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IMMUNOLOGICAL STUDIES IN
EXTRINSIC ALLERGIC ALVEOLITIS.

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A thesis submitted for the degree of
Doctor of Philosophy of the University of Glasgow

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SUMMARY

The lungs are protected against the toxic effects of inhaled dusts and infectious agents by a variety of innate and adaptive responses. However the production of antibody against seemingly innocuous organic dusts can occasionally be associated with hypersensitivity lung disease. The most commonly recognised of these pulmonary hypersensitivity, or allergic, states are acute extrinsic asthma and extrinsic allergic alveolitis. Acute asthma is an obstructive respiratory disease due to an anaphylactic reaction in the central airways and the symptoms occur classically within 30 minutes of antigen inhalation. The role of IgE antibody to the inhaled antigen in the pathogenesis of the disease is well established. Alveolitis, on the other hand, is a respiratory disease with a restrictive ventilatory defect associated with additional systemic symptoms of fever and malaise, which becomes apparent 4 - 8 hours after antigen exposure. The patients often have serum IgG antibody to the inhaled antigen but the role of this antibody in the pathogenesis of the disease is unresolved. This report examines some humoral immune parameters and their involvement in the pathogenesis of extrinsic allergic alveolitis. The patients under investigation were individuals who were regularly exposed to organic dusts during the husbandry of pigeons. This exposure can cause one syndrome of alveolitis namely pigeon breeders disease. This disease is a very useful working model of alveolitis since the population at risk can be easily identified and the relevant antigens purified.

The main antigens were the avian equivalent of secreted IgA, and fragments of this molecule, readily found in saline soluble extracts of pigeon droppings and of the fine powder coating feathers. This protein has partial immunological identity with the main pigeon serum gammaglobulin antigen, and the common antigenic determinants were on the immunoglobulin light chains.

The serum IgG antibody response against these antigens was quantified by radio-immunoassay in a population of pigeon breeders. The antibody levels in non-exposed individuals were very low; always less than 4 ug/ml, and by comparison, the levels

could be measured at approximately 500 ug/ml in the most florid responders among the pigeon breeders. Very few of the subjects had these high titres, however, and the distribution of antibody levels in a population of pigeon breeders demonstrated that increasing numbers of subjects had lower IgG levels with about half of them having insignificant levels, despite extensive avian exposure. The population could therefore be separated arbitrarily into an antibody non-responder or tolerant, group, and high and low responder groups. Some constitutional and environmental factors which might have had a bearing on this responsiveness were examined.

Cigarette smoking appeared to have a profound inhibitory effect on the ability to mount an antibody response to the inhaled antigens and this effect was reversible since some ex-smokers had raised antibody levels. The extent of the antigen exposure also affected the antibody response. The monthly serum antibody level was seen to fluctuate in parallel with the amount of time individuals spent with their birds due to seasonal requirements of husbandry. However, in population studies there was an association between increased avian exposure in decades and decreased antibody levels, which was unrelated to the age of the individuals. This suggests that either a tolerogenic or desensitisation mechanism exists or that there is some self-regulation of exposure in the symptomatic population; for example as individuals with high antibody levels develop disease, they will moderate their exposure in an attempt to remain asymptomatic thus reducing antibody levels. Thus the high antibody responders may be under-represented in the groups with progressive exposure.

The constitutional factors of age, HLA and P1 phenotypes, and the atopic status have no bearing on the antibody responsiveness and disease.

The subjects with the most vigorous antibody response in the IgG class also had the most florid responses in the IgA and IgM classes. Furthermore when antibody activity was measured in the IgG subclasses, it was found in all four in proportion to the total IgG activity. There was therefore a spectrum of responsiveness to the inhaled antigens, and the subjects with

alveolitis tended to be in the high-responder group. The high-responder group included almost all the subjects with raised total immunoglobulin of the three major classes and most of the subjects with raised acute phase proteins, moreover this group also had a high incidence of serum immune complexes and reduced mean levels of complement components, suggesting that in these subjects there was an active inflammatory response allied to the active antibody response against the antigens to which these individuals are constantly exposed.

A close association between antibody and disease was observed, with the incidence of alveolitis rising from 3.7% in the antibody negative group to 50% in the subjects with greater than 50 ug/ml, and virtually everyone with IgG antibody greater than 150 ug/ml had alveolitis. Furthermore in population studies investigating the symptoms associated with alveolitis, the antibody levels increased with an increasingly complete description of the classical symptom of the disease. In order to correlate antibody with the disease process of alveolitis, serial measurements of serum immunological parameters were made in subjects at different phases of the disease. During the acute phase of disease in individuals following inhalation challenge with antigen there was an association between symptoms, physiological changes and the formation of immune complexes with evidence of complement activation and production of C3d. Conversely, in subjects convalescing after an acute episode of alveolitis there was a very rapid fall in circulating immune complex level, the specific IgG titre falling with a half-life similar to the normal IgG turnover and there was a slow return to normal levels for the total immunoglobulins and C4 levels.

Skin testing these pigeon breeders with common allergens, specific avian antigen and tuberculin has demonstrated that the atopic status had no bearing on the disease incidence of alveolitis. There was, however, a high incidence of immediate weal and flare reactions following intracutaneous injection of pigeon serum antigens and the weal diameter correlated with the titre of specific IgG and not with total IgE. This suggested that a possible anaphylactic activity was associated with this antibody, and future considerations of disease mechanism must take

this into consideration, perhaps as an initial trigger for the subsequent immune-complex mediated disease. There was an association between cutaneous anergy to tuberculin and active disease, and there were also no delayed 48 - 72 hour reactions against the avian antigens, despite the probable presence of sensitised lymphocytes in these subjects. This suggests that cellular immune mechanisms were also involved in the disease.

CHAPTER 1.INTRODUCTION1. PULMONARY IMMUNOLOGY.

The large gas-exchanging area of the lung is protected against inhaled bacteria and the toxic effects of airbourne dusts by combinations of various mechanisms of host defence. These include the innate properties of the lung such as reflex coughing or bronchoconstriction and also the anatomical structure of the airways which cause inhaled particles to impact onto the walls of the airways which are lined with ciliated epithelium down to the level of the terminal bronchioles. These cilia constantly waft a layer of mucus ; the "mucociliary blanket", with the impacted dust up into the back of the throat. Inhaled particles which penetrate to the peripheral airways (respiratory bronchioles and alveolar spaces) are met by the phagocytic alveolar macrophages, and a range of soluble proteins which deter the settlement of micro-organisms, these include the iron scavenging properties of lactoferrin, "natural" antibody and complement components.

The lung is not just a passive framework protected by serum derived antibody and cells, but is increasingly understood to function autonomously as an immunoresponsive organ. The production of local antibody and the beneficial effects of local rather than systemic vaccination was first studied by Fazekas de St. Groth (1950) using influenza infections in ferrets as a model of respiratory disease. The comparative effects of pulmonary immunisation and parenteral immunisation on local and serum antibody and cellular immune responses was outlined in a series of papers by Waldman and colleagues (1971, 1972, 1973) in both humans and experimental animals. This work demonstrated the predominance of pulmonary antibody and cellular responses following local rather than systemic immunisation, and the relatively poor local immune responsiveness to parenteral immunisation. The benefits of respiratory vaccination for health care programmes and for the economy form the basis of recent reports (Developments in Biological Standardisation, 1975; Chanock, 1981).

The respiratory system can be arbitrarily divided into upper and lower (central or peripheral) airways, at the broncho-alveolar junction; between the terminal and respiratory bronchioles. This arbitrary division corresponds operationally to distinct immunological structure and function. The general physiological features of the central airways consist of vascularity mediated by the bronchial artery, a rich nerve supply of chiefly cholinergic fibres, atropine sensitivity and an immunological responsiveness mainly mediated by IgA and IgE, and low molecular weight mediators. Peripheral airways are characterised by a vascular supply from the system of the pulmonary artery, a poor nerve supply and a mainly cell mediated or IgG and IgM immune responsiveness.

This immune responsiveness is mediated by the extensive fixed lymphoid tissue present throughout the lung. This tissue has been described classically by Arnold in 1880, but has been recently redefined by Bienenstock, Johnson and Perey (1973) and is now known by the acronym BALT (bronchus-associated lymphoid tissue). This includes the regional lymph nodes (the mediastinal and hilar nodes) which drain the lymph of the respiratory system, the lymphoid nodules strategically sited at branchings of the large and medium-sized bronchi, and the lymphoid aggregates sited in the submucosa and in the lamina propria particularly in the lower respiratory tract.

The organisation of this tissue is progressively simpler from the proximal airways to the peripheral air spaces. The lymph nodes are complex structures similar to others of the human reticulo-endothelial system. The lymphoid nodules contain follicles but lack capsules, and they extend from subepithelium through submucosa to the peribronchial connective tissue. The nodules are covered on their air side by a single layer of flattened, nonciliated epithelium which is infiltrated by lymphocytes; and called lymphoepithelium (Chamberlain, Nopajaroonsri & Simon, 1973). Ultrastructural analysis demonstrated an abundance of lymphatic channels and blood vessels within the follicles and provided morphological evidence for both transvascular and transepithelial migration of lymphocytes. Therefore a potential pathway exists for the interchange of

lymphoid cells among vascular, interstitial and intra-alveolar compartments.

The morphological similarities between these nodules and similar structures in the gut have been drawn by Bienenstock et al (1973) and he suggested a common mucosal immune system. The lymphoid aggregates are compact accumulations of lymphocytes and mononuclear phagocytes, they are very prominent in the respiratory bronchioles and are separated from the lumen also by 'lymphoepithelium'. These lymphoid structures are in direct contact with the blood vessels and close to the respiratory membrane, and are called von Hayek's lymphoepithelial organs (von Hayek, 1960). Lymphatic capillaries originate within these aggregates as blind pouches close to the respiratory membrane and the smallest branches of blood vessels. The alveolar clearance afforded by the lymphatic system is of vital importance and is the subject of an extensive review (Lauweryns & Baert, 1977).

When an antigen is inhaled or experimentally instilled via the trachea it is taken up by these areas of lymphoepithelium (Racz et al, 1977) and processed in the local lymph nodes. This route was first demonstrated using dye uptake (Correll & Langston, 1958). The kinetics of the subsequent antibody and cell mediated immune responses in experimental animals is reported widely in the literature; one typical report is by Brownstein et al (1980). These workers instilled sheep erythrocytes into one lobe of a canine lung. They found histological evidence of an immune response within 2 days in the ipsilateral tracheobronchial lymph nodes, and on day 5 in immunized lung lobes. Pulmonary lymphoid infiltrates appeared initially around pulmonary venules and veins. There was expansion of the infiltrates into alveolar spaces, where mixed mononuclear aggregates were formed in association with alveolar macrophages 7 days after immunization. A similar but attenuated and delayed response occurred within contralateral control lung lobes, although mononuclear aggregates were not found. Activated lymphocytes in lavage samples increased prior to histological evidence of pulmonary lymphoid infiltrates. The authors suggested that after intrapulmonary immunization the lung recruits circulating immunocytes produced in ipsilateral lung-associated lymph nodes as a source of specific antibody-

forming cells in bronchoalveolar air spaces. This is in agreement with experimental lymphocyte recirculation in mice where there is pulmonary recruitment of lymphocytes after 48 hours (Emeson & Veith, 1979). Cell transfer experiments using radiolabelled thoracic duct cells from rats have demonstrated an enhances traffic of recently divided "effector" cells, predominantly B-cells, into the lung (Beacham & Daniele, 1982). Moreover, antigen specific recruitment of circulating T-cells into the lung has been shown in guinea-pigs following intratracheal administration of influenza virus (Lipscomb et al, 1982), and recruitment of specific T-cells with cytotoxic effector cell activity into the lungs of mice has been observed following immunization with alloantigenic P915 cells (Liu, Ishizaka and Plant, 1982). Earlier work has shown that pulmonary T-lymphocytes exhibit memory, since a lymphokine response can occur 2-3 days sooner following a booster (Gado, Johnson and Waldman, 1974; Clancy et al, 1978). Such information is unfortunately not available for humans. However, by using fiberoptic bronchoscopy it has become possible to lavage the human peripheral lung and obtain a profile of the local cells and proteins (Reynolds & Newball, 1974; Daniele et al, 1975). The T-B lymphocyte ratio in lung lavage is the same as in peripheral blood, but there is a larger number of null cells; up to 30%. The percentages of these cells are altered by smoking, where the total number and percentage of alveolar macrophages is increased, and in various pulmonary diseases such as sarcoidosis (Crystal et al, 1981), and also by extrinsic allergic alveolitis and cryptogenic fibrosing alveolitis (Reynolds et al, 1977) where increases in local T-cells and neutrophils are thought to account for and perpetuate disease.

The predominant local humoral antibody response involves secretory antibody, and the work in this field was pioneered by Tomasi and Grey (1972). The respiratory secretions contain IgG, IgA, IgM and IgE and the relative amounts of these differ markedly from serum. Secretory IgA is the predominant immunoglobulin in the upper respiratory fluids (saliva, nasopharyngeal and tracheobronchial secretions) whereas IgG is predominant in the serum. Lavage fluid from the lower respiratory tract contains

intermediate levels of IgG and IgA (Reynolds & Newball, 1974; Kaltreider & Chan, 1976). In general, IgA, IgM and IgE are present in secretions in amounts greater than can be explained on the basis of transudation from serum; this, therefore suggests either some local synthesis or selective active transport. There has been some transudation of radiolabelled IgG from serum measured in the airways therefore the local and systemic antibody levels cannot be strictly separated, however, local synthesis of all the immunoglobulins has been shown by immunofluorescence by Martinez-Tello, Braun & Blanc (1968), Tada & Ishizaka (1970) and Tomasi & Grey (1972). IgA producing cells have been demonstrated in the lamina propria and submucosa of the entire respiratory tract. This locally synthesized IgA is secreted from plasma cells as a dimer linked by a J-chain. This IgA dimer combines covalently to secretory component in epithelial cells and is transported across the cells in endocytic vesicles to the lumen (Goodman et al, 1981), 90% of IgA in bronchial secretions is in this form.

The BALT cells do not stain for cytoplasmic immunoglobulin but consist of predominantly B-cells and null cells, and BALT is thought to be a repository for precursors of antibody producing cells of IgA, IgG and IgE classes (Bienenstock, Clancy & Perey, 1976). BALT cells can repopulate the lamina propria of both lung and gut mucosa with IgA producing cells following lethal irradiation of recipient animals (Rudzik et al, 1975). This suggests a route of recirculation following antigen stimulation, maturation then local antibody production of specific IgA. It also suggests that there may be a common mucosal immunity (Bienenstock & Befus, 1980). This is supported by a reported link between pulmonary and urinary tract IgA production against E. coli 06 in the bladder (Mattsbj, Hanson & Kaijser, 1979).

Local pulmonary antibody production is controlled primarily by T-cells, but other non-specific controlling factors include the effects of alveolar macrophages and pulmonary surfactant. Macrophages have a central role in the immune response (Rosenthal, 1980) and a similarly important role is emerging for the alveolar macrophage (Brain et al, 1978; Hocking

& Golde, 1979). These macrophages can modulate the antibody response depending on the macrophage:lymphocyte ratio. When this ratio is the same as in normal lung there is an in vitro immunosuppressive effect on peripheral blood lymphocyte stimulation (McCoombs et al, 1982), and when the macrophage numbers are reduced the effect is stimulatory (Kaltreider, 1982).

Pulmonary surfactant material isolated from lung lavage fluid contains a potent non-cytotoxic suppressor of lymphocyte proliferation (Ansfield et al, 1979) but the nature of the active substance is still unknown even after considerable biochemical analysis. It is possible that this effect is caused by a soluble factor derived from alveolar macrophages (Yeager, Barsoum & Kagan, 1980).

The importance of the immune system in protecting the lung is best emphasised by the high incidence of pulmonary infections in situations where a patient is immunodeficient or immunocompromised either as an unwanted side effect of cancer chemotherapy or deliberately in an attempt to induce a host to accept transplants, or just non-specifically following surgery (Williams, Krick & Remington, 1976). In this latter case, there was an incidence of post-operative bronchopulmonary infections in 38% of one series of patients (Lazlo et al, 1973).

Immunodeficiency can be a severe life threatening disorder of infants, however, recently there is increasing recognition of defects both mild and severe in almost every component of the immune system in both children and adults. Antibody deficiencies are related to frequent bacterial infections, commonly Staphylococcus aureus and Streptococcus pneumoniae. By far the commonest immunoglobulin defect seen in one study of 43 young people with severe recurrent respiratory infections was a gross or partial selective IgA deficiency (Turner-Warwick, 1975). Of interest in this respect was the association between transient IgA deficiency in children and development of atopy caused by ineffective clearance of allergens (Taylor et al, 1973), however rather than being cause and effect, these may both be a consequence of a more fundamental immunological defect. A selective IgA deficiency can occur in 1 in 700 normal healthy subjects and the difference between these and IgA deficient

subjects who develop recurrent infections was found to be due to a co-existing IgG2 deficiency in 7 out of 43 patients (Oxelius et al, 1981). Selective deficiencies in IgG subclasses were found in patients who had normal IgG levels and had recurrent pulmonary infections (Schur et al, 1970). Four other patients who had frequent pneumonia and bronchiectasis had completely normal immunoglobulin profiles except for a virtual absence of IgG4 (Beck & Heiner, 1981). T-cell deficiencies, however, are associated primarily with opportunistic infections caused by viruses and fungi; Candida albicans, Aspergillus fumigatus and Pneumocystis carinii. Defects in neutrophil function along with serum complement deficiencies also predispose to repeated lung infections. Alper et al (1972) described a congenital complement deficiency of C3 in a patient with recurrent pyogenic infections, and the importance of an intact complement system for the clearance of Gram positive organisms was demonstrated experimentally by Gross, Rehm & Pierce (1978). This complement C3 requirement for efficient killing of S.aureus 502A, but not E.coli ON2, by normal human neutrophils was confirmed by Repine et al (1979). A defect in cellular chemotaxis was reported to predispose to recurrent infections (Witemeyer & Van Epps, 1976) and this type of defect was considered to be part of a common membrane disturbance which was correlated with defective opsonization and recurrent pneumonias in paediatric patients (Murphy & Van Epps, 1979). The effects of immunodeficiency on pulmonary infections are reviewed more extensively by Turner-Warwick (1978) and Williams, Krick & Remington (1976).

2. HYPERSENSITIVITY LUNG DISEASES.

Despite the obvious benefits of these immune responses in maintaining the integrity of the lung and the sterility of the peripheral airways, they can also be involved in hypersensitivity reactions resulting in local tissue damage. It is difficult to draw a concise list of the various immunologically mediated lung diseases because virtually all lung diseases have some degree of immunological involvement. However, conditions which would not strictly comply would include for example recurrent pulmonary infections which commonly occur in immunodeficient or

immunocompromised patients, adult respiratory distress syndrome, silicosis and some forms of primary lung cancer which may involve altered immune surveillance. The lung is also a common site for tissue damage in the collagen vascular diseases and although they undoubtedly involve immune hyper-reactivity the pulmonary disease is only one part of a multi-organ system, therefore this disease could be classified on the periphery of the hypersensitivity lung diseases. There are also difficulties classifying diseases which are undoubtedly hypersensitivities, for example the same antigen; Aspergillus can cause at least three patterns of disease, i.e. asthma, alveolitis or both. The diseases sarcoidosis and cryptogenic fibrosing alveolitis are also difficult to classify because there is no clear aetiological agent and the pathogenesis involves a complex interaction of both cellular and humoral hypersensitivity. However, the flow chart (Table 1) includes a simplified version of current views of the involvement of the immune system in the main hypersensitivity lung disorders.

a) The major classes of hypersensitivity lung disease.

Immediate hypersensitivity (Type 1):

This involves the release of mediators from reagin-sensitized mast cells, causing increased vascular permeability, oedema and smooth muscle contraction. The major reagin in man is IgE and the classical immediate hypersensitivity disease is extrinsic asthma where antigens recognised by the IgE can trigger an acute episode. More recently a similar anaphylactic activity for IgG has been described, however, this activity has not been conclusively designated to any of the four subclasses, although IgG4 has received considerable attention. Several other non-immunological factors contribute to the disease such as bronchial hyper-reactivity, infection, irritants, exercise, emotion and aspirin. These factors predominate in some adult asthma where no immunological sensitivity can be detected.

Cytotoxic, antibody-mediated disease (Type 2):

The clearest example of this is Goodpasture's syndrome (glomerulonephritis and pulmonary haemorrhage). This disease is produced by auto-antibody which reacts with glomerular and

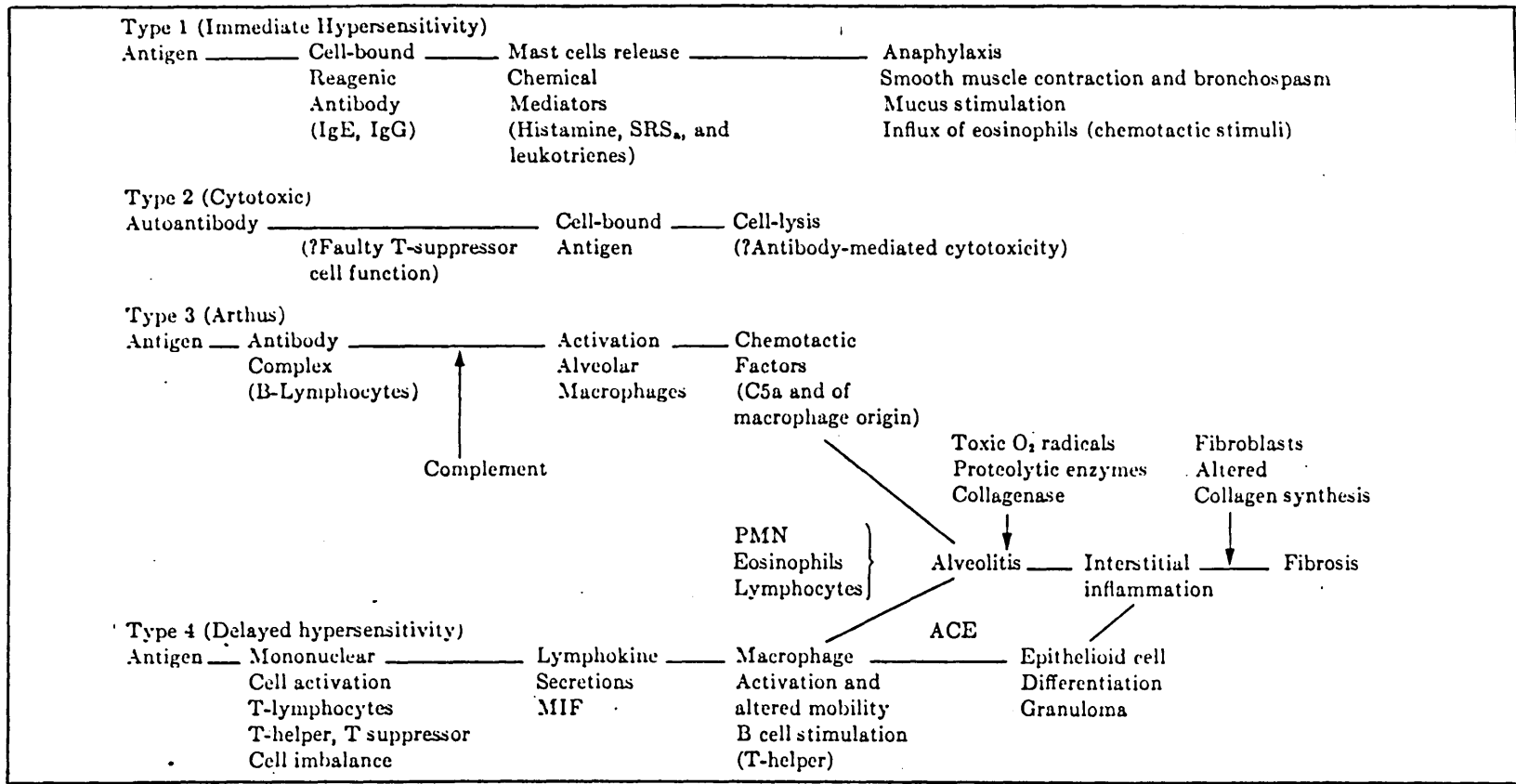


TABLE 1. Some pathways of involvement of the immune system in the disease processes of the four major classes of hypersensitivity reaction.

alveolar basement membrane (Dixon, 1968).

Immune complex, Type 3 and cellular, Type 4 hypersensitivity:

In practice there are no examples of either of these disease profiles in isolation. There is, however, a spectrum of presentation with relatively more evidence for immune complex damage at one end, and cell mediated tissue damage at the other. Cryptogenic fibrosing alveolitis (idiopathic pulmonary fibrosis) is one example where there is a consistent finding of a granular pattern of IgG and C3 shown by immunofluorescence in patients with predominantly cellular changes associated with high titres of auto-antibody (Turner-Warwick, Haslam & Weeks, 1971), but not in patients with predominantly fibrosis. IgG in the airway secretions and in blood, often in the form of immune-complexes seems to be clearly associated with the inflammatory cell reaction in lung tissue, and circulating titres of immune complexes correlate well with the degree of cellularity of histological lesions (Dreisen et al, 1978). The postulated sequence of events commences with immune complex formation with as yet an unidentified antigen, these complexes activate the pulmonary macrophages to release chemotactic factors which can cause an influx of inflammatory cells, which set in motion a cycle of tissue injury resulting in pulmonary fibrosis. This is supported in part by the clinical improvement seen in patients at the cellular, immune-complex stage of disease, on treatment with corticosteroids. There is also a possibility of Type 4, cellular hypersensitivity involvement since isolated peripheral blood lymphocytes from cryptogenic fibrosing alveolitis patients can produce a lymphokine; macrophage migration inhibition factor, and undergo blastogenesis on exposure to collagen (Kravis et al, 1976).

Isolated immune complex reactivity also appears to be the primary disease mechanism in patients with rheumatoid lung disease. The lung diseases associated with rheumatoid arthritis include pleural disease, vasculitis, nodules, Caplan's syndrome and pulmonary fibrosis. The association of rheumatoid lung disease with high-titre rheumatoid factor is suggestive of immunopathogenesis. This is supported by measurement of high

concentrations of immune complexes and decreased concentrations of complement in the pleural fluid of patients with rheumatoid pleuritis, and the presence of pulmonary vasculitis with deposits of immunoglobulin and IgM rheumatoid factor in pulmonary vascular and alveolar walls. It seems likely that immune-complex mechanisms are important also in non-infectious pulmonary manifestations of other collagen vascular diseases (Hunninghake & Fauci, 1979).

The classical example of Type 4 hypersensitivity is cell mediated immunity to tuberculin, where all the pulmonary histological finding can be accounted for by sensitised lymphocytes. Another disease is chronic beryllium disease in which sensitisation by inhaling beryllium dust results in primarily cell mediated mechanisms.

One systemic granulomatous disease which affects the lungs and in which the causative agents is unknown is sarcoidosis. The response is primarily a T-cell mediated inflammatory reaction with mononuclear pulmonary infiltrate and granuloma formation. The local pulmonary cells collected by lung lavage contain a very high percentage of T-helper cells, and these are thought to stimulate the local production of IgM as well as the high local and circulating total immunoglobulins. Patients have a decreased circulating T-helper cell population and this may account for the associated cutaneous energy (Crystal et al, 1981). There is also a high incidence of serum immune complexes in these patients but their significance is unknown.

One lung disease syndrome in which there is compelling evidence for both Type 3 and Type 4 hypersensitivities is extrinsic allergic alveolitis, which therefore occupies a central position in the spectrum of hypersensitivity lung diseases. The greater clarification of the immunopathogenesis of this disease should help in understanding the mechanisms of normal and hypersensitivity responses of the lung.

3. EXTRINSIC ALLERGIC ALVEOLITIS.

Extrinsic allergic alveolitis is a pulmonary hypersensitivity disease caused by inhaling organic dusts from a wide variety of sources: fungal, bacterial and animal proteins. The

symptoms of this disease in the acute phase include shortness of breath with no wheezing, general malaise with aching discomfort in the joints and muscles. There is an associated pyrexia with rigors and sweating and an irritating unproductive cough. These symptoms occur 4 to 8 hours after exposure to the offending dust and are similar to an active viral infection often being described as "flu-like" by affected subjects, however, these symptoms usually resolve within 24 hours. The insidious, chronic form of the disease, however, is characterised by a slow progressive increase in shortness of breath and reduction in exercise tolerance, with a chronic cough.

The disease affects the distal, gas exchanging parts of the lung, and for this reason it was called "Extrinsic Allergic Alveolitis" by Pepys (1967). However, since the disease can also affect the terminal bronchioles it has been termed "Extrinsic Allergic Pneumonia" (Nicholson, 1972), "Extrinsic Allergic Bronchiolo-Alveolitis" (Scadding, 1970 ; Seal, 1975) and in the American literature, "Hypersensitivity Pneumonitis" (Reed, 1972). The disease, therefore, is associated with a decrease in lung function with a reduction in carbon monoxide diffusing capacity (Dinda, Chatterjee & Riding, 1969; Schlueter, Fink & Sosman, 1969). With this restrictive defect, there is a loss of lung volume (decreased vital capacity) and an increase in compliance (loss of lung elasticity). In the chronic stages of the disease there can also be increasing airways obstruction (Allen, Williams & Woolcock, 1976). Radiological investigation of the disease commonly demonstrated abnormalities described as honeycombing throughout the lung parenchyma, or "ground-glass" appearance (Molina, 1976), and auscultation demonstrated the presence of fine crepitations throughout the lung fields, most prominent at the bases. The clinical and pulmonary function aspects of the disease are discussed further in appendices A and C, and Chapter 5. The clinical aspects are very similar for all syndromes of alveolitis despite the causative agent.

The number of dusts which are known to cause extrinsic allergic alveolitis form an ever lengthening list which is increasing with greater general awareness of the disease (see Table 2). The majority are organic dusts, animal derived

TABLE 2. The major disease syndromes of
Extrinsic Allergic Alveolitis 11a.

<u>SYNDROME</u>	<u>ANTIGEN</u>	<u>REFERENCE</u>
Farmer's Lung	Micropolyspora faeni	Pepys et al, 1963
Bagassosis	Thermoactinomyceete vulgaris Thermoactinomyceete sacchari	Salvaggio et al, 1969 Lacey, 1971
Suberosis	Penicillium Frequentans	Pimental & Avila, 1973
Mushroom Worker's Lung	Thermoactinomyceete sp. Agaricus hortensis spores	Sakula, 1967 Stewart & Pickering, 1974
Humidifier Fever	Micropolyspora faeni Naegleria grubii	Fink et al, 1971 Edwards et al, 1974
Malt Workers Lung	Aspergillus clavatus	Riddle et al, 1968
Maple Bark Strippers Lung	Cryptostroma corticale	Emanuel et al, 1966
Wood pulp workers Lung	Alternaria sp.	Schlueter et al, 1972
Woodworkers Lung	Ramin extract Redwood extract	Howie et al, 1976 Cohen et al, 1967
Cheese Washers Lung	Penicillium casei	de Weck et al, 1969
Enzymes	Papain Bacillus subtilis	Novey et al, 1980 Pepys et al, 1969
Snuff Taker's Lung	Bovine or Porcine pituitary extract	Pepys et al, 1966
Rat Handlers Lung	Rat serum proteins	Carroll et al, 1975
Fish Meal Workers Lung	Fish meal extract	Avila, 1971
Prawn Workers Lung	Prawn meat extract	Gaddie et al, 1980
Wheat Weevil Lung	Sitophilus granarius	Lunn & Hughes, 1967
Fungal	Streptomyces albus Merulius lacrymans Aspergillus Cryptococcus	Kagen et al, 1981 O'Brien et al, 1978 Katz et al, 1973 Miyagawa et al, 1978
Byssinosis	Leucocyanidin	Taylor et al, 1971
New Guinea Lung	Thatch extract	Blackburn et al, 1966
Sequoiosis	Graphium spp. Aureobasidium pullulans	Cohen et al, 1967
Coffee workers Lung	Coffee bean dust	Van Toorn, 1970
Furriers Lung	Animal hair	Pimental, 1970
Paprika splitter's Lung	Mucor stolanifer	Hunter, 1969
Sisal workers Lung	Sisal dust	Pepys et al, 1964
Smallpox handlers Lung	Smallpox scabs	Morris-Evans et al, 1963
Seaweed processors Lung	Alginate	Henderson et al, 1984
Animal handlers Lung	Laboratory animals Meriones unguiculatus (gerbil)	Slavin, 1978
Weavers cough	Tamarind seed powder	Tuffnel et al, 1957

TABLE 2 CONTINUED:

<u>Bird breeders Lung</u>	Duck - goose	Plessner, 1959
	Parakeet	Pearsall et al, 1960
	Pigeon	Reed et al, 1965
	Budgerigar	Hargreave et al, 1966
	Chicken	Korn et al, 1968
	Turkey	Boyer et al, 1974
	Lovebirds	Fink, 1974
	Dove	Cunningham et al, 1976
	Cockatail	Harries et al, 1980

TABLE 3. Reported prevalences of alveolitis caused by avian exposure.

NUMBER	SYMPTOMATIC	REFERENCE
43	0	Reed et al, 1965
30	0	Hargreave et al, 1966
21	0	Maloney et al, 1967
11	0	Stiehm et al, 1967
11	0	Eyckmans et al, 1968
80	0	Fink et al, 1968
14	1 (7%)	Siegal et al, 1969
54	3 (5.5%)	Jongerius, 1969
41	0	Zaman et al, 1971
51	0	Verbeke et al, 1971
146	11 (8%)	Elgefors et al, 1971
200	0	Fink et al, 1972
	1.4 per 1,000	Maesen et al, 1972
150	6%	Caldwell et al, 1973
53	11 (21%)	Christensen et al, 1975
	1 per 4-5,000	Molina, 1976
117	0.5 - 7.5%	Hendrick et al, 1978
38	1 (2.6%)	ibid
64	14 (22%)	Boyd, 1975

proteins and fungal products, and the potential for production of disease following inhalation of this type of material was recognised by Pepys (1969). Due to the widespread occurrence of the thermophilic Actinomycetes, the spores of these organisms are most frequently the causative agent of alveolitis in a variety of settings, and the popularity of different bird species has similarly resulted in widespread exposure to avian antigens (Hendrick, Faux & Marshall, 1978). Consequently, Farmer's Lung and Bird Breeder's Lung are the two most studied of the disease syndromes of extrinsic allergic alveolitis and are therefore useful models for studying the immunopathogenicity of the disease processes which could equally apply to the more individual syndromes listed on Table 2. For further information in the historical, epidemiological, clinical and experimental aspects of alveolitis the reader is directed towards the following reviews (Nicholson, 1972; Molina, 1976; Roberts & Moore, 1977; Boyd, 1978; Turner-Warwick, 1978; Salvaggio & Karr, 1979).

Similar inflammatory reactions have been described following inhalation of grain dust (Broder & McAvoy, 1981) and endotoxin rich sewage dust (Mattsby & Rylander, 1978) however these were thought to be due to the toxic nature of the dusts rather than a hypersensitivity reaction. Similarly, the toxic effects of prolonged high dose oxygen (Fox et al, 1981), irradiation, beryllium and drugs, particularly chemotherapy (Sostman et al, 1976) can result in lung inflammation and fibrosis. However, on closer examination some degree of immunological hypersensitivity has also been seen following drug administration (McCormick et al, 1980). The specific and non-specific agents causing lung fibrosis have been reviewed elsewhere (Kissler, 1983).

a) Extrinsic allergic alveolitis caused by avian exposure.

The first description of alveolitis caused by exposure to pigeons was reported in 1965 by Reed and colleagues and brought to light similar disease patterns in subjects exposed to other avian species, listed on Table 2. This first clinical report however was not the first recognition of the problem because the pigeon breeders (fanciers) themselves had long known about the

intolerance to their birds which was different from ornithosis (Lamers & Maesen, 1976; Lynch, P.P. personal communication), and the typical disease pattern was previously described in subjects exposed to avian antigens (Fawcitt, 1936; Feldman & Sabin, 1948; Emmons, 1955; Plessner, 1959; Pearsall et al, 1960). Long before these reports however, there was a typical description of the disease in the "gaveurs de pigeons"; the people who force fed pigeons in Paris which necessitated daily contact with thousands of birds (Renon, 1897). Nevertheless, Reed et al (1965) gave the first full clinical and serological report and proved the association of the disease with pigeons by provoking the symptoms in one subject after contact with his pigeon in a controlled environment. The disease was subsequently reported worldwide (Boyd, 1975; Molina, 1976).

4. IMMUNOPATHOGENESIS OF ALVEOLITIS.

The pathogenesis of extrinsic allergic alveolitis is thought to be a complex hypersensitivity reaction against inhaled antigens with involvement of both humoral and cellular responses. This is based on the finding of antigen specific antibody and lymphocytes in the circulation of affected patients. Skin testing these individuals with the appropriate antigens often results in a dual immediate (15 min. maximum) and late (4-8 hours) reaction. This late skin reaction has the histological characteristics of an Arthus reaction and the close temporal relationship between this skin reaction and the late (4-8 hours) pulmonary reaction has suggested that a similar inflammatory process may prevail. However, conventional histology of lung biopsy suggested a predominantly cellular reaction. The evidence for the involvement of antibody as well as sensitized cells is discussed below, and parallel studies in animal models are discussed in Appendix D.

a) Antibody.

The association of serum antibody with extrinsic allergic alveolitis was first described by Pepys et al (1961), when the serum of a farmer with disease was shown to precipitate with an extract of thermophilic Actinomycetes grown from mouldy hay.

Serum precipitins against avian antigens were first described by Pearsall et al (1960), in a budgerigar keeper with an unexplained febrile illness, and the significance of serum precipitins against pigeon serum was discussed by Reed et al (1965) in the first full description of an interstitial lung disease caused by exposure to pigeons.

The serum antibody is predominantly IgG, and milligram/ml quantities have been reported (Fink et al, 1969; Boren et al, 1977) and antibody activity in the other main classes has also been measured (Faux, Wells & Pepys, 1971; Patterson et al, 1976). The IgG antibody when combined with avian antigens can activate complement (Caldwell et al, 1973) and the role of antibody in disease is discussed fully in Chapter 3.

