

SPECIFIC SERUM ANTIBODIES TO BACTERIAL
ANTIGENS IN ALLERGIC LUNG DISEASE

by

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ABSTRACT

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The aim of this work was to examine aspects of the serum antibody response in man to whole bacterial cells of Staphylococcus aureus, Streptococcus pneumoniae and Haemophilus influenzae and to ascertain their possible role in certain allergic lung diseases. Most of the thesis is concerned with the development and use of assays for specific IgE, although data is also presented on the measurement of total specific IgG and specific antibody of the IgG₄ subclass to these three bacteria.

Early experiments are reported where whole bacterial cells were immobilised by heat fixation to plastic on slides in a radioimmunoassay and on individual wells in an enzyme linked immunosorbent assay. However, a more sensitive assay was required and a radio-allergosorbent test (RAST) was developed by covalently linking the antigen to cyanogen bromide activated Sepharose 4B beads. After establishing the specificity and optimum conditions of the assay it was employed throughout the study.

Patients (20-30) from each of six disease groups: chronic bronchitis, allergic broncho-pulmonary aspergillosis (ABPA), bronchial asthma, allergic rhinitis, urticaria and cystic fibrosis were skin prick tested and investigated serologically. The IgE RAST values were compared with those from non-atopic blood donors and cord blood sera containing no demonstrable total IgE.

The ABPA group gave the highest specific IgE RAST scores to all three bacteria, whilst with Haemophilus influenzae the chronic bronchitis and cystic fibrosis groups also gave raised RAST scores. Statistically significant differences between the disease groups were also found in the IgG and IgG₄ RAST scores.

There was a positive correlation between the patients' Staphylococcus aureus and Streptococcus pneumoniae immediate-type skin reactions and their RAST scores and their total serum IgE concentrations. The low incidence of immediate-type skin test positivity to Haemophilus influenzae prevented comparable correlations with this organism.

These results taken together suggest that specific IgE and IgG antibodies to bacteria can be recognised in the sera of patients from a number of respiratory diseases, especially ABPA.

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CHAPTER I

BACTERIAL ALLERGY.

1.0 INTRODUCTION

Evidences of bacterial allergy were recorded long before the existence of anaphylaxis and allergy was appreciated. Amongst these were acute infectious diseases, such as pneumonia, which were frequently found as an antecedent to and in conjunction with asthma, rhinitis and urticaria; the occasional and prompt relief of long-standing allergic manifestations following drainage of or removal of an infected focus; and the frequent reproduction of an allergic symptom following an injection of an autogenous or stock vaccine.

In general, two types of allergic response to bacteria and their products were recognised. The first was an anaphylactic response which was thought to be a "protein reaction" of the immediate oedema-hyperemia (weal) type and was considered comparable in all respects to the "non-bacterial protein sensitisation". The second was the tuberculin-type allergic response which was well documented and which most workers considered to be caused by any bacterial organism and was manifested by a delayed inflammatory reaction. These reactions were evident when intracutaneous tuberculin was given to a tuberculous guinea pig since there was both an immediate anaphylactic response followed several hours later by a delayed reaction (Koch, 1891).

There has always been some confusion over the relationship between allergy and immunity. The former term was defined by Von Pirquet in 1906 as 'an altered reactivity resulting from primary sensitivity consequent upon an animal's experience of antigenic material, living or dead including

both immunity and hypersensitivity'. In his extensive review of bacterial hypersensitivity, Rich (1951) stresses that a bland antigenic substance can initiate "immunological injury" by acting in the hypersensitive animal as though it were an acutely irritant toxin. Nowadays allergy and immunity are regarded as biologically distinct processes which may or may not co-exist. In descriptive clinical terms "immunity" refers to the protection offered by the primed immune response, whilst "allergy" is defined in terms of hypersensitivity to antigen challenge and is essentially harmful.

Atopy was defined by Coca (1923) as a "type of hypersensitiveness peculiar to man, subject to hereditary influence, presenting the characteristic wealing type reactions having circulating reagin (reaginic antibody) and manifesting peculiar clinical syndromes such as asthma and hay fever".

Since patients with asthma may show no evidence of anaphylactic allergy whilst others do, but with no clinical manifestations, Pepys (1975) suggested that the term 'atopy' should more narrowly define the capacity to develop anaphylactic sensitivity to common allergens as demonstrable by skin or serological tests. In this thesis a patient with one or more positive skin prick tests to a battery of 23 common inhalant and food allergens was placed in the atopic category.

In 1963 Gell and Coombs classified hypersensitivity reactions into four types, I-IV, based on a return to Von Pirquet's original usage of the term 'allergy' which included

both antibody-mediated decreased reactivity (immunity) and increased reactivity (hypersensitivity) phenomena.

On this basis human bacterial allergy examples could range from immunity to diphtheria, the patient response to M. tuberculosis, to the production of glomerulonephritis following streptococcal infection.

Gell and Coombs four types of hypersensitivity were I anaphylactic; II cytotoxic; III complex-mediated and IV cell-mediated (delayed type). Types I, II and III depend on the interaction of antigen with humoral antibody. The four types will be briefly described and evidence of known bacterial involvement in each discussed.

Type I. Anaphylactic sensitivity

When antigen is reintroduced into an immunologically primed or sensitised individual, it reacts with reaginic antibody ("reagin") bound to mast cells or circulating basophils through a specialized region of the Fc piece. This leads to degranulation of the mast cells and release of vasoactive amines.

Sensitivity is normally assessed by the response to skin or provocation testing with antigen. In the former the release of histamine and other mediators rapidly produces a wheal and erythema, maximal within 20 minutes and then subsiding. That a component of serum "reagin" is responsible is shown by the ability of an allergic patient's serum to passively sensitise the skin of normal humans (Prausnitz-

Kustner or 'P-K' test) or preferably, to avoid the risk of transferring human serum hepatitis, of monkeys. As this reaction can be blocked by prior injection of an IgE myeloma, the serum component responsible would appear to be IgE. Ishizaka and his co-workers (1966) showed that "reagin" was a hitherto unrecognised immunoglobulin class which was designated IgE. The same conclusion was reached independently by Johansson and Bennich (1967) who discovered an IgE myeloma (IgND). IgE has a sedimentation constant of 8.0 compared with 6.8 S for IgG. It sensitises preparations of blood leucocytes to an antigen-induced release of histamine (Lichtenstein and Osler, 1964) and also sensitises human lung tissues to histamine and SRS-A release (Parish, 1967; Orange et al. 1971). IgE antibody is heat-labile and is now called 'long-term' homocytotropic antibody because of the time interval required for optimal sensitisation of target mast cells.

In addition Parish (1970) has identified an IgG antibody capable of mediating Type I reactions in man which, in passive transfer tests, sensitises the skin for an antigen-induced "blueing reaction" which persists for only about four hours. This 'short-term' sensitising (S-TS IgG) antibody is heat-stable and there is some evidence that it is of the IgG₄ subclass, (Stanworth and Smith, 1973; Parish, 1974) but its role in vivo has yet to be defined. Parish (1971a) found anaphylactic IgG-type antibodies in the sera of some patients (i) recovering from streptococcal pneumonia, (ii) with tonsillitis associated with vasculitis, (iii) after vaccination

with tetanus toxoid and (iv) with allergy to milk (Parish, 1971b).

Evidence for the involvement of Type I allergic reactions against bacterial substances comes from a number of sources. The copious alveolar exudation that characterises the onset of acute lobar pneumonia and the lack of toxicity of autolysates in man is suggestive of an anaphylactic reaction in persons naturally hypersensitive to pneumococcal antigens. There is evidence of such a hypersensitivity from skin tests which, in those with the disease, disappear during the acute phase (Finland and Sutliff, 1931). Challenge of anaphylactically sensitised rabbits (Sharp and Blake, 1930) and guinea pigs (Lindau, 1933) with inhaled sterile pneumococcal antigens induced severe oedema and lesions resembling lobar pneumonia in man.

The resemblance between natural infective lesions and anaphylactic-type responses to the corresponding microbial antigens in sensitised animals is the basis of the suggestion that the acute E. coli bowel oedema and haemorrhagic gastro-enteritis of piglets is anaphylactic in origin (Thomlinson and Buxton, 1963). Heterocytotropic but not homocytotropic antibody to E. coli is demonstrable in the serum during the disease, indicating that if there is an anaphylactic element, it is an aggregate rather than an antibody adherent reaction (Parish, 1972).

More evidence for the involvement of Type I allergic reactions against bacterial substances comes from the development of bronchial asthma in workers producing biological

washing powders containing proteolytic enzymes and spores of B. subtilis. These patients developed both immediate and late reactions of asthma following inhalation of one to ten mg of B. subtilis enzyme and also had positive specific RAST scores to the antigen (Pepys et al. 1969)

Type II. Antibody-dependent cytotoxic

hypersensitivity involves the death of cells bearing antibody attached to a surface antigen. The cells may be ingested by phagocytic cells to which they adhere through their coating of IgG or C3b or lysed by the complement system, or non-phagocytic extracellular cytotoxicity may occur by killer cells with receptors for Ig Fc.

When microbes or their antigenic components are adsorbed to a tissue in vivo, reactions with anti-microbial antibody would induce a Type II reaction, comparable with the in vitro lysis of red blood cells coated with bacterial antigens in the presence of antibody and complement. Parish (1972) records a similar adsorption by HeLa cells and a specific, complement-dependent cytotoxic change subsequently induced by antibody. The degree of damage depended on the nature of the antigen, being slight with non-toxic protein antigens of S. aureus and E. coli and substantial with enterobacterial lipopolysaccharide and some streptococcal antigens.

Type III. Complex-mediated hypersensitivity

The combination of antibody with antigen within the body usually leads to the fixation of complement and the

release of anaphylatoxins and chemotactic factors. The anaphylatoxins release histamine from mast cells by an IgE independent mechanism. Polymorphonuclear leucocytes are attracted to the site of complement fixation and release proteolytic enzymes causing local tissue damage. In relative antibody excess the antigen is precipitated near the site of entry into the body. The reaction in the skin is characterized by polymorph infiltration, oedema and erythema maximal at 3-8 hours (Arthus reaction) e.g. Farmer's lung. In relative antigen excess, the complexes formed are soluble and are free to circulate, being deposited at certain preferred sites, e.g. the joints, the skin and the kidney glomerulus.

In glomerulonephritis immunofluorescent and ultramicroscopic techniques have demonstrated the presence of complexes of streptococcal antigen, immunoglobulin and complement proteins on the glomerular basement membrane. Burkholder (1968) and Parish (1972) showed that the damage by such deposited aggregates was not mediated by neutrophil lysosomal enzymes.

However neutrophil-mediated aggregate reactions may be the basis of the cutaneous vasculitis in man that occurs after the acute stage of a number of microbial infections. Parish (1971c) identified streptococcus A, Candida albicans, streptococcus D, Staphylococcus aureus and M. tuberculosis antigens, in that order of frequency in the vascular lesions of a small proportion of subjects he examined, sometimes in association with deposits of IgG, IgM and the β 1c component

of complement. Patients with post-streptococcal vasculitis had precipitating and complement-fixing antibodies to various streptococcal components in the serum, and aggregates of these antibodies with the appropriate antigen induced a local vasculitis in the skin of monkeys and guinea pigs.

Type IV. Cell-mediated or delayed type hypersensitivity is based upon the interaction of antigen with endogenous receptors on the surface of primed T-lymphocytes. Lymphokines are released which cause a typical delayed hypersensitivity reaction, namely the delayed appearance of an indurated and erythematous reaction which reaches a maximum at 24-48 hours and is characterized histologically by infiltration first with polymorphs and subsequently with mononuclear phagocytes and lymphocytes.

This form of hypersensitivity is encountered in many allergic reactions to bacteria, e.g. in tuberculosis (the Mantoux reaction to tuberculin) and in leprosy. In the latter the type and intensity of the allergic response influences both the nature of the tissue damage and the patient's resistance to the infection. The 'tuberculoid' form of the disease which shows strong Type IV reactivity, is relatively benign clinically. In the 'lepromatous' form however, there is weak Type IV reactivity with extensive proliferation of organisms, large amounts of precipitating antibody formed and development of allergen complexes (erythema nodosum leprosum nodules).

2.0 MORPHOLOGY AND IMMUNOLOGY OF THE THREE BACTERIA

2.1. Staphylococcus aureus

From 1884 (Rosenbach), the pathogenicity of staphylococci was denoted by the colour of colonies they produced on solid culture media - namely golden S. aureus for pathogens and white S. albus for non-pathogens. Colour however is subject to variation and staphylococci today are classified primarily on the basis of coagulase production (Wilson and Miles, 1964). The production of coagulase is characteristic of S. aureus; this substance is almost certainly an enzyme and a precursor of a thrombin-like substance which coagulates blood plasma. However, the coagulation description of pathogenic staphylococci has not universally been accepted and the term S. aureus is still used to describe pathogenic organisms and will be so used in this study. On staining, staphylococci appear as Gram-positive spherical cocci arranged in irregular clusters, the individual cells being approximately 1μ in diameter. Further properties which distinguish S. aureus from other members of the species are its ability to grow in 10% salt broth, to ferment mannitol and to produce phosphatase.

There are protein antigens common to both pathogenic and non-pathogenic staphylococci, but sera prepared against pathogenic strains do not agglutinate non-pathogenic strains and the converse is also true. At least four classes of substance have now been identified in the staphylococcal cell wall or external to it (Morse, 1965) and are involved in immunological reactions with corresponding antisera. The

main structural unit of the cell wall consists of mucopeptide and the peptide subunits are bridged by chains composed almost exclusively of glycine (Tipper and Berman, 1969). Linked to the mucopeptide but extractable with trichloroacetic acid are one or more teichoic acids, which can be detected by a precipitin reaction. More superficial, and therefore also detectable by agglutination, are a number of other antigens, some of which are proteins.

Julianelle and Wieghard (1935a and b) extracted a "polysaccharide A" from pathogenic bacteria which gave a precipitate only with rabbit antisera prepared against other pathogenic staphylococci. Since this polysaccharide contained phosphorus, Heptinstall et al. (1970) thought it could be concerned with ion exchange at the cell membrane. A similar but immunologically different substance "polysaccharide B", was obtained from non-pathogenic staphylococci. Both these substances were later shown to be teichoic acids (Haukenes et al. 1961) and to be part of a series of such haptens distributed through the species (Baddiley et al. 1962). These workers showed that teichoic acid A was a polymer of ribitol with β -linked N-acetylglucosamine and was present in most strains of S. aureus. Certain strains belonging to phage-group I have in addition an antigenically distinct ribitol teichoic acid with α -linked N-acetylglucosamine (Hofstad, 1964).

Another antigen found in nearly all strains of human origin and which also causes agglutination of S. aureus is a protein named protein A. Jensen (1958) showed that it

was responsible for a precipitin line in agar gel between crude acid extracts of S. aureus and normal human serum. It has been isolated and shown to be a protein of the cell wall from which it is readily removed by proteolytic enzymes (Yoshida, Mudd and Lenhart, 1963). It has a molecular weight of about 13000 and sensitises tanned but not untreated sheep erythrocytes (Grosv 1967, 1968).

Forsgren (1970) reported that of 700 coagulase-positive staphylococcal strains isolated from humans, 98.9% produced protein A. Strain Wood 46, which is commonly used for the preparation of staphylococcal toxoid, had previously been thought not to produce the protein, but Forsgren (1970) using a sensitive haemagglutination test demonstrated that it did contain a small amount of extracellular as well as cell-bound protein A. Although specific antibodies are present in human sera against protein A (Martin and White, 1968), it has been shown that this substance also has the facility to combine non-specifically with the Fc-portion of human γ -globulin (Forsgren and Sjoquist, 1966), thus presenting a problem when immunological mechanisms of S. aureus are being studied. Protein A may also play a role in the development of inflammation since the antigen has been shown to directly release histamine from human leucocytes in vitro (Martin and White, 1969).

Burns and May (1968) in their study of bacterial precipitins in serum of patients with cystic fibrosis demonstrated antibody to an S. aureus extract in 53% of these patients, in contrast to 29% of patients with bronchiectasis,

"significantly less" in bronchitics and in 9% of controls.

2.2. Streptococcus pneumoniae

The account by Pasteur, Chamberland and Roux (1881) of a septicaemic infection of rabbits inoculated with human saliva is probably the earliest reference to the pneumococcus, though no clearly identifiable description of the organism was published before the studies of Fraenkel in 1886.

Topley and Wilson (1975) classify the pneumococci as a separate and well defined group of streptococci. They are normally ovoid or lanceolate cocci, arranged in pairs or short chains; when in pairs the opposite ends are the more acutely pointed. A capsule surrounds each pair and possesses one of a series of antigenically specific capsular polysaccharides. They produce α -haemolysis of blood containing media.

Neufeld and Handel (1909) first established the existence of antigenically different types of pneumococci by showing that pneumococcal antisera conferred type-specific immunity on mice. Dochez and Gillespie (1913) studied the antigenic relationships of a large collection of pneumococci by means of agglutination tests. Strains isolated from cases of lobar pneumonia in man included three well differentiated types I, II and III and a large heterogeneous group IV. Dochez and Avery (1917) showed that the basis of the type separation lay in the possession of various soluble components in the bacterial capsule. In 1923 Heidelburger and Avery

showed that this type-specificity depended on the formation of capsular polysaccharides.

By means of the capsular swelling typing method (Neufeld and Etinger-Tulczynska, 1931), agglutination or immunoelectrophoretic precipitation test (Dorff et al. 1971), 84 different types of S. pneumoniae have been identified to date of which 80% belong to a small group of 14 sero types (Emmerson, 1980).

Solutions of the type polysaccharides in high dilution give a precipitate when mixed with the corresponding antisera. Although these reactions are in general specific, cross reactions between some pneumococcal types and the polysaccharides of Klebsiella and Haemophilus (Pittman, 1931) and human blood group substances have been described.

The sugar composition of many and the chemical structure of a few of the type specific soluble capsular polysaccharides have now been established, e.g. the type III polysaccharide is a polymer composed of alternating units of glucose and glucuronic acid (Stacey and Barker, 1960).

Under optimal growth conditions the pneumococcus is capsulated but in less favourable circumstances the organisms lose their capsules and with it their virulence. The capsulated form and its non-capsulated variant are termed 'smooth' (S) and 'rough' (R) respectively.

The cell-wall antigens of the pneumococci have received less attention than the capsular antigens. Tillett and Francis (1930) obtained a somatic polysaccharide antigen from an R strain which appeared to be species-specific. The

chemical composition of pneumococcal 'C-substance' as it came to be called was analysed by Liu and Gotschlich (1963) who obtained and purified extracts rich in this material and found in it muramic acid and other constituents of the cell-wall mucopeptide. Pneumococcal 'C-substance' combines in a non-immunological way with the so-called C-reactive protein that appears in the blood of patients with certain febrile illnesses (Kabat and Meyer, 1961). This C-reactive protein is a beta-globulin (Hedlund, 1961) and not an antibody but can cause some confusion when true antibodies to the C-substance are being measured. It reacts and combines with the major serological determinant of the C-substance - the N-acetylglucosamine phosphate polymer, or more probably, with a peptide attached to the polymer (Gotschlich and Liu, 1967). The reaction depends on the presence of calcium ions (Abernethy and Avery, 1941), the elimination of which by sodium citrate inhibits the reaction and prevents confusion with genuine antigen-antibody precipitation reactions. Longbottom and Pepys (1964) have shown similar reactions between C-reactive protein and C-substance-like polysaccharides in fungi, which are also inhibited by removal of calcium ions. Burns (1968) found precipitins against the species specific pneumococcal C-substance in 26% of patients with simple bronchitis and a similar 27% of patients with chronic obstructive bronchitis. In contrast only 14% of asthma patients had these precipitins and 13% of the 107 controls. Burns and May (1968) found that this pneumococcal antibody was found in cystic fibrotics no more commonly than in the

control subjects.

Austrian and Macleod (1949) extracted from both capsulated and non-capsulated pneumococci a series of type-specific proteins now referred to as type-specific pneumococcal M antigens. They are taken to be near the surface of the cell-wall since they can be destroyed by trypsin in the intact cell. Unlike the M proteins of Streptococcus pyogenes, they play no part in the virulence of the organism.

Agglutinating antibodies may be produced in infection and agglutinin titres may be determined by adding the bacterial antigenic suspension to dilutions of the patient's serum. Nicholls, Pease and Green (1975) used agglutination tests employing whole organisms to follow the antibody levels present in sera. Of 57 chronic bronchitis patients in an acute phase, 10 showed rising titres of agglutinating antibody to S. pneumoniae. Rising antibody titres were not found in normal control subjects nor in chronic bronchitics not having an acute exacerbation.

In 1970 Reichel et al. used immunofluorescent techniques to measure antibody titres to whole organisms of S. pneumoniae (and H. influenzae) in chronic bronchitics. It is usual to consider as significant a 4-fold titre rise in an acute stage. These workers detected such significant rises in titre of IgM antibody in 3 of 11 cases in exacerbation tested against S. pneumoniae.

2.3. Haemophilus influenzae

H. influenzae was first isolated by Pfeiffer (1893) from the sputum of many patients suffering from influenza. It was thought to be the causative agent in the disease, hence its name, and this error persisted until Smith, Andrewes and Laidlow (1933) finally showed that influenza was caused by a virus. H. influenzae most commonly appears as very short Gram negative rods ($1 - 1.5 \mu\text{M} \times 0.3 - 0.4 \mu\text{M}$), sometimes almost coccal, sometimes thread-like. Great pleomorphism is shown between and within strains. The studies of Pittman (1931) emphasize the importance of this distinction, suggesting that typical and atypical morphology are associated with an S $\rightarrow$ R type of variation and are not permanent characters of stable varieties of types. The complexity was to some extent resolved by Pittman who recognised that the species could be divided on a basis of cultural appearance into capsulated organisms showing smooth colonies (S) and non-capsulated organisms which had rough colonies (R). The former could be further sub-divided into 6 types on the basis of the polysaccharides present in the capsule (a - f) in an analogous way to the pneumococci. Indeed antigenic similarities have been described in the capsular polysaccharide structure of some H. influenzae and S. pneumoniae (Turk and May, 1967).

In 1937 Chandler, Fothergill and Dingle showed that there were at least 3 basic colonial forms of H. influenzae. These were M (mucoid), S (smooth) and R (rough) forms. The virulent mucoid M form is capsulated; the more common S form can also have a capsule but may not; whilst the R form is

rarely capsulated. May (1965) in experiments made with antigens and antisera prepared from 5 hour capsulated and 24 hour non-capsulated cultures concluded that there were 3 main antigens present in H. influenzae:

- (1) An M antigen - a type specific polysaccharide (types a - f)
- (2) A thermostable somatic species-specific R antigen
- (3) A thermolabile somatic type-specific S antigen

The M strains contain all three antigens, the S strains S and R and the R strains R alone.

In chronic respiratory tract infections the most frequently isolated strains of H. influenzae are non-capsulated. May (1967) isolated them from 95% of infections in chronic bronchitis patients. R strains are seldom found and the capsulated M strains, though commonly isolated from infections of the meninges, are only found in 5% of chest infections (Holdaway and Turk, 1967). For these reasons a non-capsulated strain of H. influenzae was chosen for this study. Since type-specificity depends on a polysaccharide component in the capsule of the organism, non-capsulated strains are untypable. The complex antigenic structure of these non-capsulated forms is still not fully elucidated.

An endotoxin-like substance from H. influenzae was described by Dubos (1942). More recently Branefors-Helander (1973) obtained a cell wall antigen of H. influenzae having endotoxic properties while Van Der Zwan, Orie and De Vries (1975) have demonstrated endotoxin in an antigenic preparation prepared after the method of Burns and May (1967).

Agglutination techniques have been used to detect allergens on the organism surface. In Nicholls, Pease and Green's study, referred to under the S. pneumoniae section, rising titre of agglutinating antibody to non-capsulated H. influenzae was found in 38 of 57 chronic bronchitis patients in an acute phase but not in control subjects nor in chronic bronchitics not in an acute phase.

Glynn (1959) used an indirect haemagglutination technique employing tanned red cells to assess antibodies to H. influenzae extract. He found antibodies greater than 1:500 in 66% of his chronic bronchitic group and raised titres in 21% of asthmatics and 6% of controls.

The study of Reichel et al. (1970) using immuno-fluorescent techniques, also referred to under S. pneumoniae, detected rises in titre of IgM antibody to H. influenzae in 18 of 47 chronic bronchitics in exacerbation.

Specific antigens to H. influenzae were demonstrated by Tunevall (1953) using a carbonate extraction method and by Omland (1964) who found at least 2 common antigens. He tested ultrasonic extracts of 73 strains of H. influenzae, including both capsulated and non-capsulated forms, using immunoelectrophoresis techniques. Following Omland's work, Burns (1967) was able to differentiate 5 basic groups of antigen by immunoelectrophoresing ultrasonic extracts of non-capsulated H. influenzae with human sera. These he named $H_1 \rightarrow H_5$ and are shown diagrammatically in Fig. 1. Neutralisation studies with extracts from Pseudomonas pyocyanea and Klebsiella species showed that antigens in the H_3 to

H₅ groups which migrate towards the anode were also to be found in other Gram negative bacilli, but that the H₁ and H₂ antigens which migrate towards the cathode appeared specific for non-capsulated H. influenzae. Since the precipitation reaction was prevented if the extract was initially autoclaved to denature protein, Burns (1967) suggested that the allergenic determinants might be protein in nature. H₁ is far commoner than H₂ and Burns and May (1967) proceeded to test patients' sera with this specific antigen. A high proportion (69%) of patients with advanced obstructive bronchitis gave a precipitin line to H₁ and 25% with simple bronchitis in contrast to 8% of asthmatics and 6% of the control subjects. In a further study Burns and May (1968) found similar specific H. influenzae precipitins in approximately 30% of 30 cystic fibrosis patients and in those with combined chronic bronchitis and bronchiectasis, compared with only 8% of controls. Clarke (1979) confirmed these Burns and May studies of the frequency of precipitating antibody against H. influenzae H₁ H₂ antigens in chronic bronchitis and cystic fibrotics. In addition he showed an increased frequency of reaction of asthmatic patients to the heat-stable cell wall antigens without an increased frequency of reaction to heat-labile cytoplasmic antigens. Clarke thought this might reflect an increased resistance to colonization and invasion by the organism. The same study showed that the cell wall antigen caused release of histamine from human leucocytes, while both this antigen and a culture filtrate antigen caused release from human lung.

3.0 IN VIVO REACTIONS

3.1. Skin Testing in Hypersensitivity to Bacteria

Immediate reactions to skin tests, both with common allergens e.g. pollen and with bacterial allergens, are mediated mainly in atopic subjects by the heat-labile, reaginic antibody IgE. This skin reaction consists of an urticarial type weal with erythema and irritation, developing usually within two minutes, maximal at 10-15 minutes and lasting approximately 2 hours. This is accompanied by an eosinophil cell infiltration and can be inhibited by anti-histamines but not by the inhalation of beta receptor agonists (Galant et al. 1973) nor by administration of corticosteroids (Slott and Zweiman, 1974) nor by injection of disodium cromoglycate into the test site (Cox, 1971).

Whilst the majority of immediate skin reactions are IgE mediated, a small percentage are attributed to the heat-stable short-term-sensitising-IgG described by Parish (1970).

Skin testing has been the traditional method of examining patients' immunological reactions to bacteria, but the use of non-standardised antigens and inadequate controls in many studies has often precluded any meaningful conclusions being made.

3.1.1. S. aureus

The first report of skin testing to bacterial antigens was from Chandler Walker (1916) in Boston. He used the scratch method to test 'protein' extracts of several

respiratory tract organisms in 100 asthma patients. He obtained positive reactions in 18% of the patients to S. aureus, 6% to S. albus and in only 1-2% to the other organisms (micrococcus, Streptococcus viridans, diptheroid bacillus). It was from this study that the concept of bacterial allergy arose and hyposensitization attempts with autogenous bacterial vaccines followed (Rackeman and Graham, 1923).

In subsequent studies the greatest number of positive reactions also occurred with S. aureus. In 1936 Stevens and Jordani obtained 28% positivity with this organism on intradermally testing 100 asthmatics with 'nucleoprotein' extracts of 5 upper respiratory tract pathogens. They found immediate type reactions to be present as frequently in atopic patients as non-atopics. In contrast Kraft, Mothersill and Nestman (1949) found that whilst 31% of their allergic patients had a Type I reaction to intradermal injections of fragmented bacterial extracts of S. aureus, only 8% of their non-allergic patients did so. Swineford and Holman (1949) carried out a large study consisting of 3,860 cutaneous reactions with 23 crude polysaccharide and 11 nucleoprotein fractions of 14 different bacteria. The largest number and the highest percentage of wheals were elicited by polysaccharides of S. aureus (62%) and Streptococcus faecalis (37%). These workers found that there was a tendency for polysaccharides to give immediate type reactions and for proteins to give a delayed reaction, though with the latter they were unable to differentiate between an Arthus reaction or a Type IV reaction.

In contrast to Swineford and Holman's crude antigens, Kahn, Baldwin, Zeitlin and Smart (1951) prepared 4 fractions from 10 strains of staphylococci isolated from the anterior nares of asthmatic patients. These were a purified type specific protein, a purified polysaccharide, a nucleoprotein and a crude residue. 73% of atopic patients gave Type I reactions on intradermal testing to the purified polysaccharide compared with 32% of control subjects. There were no delayed reactions to these purified polysaccharides although both allergic and control subjects equally gave such reactions to the nucleoprotein fraction.

Julianelle and Hartman (1936) studied the cutaneous reactions to their type A specific carbohydrate from pathogenic staphylococci and their type B from non-pathogenic strains. Positive skin tests to carbohydrate A were found not only in all patients with active staphylococcal infection but also in about 65% of normal adults. There were few positive cutaneous tests to carbohydrate B.

Haukenes et al. (1961) and Baddiley et al. (1962) have shown that these specific carbohydrates are teichoic acids. Strominger (1962) and Torii, Kabat and Bezer (1964) demonstrated positive intradermal tests to teichoic acid from S. aureus in normal control groups. Many earlier studies had not included testing of normal people so these findings emphasised the need for such controls. Indeed Davies (1976) found 26% of his control group had a positive Type I response to the α teichoic acid of S. aureus, which was equal or greater than the incidence in his asthma and chronic bronchitis patient groups.

3.1.2. S. pneumoniae

Most of the studies of cutaneous reactions to antigens of S. pneumoniae have been on patients suffering from lobar pneumonia.

Clough (1915) was the first to show skin test positivity in pneumonia patients using alcohol precipitated protein from pneumococci.

Tillett and Francis (1929) studied reactions in pneumonia patients to intradermal injections of pure Types I, II and III pneumococci polysaccharide and the somatic protein. The polysaccharides elicited specific weal and flare reactions only in patients post-crisis or convalescent from pneumonia. Those not recovering from the pneumococcal infection failed to show this immediate type reaction - this became known as the 'Francis Test'. The somatic protein in contrast only induced late reactions.

Sutliff and Finland (1932) also demonstrated Type I skin reactions to pneumococcal polysaccharides in lobar pneumonia cases and in a few patients without the disease. Finland with Dowling (1935) went on to show that Type I reactions could be produced in normal subjects with repeated intracutaneous injections of pneumococcal polysaccharide.

Davies (1976) found 32% of his atopic asthma group patients gave a Type I reaction to a S. pneumoniae extract compared with 12% in his control group of laboratory workers and their families.

3.1.3. H. influenzae

There have been few studies of cutaneous reactions to H. influenzae antigens.

In 1960 Mary-Katherine Hajos published her results on skin and bronchial tests with bacterial suspensions on 32 patients with bronchial asthma. She used Bencard's standard suspensions of 6 bacteria including H. influenzae and S. aureus. Positive skin prick tests occurred to three bacterial suspensions in between 34 and 59% of the asthmatic patients but only in 6-9% of the same patients when tested intradermally with the same strength suspension. The lower incidence rate of the latter was surprising and even more so was the report of 3% positivity to the control solution, a result which normally only occurs in patients with dermatographia. Hajos found late skin reactions in 65% of patients to H. influenzae but in only 31% of patients with S. aureus.

In 1975, Van Der Zwan, Orie and De Vries reported their results of skin testing with an extract of a non-capsulated strain of H. influenzae which contained all the H_1 to H_5 allergens. They tested intracutaneously five patients with chronic non-specific lung disease and two control subjects. Four of the five patients gave an immediate type reaction whilst neither of the controls did. However, all 7 subjects developed the late skin response which was maximal at 24 hours. Van Der Zwan et al. demonstrated the presence of endotoxin in the extract they used and since this has been shown to be able to activate complement directly through the alternate pathway, it is most likely that this produced the delayed skin reactions.

Another major study of bacterial hypersensitivity, though not directly concerned with the organisms under investigation here, was Hampton, Johnson and Galakatos' (1963) study of asthmatics using Neisseria catarrhalis. After sonically disrupting the organism they prepared multiple ammonium sulphate precipitated fractions and tested them in asthmatics and controls by both bronchial challenge and intradermal skin tests. Asthma, as measured by a change in vital capacity, was induced in 13 of the 16 asthmatics and in none of the 9 controls on aerosol provocation with the fractions. Twelve of the 14 patients showed immediate reactivity to one or more fractions, and 13 developed delayed reactions. Of the control subjects, only 3 out of 21 showed positive immediate reactions whilst 15 developed delayed reactions.

It is difficult however to interpret these skin reactions in immunological terms since the extracts were found to be directly toxic. This caused these workers to have to use non-optimum, low concentrations because of the occurrence of severe delayed skin reactions found in all subjects.

3.1.4. Summary

The properties possessed by certain bacterial substances to trigger immunological pathways in a non specific way cause the study of bacterial allergy to be most complex. Examples of such non-allergic mechanisms concerning the three organisms being studied include (1) the protein A

antigen of staphylococcus combining directly with the Fc fragment of immunoglobulin, (2) the pneumococcal polysaccharide adhering to cell membranes, (3) the H. influenzae endotoxin directly activating complement through the alternate pathway.

One important finding which emerged from these early studies on the cutaneous reactions to antigens from S. aureus was that whilst polysaccharides tended to give immediate skin reactions, the protein extracts usually caused delayed responses. Recent work has shown that bacterial polysaccharides, unlike proteins, may not require T-lymphocyte co-operation in the production of antibody from the B-lymphocyte. Howard et al. (1971) have shown these allergens to be T cell independent. Since T-lymphocyte involvement typifies the Type IV reaction, it is not surprising that late reactions may not be a feature of cutaneous reactions to bacterial polysaccharides. Ishizaka (1975) has suggested that T cell co-operation may be necessary for the production of IgE antibody; therefore it is possible that skin sensitising antibodies elicited by bacterial polysaccharides may be of an immunoglobulin class other than IgE.

C-substances like that of S. pneumoniae and Aspergillus fumigatus are widely distributed in nature. They induce positive skin prick tests and since they have chemical determinants which are closely related, cross reactivity may occur. This may explain Davies' relatively high number of skin prick test positive patients in his control group since the extract of S. pneumoniae he used contained the C-substance which is known to combine in a non-immunological manner with C-reactive protein.

3.2. Bronchial Provocation Tests

Bronchial provocation testing with common inhalant and food allergens has been practised since the 1930s. The technique has been shown to correlate well with specific IgE levels and skin testing (Aas and Johansson, 1971; Stenius et al. 1971).

The airways of asthmatic subjects will respond more to non-specific stimuli than those of non-asthmatics. Substances having a direct toxic effect like bacterial endotoxins could be such stimuli.

Hajos (1960) considered 40% of her 32 asthmatic subjects and none of her 10 non-allergic subjects produced asthma on inhalation of suspensions of H. influenzae and mixed streptococci (Bencard Ltd).

Similarly Hampton et al. (1963) produced episodes of asthma in 13 of 16 asthmatic patients and in none of their 9 control normal subjects on exposure to nebulised extracts of N. catarrhalis.

In 1967, Ricci et al. showed the development of immediate (62%) and late (83%) asthmatic reactions in patients with asthma (113) but not chronic bronchitis (50) following the inhalation of whole organisms and broth culture from an alpha haemolytic streptococcus they isolated from sputum.

Van Der Zwan et al. (1975) challenged five patients with chronic non-specific lung disease with extracts of H. influenzae. Four of these patients produced immediate type reactions and they all developed late reactions at 5-6 hours. Since however the two controls also gave a late

response, it was probable that a toxic component was present in the extract.

Cuevas, Marmier and Girard (1977) looked at the correlation of bronchial provocation tests with some immunological features of atopic and non-atopic asthma. In spite of negative immediate skin tests (to streptokinase-streptodornase and E. coli endotoxins), 11 immediate bronchospastic responses developed in the group of 20 intrinsic asthmatics, 4 to bacterial antigens (M.R.V. Merieux, France) and 7 to E. coli endotoxins.

The development of respiratory disease has also been reported in workers manufacturing vaccines and in those producing biological washing powders. In regard to the former, Pernis et al. (1961) studied workers developing wheezing and malaise on exposure to S. typhi bacteria which were dispersed into the air during the production of typhoid vaccine. Then in 1969, Flindt described similar symptoms amongst factory workers in the biological detergent industry due to inhalation of proteolytic enzymes and viable spores of Bacillus subtilis. In the same year, Pepys et al. reported immediate and late reactions following inhalation of 1 to 10 mg.

Though these challenge concentrations of enzyme are far greater than the levels normally present in the atmosphere when biological washing powders are used, these results do provide good evidence of a Type I response to bacterial antigens.

3.3. Bacterial Vaccines

With the aim of immunising patients against infecting organisms, Carmalt Jones (1909) gave bacterial vaccines to patients to try and prevent attacks of asthma and bronchitis. Vaccines were then used by many subsequent workers in an attempt to hyposensitise their patients. Chandler Walker (1919) reported that 40-75% of 178 patients were relieved of their symptoms and a further 20% improved on administration of vaccines prepared from bacteria isolated from the pharynx or sputum of the patients. Rackeman and Graham (1923) claimed 40-65% of cases improved with autogenous vaccines; whilst Fink and Cay (1934) reported 74% of 20 urticaria patients as cured by vaccine treatment. Other workers, e.g. Berquist (1952) also showed vaccine therapy to be beneficial in asthma, but until Frankland, Hughes and Gorrill's trial (1955) no normal subjects had been included in the studies as controls. This was a double-blind trial in asthmatics with each patient being given either the appropriate autogenous bacterial vaccine or a placebo injection. 58% of the patients improved after vaccine therapy but so also did 52% of those given the placebo. These results cast doubt on the findings of the earlier uncontrolled studies which had not taken the 'placebo effect' into account. Indeed Helander (1959) found no significant difference between vaccine and placebo therapy in 308 adults with presumptive bacterial allergy; neither did Aas et al. (1963) in controlled trials in children.

There have been several trials with H. influenzae vaccines in chronic bronchitis patients (Edwards et al. 1957)

but no real evidence was found of suppression or prevention of infection.

Cooke (1954) thought one of the most compelling pieces of evidence supporting the hypothesis of bacterial allergy was that the symptoms could be reproduced in some patients by the injection of bacterial vaccines. Such examples were reported by Flensburg (1950) who showed asthmatic reactions to bacterial vaccines in 26% of the 162 children he studied; Crump (1954) where 10% of his 112 children developed asthma after autogenous vaccines and Aas (1961) where 3 out of 15 children produced eosinophilic secretion, two of these with wheeze, on administration of bacterial vaccines to the nasal mucosa.

Currently there is a 14-valent pneumococcal vaccine on trial. Despite the advent of antibiotics there is still a need for such a vaccine, for invasive pneumococcal infections still cause high morbidity with a mortality rate approaching 30% in patients over the age of 50 years (Emmerson, 1980).

4.0 SEROLOGICAL STUDIES

4.1. Skin-sensitising antibody studies

IgE antibodies can be demonstrated by the ability of allergic patient's serum to passively sensitise the skin of a non-sensitive human (Prausnitz-Kustner or 'P-K' test) or a non-human primate. This passive sensitisation of primate skin can be blocked most effectively by prior injection of a myeloma of IgE rather than of any other class. Caulfield (1930) reported a successful 'P-K' test from an asthma patient with immediate skin test sensitivity to an autogenous staphylococcal vaccine. More evidence came by passive transfer tests in the Rhesus monkey from Schwartz and Terr (1971) who demonstrated heat-labile skin sensitising antibody to pneumococcal polysaccharide in two subjects.

In animals when intradermally sensitised sites are challenged with intravenously injected antigen plus dye (e.g. Evans blue), the test is referred to as "passive cutaneous anaphylaxis" (PCA).

Using PCA tests in monkeys Parish (1971a) demonstrated heat-stable, short-term passive sensitising antibodies against allergens from Streptococcus pyogenes in two cutaneous vasculitis patients. Here was evidence that the heat-labile IgE antibodies were not mediating the anaphylactic sensitivity.

Finland and Sutliff (1931) had found that intravenous injection of human concentrated anti-pneumococcal sera led to the development of anaphylactic skin responses but the skin sensitivity seldom persisted for more than three days. It is possible that these skin reactions were due to short-term

sensitising IgG antibodies described by Parish (1970) which confers antigen-specific sensitivity in monkey skin for approximately four hours in contrast to sensitivity caused by IgE antibodies which in human skin lasts for 21-28 days.

There have only been a few attempts to look for specific IgE antibodies to bacteria in patients' sera.

In 1971a, Parish was unable to show IgE antibodies by radio-immunoelectrophoresis in sera of patients with cutaneous vasculitis, although he was able to demonstrate these antibodies to tetanus toxoid in three patients.

Pepys et al. (1973), continuing their studies on B. subtilis enzyme, were able to demonstrate IgE antibodies to this enzyme by the radio-allergosorbent test (RAST) in those working in the industry who had positive skin prick tests, in contrast to the low or negative antibody levels found in control subjects with negative skin tests.

A study relevant to this thesis was that reported by Schopfer et al. (1979) who demonstrated specific IgE antibodies to S. aureus in sera from seven patients with hyperimmunoglobulinemia E, recurrent staphylococcal abscesses and chronic eczema. They did not detect antibodies to staphylococcal IgE in the serum of controls which included patients with (a) high levels of total IgE without chronic staphylococcal infection and with (b) chronic staphylococcal infections without high levels of total IgE.

In the RAST which Schopfer et al. developed a suspension of S. aureus Wood 46 was used as the solid phase. This strain is protein A deficient and thus its use reduces

the problem of protein-A combining non-specifically with the Fc portion of human γ -globulin (Forsgren and Sjoquist, 1966). IgE antibody assays were also performed with Staphylococcus epidermidis, Streptococcus haemolyticus Groups A and B, Escherichia coli and H. influenzae but no raised levels were found in any of the same group of patients.

4.2. STUDIES RELEVANT TO PRESENT STUDY

Reference will now be directed to the various experimental techniques employed in the present study.

4.2.1. Slide RAST

Hutchinson and Ziegler (1972) devised a radioimmunoassay to measure specific IgG antibodies to Escherichia coli, vaccinia virus and herpesvirus where the antigens were affixed to glass cover slips. This method was adapted for use in the slide RAST (Chapter IV, 2.0) by affixing the bacterial antigens to the wells of Roche plastic slides and proved satisfactory for the measurement of specific IgG antibodies to S. aureus, S. pneumoniae and H. influenzae.

4.2.2. Enzyme-linked immunosorbent test (ELISA)

In recent years ELISA has developed as an alternative to radioimmunoassay for the measurement of specific antibody or antigen. Enzymes are linked to antibodies or antigens and the resulting conjugates have both immunological and enzymic activity which can be quantified by their ability to degrade a suitable substrate. Engvall and Perlmann (1971 and 1972) and Engvall and Ljungstrom (1975) have estimated

specific antibodies quantitatively by enzyme labelling anti-immunoglobulins. They achieved a sensitivity in the 10^{-1} - 10^{-17} mole range which is therefore comparable to that of radioimmunoassay. Voller et al. (1976a and b) have used ELISA for detecting IgG antibodies in patients' sera in the diagnosis of virus and parasite infections. Sepulveda et al. (1979) using the microplate method of Voller et al. (1976b) were able to detect both specific IgG and specific IgE antibodies to protein and polysaccharide antigens of Aspergillus fumigatus. A similar assay was attempted in this study in order to detect IgE antibodies to bacteria (Chapter IV, 3.0).

Both the ELISA and slide RAST techniques were found not to be sensitive enough for the quantitation of specific IgE antibodies to the bacteria.

4.2.3. Total serum IgE

The determination of total IgE levels in serum is an essential component in the investigation of all subjects with suspected allergy.

Johansson (1967) was the first to report raised levels of IgE in atopic extrinsic asthma but not in other types of asthma. These studies have now been⁵ widely confirmed and extended to other atopic diseases. Elevated serum total IgE has been found to correlate well with clinical findings and if parasitic infestation can be excluded, is indicative of atopy to one or more allergens.

In the assay used in this study, based on the method

by Ceska and Lundkvist (1972), the normal geometric mean was found to be 33.6 I.U./ml (range 2-240 I.U./ml) based on assaying sera from 78 blood bank donors.

These figures are similar to those obtained by Nye et al. (1975) who, using a double antibody solid phase technique, estimated the mean total IgE level of their 102 normal subjects to be 36.3 I.U./ml (range 1-178 I.U./ml).

CHAPTER 2

AIMS AND OBJECTIVES.

The aim of the present study was to examine antibody responses in man to whole bacterial cells with particular emphasis on their possible involvement in allergic lung disease.

Three bacteria, representative of common pathogens in chest disease, were studied. These were a coagulase positive strain of Staphylococcus aureus (NCTC 6571); an alpha haemolytic strain of Streptococcus pneumoniae (NCTC 10319) and a non-capsulated strain of Haemophilus influenzae (NCTC 4842).

A major aim was to develop an assay sufficiently sensitive to measure specific IgE antibodies to these bacteria and to apply this technology to the study of various allergic diseases. The results were also compared with skin "prick" test reactivity and total IgE concentrations.

In addition, RAST assays directed against these bacterial antigens of the IgG (total) and IgG₄ subclass were developed. Where possible the laboratory results were compared with clinical manifestations. All the immunological findings in the different disease groups were analysed for possible correlations.

CHAPTER III

Materials and Methods

1.0 BUFFERS

A. RADIOIMMUNOASSAYS

(i) Phosphate buffered saline (PBS) pH 7.3

58.5 g NaCl

66 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

20 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

5 g NaN_3

made up to 10 litres with distilled water.

(ii) 0.5 M Phosphate buffer, pH 7.3

2 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

6.6 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

made up to 100 ml with distilled water.

(iii) "Wash" buffer, pH 7.3

PBS + 0.5% Tween-20

(iv) Incubation buffer

PBS (above) + 0.3% human serum albumin (Sigma, Poole, U.K.).

B. TOTAL IgG MEASUREMENT

(i) Veronal buffer, 0.12 M, pH 8.65

20.6 g Sodium barbitone

3.68 g Barbitone

0.1 g Merthiolate

made up to 1 litre with distilled water.

C. POLYACRYLAMIDE GEL ELECTROPHORESIS

(i) Tris-borate EDTA buffer, pH 8.35

10.75 g Tris

5.04 g boric acid

0.93 g Na_2EDTA

made up to 1 litre with distilled water.

D. ELISA USING ALKALINE PHOSPHATASE

(i) Coating buffers: 0.05 M carbonate buffer, pH 9.6

Shelf life - 2 weeks:

1.59 g Na_2CO_3

2.93 g NaHCO_3

0.2 g NaN_3

made up to 1 litre of distilled water.

(ii) Phosphate buffered saline (PBS), pH 7.4

8 g NaCl

0.2 g KH_2PO_4

2.89 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

0.2 g KCl

made up to 1 litre of distilled water.

(iii) Wash buffer, pH 7.4

PBS with 0.05% Tween-20 and 0.02% NaN_3 added.

(iv) Ethanolamine buffer, pH 9.8

97.0 ml diethanolamine

800 ml distilled water

101 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

adjusted to pH 9.8 with 1 M HCl and made up to
1 litre with distilled water. 0.02% NaN_3 added.

2.0 CHEMICALS AND REAGENTS

Reagents were obtained as follows.

- 2.1 Rabbit anti-human IgE antiserum (Behring) from Hoechst Pharmaceuticals, Watford.
- 2.2 Goat anti-human IgG labelled with alkaline phosphatase (Miles Laboratories, Stoke Poges).
- 2.3 P-nitrophenylphosphate 104 substrate (Sigma, Poole).
- 2.4 Normal goat serum (Wellcome Reagents, London).
- 2.5 Normal rabbit serum (Wellcome Reagents, London).
- 2.6 Bovine plasma albumin (Armour Pharmaceuticals Ltd., Eastbourne).
- 2.7 Goat anti-human IgE antiserum (monoclonal Fc specific), a gift from Dr. C.E. Arbesman, Buffalo (see Appendix 1).
- 2.8 Rabbit anti-human IgG antiserum specific for γ chains. Code 10-090 (Dakopatt, Mercia Brocades Ltd., Weybridge).
- 2.9 Rabbit anti-human IgG₄ antiserum (Seward, London).
- 2.10 Human serum albumin (Sigma, Poole).
- 2.11 Sodium iodide 1 mCi of ^{125}I IMS 30 (Radiochemical Centre, Amersham).
- 2.12 Cyanogen bromide (Koch-Light, Colnbrook, Bucks).
- 2.13 WHO Standard serum for IgG.
- 2.14 British Working Standard serum for IgE, No. 75/502 (Holly Hill, London).
- 2.15 High molecular weight (HMW) electrophoresis calibration kit (Pharmacia, London).

3.0 MATERIALS

- 3.1 Dynatech microtitre plates (M129A) specially manufactured for ELISA (Dynatech, Billingshurst).
- 3.2 Removawell plastic cups (M179A) (Dynatech, Billingshurst).
- 3.3 Disposable ELISA Removawell holders (Dynatech, Billingshurst).
- 3.4 Plastic slides for immunofluorescent testing (Roche Products Ltd., Welwyn Garden City).
- 3.5 Disposable polystyrene tubes 12.5 x 75 mm GW4 (Seward, London).
- 3.6 Sephadex G25 (Pharmacia).
- 3.7 Sepharose 4B (Pharmacia).
- 3.8 Activated Sepharose 4B (Pharmacia).
- 3.9 Polyacrylamide gradient gels PAA 4/30 (Pharmacia).
- 3.10 Electrophoresis apparatus GE-4 (Pharmacia).
- 3.11 Destaining bath (Shandon, Runcorn, Cheshire).
- 3.12 4 mm gel cutter.

4.0 BACTERIAL CULTURE AND EXTRACTS

4.1. PREPARATION OF BACTERIAL WHOLE CELLS

4.1.1. Batch culture of S. aureus (NCTC 6571)

Medium.

Six litres of Nutrient Broth (Oxoid No. 2) were made up and distributed into seven 1 litre plastic screw-capped Duran bottles and autoclaved 10 lbs/10 min (+).

The bottles of media were seeded with a broth culture of S. aureus NCTC 6571 grown up overnight and incubated at 37°C for 18 hr.

The bacterial suspensions (++) were then heat killed in a water bath, by exposing them to 60°C for 1½ hr.

The killed suspensions (+++) were centrifuged at 12,500 g for 20 min and the sediment obtained washed twice with PBS. The final sediment was resuspended in a small quantity of PBS, transferred into pre-weighed plastic universal bottles and freeze dried.

Sterility tests were performed by plating out a loopful of medium on to a blood agar plate and incubating at 37°C overnight as follows:

- (+) after preparation of medium - sterile.
- (++) after growth of organism - pure growth of desired organism.
- (+++) after heat killing - sterile.

4.1.2. Batch culture of *S. pneumoniae* (NCTC 10319)

Medium. 2% Glucose Broth

Ten Duran glass bottles containing 625 ml Nutrient Broth (Oxoid No. 2) were autoclaved 10 lb/10 min.

Twenty-five millilitres of a 50% sterile glucose solution was added to each bottle aseptically.

The bottles were seeded with an overnight broth culture of *S. pneumoniae* (NCTC 10319) and incubated at 37°C for 18 hr.

The bacterial suspensions were heat killed at 60°C/1½ hr and harvested as described for *S. aureus* with the same sterility test checks.

4.1.3. Batch culture of *H. influenzae* (NCTC 4842)

Medium.

Six litres of the following medium were prepared:

60 g OXOID's Lab-Lemco powder	- Code L29
60 g OXOID's Proteose peptone	- Code L46
18 g OXOID's Neutralised Liver Digest	- Code L27
18 g OXOID's Yeast extract (powder)	- Code L21
30 g Sodium chloride	
0.06 g Magnesium chloride	
6 l Tapwater	

The medium was heated to a temperature of 90°C when the pH was raised to 8.5-9 (8.8 ml saturated NaOH was required) in order to precipitate the phosphates. These were removed by filtering the media through Greens 904½ filter paper which

was then made up to the original volume with distilled water. When cold the pH was adjusted to 7.2, (8 ml conc HCl required) the media bottled into 1 litre amounts and autoclaved at 15 lbs/15 min.

After filtering through a sterile 0.22 μ Millipore filter 0.3 ml of 10 mg/ml Haemin in N/5 NaOH and 0.3 ml of 10 mg/ml NAD (nicotinamide-adenine dinucleotide) in distilled water was added aseptically to each litre bottle of medium.

The bottles of media were seeded with an overnight culture of H. influenzae (NCTC 4842) grown in the medium detailed above. After incubation at 37°C for 18 hr the bacterial suspensions were heat killed at 60°C for 1½ hr and harvested as described for S. aureus.

4.2. PREPARATION OF BACTERIAL EXTRACTS

4.2.1. S. aureus allergen extract

A coagulase positive strain of S. aureus (NCTC No. 6571) was grown in nutrient broth. The suspension was centrifuged, washed three times in isotonic saline and finally resuspended in distilled water. Aliquots of the suspension were placed in disposable universal containers and the bacteria ultrasonically disrupted using a "Soniprobe" (Type 1130, Dawe Instruments Ltd., Acton, London) at approximately 20 Kc/sec. The procedure was continued for 2.5 min in an ice cooled chamber to prevent excessive heating. The suspension was then centrifuged at 15,000 g for 30 min at +4°C and the cell sediment resuspended in a small amount of isotonic saline. This was autoclaved at 10 lbs/sq. in for 10 min, dialysed

against distilled water for 48 hr and freeze dried.

4.2.2. S. pneumoniae allergenic fraction containing C-substance polysaccharide.

A rough strain of S. pneumoniae (NCTC No. 10319) was grown in 2% glucose broth culture. The supernatant was taken, Seitz filtered and dialysed against repeated changes of distilled water at 4° for 48 hr. It was then autoclaved at 10 lbs per square inch for 10 min and the product filtered and freeze dried.

