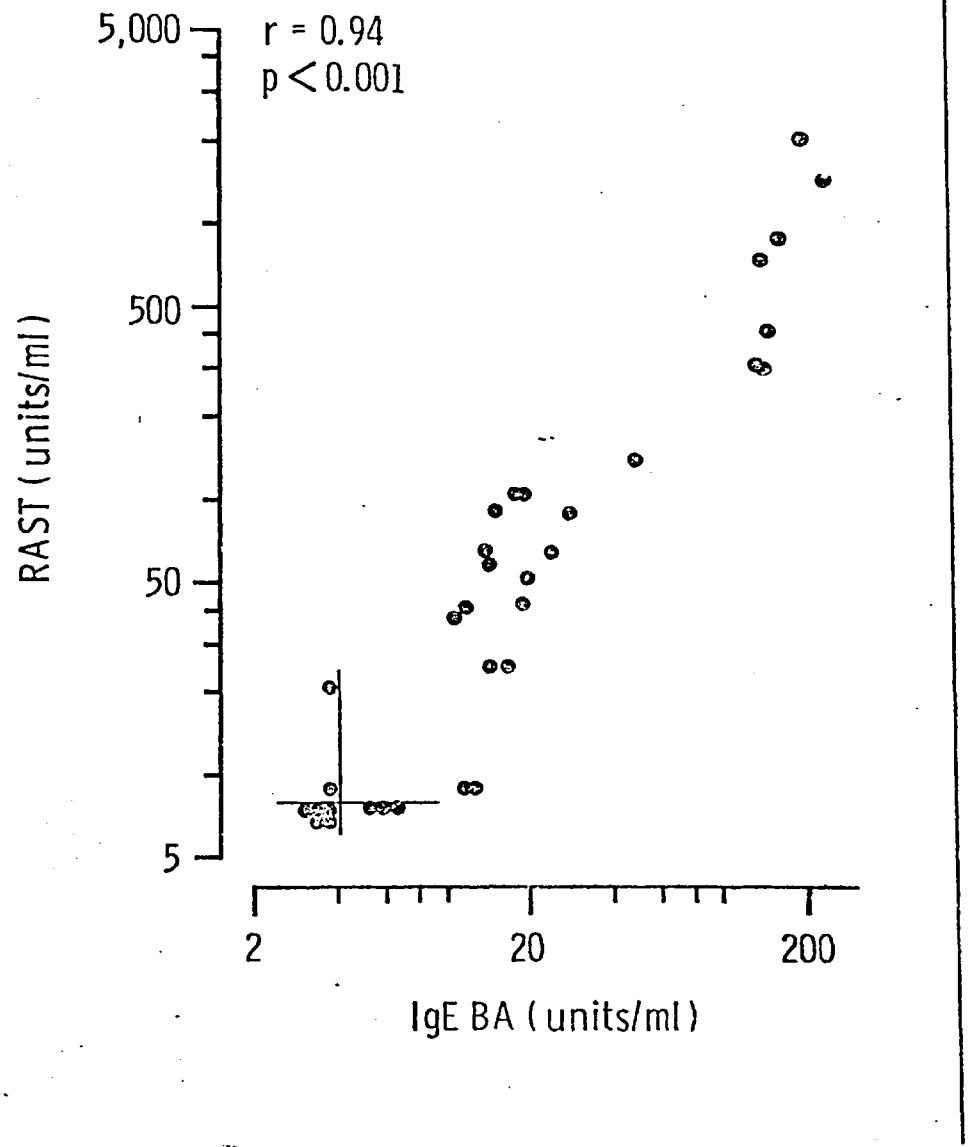


Figure 1.7

Correlation between the antigen binding assay (using ^{125}I F_4P_1) and RAST (using D.pteronyssinus Extract I) for measuring IgE ab to D.pteronyssinus in the serum of prick test positive persons. The minimal level of sensitivity of each assay is indicated (——).

and IgG BA ($r = 0.87$, $p < 0.001$) and IgE BA and total IgE ($r = 0.63$, $p < 0.001$), and a modest correlation between IgG BA and total IgE ($r = 0.38$, $p < 0.02$).

1.4 Discussion

The results describe the preparation of a partially purified allergen, F_4P_1, from an aqueous extract of D.pteronyssinus culture using conventional protein separation techniques. At each stage during the purification fractions were chosen because they showed the greatest biological activity in vivo and in vitro. This selection process was aimed at identifying the major allergen(s) from D.pteronyssinus, rather than attempting to characterize each individual allergen within the extract. The assessments of biological activity were based primarily on the interaction of allergen with specific IgE ab (skin testing and direct RAST) and also on antigenic analyses using hyperimmune rabbit antiserum. Intradermal skin tests were preferred for use in vivo because they require very small quantities (μg) of allergen and allow several different fractions to be tested in the same individual. Furthermore, they show an excellent correlation (>90%) with the results of bronchial challenge tests (Cavanaugh, Bronsky and Buckley, 1977) and are less likely to induce hazardous clinical side-effects, e.g. 'late' reactions.

The gel filtration results were in close agreement with those of previous workers who showed that the most allergenic fractions of D.pteronyssinus (assessed by skin testing, direct RAST and RAST inhibition) eluted with MW 10-30,000 daltons (Miyamoto et al, 1969;

Romagnani et al, 1972, 1976; Holford-Strevens, 1972). Further purification of the most active gel filtration fraction (F_4) by electrophoresis resulted in the isolation of the F_4P_1 preparation. The electrophoretic mobility of F_4P_1 was very similar to that reported by Ito (1975) for the main allergenic component of D.farinae. The large peak of rapidly migrating material, F_4P_2, was assumed to comprise the culture medium components which, according to the manufacturer, are only very weakly immunogenic in rabbits.

The results of skin testing and in vitro measurements of IgE ab suggested that F_4P_1 contained the major allergens from D.pteronyssinus. However, despite the very good quantitative correlation between skin tests to F_4P_1 and D.pteronyssinus ExI (Table 1.2), it was disappointing that the increased reactivity of F_4P_1 was only about 5-fold. On Laurell electrophoresis one of the antigens present in high concentration in ExI appeared to be the major antigen in F_4P_1 (Fig. 1.4). If this assumption proved correct and the antigen was also allergenic, then any "enrichment" achieved during the purification would have little effect on the skin tests because they are only sensitive to 10-fold differences in allergen concentration. The in vitro measurements of IgE ab complemented the skin test data and showed an excellent quantitative correlation between IgE ab to F_4P_1 and IgE ab to D.pteronyssinus in serum (Table 1.2, Fig. 1.7). Most allergic individuals had detectable IgG, IgA and IgE BA for F_4P_1 and the proportion of ^{125}I-labelled F_4P_1 which could be bound by IgG ab could be as high as 68% (Appendix A.4.1). This comparison of in vitro techniques suggested that individuals who made an ab response to D.pteronyssinus also made

an ab response to F_4P_1. All these data made it extremely likely that F_4P_1 contained the major allergen of D.pteronyssinus. However, the results with F_4P_1 did not exclude the possibility that other fractions of mite extract, e.g. gel filtration fractions 3 and 5 also contained potent allergens.

The use of radiolabelled F_4P_1 in an antigen binding assay to measure ab to D.pteronyssinus offered several advantages over previous methods of measuring allergen-specific ab. The most important feature was the ability to measure IgG and IgA ab as well as IgE ab. In the past, the only method for measuring IgG ab was radioimmunoelectrophoresis, using ^{125}I-labelled crude mite antigen (D'Souza et al, 1973; Ito, 1975). This semi-quantitative technique lacked sensitivity and could not detect IgG ab in the majority of sera. Most attempts to use the RAST to measure ab other than IgE were unsuccessful because the background binding by non-allergic sera was too high. The results of a modified RAST for measuring IgG and IgA ab to D.pteronyssinus are shown in Fig. 1.8. Significant differences between sera from allergic and non-allergic individuals were only obtained for IgE ab. The non-specific binding is probably due to the very high serum concentrations of these immunoglobulins (1-12mg/ml) compared to IgE ($\sim$ 1µg/ml) and the much lower proportion of immunoglobulin that is allergen-specific. Recently, solid phase assays for IgG ab have been developed using ^{125}I-labelled Staphylococcal protein A, instead of anti-IgG, to bind the allergen-ab complex (Hamilton, Sobotka and Adkinson, 1979; Delespesse, Debisschop and Flament, 1979). These assays appear to be less sensitive than the IgE RAST, but may be suitable for assaying sera with high levels of IgG ab, e.g. from patients receiving desensitization treatment.

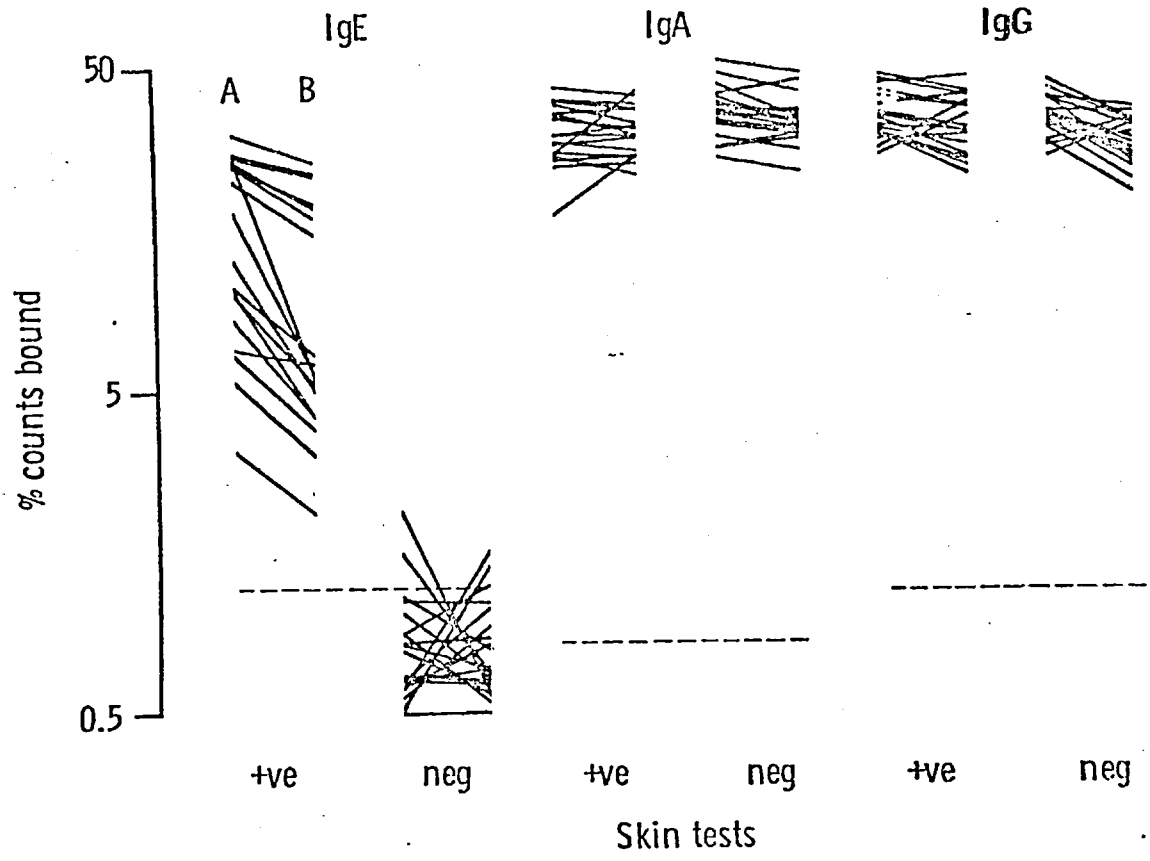


Figure 1.8

Modified RAST for measuring IgG and IgA ab to D.pteronyssinus. Sera from 18 skin test positive and 18 skin test negative individuals were incubated overnight at dilutions of 1:12.5 (A) and 1:25 (B) with D.pteronyssinus ExI RAST discs. After extensive washing the discs were incubated with 2ng ^{125}I-labelled anti-IgE, anti-IgA or anti-IgG (total radioactivity added = 37,000, 49,000 and 46,000 cpm, respectively). The percentage radioactivity bound to the disc, after further extensive washing, is shown for each class of ab. The background binding (0.8-1.2%) using bovine serum albumin instead of serum is also indicated (---).

The other advantages of the antigen binding assay include measurements of IgG, IgA and IgE ab in parallel, so that the quantities of each class can be directly compared; low non-specific binding, which is useful for assays on nasal secretions and saliva; and, finally, more economical use of allergen. Solid phase techniques require mg quantities of allergen for routine purposes, whereas antigen binding assays can be carried out for several months using only 5-10µg labelled allergen. This is particularly useful for measuring ab to highly purified allergens which are often only available in small amounts. The main disadvantage with antigen binding assays for IgE ab is the requirement for large quantities of IgE myeloma serum (20µg/assay) as carrier to form a precipitate. These myelomas are extremely rare, so the serum is unlikely to be available for general use. The IgE assay in its present form is essentially a research tool. However, " triple antibody assays" have been developed in which the first ab is incubated with a second ab derived from another species (e.g. rabbit anti-IgE) and then precipitated with a third ab (e.g. goat-anti rabbit IgG) (Platts-Mills and Ishizaka, 1975). This modification has recently been used to measure IgE ab to D.pteronyssinus using ^{125}I-labelled crude mite antigen (Sano and Ito, 1978).

The nature of the ab response to D.pteronyssinus will be discussed more fully in Chapter III. The results of these preliminary studies suggested that the response was very similar to the ab response to Rye I in serum from patients with hay fever (Platts-Mills et al, 1978).

C H A P T E R I I

Purification and characterization of the major allergen from *D. pteronyssinus* - Antigen P_1.

2.1 Introduction

Although the F_4P_1 preparation appeared to contain the most potent D.pteronyssinus allergens it was clearly not fully purified. Studies on cellular and genetic mechanisms in allergy and on allergen exposure and standardization are dependent upon the use of allergens which have been purified to homogeneity (>99.5% pure). The isolation of these "ultra-pure" allergens is essential in order to exclude immune responses to highly allergenic trace impurities (Marsh et al, 1980). This chapter describes further studies on the immunological and physico-chemical properties of the allergens present in F_4P_1 which facilitated the purification of the major allergen of D.pteronyssinus - antigen P_1.

2.2 Methods

The protein composition of F_4P_1 was investigated by analytical IEF and 'immunoisoelectricfocusing'. The components were then separated by preparative IEF and the antigenic activity of the isolated fractions was detected by inhibition radioimmunoassay and by immunodiffusion using rabbit anti-ExI. Most of the antigenic activity was distributed in two protein peaks, designated Fr.5 and Fr.14. These fractions were compared with ExI and an extract of the D.pteronyssinus culture medium by immunodiffusion. Each fraction was also radiolabelled and compared with the other by cross-inhibition of radioimmunoassay. The allergenic potency of Fr.5 and Fr.14 was compared with ExI by intradermal skin testing. The physico-chemical properties of the two fractions were determined from the IEF studies, amino acid and carbohydrate analyses and SDS polyacrylamide gel electrophoresis.

Antigen P_1 was defined on the basis of the immunological and physico-chemical criteria described above. The importance of antigen P_1 as an allergen of D.pteronyssinus was assessed using RAST absorption (Appendix A.4.2) to estimate the amount of allergen-specific IgE in sera from mite-allergic individuals.

Further details of the methods used in this chapter are given in Appendix B.

2.3 Results

2.3.1 Purified allergens from isoelectricfocusing

Although F_4P_1 showed only one major band on Laurell electrophoresis it showed multiple bands on analytical IEF over a wide pI range (pI 4-7, see Fig. 2.3). This suggested either that the preparation contained other proteins which could be separated by IEF, or that the allergen was present in multiple isoelectric forms. The latter possibility was supported by the results of 'immunoisoelectricfocusing'. When the IEF separated allergens were electrophoresed into agarose containing rabbit antiserum to ExI, a single line of antigenic identity with no spurs was demonstrated across the IEF spectrum (Fig. 2.1). There appeared to be three peaks of antigenic activity which showed complete cross-reactivity. Preparative IEF was used to obtain larger quantities of allergens for further immunochemical analysis. The antigenic activity of the preparative IEF fractions eluted almost in parallel with protein and was predominantly distributed in two peaks, fraction 5 (Fr.5) and Fr.14, with pI's of 4.7-5.4 and 6.6-7.1 respectively (Fig. 2.2). There was also a smaller intermediary peak, Fr.10, with pI 5.8-6.2. The separation of Fr.5, Fr.10 and Fr.14 was checked

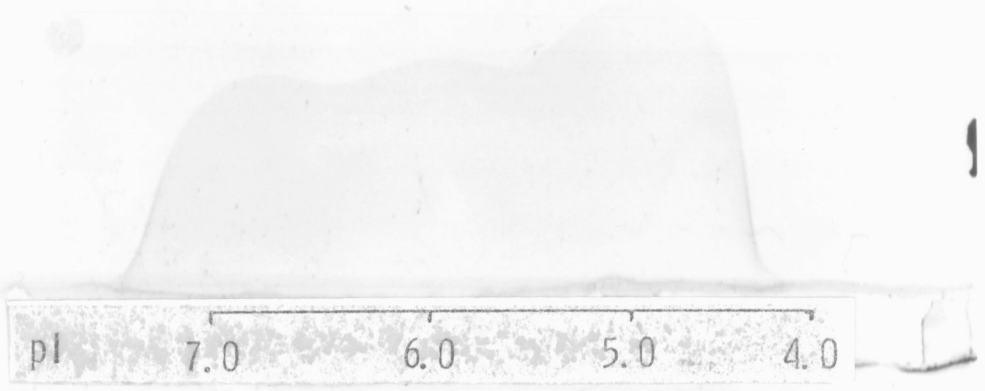


Figure 2.1

'Immunoisoelectricfocusing' of F_4P_1. Approximate pI values are shown.

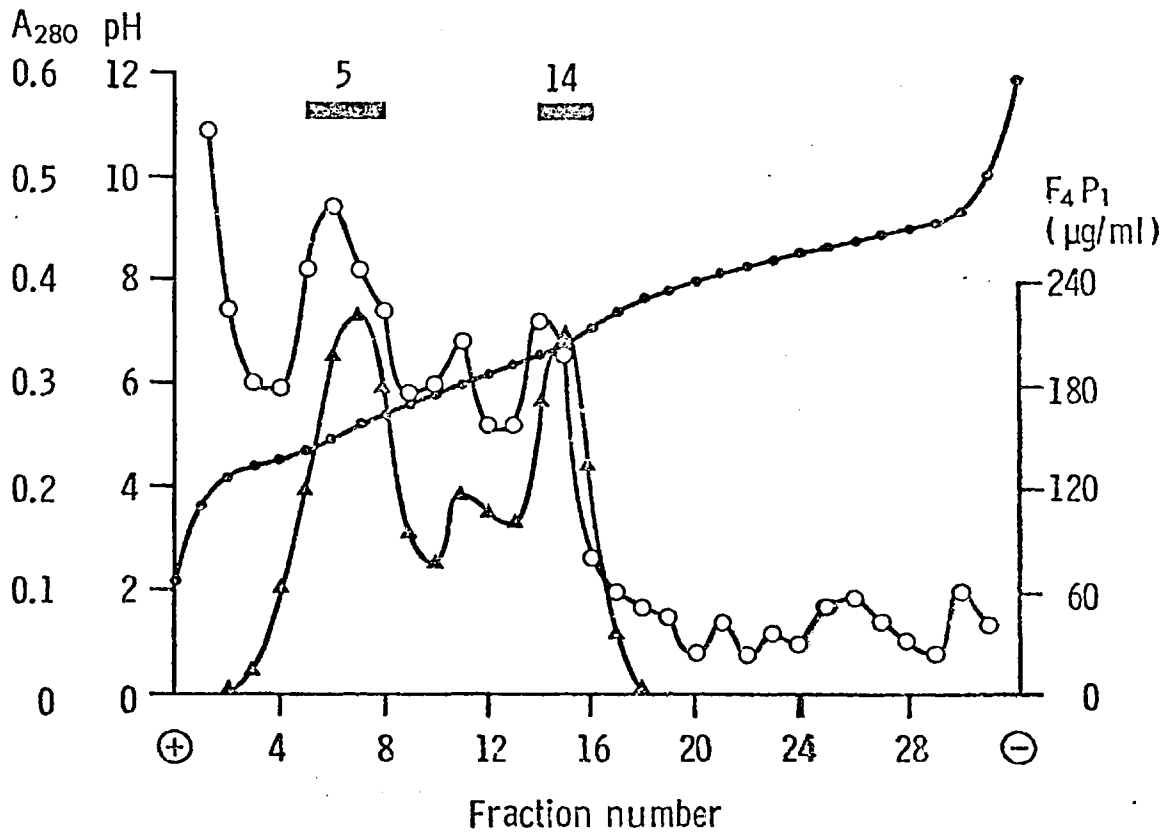


Figure 2.2

Preparative isoelectricfocusing of F_4P_1. The pH (o——o), protein content (absorbance at 280nm) (o——o) and F_4P_1 content (Δ——Δ) of each fraction is shown. The quantity of F_4P_1 was determined by inhibition radioimmunoassay using immunosorbent purified rabbit antibody to ExI. Fr.5 and Fr.14 were pooled as indicated (▭).

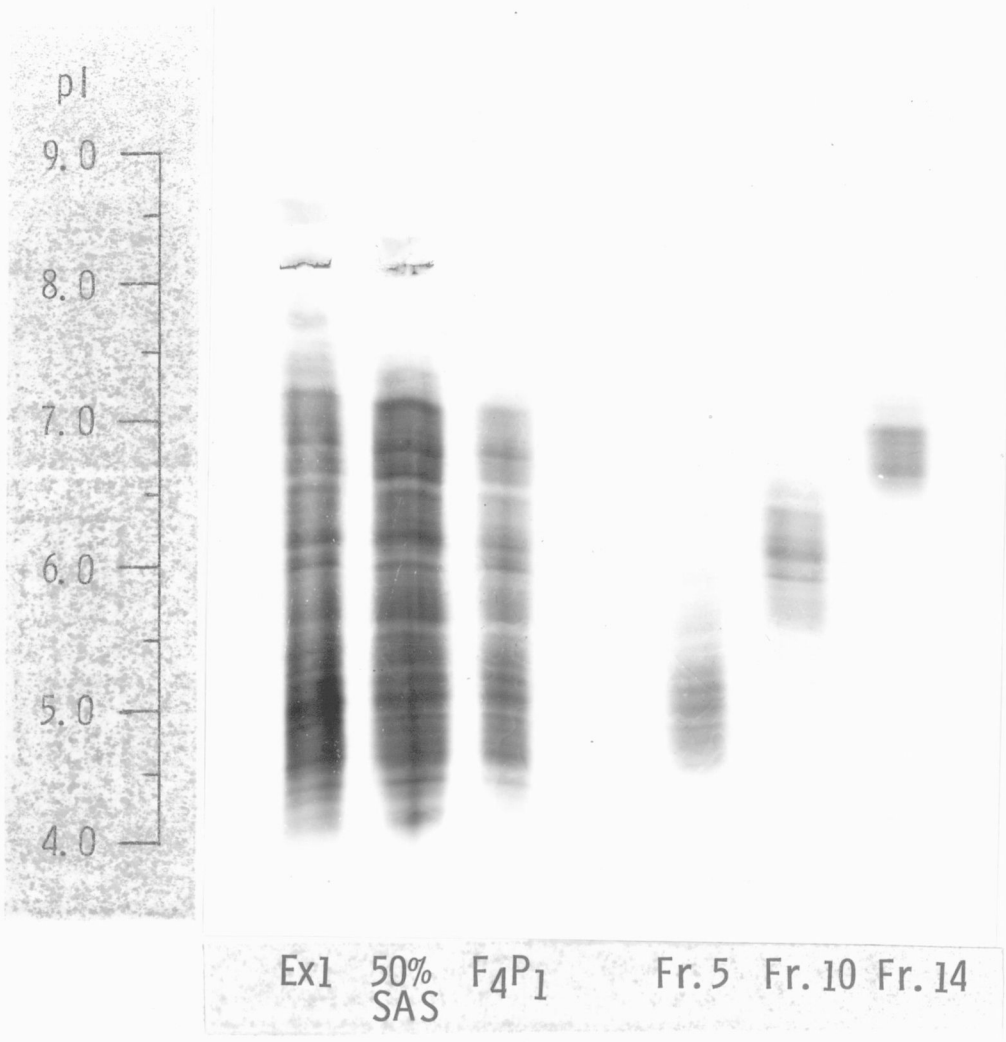


Figure 2.3

Analytical isoelectricfocusing of D.pteronyssinus allergens.

by analytical IEF in polyacrylamide gel (Fig. 2.3). The two major fractions, Fr.5 and Fr.14 were completely separated from one another though each fraction showed more than one band. Also shown are the IEF spectra of F_4P_1, ExI and a preparation of ExI which precipitated with 50% saturated ammonium sulphate. In spite of the considerable antigenic differences between F_4P_1 and ExI there were very few differences between these preparations on IEF.

2.3.2. Immunological properties of IEF purified allergens

The pooled fractions, Fr.5, Fr.10 and Fr.14, were tested on immunodiffusion against rabbit antiserum to ExI (anti-ExI). Each fraction showed a single line of complete identity with the other fractions (Fig. 2.4). This line was identical with the major line against ExI (Fig. 2.5a) but did not cross-react with highly concentrated extract of the D.pteronyssinus culture medium which also showed a single immunodiffusion line (Fig. 2.5b). The culture medium line showed complete identity with one of the lines against ExI (Fig. 2.5c). The IEF fractions were also compared using cross-inhibition of radioimmunoassay. Dilutions of cold Fr.5 or Fr.14 were used to inhibit the binding of ^{125}I-labelled Fr.5 or Fr.14 by either IgG or IgE ab from a mite-allergic person. The inhibition curves were very similar using either the homologous or the heterologous antigen as inhibitor (Fig. 2.6). In both cases binding could be inhibited by up to 94% using the opposite fraction. These findings were confirmed by RAST absorption using Fr.5 coupled to sepharose beads. Overnight incubation with this immunosorbent completely

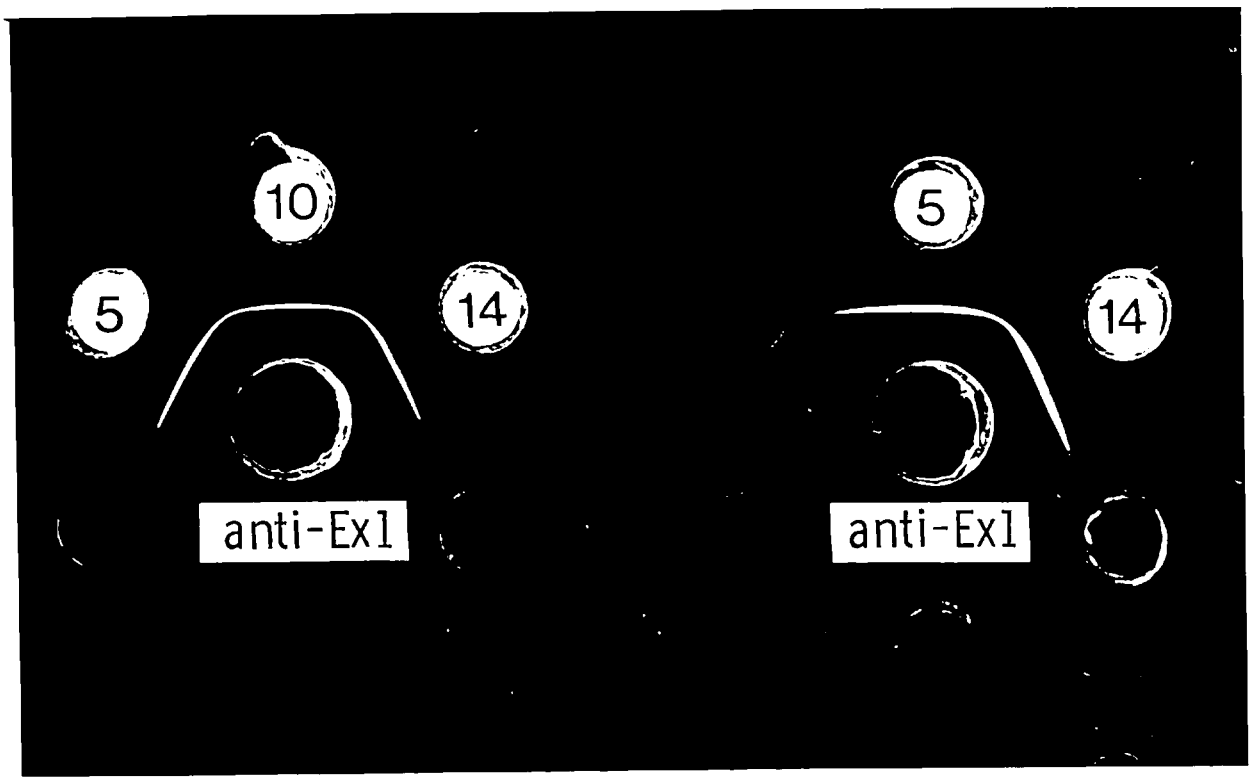


Figure 2.4

Immunodiffusion analysis I. IEF fractions of F_4P_1. Fr.5, Fr.10 and Fr.14 were tested against rabbit antisera to whole D.pteronyssinus culture (anti-ExI).

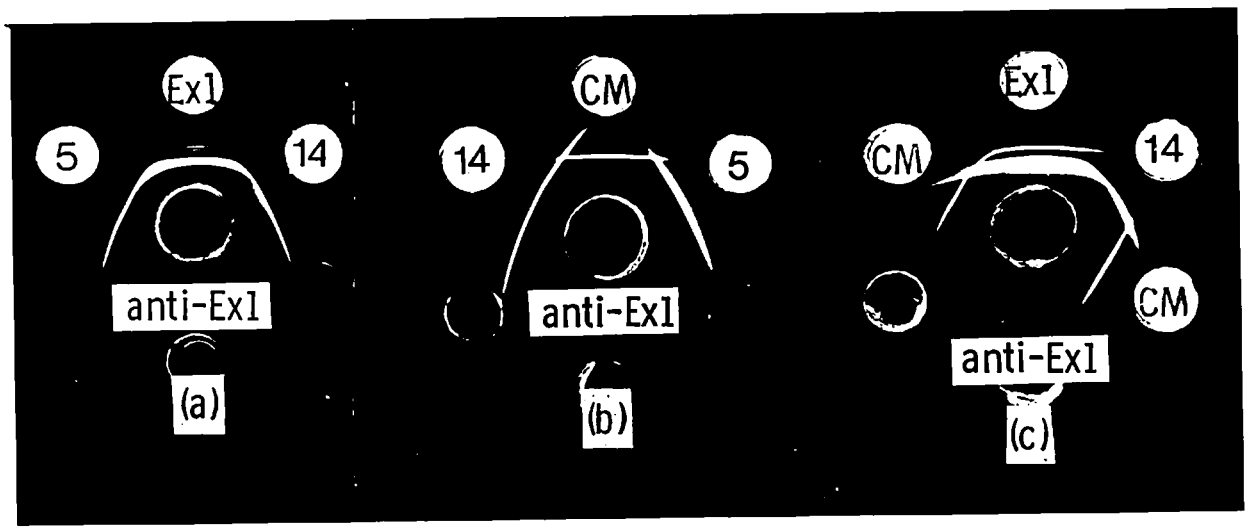


Figure 2.5

Immunodiffusion analysis II. Comparison of IEF Fr.5 and Fr.14 with ExI and an extract of the culture medium (CM). All fractions were tested against rabbit anti-ExI.

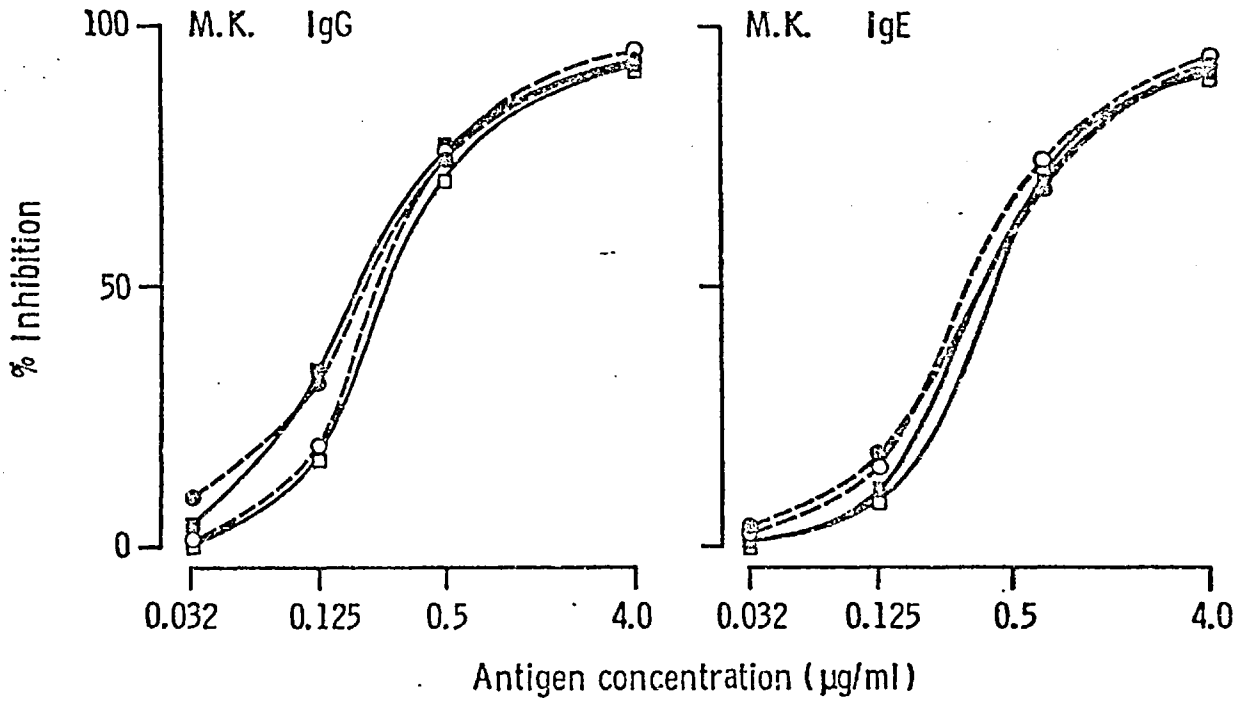


Figure 2.6

Cross-inhibition using IEF purified fractions. Binding by human IgG or IgE antibody to ^{125}I-labelled Fr.5 (circles, dotted line) or ^{125}I-labelled Fr.14 (squares, solid line) was inhibited using either the homologous (closed symbols) or heterologous antigen (open symbols). Human antibodies were precipitated using a second antibody; goat anti-IgG or goat anti-IgE.

removed (>95%) the binding activity for radiolabelled Fr.14 from all ten sera from allergic individuals tested (data not shown).

In keeping with the view that Fr.5 and Fr.14 were antigenically identical, both fractions gave similar skin test responses in allergic individuals. Quantitative intradermal skin tests on eleven skin test positive individuals and four non-allergic controls showed a good correlation between sensitivity to each purified fraction and sensitivity to ExI (Table 2.1). With Fr.5 two individuals gave positive (++) reactions using 10^{-5}µg/ml of purified allergen (i.e. ~ 5pg). One subject (N.J.) who was only weakly sensitive to mites gave a ++ skin response to a 100-fold higher dilution of ExI than of the purified fractions, suggesting that he was probably reacting to different allergens in the crude mite extract.

On the basis of all these immunological criteria Fr.5 and Fr.14 were considered to share identical antigenic determinants.

2.3.3 Physico-chemical characterization of IEF purified allergens

Both Fr.5 and Fr.14 showed the typical absorption spectra of proteins in the ultraviolet spectrophotometer with absorption maxima at 276-278nm. The amino acid composition of each fraction was very similar apart from slight differences in the numbers of glycine and valine residues (Table 2.2). The difference in amino acid content was estimated using the SΔQ system for relatedness amongst proteins (Marchalonis and Weltman, 1971). This system involves summing the squares of the differences in amino acids (expressed as residues/100

TABLE 2.1

Intradermal skin tests with D.pteronyssinus allergens

Minimum concentration of allergen giving a positive skin test (µg/ml).[a]

Subject	Extract I	Fr.5	Fr.14
W.S.	10^{-1}	10^{0}	10^{0}
T.S.	10^{-4}	10^{-4}	$10^{-3.5}$
N.D.	10^{-5}	10^{-5}	10^{-4}
A.J.	10^{-4}	$10^{-4.5}$	10^{-4}
M.C.	10^{1}	10^{0}	10^{1}
N.J.	10^{-2}	10^{0}	10^{0}
M.K.	10^{-5}	10^{-5}	$10^{-4.5}$
J.S.	$10^{-2.5}$	$10^{-2.5}$	10^{-2}
E.T.	10^{-3}	$10^{-3.5}$	10^{-3}
S.J.	$10^{-3.5}$	10^{-4}	10^{-3}
R.B.	$10^{-3.5}$	10^{-4}	$10^{-3.5}$
Controls x 4	$>10^{1}$	$>10^{1}$	$>10^{1}$

(a) Intradermal skin tests were carried out using 0.05ml of serial tenfold dilutions of allergens. A wheal of >6 x 6mm was regarded as a positive (++) response. Values were extrapolated to the nearest ½ log when the lower dilution gave a strongly positive reaction. 10^{0} = 1µg/ml. Negative skin tests at 10µg/ml are recorded as $>10^{1}$.

TABLE 2.2

Amino acid analysis of Fr.5 and Fr.14[a]

	Residues per 100 residues of amino-acids		Residues per molecule[b]
	Fr.5	Fr.14	
Asx	14.3	13.9	24
Thr	4.6	4.4	8
Ser	7.6	7.8	13
Glx	12.2	12.9	21
Pro	4.9	5.2	9
Gly	11.6	10.3	20
Ala	9.5	9.3	17
½ Cys	2.0	1.0	4
Val	4.2	5.2	7
Met	1.2	1.2	2
Ileu	6.3	6.4	11
Leu	4.3	4.4	8
Tyr	5.2	5.4	9
Phe	2.3	2.2	4
His	2.4	2.6	4
Lys	2.1	2.1	4
Arg	5.4	5.6	10

(a) Twenty micrograms of Fr.5 and Fr.14 were hydrolysed in vacuo with 1 ml 6N HCl for 24 hours at 110°C and applied to a Beckman Model 120C amino acid analyser. I am grateful to Dr. A.S.R. Donald for performing the amino-acid analyses.

(b) Calculations of the numbers of amino-acids per molecule were made for Fr.5, assuming a MW of 24,000 daltons, a carbohydrate content of 8.4% and that no other components were present. Values are given to the nearest integer.

residues of amino acids) of the two proteins being compared to obtain an 'SΔQ' value. The larger this value the greater the difference in amino acid content. Values of >100 units usually indicate completely non-identical proteins. Using the SΔQ system Fr.5 and Fr.14 differed by <4 units, indicating that they were "identical proteins". In contrast, when Fr.5 was compared with the published amino acid analysis of the purified pollen allergen Rye I (see Marsh, 1975) it differed by 140 units. Preliminary analyses using gas chromatography showed that Fr.5 and Fr.14 were glycoproteins containing galactose, mannose, N-acetylglucosamine and N-acetylgalactosamine (Dr. E.F. Hounsell - personal communication). Fucose and sialic acid were not detected in either fraction. The carbohydrate comprised roughly 8% and 5% of the total protein in Fr.5 and Fr.14 respectively.

On SDS polyacrylamide gel electrophoresis (SDS-PAGE) each fraction migrated as a single homogeneous band with an apparent molecular weight of 24,000 daltons (Fig. 2.7). The fractions migrated to the same position as the most heavily staining band in F_4P_1, ExI and the 50% SAS fraction of ExI. This suggested that the glycoprotein present in Fr.5 and Fr.14 was a major component of crude mite extract. Since Fr.5 and Fr.14 could not be distinguished immunologically, by amino-acid analysis or SDS-PAGE, it seemed likely that they represented minor structural modifications of a single glycoprotein. The common antigen present in Fr.5 and Fr.14 was designated Dermatophagoides pteronyssinus antigen P_1.