Coincidental antibody findings include a profile of raised antibody to viruses and bacteria, thought to be an anamnestic response (Boyd et al, 1967; Bach et al, 1971; Marx et al, 1977; Miyagawa et al, 1978). There was a high incidence of rheumatoid factor reported in pigeon breeders disease (Banazak & Thiede, 1974) and in farmers lung (Parrat, 1976) although Turner-Warwick & Haslam (1971) found a distribution of autoantibody in alveolitis patients sera similar to a normal population.

b) Skin testing.

The sera from sensitized pigeon breeders can mediate passive cutaneous anaphylaxis in guinea-pigs following skin testing with pigeon serum antigens (Fink et al, 1967), and on passive transfer intradermally, could also sensitize human skin for a Prausnitz-Kustner reaction, this being mediated by a short-term sensitizing antibody, which was heat stable and resistant to mercaptoethanol (Warren et al, 1977). However, positive immediate skin prick test reactions against avian antigens have been reported infrequently among bird breeders, and only usually in association with asthma (Fauz et al, 1971). Following intracutaneous injection of these antigens there are many reports of late, 4-8 hours, skin reactions with a variable incidence of preceding early reactions (Hargreave & Pepys, 1972; McSharry et al, 1983). This late reaction consisted of an

oedematous non-irritating swelling which showed histologically in 2 cases, an eosinophilic vasculitis (Jean et al, 1970). Sahn & Richerson (1972) also described oedema with a mainly perivascular infiltration of polymorphonuclear leucocytes and a few eosinophils, and Caldwell et al (1973) described perivascular inflammation with polymorphonuclear leucocytes and eosinophils, with no histological appearances suggestive of cell mediated hypersensitivity. Immunofluorescence demonstrated IgG, IgM, C3 and C4 in the capillary walls (Jean et al, 1970; Caldwell et al, 1973) and Pepys et al (1968) demonstrated the presence of IgG antibody and complement at first outside, and later inside phagocytic cells.

This type of skin reaction was first described by Maurice Arthus (1903) and the term "Arthus type" reaction has been widely used to describe local immune complex mediated tissue injury, and this mechanism was suggested to mediate the lung damage associated with alveolitis.

c) Lung biopsy.

There have been numerous histological reports from lung biopsies of individual cases of bird breeders disease (Eyckmans et al, 1968; Hensley et al, 1969; Palma-Carlos et al, 1971; Fasseke et al, 1974; Hogan, 1978; Barrios et al, 1979) whereas histological studies have been performed on greater numbers of farmers lung patients and several series have been reported (Seal et al, 1968; Reyes et al, 1976; Sutinen et al, 1983).

The most consistent histological features are an interstitial and alveolar inflammation, which may also involve the bronchioles (Nicholson, 1972; Reyes et al, 1976). There was a marked infiltrate of mononuclear cells, predominantly lymphocytes, often arranged in follicles with variable numbers of plasma cells. There are only rarely reports of neutrophils and eosinophils in the interstitium. The presence of interstitial foam cell aggregates, occasionally also in the alveolar spaces, is suggested to represent a distinctive lesion for pigeon breeders disease (Hensley et al, 1969; Hogan, 1978) and a role for activated bronchopulmonary macrophages in the pathogenesis of alveolitis has been suggested (Stankus, Cashner & Salvaggio, 1978). More

advanced cases of pigeon breeders disease are characterised by increasing fibrosis with thickening of the alveolar septa, along with capillary and alveolar basement membranes and bronchiolitis obliterans (Shannon et al, 1969; Hensley et al, 1969; Heywood, 1971; Fasske et al, 1974). A similar picture is true also for farmers' lung (Barrowcliff & Arblaster, 1968; Tukianen et al, 1980).

Immunofluorescence studies on lung biopsies from patients with alveolitis have not conclusively demonstrated the presence of immune complexes. Wenzel, Emanuel & Gray (1971), demonstrated immunoglobulins located in plasma cells throughout the diseased lung and C3 fixed in large amounts to macrophage but not in relation to capillary walls, and Turner-Warwick et al (1971), examining biopsies from 8 cases of bird breeders lung failed to demonstrate immunoglobulin or complement deposited around the capillary walls although some immunofluorescence was demonstrated in 2 cases in germinal centres and in surrounding plasma cells. In these two reports it is probable that the workers only demonstrated local immunoglobulin and complement synthesis (Colten, 1976). Furthermore, Emanuel et al (1964) could not demonstrate antigen, antibody or complement in lung biopsies from farmers with alveolitis, however 2 biopsies were shown to have IgG and complement deposited along the alveolar capillaries (Meijer et al, 1971) and only one out of 10 biopsies in a further study of bird breeders lung had demonstrable deposits of immunoglobulin and complement (Barrios et al, 1979). However, in animal models of disease, antigen antibody and complement can be readily detectable in lung tissue (Hensley et al, 1974) but these deposits can be detected only if the lesions are studied before extensive phagocytic infiltration (Cochrane & Koffler, 1973).

It is highly probable that the immunofluorescence studies mentioned above in human cases were performed either as investigations of chronic cases (as suggested by the histological findings) or long after the last antigen exposure. The normal phagocytic activity would have removed the immunoreactants and therefore negative immunofluorescence findings do not rule out the possibility that immune complexes are involved in the pathogenesis of alveolitis. Some supporting evidence for the association

between immune complexes and disease is provided by Boyd (1975) and Akoun et al (1975) who both described renal deposition of immune-complex like material suggesting this to be overspill from antigens absorbed via the respiratory tract, through the bloodstream and filtered out in the kidney. A further unusual pulmonary-renal syndrome was described by Dunn et al (1981) who reported mesangiopathic glomerulonephritis in alveolitis. Other diseases reported in conjunction with alveolitis include impotentia coeundi (Lamers & Maesen, 1976), allergic cerebral manifestations (Misur & Takac, 1978), pericarditis (Reybet-Degat, 1979), sarcoidosis (Cohen et al, 1976), jejunal villous atrophy (Berrill et al, 1975), and extrinsic asthma (Karr, Kohler & Salvaggio, 1978).

d) Cellular immunity.

The occasional finding of pigeon breeders disease in subjects with no specific serum antibody (Allen et al, 1975; Sennekamp et al, 1978; Canet et al, 1980) and the predominantly mononuclear infiltrate and granulomatous inflammatory response observed on histology, have suggested a role for cell-mediated hypersensitivity in the pathogenesis of alveolitis, despite the paucity of reports on delayed skin tests (Heywood, 1971; Warren, 1972; El Hefny et al, 1980). The first reports on cell sensitivity to pigeon derived antigens in one subject with pigeon breeders disease (Jean et al, 1970; Bach et al, 1971) demonstrated peripheral blood lymphocyte stimulation with pigeon derived antigens. Thereafter, avian antigen specific blastogenesis and lymphokine production from circulating lymphocytes of patients with pigeon breeders disease have been reported (Caldwell et al, 1973; Moore et al, 1974; Hansen & Penny, 1974; Fink et al, 1975; Allen et al, 1975; Schatz et al, 1976). It was initially thought that this lymphocyte sensitivity was a feature only of subjects with alveolitis but Allen et al (1975) demonstrated production of macrophage migration inhibition by cells from 2 symptomatic as well as 2 asymptomatic pigeon breeders, all without precipitins. Furthermore, Schatz et al (1976) demonstrated lymphocyte blastogenesis using pigeon serum antigen in lymphocyte cultures using cells from pigeon breeders

with and without alveolitis, and also in one subject with no exposure to pigeons. In this latter subject, the lymphocyte sensitivity increased following experimental intracutaneous injection of pigeon serum antigens. These workers observed the lymphocyte stimulation index at intervals after cessation of exposure in one patient with alveolitis and found a significant decline in antigen induced blastogenesis coincidental with clinical recovery. Sennekamp et al (1978) also reported a decline in lymphocyte transformation following cessation of avian contact in a precipitin-negative case of pigeon breeders disease. A further fluctuation in cell sensitivity was shown by Lazary et al (1975), in alveolitis in cattle where a fluctuation in blastogenesis on exposure to Micropolyspora faeni antigen occurred in vitro, which correlated with the seasonal rate of antigen exposure. The interpretation of studies of cell mediated immunity among patients with alveolitis has been made more difficult because there was no information on the patients' current drug therapy in the literature reports, and the effects of steroids have been shown to cause depressed blastogenesis while the phytohaemagglutinin response was unaltered (Toogood et al, 1980). This is an area which therefore requires further detailed re-examination.

e) Local pulmonary immunity.

The technique of bronchial lavage using the fiberoptic bronchoscope has greatly increased the knowledge of local events during pulmonary disease, and using this procedure Warren & Tse (1974) first obtained evidence for local antibody production in extrinsic allergic alveolitis where precipitins to chicken feather extract were found in proportionally greater amounts in bronchial washings than in the serum of one affected subject. Local antibody production in symptomatic pigeon breeders has been confirmed (Voisin et al, 1981) and this activity has been assigned to both IgA and IgG antibody classes (Patterson et al, 1979). In extrinsic allergic alveolitis, total lavage protein and immunoglobulins have been demonstrated in higher titres than in normal subjects (Reynolds et al, 1977; Voisin et al, 1981) with the importance of local production of IgM and a high IgG to

albumin ratio being stressed by Weinberger et al (1978), and increased local production of IgG subclass 4 was suggested to play a role in the pathogenesis of the disease by Calvanico et al (1980). The altered lipid profile of surfactant obtained from lavage fluid has been suggested to influence the mechanical abnormalities in the disease (Jouanel et al, 1981). Lung histology and bronchial lavage demonstrates an increase in total cell numbers, predominantly of mononuclear cells characterized as T lymphocytes (Reynolds et al, 1977; Weinberger et al, 1978; Godard et al, 1981), which is particularly marked during the acute phase (Molina et al, 1979). These local T lymphocytes have been shown to respond specifically to pigeon antigens by lymphokine production (Schuyler, Thigpen & Salvaggio, 1978) and cellular proliferation (Moore et al, 1980).

The respiratory tract is the route of antigen entry and the lung is the primary target organ. Sequential quantitative measurement of humoral and cellular immune parameters at the pulmonary level therefore is likely to increase our understanding of the disease mechanism in extrinsic allergic alveolitis and of the general capability for antigen processing by the lung in this condition.

f) Prevalence.

A true reflection of the prevalence of avian induced alveolitis is difficult to achieve because the appropriate epidemiological studies have not yet been done.

The main literature information regarding the prevalence of alveolitis among subjects exposed to pigeons is listed on table 3 and the range is from zero to 21%. There will be many reasons for the discrepancies including:-

- (a) the population selection; some studies encouraged the co-operation of subjects with symptoms whereas others, e.g. Molina (1976) based their figures on a small number of local subjects who had restricted avian exposure in well ventilated lofts, with a consequently low incidence of disease.

(b) the unwillingness of symptomatic subjects to co-operate in a study which may suggest that they have to give up their hobby in which they may have a major social and financial investment.

(c) the criteria on which a decision of alveolitis is based. For example although none of 200 subjects investigated by Fink et al (1972) were considered to have disease, some may have had chronic alveolitis since cough and breathlessness were common, and some had compatible radiographic and pulmonary function abnormalities.

The question of assessment of the disease has proved to be very difficult, and a wide experience of treating this disease is of primary importance. Various presentations are discussed by Boyd et al (1982) and it is possible that some subjects with either subacute or chronic forms of disease were overlooked in the earlier studies listed on table 3. However, even a conservative estimate of prevalence would generate a large number of cases since the numbers of subjects exposed to pigeon in various countries is: UK - 180,000; Belgium - 250,000; US - 75,000; Sweden - 1,000; France - 40,000 (Molina, 1976). The incidence in Britain of people keeping pigeons is quoted as 0.3%, and budgerigars 12% (Hendrick et al, 1978). The population at risk is, therefore, considerable. Consequently, clinicians and general practitioners should be made aware of these figures.

g) Genetic markers of alveolitis.

The genetic control of the immune response in humans and the genetic susceptibility to immunological disease has been reviewed recently by some major workers (Bodmer & Bodmer, 1978; Zinkernagel, 1979; Benacerraf, 1981). The empirical clinical findings of variable associations between the incidence of specific diseases and certain HLA antigens has provided evidence for basic immune defects associated with disease and has been used as a probe to examine the immunology of diseases, for example these HLA haplotypes have been used as disease markers.

With respect to extrinsic allergic alveolitis, the relative contribution of various constitutive factors, including genotype, and environmental factors, on the antibody response and on disease remains unknown. Attempts at HLA typing pigeon breeders with alveolitis have shown no association with HL-A in 30 patients (Boyd & Dick: personal communication), and no significant association with HLA-A, B and C in 51 symptomatic and 102 asymptomatic pigeon breeders nor HLA-DR in 32 and 29 respectively although HLA-DR3 was higher in patients (Rodey et al, 1979). Furthermore there was no correlation between pigeon breeders disease and immunoglobulin allotypes in 9 symptomatic and 14 asymptomatic subjects (Moore et al, 1975). Muers et al (1982) studied 23 patients with budgerigar fanciers disease and 154 controls, and although HLA-B8-DR3 was increased in the patient group the difference did not reach significance.

There was some early interest in a reported link between pigeon breeders disease, coeliac disease and HLA-B8 (Berrill et al, 1975; Rittner et al, 1975). However this was resolved by Faux et al (1978) who demonstrated different avian antigens associated with each condition; dietary ovalbumin with coeliac disease and inhaled avian gamma-globulin antigens with alveolitis.

There have been some reports however linking a particular genotype with alveolitis. Flaherty et al (1975) described an increase in HL-A8 in 20 farmer's lung patients, i.e. 40%, compared with 8% in controls. Some subtle genetic links with bird breeders disease were described by Allen et al (1975) in 2 families with 7 individuals affected. Three with disease in one family were all HL-A2, W15, and in the other family 2 children with severe disease were HL-A2, W10 and 2 with mild disease were HL-A1, 8. Furthermore, on further examination of 28 patients, there was a reduction of HL-A3, 7, i.e. 3.7% compared with 40% in a normal population, and these authors associated this with hyporesponsiveness. Diaz de la Vega et al (1980) report an association of HLA-BW40 with members of a family with disease, and Morell et al (1981) again report a familial incidence of alveolitis this time in 2 brothers who are HLA-BW15. A recent report (Rittner et al, 1983) supports an association between disease and HLA-DR3 when comparing 52 symptomatic and 64

asymptomatic pigeon breeders, and Berrill & van Rood (1977) suggested a link with HLA-DW6.

In relation to other respiratory disorders the hereditary component of asthma was recognised long before the awareness of the genetic principles (Cooke & Vander Veer, 1916). However, even with the current awareness, the genetic predisposition to asthma cannot be designated to any one HLA haplotype in the general population (Turton et al, 1979), and although within families some HLA haplotypes related to asthma were reported to prevail, unfortunately these were different between families. These observations are in keeping with the complexity of asthma where various contributory factors, e.g. IgE production, bronchial hyper-reactivity, etc, are all under separate genetic control.

There has been one report linking HLA-B8 with cryptogenic fibrosing alveolitis (Turton et al, 1978). This study of 167 patients demonstrated a significantly raised incidence of B8 in women, and in patients with an onset of disease at less than 50 years of age. There was no association with HLA-A or C. A further study of 33 white patients with the same disease could find no association with HLA-A or B antigens (Fulmer et al, 1978). There has been no HLA association with the other major interstitial inflammatory disease with no known cause; sarcoidosis. Nevertheless, HLA-B8 is reported to be associated with spontaneous resolution of disease (Smith et al, 1981). Goodpastures syndrome has been added to this list by Rees et al (1978) who reported an 88% incidence of HLA-DRW2 in a series of 17 patients compared with 32% in a normal control population, and in pulmonary tuberculosis there was a reported increase in HLA-DR2 in 124 patients with a concurrent decrease of DRW-6 (Singe et al, 1983).

5. SCOPE OF PRESENT THESIS.

It has been reported at an early stage in the study of alveolitis that the presence of serum precipitating antibody did not fully correlate with disease. The next logical investigative step was for studies on cellular sensitivity to be performed but this proved to be no better in delineating the symptomatic subject. This thesis attempts to reappraise the role of humoral

immunity in the hypersensitivity response associated with acute extrinsic allergic alveolitis among pigeon breeders.

Firstly, the relevant antigens were described, and these were selected for use in sensitive assays in order to quantify the serum antibody response among various populations of pigeon breeders. There was a full appraisal of the incidence and severity of symptoms in these subjects, and the antibody levels and disease were clearly demonstrated to be very closely related. Various constitutional factors such as age and atopic status along with various environmental factors such as smoking and various indices of antigen exposure were studied for their effects on the antibody response and disease.

Other serological parameters were measured and it appeared that there was an abnormal inflammatory profile in those subjects who were sensitised to the inhaled antigens. These parameters were followed serially in individuals during different phases of disease, and the changes observed suggested that these dynamic events were closely related.

Finally, the various laboratory investigations of serology, skin testing and inhalation provocation testing were assessed for their bearing on the disease process and for their diagnostic value to the clinician in the management of the disease.

CHAPTER 2

THE ANTIGENS

1. INTRODUCTION.

The sources of antigens which can cause alveolitis are widespread and form an ever increasing list which was discussed in the last chapter (Table 2). However, despite this range of antigens the clinical presentation of affected subjects is similar. This suggests that although the initial antigen recognition is highly specific, the subsequent hypersensitivity reactions are common for all affected subjects. For this simple reason it could be argued that there can be no "disease-specific antigen" as such, although there are some immunologically specific and non-specific factors common to all these antigens.

a) Non-specific factors

The antigens are generally derived from the dusts or aerosols of organic material, or from organisms growing on organic waste (Pepys, 1969). The airborne particles which can cause alveolitis are of the order of one micron in diameter (Austwich, 1966) which is the correct aerodynamic size to reach and settle in the alveoli (Brain & Valberg, 1979), and it is at the alveolar level that the disease process is most evident (Hensley et al, 1969; Seal et al, 1975).

There will inevitably be some non-specific irritant material in crude organic dust, an extreme case is with grain dust (Broder & McAvoy, 1981) where respiratory symptoms are common in most heavily exposed subjects and soluble extracts from grain dust can react non-specifically with all sera. The presence of irritant material in Micropolyspora faeni extracts has hampered the use of these extracts in the investigation of farmers lung by skin testing and inhalation provocation testing (Williams, 1963). Bacterial teichoic acid as well as "C-substance like" material have been identified in various dust extracts and these have caused false-positive precipitin reactions with sera of patients under investigation for alveolitis (Faux et al, 1970). This is now routinely avoided by the incorporation of citrate buffer in the gel (Longbottom & Pepys, 1964).

It would seem that a vigorous immunological stimulus is required to establish experimental alveolitis in animal models (discussed in Appendix D). As well as the experimental antigen, there is also a need for an adjuvant (Richerson et al, 1978), mitogen (Hollingdale et al, 1978) or non-specific inflammatory agent such as carrageenan or BCG (Peterson et al, 1977) in order to initiate disease. For alveolitis in man, a similar intrinsic immunological adjuvant activity in the antigens causing the disease has been postulated by Pepys (1978) and both mitogenic and adjuvant activity of spores of Micropolyspora faeni have been described (Bice et al, 1977; Smith, Snyder & Burrell, 1980). Some glycoprotein precipitinogens have been isolated from Aspergillus clavatus which have non-specific alveolitis-inducing activity in mice, and these have been suggested to potentiate the disease process of alveolitis in maltworkers exposed to A.clavatus (Blyth, 1978). In these cases there is the added potential of inhaled spores to grow in the lung. This has been recently confirmed by Anderson and Greatorex (1983) who demonstrated airway colonisation with M. faeni in 32 of 46 farmers lung patients who had positive cultures from sputum. This would explain the aggressive features of the lung histology of farmers lung patients which show widespread granuloma formation and activated pulmonary macrophages (Seal et al, 1975), whereas there is much less evidence for a foreign body reaction in the lungs of pigeon breeders who are exposed to primarily soluble antigens.

The antigens associated with extrinsic allergic alveolitis have been reported to directly activate complement when added to normal human serum (Marx, Boren & Moore, 1977). These antigens include soluble extracts from Micropolyspora faeni and Thermoactinomyces vulgaris (Edwards, 1976), antigens from bacteria and fungi associated with EAA (Marx & Flaherty, 1976; Huis in't Veld & Berrens, 1976) and avian antigens (Berrens, Guikers & Van Dijk, 1974; Moore & Fink, 1975; Marx, Boren & Moore, 1977; Boren, Moore & Abramoff, 1977; Berrens, 1978). Activation is via the alternative pathway although there is some evidence to suggest that the antigens can act directly at the level of C4 of the classical pathway (Marx, 1978). This worker described one further unusual feature in that these antigens did not activate

guinea-pig complement despite their efficiency in activating human complement. The mechanism of these activities awaits clarification by their proponents. The proteolytic enzymes from bacteria and fungi and the hydrolytic enzymes from avian droppings were initially implicated in the activation sequence (Berrens & Guikers, 1972) but this has now been discounted (Marx, 1978).

b) Specific factors

All alveolitis-inducing dusts have a soluble component which is immunogenic in subjects exposed to that dust, and against which most subjects with alveolitis can have high titres of serum antibody. It is this specific nature of the antigens which relates most closely with the disease incidence of alveolitis, although the non-specific activities undoubtedly have some importance in initiating and determining the extent of this specific response.

Within the soluble component of the dusts causing the major syndromes of alveolitis there have been numerous antigenic determinants described. In farmers lung, antigen extracts from Micropolyspora faeni containing up to 46 antigens have been resolved by crossed-immuno-electrophoresis and up to 36 of these reacted with farmers lung patients' sera (Treuhaft et al, 1979). In Aspergillus fumigatus extracts, 52 antigens have been similarly described with up to 17 of these reacting with sera from aspergillosis patients (Kim & Chaparas, 1978; Kim & Chaparas, 1979). Some antigens in the M. faeni extract were considered to be "disease-specific" because they would precipitate only with sera from patients with alveolitis and not with asymptomatic farmers who were exposed to the mouldy hay on which the M. faeni grew (Treuhaft et al, 1979). Some of these disease-specific antigens have been suggested to include one polysaccharide and one protein with chymotrypsin activity (Gari et al, 1983). This aspect of alveolitis is of considerable interest and was indicated as a useful area of future development by a recent international symposium (NIAID workshop, 1978).

c) Antigens associated with pigeon breeders disease.

Various antigens have been extracted from pigeon droppings, feathers, serum, egg yolk and white, crop fluid and gut wall (Barboriak et al, 1965; Edwards et al, 1970; Sennekamp et

al, 1976). Early studies which sought to characterise the various antigens demonstrated, by cross absorption, that the major antigens occurred in pigeon droppings (Barboriak et al, 1965). Subsequently pigeon droppings were considered the only "complete" source of antigens concerned with the disease (Edwards et al, 1970).

In a series of papers Berrens and coworkers (1972 a-d) firstly described a crude pigeon droppings extract (PDE) as being a very heterogeneous mixture of soluble proteins, glycoproteins and polysaccharides which could precipitate in agar gel double diffusion with normal human sera. The aselective antigens could be separated from the specific antigens by gel filtration on Sephadex G-150; the first peak eluted was designated the "A antigens" and contained the aselective antigens. The second peak contained a mixture of glycoproteins designated the "B antigens", of which the major antigen having gamma-mobility on immunoelectrophoresis (IEP), was more specific for detecting antibody among pigeon breeders by precipitin formation. These authors went on to describe complement consumption by these antigens as a simple laboratory test for the disease.

A further group using immunoelectrophoresis of crude PDE separated four distinct components designated PDE 1-4 (Fredricks & Tebo, 1977). Two additional minor constituents PDE A and B were subsequently uncovered by partial purification and concentration (Fredricks & Tebo, 1980). Antigen PDE1, an acidic glycoprotein of molecular weight 200,000 was identified as pigeon IgA; it is quantitatively the major antigen of PDE, constituting 24% of the dry weight of PDE. Antigen PDE B, a basic glycoprotein (Mwt = 51,000) was identified by papain digestion of PDE 1 to be the basic Fab fragments of IgA (Fredricks & Tebo, 1980). PDE A corresponded to the acidic Fab fragments, and PDE 2 to the Fc fragments of pigeon IgA. The exact nature of antigens PDE 3 and 4 has not been elucidated; however, present evidence suggests that PDE 3 is a highly heterogeneous acidic proteoglycan (McCormick et al, 1982). The pigeon IgA antigen had already been described as XPGG, which was an antigen in pigeon droppings which cross-reacted with the main serum gamma-globulin antigen (Edwards et al, 1969). A Dutch group working on the same antigens also

described pigeon IgA (Goudswaard, Noordzij, & Stam, 1978; De Ridder, Goudswaard & Berrens, 1979), and suggested that this may be an important antigen in pigeon breeders disease.

Pigeon serum was demonstrated to be a major antigen by serological and inhalation provocation tests in the first description of alveolitis caused by pigeon exposure (Reed, Sosman & Barbee, 1965). Since then it has become the most readily used antigen source for investigating this condition. Avian serum, when separated electrophoretically, contained many antigenic proteins detected by the sera of sensitised pigeon fanciers (Faux, Wells & Pepys, 1971), and column chromatography of pigeon serum on Sephadex G200 generated many protein fractions of different molecular sizes, and these all had complement-depleting activity when incubated with the sera of pigeon fanciers (Berrens, 1978).

The sera of many avian species have some proteins with antigenic identity (Faux, Wells & Pepys, 1971; Sennekamp et al, 1981) which could explain the positive precipitin reactions to pigeon serum in subjects with exposure to other avian species, or in patients with coeliac disease caused by ingesting hens' egg proteins (Faux, Hendrick & Anand, 1978). There were, however, specific serum antigens associated with each avian species belonging to the gamma-globulin fraction (Fink, Tebo & Barboriak, 1969a; Faux, Wells & Pepys, 1971). Therefore pigeon serum gamma-globulin was considered to be the specific antigen associated with pigeon breeders disease, and this antigen was further demonstrated to be highly immunogenic in man, more so than serum albumin (Fink, Tebo & Barboriak, 1969b).

This chapter investigated some major sources of antigen derived from the pigeon in order to assess which was the most immunogenic among pigeon breeders, and furthermore to clarify any association between a particular antigen and disease.

2. EXPERIMENTAL.

a) Pigeon serum

Pigeon blood was obtained by cardiac puncture of ether anaesthetised birds. The blood was left to clot at room temperature for 2 hours, then overnight at 4°C to allow the clot to retract. After centrifugation the serum was aspirated and

stored at -20°C until used. The serum was used neat as an antigen in Ouchterlony double diffusion (see Appendix B), and using this system 20 of 97 pigeon fanciers had serum precipitating antibody. This incidence of precipitins, 20.6%, was similar to that found using pigeon serum as antigen in 200 pigeon fanciers, where 20% had precipitins (Fink et al, 1972).

To investigate the various serum fractions for antigenic activity the pigeon serum was separated by molecular sieving on Sephadex G200 and ACA-22 (figures 1 and 2). All the protein peaks had antigenic activity except the last peak from each separation which was made up of low molecular weight proteins (<1,000 daltons).

The strongest precipitin band was formed against the peak containing the main gamma-globulin antigen; pigeon IgG. The antigenic nature of the pigeon IgG molecule was investigated further. The column-purified IgG was reduced and acetylated according to Hudson & Hay (1980) and passed through Sephadex G-75 to separate the heavy (H) and light (L) chains (Figure 3). These two fractions were used as antigens in double-diffusion, and pigeon fanciers sera precipitated predominantly with the L chain fragment.

The albumin fraction of pigeon serum was separated from the globulin using 50% saturated ammonium sulphate (Hebert, 1974) and by passing the dialysed supernatant through Sephadex G200. This purified pigeon albumin, and the purified pigeon gamma-globulin (above) were used as antigens in an ELISA system (Appendix B) for the measurement of serum IgG antibody in 58 pigeon fanciers, and 48 control subjects with no avian exposure. These results were plotted in Figure 4, where the mean IgG values against all the antigens were seen to be significantly greater in the symptomatic than the asymptomatic pigeon fanciers, and both these groups had significantly higher levels than the controls.

b) Pigeon droppings extract.

In order to investigate this particularly difficult antigen source in the laboratory, approximately 5gm of freshly voided pigeon droppings were extracted with 20ml of saline for 24 hr. The greenish suspension was centrifuged at 3,000 rpm for 30 min and the clear green supernatant was aspirated. An equal

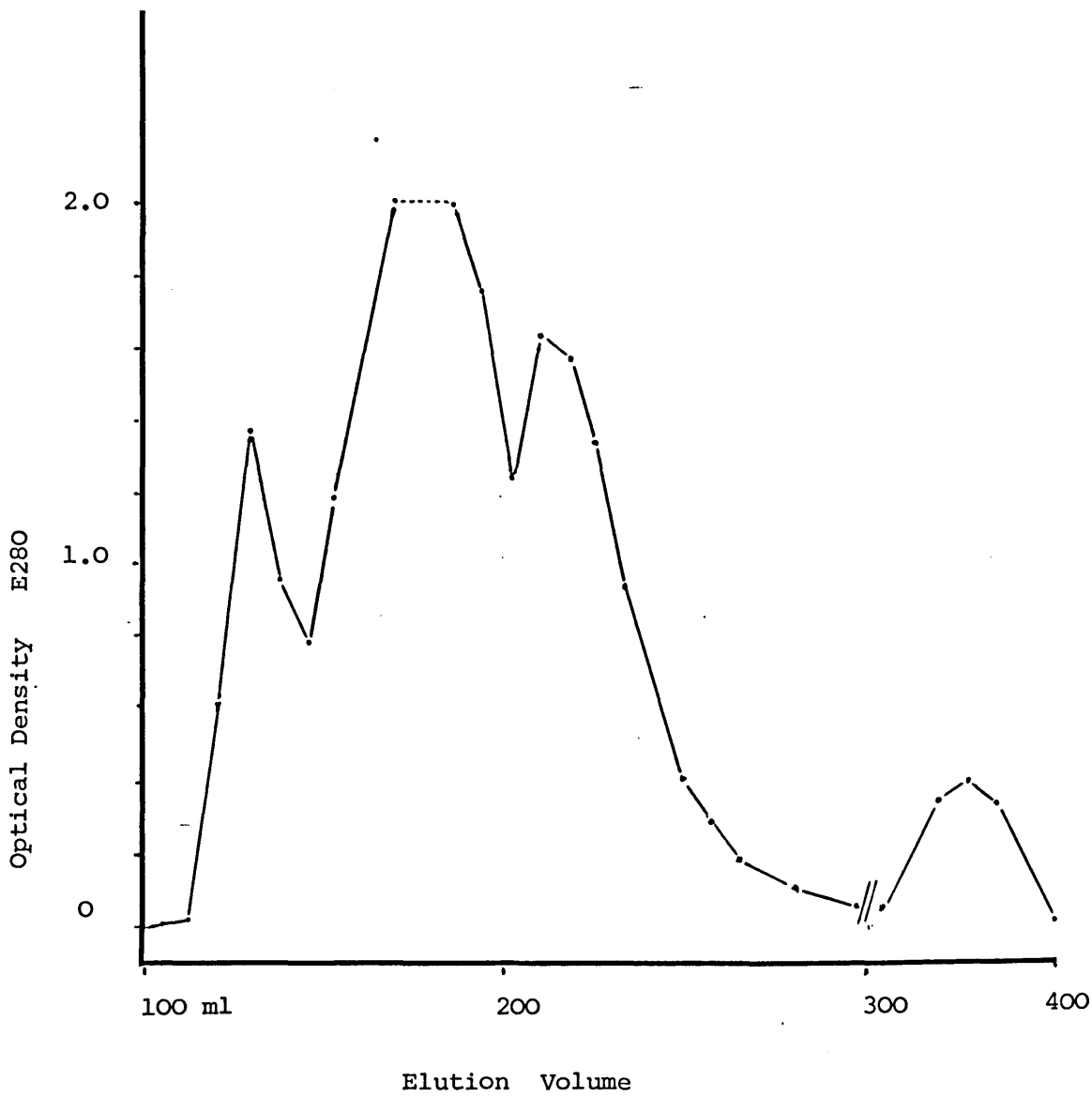
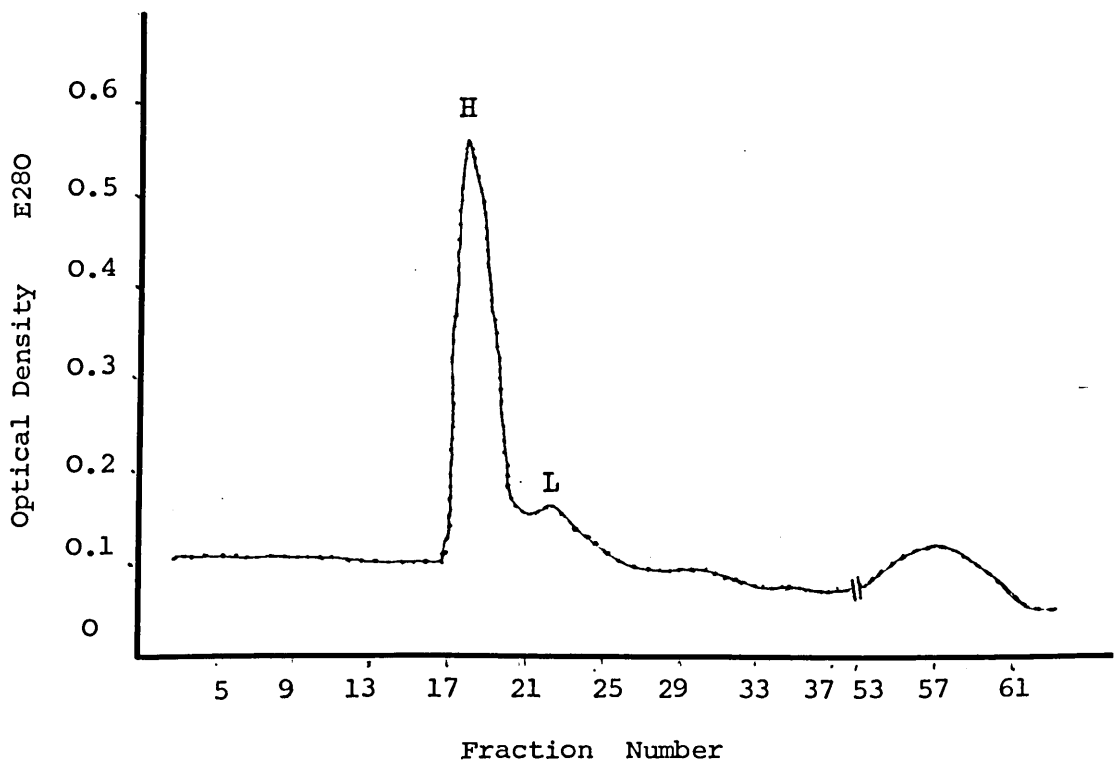


FIGURE 1: Elution profile of pigeon serum through Sephadex G-200

FIGURE 3: Elution profile of reduced and acetylated pigeon IgG heavy (H) and light (L) chains through Sephadex G-75.



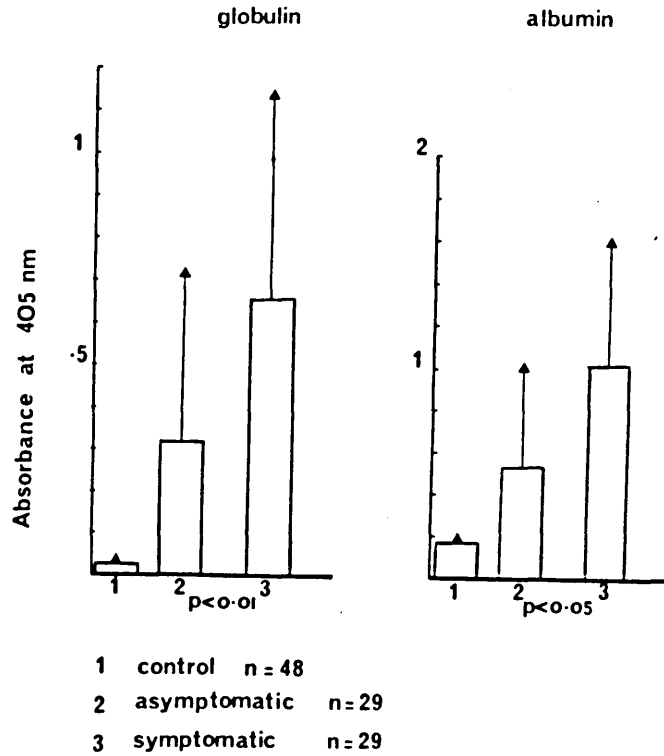


FIG. 4:

Measurement of serum IgG antibody against pigeon serum albumin and globulin antigens in symptomatic and asymptomatic pigeon breeders, and control subjects with no avian exposure.

volume of saturated ammonium sulphate was added dropwise and the globulin content precipitated. This was harvested by centrifugation, dissolved in 2ml saline, dialysed against saline and passed through Sephadex G200 (Figure 5). The first fraction collected in the exclusion volume contained pigeon IgA (Berrens & Maesen, 1972). This precipitated effectively with immune sera from pigeon fanciers and was used as an antigen for ELISA quantification of IgG antibody in 56 pigeon breeders and 48 controls (Figure 6). This antigen was highly selective for detecting antibody in pigeon breeders sera and the mean level in the symptomatic group was significantly higher than the asymptomatic group, and both of these were significantly higher than controls.

c) Feather dust (bloom) extract

Precipitins to feather extracts have been demonstrated in the sera of pigeon breeders (Sosman, Barboriak & Reed, 1965) and the clinical relevance of these antigens was demonstrated by positive late reactions following inhalation challenge tests on susceptible subjects with aqueous extracts from feathers (Reed et al, 1965; Muller et al, 1976; Warren, 1977). However, because feathers were more commonly associated with immediate hypersensitivity reactions (Barr & Sherman, 1961; Halpin & Prince, 1963), and because pigeon droppings were thought to be the most complete antigen source, this antigenic material has since received very little detailed investigation. However, Boyd (1978) reaffirmed the clinical importance of this source of antigen following discussions with pigeon breeders who admitted having symptoms more often during the moult, when the birds cast their feathers which resulted in clouds of feather dust. Symptoms were also prominent during pigeon shows when birds are in prime condition, one index of this being the amount of bloom on the feathers, and where excreta is minimal.