The presence of C-substance polysaccharide was demonstrated by the method of Longbottom and Pepys (1964) in which the allergen extract was reacted in buffered agar gel with the sera of patients known to contain C-reactive protein. The resulting precipitation lines readily dissolved when 0.1 M citric acid was flooded over the plates to remove calcium ions.

4.2.3. H. influenzae allergen extract containing the specific H₁ and H₂ groups.

First an allergenic extract from a non-capsulated strain of H. influenzae (Brompton 909) was prepared in a similar way to that described for the extract from S. aureus. The bacteria were grown on XV agar plates (Turk & May, 1967b), scraped off, washed and centrifuged three times. They were

disrupted by ultrasound and after centrifugation at 15,000 g for 30 min the supernatant was dialysed against 0.02 M ammonium bicarbonate for 48 hr and finally freeze dried.

On immunoelectrophoresis this allergen extract gave precipitin lines in the five positions H_1 to H_5 described by Burns & May (1967) (see Fig. 1).

The H_1 and H_2 groups of allergens, species specific for H. influenzae were separated from the non-species specific H_3 to H_5 allergenic groups by a modification of the method described by Davies, Laughton & May (1974). The principle that was used depended on the fact that during electrophoresis at pH 8.2 the H_1 and H_2 groups of allergens migrate to the cathode and the H_3 to H_5 group of allergens to the anode. Bulk separation was, therefore, achieved by ion exchange chromatography by absorbing the sonically disrupted allergenic extract with A 25 DEAE Sephadex.

4.3. ANALYSIS OF ANTIGEN PREPARATION

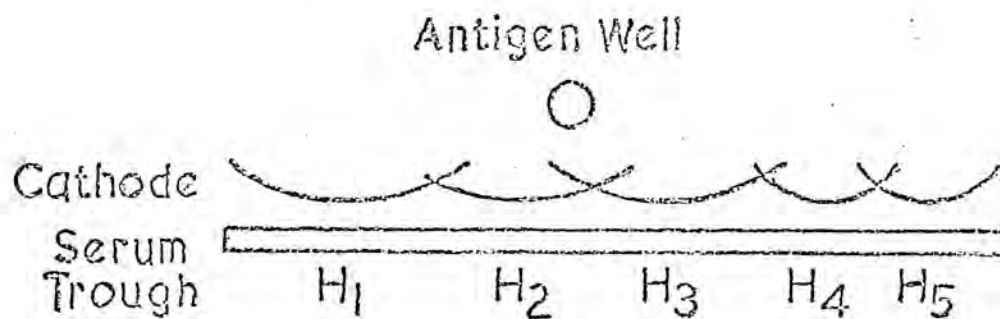
4.3.1. ELECTROPHORESIS IN POLYACRYLAMIDE GRADIENT GEL FOR THE ESTIMATION OF MOLECULAR WEIGHTS OF PROTEIN ANTIGENS

Polyacrylamide gel electrophoresis (PAGE) provides a method for the molecular characterization of proteins by combining an electrophoretic separation with the sieving effect of polyacrylamide gel. This electrophoresis in a gel of increasing acrylamide concentration (gradient gel electrophoresis) and thus decreasing pore size (Rodbard et al. 1971)

Figure 1

Position of the precipitin arcs between human antibodies against H. influenzae and electrophoresed H. influenzae antigen extract.

(After Burns and May, 1967)



produces sharp bands and good resolution of a wide range of protein sizes on the same gel.

Pharmacia's pre-formed slab Gradient Gels PAA 4/30 were used and samples were run in parallel with a high molecular weight protein standard kit (Pharmacia) to provide a series of reference molecular weights ranging from 67,000 daltons (Albumin) to 669,000 daltons (Thyroglobulin).

Procedure

Electrophoresis was performed in Pharmacia electrophoresis apparatus GE-4 loaded with one gradient gel PAA 4/30. The gel was pre-run at 125 V for 15 min to allow the casting buffer and running buffer (Tris-borate EDTA buffer, pH 8.35) to equilibrate before the samples were applied. The samples (5 μ l) were applied using a loading comb and the gel pre-electrophoresed at 70 V for 20 min. Electrophoresis was then allowed to proceed for 18 hr at 125 V, the buffer being maintained at approximately 15°C by means of circulating cold water.

Staining and destaining of gels

The gel was removed and stained in 0.7% amido black (Gurr 10B) in 7% acetic acid for 30 min.

Destaining was achieved electrophoretically at 24 volts with 7% acetic acid for 45 min in a Shandon destaining bath.

Samples

The supernatants of S. aureus, S. pneumoniae and H. influenzae whole cell saline suspensions were each electrophoresed at 60 mg/ml and 30 mg/ml in parallel with Pharmacia's high molecular weight marker and stained for protein with amido

black.

Results

A photograph of the stained plate appears as Plate 1. The five proteins in the high molecular weight marker were:

	<u>Daltons</u>	<u>Mobility</u> <u>mm</u>
Thyrogobulin	669,000	27
Ferritin	440,000	42
Catalase	232,000	59
Lactate dehydrogenase	140,000	69
Albumin	67,000	90.5

Fig. 2 shows the standard curve constructed from the mobilities of these proteins.

Neither concentration of the S. aureus and H. influenzae supernatants produced any protein bands, but both concentrations of the S. pneumoniae supernatant gave eight. These occurred at the following approximate molecular weights.

	<u>Daltons</u>	<u>Mobility</u> <u>mm</u>
1.	900,000	22
2.	385,000	45
3.	320,000	50
4.	275,000	54
5.	170,000	67
6.	147,500	70.5
7.	138,000	72.5
8.	85,000	85

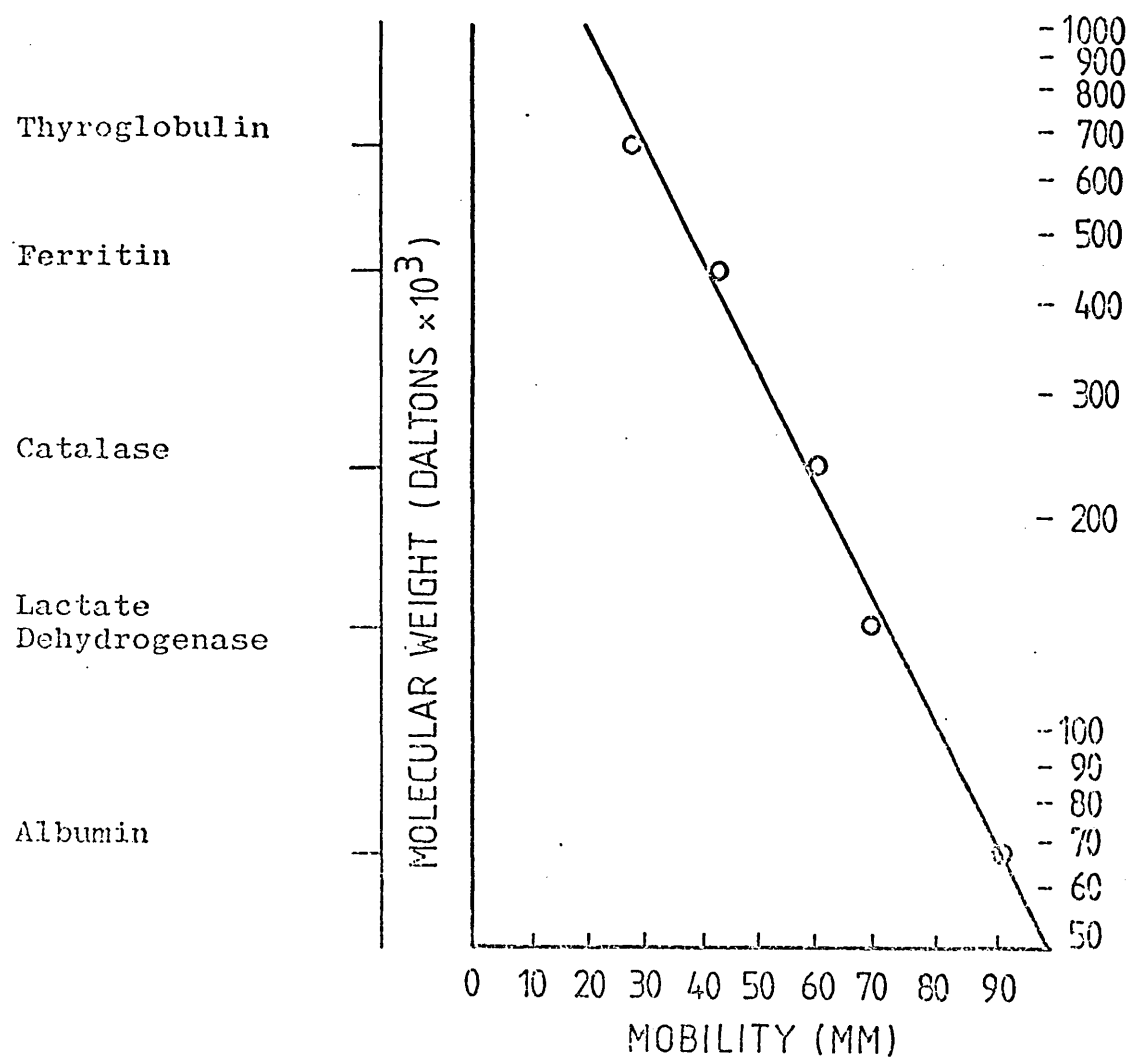
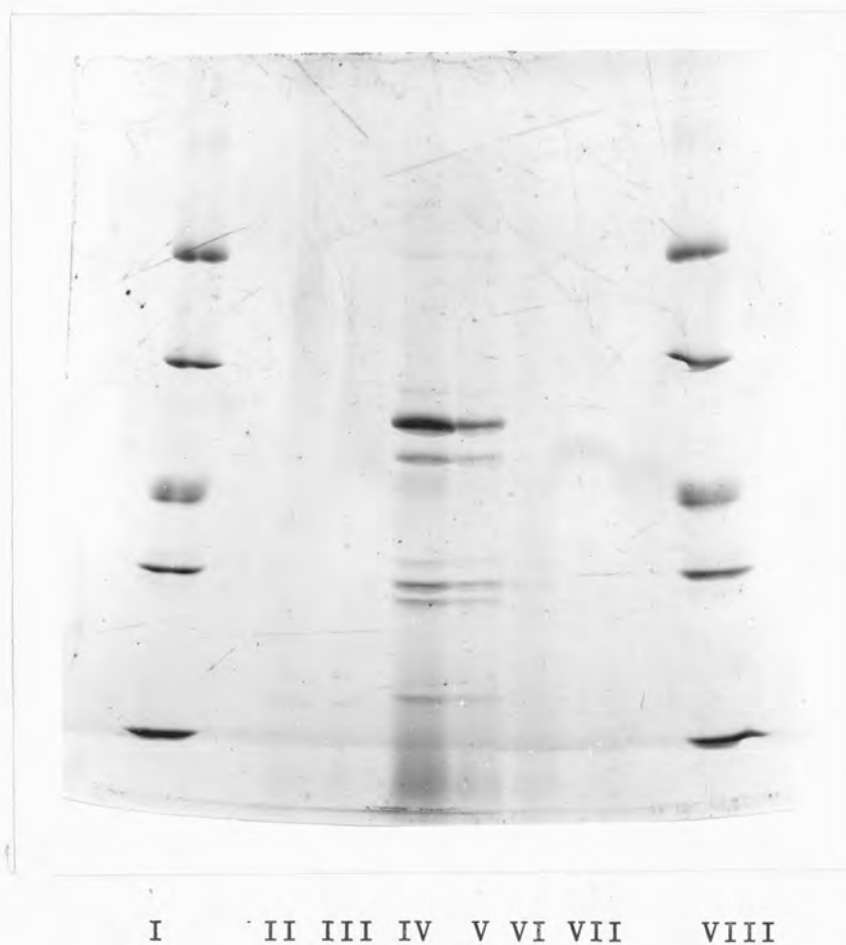


Figure 2

Calibration curve established using the HMW Calibration Kit on a Pharmacia PAA 4/30 gradient gel.

PLATE 1

Molecular weight estimation of S. aureus, S. pneumoniae and H. influenzae antigens by polyacrylamide gradient gel electrophoresis.

KEY

- | | |
|------|-------------------------------|
| I | HMW marker |
| II | <u>S. aureus</u> 60 mg/ml |
| III | <u>S. aureus</u> 30 mg/ml |
| IV | <u>S. pneumoniae</u> 60 mg/ml |
| V | <u>S. pneumoniae</u> 30 mg/ml |
| VI | <u>H. influenzae</u> 60 mg/ml |
| VII | <u>H. influenzae</u> 30 mg/ml |
| VIII | HMW marker |

Discussion

The S. aureus and H. influenzae antigens may be too insoluble to leach enough antigen into the supernatant of the cell suspension to produce any protein bands on electrophoresis. S. pneumoniae on the other hand is known to lyse easily thus the protein bands which appear on electrophoresis could be from the cell cytoplasm.

5.0 IMMEDIATE SKIN REACTIONS

5.1. Introduction

Immediate reactions to skin tests are mediated mainly in atopic subjects by the heat-labile, reaginic antibody IgE and these tests are used for the aetiological investigation of such diseases as asthma, rhinitis and urticaria. In 1970 Parish described a heat-stable IgG 'short-term homocytotropic' antibody also capable of mediating immediate test reactions, although the role of this antibody in allergic disease is still not fully defined.

5.2. Method

Skin "prick" test solutions

The bacterial extracts, at suitable concentrations, were dissolved in 50% Coca's solution (5 g sodium chloride, 2.75 g sodium bicarbonate and 4 g of phenol made up to 1 litre of distilled water) and 50% glycerol. After centrifugation, the supernatant was passed through a 0.45 μ Millipore filter (Millipore Ltd., Bedford, U.S.A.) into a glass bottle with plastic cap and applicator (Bencard Ltd.).

The bacterial suspensions were similarly made up in 50% Coca's solution: 50% glycerol but were not passed through a Millipore filter.

All the bacterial extracts and suspensions were tested for sterility before use by inoculation of samples according to the European Pharmacopoeia (1978).

5.3. Method of skin "prick" tests

A drop of the test material was placed on the volar aspect of the subject's forearm. The tip of a disposable 25 gauge hypodermic needle (Gillette, Isleworth, Middlesex) was introduced at an angle of 45° through the drop into the superficial epidermis. A gentle lifting motion was made as the needle was withdrawn, ensuring that the trauma produced was insufficient to draw blood. This introduces approximately three-millionths of one ml ($0.003 \mu\text{l}$) into the skin (Squire, 1952). Control tests produced no detectable weals, so that the presence of a weal greater than one millimetre in diameter was considered as a positive test.

A negative control skin test, of Coca's solution with glycerol in equal volumes, was always performed at the same time as allergen skin prick testing.

Tests were read after 10-15 min and the maximum diameter of the weal recorded.

6.0 RADIOALLERGOSORBENT TEST (RAST)

6.1. Introduction.

In 1967 Wide, Bennich and Johansson introduced a test for the measurement of allergen-specific serum IgE antibodies, which they termed the radioallergosorbent test (RAST).

This is a solid phase, sandwich, semi-quantitative, radio-immunoassay. An excess of solid phase immobilized allergen is incubated with the test serum, and will bind allergen-specific antibodies of all immunoglobulin classes.

After washing to remove non-specific serum proteins, the presence of specific IgE antibodies attached to the allergen is assessed by incubating labelled anti-IgE with the complex. Interference by proteins other than IgE (e.g. IgG) is minimal provided that an excess of allergen and purified radio-labelled anti-IgE is employed.

The radioactivity bound to the complex is determined after a further wash and is directly proportional to the specific IgE antibody content of the original serum. Similarly, it is possible to measure allergen-specific antibodies in IgG and IgG₄ sub-classes.

6.2. Anti-human IgE antiserum

The purified anti-human IgE antiserum used in all the RAST assays was kindly donated by Dr. C.E. Arbesman (Buffalo). It was supplied as a lyophilised gamma-globulin fraction of a goat antiserum raised to the Fc fraction of human monoclonal

IgE and reconstituted to give a protein concentration of 2.0 mg/ml. Details of Dr. Arbesman's purification of the antiserum appear in Appendix 1.

6.3. Labelling of anti-human IgE with ^{125}I

Radioactive labelling of a protein, with e.g. iodine or chromium, enables it to be traced through a reaction.

Campbell et al. (1956) and Cohen et al. (1956) have shown that carefully iodinated proteins, although essentially altered by the addition of an iodide atom, cannot be distinguished immunologically from native protein.

This iodination system was adapted from Hunter and Greenwood's method (1962) which uses chloramine-T to generate free iodine from sodium iodide. It is suitable for very small quantities of protein and is so rapid that radiation damage to the molecule is minimal.

6.3.1. Iodination procedure

The iodination was carried out in the vial containing the sodium iodide (1.0 mCi ^{125}I IMS 30) within a fume cupboard.

Sodium phosphate buffer (10 μl of 0.5 M, pH 7.3) and 50 μl of the purified goat anti-human IgE (100 μg) were added to the vial and the reaction started by the addition of

chloramine-T (10 μ l of 5 mg/ml). The vial was then capped and the contents mixed on a vortex mixer.

After 1 min the reaction was stopped by the addition of sodium metabisulphite (10 μ l of 12 mg/ml). The vial was again capped, the contents mixed and potassium iodide (50 μ l of 16.6 mg/ml) and bovine serum albumin (100 μ l of 3 mg/ml) added.

6.3.2. Desalting

The low molecular weight inorganic material including unreacted Na 125 I was separated from the labelled protein by gel filtration, which also demonstrates the proportion of non-protein bound activity.

A 13 cm column was prepared by pouring pre-swollen Sephadex G25 fine in 1/6 PBS into a chromatography column (15 x 200 mm). After allowing the Sephadex to settle, the drained column was saturated with 1 ml of normal goat serum to reduce non-specific adsorption of the radio-labelled protein on to the Sephadex. After eluting the goat serum with 1/6 PBS, the contents of the reaction vial were layered on to the drained column, and 0.5 ml aliquots were collected on a fraction collector.

6.3.3. Determination of protein-bound 125 I

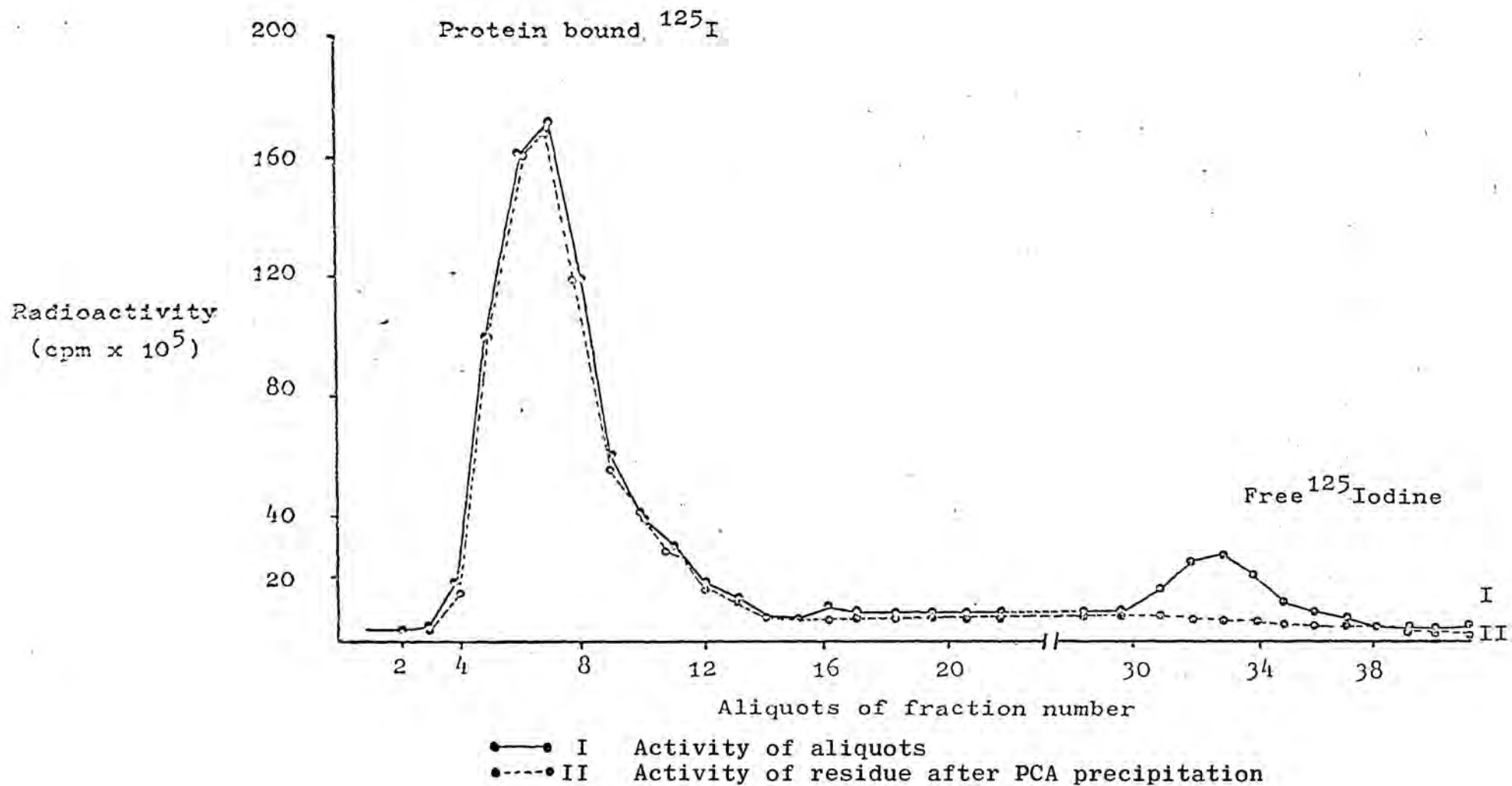
Ten microlitre aliquots of each fraction collected

were transferred to individual tubes and counted for 3 sec in a gamma counter (LKB/Wallac).

To each fraction was added 500 μ l phosphate buffered saline and 100 μ l bovine plasma albumin (3 mg/ml), followed by 500 μ l 12% perchloracetic acid to precipitate any protein. The contents of each tube were mixed on a vortex mixer, left to stand for 10 min and then centrifuged at 5000 rpm for 5 min. The supernatant in each tube was discarded, and the precipitated material counted for 3 sec. From the difference in counts obtained before and after removal of the supernatant, the percentage of radio-labelled iodine in each fraction was calculated. Fractions containing over 90% protein-bound ^{125}I were pooled. A profile of protein-bound ^{125}I versus free ^{125}I is shown in Fig. 3. An equal volume of normal goat serum was added to the labelled antiserum to prevent release of free iodine (Bogdavore and Strash, 1975) and the resultant label aliquoted and stored at -70°C until required. It was used within one half-life (60 days).

6.4. Labelling of anti-human IgG with ^{125}I

The gamma globulin fraction of a rabbit antiserum specific to the γ chain of human IgG (Dakopatt) was labelled. The preparation contained approximately 10 mg/ml of protein and was diluted with phosphate buffered saline to give 1.0 mg/ml. Ten microlitres of this was labelled with 1 m Ci ^{125}I by the chloramine-T method previously described.



Note: Absence of activity in second radioactivity peak after PCA precipitation due to lack of protein binding of isotope

Figure 3: Determination of protein bound ^{125}I Iodine after radio-iodination of goat anti-human monoclonal IgE.

Normal rabbit serum (Wellcome Reagents, London) was used in place of goat serum, both as the buffer protein and to saturate the desalting column.

6.5. Labelling of anti-human IgG₄ with ¹²⁵I

The gamma globulin fraction of rabbit antiserum raised to human IgG₄ subclass (Seward, London) was labelled. The protein content of this preparation was estimated at 280 nm to be 30 mg/ml. Ten microlitres of a 1:30 dilution of this was labelled with 1 m Ci ¹²⁵I.

7.0 DISC RAST METHOD TO MEASURE SPECIFIC SERUM IgE ANTIBODIES

7.1. Activation of cellulose discs

Cellulose discs, 6.5 mm diameter, were cut from Whatman No. 1 chromatography paper using a standard stationery punch. Activation procedures were carried out in a fume cupboard. The discs were activated by a modification of the method described by Axen et al. (1967).

Discs (5 g) were weighed out and placed in 100 ml distilled water. Cyanogen bromide (5 g) was dissolved in distilled water (100 ml) and added to the discs. The pH was maintained at 11-12 by the addition, dropwise, of sodium hydroxide solution (1.0 M). This required the addition of 40-50 ml of sodium hydroxide, over a 20 min period. When the pH was constant, the discs and solution were transferred to a beaker containing cooled sodium bicarbonate solution (1 l of 0.005 M). The discs were washed four times with 1 l volumes of cooled bicarbonate solution. After the final wash, the discs were washed with four changes of pure acetone (250 ml).

The last acetone wash was discarded and the discs were spread out to dry on a sheet of filter paper. When visible traces of moisture had evaporated, the discs were placed in a large desiccator which was evacuated and left at 4°C for 4 days to dry thoroughly.

The desiccator was brought to room temperature, and the vacuum released slowly. The discs were transferred to universal bottles and stored at -70°C until required.

7.2. Coupling of activated discs with *S. pneumoniae* extract

Freeze-dried *S. pneumoniae* extract was prepared in sodium bicarbonate solution (0.1 M) at a concentration of 200 µg/ml. One hundred dry, activated discs were added to the antigen solution (20 ml) in a universal bottle, which was rotated slowly at 4°C overnight on a rotary mixer.

The antigen solution was aspirated and the discs were washed with three changes of sodium bicarbonate solution (0.1 M) for 1 hr. The last bicarbonate wash was aspirated, and replaced with ethanolamine (0.05 M in 0.1 M sodium bicarbonate solution). The universal bottle was again placed on the rotary mixer and rotated for 3 hr at room temperature.

The discs were then washed in two changes of sodium bicarbonate solution (0.5 M) and two changes of acetate buffer (0.1 M, pH 4.0). After two final washes, of 30 min each, in acetate buffer, the discs were washed in two 15 min washes, of incubation buffer, phosphate buffered saline, pH 7.4, containing 0.05% sodium azide and 0.3% human serum albumin (Sigma, Poole). The discs were stored in incubation buffer at 4°C.

7.3. Disc RAST assay procedure

Patients' sera (50 µl) were incubated with individual antigen-coupled discs in polystyrene tubes (12.5 x 75 mm from Seward, London) which were covered with parafilm and left at room temperature for 3 hr.

The excess serum was then aspirated and the discs washed with three changes of 0.9% saline at 10 min intervals. Preabsorbed ^{125}I -labelled goat anti-human IgE antiserum* (50 μl containing 100,000 CPM after absorption) was added to each tube, the tubes covered with parafilm and incubated overnight at room temperature.

The tubes were then washed six times with wash buffer at 10 min intervals and the residual activity counted for 60 sec in a gamma counter.

* Absorption of labelled antibody

500 μl ^{125}I anti-IgE

+ 500 μl 10% normal goat serum in PBS

+ 6 antigen-coupled discs

agitated on a shaker at room temperature for 3 hr.

8.0 SLIDE RAST METHOD TO MEASURE SPECIFIC SERUM ANTIBODIES

Introduction

A slide RAST method was developed using blank Roche plastic slides (see Plate 2) similar to those used for immunofluorescent work. Bacterial suspensions were immobilised on to the 8 wells by heat fixation.

8.1. Preparation of slides

Slides were washed in a "PYRONEG" solution, rinsed and dried. Chromic acid was applied to the wells for 10 min, after which the slides were washed thoroughly in distilled water and air dried.

Bacterial cell suspension

The optical density of a suspension of freeze dried whole bacterial cells in phosphate buffered saline was adjusted to 0.2 at 650 nm measured on a Pye Unicam spectrophotometer SP 1700.

Fifteen microlitres of bacterial cell suspension (O.D. 0.2 at 650 nm) was pipetted on to the wells. The slides were then placed in a 37°C incubator for 45 min, followed by exposure at 90°C for 1 hr.

To remove any loosely attached suspension the slides were placed in a staining rack and submerged in a beaker of 1 M sodium chloride for 15 min. They were then washed in phosphate buffered saline overnight, rinsed in distilled water and air dried.

PLATE 2

(Actual size)

ROCHE plastic slide
used in the slide RAST

8.2. Slide RAST assay procedure

Twenty microlitre aliquots of serum samples tested in duplicate were applied to the bacteria coated wells. The slides were then incubated at 37°C for 1 hr in 14 cm petri dish "moist chambers" containing strips of moist "spontex".

The serum samples were aspirated off and the slides placed in a staining rack and given three washes of phosphate buffered saline with 10 min intervals between washes.

¹²⁵I-labelled goat anti-human IgE antiserum (20 µl containing 100,000 CPM), preabsorbed with the bacterial cell suspension, was applied to each well and the slides again incubated at 37°C for 1 hr.

The label was aspirated off and the slides given six washes with phosphate buffered saline containing 0.5% Tween 20 with 10 min intervals between washes.

The dried slides were cut up with wire nippers and each well placed in a disposable polystyrene tube and counted in a gamma counter (LKB/Wallac).

9.0 SEPHAROSE RAST METHODS TO MEASURE SPECIFIC SERUM ANTIBODIES

Activated Sepharose 4B enables proteins and ligands with primary amino groups to be immobilised.

9.1. Activation of Sepharose 4B

The method followed was that of March et al. (1974). The complete procedure was performed in a fume cupboard. Five grams of cyanogen bromide were dissolved in 2.5 ml acetonitrile in a stoppered glass tube.

The tube contents were then added to a beaker containing 25 ml packed volume of Sepharose 4B, prewashed with distilled water and resuspended in 50 ml 2 M sodium carbonate.

The activation was allowed to continue for 2 min with magnetic stirring.

The slurry was washed under vacuum on a sintered glass funnel No. 3 with 1 litre of 0.1 M sodium bicarbonate (pH 9.5), followed by 1 litre of distilled water, and 1 litre of 0.2 M sodium bicarbonate (pH 8.5).

The resultant compact moist cake was then ready to be coupled to antigen or antibody.

In the later part of the study, commercially activated Sepharose 4B (Pharmacia) was used and washed as follows: Twenty grams dry weight was allowed to swell in 300 ml 10^{-3} M hydrochloric acid solution for 3 hr and then washed on a Buchner funnel with Whatman No. 54 filter paper with 3,700 ml 10^{-3} M HCl. The Sepharose was then ready for coupling.

9.2. Insolubilisation to activated Sepharose 4B of

- (a) Freeze dried bacterial antigen
- (b) Rabbit anti-human IgE antiserum (Behring).

To 12.5 ml packed volume of activated Sepharose 4B resuspended in 12.5 ml 0.2 M sodium bicarbonate (pH 8.5):

- (a) 125 mg freeze dried bacterial antigen was added
- (b) 1 ml rabbit anti-human IgE was added.

The suspensions were mixed on a rock and roll mixer (Luckhams) for 18 hr at +4°C.

Any remaining active groups were blocked by the addition of 1 M ethanolamine at pH 8 for 3 hr.

The suspensions were filtered to a moist cake on a Buchner funnel with Whatman No. 54 filter paper over reduced pressure and three washing cycles were performed to remove non-covalently adsorbed protein. Each cycle consisted of a 600 ml wash at pH 4 (0.1 M acetate buffer containing 1 M sodium chloride) followed by a 600 ml wash at pH 8 (0.1 M borate buffer containing 1 M sodium chloride).

The immobilised (a) antigen and (b) antibody were stored at +4°C in incubation buffer.

9.3. Sepharose RAST assay procedure

Freeze dried bacteria (S. pneumoniae, S. aureus and H. influenzae) were immobilised by coupling to cyanogen bromide activated Sepharose 4B (as described in 9.2).

One hundred microlitre aliquots of the resulting immunoabsorbents, diluted 1/4 in incubation buffer, were

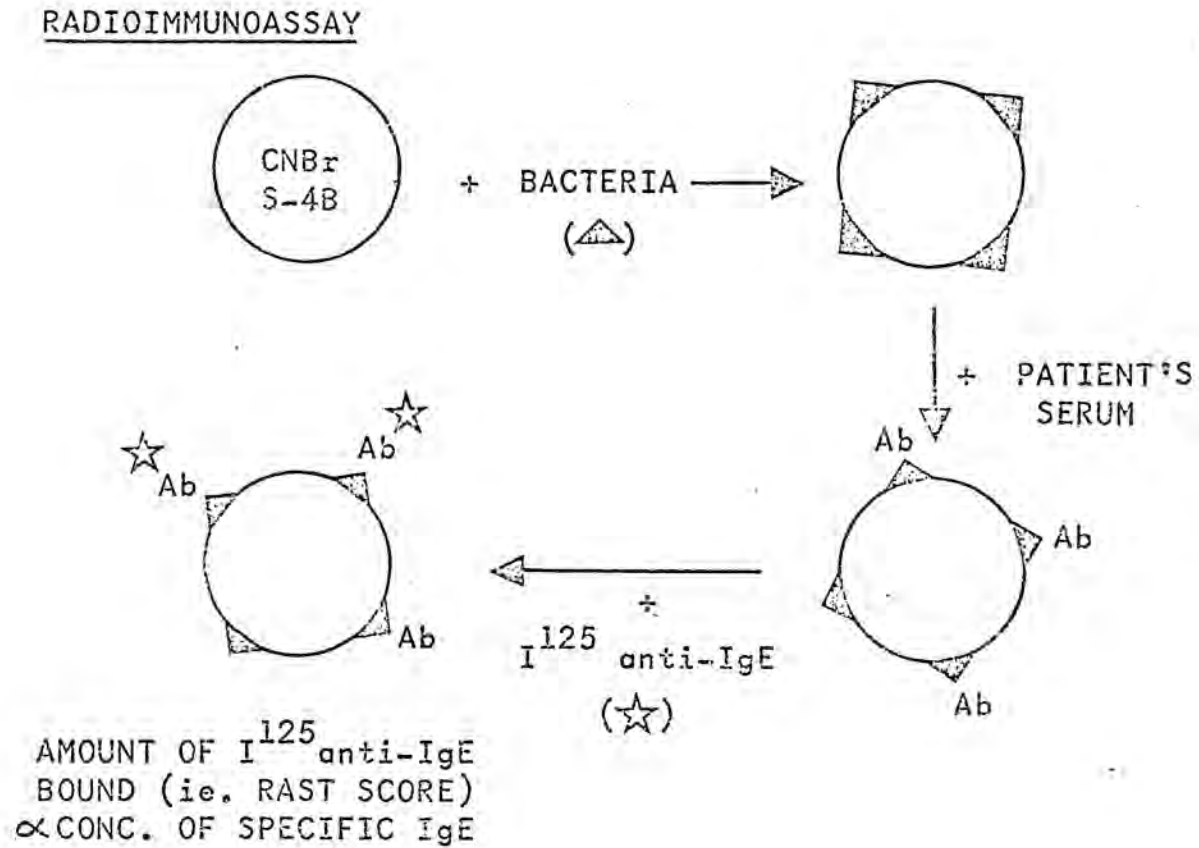
agitated in polystyrene tubes (Seward GW4, London) with 50 μ l of neat test sera for 16 hr. All tests were made in duplicate and cord blood sera (10), containing no demonstrable total IgE, were run in parallel as negative controls.

After three washes with phosphate buffered saline, achieved by successive centrifugation (2000 rpm/8 min) and aspiration, specific bacterial IgE was detected by a further incubation with agitation for 16 hr with 125 I-labelled anti-IgE (50 μ l containing 180,000 CPM, preabsorbed by agitation for 3 hr with half the label volume of solid phase 1:4). Following six washes with PBS/0.5% Tween to remove unbound radio-labelled antibody, the contents of the tubes were counted in a gamma counter. The number of radioactive counts bound to the solid phase is directly proportional to the concentration of specific bacterial IgE in the serum, i.e., RAST score.

The procedure is depicted in Fig. 4.

Figure 4.

Diagrammatic representation of the Sepharose RAST for detection of specific IgE antibody to bacteria.



10.0 DETERMINATION OF TOTAL IgE CONCENTRATION IN HUMAN SERUM

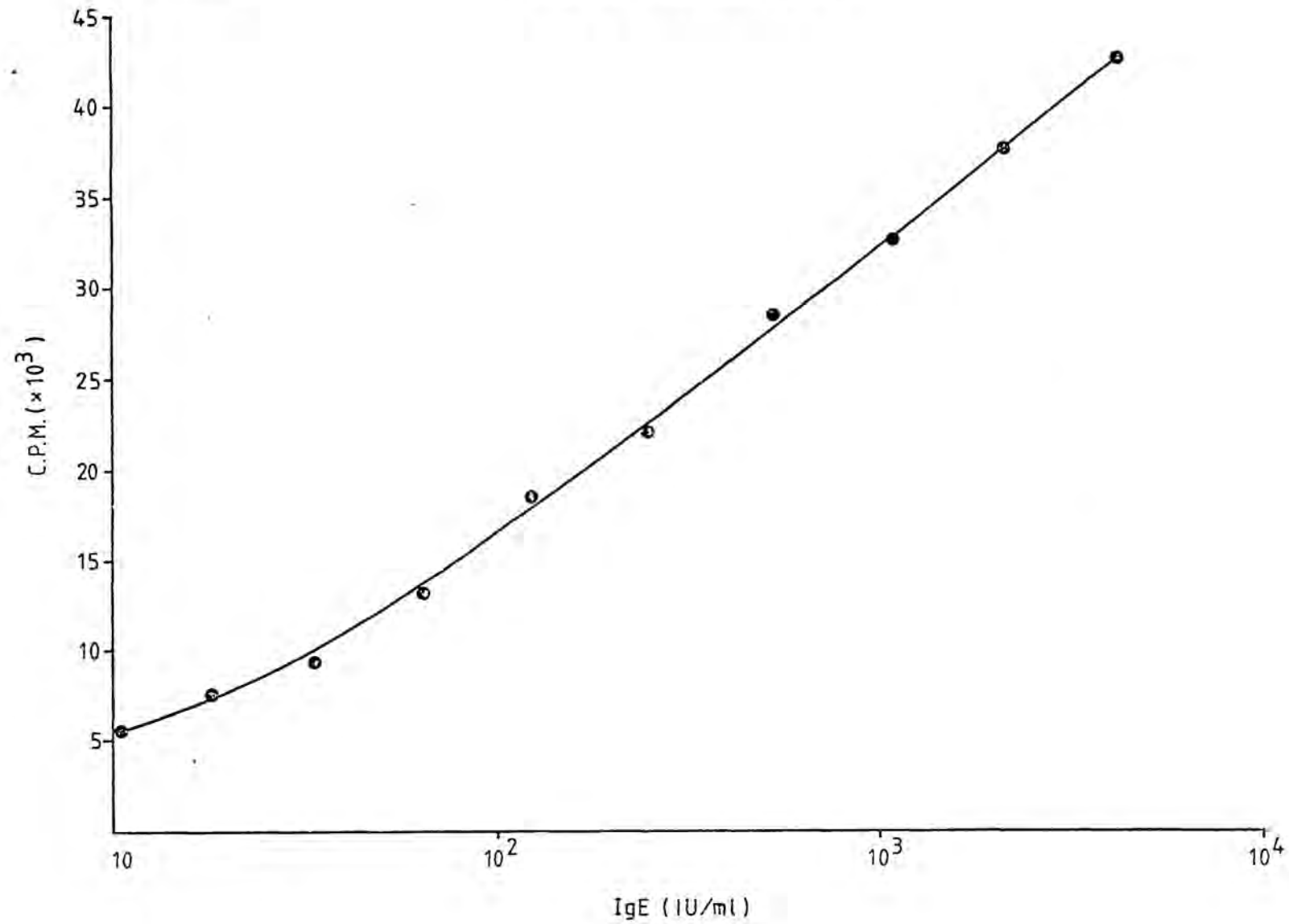
This method is based on that described by Ceska & Lundkvist (1972).

Rabbit anti-human IgE (Behring) was immobilised by coupling to cyanogen bromide activated Sepharose 4B. 100 μ l aliquots of the resulting immunoabsorbent, diluted 1:4 in incubation buffer, were agitated in polystyrene tubes (Seward GW4) with 50 μ l test sera for 16 hr. After three washes in phosphate buffered saline, total IgE was detected by a further incubation for 16 hr with 125 I-labelled anti-human IgE (50 μ l containing 180,000 CPM). Following six washes with PBS + 0.5% Tween 20 the contents of the tubes were counted in a gamma counter. The number of radioactive counts bound to the solid phase are directly proportional to the concentration of total IgE in the serum. Absolute values were derived from a standard curve constructed for each assay with a suitable reference serum (Laboratory Standard) obtained by testing doubling dilutions from 1:1 to 1:512 (see Fig. 5).

The laboratory standard was first established by running it in parallel in the assay with the WHO Standard of known total IgE content six times.

Figure 5.

TOTAL IgE STANDARD CURVE



11.0 SINGLE RADIAL IMMUNODIFFUSION FOR THE MEASUREMENT OF TOTAL SERUM IgG

11.1. Principle

Diffusion of antigen into a gel containing the corresponding antibody results in the development of a circular immunoprecipitation ring. The squared diameter of the precipitation ring is linearly proportional to the amount of antigen when the diffusion has been completed.

Total IgG levels in the patients' sera (where the immunoglobulin acts as the 'antigen') were estimated by precipitation with rabbit anti-human IgG antiserum, specific for γ chains.

11.2. Preparation of gel

Twenty-five millilitres of molten 1% Agarose (BDH) in veronal buffer 1:4 was cooled to 56°C and 170 μ l rabbit anti-human IgG (Dakopatt) (concentration of 1.0 μ l/cm²) added.

Two glass plates (200 x 120 mm), one siliconised, were clamped together separated by a rubber gasket (15 mm wide x 0.8 mm thick) along three slides. The agar containing the antiserum was poured between the glass plates via a syringe barrel with cannula attached. After leaving to set for several hours at 4°C, the clamps were removed and the upper glass plate slid off the gel.

11.3. Procedure

The slide with the antibody-containing gel was placed on a template and 45 origin wells cut with a 4 mm cutter.

To produce a standard curve, standard serum (WHO standard serum for IgG) was tested at dilutions of 1 + 30, 1 + 50, 1 + 100 and 1 + 200. Test sera were diluted 1 + 100. Five microlitre samples of test and standard sera were added to the wells and diffusion was allowed to proceed for 4 days at room temperature in a moist chamber.

11.3.1. Washing and staining of plate

After completion of diffusion the plate was washed in isotonic saline overnight. The wells were filled with molten agar, Whatman 3MM paper pressed evenly over the gel surface and the plate dried by means of a hairdryer.

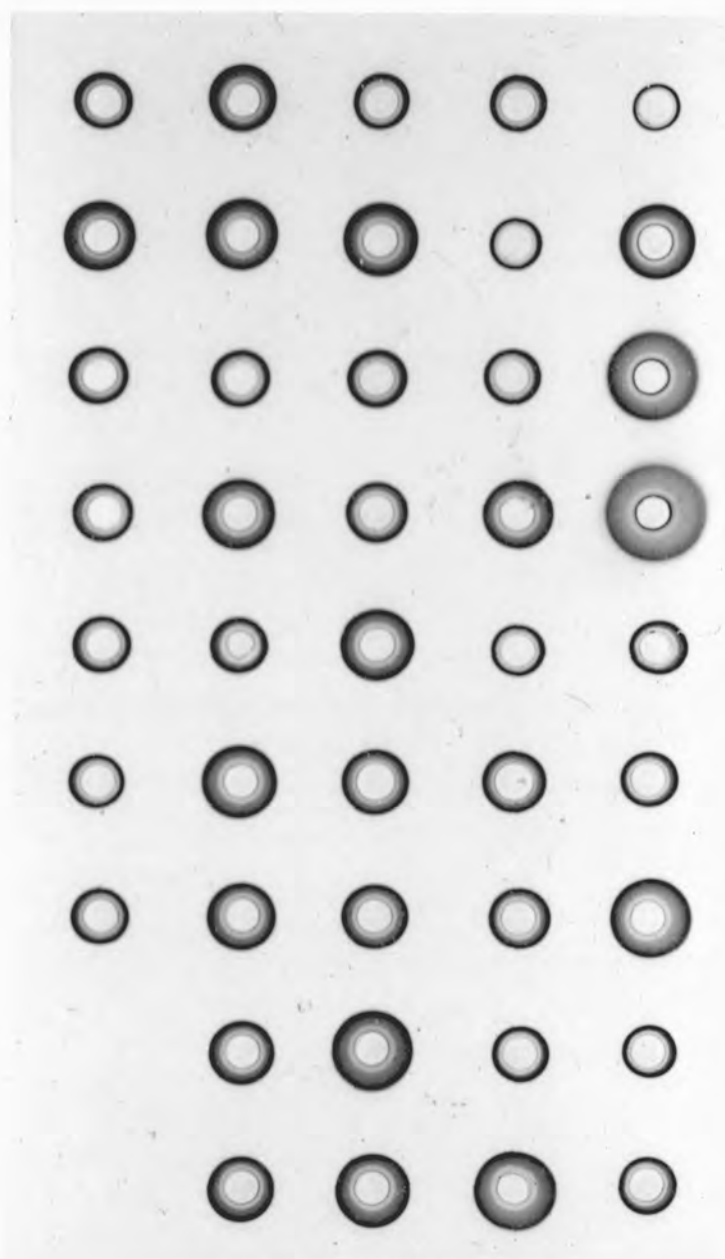
The dried plate was stained for 20 min in 0.5% Coomassie blue (Gurr 250) in a solvent mixture of methylated spirits: distilled water: glacial acetic acid in the proportions 45:45:10. The same solvent mixture was used as a destaining solution in which the plate was given several washes until a colourless background was obtained.

11.3.2. Construction of standard curve

Two diameters of each precipitation ring were measured accurately to 0.1 mm using a calibrated magnifier and a mean

value found. The squared diameters of the standard precipitation rings were plotted against the amount of antigen applied to the wells, when a linear curve with an intercept on the ordinate was obtained. By interpolation on the curve unknown samples were quantitated.

An example of a stained plate is shown in Plate 3.



1 + 200

1 + 100

1 + 50

1 + 30

Single radial immunodiffusion for estimation of total serum IgG.

Note: Wells 1-4 containing dilutions of standard human serum at 1 + 30, 1 + 50, 1 + 100 and 1 + 200. Other wells contain patients' sera diluted 1 + 100.

CHAPTER IV

DEVELOPMENT OF THE ASSAYS

1.0 INTRODUCTION

This chapter reports the experimental work with a number of assays (disc RAST, slide RAST, enzyme-linked immunosorbent assay and Sepharose RAST) together with their evaluation leading to the selection and development of the preferred assay (Sepharose RAST).

In the development of the assays, sera from patients attending the Allergy Clinic at the Brompton Hospital were employed. The patients' atopic state was determined by skin prick reactivity to common allergens and where possible the patients were also skin prick tested with extracts of S. pneumoniae, S. aureus and H. influenzae.

The Sepharose RAST was employed to detect specific IgE antibodies to bacteria. Optimum conditions were ascertained and the specificity of the assay was established by

- (1) Inhibition of binding of specific ^{125}I anti-IgE with free (i.e. unbound) bacterial antigens which was demonstrable in a dose-dependent fashion with the three bacteria under study. "Cross-over" studies revealed that there was a certain degree of cross-reactivity between the bacteria.
- (2) The demonstration of appreciable binding of the isotope in the presence of sera from patients with positive skin tests to the bacterial antigens.
- (3) The demonstration of negligible binding with cord blood sera which contained no demonstrable total IgE.

- (4) The demonstration of insignificant binding of radio-labelled antiglobulin to Sepharose coupled to human serum albumin (control protein).

1.1. PAPER DISC IgE RAST (S. pneumoniae extract)

The paper disc RAST method has been widely used for the detection of circulating specific IgE to soluble antigens. Therefore in order to attempt to demonstrate specific anti-bacterial IgE antibodies in human sera the disc RAST was performed using paper discs to which an extract of S. pneumoniae was covalently coupled (see Chapter III, 7.2).

Sera from 15 patients attending the Allergy Clinic were tested in duplicate. In addition 18 samples of cord sera pool containing no detectable total IgE were tested in parallel.

The results are shown in Table I.

1.2. DISCUSSION

The RAST was considered positive when the radioactive count was greater than that of the mean cord plus two standard deviations. By this convention two of the patients were considered to have a positive RAST to S. pneumoniae extract, thus indicating that specific IgE antibody might be present.

Since this method had the disadvantage of being restricted to cell extracts and a technique in which whole

S. PNEUMONIAE EXTRACT

SERA	IgE RAST <u>S. pneumoniae</u> extract (Mean of two duplicates)
	C.P.M.
1	3247
2	3567
3	3981 *
4	3306
5	3161
6	3781 *
7	3146
8	3216
9	3340
10	3161
11	2822
12	3653
13	3475
14	3229
15	3144
	(Mean of 18 duplicates)
CORD SERA POOL	3197
STANDARD DEVIATION (s.d.)	244
MEAN CORD + 2 s.d.	3685

TABLE I

* RAST positive (> mean cord + 2 S.D.)

cell organisms could be used was required, another form of RAST was attempted, namely the slide RAST.

2.0 SLIDE RAST

2.1. INTRODUCTION

A slide RAST technique was developed where a suspension of whole bacterial cells was immobilised on to the wells of Roche plastic slides by heat fixing at 90°C for 1 hr. IgG was estimated first as it was assumed that this class of antibody would be more readily detectable. (Previous studies had demonstrated precipitins against common pathogens by immunodiffusion techniques (Burns & May, 1967, 1968).)

2.2. DETECTION OF IgG ANTIBODIES

In Fig. 6 specific IgG antibody to H. influenzae whole cell suspension is demonstrated in the serum from seven patients and in a cord blood serum (with known precipitins). The IgG RAST results ranged from 8000 CPM (Patient 1) to 3500 CPM for the 10^{-1} dilution of the sera. In most cases the specific antibody was titrated out at a 10^{-5} dilution of the patients' sera (1000 CPM). This figure was established as the background count since human serum albumin, the negative control included in the assay, gives approximately this count with concentrations from 0.47 mg to 30 mg per ml.

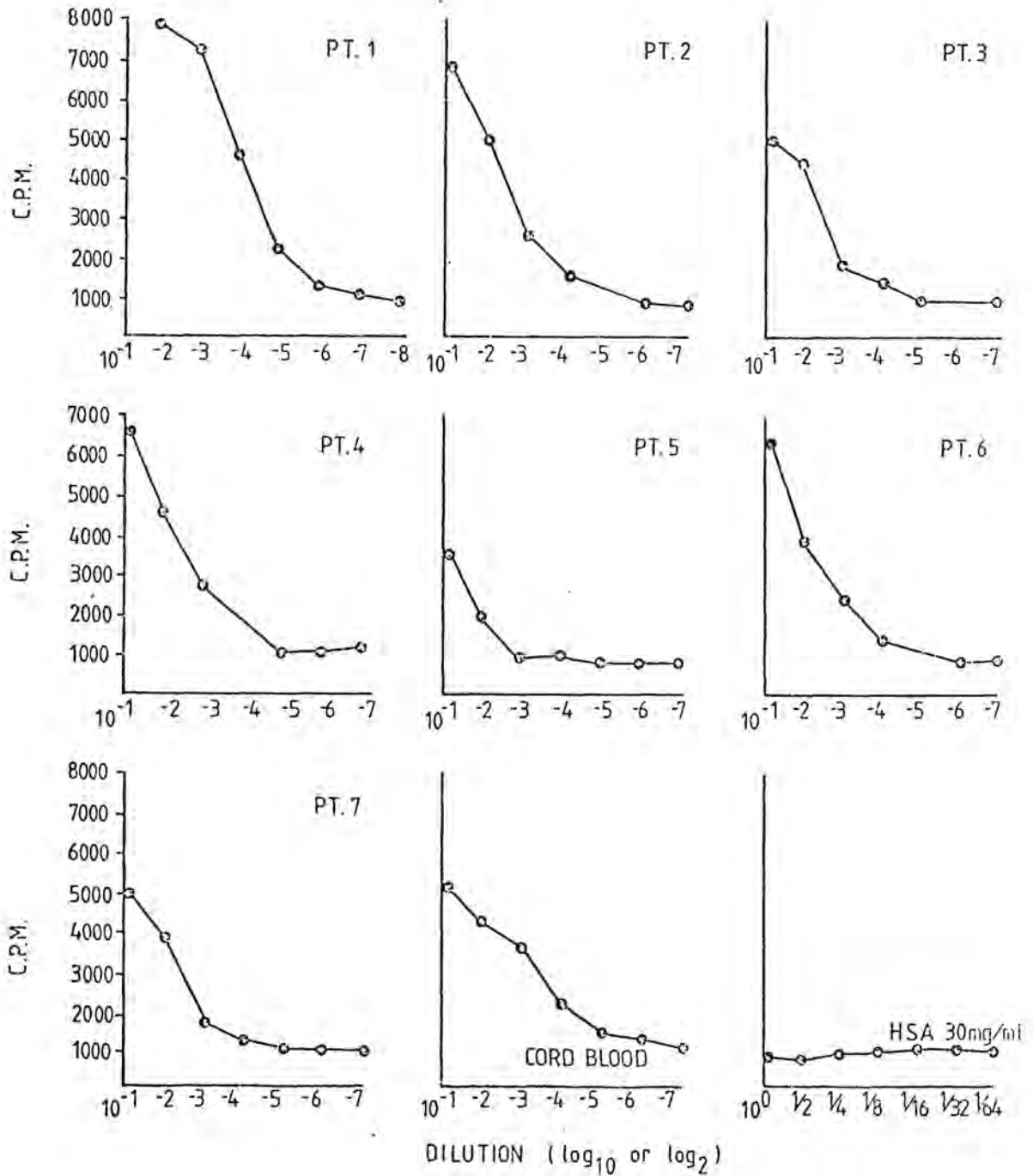
Thus specific IgG antibodies to H. influenzae were demonstrated in all seven of the patients tested. Antibodies in the cord blood serum were presumably of maternal origin.

Figure 6.**SLIDE RAST**

**Titration of specific IgG antibody to
H. influenzae (WCS).**

(7 patients, 1 cord blood; 1 HSA control).