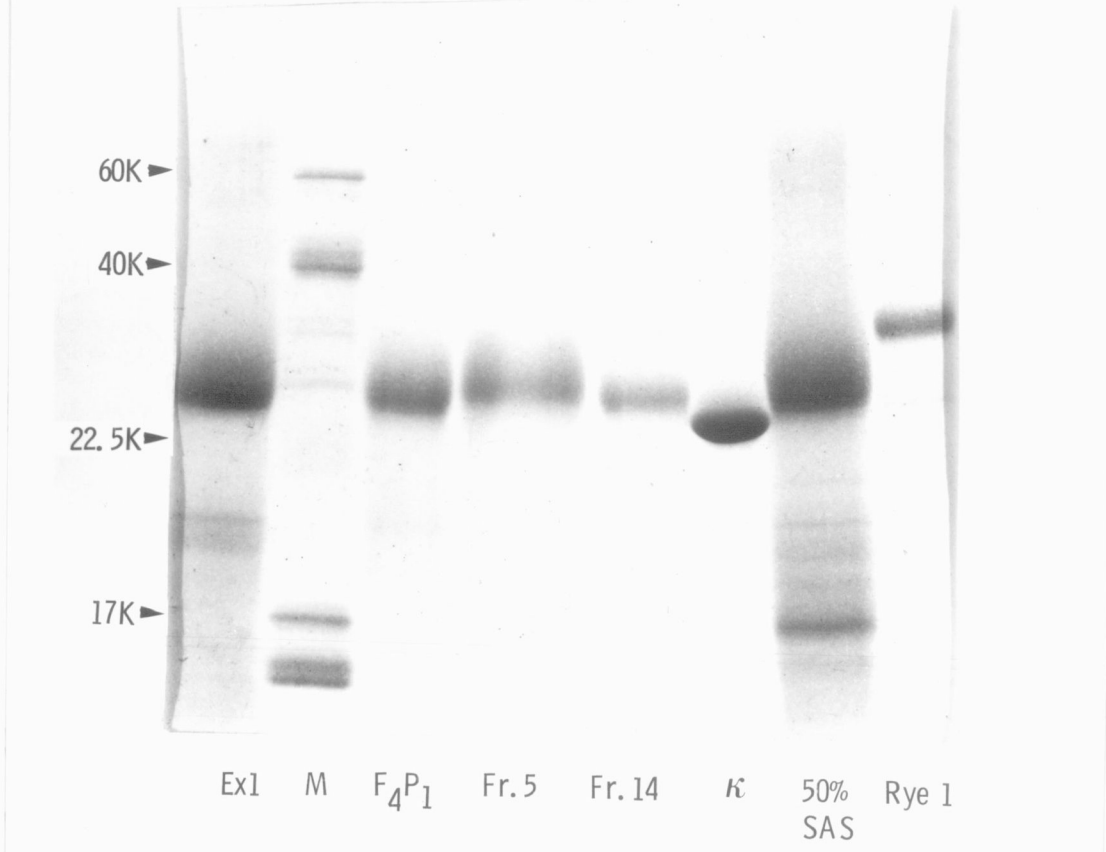


Figure 2.7

Analysis of D.pteronyssinus antigens by SDS-PAGE. The migration of several marker proteins was compared with Fr.5, Fr.14, F_4P_1 and ExI. M = marker solution containing catalase (MW 60,000), peroxidase (MW 40,000) myoglobin (MW 17,000), haemoglobin (MW 15,000) and cytochrome 'C' (MW 13,000). κ = purified kappa light chain (human Bence-Jones protein) (MW 22,500). 50% SAS = an enriched preparation of ExI which precipitated in the presence of 50% saturated ammonium sulphate. Rye I = the group I protein of rye grass pollen.

The stability of antigen P_1 was assessed by incubating small quantities (5-50µg) of ExI or F_4P_1 for a given time at different temperatures or in buffers at different pH. The P_1 content was then assayed by inhibition radioimmunoassay (Appendix B.2). The results showed that antigen P_1 was stable in aqueous solution at temperatures up to 37°C, lost 50% of its antigenicity after one hour at 56°C and was completely denatured at 80°C (Table 2.3). Following prolonged pH treatment (16 hours) the antigen remained stable at pH6-8, lost 50% of its antigenicity at pH9.4, 75% at pH4.0 and was denatured at pH2.0 or 12.0.

2.3.4 IgE antibodies to antigen P_1 in allergic individuals

The results in Table 2.1 showed that skin test reactivity to purified preparations of antigen P_1 correlated with reactivity to crude mite extract in seven symptomatic, mite allergic subjects who were highly sensitive to ExI (++ reaction with $<10^{-3}$µg/ml). In serum there was a very good quantitative correlation between IgE ab to P_1 measured by antigen binding assay and IgE ab to D.pteronyssinus measured by RAST ($r = 0.82$, $p<0.001$, $n = 30$). The quantities of IgE ab to ExI and to P_1 were directly compared by RAST absorption using six sera from mite allergic individuals (Table 2.4). In these samples IgE ab to ExI and to P_1 formed from 15-36% and 9-21% of the total IgE respectively. A much higher percentage of the total IgE was directed against each allergen in two preparations of immunosorbent purified anti-mite ab (from patients MK and DB). In all cases IgE ab to P_1 accounted for a large proportion (up to 72%) of the IgE ab to ExI. The quantities of IgE BA in unabsorbed serum

TABLE 2.3

Effect of 1 hours heat and 16 hours at different pH on the stability of antigen P_1*

(a) Antigen P_1 (µg) after 1 hour at various temperatures

Temperature (°C)	20°	37°	56°	80°	100°	Autoclave
ExI	4.8	5.1	2.6	<0.05	<0.05	<0.05
F_4P_1	26.3	28.2	14.5	0.7	<0.05	<0.05

(b) Antigen P_1 (µg) after 16 hours incubation in buffers at different pH.

pH	2.0	4.0	6.0	8.0	9.4	12.0
ExI	<0.8	2.3	11.7	10.0	6.7	<0.5
F_4P_1	<0.05	16.0	56.3	64.0	33.5	<0.5

*The effect of both parameters on the quantities of antigen P_1 in ExI or F_4P_1 was assessed by inhibition radioimmunoassay using a standard preparation of antigen P_1 (IEF Fr.14) (Appendix B.2). All assays were performed using two dilutions of allergen solutions. Interassay variation was ~ 10%. Results are the mean values of two experiments.

(a) Samples were incubated for one hour in 0.1ml BBS (pH 8.0) at the temperatures shown.

(b) Samples were incubated in 0.1ml of 0.1M buffers at the pH indicated. The pH was neutralized with 0.1ml 0.3M phosphate buffer pH 7.7 prior to assay.

TABLE 2.4

Estimation of the absolute quantities of IgE ab to Mite Allergens

Patient	Sample	(a) IgE BA for P_1 units/ml	(b) Total IgE units/ml	(c) % Total IgE directed against Ex 1	(c) % Total IgE directed against P_1	P_1/Ex 1 %	(d) Estimation of 1 unit of IgE BA (ng)
S.B.	serum	204	708	36	16	44	1.33
P.P.	serum	576	1,116	30	15	50	0.70
T.W.	serum	99	180	29	21	72	0.92
D.J.	serum	180	816	18	9	50	0.98
M.K.	serum	234	606	15	10	67	0.62
	Sp.Ab(e)	18,000	19,900	61	40	65	1.06
D.B.	serum	852	12,480	12	6	50	2.11
	Sp.Ab(e)	28,000	51,840	50	30	60	1.33

(a) Measured on unabsorbed samples by antigen binding radioimmunoassay.

(b) Measured on unabsorbed samples by double antibody inhibition radioimmunoassay. 1 unit of IgE was assumed to be equivalent to 2.4ng (Bazaral and Hamburger, 1972).

(c) The results, expressed as a percentage of the total IgE, are the quantity of IgE absorbed by Ex I or P_1 immunosorbent minus the IgE absorbed by an HSA immunosorbent.

(d) The quantity of IgE represented by 1 unit of IgE BA was calculated as follows:-

$$1 \text{ unit} = \frac{\text{Total IgE (units/ml)} \times 2.4 \times \%\text{ total IgE directed against } P_1}{\text{IgE BA for } P_1 \text{ (units/ml)}} \text{ ng}$$

(e) Immunosorbent purified specific antibody to Ex I.

samples were compared with the quantity of IgE absorbed by RAST beads coated with P_1 and used to make an estimate of the amount of IgE represented by one unit of IgE BA (Table 2.4). These calculations gave a mean value of 1.13 ± 0.44ng IgE ab/unit IgE BA. When this value was applied to the previous measurements of IgE BA on sera used in Chapter I (including 20 other sera from skin test positive individuals) IgE ab to P_1 accounted for 8.4 ± 11.5% (n = 54) of the total IgE. Calculations on sera from two other groups of mite allergic individuals (see Chapter III) showed that IgE ab to P_1 accounted for 10.8 ± 7.4% (n = 60) and 17.4 ± 12.1% (n = 44) of the total IgE. The mean value from all these studies (12%) is very similar to those reported using purified major allergens from pollens (Zeiss et al, 1973; Schellenburg and Adkinson, 1975; Platts-Mills et al, 1978).

2.3.5 Further analyses of the components of D.pteronyssinus culture

A modified scheme for the preparation of antigens and allergens from D.pteronyssinus culture is given in Fig. 2.8. Almost all the antigen P_1 (>90% by radioimmunoassay) was recovered by fractionation of ExI with 50% saturated ammonium sulphate (SAS). Gel filtration of the re-dissolved 50% SAS precipitate resulted in a sharp peak of high molecular weight material ('HMW') (>100,000 daltons) and a very broad peak of lower molecular weight material (5-50,000 daltons). Fractions comprising the 'HMW' peak were extremely turbid and showed a much higher absorbance at 280nm than the lower molecular weight fractions. The 'HMW' fraction gave two lines on immunodiffusion against rabbit anti-ExI which did not cross-react with the line against antigen P_1 (data not shown). Fractions with MW 15-25,000 daltons were further

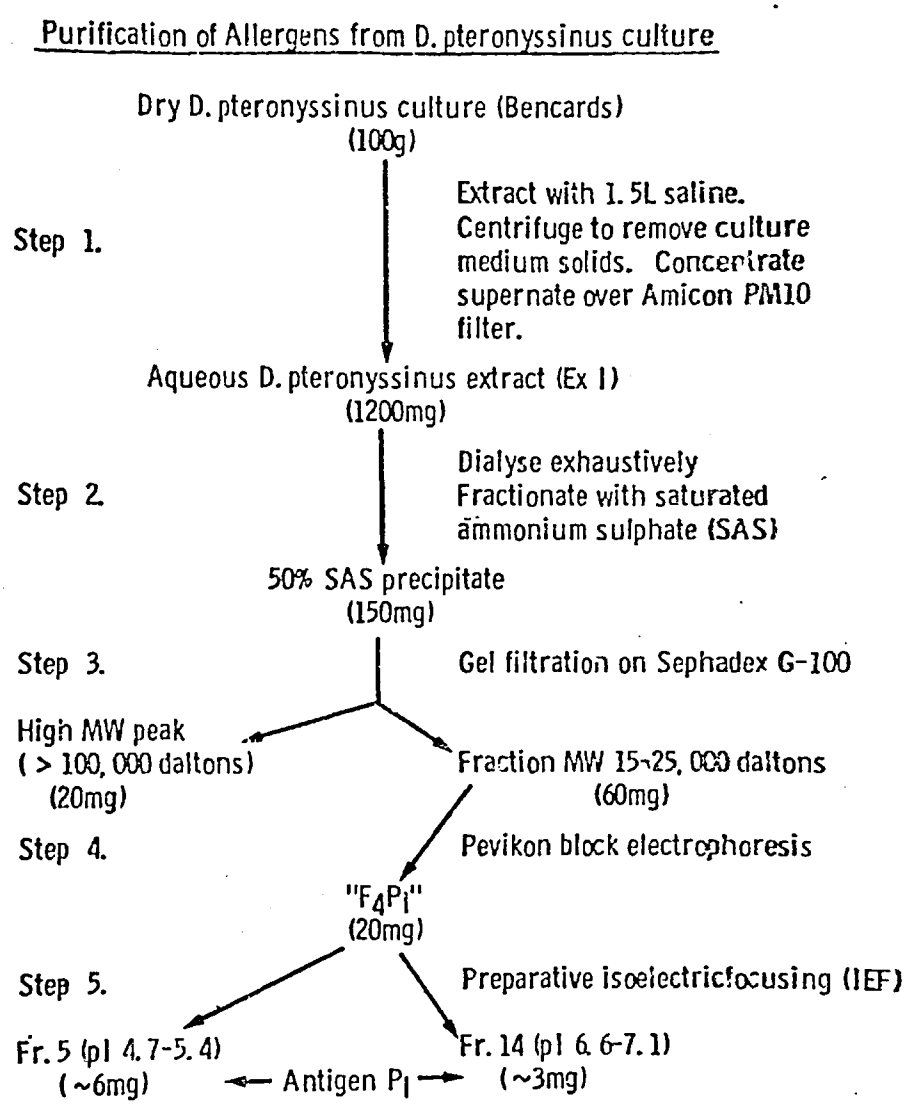


Figure 2.8

Modified procedure for the purification of allergens from D. pteronyssinus culture. Approximate yields of protein are given in parentheses. Values were determined using a trichloroacetic acid modification of the Lowry technique (Bensadoun and Weinstein, 1976).

purified by pevikon block electrophoresis. By this stage the bulk of the highly coloured material had been removed and most of the protein migrated with β-mobility as F_4P_1.

Using the modified purification procedure the yield of antigen P_1 ($\sim$ 10mg) from the total protein extracted from mite culture (1200mg) was about 1% (Fig. 2.8), i.e. very similar to the yield of purified allergens from pollen extracts (see Marsh, 1975). A yield of 6% was obtained from the 50% SAS precipitate of ExI. While these yields may not appear to be very high, in most cases the yield was sacrificed in order to obtain fractions with high antigenic purity.

Fractions isolated using the modified purification procedure were analysed by SDS-PAGE (Fig. 2.9). The samples were treated with SDS alone and boiled for 2 minutes before being applied to the gel. This resulted in more clearly defined bands, particularly for ExI and the 50% SAS fraction. The 'HMW' fraction, somewhat surprisingly, showed a major band at 17,200 daltons and three other bands below 20,000 daltons but very little higher molecular weight material. Both ExI and the 50% SAS fraction showed a densely staining band at 28,500 daltons which was not seen on the previous gel (Fig. 2.7). An extract of the D.pteronyssinus culture medium showed one major band, MW $\sim$ 55,000 daltons.

On immunodiffusion it was possible to identify most of the antigenic components of ExI; 2 lines derived from the 'HMW' fraction,

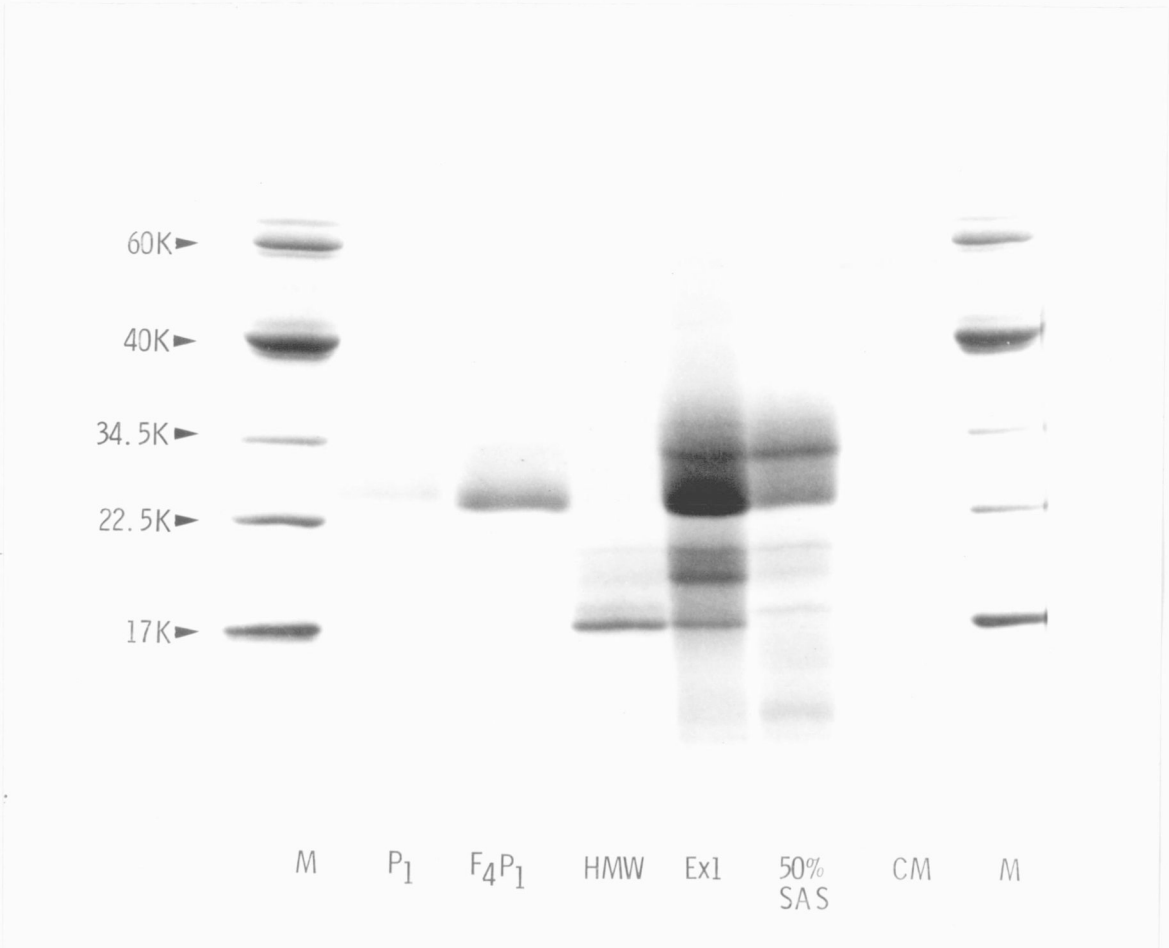


Figure 2.9

Further analysis of D.pteronyssinus allergens on SDS-PAGE. M = marker solution containing catalase, peroxidase, κ light chain and myoglobin. HMW = 'high molecular weight' fraction (>100,000 daltons on gel filtration). CM = highly concentrated culture medium extract.

2-3 lines from F_4P_1 and a single line from the culture medium component. The antigens present in D.pteronyssinus extracts have been analysed by Drs. H. Lowenstein and P. Lind in Copenhagen using crossed immunoelectrophoresis (CIE). These workers kindly offered to analyse F_4P_1 and antigen P_1 in their CIE reference system. Their results (Table 2.5) showed that antigen P_1 corresponded to antigen Dp.42 which they considered to be the major IgE binding protein in crossed radioimmunoelectrophoresis (CRIE). Antigen Dp.42 was the only allergenic component identified in either preparation. In a quantitative CIE analysis 1µg F_4P_1 and 1µg antigen P_1 contained an amount of Dp.42 corresponding to 39µg and 19µg of whole D.pteronyssinus culture extract respectively.

2.4 Discussion

Several lines of evidence suggested that antigen P_1 was the major allergen from D.pteronyssinus. There was a very good quantitative correlation both in vivo and in vitro between IgE ab to antigen P_1 and to ExI. Positive skin responses could be induced with as little as 5pg of the purified allergens (Table 2.1) and more than 90% of individuals who gave positive prick tests to D.pteronyssinus had detectable serum IgE ab to antigen P_1. The RAST absorption experiments showed that up to three-quarters of the mite-specific ab was directed against antigen P_1. These results were further supported by the independent observation that antigen P_1 was the major IgE binding protein in CRIE. Although it was clear that antigen P_1 was the major D.pteronyssinus allergen, there was some evidence that this antigen did not account for the total allergenic activity of mite extract. For example, one

TABLE 2.5

Analysis of F_4P_1 and antigen P_1 by crossed immunoelectrophoresis

Allergen	Number of antigens found	Classification*
F_4P_1	5	Dp-42, Dp-30
P_1	2	Dp-42

*Based on a standard D.pteronyssinus extract (Dp 0475) which gave 49 immunoprecipitates against rabbit antibodies to whole D.pteronyssinus culture.

subject was 100-fold more sensitive to ExI on skin testing (Table 2.1) and in some sera a somewhat lower proportion (<50%) of the IgE ab was specific for antigen P_1 (Table 2.4). This evidence suggested that there are minor D.pteronyssinus allergens which also elicit the formation of IgE ab.

The SDS-PAGE analyses showed that antigen P_1 was present on a single polypeptide chain, MW 24,000 daltons. Stewart and Turner (1980a) reported a similar molecular weight for the most potent D.pteronyssinus allergen (26,000 daltons), however, this value is slightly higher than would be expected from the results of gel filtration (7,500-20,000 daltons) (Chapter I, see also Allen et al, 1969; Kabasawa and Ishii, 1979). In preliminary sedimentation velocity experiments antigen P_1 showed a continuous boundary between 0.5-2S (data not shown). These S values are compatible with the gel filtration data and suggest that the molecular weight of antigen P_1 is slightly over-estimated on SDS-PAGE. Several factors are known to affect the mobility of proteins on SDS-PAGE, e.g. protein charge, degree of glycosylation and number of disulphide bonds (Payne, 1976). Because antigen P_1 is present on a glycoprotein it probably binds fewer SDS molecules and therefore has a reduced electrophoretic mobility.

In a previous study Romagnani et al (1976) demonstrated extensive cross-reactivity between IEF fractions of crude mite extract by RAST inhibition and concluded that the extract contained "one major allergen existing in multiple molecular forms". The results with

antigen P_1 certainly confirm those original observations. Antigen P_1 occurs in at least two isoallergenic forms which exhibit markedly different isoelectric points (pI 4.7-5.4 and 6.6-7.1). The presence of a common antigenic determinant on isoelectric variants of the same protein is not unusual, and has been reported previously for several allergens including Rye I (Johnson and Marsh, 1965b), Cladosporium herbarum allergen 32 (Aakrust and Borch, 1979) and hazel pollen allergens HI and HII (Borch, Skalhegg and Sletten, 1980). In all these cases the pI values of the isoallergens differed by <1 pI unit. Wide variations in pI occur in some molecules such as the immunoglobulins (Awdeh, Williamson and Askonas, 1968) and L amino-acid oxidase (Wellner and Hayes, 1973). However, these are larger molecules with a relatively complex sub-unit structure. The wide-spread distribution of the isoallergens of antigen P_1 seems remarkable for a molecule which has a low molecular weight and is homogeneous on SDS-PAGE. The heterogeneity of the two isoallergens, Fr.5 and Fr.14, on IEF (Fig. 2.3) could be explained in part by the differences in their carbohydrate content, though differences in amide content and conformational changes may also be involved (Williamson, Salaman and Kreth, 1973; Righetti and Drysdale, 1976). At present, it is not possible to say whether the glycoprotein bearing antigen P_1 is synthesized in a heterogeneous form, or whether the IEF changes occur post-synthetically, in the mites' digestive tract; or as a consequence of prolonged high-density culture; or during the preliminary extraction procedures employed by the manufacturer. However, it is clear from the present data that analytical IEF would not be a useful technique for standardizing mite extracts.

The finding that antigen P_1 was susceptible to heating for one hour at 56°C was surprising, since in the past mite cultures were routinely treated for six hours at 60°C to kill the live mites (Voorhorst and Spieksma, 1969) and mite allergens have been reported to be heat stable (Ricci, Biliotti and Romagnani, 1975; Nakagawa et al, 1977). At present, there is no clear explanation for these findings. It is possible that 'dry' mite cultures are less susceptible to heat than the soluble allergen. Equally, a reduction of 50% in the allergenicity of mite culture would probably not have been detected previously by skin testing.

One of the aims of studying purified allergens is to understand the mechanism by which these molecules elicit IgE ab formation. It has been proposed that allergens require a divalent structure to cross-link surface receptors on mast cells (Stanworth, 1973) and also that there are chemically-defined allergenic determinants (N-glycosidically linked protein-sugar residues) (Berrens 1970, 1974). Although these factors may make some contribution towards biological activity they are clearly not essential for allergenic activity. Thus both low molecular weight, monovalent allergens, e.g. ragweed Ra5 and allergens which lack carbohydrate, e.g. allergen M have been described (Lapkoff and Goodfriend, 1974; Elsayed and Aas, 1970). Most major allergens derived from pollens, codfish, animal danders and vespid venoms are proteins or glycoproteins with MW 10-40,000 daltons. They usually comprise a high proportion of the extractable protein in crude allergen extracts and are all rapidly soluble in aqueous solution (Stanworth, 1957; King, 1979; Borch, Skalhegg and

Sletten, 1980). Antigen P_1 has very similar physical properties to the other major allergens. It forms the most predominant component on immunodiffusion (Fig. 2.5) and SDS-PAGE (Figs. 2.7 and 2.9) and radioimmunoassay results suggest that antigen P_1 comprises 10-20% of the protein in crude mite extract. Recent work has shown that antigen P_1 elutes rapidly (>90% within 2 minutes) from mite cultures and samples of house dust (Tovey, Chapman and Platts-Mills - manuscript in preparation). There seems to be a relationship between these simple, physical properties and allergenic activity. Tracer experiments in animals suggest that antigens with a molecular weight less than 50,000 daltons would readily penetrate mucosal surfaces, whereas larger molecules would be excluded (Schneeburger and Karnovsky, 1971). Smaller molecules (<10,000 daltons) have fewer antigenic determinants and would be expected to be less immunogenic (Crumpton, 1975). In addition, these molecules would probably pass through capillary membranes and be rapidly diluted in the bloodstream in vivo (Yoffey and Courtice, 1970). Thus major allergens can penetrate mucosal surfaces, are rapidly soluble in extracellular fluids and are present in sufficient quantities to cause immediate symptoms. It has been reported that the high molecular weight fractions of mite extract (>274,000 daltons) contain lectin-binding allergens which are active in RAST and CRIE (Baldo and Uhlenbruck, 1977; Stewart and Turner, 1980b). According to the above criteria these fractions would not be expected to be allergenic. However, the SDS-PAGE analyses (Fig. 2.9) indicate that the high molecular weight material dissociates into several sub-units, MW <20,000 daltons, with a major band at 17,200 daltons. These sub-units could be the active moiety of the high molecular weight allergen.

C H A P T E R I I I

The antibody response to *D. pteronyssinus*

3.1 Introduction

Under conditions of natural exposure, antibodies to inhalant allergens, e.g. pollen grains, are induced by minute doses (ng) of allergen entering via the nasal mucosa. This type of exposure generates a response which, in most patients with hay fever, includes IgG, IgA and IgE ab (Yagi et al, 1963; Ishizaka, Ishizaka and Hornbrook, 1967; Platts-Mills et al, 1978). Once induced, the response is not thought to be affected by drugs used to alleviate allergic symptoms e.g. anti-histamines, topical steroids or di-sodium cromoglycate, and can be regarded as characteristic of essentially 'untreated' patients. The ab response may subsequently be changed following the 'systemic' administration of soluble allergen in a course of desensitization treatment (hyposensitization or immunotherapy). There are potential differences in the quantities and distribution of ab between 'untreated' and 'treated' patients. Therefore, each type of response should be considered separately. This chapter describes the ab response to D.pteronyssinus in both groups of patients.

Desensitization was first introduced by Noon (1911), with the rationale of raising neutralizing ab to "pollen toxin". The treatment was later used for a wide variety of allergens, including house dust. Early attempts to desensitize patients with asthma associated with immediate reactions to dust involved injections of high concentrations of dust extracts, often with quite dramatic (anaphylactic) side-effects (Spivacke and Grove, 1925). Nonetheless, in those uncontrolled studies, and othersinvolving a total dose of 5-20mg house dust (Maunsell, Whetnall and Rimington, 1947), there was a

marked improvement in patients' symptoms. More recently, there have been several trials of the efficacy of desensitization using dilute extracts of house dust, D.farinae or D.pteronyssinus. Treatment with house dust was ineffective in the short term (15 weeks) (British Tuberculosis Association, 1968) but caused symptomatic improvement and a reduction in bronchial sensitivity when continued for a 2-3 year period (Aas, 1971). Aqueous extracts of mite cultures were more effective than house dust (Maunsell, Wraith and Hughes, 1971) and the improvement in symptoms was, in some cases, accompanied by a reduction in either bronchial or nasal sensitivity (Smith, 1971; Assem and McAllen, 1973; D'Souza et al, 1973). Contradictory results were obtained with tyrosine adsorbed mite extract (Migen). In detailed studies Warner et al (1978) reported symptomatic improvement associated with a reduction in the use of bronchodilator drugs, and also a lower incidence of late reactions to bronchial challenge. However, other workers using the same treatment and similar methods of assessment found no improvement (Gaddie, Skinner and Palmer, 1976). It is difficult to make direct comparisons between any of these treatments because of differences in the selection of patients, treatment regimes and methods of assessment. At best, up to 80% of the treated patients were improved by desensitization.

Although desensitization has been practised for nearly 70 years the mechanism by which improvement occurs is poorly understood. It has been thought to be due to the production of IgG 'blocking' antibodies in the serum (Cooke et al, 1935; Loveless, 1940) which prevent the interaction between allergen and mast cell bound IgE ab. Increases

in IgG ab following desensitization have been demonstrated in the serum and nasal secretions of hay fever patients by antigen binding assay (Yunginger and Gleich, 1973; Platts-Mills et al, 1976; 1979) and by inhibition of histamine release *in vitro* (Lichtenstein, Norman and Winkenwerder, 1971; Levy et al, 1971; Lichtenstein et al, 1973). However, there have been very few studies on 'blocking' ab to mites because suitable assays were not available.

This chapter describes the use of the antigen binding assay for antigen P_1 to assess the ab response to *D.pteronyssinus* in patients from Hong Kong who took part in a double-blind, placebo-controlled trial of desensitization (Gabriel et al, 1977). Clinically, the *D.pteronyssinus* treated (DP) patients showed significant benefit during maintenance therapy when compared with the placebo-treated ('placebo') controls. The aim of the present studies was to study the relationship between improvement in symptoms and changes in ab production.

3.2 Methods

3.2.1 Patients

The 'London' patients presented with symptoms of rhinitis or asthma at Northwick Park Hospital Allergy Clinic. The majority had positive skin tests to *D.pteronyssinus*. Only one of these patients had received desensitization treatment. Nasal washings were collected as described by Platts-Mills et al (1976). 10ml BBS was instilled into each nostril and allowed to drain out. From 20-40ml washings were concentrated to 1-2ml using a collodion bag apparatus (UM 100, Schleicher and Schull, W. Germany) and stored at -20°C. Saliva was

collected without stimulation, by asking patients to salivate into a container without coughing and to avoid secretions from the back of the mouth. The samples were centrifuged and stored at -20°C.

The 'Hong Kong' patients were all Chinese, gave a three year history of seasonal or perennial rhinitis, and had positive skin tests and nasal challenge tests to D.pteronyssinus (Gabriel et al, 1977). Patients were stratified by age and randomly allocated to treatment with either graded doses of D.pteronyssinus extract (from 0.0004 to 0.2%, n = 29) or saline placebo (n = 29). The course consisted of 18-22 weekly injections followed by 12 monthly maintenance doses. Serum samples were taken before treatment, at the end of the weekly course (4 months) and after 12 months of treatment, i.e. about 6 months after starting the maintenance course. Patients recorded daily the severity of their symptoms of sneezing, obstruction, discharge and asthma on a four-point scale (nil, mild, moderate or severe). These values were used to calculate patients' symptom scored throughout treatment. Previously, Gabriel et al (1977) calculated symptom scores as the ratio of the number of moderate or severe symptom days to the total number of symptom days. In the present studies the symptom scores were modified from the original diary score cards as follows:-

$$\text{Symptom score} = \frac{\text{No. of moderate or severe symptom days} \times 4}{+ \text{ Total number of symptom days}}$$

The factor of 4 was introduced to give further weight to moderate or severe symptoms and to extend the range of possible scores. Thus a patient who experienced all three symptoms (sneezing, obstruction and discharge) on each of the 28 days assessed could achieve a maximum symptom score of (3 x 28 x 4) + (3 x 28) = 420.

The co-operation of Dr. L.E. Hill, Sister M. Gabriel, Dr. H.K. Ng and Dr. W.G.L. Allan in providing the sera and diary score cards from the Hong Kong patients is gratefully acknowledged. I would also like to thank Dr. A.J. Nunn for providing the original assessments of the score cards and for statistical advice.

3.2.2 Measurement of antibodies to D.pteronyssinus

IgG, IgA and IgE ab to D.pteronyssinus was measured by antigen binding assay using the partially purified allergen, F_4P_1. Assays on nasal washings and saliva were carried out using 0.1ml and 0.2ml of each undiluted sample respectively. Some mid- and post-treatment sera from DP patients contained large quantities of IgG BA and had to be assayed at high dilution (1:100 and 1:200). In sera with >10 fold excess of IgG BA over IgA BA or IgE BA it was difficult to maintain antigen excess and the minor classes might have been underestimated. For these sera IgE BA for F_4P_1 was estimated from the RAST values for D.pteronyssinus (see Appendix A.4.1). RAST assays and measurements of total IgE were carried out as described in Appendices A.4.2 and B.2.

3.2.3 Statistical Methods

Linear regression analysis was used to compare IgG BA and IgE BA, total IgE and IgE BA and the RAST values for *D.pteronyssinus* and *D.farinae*.

The χ^2 test with Yates correction was used to compare the increase in IgG BA in the DP and 'placebo' patients. The Spearman Rank correlation was used to compare pre- and post-treatment IgG BA. It was also used to compare changes in symptom scores with the differences in IgG BA and the ratios of IgG BA and IgG BA:IgE BA in pre- and post-treatment sera.

3.3 Results

The antigen binding assays were carried out using a preparation of F_4P_1 which contained >90% antigen P_1. Comparative experiments using pure antigen P_1 and F_4P_1, with different control curves for each allergen, gave exactly the same values for IgG BA and IgE BA in five sera from allergic patients (data not shown). Therefore, for the purposes of this chapter, measurements of BA for each allergen can be regarded as equivalent.

3.3.1 Antibodies to mites in sera from 'untreated' patients

Antigen binding assays on 58 pre-treatment sera from 'Hong Kong' patients showed the same pattern of ab response as the 'London' sera described in Chapter I. All but one of the sera contained IgG BA and IgE BA for antigen P_1 in quantities which were generally higher than for the 'London' sera. In both groups there was an excellent

quantitative correlation between IgE BA and IgG BA ($r = 0.87$, $p<0.001$, Fig. 3.1). The quantitative correlation between IgE BA and total IgE was also confirmed for the 'Hong Kong' sera ($r = 0.76$, $p<0.001$). IgA BA was assayed in parallel with the other classes in 20 sera: the levels were lower than IgG BA or IgE BA in all 16 sera with detectable IgA BA (Fig. 3.4).

A fully quantitative RAST, with separate control curves for each allergen, was used to compare IgE ab to D.pteronyssinus and to D.farinae. A high proportion of sera contained IgE ab to both mites, with an excellent quantitative correlation between the two species ($r = 0.90$, $p<0.001$, Fig. 3.2). These results suggest that the mites produce some cross-reacting allergen(s). In keeping with the previous study (Gabriel et al, 1977) the IgE ab levels of the 'placebo' patients were 2-3 fold higher than those of the DP patients (Table 3.1).

3.3.2 Antibodies to D.pteronyssinus in secretions from 'untreated' patients

Antibodies were assayed in nasal secretions and saliva from 'London' patients with rhinitis and/or asthma (skin test positive) or with rhinitis alone (skin test negative) (Fig. 3.3). The majority of skin test positive patients had detectable IgG, IgA and IgE BA in their nasal secretions. In contrast to the results obtained with serum (Fig. 3.4), the quantities of IgA BA in secretions were very similar to those of IgG BA and IgE BA. Nasal secretions from skin test negative patients had no detectable IgG BA or IgE BA, but 2/5 had low levels of IgA BA. In general, IgG, IgA and IgE BA were not detected in saliva,

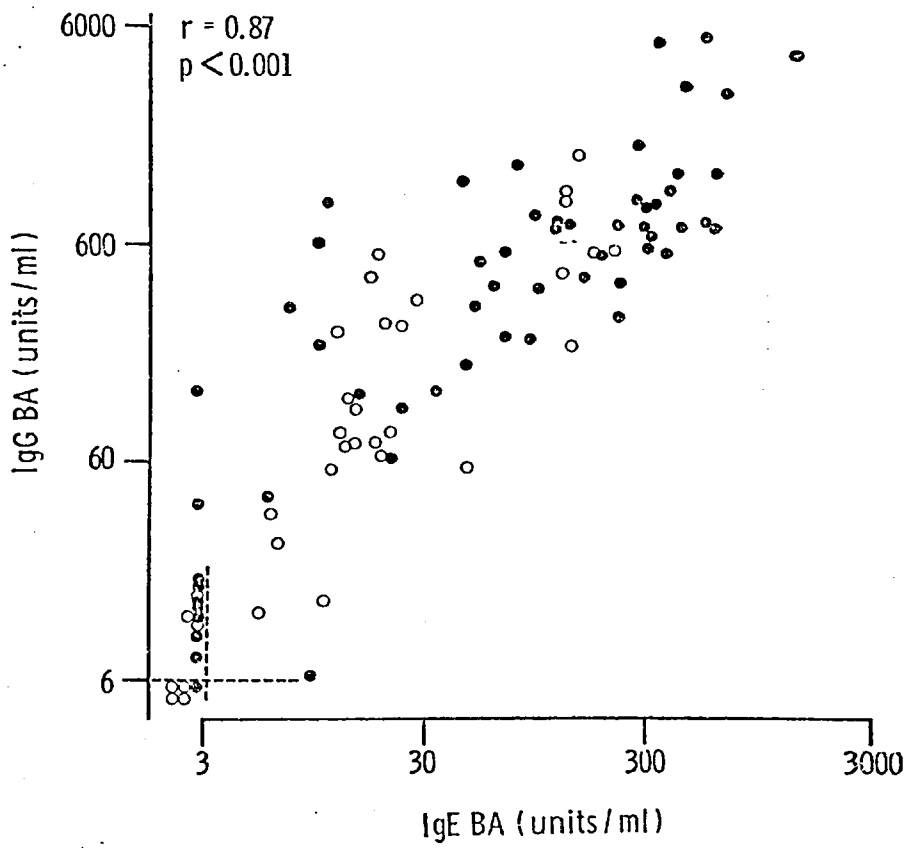


Figure 3.1

Correlation between IgE BA and IgG BA for antigen P_1 in sera from untreated patients with positive skin tests to D.pteronyssinus. The patients were included in either the 'Hong Kong' (●) or the 'London' (○) study. The minimum level of sensitivity of each antigen binding assay is indicated (---).

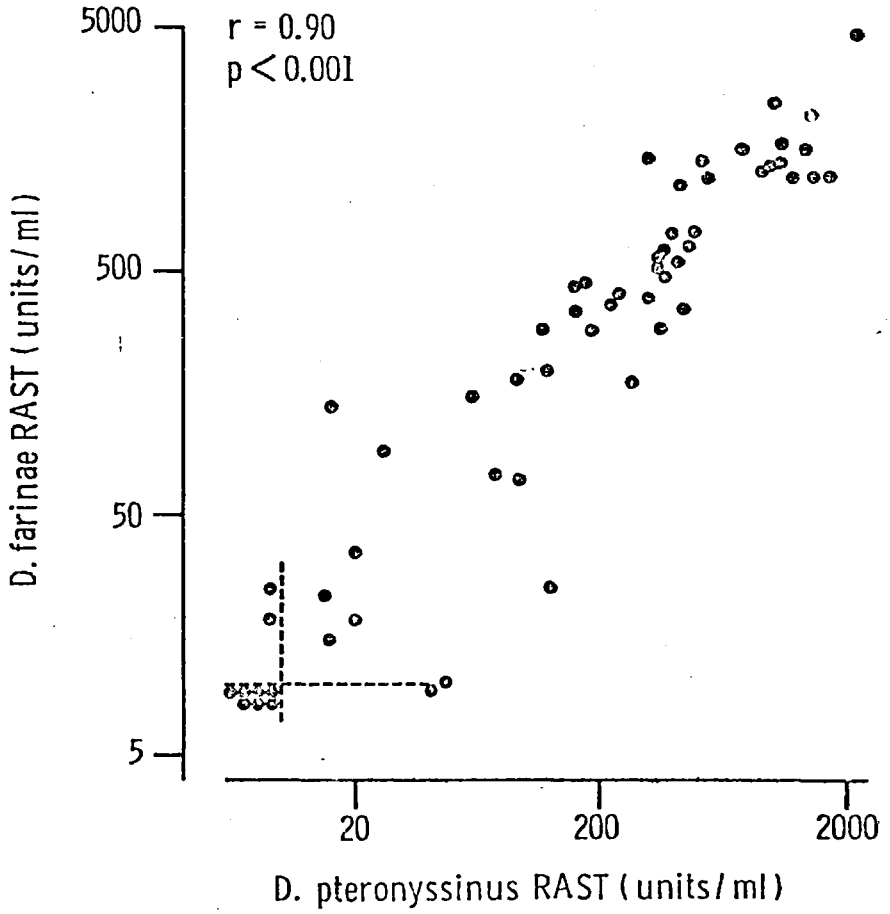


Figure 3.2

Correlation between the RAST values for IgE ab to D.pteronyssinus and IgE ab to D.farinae in pre-treatment sera from 'Hong Kong' patients. The dotted line shows the minimum level of sensitivity for each assay.