This section describes firstly the biochemical separation of bloom extract proteins and secondly the antigenic nature of these proteins:

1) Simple biochemistry of the proteins in the soluble bloom extract:

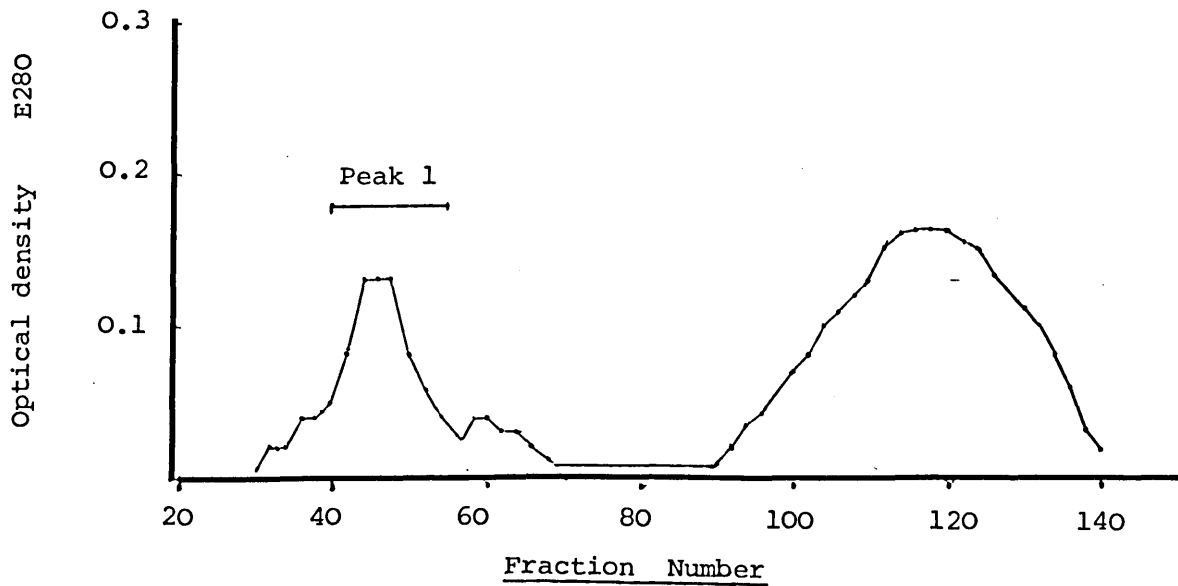


FIGURE 5: Elution profile of the secretory sIgA rich globulin fraction from pigeon droppings extract through Sephadex G-200. Peak 1 contained the sIgA antigen.

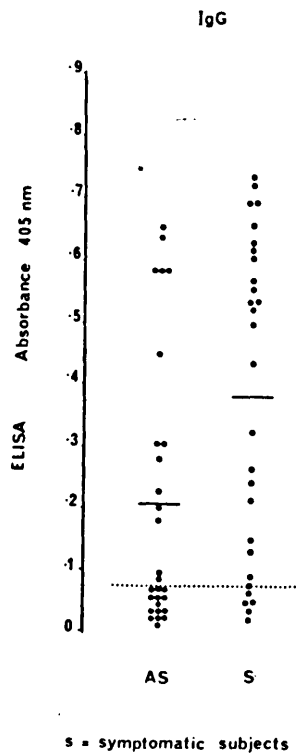


FIGURE 6:

Measurement of serum IgG antibody against pigeon droppings secreted IgA antigen in symptomatic and asymptomatic pigeon breeders.

Dotted line = upper limit of normal

Bar = mean

A soluble extract from the pigeon bloom was prepared by swabbing the chest and wings of several birds with a sterile swab and then extracting several swabs with 400ml saline. The resulting white suspension was centrifuged for 30 min at 3000 rpm and the clear aqueous layer was aspirated, concentrated by pressure dialysis to 10ml and dialysed against saline. The extract contained 2.5mg/ml protein as measured by the Lowrie method, and 0.4mg/ml carbohydrate as measured by the phenol-sulphuric acid method. There was no detectable cholesterol or triglyceride. A dilute sample of the extract had an absorption spectrum profile in the U.V. range, similar to other allergen preparations (Berrens et al, 1972), having a peak at 280nm with a shoulder at 260nm. There was no absorption in the visible range.

Allergens derived from a large number of animal species have been found to have some similarity in their electrical charge properties (Ohman, 1978). They migrated towards the anode on electrophoresis and had isoelectric points less than 7. Immuno-electrophoresis (IEP) of the bloom extract at pH 8.6, developed using the serum of a highly sensitized pigeon fancier, suggested that the main allergenic components of the bloom extract were also acidic in nature. This was confirmed by polyacrylamide gel isoelectric focusing which resolved proteins with isoelectric points at 5.5, 4.9 and several between 4.7 and 4.2. A commercial skin test preparation of pigeon feather extract (Bencard) was focused in parallel, and proteins were also resolved between 4.7 and 4.2.

When the bloom was dispersed in saline, the bulk of the white waxy powder aggregated and formed an upper, highly hydrophobic layer. A sample of this was aspirated and examined directly by scanning electron microscopy, SEM. Aggregated microgranular material consisting of unit particles of diameter between 0.3 and 1.0 microns was observed. These particles could be clearly seen by SEM on a small breast feather (Figure 7).

ii) Antigenic nature of the soluble bloom extract:

The soluble bloom extract contained antigens which would precipitate with sera from pigeon fanciers but not with sera from subjects with no avian exposure. Precipitins were demonstrated

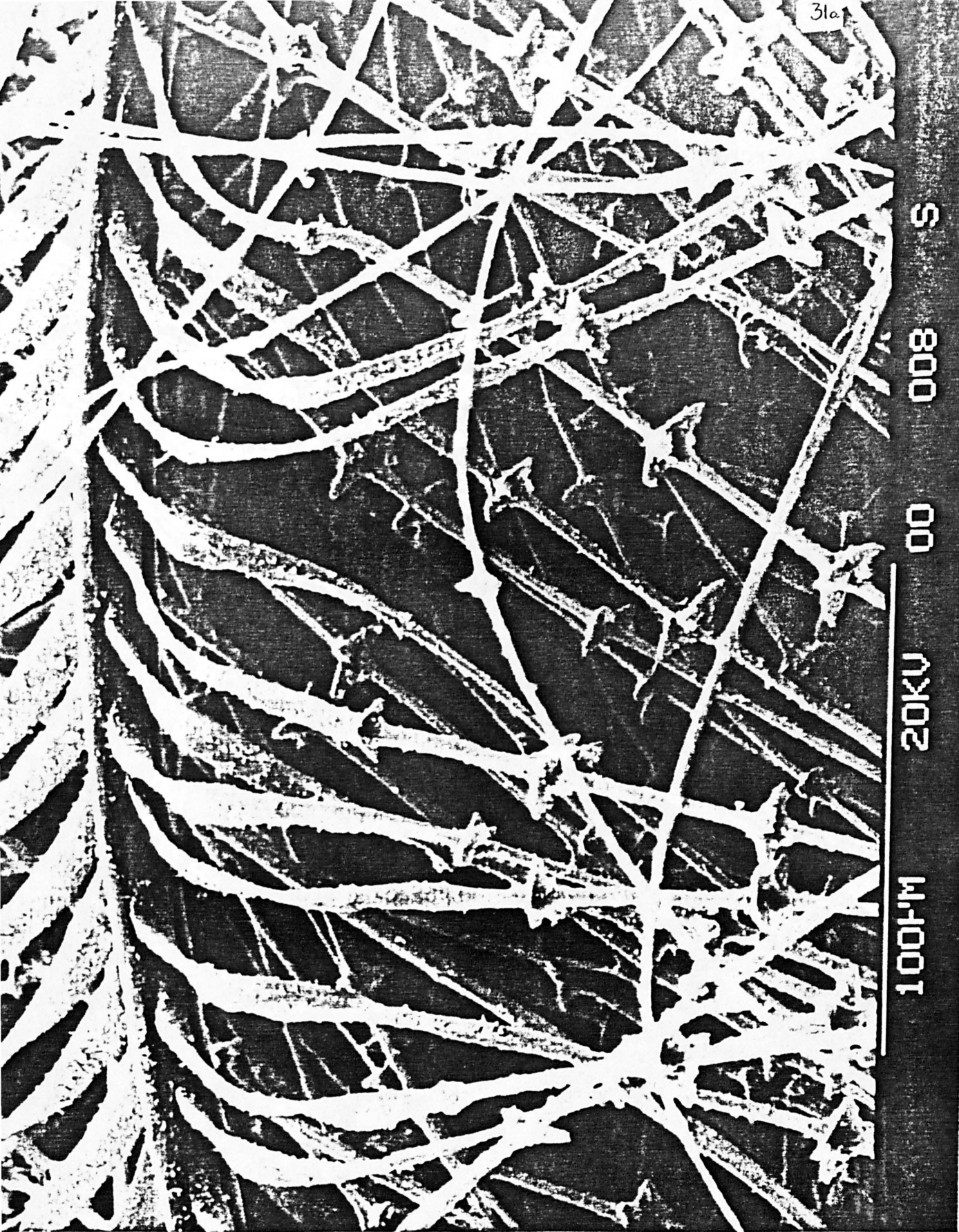


FIGURE 7a. Scanning electron micrograph showing detail of the fronds of a small breast feather of a pigeon. These are coated with the bloom particles. The size bar is 100 μ m.



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FIGURE 7b. S.E.M. showing detail of one of the barbs on the feather in Figure 7a. The individual bloom particles are clearly seen, and they approximate to 1 μ m. in diameter. The size bar is 10 μ m.

by double diffusion in agar in 59 of 97 pigeon fanciers' sera, with up to 5 clear bands resolved, whereas precipitins to pigeon serum were found in only 37. The five major antigenic components were confirmed by two dimensional IEP which demonstrated up to five arcs.

The antigenic components of the extract, taking advantage of their acidic nature, were examined by counter-current IEP against the sera of 54 active pigeon fanciers. After 2 hours at 10 volts/cm and overnight incubation, 36 sera had between one and six clear bands.

The extent of each individual's response to the bloom extract as measured semi-quantitatively by the number of precipitin bands was seen to correlate very closely with the extent of the antibody response to pigeon serum gamma-globulin antigen measured quantitatively by radioimmunoassay (figure 8).

d) Comparison of the antigens from various extracts

The correlation shown on Figure 8, between the extent of the antibody response to the bloom extract antigen as measured semi-quantitatively by the number of precipitin arcs resolved and the serum IgG antibody against the purified pigeon gamma-globulin antigen measured by radioimmunoassay, was highly significant for the 54 individual subjects ($r=0.81$, $t=9.9$, $p<0.001$). The immunogenicity of the other purified antigens was similarly compared by measuring the serum IgG antibody to each antigen by ELISA (see Appendix B) in a further 102 pigeon breeders and correlating the extent of the antibody response to each antigen. The antigens compared were the purified pigeon serum gammaglobulin, serum albumin, faecal (secreted) IgA, the pigeon erythrocyte and the crude soluble bloom extract. It was found that each individual's response to all these antigens was significantly correlated (Table 4; Figure 9).

All subjects with antibody to one antigen tended to have antibody to all antigens, and furthermore the extent of the antibody response to one antigen was similar to the extent of the response to all the antigens. There was also a clearly non-responsive group who did not respond to any of the antigens serologically.

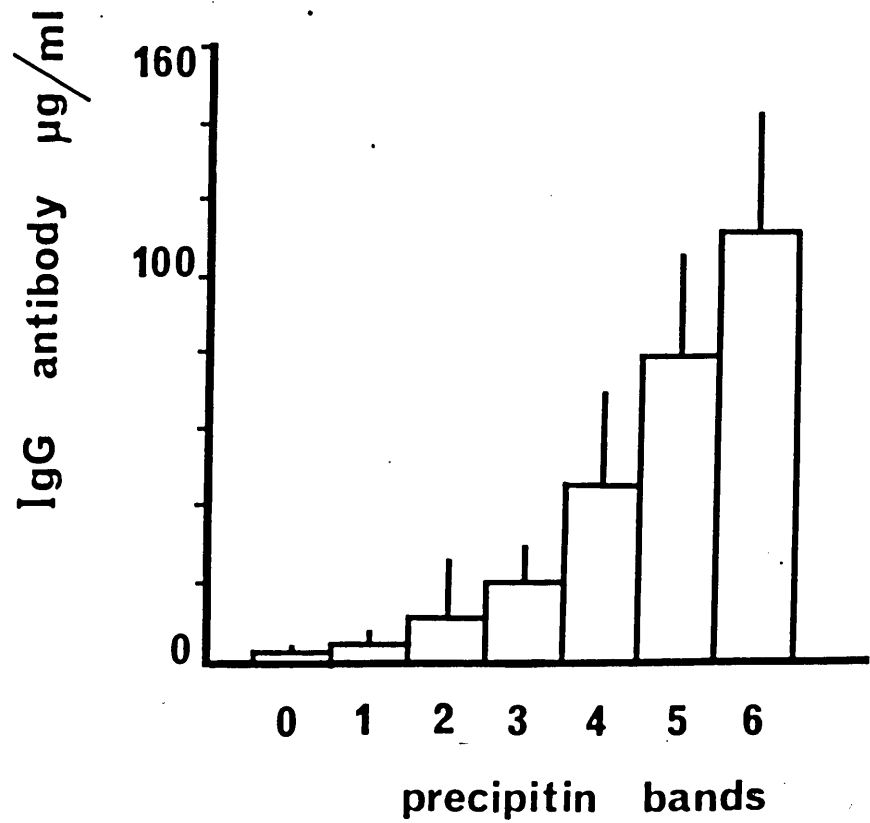


FIGURE 8:

Comparison of the quantitative titre of serum antibody against pigeon serum gamma-globulin antigen (ug/ml IgG mean + standard deviation) and the extent of the serum antibody response against pigeon bloom extract antigens (number of precipitin bands resolved) in 97 pigeon breeders.

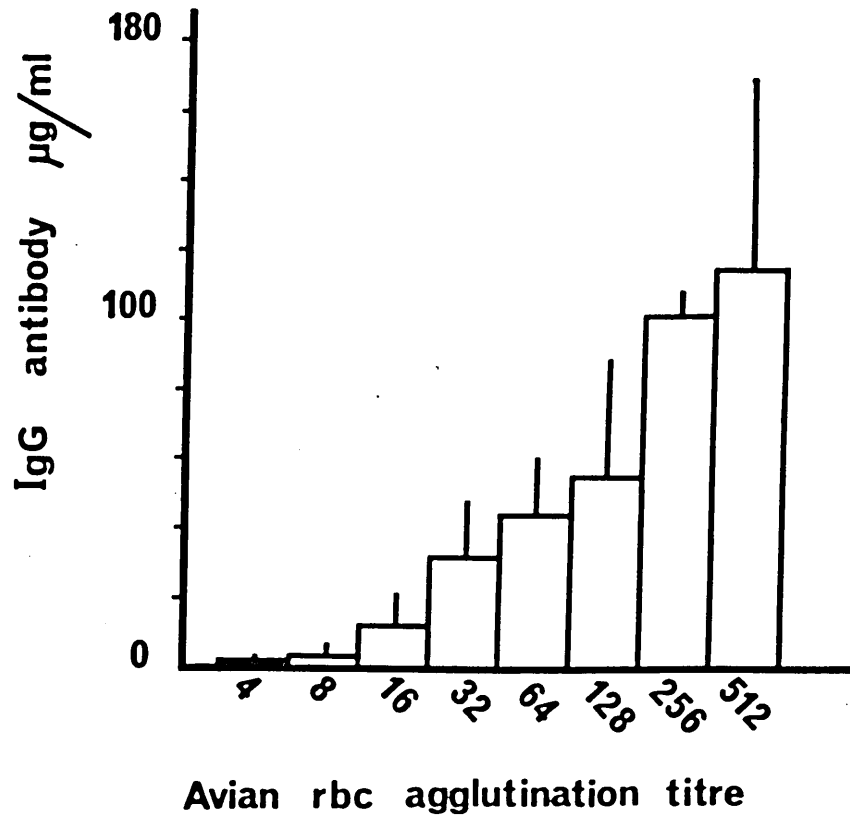


FIGURE 9:

Comparison of the quantitative titre of serum antibody against pigeon serum gamma-globulin antigen (ug/ml IgG, mean + standard deviation) and the titre of serum agglutination of pigeon erythrocytes in 100 pigeon breeders.

ANTIGENS COMPARED	CORRELATION COEFFICIENT
gamma-globulin and sIgA	0.94
sIgA and serum albumin	0.91
gamma-globulin and serum albumin	0.86
sIgA and bloom extract	0.86
gamma-globulin and bloom extract	0.83
serum albumin and bloom extract	0.76

TABLE 4. Comparison of the extent of the serum IgG antibody response in each of 102 pigeon breeders against various avian antigens:- pigeon serum gamma-globulin and albumin, droppings secreted IgA (sIgA) and soluble feather dust (bloom) extract.

ANTIGEN	SYMPTOMATIC	ASYMPTOMATIC	t	P
Serum gamma-globulin	0.656	0.322	2.76	0.01
Serum albumin	1.017	0.528	2.36	0.05
Droppings sIgA	0.381	0.208	2.72	0.01
Bloom extract	0.283	0.193	2.3	0.025

TABLE 5. Differences between the mean serum IgG antibody titres, as measured by optical density, of symptomatic and asymptomatic pigeon breeders using various pigeon derived antigens in an ELISA system.

The same antigens were tested to see which would best discriminate between pigeon breeders with and without alveolitis. The results of the comparisons between 29 symptomatic and 29 asymptomatic subjects are listed on Table 5 (also illustrated on Figures 4 and 6). In all cases the symptomatic group had a significantly higher mean antibody level than the asymptomatic group, but there was no clear separation of these groups based on their serum antibody response to any of these antigens.

In view of the very close association of antibody titres to each of the pigeon-derived antigenic materials discussed above, the possibility of a common antigen was considered. Double diffusion analysis using one high titre serum, and various combinations of antigens demonstrated multiple bands against pigeon serum, pigeon dropping extract and pigeon bloom extract. There were reactions of identity between each antigen source occurring between the major antigens as judged by the precipitin lines. There were also individual minor antigens which were specific for each antigenic source. The common antigenic material in each extract was investigated further, and it appeared to be due to the content of avian immunoglobulin, with the light-chain being common to all.

3. DISCUSSION.

Soluble antigenic extracts from pigeon serum, droppings and feathers are known to be clinically important in that they can provoke an acute episode of alveolitis when inhaled by a sensitised subject (Hargreave & Pepys, 1972; Muller et al, 1976; Warren et al, 1977). Equally in these patients, serum precipitating antibody to these extracts was prevalent. The extent of each individual's immune response to these antigens was determined by various genetic and constitutional factors (discussed in Chapter 3) however the relative responsiveness to each antigen was the same. This meant that the individual with the highest antibody titre to one antigen usually had the highest titres to all the antigens, and the subjects with low antibody levels to one antigen had similar relative titres against the other antigens. The relative responsiveness in one individual against the range of these inhaled pigeon-derived antigens was

therefore a reflection of the availability of these antigens and the immunogenicity of the antigens. For example, pigeon serum albumin appeared to be less immunogenic than pigeon serum globulin in accordance with the observations of Fink et al (1969), and also, whereas albumins could be detected in small amounts in pigeon droppings (Edwards et al, 1969), the gamma-globulin constituted up to 24% of the soluble extract from droppings. This perhaps explained why the pigeon equivalent of IgA seemed to be the most antigenic of the purified proteins. Pigeon IgA was also identified in extracts from the bloom, therefore it was likely that some immunological identity existed between the major sources of antigen, including pigeon serum which tended not to be a feature of the loft environment. This identity was predominantly due to immunoglobulin light-chain determinants which were common to the serum IgG and the excreted IgA. It has been suggested that the pigeon erythrocyte has light-chain on the surface (Diment & Pepys, 1977) and this would explain the close correlation between the red cell agglutination titre and the anti-globulin titre of the patients' sera. Selective inhibition of the red-cell agglutination by adding aliquots of light chain and the various other purified antigens would help to identify the main red cell antigens. It is of interest at this point that there is a seemingly fortuitous identity between the antigens on the pigeon erythrocyte and the antigens associated with the human P1 blood group (Munro et al, 1980). The antigenic structure of P1 is: alpha galactose(1-4)beta galactose (1-4) beta N-acetylglucosamine(1-3) beta galactose (1-4) glucose ceramide. It might be appropriate now to look for similar structures on the avian erythrocyte, and this may be an opportunity to study some of the antigens associated with this disease at the molecular level.

There have been intermittent reports on disease-specific antigens associated with alveolitis (Barboriak et al 1968; Peeters, Brombacher & Maesen, 1971; McCormick et al, 1982). This last group of workers claimed to have isolated a specific antigen which would only be precipitated by the sera from subjects with alveolitis (Calvanico, N. 1983, personal communication) however it is known that the disease process of alveolitis is very dynamic and can be acute, sub-clinical or chronic, therefore, the role of

this putative disease-specific antigen in these various disease presentations is eagerly awaited. It is noteworthy that the proposed disease-specific antigens are generally minor antigenic components, and it is possible that the observation of their "disease-specificity" is based on the fact that the subjects with disease have in general a more florid antibody response, including minor antigens, whereas asymptomatic, antibody-positive subjects will have more moderate antibody responses which would mean that the antibody titre of these minor antigens would be below the detection limit of the precipitin techniques used by the above workers. More sensitive quantitative techniques such as radioimmunoassay or ELISA (appendix B) would resolve this issue.

An alternative source of antigen to pigeon droppings has been investigated, namely pigeon bloom. This material was abundant and is readily airborne in contrast to droppings which were hygroscopic and bulky. A soluble extract of bloom contained pigeon IgA and up to five other major antigens, and the protein bands which focussed at pI 4.7 to 4.1 were remarkably similar to the protein band of PDE1 (pigeon IgA) which focussed at pI 4.6 (McCormick et al, 1982). The number of these bands which focussed between pI 4.7 and 4.2 was consistent with the charge heterogeneity characteristic of polyclonal immunoglobulin.

One further feature of bloom was its particulate nature. By microscopy the particles were on average one micron in diameter, which was in accordance with the estimate of Stettenheim (1972). This ornithologist describes the bloom as being composed of granules of keratin approximately one micron in diameter. Keratin is a highly hydrophobic protein, very resistant to enzyme degradation and may act therefore as an inflammatory or adjuvant stimulus when inhaled into the lung. This resistance to proteolysis could also cause granuloma formation in the lung. The granules are coated with the main soluble antigens associated with the disease and they are of the correct aerodynamic size to penetrate and sediment in the alveoli, therefore, they may act as a vehicle carrying the antigens to the alveoli where the disease process is most evident.

For this thesis the antigen source used for all serological testing and for skin testing and inhalation

provocation testing was pigeon serum. This antigen source was the most readily available and its use was described widely in the literature. Furthermore, when sterilised by heating at 56°C for 30 min and Seitz filtering, it was considered safe and proved to be non-toxic and non-irritant for skin testing and inhalation challenges.

CHAPTER 3THE ANTIBODY RESPONSE1. THE SERUM IgG ANTIBODY RESPONSE TO INHALED AVIAN ANTIGENS.

Since serum antibody against inhaled antigens was first observed in cases of extrinsic allergic alveolitis among farmers (Pepys, Riddell, Citron & Clayton, 1961), the association of specific serum precipitins with each disease syndrome of alveolitis has been consistently reported (Molina, 1976; Turner-Warwick, 1978; Belin, 1981). The incidence of these precipitins in the general population is very low (Elgefors, Belin & Lanson, 1971; Dodge & Barbee, 1977; doPico et al, 1976) whereas in populations of subjects with regular avian exposure the reported incidence of precipitating antibody has ranged from 11% (Boyer et al, 1974), 16% (Reed, Sosman & Barbee, 1965), 20% (Barboriak, Fink, Sosman & Dhalival, 1973), 27% (Elman, Tebo & Fink, 1968), to 60% (Elgefors, Belin & Lanson, 1971). These high incidences of serum precipitins have been used as evidence to suggest that this antibody was only a reflection of avian exposure rather than avian induced alveolitis (Fink et al, 1972), and this has brought the diagnostic significance of these precipitins into question (Salvaggio, 1972; Burrell, Law & Olenchock, 1978; Burrell & Rylander, 1982). However, a closer association between antibody and disease was suggested by Radermecker (1971) who found precipitins in 27% of healthy pigeon fanciers and in 75% of pigeon fanciers with respiratory disease, and Molina (1976) found a significantly higher incidence of precipitins in farmers with alveolitis than in their asymptomatic counterparts.

Using a sensitive quantitative radioimmunoassay technique in an attempt to improve upon the resolution of precipitins, Boyd (1978) confirmed that there was a difference in the mean IgG antibody levels against a defined pigeon-derived antigen between the sera of pigeon fanciers with and without alveolitis. Subjects with disease had a significantly higher antibody titre than the asymptomatic subjects but there was an overlap of values, with some asymptomatic subjects having high antibody titres, and a

small number of subjects describing typical symptoms without demonstrable antibody.

A systematic evaluation of the humoral immune response against inhaled antigens was therefore required. Firstly, methods with adequate sensitivity for accurately measuring the serum antibody response were evaluated (see Appendix B). The most reliable system was a radioimmunoassay based on the method of Neilson, Parratt, Boyd & White, 1974. Using this the antibody levels were measured in a large population of pigeon breeders in order to assess the normal distribution of responsiveness against a purified pigeon gamma-globulin antigen. The effects of various parameters including age, avian exposure and smoking habit on this antibody response were also studied in order to provide a background against which the quantitative antibody levels could be compared with the clinical presentation and incidence of alveolitis, and with different symptom patterns of hypersensitivity in individuals at one point in time and, more importantly in a prospective manner.

2. FACTORS AFFECTING THE ANTIBODY RESPONSE.

a) Cigarette smoking

Smoking has been reported to impair pulmonary immune mechanisms (Holt & Keast, 1977) and has been associated with an increased susceptibility to various lung diseases (Haynes, Krstulovic & Loomis, 1966). However, during epidemiological investigations of extrinsic allergic alveolitis among farmers (Morgan et al, 1975; Gruchow et al, 1981) and among pigeon fanciers (Boyd, 1977; Warren, 1977), the reported incidence of the disease was found to be lower in cigarette smokers.

In the survey of 102 pigeon fanciers (see appendix A) there were 23 smokers, 65 non-smokers and 14 ex-smokers. The IgG antibody levels against inhaled avian antigens in these subjects is plotted on Figure 10. The mean antibody level in the smokers is 2.9 ug/ml (standard deviation = 4.0) and this is significantly lower than the non-smokers' mean titre of 31.7 ug/ml (s.d. = 39.2). The mean antibody level in the ex-smokers was 25.6 ug/ml (s.d. = 37.4) therefore this antibody responsiveness appeared to be restored in subjects who stopped smoking, and the apparent

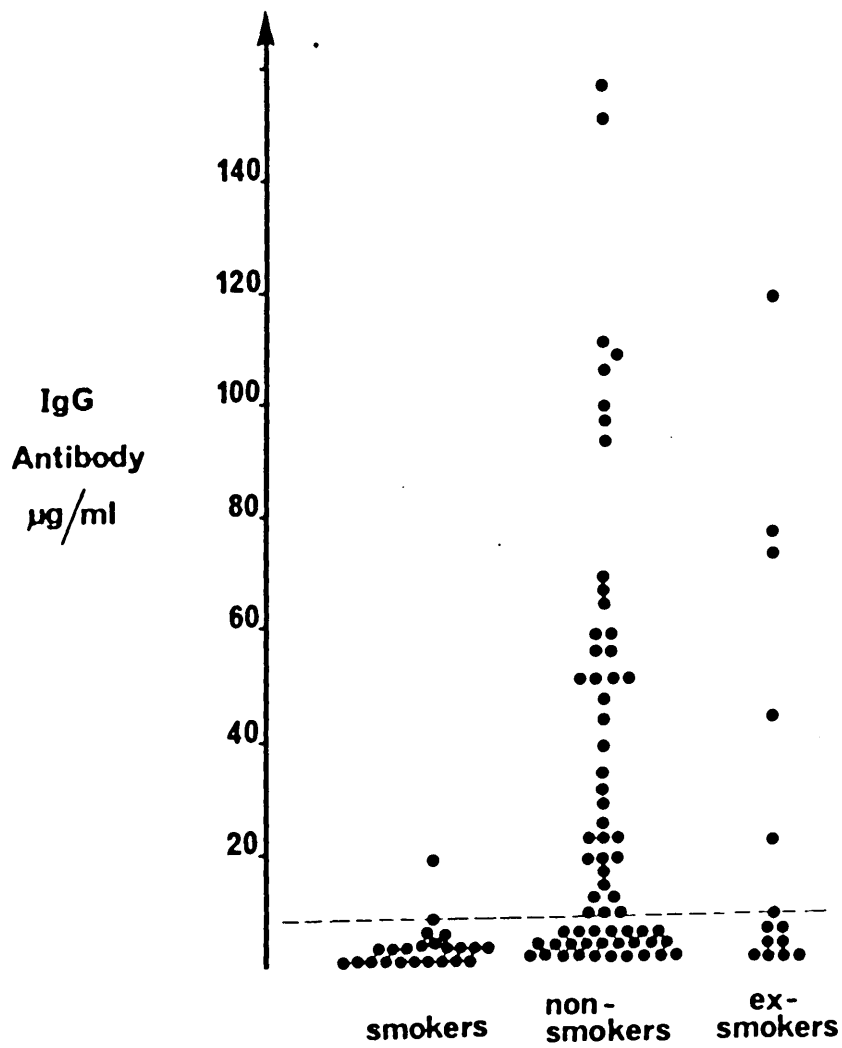


FIGURE 10:

Serum IgG antibody levels against pigeon gamma-globulin antigen in 102 subjects according to smoking history.

inhibitory effect of smoking on the antibody response to inhaled antigens seemed to be reversible.

The smoking habit of the subjects in the earlier survey of 277 subjects (see appendix A) was divided into those who smoked at least 5 pack years or not (1 pack year = 20 cig. per day for 1 year, or an equivalently shorter time with proportionally more cigarettes per day). The data from both surveys are summarised on Table 6. In this table the percentage of smokers who mounted an antibody response is clearly much lower than the non-smokers. Furthermore, the smokers who did mount an antibody response have a much reduced mean titre. This was highly significantly lower than the titre of non-smokers ($T = 2.75$, $p < 0.01$).

Smoking would appear to have a profound inhibitory effect on the ability to mount an antibody response to these inhaled antigens. It was important therefore to establish the incidence of smokers according to age and years of avian exposure before drawing any conclusions about the effects of these parameters on the antibody response; which is to be discussed in the two following sub-sections. Table 7 shows the distribution of smokers among 379 pigeon breeders divided into decades of age and avian exposure. There were no major differences in the percentage incidences of smokers with each decade group except for a lower incidence of smokers older than 71 years and similarly for subjects having kept pigeons for longer than 51 years. This was presumably due to the lower age survival of smokers.

b) Age

The ability to mount a serum antibody response to inhaled avian antigens as a function of age was investigated in 379 pigeon fanciers (Table 8). The mean age was 44.9 years and the population appeared to be normally distributed. The proportion of antibody positive subjects within each age-decade group was very similar between the ages of 21 and 70 years. This comprised the majority, 90.5%, of the study group. The youngest age group - younger than 20 years, had a lower incidence of antibody-positive subjects, whereas the oldest group - older than 71 years, had a higher incidence. The mean antibody titre was also highest in this age group and this was in accordance with the lower numbers of smokers in the group (Table 7). The mean antibody

	Smokers	Non-Smokers
Number	145	220
Antibody positive	27 (18.6%)	111 (50.5%)
Antibody level (mean plus s.d.)	17.1 (20.4%)	43.7 (49.1)*

* $t = 2.75$ $p < 0.01$

TABLE 6. The number of antibody positive subjects (IgG > 4 ug/ml) and their mean IgG antibody level (ug/ml) against pigeon gamma-globulin antigen in 365 pigeon breeders according to smoking history.

Years	Number	Smokers	%
<u>Age</u>			
1-20	19	7	36
21-30	40	18	45
31-40	84	33	39
41-50	89	36	40
51-60	83	30	36
61-70	47	20	42
71-80	17	4	23
<u>Avian Exposure</u>			
1-10	102	46	45
11-20	97	37	38
21-30	85	31	36
31-40	30	11	37
41-50	42	14	33
51-60	23	6	26

TABLE 7: The percentage of smokers within each decade increment of age and avian exposure for 379 pigeon fanciers.

	AGE (Years)						
	0-20	21-30	31-40	41-50	51-60	61-70	71-80
Number	19	40	84	89	83	47	17
Mean IgG Level	14.7	19.0	9.7	17.2	12.4	12.3	34.2
<u>Antibody Positive</u>							
Number	4	14	27	30	37	16	9
Percent	21	35	31	34	45	34	65
Mean IgG level	62.3	50.6	26.0	47.1	25.3	32.3	62.8

TABLE 8. Effect of increasing age in decades on the serum IgG antibody response (ug/ml) against avian antigen in 379 pigeon breeders.

levels in the other groups did not markedly fluctuate, and there was no correlation between antibody level and age ($r=0.03$, $t=0.32$, $p=\text{not significant}$). Moreover, when the antibody titre of the antibody-positive subjects only was compared with age there was again no correlation ($r=-0.07$, $t=0.69$, $p=\text{not significant}$). It would appear, therefore, that the individual subject's age had no bearing on the ability to mount an antibody response to inhaled avian antigens, nor the extent of this response.

c) Avian exposure

The extent of avian exposure would be expected to affect the antibody response to avian antigens. An 'exposure index' was proposed by Fink and colleagues (1972) to include the length of time spent with birds and the number of birds; they suggested that there was a higher incidence of precipitins in the subjects with a greater exposure index. This relationship was examined in this section using quantitative antibody measurement techniques to assess the effects on the IgG antibody levels of various parameters of exposure, including number of birds and exposure time in hours per day and also in numbers of years.

i) Number of birds

The number of subjects who mounted a significant immune response to inhaled avian antigens was shown to increase with exposure to an increasing number of birds (table 9). Increments of 25 birds were chosen arbitrarily to give a reasonable scale of the numbers of birds normally handled. Between these increments there were minor fluctuations of mean serum IgG antibody levels but no trends emerged. There was however a clear trend of an increasing incidence of antibody responders having contact with an increasing number of birds. However, there was an indication that the extent of the antibody response of these antibody-positive subjects was reduced with increasing avian contact.

ii) Years of avian exposure

The mean number of years of avian exposure for the group was 22 years and the distribution and range are listed on Table 10. There was a gradual decrease in the mean antibody level of the subjects in each exposure decade, but this was not significant ($r=-0.03$, $T=-0.28$, $p=\text{ns}$). There was a gradual increase in the number of subjects who were antibody positive at each increasing

	<u>AVIAN EXPOSURE (Number of birds)</u>				
	25	50	75	100	>100
Number	54	202	70	37	16
Mean IgG level	6.0	18.6	11.6	13.5	13.0
<u>Antibody Positive</u>					
Number	10	70	26	18	8
Percent	18.5	28.6	35.8	48.6	50
Mean IgG level	23.6	49.9	27.8	25.6	24.0

TABLE 9. The serum IgG antibody response (ug/ml) to avian antigens in 379 pigeon breeders as a function of the number of birds kept.

	<u>AVIAN EXPOSURE (Years)</u>					
	1 - 10	11 - 20	21 - 30	31 - 40	41 - 50	51 - 60
Number	102	97	85	30	42	23
Mean IgG Level	17.2	15.3	15.2	13.6	16.3	5.8
<u>Antibody Positive</u>						
Number	27	31	34	15	20	8
Percent	26	32	40	50	48	35
Mean IgG level	59.6	43.6	34.9	25.1	32.1	13.0

TABLE 10. Effect of increasing decades of avian exposure on the serum IgG antibody response (ug/ml) to avian antigens in 379 pigeon breeders.

exposure decade, until the oldest group. Despite this there was a significantly decreasing mean antibody level when the IgG titres of the antibody positive subjects were plotted against years of exposure ($r=-0.22$, $t=-2.3$, $p<0.025$). This is plotted on Figure 10. It would appear, therefore, that increasing avian contact in terms of years exposure is associated with reduced antibody titres.

iii) Hours of daily avian contact

The information regarding the number of hours per day in contact with pigeons was tabulated for 102 subjects on Table 11. The average time spent was 3.9 hours/day. With increasing daily hours of exposure, there was a significant decrease in both the number of subjects who were antibody responders ($r=-0.93$, $t=-5.78$, $p<0.005$) and the mean antibody level ($r=-0.88$, $t=-4.14$, $p<0.01$). It would appear therefore that increasing daily avian contact, like the other parameters of increasing avian exposure, is associated with reduced antibody titres.