IgG SLIDE RAST (H. influenzae whole cell suspension)



2.2.1. INHIBITION WITH THE SPECIFIC ANTIGEN *H. influenzae*

The specificity of the reaction is demonstrated in Fig. 7 by the inhibition of a patient's serum IgG RAST score to *H. influenzae* whole cell suspension. Two hundred microlitres of a 1:100 dilution of the serum was agitated on a rotary mixer with 200 μ l of a whole cell suspension of *H. influenzae* (optical density 0.22 at 650 nm) overnight at room temperature. Duplicate aliquots of ten-fold dilutions of the supernatant were then assayed in parallel with aliquots of the patient's non-absorbed serum similarly diluted.

The absorbed serum displays minimal antibody titration (541 CPM at 10^{-2} to 350 CPM at 10^{-4}) whilst the non-absorbed serum gives counts ranging from 2211 CPM at 10^{-2} dilution to 352 CPM at 10^{-5} dilution. Further dilution did not reduce the counts. This inhibition of the RAST reaction is achieved by absorption of the serum with specific antigen.

2.3. DETECTION OF IgE ANTIBODY

The slide RAST assay was then established to measure specific IgE antibodies to *S. pneumoniae*, *S. aureus* and *H. influenzae*. Optimal conditions were achieved by varying the conditions of fixation (e.g. 10% formalin in PBS or 90°C for 1 hr); the concentration of the whole bacterial cell suspension applied to the wells (optical densities of 0.1 to 0.5 at 650 nm); and the amounts of 125 I-anti-IgE used in the assay (50,000 CPM, 100,000 CPM or 200,000 CPM per tube).

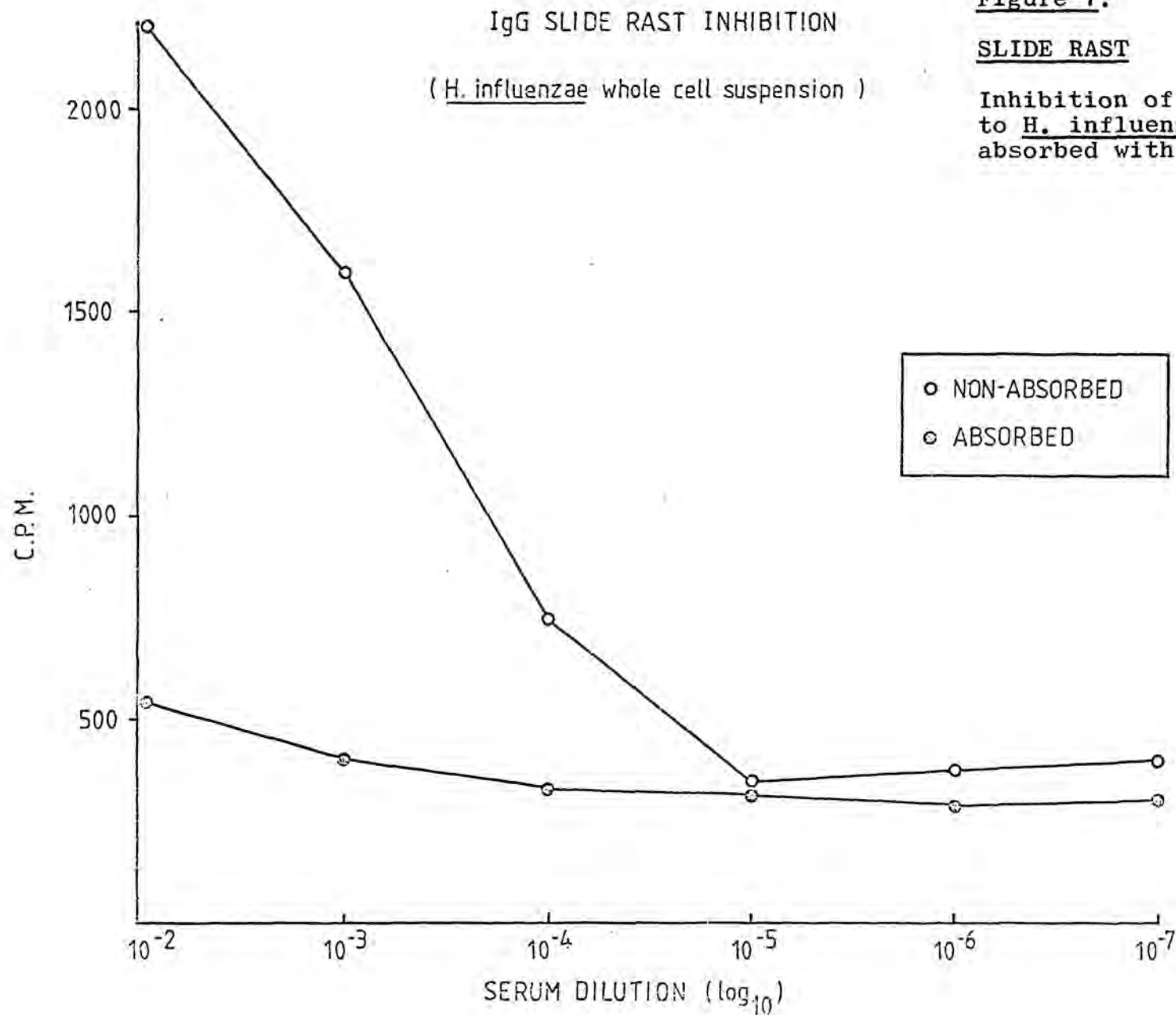


Figure 7.

SLIDE RAST

Inhibition of specific IgG antibody to H. influenzae in a patient's serum absorbed with H. influenzae antigen.

The procedure finally adopted employed heat fixation (90°C/ 1 hr) of a whole bacterial cell suspension of optical density 0.2 at 650 nm and the addition of 100,000 CPM of ^{125}I -anti-IgE to each tube. Full details of the method are described in Chapter III, 8.2.

2.3.1. INHIBITION WITH THE SPECIFIC ANTIGENS

Titration of specific bacterial IgE antibody in patients' sera was achieved and inhibition of the reaction was obtained by absorption of 150 μl of patients' sera with 150 μg of freeze dried specific antigen overnight at room temperature. RAST counts of aliquots of the supernatant were reduced to those of cord blood sera which contained no demonstrable total IgE.

2.4. PRELIMINARY (CLINICAL) STUDY USING THE SLIDE RAST

Using the above developed slide RAST technique, specific IgE antibodies to the three bacteria (S. pneumoniae, S. aureus and H. influenzae) were then measured in a preliminary clinical study of sera from 13 patients (attending the Brompton Hospital Allergy Clinic) and two sera from blood donors. Cord blood sera containing no demonstrable IgE were run in parallel as negative controls. To allow comparisons between assays results are expressed as RAST score ratios:

$$\text{RAST Score Ratio} = \frac{\text{Patient's specific RAST score}}{\text{Cord blood specific RAST score}}$$

Fourteen of these patients were skin prick tested with the three bacterial extracts.

The slide IgE RAST score ratio of the study patients to the three bacteria are shown in Table II together with the diagnosis for each patient, the immediate type bacterial skin reactivity and their total IgE levels (measured as described in Chapter III, 10.0).

2.4.1. RESULTS

The S. aureus RAST score ratios ranged from 0.9 to 1.7 with eight patients (three of whom were skin prick test positive) having a score higher than cord value. With S. pneumoniae the RAST score ratios ranged from 0.9 to 1.6 with 15 patients (seven of whom were skin prick test positive) having a score higher than cord value. The H. influenzae RAST score ratios ranged from 1.10 to 1.55 with all patients having a score higher than cord value, although only one patient was skin test positive. The level of total IgE of the study patients ranged from 18-17,500 IU/ml. The upper limit of the normal range was 240 IU/ml with the assay system employed. By this criterion 14 out of the 18 patients studied had raised total IgE levels. The total IgE level of the two blood donor sera were 165 and 30 IU/ml respectively.

These total IgE values of the study patients were

PRELIMINARY STUDY SLIDE RAST RESULTS

PAT. NO.	DIAGNOSIS	TOTAL IgE IU/ml	<u>S. aureus</u>		<u>S. pneumoniae</u>		<u>H. influenzae</u>	
			SPT	RAST	SPT	RAST	SPT	RAST
1	○	1700	-	1.15	+	1.00	-	1.40
2	⊙	1550	-	0.90	+	1.15	-	1.40
3	⊙	600	+	1.70	-	1.60	-	1.40
4	○	340	-	1.00	+	1.30	-	1.55
5	○	520	-	0.90	+	1.30	-	1.30
6	△	1450	+	1.20	+	1.10	-	1.30
7	□	210	-	1.30	+	1.35	-	1.35
8	△	8200	-	1.20	+	1.40	-	1.30
9	□	480	?	0.70	?	1.10	?	1.10
10	⊞	18	+	1.30	+	1.10	-	1.40
11	⊞	470	+	1.00	-	1.50	+	1.40
12	□	135	?	0.90	?	1.60	?	1.50
13	△	5300	-	1.20	-	1.20	-	1.40
14	△	8400	?	0.90	?	1.25	?	1.30
15	□	1120	?	0.90	?	1.20	?	1.30
16	△	17,500	-	1.20	-	1.50	-	1.35
17	○	18	-	0.90	-	0.90	-	1.10
18	□	170	-	0.90	-	1.00	-	1.30
19	▽	165	?	1.05	?	1.00	?	1.20
20	▽	30	?	1.10	?	1.10	?	1.20

SPT = Skin Prick Test

RAST = RAST Score Ratio

Key of Diseases

- Rhinitis
 ⊙ Rhinitis + Asthma
 △ Asthma + Hayfever
 ▲ Aspergillosis + Asthma
 □ Bronchial Asthma
 ⊞ Urticaria
 ▽ Blood Donor

TABLE II

compared with the specific IgE RAST score ratios in Fig. 8 (S. pneumoniae), Fig. 9 (S. aureus) and Fig. 10 (H. influenzae). The same symbols denoting disease groups are used throughout. A low correlation, $r = 0.32$ ($p < 0.1$), was obtained with S. pneumoniae on estimating the linear regression, but non-significant correlation values ($r = 0.14$ and $r = 0.05$) were obtained with S. aureus and H. influenzae respectively.

In Fig. 11 the specific bacterial skin prick test reactivity of the 14 study patients tested is displayed against their specific bacterial RAST score. Five patients gave immediate type skin reactions to S. aureus, eight to S. pneumoniae and only one to H. influenzae.

2.4.2. DISCUSSION

Whole bacterial cells were used in the RAST assays whilst only bacterial extracts were used for the skin testing because the whole bacterial cell suspension skin test solutions were unavailable.

Raised specific RAST score ratios occurred in both skin prick test positive and negative patients and indeed no statistical correlations between the two criteria could be found with any of the three bacteria, possibly because different forms of the antigen had been used.

Thus, although there had appeared to be some agreement between a positive slide RAST score and a positive specific prick test in the early work, this preliminary (clinical)

Figure 8.

Correlation of patients' slide RAST scores of specific IgE to S. pneumoniae with their total IgE levels.

IgE SLIDE RAST (S. pneumoniae whole cell suspension)

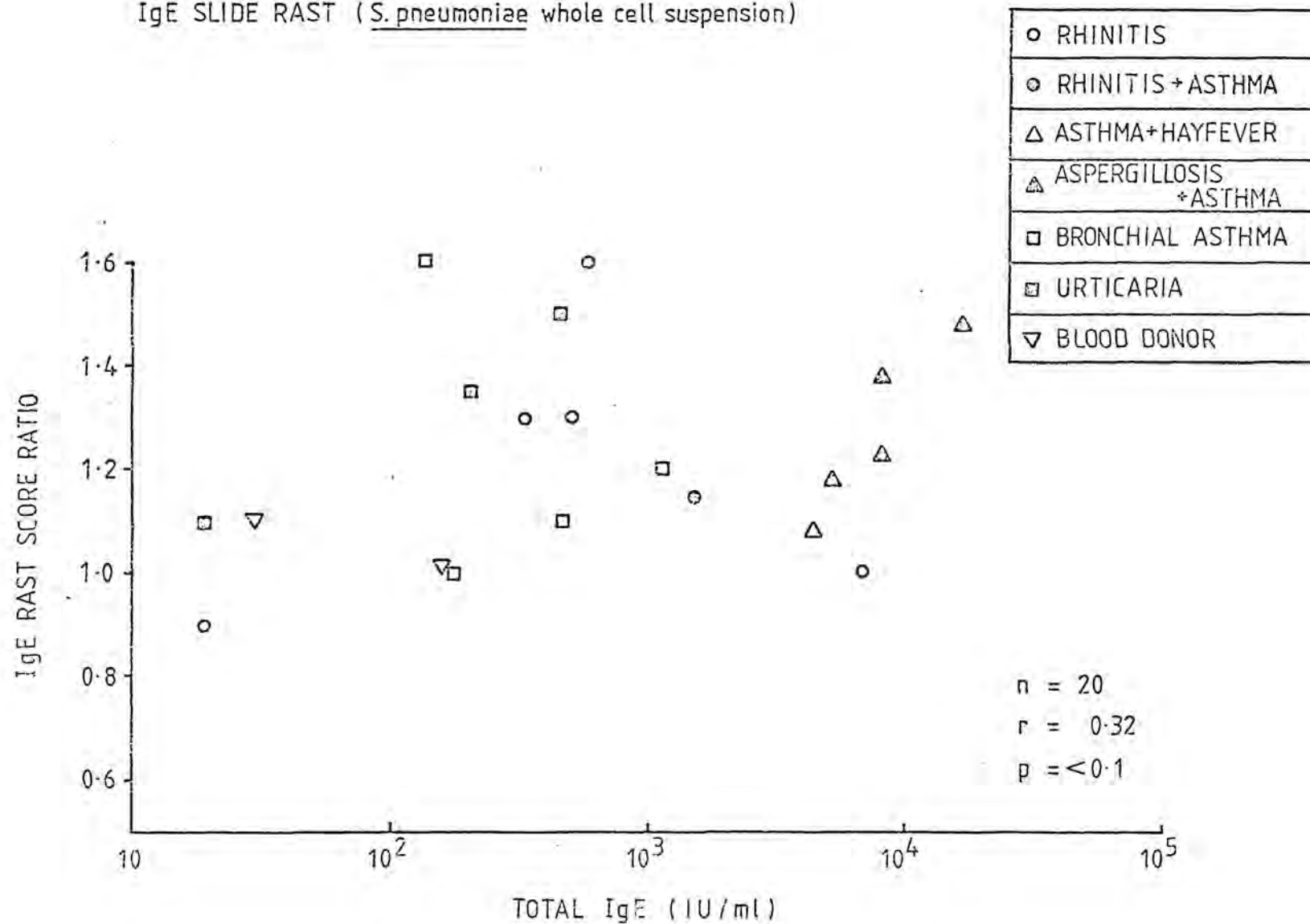


Figure 9.

Correlation of patients' slide RAST scores of specific IgE to S. aureus with their total IgE levels

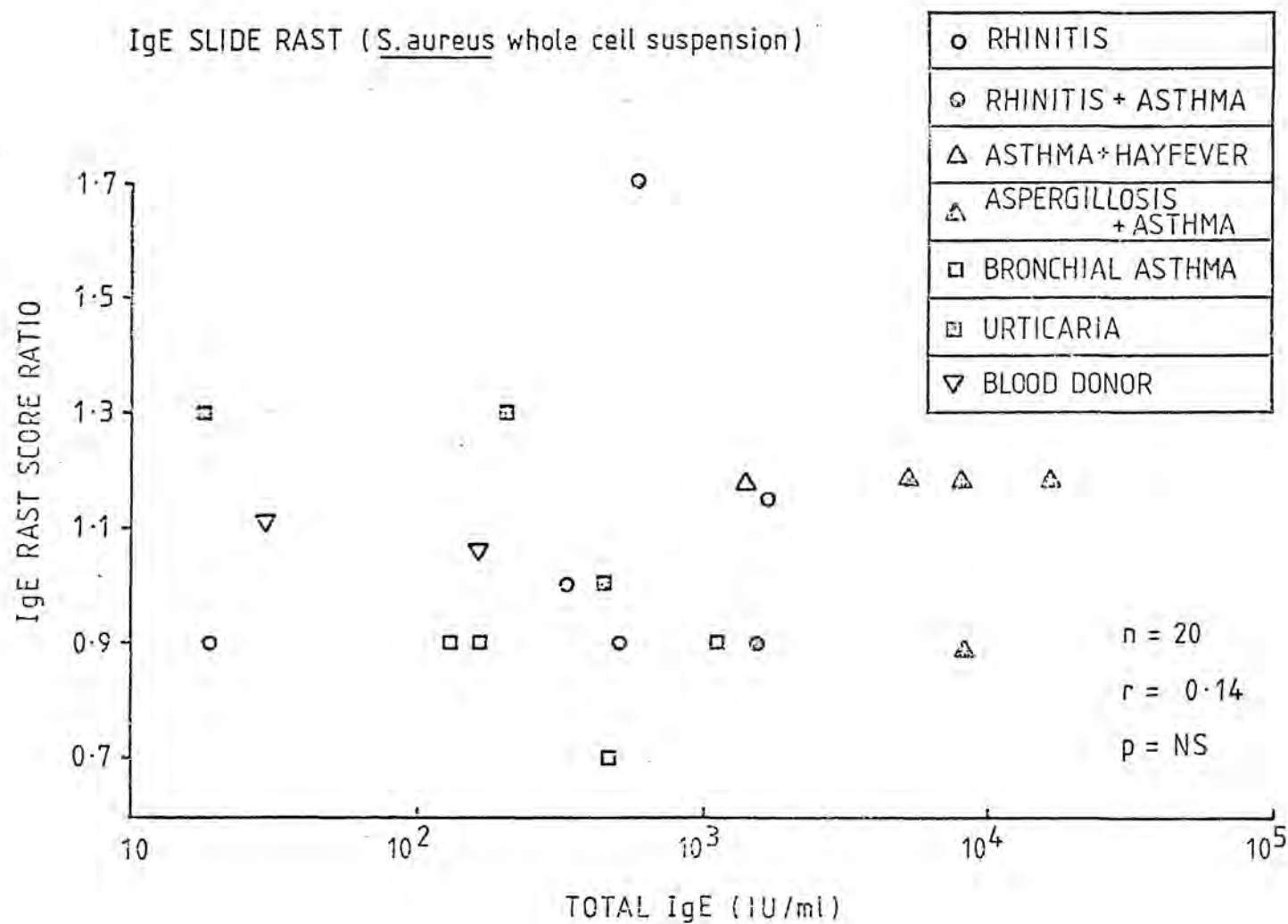


Figure 10.

Correlation of patients' slide RAST scores of specific IgE to H. influenzae with their total IgE levels.

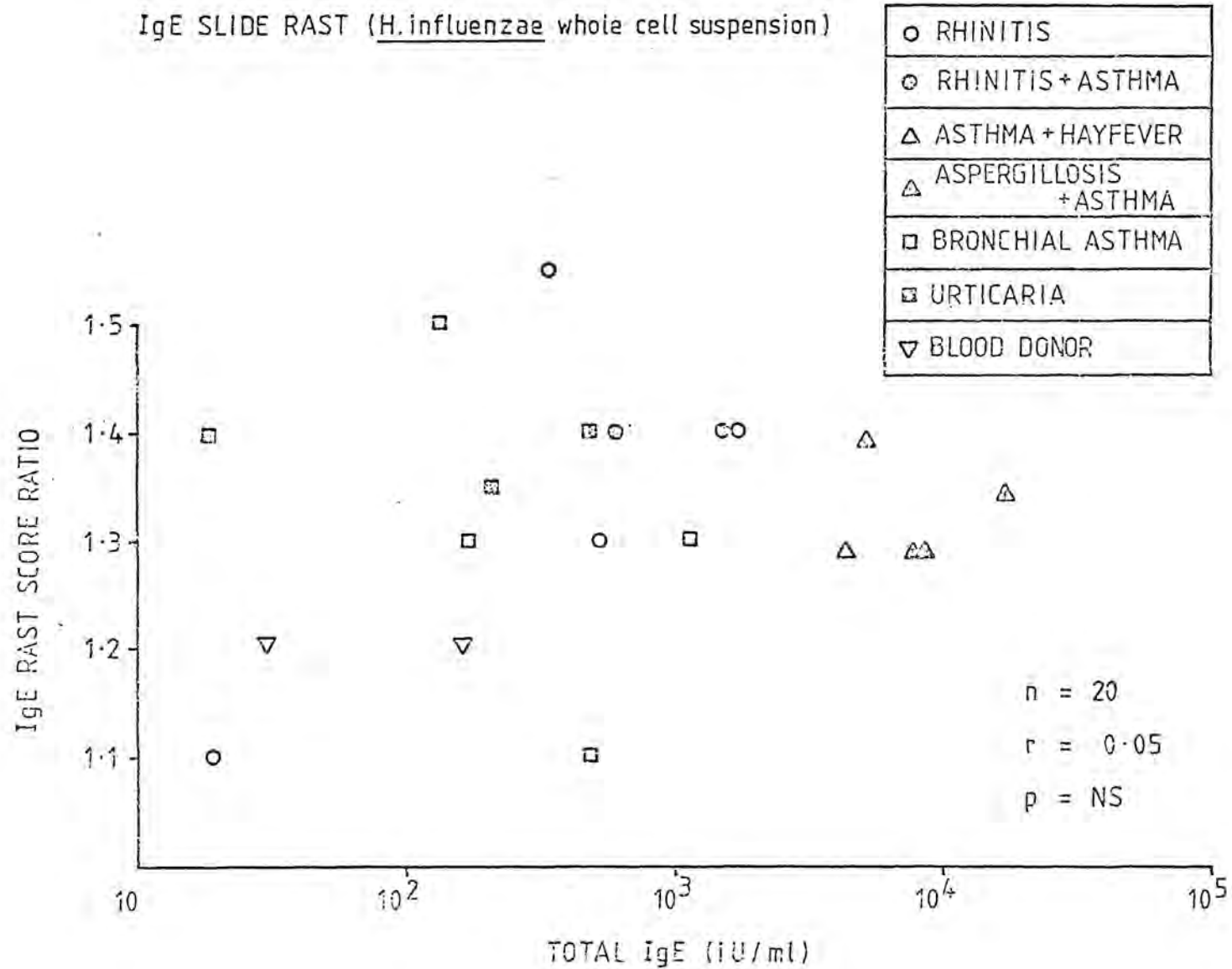
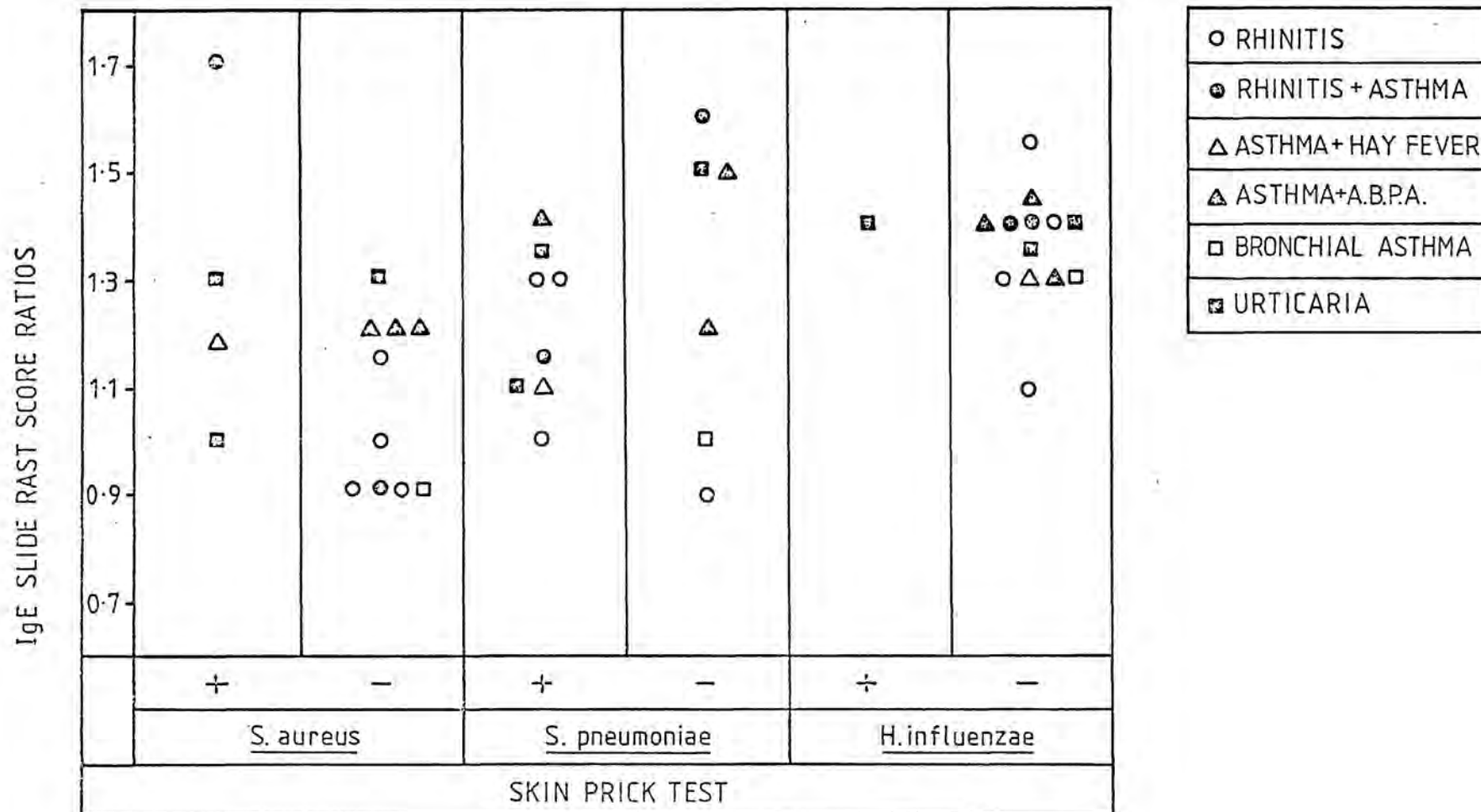


Figure 11.

Correlation of patients' slide RAST scores of specific IgE to S. aureus, S. pneumoniae and H. influenzae respectively with their specific skin prick test results.



study showed that the method was unacceptable for measurement of specific IgE, both in terms of sensitivity and reproducibility. The slide RAST was thus rejected as inappropriate for the purposes of this study.

3.0 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

In order to try and find a better method to measure serum IgE levels to whole bacterial cells, an enzyme-linked immunosorbent assay (ELISA) using an alkaline phosphatase system was employed.

3.1. Introduction

Enzyme-linked immunosorbent assays (ELISA) depend on two factors. Firstly, that antigen (or antibody) can be attached to a solid-phase support yet retain immunological activity and secondly, that either antibody (or antigen) can be linked to an enzyme and that the conjugate retains both immunological and enzymatic activities. The conjugate can then be used in a direct binding assay. The principle of the assay is that specific antibody reacts with antigen adsorbed on to the solid-phase. This antibody will in turn bind to an anti-immunoglobulin enzyme conjugate. The amount of conjugate specifically taken up, which is proportional to the concentration of the unknown, can be quantified in terms of its ability to degrade a suitable substrate. Degradation involves a colour change which is assessed visually or by spectrophotometry.

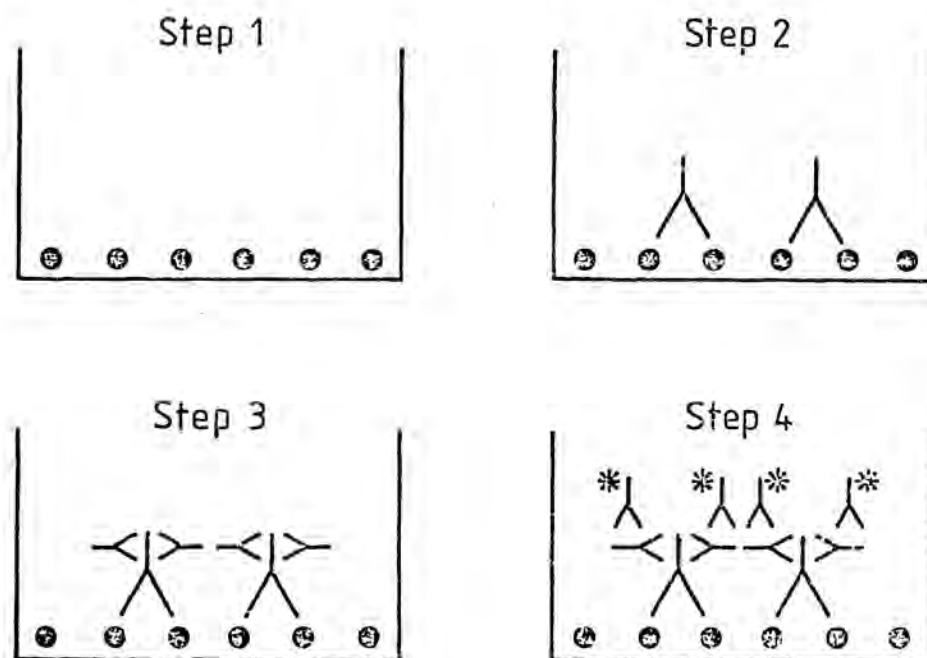
3.2. ELISA (IgE) double antibody technique

The microplate method of Voller et al. (1976b) using polystyrene microhaemagglutination plates was modified for

the measurement of IgE antibodies as depicted in Fig. 12. Additional sensitivity was imparted to the assay by using unlabelled specific antibody (1st antibody) followed by enzyme-labelled anti-species immunoglobulin as the indicator (2nd antibody).

Chequerboard titrations were performed to determine the optimum concentrations of each antigen, the patient's serum, 1st antibody and the enzyme conjugate. In developing the assay sera were included from patients with strong and weak reactions as assessed by other criteria (i.e. immediate-type skin reaction and RAST score) as well as negative sera (cord blood with no demonstrable IgE). All dilutions were made in the wash buffer.

Figure 12. Diagrammatic representation of the enzyme-linked immunosorbent assay (ELISA) for specific IgE antibody detection by a double antibody technique.



- Step 1: Antigen (●) is coated on the inside of wells.
- Step 2: Patient's serum is added and any specific antibody (Y) attaches to antigen.
- Step 3: First antibody is added (rabbit anti-human IgE) (Y) which attaches to antigen/IgE antibody complex.
- Step 4: The second antibody, the enzyme-conjugate is added (alkaline phosphatase labelled goat anti-rabbit IgG) (Y*) which attaches to the complex.
- Step 5: (not shown) Enzyme substrate (p-nitro-phenyl phosphate) is added and the amount of hydrolysis (= depth of colour) is proportional to the amount of specific IgE antibody in the patient's serum.

3.3. DEVELOPMENT OF ELISA TECHNIQUES

A series of experiments was carried out in which the optimal conditions for sensitivity and specificity of the assay were determined (Table III).

In Experiment I (Table III) an extract of S. pneumoniae was used. A chequerboard titration of the antigen was performed by sensitising the wells of the microtitre plates (Dynatech M1294) with ten-fold dilutions (200 μ l per well) of 1 mg/ml - 0.0001 mg/ml in carbonate buffer (pH 9.6) and allowing the plate to remain overnight at 4°C.

To remove non-absorbed antigen the solution was "shaken out" of the plate and each well washed individually four times by filling with "wash buffer" from a wash bottle. Three to five minute intervals were left between washes.

Two hundred microlitres of 1/10 and 1/50 dilutions of patient's serum was added to duplicate individual wells and the plate incubated in a humidity chamber for 5 hr at room temperature.

The plate was then washed four times as previously described and 200 μ l of a 1:1000 dilution of rabbit anti-human IgE (this concentration previously determined by performing a chequerboard titration of dilutions between 1:500 and 1:2000) was added to each well and the plate incubated in a humidity chamber overnight at room temperature.

After four washes the anti-human IgE bound was detected by a labelled specific 2nd antibody (the optimum concentration having been previously determined by chequerboard titration).

EXPERIMENT	I	II	III	IV	V	VI	VII
ANTIGEN	<u>S. pneumoniae</u>	<u>S. pneumoniae</u>	<u>S. aureus</u>	<u>H. influenzae</u>	<u>S. pneumoniae</u>	<u>S. aureus</u>	<u>H. influenzae</u>
Sensitisation of solid phase	Extract in CO ₃ buffer. Left at 4°C overnight.	W.C.S. O.D. 0.193 viz. 10 ⁰ (a) CO ₃ buffer v. aqua dist. (b) CO ₃ buffer 4°C/ 18 hr v. 90°C/1 hr	W.C.S. O.D. 0.2 viz. 10 ⁰ in CO ₃ buffer (a) 90°C/ 1 hr. (b) air dried + 10% HCHO	W.C.S. O.D. 0.2 viz. 10 ⁰ in CO ₃ buffer (a) 90°C/1 hr (b) air dried + 10% HCHO	W.C.S. O.D. 0.2 viz. 10 ⁰ in CO ₃ buffer. 90°C/1 hr	W.C.S. O.D. 0.2 viz. 10 ⁰ in CO ₃ buffer. 90°C/1 hr	W.C.S. O.D. 0.2 viz. 10 ⁰ in CO ₃ buffer. 90°C/1 hr
Concentration	1 mg - 0.001 mg/ml	10 ⁰ - 10 ⁻⁴	10 ⁻¹ , 10 ⁻² , 10 ⁻³	10 ⁻¹ , 10 ⁻² , 10 ⁻³	10 ⁻³	10 ⁻²	10 ⁻²
PATIENTS' SERUM CONCENTRATION	1/10 1/50 5 hr	1/5 5 hr	1:5 5 hr	1:5 5 hr	1:5 20 test sera	1:5 20 test sera	1:5 20 test sera
1st ANTIBODY Rabbit anti- IgE	1:1000 18 hr	1:1000 18 hr	1:1000 18 hr	1:1000 18 hr	1:1000 18 hr	1:1000 18 hr	1:1000 18 hr
2nd ANTIBODY CONJUGATE	1:2000	1:3000	1:3000	1:3000	1:3000	1:3000	1:3000
Alkaline phosphatase goat anti- rabbit IgG	3 hr	3 hr	3 hr	3 hr	3 hr	3 hr	3 hr
SUBSTRATE P-nitrophenyl- phosphate	30 min	40 min	45 min	45 min	25 min	45 min	45 min

TABLE III
ELISA. SUMMARY OF SELECTED EXPERIMENTS.

Two hundred microlitres of a 1:3000 dilution of conjugated goat anti-human IgG (Miles-Yeda Ltd., Stoke Poges) labelled with alkaline phosphatase was added to all wells and the plate incubated for 3 hr.

Following a further four washes 200 μ l of substrate, p-nitrophenylphosphate in diethanolamine buffer (1 mg/ml, Sigma 104 substrate) was added to each well.

This resulted in the development of a yellow colour due to p-nitrophenol release. The enzyme reaction was stopped (after 30 min) by the addition of 50 μ l of 3 M NaOH to each well.

Samples were transferred to a "Vitatron" spectrophotometer (MSE Scientific Instruments) and the absorbance recorded using a 405 nm filter.

This experiment demonstrated specific IgE antibodies to S. pneumoniae extract by ELISA in sera from patients with positive specific skin prick tests and RAST's compared with cord blood sera. Results are as shown in Figs. 13 and 14. The absorbance readings of the expected positive sera were higher than cord blood serum but were only maximally 0.52 with sera 1:10 and maximally 0.36 with sera 1:50. In order to obtain higher absorbance readings a 1:5 dilution of patients' sera was employed in the further experiments.

Figure 13.

IgE ELISA

Titration of S. pneumoniae extracts to determine the optimum concentrations of antigen.

Patients' sera diluted 1:10

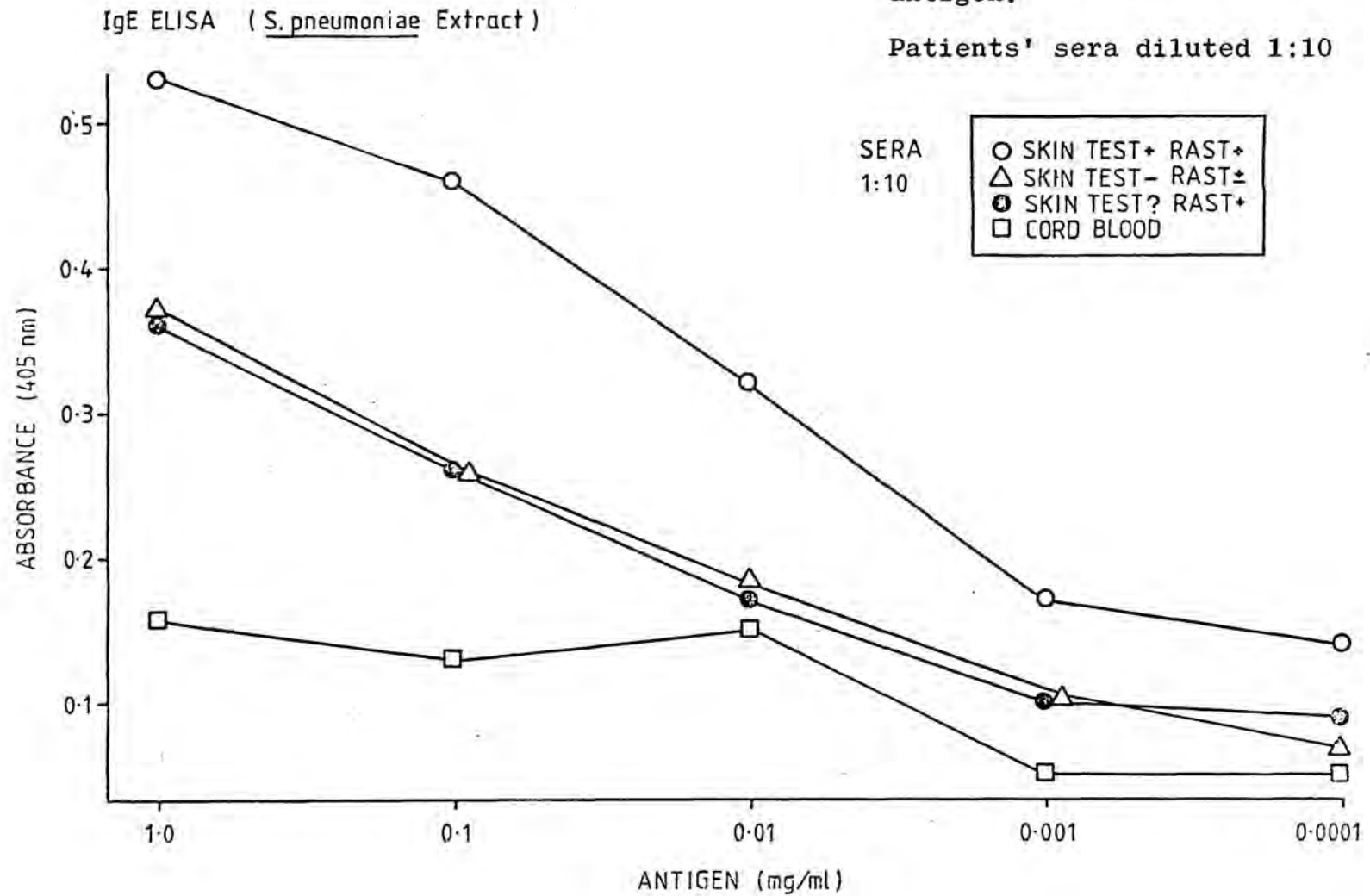
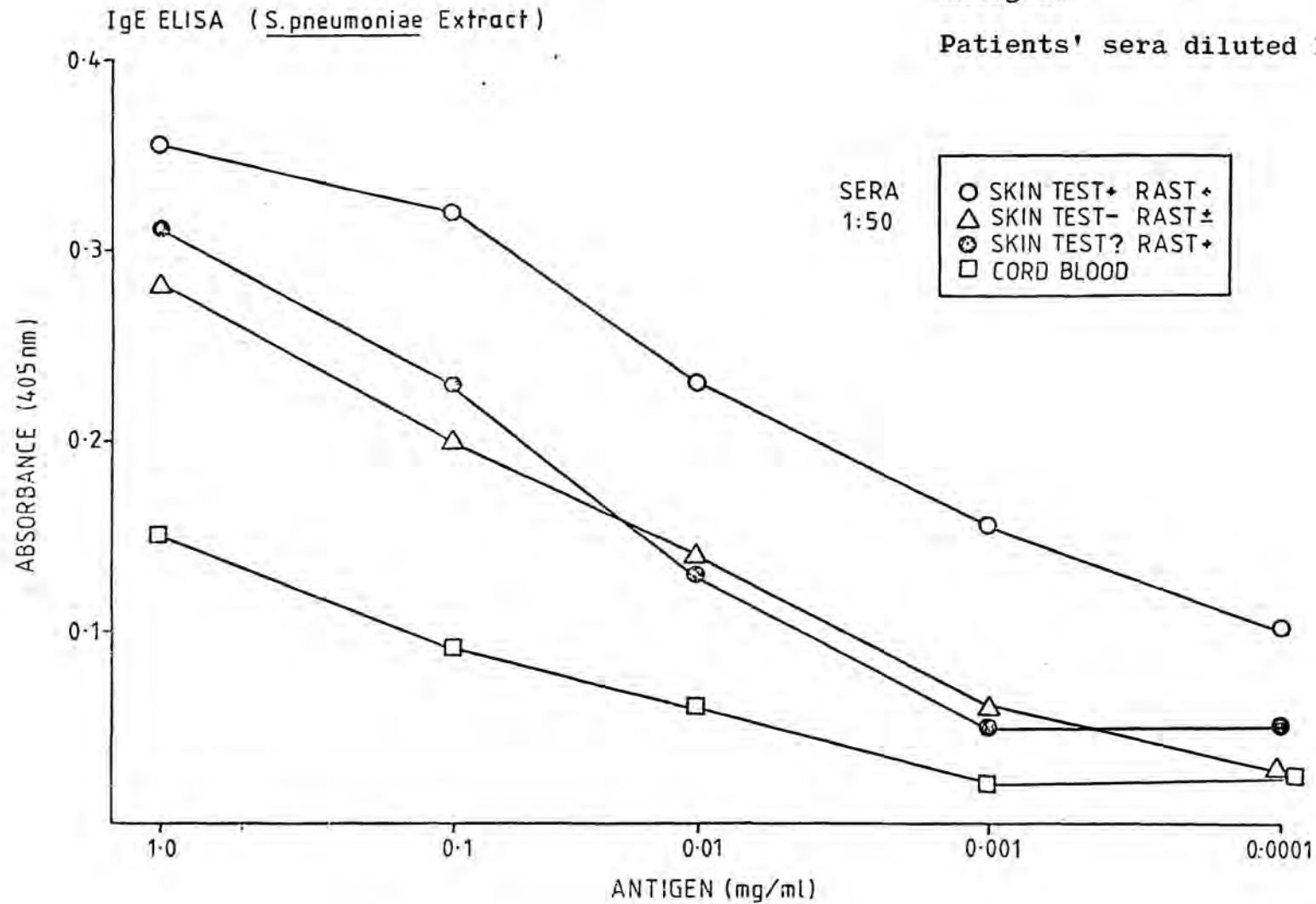


Figure 14.

IgE ELISA

Titration of S. pneumoniae extracts to determine the optimum concentrations of antigen.

Patients' sera diluted 1:50



3.3.1. WHOLE BACTERIAL CELL ASSAYS

Having detected specific IgE antibodies to an extract of S. pneumoniae, assays using whole cell suspensions (W.C.S.) were attempted.

It was found that only low absorbance readings were obtained when the bacterial suspension was left to stand overnight in the wells without fixation. The suspension was heat fixed at 90°C for 1 hr in individual plastic Dynawell cups, which unlike the microtitre plates, were able to withstand this temperature. The individual cups were then placed in an ELISA holder and the assay continued as described above.

In Experiment II (Table III) a "chequerboard titration" of a suspension of S. pneumoniae in carbonate buffer (optical density 0.193 at 650 nm) was performed with dilutions of 10^0 to 10^{-4} , in order to find the optimal concentration of antigens for use.

A comparison was made between the bacterial suspension fixed at 90°C for 1 hr made up in

- a. (i) the carbonate coating buffer, and
- a. (ii) distilled water.

The absorbance readings were lower in (ii) by as much as 83%, thus demonstrating the requirement of the carbonate buffer, pH 9.6.

In the comparison made between leaving the bacterial suspensions in carbonate buffer

- b. (i) overnight in the wells at 4°C, and
- b. (ii) exposing them to 90°C for 1 hr

the former gave absorbance readings 66-87% lower than those

with heat fixed antigens.

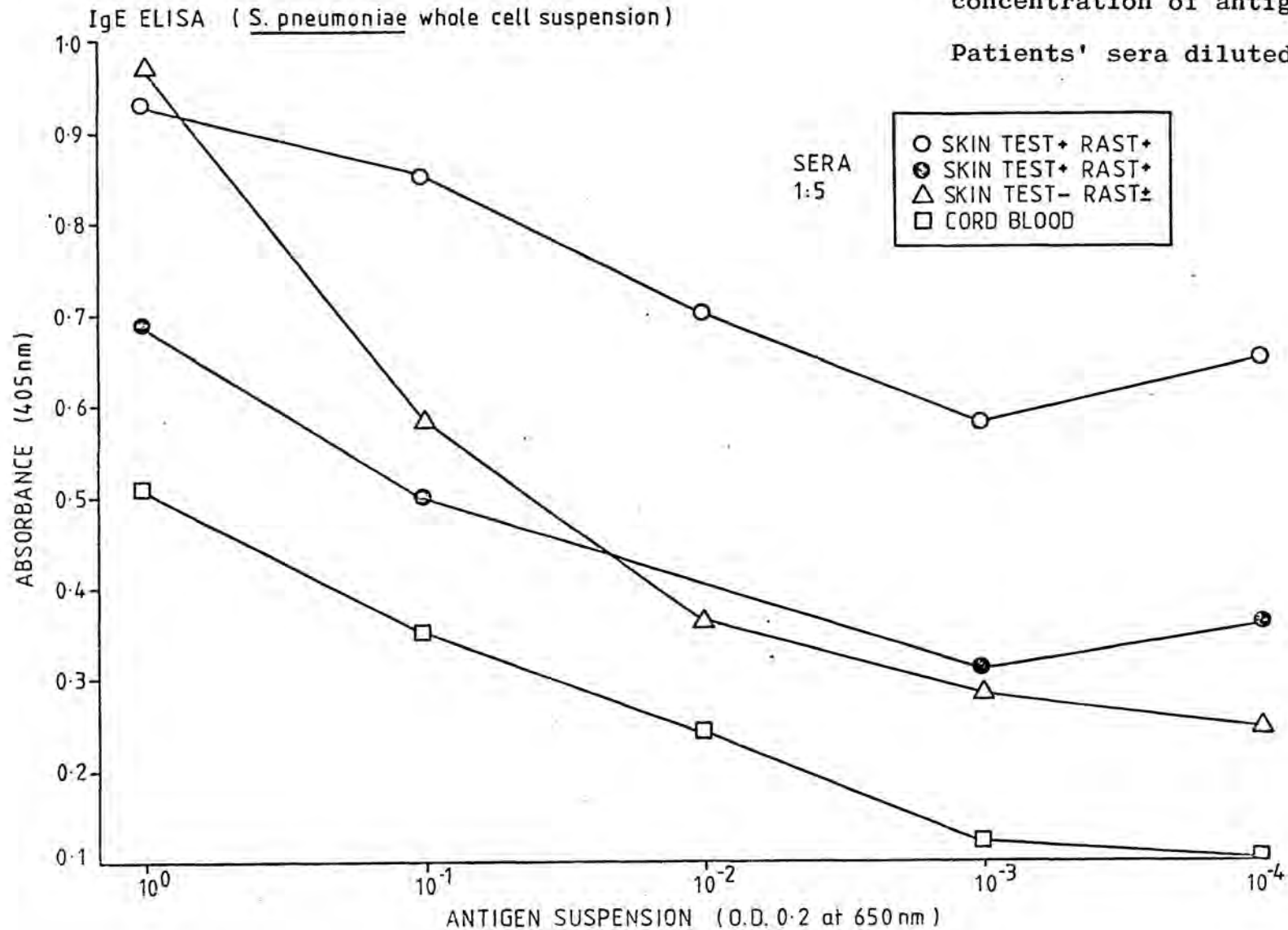
Figure 15 shows the results of known positive (patients with positive specific skin prick test) and negative sera (1:5) with heat fixed antigen in carbonate buffer in an IgE ELISA to S. pneumoniae whole cell suspension. A concentration of 10^{-3} of the antigen suspension was chosen for further experiments as that giving reasonably high absorbance readings for positive sera and an acceptably low reading for the cord blood serum.

Figure 15.

IgE ELISA

Titration of S. pneumoniae whole cell suspension to determine the optimum concentration of antigen.

Patients' sera diluted 1:5

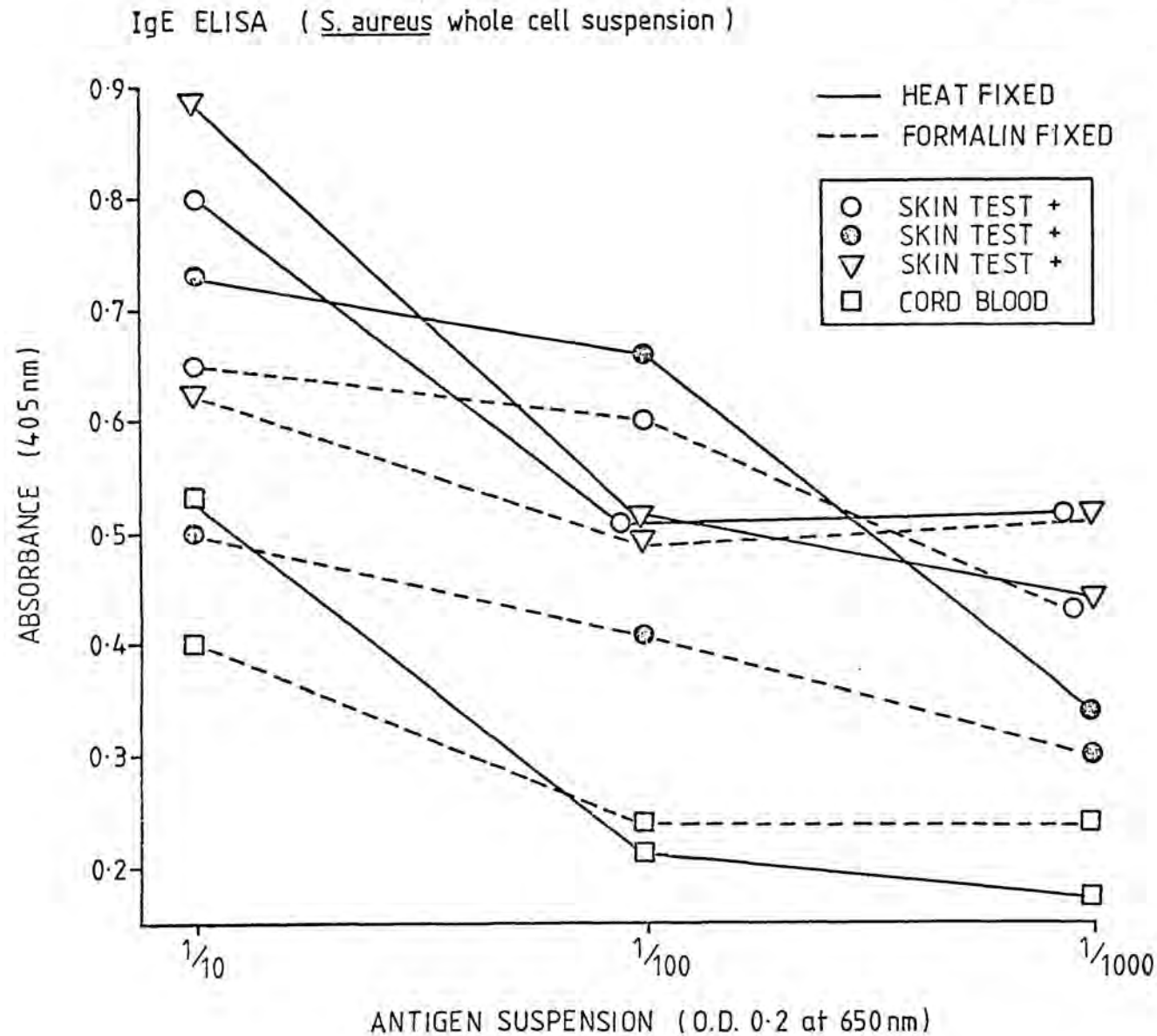


In Experiments III and IV (Table III) the determination of optimal conditions for antigen concentration and fixation for S. aureus and H. influenzae respectively was carried out, using the same basic techniques of Experiment II. In addition the relative efficacy of the prior heat treatment was compared with a formalin fixation procedure in which the antigen suspension was added to the Dynawell cups, air dried and then treated with a 10% solution of formalin for 15 minutes. The results are displayed in Figs. 16 and 17 respectively.

In these figures the solid line represents the heat fixed antigen and the dotted line represents the formalin fixed antigen for the same serum as indicated by the symbols.

A dilution of 10^{-2} was chosen as optimum for both S. aureus and H. influenzae cell suspensions for future experiments, for the reasons given above for S. pneumoniae. In the comparison of the individual cups with heat fixed and 10% formalin fixed cell suspensions at the optimum 10^{-2} antigen dilution, the absorbance readings with the latter were 96, 91 and 62% for S. aureus and 101, 76 and 57% for H. influenzae of the absorbance readings with the heat fixed antigen. Thus the formalin fixation appeared to offer no advantage and heat fixation was used in future assays.

Figure 16. IgE ELISA



Titration of *S. aureus* whole cell suspension to determine the optimum concentration of antigen.