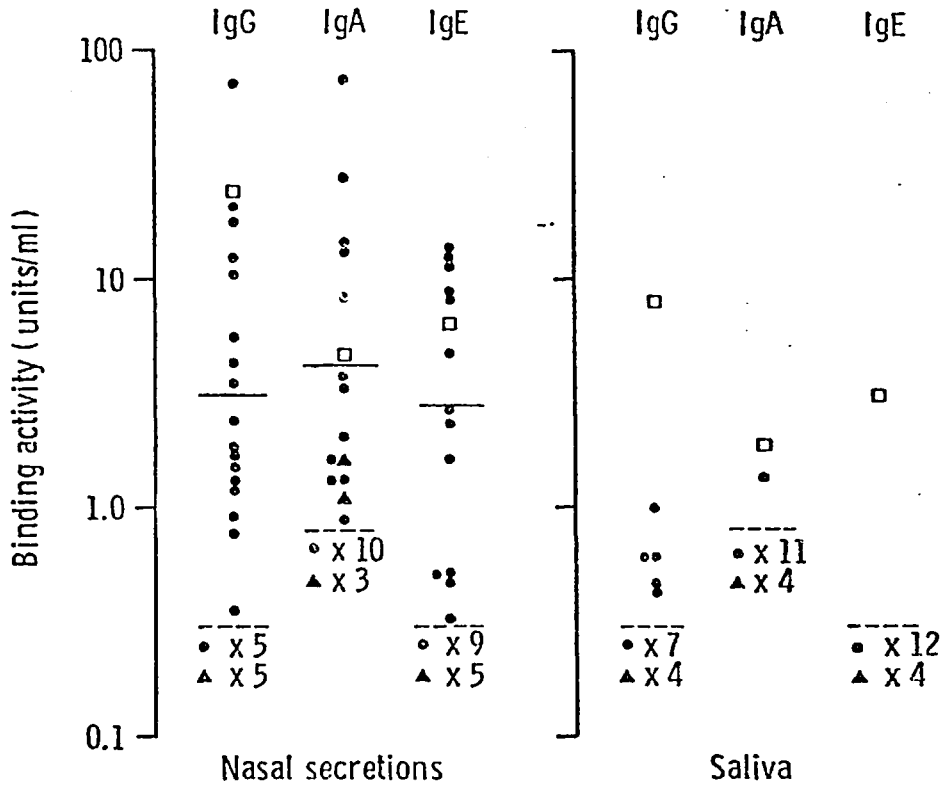


Figure 3.3

IgG BA, IgA BA and IgE BA for antigen P_1 in nasal secretions and saliva from 'London' patients. Samples were obtained from 22 skin test positive patients with perennial rhinitis and/or asthma (●); 5 skin test negative patients with rhinitis alone (▲); and 1 skin test positive asthmatic who had received desensitization treatment (□). Geometric mean values for each class of BA are shown (——). The number of samples which lacked detectable BA are shown below the dotted line.

though one sample (from a patient who had been desensitized) contained good levels of all three classes (Fig. 3.3). Nasal secretions and saliva from five non-allergic controls contained no detectable BA.

3.3.3 Antibody response following desensitization

Table 3.1 shows geometric mean values for the serum ab levels of 29 DP and 29 'placebo' patients during treatment. The DP patients showed a mean rise in IgG BA of six-fold with a maximum rise of fifty-fold. An increase of more than two-fold was found in 27 of the DP patients and in only one of the 'placebo' patients ($\chi^2 = 43.2$, $p<0.001$). There was little change in IgE BA (measured either by antigen-binding assay or by extrapolation from RAST) in either the DP or 'placebo' patients. For a selected group of 10 DP and 10 'placebo' patients (all with pre-treatment IgG BA:IgE BA of less than 8:1) IgG, IgA and IgE BA was measured in parallel (Fig. 3.4). The results demonstrate that the response to active treatment was a selective rise in IgG BA. The maximum rise occurred during and at the end of the weekly course of injections (mean increase 3.9 fold) with an apparent plateau during the maintenance treatment (mean increase 1.5 fold). In general, the levels of IgA BA were very low and only 2 DP patients showed an increase in IgA BA of more than two-fold.

The ab response of the 'Hong Kong' patients was compared with that of 44 patients resident in the U.K. who had been treated with tyrosine adsorbed mite extract (Migen) (Warner et al, 1978). The relationship between classes in pre-treatment sera was very similar to that of both

TABLE 3.1

Antibody levels in DP and 'Placebo' Patients[a]

Geometric Mean Data

Treatment Group	Serum sample	IgG BA units/ml	IgE BA units/ml	IgG BA:IgE BA	D.pteronyssinus RAST units/ml	D.farinae RAST units/ml
DP	Pre-treatment	300	74	4.0:1	83	129
DP	Mid-treatment	1,171	84	13.9:1	119	166
DP	Post-treatment	1,795	75	23.9:1	101	150
'Placebo'	Pre-treatment	390	210	1.9:1	228	305
'Placebo'	Mid-treatment	328	145	2.3:1	195	259
'Placebo'	Post-treatment	323	137	2.3:1	199	265

(a) In both DP and 'Placebo' patient groups 29 patients were assessed for IgG BA, D.pteronyssinus RAST and D.farinae RAST, and 24 patients were assessed for IgE BA.

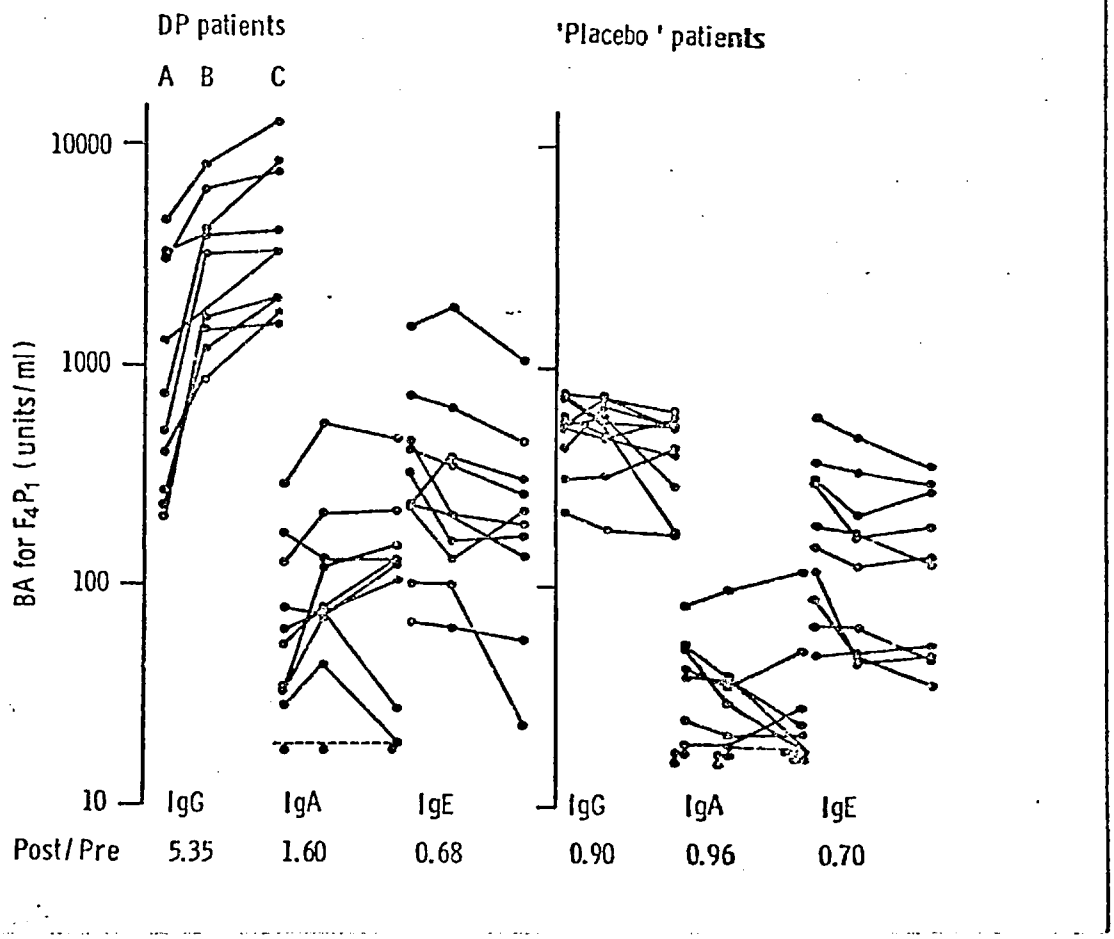


Figure 3.4

IgG BA, IgA BA and IgE BA for antigen P_1 in sera from a selected group of DP and 'placebo' patients. The serum samples are indicated: A = pre-treatment, B = mid-treatment (4 months), C = post-treatment (12 months). Sera without detectable IgA BA are shown below the dotted line. The mean post-treatment:pre-treatment ratio (post:pre) for each class of BA is shown.

the 'London' and 'Hong Kong' patients and, again, there was a very good correlation between IgG BA and IgE BA ($r = 0.77$, $p<0.001$). In marked contrast to the 'Hong Kong' study, patients treated with Migen did not show a selective increase in IgG BA during treatment (Fig. 3.5). There was a small rise in all the ab levels of Migen-treated patients compared with a slight fall in 'placebo'-treated patients. However, only two Migen-treated patients showed an increase in IgG BA of more than two-fold.

I am very grateful to Dr. M.W. Turner for providing serum samples from patients treated with Migen.

3.3.4 Relationship between IgG BA response and symptoms

In their original study Gabriel et al (1977) compared the effect of desensitization on the symptoms of 23 DP and 27 'placebo' patients. The DP patients showed significant improvement from four months onwards and were improved in all symptoms over the last three months of treatment ($p<0.01$). The present studies include data from a further 6 DP and 2 'placebo' patients, for whom both symptom scores and sera were available at one year. There was a fault in the design of the 'Hong Kong' study in that there was no 'run in' period prior to treatment to assess whether the symptoms of the two groups of patients were comparable. However, both groups recorded very similar symptoms during the first 28 days of treatment. Therefore, symptom scores during this 'baseline' period were compared with those obtained during the final month of the trial, i.e. one year after commencing treatment. The symptom scores were based on patients own assessment of symptoms (see

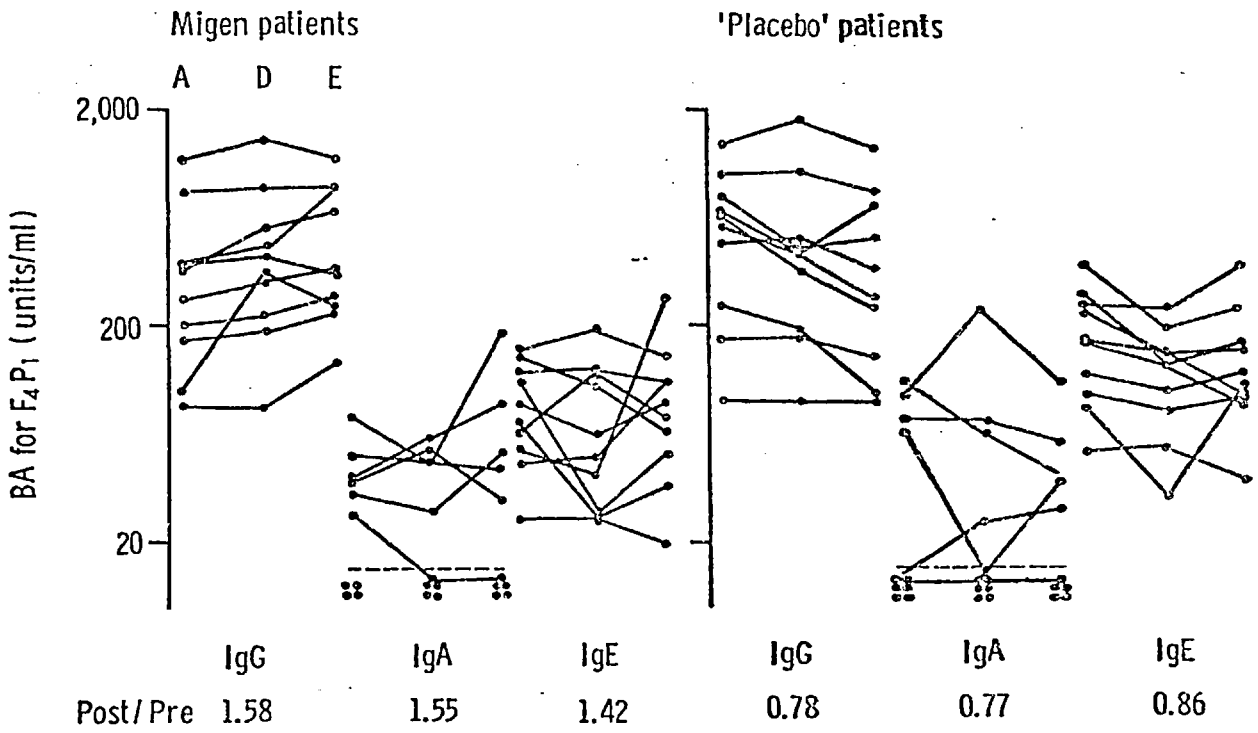


Figure 3.5

IgG BA, IgA BA and IgE BA for antigen P_1 in sera from a selected group of Migen-treated and 'placebo'-treated patients. Serum samples were taken pre-treatment (A), at 6 months (D) and at 12 months (E). The minimum level of sensitivity for IgA BA (----) and post-treatment:pre-treatment ratios (post:pre) for each class of BA are shown.

Methods) and ranged from 0-360 out of a maximal possible range of 0-420. These values were ranked and compared with the changes in IgG BA (Table 3.2). There was a slight inverse correlation between IgG BA and symptom scores pre-treatment ($p<0.02$) but no correlation after treatment. The changes in symptom scores during treatment (ΔS) were compared with the absolute increase in IgG BA and with the ratio of IgG BA post-treatment:pre-treatment. Neither comparison correlated with the change in symptom scores in either the DP or 'placebo' patients. Similarly, there was no correlation between symptom scores and the ratio of IgG BA:IgE BA post-treatment to IgG BA:IgE BA pre-treatment. Thus there was no evidence that the improvement in symptoms was mediated by feedback inhibition of IgE ab by IgG ab as had been suggested from work in experimental animals (Tada and Okumura, 1971).

3.3.5 Time course of binding by IgG ab

In view of the lack of correlation between IgG BA and symptom scores, further studies were designed to assess whether excess IgG ab would be able to 'block' the binding of antigen to mast cells *in vivo*. Allergic symptoms usually develop within 15 minutes of exposure to very small quantities of allergen (<1ng). The aim of these experiments was to use the antigen binding assay as a model to investigate the rate at which IgG ab could bind limiting quantities of allergen, i.e. 7ng, 1ng and 0.2ng ^{125}I-labelled F_4P_1. Initially, the antigen-ab reaction was stopped using the standard method of precipitation with monospecific anti-IgG (Appendix A.2.2; A.4.1). In those experiments 40-90% of the maximal antigen binding occurred within the first 15 minutes (data not shown) suggesting either that binding occurred very

TABLE 3.2

Correlation between changes in IgG BA and changes in symptom scores (ΔS) in patient groups

Treatment Group	Comparison	No. of patients assessed	Correlation coefficient	P value
All patients	Pre-treatment vs Pre-treatment IgG BA vs symptom score	58	-0.25	>0.05*
All patients	Post-treatment vs Post-treatment IgG BA vs symptom score	58	-0.09	>0.1
DP	IgG BA post. - IgG BA pre. vs ΔS	29	-0.09	>0.1
DP	IgG BA post./IgG BA pre. vs ΔS	29	0.28	>0.05
DP	$\frac{\text{IgG BA:IgE BA post.}}{\text{IgG BA: IgE BA pre.}}$ vs ΔS	24	0.12	>0.1
'Placebo'	IgG BA post. - IgG BA pre. vs ΔS	29	-0.16	>0.1
'Placebo'	IgG BA post./IgG BA pre. vs ΔS	29	-0.13	>0.1
'Placebo'	$\frac{\text{IgG BA:IgE BA post.}}{\text{IgG BA:IgE BA pre.}}$ vs ΔS	24	-0.24	>0.05

* When all the patients included in the previous study were assessed a slightly better correlation was obtained (n = 66, r = -0.3, p<0.02) (Gabriel et al, 1977).

rapidly or that it continued after the addition of anti-IgG. To investigate these possibilities the reaction was stopped by the addition of a 50-fold excess of cold antigen prior to the anti-IgG. Under those conditions less than 20% of maximal binding occurred within 15 minutes, suggesting that anti-IgG alone was not as effective in stopping the reaction in very short time periods. The results obtained using the cold antigen system showed that the binding of very low concentrations of ^{125}I-labelled allergen occurred slowly, even in post-treatment sera with 10-30 fold higher levels of IgG BA (Fig. 3.6). The reaction curves for pre- and post-treatment sera from the same individual were very similar, with maximal binding after eight hours incubation. The quantities of F_4P_1 bound by two of the sera shown in Fig. 3.6 are given in Table 3.3. Post-treatment sera from both patients could bind more than 1ng out of 7ng after 120 minutes. However, when the antigen concentration was reduced less binding occurred, so that <0.5ng was bound when 1ng was added and much lower quantities, 0.15 and 0.36ng, were bound in the first 30 minutes. Mygind (1979) has shown that allergic symptoms occur within a minute of nasal challenge with allergen. The present results show that excess IgG ab would not bind sufficient allergen to prevent symptoms in such a short time.

3.4 Discussion

The advantages of antigen binding assays compared with other methods of assessing allergen-specific ab were discussed in Chapter I. The results described in this Chapter illustrate the use of these assays for measuring ab in serum and secretions from both 'untreated'

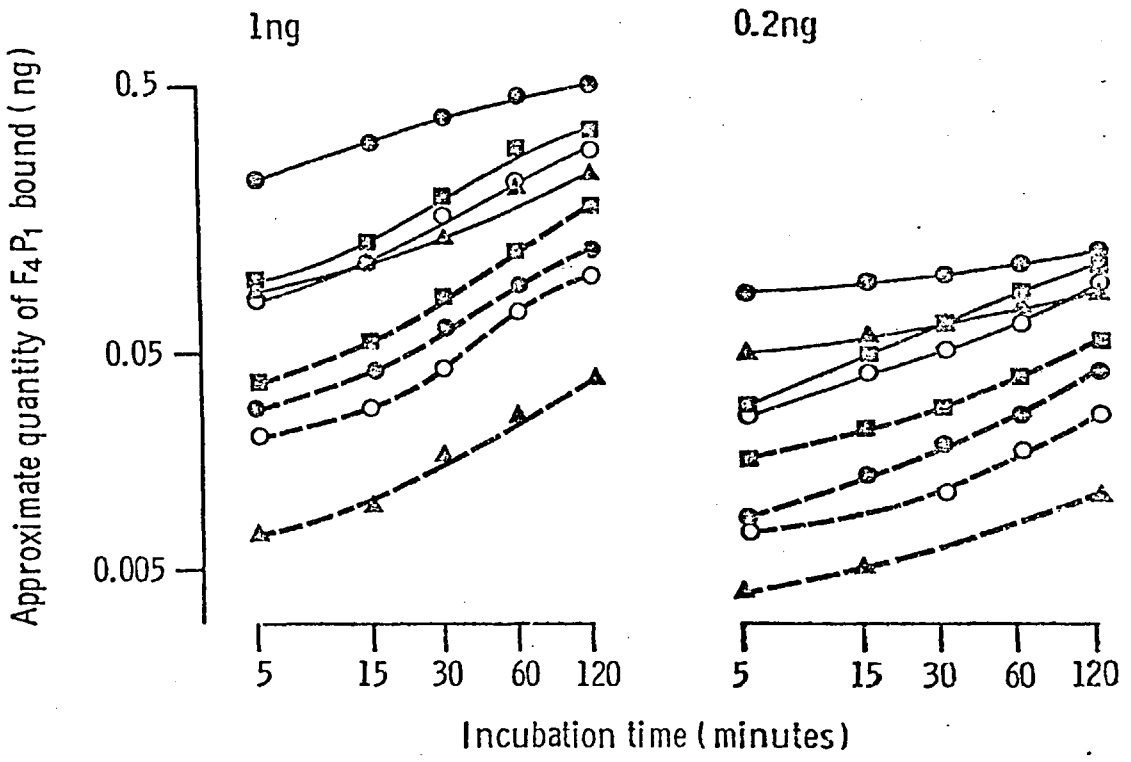


Figure 3.6

Time course for binding F_4P_1 by sera from DP treated 'Hong Kong' patients. Pre-treatment (---) or post-treatment (——) sera from 4 patients, No's 410 (O), 417 (●), 430 (▲) or 454 (■), were incubated with 1ng (24,000 cpm) or 0.2ng (5,300 cpm) of ^{125}I-labelled F_4P_1 for the given times. The assay was stopped by the addition of a 50-fold excess of cold antigen then IgG ab was precipitated with anti-IgG for 2 hours at 37°C. The post-treatment:pre-treatment ratios of IgG-BA in different sera were 4:1 (O), 10:1 (●), 33:1 (▲) and 3:1 (■).

TABLE 3.3

Quantities of F_4P_1 bound[1] by IgG ab[2] in pre- and post-treatment sera using different antigen concentrations

Patients[3]	Incubation Time	Quantity of ^{125}I F_4P_1 added to:-					
		Pre-treatment sera			Post-treatment sera		
	(min.)	7ng	1ng	0.2ng	7ng	1ng	0.2ng
Patient No. 410	5	0.06	0.02	0.007	0.25	0.07	0.02
	30	0.14	0.04	0.01	0.57	0.15	0.04
	120	0.27	0.08	0.02	1.07	0.27	0.07
Patient No. 417	5	0.07	0.02	0.008	0.78	0.21	0.07
	30	0.14	0.05	0.02	1.47	0.36	0.08
	120	0.23	0.11	0.03	1.99	0.50	0.10

Footnotes

1) Values expressed as ng F_4P_1 bound, and based on the protein content of a standard preparation of F_4P_1 determined using the method of Lowry et al (1951).

2) Sera were assayed in the presence of 7ng, 1ng or 0.2ng ^{125}I-labelled F_4P_1, and the assay was stopped at 5, 30 or 120 min. by the addition of a 50-fold excess of cold antigen. IgG ab was then precipitated with anti-IgG for 2 hours at 37°C.

3) Sera from patients No. 410 and No. 417 were assayed at dilutions of 1:25 and 1:50 respectively.

and 'treated' patients. The only limitation concerned measurements of IgA BA and IgE BA in the presence of very high levels of IgG BA, usually in post-treatment sera. This problem was overcome for IgE ab by extrapolating values for IgE BA for F_4P_1 from the RAST values for D.pteronyssinus. Interference by IgG ab can be a problem in some RAST systems, but only when 'low capacity' immunosorbents are used, or when the IgG ab is present in 10-1000 fold molar excess (Lynch et al, 1975; Paull et al, 1978). The ratios of IgG BA:IgE BA in post-treatment 'Hong Kong' sera were never greater than 50:1. Moreover, at lower ratios there was good agreement between values for IgE BA obtained by antigen binding assay and RAST conversion (Results, section 3.3.3 and Appendix A.4.1). These observations suggested that in the given circumstances the RAST was not unduly affected by IgG ab and that the RAST conversion system provided a useful assessment of IgE ab in mid- and post-treatment sera.

In previous chapters it was noted that there was a marked similarity between the ab response to purified D.pteronyssinus allergens (F_4P_1 and antigen P_1) and purified pollen allergens (e.g. Rye I and antigen E) in 'untreated' patients. The essential features of this response may be summarized as follows:-

(i) Most allergic individuals have allergen-specific IgG, IgA and IgE ab in their serum and nasal secretions (Results, sections 3.3.1, 3.3.2) (Ishizaka, Ishizaka and Hornbrook, 1967; Tse, Wicher and Arbesman, 1973; Platts-Mills et al, 1976, 1978; Deuschl, Johansson and Fagerberg, 1977).

(ii) There is an excellent quantitative correlation between IgG ab and IgE ab (Fig. 3.1).

(iii) Allergen-specific IgE accounts for a high proportion of the total serum IgE (Chapter II, section 2.3.4) (Gleich and Jacob, 1975; Schellenburg and Adkinson, 1975).

(iv) Non-allergic individuals generally have no detectable IgA ab or IgE ab in either serum or nasal secretions, but 20-30% have low levels of serum IgG ab (Chapter I, Fig. 1.6) (Levine, Stember and Fotino, 1972; Black et al, 1976).

This common pattern of humoral immune responses to inhalant allergens has implications for the site of ab production and for genetic and other factors which influence the development of atopy. There is good evidence that ab to pollen allergens are produced locally, either in the nasal mucosa or the draining lymph nodes (Nakajima, Gillespie and Gleich, 1975; Huggins and Brostoff, 1975; Merrett et al, 1976; Platts-Mills, 1979). Okuda (1975) first demonstrated IgE ab to D.farinae in nasal fluid using the RAST. The present results show that nasal secretions contain IgG ab and IgA ab as well as IgE ab. The presence of ab in nasal secretions but not in saliva suggested that ab to D.pteronyssinus was locally produced. However, to be certain of this, one would want to compare the "specific activities" (ratio of total immunoglobulin to ab of that class) of ab to D.pteronyssinus in serum and secretions as has been done for Rye I (Platts-Mills, 1979).

It has been proposed that transient IgA deficiency in infancy is one of the factors influencing the development of atopy in later life (Taylor et al, 1973; Soothill et al, 1976). In support of this view it was reported that serum from non-allergic individuals contained higher levels of IgA ab to allergens than that from allergic individuals (Stokes, Taylor and Turner, 1974; Taylor, 1974). The present results clearly conflict with those earlier observations since both serum and nasal secretions from allergic individuals contained IgA ab to D.pteronyssinus, whereas samples from non-allergic individuals did not (Results, sections 3.3.1 and 3.3.2). It seems likely that the previous assessments of IgA ab, using the RAST, represented minor variations in the RAST background, as has been discussed in Chapter I (Fig. 1.8). The results obtained by antigen binding assay, particularly the highly significant correlations between IgE ab and IgG ab, suggest that factors controlling the development of inhalant allergy act upon all three ab classes, rather than on any one class alone.

It was clear from the 'Hong Kong' study that there was a selective rise in serum IgG ab following desensitization with aqueous D.pteronyssinus extract. However, several lines of evidence suggested that increases in IgG ab were not directly related to improvement in clinical symptoms. Firstly, patients' symptoms were most improved during the last three months of treatment, whereas the increase in IgG ab was most marked during the first four months (Fig. 3.4). Secondly, there was no correlation between changes in symptom scores and the levels of IgG ab in pre- or post-treatment sera. Thirdly, patients treated with Migen showed no significant increase in serum IgG ab (Fig. 3.5) but nonetheless showed significant clinical improvement (Warner et al, 1978). Apart from these studies there have been

very few reports of quantitative changes in IgG ab during desensitization with mite extracts. However, many such studies have been carried out on desensitization in grass pollen and ragweed hay fever, though the results are equivocal. Several studies showed an association between increases in serum IgG ab and improvement in symptoms (Sadan et al, 1968; Levy et al, 1971) and in some cases there was a direct correlation (Starr and Weinstock, 1970; Lichtenstein, Norman and Winkenwerder, 1971). Other workers failed to correlate rises in IgG ab with improvement in symptoms (Irons, Pruzansky and Patterson, 1975; Ahlstedt and Eriksson, 1977). There is evidence that IgG ab, whether induced by desensitization or by passive immunization, offers protection against systemic reactions in bee sting allergy (Sobotka et al, 1976; Valentine et al, 1976; Lessof, Sobotka and Lichtenstein, 1977). However, the ab response to bee venom allergens is slightly different from that to inhalant allergens (Kemeny, Lessof and Trull, 1980) because the quantities of allergen involved are much larger (10-100µg doses) and the route of exposure is different.

Traditionally, the antigen-antibody interaction has been regarded as a very fast reaction (Hooker and Boyd, 1935), for which the rate-limiting step in vitro is the time of mixing the reagents (Steward, 1977). On this basis, it seemed reasonable to assume that high levels of serum IgG ab (produced following desensitization) would 'block' the binding of allergen to mast cells at mucosal surfaces. Previously, in antibody-hapten systems, it was difficult to perform kinetic experiments because of technical problems in measuring the very low concentrations of hapten ($<10^{-6}$M) at which the time of mixing was not rate-

limiting (Froese and Sehon, 1971). The present studies demonstrate that using ^{125}I-labelled allergens of high specific activity it is possible to assess reaction rates at very low antigen concentrations (10^{-10}M). The results in Fig. 3.6 and Table 3.3 show that at such concentrations the antigen-antibody reaction occurs quite slowly. Thus, even in the presence of an apparent excess of IgG ab, the amount of allergen left unbound after 5 minutes would probably be sufficient to cause symptoms in highly allergic individuals (Table 3.3). These results certainly throw doubt on the ability of IgG ab to block histamine release *in vivo*. Similar doubts have also been raised by other workers using rabbit antisera to inhibit histamine release from human basophils (Schumacher and Jeffery, 1979). It is possible that IgG ab in nasal secretions would have more effective 'blocking' activity than that found in serum. However, the levels of IgG BA and IgA BA in nasal secretions are low, for both *D.pteronyssinus* (compare Figs. 3.1, 3.3 and 3.4) and pollen allergens, and show only a modest rise during treatment (Deuschl, Johannson and Fagerberg, 1977; Platts-Mills et al, 1976). These results suggest that the quantity of local ab produced in response to systemic immunization would not be effective in preventing allergens from releasing histamine *in vivo*.

Obviously, 'blocking' by IgG ab is not the only possible mechanism of desensitization. Experiments in animals have suggested that the production of IgE ab might be specifically suppressed (Tada, 1975; Takatsu and Ishizaka, 1976) and in humans prolonged desensitization can cause a reduction in IgE ab (Levy et al, 1971; Lichtenstein et al, 1973). There was no evidence from the present studies for specific

suppression of IgE ab. In the 'Hong Kong' study there was a progressive fall in IgE ab in both groups of patients (Fig. 3.4) and in the 'Migen' study IgE ab was slightly elevated in the treated group (Fig. 3.5). The interpretation of changes in serum IgE ab is fraught with difficulties, since neither the level of ab nor total IgE is necessarily related to the severity of symptoms (Lichtenstein et al, 1973). Furthermore, the levels of IgE ab may be boosted by seasonal exposure to allergens (Yunginger and Gleich, 1973). Exposure to mite allergens is less clearly defined on a seasonal basis than pollen allergens and this further complicates the interpretation of changes in IgE ab in these patients.

Studies on desensitization in ragweed hay fever suggest that changes in cellular sensitivity and reactivity show the best correlation with improvement in clinical symptoms (Sadan et al, 1968; Pruzansky and Patterson, 1968; Levy et al, 1971; Irons, Pruzansky and Patterson, 1975). In most cases a reduction in symptoms was associated with decreased histamine release from circulating leucocytes.
It has also been suggested that reductions in cellular sensitivity are non-specific and could affect reactivity to other allergens (Lichtenstein and Levy, 1972). So far, no consistent changes in cellular histamine release have been observed following desensitization with mite extracts (Assem and McAllen, 1973; D'Souza et al, 1973). However, the availability of a purified allergen from D.pteronyssinus (antigen P_1) should greatly improve future studies on cellular reactivity.

C H A P T E R I V

Antibodies to inhalant allergens in atopic dermatitis

4.1 Introduction

Atopic dermatitis (Coca, 1934) is an inflammatory disease of the skin, characterized by pruritis, scaling, vesiculation and lichenification as well as oedema of the epidermis. Mild forms of the disease are localised to certain areas, notably the flexures. However, in severe cases the eczema can involve most parts of the body. Atopic dermatitis is very common in infants, often causing retarded growth because of protein loss through the skin, excessive use of steroid drugs and recurrent bacterial infection. Some children may 'outgrow' the disease at an early age; in others it may persist into adolescence and adulthood.

Atopic dermatitis commonly occurs in persons who have a family history and a personal history of immediate hypersensitivity to environmental allergens. Following the discovery of IgE it soon became clear that patients with atopic dermatitis had very high levels of this immunoglobulin in their serum (Juhlin et al, 1969; Bennich and Johannson, 1971). Subsequent studies confirmed these observations and established that the levels of IgE were directly related to the severity of the disease (Ogawa et al, 1971; Stone, Muller and Gleich, 1973; Clendenning et al, 1973; Gurevitch, Heiner and Reisner, 1973; Church, Kleban and Bellanti, 1976; O'Loughlin et al, 1977). Thus patients with the most severe and widespread eczema had the highest levels of serum IgE (up to 30,000 International Units/ml).

Despite the strong correlation with atopic sensitivity, it has been impossible to prove any specific role for IgE in the aetiology

of the disease. Indeed, the failure to consistently produce eczematous lesions on challenge with allergen and the often inconclusive effects of allergen avoidance, has lead many dermatologists to reject IgE-mediated mechanisms (Norins, 1971). There are difficulties in the diagnosis of immediate hypersensitivity in eczema patients. While skin tests provide an excellent diagnostic tool for patients with hay fever and asthma, they are less useful in atopic dermatitis because generalized sensitivity of the skin may give 'false positive' results. In severe cases, the skin may be so inflamed that it is impossible to perform adequate skin tests and these may give 'false negative' results. Very high levels of serum IgE also increase the non-specific binding in the RAST (Kemeny, Lessof and Trull, 1980) such that 'false positive' results may be obtained, particularly with ill-defined allergens, e.g. food allergens. In general, there is a poor correlation between skin tests and RAST determinations for IgE ab to food allergens (Hoffman and Haddad, 1974; Hoffman et al, 1975).

It has been demonstrated in previous chapters that allergen-specific IgE can account for a high proportion of total IgE. Therefore, accurate measurements of IgE ab should give a better guide to patients' sensitivity and allow a more critical evaluation of the importance of specific allergens. This chapter describes measurements of ab to inhalant and non-inhalant allergens, and to other protein antigens, in serum from patients with atopic dermatitis. All the antibodies were measured using antigen binding assays, the results of which are unaffected by the total concentration of IgE in the serum (Chapter I).

4.2 Methods

4.2.1 Patients

All the patients (aged 2-60) presented with atopic dermatitis at dermatology or allergy clinics either in New Addenbrookes Hospital, Cambridge or Northwick Park Hospital, Harrow. There were 21 patients from 'Cambridge' and 29 patients from 'Harrow'. Some patients also suffered respiratory symptoms, i.e. hay fever, perennial rhinitis or asthma. Wherever possible the patients were prick tested, as part of their clinical assessment, with a battery of common allergens including house dust, D.pteronyssinus, mixed grass pollens, tree pollens, animal danders (horse, cat, dog), milk and egg (Bencard Ltd. Betchworth, Surrey). The skin tests were classified according to the diameter of the wheal on a semi-quantitative basis, as follows:-

Wheal diameter (mm)	1-2	3-4	5-6	>7
Classification	±	+	++	+++

Serum was obtained from each patient and stored at -20°C prior to use.

Control sera were obtained from 20 laboratory personnel (aged 20-50) with no allergic symptoms, though two individuals gave positive prick tests to grass pollen.

I am very grateful to Drs. M. di Prisco Fuenmajor and R.H. Champion for supplying serum samples and all the skin test data on patients from 'Cambridge'.

4.2.2 Antibody measurements

The IgG and IgE ab measurements were made by double antibody antigen binding assay (Appendix A.4.1). Antibodies to D.pteronyssinus Antigen P_1, Rye I, bee venom phospholipase A and horse dander allergens Nos. 6 and 11 (Lowenstein, Markussen and Weeke, 1976) were measured in serum samples. Antibodies to diphtheria toxin and β-lactoglobulin were measured in ammonium sulphate fractions of serum, enriched for either IgG ab or IgE ab (see Appendix A.4.1).

Total serum IgE was measured by double antibody inhibition radioimmunoassay (Appendix B.2).

4.3 Results

Serum samples were obtained from all 50 patients with atopic dermatitis. Some of the samples were rather small (<0.5ml), so that it was not possible to assay ab to each allergen in all cases. The numbers of sera assayed ranged from 44-50.

4.3.1 Antibodies to inhalant allergens

The response to inhalant allergens, particularly D.pteronyssinus, was the most striking feature of the ab response in the atopic dermatitis patients. Twenty-six of the thirty-five patients with positive skin tests (+ to +++) to D.pteronyssinus had detectable IgG BA and IgE BA for antigen P_1 in their serum (Fig. 4.1). In contrast, only one of the eleven patients with a negative or equivocal (±) skin test had detectable BA for antigen P_1. The ab levels of the skin test

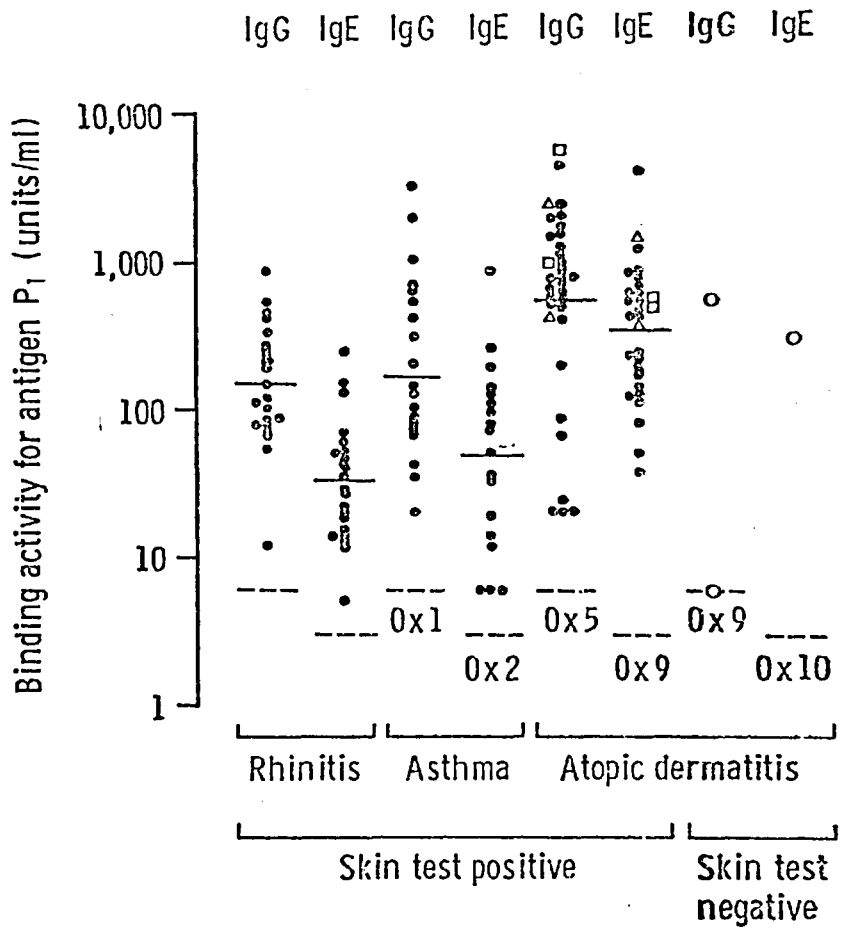


Figure 4.1

IgG BA and IgE BA for antigen P_1 in sera from patients with rhinitis, asthma or atopic dermatitis. Patients were classified as skin test positive (●), skin test negative (○) or skin tests not done (Δ). The eczematous lesions on 2 patients (□) did not permit adequate skin testing. Also shown are the geometric mean values (——) and the minimum level of sensitivity (---) for each class of BA. Sera without detectable BA are shown below the dashed line.

positive patients were on average much higher than those of patients with perennial rhinitis or asthma alone (Fig. 4.1). The geometric mean values for IgG BA and IgE BA (Table 4.1) were 3-4 fold and 6-fold higher, respectively, than for patients with rhinitis or asthma. The results were further analysed according to whether or not patients with atopic dermatitis had additional respiratory symptoms (rhinitis or asthma). Twice as many (19/24) of the patients with respiratory symptoms had detectable IgG BA and IgE BA. Quantitatively, the mean levels of IgG BA were $\sim$2-fold higher in the group with respiratory symptoms, but there was very little difference in the levels of IgE BA (Table 4.1). The quantities of IgE BA produced by many atopic dermatitis patients were similar to, and in a few cases exceeded, the quantities of IgG BA. Although all sera with detectable IgE BA contained IgG BA, the mean ratio of IgG BA:IgE BA in the atopic dermatitis group was lower than for patients with rhinitis or asthma (Fig. 4.1 and Table 4.1).

Also shown in Table 4.1 are the geometric mean values for total serum IgE in the various patient groups. As expected, the mean levels for patients with atopic dermatitis were several fold higher than for patients with rhinitis or asthma alone and were most highly elevated in the group with additional respiratory symptoms. The latter findings are in agreement with those of Johnson et al (1974). Despite the significant differences in absolute levels of IgE, the percentage of the total IgE directed against antigen P_1 was very similar in all patient groups (Table 4.1).

TABLE 4.1

Comparison of total IgE levels and IgG BA and IgE BA for antigen P_1 in patients with rhinitis, asthma or atopic dermatitis - mean data

	BA for antigen P_1 [a] (units/ml)		Total IgE [b] (International units/ml)	IgE BA [c] % total IgE
	IgG BA	IgE BA		
Rhinitis (n = 22)	148 (22/22)	33 (22/22)	276 (39- 1,880)	8.1 ± 7.9
Asthma (n = 20)	168 (19/20)	48 (18/20)	473 (61-12,500)	6.2 ± 5.2
Atopic dermatitis (all patients, n = 50)	550 (35/50)	340 (29/50)	1,746 (18-25,500)	6.8 ± 5.4
Atopic dermatitis, [d] minimal respiratory symptoms, (n = 25)	313 (13/25)	294 (10/25)	948 (18-20,000)	6.6 ± 4.9
Atopic dermatitis, with rhinitis, or asthma, (n = 24)	584 (22/24)	333 (19/24)	3,076 (269-25,500)	6.9 ± 5.9

(a) Geometric mean data based on positive results. The incidence of positive BA is shown in parentheses.

(b) Geometric mean data. Values given in parentheses indicate the range of results obtained.