3. THE ASSOCIATION OF ANTIBODY WITH DISEASE.

a) The profile of antibody levels in a population of pigeon breeders:

The serum IgG antibody to pigeon gamma-globulin was measured in 507 pigeon breeders (see Appendix A), and the number of individuals occurring within 10 ug/ml IgG antibody increments are plotted in histogram form on Figure 12. The upper limit of normal had been previously established at 4 ug/ml of antibody, and 257 of the pigeon breeders, 51%, had levels above this limit. The mean antibody level for the whole group was 19.9 ug/ml and 38.2 ug/ml for the 257 antibody positive subjects. The highest individual values of specific antibody were measured up to approximately 500 ug/ml.

b) The incidence of alveolitis relating to the antibody levels in a population of pigeon breeders:

One hundred and ten subjects (22%) fulfilled the criteria for avian related extrinsic allergic alveolitis (see Appendix A), and the percentage of these subjects within each of the above antibody-increment groups is illustrated on Figure 13. The incidence of disease was seen to increase from 3.7% in the

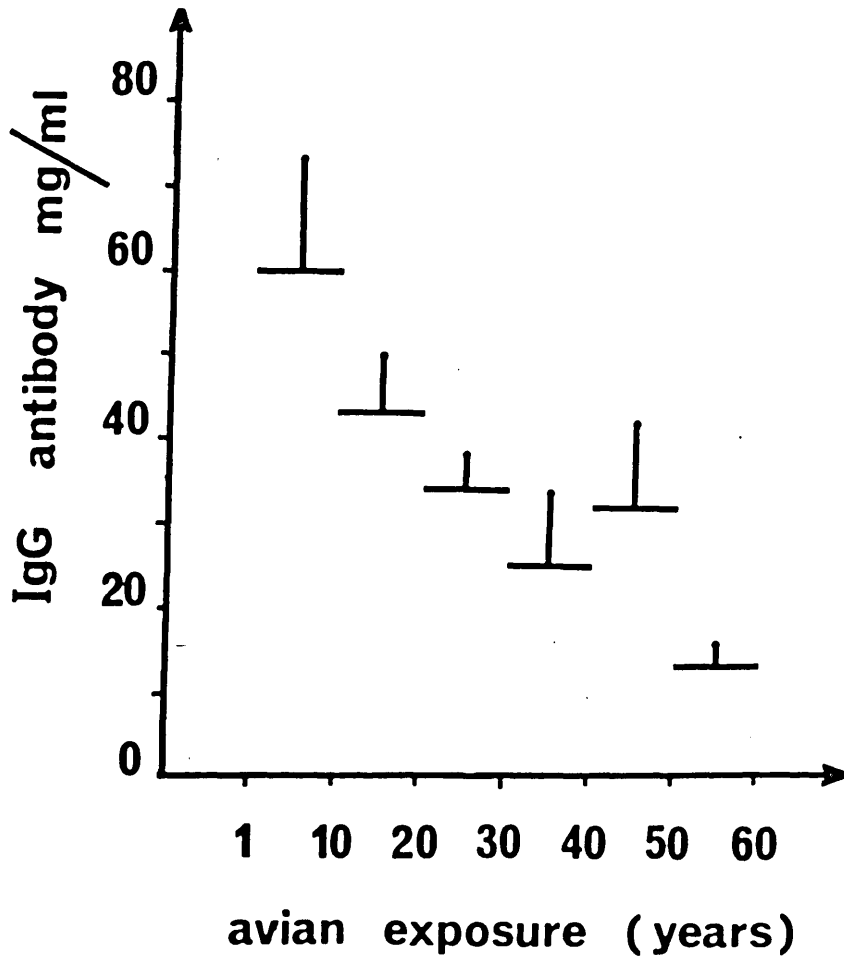


FIGURE 11:

The decrease of serum IgG antibody levels (mean + S.D.) of 135 antibody-positive pigeon breeders according to increasing decades of avian exposure.

	<u>AVIAN EXPOSURE (Hours per day)</u>						
	1	2	3	4	5	6	7
Number	6	25	19	8	25	11	7
Mean IgG level	19.0	27.7	14.1	11.0	5.6	3.3	3.1
<u>Antibody Positive</u>							
Number	4	16	9	3	7	2	2
Percent	66.6	64	47	37.5	28	18.2	28.6
Mean IgG level	27.5	42.2	27.5	25.9	14.9	9.2	5.7

TABLE 11. Daily avian exposure and the mean IgG antibody response (ug/ml) against avian antigens in 102 pigeon breeders.

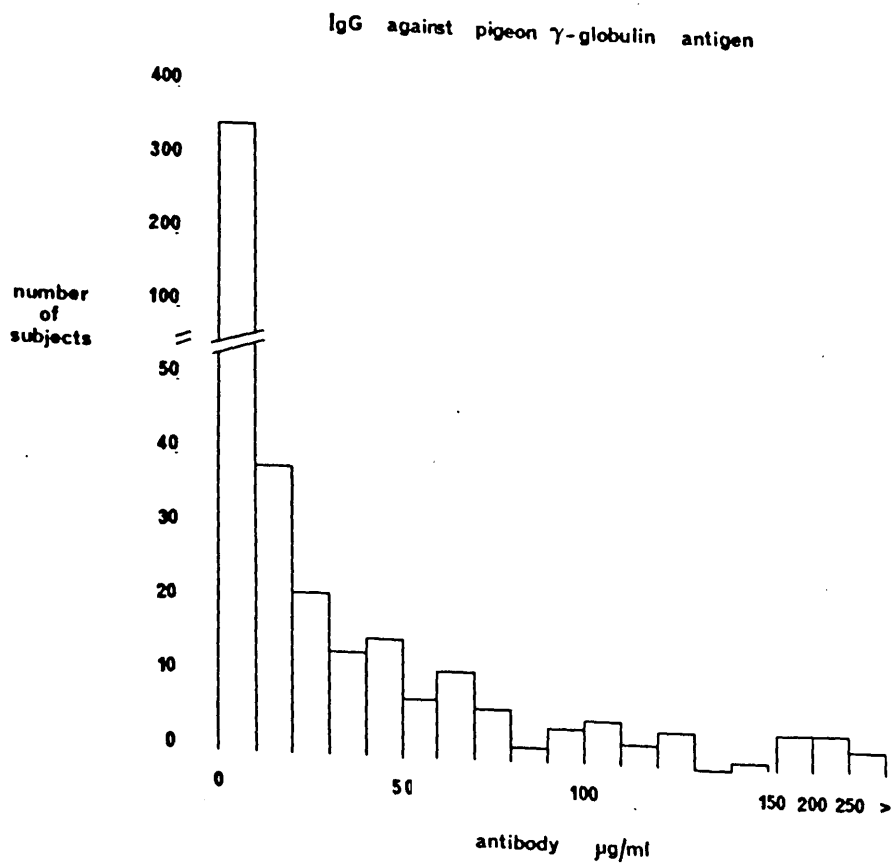


FIGURE 12:

Histogram of the distribution of serum levels of IgG antibody to avian antigens in a population of 507 pigeon breeders.

Distribution of symptomatic subjects

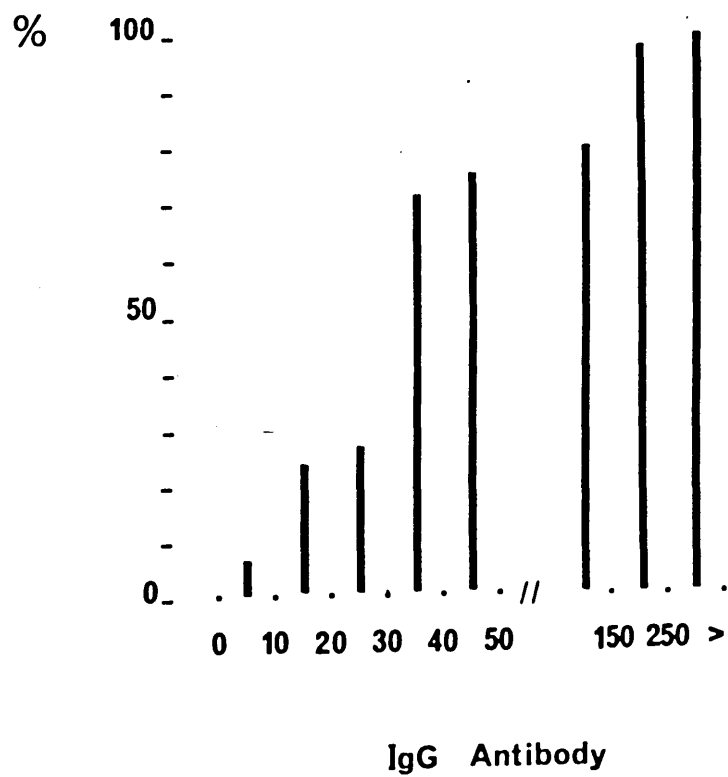


FIGURE 13:

The incidence of extrinsic allergic alveolitis within groups of pigeon breeders with increasing increments of serum antibody levels (ug/ml).

antibody-negative group to 78% with antibody greater than 30 ug/ml, and virtually all subjects with antibody levels greater than 150 ug/ml had pigeon breeders disease.

The association between antibody and pigeon breeders disease was statistically significant (chi-squared = 57.7, $p < 0.001$). The largest subgroup of the 507 subjects, 46%, had neither antibody nor disease, and the majority of the 110 subjects with PBD, 83%, had significant antibody levels. However, there was antibody without symptoms in 33% of the subjects and a small percentage, 3.7%, claimed symptoms without antibody (Table 12).

c) Correlation of symptoms of EAA with antibody levels:

In the group of 102 pigeon fanciers who were interviewed extensively for the occurrence of pulmonary and systemic hypersensitivity symptoms related to avian exposure (see Appendix A, Table 39), 53 denied any late symptoms whatsoever and 49 admitted to a range of between one and all eight symptoms investigated.

The extent of the antibody response to avian antigens in the asymptomatic group and the groups describing increasingly broader symptoms is outlined in Figure 14. This shows that a progressively complete symptomatic picture of classical alveolitis symptoms in a population is closely associated with higher antibody levels.

The same group of subjects also responded to questions relating to immediate hypersensitivity symptoms, and a similar comparison of IgG antibody level showed no correlation with the extent of immediate symptoms following avian contact. Each group had a mean IgG antibody value similar to that of the whole study group population (group mean=23.2 ug/ml, sd=35.0).

102 subjects were divided into symptomatic groupings according to the clinical criteria outlined in Appendix A. Four groups were categorised as having - A: alveolitis, B: no symptoms, C: indeterminate symptoms and D: asthmatic symptoms caused by avian exposure. The IgG antibody levels of the subjects in each subgroup are illustrated in Figure 15, and summarised along with the values of control groups on Table 13. The alveolitis group had a significantly higher mean antibody

	<u>Alveolitis</u>	
	Negative	Positive
Less than 4 ug/ml	296 (58.4%)	19 (3.7%)
Greater than 4 ug/ml	101 (19.9%)	91 (17.9%)
Chi-squared = 120.1		
p < 0.001		

TABLE 12. Correlation between the presence of IgG antibody (greater than 4 ug/ml) and extrinsic allergic alveolitis in 507 pigeon fanciers.

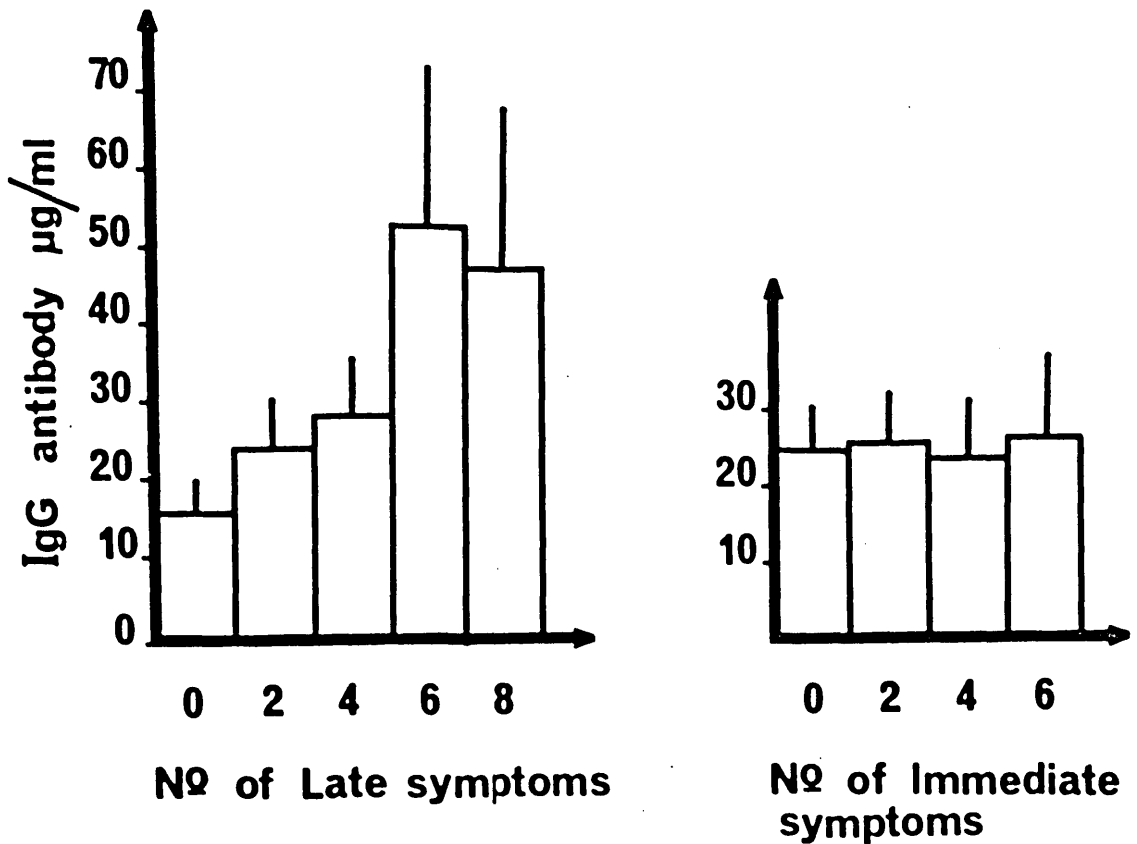


FIGURE 14:

The mean (plus s.d.) IgG antibody level against avian antigens in 102 pigeon breeders according to a progressively more complete symptomatic picture of alveolitis (late symptoms) or asthma (immediate symptoms).

GROUPS	NUMBER	MEAN	S.D.	POSITIVE
<u>Subjects</u>				
Alveolitis	32	41.4	43.3	22
Asymptomatic	28	14.0	25.5	8
Indeterminate	34	13.2	22.2	10
Asthma	8	7.2	11.2	2
<u>Controls</u>				
Normals	58	3.7	2.1	0
Myeloma	32	4.37'	2.47	0
S.L.E.	12	4.2	2.7	0
Asthma	40	4.1	2.1	0

TABLE 13. The mean and standard deviation of serum IgG antibody levels (ug/ml) against pigeon gamma-globulin antigen in 102 pigeon breeders subdivided into symptomatic groupings, in 58 normal subjects with no avian exposure and in 83 pathological sera from patients with myeloma, systemic lupus erythematosus and asthma.

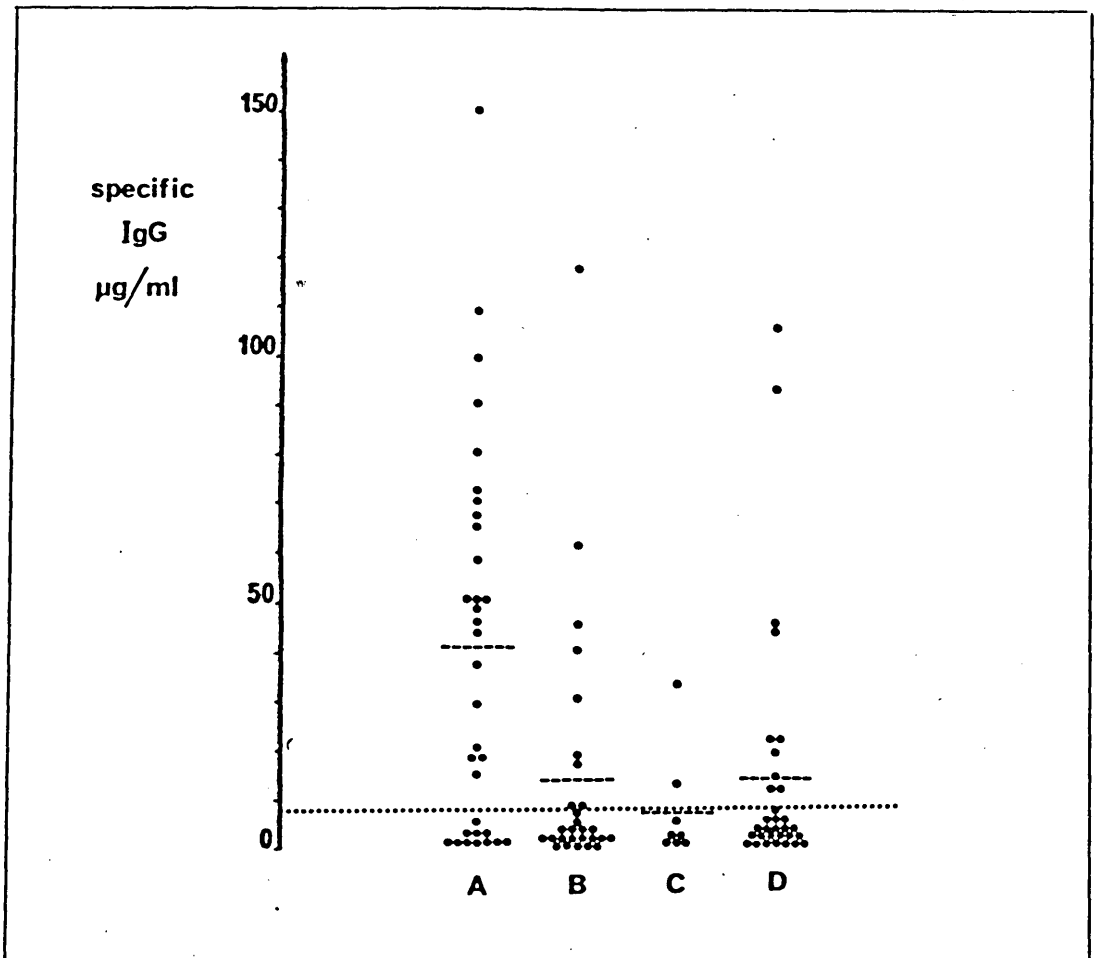


FIGURE 15. The serum IgG antibody levels against pigeon gamma-globulin antigen in four groups of pigeon breeders: A, with alveolitis; B, with no symptoms; C, with indeterminate symptoms; and D, with asthmatic symptoms. The mean bar is indicated by the hatched line in each group, and the dotted line is the upper limit of normal.

titre than the other subgroups. Sera from healthy control subjects with no avian exposure had insignificant levels of antibody, and control pathological sera from patients with myeloma, systemic lupus erythematosus, S.L.E., and asthma also had insignificant levels. Thus the presence of high levels of serum immunoglobulin in the myeloma sera and the high levels of immune complexes in S.L.E. sera did not affect the measurement of specific antibody. There was also no specific IgG antibody to avian antigens associated with asthma, and subsequently with other respiratory disorders including sarcoidosis and alveolitis caused by M. faeni and Aspergillus sp.

4. DISCUSSION.

The diagnostic importance of precipitins against a panel of likely antigens has been stressed in a recent report of the experience of one laboratory which had tested the sera of approximately 10,000 patients for whom EAA was a possible diagnosis (Belin, 1981), and similar antigen screening has demonstrated a low incidence of positive precipitins in the general population; with 1.8% of 3047 subjects reported positive (Dodge & Barbee, 1977) and 4.9% of a further 1072 subjects (Chmelik, Flaherty & Reed, 1974); these antibodies being predominantly against ubiquitous thermophilic Actinomycetes, although a small proportion, 0.8%, of the last group had precipitins to pigeon serum. There was no reference to any avian exposure in this small precipitin positive group which would have been important to know since there is extensive antigenic similarity between the sera of different avian species (Sennekamp et al, 1981) mainly due to the albumin fraction. Thus, the absorption of dietary hen's egg antigens from the gut in certain inflammatory bowel diseases (Taylor et al, 1964; Ferguson & Carswell, 1972) could result in serum antibody which would precipitate with pigeon serum antigens, however specific antigenic determinants on the serum immunoglobulin molecule of the pigeon can be used to differentiate antibody production due to pigeon exposure from these dietary antigens (Faux, Hendrick & Anand, 1978). This specific pigeon gammaglobulin antigen was used in the present study. However, false positive precipitin

reactions could occur against teichoic acids present in various antigenic extracts particularly pigeon droppings extract (Faux et al, 1970) and non-specific, calcium-dependent precipitations by the serum acute phase protein, C - reactive protein (Longbottom & Pepys, 1964).

The various reported prevalence of precipitins have been discussed earlier (Chapter 1, 4f), and in the study population of this report, using a more sensitive assay method, the prevalence was 51%. About half of the subjects therefore were non-responders to inhaled avian antigens despite similar rates of avian exposure as in the antibody positive group. The questions which now arise include (a) what factors determine whether a subjects will be an antibody responder or non-responder; (b) what factors affect the extent of the antibody response; and (c) is the presence of serum antibody of any diagnostic significance?

The ability to mount an antibody response is controlled fundamentally by the Ir genes (Munro & Waldmann, 1978) and subsequently by T-lymphocytes (Virelizier et al, 1974). The genetic disposition of subjects with alveolitis has been investigated and association have been reported with various HLA haplotypes (discussed in Chapter 1, 4g), however, previous work from this laboratory (unpublished observations) and other centres (Schatz et al, 1976; Rodey et al, 1979), did not support an association between a particular HLA haplotype and disease. Nevertheless, like the genetics of the immune response to allergens (Marsh et al, 1980), the responsiveness to inhaled antigens and the development of extrinsic allergic alveolitis are likely to be a function of several genetic factors, both antigen specific (Wilson et al, 1982) and antigen non-specific (Wilson et al, 1982; Keller, Calvanico & Stevens, 1982). These control firstly the ability to mount an antibody response to specific inhaled antigens, and the extent of this response, and secondly the development of alveolitis.

The purpose of the present study was to investigate other parameters thought to affect this antibody response. One of the strongest environmental factors influencing the development of a serum antibody response to inhaled antigens was the smoking habit. Cigarette smoking was associated with a lower incidence

of antibody-responsiveness, and even in the few responders the extent of the response was significantly lower. The reasons for this are obscure; it has been reported that smoking causes small airways obstruction (Dolovitch et al, 1976) resulting in more proximal deposition of inhaled particles (Bohring et al, 1975). Thus, the access of organic dusts to the alveoli where the disease process of avian hypersensitivity is most evident (Hensley et al, 1969) may be hindered.

There are, however, more fundamental effects of smoking on the development of antibody. The products derived from cigarette smoke have immunosuppressive activity (Roszman & Rogers, 1973) in vitro, and in vivo (Thomas, Holt & Keast, 1974), and smokers have an increased susceptibility to infection (Haynes, Krstulovic & Loomis, 1966: Nymanol, 1974), with lower subsequent antibody titres (Finklea et al, 1971) and a general suppression of total IgG and IgA has been reported (Gulsvik & Fagerhod, 1979). Within the lung compartment, smoking has been shown to markedly increase the local number of alveolar macrophage (Warr et al, 1976) and one consequence of increasing the alveolar macrophage: lymphocyte ratio was immuno-suppression in vitro (Schuyler & Todd, 1981). These mechanisms may be responsible for the impaired ability of smokers to respond to inhaled avian antigens.

The age of the individual did not determine the antibody responsiveness, as has been shown experimentally in mice (Crousle, Miller & Grove, 1981). However other literature would suggest that older subjects have a decreased capacity for humoral responsiveness (Heidrick & Makinodan, 1973: Pahwa, Pahwa & Good, 1981). In fact, the proportion of responders in the older group in this study, i.e. older than the mean of 44.9 years, was higher than the younger group (40.3% vs 31.9%, Chi-sq=2.9, $0.05 < p < 0.1$), however, the extent of this antibody response was less, (38.6 ug/ml vs 44.0 ug/ml, p=ns).

Having established, therefore, that there was no significant effect of age on the antibody response, it was possible to measure the effects of varying lengths of time of avian exposure on the antibody response. With increasing years of exposure, and with increasing numbers of birds kept, the

incidence of antibody-responders increased. However, the extent of the antibody response diminished significantly with continued exposure. This effect was also noticed in the daily exposure rates where subjects who spent more hours per day with their pigeons had significantly reduced antibody levels. Since these changes in antibody level were not due to either age or smoking habit then it is possible that the decreasing levels of antibody with time may be due to a desensitising process as described in animal models of the disease by Richerson and colleagues (1981) and Wilkie & Neilson (1981).

There was, however, evidence that subjects with symptoms of extrinsic allergic alveolitis had reduced daily exposure to birds (Banham et al, 1984). This EAA group did not differ significantly from the whole study population in age, number of birds kept, nor years of avian exposure, but the lower mean hours-per-day exposure (2.3 hr. vs. 4.2 hr. for the whole group) would suggest that they were aware of their symptoms and that this reduction in exposure-time was a compromise between their health and their hobby. It may be appropriate at this point to underline the intense committment these pigeon fanciers have for their birds. It is often a life-long pastime that has been in the family for generations, and apart from the considerable financial investment involved, the sport is often the main focus for social activity. This means that they generally ignore any advice to give up the hobby until their allergy is particularly troublesome; or they may find an arbitrary balance by wearing a mask and reducing their daily exposure, thereby remaining asymptomatic.

The antibody levels against pigeon derived antigens were shown to be a good indicator of the probability of having pigeon breeders disease, and also to be a useful parameter to monitor clinical progress. However, the value of antibody measurement using precipitin techniques was questioned (Salvaggio, 1972) principally due to the high incidence of precipitins in asymptomatic subjects (Fink et al, 1972). The present study has shown that 52.6% of subjects with significant antibody levels were asymptomatic, however, when the level of this antibody response was quantified, a clear association between increased

disease incidence and progressively higher antibody levels became apparent. Furthermore, several subjects with high antibody titres developed functional and serological changes following controlled inhalation challenges with nebulised pigeon serum, without overt symptoms (see Chapter 5), and other workers have reported the association of antibody in asymptomatic subjects with pulmonary function abnormalities (Allen et al, 1975, 1976; Braun et al, 1980; Hapke et al, 1968), and with evidence of an inflammatory process (Sennekamp et al, 1978, Turton et al, 1981). It would seem therefore that the antibody positive subjects, particularly those with high titres, form more of an "at-risk" population than is presently considered.

By far the exceptional situation was the antibody-negative subjects with alveolitis; 3.7% of our study group fell into this category, and it has been described also by Edwards, Baker & Davis (1974); Sennekamp et al, (1978) and Canet et al (1980). Again, confirmation by positive late alveolitis reactions to inhalation challenges with appropriate antigen, in subjects who were antibody-negative by radio-immunoassay, would resolve the controversy about whether or not antibody-negative subjects have disease.

These observations reaffirm the importance of determining the presence and quantifying the serum IgG antibody in the investigation of the epidemiology and natural history of extrinsic allergic alveolitis. Moreover, these measurements are a clear guide to the therapeutic progress of patients with acute disease, and are of value in monitoring subjects who may otherwise develop insidious disease.

CHAPTER 4THE ASSOCIATION OF SOME SEROLOGICAL MEASUREMENTS WITH ALVEOLITIS AND WITH ANTIBODY RESPONSIVENESS TO INHALED ANTIGENS.1. SERUM IgM and IgA ANTIBODY.a) Introduction.

The serum antibody of alveolitis patients is usually demonstrated by precipitin reactions using the appropriate causative antigens. The precipitin technique precludes quantitative measurement of the serum antibody, as discussed in Chapter 3, and furthermore it gives no information of the immunoglobulin class distribution of the antibody activity. The presence of IgG antibody against avian antigens did not correlate directly with the presence of alveolitis among pigeon breeders, therefore, the serum antibody in the other major classes IgA and IgM was measured to see if they could better discriminate between symptomatic and asymptomatic subjects. Moreover, the antibody activity was measured in IgA and IgM classes in a large population of pigeon breeders, and was compared with their IgG antibody levels (and IgE antibody - see section 3) in order to obtain a full profile of the antibody responsiveness against inhaled antigens.

b) Antibody activity in the major classes.

The serum IgG, IgM and IgA antibody activity against pigeon gamma-globulin antigen was measured by an amplified enzyme-linked immunosorbent assay, ELISA, using class specific antisera (Appendix B). Serum was obtained from 54 pigeon breeders and from 58 control subjects with no avian contact. The antibody measurements in optical density units are graphed on Figure 16. Forty subjects (74.1%) had higher than normal IgG antibody levels, and 20 (37.0%) and 5 (9.3%) had significantly raised IgA and IgM antibody levels respectively.

Having established that there was antibody activity in the IgM and IgA classes, the levels of these were compared in two groups of pigeon breeders - with and without alveolitis. The results using pigeon gamma-globulin antigen are illustrated on

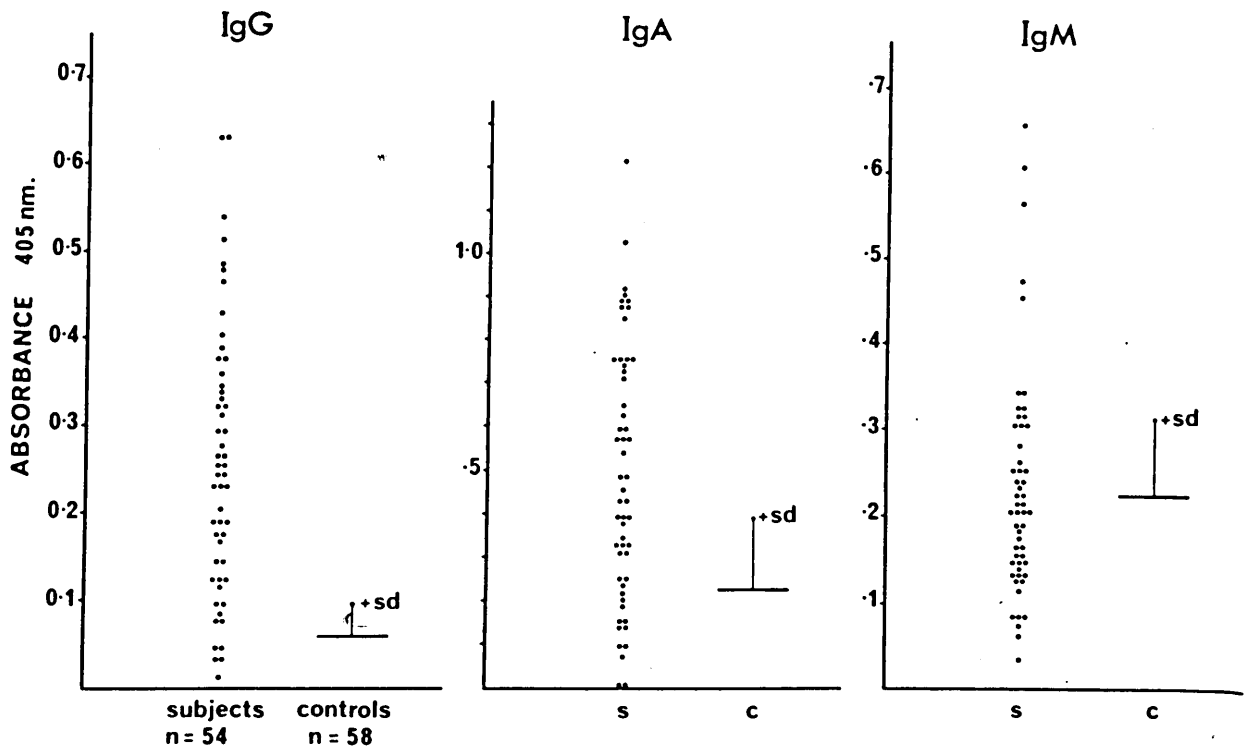


FIGURE 16:

The serum antibody activity in the 3 major classes against avian antigens in 54 pigeon breeders and 58 control subjects with no avian exposure.

Figure 17, and using purified pigeon droppings sIgA antigen on Figure 18. There were significant differences between the two groups in all the antibody classes, and despite this, there are still areas of overlap between the ranges of the antibody levels between the two groups.

It became apparent that the subjects with the highest antibody activity in the IgG class also had the highest antibody titres in the other classes. The relative extent of the antibody activity in all three classes was compared in 102 pigeon breeders against gamma-globulin antigen. The correlations between the classes were, IgG and IgA:- $r=0.83$, IgG and IgM:- $r=0.70$, and for IgA and IgM:- $r=0.77$. These were all highly significant, and similar correlation coefficients were measured for the class specific antibody titres to the other major antigens.

c) Discussion.

Antibody activity against avian antigens can be measured in the IgA and IgM classes in the serum of pigeon breeders if a suitably sensitive technique is used. Patterson and colleagues (1976) using a tube radioimmunoassay method and Faux and colleagues (1971) using a radioimmuno-electrophoresis technique also demonstrated antibody activity in the three major classes against avian antigens. These workers commented that the measurement of IgM and IgA antibody may have diagnostic value.

In this section it would appear that measuring these antibody classes does not resolve the paradox of antibody-positive subjects who are asymptomatic and equally, the subjects with no antibody but who describe typical symptoms. In quantitative terms, these antibody levels are indeed significantly higher in symptomatic subjects, but they provide no better discrimination between subjects with and without disease than the simple IgG antibody measurements.

The titres of serum IgM and IgA antibody would appear to be considerably lower than IgG, based on the numbers greater than the normal range. However, the extent to which an individual will make an antibody response in the IgG, IgM and IgA class seems to be related. Therefore the high-responder subjects will have the highest titres of all antibody classes, and the low-responders will have low or absent titres of antibody in all three

Antibody Class response to pigeon γ globulin antigen

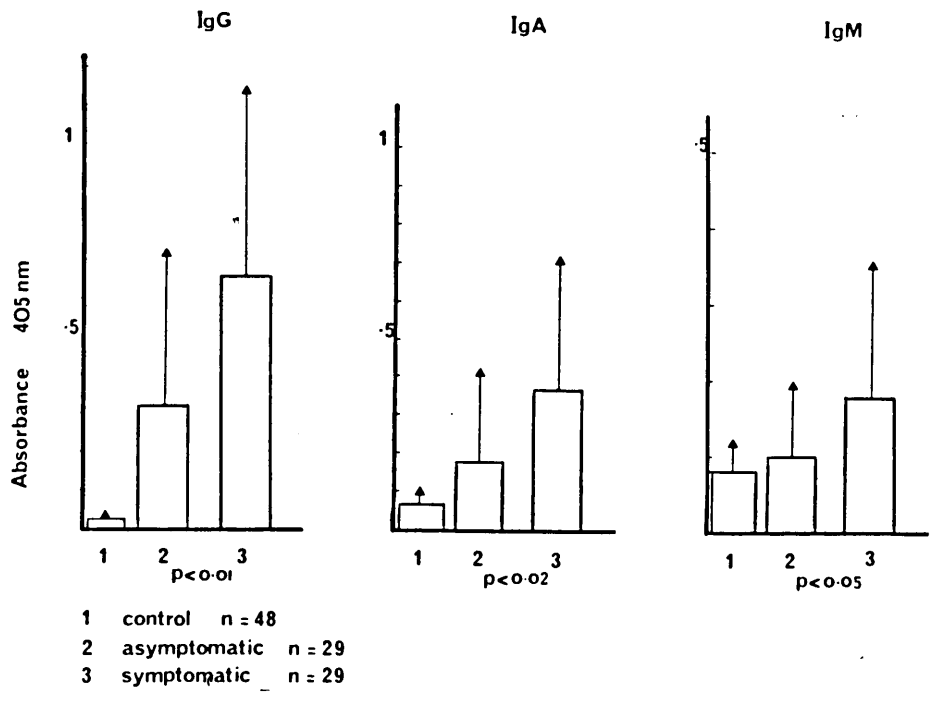


FIGURE 17:

The serum antibody activity in the 3 major classes against pigeon gamma-globulin antigen: a comparison of titres between symptomatic and asymptomatic pigeon breeders.

ANTIBODY AGAINST PIGEON IgA ANTIGEN

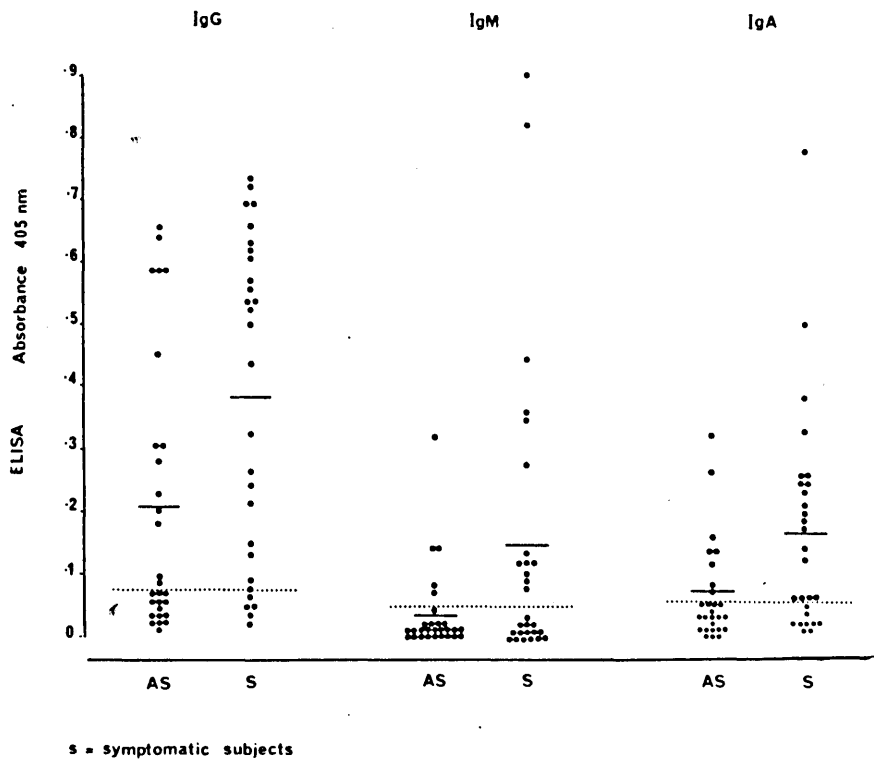


FIGURE 18:

The serum antibody activity in the 3 major classes against pigeon IgA antigen: the difference in titres between symptomatic and asymptomatic pigeon breeders.

immunoglobulin classes.

The ability to mount such a broad antibody response to antigens which are inhaled was of interest because it might be expected that the response would be more localised in the lungs, and that the immune-response of the secretory system would have been preferentially stimulated. The predominant class of antibody stimulated at mucosal surfaces is IgA, and antibody activity in the IgG and IgA classes directed against pigeon antigens was measured by RIA in bronchial lavage fluid from 6 symptomatic and 10 asymptomatic pigeon breeders who volunteered for fiberoptic bronchoscopy (Patterson et al, 1979). These workers found local levels of IgA antibody in proportionally higher titres than serum, and reported differences between symptomatic and asymptomatic subjects based on this local IgA antibody titre. Local lung IgG antibody was also measured but no comparisons between the IgG and IgA titres were possible. This approach is unrealistic in practical terms for repeated individual measurements, for large scale studies, nor indeed for individuals who have compromised pulmonary function. It was of interest to note that the serum IgG and IgA antibody activity in these subjects were approximately of the same proportional titre as the bronchial fluid titres. This suggests strongly that serological investigation of these subjects' serum is far easier and gives a reasonable approximation to the parameters in the lung.

2. TOTAL IMMUNOGLOBULINS.

a) Introduction.