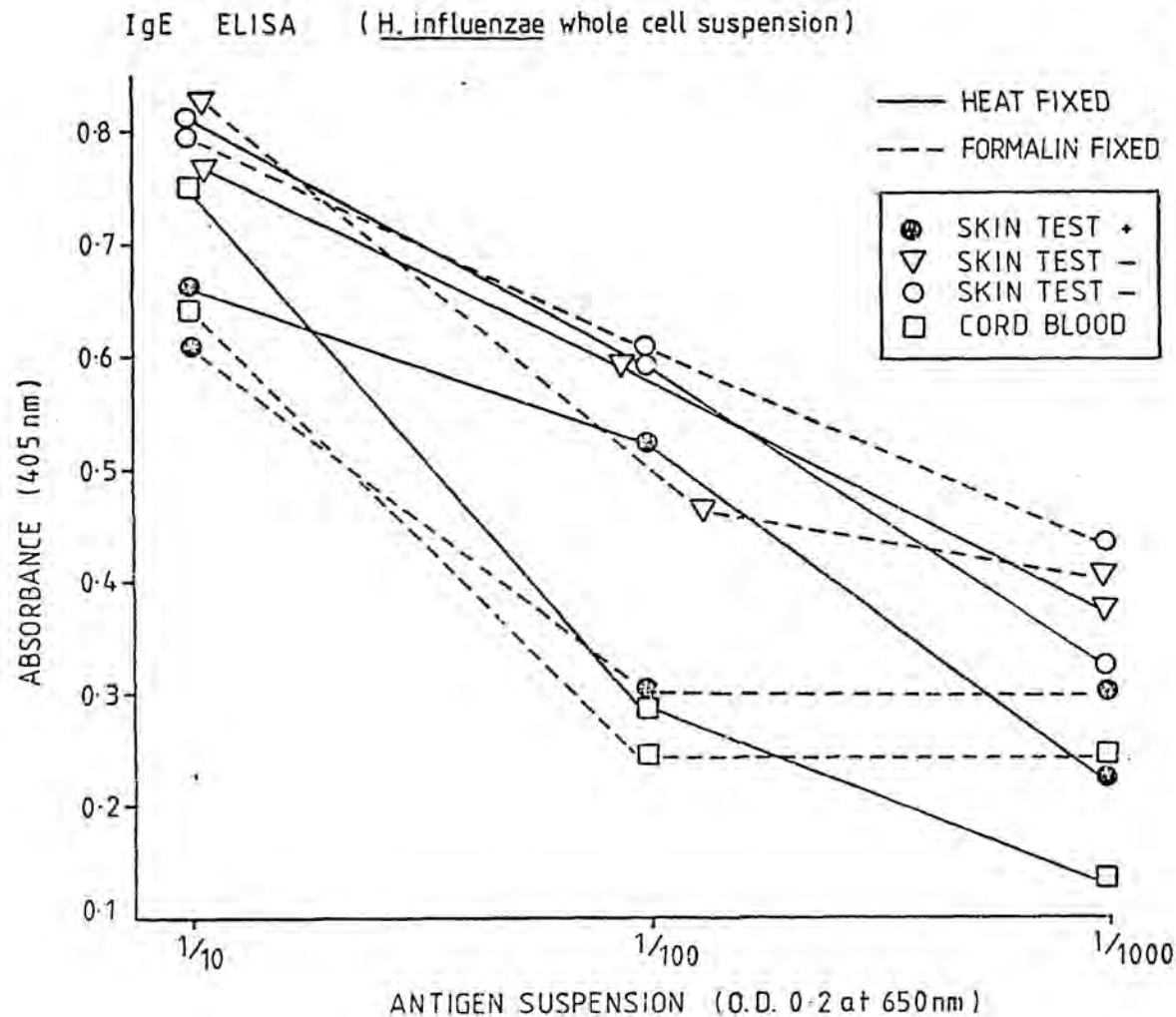
Comparison of results using heat-fixed/formalin-fixed antigens.

(4 sera diluted 1:5)

Figure 17. IgE ELISA

Titration of H. influenzae whole cell suspension to determine the optimum concentration of antigen.

Comparison of results using heat-fixed/formalin-fixed antigens.



3.4. PRELIMINARY (CLINICAL) STUDY USING ELISA

Having established the optimum conditions for whole cell suspension ELISA assays, sera from 17 of the Allergy Clinic patients previously tested by slide RAST and the same two blood donor sera were tested in triplicate and compared with cord blood control sera.

IgE ELISA whole cell suspension assays were performed with S. pneumoniae (Experiment V), S. aureus (Experiment VI) and H. influenzae (Experiment VII) - Table III. Results were correlated with the diagnosis for each patient, the immediate type bacterial skin reactivity and their total IgE levels. This data is shown in Table IV.

3.4.1. RESULTS

Specific bacterial IgE ELISA scores of the two blood donor sera and all but patient No. 18 (with bronchial asthma and having low ELISA scores to all three bacteria) were greater than the ELISA scores of cord blood sera.

These specific IgE values were compared with the concentration of the patients' total IgE in Fig. 18 (S. pneumoniae), Fig. 19 (S. aureus) and Fig. 20 (H. influenzae). Negative correlations were obtained between the two criteria with S. aureus and H. influenzae but a correlation of $r = 0.62$ ($p < 0.005$) was calculated with S. pneumoniae on estimating the linear regression.

In Fig. 21 the specific bacterial skin prick test

PRELIMINARY STUDY IgE ELISA WHOLE CELL SUSPENSION RESULTS

PAT. NO.	DIAGNOSIS	TOTAL IgE IU/ml	<u>S. aureus</u> E ^o		<u>S. pneumoniae</u> E ^o		<u>H. influenzae</u> E ^o	
			SPT	405 nm	SPT	405 nm	SPT	405 nm
1	○	1700	-	0.63	+	1.25	-	1.20
2	⊙	1550	-	0.58	+	0.77	-	1.20
3	⊙	600	+	0.77	-	0.76	-	1.30
4	○	340	-	0.69	+	1.10	-	1.10
5	○	520	-	0.60	+	0.62	-	0.90
6	△	1450	+	0.59	+	0.58	-	1.00
7	⊠	210	-	0.50	+	0.82	-	1.30
8	△	8200	-	0.67	+	0.62	-	1.30
9	□	480	?	0.46	?	0.63	?	1.10
10	⊠	18	+	0.66	+	0.60	-	1.00
11	⊠	470	+	0.48	-	0.55	+	1.10
12	□	135	?	0.54	?	0.53	?	1.40
13	△	5300	-	0.72	-	0.73	-	1.50
14	△	8400	?	N.D.	?	N.D.	?	N.D.
15	□	1120	?	0.74	?	0.50	?	1.30
16	△	17,500	-	0.62	-	0.70	-	1.20
17	○	18	-	0.61	-	0.71	-	1.20
18	□	170	-	0.39	-	0.40	-	0.70
19	▽	165	?	0.56	?	0.50	?	1.10
20	▽	30	?	0.59	?	0.53	?	1.10
	CORD BLOOD	-		0.47		0.43		0.87

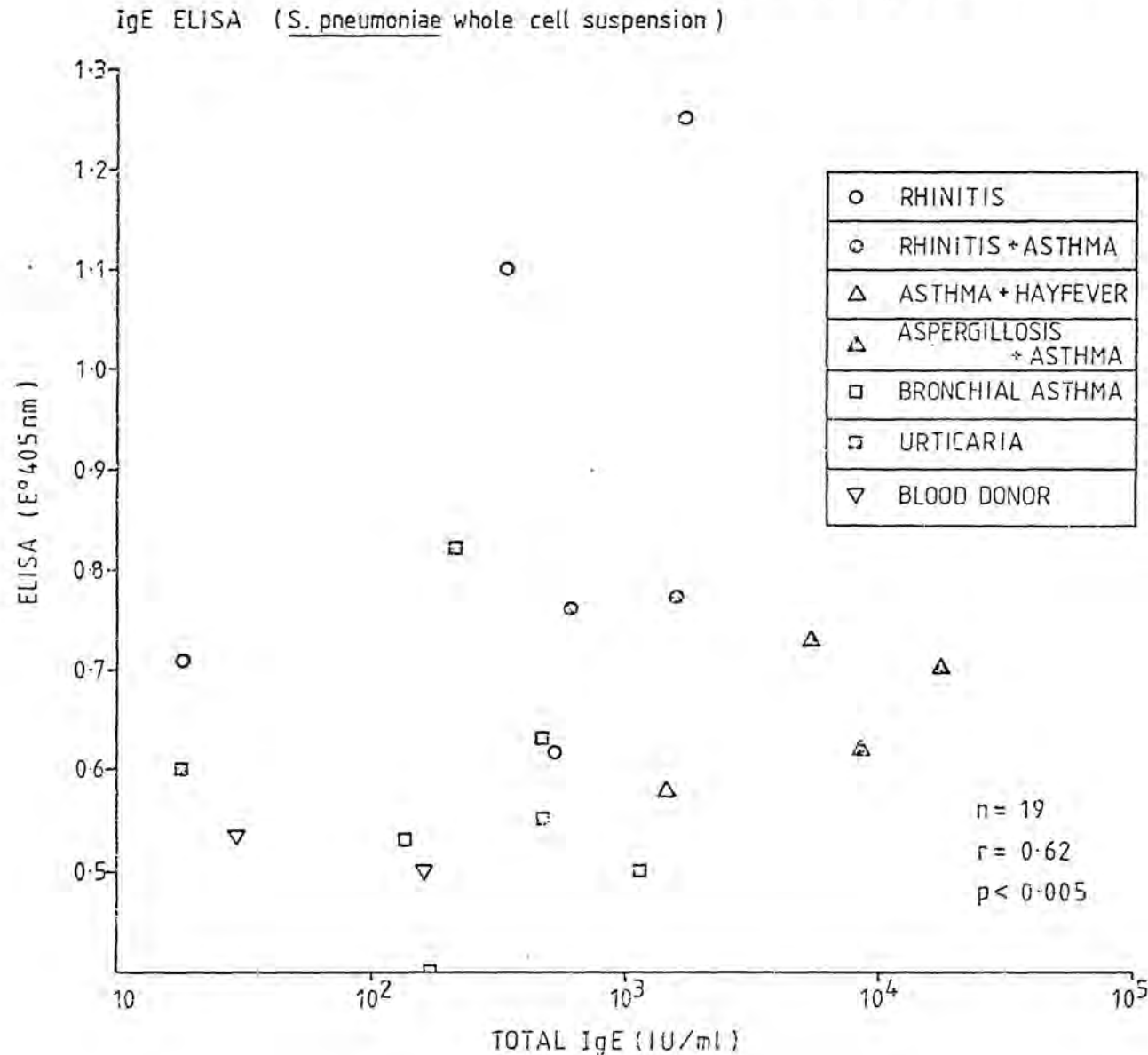
SPT = Skin Prick Test

Key of Diseases

- Rhinitis
- ⊙ Rhinitis + Asthma
- △ Asthma + Hayfever
- △ Aspergillosis + Asthma
- Bronchial Asthma
- ⊠ Urticaria
- ▽ Blood Donor

TABLE IV

Figure 18. Correlation of patients' specific IgE antibody to S. pneumoniae measured by ELISA and their total IgE level.



IgE ELISA (*S. aureus* whole cell suspension)

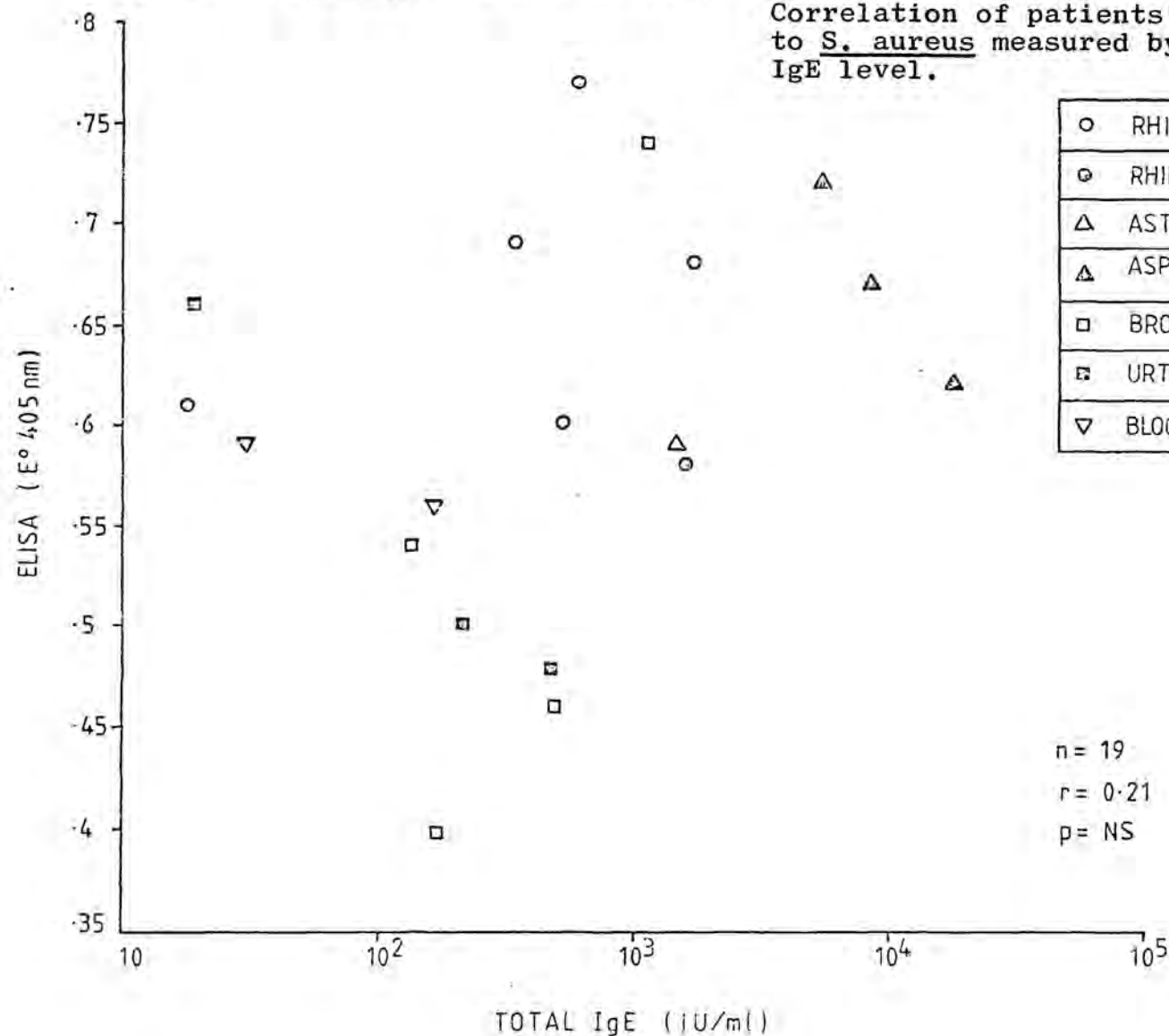


Figure 19.

Correlation of patients' specific IgE antibody to *S. aureus* measured by ELISA and their total IgE level.

Figure 20. Correlation of patients' specific IgE antibody to H. influenzae measured by ELISA and their total IgE level.

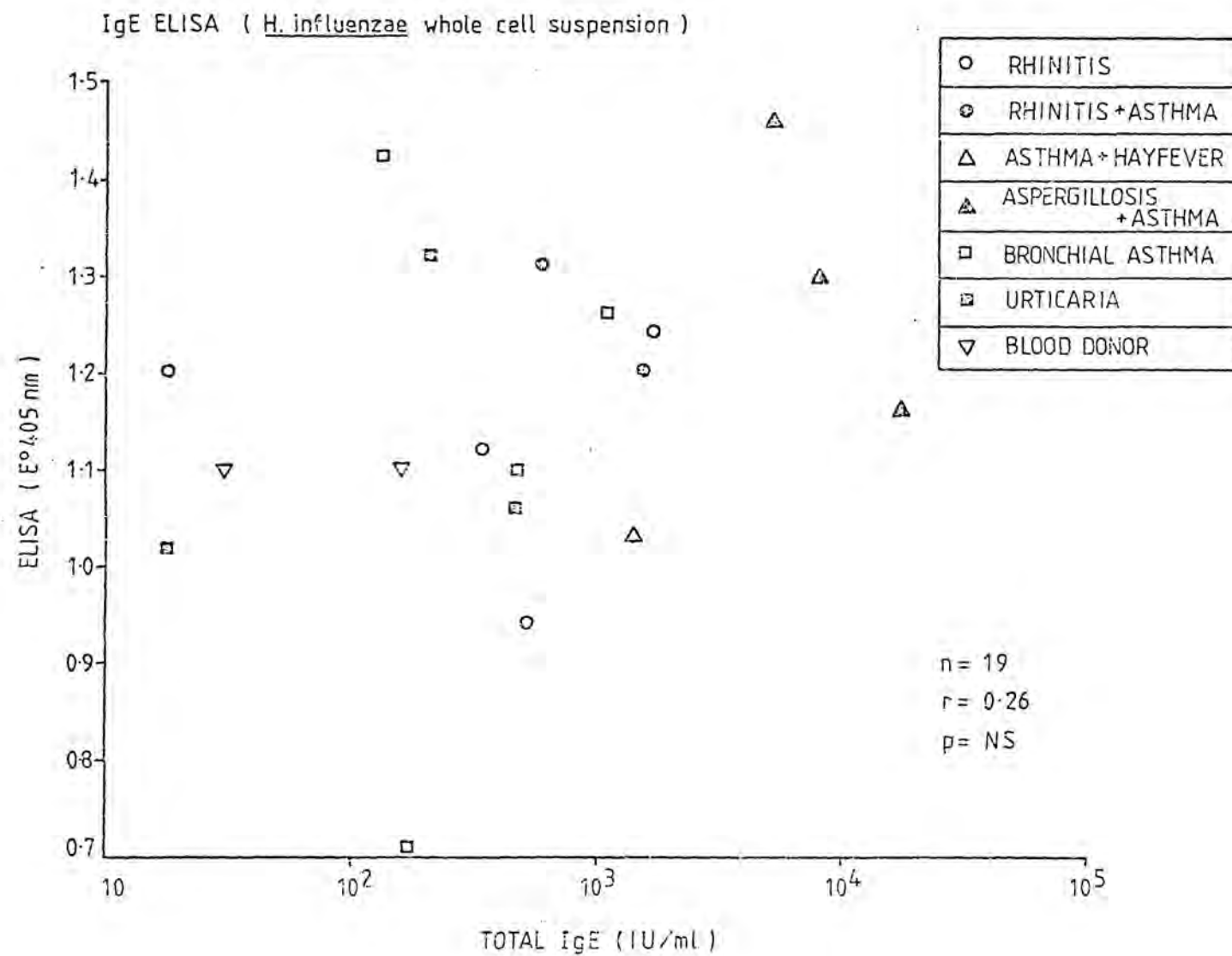
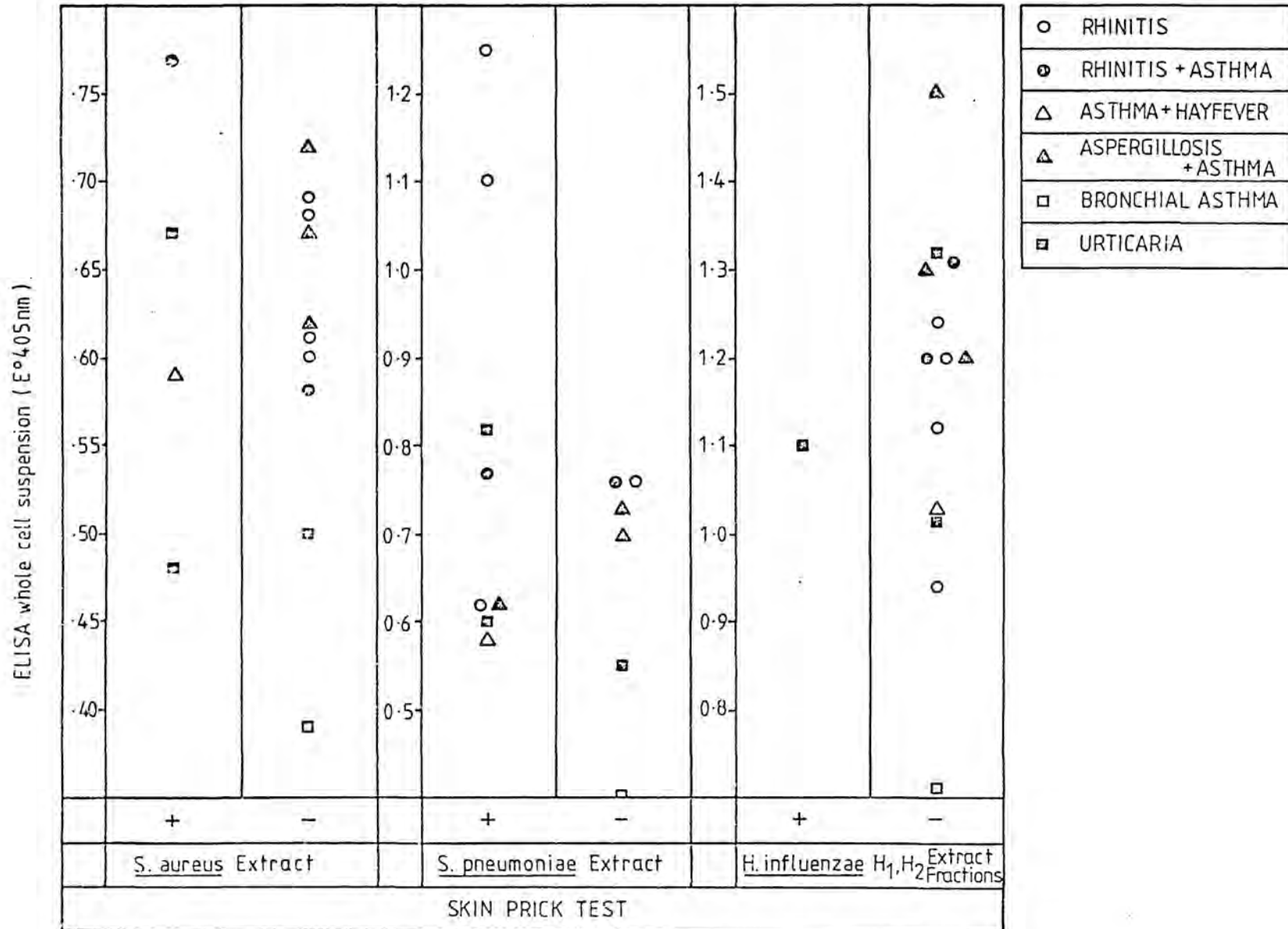


Figure 21. Correlation of patients' specific IgE antibody to S. aureus, S. pneumoniae and H. influenzae measured by ELISA with their specific skin prick test results.



reactivity of the patients is displayed against their specific bacterial ELISA scores, but no correlations could be found.

3.4.2. DISCUSSION

When linear regressions were estimated between the slide RAST and ELISA results of this preliminary study a negative correlation was found with S. pneumoniae but a correlation of $r = 0.41$ ($p < 0.05$) was found with both S. aureus and H. influenzae.

Although a skin prick test positive asthma patient with rhinitis (No. 3) gave the highest S. aureus ELISA reading of 0.77 (the patient also gave the highest S. aureus slide RAST score of 1.7) and two skin prick test positive rhinitis patients (Nos. 1 and 4) gave the highest S. pneumoniae ELISA readings of 1.25 and 1.10 respectively; looking at the preliminary study overall there was poor correlation of specific IgE measurement both by ELISA and the slide RAST with skin prick test results.

Thus although specific IgE antibodies to the three bacteria had been demonstrated by both techniques, they were of relatively low titre and, therefore, would require a more sensitive, less micro-method for measurement.

An added disadvantage to both the described techniques was the necessity to subject the bacterial antigens to 90°C for 1 hr in order to fix them to the solid phases. Since this study was to examine responses to whole bacterial cells

in the lung, such extreme treatment of the antigen was not desirable for the simulation of conditions in vivo.

4.0 SEPHAROSE RAST

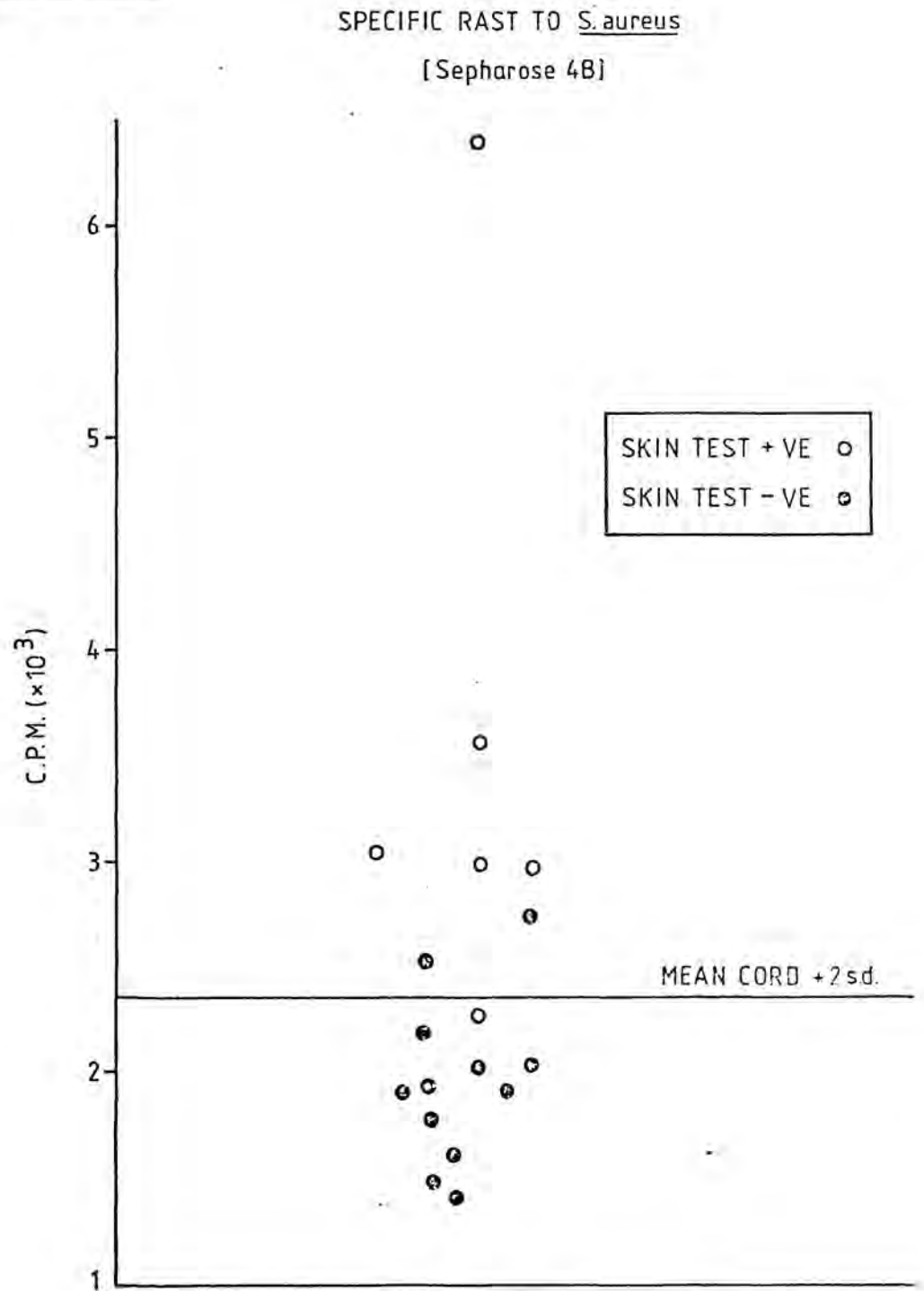
4.1. Preliminary studies

A successful radioimmunoassay was developed by insolubilizing the freeze dried whole bacterial cells by coupling them to cyanogen bromide activated Sepharose 4B. The latter enables proteins and ligands with primary amino groups to be immobilized.

The methods used to activate the Sepharose 4B, insolubilize the bacterial antigen and that of the assay itself are described in Chapter III, 9.1, 9.2 and 9.3 respectively.

Fig. 22 shows the distribution of the specific IgE RAST scores to S. aureus of 18 patients chosen at random from the Allergy Clinic. These patients were skin prick tested and six of them had a positive skin test to S. aureus extract. Five of the skin prick test positive patients had the highest RAST scores (maximum 6434 CPM) and the other skin prick test positive patient had a RAST score only 123 CPM below the line representing the mean of the RAST score of the cord blood sera plus two standard deviations of their scores (mean cord blood = 1950 CPM, S.D. = 195 CPM). Ten of the specific skin prick test negative patients had RAST scores below this line and the other two were 320 CPM and 86 CPM above the line. Thus this Sepharose RAST method appeared to give reasonable separation of RAST scores in relation to the patient's specific skin prick test positivity and further examination of the assay was made.

Figure 22. Sepharose RAST showing distribution of scores of specific IgE to S. aureus of 18 patients, 6 of whom were skin prick test positive to S. aureus.



4.2. Comparison of solid phases

A comparison was made of two different solid phases in order to see if a non-activated matrix could act as a carrier for the antigen. S. aureus coupled to activated Sepharose 4B was compared with S. aureus mixed with non-activated cellulose.

Ten patients, seven of whom had a positive skin prick test to S. aureus, and four cord blood sera were assayed against both the two solid phases in parallel with the same addition of radiolabelled anti-human IgE (185,497 CPM/tube). Results are shown in Fig. 23.

With the S. aureus/activated Sepharose 4B assay the skin prick test positive patients all had higher RAST scores than all but one of the skin test negative patients and of all the cord blood sera (mean of eight cord blood samples = 2024 CPM).

With the S. aureus/non-activated cellulose assay all RAST counts were below the mean cord of the S. aureus/activated Sepharose 4B assay thus indicating that immobilising the antigen on the activated matrix gives a satisfactory solid phase.

Another comparison of different solid phases was made to show that specific antibody was being measured and not non-specific protein. For this purpose human serum albumin was compared with S. aureus, both coupled to activated Sepharose 4B. Five patients' sera and two cord blood sera were tested against both the two solid phases in parallel. Fig. 24 shows the results of the assays and of the three patients with a

Figure 23. IgE/S. aureus specific RAST comparing sensitivity and discrimination of matrices using non-activated cellulose and activated Sepharose respectively, coupled to S. aureus as antigens.

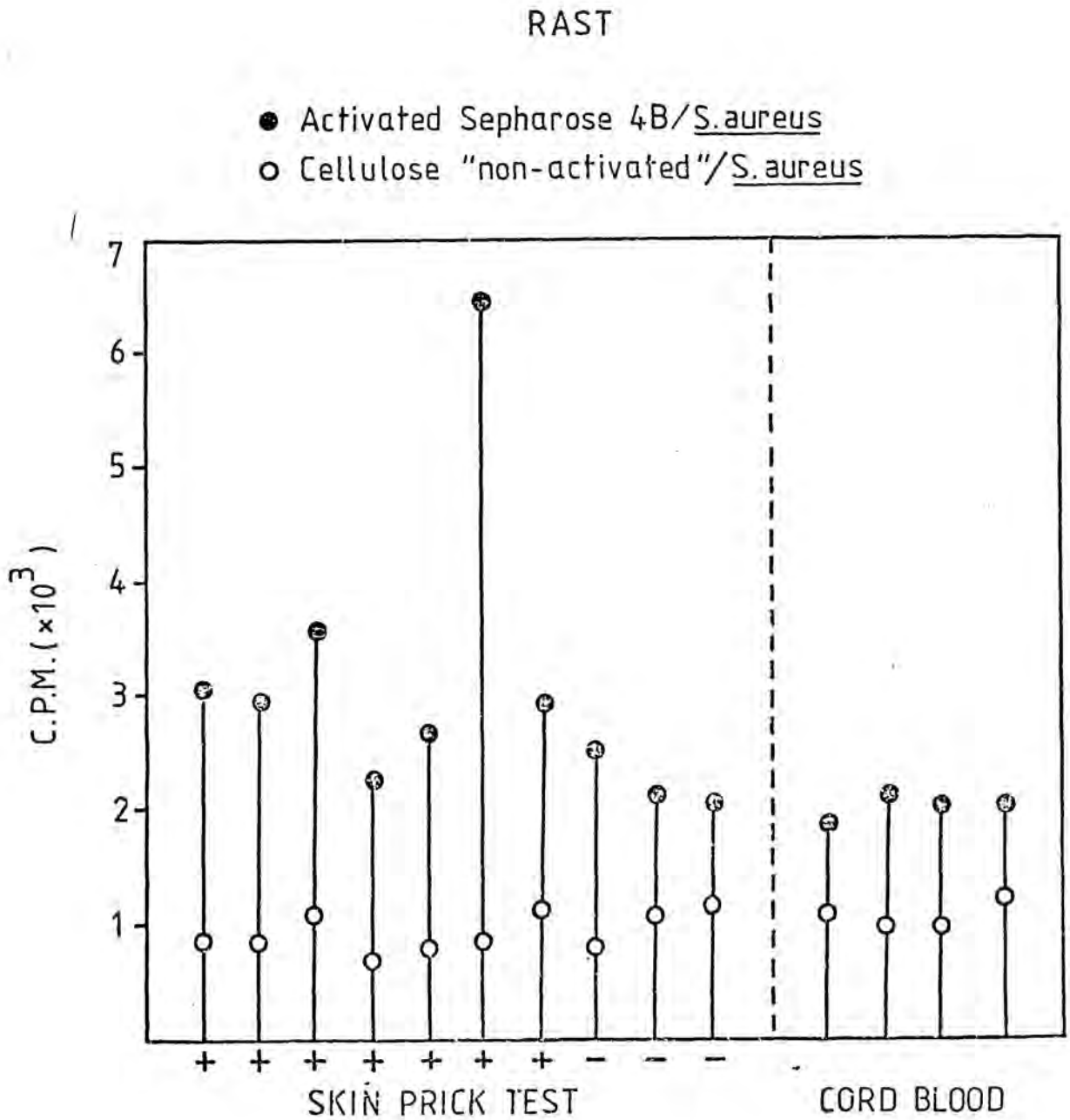
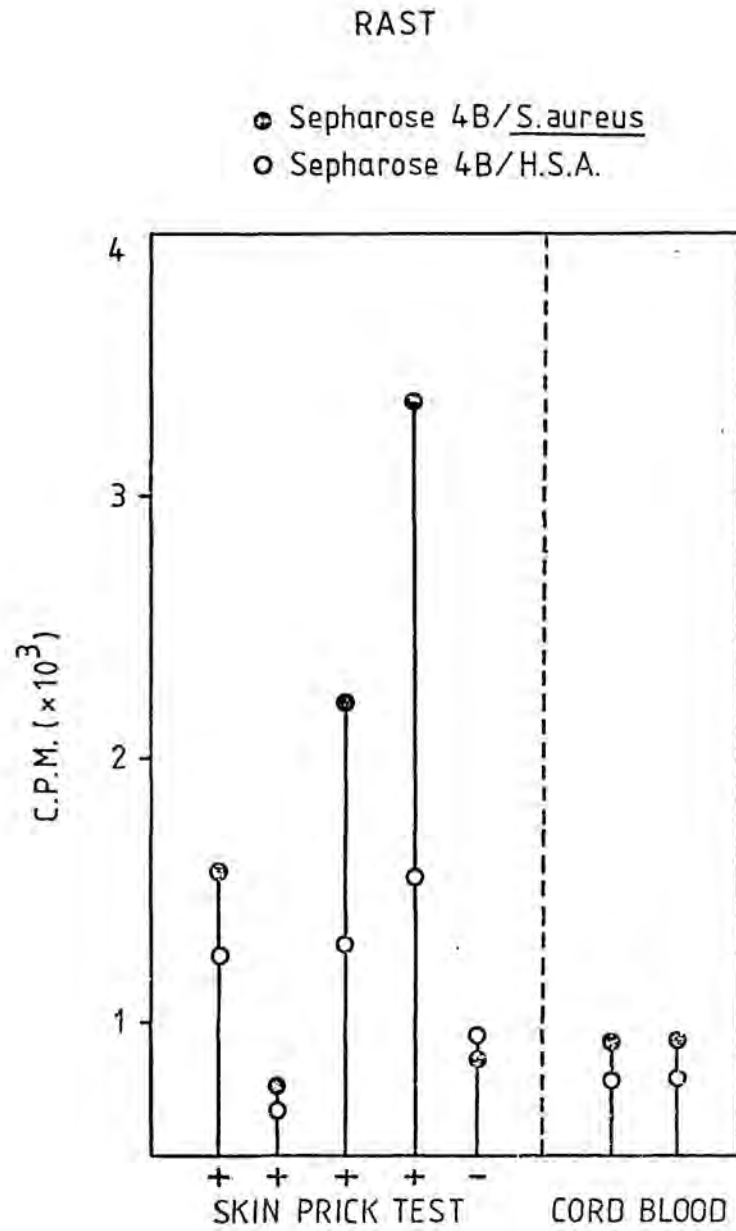


Figure 24. IgE Sepharose RAST demonstrating the specificity of Sepharose 4B/S. aureus coupled solid phase.



raised RAST score to S. aureus. Whilst there was appreciable binding of the isotope with the Sepharose/S. aureus phase there was insignificant binding of radiolabelled antiglobulin to Sepharose/HSA, thus demonstrating that specific antibody was being measured in the main assay.

4.3. Pilot study of sera from three disease groups

A pilot study estimating specific IgE antibodies to S. aureus in three disease groups was then undertaken. These were allergic bronchopulmonary aspergillosis (ABPA) (25 sera), bronchial asthma (35 sera) and cystic fibrosis (19 sera) and the results are displayed in Fig. 25. Raised RAST scores occurred in all three groups but mainly in the ABPA, where all counts were above the mean cord blood RAST (2054 CPM).

Similar radioimmunoassays were established with S. pneumoniae and H. influenzae.

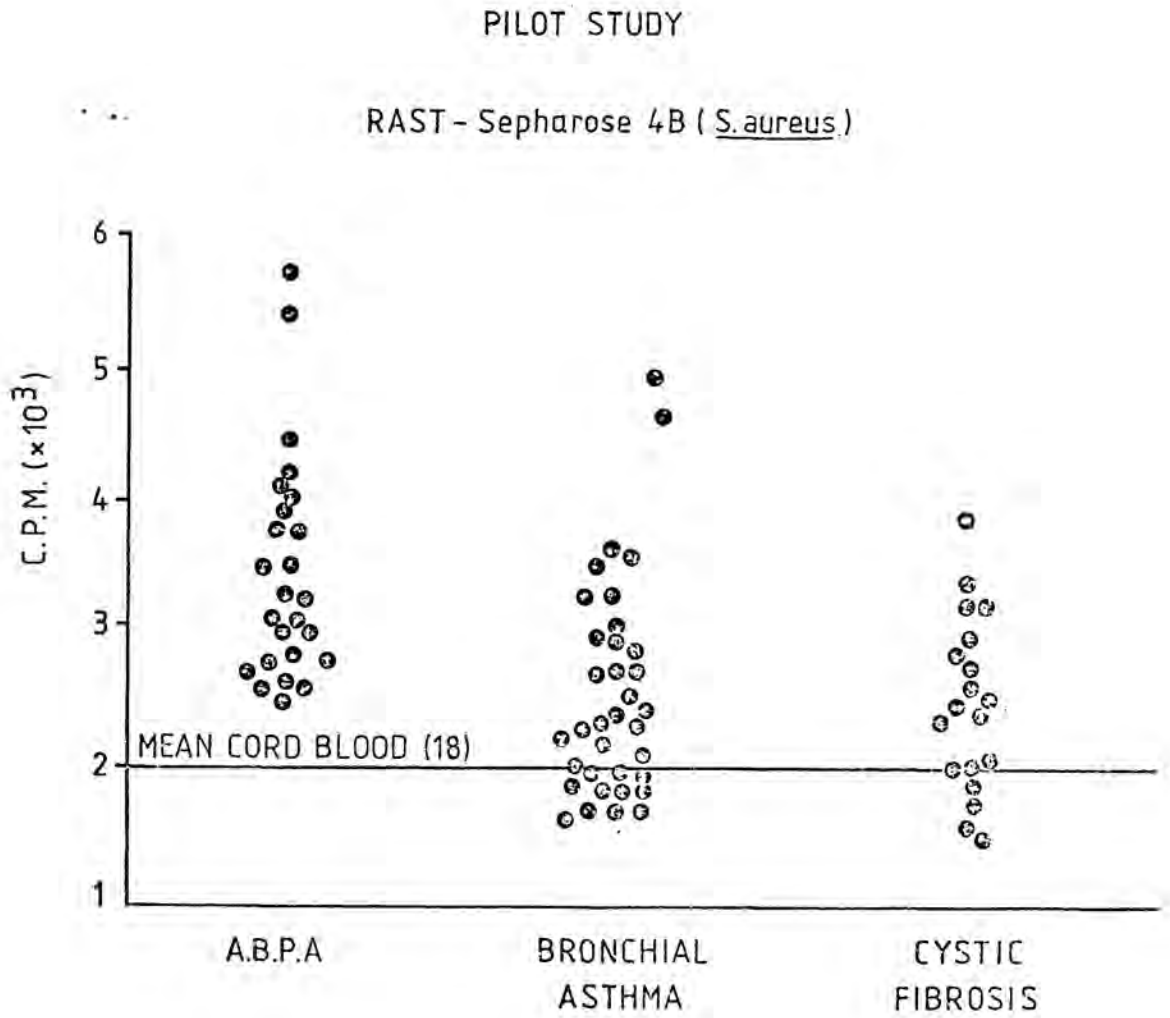
4.4. Titration of solid phases

Optimum conditions for the three bacterial assays were ascertained by titrating the solid phases and positive RAST sera. In Figs. 26-34 the symbols denote different sera and the open squares represent cord blood sera. The titration of solid phase of S. pneumoniae, S. aureus and H. influenzae

Figure 25.

Sepharose RAST

Specific IgE antibody to S. aureus
in 3 disease groups.



are depicted in Fig. 26, 27 and 28 respectively. Ten milligrams of freeze dried antigen was coupled to 1 gm wet weight of activated Sepharose 4B. An equal volume of phosphate buffered saline containing 0.3% human serum albumin was added to the coupled solid phases to give the 1:2 dilution and further doubling dilutions to 1:16 were made in the same diluent.

The 1:2 dilution would appear to be optimum for use but is too thick to dispense reproducibly. Therefore, for practical reasons, a 1:4 concentration of the solid phase was used in all assays. The figures show this is an acceptable level.

4.5. Titration of specific antibody in sera

Titration of specific antibody in positive RAST sera is shown in Fig. 29 to S. pneumoniae, in Fig. 30 to S. aureus and in Fig. 31 to H. influenzae. Doubling dilutions from neat to 1:16 of five sera with previously raised RAST scores and one cord blood serum were assayed for the three bacteria. In each case the cord blood serum did not titrate. In the RAST positive sera the specific antibody was titrated out by a 1:16 serum dilution; poor discrimination between positive sera and cord blood serum was obtained with a 1:4 serum dilution; whilst the clearest differentiation was achieved with neat sera (maximally 8210 CPM with S. pneumoniae, 10,420 CPM with S. aureus and 8709 CPM with H. influenzae) which was,

Figure 26.

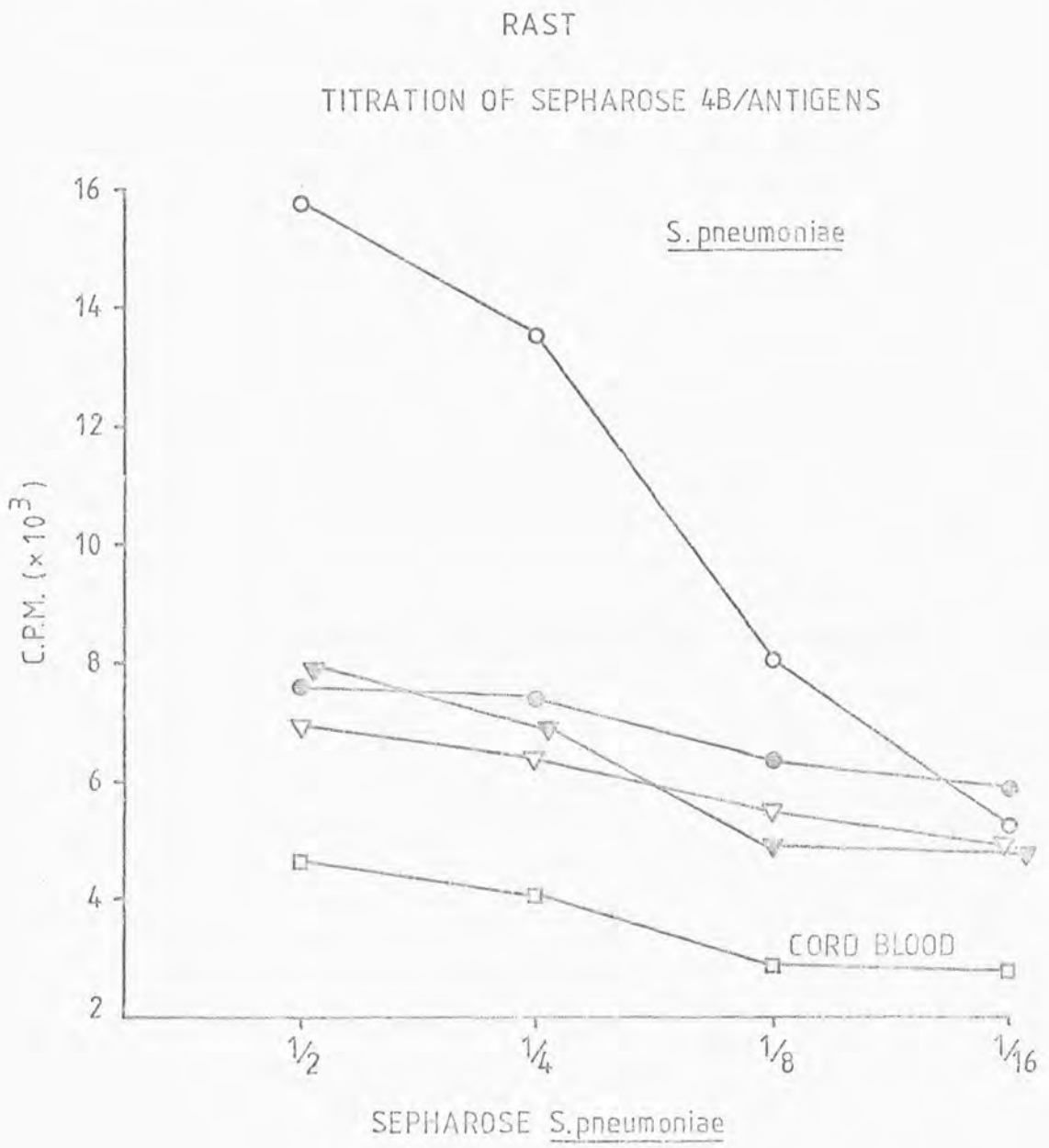


Figure 27.

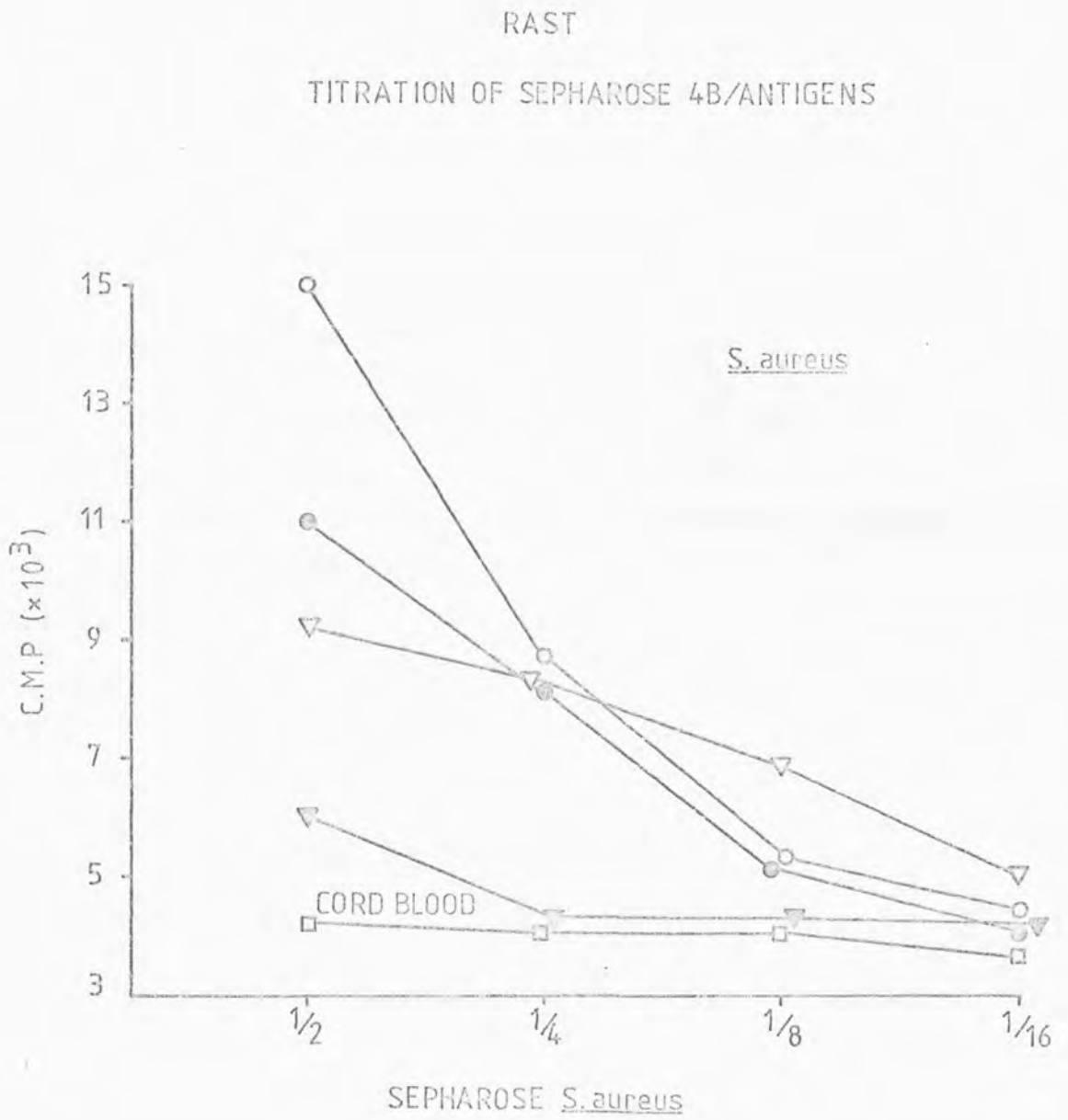


Figure 28.

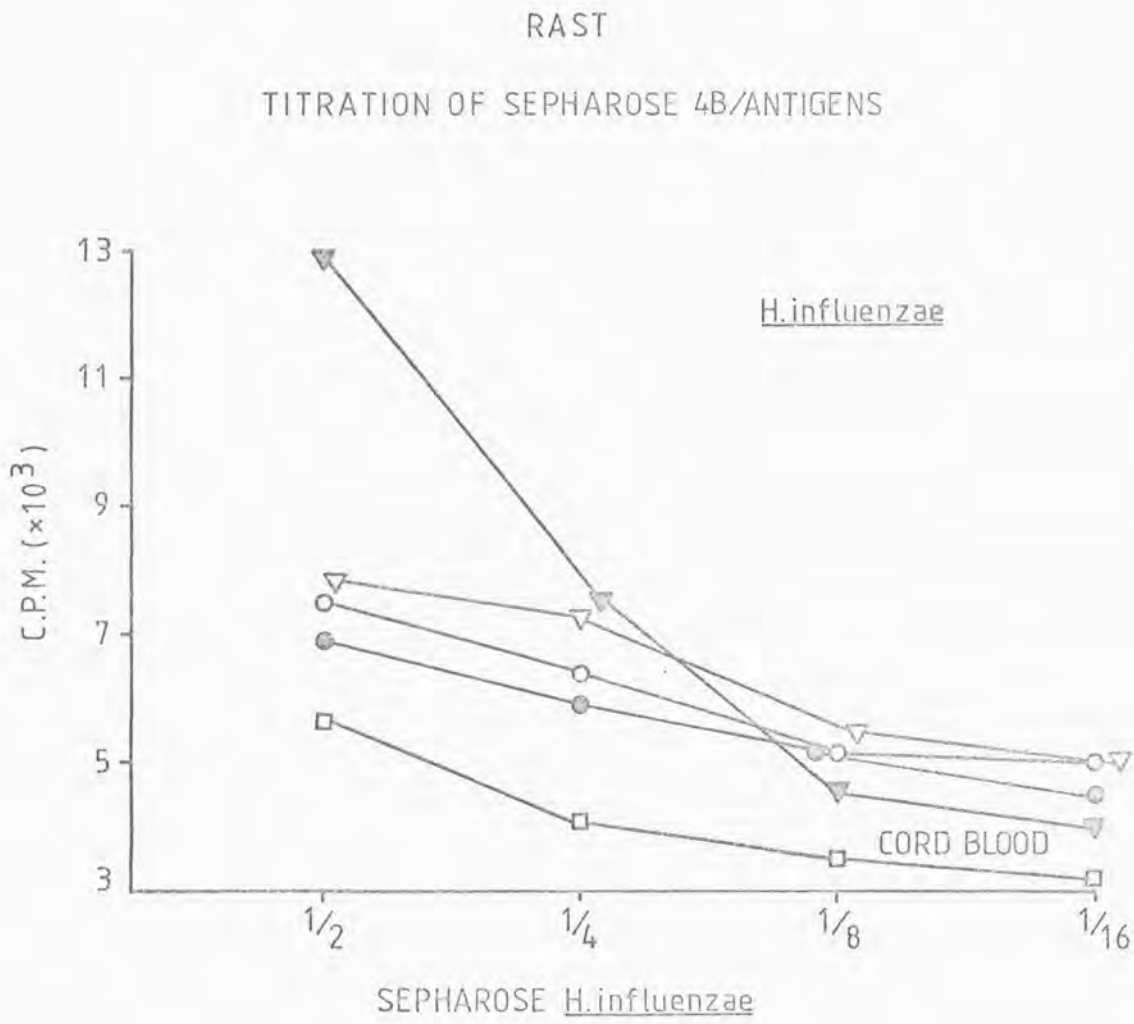


Figure 29.