(c) Calculated as described in Chapter II (Results, section 2.3.4), assuming 1 unit of IgE BA for antigen P_1 to be equivalent to 1ng IgE. Values represent the arithmetic mean ± one standard deviation.

(d) Patients with no history of respiratory symptoms. On further questioning, some patients reported occasional mild sumptoms e.g. nasal obstruction and discharge.

The levels of IgG BA and IgE BA for Rye I, the major allergen from rye grass pollen, in sera from skin test positive and skin test negative patients with atopic dermatitis are shown in Fig. 4.2. Most of the skin test positive patients had very high levels of BA; the geometric mean values for both IgG BA (1245 units/ml) and IgE BA (802 units/ml) being greater than those reported for patients with grass pollen hay fever (IgG BA, 869 units/ml and IgE BA, 248 units/ml) (Platts-Mills et al, 1978). In keeping with the results obtained with antigen P_1, the ratio of IgG BA:IgE BA for Rye I was much lower in the atopic dermatitis group (1.5:1, Fig. 4.2). One of the skin test negative patients had high levels of IgG BA (2,500 units/ml) and IgE BA (588 units/ml) for Rye I, another had high IgG BA (825 units/ml) without detectable IgE BA. The other values for IgG BA in patients with negative skin tests to grass pollen were quite low (<250 units/ml). Similar low levels of IgG BA are found in 20-30% of sera from normal individuals (Discussion, Chapter III).

Several of the 'Cambridge' patients had strongly positive skin tests (+++) to horse dander allergens which were assumed to reflect increased exposure in that predominantly rural area. None of the 'Harrow' patients had been skin tested to horse dander. Nonetheless, it was considered worthwhile to investigate the ab response to two purified horse dander allergens (No. 6 and No. 11) (Lowenstein, Markussen and Weeke, 1976) in both groups of patients to see whether this might be related to allergen exposure. The lack of a suitable standard serum meant that it was not possible to construct a control

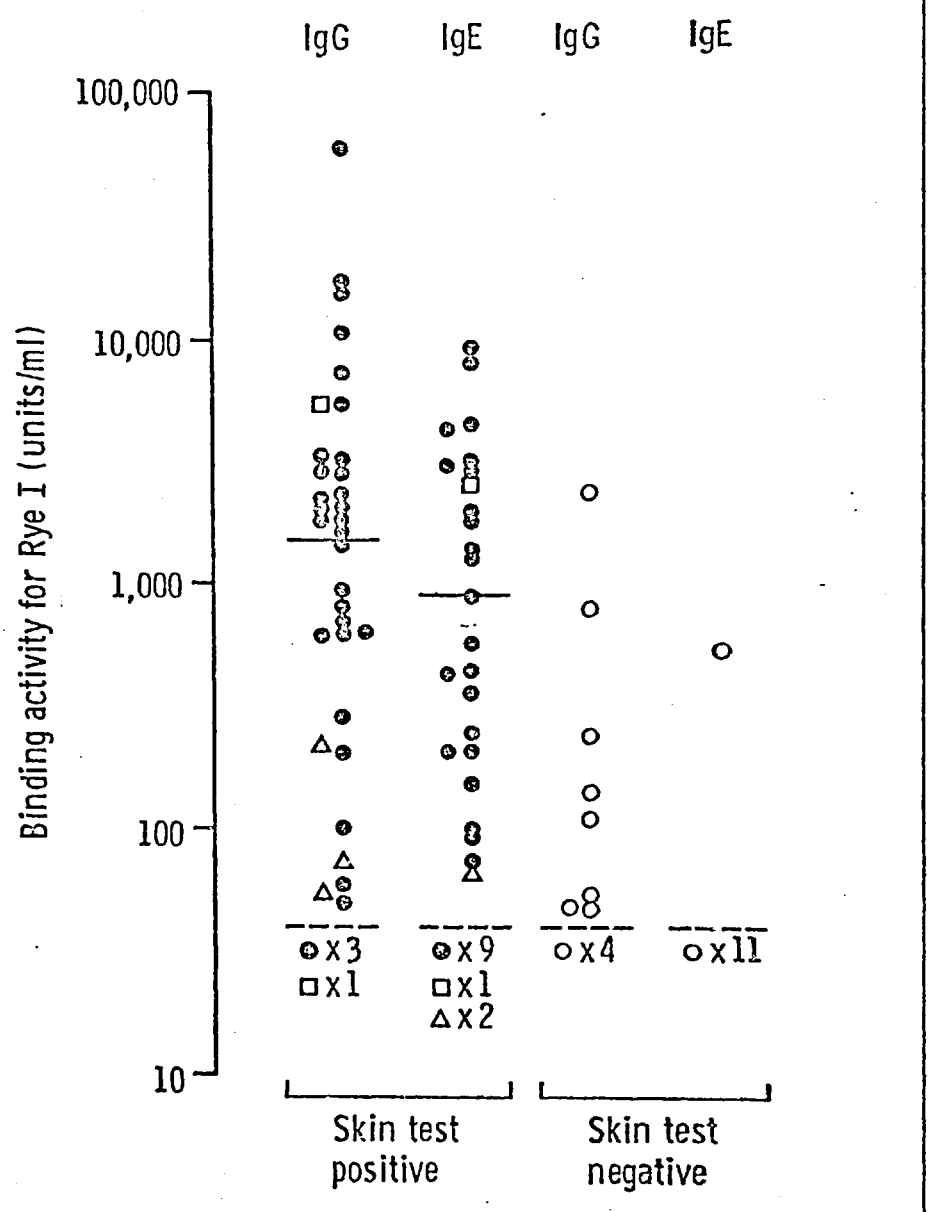


Figure 4.2

Serum antibodies to Rye I. Patients with atopic dermatitis were skin tested with grass pollen and classified as skin test positive (●), skin test negative (○), skin tests not done (△) or not possible (□). Sera without detectable BA are shown below the minimal level of sensitivity for each assay (---).

curve to make the antigen-binding assays for horse allergens fully quantitative. However, semi-quantitative assessments of binding activity for each allergen were made, based on the increase in binding relative to five sera from non-allergic individuals (Fig. 4.3). All of the six sera from 'Cambridge' patients with strongly positive skin tests had detectable IgG or IgE ab to horse allergens No. 6 or No. 11, or both. Two 'Cambridge' patients with equivocal skin tests had detectable IgG ab but lacked IgE ab. The majority of sera, from patients on whom skin tests were not done, did not contain ab to horse allergens. However, three of these sera (two from 'Harrow', one from 'Cambridge') contained both IgG and IgE ab and a further two samples contained IgG ab without detectable IgE ab (Fig. 4.3).

Table 4.2 summarizes the relationship between skin tests and IgE ab levels in a representative series of 15 patients with atopic dermatitis. The patients were characterized by multiple positive skin tests associated with very high levels of serum IgE. With a few exceptions (C.T., N.G., Table 4.2), most patients with positive skin tests to house dust, D.pteronyssinus and grass pollens also had detectable IgE BA for antigen P_1 and Rye I. Furthermore, the overall incidence of positive skin tests to inhalant allergens was much greater than that to food allergens (milk and egg). Notable amongst these figures, was the high incidence of positive skin tests to cat allergens and several patients reported exacerbation of symptoms following exposure to cats. A smaller group of patients showed positive skin tests to milk and eggs, one of whom was also milk intolerant (R.L., Table 4.2). Some of these patients appeared to be almost exclusively positive to skin test

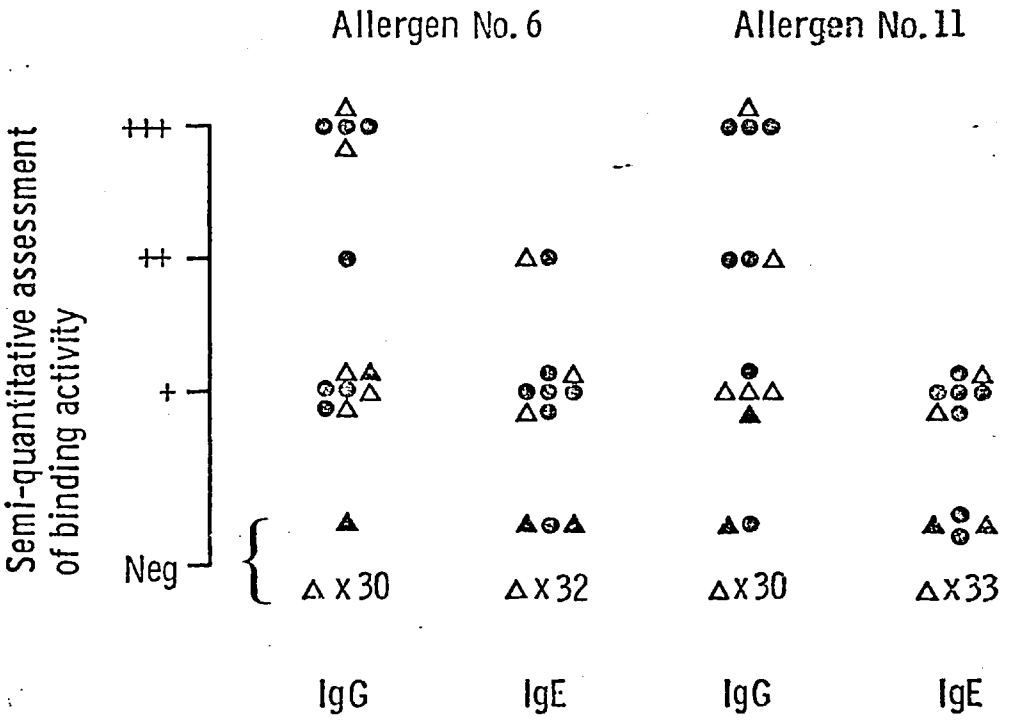


Figure 4.3

Semi-quantitative assessment of IgG and IgE antibodies to horse allergens, No.6 and No.11. Positive binding activity was graded as >2 standard deviations above background (+); >5 x background (++); and >10 x background (+++). The negative background (-) was defined as the mean ±2 standard deviations of the values obtained from 5 sera from non-allergic controls. Patients were classified as skin test strongly positive (++ to +++) (⊙), skin test equivocal (±) (◭), or skin test not done (Δ).

TABLE 4.2

Comparison of skin tests, total IgE and IgE BA in 15 patients with atopic dermatitis

Subject, age/sex	Skin test responses to:-[a] H.D.	D.pt.	Pollen	Milk	Egg	Cat	Horse	Total IgE units/ml	IgE BA (units/ml)[b] for Antigen P_1	Rye I
DB, 22/M	++	+++	+++	-	-	+++	+++	12,500	1,225	3,000
PL, 35/F	++	+++	++	±	±	+++	+++	6,240	240	101
CH, 14/M	n.d.	+++	+++	-	±	+	n.d.	20,000	800	4,438
CT, 10/F	+	++	++	+++	+++	-	++	5,750	575	<40
SC, 6/M	+	+	+	-	-	+	-	512	142	444
RP, 26/F	+	++	++	n.d.	±	+	n.d.	3,875	432	444
RB, 35/F	++	++	+++	+	+	++	n.d.	25,500	420	9,563
NG, 10/F	+	++	-	-	-	+++	+++	11,250	544	588
RS, 7/M	+	+	±	n.d.	n.d.	++	n.d.	2,700	<3	<40
GY, 6/M	±	±	+++	-	+	+++	n.d.	800	<3	1,807
EG, 34/F	-	-	+	++	++	++	-	3,625	<3	<40
RL, 3/M	n.d.	±	±	+++	++	n.d.	n.d.	345	<3	<40
MW, 2/F	±	++	-	-	++	-	n.d.	8,280	128	<25
CF, 10/M	++	+++	+++	-	-	++	n.d.	1,550	469	213
JH, 60/M	++	++	++	-	-	++	n.d.	14,500	175	3,163
Prevalence[c]		70%	66%	16%	32%	66%				

Footnotes to Table 4.2

(a) Skin tests were performed using the 'prick' technique. Gradings represent >7mm wheal (+++), 5-6mm wheal (++), 3-4mm wheal (+), 1-2mm wheal (±) or negative (-). H.D. = house dust, D.pt. = D.pteronyssinus, n.d. = not done.

(b) Values for IgE BA for antigen P_1 and Rye I were obtained from different control curves (see Appendix A.4.1) and are not directly comparable. One unit IgE BA, for antigen P_1 approximates to 1ng IgE (Chapter II) whereas 1 unit IgE BA for Rye I represents 0.1-0.3ng IgE (Platts-Mills et al, 1978).

(c) Overall prevalence of positive skin tests (+ to +++) for all patients on whom skin tests were performed.

foods (e.g. E.G., R.L., M.W., Table 4.2) and most had undetectable levels of IgE BA for antigen P_1 and Rye I in their serum.

4.3.2 Antibodies to non-inhalant allergens

These studies were undertaken to investigate potential differences in the ab response to inhalant allergens and to other proteins administered in larger doses by different routes (ingestion or injection). The proteins included two non-inhalant allergens, β-lactoglobulin and phospholipase A (the major allergen from bee venom) and another protein to which most individuals are commonly exposed, that is diphtheria toxoid. In each case, the ab response of the patients with atopic dermatitis was compared with that of 20 non-allergic controls.

Measurements of IgG BA and IgE BA for β-lactoglobulin are shown in Fig. 4.4. There was a high incidence of IgG BA (>80%) in both skin test positive and skin test negative patients, with G.M. values of 466 units/ml and 183 units/ml, respectively. A smaller percentage ($\sim$50%) of non-allergic controls had detectable IgG BA and the levels were lower than for patients with atopic dermatitis (G.M. 81 units/ml). The findings for IgE BA illustrate some of the problems in assessing IgE ab to food allergens. Six of the ten patients with positive skin tests to milk had IgE BA for β-lactoglobulin. Using sera from three of these patients, the presence of IgE ab to β-lactoglobulin was confirmed by P-K testing (P in Fig. 4.4); the sera giving positive P-K tests at dilutions of 1:50-1:500. Some of the patients with negative skin tests (8/26) also showed positive binding in the assay

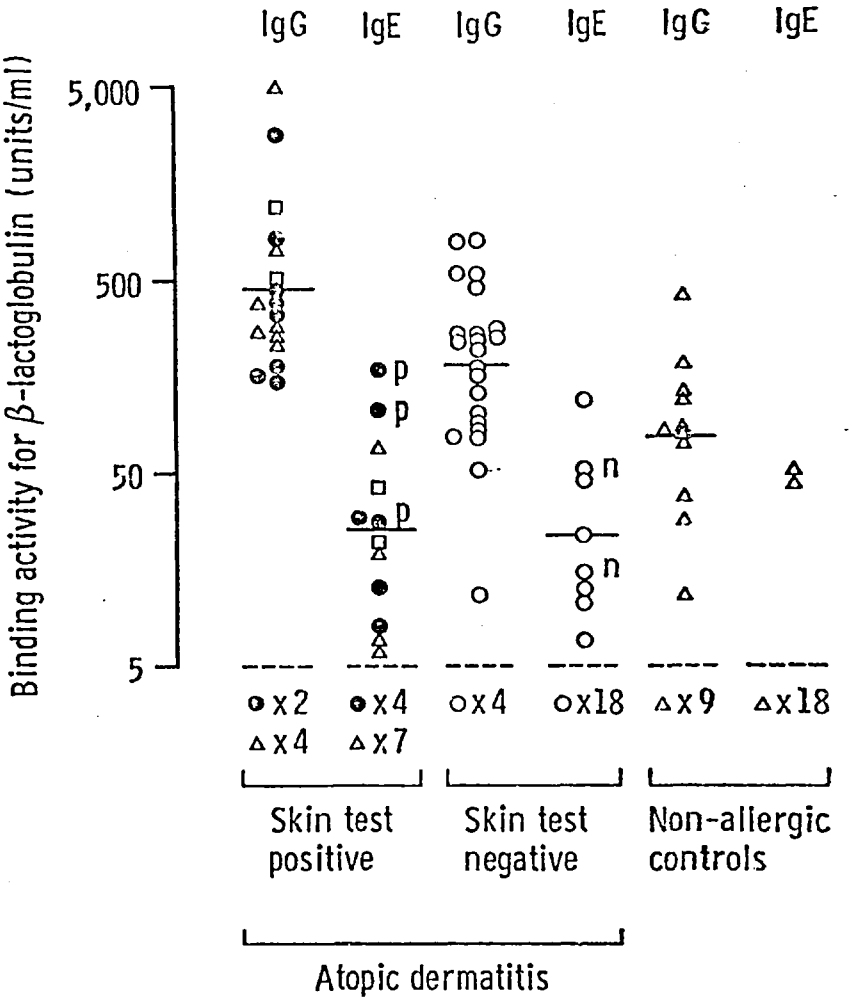


Figure 4.4

IgG BA and IgE BA for β-lactoglobulin in ammonium sulphate fractions of sera from patients with atopic dermatitis and from non-allergic controls. Skin test responses to milk are shown as positive (●); negative (○); not done (△); or not possible (□). Also shown are the geometric mean values (——) and the minimal level of sensitivity for each class of BA (---). Five sera were also used in P-K tests and are recorded as P-K positive ('p') or P-K negative ('n').

for IgE ab, but two of these sera did not react in P-K tests at a 1:4 dilution (n in Fig. 4.4). The majority of sera from non-allergic controls contained no detectable IgE BA. However, two sera from persons with elevated total IgE (300 and 3,000 I.U./ml) and positive skin tests to grass pollen did show positive IgE binding. The presence of IgE ab to β-lactoglobulin in sera from children and adults with no allergic symptoms to milk has been demonstrated previously by Parish (1971) using radioimmunodiffusion. By an interesting analogy with the present findings, some of those sera could not passively sensitize monkey skin or isolated monkey basophils (Parish, 1971).

Neither patients nor controls showed a significant ab response to phospholipase A. Very low levels of IgG BA (<50 units/ml) were detected in sera from two patients and one control, but none of the sera contained detectable IgE BA.

The ab response to diphtheria toxoid was assessed using radiolabelled diphtheria toxin (the toxoid labels poorly using the standard chloramine T technique). The results showed a high incidence of IgG antitoxin in sera from both patients and controls (Fig. 4.5), suggesting that most individuals had been immunized against diphtheria. Patients with atopic dermatitis appeared to have higher levels of IgG antitoxin. However, the quantities of IgG antitoxin in control sera were similar to those in sera from atopic dermatitis patients aged over 20 (G.M. values 48 units/ml and 80 units/ml respectively). This suggested that the higher levels of IgG antitoxin occurring in

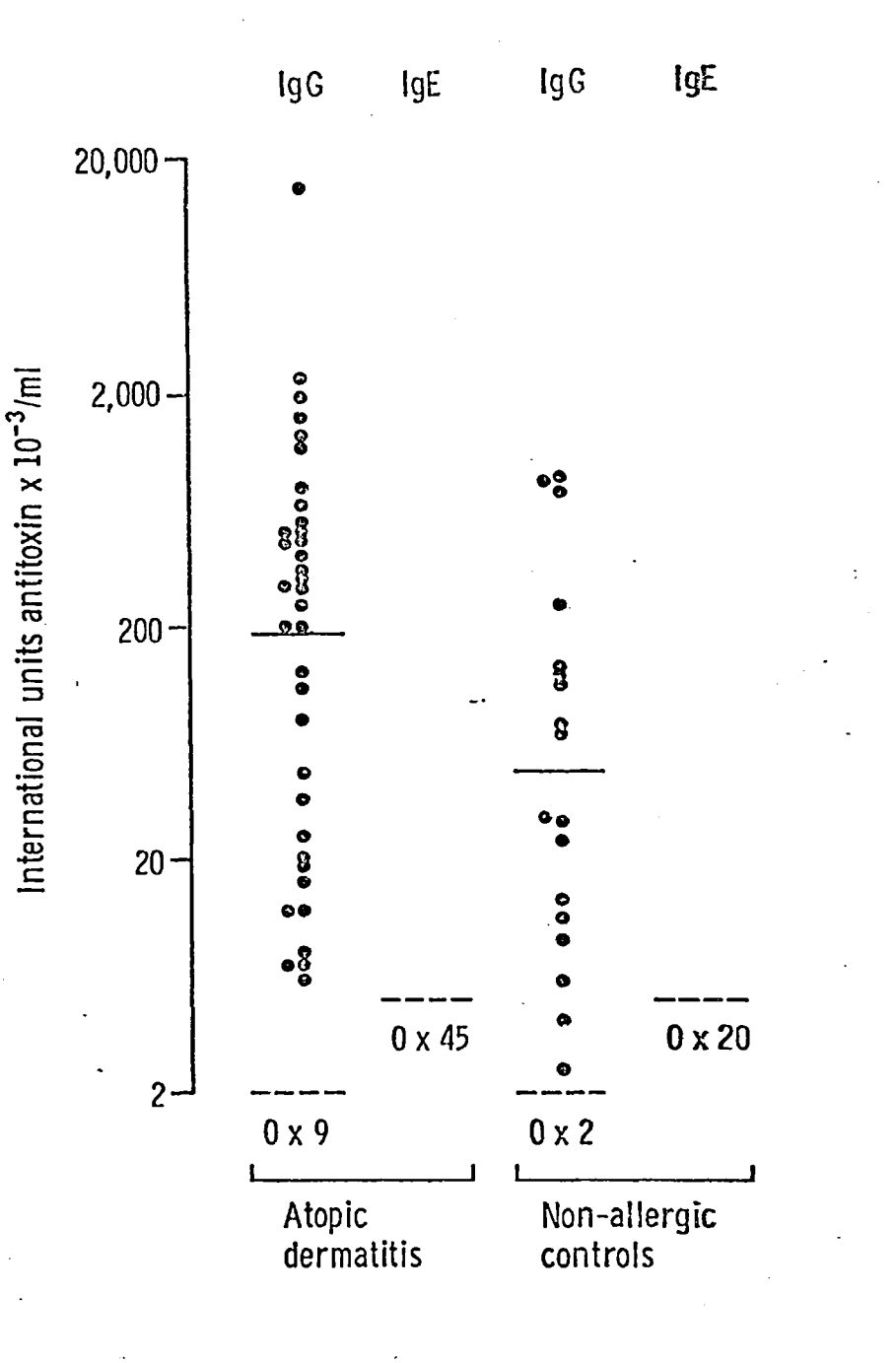


Figure 4.5

IgG and IgE diphtheria antitoxin levels in ammonium sulphate fractions of sera from patients with atopic dermatitis and from non-allergic controls.

the younger patients (aged under 20) were the result of more recent immunization. In contrast to previous results obtained using RAST (Nagel et al, 1979), neither patients nor controls had detectable IgE antitoxin. Some of the adult patients developed eczema long after diphtheria immunization and might not be expected to make an IgE antitoxin response. But at least five infants, under five years old, had eczema at the time of maximum exposure to diphtheria toxoid, yet none made IgE antitoxin.

4.4 Discussion

The results show that patients with atopic dermatitis make IgG and IgE ab responses to a variety of common environmental allergens. This was illustrated by the response to two major inhalant allergens, antigen P_1 and Rye I. There was a high incidence of BA for both allergens which showed a good correlation with the results of skin prick tests. The quantities of BA were significantly higher in patients with atopic dermatitis than in other, non-eczematous, allergic patients. It was also apparent that there was a lower ratio of IgG BA:IgE BA in the atopic dermatitis group. Subsequent analyses of data from patients with eczema, asthma or rhinitis (Fig. 4.1), showed that there was an inverse correlation between the ratio of IgG BA:IgE BA for antigen P_1 and total serum IgE ($r = -0.48$, $p<0.01$, $n = 71$). Comparison of the values obtained on pre-treatment sera from the 'Hong Kong' and 'Migen' patients (Chapter III) confirmed this correlation ($r = -0.50$, $p<0.01$, $n = 103$). These findings could reflect progressive saturation of mast cell receptors, resulting in higher levels of serum IgE ab and hence a reduction in the ratio of

IgG BA:IgE BA. Alternatively, it has been suggested that the serum half-life of IgE is longer in patients with very high serum IgE (Waldman et al, 1976), so the lower ratio could be due to slower removal of IgE from the serum of these patients.

The results confirmed that allergens No.6 and No.11 are important allergens of horse dander (Lowenstein, Markussen and Weeke, 1976). None of the 'Harrow' patients gave a clinical history of horse dander allergy and only 2 out of 23 of these patients had detectable IgG and IgE ab. By contrast, one third (7/21) of the 'Cambridge' patients had positive skin tests and detectable IgG and IgE ab to horse allergens (Fig. 4.3). Assuming, as seems likely, that the 'Harrow' and 'Cambridge' patients differ in their exposure to horse allergens, then the results suggest that the development of allergen-specific ab responses in atopic dermatitis is dependent upon adequate allergen exposure.

The ab response to non-inhalant allergens was clearly distinct from that to inhalant allergens. In keeping with previous findings (Dannaeus et al, 1977, 1978), there was a high incidence of IgG ab to β-lactoglobulin in sera from both skin test positive and skin test negative individuals. Atopic patients tended to have higher IgG ab levels than the non-allergic controls (Fig. 4.4). However, as with the response to diphtheria toxin, this was to some extent age related since patients aged under 20 had higher IgG BA for β-lactoglobulin (G.M. 358 units/ml) than those aged over 20 (G.M. 172 units/ml). A striking feature of the response was the very high ratio of IgG BA:

IgE BA (18:1 in the skin test positive group, Fig. 4.4). This finding differs from that of Dannaeus et al (1977, 1978) who found similar quantities of IgG ab and IgE ab. However, those workers were using two different solid phase assays to compare the quantities of ab.

Previously, IgE ab to β-lactoglobulin, and other food allergens, has been assessed by radioimmunodiffusion, passive sensitization techniques, the RAST and enzyme-linked immunosorbent assays (Parish, 1971; Kletter et al, 1971; Hoffman et al, 1975; Dannaeus et al, 1977, 1978; Fallstrom, Ahlstedt and Hanson, 1978). In general, there has been a relatively poor correlation between in vitro assessments of IgE ab, skin tests and clinical symptoms. This in part represents technical problems in the assay systems and also the different immunological and non-immunological mechanisms which may be responsible for food allergy (Matthews and Soothill, 1970; Walker-Smith, 1975). In the present studies, IgE BA for β-lactoglobulin was found in sera from most individuals with positive skin tests to milk. The finding that ~30% of sera from skin test negative, milk tolerant, allergic individuals also had IgE ab which bound β-lactoglobulin is in close agreement with the studies of Parish (1971). The interpretation of these findings is difficult because some of the sera were not active in P-K tests (Fig. 4.4). It has been suggested that these antibodies represent "non-anaphylactically sensitizing IgE" (Parish, 1971). The current data is inadequate to resolve this issue.

In recent years the role of inhalant allergens in atopic dermatitis has been largely overlooked. However, several early investigators recognised the importance of house dust, pollen and animal dander allergens in causing symptoms and used allergen avoidance and appropriate desensitization as treatment (Hill, 1937; Zakon and Taub, 1938; Tuft, 1949). Recently, many dermatologists and allergists have observed that patients with atopic dermatitis often have positive skin tests to house dust mites, pollens and dander (see Table 4.2), but have failed to relate this to the aetiology of the disease. Inhalant allergens are the most common allergens in the U.K. It is not surprising, therefore, that these patients should make ab responses, but the magnitude of these responses is much greater than for other allergic patients (Fig. 4.1) and might well be of some significance. The results with horse allergens suggest that the specificity of response is related to allergen exposure. In addition, the response to non-inhalant allergens suggests that allergen dosage is important. The responses to β-lactoglobulin and to diphtheria toxin were primarily IgG ab and are probably typical of the response to high dose immunization. The failure of eczema patients to make IgE ab responses to diphtheria toxin or to phospholipase A, provides good evidence that the hyper-responsiveness (in terms of IgE production) is not related to 'polyclonal' activation of IgE B cells. Furthermore, the relatively poor response to β-lactoglobulin suggests that there is no 'adjuvant' effect on existing IgE ab responses, analagous to that caused by parasitic infection (Jarrett and Stewart, 1972; Strejan, 1975; Turner, Feddema and Quinn, 1979).

It is clear that patients with atopic dermatitis make IgG and IgE ab responses to a large number of allergens in the environment. In particular, they produce large quantities of allergen-specific IgE. Clinically, it seems that any attempt to either exclude or implicate a role for IgE in eczema must take these allergens into account. To this end, the availability of purified allergens for use in antigen binding assays should facilitate more quantitative assessments of patients' sensitivity. Although more work needs to be done on assays for food allergens, it should be possible to use antigen binding assays to measure the ab responses of individual patients to a battery of highly purified allergens. This will reduce the possibility of 'false positive' results which can be obtained in some IgE RAST systems. Further work on the role of IgE in eczema will require more accurate assessments of allergen exposure, to study the effects of allergen avoidance, and also functional assessments of patient's cellular reactivity.

GENERAL DISCUSSION

Over the past 20 years, allergens have been purified from many diverse source materials and the properties of some 20-30 major and minor allergens have been described in detail. Only one of these allergens, ragweed antigen E, is available commercially. This underlines the fact that purified allergens have found little place in routine clinical allergy. Most clinical tests, e.g. skin testing and the RAST are carried out using ill-defined crude allergen extracts. In most cases, purified allergens have been used for research studies on cellular, humoral and genetic factors which influence immediate hypersensitivity. This discussion will be concerned with some of the clinical applications of antigen P_1, but will concentrate mainly on the biological significance of the properties of this major inhalant allergen.

1. Clinical relevance of antigen P_1

Purified allergens could be used to bring about substantial improvements in allergen standardization. At present, there is no internationally accepted method for comparing the allergenic potency of mite extracts. Commercial manufacturers use different conditions for growing and extracting mites and produce extracts which vary in allergen content (Tovey and Vandenburg, 1979) and in the amount of culture medium derived impurities (Chapter I). Traditionally, allergen extracts have been standardized according to their protein nitrogen content (1 protein nitrogen unit, PNU = 0.01µg protein nitrogen) or the quantity of allergen used for extraction (the allergen extracted from 1µg source material = 1 Noon unit). Both PNU and Noon units have proved unreliable methods of assessment. For example, two commercial mite extracts analysed during the present studies had the same PNU, yet differed by 22-fold in their

antigen P_1 content determined by radioimmunoassay. Modern approaches to standardization have been aimed at either comparing the major allergen content or the total allergenic activity within an extract.

Antigen P_1 could be used to standardize D.pteronyssinus extracts in the same way that antigen E and Rye I have been used to standardize pollen extracts (Baer et al, 1970, 1974). As shown above, quantitative differences between the antigen P_1 content of mite extracts can be demonstrated using the inhibition radioimmunoassay (Appendix B.2). However, for routine use radialimmunodiffusion would probably be the method of choice. The limitations which previously applied to radialimmunodiffusion, i.e. the production of large quantities of monospecific antiserum, have become less important with the advent of techniques for the continuous production of monoclonal antibodies (Kohler and Milstein, 1975). Attempts to produce monoclonal antibodies to antigen P_1 have already begun. These antibodies could be used in either radialimmunodiffusion or radioimmunoassay and offer one of the best long term prospects for standardizing mite extracts.

There is some concern that standardization using major allergens does not reflect the total allergenic activity of an extract. Other methods are available which satisfy this requirement, notably quantitative immunoelectrophoresis (Lowenstein, 1978) and RAST inhibition (Aalberse, 1974; Gleich et al, 1974). However, these techniques also have their problems: quantitative electrophoresis assumes that all the allergens are antigenic in rabbits and RAST inhibition assumes that all the allergens will bind to cyanogen bromide activated immunosorbents.

Nonetheless, quantitative differences between mite extracts have been demonstrated by RAST inhibition (Tovey and Vandenburg, 1978, 1979). In future it should be possible to standardize mite extracts according to both their antigen P_1 content (in µg/ml) and the dilution required to cause 50% RAST inhibition of a pooled international reference serum.

The other areas where it might be considered that antigen P_1 has clinical application are in diagnosis, e.g. skin testing and in vitro assessments of IgE ab, and in desensitization treatment. In general practice, the diagnosis of allergy is usually based on clinical history and skin tests. Whilst there are good reasons for using D.pteronyssinus extracts instead of house dust extracts for skin testing, there is no real case for using antigen P_1 in place of a properly standardized D.pteronyssinus extract. Indeed, it would be unadvisable to skin test with antigen P_1 alone because of dangers of underdiagnosis of mite allergy in some 5% of cases. The question as to whether antigen P_1 should be used in in vitro measurements of mite specific ab is less clear cut. At present, a D.pteronyssinus RAST is marketed commercially as an aid to diagnosis, yet yields no more clinical information than a skin test. If ab measurements are to be used in diagnosis of allergy it could be argued that it would be more worthwhile to measure both IgG and IgE ab by antigen binding assay. The problems with establishing routine antigen binding assays, i.e. excessive use of antisera and IgE myeloma sera (Chapter I) may well be overcome by the development of 'micro' assays performed in microtitre plates (Newton, Pereira and Farrant - submitted for publication). Apart from requiring smaller quantities of reagents, these assays can be washed, without centrifugation,

on an automated harvester suitable for processing several hundred samples/day. It is quite conceivable, therefore, that 'micro' assays for measuring IgG and IgE ab to several common allergens could be established in 'allergy referral' laboratories. As was mentioned in Chapter IV, this type of assessment might be a particularly useful aid to the diagnosis of atopic dermatitis.

The use of antigen P_1 in desensitization would not appear to be justified until the efficacy of this treatment is more clearly established. It is still not clear which patients are likely to benefit from desensitization. Moreover, the mechanisms of desensitization remain poorly understood. Desensitization using ragweed antigen E or partially purified grass pollen components was found to be no more effective than using crude pollen extracts (Frankland and Augustin, 1954; Norman, Winkenwerder and Lichtenstein, 1968). Theoretically, desensitization with major allergens alone might exclude other allergens which may also contribute towards symptoms (Norman, 1980).

2. The properties of antigen P_1

It was concluded in Chapter II that the physical properties of antigen P_1, i.e. molecular size, solubility and concentration in crude mite extracts determine its importance as an allergen. Comparisons of the properties of antigen P_1 with those of other purified allergens support the view that these molecules do not share physico-chemically defined, allergenic determinants (Marsh, 1975; Aas, 1978; King, 1979). It seems unlikely, therefore, that further biochemical analyses, e.g. amino acid sequencing or carbohydrate analysis will yield much more

information about the ability of antigen P_1 to stimulate IgE ab production. More detailed biochemical studies will obviously be useful in determining the structure of antigen P_1, it's susceptibility to enzymic and chemical degradation and the molecular interactions between its isoallergens. Greater knowledge of the physico-chemical properties of antigen P_1 will also make it easier to analyse D.pteronyssinus fractions for minor allergens. The widespread distribution of antigen P_1 on IEF will undoubtedly complicate further separation procedures. However, using the radioimmunoassay it will be possible to ensure that potential minor allergens are not contaminated with trace amounts of antigen P_1 before they are tested for allergenic activity in mite allergic individuals.

In their early work, Voorhorst and colleagues (1964, 1967) suggested that mites "secreted or excreted" allergens. Since then there has been much speculation about the origins and functions of mite allergens, though very little experimental evidence has been forthcoming. Using inhibition radioimmunoassay it has been possible to assay the antigen P_1 content of isolated components of D.pteronyssinus culture, i.e. male, female and juvenile mite bodies, mite cuticles, eggs and faeces (Tovey, Chapman and Platts-Mills, 1981). The results showed that >95% of the antigen P_1 accumulating in culture was derived from mite faeces - spherical particles about 10-40μ in diameter (see General Introduction, Fig. I.1). These particles are analogous to pollen grains in that they are roughly the same size and shape, readily become airborne and contain highly concentrated, soluble allergen. The deposition of faecal particles on the nasal mucosa could well cause rhinitis. It is also possible that mite allergens could be carried to the lungs on faecal particles of

$\leqslant 10\mu$ diameter and thereby initiate the symptoms of asthma (Tovey, Chapman, Wells and Platts-Mills - submitted for publication). Thus, there is a striking similarity between both the physical properties and the 'packaging' of the two most important inhalant allergens which appears to be directly related to their ability to induce immediate hypersensitivity.

Faecal particles comprise several compacted foodballs surrounded by a peritrophic membrane (Wharton, 1976). Antigen P_1 could be derived from either of these components as a product of digestion or as a digestive enzyme. These possibilities are currently under investigation using fluorescent and peroxidase labelling techniques with purified human anti-mite antibody to locate the allergen by electron microscopy.

It remains to be established how far the relationship between antigen P_1 and D.pteronyssinus pertains to other mite species. That is, whether antigen P_1 cross-reacts with other mite allergens and whether those allergens are also carried in faeces. Recently, there has been renewed interest in occupational asthma induced by 'storage' mites (Acarus, Tyrophagus and Glycyphagus sp; Appendix A.1). This complaint is common amongst farmers, millers and bakers, who become sensitized by handling mite-infested hay or cereal products and show positive skin tests and bronchial provocation tests to 'storage' mite extracts (Cuthbert et al, 1979; Ingram et al, 1979; Wraith, Cunnington and Seymour, 1979). In addition to occupational exposure, individuals may encounter different populations of mites in their own house dust, despite the predominance of Dermatophagoides sp. Appreciable numbers

of 'storage' mites and Tarsonemidae are sometimes found (Korsgaard and Hallas, 1979; Wraith, Cunnington and Seymour, 1979) and in some houses in the U.K. E.maynei can replace D.pteronyssinus as the dominant species (Blythe, Williams and Morrison-Smith, 1974; Burr et al, 1980). Dermatophagoides sp have been studied in preference to these other mites because, allergenically, they are most important and because they could readily be grown in in vitro cultures. Comparable quantities of 'storage' mite cultures have not been available and some mite species, e.g. E.maynei have proved almost impossible to culture.

Clinical and immunological investigations of allergic reactions to E.maynei and 'storage' mites will require further assessments of their antigenic and allergenic cross-reactivity. Previous studies, using skin testing and immunodiffusion (Pepys, Chan and Hargreave, 1968; Voorhorst, Spieksma and Varekamp, 1969; Dasgupta and Cunliffe, 1970; Biliotti, Romognani and Ricci, 1975), suggest that pyroglyphid mites produce common allergens that are not shared with 'storage' mites and that cross-reactivity amongst mite species is phylogenetically related (Blythe, 1976). The RAST results (Chapters I-III), which showed an excellent correlation between IgE ab to antigen P_1, D.pteronyssinus and D.farinae, suggested that antigen P_1 might be an antigenic determinant common to most mite species. However, the D.farinae extract used for RAST contained very small quantities of antigen P_1 ($\sim$ 10µg/ml) and gave very poor lines against rabbit antiserum to D.pteronyssinus (anti-ExI) on immunodiffusion. On the basis of this evidence, it seems that other allergens also cross-react between Dermatophagoides sp. Small scale cultures of other mites are available (Wraith, Cunnington

and Seymour, 1979) and it will be possible to determine their antigen P_1 content. A better assessment of the allergenic importance of these mites will also be possible, using the antigen binding assay to measure IgE ab to antigen P_1 in sera from individuals who give skin test reactions to different mites. Reports that there may be cross-reactions between dust mites and scabies mites and locusts (Falk and Bolle, 1980; Burge et al, 1980) need further investigation. However, extracts of two locust species (Locusta migratoria and Schistocerca gregaria) did not react on immunodiffusion when tested with the anti-D.pteronyssinus serum used in the present studies.