Hypergammaglobulinaemia has been frequently documented in extrinsic allergic alveolitis (Elgefors, Belin & Lanson, 1971; Molina, 1976; Haslam, 1978). However, there have been few attempts to explain this, other than as a generalised hypersensitivity response following host tissue damage. Other conditions with raised polyclonal immunoglobulins include chronic active hepatitis and cirrhosis (Thomas, McSween & White, 1973), infectious diseases, some neoplastic conditions and autoimmune diseases (Hobbs, 1970); and the fundamental mechanism proposed for these was as a response to chronic antigenaemia.

The purpose of this study was to establish the effect of continuous avian antigen exposure on the immunoglobulins G, M and A (total IgE is to be discussed in the next section). The pigeon breeders under investigation could be divided into two groups; the antibody responders and non-responders against these avian antigens. This specific responsiveness, as well as the extent of the response, was compared with the total immunoglobulins. The incidence of raised immunoglobulins was also measured in the various hypersensitivity diseases associated with avian exposure and during the different phases of disease.

b) Total immunoglobulins in a groups of pigeon breeders with extrinsic allergic alveolitis.

A group of 32 pigeon breeders with alveolitis of varying degrees of severity had their total serum IgG, IgM and IgA measured. Using the normal range for IgG (5-16 mg/ml), IgM (0.5-1.9 mg/ml) and IgA (0.5-3.5 mg/ml) established by Brown (1982), there were 16 subjects with raised IgG and 11 with raised IgA (Figure 19). The IgM was raised in only three cases, the rest were normal. There was an indication from the graph that the IgG and IgA levels were correlated and this was statistically significant ($r=0.60$, $t=4.40$, $p<0.01$).

c) Correlation of the total immunoglobulins with the specific antibody response to inhaled antigens.

The total IgG and IgA levels were measured in 230 and 210 subjects respectively, and 146 and 139 of these were positive for serum IgG antibody against avian antigens. The incidence of antibody-responders was measured in increasing levels of total immunoglobulin as outlined on Figure 20. This suggested that higher levels of total IgG and to a lesser extent, higher levels of IgA, were associated with increased responsiveness to inhaled avian antigens.

This correlation between specific IgG and total immunoglobulins was investigated further in 102 subjects. In the antibody positive group the mean titres of IgG, IgM and IgA were higher than the antibody negative group (IgG = 14.1 and 11.0, IgM = 1.08 and 0.99, IgA = 2.78 and 2.20 respectively, all values in mg/ml). The correlation between the total IgG and the specific IgG antibody levels was shown to be statistically significant on

Hyper gamma-globulinaemia associated with
Pigeon Breeders' Disease

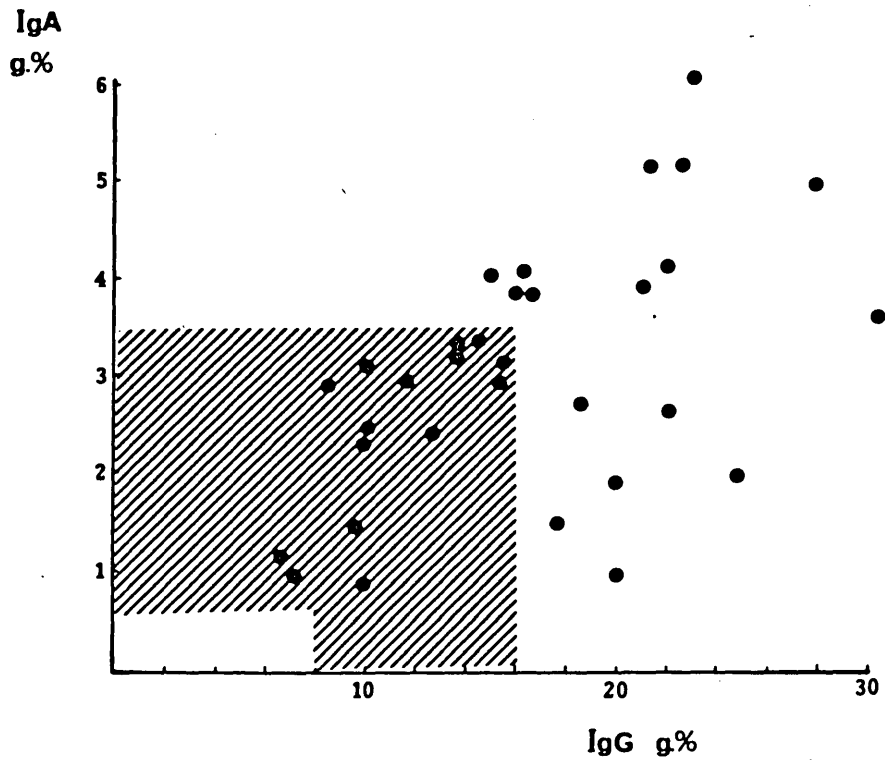


FIGURE 19:

Comparison of serum levels of total IgA and IgG in
32 pigeon breeders.

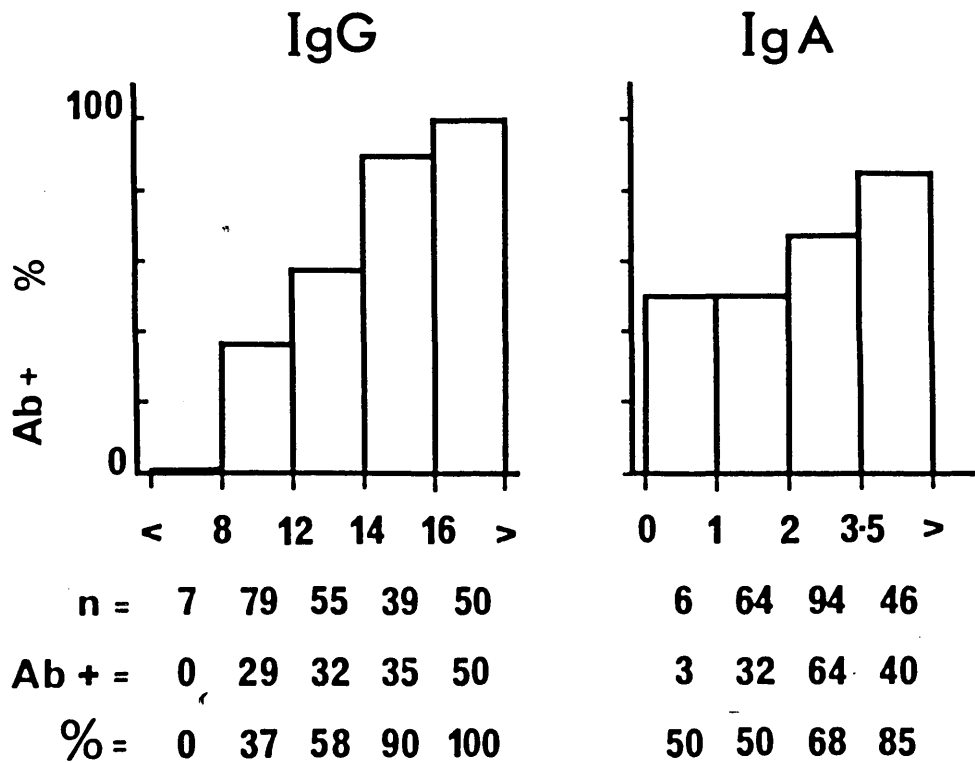


FIGURE 20:

The numbers and incidence of antibody-positive subjects with increasing levels of total serum IgG and IgA.

Figure 21, ($r = 0.60$, $t = 7.45$, $p < 0.001$).

d) The effect of cigarette smoking on the total immunoglobulin levels.

During the investigation of the incidence of specific antibody in the raised IgG subjects it became apparent that non-smokers, who comprised the majority of the antibody responders (see Chapter 3) had higher IgG levels. The mean values in mg/ml for immunoglobulins, and international units/ml for IgE are listed on Table 15. The IgG and IgA levels were greater in non-smokers ($t = 3.19$, $p < 0.005$; and $t = 1.73$, $0.05 < p < 0.1$, respectively).

e) Discussion

Raised serum immunoglobulins, particularly IgG, were remarkably common among pigeon breeders irrespective of whether or not they described typical symptoms of alveolitis. This hypergammaglobulinaemia was more closely associated with the individual's antibody responsiveness to the antigens associated with pigeons, and because this was primarily a feature of the subjects with alveolitis, then these patients also generally had raised serum immunoglobulins.

Many subjects with no overt symptoms, who had high serum antibody, also had equally high total serum immunoglobulin levels. Assuming that raised serum immunoglobulins were a feature of inflammatory reaction, this would suggest that there was an active process ongoing in these subjects despite the lack of overt symptoms. A similar profile of inflammatory parameters including acute phase proteins and immune-complexes in these asymptomatic antibody-positive subjects has emerged in this chapter (see below), which suggests that these subjects have subclinical reactivity to the antigens to which they are constantly exposed.

The raised immunoglobulin titres were primarily a feature of the antibody responders, and furthermore the extent of the non-specific inflammatory response measured by total immunoglobulins seemed to be related to the extent of the specific antibody response. There were no non-specific effects of high serum IgG on the assay procedure for measuring the specific antibody (Appendix B), so it would seem that there is a common stimulatory mechanism for the specific and total IgG. Other raised titres of antibody to respiratory viruses in alveolitis patients have been reported (Marx et al, 1977; Boyd, 1978) and a

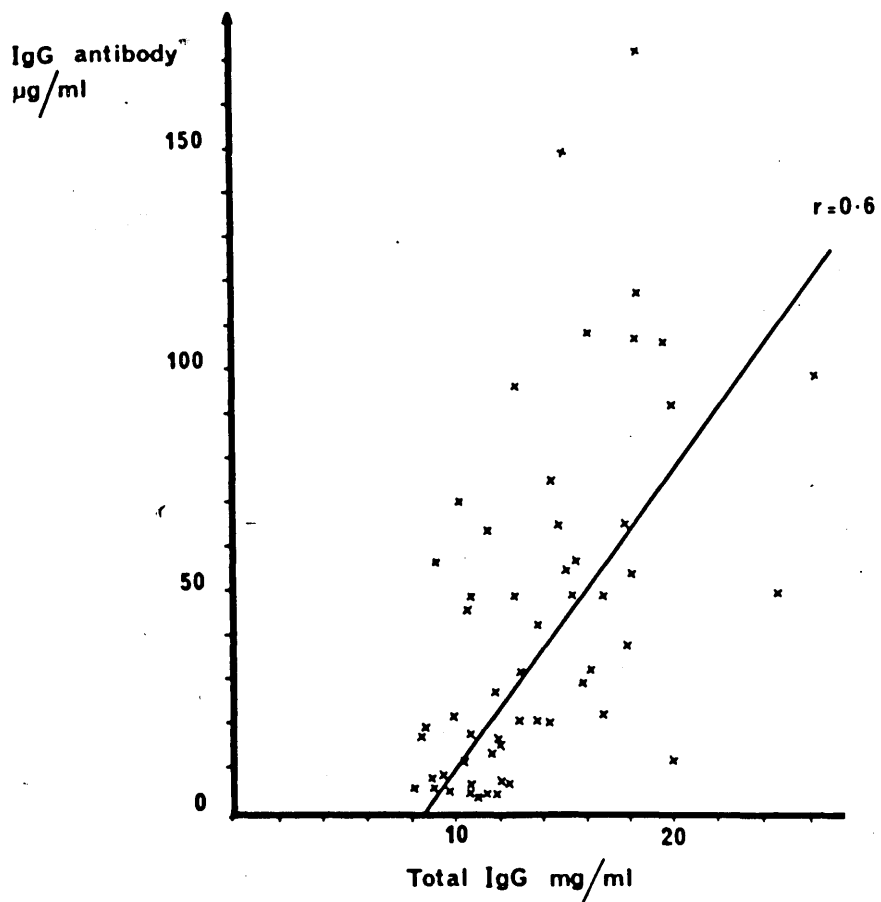


FIGURE 21:

Comparison of levels of specific IgG antibody to avian antigens and total serum IgG.

	Smokers n = 23		Non-smokers n = 65	
	Mean	s.d.	Mean	s.d.
IgG	10.8	1.8	13.5	3.9
IgA	2.2	0.8	2.7	1.3
IgM	1.1	0.4	1.1	0.7
IgE	142	106	140	163

TABLE 15. Total immunoglobulin levels in smoking and non-smoking pigeon fanciers

similar anamnestic response was seen in the sera of sarcoidosis patients (Hirshault et al, 1970; Daniele et al, 1978).

The serum IgA levels can also be raised in these individuals, and a significant correlation between total IgG and IgA levels was noticed. The IgM levels were normal or low. The IgA and IgG levels rose and fell in tandem with avian exposure and antibody levels in one yearly study of monthly levels of these parameters in 20 pigeon breeders (McSharry et al, 1983), and the raised total IgG and IgA fell in tandem in one patient during a period of avian antigen avoidance, a short period after the antibody titres dropped (Chapter 5). This suggests that there is a general immuno-stimulation associated with the extensive antigen exposure among pigeon breeders which affects both total and specific antibody.

Other pulmonary diseases which are associated with raised serum immunoglobulin include fibrosing alveolitis where 21 of 32 patients were reported to have above normal levels of at least one class of antibody (Hobbs & Turner-Warwick, 1967). Increased serum levels of IgG and IgA was reported in 49 patients with chronic bronchitis, and emphysema (Falk, Siskind & Smith, 1970). Hodson (1977) reviewed 124 cystic fibrosis patients and reported a 47% incidence of raised serum IgG levels, 33% with increased IgM and 23% with increased IgA. Interestingly, he also reported a 68% (51 of 74) incidence of raised IgE, but found that allergic symptoms were uncommon. A high incidence of raised serum IgG occurs in pulmonary sarcoidosis and the source of this immunoglobulin was suggested to be from intrapulmonary produced immunoglobulin, controlled by lung T lymphocytes, (Hunninghake & Crystal, 1981). These workers, as well as Lawrence et al (1980) described increased local bronchoalveolar IgG secreting cells in sarcoidosis and other interstitial lung diseases including one patient with EAA.

In alveolitis, the local lung fluid immunoglobulins were measured in higher titres than normal subjects (Reynolds et al, 1977; Voisin et al, 1981) and this was suggested to be predominantly locally synthesised because of the high titres of IgM and the high IgG to albumin ratio (Weinberger et al, 1978). These observations support the histological evidence of increased

plasma cells in lung sections from alveolitis patients (Boyd, 1975), and suggest that the lung might be the source of the high immuno-globulins associated with alveolitis. In normal individuals, the lung has been estimated to synthesis 8% of the serum pool of IgG (Deuschl & Johansson, 1974), which could be expected to increase given the large increase in cell numbers in the lung of alveolitis patients (Reynolds et al, 1977).

The lower serum levels of immunoglobulins among smokers supports the data of Gulsvik & Fagerhod (1979). However, the mechanism of the effect is unknown. The products derived from tobacco smoke have in vitro immunosuppressive activity (Roszman & Rogers, 1973), and this is also seen in vivo (Thomas, Holt & Keast, 1974). With respect to the pigeon breeders the habit of cigarette smoking seems to abrogate all the immuno-responsiveness to avian antigens both in the specific serum antibody (Chapter 3) and total immuno-globulins. This may be a local pulmonary effect, because one consequence of cigarette smoking is to increase the local alveolar macrophage population which is known to cause immunosuppression (Schulzer & Todd, 1981).

3. TOTAL SERUM IgE AND IgE ANTIBODY

a) Introduction.

Constitutional factors are thought to influence the antibody responsiveness of an individual to inhaled antigens and the development of alveolitis. Innocuous antigens when inhaled by atopic subjects can result in allergic states mediated by IgE antibody. This mechanism results in the well characterised disease picture of immediate hypersensitivity such as asthma and hay fever. We sought to evaluate the role of atopy in the disease process of alveolitis by measuring IgE in a large number of pigeon breeders some with different hypersensitivity diseases associated specifically with avian exposure.

b) Total IgE.

The IgE level was measured initially in 164 sera from pigeon breeders being investigated for alveolitis. Of these, 22 (13.3%) had raised total IgE greater than 200 International Units/ml.

A further 102 pigeon fanciers were interviewed extensively concerning their hypersensitivity symptoms

specifically related to avian exposure (Appendix A). The symptom categories and the total IgE are listed on Table 16. The "hay fever" subjects were a sub-group of the indeterminate symptom group, who described both rhinitis and conjunctivitis immediately (within 30 minutes) of contact with pigeons.

The subjects with alveolitis had an incidence of individual raised IgE (and group mean IgE levels) which did not differ significantly from the whole study group.

The study group was divided into antibody responders and non-responders to inhaled avian antigens, and the mean and standard deviation of the total IgE levels in these two groups were 144 iu/ml (sd = 177) and 113 iu/ml (sd = 109) respectively ($t = 1.2$, $P = ns$). The extent of this antibody response did not correlate with the individual IgE levels ($r = -0.05$, $t = -0.47$, $P = ns$).

c) Total IgE, IgE antibody and immediate symptoms related to avian exposure.

The total IgE (and IgE antibody) in subjects who describe an increasingly complete picture of immediate symptoms related to avian exposure is illustrated on Figure 22. This demonstrated an association of immediate symptoms with IgE and not IgG antibody.

The serum IgE antibody to avian antigens was measured by RAST using sepharose-pigeon globulin antigen (Appendix B) and commercially available antigen discs coated with pigeon droppings extract (Pharmacia, e7 discs). Using these, 14 of the above 102 subjects were positive. These were:- 4 with a low level (RAST grade 1) of IgE antibody against droppings extract, and 12 positive against pigeon globulin; 9 grade 1, and 3 grade 2 (moderate level of IgE antibody). Two subjects were positive for both antigens and those were included in the asthma group (Table 16). The other RAST positives included 5 with EAA, 1 with asthma, 4 with rhinitis-conjunctivitis and 2 with no symptoms.

d) Discussion.

Serum IgE is raised specifically in allergic diseases (Johansson et al, 1968) and non-specifically in allergic bronchopulmonary aspergillosis (Patterson, Rosenberg & Roberts, 1977) and in patients with some tropical parasitic infections such as filariasis and schistosomiasis. Our investigation into

	Number	Mean IgE (sd)
All	102	138 (159)
Asthmatic	8	327 (332)
Asymptomatic	29	108 (95)
Alveolitis	33	144 (138)
Indeterminate	32	109 (135)
(Hay Fever)	8	268 (296)

TABLE 16. Serum IgE levels (iu/ml) in pigeon breeders according to their symptomatic status

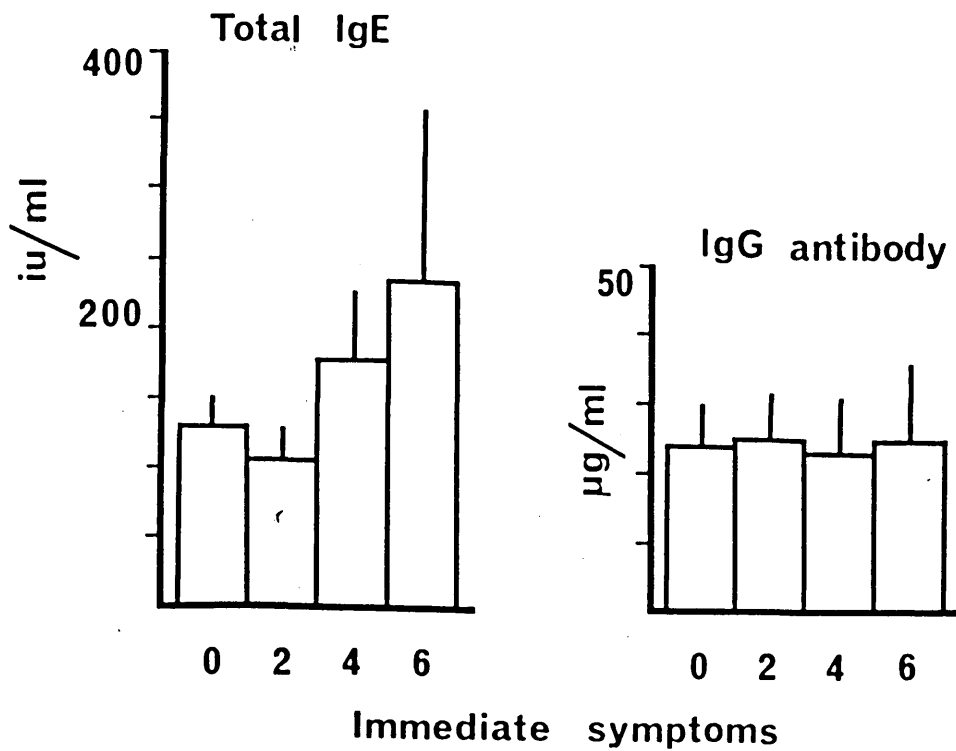


FIGURE 22:

The total serum IgE and specific IgG antibody levels in 102 pigeon breeders with increasingly complete symptomatic profiles of immediate hypersensitivity following avian contact.

abnormalities of IgE levels in subjects with EAA caused by avian exposure revealed no particular role for this class of immunoglobulin in the disease process of alveolitis. This is in general agreement with Patterson and colleagues (1973). Similarly, the sera from alveolitis patients had no IgE antibody activity, thus confirming the reports of Patterson et al (1976), Warren et al, (1977); Cohen, Yunginger & Fink (1979); Faux, Wells & Pepys (1971). This last group did find IgE antibody in some subjects but this was associated with symptoms of immediate hypersensitivity. The present report confirmed the general lack of IgE antibody among pigeon fanciers and also that the few positive IgE antibody subjects had immediate symptoms associated with avian exposure. These subjects will be discussed in terms of skin testing and IgE antibody in Chapter 6.

4. TOTAL AND SPECIFIC IgG SUBCLASSES

a) Introduction.

Human serum IgG is made up of at least 4 subclasses which have structurally different heavy chains, and consequently each can have different effector functions following combination with antigen (Shakib & Stanworth, 1980). These authors reviewed the selective changes in IgG subclasses which were noticed in association with many clinical conditions and the IgG subclass restriction which could be demonstrated during immunization with various antigens.

Since the route of antigen administration was suggested to alter the subclass distribution of the IgG antibody, and because there was no information on the effect of immunizing via the respiratory tract then the antibody activity in the IgG subclasses was measured against the major inhaled avian antigen in a population of pigeon breeders. This was also undertaken to quantify any qualitative difference in the antibody response between pigeon breeders with and without alveolitis.

Allergic alveolitis is associated with raised total IgG (see above), and because of the apparent independence of synthesis of each IgG subclass, the level of each was measured to clarify which would account for the raised total levels.

b) Total subclasses.

The levels of the total serum IgG subclasses were measured in 99 pigeon breeders by radial immunodiffusion (see Appendix B). The sum of the individual subclasses correlated very closely with the total IgG immunoglobulin ($r = 0.79$, $p < 0.001$). This was primarily due to the contribution of IgG1, which correlated significantly with the total level ($r = 0.75$, $p < 0.001$), and to a lesser extent due to the contribution of IgG2 which again correlated significantly with the total IgG, ($r = 0.56$, $p < 0.001$). The values of these individual subclass totals are listed on Table 17. The levels of subclasses 3 and 4 did not make a significant contribution to the total IgG fluctuations. Of the 99 subjects investigated, 51 had detectable serum antibody to the inhaled avian antigen. These positive responders had significantly higher mean levels of total serum IgG and total subclasses 1 and 2 (Table 18). There were no significant mean differences between the groups in the IgG 3 and 4 subclasses.

Within these antibody responders there were 22 subjects with alveolitis and 29 without. The distribution of total subclasses within these disease subgroups is listed on Table 19. The mean values for IgG1, 2 and 4 were similar for both groups but the group with alveolitis had a significantly higher mean IgG3 than the group with no disease.

c) The subclass distribution of antibody activity.

The specific subclass antibody activity against pigeon gamma-globulin antigen was measured by an amplified ELISA method (see Appendix B). Using this technique antibody activity could be measured in all 4 subclasses and the extent of the response in each subclass was quantitatively related to the total IgG antibody activity (Figure 23). The correlations between each of the subclasses (1-4 on the figure) and the total were all highly significant, with the closest being IgG3, $r = 0.92$.

Supporting data from antibody measurement by radioimmunoassay (Appendix B) in 102 subjects using radiolabelled protein A (specific for IgG1, 2 and 4), has shown a close correlation between this and the total IgG (all subclasses) measured by ELISA ($r = 0.96$). Furthermore, using the avian red cell agglutination test (Appendix B), with positive sera at

Subclass	Correlation Coefficient	t	p
1 - 4	0.79	6.0	0.001
1	0.75	5.37	0.001
2	0.56	3.17	0.001
3	-0.13	-0.61	n.s.
4	0.01	0.05	n.s.

TABLE 17. Correlation between the levels of each of the total IgG subclasses, including the sum of these, and the total IgG.

	Antibody Positive n=51	Antibody Negative n=48	
Total IgG	14.1	11.0	p<0.001
IgG - 1	10.7	9.5	p<0.001
IgG - 2	6.8	5.4	p<0.01
IgG - 3	0.511	0.472	p = n.s.
IgG - 4	0.766	0.955	p = n.s.

TABLE 18. Immunoglobulin G subclass levels (mg/ml) in subjects with and without avian antigen specific serum IgG antibody.

	Alveolitis n=22	No Alveolitis n=29	
IgG - 1	10.8	10.6	p = n.s.
IgG - 2	7.1	6.8	p = n.s.
IgG - 3	0.57	0.46	p < 0.02
IgG -4	0.77	0.78	p = n.s.

TABLE 19. IgG subclass levels in antibody positive pigeon breeders with and without alveolitis.

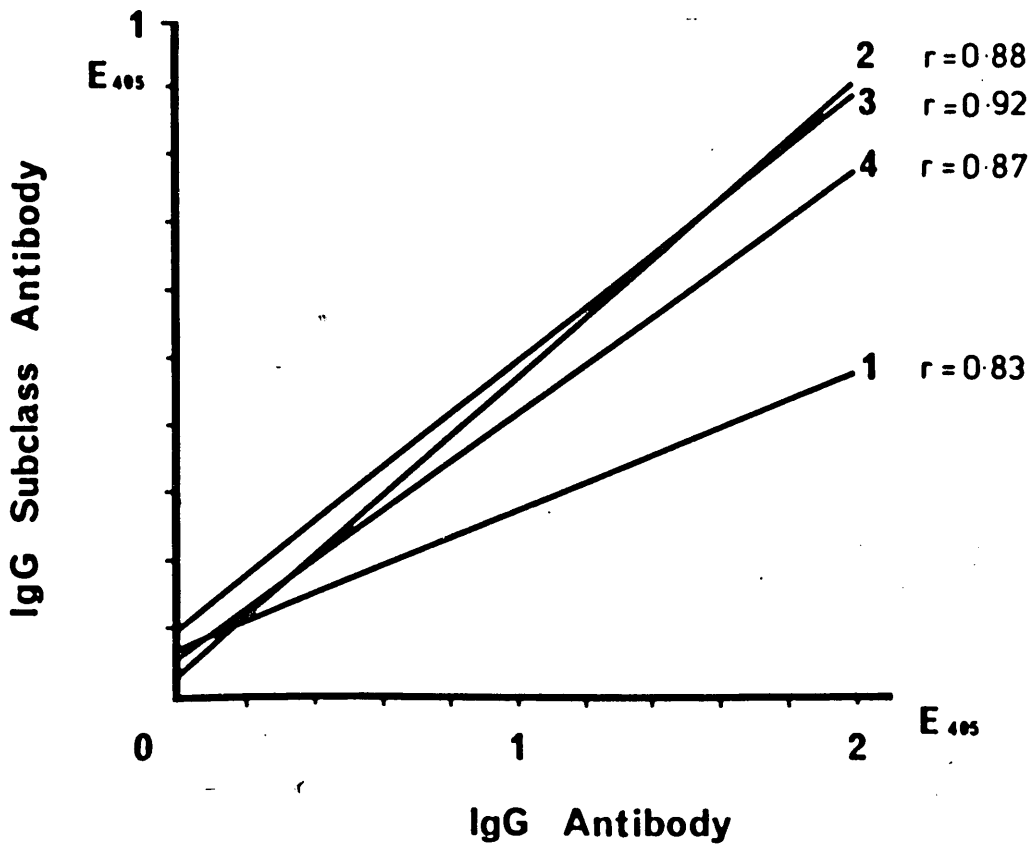


FIGURE 23:

Correlations between the extent of the serum antibody activity, in each of the four IgG subclasses with the total IgG antibody activity against avian antigens.

subagglutination titres and using subclass specific antisera as a Coombs reagent, agglutination could be demonstrated equally by all 4 subclass antisera at low dilution.

d) Discussion.

The close correlation between the sum of the individual IgG subclasses and the total IgG measured independantly was an indication of the validity of the system. The commercial source of the subclass antisera also supplied standard sera with reference levels of IgG subclasses, however the consistent finding that quantitatively the sum of the subclasses was approximately 25% greater than the total IgG suggested that either the reference values were not accurate or that the antisera have some cross-reactivity. On discussion with the suppliers it would appear that the former reason may prevail. However, for comparisons within the study group on which this section is based, the absolute values are not necessary so long as each individual's results are calculated similarly using the same standards.

The good correlation between IgG1 and the total IgG confirms that the hypergammaglobulinaemia associated with alveolitis was due to increases in IgG1 and to a lesser extent IgG2. There was no significant contribution from IgG3 and IgG4 to the IgG fluctuations.

The individuals who had serological evidence of an antibody response to inhaled avian antigens also had a clear association with raised total IgG, and this was significant for raised mean levels of IgG1 and 2. It would appear therefore that the genetic or constitutional disposition to respond to inhaled antigens is associated with the production of increased amounts of IgG1 and 2. The production of subclasses is known to be partly under genetic control (Morrell et al, 1972) and it is possible also that the antibody responsiveness to inhaled antigen is also under similar genetic control although the nature of this at present in humans is obscure. Experiments in mice have shown multigenic control (see Appendix D) involving both H-2 and non H-2 linked genes. However, it could equally follow that during continuous antigen exposure, the subjects with serological evidence of an immune response, i.e. with antibody, may mount an inflammatory response, one consequence of which would be a

generalised increase in total IgG, predominantly IgG1.

Notwithstanding these differences between antibody responders and non-responders, the distribution of IgG subclasses between antibody positive subjects with and without alveolitis has shown a difference in total IgG3, which was significantly higher in the disease group. Although the reasons for this are obscure, a similar finding was reported for farmers with and without alveolitis (Stokes, Turton & Turner-Warwick, 1981). It would appear therefore that over and above the genetic control of antibody production to inhaled antigen which may also control IgG1 and IgG2 synthesis, there may also be a genetic link between IgG3 production and susceptibility to hypersensitivity reactions to inhaled antigens. It is of interest that the IgG3 subclass is structurally dissimilar to the other subclasses and has a much faster turnover rate with an average biological half-life of 7.1 days compared with 21 days for the other subclasses (Morrell, Terry & Waldmann, 1970) suggesting perhaps a separate genetic control. This subclass is also known to be the most potent activator of complement, underlining the importance of the role of antibody activity in this subclass in a disease process which involves immune complex formation and complement mediated tissue damage.

An increase in total IgG4 within fluid obtained from lung lavage from symptomatic as opposed to asymptomatic pigeon breeders was reported by Calvanico et al (1980). However, the significance of this observation was uncertain because they did not measure the other subclasses.

The most relevant investigation was the measurement of antibody activity in each subclass against the specific antigens relating to pigeon breeders disease. There was antibody activity measurable in all 4 subclasses in the subjects where the antibody was found in the total IgG. Furthermore, the extent of the activity in each subclass was closely related to the titre of total IgG antibody. However, it was not possible to estimate the relative titres of antibody activity between each subclass, which would be of interest to determine which subclass contributed the highest specific activity.

Subclass restriction has been reported in the antibody response to defined antigens, for example the human antibody response to dextrans and certain polysaccharides are restricted to IgG1 and IgG2, anti-rhesus antibodies are IgG1 and IgG3, anti-tetanus toxoid in IgG1, and haemolytic disease is apparently mediated by IgG3 antibody alone. However, when suitably sensitive techniques are used then antibody activity is usually demonstrable in all subclasses (Urbanek, 1980; Shakib and Stanworth, 1981).

5. IMMUNE COMPLEXES

a) Introduction.

The role of immune complexes in disease has been the subject of numerous general reviews (WHO Scientific Group Report, 1977; Theofilopoulos & Dixon, 1980; Inman & Kay, 1981), and several reviews of the involvement of immune complexes in lung diseases have been published (Cooper, Moore & Hilton, 1981; Lewiston, 1981; Daniele, 1981). More specifically, serum immune complexes have been measured in the following:

Sarcoidosis:- Hedfors & Norberg, 1974; Gupta et al, 1977; Daniels et al, 1978; Selroose et al, 1980; Johnson et al, 1980.

Cryptogenic fibrosing alveolitis (or idiopathic pulmonary fibrosis in the American Literature):- Nagaya, Elmore & Ford, 1973; Dreisen et al, 1978; Haslam et al, 1979.

Cystic fibrosis:- McFarlane et al, 1975; Church et al, 1981.

Tuberculosis:- Johnson et al, 1981.

Aspergillus infections:- Katz & Knicker, 1973; Geha, 1977.

Pulmonary aspects of the collagen vascular diseases:- Hunninghake & Fauci, 1979.

These authors have implicated immune complexes in the various disease processes based on their detection in the patients' sera. The same approach therefore was used here to demonstrate the presence of immune complexes in the sera of pigeon breeders. A role for circulating immune complexes in the pathogenesis of extrinsic allergic alveolitis is then discussed.

b) Immune complexes in control and test sera.

The serum immune complexes were measured by protein A

binding activity (prAba), according to the method of Barkas (1981). The details of this technique are outlined in appendix B, but briefly it involves the precipitation of high molecular weight protein from serum using polyethylene glycol, adsorbing the soluble component of this precipitate which contains immunoglobulin onto the surface of protein A-rich staphylococci (solid phase) and rendering this measurable by the extent of binding of radiolabelled free protein A.

To validate this method, sera from 23 normal healthy blood donors was processed to establish a normal range. This was measured as the percentage of added radiolabelled protein A which bound, and a low mean level was recorded, mean = 6.9%, standard deviation 3.3%. A normal range of the mean plus three standard deviations was established, and sera with prAba of greater than 16.8% were considered positive. Sera from patients with diseases associated with high levels of serum complexes were included as positive controls. Ten SLE patients and 42 myeloma patients were studied, and pathological sera from 40 asthmatics were also measured. The results for these control groups are listed on Table 20.

The sera from 101 pigeon breeders divided into groups according to their clinical assessment (see Appendix A) were measured for prAba. The results are listed on the upper half of Table 20. It appeared that there was a high incidence of complex activity in the group as a whole (39% positive) and this was accounted for primarily by the high incidence of activity in the sera of the alveolitis patients (61% positive).

The mean prAba for the EAA group was significantly higher than the asymptomatic group ($p < 0.025$), and the asthmatic group ($p < 0.02$), and higher ($p = n.s.$) than the indeterminate group.

c) Relationship between protein A binding and specific IgG antibody.

It became apparent that within the subgroups or symptom categories the subjects with positive prAba had specific antibody. There was a strong association between the presence of specific Ab (>4 ug/ml) and the presence of immune complexes (serum prAba $> 16.8\%$) illustrated on Figure 24. This relationship proved to be quantitative (Figure 25). These parameters were significantly correlated ($r = 0.71$, $t = 10.0$, $p < 0.001$).

Group	Number	Mean	S.D.	Positive	%
<u>Subjects</u>					
EAA	32	21.8	14.8	20	61
Asymptomatic	29	13.9	10.5	8	28
Asthma	8	8.5	6.4	1	12
Indeterminate	32	15.5	12.1	10	30
<u>Controls</u>					
Normal	23	6.9	3.3	0	0
Myeloma	42	23.9	13.3	26	62
SLE	10	21.2	13.6	7	70
Asthma	40	13.4	8.3	12	30

TABLE 20. Serum immune complex activity, measured as percentage protein A binding, in pigeon breeders with different clinical presentations and in control groups of sera from healthy normal donors and pathological sera.

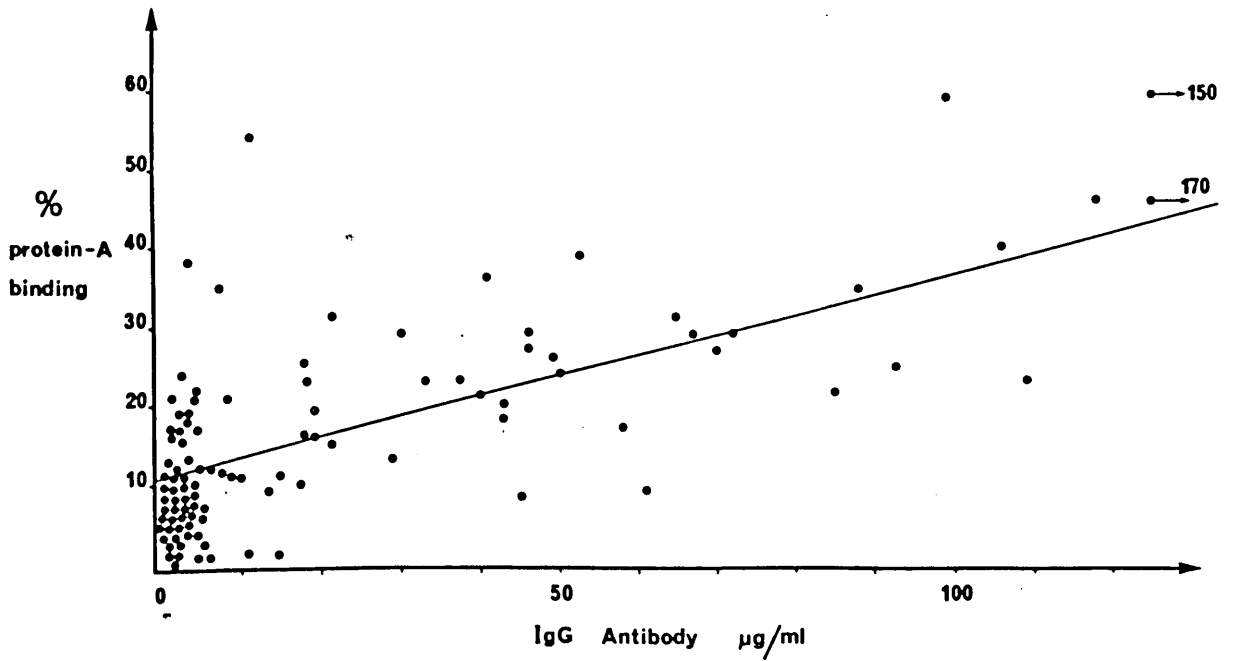


FIGURE 25:

Correlation between the level of serum immune complex activity and the serum IgG antibody titre against avian antigens.

d) Discussion.