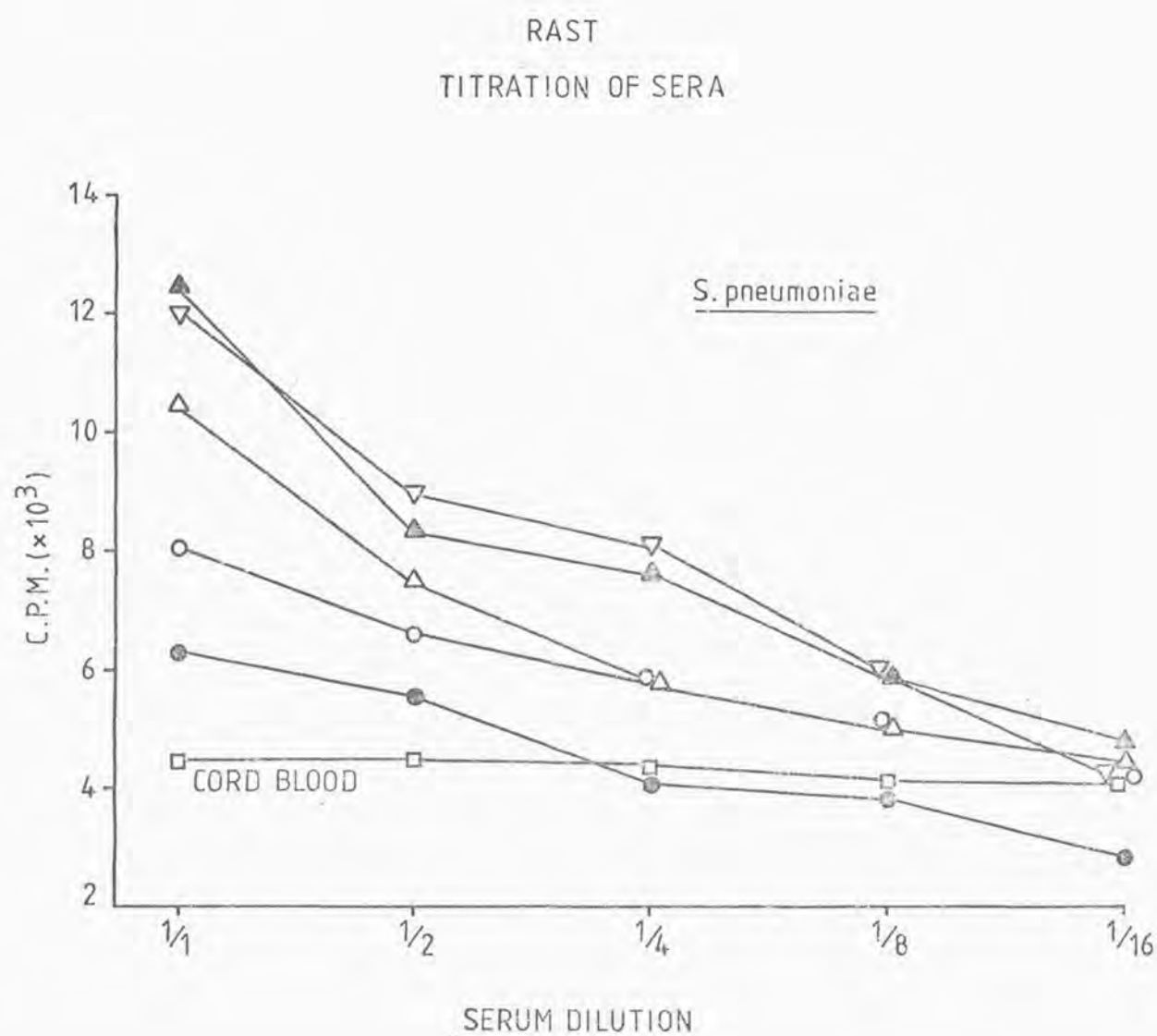


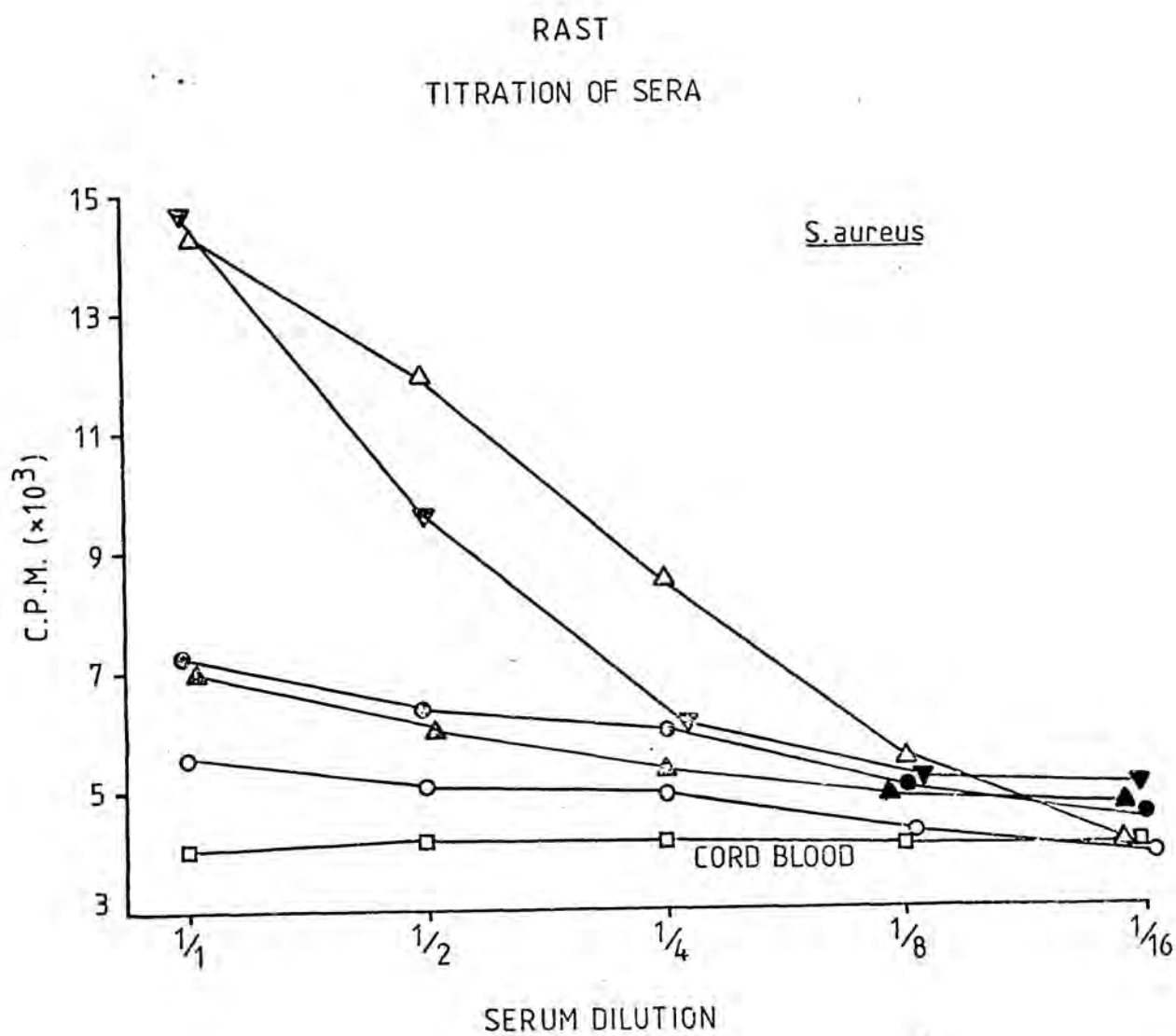
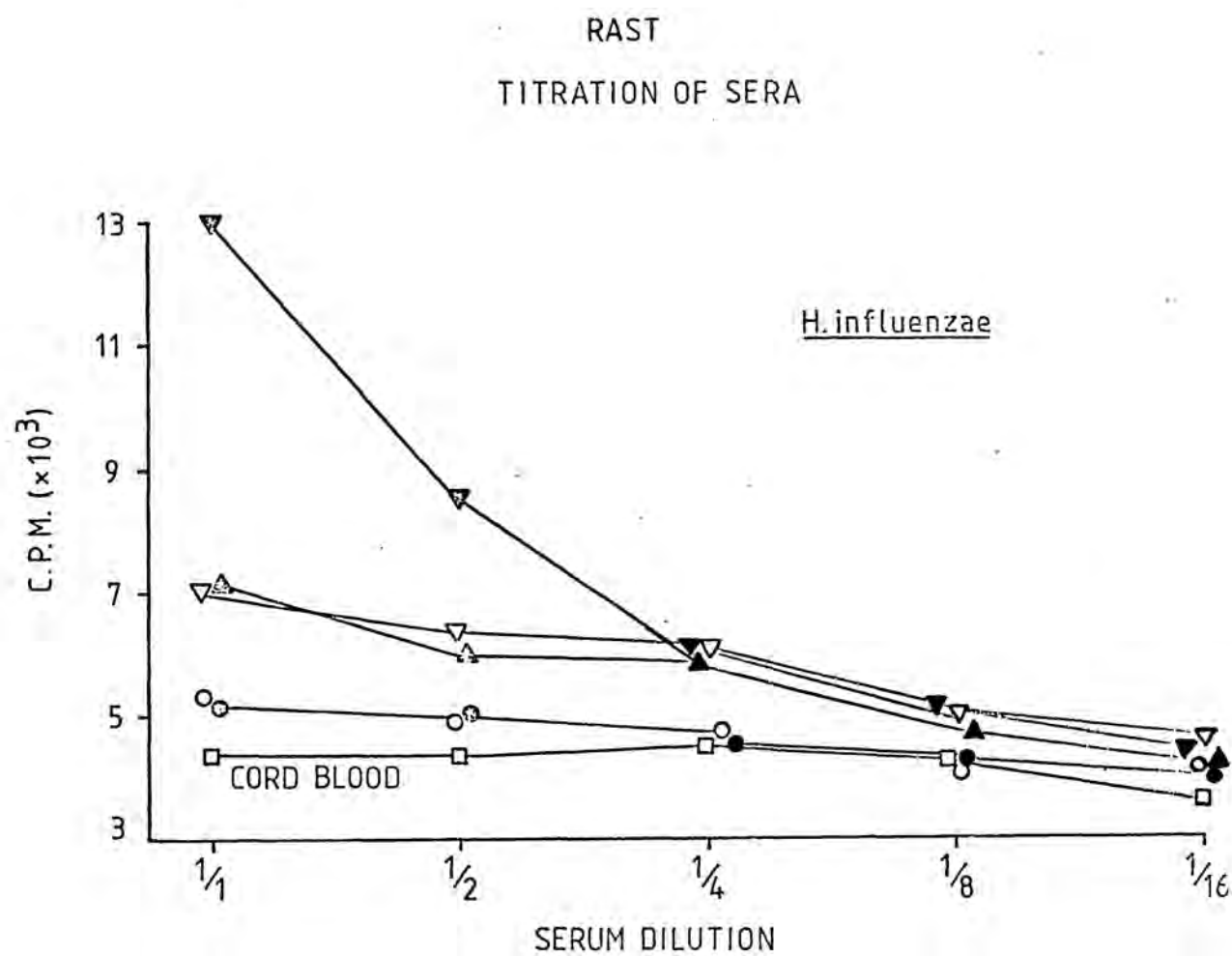
Figure 30.

Figure 31.

therefore, used in future assays.

4.6. INHIBITION STUDIES

The specificity of the assay was established by the inhibition of binding of specific ^{125}I -anti-IgE with freeze dried bacterial antigen which inhibition was demonstrable in a dose-dependent fashion with the three bacteria under study.

Two hundred and fifty microlitres of sera previously giving a positive RAST score to S. pneumoniae (5 sera), S. aureus (8 sera) or H. influenzae (5 sera) were absorbed with 1 mg, 3 mg, 10 mg and in some cases 20 mg of specific freeze dried antigen, and also with 10 mg of the other two freeze dried antigens to test for cross-reactivity between the bacteria. Cord blood sera were treated similarly. The absorptions were agitated in tubes at room temperature for 24 hr and after centrifugation (2000 rpm/10 min) the supernatants were assayed in duplicate.

Percentage inhibition figures were calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{Neat serum RAST} - \text{Absorbed serum RAST}) - (\text{Neat cord RAST} - \text{Absorbed cord RAST})}{\text{Neat serum RAST} - \text{Neat cord RAST}} \times 100$$

4.6.1. Results

The S. pneumoniae RAST inhibitions are depicted in

Figs. 32 and 35 which show dose response curves of RAST scores vs amount of absorbing antigen and percentage inhibition vs amount of absorbing antigen respectively. With all but one serum the specific antibody titre is reduced to cord blood value (100% inhibition) by absorption with 10 mg of freeze dried specific antigen.

The S. aureus RAST inhibitions are similarly shown in Figs. 33 and 35. Dose response curves were obtained with all the positive sera but not with the cord blood serum. However, the maximum inhibition obtained was only 68%.

The H. influenzae RAST inhibitions are shown in Fig. 34 and 35, and whilst dose response curves were again obtained, more antigen (20 mg) was required to reduce the H. influenzae RAST score to cord blood values and 100% inhibition was only achieved with one serum.

Figure 36 displays the percentage inhibition in histogram form of the other two bacterial antigens compared with that of the specific antigen. A certain degree of cross-reactivity is found to occur between the bacteria, for whilst the inhibition is always greatest with the specific antigen, a varying amount of RAST inhibition (0-57%) does occur with the other two bacteria, especially in the S. aureus and H. influenzae RAST systems.

4.6.2. Discussion

RAST inhibition studies provide a means of determining the specificity of the assay in terms of both the antibody class and the antigen. The assays described here rely on

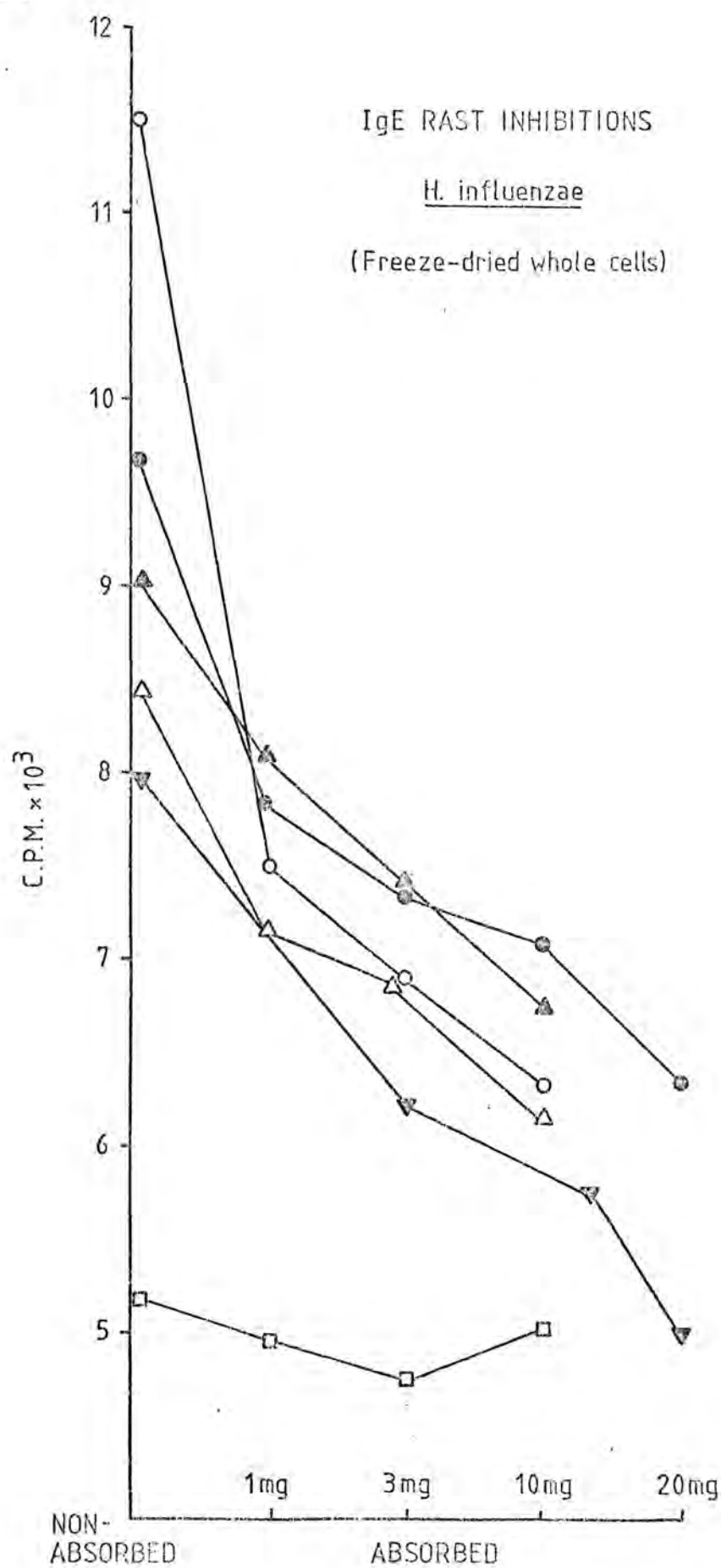
Figure 34.

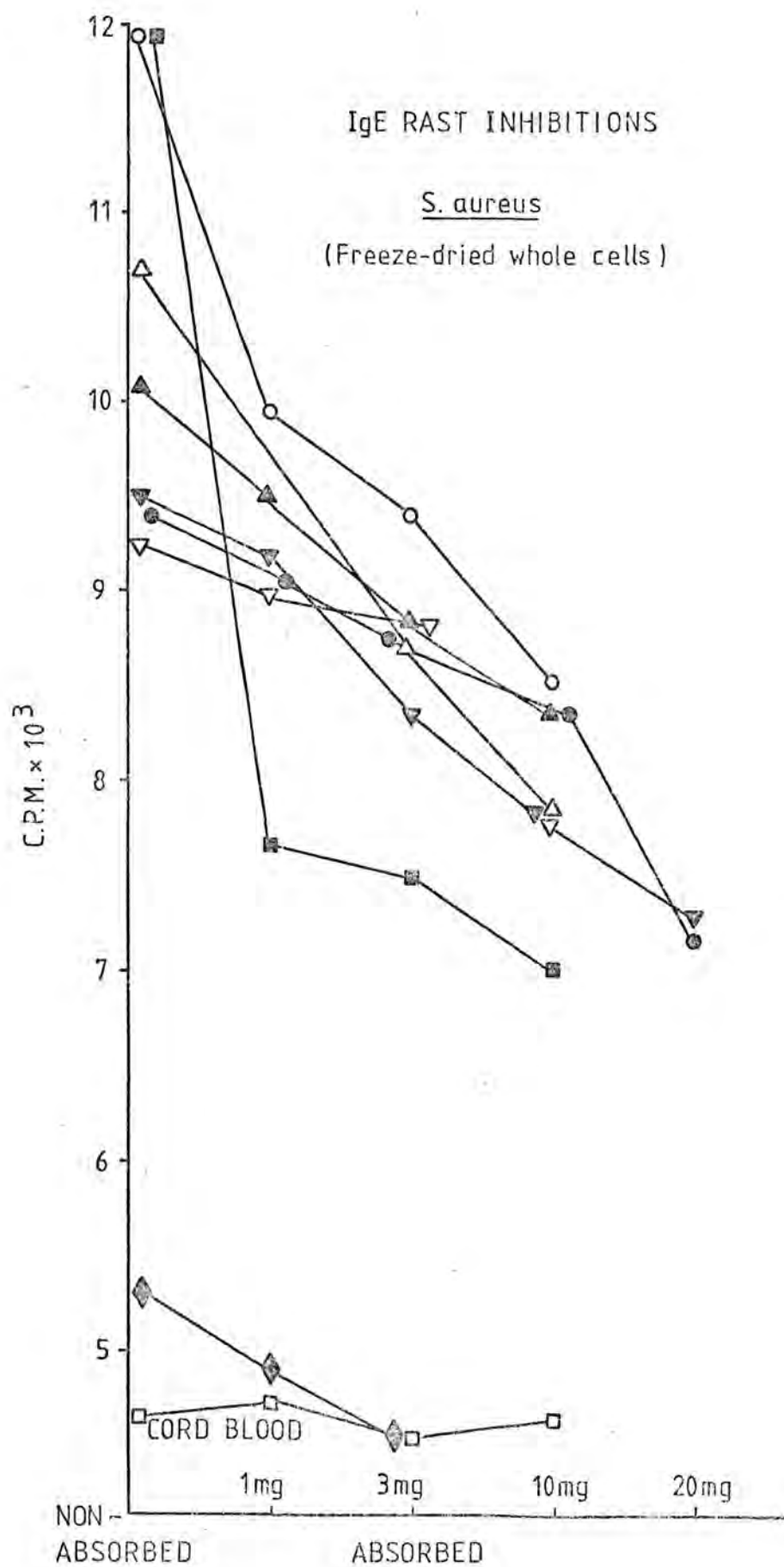
Figure 33.

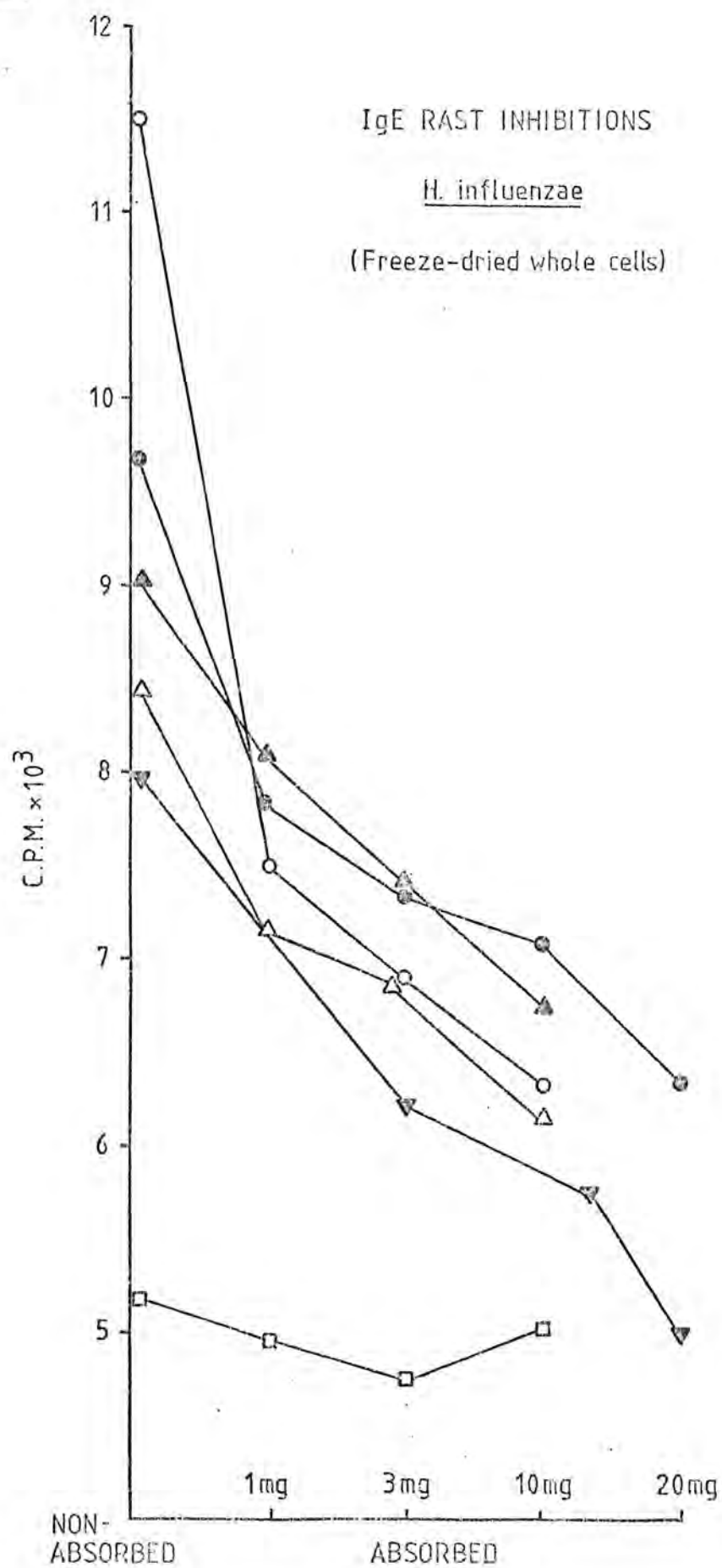
Figure 34.

Figure 35.

IgE Sepharose RAST Assays.

SPECIFIC ANTIGEN INHIBITION

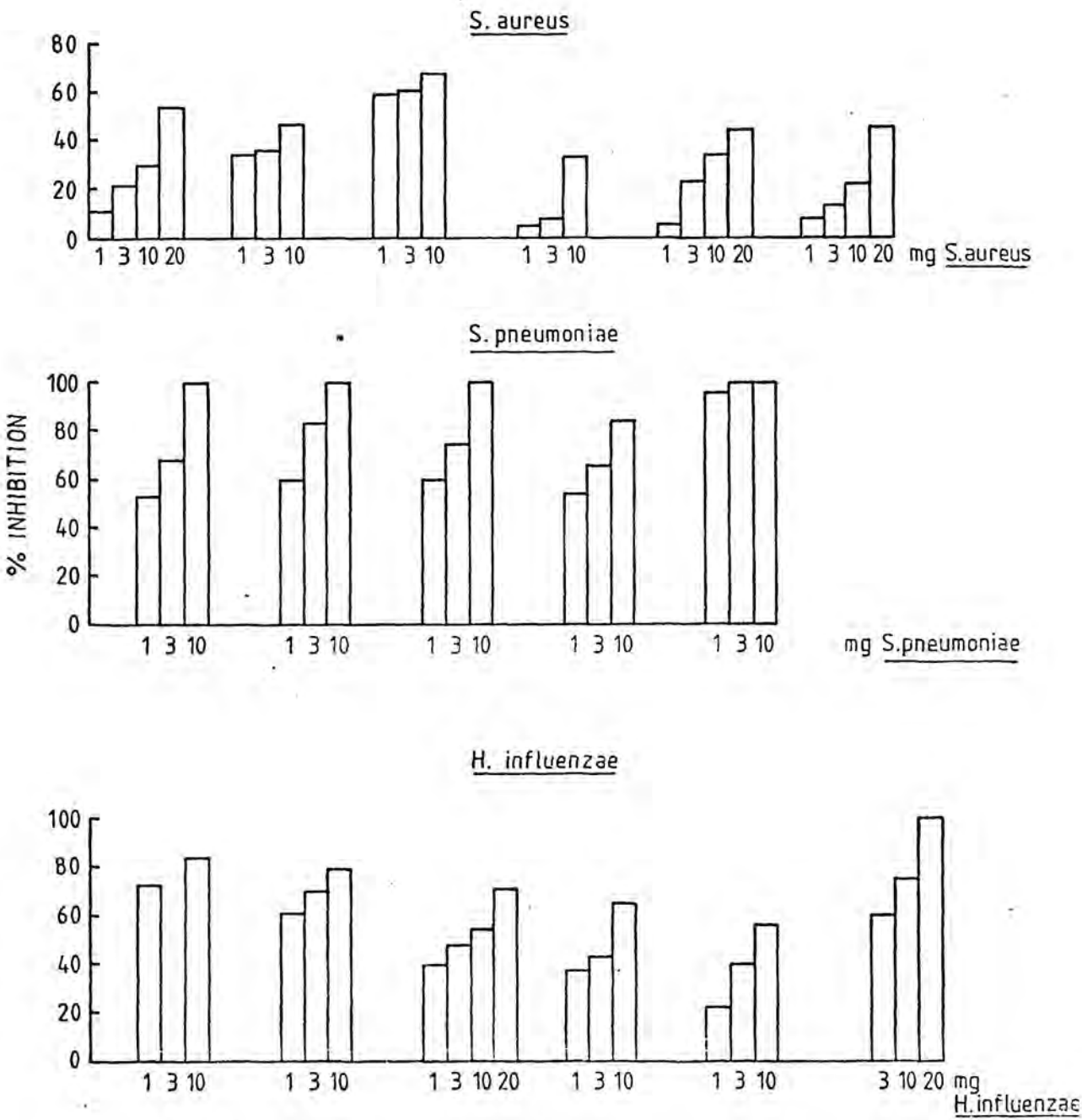
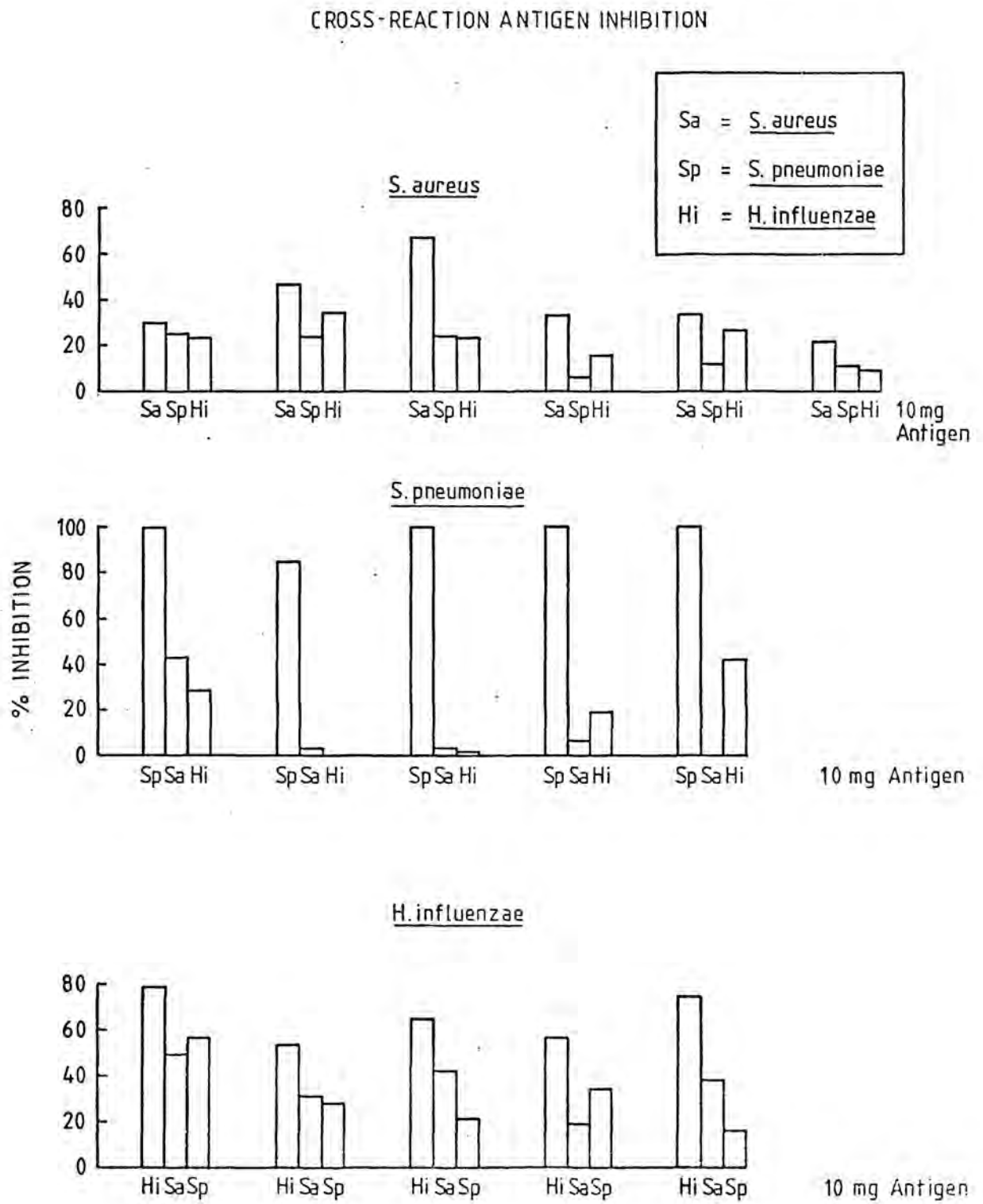


Figure 36.

IgE Sepharose RAST Assays.



indirect inhibition of binding of antigen specific antibody in that this is removed from the serum by adsorption to the immobilized antigen prior to the serum being used in a RAST assay. A direct inhibition would involve addition of free antigen to the assay system in order that it could compete with the immobilized preparation to bind specific antibody. This approach is not feasible in this case because the antigen is already in an insoluble form and its separation from material coupled to Sepharose is not practical as far as the assay is concerned.

The absorption of RAST positive sera with preparations of the three bacteria used in this study has resulted in reductions in the amount of antigen specific IgE antibody detectable by the RAST in terms of uptake of radioisotopically labelled anti-IgE. Dose response curves have been obtained using increasing amounts of the antigen. A high percentage inhibition with sera adsorbed with the smallest quantity of bacteria indicates a high concentration of the most relevant antigen in the preparation. If the inhibition is low this may point to either a low concentration of the most relevant antigen or high concentrations of specific antibody in other immunoglobulin classes. The latter would appear to be less of a problem with S. pneumoniae in that 100% inhibition was achieved with all the five sera tested. In the case of these five sera and those tested to S. aureus and H. influenzae IgE antibody inhibition increased in a dose-dependent fashion which either achieved 100% inhibition or extrapolated to it. In the cases of the two sera which gave the highest RAST scores

to S. aureus, inhibition of 32% and 60% were not greatly improved upon by absorbing the sera with larger quantities of the bacteria. One explanation would be that the RAST preparation contained antigens which were not present in the absorbing material. This is most unlikely since the absorbing material came from the same preparation as that coupled to the Sepharose. A more likely explanation would seem to be that there is some degree of immunoglobulin class specificity in the immune response. The initial reduction on RAST score may be due to prior absorption by antigen of antibody which has been produced exclusively in the IgE class. Remaining RAST activity may be due to specific IgE which is present together with antibody of the same specificity from other immunoglobulin classes.

The cross-reaction inhibition studies indicate that there may be shared antigenic determinants between the three organisms. S. pneumoniae would seem to be the only one of the three which shows significant specificity. Those antigenic determinants that it shares with the other two micro-organisms are presumably less important in the immune response to S. pneumoniae than they are in that of S. aureus and H. influenzae.

This concludes the developmental work of the assay.

The Sepharose RAST was then used to detect anti-bacterial IgE in the serum of patients from six disease groups.

CHAPTER V

STUDY OF IgE ANTIBODIES IN
VARIOUS ALLERGIC LUNG DISEASES.

1.0 Introduction

Twenty to 30 patients in each of six disease groups were skin prick tested with extracts of S. pneumoniae, S. aureus and H. influenzae and were also investigated serologically. The disease groups were chronic bronchitis, allergic bronchopulmonary aspergillosis (ABPA), bronchial asthma, allergic rhinitis, urticaria and cystic fibrosis.

The patients' specific IgE antibodies to the three bacteria were measured by the Sepharose RAST and the values compared with those from blood donors with normal total IgE levels and cord blood sera containing no demonstrable total IgE. The results are expressed as a ratio of the patient's RAST to cord blood RAST, viz.

$$\text{RAST SCORE RATIO} = \frac{\text{Patient's specific RAST score}}{\text{Cord blood specific RAST score}}$$

Total serum IgE estimations were also made on the sera and was determined in an assay employing anti-human IgE immobilized by coupling to cyanogen bromide activated Sepharose 4B (as described in Chapter III, 10.0).

The detailed data, i.e. skin prick test results, bacterial IgE RAST score ratios and total IgE levels for each patient are found in Appendix 2.

Statistical analysis of the results obtained in this study were performed and the findings discussed.

2.0 ORIGIN OF PATIENTS

Patients with a clinical diagnosis of chronic bronchitis were attending the "chronic bronchitis clinic" at Wandsworth Chest Clinic.

Patients with allergic bronchopulmonary aspergillosis (ABPA), bronchial asthma, allergic rhinitis and urticaria were attending the Allergy Clinic, Brompton Hospital.

Patients with cystic fibrosis (CF) were attending Dr. Margaret Hodson's Cystic Fibrosis Clinic at the Brompton Hospital. Due to limitation on the amounts of sera available from these patients, a second group of CF patients from the Institute of Child Health, London were studied. These patients were not skin prick tested and their sera were used for the specific IgG and IgG4 RAST estimations only.

The control subjects, matched where possible for age and sex, were blood donors who had not been skin prick tested but whose serum total IgE levels were less than 200 I.U./ml. These samples were kindly supplied by the South London Blood Transfusion Centre, London, S.W.17 for serological investigation.

2.1. DEFINITION OF THE DISEASE GROUPS

2.1.1. Chronic bronchitis

Patients were included in the chronic bronchitis group on fulfilment of criteria laid down in a report by the Medical Research Council (1965). These features are cough and sputum occurring on most days for at least three months

in the year for at least two successive years. This chronic or recurrent excess mucus secretion in the bronchial tree should not be brought about by other diseases, such as pulmonary tuberculosis, bronchiectasis or bronchial carcinoma. Two other manifestations of chronic bronchitis which frequently occur are bacterial infection, which may result in mucopurulent sputum and generalised airways obstruction.

2.1.2. Allergic Bronchopulmonary Aspergillosis (ABPA)

In the U.K. allergic bronchopulmonary aspergillosis (ABPA) is a common complication of asthma.

Mycelial growth of Aspergillus fumigatus in the lung acts as a source of antigen in atopic subjects. The Type I allergy becomes complicated by Type III allergy, with dual responses on skin or inhalation testing. Precipitins are found in the patients' sera in most cases.

The clinical features of ABPA are feverish bouts with asthma; transient lung shadows; bronchial damage (resulting in characteristic cylindrical bronchiectasis with normal peripheral filling); expectoration of plugs in which Aspergillus mycelium may be demonstrated and pulmonary and blood eosinophilia.

2.1.3. Bronchial Asthma

Bronchial asthma may be defined as a condition

characterised by widespread partial narrowing of the bronchial airways giving rise to dyspnoea of varying severity. This airway narrowing is reversible with an increase of 30% or more of the initial F.E.V.₁, either spontaneously or as a result of treatment (Ciba Foundation Guest Symposium, 1971).

There is no generally accepted classification of asthma. Patients with bronchial asthma, however, can be divided into two types: those in whom the provoking allergen can be identified (extrinsic asthma) and those in whom allergic factors cannot be recognised (cryptogenic or intrinsic asthma). Extrinsic asthma may be caused by inhalation and sensitization of the respiratory tract by wind-carried pollen, dusts, moulds and animal epithelia. Foods and drugs are other potential allergens.

2.1.4. Allergic Rhinitis

Allergic rhinitis can be either seasonal or perennial. Symptoms of seasonal allergic rhinitis are rhinorrhea, varying degrees of congestion and nasal itching. The symptoms occur at specific times of the year, coinciding with the presence of pollens or mould spores in the environment. This diagnosis is confirmed by positive skin tests or other methods for demonstrating IgE-specific antibody. Eosinophils are found in the nasal smears of about 70% of patients.

Some patients with a non-seasonal and chronic pattern of these symptoms are thought to have a perennial form of the disease. The antigens commonly associated with perennial symptoms are the house dust mite (Dermatophagoides pteronyssinus),

feather pillows and animal dander, but may be almost any airborne particle in the environment capable of sensitizing an individual. The diagnosis of perennial allergic rhinitis is much more tenuous than that of seasonal rhinitis for many patients have positive skin tests (Hagy and Settupane, 1976) or RAST but do not have overt clinical symptoms.

2.1.5. Urticaria

Urticaria is a common disorder characterized by recurrent subcutaneous swelling and affects approximately 15-20% of the population at least once during their lifetime (Sheldon et al. 1954; McKee, 1966). Urticarial lesions result from localized dilation and transudation of fluid from small venules, and in their simplest form are thought to represent a reaction similar to the wheal and flare observed when histamine is injected into the skin.

The cause of urticaria is often difficult to identify. It may be caused by foods (e.g. strawberries and shellfish), drugs (e.g. penicillin, aspirin), environmental factors (e.g. change in temperature, sun) or by a C1 esterase deficiency.

2.1.6. Cystic Fibrosis

Cystic fibrosis, first described by Dorothy Anderson in 1938, is an hereditary disease which affects mainly children and young adults. The most important clinical features are pulmonary disease, pancreatic insufficiency and

abnormal sweat electrolytes. It was noted that patients with cystic fibrosis have an increased sodium and chloride content in their sweat (di Sant'Agnese et al. 1953) and this is now a useful quantitative diagnostic test in children. The disease is characterized by the presence in the patient's serum of a protein factor (cystic fibrosis factor) which when added to a preparation of ciliated rabbit tracheal mucosa, results in a disorganized ciliary beat (Spock et al. 1967).

Pulmonary symptoms usually start during the first year of life and death in most patients is due to lung complications. The common pathogens are Staphylococcus aureus and Haemophilus influenzae and later Pseudomonas aeruginosa becomes more important (May, Herrick and Thompson, 1972). Many patients have raised serum levels of IgG, IgM and IgA, which is thought to be due to repeated and intense antigenic stimuli from numerous bacterial infections (Hodson, M., M.D. Thesis, 1977).

3.0 SKIN PRICK TESTING

The patients in all but the chronic bronchitis disease group were skin prick tested with 23 common inhalant and food allergens (Bencard, Brentford, U.K)*, together with a negative control solution (Coca's:glycerol). Since the chronic bronchitis group were skin prick tested especially for this study, only a reduced number of allergens (Bencard) were used, viz. Group B3 tree pollens, Group B2 grass pollens, D. pteronyssinus, cat fur, Aspergillus fumigatus. Hendrick et al. (1975) showed that when screening solely for the presence of the atopic state only three allergens (D. pteronyssinus, grass pollen and Aspergillus fumigatus) needed to be used.

The presence of one or more positive skin prick tests placed the subject in the atopic category, whilst those with wholly negative tests were recorded as being non-atopic.

* 1. Moulds

Alternaria, Aspergillus fumigatus, Aspergillus terreus, Cladosporium herbarium, Merulius lacrymans, Sporobolomyces roseus, yeast, Candida albicans.

2. Mixed Pollens

Grasses (B2), shrubs (B1), trees (B3).

3. Foods

Milk, egg, wheat, fish, nuts.

4. Animals

Cat, dog, feathers, horse.

5. House dust and mites

House dust, Dermatophagoides farinae or Dermatophagoides pteronyssinus.

3.1. Bacterial allergens used in skin prick testing

The bacterial allergens used for skin prick testing in this study ranged from whole cell suspensions of S. pneumoniae, S. aureus and H. influenzae to the species specific allergenic extracts from these bacteria and are detailed in Table V.

Initial tests with Bencard's (Brentford, U.K.) whole cell suspensions indicated that they were too dilute. 112 patients attending the Brompton Allergy Clinic were skin prick tested with Bencard's (Brentford, U.K.) S. aureus (NCTC No. 6571), S. pneumoniae (NCTC No. 7465) and H. influenzae (NCTC No. 7279) whole cell suspensions containing 2.5×10^9 organisms per ml; the bacteria being previously heat killed at 60°C for 1 hr. Since there was only one positive immediate type reaction (to H. influenzae) amongst these patients, testing with these commercial suspensions was discontinued.

Until whole cell suspensions of greater antigen concentration were available, bacterial extracts prepared at the Cardiothoracic Institute (Chapter III, 4.2) were used and all the patients in the study were skin prick tested with them, viz. S. aureus extract 100 mg/ml; S. pneumoniae extract 100 mg/ml and H_1H_2 extracts of H. influenzae 10 mg/ml.

Subsequently arrangements were made with the Microbiological Research Establishment at Porton, Wiltshire, to supply freeze dried whole cells of the following strains: S. aureus (NCTC 6571); H. influenzae (NCTC 4842), S. pneumoniae (NCTC 10319). The whole cell cultures were heat killed at

DETAILS OF MATERIALS USED FOR SKIN PRICK TESTING

SKIN PRICK TEST MATERIALS	TEST CONCENTRATION
<u>S. aureus</u> (NCTC 6571)	
Bencard whole organism suspension	2.5×10^9 organisms
Bencard whole organism suspension	1×10^{10} organisms
Allergen extract	100 mg/ml
Brompton) Porton) whole cell suspension	100 mg/ml
<u>S. pneumoniae</u>	
Bencard whole organism suspension (NCTC 7465)	2.5×10^9 organisms
Allergen extract (NCTC 10319)	100 mg/ml
Brompton) Porton) whole cell suspension (NCTC 10319)	100 mg/ml
<u>H. influenzae</u>	
Bencard whole organism suspension (NCTC 7279)	2.5×10^9 organisms
H ₁ H ₂ allergenic groups (Brompton 909)	10 mg/ml
Brompton) Porton) whole cell suspension (NCTC 4842)	100 mg/ml
<u>CONTROL</u>	
50% Coca's solution:	
50% Glycerol	

TABLE V

60°C for 1½ hr, washed twice with phosphate buffered saline and freeze dried.

These antigens were used for all the serological tests performed on the patients in the main study and in the repeated skin prick testing in the ABPA patient group at 100 mg/ml. Additionally, this latter group were skin prick tested with a S. aureus whole cell suspension of 2.5×10^9 organisms supplied by Bencard's.

4.0 RESULTS

4.1. SPECIFIC BACTERIAL IgE RAST ASSAYS

The IgE RAST score ratios to the three bacteria studied are plotted in Figs. 37-39. Results are shown for each of the six disease groups and the blood bank controls. The horizontal bars represent the arithmetic means for each group and χ^2 tests were applied to test for any statistically significant differences in RAST scores between the disease groups.

S. aureus IgE RAST scores (Fig. 37)

The blood bank control group had a mean RAST score ratio of 1.0 and a range from 0.81 to 1.22, with one higher score of 1.49. The chronic bronchitis group scores (mean 0.8) had little spread and were significantly lower ($p < 0.001$) than the other disease groups. In the ABPA (mean 1.4), asthma (mean 1.2), rhinitis (mean 1.0), urticaria (mean 1.1), and cystic fibrosis groups (mean 1.2) there was a greater range of RAST scores, some patients having demonstrable specific antibody to S. aureus. The patients' RAST scores in the ABPA group (mean 1.4) were significantly higher than those in all other groups ($p < 0.01$). On application of a median test the ABPA was greater than all the other disease groups ($p < 0.001$). There were no significant differences between the asthma, rhinitis, urticaria and cystic fibrosis groups.

S. pneumoniae IgE RAST scores (Fig. 38)

The RAST score ratios of the ABPA group (mean 1.7) were again higher than all other groups (χ^2 test $p < 0.01$) and higher than all the disease groups (median test $p < 0.001$). RAST score

Figure 37

IgE RAST SCORES TO WHOLE CELLS OF Staphylococcus aureus

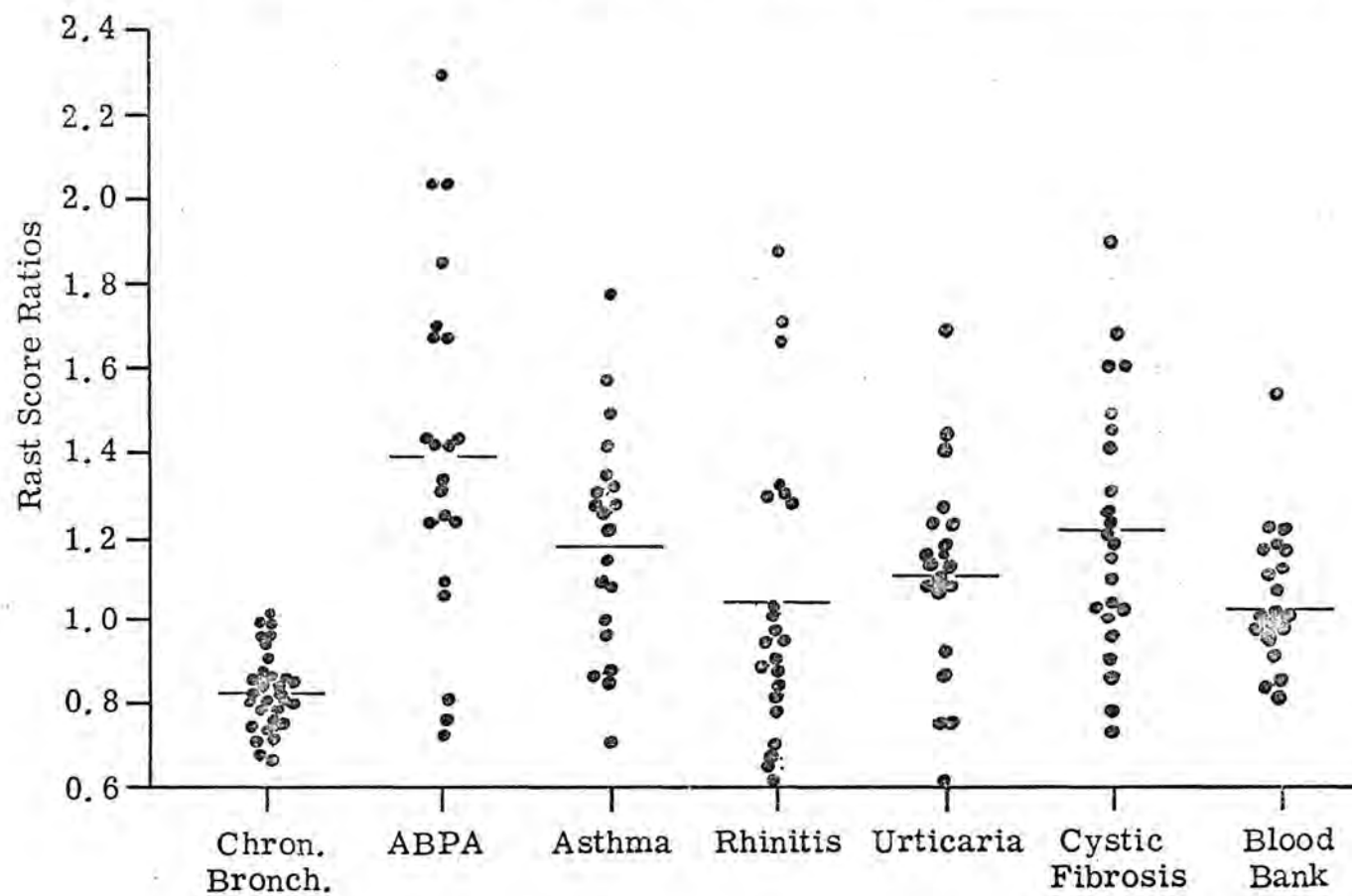
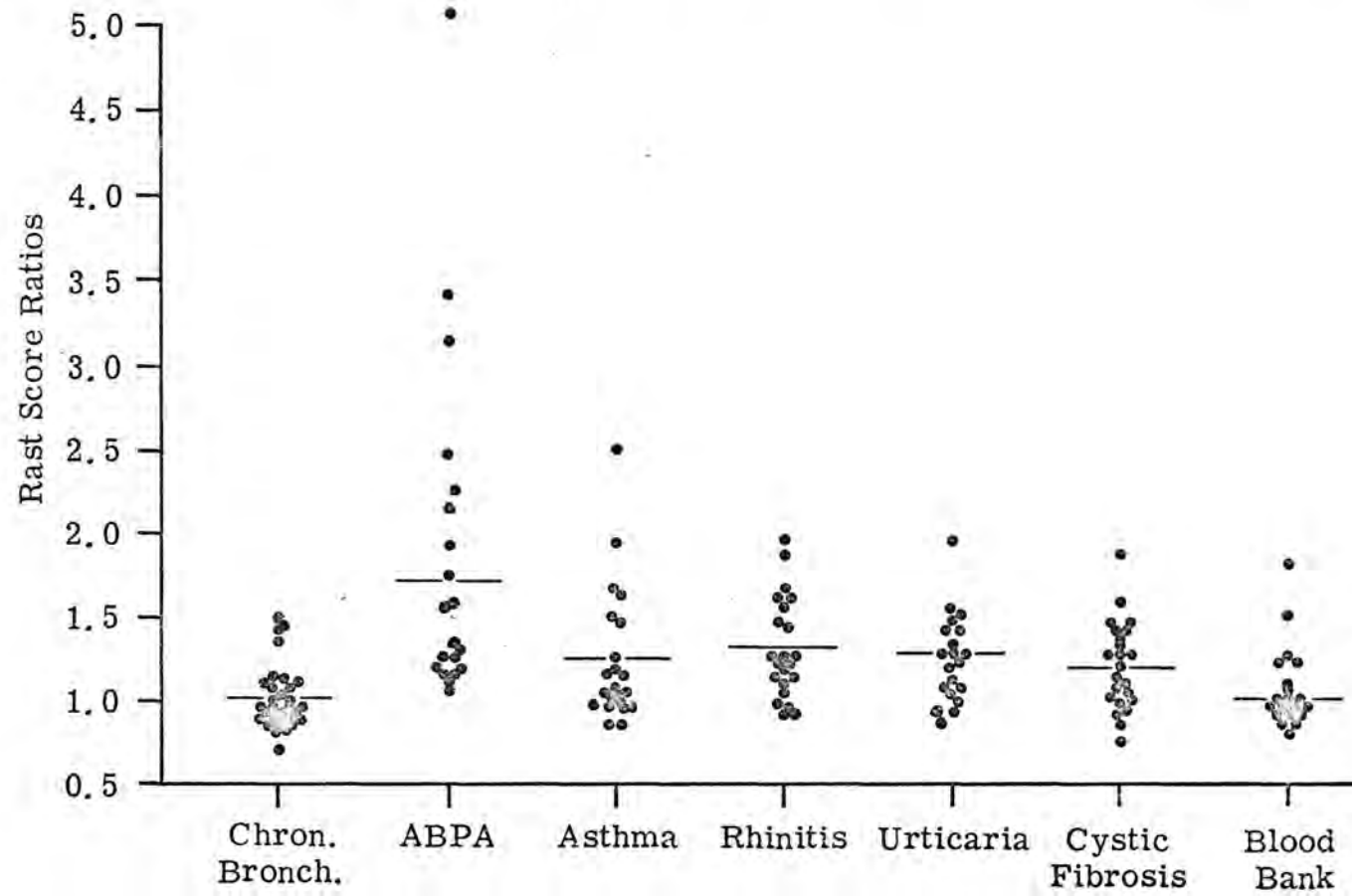


Figure 38

IgE RAST SCORES TO WHOLE CELLS OF Streptococcus pneumoniae



ratios were higher generally with S. pneumoniae than with S. aureus with ratios >1.4 occurring in all groups. Ratios ranged from 0.7 in the chronic bronchitis group to 5.03 in the ABPA group. The scores of the chronic bronchitis group (mean 1.0) were significantly lower than all the other disease groups (median test $p < 0.001$). There were no significant differences between the asthma (mean 1.3), rhinitis (mean 1.3), urticaria (mean 1.3) and cystic fibrosis (mean 1.2) groups.

H. influenzae IgE RAST scores (Fig. 39)

With this organism, the chronic bronchitis, ABPA and cystic fibrosis groups all had significantly raised RAST scores. Those of the chronic bronchitis group (mean 1.7) were significantly higher than the asthma (mean 1.2), rhinitis (mean 0.9), and urticaria (mean 0.9) groups (median test $p < 0.001$). Those of the ABPA (mean 1.9) and asthma groups were significantly higher than the rhinitis group ($p < 0.005$) and the urticaria group ($p < 0.001$). Those of the cystic fibrosis group (mean 1.7) were also significantly higher than these two groups at the 0.1% level.

4.2. Total IgE Measurements (Fig. 40)

Fig. 40 shows the total IgE levels for the seven study groups and the horizontal bars denote the geometric means of the groups.