It is well established that the symptoms of hay fever roughly correlate with assessments of allergen exposure made using 'rotoslide' pollen counts. Although it is possible to detect mite faeces in dust, the 'counting' of faecal particles would not be sufficiently accurate to provide an assessment of allergen exposure similar to pollen counts. Usually, mites are physically separated from dust samples and then counted under light microscopy. Mite counts show a reasonable correlation with the skin test reactivity of dust extracts (Voorhorst et al, 1967; Maunsell, Wraith and Cunnington, 1968), but are too time consuming for large numbers of samples and require considerable technical expertise. The inhibition radioimmunoassay for antigen P_1 provides a quantitative method for measuring the allergen content of dust extracts which shows an excellent correlation with mite counts (Fig. D.1). The results show that the antigen P_1 content of different dust extracts varies considerably, extending over a four log range. Some samples contain few mites (<10 mites/g dust) but appreciable quantities of allergen (>200 ng), suggesting that antigen P_1 persists in the environment when

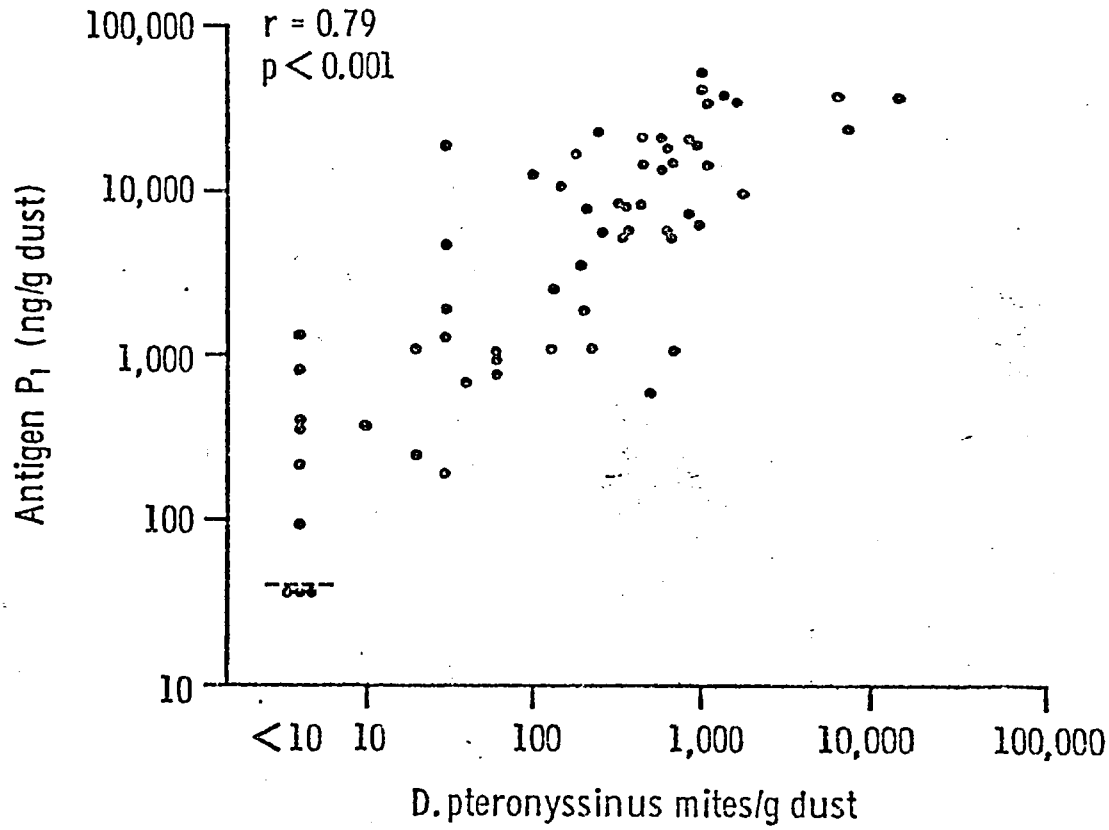


Figure D.1

Mite allergen content of house dust extracts assessed using the inhibition radioimmunoassay for antigen P_1 and by counting the numbers of D.pteronyssinus. Samples of house dust were sieved through an 0.3mm mesh to obtain the fine dust. For radioimmunoassay, 100mg each dust was extracted in 2ml borate buffered saline (pH 8.0) for 2 hours, centrifuged and assayed at appropriate dilutions. For counting, mites were recovered from another 100mg dust by flotation in saturated NaCl/ 20% sucrose, stained with crystal violet and examined on lined filter paper under a stereo microscope. The two methods were compared by linear regression analysis of the logged data.

mite populations are reduced. The results obtained with these samples suggest that the allergen content of some houses would be underestimated if it were based on mite counts alone.

Air sampling experiments have shown that in airborne dust antigen P_1 is not associated with mite bodies, but with particles <50μ in diameter, some of which have been identified as mite faeces (Tovey, Chapman, Wells and Platts-Mills - submitted for publication). These experiments, carried out in houses in the Harrow area, suggest that exposure to antigen P_1 can be of the order 2-50 ng/hour when the dust in a room is disturbed. Several factors are likely to affect the degree of exposure, including the total quantity of dust, the allergen content of the dust (both of which may vary in different parts of the same house) and the extent to which the dust is disturbed. Some houses contain very little antigen P_1 (<50 ng/g dust), so that it is impossible to detect airborne allergen after disturbing the dust or after prolonged sampling (8 hours). On the other hand, in houses containing 10-70,000 ng antigen P_1/g dust exposure to 2-30 ng allergen would not be uncommon if the dust were disturbed for 1 hour. It has been estimated that exposure to pollen allergens is about 1μg/year (Marsh, 1975). Clearly, any one figure could not accurately reflect exposure to mites. On a yearly basis people living in heavily infested houses are likely to be exposed to larger quantities of mite allergens than pollen allergens. Nonetheless, the total dose of these inhalant allergens is minute when compared with the doses of antigens, e.g. toxoids used for theraputic immunizations.

Accurate measurements of the allergen content of dust samples are an essential prerequisite for controlled studies on the use of allergen

avoidance in the treatment of asthma. Successful studies on allergen avoidance were first reported over 50 years ago by Storm van Leeuwen (1927). He treated 500 asthmatic patients by admission to an 'allergen-free' chamber maintained at low humidity (20-30%) and found that 75% of patients were 'cured' of asthma within a few weeks. Recently, physical methods, such as wrapping mattresses in polythene, removing furnishings and carpets, and regular vacuum cleaning, have been used to reduce overall mite infestation (Sarsfield et al, 1974). These methods have been studied in a controlled trial (Burr et al, 1980), but were not considered greatly beneficial. Specific eradication of the mite has been attempted using acaricides, perticides or disinfectants (van Bronswijk and Koekkoek, 1971; Leysen, Reybrouck and Van der Voorde, 1974; Penaud et al, 1975). It has also been suggested that spraying mattresses with light oil effectively reduces the concentration of airborne allergen (Tovey, Tamm and Vandenburg, 1977). However, so far neither physical nor chemical methods of mite control have been shown to cause a significant reduction in allergen concentrations.

Ultimately, the demonstration that inhalant allergens are an important cause of asthma or eczema will require that the experiments of Storm van Leeuwen be repeated under carefully controlled, allergen-free conditions. These facilities can quite easily be established in hospitals or research institutes. Thus in future it would be possible to challenge mite allergic individuals with dust containing known concentrations of antigen P_1 under conditions which resemble natural exposure.

3. Immune responses to antigen P_1

The results in Chapter III clearly demonstrate that mite allergic individuals produce IgG, IgA and IgE antibodies to antigen P_1 in their serum and nasal secretions. The role of IgE ab in causing symptoms is well established, but comparatively little is known of the functional significance, if any, of the other classes. Traditionally ascribed 'blocking' roles for both IgG and IgA ab are not borne out by the present studies (Chapter III). On the other hand, more recent functions attributed to non-reaginic, allergen-specific ab, such as immune exclusion via IgA ab (Stokes, Soothill and Turner, 1975) and mediation of symptoms via heat stable, 'short term sensitizing' IgG ab (Parish, 1970; Bryant, Burns and Lazarus, 1973, 1975; Pepys et al, 1979) deserve consideration. Secretory IgA is certainly involved in the exclusion of 'high dose' antigens, e.g. virus particles, bacteria and bacterial enterotoxins at mucosal surfaces (Tomasi and Bienenstock, 1968; Heremans, 1974). At this level, antigen uptake is prevented by the formation of immune complexes which are then cleared or degraded in the mucous (Walker and Isselbacher, 1977). For high dose antigens these functions appear to be common to both allergic and non-allergic individuals. It is not clear whether exclusion of 'low dose' inhalant allergens could operate in a similar manner. Non-allergic adult individuals do not produce allergen-specific IgA ab in either their serum or nasal secretions (Chapter III). Moreover, the quantities of IgA ab produced by allergic individuals are very low. Thus the present results suggest that allergen exclusion by IgA ab is unlikely, although it is possible that measurements of IgA ab in serum and secretions do not accurately reflect the local concentration of IgA ab in the sub-mucosa.

'Short term sensitizing' antibodies have been implicated in allergic disease because they can passively sensitize human or monkey skin for 2-4 hours. They are thought to be of the IgG_4 sub-class (Stanworth and Smith, 1973) and it has been reported that IgG ab to pollens, castor bean and mites is predominantly IgG_4 (Devey and Panzani, 1975; Van der Giessen et al, 1976; Etievant et al, 1979). The antigen binding assay could not be used to measure IgG sub-class antibodies to antigen P_1 because it is difficult to obtain sufficient quantities of sub-class specific antisera. It is possible that allergen-specific IgG ab responses show sub-class restriction, though as yet there is no direct evidence that 'short term sensitizing' antibody causes symptoms in either mite or pollen allergy.

Interestingly, it has not been possible to demonstrate precipitins to antigen P_1 in mite allergic patients' sera. At first, it was assumed that this reflected the low concentration of allergen-specific ab, though some workers claimed to detect anti-mite precipitins using micro-immunodiffusion techniques (Holford-Strevens et al, 1970; Romagnani et al, 1973). Some sera contained large quantities of IgG BA and IgE BA for antigen P_1 (5-15,000 units/ml, Chapter III), equivalent to 1-10µg antibody, but consistently failed to precipitate on immunodiffusion or Laurell electrophoresis against different concentrations of antigen P_1 or crude mite extract. Furthermore, several preparations of immunosorbent purified human anti-mite ab, containing 20-100,000 units IgG BA/ml, also failed to precipitate. These results suggest that the reasons for 'non-precipitation' are more fundamental than low ab concentration. Very low dose immunization, without adjuvant, might result in a

restricted ('oligoclonal') IgG and IgE ab response, directed against only a few determinants on the allergen molecule. This also implies that the IgG and IgE antibodies are directed against the same determinants. The size of antigen P_1 (24,000 daltons) is consistent with having few antigenic determinants, assuming 1-5 determinants per 10,000 daltons for a typical protein (Crumpton, 1975; Arnon and Geiger, 1977). In such circumstances, the antigen-antibody reaction would lead to the formation of linear complexes, without the cross-linkage required for precipitation. This possibility could be tested using monoclonal antibodies to different determinants on antigen P_1 to inhibit the binding of the allergen to human antibodies.

Marsh (1975) has proposed that the ability to recognise limiting low doses (ng) of environmental allergens is crucial to the development of allergy. The effect of antigen dosage is exemplified by the results in Chapters III and IV showing that atopic individuals differed from non-atopic individuals in their response to inhalant allergens, but not in their response to systemic immunization with protein antigens. Low dose immunization is also necessary for the production of IgE ab responses in experimental animals. The classic animal model for IgE ab production is that devised by Levine and co-workers (Levine and Vaz, 1970; Vaz, Vaz and Levine, 1971). They showed that certain strains of mice, e.g. SWR, C57BL and C_3H produced persistent IgG and IgE ab responses to ovalbumin when immunized with 0.1-1µg antigen in alum, but not at higher doses (10-100µg). Other mouse strains, e.g. A/J, CBA/J failed to produce persistent IgE ab responses whatever antigen dose was used, though transient responses were obtained at the higher

doses. Apart from antigen dose, the other features of the mouse model that relate to human allergy are that the same strains tend to produce IgE ab and that IgG ab is always produced in parallel with IgE ab (Prouvost-Danon et al, 1977). There is a clear analogy between the production of IgG, IgA and IgE ab to allergens in atopic but not in non-atopic humans and the 'high responder' and 'low responder' mouse strains. In man, the genetic factors related to atopy are thought to include gene(s) controlling total serum IgE (not linked to the major histocompatibility complex - HLA); specific HLA-linked immune response (Ir) genes; and antigen non-specific 'generalized hyper-responsiveness' (Levine, Stember and Fotino, 1972; Bazaral, Orgel and Hamburger, 1974; Marsh, Bias and Ishizaka, 1974; Marsh et al, 1980). The excellent quantitative correlation between IgG and IgE ab to major inhalant allergens (Chapter III) suggests that the putative Ir genes control the production of all three classes of ab, in parallel. Recent work on ab responses to the minor ragweed allergen, Ra_3, illustrates the relationship between allergen dosage and exposure and genetic factors. Compared to antigen E, Ra_3 is a minor component of ragweed pollen and only 60% of antigen E sensitive individuals become allergic to Ra_3. However, the ab response to Ra_3 is similar to that of the major allergen. It includes both IgG and IgE ab and can only be boosted (following desensitization) in skin test positive individuals (Platts-Mills, Chapman and Marsh, 1981).

Genetic differences between atopic and non-atopic individuals must, in part, be expressed at the cellular level, though the exact mechanisms involved remain unclear. Most cellular studies in man have involved the

use of allergen-stimulated peripheral blood leucocytes either in histamine release experiments or in lymphocyte transformation experiments. There have also been studies enumerating different cell types in allergic and non-allergic individuals. Preliminary experiments have shown that antigen P_1 will induce histamine release from the leucocytes of mite allergic subjects, but not from those of non-allergic controls. These results are in keeping with the behaviour of antigen P_1 in *in vivo* challenge tests and provide further evidence that the properties of antigen P_1 are consistent with those of other major allergens. Although the histamine release assay provides a useful method for monitoring cellular reactivity, e.g. during desensitization (Discussion, Chapter III), it obviously cannot be used to assess the role of non-histamine containing cells and does not necessarily reflect any immunological function.

Allergic reactions are the result of complex interactions between several cell types, including T and B lymphocytes, basophils, eosinophils and mast cells. The results of lymphocyte transformation experiments, using pollen extracts (Brostoff and Roitt, 1969; Black et al, 1976) and mite extracts (Romagnani et al, 1973; Ricci, Biliotti and Romagnani, 1975); suggest that allergic individuals have allergen-specific, sensitized T cells in the circulation. There is also good evidence that IgE production in experimental animals is under T cell control (Tada, 1975; Ishizaka, 1976). The relationship between T cell function and atopy is still disputed. Katz (1978, 1980) has proposed that IgE production in non-allergic individuals is actively suppressed by T cells. He suggests that in allergic individuals the suppressor mechanism fails, possibly as a result of environmental influences, e.g. viral infection,

resulting in "allergic breakthrough"; a concept based only on changes in IgE production. Alternatively, it has been suggested that since antibodies to inhalant allergens are produced locally, in lymphoid tissue draining the nasal mucosa, that atopic individuals have highly sensitive local immunity (Platts-Mills, 1979). This is thought to be a function of improved antigen recognition by T cells or macrophages, particularly for the low doses of inhalant allergens that are encountered during natural exposure. The concept of improved local immunity is supported by the present studies (Chapter III). It also takes into account recent work suggesting that there is extensive co-operation between T cells, basophils and mast cells (Askenase, 1979). Hay fever subjects show increased recruitment of mast cells and basophils to the nasal mucosa during the pollen season (Okuda and Otsuka, 1977; Hastie, Heroy and Levy, 1979) which could be influenced by chemotactic factors produced by T cells.

One of the aims of investigating cellular aspects of allergy is to develop better methods of controlling IgE ab responses for therapeutic purposes. Several years ago it was suggested that local desensitization might be a better form of treatment than the systemic administration of allergen (Taylor and Shivalkar, 1972). There have been preliminary reports that local desensitization is successful in treating hay fever (Johansson, Deuschl and Zetterstrom, 1979), though few direct comparisons with conventional desensitization have yet been made. The results in Chapter III indicate that even if locally applied allergen substantially increased IgG and IgA ab production in the nose, these antibodies would probably still be ineffective in 'blocking' the interaction between allergen and local histamine containing cells. Local desensitization

is more likely to operate via a cellular mechanism, e.g. by priming local 'suppressor T cells' or by reducing the reactivity of mast cells and basophils in the mucosa, than by inducing significant changes in ab production. There was no evidence from the present studies that systemic desensitization caused suppression of IgE ab responses. However, experiments in animals have shown that the administration of urea denatured allergens or allergen-hapten conjugates can inhibit IgE ab production (Ishizaka, Okudaira and King, 1975; Liu and Katz, 1979; Kudo et al, 1978). It has also been shown that IgE B cells can be made tolerant by giving allergens linked to high molecular weight carriers, e.g. polyethylene glycol (Lee and Sehon, 1977). These procedures are critically dependent on the immunization schedules used and there is little evidence that they can be used to suppress IgE responses which have been established for 1-2 months. Unless the latter situation can be achieved, it remains doubtful whether any of these treatments will be of practical use in man.

It is clear that further studies on the cellular basis of immediate hypersensitivity in man will require better assessments of functional activity in vitro. Recent studies have shown that the minute quantities of IgE produced by peripheral blood lymphocytes in vitro can be measured using highly sensitive radioimmunoassays (Urena et al, 1978; Fiser and Buckley, 1979; Tjio et al, 1979; Saxon and Stevens, 1979). It will now be possible to use antigen P_1, and other purified allergens, to induce IgE ab production in vitro to determine whether the controls on IgE production observed in animals are relevant to man. The improvements in cell separation techniques

that have occurred during the last decade will facilitate studies on cellular co-operation using purified populations of T cells, B cells, basophils and eosinophils. These studies also offer the opportunity to develop a functional assessment of allergen-mediated T cell reactivity which can be used in clinical investigations of allergic disease.

In conclusion, antigen P_1 can be used as a model for the investigation of mite allergy and house dust allergy. The present studies have concentrated on the properties of antigen P_1 which are of allergenic importance and on quantitative assessments of antibody responses to antigen P_1 in different patient groups. Preliminary work suggests that antigen P_1 will be useful in assessing allergen exposure, in designing suitable allergen avoidance regimes and in cellular studies on the control of IgE production. In this respect, one of the main advantages of antigen P_1, compared with pollen allergens, is that it is an allergen suitable for studying the aetiology of perennial rhinitis, asthma and atopic dermatitis in parallel.

A P P E N D I X A.

A.1 Classification of Mites

The order Acarina comprises all the families of mites (Fig. A.1.1). The most potent allergens have been associated with the house dust mites and the 'stored food' mites. The distribution of house dust mites is discussed in the General Introduction. Storage mites usually infest grain, flour, cereal products and animal foodstuffs kept in damp conditions. They are also found in old hay and in barn dust and some species often occur in house dust (including bedroom and mattress dust). The Cheyletidae or 'predator' mites are large mites ($\sim$ 600μ in length) which feed on other species present in house dust. They are not considered to be allergenic. In addition to the prominent allergens the order Acarina contains many more species of medical, horticultural and veterinary importance, including the scabies mite, the red spider mite (an orchard pest) and the chicken mite.

A.2 Preparation of reagents

A.2.1 Rabbit antiserum to D.pteronyssinus

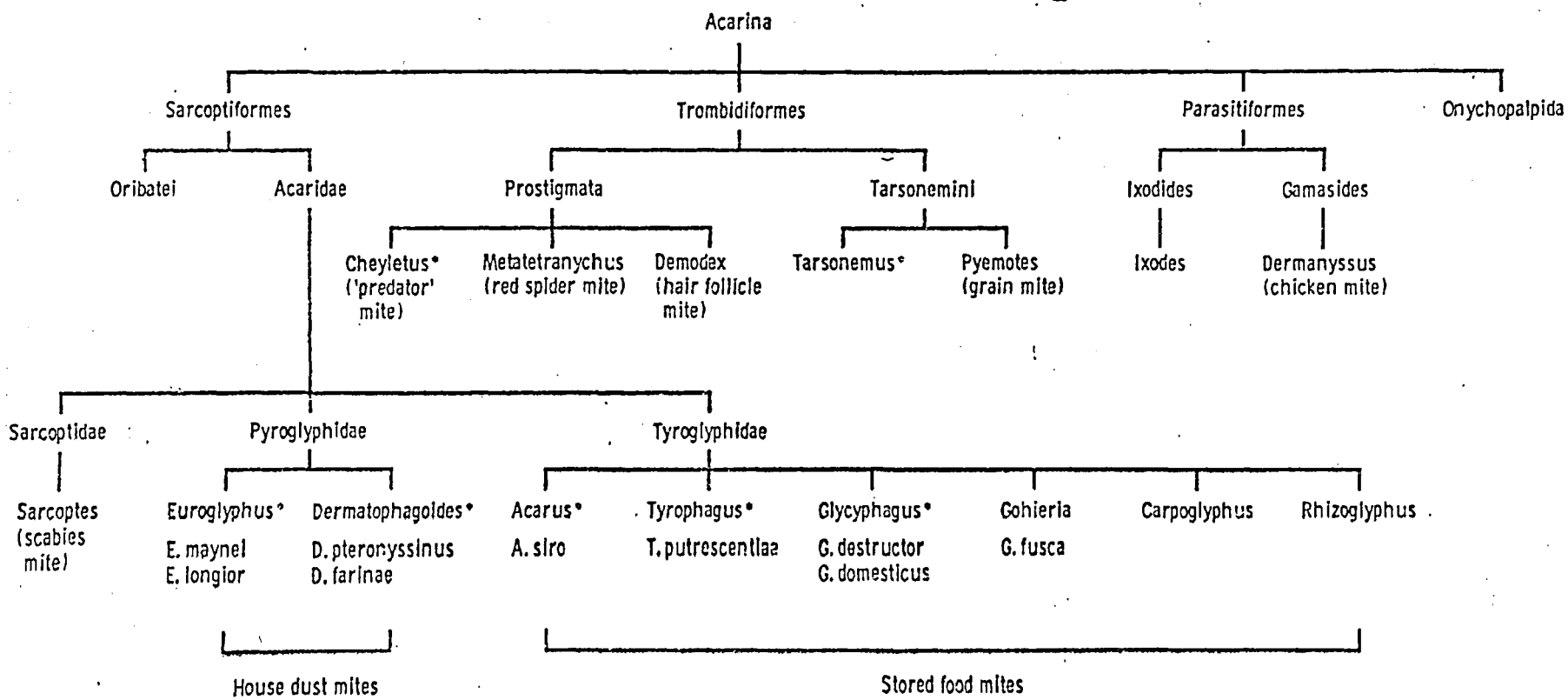
Two New Zealand White rabbits were injected intramuscularly with 1mg D.pteronyssinus Extract I in complete Freund's adjuvant (Difco Labs, Detroit, Michigan, U.S.A.) at fortnightly intervals. The rabbits were bled eleven days after the fifth injection and the serum (rabbit anti-ExI) was separated and stored at -20^{o}C.

A.2.2 Class specific antisera for use in antigen-binding radioimmunoassays

The specificity of antigen-binding assays is determined by the use of antisera directed against immunoglobulin heavy chains. These reagents

Figure A.1.1

Classification of the order Acarina



* Genera commonly found in house dust

This classification is based on that of Voorhorst, Spieksma and Varekamp (1969)

are best prepared using Fc fragments or purified myeloma proteins as immunogens (Platts-Mills and Ishizaka, 1975; Platts-Mills et al, 1978). The preparations sometimes contain trace amounts of impurities or irrelevant antigens, e.g. light chains which can be recognised by an animals immune system, particularly when immunized in adjuvants. The resulting antisera usually contain small quantities of antibodies directed against contaminating determinants and these have to be absorbed out. Antigen-binding assays, especially for the IgG class, require substantial quantities of antiserum; of the order 0.05-0.2ml per assay. High titre antisera are conveniently prepared by immunizing a large mammal with antigen emulsified in complete Freund's adjuvant.

For the present studies goats were immunized with the Fc fragment of IgG from normal serum; IgA myeloma protein; or IgE myeloma protein (PS). The Fc fragment was prepared by papain digestion (Porter, 1959) using human IgG (Lister γ-globulin, Elstree, Herts) which had been purified by DEAE ion-exchange chromatography. The Fc and Fab fragments were separated from undigested IgG by gel filtration and isolated in pure form by pevikon block electrophoresis. The purified IgA and IgE myeloma proteins were already available within the laboratory. Three goats were injected intramuscularly with 0.2-1.0mg antigen emulsified in complete Freund's adjuvant at fortnightly intervals. Eleven days after the third injection the goats were test bled and the antisera checked for specificity and titre. Each animal was bled 200-400ml at

2-3 weekly intervals and boosted periodically until hyperimmune. The antisera were made monospecific by passage over immunosorbent columns. Anti-IgG was absorbed with Fab, IgA and IgE. Anti-IgA was absorbed with serum from an IgA deficient patient whose serum contained <0.01µg IgA/ml (Cassidy, Oldham and Platts-Mills, 1979). Anti-IgE was absorbed with normal human IgG.

A.2.3. Immunosorbents

Immunosorbents were prepared by coupling the proteins to Sepharose-2B using the cyanogen bromide technique (Axen, Porath and Ernbach, 1967). The reaction betweeen CNBr and the polysaccharide matrix of Sepharose produces reactive imidocarbonates which form covalent bonds with protein amino groups (Axen and Ernbach, 1971). The protocol for cyanogen bromide activation is given below:-

(i) 20ml Sepharose-2B (Pharmacia, Uppsala, Sweden) was washed three times (2,000g x 10 mins.) with 20ml distilled water and poured into a 100ml beaker.

(ii) 1g CNBr (Eastman-Kodak, Liverpool, U.K.) was weighed out on a pan balance in a fume cupboard, and transferred to a 50ml flask containing 20ml distilled water. The flask was stoppered and the solution was stirred until all the CNBr had dissolved (about 20 mins.).

(iii) The CNBr solution was poured into the beaker containing the washed Sepharose and stirred gently. The pH was monitored immediately and maintained at pH 10-11 by adding drops of 2MNaOH as the activation proceeded.

(iv) After 10 mins the activated Sepharose was transferred into a sintered glass funnel on a side-arm vacuum flask and washed with 15 vols. (i.e. 300ml) distilled water followed by 15 vols. 0.1M carbonate-bicarbonate buffer pH 9.0. The Sepharose was then air-dried to a moist cake and transferred to 20ml universal tubes.

(v) A solution of protein in 0.1M carbonate-bicarbonate pH 9.0 was added to the activated Sepharose and rotated overnight at $4^{o}C$ on a vertical rotator. Between 5-50mg of protein was generally used. Extra buffer was added to ensure that the mixture remained fluid while being rotated.

(vi) The coupled Sepharose was transferred to the sintered glass funnel and washed with 100ml volumes of 0.1M borate buffer pH 8.5, 0.1M acetate buffer pH 4.1 and tris-HCl pH 8.0. The borate and acetate buffers also contained 1M sodium chloride. The immunosorbent was poured into a column and washed with 200ml BBS prior to use.

N.B. Cyanogen bromide is extremely poisonous and steps (ii) to (iv) were carried out in a fume cupboard. Washings containing cyanide residues were stored in strong sodium hypochlorite solution ('Chloros' Durham Chemical Distributors, Birtley, Co. Durham) for one week before being washed down the sink.

The pH titration step (iii) can be omitted by using CNBr dissolved in acetonitrile (2g CNBr/ml acetonitrile) (March, Parikh and Cuatrecasas, 1974).

A.2.4. Purified antibodies to D.pteronyssinus

The use of immobilized antigen to prepare specific ab by 'immuno-adsorption' was first described by Campbell, Leuscher and Lerman (1951). The method involves the adsorption of antiserum onto antigen coupled to a solid matrix, e.g. agarose or cellulose. After washing to remove non-specifically bound protein the specific ab is eluted using buffers at extremes of pH or high ionic strength salt solutions. The principle of the technique has been used in other systems involving a specific interaction between a ligand and its combining site, e.g. enzyme-substrate or hormone-receptor and is commonly known as affinity chromatography (Cuatrecasas and Anfinsen, 1971).

Rabbit ab to ExI was prepared by passing 20ml hyperimmune anti-serum over a 25ml column of ExI immunosorbent at a flow rate of 10-15 ml/hr. The column was washed with 200ml BBS and then eluted with 100ml 0.5M glycine-NCl pH 2.8. Fractions of 5ml were collected in tubes containing 1ml 2M tris-HCl pH 8.5 and mixed immediately to neutralize the pH. The fractions were pooled on the basis of protein content (absorbance at 280nm) and concentrated by negative pressure dialysis.

Human ab to ExI was prepared using 220 ml serum from several different allergic donors. The serum from each donor was equilibrated in 0.1M ethylenediaminetetracetic acid and passed over a 2.5ml column. The eluted fractions were assayed for IgEab by antigen binding assay, pooled and concentrated down to 1ml. In each case $\sim$ 20% of the applied ab was recovered.

A.2.5. Iodination of proteins

Both antigen binding assays and the RAST rely on the use of proteins labelled to high specific activity with ^{125}I. This isotope is preferred because of its long half life (60 days) high isotopic abundance and greater counting efficiency (Hunter, 1978). It is a lower energy γ-emitter than ^{131}I and hence is much safer for routine use in the laboratory. Most proteins can be labelled directly with ^{125}I by a substitution reaction in which iodine atoms replace hydrogen atoms in the aromatic ring of tyrosine groups. Free iodine is generated by the oxidation of carrier-free isotope (Na ^{125}I) using chloramine T. The reaction is stopped by adding either a reducing agent e.g. sodium metabisulphite or a saturated solution of tyrosine. Milder labelling procedures have been developed for proteins which may be denatured by chloramine T (a powerful oxidising agent). These include enzymic oxidation using lactoperoxidase (Morrison, Bayse and Webster, 1971), and direct substitution using chloroglycouril instead of chloramine T (Markwell and Fox, 1978). Proteins which lack tyrosine can be labelled indirectly using the Bolton-Hunter reagent; the N'hydroxysuccinimide ester of p-hydroxyphenyl propionic acid. This ester is labelled independently with chloramine T and is subsequently condensed with protein amide groups (Bolton and Hunter, 1973).

The procedure for radiolabelling with chloramine T used in these studies was developed by Dr. T. Kishimoto as a modification of the technique described by Klinman and Taylor (1969). The reaction was started by the addition of 40μg freshly prepared chloramine T to a reaction mixture containing 10-50μg protein (i.e. 10-50μL of a 1mg/ml

solution), 0.05ml 0.3M phosphate buffer pH 7.5 and 1.0mCi Na ^{125}I in a volume of <0.2ml. After 65 seconds the reaction was stopped by adding 40µg sodium metabisulphite and three minutes later the reactants were diluted with 2ml 0.4% bovine serum albumin in BBS (BSA BBS) and 0.05ml 1% potassium iodide. Unbound iodine was separated from the labelled material by dialysis against two changes of BBS over 40 hours. Estimates of the labelling efficiency were obtained by comparing the counts per minute (cpm) of the dialysis fluids and those of the labelled protein as a percentage of the total radioactivity. The specific activity of the labelled proteins is given in Table A.2.5.1. The specific activities of some of the allergens, e.g. Rye I and Phospholipase A were lower than for hormones with similar molecular weights, e.g. growth hormone, which can have specific activities of >100µCi/µg protein. However, the quantities of allergens used for labelling (10-50µg) were much higher than those normally used for hormones (1-2µg) and when smaller quantities were used higher specific activities were obtained, e.g. 10µg P_1 had a specific activity of 67.1µCi/µg. Heavily iodinated proteins, with a mean number of >2 atoms ^{125}I/molecule, generally show reduced immunoreactivity and are more likely to be affected by radiation damage (Bolton, 1977). The ^{125}I substitution values for the proteins described here were well below the limits at which this type of damage might become a problem.

TABLE A.2.5.1

Radiochemical properties of ^{125}I-labelled proteins[a]

Protein (M.W.)	Radioactivity incorporated into protein %	μCi ^{125}I per μg protein	Mean number[b] of ^{125}I atoms per molecule	cpm/ng protein[c]
F_4P_1 (24,000)	53	35.4	0.38	43,000
Antigen P_1 (24,000)	67	67.1	0.74	78,500
Rye I (27,500)	60	11.9	0.15	10,400
Phospholipase A (19,500)	85	21.3	0.19	24,150
Horse Allergen No.6 (19,000)	70	17.3	0.15	24,000
Horse Allergen No.11 (50000)	35	34.5	0.82	40,600
Diphtheria Toxin (72,000)	80	26.7	0.86	23,300
Anti-IgE (150,000)	88	22.1	1.56	32,500
IgE (ND) (190,000)	84	13.9	1.22	18,400

Footnotes to Table A.2.5.1

(a) All calculations were made on the third day after labelling (not corrected for decay).

(b) Assuming 100% isotopic abundance.

(c) From 85-90% of the radiolabelled material was precipitable with trichloroacetic acid.

A.3. Electrophoretic techniques

A.3.1. Pevikon block electrophoresis

The main advantages of Pevikon (a copolymer of polyvinyl chloride and polyvinyl acetate) over other supporting media, e.g. starch for electrophoresis are that it causes low electroendosmotic effects and does not interfere with assays for proteins or carbohydrates (Müller-Eberhard, 1960). Full details of the preparation of pevikon and the apparatus used in this study are given by Campbell et al (1970). Briefly, 500g Pevikon C-870 (Shandon Southern Ltd., Runcorn, Cheshire) was washed three times in 4L distilled water and once in 4L 0.1M barbital buffer, pH 8.6. The pevikon was resuspended in ~ 200ml buffer and the thick slurry was poured into a 50 x 10 x 1cm electrophoresis frame covered with plastic sheeting. Surplus buffer was absorbed with thick wads of paper towels and excess pevikon was removed with a wide spatula. The block was trimmed so that a flat, level surface was obtained. Absorbent cotton wicks (10 x 20cm) were applied to each end of the block and secured with damp pevikon. The ends of the plastic sheeting were folded over the pevikon and the block was equilibrated in buffer for 30 minutes prior to use.

Application of sample A 1cm segment of pevikon was removed to form a trough 10cm from the cathodic end of the block. Approximately 30mg of mite fraction 4 (F4 - Chapter I) in 5ml barbital buffer was mixed with washed, dry pevikon and filled in the trough. The sample was electrophoresed for 26 hours at 260V and 50mA at 4°C. The block was then divided into 1cm segments which were eluted twice

with 5ml BBS. The eluates were pooled on the basis of their protein content (Lowry et al, 1951) and reactivity on immunodiffusion against rabbit antiserum to crude mite extract.

A.3.2. Laurell Electrophoresis

Crossed immunoelectrophoresis (Laurell, 1965) has been widely used to analyse allergen extracts. The technique involves two dimensional electrophoresis in agarose, with the second dimension gel containing antiserum directed against the whole extract. During electrophoresis the antigen-antibody complexes form precipitin arcs at equivalence which may be visualized using conventional protein staining techniques. The method is outlined in Fig. A.3.2.1. Semi-quantitative assessments of the concentration of individual components can be made by comparing the area beneath the precipitin arcs with that of a known standard (Axelson, Kroll and Weeke, 1973). Modifications of the technique have been developed for allergen assay and to make qualitative assessments of allergen-specific IgE (see Lowenstein, 1978).

Protocol

Twenty millilitres 1% agarose (Indubiose A37, L'Industrie Biologique Francais, Clichy, France) in 0.1M barbital buffer pH 8.6 (the electrophoresis buffer) was poured onto a clean 8 x 8 cm glass plate to form a gel 1-2 mm thick. Allergen extracts were applied to sample wells cut 1.0 cm from the cathode and 1.5 cm apart. Following electrophoresis in the 1st dimension (see Fig. A.3.2.1. for details) each gel segment containing separated allergens was transferred to

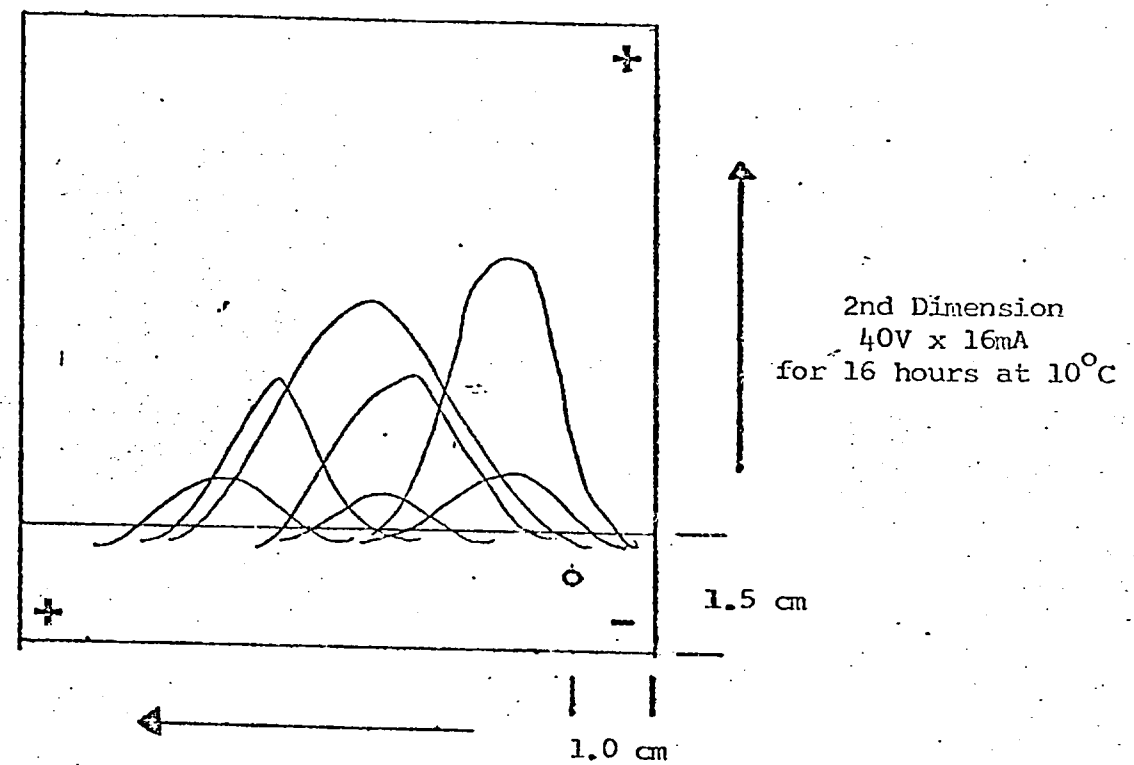


Figure A.3.2.1 Laurell Electrophoresis

another 8 x 8 cm glass plate coated with 'sticking agar'. Twelve millilitres 1% buffered agarose at 56°C was carefully mixed with 0.1ml rabbit anti-ExI (so that the formation of air bubbles was avoided) and poured onto each plate on a level surface. When the gels had set (after about 10 mins.) electrophoresis in the 2nd dimension was carried out. The gels were then dried and stained with Coomassie Blue R-250.

The technique was modified to include isoelectricfocusing in the first dimension ('immunoisoelectricfocusing'). Analytical IEF in thin layer polyacrylamide gel was carried out as described in Appendix B.1.1. The 0.5 cm strip of IEF gel containing F_4P_1 was transferred to a trough cut into a pre-formed second dimension gel (Fig. A.3.2.2.). The IEF strip was secured by filling the trough with 1% buffered agarose.

A.4. Methods for measuring allergen-specific antibodies

A.4.1. Antigen binding radioimmunoassays

Specific IgG, IgA and IgE ab to allergens were measured by double antibody antigen binding radioimmunoassays based on the technique used for measuring class-specific ab to diphtheria toxin (Platts-Mills and Ishizaka, 1975). All the assays followed a similar protocol to that outlined in Chapter I. The partially purified allergen F_4P_1 was used for measuring ab to D.pteronyssinus. Serum dilutions were incubated with 7ng ^{125}I-labelled F_4P_1 for 4 hours at room temperature. Specific ab was precipitated overnight at 4°C by adding monospecific goat antisera. The precipitates were washed four times with BBS and counted in a γ-counter (NE 1600, Nuclear Enterprises Ltd. Edinburgh, U.K.). All assays were performed in plastic tubes (75 x 12mm) coated with

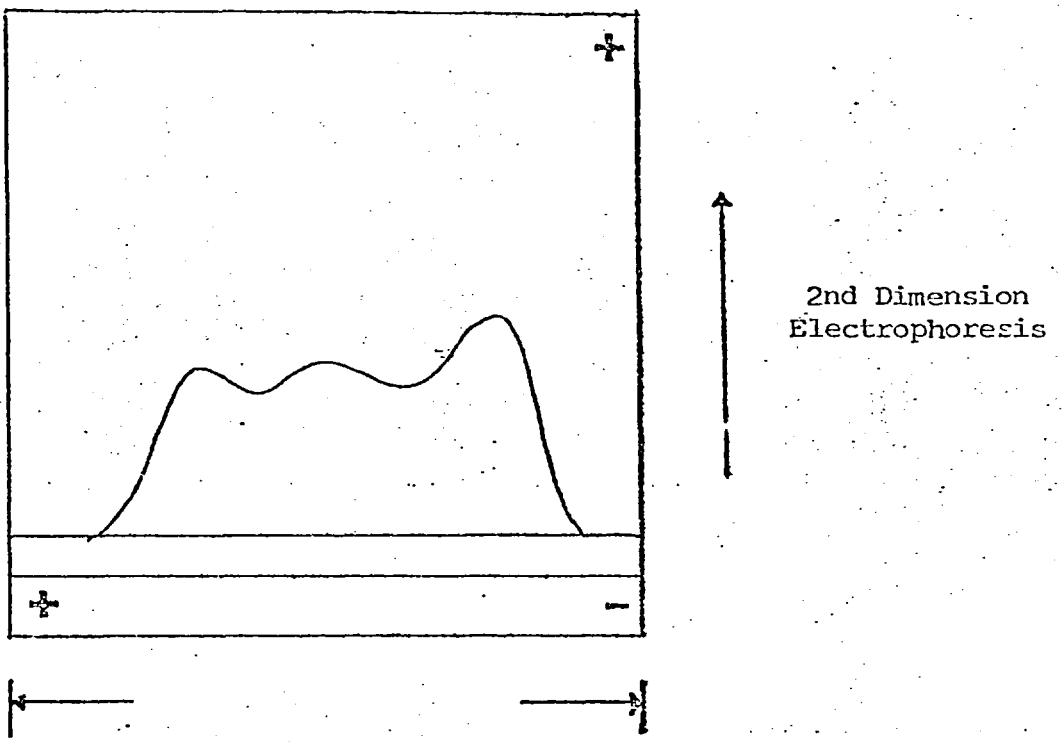


Figure A.3.2.2 'Immunoisoelectricfocusing'

The IEF gel was placed in a trough cut 0.8 cm from the cathode. The second dimension agarose gel consisted of 20ml 1% agarose and 0.1ml rabbit anti-ExI in 0.1M barbital buffer, pH 8.6. Electrophoresis at 40V x 16mA was carried out for 16 hours at 10°C.