Experimental lung disease can result from direct intratracheal administration of performed immune complexes (Scherzer & Ward 1978a) or as a consequence of high serum titres of immune complexes maintained by daily infusion of sufficient antigen in hyperimmune rabbits (Brentjens et al, 1974). The extent of pulmonary injury is related to the antigen content of the complex (Scherzer & Ward, 1978b) and is maximal at antigen equivalence, correlating with optimal complement fixing activity, as has been described in other tissues (Ishizaka, 1963).

The role for such a mechanism in extrinsic allergic alveolitis is unresolved. The acute alveolitis reaction in sensitized guinea pigs on inhalation of nebulised pigeon serum is seen to be a complement dependant, humoral immune process consistent with immune complex disease (Roska et al, 1979). Immunofluorescent studies on lung biopsy tissue from alveolitis patients with pigeon breeders disease (Hogan et al, 1978, Barrios et al, 1979) and farmers lung (Ghose et al, 1974) have demonstrated diffuse deposits of immunoglobulin and complement in alveolar spaces. Other workers have reported negative findings in similar biopsy specimens of pigeon breeders disease and farmers lung (Emanuel et al, 1964; Wenzell et al, 1971; Hensley et al, 1969). These could be explained in part by rapid clearance of intra-alveolar immune complexes by macrophage (Cochrane & Koffler, 1973; Pratt et al, 1979).

Only very rarely would taking a transbronchial biopsy be justified in the diagnosis of EAA, therefore we sought indirect evidence for the presence of immune complexes in the serum of subjects regularly exposed to pigeons and therefore at risk of developing alveolitis following inhalation of avian antigens. The data presented here concerning serum protein A binding activity would suggest that thirty-nine of a group of 102 pigeon breeders had circulating serum immune complexes and that these were predominantly subjects with evidence of EAA.

Subjects were assessed for serum IgG antibody to pigeon gamma-globulin; a major specific antigen for pigeon breeders' disease (Faux et al, 1971). Despite claims that precipitins are of limited diagnostic value in extrinsic allergic alveolitis

(Salvaggio, 1972), we have found a good correlation between disease and specific antibody levels using quantitative radio-immunoassay (Chapter 3). Similarly the alveolitis subgroup of the pigeon breeders investigated here had a higher incidence and mean titre of IgG antibody than similarly exposed subjects with mainly asthmatic symptoms, indeterminate respiratory symptoms, or no symptoms at all. Pathological sera from patients with unrelated respiratory problems, mainly asthma, had a mean specific IgG level within the normal control range, and any non-specific effects that high total immunoglobulin and immune complexes had on this assay were discounted when sera from myeloma and SLE patient were found to have insignificant amounts of avian antigen specific IgG.

Protein A binding activity was measured at low levels in serum from healthy control subjects and was raised in sera from SLE and myeloma patients, confirming the results of Barkas (1981). These sera were therefore used as negative and positive controls. The prABA was measured in a group of asthmatic patients and a low positive incidence of immune complexes was noted similar to the results of Cooper et al (1981). In the 102 pigeon breeders under investigation the highest prABA was associated with the group describing extrinsic allergic alveolitis where 20 of the 32 subjects (61%) had evidence of circulating immune complexes. However, 10 of the 32 subjects describing indeterminate symptoms had significantly raised prABA, perhaps related to infections common amongst bronchitics, and 8 of the 28 asymptomatic subjects also had raised prABA despite observation of low serum immune complex levels in the general healthy population (Walker & Al Khateeb, 1981).

On closer examination, most subjects with significant prABA, were those with a high titre of serum antibody to avian antigens. These included the majority of the EAA group, although the presence of specific antibody and immune complexes in the serum of subjects who described no symptoms may be an indication of subclinical immunological and inflammatory events. Insidious development of alveolitis has been described (Hensley et al, 1969) and workers have looked for humoral parameters to monitor this subclinical disease (Turton et al, 1981). The role of the

measurement of specific antibody levels and immune complexes in these cases would seem appropriate and some prospective studies are discussed in Chapter 5.

Previously, the only data on immune complexes in extrinsic allergic alveolitis was provided by Girard et al (1978). Using a Clq binding assay this group demonstrated serum immune complexes in 13 of 15 subjects with EAA related to various avian exposures and also in 6 out of 23 asymptomatic controls; these six being precipitin positive. A short communication by Yang et al (1978) demonstrated immune complexes in only two of ten bird breeders with alveolitis, also in two out of 10 bird breeders without alveolitis and in one out of eleven farmers lung patients. This group used an Fc binding assay with Raji cells and murine L-1210 cells, and a low incidence of complexes was found despite initially heat depleting the sera, which would aggregate the IgG (Soltis et al, 1979), especially since raised total IgG is common in alveolitis.

There is a correlation between the prABA and total IgG, which may be a feature of polyethylene glycol (PEG) precipitation, as seen in other reports (Haslam et al, 1978). However, Johnson et al (1981) found that the absolute amount of immunoglobulin precipitated by 2% PEG was largely independent of serum immunoglobulin levels. The total IgG levels in myeloma sera did not correlate significantly with prABA as has been described also by Barkas (1981), so it is possible that both hyper-gammaglobulinaemia and immune complex formation are a feature of the inflammatory reactions due to the chronic antigen exposure associated with extrinsic allergic alveolitis.

A central role for immune complexes in the disease process of extrinsic allergic alveolitis could be postulated, similar to the mechanism of immune complex mediated pulmonary injury in vasculitis (reviewed by Hunninghake & Fauci, 1979). The main histological features in common include granuloma formation, a small mononuclear cell infiltrate and macrophage activation; all features which can result from immune complex formation (Spector & Heesom, 1969; Fauci & Wolff, 1977, Pestel et al, 1981).

Data reported here would suggest that immune complex formation can occur among antibody positive pigeon breeders, presumably related to the constant inhalation of the appropriate antigens. However, ingestion of related antigens, e.g. hens eggs, is a possibility that cannot be discounted, since antigenic similarity has been shown between the hen, Gallus domesticus, and pigeon, Columbia livia, (Sennekamp et al, 1981), and post-prandial immune complexes have been demonstrated in normal and atopic subjects (Paganelli et al, 1979).

The role for immune complexes in the active disease process requires further study and parallel involvement of sensitised lymphocytes must also be considered. The marked steroid sensitivity of the disease (Pepys, 1969) may perhaps underline an immune-complex pathology, however prospective studies with subjects in various phases of the disease are necessary to clarify this further.

6. COMPLEMENT.

a) Introduction.

The role of complement in the disease process of EAA is described as "enigmatic" (Schatz et al, 1976). The subject's serum IgG is complement fixing (Caldwell, 1973; Moore & Fink, 1975; Boren, Moore & Abramoff, 1977), although the titres are reported to be as high in some asymptomatic subjects as in most of the subjects with EAA. Furthermore, tissue deposits of complement are only rarely visualised by immunofluorescence in EAA patients (Emanuel et al, 1964) most probably related to their removal by phagocytic activity following the rapid influx of inflammatory cells (Pepys et al, 1968; Cochrane & Koffler, 1973; Richerson, 1974).

Various groups of workers report conflicting results of the fluctuations in serum complement levels and CH-50 in experimental antigen inhalation provocation challenges in man and animals (reviewed in Chapter 5), and the inflammatory process following these challenges in animals has been successfully inhibited by prior decompementation with cobra venom factor (Olenchoch & Burrell, 1976; Roska et al, 1977).

These observations plus the previous data from immune complex involvement appear to be compelling evidence for the involvement of complement in the disease process of EAA, however, only one published report has indicated reduced resting complement levels in only four patients (Baur, Dorsch & Becker, 1980).

This section is concerned with the measurement of the serum profile of complement proteins, C1q, C3, C4 and Factor B in various groups of pigeon breeders, with and without serum antibody, and relates the C4 level to the extent of this antibody response.

b) Experimental.

Fifty-eight pigeon fanciers donated a blood sample from which the serum was separated by centrifugation, snap frozen and stored at -70°C . The serum levels of the complement proteins C1q, C3, C4 and Factor B were measured by radial-immunodiffusion. The subjects were divided into those who mounted a significant serum IgG antibody response to the inhaled antigens associated with pigeon breeders disease and those who did not. The profile of the complement levels in these two groups is outlined on Table 21. There were no significant differences between these groups in age and years of avian contact, but there were lower mean titres of all the complement components in the antibody positive group, which was statistically significant for C3c, and Factor B. Most individual values were within the normal range, but five of the antibody positive group has significantly low levels, below the normal range, for at least two of the four components.

The fourth complement component is reported to be depleted in proportion to the levels of immune complexes in disease (Scullion et al, 1979) therefore, we investigated the quantitative relationship between the extent of specific antibody production as a measure of disease susceptibility, and the C4 level in a further 102 subjects. The C4 level is plotted on the abscissa of Figure 26, with the quantitative value of serum IgG against avian antigens on the ordinate. The lower mean level of C4 in the antibody positive group was confirmed, again not statistically significant, (24.9mg%, s.d. = 6.8, and 29.1, s.d. = 8.8 respectively). There was no correlation between the extent of the antibody response and the C4 level.

	Antibody		t	p
	Positive	Negative		
Number	35	23		
Age	42.8	38.9	1.0	NS
Exposure	19.7	17.8	0.4	NS
C1q (% normal)	98.5	106.7	1.0	NS
C3c (mg/dl)	64.4	72.4	2.2	0.05
FacB (mg/dl)	34.4	39.1	2.2	0.05
C4 (mg/dl)	29.7	33.3	1.1	NS

TABLE 21. The serum complement profile in pigeon breeders with and without serum antibody.

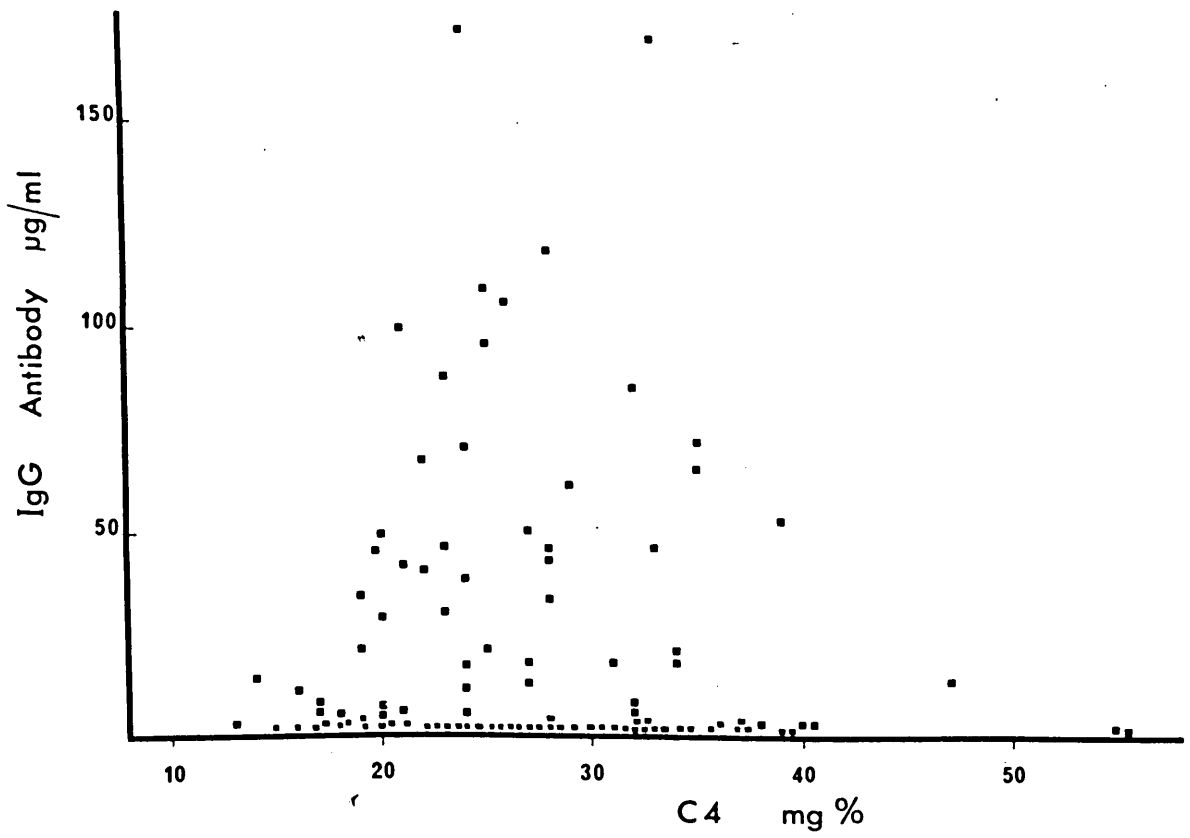


FIGURE 26:

Levels of serum complement C4 in 102 pigeon breeders compared with their serum IgG antibody level to avian antigens.

c. Discussion.

Local lung fluid contains adequate complement proteins to mediate an inflammatory reaction (Reynolds & Newball, 1974). These proteins can be serum derived or locally synthesised (Colten, 1976; Alpert et al, 1978). This, therefore, with the presence of high titres of local specific antibody (Warren & Tse, 1974) would allow complement activation within the lung compartment following inhalation of the appropriate antigens associated with alveolitis. Some evidence for this local hypersensitivity reaction involving complement was suggested by observing positive immunofluorescence for deposits in a few lung biopsies of farmers with alveolitis (Emanuel et al, 1964; Seal et al, 1968; Barrowcliff & Arblaster, 1968; Wenzel et al, 1971; Ghose et al, 1974) and pigeon breeders with alveolitis (Hogan, 1978; Barrios et al, 1979), and some animal models of disease (see Appendix C).

We sought in this section to quantify differences of serum complement levels between pigeon fanciers with and without antibody in order to investigate the involvement of complement in alveolitis. The mean levels were markedly lower in those subjects with evidence of an antibody response to the inhaled antigens associated with the disease. The C1q level was reduced by 8%; C4 by 11% (and by 14% in a further population of 102 subjects); C3c by 11% and Factor B by 12%. The reductions in C3c and Factor B were statistically significant. Moreover, all the individuals with significantly reduced levels of at least two components of complement were antibody responders. This suggests that there was evidence of an inflammatory response associated with a low level of complement consumption in subjects who have actively responded by making antibody to inhaled antigen. The measurement of these complement levels at one point in time reflects the product of activation, catabolism and synthesis. Confirmation of complement involvement in acute alveolitis therefore would require an index of complement activation; for example the production of a stable end product C3d, and also serial measurements correlating with the various dynamic stages of disease. These are discussed in Chapter 5.

Only very rarely are complement levels reduced in lung diseases unassociated with other organ involvement (Turner-Warwick, 1978) and indeed C3 levels are increased in cryptogenic fibrosing alveolitis and sarcoidosis. Complement levels in asthmatics are normal, but intrinsic asthmatics have significantly higher levels of complement components C1s, C4, C5 and C3b inactivator, than atopic asthmatics, with both groups having normal C3 turnover as measured by free C3d (C.J.Lewis, personal communication). Patients with bronchitis have lower levels of serum complement components (Millar, Kueppers & Offord, 1980) and cigarette smokers have increased serum levels of C5, C9 and C1-inhibitor (Wyatt, Bridges & Halater, 1981) and increased local pulmonary levels of C3 and C4 (Bell et al, 1981). To this list of complement levels in pulmonary disease should be added the reduced levels associated with alveolitis reported here.

The antigens associated with extrinsic allergic alveolitis have been reported to directly activate complement when added to normal human serum (Marx, Boren & Moore, 1977). These antigens include soluble extracts from Micropolyspora faeni and Thermoactinomyces vulgaris (Edwards, 1976), antigens from bacteria and fungi associated with EAA (Marx & Flaherty, 1976; Huis in't Veld & Berrens, 1976) and avian antigens (Berrens, Guikers & Van Dijk, 1974; Moore & Fink, 1975; Marx, Boren & Moore, 1977; Boren, Moore & Abramoff, 1977; Berrens, 1978). Activation is via the alternative pathway although there is some evidence to suggest that the antigens can act directly at the level of C4 of the classical pathway (Marx, 1978). This worker describes one further unusual feature that these antigens do not activate guinea-pig complement despite their efficiency in activating human complement. The mechanism of these activities awaits clarification by their proponents. The proteolytic enzymes from bacteria and fungi and the hydrolytic enzymes from avian droppings were initially implicated in the activation sequence (Berrens & Guikers, 1972) but this has now been discounted (Marx, 1978).

The inactivation of complement by adding soluble antigen to patients serum was considered a sensitive test for pigeon breeders disease (Berrens, Guikers and Van Dijk, 1974). These workers also described an "autoantigen", a modified human serum

gamma-globulin which stimulated continued production of complement consuming antibodies in subjects who had no further avian contact. They also suggested that subjects "predisposed" to having pigeon breeders disease had an intrinsically labile complement system (Berrens, Guikers & Van Dijk, 1974; DeRidder, Van Dijk & Berrens, 1979), however, these results could also be explained by the presence of low titres of complement fixing serum antibody in these patients. Equally, where alveolitis has been described in precipitin negative subjects, the possibility of direct complement activation was postulated (Edwards, Baker & Davies, 1974; Sennekamp et al, 1978; Canet et al, 1980). However, it is also possible that these subjects had antibody in titres less than the detection threshold of precipitin techniques. If this non-specific complement activation were involved in the pathogenesis of alveolitis then the symptoms would be more prevalent in all exposed subjects dependent only on the dose of antigen, and the immunological specificity of the development of symptoms would not apply.

7. NON-SPECIFIC MARKERS OF INFLAMMATION.

a) Introduction.

Many acute phase proteins and enzymes in both serum and bronchoalveolar lavage fluid have some diagnostic significance in the investigation of pulmonary diseases: for example, angiotensin-converting enzyme (ACE) in sarcoidosis (Lieberman et al, 1979), collagenase in idiopathic pulmonary fibrosis (Gadez et al, 1979), rheumatoid factor in rheumatoid lung and alpha 1 - antitrypsin in emphysema. In this latter case a genetically programmed reduction of this protein is associated with poor control of proteolytic enzyme activity in the lung resulting in pulmonary fibrosis. It would be appropriate therefore to assess the importance of this protein in the disease process of alveolitis. A report by Muller et al (1976) demonstrated elevated titres of alpha 1 - antitrypsin in 11 of 15 alveolitis cases and transiently diminished levels in 4; 2 of these had a genetic heterozygous deficiency.

This enzyme and also ACE are synthesised by activated pulmonary macrophage (White et al, 1981; Hinman et al, 1979)

therefore since activated macrophages are thought to be involved in the disease process of alveolitis (Stankus et al, 1978) it might be appropriate to assess the role of serum ACE in this condition. Lieberman et al (1979) reported that there was no significant association between raised serum ACE and farmers lung in 17 patients although 5 of these had raised levels, but there was no indication of the stage of disease of these patients. Further studies by Turton et al (1981) suggested that serum ACE was raised in farmers lung patients compared with control farmers. Teuksbury et al (1981) however found a significantly reduced serum ACE level in acutely ill farmers, but during active responses to challenge with Micropolyspora faeni in 3 patients there were no consistent fluctuations in serum ACE levels, and furthermore there were no differences between stable farmers lung patients and controls. In a survey of pigeon breeders with (n = 17) and without (n = 10) alveolitis there was no difference in serum ACE levels between these groups and normal controls (n = 32), (McCormick et al, 1981). The evidence, therefore, is strongly against involvement of this particular enzyme in disease, and for this reason it was not pursued in this section.

Serum C-reactive protein (CRP) is the prototype acute-phase plasma protein which can rise rapidly following infection or tissue injury resulting from a variety of inflammatory diseases (Morley & Kushner, 1982) and was therefore investigated for its usefulness as a disease marker in alveolitis. The serum rheumatoid factor was also measured in order to define one parameter of autoimmunity in a population of subjects with alveolitis.

b) Experimental.

Rheumatoid factor measured by latex agglutination (Appendix B) was undetectable in all but one of a survey group of 102 pigeon breeders. This subject had particularly high levels of serum antibody to avian antigens and raised total immunoglobulins, but had no symptoms of alveolitis.

Raised levels of total serum alpha 1 - antitrypsin, measured by radial-immunodiffusion and C-reactive protein, measured by latex agglutination occurred in 17% and 48% respectively. The distribution of these parameters according to

the individuals antibody responsiveness is listed on Table 22(a). Raised levels of CRP occurred with equal frequency in antibody positive and negative pigeon breeders, but the incidence of raised alpha 1 - antitrypsin was much higher in the antibody positive group. Similarly when subjects with and without alveolitis were compared there was no difference in CRP levels, but again raised alpha 1 - antitrypsin levels occurred more frequently in subjects with disease (Table 22b). Interestingly when the asymptomatic subjects were subdivided into antibody positive and negative, Table 22c, then the incidences of raised markers were predominantly accounted for by the antibody positive subjects.

The occurrence of these serum parameters according to the individual's smoking habit is listed on Table 23. There was no indication that smoking affected the occurrence of raised levels in this population.

c) Discussion.

The major problems in assessing the diagnostic value of acute phase proteins in this disease are that they are modulated by many factors and that their levels are very dynamic. Perhaps for these reasons the CRP levels were a poor discriminator of disease. Notwithstanding, the other obvious stimulus, i.e. cigarette smoking did not appear to alter the serum profile of these proteins. This is in accordance with Warr et al (1978), although the activity of the alpha 1 - antitrypsin is suggested to be reduced in smokers (Janoff et al, 1981).

The alpha 1 - antitrypsin levels were increased in subjects with serum antibody to inhaled avian antigens, and this association was also true for the enzyme in bronchopulmonary lavage fluid from pigeon breeders (Voisin et al, 1979). The extent of the serum antibody response in the 101 pigeon breeders, however, was not quantitatively related to the alpha 1 - antitrypsin level ($r = 0.13$, $p = 0.185$, not significant).

Of particular interest is the observation of a high incidence of these serum parameters of inflammation in asymptomatic pigeon breeders who have serum antibody to inhaled avian antigens; these subjects have already been shown to have a higher incidence of raised immunoglobulins and immune complexes than the antibody negative asymptomatic subjects. This suggests

	(a)		(b)		(c)	
	ANTIBODY		ALVEOLITIS		ASYMPTOMATIC	
	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	ANTIBODY POSITIVE	ANTIBODY NEGATIVE
Number	51	50	33	29	9	20
Rheumatoid factor	1	0	0	1	1	0
C-reactive protein	16	18	15	14	55	15
Alpha 1-antitrypsin	53	35	63	37	57	0

TABLE 22: Distribution of positive rheumatoid factor and percentages of raised serum levels of C-reactive protein and alpha 1-antitrypsin in pigeon breeders, (a) with and without serum antibody to inhaled avian antigens, (b) with and without symptoms of alveolitis and (c) asymptomatic subjects with and without antibody.

	Non-Smoker	Smoker	Ex-Smoker
Number	65	23	14
Rheumatoid factor	1	0	0
C-reactive protein	13.8%	13%	35.7%
Alpha 1-antitrypsin	54.1%	44.4%	16.7%

TABLE 23: Distribution of positive rheumatoid factor and percentages of raised serum levels of C-reactive protein and alpha 1-antitrypsin in pigeon breeders according to smoking habit.

that active inflammation may be present in subjects with evidence of an active immune response but without acute symptoms. This possibility was also raised by Turton et al (1981) who found significantly higher serum lysozyme and ACE levels in antibody positive farmers lung patients during periods of active antigen exposure despite the absence of acute symptoms.

It would appear that evidence for an inflammatory response can occur in subjects who have evidence of an antibody response despite symptoms, and this re-emphasises the importance of the measurement of serum antibody, even in asymptomatic subjects, since the development of alveolitis can often be insidious.

Over and above any diagnostic use of the acute phase proteins they may also have a biological role in limiting the pulmonary response following antigen exposure (Baseler & Burrell, 1981). These workers have found that rabbits exposed to aerosols of appropriate fungal spores demonstrated a significant increase in haptoglobin level, and that this was a reliable and consistent indicator of pulmonary inflammation. Furthermore, when the role of haptoglobin in this response was investigated it was found that rabbits with actively or passively augmented haptoglobin levels did not respond with typical depressions in arterial oxygen tension following challenge. The authors also pointed out that haptoglobin was only one parameter of the acute phase response and that the apparent protective effect may be due to a combination of any number of parameters.

8. P1 ANTIBODY.

a) Introduction.

The main phenotypes of the P1 red blood group antigen system are P1(+) and P1(-), sometimes called P2. The P2 phenotype occurs in approximately 20% of the white population and the serum of these subjects may contain anti-P1 antibodies. This anti-P1 is usually IgM class antibody in low titres and is rarely active at body temperature. Various substances have been shown to have P1 antigenic activity including hydatid fluid, house dust and some Gram negative bacilli. Recently P1 antigens have been described on pigeon erythrocytes and in pigeon serum, and a higher

incidence of anti-P1 was noted in P1(-) pigeon breeders. This increase in P1 antibody however was thought to have no bearing on the incidence of disease.

This section examines the P1 phenotype of a population of pigeon breeders and the extent of the P1 antibody response was compared with the antibody levels against pigeon serum antigens. The bearing of this genotype on the disease incidence of alveolitis was also examined.

b) Results and discussion.

The incidence of P1 was established by erythrocyte agglutination using anti-P1 antisera on fresh heparinised blood from 223 pigeon breeders. The incidence was not significantly different from controls (Table 25), and both were similar to the general published phenotype distribution of 80% P1(+) and 20% P1(-).

The incidence of the cold agglutinin antibody (IgM, anti-P1) in the P1(-) subjects was much greater among the pigeon breeders than the controls (53.9% vs 11.9%), and was particularly high in pigeon breeders with alveolitis (75.0%). In parallel with this increasing incidence, the mean titres of the antibody increased, thus the extent of the antibody response to P1 antigens was much greater in pigeon fanciers, and more so in those with alveolitis (Table 25).

There was no detectable IgM anti-P1 measured at room temperature in any of the control subjects, but this was detected in 21.2% of the P1(-) pigeon fanciers and in 58.3% of those with symptoms of alveolitis.

The incidence of antibody in populations of pigeon breeders and controls and the subgroups with detectable P1 cold agglutinins are listed on Table 24. These figures suggest that there is a slightly higher incidence of detectable antibody against avian antigens in subjects with "natural" antibody against P1 erythrocytes, but the differences are not significant.

Sera from five pigeon fanciers with high antibody levels to pigeon gamma-globulin antigens were extensively absorbed with P1(+ve) erythrocytes. There was a reduction of antibody titres in post-absorbed sera of between 6% and 37%, with a mean value of 26%, which was probably due to the dilution effect of adding the

	IgG Antibody to Pigeon Serum Antigens	
	Positive	Negative
Pigeon Breeders (n = 507)	257 (51%)	250 (49%)
P1 - antibody +ve (n = 25)	15 (60%)	10 (40%)
Controls (n = 42)	0 (0%)	42 (100%)
P1 - antibody +ve (n = 25)	3 (12%)	22 (88%)

TABLE 24: A comparison of the antibody responsiveness against pigeon antigens and IgM anti-P1 among pigeon breeders and control unexposed individuals.

	Control Blood Donor	Scottish Pigeon Fanciers	Pigeon Symptomatic	Fanciers Asymptomatic
Number	200	223		
P1 (-) individuals	42 (21.0%)	52 (23.3%)	12	37
P1 - antibody (4°C)	5 (11.9%)	28 (53.9%)	9 (75.0%)	19 (51.4%)
P1 - antibody (ambient temp.)	0 (0%)	11 (21.2%)	7 (58.3%)	4 (10.8%)
Mean titre score of				
P1 - antibody (4°C)	11.0	23.3	31.6	19.4

TABLE 25: The incidence of P1 (-) phenotype and anti-P1 antibody among normal adults, and pigeon fanciers with and without alveolitis.

red cells. This suggest that the P1 antigenic determinant on human red cells is different from the main antigen in pigeon serum and that the associated "natural" IgM cold agglutinin antibody in P1(-ve) subjects is unrelated to the IgG anti-pigeon gamma-globulin response, and that the P1 phenotype had no bearing on pigeon breeders disease.

CHAPTER 5FLUCTUATIONS OF IMMUNOLOGICAL PARAMETERS DURING VARIOUS DISEASE
PHASES OF ALVEOLITIS1. INTRODUCTION.

The association between various immunological parameters and the incidence of alveolitis which were discussed in Chapters 3 and 4 were derived from the symptom patterns and serology of individuals at one point in time. In order to confirm these associations, they were followed prospectively in individuals during various phases of disease. The most clearly defined phases of alveolitis include firstly the acute phase which occurs in sensitized subjects 4 to 8 hours after contact with antigen which consists of a typical symptomatic period, the duration and severity of which is variable but usually resolves within 24 hours and secondly the convalescent phase, where the various clinical, physiological and serological parameters could be followed during the recovery period of sensitized individuals over several weeks following complete avian antigen avoidance. These phases of disease were followed in this chapter to assess the involvement of the immune system in both the development and resolution of disease.

2. THE ACUTE PHASE: ANTIGEN INHALATION PROVOCATION TESTS.

The study of allergic lung disease is facilitated by the availability of the lung and skin as experimental target organs for antigen challenge. Skin testing can only be considered as an extrapolation of reactions occurring locally in the lung, but can give complementary information, and furthermore the skin is readily accessible for simple investigative procedures. The role of skin testing in alveolitis is discussed in Chapter 6.

This section is concerned with the use of allergen inhalation provocation tests in the investigation of the immunological and physiological events associated with acute alveolitis. These inhalation provocation tests can consist of a 'natural' exposure to the causative allergens in their normal environment, e.g. farmers with alveolitis strewing hay on the farm, or patients with PBD in contact with pigeons during normal

husbandry procedures in a pigeon loft. However, the allergen exposure can be more closely regulated in the laboratory, and this more controlled environment allows better observation of the subsequent changes of symptoms, pulmonary function, physiology, haematology and serology. The primary concern during these challenges was the safety of the patient, and very conservative protocols were drawn up controlling the exposure to potentially potent allergens. The various responses to antigen inhalation provocation to challenges are summarised on Table 26. The 'late' reactions of acute alveolitis are the concern of this section.

a) Natural challenge in a pigeon loft.

Pulmonary function, white blood cell count, oral temperature and serological changes were followed for up to 24 hours in a group of 12 active pigeon breeders, with the clinical criteria for alveolitis, following a natural exposure consisting of one hour in a loft with 50 birds. All these subjects had serum antibody to avian antigens (this and other details are listed on Table 27).

Only one subject (TC) described typical symptoms of chills, fever, muscle pains and general malaise four hours post exposure. These symptoms were accompanied by leucocytosis, fever and a decrease in pulmonary function as measured by a significant decrease of both vital capacity (VC) and carbon monoxide diffusing capacity (TLCO), see Table 27. This table also lists these parameters in the other individuals who, despite similar antigen exposure, did not develop any recognisable symptoms of acute alveolitis.

Despite the lack of overt symptoms, individuals developed pyrexia, leucocytosis, and pulmonary function abnormalities during the 8 hour period after exposure. Four subjects had a significant decrease (by more than 20% of the initial, prechallenge value) of both VC and TLCO and two others changed in one each. Six subjects developed a temperature of 37.2°C or greater (Hendrick et al, 1980) and five had a leucocytosis of greater than a 30% increase above the prechallenge counts. No subject had changes in all three parameters simultaneously. In fact, although there was a very close correlation between the development of both pyrexia and leucocytosis together, these did

76a.

	<u>A S T H M A</u>		<u>ALVEOLITIS</u>
	IMMEDIATE	LATE	
ONSET	10-30 min	6-12 hr. Requires preceding immediate reaction	4 - 8 hr.
DURATION	1 - 2 hr.	24 - 48 hr.	Usually less than 24 hr.
SITE OF REACTION	Central airways	Central airways	Peripheral airways
PULMONARY DEFECT	Obstruction Wheeze	Obstruction Wheeze	Restriction Dyspnoea. Crepitations.
<u>IMMUNOPATHOGENESIS:</u>			
ANTIBODY	IgE	IgE	IgG
CELLS	Mast cell	?	Lymphocytes
<u>THERAPY:</u>			
STEROIDS	Not acute asthma	Stabilises chronic asthma	YES
BETA AGONISTS	+	+	-
ADRENALIN	+	+	-
DSCG	+	+	+
<u>GENETIC FEATURES:</u>			
	No HLA link Multigenic control of a)IgE production b)bronchial hyper-reactivity		No HLA link Presumed multigenic control of a)IgG antibody responsiveness b)disease susceptibility
<u>SYSTEMIC FEATURES:</u>			
PYREXIA	-	-	+
LEUCOCYTOSIS	Eosinophilia, 24hr	-	Polymorph.leucocytosis, 4-8 hr.
SYMPTOMS	-	-	General malaise, Flu-like symptoms.

TABLE 26. Summary of the features associated with the acute pulmonary responses following exposure to antigen.

Subject	Age years	Duration of disease years	IgG antibody ug/ml	WBC	Response to Challenge		
					Temperature °C	Pulmonary Function V.C. , TLCO	
1. AW	63	7	113	no change	36.9	+	+
2. HM	31	5	63	no change	37.0	-	-
3. WO	41	1	62	increase 68%	37.2	-	-
4. AB	60	7	54	no change	36.5	-	-
5. JBr	58	4	48	no change	37.0	+	+
6. JH	63	1	36	increase 62%	37.7	-	-
7. JBe	57	5	30	increase 26%	37.0	+	+
8. PB	67	2	21	increase 12%	37.5	+	+
9. TC*	32	1	17	increase 30%	37.4	+	-
10. JMK	55	1	14	increase 33%	37.2	-	-
11. WR	59	1	11	increase 38%	37.3	-	+
12. JM	52	1	9	increase 16%	36.9	-	-

* symptomatic

TABLE 27. Individual details and response to natural challenge in 12 symptomatic pigeon breeders.

not occur with the pulmonary function defects. Only three subjects did not show any of these responses. Therefore, although symptoms of alveolitis were uncommon, even in sensitized subjects, to a mild although typical episode of avian exposure, there were changes suggestive of subclinical reactions.

Although this study group was small there was some suggestion of trends of reactivity. For example, the subjects with the highest antibody levels were not the most florid reactors. The mean antibody levels of the 6 subjects who developed pyrexia and the 6 who did not were 27 ug/ml and 53 ug/ml respectively. The mean antibody levels of the 5 subjects who developed a leucocytosis and the 7 who did not were 28 ug/ml and 48 ug/ml respectively. However, the trend was opposite for pulmonary function changes, the four subjects who developed a significant deterioration in lung function had a mean antibody level of 52.5 ug/ml whereas the eight subjects with no deterioration had a mean antibody level of 33.3 ug/ml.

It also appeared that the most acute reactions (involving both fever and leucocytosis) occurred in 5 subjects with comparatively recent onset of disease; all claimed to have first noticed symptoms about one year before the challenge. The 4 subjects who only had pulmonary function changes with no leucocytosis and only one pyrexial response, had durations of disease of 2,4,5 and 7 years, and these could be considered as a more chronic disease group.

b) Complement and immune complexes.

Serial measurements of complement components C3, C4 and Factor B, along with a measure of serum immune complex activity were made in these 12 subjects before, and 4 hour and 8 hour post-exposure (Figure 27). The values of each complement protein were remarkably constant over the 8 hour period for each individual, and all were within normal limits except for subject TC, (who responded positively to exposure) and one other, both of whom had significantly low Factor B. The immune complex levels fluctuated marginally in all subjects but there was no discernable pattern. Only the symptomatic subject TC demonstrated a distinctive change with an increase during the study period. This, along with the values of complement components of TC is

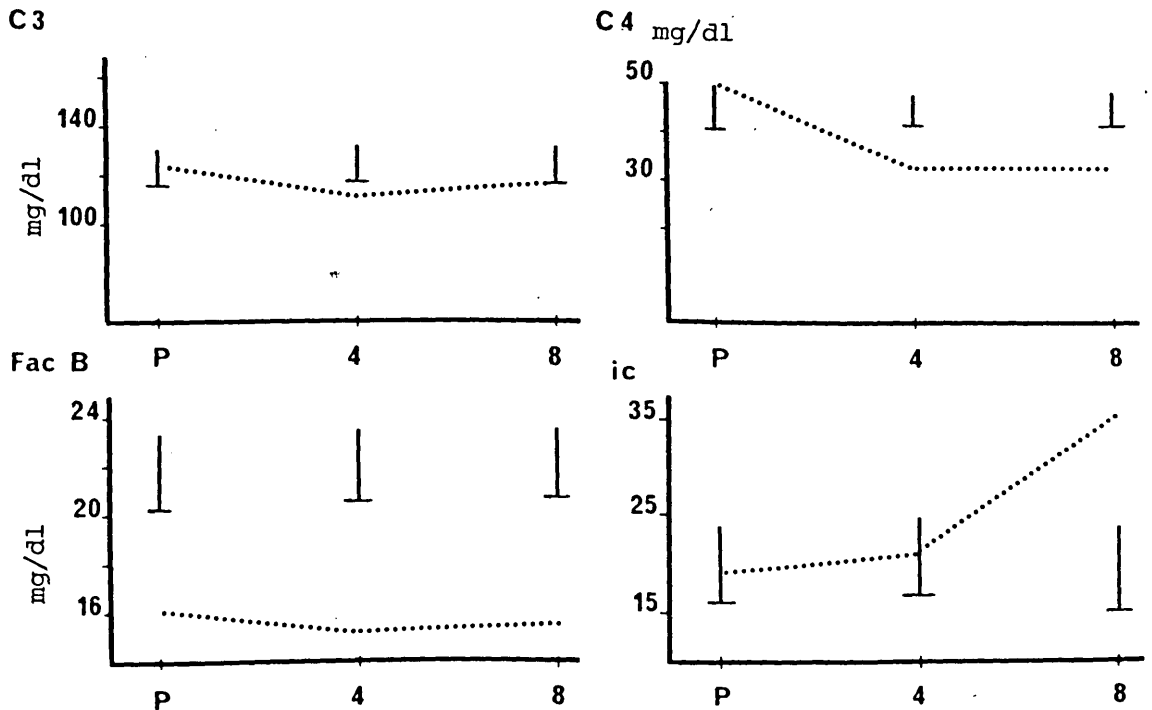


FIGURE 27:

Serial measurements of serum complement components C3, C4 and Factor B along with immune complex activity (%) before a natural antigen-exposure challenge and during the 8 hour period post-challenge, for 11 asymptomatic pigeon breeders (mean + S.D.) and one symptomatic responder (-----).

compared with the mean plus standard deviations of these parameters of the other 11 individuals who did not have a symptomatic response to exposure, and is illustrated on Figure 27. Subject TC had a marked drop in C3 and C4 levels, with a modest drop in Factor B level along with a marked increase in circulating immune complexes coincidental with the development of symptoms of alveolitis. These fluctuations were particularly marked in comparison with the stable values of the rest of the study group.