Using the same assay (Chapter III, 10.0) the total IgE levels of 78 blood bank donor sera had been estimated to range from 2-240 I.U./ml (geometric mean 33.6 I.U./ml).

Figure 39

IgE RAST SCORES TO WHOLE CELLS OF H. influenzae

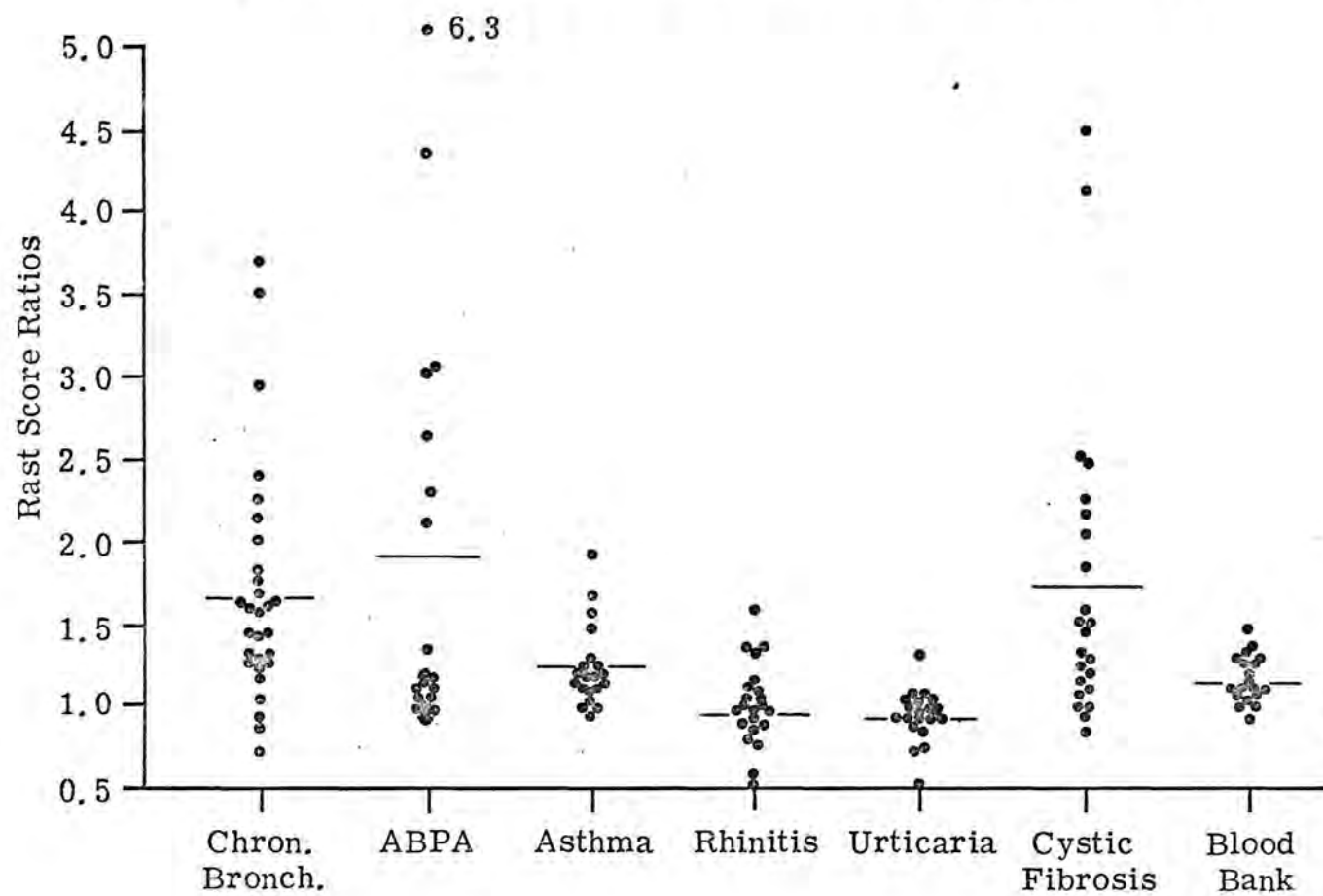
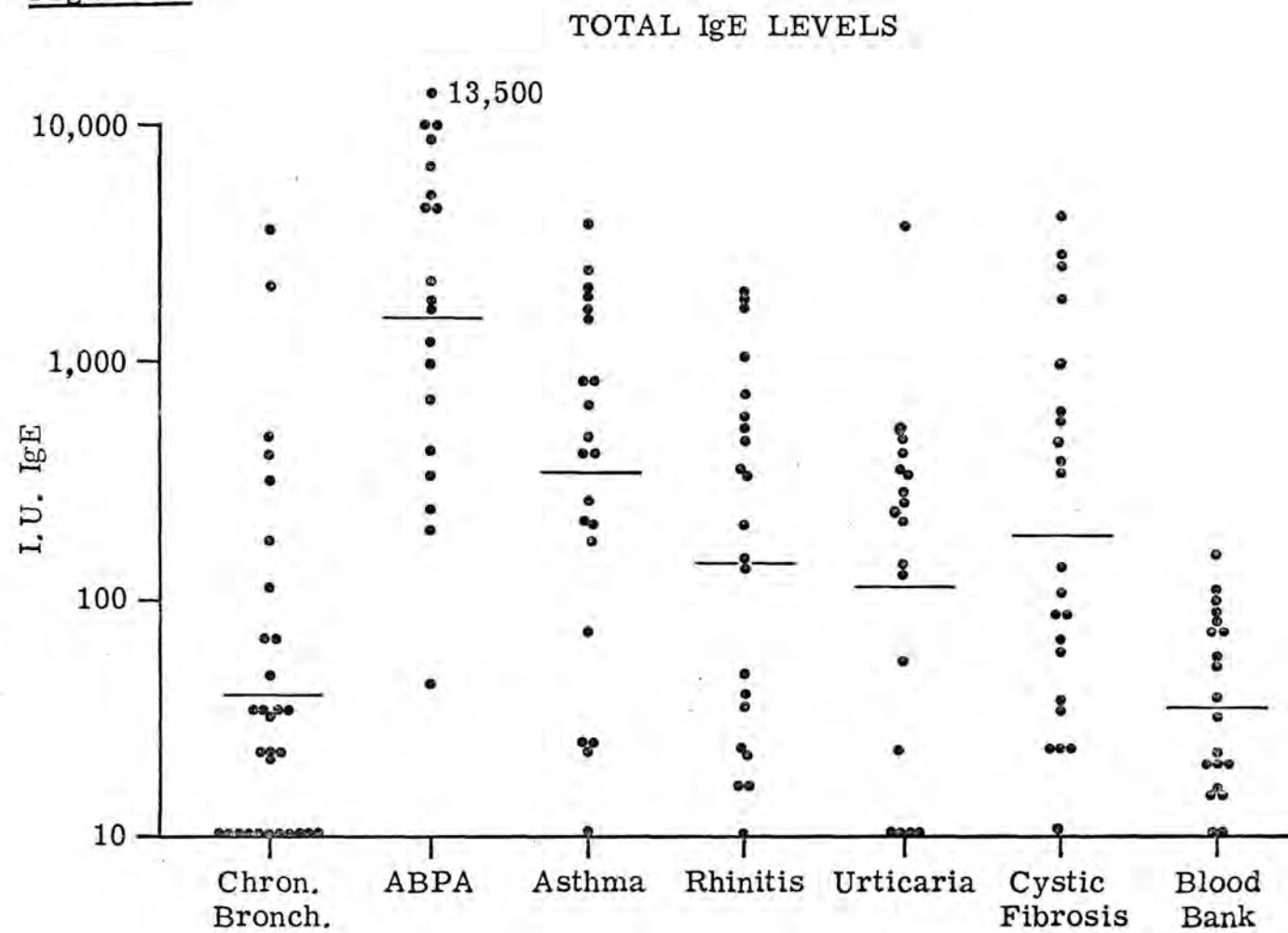


Figure 40



Taking 240 I.U./ml as the upper limit of normal, none of the current blood bank study patients had a raised total IgE level. Four of the chronic bronchitis patients had raised levels (maximum 3700 I.U.) as did 17 of the ABPA group (maximum 13,500 I.U./ml); 15 of the asthma patients (maximum 3800 I.U.), 11 of the rhinitis patients (maximum 1800 I.U.), 10 of the urticaria patients (maximum 3700 I.U.) and 10 of the cystic fibrosis patients (maximum 4000 I.U.).

On application of a median test to the results the mean of the total IgE of the blood bank sera was significantly lower than all the other disease groups except the chronic bronchitis group ($p < 0.001$), whilst the ABPA total IgE mean was significantly higher than all other groups ($p < 0.001$). There were no significant differences between the means of the remaining groups.

The number of patients in each disease group with RAST score ratios greater than 1.5 are shown in Table VI.

When individual patients were considered only five patients (three in the ABPA group, one in the rhinitis group and one in the cystic fibrosis) had RAST score ratios greater than 1.5 to all three bacteria. Three patients (two ABPA, one rhinitis) had RAST score ratios of this level to both S. aureus and S. pneumoniae. Three patients (two ABPA, one asthma) had scores >1.5 to S. pneumoniae and H. influenzae whilst four patients had scores >1.5 to S. aureus and H. influenzae (one asthma, three cystic fibrosis). The total IgE levels of these patients ranged from 460 I.U. to 9900 I.U./ml.

DISEASE GROUP	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>
Chronic bronchitis	0	0	14
ABPA	6	10	7
Asthma	2	4	2
Rhinitis	3	6	1
Urticaria	1	2	0
Cystic fibrosis	4	2	10
Blood bank	0	1	0

No. of patients with IgE RAST scores >1.5

TABLE VI

4.3. Discussion

In this study the blood bank control group had the lowest geometric mean total IgE (35 I.U./ml) and did not give raised specific RAST scores to any of the three bacteria studied.

In contrast the ABPA group, whilst showing a wide variation in individual RAST scores, gave the highest specific mean RAST scores to all three bacteria and also had the highest geometric mean total IgE (1520 I.U./ml).

It is interesting that the mean H. influenzae RAST score ratios in the chronic bronchitis and the cystic fibrosis group were also significantly higher than in the other disease groups, since H. influenzae is known to be the principal pathogen involved in chronic bronchitis (May, 1967) and with S. aureus and Pseudomonas aeruginosa is the commonest pathogen found in cystic fibrosis.

4.4. Skin Prick Test Results

Data on numbers, age, atopic status and bacterial skin prick test reactivity of the seven groups of subjects studied are shown in Table VII.

Atopic state

The numbers of patients who had one or more positive skin prick tests to common allergens amounted to 10% in the chronic bronchitis group, 100% in the ABPA group, 85% in the bronchial asthma group, 73% in the allergic rhinitis group, 47% in the urticaria group and 70% in the cystic fibrosis group.

	No. IN GROUP	AVERAGE AGE Yrs.	AGE RANGE Yrs.	ATOPIC STATE (1 or >1 SPT)	SKIN PRICK TEST POSITIVITY		
					<u>S. aureus</u> EXTRACT	<u>S. pneumoniae</u> EXTRACT	<u>H. influenzae</u> H ₁ H ₂ EXTRACT
Chronic Bronchitis	30	66	55 - 80	3:30	2:30	2:30	1:30
ABPA	20	51	24 - 71	20:20	12:20	9:20	3:20
Bronchial Asthma	20	37	22 - 73	17:20	2:19	2:19	0:19
Rhinitis	20	33	19 - 67	16:22	2:22	6:22	0:22
Urticaria	20	35	16 - 69	9:19	2:15	4:15	1:15
Cystic Fibrosis	23	22	9 - 41	16:23	0:22	2:22	1:22
Blood Bank	20	36	19 - 60	-	-	-	-
TOTAL					20:128	25:128	6:128
% +ve					15.6	19.5	5.0

TABLE VII

Data on study patients

Bacterial skin prick test reactions

Immediate whealing Type I skin reactions were elicited by the skin prick method against extracts of all three bacteria studied. The macroscopic appearance of these reactions was similar to that following testing with common inhalant allergens.

Thirty three of all the patients in the study had a positive skin prick test to one or more of the bacterial antigens. Four of these patients were from the non-atopic category.

19.5% of patients overall gave positive skin prick tests to S. pneumoniae extract, 15.6% were positive to S. aureus extract and only 5% were positive to the H_1H_2 fractions of H. influenzae extract.

Correlation with bacterial IgE RAST score ratios

The bacterial IgE RAST scores of all the patients in the study were considered in relation to their skin prick test positivity to the bacterial extracts. Data are presented in Table VIII. Mean RAST scores with all three bacteria were higher in patients with positive specific skin prick tests, viz. with S. pneumoniae, mean RAST score ratios for the skin test positive group were 1.52 compared with 1.21 in the skin test negative group. Similarly with S. aureus they were 1.29 as against 1.06 and with H. influenzae 2.03 as against 1.38.

On application of a Mann-Whitney U test significant correlations were found between specific positive skin prick tests and specific bacterial RAST scores to S. pneumoniae and S. aureus at the 0.1% and 2% levels respectively, but not with H. influenzae.

RAST SCORE RATIOS (Mean $\pm$ SEM)

ANTIGEN	SKIN PRICK TEST		Mann Whitney p value
	POSITIVE	NEGATIVE	
<u>S. pneumoniae</u>	1.52 $\pm$ 0.09 (n = 25)	1.21 $\pm$ 0.04 (n = 103)	<0.001
<u>S. aureus</u>	1.29 $\pm$ 0.08 (n = 20)	1.06 $\pm$ 0.03 (n = 108)	<0.02
<u>H. influenzae</u>	2.03 $\pm$ 0.67 (n = 6)	1.38 $\pm$ 0.06 (n = 122)	NS

TABLE VIII

Correlation of skin "prick" test positivity of study patients with their specific bacterial RAST score ratios (mean $\pm$ SEM).

Correlation with total IgE levels

The total IgE levels of the patients in the study were also considered in relation to their skin prick test reactions to bacterial extracts. Data are presented in Table IX.

Patients with a positive skin prick test to S. pneumoniae, S. aureus or H. influenzae had higher mean total IgE levels than patients with negative bacterial skin prick tests. By Mann-Whitney assessment a significant correlation was found between positive skin prick tests to S. pneumoniae and S. aureus and total IgE at the 1% and 0.1% level respectively, but not with H. influenzae.

4.4.1. Discussion

Results to skin prick testing to S. aureus and S. pneumoniae extracts correlated significantly with both the patient's bacterial whole cell RAST scores and total IgE levels. With the low incidence of skin prick test positivity to the H_1H_2 fractions of H. influenzae extract similar correlations could not be recognised. However, the mean values given for the skin prick test positive patients and those given for the skin prick test negative patients differ sufficiently to suggest that the correlation is not statistically significant more because of the small number of positives than because an underlying correlation is not present.

4.5. Study of repeated skin prick test with bacterial allergens

It was possible to study the incidence of positivity

TOTAL IgE IU/ml (Mean $\pm$ SEM)

ANTIGEN	SKIN PRICK TEST		Mann Whitney p value
	POSITIVE	NEGATIVE	
<u>S. pneumoniae</u>	2092 $\pm$ 706 (n = 24)	794 $\pm$ 176 (n = 100)	<0.01
<u>S. aureus</u>	2299 $\pm$ 548 (n = 20)	778 $\pm$ 203 (n = 104)	<0.001
<u>H. influenzae</u>	2568 $\pm$ 1623 (n = 6)	941 $\pm$ 187 (n = 118)	NS

TABLE IX

Correlation of skin "prick" test positivity of study patients with their total IgE levels (mean $\pm$ SEM).

of repeated skin prick testing in the 20 ABPA patients. This group were skin prick tested with extracts and whole cell suspensions of the three bacteria (plus a 10^{10} organism/ml suspension of S. aureus supplied by Bencard) at monthly intervals over a seven month period whilst the patients were taking part in a drug trial with Miconazole, (Janssen Pharmaceuticals Ltd.), an imidazole derivative. The serological and skin prick test results already discussed were from the patient's visit prior to starting the trial. The incidence of skin prick test positivity is recorded for each of the 20 patients in Table X.

4.5.1. Results

With S. aureus 48% of tests were positive to the extract at 100 mg/ml compared with 46% of tests to the whole cell suspension at 100 mg/ml. Whilst these results were comparable, only 31% of tests were positive with the Bencard 10^{10} organism/ml whole cell suspension.

With S. pneumoniae there was a greater discrepancy between the number of tests positive to the extract at 100 mg/ml (37%) and those of the whole cell suspension at 100 mg/ml (18%).

With H. influenzae 17% of tests were positive to the whole cell suspension compared with 7% to the H_1H_2 extract.

4.5.2. Discussion

Only in the case of H. influenzae did the whole cell suspensions give a greater number of skin prick test positives than did the extracts. Since the extract contained only the H_1H_2 antigens specific to H. influenzae the lower concentration

Pat. No.	<u>S. aureus</u>			<u>S. pneumoniae</u>		<u>H. influenzae</u>	
	Extract 100 mg/ ml	Bencard Whole Cell 10^9 orgs./ ml	Whole Cell Susp. 100 mg/ ml	Extract 100 mg/ ml	Whole Cell Susp. 100 mg/ ml	H ₁ H ₂ Extract 10 mg/ ml	Whole Cell Susp. 100 mg/ ml
1	4/6	5/6	5/5	4/6	0/4	1/6	0/5
2	6/7	3/7	0/5	5/7	0/4	0/7	0/5
3	0/6	0/6	0/6	0/6	0/3	0/6	0/6
4	7/7	5/7	5/6	1/7	0/4	0/7	0/6
5	7/8	0/8	1/6	6/8	1/5	0/8	1/6
6	0/7	0/7	4/6	1/7	1/4	2/7	2/6
7	2/7	1/7	1/6	1/7	0/3	0/7	0/6
8	0/7	0/7	1/6	0/7	1/4	0/7	0/6
9	2/7	1/7	5/6	2/7	0/5	0/7	2/6
10	3/7	3/7	3/5	3/7	1/4	0/7	0/5
11	3/7	7/7	5/6	5/7	3/4	1/7	0/6
12	2/6	1/6	2/4	1/6	2/4	1/6	1/4
13	5/8	1/8	6/6	1/8	0/5	0/8	0/6
14	7/8	4/8	4/6	7/8	1/6	1/8	6/6
15	2/7	2/7	0/4	1/7	2/4	1/7	0/4
16	4/7	3/7	3/6	4/7	0/4	1/7	3/6
17	5/7	5/7	3/6	1/7	2/5	0/7	2/6
18	2/7	1/7	0/5	2/7	0/4	0/7	0/5
19	2/7	0/7	0/6	3/7	0/4	2/7	0/6
20	2/2	0/2	1/1	2/2	-	0/2	1/1
No. of tests positive	65/135	42/135	49/107	50/135	14/80	10/135	18/107
% positivity	48	31	46	37	18	7	17

TABLE X

Incidence of positivity of repeated skin prick tests on the ABPA group patients.

of 10 mg/ml had been used for skin prick testing. However, since so few tests proved to be positive, even in this highly atopic patient group, it would probably have been more informative to Type I skin hypersensitivity to test the H_1H_2 extract also at the 100 mg/ml strength of the S. aureus and S. pneumoniae extracts.

CHAPTER VI

STUDY OF IgG AND IgG₄ ANTIBODIES
IN VARIOUS ALLERGIC LUNG DISEASES.

1.0 INTRODUCTION

A Sepharose RAST was employed to measure specific IgG and IgG₄ antibodies to S. pneumoniae, S. aureus and H. influenzae in the serum of the same patients whose IgE antibodies had been studied as previously described. Due to the shortage of serum from the original cystic fibrosis patients, the IgG and IgG₄ estimations were made on a second group of cystic fibrosis patients as indicated in Chapter V, 2.0. The values obtained from 20 subjects in the six disease groups and from the blood bank donors were compared with those of human serum albumin run in parallel in all assays as a negative control.

Results are expressed as a ratio of the patient's IgG or IgG₄ RAST to the human serum albumin (HSA) RAST, e.g.

$$\text{IgG RAST Score Ratio} = \frac{\text{Patient's specific IgG RAST score}}{\text{HSA specific IgG RAST score}}$$

Total serum IgG estimations were also made on these sera using a radial immunodiffusion technique (as described in Chapter III, 11.0). Detailed data of IgG RAST score ratios and total IgG levels for each patient are found in Appendix 3.

For the specific bacterial IgG and IgG₄ Sepharose RAST assays a similar procedure was adopted to the IgE assay (see Chapter III, 9.3) including the use of the same solid phase, namely freeze dried whole bacterial cells coupled to cyanogen bromide activated Sepharose 4B.

2.0 SPECIFIC BACTERIAL IgG RAST ASSAYS

2.1. OPTIMIZATION OF ASSAY CONDITIONS

Fig. 41 depicts the titration of the S. pneumoniae, S. aureus and H. influenzae solid phases with a serum giving raised IgG RAST scores to the three bacteria. Since it is not possible to dispense the 1:2 dilution of solid phase reproducibly, the 1:4 dilution was chosen for future assays. This 1:4 dilution of solid phase was considered to be acceptable since the radioactive counts were 86% for S. pneumoniae, 93% for S. aureus and 83% for H. influenzae of those with 1:2 dilutions. Human serum albumin gave background RAST score counts of 823 CPM with S. pneumoniae, 929 CPM with S. aureus and 954 CPM with H. influenzae.

Titration of specific IgG bacterial antibody in another serum with raised IgG RAST scores to the three bacteria is shown in Fig. 42. The IgG RAST counts ranged from 7563 CPM in neat serum to 1088 CPM for a 10^{-6} serum dilution with S. pneumoniae. With S. aureus similar dilutions ranged from 6381 CPM to 876 CPM and with H. influenzae from 5625 to 943 CPM. Specific antibody was titrated out at a 10^{-4} dilution of the patient's serum with all three bacteria.

The optimum number of counts of the ^{125}I anti-IgG to add to each assay tube in order to differentiate between RAST positive sera and human serum albumin (negative control) was found to be 100,000 CPM.

2.2. Results of specific bacterial IgG RAST assays

The IgG RAST score ratios to the three bacteria

Figure 41

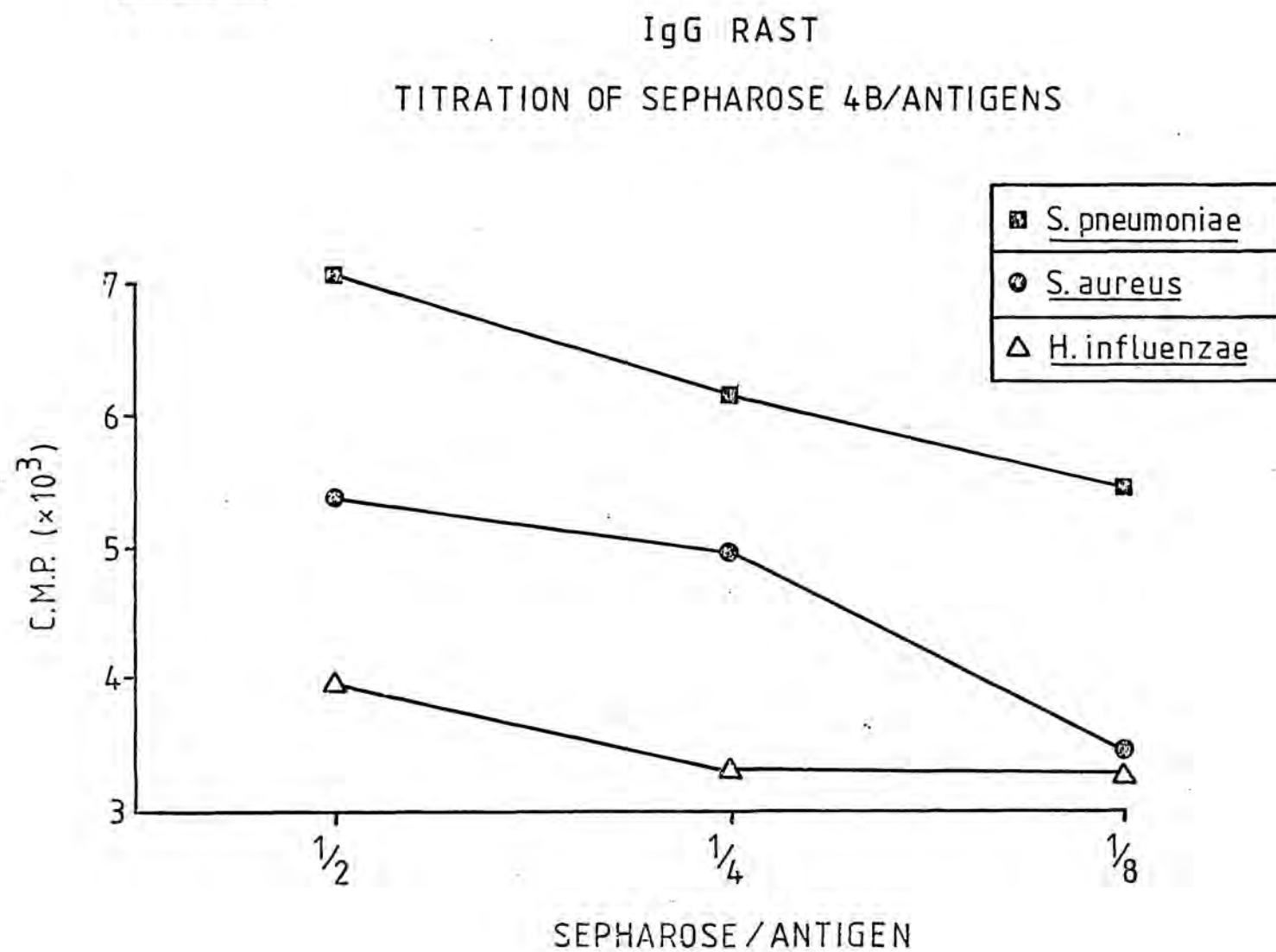
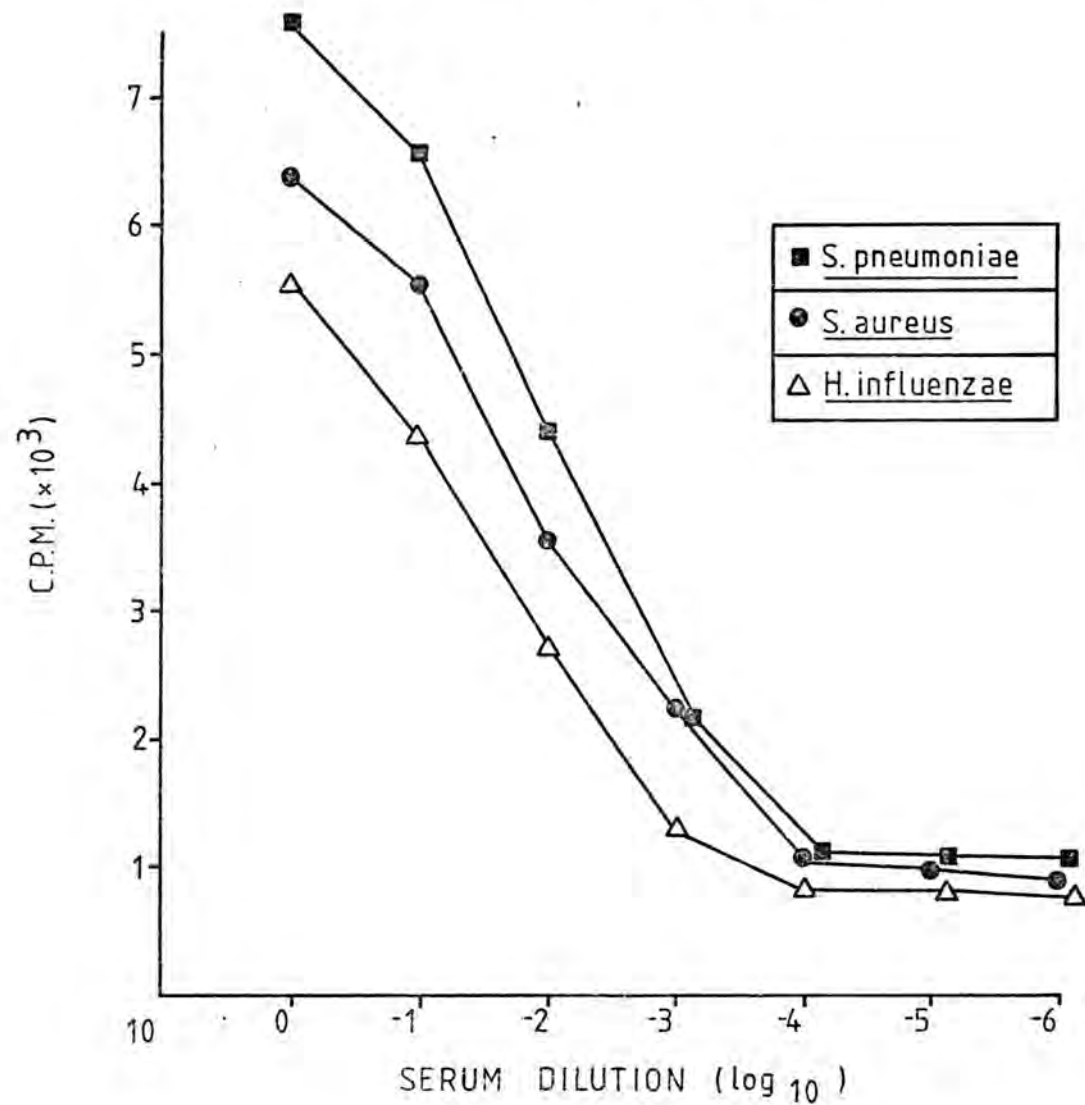


Figure 42

IgG SEPHAROSE RAST
TITRATION OF SERUM



studied are plotted in Figs. 43-45. Results are shown for each of the six disease groups and the blood bank controls. The horizontal bars represent the arithmetic means for each group.

All subjects had RAST score ratios greater than 1.5 against all three bacteria.

S. aureus IgG RAST scores (Fig. 43)

The majority of the IgG RAST scores to S. aureus range between 1.5 and 4. The mean RAST score ratios of the cystic fibrosis (3.05) and ABPA groups (2.99) were highest and, on application of a one way analysis of variance test, were significantly higher than the chronic bronchitis group (mean 2.37) at the 0.1% level and the asthma (mean 2.62) and rhinitis (mean 2.57) groups at the 2.5% level. The mean RAST score ratios of the urticaria (2.84) and blood bank (2.83) groups were also higher than the chronic bronchitis group at the 1% level.

S. pneumoniae IgG RAST scores (Fig. 44)

Again the majority of the IgG RAST score ratios range between 1.5 and 4. Only the ABPA (mean 3.19) and asthma (mean 3.07) groups had patients with RAST score ratios greater than 4. These two groups were significantly higher than all the other groups ($p < 0.025$). The chronic bronchitis (mean 2.82), blood bank (mean 2.66), rhinitis (mean 2.64) and urticaria (mean 2.54) groups were all significantly higher than the cystic fibrosis group (mean 2.28) ($p < 0.05$).

Figure 43

IgG RAST (S. aureus whole cell suspension)

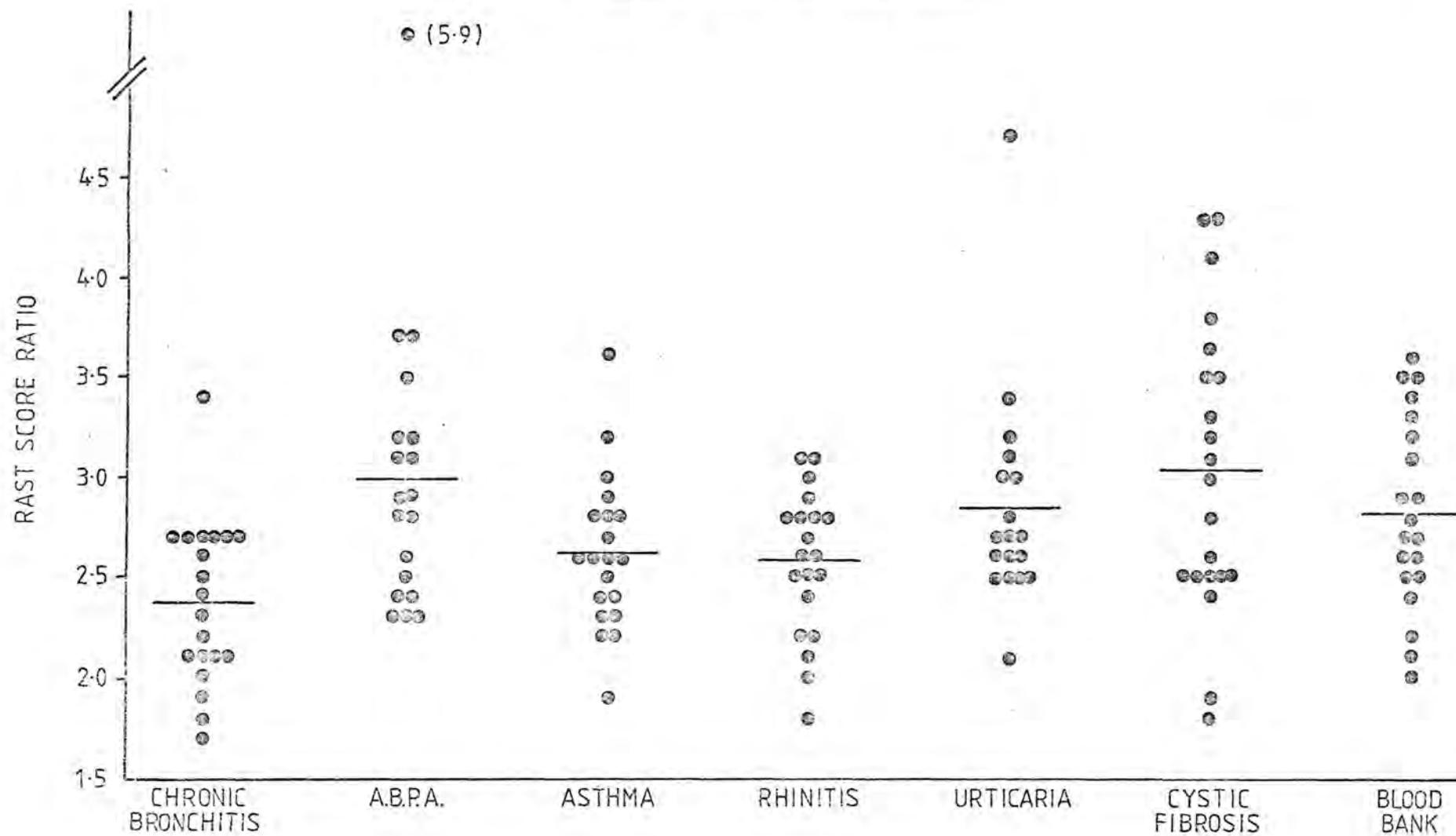
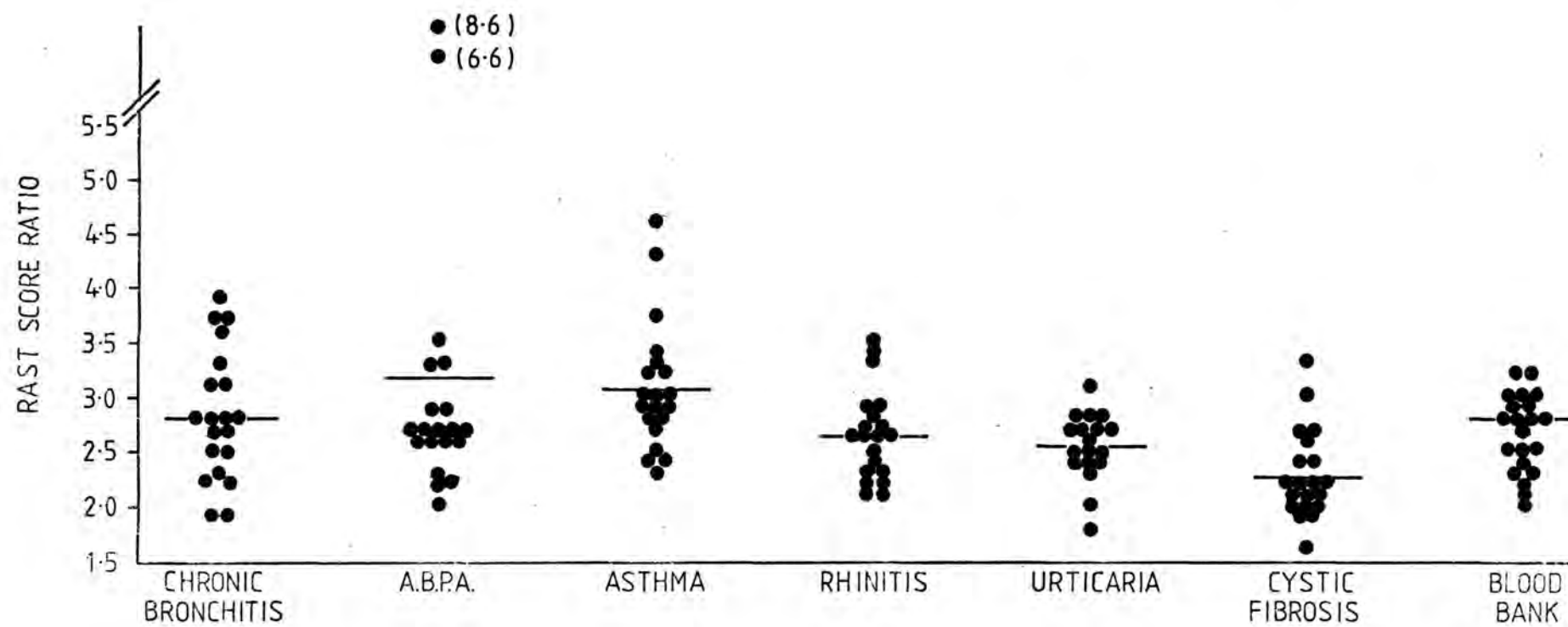


Figure 44

IgG RAST (S. pneumoniae whole cell suspension)



H. influenzae IgG RAST scores (Fig. 45)

All but five raised IgG RAST scores fall between 1.6 and 4. The mean of the cystic fibrosis group (3.21) was significantly higher than all other groups ($p < 0.001$). The ABPA (mean 2.63), blood bank (mean 2.56), urticaria (mean 2.54), chronic bronchitis (mean 2.51) and rhinitis (mean 2.45) groups were in turn significantly higher than the asthma group (mean 2.14) ($p < 0.05$).

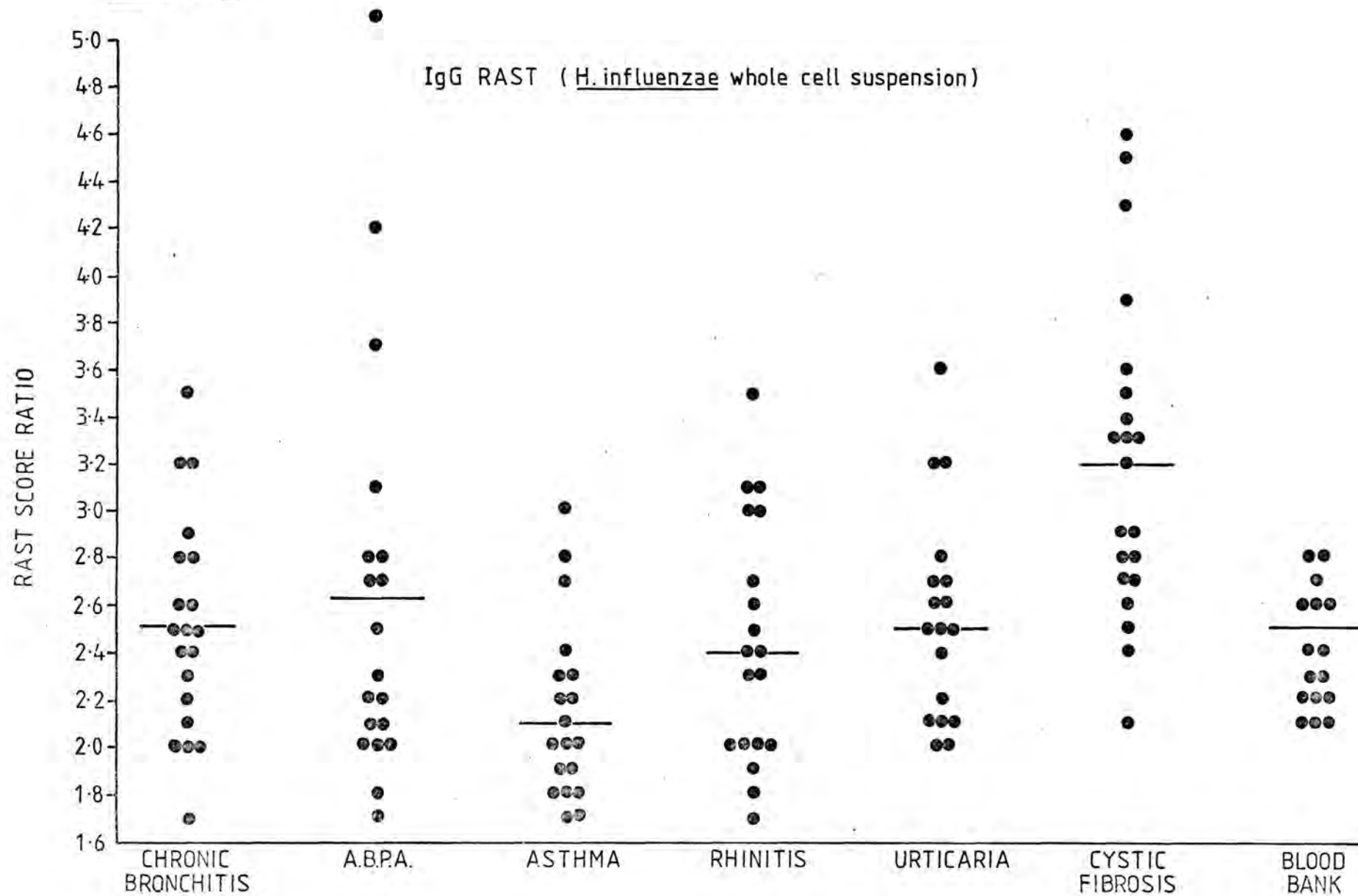
2.2.1. Results of total IgG measurements

The mean levels of total IgG in the six disease groups were all within normal limits (normal range 800-1800 mg/100 ml). The mean of the blood bank donor group was above this normal range, viz. 1847 mg/100 ml., five subjects having a level over 2000 mg/100 ml.

2.3. Discussion

Ratios greater than unity indicate some degree of immunity acquired from past exposure to the organisms. The two very raised individual scores (5.9 in the ABPA group and 4.7 in the urticaria group) were probably due to an acute infection involving S. aureus at the time the patients were bled. Neither of these patients had a raised IgE RAST to S. aureus. Whereas S. aureus appears to be a problem in cystic fibrosis, (e.g. RAST score ratios of 4.3, 4.3, 4.1, 3.9, 3.8) and in the ABPA groups it does not appear to be a major pathogen involved in chronic bronchitis or rhinitis.

Figure 45



The two highest IgG RAST score ratios to S. pneumoniae were found in the ABPA group. The patient with an IgG RAST score ratio of 8.6 had a raised IgE specific RAST score of 1.58, whilst the patient with an IgG RAST score ratio of 6.6 had an IgE RAST score ratio of 3.39. The next highest IgG RAST score ratios of 4.6 and 3.7 were from patients in the asthma group, the former not having a correspondingly raised IgE RAST score ratio but the latter with one of 1.46.

With H. influenzae three patients had high IgG RAST score ratios of 5.1 (IgE 1.32), 4.2 (IgE 0.99) and 3.7 (IgE 3.05) in the ABPA group and four patients in the cystic fibrosis group with scores of 4.6, 4.5 (IgE 2.46), 4.3 and 3.9, thus emphasizing the importance of the organism in these diseases.

3.0 SPECIFIC BACTERIAL IgG₄ RAST ASSAYS

3.1. Procedure

The procedure for the assay was as described for the IgE Sepharose RAST (see Chapter III, 9.3). On assaying test sera and human serum albumin in parallel with the addition of 100,000 CPM, 200,000 CPM or 300,000 CPM to each tube, 200,000 CPM was found to be optimum to differentiate between RAST positive and the background count with human serum albumin. Since all sera were tested against a bacterium at one time, RAST results were plotted as counts per minute as the problems of interassay variation did not arise. Human serum albumin was, however, run in parallel in all assays, so as to conform with the IgE and IgG RAST results, the IgG₄ results are also tabulated as RAST score ratios, viz.

$$\text{IgG}_4 \text{ RAST score ratio} = \frac{\text{Patient's specific IgG}_4 \text{ RAST score}}{\text{HSA specific IgG}_4 \text{ RAST score}}$$

Tables containing the bacterial IgG₄ RAST score ratios for each patient appear in Appendix 4.

3.2. Results

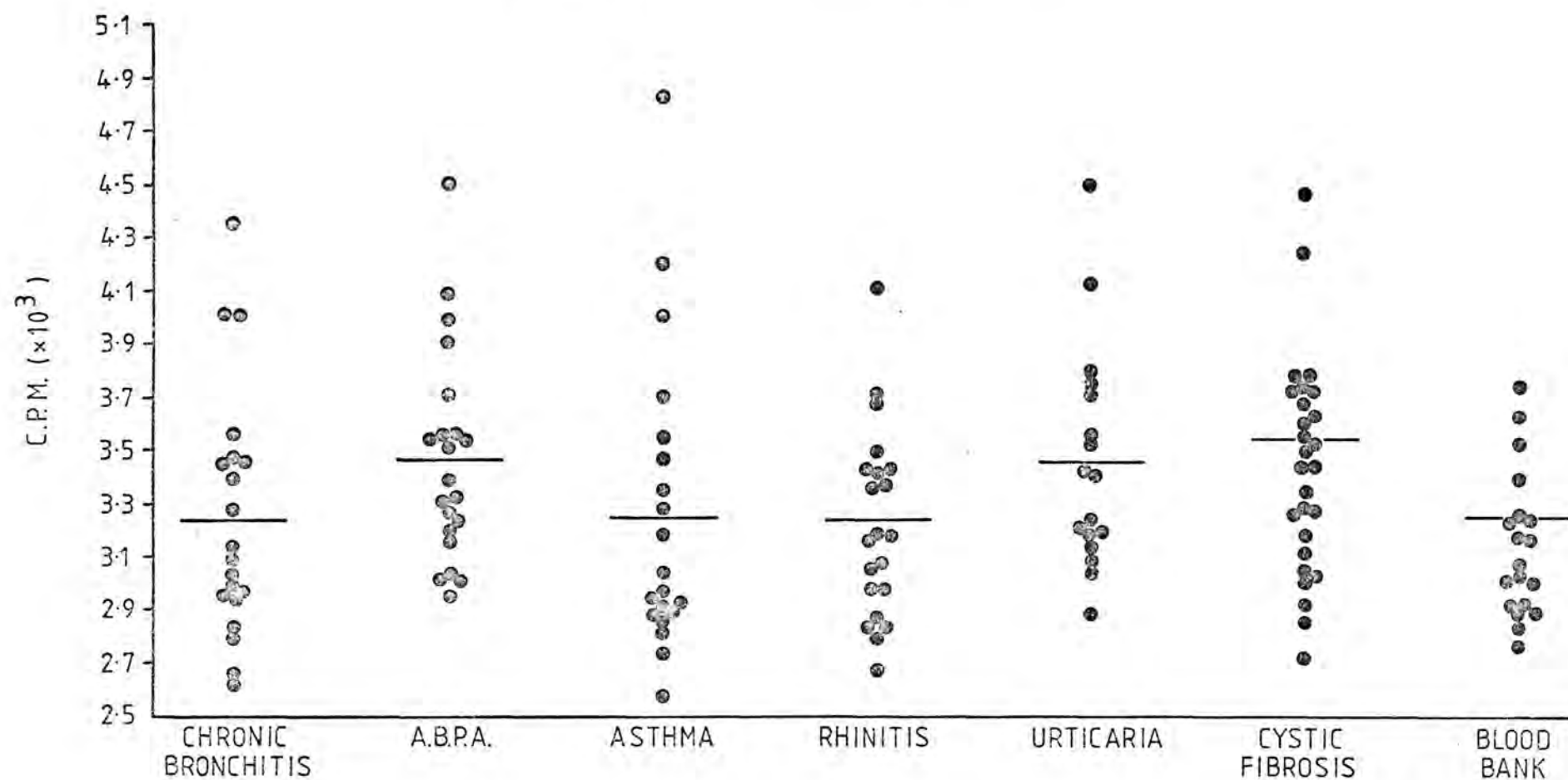
The IgG₄ RAST score counts to the three bacteria studied are plotted in Figs. 46-48. Results are shown for each of the six disease groups and the bars represent the arithmetic means for each group.

S. aureus IgG₄ RAST scores (Fig. 46)

The mean IgG₄ RAST count for human serum albumin was 2928 CPM. RAST counts of the patients ranged from 2575 to 4822 CPM (RAST score ratios 0.88 to 1.65) and only in the

Figure 46

IgG₄ RAST (*S.aureus* whole cell suspension)



blood bank group were there no subjects with RAST counts above 3900 CPM. Counts above this level were found in three chronic bronchitis patients, four ABPA patients, three asthma patients, one rhinitis patient, two urticaria patients and in two cystic fibrosis patients.

The mean RAST score ratios of the ABPA, urticaria and cystic fibrosis groups were higher than those of the chronic bronchitis, asthma, rhinitis and blood bank groups but on application of a one way analysis of variance test no group was found to be significantly different from the others.

S. pneumoniae IgG₄ RAST scores (Fig. 47)

The mean IgG₄ RAST count for human serum albumin was 4181 CPM. RAST counts of the patients ranged from 3170 to 5729 CPM (RAST score ratios 0.76 to 1.37). RAST counts greater than 4600 CPM were found in the asthma group (six patients), the ABPA group (five patients), the urticaria group (two patients), the cystic fibrosis group (one patient) and the rhinitis group (one patient).

On application of a one way analysis of variance test the mean RAST score ratios of the asthma, ABPA and urticaria groups were significantly higher than those of the blood bank, chronic bronchitis, cystic fibrosis and rhinitis groups, $p < 0.001$.

H. influenzae IgG₄ RAST scores (Fig. 48)

The mean IgG₄ RAST count for human serum albumin was 4738 CPM. RAST counts of the patients ranged from 3579 to 8828 CPM (RAST score ratios 0.76 to 1.86) and only the blood

Figure 47

IgG₄ RAST (S. pneumoniae whole cell suspension)

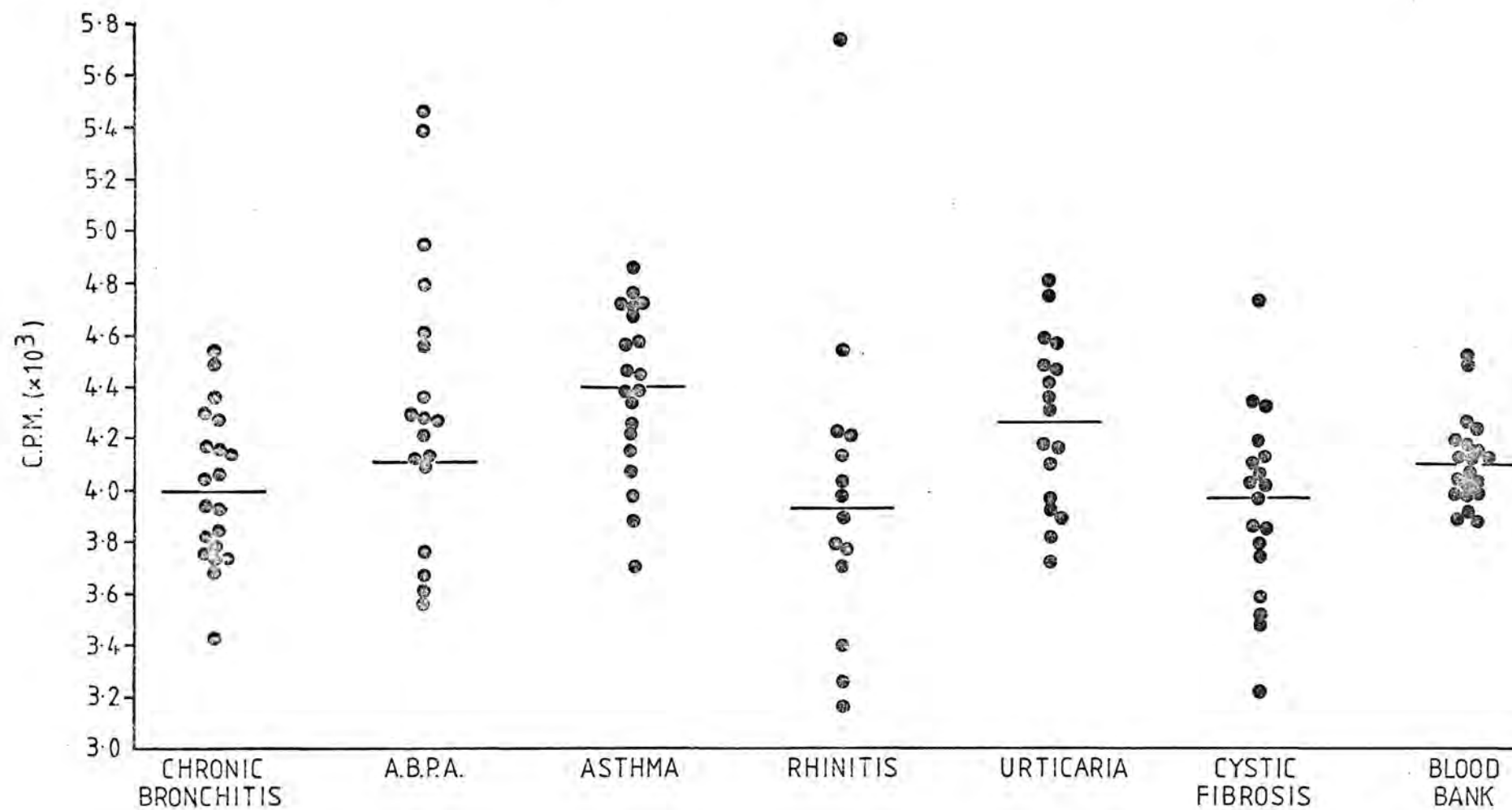
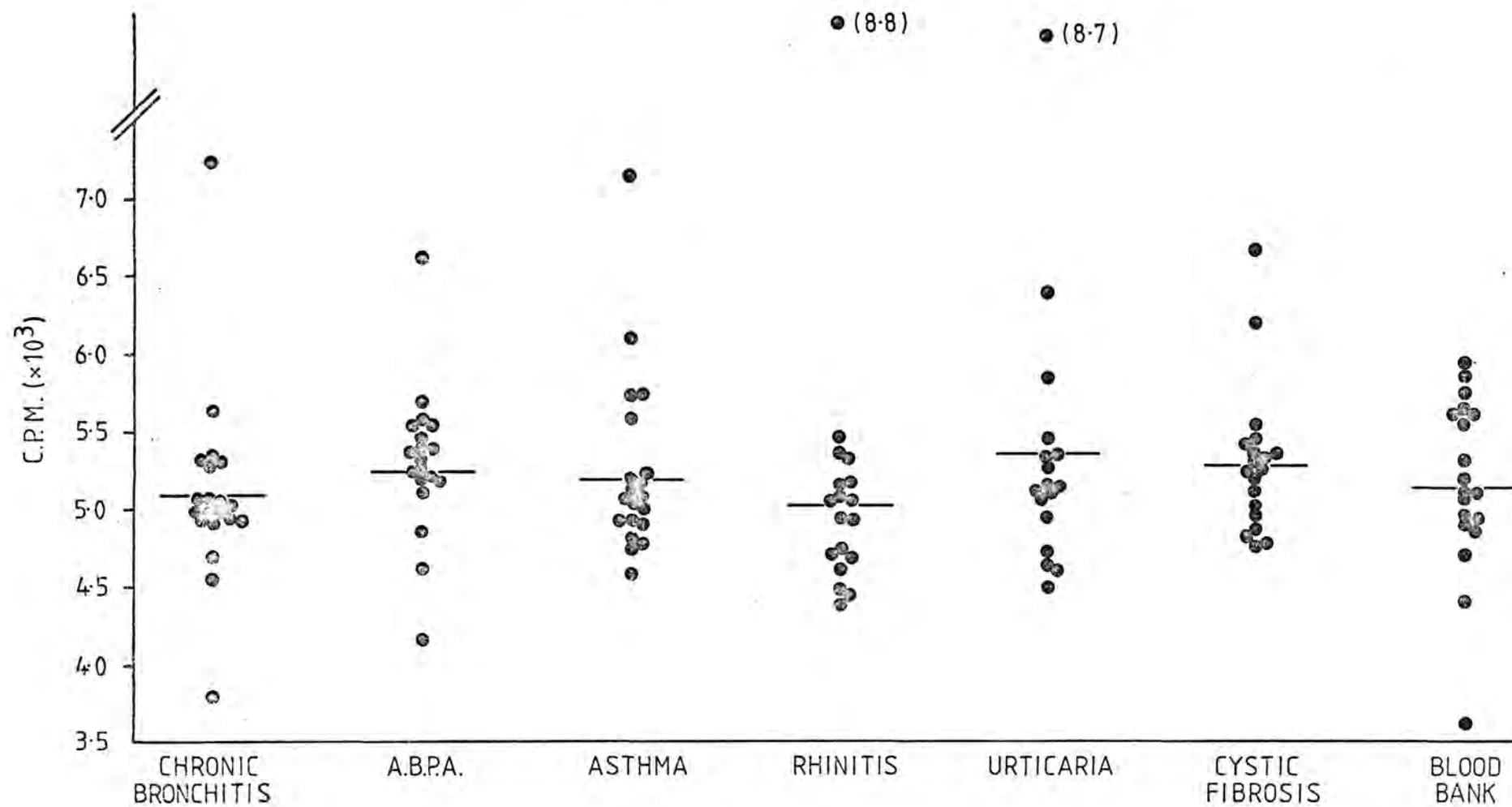


Figure 48

IgG₄ RAST (H. influenzae whole cell suspension)



bank group had no subjects with RAST counts above 5900 CPM. Such counts were found in one chronic bronchitis patient, one ABPA patient, two asthma patients, one rhinitis patient, two urticaria patients and two cystic fibrosis patients.