BSA to reduce non-specific binding. For coating, tubes were filled with 2% BSA BBS, emptied and drained on paper towels until dry. They could be stored dry at room temperature for up to 6 months prior to use.

The quantity of antiserum necessary to form a precipitate was estimated by a semi-quantitative precipitin test (Campbell et al, 1970). Carrier immunoglobulins were added to samples containing insufficient immunoglobulin to form a precipitate. The carrier protein for the IgE assay was 0.1ml of a 1:100 dilution of PS myeloma serum (19.5 mg/ml IgE). For the IgA assays 0.1ml of a 1:50 dilution of an IgA myeloma serum was used. In general, IgG carrier (0.1ml of a 1:100 dilution of IgG myeloma serum) was necessary only for the control curve and for sera diluted more than 1:50. The radioactivity bound by precipitates of carrier immunoglobulins was <0.4% of the radioactivity added.

Calculation of results

Initially, sera from mite allergic patients were screened to find samples which bound a high proportion (>60%) of ^{125}I-labelled F_4P_1. A pool of serum from three patients who had been desensitized with aqueous D.pteronyssinus extract could bind 68% of the radioactivity added. This pool (control serum) was arbitrarily defined as containing 13,000 units IgG binding activity (BA) for F_4P_1/ml. Serial two-fold dilutions of the control serum from 1:100 to 1:51,200 were assayed for IgG BA and the results (control curve) were plotted on a log-linear scale. The radioactivity bound by serum samples was converted to values for IgG BA by reference to this control curve set up in parallel with each assay

(Fig. A.4.1.1.). Assays for IgG BA were performed using 0.1ml serum diluted 1:12.5, 1:25 and 1:50. Results were calculated as the mean of the three values. The quantities of IgE BA (and IgA BA) were lower than for IgG BA. However, the dilution curves for IgE BA could be superimposed on those for IgG BA (Fig. A.4.1.2.), and therefore quantitative results for all three classes were obtained by extrapolation from the IgG BA control curve. Assays for IgE and IgA BA were carried out using lower dilutions of serum (1:6, 1:12.5 and 1:25). Sera containing high levels of BA were diluted further so that >80% of the radioactivity was not bound by any class of ab, i.e. the antigen excess was at least four-fold. Four sera from non-allergic persons were tested in each assay to define the negative background. A result two standard deviations above this background was regarded as positive.

The antigen binding assays for measuring ab to Rye I, phospholipase A (PLA) and diphtheria toxin were based on those described by Platts-Mills et al (1978), Sobotka et al (1976) and Platts-Mills and Ishizaka (1975). I am very grateful to Dr. D. Marsh for providing Rye I and to Professor M. Lessof for providing PLA. The assays for ab to horse allergens No.6 and No.11 (kindly provided by Dr. H. Löwenstein) and to β-lactoglobulin (BDH, Poole, Dorset) were developed using 10ng, 5ng and 3ng of each ^{125}I-labelled allergen respectively. A high proportion ($\sim$5%) of the ^{125}I-labelled β-lactoglobulin bound non-specifically to the plastic assay tubes, thus reducing the sensitivity of the assay. This background was reduced to <0.4% of the radioactivity added by transferring each precipitate to another tube after the second wash.

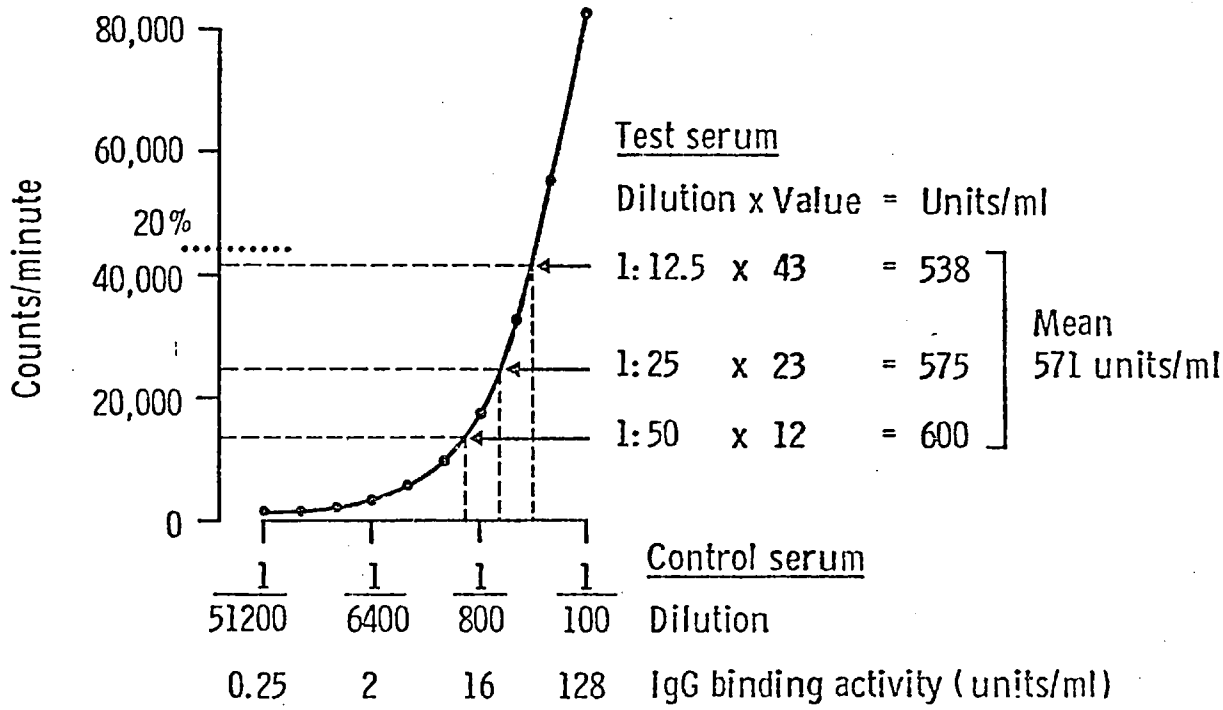


Figure A.4.1.1

Control curve for IgG BA for F_4P_1. Serial dilutions of the control serum containing 13,000 units IgG BA/ml were assayed using 7ng ^{125}I-labelled F_4P_1 (208,000 cpm ^{125}I) and anti-IgG to precipitate (o——o). The dashed lines show the radioactivity bound by 3 dilutions of a test serum and the method of extrapolation used to obtain units of BA. Also shown is the value representing ~20% binding of the added F_4P_1 (••••). Values below this level lie within the useful limits of the assay (i.e. there is >4-fold antigen excess). The IgG BA of a 1:12.5 dilution of the test serum lies just within the 20% limit. At lower dilutions, e.g. 1:6 values for IgA BA or IgE BA would be less reliable and might be underestimated because the overall antigen excess would be <4-fold. This control curve would be obtained soon after labelling F_4P_1. Because the quantity of antigen added is constant the counts will fall progressively, i.e. 40,000 cpm bound after 60 days, 20,000 cpm bound after 120 days, etc. The labelled allergens can usually be used for 6 months though the sensitivity gradually becomes reduced.

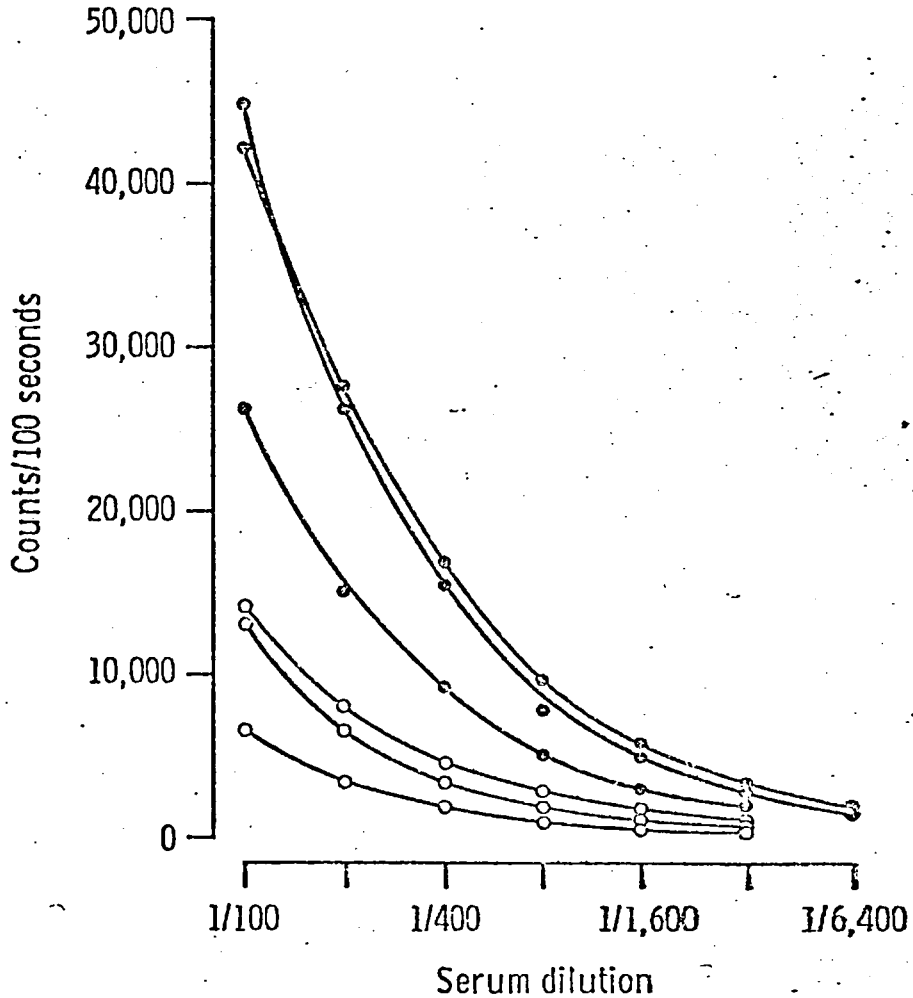


Figure A.4.1.2

Radioactivity bound by dilutions of three sera from mite-allergic persons precipitated by anti IgG (o——o) or anti IgE (o——o). 7ng ^{125}I F_4P_1 were added to each tube.

A description of the control sera used as Standards in antigen binding assays is given in Table A.4.1.1.

Accurate measurements of BA can only be obtained if the antigen excess over all classes is at least four-fold. It was difficult to maintain antigen excess in the β-lactoglobulin and diphtheria toxin assays because of the high levels of IgG ab. These assays were therefore repeated on serum fractions which were enriched for IgG or IgE to ensure that measurements of IgE BA were not underestimated. The fractions were obtained by precipitation with 33% and 33-50% saturated ammonium sulphate (SAS) (Carson, Metzger and Bazin, 1975). Briefly, 0.2ml serum was diluted with 0.2ml BBS, precipitated with 0.2ml SAS and stirred vigorously on a vortex mixer. After 30 mins. the precipitate (33% cut) was removed by centrifugation (3,000g x 15 mins.) and resuspended with 0.4ml BBS. The supernatant was mixed with a further 0.2ml SAS to obtain a 33-50% SAS cut. Dilutions of the 33% and 33-50% SAS fractions were used in assays for IgG BA and IgE BA respectively.

The problem of maintaining antigen excess over all classes of ab also arose with some mid- and post-treatment sera from patients who had received desensitizing injections of D.pteronyssinus extract. These sera contained very high levels of IgG BA for F_4P_1 and the ratio of IgGBA:IgEBA could be >20:1. Values for IgE BA were estimated from the RAST results for D.pteronyssinus which were not affected to the same extent by IgG ab (see Chapter III). The RAST results were divided by 2:39 (the mean ratio of RAST:IgE BA in pre-treatment sera) to obtain

TABLE A.4.1.1

Control sera used in antigen binding assays

Assay for ab to:-	Control serum[a]	Estimate of the quantity of IgG ab represented by 1 unit of activity
F_4P_1 (or antigen P_1)	13,000 units BA/ml*	1.1 ng [b]
Rye I	100,000 units BA/ml*	0.2-0.7 ng [c]
Phospholipase A	10,000 units BA/ml*	n.d.
Diphtheria toxin	40 International Units of Antitoxin x 10^{-3}/ml	10 ng [d]
β-lactoglobulin	5,000 units BA/ml	n.d.

(a) Values represent units of IgG binding activity.

(b) Calculated from RAST absorption experiments (Chapter II).

(c) Range of estimates obtained by direct binding, RAST elution and RAST absorption (Platts-Mills et al, 1978).

(d) Based on a serum which had been substandardized against horse antitoxin by toxin neutralization in rabbit skin (Platts-Mills and Ishizaka, 1975).

*Serum obtained from patient(s) who had received desensitizing injections of allergen extracts.

estimated units of IgE BA for F_4P_1. The mean difference between the measured IgE BA and that estimated by RAST conversion was <20% in post-treatment sera from 10 DP and 10 'placebo' patients (Chapter III).

A.4.2. The radioallergosorbent test (RAST)

The RAST is the most widely used in vitro test for measuring IgE ab to allergens. In its original form (Wide, Bennich and Johansson, 1967) allergen-coupled sepharose beads were incubated for several hours with serum then washed extensively and incubated with ^{125}I-labelled anti-IgE. The counts bound by the beads (after further washing to remove excess ^{125}I) were directly related to the concentration of allergen specific IgE in the serum. For most allergens there was a very good correlation between the RAST and in vivo methods of assessing IgE ab, e.g. skin tests and provocation tests (Wide et al, 1967; Berg, Bennich and Johansson, 1971; Bennich and Johansson, 1971). The technique achieved widespread clinical application following the introduction of paper discs as immunosorbent (Ceska, Eriksson and Varga, 1972). Using the disc RAST in microtitre plates large numbers of samples could be processed quickly without loss of sensitivity. Furthermore, the use of a single radiolabelled reagent (anti-IgE) to measure IgE ab to different allergens was ideal for clinical purposes.

The results of the RAST are often expressed semi-quantitatively either as '% counts bound' or on a 1 to 4 plus basis, e.g. 'Phadebas' RAST (Pharmacia, Sweden). This makes it difficult to compare results obtained in different laboratories and reduces the sensitivity of the

assay. The RAST can be quantitated using a control serum given units of RAST activity to form a control curve. Values for unknown sera are extrapolated from the curve and expressed in RAST units/ml. This method gives extremely accurate quantitative results and can distinguish levels of IgE ab in different sera over a range of 2-3 orders of magnitude. Recently, RAST techniques have been developed (for experimental purposes) which allow the absolute amount of allergen-specific IgE to be determined in ng. These methods, RAST absorption (Gleich and Jacob, 1975) and RAST elution (Schellenburg and Adkinson, 1975), involve measuring the IgE either absorbed by or eluting from allergen-coupled sepharose beads.

RAST for measuring IgE antibody

The method of Ceska et al (1972) was used. Paper discs were activated with cyanogen bromide using a modification of the technique described in Appendix A.2.3. 5g pre-swollen paper discs (Whatman Grade 541, 0.6cm diameter) were activated with 5g CNBr in 150ml distilled water, washed with 4L 0.005M sodium bicarbonate followed by 1L acetone and stored in a dessicator until dry. The activated discs can be stored dry at -20°C for up to one year prior to use. 15mg allergen was coupled to about 1000 discs (2-3g) in 25ml 0.1M sodium bicarbonate for 24 hours at 4°C. The discs were incubated with 0.1M ethanolamine for 2 hours, washed successively with 0.1M sodium bicarbonate, 0.1M sodium acetate pH 4.0 and BBS and then dessicated.

The protocol for the RAST is shown in Fig. A.4.2.1. Assays were carried out in the wells of flat-bottomed microtitre plates (Sterilin,

Allergen coupled paper disc

Add 0.1ml serum dilution

Incubate overnight at room temperature

Wash five times with RAST buffer

Add 2ng ^{125}I-labelled anti-IgE (0.05ml)

Wash five times with RAST buffer

Transfer the disc to a plastic tube and
count the radioactivity bound to the disc

Figure A.4.2.1 The radioallergosorbent test

Teddington, U.K.). Sera were routinely assayed at 1:12.5 and 1:25 dilutions or a higher or lower dilutions where necessary. The RAST buffer contained 0.5% sodium azide and 0.5% Tween-20 (Sigma, Poole, Dorset) in 0.2% BSA BBS, pH 7.5. The discs were washed by filling the wells with RAST buffer and then aspirating the supernatant. Anti-IgE was purified from goat antiserum by affinity chromatography using an immunosorbent column linked to pure PS myeloma protein.

The RAST results were quantitated by reference to a control curve analagous to those used for antigen binding assays. The RAST curve was obtained by assaying IgE ab in two-fold dilutions of an allergic serum with high levels of IgE ab. This control serum could bind ~50% of the radioactivity added at a 1:6 dilution and showed significant binding (2-3%) at a 1:6400 diltuion. The control serum was defined to contain 6400 RAST units/ml for both D.pteronyssinus and D.farinae on the basis of the dilution curves (Fig. A.4.2.2). The negative background was defined by the mean level for sera from five non-allergic individuals. Values which were greater than two standard deviations above the mean were regarded as positive. The non-specific binding by control sera was ~0.8% of the radioactivity added.

RAST absorption

RAST absorption was carried out as described by Gleich and Jacob (1975). Dilutions of serum (1:2-1:20) or specific ab (1:200-1:400)

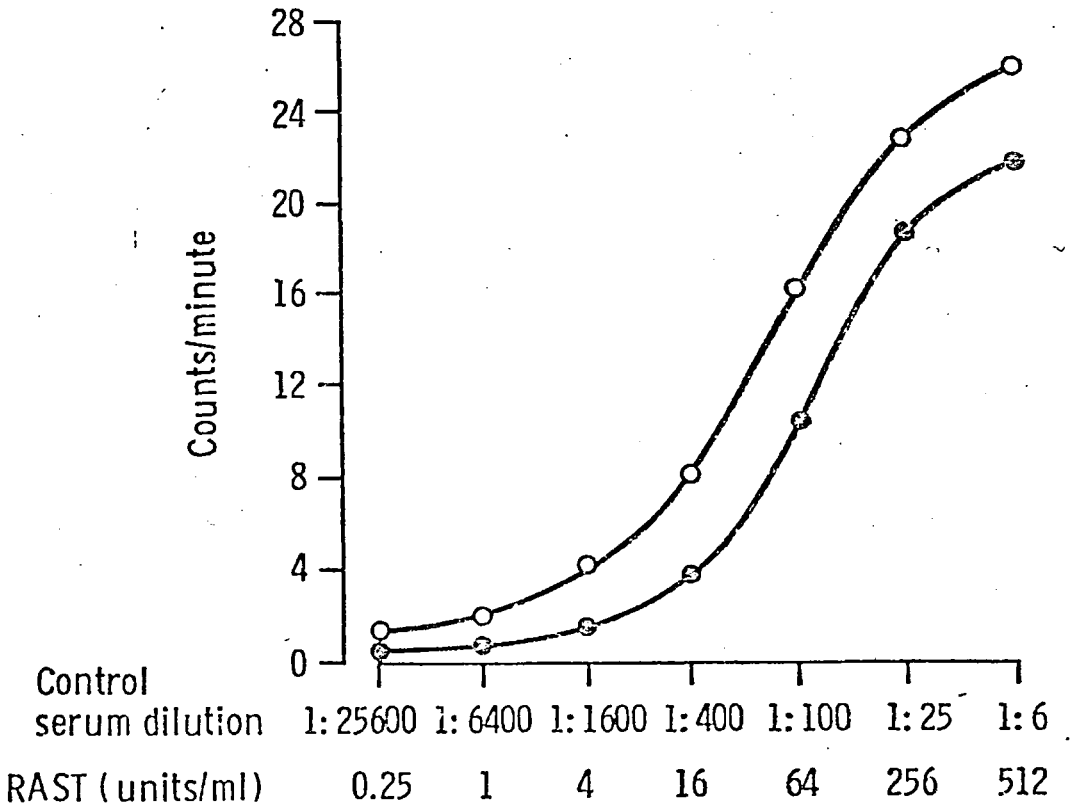


Figure A.4.2.2

Typical RAST control curves. Serial dilutions of the control serum were incubated with RAST discs coupled to crude extracts of either D.pteronyssinus (O——O) or D.farinae (●——●). The quantity of ^{125}I-labelled anti-IgE bound at different serum dilutions and the equivalent value in RAST units/ml is shown. Total radioactivity added = 56,000 cpm.

were absorbed overnight with 0.5ml 2% sepharose beads coupled tc ExI, antigen P_1 (IEF Fr.5) or human serum albumin. The beads were sedimented by centrifugation (2,000g x 15 mins.) and the supernatant was removed and assayed for total IgE (Appendix B.2.).

A P P E N D I X B.

B.1. Preparation and analysis of proteins

B.1.1. Isoelectricfocusing

Isoelectric focusing is a method of separating proteins according to their behaviour on electrophoresis in a pH gradient. Proteins, being amphoteric, migrate until they reach the pH at which their net change is zero and concentrate or 'focus' at that point (the isoelectric point, pI) (Kolin, 1958). The pI is an intrinsic property of a given protein which is exploited by IEF. The technique is dependent on the generation of stable, continuous pH gradients using carrier ampholytes. These molecules should have good buffering capacity, high conductivity and solubility (Svensson, 1962). Other desirable properties include low light absorption at 280nm and low molecular weight. Molecules with such properties were first synthesized as isomers of polyamino-polycarboxylic acids (ampholine) (Vesterburg, 1969). Ampholine has been shown to be non-toxic (Wadstrom et al, 1974) and is, therefore, particularly suitable for use in allergen purification. IEF is an extremely high resolution technique which can separate proteins that differ by <0.01 pI units. It is also the easiest method for determining the pI. The results are usually reported as 'apparent' pI because results may vary slightly with different IEF techniques and may also differ from values obtained by other physicochemical methods.

In these studies both analytical and preparative IEF was carried out using the LKB 2117 Multiphor with a constant wattage power pack (LKB 2103 - LKB-Producktor RB Bromma, Sweden). Carrier ampholine, analytical polyacrylamide gels and Ultrodex were also obtained from LKB.

Analytical IEF

Thin layer polyacrylamide gel IEF (Awdeh, Williamson and Askonas, 1968) was carried out as described by Karlsson et al (1973). Allergen solutions (1-2 mg/ml) were absorbed onto 0.5 x 1.0cm strips of filter paper (Whatman 3M) and applied to the surface of a polyacrylamide IEF gel. This gel was 1mm thick and contained 5% acrylamide and 2.4% ampholine pH 3.5-10. Cellulose electrode strips soaked in 1M NaOH or 1M H_3PO_4 were used to form the cathode and anode respectively. The gel was initially focused at 10W for 15 minutes. The sample strips were then removed and the focusing was continued at 15W for 75 minutes. The gel was stained with 0.5% Coomassic Blue R-250 at 56°C for 20 minutes and destained in ethanol-acetic acid-water (3:1:8).

Preparative IEF

Preparative IEF was carried out in flat beds of granulated gel (Radola, 1969). A gel containing 4.0% (w/v) Ultrodex, 3.5% (v/v) ampholines pH 3.5-10, 0.5% (v/v) ampholines pH 9-11 and 12mg F_4P_1 in 100ml distilled water was poured into a 24.5 x 11.0 x 0.5cm acrylic frame containing electrode strips soaked in either 1M NaOH (-) or 1M H_3PO_4 (+). The gel was air dried on a level surface until the moisture content was reduced by $\sim$35% using a hair drier positioned 30cm above the gel. It was then focused at a constant 8W for 15-16 hours at 10°C. After focusing the gel was cut into 0.5cm sections and each section was eluted twice with 2.5ml BBS. The pH, protein content (absorbance at 280nm) and F_4P_1 content of the first eluate was measured. Two fractions, Fr.5 and Fr.14 were pooled and concentrated down to 2ml. Carrier ampholines were separated from protein

by gel filtration through a 35.0 x 1.0cm column of Sephadex G-50. This method was not wholly successful because the protein fractions still contained trace amounts of ampholine. The results were in agreement with those of Baumann and Chrambach (1975) who showed that some ampholine preparations contained high molecular weight species (20-30,000 daltons). This problem may be resolved by the use of low molecular weight ampholytes (<1,000 daltons) with very good isoelectric properties which have recently been described (Laas, Olsson and Söderburg, 1980).

B.1.2. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE) is commonly used for determining the molecular weights of proteins and other macromolecules. Most proteins form negatively charged, rodlike particles when they bind SDS which separate accroding to molecular weight on electrophoresis (Shapiro, Vinuela and Maizel, 1967). Accurate values for the molecular weight are obtained by comparing the electrophoretic mobility of the test sample with that of known standards. The concentration of acrylamide can be adjusted to make gels with a range of pore sizes especially designed for the separation of proteins in a given molecular weight range. SDS-PAGE can also be used to assess the homogeneity and sub-unit structure of proteins.

Analytical SDS-PAGE was performed in a slab gel apparatus (Studier, 1973) using a discontinuous buffer system (Laemmli, 1970).

The reagents used were of analytical grade; acrylamide was obtained from Eastman Kodak, Liverpool, U.K., TEMED (NNN'N' - tetramethylethylene diamine) and sodium dodecyl sulphate "specially purified for biochemical work" were obtained from B.D.H. Poole, Dorset. Acrylamide monomer was cross-linked using N-N'-diallyl-tartardiamide (DATD, Aldrich Chemical Co. Milwaukee, Wisconsin, U.S.A.) at an acrylamide:DATD ratio of 38:1 (Anker, 1970). The resolving gel contained 15% acrylamide in 0.38M tris-HCL pH 8.8. It was overlaid with a spacer gel containing 5% acrylamide in 0.13M tris-HCL pH 6.8. Both gels contained 0.1% SDS and were polymerized using freshly prepared ammonium persulphate solution as catalyst. The gel formulations were modified from those of Payne (1976). Sample wells (0.5cm) were formed in the spacer gel between the 'fingers' of a teflon comb. Each well was washed free of unpolymerized acrylamide prior to use. Allergen solutions containing 5-10µg protein (1 mg/ml) were boiled for 2 minutes in 4% SDS, 0.1M dithiothreitol and 8M urea and applied to the sample wells. Electrophoresis was carried out for 3-4 hours at 15mA using electrode buffer containing 0.025M tris, 0.19M glycine and 0.1% SDS pH 8.3.

B.2. Inhibition radioimmunoassays for allergen assay and the measurement of total IgE

Inhibition radioimmunoassay (RIA) was first developed to assay hormones (Yalow and Berson, 1960) and has since achieved widespread application in many branches of scientific research. The assays rely on the use of cold antigen to inhibit the binding of excess radiolabelled antigen to a fixed amount of ab. The antigen-antibody complex is

separated from free antigen (e.g. by precipitation using a second ab) and the radioactivity counted; the number of counts bound being inversely proportional to the concentration of inhibitor. Dilutions of known concentrations of cold antigen are used to form a control curve from which values for unknown samples are obtained.

Double antibody inhibition radioimmunoassays usually require purified antigens but only in sufficient quantities (μg) to radiolabel. The inhibition RIA for F_4P_1 was developed using immunosorbent purified rabbit anti-ExI, ^{125}I-labelled F_4P_1 and goat anti-rabbit IgG (goat-anti Ra IgG) to precipitate. Initially, direct binding curves were set up to determine the dilution of rabbit anti-ExI which would bind $\sim$ 60% added ^{125}I-labelled F_4P_1 in the absence of inhibitor (Fig. B.2.1). The curve shows that above 60% binding there is progressive antibody excess. Therefore, much larger quantities of cold antigen would be required to inhibit the assay and the sensitivity would be reduced. Conversely, at <25% binding ('antigen excess') maximum inhibition would be achieved with smaller quantities of antigen thereby reducing the range of possible results. On the basis of these criteria 0.1ml of 1:20,000 rabbit anti-ExI was used to assay for F_4P_1. The ab was diluted in 1:50 normal rabbit serum and incubated successively with 0.1ml diluted sample followed by 7ng ^{125}I-labelled F_4P_1 (in 0.1ml) for 2 hours. The complex was precipitated with 0.1ml goat-anti Ra IgG diluted 1:6, sufficient to precipitate all the rabbit IgG, for 16 hours at 4^{o}C. The precipitates were then washed three times in BBS and the radioactivity counted in a γ-counter. Samples were

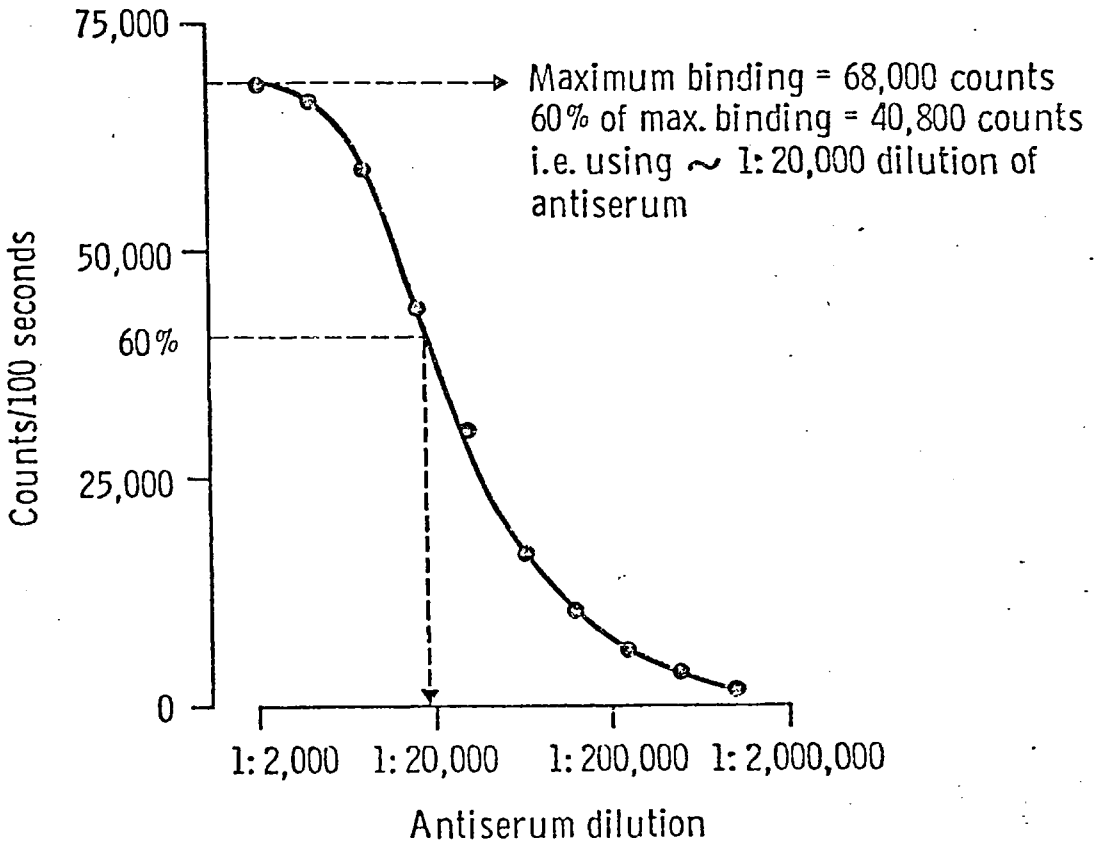


Figure B.2.1

Antigen binding capacity of rabbit anti-ExI. Serial dilutions of rabbit anti-ExI were incubated with 7ng ^{125}I-labelled F_4P_1 for 4 hours and then precipitated with goat anti-RaIgG. The radioactivity bound by the washed precipitates is shown (o——o). Total radioactivity added = 120,000 counts/100 seconds.

diluted throughout in 0.3% BSA BBS. Serial two-fold dilutions of cold F_4P_1 were made from 1µg/ml to 1ng/ml and used to construct a control curve from which values for unknown samples were obtained (Fig. B.2.2). The assay was modified for the determination of antigen P_1 by using 2ng ^{125}I-labelled Fr.14 and dilutions of cold Fr.14 for the control curve. Both assays were dependent on the availability of standard preparations of cold antigen with a defined protein content. The method of Lowry et al (1951) was used to determine the protein concentration of F_4P_1 and amino-acid analysis was used for the determination of protein in Fr.14 (Chapter II).

The double antibody inhibition radioimmunoassay for IgE (Ishizaka et al, 1970; Gleich et al, 1971) was carried out using immunosorbent purified rabbit anti-IgE, 3ng ^{125}I-labelled IgE (ND) and goat anti-Ra IgG to precipitate. All the reagents were already available within the laboratory. The rabbit antibody had been raised against a different IgE myeloma protein (PS) to avoid idiotypic cross-reactivity. The serum used for the control curve had been substandardized against the W.H.O. reference standard 75/502 (National Institute for Biological Standards and Control, London) and contained 5,000 International Units IgE/ml.

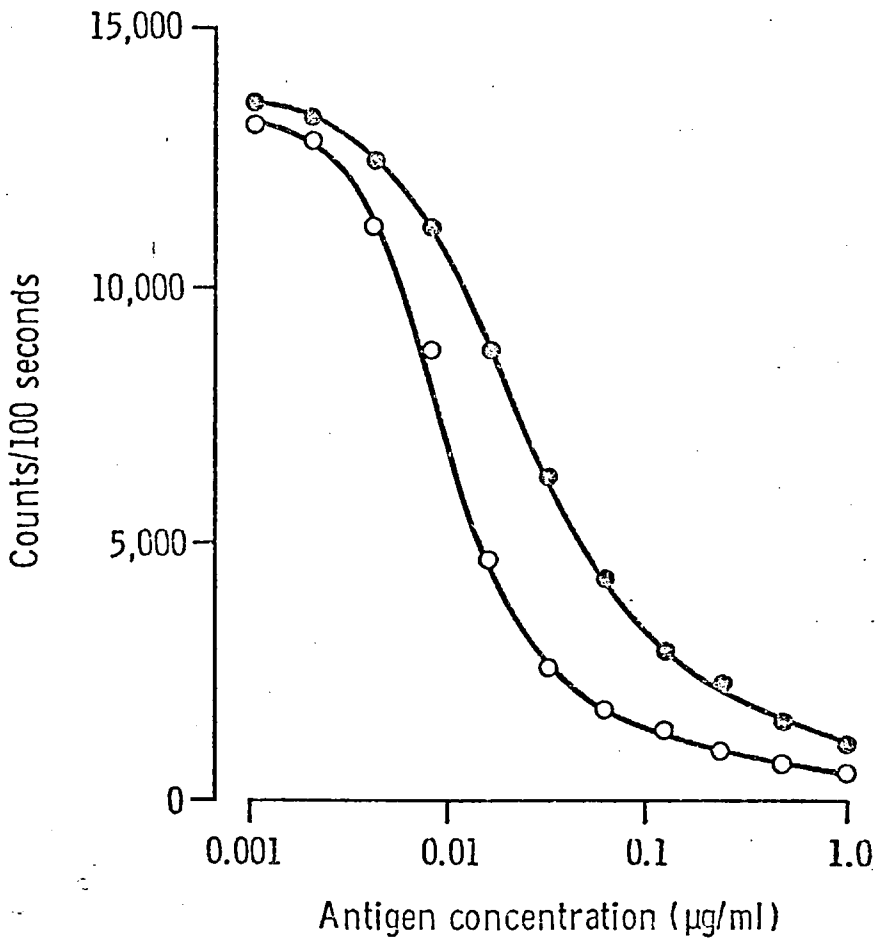


Figure B.2.2

Inhibition curves for allergen assay. Different concentrations of cold F_4P_1 of P_1 were used to inhibit the binding of 7ng ^{125}I-labelled F_4P_1 (●——●) or 2nd ^{125}I-labelled P_1 (○——○) to rabbit anti-ExI, respectively. The complexes were precipitated with goat-anti RaIgG. Total radioactivity added = 50,000 counts/100 seconds (F_4P_1) and 44,000 counts/100 seconds (P_1).

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A D D E N D U M

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Measurement of IgG, IgA and IgE antibodies to *Dermatophagoides pteronyssinus* by antigen-binding assay, using a partially purified fraction of mite extract (F_4P_1)

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SUMMARY

An extract of *Dermatophagoides pteronyssinus* culture has been fractionated by chromatography on Sephadex G-100 and Pevikon block electrophoresis to obtain a partially purified allergen (F_4P_1). This preparation has a molecular weight of between 15–25,000 Daltons, migrates slowly on electrophoresis, and is colourless in solution. The skin-test reactivity of F_4P_1 was comparable to that of crude *D. pteronyssinus* extract. F_4P_1 was radio-labelled with ^{125}I and used in an antigen-binding radioimmunoassay to measure IgG, IgA and IgE antibody (ab) to *D. pteronyssinus*. IgG, ab was detected in serum from 32/34 (94%) mite-allergic persons, and from 10/31 (30%) non-allergic persons. IgA ab and IgE ab were found in sera from 22/34 (65%) and 27/34 (79%) allergic persons respectively. Neither IgA nor IgE ab could be detected in sera from non-allergic persons. An excellent correlation was found between radioallergo-sorbent technique (RAST), using crude *D. pteronyssinus* extract and IgE-binding activity (BA) for F_4P_1, ($r = 0{\cdot}94$, $P < 0{\cdot}001$). The antigen-binding assay for IgE BA was as sensitive as RAST, but less sensitive than PK testing. There was a very good quantitative correlation between IgG BA and IgE BA ($r = 0{\cdot}84$, $P < 0{\cdot}001$). IgG BA was shown to rise in the serum of three patients treated with injections of *D. pteronyssinus* extract.

INTRODUCTION

The role of mites of the Dermatophagoides species, notably *Dermatophagoides pteronyssinus* and *D. farinae* as allergens of house dust have been well established (Voorhorst, Spieksma-Boezema & Spieksma, 1964; Voorhorst *et al.*, 1967; Maunsell, Wraith & Cunnington, 1968; Holford-Strevens *et al.*, 1970; Miyamoto *et al.*, 1968). Mite sensitivity is thought to be of great importance in the aetiology of perennial rhinitis and extrinsic asthma (Morrison-Smith *et al.*, 1969; McAllen, Assem & Maunsell, 1970; Frankland, 1971). In the past, crude extracts of mite culture have been used both for skin-testing and for measuring serum IgE antibodies (ab) in the radioallergo-sorbent technique (RAST) (Wide, Bennich & Johansson, 1967; Stenius & Wide, 1969). RAST results have been shown to correlate well with other methods of assessing IgE ab, i.e. skin-testing, PK and provocation tests (Wide *et al.*, 1967; Berg, Bennich & Johansson, 1971; Bennich & Johansson, 1971). In addition, IgG ab to Dermatophagoides has been demonstrated in sera from patients by radioimmunoelectrophoresis (D'Souza *et al.*, 1973; Gabriel *et al.*, 1977).

The most sensitive method for measuring IgG ab to allergens is the double antibody or antigen-binding technique (Osler, Mulligan & Rodriguez, 1966; Newcomb & Ishizaka, 1967; Yunginger &

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Gleich, 1973; Platts-Mills *et al.*, 1976). Antigen-binding activity associated with IgE has also been demonstrated (Ishizaka, Ishizaka & Hornbrook, 1967). Recently we have reported the use of an antigen-binding technique for measuring IgE ab in parallel with IgG and IgA ab (Platts-Mills *et al.*, 1978). However, antigen-binding techniques require purified or at least partially purified allergens (Marsh, 1975; King, 1976). As yet no purified allergen has been described for either *D. farinae* or *D. pteronyssinus*. On the other hand, several estimates of the molecular weights of mite allergens have been made, based on the skin-test reactivity of fractions of mite extract separated by gel filtration (Miyamoto, Oshima & Ishizaki, 1969; Romagnani *et al.*, 1972, 1976).

The present report describes the partial purification of a *D. pteronyssinus* allergen which has good skin-test reactivity and is suitable for radiolabelling. Using this allergen an antigen-binding radioimmunoassay has been developed for measuring IgG, IgA and IgE ab to *D. pteronyssinus*.

MATERIALS AND METHODS

Allergen preparation. Dermatophagoides pteronyssinus whole culture was kindly provided by Benéard (Betchworth, England). 117 g culture was extracted in 1L PBS by stirring overnight at 4°C. The extract was centrifuged at 23,000 *g* for 60 min, and the supernate concentrated down to 50 ml over an Amicon PM10 filter (Amicon, Lexington, Massachussetts, U.S.A.). The dark brown concentrate (Extract I) was then dialysed for 24 hr against two changes of borate-buffered saline pH 8·0 (BBS), and sterilized through an 0·45 μm Millipore filter. 7·5 ml Extract I was applied to an upward flow, gravity fed, 75 × 2·5 cm Sephadex G-100 column, equilibrated in BBS at 4°C. Samples of 6·3 ml were collected at a flow rate of 10 ml/hr, and the absorbance of each sample was measured at 280 nm. The eluted samples were then pooled in six fractions designated F1-6. These fractions were freeze-dried, resuspended in 4 ml BBS and centrifuged at 12,000 *g* for 30 min. The protein content of the fractions was determined by the method of Lowry *et al.* (1951) using ovalbumin as a standard. On the basis of skin-testing (see Results), fraction 4(F_4) was used for further purification.