It might have been optimistic to expect any marked changes in serum complement levels caused by such modest antigen exposure in basically healthy individuals. However, the minor fluctuations in the one symptomatic subject suggested that perhaps more sensitive measurement of complement activation, for example turnover of complement or the appearance of stable fragments of complement activation might be useful, especially since minor changes in complement levels across the pulmonary circulation, which would be undetectable by venous sampling, have been measured following inhalation challenge (Halprin et al, 1973).

c) Treatment with disodium cromoglycate (Intal).

These natural exposure challenges were designed to include preliminary experiments in the investigation of disodium cromoglycate therapy (Boyd et al, 1980). Each subject was pretreated with either 40mg disodium cromoglycate or placebo (supplied by Fison's Pharmaceuticals PLC) administered by inhalation. These were given in double blind fashion. The various measurements post-challenge in the active and placebo groups are listed on Table 28. There appeared to be a net protective effect conferred by this drug since the reaction "score" was reduced in the treated group and none developed symptoms. Without treatment, 9 of the 12 subjects showed some degree of reaction, and this was reduced to 4 following Intal administration. The main effect was to reduce the pulmonary function abnormalities associated with exposure.

There was no follow-up of complement or immune complexes because there was little change observed in the untreated subjects. The subclinical changes of temperature and leucocytosis observed in the treated group were again closely associated. The protective role of cromoglycate is well documented for asthma

SUBJECT	PLACEBO			ACTIVE		
	Temp.	WBC	PFT	Temp	WBC	PFT
1. AW	-	-	+	-	-	-
2. HM	-	-	-	+	+	-
3. WO	+	+	-	-	-	-
4. AB	-	-	-	+	+	-
5. JBr	-	-	+	-	-	-
6. JH	+	+	-	-	-	-
7. JBe	-	-	+	-	-	-
8. PB	+	-	+	+	+	+
9. TC	++	+	-	-	-	-
10. JMK	+	+	-	-	-	-
11. WR	+	+	-	+	+	-
12. JM	-	-	-	-	-	-
Reaction "score"	7	5	4	4	4	1
Net Score	16			9		

TABLE 28. The effect of pretreatment with disodium cromoglycate or placebo on the development of significant changes in temperature, white blood cell count (WBC) and pulmonary function tests (PFT) in 12 symptomatic pigeon breeders following a natural challenge.

(Altounyan, 1967) but has had no serious investigation in alveolitis despite the obvious economic benefits.

The mechanism of the protection which is suggested by the current small study on 12 subjects is speculative, primarily because the actual protective role of cromoglycate in asthma is still unresolved. One reported activity of cromoglycate is the inhibition of chemotactic factor release and this may have a bearing in the case of alveolitis, where the pathogenesis is thought to involve an influx of cells into the lung as part of the inflammatory process. This influx would compromise the lung function, and is in keeping with the observation that it is the lung function which appears to be most protected by cromoglycate in the treated group.

3. LABORATORY CONTROLLED ANTIGEN INHALATION CHALLENGE.

To obviate the problems of controlling the administered antigen dose and for closer monitoring of the subsequent reaction, particularly for any immediate changes, each subject was challenged individually in a controlled laboratory environment. The antigen dose was determined by first performing a skin prick test with the antigen preparation (sterile pigeon serum, approximately 50 mg/ml protein). This identified any individuals at risk of developing immediate hypersensitivity reactions (Hendrick, Marshall, Faux and Krall, 1980). Depending on this skin reactivity, subjects were given between 0.1 to 0.5 ml of pigeon serum, diluted in 5 ml sterile saline, and nebulised via a mini-nebuliser and face mask with air at 8 L/min. Following baseline pre-challenge pulmonary function tests and physiological measurements, the nebulised antigen was administered for 2 minutes. After 10 minutes, if there were no changes in peak expiratory flow rate (PEFR), the antigen was given for a further 3 minutes and the PEFR was checked every 5 minutes over the next 20 minutes. The physiological measurements included helium dilution lung volumes, single breath TLCO, spirometry (including flow-volume loop), oral temperature and peripheral blood white cell count. Regular venous blood samples were taken. In addition some subjects had an indwelling arterial cannula and blood gas measurement were recorded over a 4 hour period. Local

hospital ethical committee approval was obtained for these studies.

Fourteen subjects with documented EAA caused by avian exposure were challenged by inhaling a set volume of pigeon serum (listing on Table 29). This table summarises each individual's details and their responses to this challenge.

a) Clinical response.

Nine subjects (Nos. 1 - 9) developed clinical symptoms which they recognised as typical of an acute response following prolonged avian contact. Five others (Nos. 10 - 14) had no symptoms. The severity of the symptoms ranged from mild (feeling warm and uncomfortable, see Table 30) in two subjects, to marked general malaise and dyspnoea. In general the reactions did not proceed this far; they were either self-limiting or were terminated by oral prednisolone. No reactions were unduly untoward, and all subjects were symptom-free the next morning.

No subject had any significant immediate symptoms within 30 minutes of challenge.

b) Leucocytosis and pyrexia.

Eleven of the fourteen subjects developed an increase in blood leucocytes of greater than 30% of the pre-challenge counts during the eight hour period post-challenge. These included all 9 symptomatic subjects. Eight subjects had a pyrexia of greater than 37.2°C , these were all in the symptomatic group and all of them had a significant leucocytosis.

c) Pulmonary function.

During the first 15 minutes post-challenge the peak expiratory flow rate (PEFR) was monitored and this remained constant in all but two subjects. Some further sensitive indices of airway obstruction, viz, RV, FEV_1/FVC ratio and $\text{Vmax}_{25}/\text{Vmax}_{75}$ ratio from flow-volume loops were measured at frequent intervals in the 4 hours after exposure and these did not appreciably alter, even in the individuals who had marked immediate prick test responses to pigeon serum, or those with an atopic history.

Over the eight hour period following challenge there was an 11% drop in forced vital capacity compared with pre-challenge levels in the group as a whole ($p = \text{n.s.}$). This was evident in 10 of the 14 subjects, including all but one of the symptomatic

Sub.	Age	Dur. of dis. years	IgG antibody ug/ml	Atopic	Skin test(mm) pigeon serum		Challenge dose of pigeon serum(ml)	RESPONSE TO CHALLENGE						
					1/50	1/10		Pulmonary function			Leucocytosis	Pyrexia	Sympt.	
PEFR	VC	SB												
1	FB	30	8	205	yes	0	1	0.2	n.k.	+	+	+	+	+
2	JS	45	3	173	no	0	0	0.4	-	+	-	+	+	+
3	RS	33	7	152	yes	0	3	0.25	-	+	-	+	+	+
4	TP	50	1	122	yes	0	4	0.15	+	-	-	+	+	+
5	HM	33	7	95	yes	0	2	0.25	n.k.	+	-	+	+	+
6	RD	35	3	89	n.k.	0	0	0.2	n.k.	+	-	+	+	+
7	JP	25	1	35	yes	0	2	0.15	-	+	-	+	+	+
8	WO	43	3	32	yes	0	1	0.3	-	+	-	+	+	+
9	JMK	59	5	18	yes	0	7	0.2	-	+	-	+	n.k.	+
10	DS	32	1	121	no	0	5	0.2	+	+	-	+	-	-
11	JB	60	6	55	no	1	3	0.15	-	+	-	n.k.	-	-
12	JC	50	2	39	n.k.	0	0	0.25	n.k.	-	-	+	-	-
13	WR	61	3	16	no	0	3	0.2	-	-	-	-	-	-
14	RJ	37	1	14	no	0	1	0.2	-	-	-	-	-	-

TABLE 29. Individual details and response to laboratory controlled inhalation provocation with pigeon serum in 14 symptomatic pigeon breeders.

SUBJECT	LEUCOCYTOSIS			PYREXIA				CLINICAL		
	WBC x10 ³	% increase	Onset hours post- challenge	Peak hrs.	°C	Onset hrs.	Peak hrs.	Symptoms	Onset hrs.	Peak hrs.
1. FB	11.8	52	3	6	38.2	6	7	a.b.c.	3	7
2. JS	13.5	69	n.k.	7	37.8	4	7	d(mild)	7	*
3. RS	13.7	n.k.	4	n.k.	38.2	4.5	9	a.b.c.	7	*
4. TP	10.2	48	7	n.k.	37.4	7	n.k.	e(marked).f.	7	*
5. HM	11.8	103	3	6	37.2	6	7	a.e.g.	4	7
6. RD	16.0	84	2	7	37.6	6	7	a.e(marked).	4	7
7. JP	13.2	89	6	n.k.	37.6	6	n.k.	e(marked).	7	*
8. WO	8.7	34	2	2	37.3	2	2	d(mild).	2.5	2.5
9. JMK	12.1	69	7	n.k.	37.0	nil	n.k.	a.e.	12	*

a. shivering; b. muscle aches; c. cough; d. felt "warm and uncomfortable"; e. general malaise; f. dyspnoea; g. headache.

*Reaction terminated with oral steroids.

n.k. not known.

TABLE 30. Clinical and physiological details of the 9 symptomatic responders following inhalation provocation testing.

group. However, despite the reports of an association between a reduced carbon monoxide diffusing capacity and disease (Dinda et al, 1969; Petro et al, 1980) this was only found in one subject (FB). In the other 13 there were no significant changes in the coefficient of transfer of carbon monoxide when this was corrected for alveolar volume.

Six subjects had sequential measurements of alveolar-arterial oxygen tension (A-a) gradient (Habte et al, 1982), including 3 without a definite clinical response. In 5 of these a 191% increase in the gradient was found indicating a degree of ventilation/perfusion mismatching, similar to the report of Gaultier et al (1980).

d) Complement levels.

The values of complement components C3, C3d, C4, C1q and Factor B were measured at intervals during the eight hours post-challenge. The individual levels fluctuated markedly, presumably reflecting both complement activation and simultaneous synthesis as part of an acute-phase response associated with the inflammatory process. Moreover, because these changes would presumably be temporally related to the active response and since this also varied (see Table 30) it would be more meaningful to measure these parameters relating to individual responsiveness. One of these, RD, is listed on Table 31 along with a full description of his other parameters of reaction. This will be discussed fully below, but indicates an involvement of the complement system. This is also the indication from a summary of the values obtained from 5 symptomatic subjects for whom the data could be pooled due to the timing of blood samples (Figure 28).

This clearly shows a drop in serum C3 levels which reaches significance at 3 hours post-challenge compared with pre-challenge values. At 7 hours post-challenge the C3 levels had recovered, but this would appear to be due to enhanced synthesis because the serum levels of C3d, a breakdown product of the activation of C3, continued to rise. There was a marked reduction of Factor B levels from pre-challenge levels which was maintained during the 7 hours post-challenge. There was very little change in the mean C4 levels, and a slight increase in C1q over the first 3 hours.

	PRE-CHALLENGE	TIME POST CHALLENGE							
		15 Min	1	2	3	4	5	6	7 hrs.
IgG	12.4	12.5	13.1	13.6	13.6	14	14.8	14.9	13.8
IgA	2.4	2.3	2.3	2.2	2.3	2.3	2.3	2.3	2.3
IgM	1.4	1.1	1.7	1.5	1.5	1.5	1.4	1.4	1.2
C3	151		186	126	126	177	151	168	135
C4	30		28	28	30	28	28	28	30
C1q	92		108	124	164	172	100	164	132
FacB	20		22	23	23	23	25	25	23
CRP alpha ¹ -antit.	Negative 220	neg 248	neg 258	neg 266	neg 266	neg 266	neg 272	neg 272	neg 280
FEV ₁	4.03		4.09	4.26	4.14	4.19	3.81	3.5	3.5
FVC ₁	5.45		5.45	5.45	5.28	5.28	5.07	4.79	4.64
Ratio %	74		75	78	78	79	75	73	75
S.B.	9.9			10.2		10.9		10.4	
A-aO ₂	1.2		1.41	1.23	1.37	2.7	3.05	3.41	3.68
Temperature	36.3		36.6	36.6	36.9	36.8	36.9	37.6	37.6
WBC	8700	9200	11700	12600	13100	12300	11300	13200	16000
Symptoms									onset (shivering) - progressive

TABLE 31. Serial measurements of various serological and physiological parameters during the course of acute alveolitis in one subject (RD) following antigen inhalation.

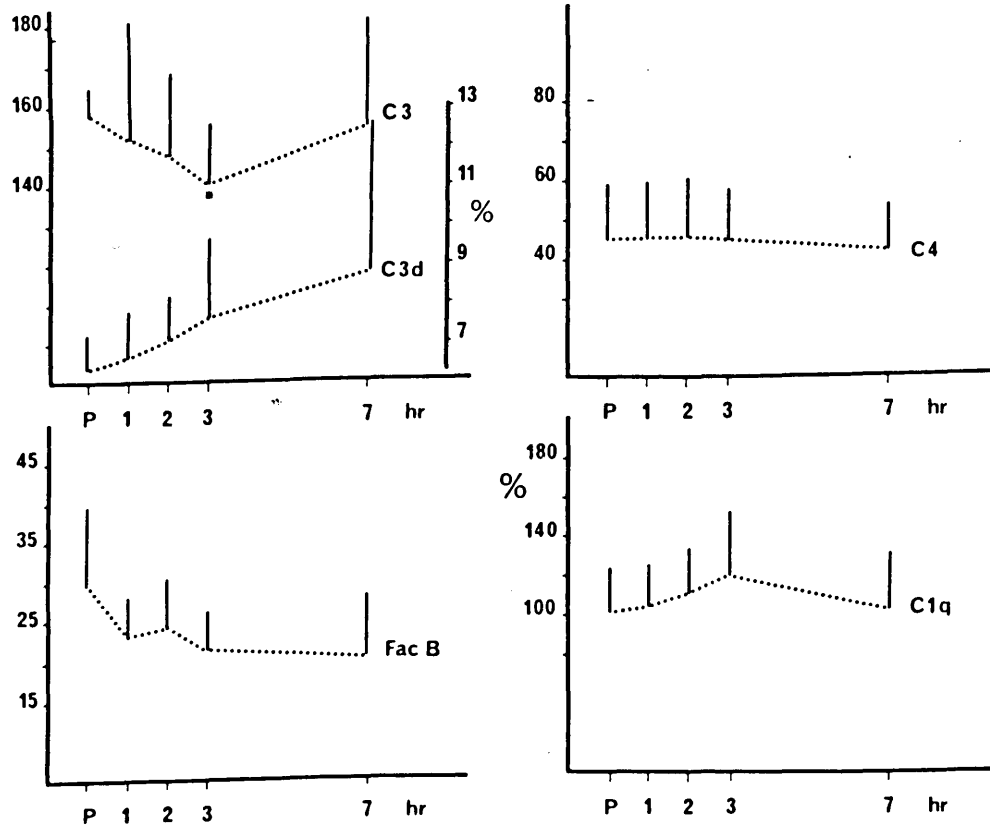


FIGURE 28:

Serial mean levels of serum complement components C3, C4, Factor B (mg/dl) and C3d, C1q (% Normal) in 5 symptomatic responders before and for 7 hr. after antigen inhalation provocation testing.

P = pre-challenge.

e) A composite view of the changes in the various parameters during the acute phase of reaction.

e(i): One individual - subject RD - had the most complete investigation and the summary of the pulmonary function tests during the acute phase is graphed on Figure 29, and the corresponding physiological and immunological parameters are listed on Table 31.

The first parameter to change was the total peripheral WBC count. This increased progressively by 34.5%, 44.8% and 50.6% of pre-challenge levels at 1, 2 and 3 hours post-challenge, and at 7 hours, the count had risen by 84%. The sequence of events started with an 8% fall in C3 levels at 2 and 3 hours post-challenge, followed by a sharp increase in A-a oxygen gradient between 3 and 4 hours. Symptoms commenced with shivering at 4 hours and there was a progressive fall in FEV₁ and FVC from 4 hours onward. There was a sharp increase in temperature from 36.9 to 37.6°C between 5 and 6 hours. Symptoms persisted, becoming increasingly marked until the reaction was terminated at 7 hours with oral prednisolone. Throughout the acute phase of reaction there was an increase in total IgG of 20% over a 6 hour period and this progressive increase was similar to an increase in serum alpha 1 - antitrypsin of 27% over the same period. During this time there was very little change in serum C4, Factor B, total IgA and IgM, and serum C-reactive protein was undetectable throughout.

e(ii): Group results:

In general a peripheral blood leucocytosis was the first evident change. This tended to precede both the pyrexial response and the development of symptoms (Table 30). The time of onset of these changes could not be predicted by the antibody level nor the challenge dose of antigen. Two subjects with no overt symptomatic response also had a leucocytosis and in one of these there were other pulmonary function changes which together suggested an active response. The extent of the leucocytosis could not be predicted by either the individuals antibody level nor the antigen challenge dose, this was true also for the pyrexial response.

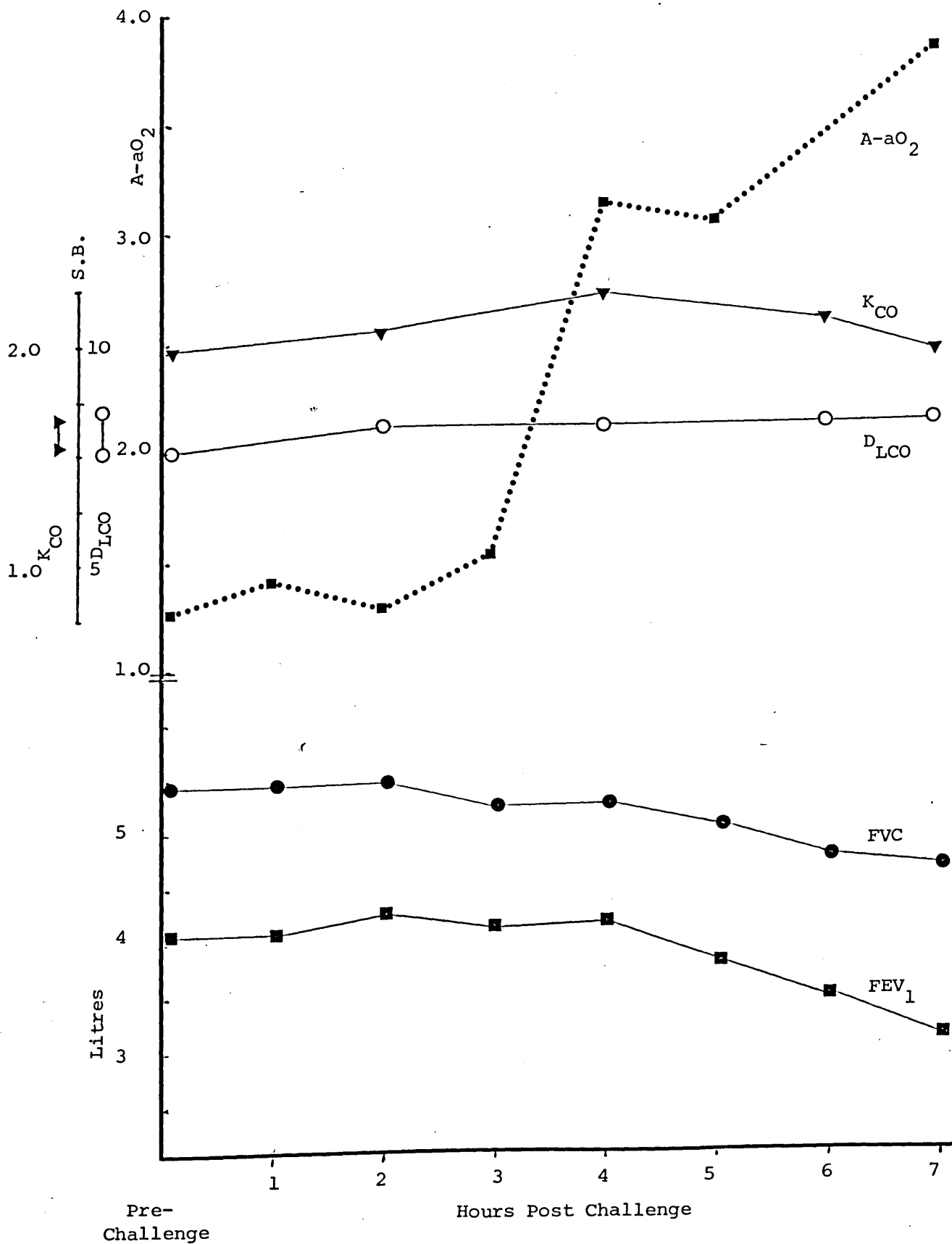


FIGURE 29. Changes in pulmonary function FVC, FEV₁, single breath carbon monoxide diffusing capacity (K_{CO} and D_{LCO}) and the alveolar-arterial oxygen tension gradient in one symptomatic responder during the 7 hour period following antigen inhalation provocation testing.

4. THE CONVALESCENT PHASE.

The converse of the acute-phase reaction discussed above, where the various parameters of physiology and serology were measured during an induced episode of disease, is the convalescent phase where these parameters are measured in symptomatic subjects who are completely removed from the antigen contact to which they had previously been continuously exposed. This was the case in three subjects who had been hospitalised due to the severity of their symptoms of alveolitis, thus guaranteeing complete antigen avoidance, and 4 others who accepted medical advice to avoid further avian contact.

a) Serial antibody measurements.

The IgG antibody level against pigeon gamma-globulin antigen was measured serially in 7 subjects with alveolitis who avoided further avian contact. Figure 30 illustrates the decrease in the specific antibody levels in all subjects. The maximum rate of decrease was soon after the initial antigen avoidance, and the rate of decrease was more pronounced in subjects with the highest antibody levels. In several of these subjects (JSI, SH and JW) there was a suspicion that avian contact was reduced rather than ceased, but in the case of the others (DD, EG and JP) the antigen avoidance was complete.

In most of the subjects the reduction in antibody levels was an exponential function rather than linear, and the half-life of decay was approximately 22 days for the subjects with complete antigen avoidance and 48 days for the others. The antibody levels were not followed until they approximated to the normal range, but it appeared that they would remain "low-positive" despite no further exposure to inhaled antigen. The reasons for this are unclear; it could be a low level of turnover of what at one time was an active plasma cell population or it could be due to a low level of cross-reactive stimulation from ingested hen's egg antigens (Lee et al, 1983).

In the normal situation, however, there is already a yearly fluctuation in antibody levels among pigeon breeders which is closely associated with seasonal rates of avian exposure (McSharry et al, 1983). There is most contact during the peak of the pigeon racing season in August and September, thereafter there

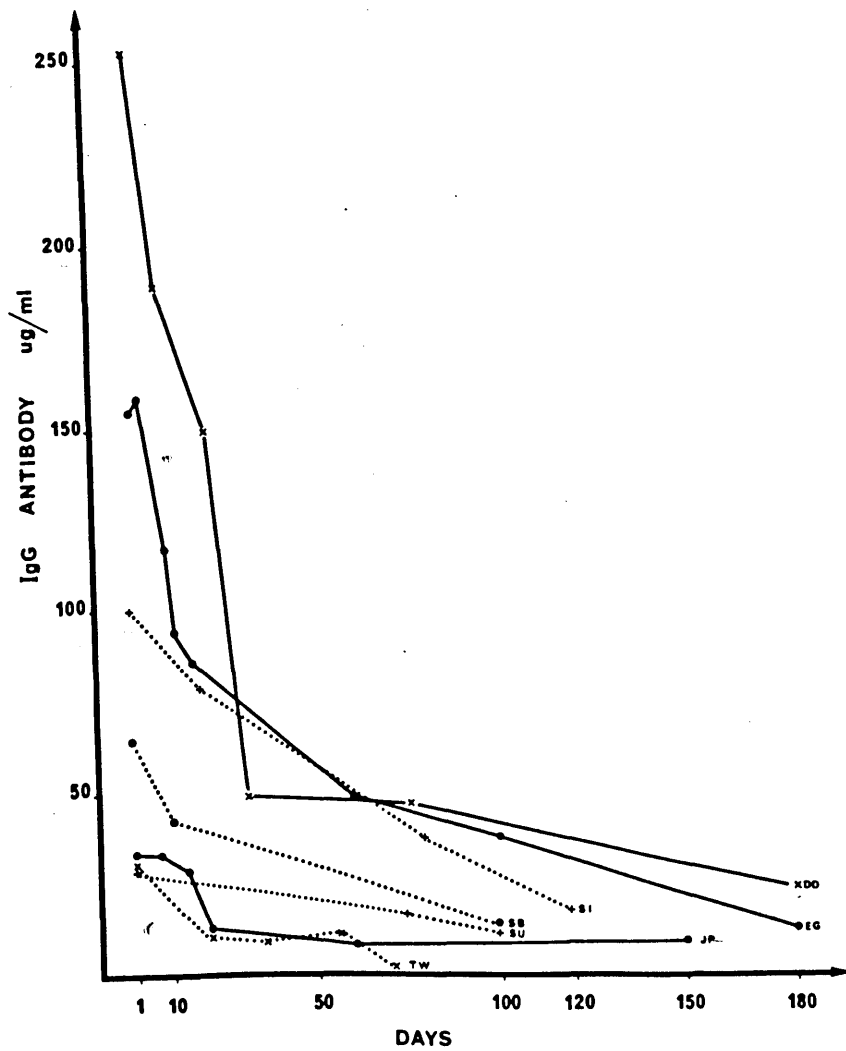


FIGURE 30:

Serial measurement of serum IgG antibody to avian antigens in 7 pigeon breeders who avoided further avian contact.

is minimal daily contact for the purposes of husbandry during the winter. The breeding season starts in March and the exposure increases until August. The effects of this natural fluctuation in exposure on the antibody levels were studied in 20 pigeon breeders who donated regular blood samples over a one year period. The profile of the monthly antibody levels is illustrated in Figure 31. Eight of the subjects with significant antibody levels, 6 of whom are illustrated, showed a marked fluctuation in antibody levels with maximum values occurring in August and September. These peaks coincided with the time of maximum avian contact which was between May and September when the average weekly hours spent with pigeons was 22, compared with 9 hours per week during winter. The 12 subjects with insignificant antibody levels displayed minimal changes despite similar avian exposure, and for clarity only one of these, CH, is illustrated.

Both these situations of varying avian exposure illustrate clearly the dynamic nature of the antibody response to inhaled avian antigen, and also the necessity for continued exposure to maintain the antibody titres. During the convalescent period, where clinical improvement was evident, illustrated by the recovery of lung function in 4 subjects (Figure 32), some further immunological parameters were followed.

b) Serial measurements of various immunological parameters.

One subject who was hospitalized with acute alveolitis and who did not receive steroid therapy had serial blood samples taken. The measurements of various immunological parameters during the convalescent phase are recorded on Figure 33 and Table 32.

There was an initial rapid fall in circulating immune complex levels, presumably reflecting the lack of antigen, and the antibody level at this stage seemed to rise, perhaps the previous titre had been reduced by complex formation. Thereafter the antibody levels fell exponentially. The total serum IgG fell initially in parallel, but levelled off while the specific antibody levels continued to fall. During this early period of 11 days the patient's serum C4 level was at the low end of normal, but increased thereafter. During this period there was a gradual clinical recovery.

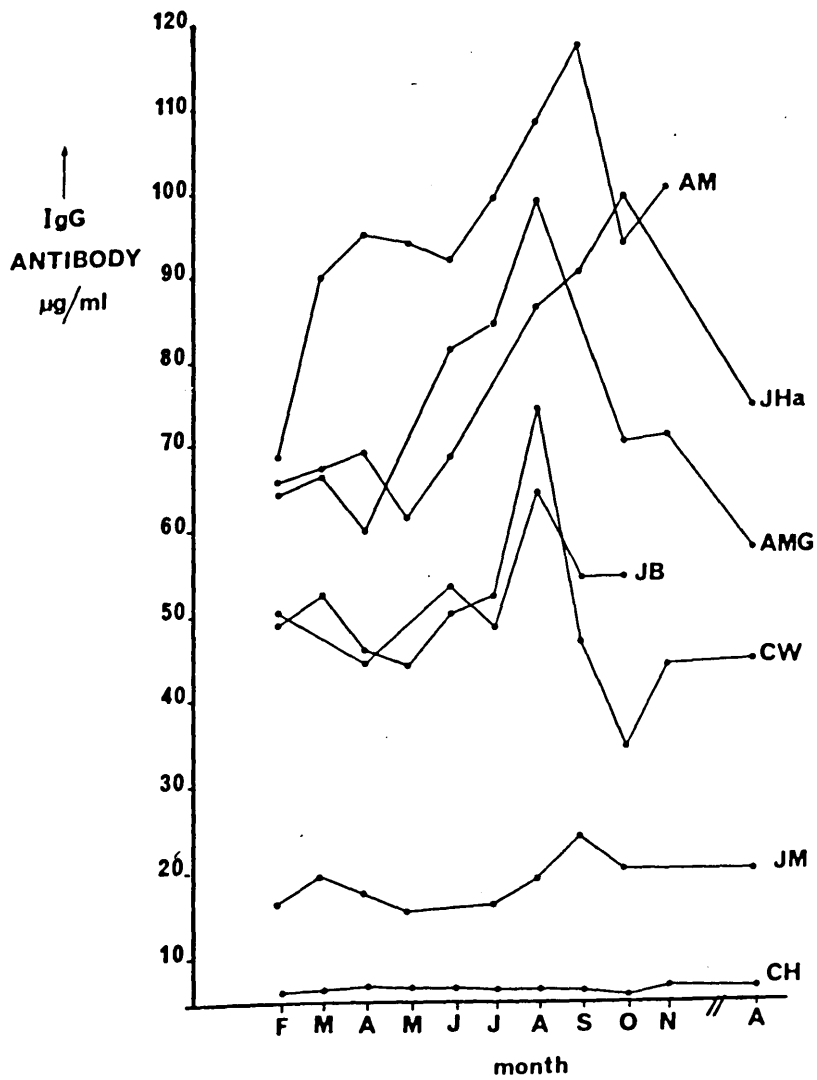


FIGURE 31:

Monthly fluctuation in serum IgG antibody levels to avian antigen over a one year period from February (F) to November (N) and in some, to the following April (A).

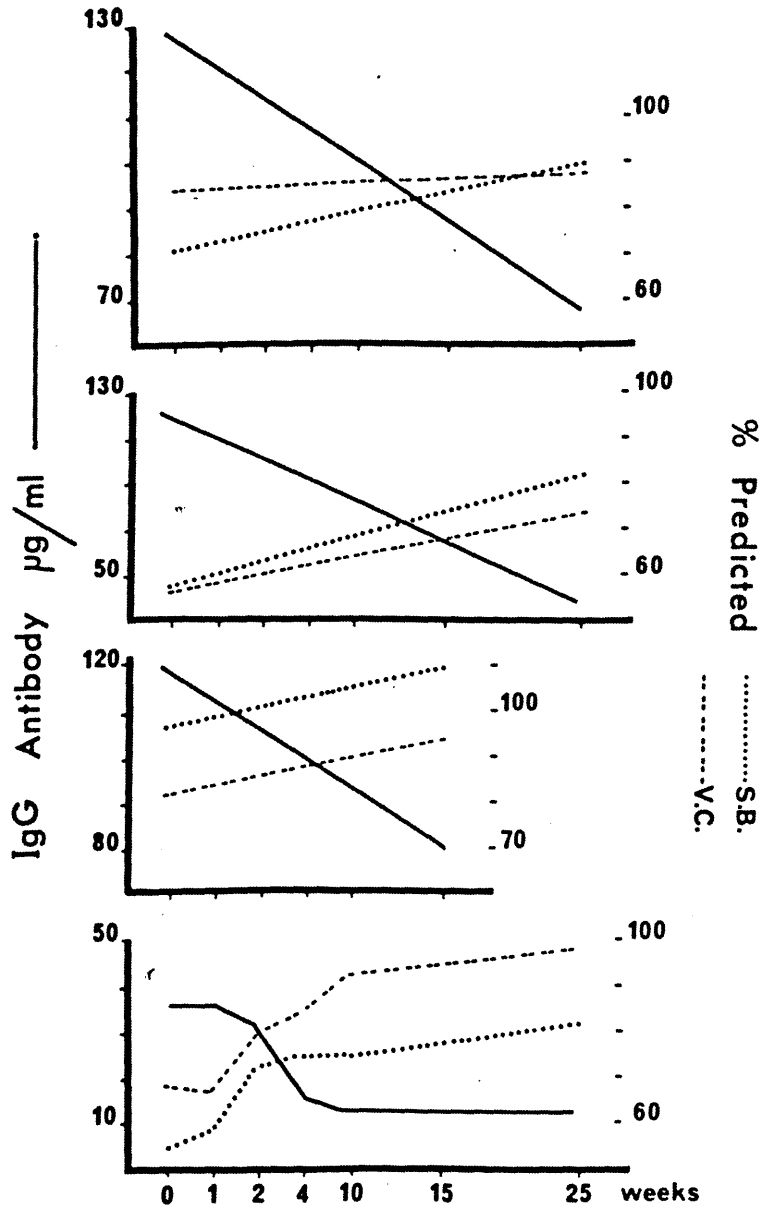


FIGURE 32:

The recovery of pulmonary function and the fall in serum IgG antibody to avian antigens in 4 symptomatic pigeon breeders who avoided further avian contact.

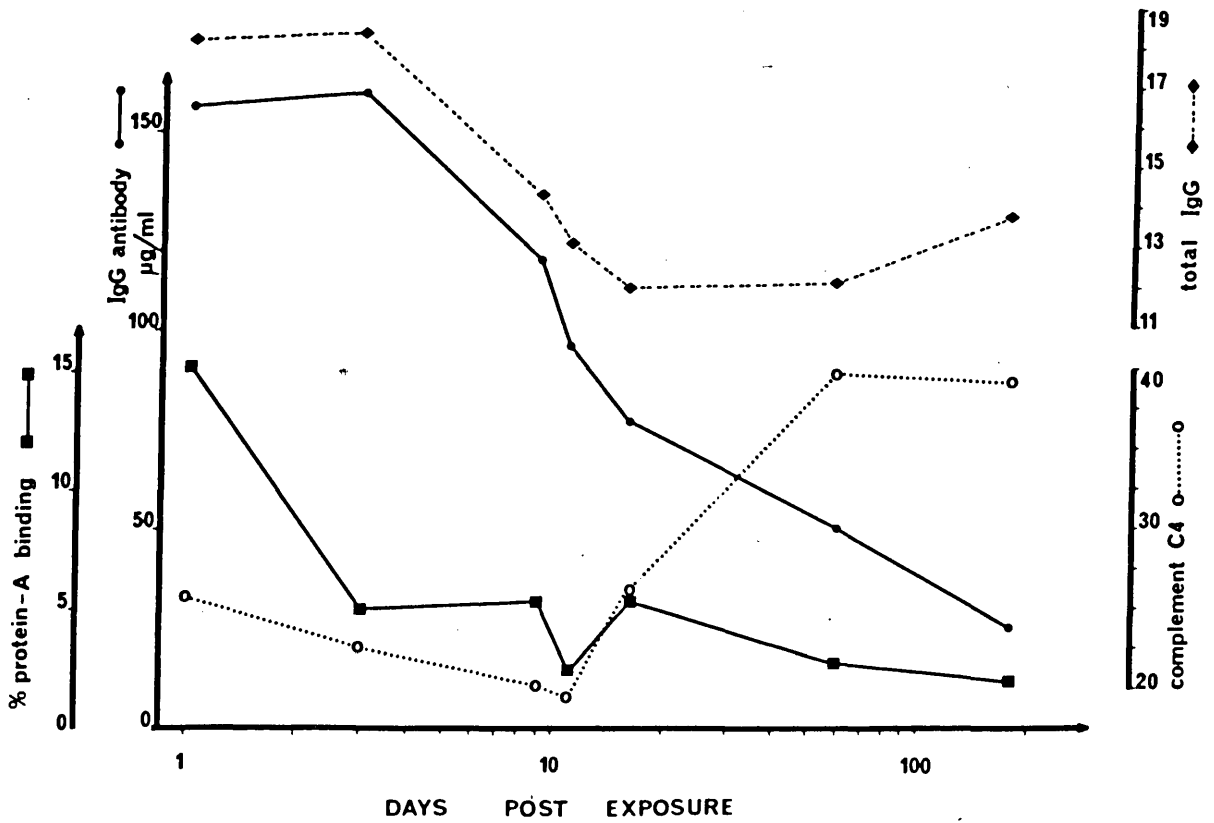


FIGURE 33:

The return to normal of various serological parameters in one patient (E.G.) during convalescence following a severe episode of alveolitis.

		DAYS OF ANTIGEN AVOIDANCE						
		0	2	8	11	13	60	180
IgG Antibody	(ug/ml)	156	160	118	95	77	50	25
IgG	(mg/ml)	18.2	18.4	14.4	13.2	12	12.2	13.8
IgA	(mg/ml)	4.2	4	3.7	3.4	3.1	2.8	3.3
IgM	(mg/ml)	low	low	low	1.4	2	1.5	1.6
IgE	(iu/ml)	1500	1700	400	280	160	180	170

TABLE 32. Prospective changes in serum immunoglobulins and specific IgG antibody to avian antigens in one symptomatic subject (EG) during convalescence, with no avian contact.

The stimulus which caused the raised total IgG levels, and which was removed following antigen avoidance, acted similarly on the IgA levels, and apparently inversely on the IgM levels (listed on Table 32). This may be the reversal of some form of polyclonal activation induced by the inflammatory process associated with antigen exposure. Interestingly this appeared to have a profound effect on the serum IgE levels which fell in this patient to 10% of the initial value.