The mean RAST score ratios of the urticaria, ABPA and asthma groups were higher than those of the cystic fibrosis, rhinitis, blood bank and chronic bronchitis groups but not significantly so.

3.3. Discussion

The ABPA and asthma groups had the greatest number of patients (10 each) with raised IgG₄ RAST scores to one of the three bacteria (criteria for raised scores were: S. aureus >3900 CPM: S. pneumoniae >4600 CPM: H. influenzae >5900 CPM). By these criteria six urticaria patients, five cystic fibrosis patients, four chronic bronchitis patients, and three rhinitis patients had raised IgG₄ RAST scores to one of the bacteria. None of the blood bank subjects had a raised IgG₄ RAST to any of the three bacteria.

Looking at the patients individually, three of the ABPA and one of the cystic fibrosis groups had raised IgG₄ RAST scores to both S. aureus and S. pneumoniae, whilst one ABPA and one urticaria patient had raised IgG₄ RAST scores to both S. pneumoniae and H. influenzae. No patient had a raised IgG₄ RAST score to both S. aureus and H. influenzae.

Table XI shows the IgE RAST score ratios of those patients with raised specific IgG₄ RAST scores. It would appear that patients with the most raised specific IgG₄ scores

COMPARISON OF IgE RAST SCORE RATIOS IN PATIENTS WITH
RAISED IgG₄ RAST SCORE RATIOS

DISEASE OF PATIENT	<u>S. aureus</u>		DISEASE OF PATIENT	<u>S. pneumoniae</u>		DISEASE OF PATIENT	<u>H. influenzae</u>	
	RAST SCORE RATIO			RAST SCORE RATIO			RAST SCORE RATIO	
	IgG ₄	IgE		IgG ₄	IgE		IgG ₄	IgE
CB	1.37	0.79	ABPA	1.10	3.11	CB	1.52	1.28
CB	1.48	0.79	ABPA	1.29	1.58	ABPA	1.40	2.60
CB	1.37	0.79	ABPA	1.15	1.11	Asth	1.28	1.08
ABPA	1.36	1.08	ABPA	1.30	3.39	Asth	1.50	1.24
ABPA	1.54	1.64	ABPA	1.18	1.16	Rhin	1.86	0.46
ABPA	1.34	1.39	Asth	1.13	1.07	Urt	1.83	1.05
ABPA	1.36	2.0	Asth	1.16	1.17	Urt	1.34	0.92
Asth	1.65	1.75	Asth	1.12	1.61	CF	1.40	-
Asth	1.43	1.14	Asth	1.13	0.95	CF	1.25	-
Asth	1.34	1.30	Asth	1.13	0.99			
Rhin	1.40	1.44	Asth	1.13	1.91			
Urt	1.53	1.15	Rhin	1.37	1.93			
Urt	1.41	0.94	Urt	1.13	1.41			
CF	1.52	-	Urt	1.14	0.92			
CF	1.45	-	CF	1.14	-			

TABLE XI

also had correspondingly raised IgE scores, but on applying a one way analysis of variance test to all the patients in the study, only a low correlation with S. pneumoniae alone was obtained ($r = 0.3202$, $p < 0.01$). Correlations between IgE and the IgG and IgG₄ RAST scores have not been made in the case of the cystic fibrosis group since sera from different patients were used to make the determinations.

Correlation values between the specific immunoglobulin levels estimated for the disease groups are given in Table XII and will be considered in the general discussion.

CORRELATION BETWEEN MEASURED SPECIFIC IMMUNOGLOBULIN LEVELS
OF THE SIX DISEASE GROUPS

	IgE v IgG	p value	IgE v IgG ₄	p value	IgG v IgG ₄	p value
<u>S. aureus</u>	0.2891	<0.05	0.1231	NS	0.2189	<0.05
			Asthma alone 0.4873	<0.05		
<u>S. pneumoniae</u>	0.3056	<0.01	0.3202	<0.01	0.4782	<0.001
			Rhinitis alone 0.5141	<0.05	ABPA alone 0.8135	<0.001
<u>H. influenzae</u>	-0.048	NS	-0.1463	NS	0.1264	NS

TABLE XII

CHAPTER VII

GENERAL DISCUSSION.

INTRODUCTION

In the present study, attempts have been made to identify antibodies of the IgE and IgG class to the three common respiratory pathogens: S. pneumoniae, S. aureus and H. influenzae.

To a large extent this has involved the development of a suitable in vitro method of measuring immunoglobulin levels in patients with allergic lung disease and other hypersensitivity states.

In this section the work presented in this thesis will be discussed in relation to more recent work in allergy.

ROLE OF BACTERIA IN LUNG DISEASE

There are many problems concerning the possible role of the three bacteria Streptococcus pneumoniae, Staphylococcus aureus and Haemophilus influenzae in the production of allergic lung diseases. Their almost universal presence and the frequent evidence of sensitisation in the form of antibodies and skin test reactions, make it difficult to decide when these are clinically relevant.

Much of the support for a bacterial factor in asthma has come from the apparent demonstration of allergic reactions against bacteria and their components. Cooke (1932) considered there was an association between respiratory infection and asthma in cases of 'intrinsic' asthma and postulated that this form of asthma was caused by bacteria. Crofton and Douglas (1975) state that whilst it is not usually possible to demonstrate an allergic reaction to any specific infecting agent, inflammation of the bronchi by an infection starts off

a chain of reactions resulting in asthma. Bacterial infection would provide a persisting source of allergen in the respiratory tract and it may be that the asthma which occurs in non-atopic patients is related to this allergen retention. Bacterial infection is indeed known to be involved in many of the acute exacerbations and episodes of airflow obstruction in chronic bronchitis, viz. increased prevalence of precipitating antibody against H_1H_2 allergens of H. influenzae over control patients (Burns and May, 1967).

Bull and Smith (1977) reported that 50% of the chronic bronchitics they studied had persistently high levels of serum IgG to H_1 antigen. They also found "indications of IgE to bacterial components" which they thought might be related to the asthmatic symptoms occurring in some chronic bronchitics.

There has been little evidence to suggest that bacterial infection is any more common in patients with asthma than in healthy people although Clarke (1979) showed an increased frequency of precipitins in asthmatic patients to the heat-stable cell wall antigen of H. influenzae. However, whilst allergic responses to bacterial allergens occurring in the respiratory tract of normal patients may have little effect, their occurrence in those with bronchial hyper-reactivity could trigger widespread obstruction of airflow. A hypersensitivity reaction on the part of the host to an organism or its products may indeed render it pathogenic (Coombs et al, 1975). Bronchial hyper-reactivity demonstrated by inhalation of prostaglandins (Mathe et al, 1973), by histamine or by exercise (Anderson et al, 1975) has been shown to be an important factor in asthma. It has also been

demonstrated in chronic bronchitis (De Vries, 1961).

H. influenzae seems to be the bacterial species that can most readily become established in damaged bronchi. It also heads the list of secondary invaders in other types of chronic broncho-pulmonary disorder, e.g. bronchial carcinoma, T.B.

It is not known why H. influenzae can so readily invade the bronchial mucosa in almost any chronic respiratory disease while S. aureus and Ps. aeruginosa can do so with significant frequency only in cystic fibrosis and bronchiectasis and not in chronic bronchitis. H. influenzae has been shown to have an inhibitory effect on mouse alveolar macrophages (Degre, 1969) and on ciliary activity in tracheal organ cultures from human fetus, chick embryo, rats and hamsters (Denny, 1974).

Clarke (1979) found H. influenzae antigens caused histamine release from human leucocytes and from human lung and surmised that if this release occurred in vivo, it could contribute to the clinical and pathological features of disease. Support for in vivo release of histamine in chronic bronchitis and extrinsic asthma has come from a study by Turnbull et al (1977) in which histamine was demonstrated in the sputum of all patients with these diseases.

CHOICE AND PROBLEMS ASSOCIATED WITH THE USE OF THE THREE BACTERIA

Since initially any allergic response in the lung is probably to the whole bacterial cell, this was the form of the antigen selected for this study.

Strains of the bacteria were chosen as being amongst those most commonly found in chest disease and not necessarily the most convenient strain to study. Thus the Wheatley Oxford strain of S. aureus (NCTC 6571) was studied although it was known to contain Protein A. Wood 46 strain, containing only a small quantity of Protein A, would have reduced the problem with this antigen but this strain was not used in the present study because it does not commonly occur in chest disease. Specificity of the reaction was demonstrated, however, by inhibition studies and differentiation was achieved in the various disease groups, with the allergic bronchopulmonary aspergillosis group having the most raised IgE RAST scores to S. aureus and the chronic bronchitis, allergic rhinitis and blood bank donor groups not having raised levels.

Choosing to work with an uncapsulated strain of H. influenzae had the disadvantage that the organisms used could not be typed, but as capsulated H. influenzae are isolated in only 5% of chest infections (Holdaway and Turk, 1967) the results would have been of limited interest.

Davies (1976) worked with Type III polysaccharide extracts of S. pneumoniae and with the H_1H_2 antigens of H. influenzae but found that less than 1-5% of his asthmatic and chronic bronchitis patients gave positive skin prick tests. In contrast, skin prick testing to the alpha teichoic acid of S. aureus resulted in positivity in the control groups (26%) being equal or greater than in the disease groups. Concentration of sera revealed that 90% of control subjects had precipitins in their serum to alpha teichoic acid. Similarly Martin et al (1965) found precipitins to this antigen in 80% of normal subjects, increasing to 100% in patients infected with S. aureus.

It seems, therefore, that the use of whole cell organisms, whilst involving problems of experimental procedure, may enable more definitive results to be obtained with the organisms in the form in which they are to be found in patients.

One such problem in the use of whole cell organisms rather than refined fractions is that certain bacterial substances can activate 'allergic' pathways by non-specific methods. All three bacteria being studied involve problems of this nature.

Nearly all coagulase positive strains of S. aureus contain Protein A antigen which can fix non-specifically to the Fc fragment of three of the four human IgG subclasses (Forsgren and Sjoquist, 1966). Protein A has been found to release histamine directly from human leucocytes (Martin and White, 1969) and thus may also play a role in the development of inflammation.

S. pneumoniae polysaccharides have been found to adhere non-specifically to cell walls (Dishon et al., 1967) whilst a somatic polysaccharide 'C-substance' antigen (Tillet and Francis, 1930) can combine non-specifically with C-reactive protein which appears in the blood of patients with certain febrile illnesses (Kabat and Meyer, 1961).

H. influenzae contains an endotoxin and endotoxins from the cell wall of Gram negative bacilli can directly fix complement via the alternative pathway (Gewurz et al., 1968). McLean (1977) by challenging ileal segments of sensitised animals and obtaining dose-dependent contractions with the endotoxin, has demonstrated that E. coli endotoxin can elicit anaphylactic reactions.

These properties of the bacteria make the study of

reactions following contact with bacterial substances more difficult than those involving common inhalant allergens.

SHORT-TERM SENSITISING IgG

Short-term sensitising IgG (S-TS IgG) has been shown to give rise to immediate-type responses which can be confounded with those of IgE. Evidence for this statement includes:

- (1) Occurrence of an IgE type of immediate wheal and flare skin reaction following injection of anti-IgG (Parish, 1970).
- (2) Release of histamine from human basophils, e.g. Grant and Lichtenstein (1972), Ishizaka et al (1972) and from human lung tissue on exposure to anti-IgG, e.g. Paul and Weir (1969), Parish (1974).
- (3) Demonstration of histamine release from human leucocytes mediated by human IgG₄ subclass in allergic individuals who had positive skin tests and negative IgE RAST, e.g. Assem and Turner-Warwick (1976), Vijay and Perelmutter (1977).
- (4) Inhibition of IgE-mediated passive cutaneous anaphylaxis in baboons by human IgG₄ subclass myeloma has been reported by Stanworth and Smith (1973). This paraprotein bound to the primate tissue for less than 24 hr.
- (5) Loss of ability of allergic human sera to sensitise monkey skin to passive cutaneous anaphylaxis (PCA) after absorption with anti-IgG (Parish, 1970).

These results suggest that IgG (probably IgG₄) is a functional component of some immediate hypersensitivity reactions.

The most extensive studies of the occurrence of the human IgG heat-stable short-term skin-sensitising antibody are those by Parish (1970, 1971a). In a total of 359 sera tested by PCA in monkeys, S-TS IgG antibody was present in 19 (5%) e.g. to milk, horse serum, tetanus toxoid, grass. Parish also demonstrated S-TS IgG to Streptococcus pyogenes allergens in two patients with cutaneous vasculitis whilst he could not detect antibody of the IgE class. A study by Longbottom et al (1976) showed that S-TS IgG antibody mediates Type I reactions to the polysaccharide antigens of Candida albicans.

Bryant, Burns and Lazarus (1973, 1975) employing PCA testing in monkeys, suggested that S-TS IgG antibody might mediate the allergic reaction to house dust mite in 20% to 30% of skin prick test positive patients with bronchial asthma. Of 21 sera tested, eight contained only S-TS IgG and two of the remaining 13 sera had this antibody in addition to IgE.

THE PRESENCE OF SPECIFIC IgE AND IgG₄ ANTIBODIES TOGETHER IN PATIENTS

The difficulties of allocating a definitive role in clinical studies to IgE and IgG₄ is emphasized by the following reported recent work.

Berrens et al (1977) found a close parallelism between IgE and IgG₄ antibodies determined by RAST in the serum of patients with mono-specific allergies, e.g. grass pollens. These workers thought that raised IgE and IgG₄ levels might reflect an enhanced responsiveness of the entire system in atopy.

In 1979, Bruynzeel and Berrens reported on IgE and

IgG₄ antibody levels measured by disc RAST in the sera of atopic dermatitis patients, with or without allergic rhinitis. They again found close parallelism with grass pollen where 63% of patients had a positive IgG₄ RAST and 68% had a positive IgE RAST. With cat dander 64% of patients had a raised IgG₄ RAST and 54% a raised IgE RAST, whilst with guinea pig dander many more patients had a raised IgG₄ RAST (73%) than an IgE RAST (27%).

By using the heat stability of IgG₄ as a differential physical property, Vijay and Perelmutter (1977) in their studies on inhibition of PCA reactions in monkeys and histamine release from human leucocytes by human IgG₄, concluded that reaginic sera from some of their patients possessed both IgE and IgG₄ antibodies against specific allergens.

In the present study the majority of the patients who had raised RAST scores to both specific IgE and IgG₄ were found in the asthma and ABPA groups (Table XI). Overall the number of patients who had these combined raised RAST scores were six to S. aureus, seven to S. pneumoniae and one to H. influenzae.

Data relating to both total IgG and IgG₄ were, therefore, necessarily pertinent to the present study and are discussed later in the context of the results of the Sepharose RAST assay technique developed to determine the immunoglobulin content of the sera.

SKIN TESTING

The use of skin prick tests to measure atopic reactions is well established. The skin prick test is safer

and more reproducible than intradermal challenge and less likely to give false positive results due to the presence of non-specific wealing agents and to trauma associated with the test. In groups of asthmatic patients, however, a good correlation ($p < 0.001$) has been found with four allergens between positive skin prick tests and a history of provocation (Hendrick et al, 1975). Belin and Norman (1977) also found good correlation ($p < 0.01$) between skin prick and intradermal tests and 'symptom severity scores' with B. subtilis antigens. Stenius et al (1971) demonstrated a good correlation between skin prick test results and specific IgE to Dermatophagoides spp. and grass pollen measured by RAST.

Skin prick tests fail to distinguish between immediate type I reactions involving IgE and the short-term reactions attributable to IgG₄ and, therefore, skin prick tests, whilst a valuable clinical tool, should be used in conjunction with other tests.

In this study with S. aureus and S. pneumoniae significant correlations were found between patients' skin prick test positivity, total IgE levels and specific IgE levels as determined by Sepharose RAST and point very strongly to an allergic response. Due to the very low skin prick test positivity to H. influenzae (5%) significant correlations were not demonstrated.

No statistically valid correlation was found between patients' skin prick test positivity and their specific IgG₄ levels in any of the five disease groups (chronic bronchitis, ABPA, asthma, rhinitis, urticaria) whose patients had had both determinations made. With S. pneumoniae alone there was a

low correlation (Mann-Whitney U test $p < 0.025$) in the patients overall with regard to these criteria. Since skin prick test results correlated with specific IgE RAST levels but not with specific IgG₄ RAST levels, this indicates that IgE is the major skin-sensitising antibody involved in the patients' responses to the three bacteria studied.

However, in the case of the few patients who gave a positive skin prick test without a correspondingly raised specific IgE RAST score, this may have been attributable to IgG₄ antibodies. It is possible that antibody of this type is more widely involved in asthma (Parish, 1971b), particularly in patients with lower levels of total IgE antibody in the serum, as has been suggested by the work of Bryant *et al* (1973).

The repeated skin prick testing with bacterial antigens of the ABPA patients over a seven month period shows fluctuation in their skin-sensitising antibody response (Table X).

Hampton *et al* (1963) also found an inconsistency of skin-sensitising antibody in their passive transfer tests which they thought was suggestive of transient sensitivity, since the antibody appeared and disappeared under influence of exposure to aerosol and by injection.

More work is needed before any conclusion can be reached but it may be that these fluctuating results are due to an exacerbation of symptoms superimposed upon the steady chronic condition. Such a history is, for example, characteristic of asthma.

ESSENTIALS FOR RAST ASSAYS

Calculations by Ishizaka and Ishizaka (1973) indicate that more than 99% of the IgE is not fixed to mast cells or basophils but free in the circulation. Thus a serological test for IgE antibodies should theoretically reflect a clinical allergy in the respiratory or the gastrointestinal tract at least as well as a skin test.

Previous work using RAST tests have shown this technique to be particularly suitable for measuring the level of IgE in patients' serum, for the results are not affected by a high content of non-allergic constituents in the antigen which, in in vivo tests, may give rise to false positives.

Nevertheless, the quality of the allergen and the specificity of the anti-IgE employed are both critical for reproducible and meaningful results.

Allergens

Great variability in the quantitative composition of available allergen extracts has been reported, e.g. variations in allergen potency of up to 100 to 1000-fold among pollen extracts designed to have the same concentration (Foucard et al, 1972).

Many species, e.g. grasses, have closely related allergenic determinants so that an extensive cross-reaction can occur in vivo and also in the RAST. In contrast, a species may contain several non-cross-reacting allergenic entities, e.g. ragweed pollen. Both species-specific and non-specific allergenic determinants probably occur simultaneously. A close relationship between allergy to birch pollen and hazel nut is well known, and a partial neutralization of a reaginic reaction to birch pollen by extracts of hazel nut has been reported (Belin, 1972).

In contrast to the freshly grown S. aureus cells of Schopfer et al (1979), any batch variation was obviated in this study by each of the three bacteria studied being coupled to activated Sepharose 4B from the same freeze dried culture prepared at the Microbiological Research Establishment, Porton. Keeping the composition of the antigens constant was essential in order that the various determinations, carried out over a period of time, were comparable.

In addition to close control over the quality of the antigens, it is important that the allergen in the allergen-polymer complex (APC) be present in relative excess because a reaginic serum often, and certainly in many of the sera involved in this study, contains allergen-specific antibody other than reagin.

Anti-IgE antiserum

Two non-cross-reacting antigenic determinants D_1D_2 have been identified on the epsilon chain of IgE (Bennich and Johansson, 1970). Antisera raised to the D_2 antigen was found to give the best technical results, but because of its heat lability, Johansson recommends that the antiserum used in RAST in addition contain activity to the heat stable D_1 specificity.

The anti-IgE goat antiserum provided by Dr. C.E. Arbesman was raised to the complete Fc fragment of the purified IgE myeloma, i.e. containing both D_1 and D_2 determinants.

RAST results have been found to correlate well with those of PK tests with purified codfish allergen (Foucard et al, 1973) and with passive sensitisation of chopped human lung with extracts of Timothy and birch pollen and horse dander (Foucard,

1972). Results with complex or impure allergens have not been so clear-cut, e.g. D. farinae. Thus a correlation of 60-75% was found between RAST and skin testing using 14 antigens (Wide et al, 1967) and of 74-100% with provocation challenge test, depending on the type and purity of the allergen preparations used (Berg et al, 1971; Wide, 1973).

Stenius et al (1971) also obtained good correlation between RAST and skin prick testing with Dermatophagoides spp. and grass pollen.

Belin and Norman (1977) obtained significant correlation between RAST and symptom severity scores, which in turn correlated with skin tests ($p < 0.01$) with B. subtilis.

RAST EVALUATION

Any immunological technique for determining IgE must be evaluated first in terms of the specificity and sensitivity of the test and then in accordance with the degree of correlation of the results obtained with clinical studies.

The immune system is not a simple 'lock and key' in which only one antigen can bind to IgE, but instead there is often a broad range of antigens of better or worse fit and consequently of greater or lesser avidity. In the absence or relative paucity of the 'best fit', binding to less well fitted antigens will inevitably occur. Thus specificity is more properly a function of the antigen/antibody system and not of the test per se. The inherent specificity of the test is thus largely a theoretical concept.

Depending upon the relative proportions of the specific IgE present in the serum and of other closely

compatible IgE species, a degree of cross-reactivity will be inevitable. This will be exacerbated if the antigens towards which the IgE is directed possess common or closely related antigenic sites.

Nevertheless, the inhibition studies previously reported in Chapter IV, 4.6 show that 100% inhibition was obtained with S. pneumoniae (Figs. 32 and 35) and with H. influenzae (Figs. 34 and 35) and that inhibition with S. aureus was 68% (Figs. 33 and 35) (see Discussion, Chapter IV, 4.6.2). In the cross-reactivity studies (Fig. 36) the reaction to S. pneumoniae is clearly distinguished from that of the other two competing antigens and this indicates the potential of the RAST test developed in this study. Although the discrimination is not so good for H. influenzae and S. aureus, in each case the desired antigen is shown to bind preferentially to the Sepharose-antigen solid phase. Thus although it may be possible to improve the discrimination of the test, the RAST assay developed seems to be a useful tool.

Further evidence for specificity comes from the lack of correlation between the patients' total IgE levels and their RAST scores for specific IgE. This matter of specificity is also relevant in considering the sensitivity of the test. The total IgE standard curve (Fig. 5) shows a substantially linear response down to 60 IU/ml whilst cord serum (the background score) was estimated to contain 11 IU/ml. This figure compares favourably with the level of 240 IU/ml calculated as the upper limit for normal levels (Chapter V, 4.1).

The correlation values between the various immunoglobulin RAST scores are shown in Table XII. In order to see if raised specific IgG occurred when raised specific IgE was

found, comparisons were made between these two parameters. There was not a significant correlation with H. influenzae, but with S. aureus and S. pneumoniae low positive correlations were found of $r = 0.2891$ ($p < 0.05$) and $r = 0.3056$ ($p < 0.01$) respectively.

When the correlation between IgE and IgG₄ alone was considered, a significant correlation was found in the case of S. aureus only in the asthmatic group, $r = 0.4873$ ($p < 0.05$). Non-significant negative correlations were found with H. influenzae. These low correlations between IgE and IgG₄, together with elevated values of specific IgG₄ to these organisms not being found, suggest that Type I reactions are due mainly to mediation of IgE and not to S-TS IgG. Only with S. pneumoniae were significant correlations found between IgE and IgG₄ RAST scores, $r = 0.3202$ ($p < 0.01$) for all the disease groups and $r = 0.5141$ ($p < 0.05$) for rhinitis alone, thus suggesting that IgG₄ may have a role in the immediate type response with this organism.

Another operational evaluation of the RAST technique can be made in terms of its correlation with the skin prick tests. Despite the reservations about the efficacy of these tests that have been mentioned earlier, they are widely regarded as relevant indicators of clinical condition. Correlations of $p < 0.001$ and $p < 0.02$ were obtained between RAST and skin prick tests specific to S. pneumoniae and S. aureus respectively. However, no significant correlation was obtained for H. influenzae. It appears likely from the trend of the figures that this lack of correlation is due mainly to the low number of positive skin prick tests (5%). As previously stated (Chapter V, 4.5), the concentration of

antigen used in skin prick testing to H. influenzae was probably too low. Further work with a stronger H. influenzae antigen would elucidate this point.

In the study certain patients have been found to have raised specific IgE RAST scores but a negative skin prick test. This may be due to circulating IgE antibodies being detected without concurrent fixation to mast cells in the skin. Where patients had multiple skin test reactivity, it is possible that receptor sites of skin mast cells were completely saturated with other allergen-specific IgE or IgG₄ molecules at the time of skin testing.

In this situation, with a raised RAST score and a negative skin prick test, more information of the allergic reaction could be obtained by bronchial provocation testing. This on its own tells one little about whether the mechanisms involved are allergic, irritant, pharmacological, etc., but does give a more determinate causal answer, especially when information from other tests is taken into account. Indeed Citron et al (1958) showed bronchial provocation testing to be better than skin testing in ascertaining which antigens caused asthma in particular individuals.

Studies by other workers have shown some evidence for local production of IgE where sensitisation of an organ has occurred with no generalised distribution of specific IgE antibody into the serum or the skin, e.g. Tada and Ishizaka (1970) showed maximum concentration of IgE plasma cells to be present in infected tonsils.

Evidence for this local production of IgE has been found in some cases of allergic rhinitis, e.g. Huggins and Brostoff (1975) and Merrett et al (1976). In the latter

study, of 19 patients with low serum IgE and no detectable specific IgE, five were found to have nasal specific IgE and a further eight had increased nasal total IgE.

This raises the possibility that the local level of IgE fixed in the lung may not be truly reflected by serum levels generally. This effect is probably of only minor importance in the present study because skin prick tests were found to correlate well with RAST scores and total IgE levels. Direct measurement of IgE levels in the lung (e.g. from lung lavages) would shed more light on this matter, but this material can not be obtained as a routine experimental procedure because of ethical problems.

CLINICAL CORRELATIONS OF THE STUDY

Blood bank donor group

In this study the blood bank control group had the lowest geometric mean total IgE (35 IU/ml) and did not have raised mean specific IgE, IgG nor IgG₄ RAST scores to any of the three bacteria studied.

Chronic bronchitis

H. influenzae is by far the most common pathogen implicated in chronic bronchitis, May (1972) reporting that it was present in at least 80% of patients with persistently or recurrently purulent sputum. In the present study the specific IgG levels to H. influenzae were not significantly raised (RAST score mean 2.51) compared with the blood bank and other groups, although evidence of the importance of H. influenzae in chronic bronchitis was found as the specific IgE levels (mean 1.7) were significantly higher than those of

the asthma, rhinitis, urticaria and blood bank groups. Turnbull et al (1978) found appreciable amounts of IgE in the sputum from both many chronic bronchitics and skin test positive asthmatics, thus it would be of interest to use the RAST developed here to measure specific IgE levels in sputum to bacteria, especially H. influenzae.

The other pathogen commonly found in chronic bronchitics is S. pneumoniae which assumes its greatest importance in acute exacerbations. Since only five of the 30 chronic bronchitics studied were on antibiotics and were fit enough to attend an out-patient clinic, it can be taken that few, if any, were in an acute phase. This is reflected in this study with a specific IgG RAST score group mean of 2.82 (not significantly raised) and neither a raised IgE nor IgG₄ RAST score group mean.

May and May (1963) concluded that S. aureus was a non-pathogen in chronic bronchitis on the basis that its suppression by chemotherapy did not affect the clinical picture. They found S. aureus in only 9% of chronic bronchitics with purulent sputum. The present results show that IgE RAST score mean to S. aureus was significantly lower than that of the other disease groups. The specific IgG and IgG₄ RAST scores were also the lowest in any disease group.

Allergic bronchopulmonary aspergillosis (ABPA)

Dual skin reaction (Types I and III) reaction to Aspergillus fumigatus is accompanied by positive precipitin tests in more than 90% of ABPA cases (McCarthy and Pepys, 1971). A similar dual response was found in this disease group to the bacterial allergens studied. These patients with ABPA were seen to be the most reactive group, giving the highest specific

IgE RAST scores to all three bacteria and also having the highest geometric mean total IgE (1520 IU/ml). Additionally, they had the significantly highest IgG RAST score to S. pneumoniae (mean 3.19); with the cystic fibrotics they were significantly higher than the other groups to S. aureus (mean 2.99), whilst with H. influenzae (mean 2.63) they were second to the cystic fibrosis group (mean 3.21), which was significantly higher than all other groups. The ABPA group also had a significantly raised IgG₄ RAST mean score to S. pneumoniae, thus this antibody may be a contributing factor to the Type I response in this disease group.

Bronchial asthma

The IgE RAST scores were elevated for all three organisms when compared to the blood bank donor group, but not significantly so.

Secondary bacterial infection is thought to be of little importance in uncomplicated asthma and Burns and May (1967) confirmed this for H. influenzae by demonstrating that the incidence of H₁ antibody was no higher in the serum of such patients than in that of control subjects. In patients with chronic asthma, however, persistent expectoration is the rule and susceptibility to infection could be as high as in chronic bronchitis.

In this study the specific IgG and IgG₄ mean RAST scores to S. pneumoniae (3.07 and 1.05 respectively) were both significantly higher than the patients without asthma. Together with the evidence of raised IgE levels this suggests that there may be both a Type I (IgE and IgG₄-mediated) and Type III response to S. pneumoniae in asthmatics.

Allergic rhinitis

There were no significantly raised mean RAST score levels in any category in this disease group although the mean specific IgE to S. pneumoniae was raised (1.3).

However, evidence has been cited, e.g. Huggins and Brostoff (1975) and Merrett et al (1976), for the local production of IgE in this disease and further work including testing nasal washings by the RAST technique would be useful in establishing the allergic status of rhinitis.

Urticaria

In this group there was a significantly raised mean IgG₄ RAST score together with a non-significantly raised mean IgE RAST score to S. pneumoniae. These results are suggestive of a Type I hypersensitivity reaction mediated by S-TS IgG and possibly also by IgE.

Cystic fibrosis

It is held that S. aureus is the commonest pathogenic bacterium found in the lungs in this condition (Anderson, 1938; Farber, 1944; Di Sant'Agnese and Anderson, 1946; Burns and May, 1968) and indeed some authorities, notably Lawson (1969), believe that Staphylococcal infection is responsible for the initial lung damage upon which secondary infection by *Pseudomonas* or *Haemophilus* is subsequently superimposed. Of the 30 cystic fibrosis patients Burns and May (1968) studied, 80% were found to have precipitins to H. influenzae and 53% to S. aureus (compared with 8% and 9% respectively of controls).

These results are substantiated in the present study where the cystic fibrosis group had the most significantly raised specific IgG RAST scores to both S. aureus (group mean 3.05) and to H. influenzae (group mean 3.21). The IgE mean

RAST score to S. aureus was not raised significantly (group mean 1.2), but the IgE mean RAST score to H. influenzae (group mean 1.7) was significantly higher than those of the rhinitis, urticaria and blood bank groups. Since there was no correspondingly raised IgG₄ RAST score to H. influenzae the Type I response appears to be mediated by IgE.

Seventy per cent of the present cystic fibrosis patients were atopic which is in agreement with Warren et al (1975) in their study of 43 cystic fibrotics. The capacity to produce IgE antibody to common allergens in atopic subjects has been attributed in part to increased permeability of mucosal membranes (Leskowitz, Salvaggio and Schwartz, 1972). The mucosal abnormality seen in cystic fibrosis patients may be responsible for penetration of common allergens with the stimulation of IgE antibody by the IgE producing plasma cells, which have been shown by Tada and Ishizaka (1970) to lie in the lymphoid tissues of mucosal membranes.

SUMMARY OF RESULTS

In this study evidence favouring the involvement of Type I allergic reactions has been given by the significant association between the presence of positive skin prick tests and raised levels of specific IgE antibody in the serum.

With S. aureus and S. pneumoniae the IgE RAST scores of all the ABPA patients were significantly higher than those in all other groups, whereas in chronic bronchitis the scores were significantly lower. In contrast, with H. influenzae the IgE RAST scores in the chronic bronchitis and cystic fibrosis groups were significantly raised in addition to those of the ABPA group.

There was a positive correlation between the patients' S. aureus and S. pneumoniae immediate-type skin reactions and their RAST scores and their total serum IgE concentrations. The low incidence of immediate-type skin test positivity to H. influenzae prevented comparable correlations with this organism.

RAST is seen to give a more definitive answer in the measurement of the patient's allergic reaction to an allergen and the test can be adapted for quantifying other immunoglobulins.

With regard to the IgG RAST scores, the ABPA and asthma groups had significantly higher scores to S. pneumoniae than other groups. With H. influenzae the cystic fibrosis group were significantly highest, whilst with S. aureus the ABPA and cystic fibrosis groups together were significantly higher than the remainder.

Of the Sepharose IgG₄ RAST scores estimated, those for S. aureus and H. influenzae showed no significant differences between the groups. However, with S. pneumoniae the values for the asthma, ABPA and urticaria groups were found to be significantly higher than the rest.

These results, taken together, suggest that specific IgE and IgG antibodies to bacteria can be recognised in the sera of patients from a number of respiratory diseases, especially ABPA. With further development of the technology the role of bacteria in atopic and allied disease may be more clearly defined.

PLANS FOR FUTURE WORK

The findings of this study suggest several avenues which should be explored further.

Further evidence that the bacteria mediated an immediate allergic reaction could be obtained by the bronchial provocation testing of selected patients from this study who had raised specific IgE bacterial RAST scores.

More direct evidence for the anaphylactic nature of positive specific skin prick tests could be obtained by the measurement of histamine release from allergen challenge of washed leucocytes in those patients giving a positive specific skin test.

In addition to the identification of serum IgE antibodies, the RAST developed in this thesis could be used for the detection and measurement of IgE antibodies to bacteria in other body secretions, such as sputum, and nasal and lung washings. It would also be of interest to relate the IgE concentrations found in these secretions with levels of Type I mediators released, e.g. histamine, SRS-A.

Since there is now considerable evidence that IgG₄ is the short-term sensitising anaphylactic antibody responsible for a minority of positive skin prick tests, further studies could encompass the measurement of total levels of serum IgG₄ to see if there is any correlation between these two criteria.

The demonstration of specific IgE serum antibodies to bacteria in the present study and their correlation with positive specific skin prick test suggests the need for the evaluation of such antibodies in relation to other diseases,

e.g. eczema, and to additional bacteria in the disease groups already studied, e.g. Pseudomonas aeruginosa in cystic fibrosis.

If the antigens can be characterised and the groups at risk identified, there may well be a case for reconsidering the role of desensitisation.

BIBLIOGRAPHY

- AAS, K. (1961).
Nasal eosinophilia in so called bacterial hypersensitivity in asthmatic children. *Acta paediat.*, Stockh. 50, 1-6.
- AAS, K., BERDAL, P., HENRIKSEN, S.D., and GARBORG, O. (1963).
'Bacterial Allergy' in childhood asthma and the effect of vaccine treatment. *Acta paediat.*, Stockh. 52, 338-344.
- AAS, K., and JOHANSSON, S.G.O. (1971).
The radioallergosorbent test in the in-vitro diagnosis of multiple reaginic allergy. *J. Allergy clin. Immunol.*, 48, 134-142.
- ABERNETHY, T.J., and AVERY, O.T. (1941).
Occurrence during acute infections of protein not normally present in blood; distribution of reactive protein in patients' sera and effect of calcium on flocculation reaction with C polysaccharide of pneumococcus. *J. exp. Med.*, 73, 173-182.
- ANDERSON, D.H. (1938).
Cystic fibrosis of the pancreas and its relation to coeliac disease: A clinical and pathological study. *Amer. J. of Diseases of Children.*, 56, 344.
- ANDERSON, S.A., SILVERMAN, M., KONIG, P., and GODFREY, S. (1975).
Exercise induced asthma. *Brit. J. dis. Chest.*, 69, 1-39.
- ASSEM, E.S.K., and TURNER-WARWICK, M. (1976).
Cytophilic antibodies in bronchopulmonary aspergilloma and cryptogenic pulmonary eosinophilia. *Clin. exp. Immunol.*, 26, 67-77.
- AUSTRIAN, R., and MACLEOD, C.M. (1949).
Type-specific protein from pneumococcus. *J. exp. Med.*, 89, 439-450.
- AXEN, R., PORATH, J., and ERNBACK, S. (1967).
Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature* 214, 1302.
- BADDILEY, J. (1962).
Teichoic acid in walls and cells of gram positive bacteria. *Fed. Proc.*, 21, 1084-1088.
- BELIN, L. (1972).
Immunological analyses of birch pollen antigens, with special reference to the allergenic components. *Int. Arch. Allergy Appl. Immunol.*, 42, 300-322.
- BELIN, L.G., and NORMAN, P.S. (1977).
Diagnostic tests in the skin and serum of works sensitized to Bacillus subtilis enzymes. *Clin. Allergy.*, 7, 55-68.

- BENNICH, H., and JOHANSSON, S.G.O. (1970).
Immunoglobulin E and immediate hypersensitivity.
Vox Sang., 19, 1-13.
- BERG, T., BENNICH, H., and JOHANSSON, S.G.O. (1971).
In vitro diagnosis of atopic allergy. I. A comparison
between provocation tests and the radioallergosorbent
test. *Int. Arch. Allergy*, 40, 770.
- BERQUIST, G. (1952).
Analysis of a series of asthma cases treated with
autovaccine. *Acta med. Scand.*, 144, 101-106.
- BERRENS, L., KOERS, W.J., and BRUYNZEEL, P.L.B. (1977).
IgE and IgG₄ antibodies in specific allergies. *Lancet*
ii, 92.
- BOGDANOVE, E.M., and STRASH, A.M. (1975).
Radioiodine escape as an unexpected source of RIA error
and chronic low level environmental contamination.
Nature 257, 426.
- BRANEFORS-HELANDER, P. (1973).
Serological studies of Haemophilus influenzae. III. The
endotoxic effect of various antigen preparations and
the relation between this effect and demonstrable
precipitinogens. *Int. Archs. of Allergy Appl. Immunol.*,
44, 585.
- BRUYNZEEL, P.L.B., and BERRENS, L. (1979).
IgE and IgG₄ antibodies in specific human allergies.
Int. Arch. Allergy Appl. Immunol., 58, 344-350.
- BRYANT, D.H., BURNS, M.W., and LAZARUS, L. (1973).
New type of allergic asthma due to IgE 'reaginic'
antibody. *Brit. med. J.*, 4, 589-592.
- BRYANT, D.H., BURNS, M.W., and LAZARUS, L. (1975).
Identification of IgG antibody as a carrier of reaginic
activity in asthmatic patients. *J. Allergy clin.*
Immunol., 56, 417-428.
- BULL, F.G., and SMITH, R. (1977).
Local and systemic antibody responses to Haemophilus
influenzae in chronic respiratory disease. *Thorax*,
32, 235.
- BURKHOLDER, P.M. (1968).
"Structural Basis of Renal Disease". Becker, E.L. (ed),
p. 197. Harper, New York.
- BURNS, M.W. (1967).
Immunological aspects of non-tuberculous respiratory
infections. Ph.D. Thesis (Univ. London).

- BURNS, M.W. (1968).
Precipitins to pneumococcal C-substance polysaccharide in the serum of patients with chronic bronchial disorders. *Lancet* i, 223-225.
- BURNS, M.W., and MAY, J.R. (1967).
Haemophilus influenzae precipitins in the serum of patients with chronic bronchial disorders. *Lancet* i, 354-358.
- BURNS, M.W., and MAY, J.R. (1968).
Bacterial precipitins in serum of patients with cystic fibrosis. *Lancet* i, 270-272.
- CAMPBELL, R.M., CUTHBERTSON, D.P., and MATHEWS, C.M.E. (1956).
Behaviour of ^{14}C and ^{131}I labelled plasma proteins in the rat. *Int. J. Appl. Radiation & Isotopes*, 1, 66.
- CARMALT-JONES, D.W. (1909).
The treatment of bronchial asthma by a vaccine. *Brit. med. J.*, 2, 1049-1050.
- CAULFIELD, A.H.W. (1930).
Local passive transfer to a frequently recovered strain of Staphylococcus haemolyticus. Case report. *J. Allergy*, 2, 372-374.
- CESKA, M., and LUNDKVIST, U. (1972).
A new and simple radioimmunoassay method for the determination of IgE. *Immunochemistry*, 9, 1021-1030.
- CHANDLER, C.A., FOTHERGILL, L.D., and DINGLE, J.H. (1937).
Studies on Haemophilus influenzae; comparative study of virulence of smooth, rough and respiratory strains of Haemophilus influenzae as determined by infection of mice with mucin suspensions of organism. *J. exp. Med.*, 66, 789-799.
- CIBA FOUNDATION, STUDY GROUP, No. 38 (1971).
Identification of asthma. Edinburgh & London, Churchill Livingstone.
- CITRON, K.M., FRANKLAND, A.W., and SINCLAIR, J.D. (1958).
Inhalation tests of bronchial hypersensitivity in pollen asthma. *Thorax*, 13, 229-232.
- CLARKE, C.W. (1979).
Relationship of bacterial and viral infections to exacerbations of asthma. *Thorax*, 34, 344-347.
- CLOUGH, P.W. (1915).
Some observations on hypersensitiveness to pneumococcus protein, with special reference to its relation to immunity. *Bull. Johns Hopkins Hosp.*, 26, 37-44.

- COCA, A.F., and COOKE, R.A. (1923).
On the classification of the phenomena of hypersensitiveness. *J. Immunol.*, 8, 163-182.
- COHEN, S., HOLLOWAY, R.C., and MATHEWS, C.M.E. (1956).
Distribution and elimination of ^{131}I and ^{14}C labelled plasma proteins in the rabbit. *Biochem. J.*, 62, 143.
- COOKE, R.A. (1932).
Infective asthma: Indication of its allergic nature. *Amer. J. Med. Sci.*, 183, 309-317.
- COOKE, R.A. (1954).
In: "Practice of Allergy". Vaughan, W.T. (ed), p. 730, St. Louis, Mosby., 3rd ed.
- COOMBS, R.R.A., GORDON-SMITH, C.E., and LACHMANN, P.J. (1975).
The allergic reactions as factors determining and influencing microbial pathogenicity. In: "Clinical Aspects of Immunology". Gell, G.H., Coombs, R.R.A., & Lachmann, P.J. (eds), p. 987, Oxford: Blackwell Scientific., 3rd ed.
- COX, J.S.G. (1971).
Disodium cromoglycate-mode of action and its possible relevance to the clinical use of the drug. *Brit. J. dis. Chest.*, 65, 189-204.
- CROFTON, J., and DOUGLAS, A. (1975).
Respiratory Diseases. p. 433., Oxford: Blackwell Scientific Publications., 2nd ed.
- CRUMP, J. (1954).
Value of vaccine treatment in allergic respiratory conditions. *Paed. Clinics, N. Amer.*, Nov, 987-993.
- CUEVAS, M., MARMIER, S., and GIRARD, J.P. (1977).
Analysis of immunological features of intrinsic and extrinsic asthma and clinical response to DSCG. *Clin. Allergy*, 7, 516.
- DAVIES, J.L., LAUGHTON, C.R., and MAY, J.R. (1974).
An improved test for Haemophilus influenzae precipitins in the serum of patients with chronic respiratory disease. *J. clin. Path.*, 27, 265-268.
- DAVIES, R.J. (1976).
Asthma, chronic bronchitis and bacterial allergy. M.D. Thesis (Cambridge).
- DEGRE, M. (1969).
Phagocytic and bactericidal activities of peritoneal and alveolar macrophages from mice. *J. med. Microbiol.*, 2, 353.

- DENNY, F.Y. (1974).
Effect of a toxin produced by H. influenzae on ciliated respiratory epithelium. *J. infect. Dis.*, 129, 93.
- DE VRIES, K. (1961).
Discussions on the physiopathology of chronic bronchitis. In: "Bronchitis". Orie, N.G.M., and Sluiter, H.J. (eds), p. 221-226, Assen, Netherlands: Royal Van Gorcum.
- DI SANT'AGNESE, P.A., and ANDERSON, D.H. (1946).
Celiac syndrome. IV. Chemotherapy in infection of the respiratory tract associated with cystic fibrosis of the pancreas: Observations with penicillin and drugs of the sulphonamide group, with special reference to penicillin aerosols. *Amer. J. Dis. Child.*, 72, 17-61.
- DI SANT'AGNESE, P.A., DARLING, R.C., PERERA, G.A., and SHEA, E. (1953).
Abnormal electrolytes composition of sweat in cystic fibrosis of the pancreas: Clinical significance and relationship to disease. *Paediatrics*, 12, 549.
- DISHON, T., FINKEL, R., MARCUS, Z., and GINSBERG, I. (1967).
Cell sensitising products of streptococci. *Immunology*, 13, 555-564.
- DOCHEZ, A.R., and AVERY, O.T. (1917).
The elaboration of specific soluble substance by pneumococcus during growth. *J. exp. Med.*, 26, 477-493.
- DOCHEZ, A.R., and GILLESPIE, L.J. (1913).
A biological classification of pneumococci by means of immunity reactions. *J. Amer. Med. Ass.*, 61, 727-732.
- DORFF, G.J., COONROD, J.D., and RYTEL, M.W. (1971).
Detection by immunoelectrophoresis of antigen in sera of patients with pneumococcal bacteraemia. *Lancet* i, 578-579.
- DUBOS, R.J. (1942).
A soluble toxin produced by Haemophilus influenzae. *J. Bact.*, 43, 77.
- EDWARDS, G., BUCKLEY, A.R., FEAR, E.C., WILLIAMSON, G.M., and ZINNERMAN, K. (1957).
Adult chronic bronchitis - the infective factor and its treatment. *Brit. med. J.*, 2, 259-264.
- EMMERSON, A.M. (1980).
The need for a pneumococcal vaccine. *J. antimicrobial Chemotherapy*, 6, 301-309.
- ENGVAL, E., and PERLMANN, P. (1971).
Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry*, 8, 374.

- ENGVALL, E., and PERLMANN, P. (1972).
Enzyme-linked immunosorbent assay. ELISA. III.
Quantitation of specific antibodies by enzyme-labelled
anti-immunoglobulin in antigen-coated tubes. *J. Immunol.*,
109, 129.
- ENGVALL, E., and LJUNGSTROM, I. (1975).
Detection of human antibodies to Trichinella spiralis
by enzyme-linked immunosorbent assay, ELISA. *Acta path.*
microbiol. scand., Sect. C, 83, 231.
- FARBER, S. (1944).
Pancreatic function and disease in early life; pathologic
changes associated with pancreatic insufficiency in early
life. *Arch. Path.*, 37, 238-250.
- FINK, A.I., and GAY, L.N. (1934).
Critical review of 170 cases of urticaria and angioneurotic
oedema followed for period of from 2 to 10 years. *Bull.*
Johns Hopkins Hosp., 55, 280-292.
- FINLAND, M., and DOWLING, H.F. (1935).
Cutaneous reactions and antibody response to intracutaneous
injections of pneumococcus polysaccharides. *J. Immunol.*,
29, 285-299.
- FINLAND, M., and SUTLIFF, W.D. (1931).
Specific cutaneous reactions and circulating antibodies
in the course of lobar pneumonia. 1. Cases receiving
no serum therapy. *J. exp. Med.*, 54, 637-652.
- FLENSBERG, E.W. (1950).
Universal reactions in treatment with bacterial vaccines.
Acta paediat., Stockh., 39, 192-214.
- FLINDT, M.L.H. (1969).
Pulmonary disease due to the inhalation of derivatives
of Bacillus subtilis containing proteolytic enzyme.
Lancet i, 1177-1181.
- FORSGREN, A. (1970).
Significance of protein A production by staphylococci.
Infect. Immun., 2, 672-673.
- FORSGREN, A., and SJOQUIST, J. (1966).
'Protein A' from S. aureus. 1. Pseudo-immune reaction
with human γ -globulin. *J. Immunol.*, 97, 822-827.
- FOUCARD, T., AAS, K., and JOHANSSON, S.G.O. (1973).
Concentration of IgE antibodies, PK titers, and chopped
lung titers in sera from children with hypersensitivity
to cod. *J. Allergy Clin. Immunol.*, 51, 39-44.
- FOUCARD, T., JOHANSSON, S.G.O., BENNICH, H., and BERG, T. (1972).
In vitro estimation of allergens by a radioimmune
antiglobulin technique using human IgE antibodies. *Int.*
Arch. Allergy Appl. Immunol., 43, 360-370.

- FRAENKEL, A. (1886).
Z. Klin. Med., 10, 401.
Cited in Topley and Wilson's "Principles of Bacteriology, Virology and Immunity", 1975, p. 712, 6th ed.
- FRANKLAND, A.W., HUGHES, M.W., and GORRILL, R.H. (1955).
Autogenous bacterial vaccine in the treatment of asthma.
Brit. med. J., 2, 941-944.
- GALANT, S.P., BULLOCK, J., WONG, D., and MAIBACH, M.I. (1973).
The inhibitory effect of anti-allergy drugs on allergens and histamine induced wheal and flare responses. J. Allergy clin. Immunol., 51, 11-21.
- GELL, P.G.H., and COOMBS, R.R.A. (1963).
The classification of allergic reactions underlying disease. In: "Clinical Aspects of Immunology". Gell, P.G.H., and Coombs, R.R.A. (eds)., Blackwell Scientific Publications, Oxford, 2nd ed., p. 575.
- GEWURZ, H., SKIN, H.S., and MERGENHAGEN, S.E. (1968).
Interactions of the complement system with endotoxin lipopolysaccharides: consumption of each of the six terminal complement components. J. exp. Med., 128, 1049-1057.
- GLYNN, A.A. (1959).
Antibodies to Haemophilus influenzae in chronic bronchitis. Brit. med. J., 2, 911-914.
- GOTSCHLICH, E.C., and LIU, T.Y. (1967).
Structural and immunological studies on the pneumococcal C polysaccharide. J. biol. Chem., 242, 463-470.
- GRANT, J.A., and LICHTENSTEIN, L.M. (1972).
Reversed in vitro anaphylaxis induced by anti-IgG: specificity of the reaction and comparison with antigen-induced histamine release. J. Immunol., 109, 20-25.
- GROV, A. (1967).
Studies on antigen preparations from Staphylococcus aureus. 3. On the homogeneity and structure of protein A. Acta path. microbiol. scand., 69, 567-575.
- GROV, A. (1968).
Studies on antigen preparations from Staphylococcus aureus. 4. Separation and purification of protein A and a related precipitinogen. Acta path. microbiol. scand., 73, 400-406.
- HAGY, W.H., and SETTIPANE, G.A. (1976).
Risk factors for developing asthma and allergic rhinitis. J. Allergy clin. Immunol., 58, 330-336.
- HAJOS, M.K. (1960).
A comparative study of skin tests and bronchial tests with bacterial solutions in infective bronchial asthma. Acta Allerg. (Kbh), 15, 517-524.