Preparative block electrophoresis of F_4 using Pevikon C-870 (Shandon Southern Ltd., Runcorn, Cheshire) was carried out as described by Müller-Eberhard (1960). Approximately 30 mg F_4 was applied to a 50 × 10 × 1 cm block of Pevikon equilibrated in 0·1M barbital buffer pH 8·6 at 4°C. Electrophoresis at 260V and 50 mA was continued for 26 hr. The protein concentration of 10 ml eluates of 1 cm segments of the block was then determined. The eluates were pooled and concentrated on the basis of their protein content and reactivity on immunodiffusion against rabbit anti-D. pteronyssinus serum (see Results). The F_4P_1 fraction was rechromatographed over Sephadex G-100 and the single, apparently homogeneous, peak was concentrated by negative pressure dialysis. After pooling, all fractions were stored at −70°C.

Antisera. Rabbit anti-*D. pteronyssinus* serum was prepared by injecting two NZW rabbits with 1 mg *D. pteronyssinus* Extract I in FCA (Difco Labs, Detroit, Michigan, U.S.A.), at fortnightly intervals. The rabbits were bled 11 days after the fifth injection, and the serum stored at −20°C. The preparation of mono-specific anti-IgG, anti-IgA and anti-IgE has been described previously (Platts-Mills & Ishizaka, 1975; Platts-Mills *et al.*, 1976, 1978). The antisera were raised in goats, by repeated immunization with Fc fragment of IgG, IgA myeloma protein or Fc fragment of IgE (PS) myeloma protein (Takatsu, Ishizaka & Ishizaka, 1975). Each antiserum was made monospecific by repeated passage over immunosorbent columns of Sepharose 2B, linked to human proteins by the cyanogen bromide technique (Axen, Porath & Ernbach, 1967). Anti-IgG was absorbed with Fab, IgA and IgE. Anti-IgA was absorbed with IgG and serum from an IgA-deficient patient, and anti-IgE was absorbed with IgG.

Skin testing. Intradermal skin-testing with *D. pteronyssinus* Extract I, Sephadex G-100 fractions, F_4P_1, and the Group I protein of Rye grass pollen, Rye I (Marsh, 1975) was carried out on the arms of twenty-one volunteers, who had been informed of the risks involved. The preparations were sterilized through an 0·45 μm Millipore filter. Serial ten-fold dilutions of allergens were made from 10 μg/ml down to 10^{-6} μg/ml in 0·05% human serum albumin (HSA) in phenol saline. Skin tests were performed by successive intradermal injection of 0·05 ml of increasing concentrations of allergen. The diameter of the wheal and flare was recorded 20 min after injection.

PK testing was performed with five sera from healthy individuals (Australia antigen negative) using only two recipients, M.C. and T.P.M. whose total serum IgE was 31 ng/ml and 175 ng/ml respectively. The sera were fractionated with saturated ammonium sulphate (SAS) and dialysed exhaustively against BBS. The 33–50% SAS fraction, which has been shown to be enriched for IgE (Carson, Metzger & Bazin, 1975), was diluted from 1:10 to 1:1600 in 0·05% HSA phenol saline, and sterilized through an 0·45 μm Millipore filter. 0·05 ml aliquots of suitable dilutions were injected intradermally. After 24 hr each site was challenged with 0·05 ml of a 1 μg/ml solution of *D. pteronyssinus* Extract I, and the wheal and flare reactions recorded.

^{125}I labelling of F_4P_1. Iodination was carried out using the Chloramine-T technique in a modification of the method of Klinman & Taylor (1969). To 40 μg F_4P_1 was added 50 μl 0·3M phosphate buffer pH 7·5, 1·0 mCi ^{125}NaI and 40 μg Chloramine T. The reaction was stopped after 65 sec by adding 40 μg sodium metabisulphite and 3 min later the reaction mixture was diluted with 2 ml 0·4% BSA BBS containing 50 μL 1% potassium iodide. After dialysis against three changes of BBS the

^{125}I-labelled F_4P_1 was 85% precipitable by trichloracetic acid, and 68% could be bound by hyperimmune human serum (see below).

Measurements of antibodies to D. pteronyssinus. Specific IgG, IgA and IgE ab to *D. pteronyssinus* were measured by a double antibody antigen-binding radioimmunoassay, analogous to those used for measuring IgG and IgA ab to Ragweed antigen E (antigen E), and IgG, IgA and IgE antibody to Rye I (Platts-Mills *et al.*, 1976, 1978). Patient's serum was incubated with 7 ng ^{125}I-labelled F_4P_1 for 4 hr at room temperature. Specific antibody was precipitated overnight at 4°C by adding monospecific goat anti-IgG, anti-IgA or anti-IgE. The quantity of antiserum necessary was estimated by a semi-quantitative precipitation technique (Campbell *et al.*, 1970). The precipitates were washed four times in BBS and radioactivity counted in a 16-channel γ counter (NE 1600, Nuclear Enterprises Ltd., Edinburgh). All assays were performed in plastic tubes coated with 2% BSA to reduce non-specific binding. Carrier immunoglobulins were added to samples containing insufficient immunoglobulin to form a precipitate. The carrier protein for IgE assays was 0·1 ml of a 1:100 dilution of PS myeloma serum (19·5 mg/ml IgE). For the IgA assays 0·1 ml of a 1:50 dilution of IgA myeloma was used. In general, IgG carrier (0·1 ml of a 1:100 dilution of IgG myeloma serum) was necessary only for the control curve and for sera diluted more than 1:50. The radioactivity bound by precipitates of carrier immunoglobulins was < 0·4% added radioactivity. A pool of sera from three patients who had been desensitized with aqueous extracts of *D. pteronyssinus* for several years was arbitrarily defined as containing 13,000 units IgG BA/ml. Serial two-fold dilutions of this pool from 1:100 to 1:51,200 were assayed for IgG BA and the results (control curve) were plotted on a log linear scale. The radioactivity bound by patient's serum was converted to values for IgG-, IgA- or IgE-binding activity (BA) by reference to this control curve set up in parallel with each assay. Assays were performed routinely using 0·1 ml allergic serum diluted 1:12·5, 1:25 and 1:50 for IgG BA, and 1:6, 1:12·5 and 1:25 for IgA and IgE BA. Results were calculated as the mean of the three values. Sera containing high levels of BA were diluted further so that at least 80% of the added radioactivity was not bound by any class of ab, i.e. the antigen excess was maintained at four-fold. Four sera from non-allergic persons were assayed at 1:12·5 and 1:25 in each assay to define the negative background. A result was regarded as positive if it was at least two standard deviations above this background.

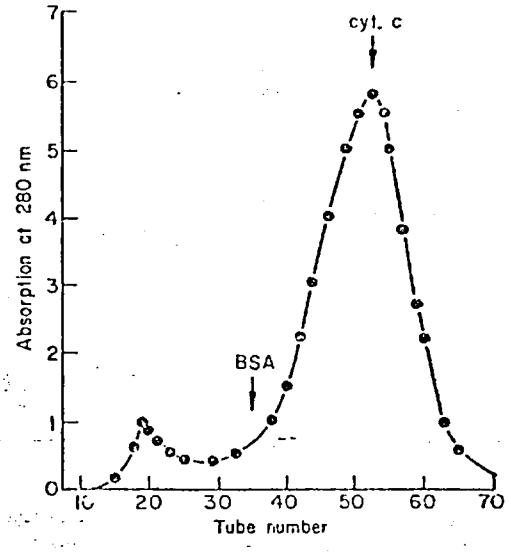
RAST assay. The RAST of Ceska, Eriksson & Varga (1972) was carried out using *D. pteronyssinus* Extract I coupled to filter-paper discs (Whatman Grade 541, 0·6 cm diameter). All RAST estimations were performed in duplicate and standardized by reference to a control curve. The control curve was obtained by assaying IgE ab in two-fold dilutions of an allergic serum which had been arbitrarily designated to contain 6500 IgE RAST units/ml. A positive RAST was defined as two standard deviations above the mean levels for sera from five non-allergic persons. RAST was also used to assess the allergenicity of Sephadex G-100 fractions of *D. pteronyssinus* Extract I, using 1 μg of each fraction coupled to a cyanogen bromide activated disc. The RAST was performed with each fraction using eight sera from allergic persons, and four sera from non-allergic persons.

Total IgE. A double antibody inhibition radioimmunoassay was used to measure total IgE as described previously (Gleich, Averbach & Svedlund, 1971; Platts-Mills *et al.*, 1978).

RESULTS

The elution profile for Sephadex G-100 fractionation of *Dermatophagoides pteronyssinus* Extract I showed two peaks at 280 nm. A small peak eluted with the void volume, and a very broad peak eluted over the 5–65,000 Daltons molecular weight range, indicated by the positions of BSA and cytochrome c markers (arrows in Fig. 1). The fractions were pooled and tested by quantitative intradermal skin-testing; maximal skin-sensitivity was shown with fractions 3 and 4. In general, the skin-reactivity of these fractions was ten- to one-hundred-fold greater than that of the other fractions in each of the six individuals tested. When each of the fractions were coupled to RAST discs 8/8 sera from allergic persons showed maximal binding towards fraction 4 (see Materials and Methods, Fig. 1). There was no significant binding to any fraction by sera from non-allergic persons. Separation of fraction 4 by Pevikon block electrophoresis showed two protein peaks (Fig. 2). The major peak, F_4P_2, migrated faster than albumin, was dark brown in colour, and by immunodiffusion showed no reactivity with a rabbit antiserum to Extract I of *D. pteronyssinus*. The smaller peak, F_4P_1, showed only a faint brown colour, migrated slowly, and was strongly reactive on immunodiffusion (Fig. 2). This fraction, F_4P_1, was passed through Sephadex G-100 and eluted with a molecular weight of between 15–25,000 Daltons. This rechromatographed material was used for skin-testing and radiolabelling and is hereafter referred to as F_4P_1.

Radiolabelled F_4P_1 was used in a double antibody antigen-binding assay to measure specific IgG, IgA and IgE ab to *D. pteronyssinus*. The effect of the amount of antigen added to this assay was investigated over the range 0·5 ng–5 μg F_4P_1 added/tube. Five μg ^{125}I, F_4P_1 was diluted with 95 μg cold F_4P_1, and serial ten-fold dilutions of this solution were made. One hundred μl of each dilution of antigen was used to assay IgG and IgE ab in six sera diluted 1:12·5. The results in Fig. 3a and 3b show that



Fraction number	1	2	3	4	5	6
Skin reactivity	+	++	+++	+++	++	+
RAST	+	++	++	+++	++	+

FIG. 1. Fractionation of *Dermatophagoides pteronyssinus* Extract I on Sephadex G-100. Arrows indicate the positions of BSA (MW 65,000) and cytochrome c (MW 12,500) markers. For each fraction skin-test reactivity was assessed by quantitative intradermal skin-testing. Each fraction was also coupled to cellulose discs and used for RAST. Typical results are shown on a semi-quantitative basis (+ to +++++).

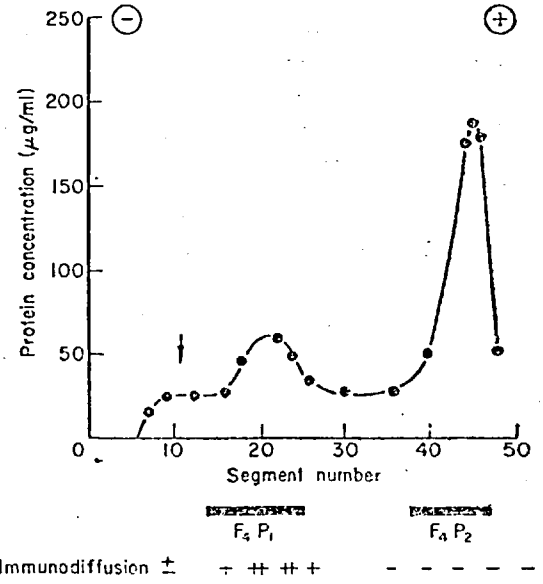
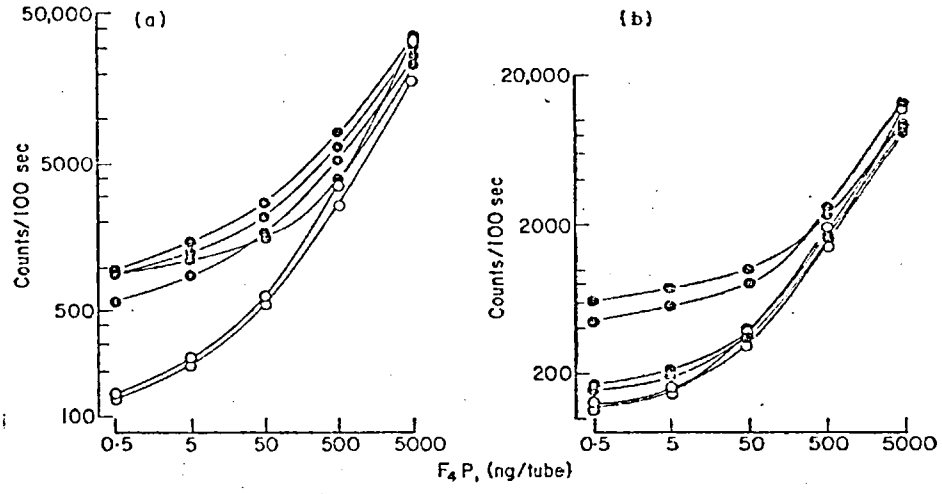


FIG. 2. Pevikon block electrophoresis of Sephadex G-100 Fraction 4. Arrow indicates the position at which 30 mg F_4 was applied to block.



	Percentage radioactivity bound by IgG					Percentage radioactivity bound by IgE				
Allergic	28·80	6·06	2·13	1·00	0·44	18·10	3·19	0·78	0·29	0·16
Non-allergic	3·74	1·01	0·43	0·34	0·23	3·65	0·66	0·26	0·18	0·16

FIG. 3. Radioactivity bound by serum from skin-test-positive (●———●) and skin-test-negative (○———○) individuals. Sera were incubated with various quantities of ^{125}I F_4P_1 over the range 0·5–5000 ng/tube and then precipitated with anti-IgG (Fig. 3a) or anti-IgE (Fig. 3b). Also shown for each quantity of F_4P_1 is the percentage of radioactivity bound by allergic sera (top line in Fig.) and by non-allergic sera (bottom line in Fig.)

maximum differences between allergic and non-allergic sera were achieved by adding 0·5–5 ng radiolabelled antigen for both IgG and IgE ab. With increasing quantities of antigen (over 50 ng F_4P_1/tube) although the percentage radioactivity bound by non-allergic sera decreased, the absolute levels of radioactivity (counts/100 sec, Fig. 3a and 3b) increased dramatically. This resulted in a progressive loss of sensitivity because the sensitivity of the assay depends on the absolute background radioactivity with serum from non-allergic persons. Assays for IgG, IgA and IgE BA were carried out routinely using 7 ng ^{125}I, F_4P_1/tube. The other details of this assay were very similar to those used previously for antigen E and Rye I (see Materials and Methods). Typical dilution curves for IgG and IgE BA are shown in Fig. 4. The IgE BA curves could be superimposed on those for IgG BA, and therefore all quantitative results were obtained by extrapolation from a single IgG BA control curve.

Specific IgG, IgA and IgE ab to *D. pteronyssinus* were measured by antigen-binding assay in the serum of thirty-four persons who had positive prick tests with *D. pteronyssinus* extract (Bencards Ltd.) Fig. 5. Sera were also studied from thirty-one non-allergic persons with negative skin tests. The results show that IgG BA was detectable in 32/34 sera from prick-test-positive persons, IgE BA was detectable in 27/34 and 22/34 sera contained IgA BA. In sera from prick-test-negative persons IgE and IgA BA were not detectable, but in 10/31 sera IgG BA could be detected. However, the levels of IgG BA were much lower than those in most prick test positive persons (Fig. 5). Twenty-eight of the prick-test-positive persons had perennial rhinitis suggestive of house dust sensitivity, and the remaining six persons had rhinitis, but their symptoms did not seem to be related to house dust. In sera from these six 'asymptomatic' persons IgE BA and IgA BA were not detectable, but 4/6 contained low levels of IgG BA. In Table 1 the clinical symptoms, intradermal skin-test reactivity and serum ab levels of fifteen prick-test-positive volunteers are shown. The results of intradermal skin-testing with *D. pteronyssinus* Extract I and F_4P_1 showed that all fourteen persons tested were sensitive to both preparations. In general, the minimum quantity of F_4P_1 necessary to give a positive skin-test was about one-fifth the quantity of Extract I. To check the specificity of the skin-tests non-allergic persons were skin-tested with 10 μg F_4P_1: none of these five gave a positive reaction. In addition, two persons with grass-pollen hay fever who gave positive skin-tests of 10^{-6} μg Rye I failed to react to 10 μg F_4P_1. Using the IgE-rich fractions of five sera from skin-test-positive persons, there was a good correlation between PK titre tested with *D. pteronyssinus*

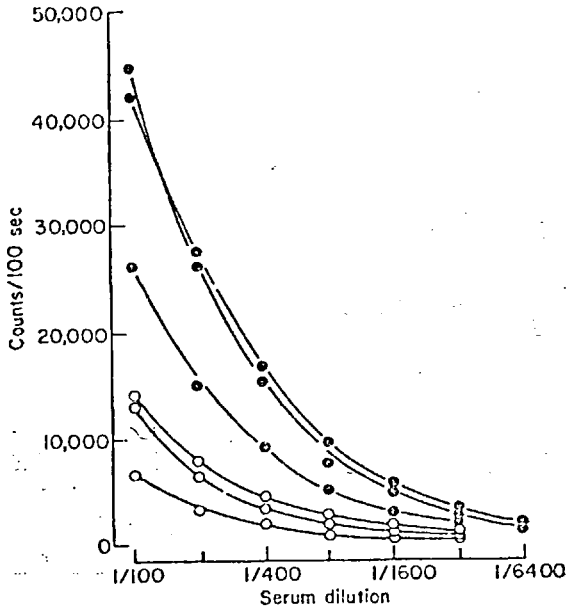


FIG. 4. Radioactivity bound by dilutions of sera from skin-test-positive persons precipitated by anti-IgG (●———●) or anti-IgE (○———○). 7 ng ^{125}I F_4P_1 were added to each tube.

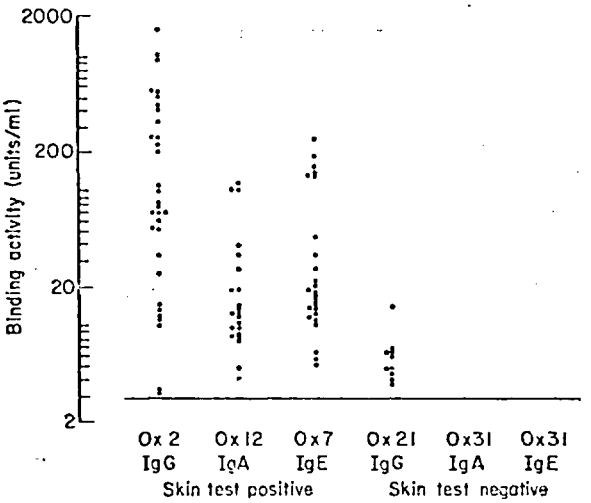


FIG. 5. IgG, IgA and IgE BA for F_4P_1 in the serum of skin-test-positive and skin-test-negative persons measured by antigen binding radioimmunoassay. The minimum detectable level of BA (units/ml) is indicated by the solid line. The number of sera without detectable BA is shown below this line.

Extract I and IgE BA for F_4P_1 (Table 1). PK tests were completely negative using two sera from skin-test-negative persons, and sera from the two persons with grass-pollen hay fever described above.

IgE ab was measured by both antigen-binding assay and RAST in thirty-three of the sera from skin-test-positive persons. An excellent quantitative correlation between the results of the two methods was found by linear regression analysis ($r = 0{\cdot}94$, $P<0{\cdot}001$, Fig. 6). Analysis of the results in Fig. 5 shows an excellent correlation between IgE BA and IgG BA ($r = 0{\cdot}87$, $P<0{\cdot}001$). The mean ratio of IgG BA: IgE BA was 7·4:1. We also found a good correlation between IgE BA and total IgE ($r = 0{\cdot}63$, $P<0{\cdot}001$), and a modest correlation between IgG BA and total IgE ($r = 0{\cdot}38$, $P<0{\cdot}02$). IgG BA was assayed in pre- and post-treatment sera from three patients who had received at least six graded injections of aqueous

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TABLE 1. Antibody levels in fifteen prick-test-positive subjects assessed by intradermal skin-testing, antigen-binding assay, RAST, and P.K. testing

Subject (Age)	Symptoms attributable* to house dust allergy*	Intradermal skin-test† Extract I 'μg/ml'	Intradermal skin-test† F_4P_1 'μg/ml'	RAST‡ Rast units/ml	BA for F_4P_1 IgG units/ml	BA for F_4P_1 IgE units/ml	BA for F_4P_1 IgA units/ml	Ratio IgG BA:IgE BA	P.K. titre§	Total IgE (ng/ml)
J.S. (17)	Rhinitis (grass, egg)	n.d.	n.d.	2125	563	184	27	3.1:1	1600 (666)	8850
J.L. (19)	Rhinitis, asthma	10^{-5}	$10^{-5\cdot5}$	775	1040	135	119	7·7:1	400 (136)	2059
M.B. (18)	Rhinitis, facial swelling	10^{-5}	$10^{-5\cdot5}$	91	330	28	< 4	11·8:1	200 (43)	1728
S.J. (39)	Rhinitis, asthma (grass)	$10^{-4\cdot5}$	10^{-5}	< 8	34	6	< 4	5·9:1	25 (17)	146
R.G. (30)	Asymptomatic (grass)	10^{-3}	10^{-3}	10	< 4	< 4	< 4	—	10 (< 4)	1620
M.K. (24)	Rhinitis	10^{-5}	10^{-6}	325	443	130	13	3·4:1		1824
S.B. (16)	Asthma	10^{-5}	10^{-6}	425	201	143	104	1·4:1		1162
S.M. (27)	Rhinitis	10^{-4}	10^{-5}	53	258	20	19	13·6:1		192
W.M. (50)	Rhinitic (grass)	10^{-5}	$10^{-5\cdot5}$	n.d.	81	21	14	3·85:1		1483
P.J. (19)	Mild rhinitis (grass)	10^{-4}	10^{-5}	66	113	14	< 4	8·1:1		2880
D.T.R.(45)	Rhinitis	10^{-4}	10^{-5}	25	72	14	8	5·1:1		643
K.B. (20)	Asymptomatic (grass)	$10^{-2\cdot5}$	10^{-3}	< 8	10	< 4	< 4	—		1248
P.M. (40)	Asymptomatic (cat)	10^{-3}	10^{-3}	< 8	< 4	< 4	< 4	—		1474
S.S. (39)	Rhinitis (grass)	10^{-5}	$10^{-5\cdot5}$	10	236	12	9·8	19·6:1		377
K.M. (26)	Rhinitis (grass)	10^{-6}	10^{-6}	144	55	47	11	1·2:1		655

* Other allergens giving clinical symptoms and positive skin-tests are indicated in parentheses. Grass = grass pollen.

† For both intradermal skin-testing and PK-testing a wheal of 6 × 6 mm was regarded as positive. Log dilutions of allergen solutions were used—intradermal skin-tests were assessed to the nearest ½ log to distinguish strongly positive results.

‡ RAST units were obtained by reference to a control curve (see Materials and Methods).

§ PK titres are the mean values obtained from tests on two recipients. The IgE BA (units/ml) of the IgE-rich serum fractions used in PK-testing is given in parentheses.

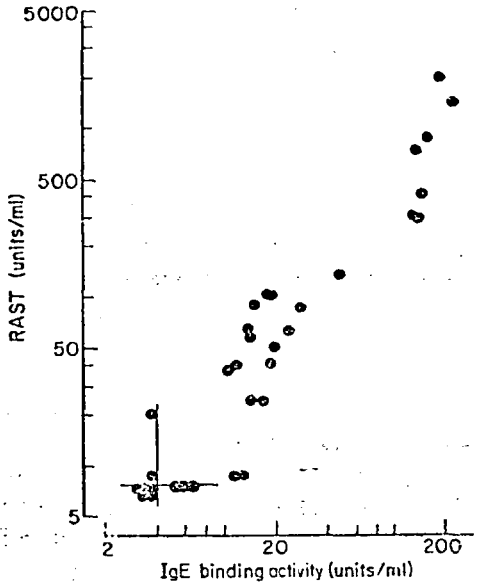


FIG. 6. Correlation between the antigen binding assay (using ^{125}I F_4P_1) and RAST (using *Dermatophagoides pteronyssinus* Extract I) for measuring IgE ab to *D. pteronyssinus* in the serum of prick-test-positive persons. The minimal level of sensitivity of each assay is indicated (———).

D. pteronyssinus extract between January and July. The levels of IgG BA rose from 41, 204 and 750 units/ml in pre-treatment sera, to 562, 2600 and 8000 units/ml in post-treatment sera, respectively.

DISCUSSION

The results of gel filtration of mite allergens reported here suggest a molecular weight of between 15–25,000 Daltons, and are similar to those reported by previous workers (Miyamoto *et al.*, 1969; Romagnani *et al.*, 1972, 1976; Holford-Strevens, 1972). Attempts to further purify the allergens in mite extracts have previously been unsuccessful. Neither isoelectric-focusing (Ricci, Biliotti & Romagnani, 1975), chromatography on DEAE-Sephadex (Romagnani *et al.*, 1976) or affinity chromatography on lectins (Baldo & Uhlenbruck, 1977) have yielded an allergen with skin-test reactivity comparable to that of the crude extract. The electrophoresis of mite extract in Pevikon reported here has given very good yields of allergenic material. The large peak of rapidly migrating material, F_4P_2, was not found to react with rabbit antisera. This highly coloured material was assumed to be made up predominantly of the culture medium in which *Dermatophagoides pteronyssinus* was grown. The culture medium was described by the manufacturer as an animal protein of non-dermal origin which was only very weakly immunogenic in rabbits. The slowly migrating fraction F_4P_1 was compared with crude mite extract by skin-testing, reactivity with rabbit antisera, and by its properties when radiolabelled with ^{125}I. There was an excellent correlation between the two preparations when compared by quantitative intradermal skin-testing (Table 1). It was disappointing that the increased skin-reactivity of F_4P_1 was only about five-fold. However, our estimates of the protein concentration of crude mite extract are only approximate because of the complex mixture of the proteins present. When tested by immunodiffusion and Laurell electrophoresis F_4P_1 gave one major line, and two faint lines. By contrast, *D. pteronyssinus* Extract I gave six lines on immunodiffusion, and at least fifteen on Laurell electrophoresis (data not shown). F_4P_1 could be radiolabelled with ^{125}I approximately three times more efficiently than Extract I. Furthermore, using serum from patients who had been treated with mite extract a large proportion (68%) of the radioactivity in ^{125}I-labelled F_4P_1, could be bound by IgG ab. The properties of F_4P_1 were as good as those we have obtained in antigen-binding assays with other inhalant allergens, e.g. antigen E and Rye I (Platts-Mills

et al., 1976, 1978). The allergen preparation of F_4P_1 is clearly not fully purified. However, two lines of evidence suggest that this fraction includes the major allergen or allergens present in *D. pteronyssinus* Extract I. Firstly, no patient gave greater skin-reactivity with the crude material than with F_4P_1. Secondly, there was an excellent correlation between estimates of IgE BA for F_4P_1 and IgE ab against Extract I measured by RAST (Fig. 6). Further purification and characterization of the proteins in F_4P_1 is in progress.

The antigen-binding radioimmunoassay used here was arranged to give maximum sensitivity. This was made possible by using small amounts of radiolabelled F_4P_1 (7 ng/tube). Results obtained with larger quantities of antigen have given very similar quantitative relationships both between classes and between different sera (data not shown). However, using higher quantities of antigen the ab levels in many of the untreated sera would not have been detectable. Because we can quantitate IgG BA in sera from untreated patients the assay is suitable for measuring the IgG ab responses to desensitizing injections. This will allow a direct evaluation of the relationship between clinical effects and the IgG ab response produced by injections of mite extract (M. D. Chapman, T. A. E. Platts-Mills, Sister M. Gabriel, H. K. Ng, W. G. L. Allan, L. E. Hill & A. J. Nunn; also M. W. Turner, J. F. Soothill, J. F. Price, M. Chapman, T. A. E. Platts-Mills, E. Hey & J. F. Mowbray—both manuscripts in preparation). The sensitivity of the assay for IgE BA was very similar to that of RAST, but approximately five-fold less sensitive than PK testing. The quantitative relationship between IgG, IgA and IgE BA to *D. pteronyssinus* in serum from untreated persons was similar to that reported previously for antibodies to Rye I in serum from patients with hay fever (Platts-Mills *et al.* 1978). Thus in most cases IgG BA > IgE BA > IgA BA and IgE BA was never found in the absence of IgG BA. The finding that a significant proportion of non-allergic persons had IgG BA to *D. pteronyssinus* in the absence of IgE or IgA BA, was not surprising, since similar results have been described for both Antigen E and Rye I (Levine, Stember & Fotino, 1972; Black *et al.*, 1976). A previous report using a modified RAST technique suggested that IgA ab to *D. farinae* were present at higher levels in sera of non-allergic individuals than in sera of allergic individuals (Stokes, Taylor & Turner, 1974). Our results show that IgA BA was present in serum from the majority of allergic persons (22/34) but was not detectable in serum from non-allergic persons. It seems possible that the previous results represent variations in the IgA ab RAST background, because several groups have found that non-specific binding of human IgA, and IgG, to RAST beads is frequently too high to allow sensitive assays (Johansson *et al.*, 1974; Soothill *et al.*, 1976). Although transient low levels of serum IgA have been shown to precede atopic disease in infancy (Taylor *et al.*, 1973; Soothill *et al.*, 1976), there is no evidence from the present investigations to suggest that poor IgA responses are involved in the aetiology of allergic illnesses of late childhood and adult life (Taylor, 1974). This may indicate that transient, low levels of serum IgA in infancy are merely a marker for some other mechanism which is related to the onset of allergy (Soothill *et al.*, 1976). The data reported here support the view that allergic patients make an immune response to inhalant allergens which includes IgG, IgA and IgE ab. By contrast, non-allergic persons make a small IgG ab response or no detectable ab response at all. The very good correlation found between IgG BA and IgE BA ($r = 0{\cdot}84$, $P < 0{\cdot}001$) adds further support to the view that the factors that control the occurrence of inhalant allergy are acting over antibody responses in general, and not over IgE ab alone.

We are very grateful to Benard for generous supplies of *D. pteronyssinus* culture. We are also grateful to Dr D. Marsh for kindly providing Rye I, and to Dr K. Ishizaka for providing serum from a myeloma patient (P.S.).

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PURIFICATION AND CHARACTERIZATION OF THE MAJOR ALLERGEN FROM *DERMATOPHAGOIDES PTERONYSSINUS*-ANTIGEN P_1[1]

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A purified allergen was obtained from *D. pteronyssinus* culture by gel filtration, block electrophoresis, and preparative isoelectricfocusing (IEF). The antigenic activity on IEF was predominantly distributed in two peaks (pI 4.7 to 5.4 and pI 6.6 to 7.1), which were completely cross-reactive on immunodiffusion and by cross-inhibition of radioimmunoassay. These fractions had the same m.w. (24,000) and a very similar amino acid composition. The common antigen in each fraction was designated *Dermatophagoides pteronyssinus* antigen P_1. In seven, symptomatic mite allergic subjects skin test reactivity to antigen P_1 and crude mite extract was closely correlated. There was also a very good correletion between IgE-binding activity (BA) for P_1 measured by antigen-binding radioimmunoassay and IgE antibody (ab) to *D. pteronyssinus* measured by using the radioallergosorbent test (RAST) ($r = 0.32$, $p < 0.001$). RAST experiments showed that up to three-quarters of the IgE ab to *D. pteronyssinus* was directed against P_1, and that IgE ab to P_1 accounted for on average 12% of the total serum IgE. These data strongly suggest that P_1 is the major allergen of *D. pteronyssinus*. By immunodiffusion, SDS-PAGE, and radioimmunoassay antigen P_1 appeared to be present as a large proportion (10 to 20%) of the protein in crude mite extract. Our results support the view that the importance of an inhalant allergen is largely determined by its physical properties, and by the quantities inhaled during natural exposure.

Positive immediate wheal and flare responses to prick tests with extracts of house dust were first reported over 50 years ago (1, 2). In the United Kingdom 5 to 10% of the population develop symptoms from dust, and in many parts of the world up to 85% of patients with extrinsic asthma show positive skin tests with dust extracts (3–5). The identification of a pyroglyphid mite, *Dermatophagoides pteronyssinus*, as the most important source of allergens in house dust by Voorhorst *et al.* (6) was a major breakthrough in house dust allergy. The occurrence of this mite in dust samples from many countries appeared to explain the "universal" nature of house dust allergens (6). An analysis of over 100 dust samples showed that mite numbers correlated well with the allergenic potency of dust extracts (7). Moreover, reactivity to house dust and to mites of the genus *Dermatophagoides* has been closely correlated by using skin testing, the radioallergosorbent test (RAST)[3] and provocation tests (3–9). There is good evidence that allergy to *Dermatophagoides* sp accounts for a large proportion of allergic reactions to dust in Europe (8, 10, 11), Asia (4), and the west coast of the United States (12). On the other hand house dust may also contain other important allergens, e.g., animal danders and fungal spores (13). Mites are probably a less common source of dust allergens in the eastern United States and in mountainous areas of Switzerland (6, 14).

Many different pollen allergens have been obtained in a highly purified form. These allergens have been widely used for studies on antibody (ab) responses and cellular responses, and also as standards for the allergen content of pollen extracts (15–17). The characterization of mite allergens by isoelectricfocusing (IEF) (18, 19), gel filtration (10, 20), ion-exchange (10, 19), or affinity chromatography (21) has not resulted in the isolation of a purified allergen. Recently, we reported the partial purification of a mite allergen, F_1P_1, which could be used in an antigen-binding radioimmunoassay (RIA) to quantitate IgG-, IgA-, and IgE-binding activity (BA) for *D. pteronyssinus* (22). The F_1P_1 preparation (m.w. 15 to 25,000) had very good skin test reactivity and up to 68% of the ^{125}I-labeled material could be bound by hyperimmune sera in RIA. We report here the purification and characterization of the major allergen to *D. pteronyssinus*-antigen P_1. The antigen is present on a heat-sensitive glycoprotein that has a m.w. of 24,000 and exhibits a wide range of isoelectric points. Antigen P_1 appears to comprise a substantial proportion of the protein in crude mite extract.

MATERIALS AND METHODS

Purification of antigen P_1. The preparation of a partially purified *D. pteronyssinus* allergen, F_1P_1, from an aqueous extract of *D. pteronyssinus* culture, Extract 1 (Ex1), has been described in detail previously (22). Briefly, gel filtration of Ex1 on Sephadex G-100 yielded six fractions that were tested by skin testing and direct RAST. The most allergenic fraction, F_1 (m.w. 15 to 25,000), gave two protein peaks after pevikon block electrophoresis one of which migrated with β-mobility and reacted strongly with rabbit antiserum to Ex1 on immunodif-

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[3] Abbreviations used in this paper: RAST, radioallergosorbent test; BA, binding activity; Ex1, an aqueous extract of whole *D. pteronyssinus* culture; IEF, isoelectric focusing; BBS, borate-buffered saline, pH 8.6; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; RIA, radioimmunoassay; HSA, human serum albumin; Rye I, the group I protein from rye grass pollen; CM, culture medium.

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fusion. This fraction, F_4P_1, was rechromatographed over Sephadex G-100 and further purified by preparative IEF using the LKB 2117 Multiphor (23). The reagents and apparatus for IEF were obtained from LKB, Bromma, Sweden. A gel containing 4.0% (w/v) Ultrodex, 3.5% (v/v) ampholines, pH 3.5 to 10, 0.5% (v/v) ampholines, pH 9 to 11, and 12 mg F_4P_1 in 100 ml distilled water was poured into a 24.5 x 11.0 x 0.5 cm acrylic frame containing electrode strips soaked in either 1 M NaOH (−) or 1 M H_3PO_4 (+). The gel was air dried for 4 to 5 hr and focused at a constant 8 watts for 15 to 16 hr at 10°C. Gel sections (0.5 cm) were then eluted twice with 2.5 ml borate-buffered saline, pH 8.0 (BBS), and centrifuged at 2000 × G for 15 min. The pH, protein content, and F_4P_1 content of the first eluate were measured. Two peaks designated Fr.5 and Fr.14 were pooled on the basis of their F_4P_1 content (see *Results*, Fig. 1). Carrier ampholines were removed from these samples by gel filtration through a 35.0 x 1.0 cm column of Sephadex G-50. The final yield of Fr. 5 and Fr. 14 was 4 mg and 2 mg, respectively. For RIA 10 to 25 μg of allergen solutions were labeled with 1.0 mCi of ^{125}I by using a modified chloramine-T technique (24). The specific activity of the labeled allergens was 20 to 40,000 cpm/ng and up to 85% of the radioactivity was precipitable with trichloroacetic acid.

Gel electrophoresis. Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a slab gel apparatus by using a discontinuous buffer system (25). The resolving gel contained 15% acrylamide in 0.38 M Tris-HCl, pH 8.8. It was overlaid with a spacer gel containing 5% acrylamide in 0.13 M Tris-HCl, pH 6.8. Both gels contained 0.1% SDS. Allergen solutions containing 5 to 10 μg of protein were boiled for 2 min in 4% SDS, 0.1 M dithiothreitol, and 8 M urea and applied to sample wells. Electrophoresis was carried out for 3 to 4 hr at 15 mA with electrode buffer containing 0.025 M Tris, 0.19 M glycine, and 0.1% SDS, pH 8.3.

Preparation of purified human antibodies to D. pteronyssinus. Serum was obtained from two mite allergic individuals. The ab from each donor was prepared by passing 220 ml serum in 0.1 M ethylenediaminetetraacetic acid over a 2.5-ml column of Sepharose 2B linked to ExI by using the cyanogen bromide technique (26). The flow rate was 10 to 15 ml/hr. The column was washed with 500 ml BBS and then eluted with 40 ml 0.5 M glycine, pH 2.8. Fractions of 2 ml were collected in tubes containing 0.5 ml 2 M Tris-HCl, pH 8.5. The fractions was assayed for IgE ab by antigen-binding RIA (22), pooled and concentrated down to 1 ml. In each case, approximately 20% of the applied ab was recovered.

Double antibody inhibition RIA for F_4P_1 or P_1. One-tenth milliliter of 1/20,000 rabbit antibody to ExI in 1/50 normal rabbit serum was incubated with 0.1 ml of diluted sample for 2 hr in plastic tubes. Seven nanograms of ^{125}I-labeled F_4P_1 in a volume of 0.1 ml were added for a further 2 hr. The complex was then precipitated with 0.1 ml of goat anti-rabbit IgG diluted 1:6, sufficient to precipitate all the rabbit IgG, for 16 hr at 4°C. The precipitates were washed three times in BBS and counted in a gamma-counter (NE 1600, Nuclear Enterprises Ltd., Edinburgh, United Kingdom). Samples were diluted in 0.3% BSA BBS. Serial 2-fold dilutions of a preparation of F_4P_1 (22) were made from 1 μg/ml to 1 ng/ml and used to construct a control curve from which values for unknown samples were obtained. This assay was modified for the determination of antigen P_1 by using 2 ng ^{125}I-labeled Fr. 14 and dilutions of cold Fr. 14 for the control curve. In cross-inhibition studies cold Fr. 5 or Fr. 14 at 4.0, 0.5, 0.125, or 0.032 μg/ml was used to inhibit the binding of 2 ng of ^{125}I-labeled Fr. 5 or Fr. 14 by either IgG or IgE ab in a preparation of specifically purified ab from an allergic subject. The ab was diluted 1/160 in 1/50 normal human serum. Monospecific goat antiserum to the Fc fragment of either human IgG or IgE was used as a second antibody to precipitate.

In the stability experiments 20 μg ExI or F_4P_1 in 0.1 ml BBS (pH 8.0) was incubated for 1 hr at room temperature, 37, 56, or 80°C. The same quantities of allergen were incubated in 0.1 M buffers at pH 2.0, 4.0, 7.0, 9.4, and 12.0 for 16 hr at 37°C. The pH was neutralized with 0.1 ml 0.3 M phosphate buffer, pH 7.7. All the experiments were performed in duplicate. Dilutions of the heat- or pH-treated samples were assayed for P_1 content as described above.

RAST, RAST absorption. The RAST was performed with paper discs coated with ExI as described previously (22). RAST absorption was carried out as described by Gleich and Jacob (27). Dilutions of serum (1:2 to 1:20) or specific ab (1:200 to 1:400) were absorbed overnight with 0.5 ml 2% Sepharose beads coupled to ExI, antigen P_1 (IEF Fr. 5) or human serum albumin (HSA). The beads were removed by centrifugation and the supernatant was assayed for total IgE.

Skin testing. Intradermal skin tests were carried out by injecting 0.05 ml of serial 10-fold dilutions of each allergen preparation in 0.05% HSA in phenol saline. The allergen solutions were sterilized through an 0.22-μm filter (Millipore Corporation, Bedford, Mass.) before use. The skin response was measured after 15 min and a wheal of >6 x 6 mm was regarded as a positive (++) response.