- HAMPTON, S.F., JOHNSON, M.C., and GALAKATOS, E. (1963).
Studies of bacterial hypersensitivity in asthma.
I. The preparation of antigens of *Neisseria catarrhalis*, the induction of asthma by aerosols, the performance of skin and passive transfer tests. *J. Allergy*, 34, 63-95.
- HAUKENES, G., ELLWOOD, D.C., BADDILEY, J., and OEDING, P. (1961).
Serological cross reactivity between polysaccharide A and teichoic acid of *Staphylococcus aureus*. *Biochem. Biophys. Acta.*, 53, 425-426.
- HEDLUND, P.E.R. (1961).
Clinical and experimental studies on C-reactive protein (acute phase protein). *Acta med. scand.*, 169, Suppl. 361.
- HEIDELBERGER, M., and AVERY, O.T. (1923).
The specific soluble substance of pneumococcus. *J. exp. Med.*, 38, 73-79.
- HELANDER, E. (1959).
Bacterial vaccines in the treatment of bronchial asthma. *Acta Allerg. (Kbh)*, 13, 47-66.
- HENDRICK, D.J., DAVIES, R.J., D'SOUZA, M.F., and PEPYS, J. (1975).
An analysis of skin prick test reactions in 656 asthmatic patients. *Thorax*, 30, 2-8.
- HEPTINSTALL, S., ARCHIBALD, A.R., and BADDILEY, J. (1970).
Teichoic acids and membrane function in bacteria. *Nature, (Lond.)*, 225, 519-521.
- HODSON, M.E. (1977).
Immunological Features of Cystic Fibrosis. M.D. Thesis (Leeds University).
- HOFSTAD, T. (1964).
Studies on the antigenic structure of the 30/81 complex of *Staphylococcus aureus*. I. Agglutinogens. *Acta path. microbiol. scand.*, 61, 558-570.
- HOLDAWAY, M.D., and TURK, D.C. (1967).
Capsulated *Haemophilus influenzae* and respiratory tract disease. *Lancet* i, 358-360.
- HOWARD, J.G., CHRISTIE, G.H., COURTENAY, B.M., LEUCHARS, E., and DAVIES, A.J.S. (1971).
Studies on immunological paralysis. IV. Thymic-independence of tolerance and immunity to type III pneumococcal polysaccharide. *Cell Immunol.*, 2, 614-626.
- HUGGINS, K.G., and BROSTOFF, J. (1975).
Local production of specific IgE antibodies in allergic rhinitis patients with negative skin tests. *Lancet* ii, 148-150.

- HUNTER, W.M., and GREENWOOD, F.C. (1962).
Preparation of I^{131} labelled growth hormone of high specific activity. *Nature (Lond.)*, 194, 495-496.
- HUTCHINSON, H.D., and ZIEGLER, D.W. (1972).
Simplified Radioimmunoassay for diagnostic serology. *Appl. microbiol.*, 24, 742-749.
- ISHIZAKA, K. (1975).
Cellular mechanisms of IgE antibody response. *Int. Arch. Allergy Appl. Immunol.*, 49, 255-270.
- ISHIZAKA, T., DE BERNARDO, R., TOMIOKA, H., LICHTENSTEIN, L.M., and ISHIZAKA, K. (1972).
Identification of basophil granulocytes as a site of allergic histamine release. *J. Immunol.*, 108, 1000-1008.
- ISHIZAKA, K., and ISHIZAKA, T. (1973).
Role of IgE and IgG antibodies in reaginic hypersensitivity in the respiratory tract. In: "Asthma". Austen and Lichtenstein (eds). *Physiology, Immunopharmacology and Treatment*. p. 55-70, Academic Press, New York.
- ISHIZAKA, K., ISHIZAKA, T and HORNBROOK, M.M. (1966).
Physiochemical properties of reaginic antibody. IV. Presence of a unique immunoglobulin on a carrier of reaginic activity. *J. Immunol.*, 97, 75-85.
- JENSEN, K. (1958).
A normally occurring streptococcal antibody in human serum. *Acta path. microbiol. scand.*, 44, 421-428.
- JOHANSSON, S.G.O. (1967).
Raised levels of a new immunoglobulin class (IgND) in asthma. *Lancet* ii, 951-953.
- JOHANSSON, S.G.O., and BENNICH, H. (1967).
Immunological studies of an atypical (myeloma) immunoglobulin. *Immunology*, 13, 381-394.
- JULIANELLE, L.A., and HARTMANN, A.F. (1936).
The immunological specificity of staphylococci. IV. Cutaneous reaction to the type specific carbohydrate. *J. exp. Med.*, 64, 149-159.
- JULIANELLE, L.A., and WIEGHARD, C.W. (1935a).
The immunological specificity of staphylococci. I. The occurrence of serological types. *J. exp. Med.*, 62, 11-21.
- JULIANELLE, L.A., and WIEGHARD, C.W. (1935b).
The immunological specificity of staphylococci. III. The interrelationship of cell constituents. *J. exp. Med.*, 64, 149-159.

- KABAT, E.A., and MAYER, M.M. (1961).
In: "Experimental Immunochemistry", Thomas, C.C. (ed),
p. 373, Springfield, Illinois.
- KAHN, M.C., BALDWIN, H.S., ZEITLIN, R., and SMART, M. (1951).
Cutaneous reactions to staphylococcal polysaccharide,
protein, and an unfractionated extract in hypersensitive
and normal individuals. *J. Allergy*, 22, 237.
- KOCH, R. (1891).
Weitere mittheilungen über ein heilmittel gegen tuberculose.
(Further communications on a remedy for tuberculosis).
Dtsch. Med. Wschr., 17, 101.
Cited in Topley and Wilson's "Principles of Bacteriology,
Virology and Immunity", 1975, p. 1755, 6th ed.
- KRAFT, B., MOTHERSILL, M.H., and NESTMAN, R.H. (1949).
Immediate urticarial reactions to intradermal injections
of bacterial antigens. *Ann. Allergy*, 7, 162-165.
- LAWSON, D. (1969).
Proc. 5th International Cystic Fibrosis Conference
(Cambridge), p. 225.
- LESKOWITZ, S., SALVAGGIO, J.E., and SCHWARTZ, H.E. (1972).
An hypothesis for the development of atopic allergy in
man. *Clin. Allergy*, 2, 237.
- LICHTENSTEIN, L.M., and OSLER, A.G. (1964).
Studies on the mechanisms of hypersensitivity phenomena.
IX. Histamine release from human leucocytes by ragweed
pollen antigen. *J. exp. Med.*, 120, 507-530.
- LINDAU, A. (1933).
Studies on varying responses to pneumococcal infections,
especially lobar pneumonia. *Acta path. microbiol. scand.*,
10, 1-59.
- LIU, T.Y., and GOTSCHLICH, E.C. (1963).
The chemical composition of pneumococcal C-polysaccharide.
J. biol. Chem., 238, 1928-1934.
- LONGBOTTOM, J.L., BRIGHTON, W.D., EDGE, G., and PEPYS, J. (1976).
Antibodies mediating type I skin test reactions to
polysaccharide and protein antigens of *Candida albicans*.
Clin. Allergy, 6, 41-49.
- LONGBOTTOM, J.L., and PEPYS, J. (1964).
Pulmonary aspergillosis: diagnostic and immunological
significance of antigens and C-substance in *Aspergillus*
fumigatus. *J. Path. Bact.*, 88, 141-151.
- MARCH, S.C., PARIKH, I., and CUATRECASAS, P. (1974).
A simplified method for cyanogen bromide activation of
agarose. *Anal. Biochem.*, 60, 149-152.

- MARTIN, R.R., DAUGHERTY, H., and WHITE A. (1965).
Staphylococcal antibodies and hypersensitivity to
teichoic acid in man. *Antimicrob. Agents Chemother.*,
5, 91-96.
- MARTIN, R.R., and WHITE, A. (1968).
Prevention of staphylococcal bacteriophage activity by
antigens. A precipitin in human sera. *J. Bact.*, 95,
2177-2181.
- MARTIN, R.R., and WHITE, A. (1969).
The in-vitro release of leukocyte histamine by
staphylococcal antigens. *J. Immunol.*, 102, 437-441.
- MATHE, A.A., HEDQUIST, P., HOLMGREN, A., and SVANBORGE, N. (1973).
Bronchial hyper-reaction to prostaglandin $F_{2\alpha}$ and
histamine in patients with asthma. *Brit. med. J.*, 1,
193-196.
- MAY, J.R. (1965).
Antibodies to Haemophilus influenzae in the sera of
patients with chronic bronchitis. *J. path. Bact.*, 90,
163-174.
- MAY, J.R. (1967).
Colonial morphology, antigens and pathogenicity of
Haemophilus influenzae. *Sci. basis Med. Ann. Rev.*,
211-226.
- MAY, J.R. (1972).
Chemotherapy of chronic bronchitis and allied disorders.
p. 26, The English Universities Press Ltd., 2nd ed.
- MAY, J.R., HERRICK, N.C., and THOMPSON, D. (1972).
Bacterial infection in cystic fibrosis. *Arch. Dis. in
Childhood.*, 47, 908.
- MAY, J.R., and MAY, D.S. (1963).
Bacteriology of sputum in chronic bronchitis. *Tubercle*,
44, 162-173.
- MCCARTHY, D.S., and PEPYS, J. (1971).
Allergic bronchopulmonary aspergillosis. *Clinical
Immunology: I. Clinical features.* *Clin. Allergy*, 1,
261-286.
- McKEE, W.D. (1966).
The incidence and familial occurrence of allergy.
J. Allergy, 38, 226-235.
- McLEAN, A.J. (1977).
Anaphylactic reactions to endotoxin in guinea-pig tissues:
relationship to endotoxin toxicity. *Br. J. Pharmacol.*,
60, 369-373.

MEDICAL RESEARCH COUNCIL (1965).

Committee on the aetiology of chronic bronchitis.
Definition and classification of chronic bronchitis
for clinical and epidemiological purposes. *Lancet* i,
775-779.

MERRETT, T.G., HOURI, M., MAYER, A.L.R., and MERRETT, J.
(1976).

Measurement of specific IgE antibodies in nasal
secretion - evidence for local production. *Clin.*
Allergy, 6, 69-73.

MORSE, S.I. (1965).

Biological attributes of staphylococcal cell walls.
Ann. N.Y. Acad. Sci., 128, 191-214.

NEUFELD, F., and ETINGER-TULCZYNSKA, R. (1931).

Nasale pneumo kokkeninfektionen und pneumokokkenkeimträger
im tierversuch. *Z. Hyg. Infektkr.*, 112, 492-526.
Cited in Topley and Wilson's "Principles of Bacteriology,
Virology and Immunity", 1975, p. 748, 6th ed.

NEUFELD, F., and HÄNDEL, L. (1909).

Arb. Reichsgesundh Amt., 34, 293.
Cited in Topley and Wilson's "Principles of Bacteriology,
Virology and Immunity", 1975, p. 748, 6th ed.

NICHOLLS, A.C., PEASE, P.E., and GREEN, I.D. (1975).

Agglutinin response to bacterial infection in acute
exacerbations of chronic bronchitis. *J. clin. Path.*,
28, 279-283.

NYE, L., MERRETT, T.G., LANDON, J., and WHITE, R.J. (1975).

A detailed investigation of circulating IgE levels in
a normal population. *Clin. Allergy*, 5, 13.

OMLAND, T. (1964).

Serological studies on Haemophilus influenzae.
6. Examination of a selection of previously untypable
H. influenzae M and S-strains for evidence of type
specific antigens. *Acta path. microbiol. scand.*,
62, 73-98.

ORANGE, R.P., and AUSTEN, K.F. (1971).

The immunological release of chemical mediators of
immediate type hypersensitivity from human lung.
In: "Progress in Immunology, 1". Amos, B. (ed.),
p. 173-192, New York and London; Academic Press.

PARISH, W.E. (1967).

Release of histamine and slow reacting substance with
mast cell changes after challenge of human lung sensitized
passively with reagin in vitro. *Nature (Lond.)*, 215,
738-739.

- PARISH, W.E. (1970).
Short-term anaphylactic IgG antibodies in human sera.
Lancet 2, 591-592.
- PARISH, W.E. (1971a).
Studies on vasculitis. IV. The low incidence of
antibacterial anaphylactic antibodies in the sera of
patients with cutaneous vasculitis following bacterial
infection. *Clin. Allergy*, 1, 433-446.
- PARISH, W.E. (1971b).
Detection of reaginic and short-term sensitizing
anaphylactic or anaphylactoid antibodies to milk in
sera of allergic and normal persons. *Clin. Allergy*,
1, 369-380.
- PARISH, W.E. (1971c).
Studies on vasculitis. I. Immunoglobulins, β 1c,
C-reactive protein and bacterial antigens in cutaneous
vasculitis lesions. *Clin. Allergy*, 1, 97.
- PARISH, W.E. (1972).
Host damage resulting from hypersensitivity to bacteria.
Symposia of the Society for General Microbiology.
Number XXII. Microbial Pathogenicity in Man and Animals,
1972.
- PARISH, W.E. (1974).
Skin sensitizing non-IgE antibodies association between
human IgG S-TS and IgG₄. In: "Progress in Immunology,
II", 4, Brent, L., and Holborow, J. (eds).
North-Holland Publishing Company.
- PASTEUR, L., CHAMBERLAND, C., and ROUX, E. (1881).
C.R. Acad. Sci., 92, 159.
Cited in Topley and Wilson's "Principles of Bacteriology,
Virology and Immunity", 1975, p. 712, 6th ed.
- PAUL, W., and WEIR, D.M. (1969).
Histamine release from human lung by specific antisera.
Clin. exp. Immunol., 5, 311-316.
- PEPYS, J. (1975).
Atopy. In: "Clinical Aspects of Immunology". Gell,
P.G.H., Coombs, R.R.A., and Lachmann, P.J. (eds), p. 887,
Oxford: Blackwell Scientific Publications, 3rd ed.
- PEPYS, J., HARGREAVE, F.E., LONGBOTTOM, J.L., and FAUX, J.
(1969).
Allergic reactions of the lungs to enzymes of Bacillus
subtilis. *Lancet* i, 1181-1184.
- PEPYS, J., WELLS, I.D., D'SOUZA, M.F., and GREENBERG, M.
(1973).
Clinical and immunological responses to enzymes of
Bacillus subtilis in factory workers and consumers.
Clin. Allergy, 3, 143-160.

- PERNIS, B., VIGLIANI, E.C., CAVAGNA, C., and FINULLI, M. (1961).
The role of bacterial endotoxins in occupational diseases caused by inhaling vegetable dusts. *Brit. J. industr. Med.*, 18, 120-129.
- PFEIFFER, R. (1893).
Die aetiologie der influenza. *Z. Hyg. Infektkr.*, 13, 357-386.
- PITTMAN, M., (1931).
Variation and type specificity in the bacterial species Haemophilus influenzae. *J. exp. Med.*, 53, 471-492.
- RACKEMAN, F.M., and GRAHAM, L.B. (1923).
The vaccine treatment of asthma. *J. Immunol.*, 8, 295-315.
- REICHEK, N., LEWIN, E.B., RHODEN, D.L., WEAVER, R.R., and CRUTCHER, J.C. (1970).
Antibody responses to bacterial antigens during exacerbations of chronic bronchitis. *Amer. rev. resp. Dis.*, 101, 238-244.
- RICCI, M., FISHLEWITZ, J., RICCA, M., and PASSALEVA, A. (1967).
A study on bacterial sensitization in the infective asthma. *Acta allerg. (Kbh.)*, 22, 47-56.
- RICH, A.R. (1951).
The Pathogenesis of Tuberculosis. Blackwell, Oxford, 2nd ed.
- RODBARD, D., KAPADIA, G., and CHRAMBACH, A. (1971).
Pore gradient electrophoresis. *Anal. Biochem.*, 40, 135.
- ROSENBACH, F.J. (1884).
Microorganismen bei der wundinfektious kranheiten des menschen.
Weisbaden. Cited by Wilson, G.S., and Miles, A.A. in "Principles of Bacteriology and Immunity", 1964, p. 746, London: Arnold, 5th ed.
- SCHOPFER, K., BAERLOCHER, K., PRICE, P., KRECH, U., QUIE, P.G., and DOUGLAS, S.D. (1979).
Staphylococcal IgE antibodies, hyperimmunoglobulinemia E and Staphylococcus aureus infections. *New Eng. J. Med.*, 300, 835-838.
- SCHWARTZ, H.J., and TERR, A.I. (1971).
The immune response of allergic and normal subjects to pneumococcal polysaccharide. *Int. Arch. Allergy appl. Immunol.*, 40, 520-525.
- SEPULVEDA, R., LONGBOTTOM J.L., and PEPYS, J. (1979).
Enzyme linked immunosorbent assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigens of Aspergillus fumigatus. *Clin. Allergy*, 9, 359-371.

- SHARP, E.A., and BLAKE, F.G. (1930).
Host factors in the pathogenesis of pneumococcus pneumonia. *J. exp. Med.*, 52, 501-517.
- SHELDON, J.M., MATHEWS, K.P., and LOVELL, R.G. (1954).
The vexing urticaria problem: Present concepts of etiology and management. *J. Allergy*, 25, 525-560.
- SLOTT, R.I., and ZWEIMAN, B. (1974).
A controlled study of the effects of corticosteroids on immediate skin test reactivity. *J. Allergy clin. Immunol.*, 54, 229-234.
- SMITH, W., ANDREWES, C.H., and LAIDLAW, P.P. (1933).
Virus obtained from influenza patients. *Lancet* ii, 66-68.
- SPOCK, A., HEICK, H.M.C., CRESS, H., and LOGAN, W.J. (1967).
Abnormal serum factor in patients with cystic fibrosis of the pancreas. *Pediatric Research*, 1, 173.
- SQUIRE, J.R. (1952).
Tissue reactions to protein sensitization. *Brit. med. J.*, i, 1-7.
- STACEY, M., and BARBER, S.A. (1960).
Polysaccharides of micro-organisms. Oxford: Oxford University Press.
- STANWORTH, D.R., and SMITH, A.K. (1973).
Inhibition of reagin-mediated PCA reactions in baboons by the human IgG₄ subclass. *Clin. Allergy*, 3, 37.
- STENIUS, B., WIDE, L., SEYMOUR, W.H., HOLFORD-STREVEENS, V., and PEPYS, J. (1971).
Clinical significance of specific IgE to common allergens. I. Relationship of specific IgE to *Dermatophagoides* spp. and grass pollen, to skin and nasal tests and history. *Clin. Allergy*, 1, 37-55.
- STEVENS, F.A., and JORDANI, L. (1936).
Reactions to intracutaneous injections of nucleoproteins of the upper respiratory pathogenic bacteria in asthmatic patients. *J. Allergy*, 7, 442-450.
- STROMINGER, J.L. (1962).
Biosynthesis of bacterial cell walls. *Fed. Proc.*, 21, 134-143.
- SUTLIFF, W.D., and FINLAND, M. (1932).
Anti-pneumococci immunity reactions in individuals of different ages. *J. exp. Med.*, 55, 837-852.
- SWINEFORD, O., and HOLMAN, J. (1949).
Studies in bacterial allergy; results of 3860 cutaneous tests with 34 crude polysaccharide and nucleoprotein fractions of 14 different bacteria. *J. Allergy*, 20, 420-427.

- TADA, T., and ISHIZAKA, K. (1970).
Distribution of γ E forming cells in lymphoid tissues of the human and monkey. *J. Immunol.*, 104, 377-387.
- THOMLINSON, J.R., and BUXTON, A. (1963).
Anaphylaxis in pigs and its relationship to the pathogenesis of oedema, disease and gastroenteritis associated with Escherichia coli. *Immunology*, 6, 126-139.
- TILLET, W.S., and FRANCIS, T. (1929).
Cutaneous reactions to the polysaccharide and proteins of pneumococcus in lobar pneumonia. *J. exp. Med.*, 50, 687-701.
- TILLET, W.S., and FRANCIS, T. (1930).
Serological reactions in pneumonia with a non protein somatic fraction of pneumococcus. *J. exp. Med.*, 52, 561-571.
- TIPPER, D.J., and BERMAN, M.F. (1969).
Structures of the cell wall peptidoglycans of Staphylococcus epidermidis Texas 26 and Staphylococcus aureus Copenhagen. I. Chain length and average sequence of cross-bridge peptides. *Biochemistry*, 8, 2183-2192.
- TOPLEY, W.W.C., and WILSON, G.S. (1975).
The Streptococci. In: Topley and Wilson's "Principles of Bacteriology, Virology and Immunity", Wilson, Sir G., and Miles, Sir A. (eds), p. 745, Edward Arnold, 6th ed.
- TORTI, M., KABAT, E.A., and BEZER, A.E. (1964).
Separation of teichoic acid of Staphylococcus aureus into two immunologically distinct specific polysaccharides with alpha and beta N. acetyl glucosaminyl linkages respectively. Antigenicity of teichoic acids in man. *J. exp. Med.*, 120, 13-29.
- TUNEVALL, G. (1953).
Studies on Haemophilus influenzae. H. influenzae antigens studied by gel precipitation method. *Acta path. microbiol. scand.*, 32, 193-197.
- TURK, D.C., and MAY, J.R. (1967).
Haemophilus influenzae. Its clinical importance. London: English Universities Press.
- TURNBULL, L.S., TURNBULL, L.W., CROFTON, J.W., and KAY, A.B. (1978).
Immunoglobulins, complement and arylsulphatase in sputum from chronic bronchitis and other pulmonary diseases. *Clin. exp. Immunol.*, 32, 226-232.
- TURNBULL, L.S., TURNBULL, L.W., LEITCH, A.G., CROFTON, J.W., and KAY, A.B. (1977).
Mediators of immediate-type hypersensitivity in sputum from patients with chronic bronchitis and asthma. *Lancet* ii, 526-529.

- van der ZWAN, J.C., ORIE, N.G.M., and de VRIES, K. (1975).
Biphasic reaction after inhalation of Haemophilus influenzae in patients with chronic non-specific lung disease. Clin. Allergy, 5, 224-232.
- VIJAY, H.M., and PERELMUTTER, L. (1977).
Inhibition of reagin-mediated PCA reactions in monkeys and histamine release from human leukocytes by human IgG₄ subclass. Int. Archs. Allergy appl. Immunol., 53, 78-87.
- VOLLER, A., BARTLETT, A., and BIDWELL, D.E. (1976a).
Enzyme immunoassays for parasitic diseases. Transactions of the Royal Society of Tropical Medicine and Hygiene, 70, 98.
- VOLLER, A., BIDWELL, D., and BARTLETT, A. (1976b).
Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In: "Manual of Clinical Immunology", Rosen, N., and Feldman, H. (eds), p. 506, American Society for Microbiology.
- VON PIRQUET, C. (1906).
Allergie. Munch med. Wschr., 30, 1457.
(Appendix in "Clinical Aspects of Immunology", Gell, P.G.H., Coombs, R.R.A., and Lachmann, P.J. (eds), 1975, p. 1723, Blackwell, 3rd ed.).
- WALKER, I.C. (1916).
Studies on the sensitization of patients with bronchial asthma to bacterial proteins as demonstrated by the skin reaction and the methods employed in the preparation of these proteins. J. med. Res., 35, 487-495.
- WALKER, I.C. (1919).
The treatment of bronchial asthma with vaccines. Arch. int. Med., 23, 220-234.
- WARREN, C.P.W., TAI, E., BATTEN, J.C., HUTCHCROFT, B.J., and PEPYS, J. (1975).
Cystic fibrosis-immunological reactions to A. fumigatus and common allergens. Clin. Allergy, 1, 1-12.
- WIDE, L. (1973).
Clinical significance of measurement of reaginic (IgE) antibody by RAST. Clin. Allergy, 3, 583-595.
- WIDE, L., BENNICH, H., and JOHANSSON, S.G.O. (1967).
Diagnosis of allergy by an IN VITRO test for allergen antibodies. Lancet 2, 1105.
- WILSON, G.S., and MILES, A.A. (1964).
Staphylococcus and micrococcus. In: "Principles of Bacteriology and Immunity", Wilson, G.S., and Miles, A.A. (eds), p. 746, London: Arnold, 5th ed.

YOSHIDA, A., MUDD, S., and LENHART, N.A. (1963).

The common protein agglutinogen of Staphylococcus aureus.

II. Purification chemical characterisation and serological comparison with Jensen's antigen.

J. Immunol., 91, 777-782.

APPENDIX I

DETAILS OF PREPARATION OF IMMUNOABSORBANT PURIFIEDANTI-IgE FROM DR. C.E. ARBESMAN (BUFFALO)Major Reference

Bennich, Hans, and Johansson, S. Gunnar O. Structure and function of human immunoglobulin E. In, J.J. Dixon and Henry G. Kunkel, eds., Advances in Immunology, V. 13, Academic Press, 1971, pp 1-55.

Starting Point

20 mg 99.9% pure myeloma P.S. IgE, obtained from Dr. K. Ishizaka.

Five hour papain digest fractionated on G-200, using PBS, pH 7.5. Five millilitre portions collected and O.D. read at 280 nM.

Individual tubes checked in immunodiffusion vs. antiserum specific for Fc of IgE and antiserum specific for Fab. Fractions containing maximal Fc activity and minimal Fab activity were pooled and concentrated.

- A. Fc and Fab fragments were separated in gel electrophoresis. Barbitol buffer, pH 8.6, ionic strength of 0.05 using 40 ml of agarose on a 5 x 7 inch glass plate. A trough 3/16 inch wide x 6 inches in length, was filled with about 1.5 ml of concentrated fragments and electrophoresed for 3 hrs, about 150 V. Initially, immunoelectrophoresis was done on the pooled sample and developed using both anti-Fc of IgE and anti λ antisera.
- B. The trough was evacuated and excised, taking about 1/4 inch of agarose on each side. The two halves of the gel were each frozen in test tubes, and centrifuged.

Denatured gel was washed 2 x with barbital buffer then discarded. The washings were pooled with the corresponding supernatant, tested in I/D and frozen. Several runs were done until the initial pool was all separated into Fc and Fab fragment pools. Protein was estimated by Biuret Method - 3 mg of protein.

A female goat was injected intradermally with 200 μ g of Fc protein in Complete Freund's Adjuvant, at 2-3 intervals for 4 injections. Two hundred millilitres of blood were then harvested 2 weeks after the fourth injection and 200 more after 18 days. Injections were then repeated 2 weeks after the last trial bleeding, followed by similar harvesting. This sequence of injection and harvest continued over the next 12 months until about 3 litres of serum were obtained. The serum titre in immunodiffusion against 100 μ g/ml IgE varied from 1-8 to 1-32 initially, and then levelled off at 1-16 to 1-32 over the balance of the experiment. The entire harvest was pooled to obtain an average titre of 1:32. The goat was never sacrificed since it became pregnant, delivering two kids. The serum of each contained a substantial titre of antibody nearly specific for Fc of IgE.

Serum of the main pool contained minimal reactivity with Normal Human Serum which was absorbed by adding to the pool, copolymerized normal human serum prepared using Ethyl-Chloroformate by the method of Avrameas and Ternyk. After absorption the antiserum failed to

react with NHS, but with the myeloma serum P.S., it reacted to give two lines against 100 μ g IgE and one line against higher concentrations. When a K-IgE myeloma serum was later available, only one well-defined line could be seen, indicating the presence of some anti-ideotope antibody. When radiolabelled for use in the measuring serum of IgE by the radioradial immunodiffusion technique, the antiserum gave a double ring with an occasional normal or allergic human serum.

Antibody to IgE was further purified for use in the RAST and PRIST, initially by mixing the antiserum with Sepharose 4B coupled to myeloma P.S. IgE (10 mg IgE protein/g of CNBr Sepharose 4B). The directions provided by Pharmacia with their CNBr-activated Sepharose 4B were followed closely. A batch technique was chosen over the column method, to ensure better control of the washing away of excess non-Ab proteins.

Elution was performed in the batch method using an excess of 6M-urea in 1 M NaCl after aspiration of wash fluid using a vacuum line (sintered glass funnel).

Recovery of anti-IgE is maximized if the eluate is filtered to remove Sepharose IgE fragments prior to

dialysis and concentration. At least initially, anti-IgE is recovered at a $1\frac{1}{2}$ ratio to 1 of the IgE present in the solid phase. Testing of the concentrated effluent for remaining anti-IgE allows one to maximize the amount of antiserum used for absorption in subsequent runs.

Protein content was determined for the concentrated eluate. After radiolabelling, binding to IgE myeloma-Sepharose approaches 80%, while maximum binding in PRIST with Normal or Allergic serum approaches 50%. An assumption of 50% purity was then assumed, and aliquots of 4 mg protein were then prepared and lyophilized.

APPENDIX 2

IGE RAST SCORE RATIOS.

CHRONIC BRONCHITIS GROUP : IgE RAST SCORE RATIOS

Pt. No.	<u>S. aureus</u>		<u>S. pneumoniae</u>		<u>H. influenzae</u>		Total IgE IU/ml
	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	
1	-	0.84	-	0.95	-	1.39	10
2	-	0.73	-	0.97	-	1.99	70
3	+	0.84	-	1.06	+	1.25	10
4	-	0.80	-	1.32	-	2.38	50
5	-	0.67	-	0.96	-	1.28	23
6	-	0.79	-	0.99	-	1.82	35
7	-	0.79	-	0.81	-	1.62	10
8	-	0.72	-	0.90	-	2.24	35
9	-	0.79	-	0.87	-	1.31	10
10	-	0.79	-	0.92	-	1.26	10
11	-	0.74	-	0.84	-	1.18	10
12	-	0.75	-	0.84	-	1.57	22
13	-	0.82	-	0.70	-	0.89	10
14	-	0.84	-	0.92	-	1.33	35
15	-	0.83	-	0.83	-	0.73	10
16	-	0.89	-	1.04	-	1.76	500
17	-	0.85	-	0.97	-	2.13	420
18	-	0.81	-	1.11	-	1.61	115
19	-	0.93	-	0.92	-	1.44	10
20	-	0.97	-	1.45	-	3.47	2200
21	-	0.71	+	1.39	-	1.68	23
22	-	0.96	-	1.03	-	2.93	320
23	-	0.85	-	1.05	-	0.91	35
24	-	0.82	-	0.89	-	1.59	23
25	-	0.93	-	1.08	-	1.42	34
26	-	0.85	-	0.95	-	1.60	180
27	-	0.83	-	0.96	-	1.01	10
28	-	0.69	-	0.91	-	1.44	10
29	+	1.04	+	1.43	-	3.68	3700
30	-	0.82	-	0.87	-	1.23	70
GROUP	+		+		+		Geom. mean
	2/30	0.8	2/30	1.0	1/30	1.7	41

ABPA GROUP : IgE RAST SCORE RATIOS

Pt. No.	<u>S. aureus</u>		<u>S. pneumoniae</u>		<u>H. influenzae</u>		Total IgE IU/ml
	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	
31	+	2.0	-	2.46	-	4.32	1800
32	+	1.66	-	3.11	-	2.60	4350
33	+	1.22	+	1.10	-	0.94	2220
34	-	1.08	-	1.28	-	0.89	330
35	+	0.74	-	1.38	-	3.01	960
36	+	0.80	-	1.34	-	1.12	420
37	-	1.40	-	1.28	-	0.91	-
38	+	1.39	+	1.90	-	2.27	5100
39	+	1.23	+	1.56	+	1.02	4500
40	+	0.72	+	1.20	+	0.99	198
41	+	1.41	-	2.24	-	1.14	6600
42	-	2.26	-	1.71	-	1.17	9900
43	+	1.64	+	1.58	-	1.22	8400
44	-	1.82	+	2.14	+	6.32	9900
45	-	1.04	-	1.11	-	0.96	45
46	+	1.29	+	5.03	-	2.09	240
47	-	1.38	-	1.16	-	3.05	1635
48	-	1.39	+	3.39	-	1.32	13500
49	+	2.00	+	1.16	-	1.09	690
50	-	1.22	-	1.06	-	1.10	1200
GROUP 12/ 20 ⁺		1.4	9/ 20 ⁺	1.7	3/ 20 ⁺	1.9	Geom. mean 1520

BRONCHIAL ASTHMA GROUP : IgE RAST SCORE RATIOS

Pt. No.	<u>S. aureus</u>		<u>S. pneumoniae</u>		<u>H. influenzae</u>		Total IgE IU/ml
	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	
51	+	1.56	+	1.07	-	1.19	1450
52	?	1.48	?	1.17	?	1.23	830
53	-	1.32	-	1.61	-	1.26	650
54	+	1.75	+	1.02	-	1.91	3800
55	-	1.19	-	1.04	-	1.11	25
56	-	1.29	-	0.95	-	1.19	2100
57	-	1.14	-	0.85	-	1.15	260
58	-	1.07	-	1.04	-	1.15	23
59	-	1.25	-	1.14	-	1.08	10
60	-	0.92	-	0.86	-	0.99	25
61	-	1.08	-	1.00	-	1.20	800
62	-	1.26	-	0.99	-	0.93	400
63	-	0.85	-	1.91	-	1.57	72
64	-	0.96	-	1.13	-	1.18	420
65	-	0.86	-	1.64	-	1.48	1700
66	-	1.12	-	1.24	-	1.09	220
67	-	0.87	-	1.46	-	1.24	500
68	-	0.70	-	1.46	-	1.29	2500
69	-	1.26	-	2.47	-	1.65	1900
70	-	1.30	-	1.04	-	0.98	215
GROUP	$\frac{+}{2/19}$	1.2	$\frac{+}{2/19}$	1.3	$\frac{+}{0/19}$	1.2	Geom. mean 340

ALLERGIC RHINITIS GROUP : IgE RAST SCORE RATIOS

Pt. No.	<u>S. aureus</u>		<u>S. pneumoniae</u>		<u>H. influenzae</u>		Total IgE IU/ml
	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	
71	-	0.67	+	1.24	-	1.07	1700
72	+	1.02	-	1.03	-	1.35	600
73	-	0.46	+	1.55	-	1.09	340
74	-	0.66	+	1.42	-	0.97	210
75	+	1.86	-	1.68	-	1.36	460
76	-	0.85	+	1.13	-	0.93	150
77	-	1.03	-	1.14	-	0.50	740
78	-	1.27	-	1.93	-	0.77	1000
79	-	0.89	-	1.19	-	0.46	520
80	-	0.90	-	0.93	-	0.57	50
81	-	0.80	-	1.08	-	0.53	10
82	-	0.98	-	1.23	-	0.48	140
83	-	1.29	-	0.97	-	1.04	16
84	-	1.65	-	1.20	-	1.17	1800
85	-	0.96	-	1.44	-	1.03	22
86	-	0.82	-	0.90	-	0.85	40
87	-	1.28	-	1.59	-	0.96	16
88	-	0.71	-	1.18	-	0.91	23
89	-	0.96	-	1.13	-	0.76	350
90	-	0.92	-	1.26	-	0.91	35
149	-	1.69	+	1.86	-	1.58	-
150	-	1.31	+	1.59	-	1.34	1555
GROUP	$\frac{+}{2/22}$	1.0	$\frac{+}{6/22}$	1.3	$\frac{+}{0/22}$	0.9	Geom. mean 145

URTICARIA GROUP : IgE RAST SCORE RATIOS

Pt. No.	<u>S. aureus</u>		<u>S. pneumoniae</u>		<u>H. influenzae</u>		Total IgE IU/ml
	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	
91	-	0.76	+	1.22	-	1.01	210
92	+	1.13	+	1.51	-	0.90	10
93	+	1.15	+	1.07	+	1.31	470
94	-	1.09	-	1.08	-	0.94	
95	-	1.23	-	1.30	-	1.00	280
96	-	1.16	-	1.00	-	0.97	10
97	-	1.15	-	1.39	-	1.05	55
99	-	1.18	-	0.92	-	0.92	23
100	-	1.08	+	1.26	-	0.87	140
101	-	1.44	-	1.45	-	0.90	10
102	-	0.88	-	1.92	-	0.73	18
103	-	0.76	-	1.49	-	0.89	260
104	-	0.63	-	1.25	-	0.85	400
105	?	1.10	?	1.25	?	0.96	3700
106	?	1.27	?	1.11	?	0.92	10
107	?	1.23	?	1.03	?	0.92	125
108	?	0.94	?	1.19	?	0.51	330
109	?	1.68	?	1.23	?	0.92	340
110	-	1.08	-	0.90	-	1.10	236
111	-	1.40	-	1.13	-	1.15	485
GROUP	2/15	1.1	4/15	1.3	1/15	0.9	Geom. mean 115

CYSTIC FIBROSIS GROUP : IgE RAST SCORE RATIOS

Pt. No.	<u>S. aureus</u>		<u>S. pneumoniae</u>		<u>H. influenzae</u> H1H2		Total IgE IU/ml
	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	
116	-	1.47	-	1.06	-	1.84	34
117	-	1.38	-	1.11	-	2.46	37
118	-	1.00	-	1.34	-	1.17	340
119	-	1.90	-	1.88	-	4.48	2500
120	-	1.68	-	1.43	-	4.12	4000
121	-	1.59	-	1.45	-	2.23	1800
122	-	0.92	-	0.84	-	0.81	23
123	-	1.29	-	1.07	-	1.50	600
124	-	1.22	-	1.01	-	0.97	11
125	-	1.58	-	1.26	-	2.16	2800
126	?	1.10	?	1.32	?	1.30	105
127	-	1.02	-	0.98	-	1.23	60
128	-	1.03	-	0.99	-	1.55	23
129	-	1.20	-	1.25	-	1.06	370
130	-	1.02	+	1.58	+	0.97	980
131	-	0.87	-	1.02	-	1.49	135
132	-	1.17	+	1.43	-	2.49	560
133	-	0.95	-	0.96	-	0.91	23
134	-	0.78	-	1.45	-	2.04	65
135	-	0.74	-	1.26	-	1.33	86
136	-	1.42	-	0.93	-	1.09	-
137	-	1.27	-	0.76	-	1.45	85
151	-	1.15	-	1.19	-	1.18	430
GROUP	⁺ 0/22	1.2	⁺ 2/22	1.2	⁺ 1/22	1.7	Geom. mean 178

BLOOD BANK GROUP : IgE RAST SCORE RATIOS

Pt. No.	<u>S. aureus</u>		<u>S. pneumoniae</u>		<u>H. influenzae</u>		Total IgE IU/ml
	Extract Skin Prick Test	RAST Score Ratio	Extract Skin Prick Test	RAST Score Ratio	H ₁ H ₂ Extract Skin Prick Test	RAST Score Ratio	
154	?	0.81	?	0.99	?	1.45	89
155	?	1.49	?	1.48	?	1.28	32
156	?	0.94	?	1.20	?	1.27	6
157	?	1.01	?	1.04	?	1.29	57
158	?	1.11	?	1.00	?	1.16	21
159	?	0.84	?	1.27	?	1.10	74
160	?	1.19	?	1.02	?	1.02	108
161	?	0.83	?	0.96	?	1.32	155
162	?	1.01	?	0.84	?	1.06	15
163	?	1.15	?	0.98	?	1.11	23
164	?	1.12	?	0.86	?	1.27	54
165	?	1.15	?	0.80	?	0.93	15
166	?	0.98	?	1.79	?	1.12	8
167	?	1.00	?	0.92	?	1.01	82
168	?	0.95	?	0.94	?	1.19	21
169	?	1.06	?	0.90	?	1.05	39
170	?	0.98	?	1.23	?	1.04	98
171	?	1.16	?	0.84	?	1.08	16
172	?	0.99	?	0.89	?	1.00	21
173	?	1.22	?	1.07	?	1.36	74
MEAN	-	1.0	-	1.0	-	1.1	Geom. mean 35

APPENDIX 3IgG RAST SCORE RATIOS.

CHRONIC BRONCHITIS

IgG RAST SCORE RATIO

Patient No.	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>	Total IgG mg/100 ml
1	2.1	2.8	2.1	970
2	1.9	2.7	2.6	1190
3	2.0	2.2	3.5	1130
4	2.1	2.7	2.6	1260
5	2.2	3.1	2.5	1320
6	2.1	1.9	2.0	1320
7	2.3	3.3	2.9	1190
8	1.8	1.9	2.2	750
9	2.7	2.5	2.5	830
10	2.1	3.9	3.2	1320
11	2.7	2.8	2.4	1290
12	1.7	2.3	1.7	1220
13	2.7	2.8	2.8	1500
14	2.7	2.5	2.4	1160
15	2.7	2.2	2.0	1360
16	2.4	3.1	2.8	1130
17	2.6	2.8	2.3	1190
18	2.7	3.7	2.0	3680
21	3.4	3.6	3.2	1290
29	2.5	3.7	2.5	1390
MEAN	2.37	2.82	2.51	1324

ABPA

Patient No.	IgG RAST SCORE RATIO			Total IgG mg/100 ml
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>	
31	2.9	2.6	2.0	1720
32	2.3	2.9	2.2	1360
33	2.4	2.3	1.7	1030
34	2.6	2.7	3.1	1060
35	2.9	2.6	2.0	1100
36	2.3	2.2	2.5	940
37	2.5	2.0	2.0	830
38	3.1	2.6	2.2	
39	2.8	2.2	2.8	1800
40	3.2	2.6	4.2	
41	3.1	2.9	2.7	1500
42	3.2	2.7	2.1	2200
43	3.7	8.6	2.8	890
44	3.7	2.7	2.7	1530
45	2.4	2.7	2.1	890
46	2.8	3.5	2.8	1720
47	3.5	3.3	3.7	1190
48	5.9	6.6	5.1	2230
49	2.3	3.3	2.3	1360
50	2.2	2.7	1.8	1060
NEAN	2.99	3.19	2.63	1356

ASTHMA

Patient No.	IgG RAST SCORE RATIO			Total IgG mg/100 ml
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>	
51	2.2	2.7	2.0	1530
52	2.8	3.2	3.0	1430
53	3.6	3.0	2.7	1430
54	2.6	2.3	2.2	1030
55	2.8	2.7	2.1	800
56	3.0	3.4	2.3	1130
57	2.3	2.4	2.3	1060
58	1.9	3.0	2.2	830
59	2.6	2.8	2.0	1240
60	2.4	2.5	1.8	1790
61	2.8	3.2	1.9	2140
62	2.3	4.6	2.0	1350
63	2.2	2.4	1.7	1590
64	2.4	3.3	1.7	1290
65	2.5	3.0	1.8	1210
66	2.7	2.9	2.8	1510
67	2.6	3.7	2.4	1590
68	2.6	2.8	1.9	1350
69	3.2	2.9	1.8	1140
70	2.9	2.9	2.1	1320
MEAN	2.62	3.07	2.14	1338

RHINITIS

Patient No.	IgG RAST SCORE RATIO			Total IgG mg/100 ml
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>	
71	2.8	3.3	2.0	1550
72	2.1	2.5	1.8	1140
73	2.9	3.4	3.3	1390
74	2.5	2.9	1.9	-
75	2.8	2.2	2.0	1670
76	3.0	2.6	2.7	2100
77	2.7	2.8	2.0	1790
78	2.2	2.7	1.7	970
79	2.5	2.2	2.6	1630
80	2.4	2.9	2.3	1630
81	1.8	2.1	2.0	1240
82	3.1	3.5	3.0	1670
83	2.0	2.6	2.5	1510
84	2.8	2.6	2.4	940
85	2.5	2.3	2.3	970
86	2.2	2.4	2.4	1790
87	2.6	2.1	2.4	2280
88	3.1	2.3	3.1	1470
89	2.8	2.6	3.0	1510
90	2.6	2.7	3.5	1350
MEAN	2.57	2.64	2.45	1505

URTICARIA

Patient No.	IgG RAST SCORE RATIO			Total IgG mg/100 ml
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>	
91	3.0	2.7	2.6	-
92	2.5	2.7	2.5	750
94	2.1	2.8	3.6	1030
95	2.5	2.0	2.6	1100
96	2.6	2.5	2.1	1470
97	2.5	2.5	2.0	1100
98	3.2	2.8	2.8	1510
99	4.7	3.1	2.7	2850
100	2.7	2.7	2.5	2240
101	2.7	2.8	2.4	1210
102	2.5	2.7	2.7	1320
103	2.7	2.6	3.2	1590
104	2.6	2.4	3.2	1590
105	3.4	2.4	2.1	1590
106	3.0	2.4	2.0	1710
107	2.6	1.8	2.1	1750
108	2.8	2.3	2.2	1170
109	3.1	2.5	2.5	1710
MEAN	2.84	2.54	2.54	1352

CYSTIC FIBROSIS

Patient No.	IgG RAST SCORE RATIO			Total IgG mg/100 ml
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>	
175	3.2	2.0	2.5	780
176	4.1	2.1	3.9	-
177	3.5	3.3	4.3	1710
178	3.1	1.9	2.8	1350
179	4.3	2.2	3.3	1030
180	2.5	2.6	2.9	970
181	3.0	2.7	2.4	1210
182	3.8	2.7	3.2	2240
183	3.5	2.4	3.4	1350
184	3.3	2.2	2.8	1750
185	3.9	2.0	3.3	1880
186	4.3	2.1	3.5	2050
187	2.5	2.2	2.7	1390
188	2.5	2.1	3.6	1510
189	2.4	3.0	2.1	1000
190	2.5	2.4	3.4	1430
191	2.5	1.9	2.6	1270
192	1.9	1.6	2.7	-
193	2.6	2.0	2.9	1510
194	2.8	2.2	4.6	1030
117	1.8	-	4.5	-
MEAN	3.05	2.28	3.21	1414

BLOOD BANK

IgG RAST SCORE RATIO

Patient No.	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>	Total IgG mg/100 ml
154	3.3	3.0	2.6	1750
155	2.5	2.8	2.4	2850
156	2.8	2.4	2.2	1790
157	2.5	2.5	3.6	2010
158	2.7	2.5	2.7	2120
159	3.4	3.0	-	-
160	2.7	2.9	2.1	-
161	2.6	2.8	2.8	1510
162	2.4	2.8	4.0	1470
163	3.5	2.8	-	1670
164	2.6	2.9	2.4	1710
165	2.2	2.7	2.1	1590
166	3.1	2.3	2.6	2480
167	2.1	2.2	2.8	2620
168	2.9	3.2	2.2	1390
169	2.9	2.0	2.3	1790
170	2.0	2.3	2.3	1590
171	3.6	3.2	2.1	1710
172	3.5	2.1	2.6	1350
173	3.2	2.5	2.2	-
MEAN	2.83	2.66	2.56	1847

APPENDIX 4IgG₄ RAST SCORE RATIOS.

CHRONIC BRONCHITIS

Patient No.	IgG ₄ RAST SCORE RATIO		
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>
1	1.18	0.99	1.13
2	1.22	0.91	1.05
3	1.05	1.03	1.11
4	1.16	1.00	1.04
5	1.00	0.91	1.52
6	1.02	0.89	1.12
7	1.37	0.97	1.07
8	1.04	0.89	0.95
9	1.48	0.90	1.05
10	1.37	0.97	1.04
11	1.07	1.04	1.06
12	1.01	0.93	0.99
13	0.90	0.88	1.05
14	1.00	1.02	1.07
15	0.97	0.92	1.04
16	0.95	0.94	1.12
17	0.89	0.82	0.80
18	1.18	1.07	1.08
21	1.18	1.08	1.18
29	1.12	0.95	1.03
MEAN	1.11	0.96	1.07

ABPA GROUP

Patient No.	IgG ₄ RAST SCORE RATIO		
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>
31	1.09	1.01	1.12
32	1.15	1.10	1.40
33	1.20	1.02	1.11
34	1.36	0.90	1.17
35	1.08	0.86	1.09
36	1.21	-	1.10
37	1.13	0.88	1.11
38	1.03	-	-
39	1.10	0.99	1.08
40	1.03	0.81	1.13
41	1.20	1.02	1.20
42	1.12	0.99	1.13
43	1.54	1.29	1.16
44	1.27	1.04	1.09
45	1.21	1.15	1.17
46	1.01	1.02	0.97
47	1.21	0.98	1.15
48	1.34	1.30	1.13
49	1.36	1.18	0.87
50	1.03	1.09	1.02
153	-	0.96	1.10
MEAN	1.18	1.03	1.12

ASTHMA GROUP

Patient No.	IgG ₄ RAST SCORE RATIO		
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>
51	1.09	1.13	1.09
52	1.21	1.16	1.09
53	0.95	1.12	1.06
54	1.65	0.88	1.08
55	0.98	1.09	1.10
56	0.99	1.13	0.96
57	1.43	0.99	1.01
58	0.98	0.98	1.05
59	1.01	1.05	1.28
60	0.88	1.09	1.17
61	1.00	1.02	1.00
62	1.00	1.13	1.03
63	0.93	1.13	1.03
64	1.26	1.01	1.06
65	0.97	1.05	1.21
66	1.18	1.04	1.50
67	1.14	1.06	1.07
68	1.03	0.93	1.04
69	1.12	1.06	1.21
70	1.34	0.95	1.00
MEAN	1.11	1.05	1.10

RHINITIS GROUP

Patient No.	IgG ₄ RAST SCORE RATIO		
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>
71	1.19	-	-
72	1.17	1.01	1.08
73	1.27	1.00	1.07
74	1.15	0.94	1.15
75	1.26	0.93	1.08
76	1.09	1.08	1.12
77	1.02	0.99	1.06
78	0.97	1.37	0.99
79	1.17	0.81	1.86
80	0.91	0.88	0.93
81	1.02	0.81	0.99
82	1.15	0.92	1.13
83	1.04	0.95	0.92
84	0.98	0.76	0.94
85	1.40	-	-
86	1.08	0.90	1.04
87	1.05	0.89	1.06
88	1.16	0.78	1.00
89	1.09	0.96	0.97
90	0.96	0.91	1.04
MEAN	1.11	0.94	1.08

URTICARIA GROUP

Patient No.	IgG ₄ RAST SCORE RATIO		
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>
91	-	-	-
92	1.21	1.09	0.97
93	-	-	-
94	1.05	0.89	0.97
95	1.21	0.98	0.95
96	1.16	1.05	1.12
97	1.53	1.00	1.11
98	1.27	1.13	1.83
99	1.29	1.14	1.14
100	1.09	1.09	1.08
101	0.99	0.91	1.06
102	1.28	0.95	1.04
103	1.07	1.07	1.23
104	1.11	0.94	1.08
105	1.09	1.07	0.99
106	1.10	1.04	1.07
107	1.04	1.00	1.07
108	1.41	0.93	1.12
109	1.17	1.03	1.34
MEAN	1.18	1.02	1.13

CYSTIC FIBROSIS GROUP

Patient No.	IgG ₄ RAST SCORE RATIO		
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>
175	1.21	-	-
176	1.27	-	1.12
177	1.06	-	1.13
178	1.03	-	-
179	1.17	-	-
180	1.24	0.91	-
181	0.93	0.92	-
183	1.52	1.00	1.14
184	1.04	1.03	1.01
185	1.12	0.84	1.02
186	1.17	0.86	1.05
187	1.00	0.90	1.04
188	1.04	0.92	1.08
189	1.11	0.98	1.10
190	1.26	-	-
191	1.27	-	-
192	1.23	0.83	1.09
193	1.20	-	1.40
195	1.15	1.04	1.00
196	1.22	1.04	1.08
197	1.45	1.14	1.12
198	0.97	1.01	0.96
199	1.25	0.95	1.06
200	1.07	0.98	1.08
201	1.22	0.98	1.05
202	1.16	0.80	1.10
203	1.14	0.96	1.25
MEAN	1.17	0.95	1.09

BLOOD BANK GROUP

Patient No.	IgG ₄ RAST SCORE RATIO		
	<u>S. aureus</u>	<u>S. pneumoniae</u>	<u>H. influenzae</u>
154	1.24	0.96	1.16
155	1.15	0.96	0.93
156	1.10	0.95	1.07
157	1.08	1.07	1.22
158	1.03	0.95	1.03
159	1.27	0.95	-
160	0.97	1.01	1.03
161	1.10	0.98	1.09
162	1.08	1.00	1.24
163	1.11	0.99	1.21
164	0.99	0.96	1.07
165	0.99	0.93	1.11
166	-	1.02	1.18
167	1.20	0.92	0.99
168	1.02	0.99	1.02
169	0.94	0.98	1.04
170	0.99	0.92	1.03
171	0.98	0.97	1.17
172	1.05	1.08	1.17
173	1.03	-	-
174	-	0.96	0.75
MEAN	1.07	0.98	1.08

APPENDIX 5STATISTICAL METHODS.

Ms. Margaret Rehahn, the group Statistician, at the Cardiothoracic Institute kindly advised on the following statistical methods.

Method 1 (a). CHI SQUARE (X^2) test

Function: To assess whether the frequency of occurrence of a single characteristic in k different samples was the same in all cases.

Null hypothesis: That the k samples have come from the same population or identical populations with respect to the proportion of cases in the various categories.

NB. Samples depart from normality. Expected result was that the disease groups' scores would be greater than the blood bank donor group's score, therefore a one tailed test was used.

e.g. Incidence of a raised specific IgE level to H. influenzae in disease groups compared with the blood bank donor group.

Method 1 (b). EXTENSION OF MEDIAN TESTS.

Function: To assess whether k independent groups have been drawn from the same population or identical populations with equal medians.

Null hypothesis: That the k samples have come from the same population or identical populations with respect to the median.

NB. Samples depart from normality. No prediction as to

direction of variation, therefore a two tailed test was used.

e.g. Comparison of the incidence of a raised specific IgE level to H. influenzae between the six disease groups.

Method 2. MANN-WHITNEY U-test

Function: To assess whether two samples have been drawn from a single population, where the samples are known not to be normal.

Null hypothesis: That the two samples are drawn from a single population or two populations having the same median.

NB. Samples depart from normality. No prediction as to direction of variation, therefore a two tailed test was used.

e.g. Incidence of a positive skin prick test to S. pneumoniae in patients with bronchial asthma and in patients with urticaria.

Method 3. KRUSKAL -WALLIS one way analysis of variance

Function: To assess whether k independent variables are from different populations.

Null hypothesis: That the k samples come from the same population or from identical populations with respect to averages.

NB. No extreme departure from normality (test is robust in this respect). No predicted direction of variation, therefore a two tailed test was used. This is the most

powerful of the non-parametric tests used.

e.g. Levels of specific IgG to H. influenzae between the disease group patients.

Method 4. PEARSON Product-Moment Correlation coefficient.

Function: To measure the degree of association between two populations that are normally distributed and to determine the significance of degree of association found.

Null hypothesis: That the values of the two properties being measured are unrelated.

NB. The data does not depart from normality sufficiently to render this test unusable. This is the only parametric test used.

e.g. Correlation between specific IgE and IgG₄ levels to S. aureus in the study patients overall.

Significance levels of all tests

The level of rejection was taken to be 0.05 (i.e. 5%) for all tests. Significance was accepted when $p < 0.05$.