RESULTS

Purified allergens from IEF. The partially purified allergen, F_4P_1, showed only a single major band on Laurell electrophoresis but multiple bands on IEF over a wide pI range. This suggested two possibilities; either that the preparation contained other proteins that could be separated by IEF, or that the allergen was present in multiple isoelectric forms. Preparative IEF was used to determine the distribution of allergen at different isoelectric points. The antigenic activity detected by inhibition RIA eluted almost in parallel with protein and was predominantly distributed in two peaks, fraction 5 (Fr. 5) and Fr. 14 with pI of 4.7 to 5.4 and 6.6 to 7.1, respectively (Fig. 1). On immunodiffusion with rabbit antiserum a single line was seen against Fr. 5 and Fr. 14, which showed complete identity. This line was identical with the major line against ExI (Fig. 2). Highly concentrated extract of the *D. pteronyssinus* culture

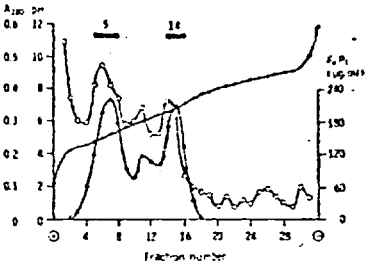


Figure 1. Preparative isoelectric focusing of partially purified *D. pteronyssinus* extract, F_4P_1. The pH (○——○), protein content (absorbance at 280 nm) (○——○), and F_4P_1 content (▲——▲) of each fraction is shown. The quantity of F_4P_1 was determined by inhibition RIA by using immunosorbent purified rabbit antibody to ExI. Fr. 5 and Fr. 14 were pooled as indicated [illegible].

medium (CM) also showed a single line, but this line showed no cross-reaction with either Fr. 5 or Fr. 14 (Fig. 2). The fractions were also compared by using cross-inhibition of RIA. Dilutions of cold Fr. 5 or Fr. 14 were used to inhibit the binding of ^{125}I-labeled Fr. 5 or Fr. 14 by either IgG or IgE ab from an allergic person (see *Methods*). The inhibition curves were very similar when using either the homologous or the heterologous antigen as inhibitor (Fig. 3). In both cases binding could be inhibited by up to 94% with the opposite fraction. Furthermore, absorption of allergic sera with Fr. 5 linked to Sepharose beads completely removed (>95%) the BA for radiolabeled Fr. 14 (data not shown). In keeping with the view that Fr. 5 and Fr. 14 showed antigenic identity both fractions gave very similar skin test responses in allergic individuals. Quantitative intradermal skin tests on 11 skin test-positive individuals and four nonallergic controls showed a good correlation between sensitivity to each purified fraction and sensitivity to ExI (Table I). With Fr. 5 two individuals gave positive (++) reactions using 10^{-3} μg/ml of purified allergen (i.e., ~5 pg). One subject (N.J.) who was only weakly sensitive to mites gave a ++ skin response to a 100-fold higher dilution of ExI than of the purified fractions, suggesting that he was probably reacting to different allergens in the crude mite extract. On the basis of all these criteria Fr. 5 and Fr. 14 appeared to be antigenically identical.

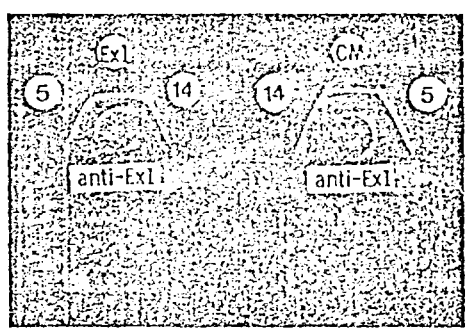


Figure 2. Immunodiffusion analysis of purified fractions. The center well contained hyperimmune rabbit antiserum to extract of whole *D. pteronyssinus* culture (anti-Ex I) (22). Immunodiffusion against ExI, IEF fractions 5 and 14, and extract of the culture medium (CM) is shown.

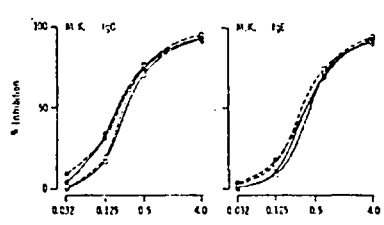


Figure 3. Cross-inhibition by using IEF purified fractions. Binding by human IgG or IgE antibody to ^{125}I-labeled Fr. 5 (*circles, dashed line*) or ^{125}I-labeled Fr. 14 (*squares, solid line*) was inhibited by using either the homologous (*closed symbols*) or heterologous antigen (*open symbols*). Human antibodies were precipitated by using a second antibody: goat anti-IgG or goat anti-IgE, respectively.

TABLE I

Intradermal skin tests with D. pteronyssinus allergens

Subject	Minimum Concentration of Allergen Giving a Positive Skin Test (μg/ml)*		
	Extract I	Fr. 5	Fr. 14
W.S.	10^{-1}	10^{0}	10^{0}
T.S.	10^{-1}	10^{-1}	$10^{-2.5}$
N.D.	10^{-2}	10^{-2}	10^{-3}
A.J.	10^{-1}	$10^{-2.5}$	10^{-3}
M.C.	10^{1}	10^{0}	10^{1}
N.J.	10^{-2}	10^{0}	10^{0}
M.K.	10^{-2}	10^{-2}	$10^{-2.5}$
J.S.	$10^{-2.5}$	$10^{-2.5}$	10^{-2}
E.T.	10^{-2}	$10^{-2.5}$	10^{-2}
S.J.	$10^{-2.5}$	10^{-3}	10^{-2}
R.B.	$10^{-2.5}$	10^{-1}	$10^{-2.5}$
Controls × 4	$>10^{1}$	$>10^{1}$	$>10^{1}$

* Intradermal skin tests were carried out by using 0.05 ml of serial 10-fold dilutions of allergens. A wheal of >6 × 6 mm was regarded as a positive (++) response. Values were extrapolated to the nearest ½ log when the lower dilution gave a strongly positive reaction. 10^{0} = 1 μg/ml. Negative skin tests at 10 μg/ml are recorded as $>10^{1}$.

TABLE II

*Amino acid analysis of Fr. 5 and Fr. 14**

	Residues/100 Residues of Amino Acids		Residues/Molecule†
	Fr. 5	Fr. 14	
Asx	14.3	13.9	24
Thr	4.6	4.4	8
Ser	7.6	7.8	13
Glx	12.2	12.9	21
Pro	4.9	5.2	9
Gly	11.6	10.3	20
Ala	9.5	9.3	17
½ Cys	2.0	1.0	4
Val	4.2	5.2	7
Met	1.2	1.2	2
Ileu	6.3	6.4	11
Leu	4.3	4.4	8
Tyr	5.2	5.4	9
Phe	2.3	2.2	4
His	2.4	2.6	4
Lys	2.1	2.1	4
Arg	5.4	5.6	10

* Twenty micrograms of Fr. 5 and Fr. 14 were hydrolysed *in vacuo* with 1 ml 6 N HCl for 24 hr at 110°C and applied to a Beckman Model 120C amino acid analyser.

† Preliminary calculations of the numbers of amino acids/molecule were made for Fr. 5, assuming a m.w. of 21,000, a carbohydrate content of 8.4% (Dr. E. F. Hounsell—personal communication), and that no other components were present. Values are given to the nearest integer.

The amino acid composition of each fraction was very similar apart from slight differences in the numbers of glycine and valine residues (Table II). With the SAQ system (30) for relatedness among proteins, Fr. 5 and Fr. 14 differed by 4 units, indicating that they were "identical proteins". In contrast, when Fr. 5 was compared with the published amino acid analysis of the purified pollen allergen Rye I (16) it differed by 140 units. On SDS electrophoresis each fraction migrated as a single homogeneous band with an apparent m.w. of 24,000 (Fig. 4). It was also clear that the fractions migrated to the same position as the most heavily staining band in ExI. Since Fr. 5 and Fr. 14 could not be distinguished by their reaction with antibodies, by amino acid analysis or SDS-PAGE it seemed likely that they

represented minor structural modifications of a single protein or glycoprotein.

The common antigen in each fraction has been designated *Dermatophagoides pteronyssinus* antigen P_1. The stability of the antigen was assessed by using a double antibody inhibition RIA (see *Methods*). The antigen is stable at temperatures up to 37°C, loses 50% of its antigenicity after 1 hr at 56°C, and is rapidly denatured at 80°C. Antigen P_1 is stable at neutral pH, but after 16 hr loses more than 50% of its antigenicity at pH 4.0 or pH 9.4, and is completely denatured at pH 2.0 or pH 12.0 (data not shown).

IgE antibodies to P_1 in allergic individuals. Skin test reactivity to purified preparations of P_1 correlated with reactivity to crude mite extract in seven symptomatic, mite-allergic subjects who were highly sensitive to ExI (++ reaction with 10^{-3} μg/ml) (Table I). In serum there was an excellent quantitative correlation between IgE ab to P_1 measured by antigen binding assay and IgE ab to *D. pteronyssinus* measured by RAST ($r = 0.62$, $p < 0.001$, $n = 30$). For six sera from allergic persons the quantities of IgE ab to ExI and to P_1 were compared by RAST absorption (Table III). In these samples IgE ab to ExI and to P_1 formed from 15 to 36% and 9 to 21% of the total IgE, respectively. A much higher percentage of the total IgE was directed against each allergen in the two preparations of specific antibody. In all cases IgE ab to P_1 accounted for a large proportion (up to 72%) of the IgE ab to ExI. The quantities of IgE BA and total IgE absorbed by RAST beads coated with P_1 were used to make an estimate of the amount of IgE represented by 1 unit of IgE BA (Table III). These calculations gave a mean value of 1.13 ± 0.44 ng IgE ab/unit IgE BA. When this value was applied to the previous measurements of IgE BA on sera from three groups of mite allergic individuals, IgE ab to P_1 accounted for 8.4 ± 11.5% (n = 54), (31), 10.8 ± 7.4% (n = 60) (32), and 17.4 ± 12.1% (n = 44) (22) of the total IgE. These values (mean 12%) are very similar to those reported by using purified major allergens from pollens (33–35).

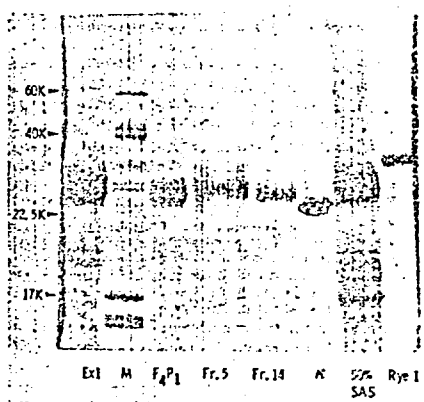


Figure 4. Analysis of *D. pteronyssinus* antigens by SDS-PAGE. A variety of markers were compared with Fr. 5, Fr. 14, F_4P_1, and ExI. *M*, marker solution containing catalase (m.w. 60,000), peroxidase (m.w. 40,000), myoglobin (m.w. 17,000), hemoglobin (m.w. 15,000), and cytochrome C (m.w. 13,000). κ, purified kappa light chain (human Bence-Jones protein) (m.w. 22,500). 50% SAS, an enriched preparation of ExI that precipitated in the presence of 50% saturated ammonium sulfate.

TABLE I

Estimation of the absolute quantities of IgE ab to mite allergens

Patient	Sample	IgE BA for P_1[a]	Total IgE[b]	% Total IgE Directed against[c]		P_1/Ex I	Estimation of 1 Unit of IgE BA[d]
				Ex I	P_1		
		units/ml	units/ml	%		%	ng
S.B.	Serum	204	708	36	16	44	1.33
P.P.	Serum	576	1,316	30	15	50	0.70
T.W.	Serum	99	180	29	21	72	0.92
D.J.	Serum	189	816	18	9	50	0.98
M.K.	Serum	234	696	15	10	67	0.62
	Sp. Ab.[e]	18,000	19,900	61	40	65	1.06
D.B.	Serum	852	12,480	12	6	50	2.11
	Sp. Ab.[e]	28,000	51,840	50	30	60	1.33

[a] Measured on unabsorbed samples by antigen binding radioimmunoassay (21) by using 2 ng ^{125}I-labeled Fr. 14, serum from a myeloma patient (P.S.) as carrier and monospecific goat antiserum to Fc fragment of IgE to precipitate. Results were obtained from a control curve and expressed in arbitrary units of binding activity (BA)/ml.

[b] Measured on unabsorbed samples by double antibody inhibition radioimmunoassay (23). 1 unit of IgE was assumed to be equivalent to 2.4 ng (29).

[c] The results, expressed as a percentage of the total IgE, are the quantity of IgE absorbed by ExI or P_1 immunosorbent minus the IgE absorbed by an HSA immunosorbent.

[d] The quantity of IgE represented by 1 unit of IgE BA was calculated as follows:

$$1 \text{ unit} = \frac{\text{Total IgE (units/ml)} \times 2.4 \times \% \text{ total IgE directed against } P_1}{\text{IgE BA for } P_1 \text{ (units/ml)}} \text{ ng}$$

[e] Immunosorbent purified specific antibody to ExI.

DISCUSSION

Several lines of evidence suggested that antigen P_1 was the major allergen of *D. pteronyssinus*. Reactivity to the purified allergen and crude mite extract was closely correlated by skin testing and showed an excellent correlation in serum. In addition, IgE ab to P_1 accounted for up to three-quarters of the IgE ab to ExI. We have also found that more than 90% of individuals who give positive prick tests to *D. pteronyssinus* extract have detectable serum IgE ab to antigen P_1. In keeping with our previous studies (22) responses to intradermal skin tests with antigen P_1 and ExI were very similar when tested at the same protein concentration. These results correlate surprisingly well with other data which suggest that antigen P_1 represents 10 to 20% of the protein in ExI. However, in agreement with other workers (21), we believe that crude mite extract also contains minor allergens as indicated by one subject (N.J.) who was 100-fold more sensitive to ExI than to antigen P_1 (Table I). The distribution of ab against P_1 is very similar to that for ab to purified pollen allergens (34, 36, 37) in both serum (22, 32) and nasal secretions (M. D. Chapman and T. A. E. Platts-Mills, unpublished data) and includes IgG, IgA, and IgE ab. The evidence suggests that this response, like that to pollen allergens, occurs locally in the nasal mucosa (38).

The apparent m.w. of antigen P_1 on SDS-PAGE (24,000) is very similar to that suggested by Stewart and Turner (21) but a little higher than that suggested by others using gel filtration (7,500 to 20,000) (19, 39). Comparison of the gel filtration data and that derived under reducing conditions on SDS-PAGE confirms that antigen P_1 is a single amino acid chain. It remains possible that the m.w. of antigen P_1 may be slightly overesti-

mated on SDS-PAGE. The presence of a common antigen on proteins with different isoelectric points is not unusual, and has been reported previously for several allergens including Rye I (16), codfish allergen M (40), and *Cladosporium herbarum* allergen 32 (41). However, in almost all cases the pI values of these "isoallergens" differed by less than one pI unit. Our results demonstrate that antigen P_1 occurs in at least two isoallergenic forms that exhibit markedly different isoelectric points. On analytical IEF the two preparations of purified P_1 (Fr. 5 and Fr. 14) each showed more than one band. The heterogeneity on IEF could be explained by differences in carbohydrate content, although several other factors such as conformational changes or differences in amide content may also be involved (42). Recent work has shown that both Fr. 5 and Fr. 14 are glycoproteins, containing galactose, mannose, *N*-acetylglucosamine and *N*-acetylgalactosamine (Dr. E. F. Hounsell, personal communication). The presence of multiple isoallergens over a wide pI range suggests that analytical IEF would not be a useful technique for standardizing mite extracts.

It has previously been thought that allergens required a particular molecular structure (43) or chemically defined determinants (44) in order to elicit IgE ab formation. Highly purified allergens from pollens, codfish, and vespid venoms do not appear to have any chemical properties in common. The major allergens are generally the "most abundant" soluble proteins or glycoproteins with m.w. of between 10,000 and 40,000 (45, 46). Antigens with these properties would readily penetrate the nasal mucosa (47) whereas larger molecules are excluded, and smaller molecules (having fewer determinants) would be expected to be less immunogenic (48). Antigen P_1 has very similar physical properties to the other major allergens. In addition, RIA results suggest that P_1 represents about 10 to 20% of the protein in the crude extract. It also forms the strongest line on immunodiffusion (Fig. 2) and is coincident with the major band on SDS-PAGE (Fig. 4). Recent work has shown that house dust samples contain up to 50 μg P_1/g dust, and that more than 90% of the P_1 can be eluted within 2 min (E. R. Tovey, M. D. Chapman and T. A. E. Platts-Mills, manuscript in preparation). Thus, antigen P_1 is a rapidly eluting glycoprotein, m.w. 24,000, which is "abundant" in extracts of mite culture and house dust. These results strongly support the view that the importance of a protein as an allergen for immediate hypersensitivity is determined by its physical properties. The availability of a purified allergen from *D. pteronyssinus* should greatly facilitate studies on cellular and humoral immunity in patients with perennial rhinitis and extrinsic asthma.

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Antibody Response following Prolonged Hyposensitization with *Dermatophagoides pteronyssinus* Extract

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Abstract. An antigen-binding radioimmunoassay was used to measure IgG, IgA and IgE antibody (ab) to *Dermatophagoides pteronyssinus* in sera from patients who had been hyposensitized with either aqueous *D. pteronyssinus* extract or placebo. An allergen, F_4P_1, was obtained from *D. pteronyssinus* culture and ^{125}I-labelled for use in the assay. The radioallergosorbent test was also used to measure IgE ab to *D. pteronyssinus* and IgE ab to *Dermatophagoides farinae*. The radioimmunoassay results showed a selective rise in IgG ab in sera from *D. pteronyssinus*-treated (DP) patients. An increase of greater than twofold in IgG ab in post-treatment sera was found in 27 of 29 DP patients compared to 1 of 29 placebo-treated ('placebo') patients ($p << 0.001$). The mean rise in IgG ab in DP patients was sixfold, and the maximum rise was 50-fold. There was little change in the levels of IgA ab or IgE ab in either DP or 'placebo' patients during treatment. The DP patients showed significant clinical improvement compared with the 'placebo' patients ($p < 0.01$). However, no correlation was found between post-treatment IgG ab and post-treatment symptom scores, or between the changes in IgG ab and the changes in symptom scores during treatment. The time course of the antigen-binding assay was investigated using very low concentrations of F_4P_1. The results showed that antigen-binding was slow, even in the presence of an excess of IgG ab. The role of IgG ab in hyposensitization is discussed.

Introduction

In 1964 *Voorhorst et al.* [1] identified the house dust mite, *Dermatophagoides pteronyssinus* as a major source of allergen in house dust. Subsequently the importance of allergy to *Dermatophagoides* species in the aetiology of perennial rhinitis and asthma has become well established [2–4]. In addition it has been found that hyposensitization (or desensitization) with extracts of house dust, *D. pteronyssinus* or *Dermatophagoides farinae*, may result in clinical improvement. In some studies up to 80% of treated patients were improved by hyposensitization, although generally the improvement was not dramatic [5–10]. Other re-

[1] Supported by the Asthma Research Council.

ports did not find significant improvement, and most clinicians would consider that the results of systemic hyposensitization therapy for house dust allergy are unsatisfactory [11, 12]. The mechanism by which improvement occurs is poorly understood, however, the production of IgG-'blocking' antibody (ab) has been thought to be important. Using purified pollen allergens in antigen-binding radioimmunoassays it is possible to demonstrate increases in serum IgG ab following hyposensitization [13–15]. These techniques could not be applied to measure IgG ab to *D. pteronyssinus* because purified mite allergens were not available. The production of IgG ab to mite extract has been demonstrated using radioimmunoelectrophoresis [9]. However, that technique gave only a semi-quantitative assessment and could not detect IgG ab in the majority of pre-treatment sera.

We have recently described the use of an antigen-binding assay to measure IgG, IgA and IgE ab to *D. pteronyssinus* in sera from untreated allergic individuals [16]. This assay used a partially purified allergen preparation, F_4P_1, derived from *D. pteronyssinus* culture. In the present study we have used this assay to measure the ab response in a double-blind trial of prolonged hyposensitization with aqueous *D. pteronyssinus* extract. The clinical results of this study on patients from Hong Kong with seasonal or perennial rhinitis have been reported previously by *Gabriel et al.* [17]. The *D. pteronyssinus*-treated (DP) patients showed consistent benefit during maintenance therapy when compared with the placebo-treated ('placebo') patients. In the present study the relationship between the production of IgG ab and improvement in clinical symptoms has been investigated.

Materials and Methods

Patients

Full clinical details of the patients entering the trial have been described previously [17]. All the patients were Chinese, gave a 3-year history of seasonal or perennial rhinitis, and had positive skin tests and nasal challenge tests to *D. pteronyssinus*. Patients were stratified by age and randomly allocated to treatment with either graded doses of aqueous *D. pteronyssinus* extract (from 0.0004 to 0.2%, n = 29), or saline placebo (n = 29). The course consisted of 18–22 weekly injections followed by 12 monthly maintenance doses. Serum samples were taken before treatment, at the end of the weekly course (4 months) and after 12 months of treatment, i.e., approximately 6 months after starting the maintenance course. Patients recorded daily the severity of their symptoms of sneezing, obstruction, discharge and asthma on a four-point scale (nil, mild, moderate or severe). These values were used to calculate patients' symptom scores throughout treatment.

Measurement of Antibodies to D. pteronyssinus

IgG, IgA and IgE ab to *D. pteronyssinus* were measured by an antigen-binding radioimmunoassay, using a partially purified *D. pteronyssinus* allergen, F_4P_1. This assay has been described in full previously and for these experiments was carried out using randomized coded samples [16]. 0.1 ml of a dilution of patient's serum was incubated with 7 ng ^{125}I-labelled F_4P_1 for 4 h at room temperature. Specific ab was precipitated overnight at 4 °C by the addition of monospecific goat anti-IgG, anti-IgA or anti-IgE. The precipitates were then washed four times with borate-buffered saline pH 8.0 (BBS), and the residual radioactivity counted in a γ-counter (NE 1600, Nuclear Enterprises, Edinburgh). The assay was quantitated by reference to a standard curve which was obtained by assaying IgG-binding activity (BA) in serial twofold dilutions of a hyperimmune serum. This serum was pooled from 3 patients who had been hyposensitized with aqueous *D. pteronyssinus* extract, and was arbitrarily defined as containing 13,000 units IgG BA for F_4P_1/ml. The values for IgG, IgA and IgE BA in patients' serum were obtained by reference to the IgG BA control curve.

Sera from non-allergic persons were used in each assay to define the negative background.

Some mid- and post-treatment sera from DP patients contained large quantities of IgG BA (see 'Results') and had to be re-assayed at higher dilutions (1:100 and 1:200). In sera with > 30-fold excess of IgG BA over IgA BA or IgE BA it was difficult to maintain antigen excess and the minor classes may have been underestimated. For these sera IgE BA for F_1P_1 was estimated from the radioallergosorbent test (RAST) values for *D. pteronyssinus*. At dilutions of 1:10 to 1:200 the RAST assay was not affected by IgG BA. The RAST results were divided by 2.39 (the mean ratio of RAST:IgE BA in pre-treatment sera) to obtain estimated units of IgE BA. In post-treatment sera from 10 DP and 10 'placebo' patients the mean difference between the measured IgE BA and that estimated by RAST conversion was < 20%. The values for IgE BA given in figure 3 were obtained by antigen-binding assay, whilst those used for correlating symptom scores were obtained by RAST conversion.

Measurement of IgE ab by RAST

The paper disc RAST [18] was carried out as described previously [16]. Allergen-coated discs were prepared using 15 mg of either *D. pteronyssinus* or *D. farinae* extract in BBS coupled to 1,000 filter paper discs. RAST results for both allergens were standardized by reference to a control curve. This was obtained by assaying IgE ab in twofold dilutions of an allergic serum which was arbitrarily defined to contain 6,500 IgE RAST units/ml for both *D. pteronyssinus* and *D. farinae*. All RAST estimations were performed using two dilutions of serum which gave results on the linear part of the control curve. The negative background was defined by the mean level for sera from 5 non-allergic individuals, and values above 2 standard deviations of this level were regarded as positive.

Total IgE

Total IgE was measured by double antibody inhibition radioimmunoassay [19].

Statistical Methods

The χ^2 test with Yates correction was used to compare the increase in IgG BA in the DP-treated and 'placebo' groups. The Spearman Rank correlation was used to compare pre- and post-treatment IgG BA. It was also used to compare changes in symptom scores with the differences in IgG BA and the ratios of IgG BA and IgG BA:IgE BA in pre- and post-treatment sera. Linear regression analysis was used to compare IgG BA and IgE BA, total IgE and IgE BA, and the RAST values for *D. pteronyssinus* and *D. farinae*.

Results

Antibodies to Mites in Sera from Untreated Patients

Ab were assayed in 58 sera from untreated DP and 'placebo' patients using both the RAST and the antigen-binding radioimmunoassay. The RAST results for both *D. pteronyssinus* and *D. farinae* confirmed that a high proportion of the patients had IgE ab to mites, and showed a very good quantitative correlation between values for the two

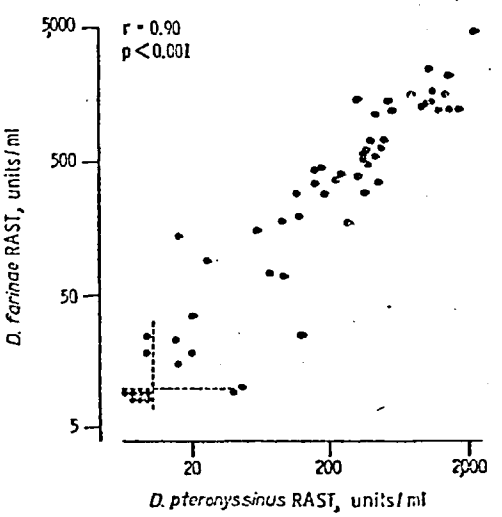


Fig. 1. Correlation between the RAST values for IgE ab to *D. pteronyssinus* and IgE ab to *D. farinae* in the pre-treatment serum of DP and 'placebo' patients. The dotted line indicates the minimum level of sensitivity for each assay.

species ($r = 0.90$, $p < 0.001$; fig. 1). The IgE ab levels of the 'placebo' patients were two- to threefold higher than those of the DP patients (table I) as had been noted previously using a semi-quantitative RAST [17]. The results for IgG BA and IgE BA were compared with results for a group of *D. pteronyssinus* skin test positive individuals in the UK (fig. 2) [16]. These results show that, although many of the Hong Kong patients had higher levels of BA, the relationship between IgG BA and IgE BA was very similar in the two groups. IgA BA was assayed in parallel with the other classes in 20 sera; the level of IgG BA was greater than IgE BA and greater than IgA BA in all 20 sera (fig. 3). Finally, there was an excellent correlation between IgE BA and total IgE ($r = 0.76$, $p < 0.001$) in pre-treatment sera. These results are very similar to those obtained previously using purified allergens (the group I protein of rye grass pollen and ragweed antigen E) [14, 20]. This comparison suggests that F_4P_1 is a suitable allergen preparation for studying ab in sera from mite allergic patients in Hong Kong.

Antibody Response to Hyposensitization

IgG BA was measured in sera from 29 DP and 29 'placebo' patients (table I). The mean rise in IgG BA in DP patients was sixfold with a maximum rise of 50-fold. An increase of more than twofold was found in 27 of 29 DP patients and in only 1 of 29 'placebo' patients ($p << 0.001$). In mid- and post-treatment sera IgE ab was measured either by antigen-binding assay or by extrapolation from RAST results (see 'Methods'). The results showed little change in IgE ab in either 'placebo' or DP patients (table I). For 10 DP patients and 10 'placebo' patients pre-, mid- and post-treatment sera were also assayed for IgA BA (fig. 3). In general, the levels of IgA BA were very low, and only 2 DP patients showed a rise in IgA BA of more than twofold. From the results in figure 3 it is clear that the response to active treatment was a selective rise in IgG BA. They also show clearly that the maximum rise occurred during and at the end of the weekly course of hyposensitization (mean increase 3.9-fold) with an apparent plateau during the maintenance

Table I. Antibody levels in DP and 'placebo' patients (geometric mean data)

Treatment group	Serum sample	IgG BA units/ml	IgE BA units/ml	IgG BA:IgE BA	*D. pteronyssinus* RAST units/ml	*D. farinae* RAST units/ml
DP	pre-treatment	300	74	4.0:1	83	129
DP	mid-treatment	1,171	84	13.9:1	119	166
DP	post-treatment	1,795	75	23.9:1	101	150
'Placebo'	pre-treatment	390	210	1.9:1	228	305
'Placebo'	mid-treatment	328	145	2.3:1	195	259
'Placebo'	post-treatment	323	137	2.3:1	199	265

In both DP and 'placebo' patient groups 29 patients were assessed for IgG BA, *D. pteronyssinus* RAST and *D. farinae* RAST, and 24 patients were assessed for IgE BA.

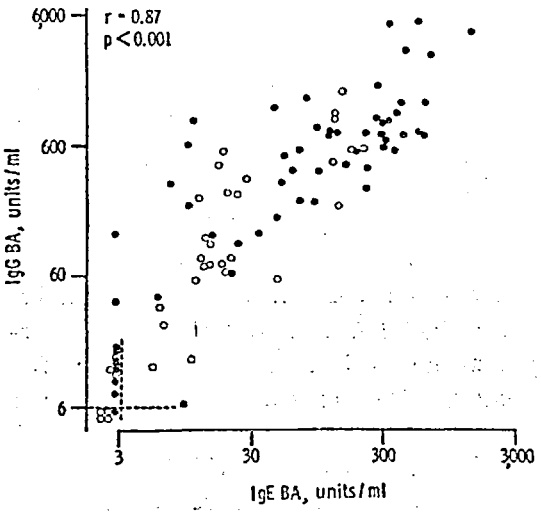


Fig. 2. Correlation between IgE BA and IgG BA for F_4P_1 in serum from untreated allergic individuals with positive skin tests to *D. pteronyssinus*, measured by antigen-binding radioimmunoassay. The minimum level of sensitivity of each assay indicated (- - -). ⊙ = Data from the present study; O = the 'London' data from a previous study [16] for comparison.

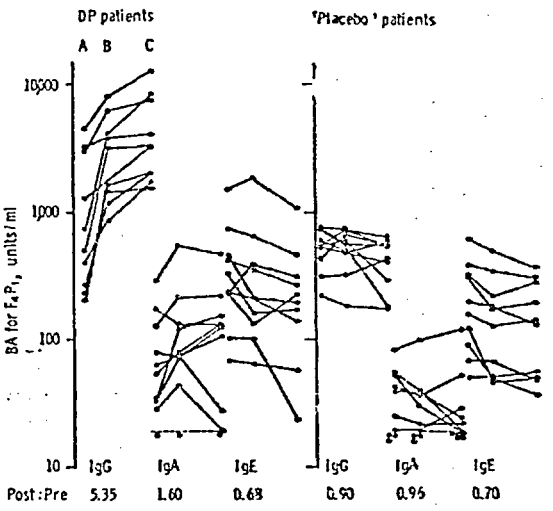


Fig. 3. IgG BA, IgA BA and IgE BA for F_4P_1 in serum samples from a selected group of DP and 'placebo' patients. The serum samples are indicated: A = pre-treatment; B = mid-treatment (4 months) C = post-treatment (12 months). Sera without detectable IgA BA are shown below the dotted line. The mean post-treatment : pre-treatment ratio (post : pre) for each class of BA is shown.

Table II. Correlation between changes in IgG BA and changes in symptom scores (ΔS) in patient groups

Treatment group	Comparison	Number of patients assessed	Correlation coefficient	p value
All patients	pre-treatment IgG BA vs. pre-treatment symptom score	58	−0.25	>0.05[1]
All patients	post-treatment IgG BA vs. post-treatment symptom score	58	−0.09	>0.1
DP	IgG BA post. − IgG BA pre. vs. ΔS	29	−0.09	>0.1
DP	IgG BA post. : IgG BA pre. vs. ΔS	29	0.28	>0.05
DP	$\frac{\text{IgG BA:IgE BA post.}}{\text{IgG BA:IgE BA pre.}}$ vs. ΔS	24	0.12	>0.1
'Placebo'	IgG BA post. − IgG BA pre. vs. ΔS	29	−0.16	>0.1
'Placebo'	IgG BA post.:IgG BA pre. vs. ΔS	29	−0.13	>0.1
'Placebo'	$\frac{\text{IgG BA:IgE BA post.}}{\text{IgG BA:IgE BA pre.}}$ vs. ΔS	24	−0.24	>0.05

[1] When all the patients included in the previous study were assessed, a slightly better correlation was obtained (n = 66, r = −0.3, p <0.02) [17].

treatment (mean increase 1.5-fold) (table I, fig. 3).

Relationship between IgG BA Response and Symptoms

The effect of hyposensitization on the symptoms of 23 DP patients and 27 'placebo' patients has been described previously [17]. In the present study we included a further 6 DP and 2 'placebo' patients for whom sera and symptom scores were available at 1 year, i.e. after approximately 6 months of maintenance therapy. When the patients' own assessments of their symptoms were compared, the DP patients showed a progressive improvement in symptom scores from 4 months onwards. The DP patients were improved in all symptoms for the last 3 months of treatment ($p<0.01$) [see also fig. 1 and 2 in ref. 17]. During the first 28 days of treatment, symptom scores were very similar in the two groups. This period has been used as the baseline to assess subsequent changes in symptoms. We attempted to correlate the production of IgG ab and improvement in symptom scores in individual patients. The results showed no correlation (table II). There was a slight inverse correlation between IgG BA and symptom scores pre-treatment ($p < 0.02$) but no correlation after treatment. The change in symptom scores during treatment (ΔS) was compared with the absolute increase in IgG BA, the ratio of IgG BA post-treatment : pre-treatment and the ratio of IgG BA : IgE BA post-treatment to IgG BA : IgE BA pre-treatment. None of these assessments of IgG BA showed any correlation with changes in symptom scores either in DP or 'placebo' patients (table II).

Table III. Quantities of F_4P_1 bound[1] by IgG ab[2] in pre- and post-treatment sera using different antigen concentrations

Patients[3]	Incubation time	Quantity of ^{125}I-F_4P_1 added to					
		pre-treatment sera			post-treatment sera		
	min	7 ng	1 ng	0.2 ng	7 ng	1 ng	0.2 ng
Patient No. 410	5	0.06	0.02	0.007	0.25	0.07	0.02
	30	0.14	0.04	0.01	0.57	0.15	0.04
	120	0.27	0.08	0.02	1.07	0.27	0.07
Patient No. 417	5	0.07	0.02	0.008	0.78	0.21	0.07
	30	0.14	0.05	0.02	1.47	0.36	0.08
	120	0.23	0.11	0.03	1.99	0.50	0.10

[1] Values expressed as ng F_4P_1 bound, and based on the protein content of a standard preparation of F_4P_1 determined using the Lowry technique [16].

[2] Sera were assayed in the presence of 7, 1 or 0.2 ng ^{125}I-labelled F_4P_1, and the assay was stopped at 5, 30 or 120 min by the addition of a 50-fold excess of cold antigen. IgG ab was then precipitated with anti-IgG for 2 h at 37°C.

[3] Sera from patients No. 410 and No. 417 were assayed at dilutions of 1:25 and 1:50, respectively.

Time Course of Binding by IgG ab

In view of the apparent lack of correlation between IgG BA and improvement in symptoms, further experiments were designed to assess whether excess IgG ab would be able to 'block' the binding of antigen to mast cells *in vivo*. The antigen-binding assay used here is routinely carried out by incubating a dilution of patient's serum with 7 ng ^{125}I-labelled F_4P_1 for 4 h. The onset of symptoms following natural exposure occurs within 15 min and may well involve much smaller quantities of allergen (<1 ng). Antigen-binding assays were carried out using constant amounts of IgG ab with smaller quantities of antigen and shorter incubation times. The results show that the antigen bound increased progressively both with time and antigen concentration (table III) [16]. However, the apparent relationship between different sera was not markedly affected by altering the conditions of the assay. It was also clear that in the presence of very small concentrations of allergen these sera still bound F_4P_1 slowly. IgG ab which could bind 1.07 or 1.99 ng out of 7 ng in 120 min bound only 0.04 or 0.08 ng in 30 min when 0.2 ng was added. Thus even in the presence of an apparent large excess of IgG ab the binding of F_4P_1 might not occur sufficiently rapidly to prevent interaction with mast cells.

Discussion

In this study injections of aqueous *D. pteronyssinus* extract produced a selective rise in serum IgG ab and an improvement in symptoms. However, the evidence suggests that the production of IgG ab was not directly related to the improvement in clinical symptoms. Firstly, the time course for ab production did not correlate with the time at which patients were observing symptomatic relief. Patients' symptoms were most improved over the last 3 months of treatment whilst most of the rise in IgG ab occurred during the first 4 months. Secondly, we could find no correlation between symptom scores and the levels of IgG ab in pre- and post-treatment sera. Other evidence also suggests that the effects of hyposensitization may not be directly related to IgG ab. In a similar study [21] we have assayed IgG, IgA and IgE ab to *D. pteronyssinus* in sera from asthmatic children who were hyposensitized with tyrosine-adsorbed mite extract (Migen). In that study the treated group showed a reduction in both their symptoms and the incidence of late reactions to bronchial challenge. However, there was no significant difference between the IgG ab levels of the Migen-treated or placebo-treated patients. Several studies of hyposensitization in grass pollen and ragweed hay fever have demonstrated a selective rise in serum IgG ab [13–15, 22–26]. In some of those studies the quantitative increase in IgG ab was weakly associated with patients showing the best clinical improvement [23–26]. However, despite considerable effort, other authors have failed to correlate rises in IgG or 'blocking' ab with improvement in symptoms [27, 28]. There is evidence that IgG ab has a role in hyposensitization for bee sting allergy [29, 30]. However, the quantities of allergen involved are much larger than those of inhalant allergens, and the route of exposure is different.

Several alternative mechanisms of the beneficial effects of hyposensitization have been proposed. Firstly, IgE ab production might be suppressed. Certainly cells which

specifically suppress IgE ab have been demonstrated in animals [31, 32]. Furthermore, prolonged hyposensitization may lead to a fall in IgE ab levels [14]. However, in the present study we found no evidence for specific suppression of IgE ab in actively treated patients. Recent studies on hay fever have suggested that the IgG ab response to injected antigen does not occur in the same place as the ab response following natural exposure [13]. If injected antigen raised IgE suppressor cells in 'central' lymphoid tissue, e.g. spleen, it is possible that these cells would fail to act on IgE production occurring at local sites. A second alternative is that hyposensitization acts on mast cells and they become less responsive to antigen. In some ragweed allergic patients improvements in symptoms have been associated with decreased histamine release from circulating basophils [14, 24]. However, no consistent evidence of this kind has been obtained for the house dust mite [8, 9].

It has often been assumed that the role of IgG ab following hyposensitization was to 'block' the binding of allergen to mast cells at mucosal surfaces. The results in table III suggest that the IgG ab in post-treatment sera, even when present in excess, might not be able to 'block' histamine release in the short periods during which symptoms develop. A similar conclusion has also been reached by other workers using rabbit antisera to inhibit histamine release from human basophils [33]. It is possible that IgG ab in nasal secretions would have more effective 'blocking' activity than that found in serum. The levels of IgG BA and IgA BA found in nasal secretions are not high, neither for pollen antigens [34, 35] nor for *D. pteronyssinus* [*Chapman and Platts-Mills*, unpublished data], and show

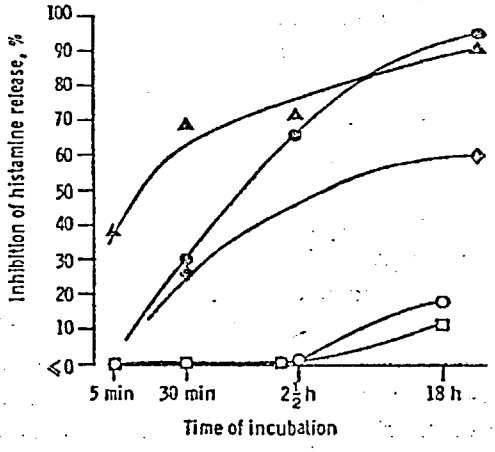


Fig. 4. Time course of inhibition of ragweed antigen E-induced histamine release by pooled nasal washings from allergic (△, ◭, ⊖) and non-allergic individuals (○, □).

only a modest rise during treatment [13, 34, 35]. Furthermore, the blocking of histamine release by IgG and IgA anti-antigen E in nasal secretions also occurs very slowly (fig. 4) [*Platts-Mills and Lichtenstein*, unpublished data]. Thus, even concentrated nasal washings which could cause $> 90\%$ blocking after 18 h achieved $< 45\%$ of this level in 30 min. These results suggest that the quantity of local ab produced in responce to systemic hyposensitization would not be effective in preventing allergens from releasing histamine *in vivo*. There is considerable evidence that the antibodies to inhalant allergens are produced locally [13], and it is possible that local hyposensitization [36] would be a more effective form of treatment. Directly applying allergen to the nose might improve symptoms either by raising 'blocking' ab levels in nasal secretions or by priming local IgE-specific suppressor cells [13, 37]. However, symptoms could be made worse either by increased local production of IgE ab, or by the recruit-

ment of basophils and mast cells to the nasal mucosa [38]. In the present study systemic injections produced modest clinical improvement, but the production of IgG ab did not appear to be the mechanism by which this improvement was mediated.

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