5. DISCUSSION.

Longterm inhalation of dusts can have an adverse effect on the lungs. This is certainly the case in coal-workers pneumoconiosis and asbestos inhalation, where the lung is unable to clear the inorganic insoluble dusts. Soluble organic dusts from grain (Broder & McAvoy, 1981) and sewage (Mattsby & Rylander, 1978) can also cause non-specific pulmonary reactions in exposed workers and these are thought to be due in the first case to a protein in grain which efficiently binds and activates human immunoglobulin, and in the second case to bacterial endotoxin in sewage. Emanuel, et al (1964), during studies on farmer's lung, also described non-specific lung reactions following the inhalation of dusts, especially to fungi, and they called this "mycotoxicosis". Similarly, soluble extracts from these fungi were equally non-specifically toxic when used in skin testing of farmers (Williams, 1963).

Despite these non-specific effects, controlled exposure to aerosols of antigens which are considered to cause alveolitis are of value for establishing a diagnosis and for investigating the mechanism of disease. Some of the main literature reports using this technique are summarised in Appendix C. The method is time and effort intensive, requiring one full day per challenge, and good patient cooperation is essential, especially for the forced respiratory function manoeuvres. Conservative protocols have been devised in order to deliver the minimum inhaled antigen dose to elicit a reaction (Hargreave & Pepys, 1972) and this procedure was followed in this chapter. Similarly for natural challenges, a typical exposure period in a pigeon loft going through the normal procedures of husbandry was used. These

protocols were considered to be valid since control individuals had no symptomatic, physiological or serological responses. However, before appropriate antigen doses had been established, some workers had overestimated the amounts required and caused pyrexial responses of up to 37.5°C but no symptoms in control subjects who inhaled 3ml of neat pigeon serum (Reed et al, 1965). At this dose the symptomatic subjects developed a temperature of 39.2°C and exertional dyspnoea which persisted for several weeks. Similar pyrexial responses to Micropolyspora faeni antigens in high doses were also observed in controls (Barbee et al, 1965). Subsequent workers have tended to use one or two orders of magnitude less of antigen, and it has been reported that acute episodes of alveolitis can occur following exposure to minute amounts of antigen, for example, as in briefly handling just one bird (Fink, 1974; Duchet-Suchaux et al, 1969). This was a reflection of how sensitive some individuals could be.

Following natural exposure to 50 pigeons for one hour in a loft, only one out of 12 pigeon breeders with alveolitis developed overt symptoms. A symptomatic response therefore seemed to be uncommon following normal day-to-day avian exposure. Although this exposure was mild, and almost all subjects remained asymptomatic, there were some measurable physiological changes. Five subjects developed both a significant pyrexia and leucocytosis, these subjects did not have a significant decrease in lung function; they had a lower mean antibody level than the others, but all had recent onset of disease. The four subjects who had pulmonary function changes were a more chronic group in that they had a much longer history of disease and also a much higher mean antibody level. Interestingly, some of the asymptomatic subjects had much more pronounced fever, leucocytosis and decrease in pulmonary function than the one symptomatic subject. There was no reason in this group for the individuals to hide any symptoms, therefore it could be that they were either accustomed to mild symptoms which might have been expected with a pyrexia of up to 37.7°C and compromised lung function, or that the development of symptoms was a separate but related event following antigen exposure. Similarly in this group, the pyrexial and leucocytosis responses seemed to occur together, but were

unassociated with decreases in pulmonary function.

All these aspects of responsiveness were seen to be dose-dependent in that they were all increased in incidence and severity when the administered dose of antigen was increased during laboratory inhalation challenges. One assumption was that the inhalation of pigeon serum in the laboratory was equivalent to the natural exposure. This appeared to be the case in that the subjects described what would be typical symptoms in both types of challenge. Following laboratory challenge 9 out of 14 pigeon breeders with alveolitis developed symptoms. The individual's serum antibody level against avian antigens could not be used to predict who did or did not develop symptoms in this group. Furthermore, the antibody level did not predict the onset or severity of symptoms, nor the extent of pyrexia and leucocytosis, nor the decrease in pulmonary function. There was an indication, which was difficult to tabulate, that the subjects who had most recently had an acute episode of alveolitis were those who reacted most vigorously to challenge.

The changes in pulmonary function were also more marked in the laboratory challenge group, and the most common abnormality which developed was a fall in forced vital capacity. The loss in lung volume associated with this could account for the different literature reports of changes in carbon monoxide diffusing capacity following challenge (Appendix C). Where the diffusing capacity was reduced in the present subjects challenged, this could be accounted for by a reduction of alveolar volume in all but one subject

In all subjects the pyrexia and leucocytosis tended to occur together and these preceded the development of pulmonary function changes and symptoms. The maximum change in pulmonary function was a mean reduction of 16% in VC after 6 hours compared with pre-challenge levels. However, there was a more immediate change noted in 5 of the 6 subjects who had their arterial-alveolar oxygen tension gradient measured. In these cases a clear increase of 41.2% was measured between 2 and 3 hours post-challenge, and this was an earlier response than either the leucocytosis or pyrexia. There were no immediate (less than 30 min) symptoms in any of the subjects despite a high incidence of

atopy in the group (58.3%), and despite immediate skin test responses to pigeon serum in 11. This is in general agreement with Warren et al (1977), who could not find an early response even using sophisticated pulmonary function measurements.

The sequence of events which appear to follow an inhalation challenge on a sensitised subject are firstly an increase in the A-a oxygen gradient, suggesting a local pulmonary reaction, followed by a systemic reaction of neutrophil leucocytosis which seems to slightly precede the pyrexial response. Thereafter there can be development of symptoms and then a compromise in pulmonary function. The extent to which each individual exhibits these changes will be a function of the antigen dose and the individual susceptibility. A positive challenge therefore need not go as far as compromising the pulmonary function.

These pulmonary function tests were too insensitive an index for following an acute reaction, and this was also the conclusion of Hendrick et al (1980). They were, however, a useful indicator, along with the antibody level, of the current status of an individual with pigeon breeders disease. Persistent positive precipitins correlated well with a decrease in CO diffusing capacity in one longterm follow-up of farmers lung patients (Braun et al, 1979), and during antigen avoidance the 4 subjects discussed in this chapter had good recovery of pulmonary function over a period of 25 weeks, although one report which followed up 9 patients prospectively suggested that the lung damage could be irreversible, and progressive decrease in lung function could occur despite antigen avoidance if the disease had been established beyond a critical point (Allen, Williams & Woolcock, 1976). This confirmed the work of Nash (1970), Heywood (1971) and Riley & Saldana (1973) who stated that the longterm effects of established pigeon breeders disease resulted in chronic disability, ultimately with respiratory failure.

The involvement of humoral immune parameters in the initiation and development of the physiological responses discussed above was suggested by the coincidental changes of serum complement and immune-complex levels. The levels of these components were remarkably conserved during the eight hour period

following a mild natural challenge in 12 subjects, and in contrast to this the levels fluctuated markedly in some of the 14 subjects who had the more vigorous challenge in the laboratory. When the stable breakdown products of complement activation were sought following challenge in 5 subjects, C3d was found to increase during the first 3 hours coincidental with a significant decrease in C3 levels. The C3 levels returned to normal after 7 hours, but the C3d levels continued to rise suggesting an increase in synthesis of C3, perhaps as a consequence of the inflammatory response, occurring along with catabolism. The inability to differentiate between synthesis and degradation of complement by the simple measurement of total serum levels, or haemolytic complement titres, may explain why there is conflicting evidence in the literature for the involvement of complement in the disease process. A further complication would be that any changes in complement would be presumably related to the antibody titre to the inhaled antigens, and also the amount of antigen inhaled. Other undetermined constitutional factors may also prevail going by the observed variability of the physiological responses to challenge in the study group. In literature reports of antigen inhalation challenge of alveolitis patients it was first suggested that a drop in CH-50 occurred in non-responders (Moore et al, 1974) but then Schatz et al (1976) reported a drop in CH-50 in all challenged subjects. Voisin (1979) only reported one out of six challenged subjects with a decreased CH-50, interestingly this was the subject with by far the most florid symptomatic and physiological response. Baur et al (1980) in contrast reported an increase in CH-50 in 4 subjects following inhalation challenge and he also did not find any C3 split products. The most recent reports (Edwards & Davies, 1981; Lozano et al, 1982) find no significant differences in control or alveolitis patients following exposure to antigen. Thus the situation has not progressed much since Schatz et al (1976) described the role of complement in alveolitis as "enigmatic". However, the work in this section would suggest that complement is involved, and that differences in serum complement are manifest only after relatively high challenge doses, or are masked by concomitant synthesis. Moreover, minor changes in complement activity have been measured

across the pulmonary circulation in association with inhalation challenges (Halprin et al, 1973). These complement changes would be too small to be detected by venous sampling alone.

Further evidence for the involvement of complement and immune complexes in the disease process was suggested by the rapid fall of serum levels of complexes when the antigen source was avoided, and by the return to normal of C₄ in one subject coincidental with clinical recovery. The serum antibody level appeared to require continuous antigen contact, otherwise the titres fall exponentially. Further non-specific parameters of inflammation including total immunoglobulins IgG and IgA and interestingly occasionally IgE, but not IgM, seem to rise following challenge and fall when the antigen is avoided.

These parameters are, therefore, at least associated with disease, if not actually involved. This is notable in that they were also raised in some asymptomatic subjects discussed in Chapter 4. Following natural challenge it was noticed that symptoms were rarely seen, and only when the challenge dose was substantially increased by laboratory challenge did symptoms become evident. These observations suggest that there can be definite physiological changes associated with avian exposure without overt symptoms and that subclinical disease may be prevalent, the extent of which is unknown. However, if humoral parameters such as antibody are used as a guideline then incidences of disease may be far greater than indicated in Chapter 1. This question could be resolved by subjecting asymptomatic subjects who have an inflammatory serum profile to a laboratory controlled challenge in order to assess the responsiveness to higher antigen doses. This was done in 2 subjects with serum antibody to inhaled antigens from a humidifier system, and although they had always been asymptomatic, a laboratory challenge with an equivalent daily dose of antigen was enough to cause a typical alveolitis response (Edwards & Cockcroft, 1981). This approach would be of value because it is primarily the insidious, subclinical disease which results in irreversible lung damage.

It is highly probable that cellular events also occur during the acute phase of alveolitis. Both the lung histology and lung lavage demonstrate an increase in total cell numbers,

predominantly of mononuclear cells characterised as T lymphocytes (Reynolds et al, 1977; Weinberger et al, 1978; Godard et al, 1981), which is particularly marked during the acute phase (Molina et al, 1979; Bernardo et al, 1979). The peripheral blood leucocytosis during the acute phase of disease was predominantly a neutrophilia, with occasional reports of eosinophilia (Hargreave & Pepys, 1972; and one patient in this study - subject JC). A mild lymphopenia has also been reported in conjunction with the rise in polymorphs (Hargreave & Pepys, 1972; Geller et al, 1977; Voisin et al, 1979; Hendrick et al, 1980), the inference being that the active lymphocytes are somehow sequestered in the lung. This is thought to be the case in pulmonary sarcoidosis where local lung lymphocytes are extremely active, but there is anergy in the peripheral cells (Hunninghake & Crystal, 1981). A similar anergy has been reported against tuberculin in farmers (Fuller, 1962) and pigeon breeders (McSharry et al, 1983) and there are very few reports of cell-mediated delayed type skin reactions in these subjects to avian antigens. This topic will be discussed in Chapter 6.

It is particularly pertinent to note that systemic delayed hypersensitivity in the guinea pig is characterised by fever which begins approximately 2 hours after an intravenous challenge (Uhr & Brandriss, 1958) and perhaps after an aerosol challenge as well (Miyamoto & Kabe, 1971; Miyamoto et al, 1971). The fever associated with the acute phase may well be related to interleukin-1 production by alveolar macrophages (Murphy, Simon & Willoughby, 1980). These workers pointed out that the endogenous pyrogens were identical to lymphocyte activating factor. This suggests that the macrophage could provide the trigger for the systemic reaction and also the local pulmonary T-cell activation. The depletion of an intracellular biologically active factor such as this could account for the refractory periods which were reported in humidifier fever (Edwards, 1980), in farmers lung (Williams, 1963) and in aspergillosis (Pepys et al, 1968). Although a similar refractory period has not been demonstrated following inhalation challenge of pigeon breeders, it could explain the inconsistency of the responsiveness to experimental aerosol challenges reported here and in the literature. Some supporting

evidence for this was reported by Schuyler et al (1978) who noticed that the bronchial lymphocytes were refractory to avian antigen stimulation for up to 7 days in one pigeon breeder following a particularly vigorous inhalation challenge.

The treatment of alveolitis would be of considerable benefit, and a model for testing the efficacy of drugs using challenges on pigeon breeders would appear to be the simplest system. Isoprenaline had little effect on the response (Williams, 1963; Hargreave et al, 1966; Hargreave & Pepys, 1972), and although corticosteroid treatment has been widely used to relieve acute symptoms (Emanuel et al, 1964; Hapke, Seal & Thomas, 1968) and to reduce the circulating antibody levels (Boyd, 1978), it seemed to have no marked influence on the course of lung function or the prognosis of working capacity in one follow-up study of 93 patients with farmers lung (Monkare, 1983), however it did diminish fibrotic appearances in chest radiographs.

There seems to be, therefore, a place for a drug in the treatment of this condition. Disodium cromoglycate (DSCG) has been extensively used in asthma, and its use in alveolitis has only been reported by Pepys et al (1968) who inhibited the early and late reaction associated with the inhalation of Aspergillus fumigatus antigens in two patients and the late reaction in 3 pigeon breeders after inhaling pigeon serum. In this section the use of DSCG was shown to reduce the responsiveness to challenge of a group of sensitized pigeon breeders. These results should prove to be a useful platform from which to conduct a more thorough investigation of the efficacy of the drug in alveolitis. However, until such time, the only protection afforded to the alveolitis patient is complete antigen avoidance, or at least minimal contact, usually by wearing a mask (Siegel & Oullette, 1969).

The diagnostic usefulness of inhalation challenges discussed above have been questioned, and indeed were suggested to be dangerous (Hendrick et al, 1980). In our experience they are very time and labour intensive and require considerable goodwill on the part of the subject under investigation. The results can be far from conclusive, and therefore better diagnostic aids would be useful. These could include along with the serological

profile, measuring the arterial oxygen tension by transcutaneous oxygen electrode and gallium-67 scanning to stage the alveolitis (Line et al, 1981). These techniques however require sophisticated machinery, therefore, a more readily available and simple technique would be needed. To fulfill this need we looked at skin testing of patients using specific antigens.

CHAPTER 6

SKIN TESTING

1. INTRODUCTION

The late (4 to 8 hours) skin test reaction which can occur in patients with EAA, when the appropriate antigens are injected intradermally, has received considerable interest in the study of the disease (Fink, Barboriak & Sosman, 1967; Moore et al 1974; Hargreave & Pepys, 1972). There is a close temporal association between this reaction and the late pulmonary inflammatory reaction following inhalation provocation test using the same antigens, and a common, type III Arthus reaction has been postulated (Pepys et al, 1968; Pepys, 1969). It has been noted that this reaction may be preceded by an immediate weal and flare skin reaction (Hargreave & Pepys, 1972), and although this is of considerable diagnostic value in Type I hypersensitivity diseases such as asthma and hay fever (Bryant, Burns & Lazarus, 1975), the significance of this early reaction in alveolitis has received little attention.

This chapter investigated the prevalence of the immediate and late skin reactions following prick testing with, and intracutaneous injection of a sterile, non-irritant avian antigen preparation among pigeon breeders. The incidence of skin test reactions to common inhalant allergens and to tuberculin was also investigated. These reactions, together with the atopic status, total IgE and circulating avian antigen specific IgG and IgE antibody were assessed for their usefulness to the clinician investigating respiratory symptoms amongst pigeon breeders and are discussed in the light of current views on the disease mechanism of extrinsic allergic alveolitis.

2. RESULTS

a) Skin testing with common allergens.

One hundred active pigeon breeders were skin prick tested with six common allergens, i.e. Dermatophagoides pteronyssinus, grass pollen, cat fur, mixed feathers, Sporobolomyces and Aspergillus fumigatus, along with the appropriate skin test control solution (supplied by Bencard Ltd).

The incidence of positive skin tests to the individual

allergens in the test population of pigeon breeders was compared with control and atopic populations on Table 33. The incidence of atopy was higher compared with the figures from an unselected control population. This perhaps reflected the bias caused by encouraging as many pigeon breeders with any respiratory symptoms to participate in the study. However, the profile of skin-test responsiveness among the atopic pigeon breeders was similar to that of other atopic groups, and the incidence and profile of skin-test responses in the alveolitis sub-group was very similar to the whole study group.

Fifty of the subjects had one or more positive immediate weal and flare reactions, however 15 of these had only modest skin reactions (1mm or 2mm reaction to only one allergen, or 1mm to 2 allergens, and these were considered as a separate sub-group on Table 34. From this table the skin test reactivity was seen to correlate with total IgE levels and with a personal history of symptoms of atopic diseases. However, the incidence of hypersensitivity symptoms related to avian exposure was only marginally greater in the skin test positive group. Therefore, atopy, as judged by skin testing with common allergens, was not a feature of extrinsic allergic alveolitis due to avian exposure, however, the extent of the IgG antibody response to avian antigens was lower in the skin test negative group ($t=3.1$, $p<0.005$).

b) Skin testing with pigeon serum antigens.

A summary of the main literature reports concerning skin testing with specific antigens in extrinsic allergic alveolitis is listed on Table 35. The present study is more comprehensive in terms of numbers; 102 pigeon fanciers were investigated and full clinical details were taken. Skin prick tests and intracutaneous tests were performed using a pigeon serum antigen preparation, and the serum antibody levels were quantified.

c) Prick test.

Twenty-two of the 102 subjects had an immediate, 15 min., weal and flare reaction to neat pigeon serum. This group had a significantly higher mean IgG antibody level against pigeon serum antigens than the whole group (Table 36), was associated with a

Table 33: Percentage positive skin tests to common allergens in various populations of normal subjects, atopic subjects and pigeon breeders

	Unselected* Population n = 1300	Atopic Asthmatics* n = 554	Atopic Asthmatics** n = 220	Pigeon Breeders n = 100		
				Atopic n = 50	All subjects n = 100	Alveolitis n = 32
House dust +/- D farinae	—	82	74	—	—	—
House dust	16	70	72	—	—	—
Dermatophagoides farinae	—	69	67	—	—	—
Dermatophagoides pteronyssinus	14	—	—	72	36	42
Pollens	—	66	53	—	—	—
Grass pollen	10	—	46	52	26	25
Animal dander	—	38	78	—	—	—
Cat fur	—	—	61	58	29	25
Mixed feathers	—	—	55	58	29	31
Moulds	—	21	40	—	—	—
Sporobolomyces	—	—	8	10	5	6
Aspergillus fumigatus	4	16	20	18	9	9
Foods	—	16	30	—	—	—

* from Davies, R.J. (1979)

** from skin test clinic, Centre for Respiratory Investigation, Glasgow Royal Infirmary.

Table 34. Skin prick test response to common allergens compared with the total IgE (iu/ml) and the personal history of atopic diseases. Also with the mean specific IgG level (ug/ml) and IgE (RAST positive) for avian antigens, and the incidence of immediate and late symptoms following avian exposure.

	Immediate reactions (15 min weal)		
	Negative	Positive	
		Marginal	Definite
Number	50	15	35
IgE (mean + sd)	75.5 (66.9)	129.3 (128.0)	225.9 (215.4)
Atopic history	20%	27%	54%
Avian hypersensitivity symptoms:			
Immediate - asthmatic	3 (6%)	5 (10%)	
Late - alveolitis	16 (32%)	17 (34%)	
Avian antigen specific:			
IgG mean + sd	13.3 (28.4)	35.4 (38.7)	
IgE RAST positive	2 (4%)	12 (24%)	

Number	Precipitin		Prick Test	Intradermal		Reference
	Positive	Negative	15 min	15 min	6 hr.	
12	12		5/11	ND	9/11	Hargreave et al 1966
3	3		ND	1	2	Fink et al 1968
22	22		7	19	14	Pepys, 1969
9 10	9	10	ND ND	4 0	9 0	Warren, 1972
81	32		15	23/55	29/55	Hargreave et al 1972
30 15	10	15	9 0	17 1	12 0	El-Hefny et al 1980
16 17	11	5 17	ND ND ND	10 .1 0	8 1 0	Edwards & Davies 1981
55 21	38	17 21	ND ND ND	29 1 1	8/14 ND 0/10	Freedman et al 1981

ND = Not Done

TABLE 35. The main literature reports of results from skin testing with specific antigens in extrinsic allergic alveolitis.

Table 36. The IgG (ug/ml) and IgE (RAST positive) antibody titres to pigeon antigens and the atopic status of 22 subjects prick test positive to pigeon serum.

	Test Population n = 102	Skin Test Positive to Pigeon Serum n = 22
IgG antibody (mean + sd)	24.0 (36.1)	52.5 (43.0)
IgE RAST positive	14 (13.7%)	8 (36.4%)
Total IgE (mean + sd)	135.5 (159.0)	215.4 (210.2)
Atopic history	33%	50%
Positive prick test to common allergens	35%	82%

Table 37. Avian antigen specific serum IgG antibody titre (ug/ml) and alveolitis (EAA) in relation to tuberculin sensitivity, by Tine and Mantoux skin testing.

	Tuberculin (Tine test) reactivity		
	Negative Grade 0	Intermediate Grades 1 and 2	Positive Grades 3 and 4
Number	39	36	16
IgG antibody	33.8	18.8	16.6
Alveolitis	16	12	2
	Mantoux Skin Test		
	Negative	Positive	
Number	36	31	
IgG antibody	44.5	24.0	
EAA Positive	27	9	
EAA Negative	9	12	

higher total IgE level ($t=3.9$, $p<0.001$) and occurred predominantly in subjects with positive skin tests to common allergens.

This immediate skin reactivity was correlated with serum IgE antibody by RAST measurements against pigeon dropping extract (PDE) and pigeon serum antigens. Of the 102 subjects, 4 had a low level (RAST grade 1) of IgE antibody against PDE, and 12 were positive against pigeon serum; nine grade 1 and three grade 2 (moderate level of IgE antibody). Two subjects were positive for both antigens. Of this group of 14 RAST positive subjects, there were 8 with a positive prick test to pigeon serum (Chi-squared = 12.1, $p<0.001$).

d) Intradermal testing: immediate, 15 min., skin reaction.

Ninety-eight subjects were skin tested intracutaneously on the flexor aspect of the forearm with pigeon serum diluted 1 in 5 with sterile saline, (0.01ml, approximately 50 ug of protein). The reactions were measured at 15 mins. and 6 hours. These reactions were graded as follows:- grade 0 was no reaction or reaction equal to the control intradermal test, grade 1 was 1-3mm greater than control, grade 2 was 3-6mm, grade 3 was 7-9mm, and grade 4 was 10mm or greater. The antigen preparation was non-irritant when administered by this route and produced only grade 0 reactions in control subjects with no avian exposure.

Sixty-nine subjects (70%) had an immediate weal and flare reaction. The extent of this skin reaction as measured by weal diameter was quantitatively related to the serum IgG antibody level against pigeon serum gammaglobulin antigen (Figure 34), and not to total IgE. There was no correlation between this skin reaction and immediate skin reactivity to common allergens nor with an allergic history.

The stronger positive skin reactions were progressively more selective for subjects with avian induced extrinsic allergic alveolitis (Figure 35). Thirteen percent of all subjects with no skin reactivity had symptoms of alveolitis and this increased to 82% of the grade 4 reactors.

e) Intradermal testing: late 4-8 hour skin reaction.

Of the 98 subjects skin tested with pigeon serum, 10

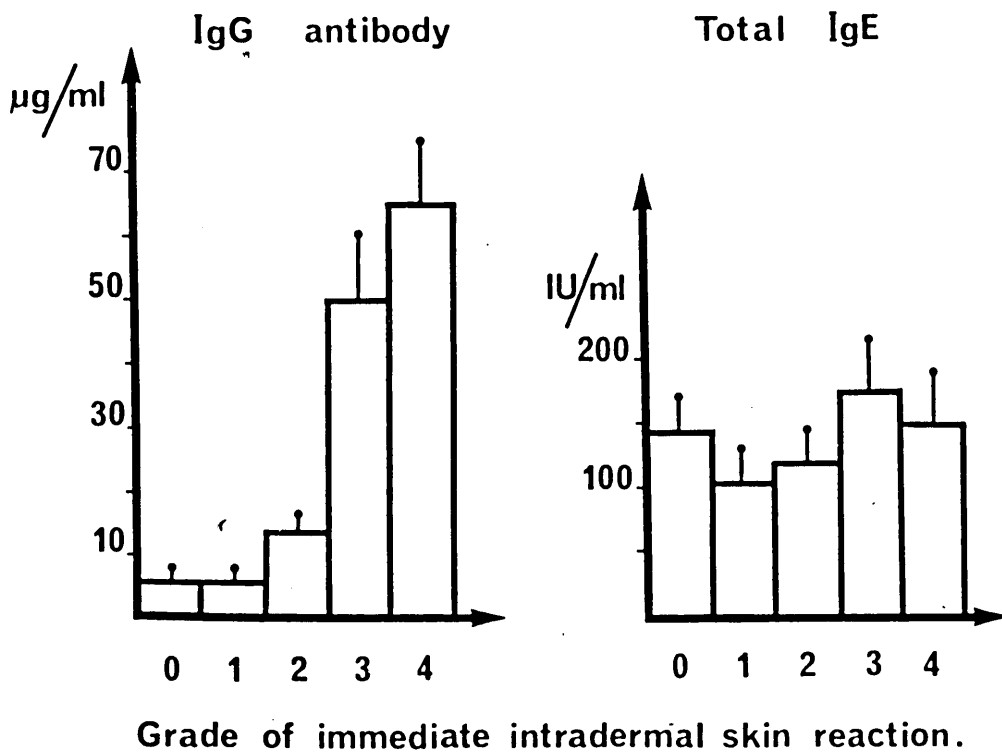


FIGURE 34:

The mean plus standard error of (a) serum IgG antibody levels specific for avian antigen, and (b) total IgE, within each increasing skin reaction grade against intradermally applied pigeon serum antigen.

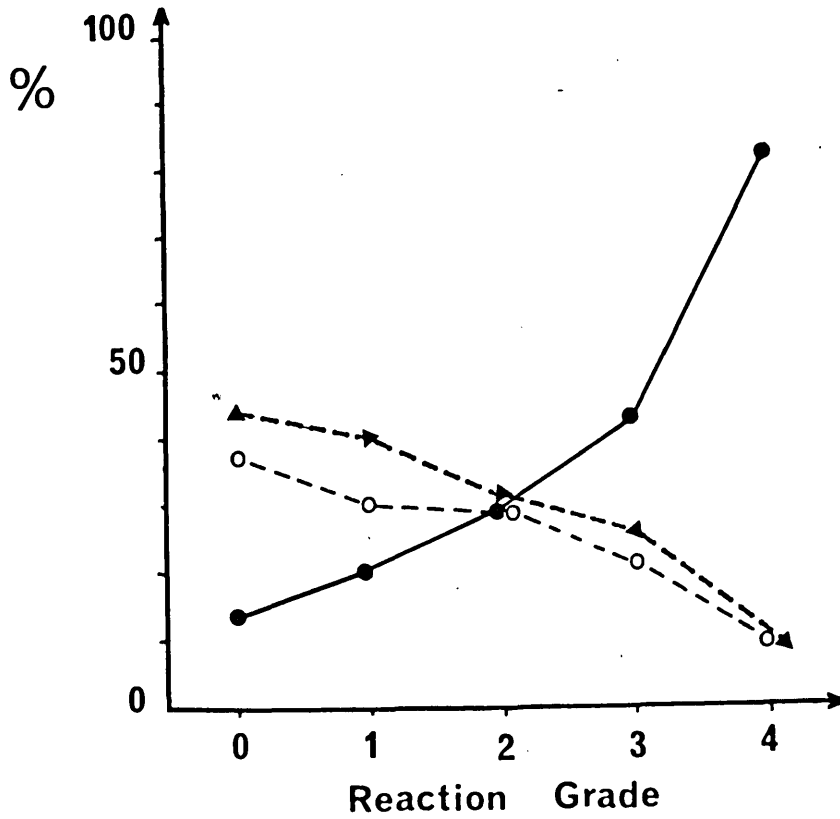


FIGURE 35:

The percentage of subjects in each of the immediate intradermal skin reaction grades with alveolitis (●---●), with indeterminate symptoms (▲---▲) and with no symptoms (○----○) related to avian exposure.

reported a late skin reaction, maximal between 4 to 8 hours. All of these subjects had had strong early reactions and could be considered as "dual" skin reactors. This late reaction was a feature of subjects with particularly high IgG antibody titres (mean = 66.1 ug/ml). One subject however had insignificant IgG antibody to avian antigens but described prominent avian related asthmatic symptoms, and 8 of the 9 remaining had symptoms of alveolitis.

f) Delayed, 48-72 hour tuberculin skin reactions.

The 102 subjects were skin tested with a tuberculin Tine test and the reactions were read after 72 hours by each subject, who indicated the size, if any, of the reaction on an illustrated returnable postcard. The results for the Tine test were graded 0 for no reaction, grades 1 and 2 for increasingly positive reactions before the 4 individual weals from the 4 small tuberculin - coated spikes coalesce, grade 3 when the weals coalesce and grade 4 for any larger reaction. Grades 3 and 4 were considered as positive reactions.

There was an 89% return of the score cards and the grades of the reactions were listed on Table 37. There was a markedly lower mean avian-antigen specific IgG antibody response in tuberculin positive subjects and this was associated with a lower incidence of alveolitis. However, due to reports of occasional false-negative tuberculin skin reactions using the Tine test, the association between antibody responsiveness, disease incidence and tuberculin sensitivity was followed up using a 1:1000 Mantoux test on a further 57 pigeon fanciers (Table 37). The reaction was positive after 72 hours if the reaction was 5mm or greater. The lower mean antibody levels in the tuberculin positive subjects was confirmed ($0.05 < p < 0.1$) and there was a significant correlation between alveolitis and cutaneous anergy to tuberculin (Chi-squared = 5.9, $p < 0.02$).

3. DISCUSSION

Positive skin tests to common allergens and raised IgE levels were no more prevalent among the 32 pigeon fanciers with extrinsic allergic alveolitis than the whole study group,

suggesting that the individuals' atopic status (Pepys, 1975) did not predetermine the development of alveolitis caused by avian exposure.

There was however a highly significant association between positive skin test responsiveness to common allergens and raised serum IgG antibody to inhaled avian antigens suggesting that there may be a common control mechanism. This could be T-cell mediated because skin test responsiveness is known to deteriorate with age (Tuft, Heck & Gregory, 1955) due to decline in T-helper cell function (Allison, Denman & Barnes, 1971). A further involvement in T-cell function was suggested by the higher IgG antibody titres against inhaled avian antigens in subjects with cutaneous anergy to tuberculin. An early report (Fuller, 1962), published when positive tuberculin responses were more prevalent in the general population, had suggested that extrinsic allergic alveolitis amongst farmers (farmers lung) would be unlikely if subjects had a positive mantoux. This view was confirmed by our results which significantly correlated EAA with cutaneous anergy to tuberculin. This would tend to support the view that both the humoral and cellular arms of the immune response are involved in the pathogenesis of EAA (Roberts & Moore, 1977).

An immediate skin prick test to pigeon serum in this study was a feature of atopic subjects with high IgG antibody titres, and serum IgE antibody to avian antigens measured by RAST. This is in agreement with Faux and co-workers (1971) who described IgE antibody and positive immediate prick tests in subjects who developed immediate asthmatic symptoms only, when challenged with avian antigens.

In the present study, however, there was little evidence of strong IgE responsiveness to avian antigens among all the pigeon fanciers, despite a high incidence of atopy and extensive avian contact (mean years exposure = 22 years). Other workers have been unable to detect specific IgE among pigeon fanciers (Patterson et al, 1976; Cohen, Yunginger & Fink, 1979) however this may be explained by the high titre serum IgG antibody blocking the RAST system. This potential for IgG to block the RAST test has been discussed by Aalberse et al, (1972), and has

been demonstrated in patients allergic to *Aspergillus* and budgerigar proteins by Pepys, Parish, Stenius-Aarniala and Wide (1979), who showed that short-term sensitizing antibody (IgG S-Ts) could interfere with the specific IgE activity by blocking RAST tests and passive cutaneous anaphylaxis (PCA) testing in baboons.

The late (4 to 8 hour), type III skin reaction against intracutaneously administered avian antigens has received considerable attention in the study of extrinsic allergic alveolitis because it has a similar time course to the acute pulmonary and systemic reactions which follow inhalation challenges with these same antigens (Hargreave & Pepys, 1972). These authors demonstrated that the majority of subjects in their study with late respiratory symptoms after an inhalation challenge with avian antigens had late skin test reactions (85%) and just over half had preceding immediate reactions (58%); whereas all those with dual, immediate and late, respiratory reactions had dual skin tests.

Of the ten subjects who reported a late skin reaction to intradermal pigeon serum antigen in the present study, eight had evidence of avian related extrinsic allergic alveolitis, and this skin reactivity appeared to be associated with a particularly high specific IgG titre to pigeon serum gamma-globulin antigen. One subject who had insignificant IgG against avian antigen but with a positive RAST had a similar late skin reaction. He described significant immediate and late asthmatic symptoms related to avian exposure, and it was possible that this late skin reaction was mediated therefore by IgE antibody, as has been described in other atopic conditions (Dolovitch et al, 1973).

A high percentage (70%) of the study population developed an immediate weal and flare reaction following intracutaneous administration of diluted pigeon serum. This skin reactivity was not associated with atopy as judged by total IgE levels, allergic history or skin reactivity to common allergens, but was quantitatively associated with the titre of IgG antibody to pigeon serum antigens. Anaphylactic IgG was first described by Parish (1970), and an IgG associated skin sensitizing factor has been reported in extrinsic allergic alveolitis among farmers (Freedman et al, 1981; Edwards & Davies, 1981). The present work with

subjects exposed to definable avian antigens which can be purified, and in whom the IgG antibody response can be quantified, offers an opportunity to isolate the class and subclass of this anaphylactic antibody activity (see Chapter 4).

A role for a possible IgG anaphylactic antibody in the pathogenesis of extrinsic allergic alveolitis should now be considered, perhaps in combination with the traditionally postulated immune complex aetiology (Pepys, 1969). Certainly, Fink and his colleagues (1968) found a clear difference in the PCA antibody titre between pigeon breeders with and without alveolitis, and Morell-Brotad and colleagues (1981) found that positive immediate intradermal skin tests using appropriate antigens were highly selective for farmers lung patients, more so than other diagnostic tests including lymphocyte transformation using the same antigens. Similarly, in the present study, strong positive immediate intradermal skin tests using pigeon serum as an antigen were highly selective for patients with alveolitis caused by avian exposure, and this simple and safe technique may prove a useful adjunct in the diagnosis and management of alveolitis.

CONCLUSIONS

1. Pigeon derived dusts which can be readily airborne consist of primarily the feather dust and the dust from desiccated droppings. The major protein component in soluble extracts from these dusts is also the major antigen and this is suggested to be the avian equivalent of secreted IgA. This has antigenic determinants in common with pigeon serum and antigenic identity between all these materials appears to reside in the gamma globulin light-chain.

Pigeon serum, therefore, contained the main antigen and was a readily available and relatively clean antigen source. This material was used successfully in serology, skin testing and inhalation provocation testing, and for future coordinated work on these aspects of alveolitis it would be useful to have general agreement to use pigeon serum as a standard antigen in standardised amounts for each procedure.

2. The particulate, chemical and aerodynamic properties of the pigeon feather dust makes this a possible vehicle for carrying the soluble antigens to the peripheral airways and to initiate and stimulate an immune and inflammatory response.

3. Serum IgG antibody to these avian antigens could be measured quantitatively by RIA and ELISA. However, a semi-quantitative estimate of antibody could be efficiently obtained by avian RBC agglutination and this rapid technique is probably the best compromise between simplicity and sensitivity for use in a routine laboratory.

4. The distribution of serum IgG antibody levels against avian antigens in a population of pigeon breeders has demonstrated a spectrum of responsiveness, with approximately half of the subjects having no serum antibody despite extensive avian exposure. The mechanisms of this non-responsiveness, whether it is an active tolerance or a progressive desensitisation, would be of interest to clarify some of the normal immune functions of the lung following repeated antigen exposure, and also because these subjects tend not to have alveolitis.

5. The presence of serum antibody in a patient confirmed avian exposure, but when this antibody was quantified there was an association between higher levels of antibody and an increasing incidence and severity of symptoms. Serial measurements of antibody in individuals was of value in monitoring therapeutic progress, and persistently low values thereafter confirmed that the patient has avoided further avian contact.

6. The subjects with serum antibody also had increased serum levels of some immunoglobulins, some acute phase proteins, and immune complexes, along with lower levels of some complement components. These abnormalities suggest an active inflammatory response. The levels of some of these non-specific parameters were raised in tandem with the specific antibody level, which suggests that the inflammatory response was a consequence of the continuous antigen exposure to which these individuals submit themselves. Furthermore, these antibody positive subjects should be considered as an at-risk population and should be carefully monitored for the development of subclinical, insidious disease.

7. Subclinical physiological changes were observed in sensitized individuals following exposure to antigens. Moreover, when exposure was extensive enough to provoke mild symptoms, there were marked changes in leucocytosis and pyrexia which preceded the onset of symptoms. There were also some fluctuations in immunological parameters associated with these changes in some individuals during the 8 hours following exposure. These included the formation of immune complexes and activation of complement. There was also an increase in total serum IgG and alpha 1 - antitrypsin. The same parameters returned to normal during convalescence in individuals with alveolitis who avoided further avian contact. All these changes, which occurred in tandem, suggested that the humoral immune system was involved in the active disease process.

8. Various genetic and environmental factors were examined to see which would delineate individuals who were predisposed to developing alveolitis, and also to being antibody responders.

The atopic status, as measured by total serum IgG and IgE

antibody to avian antigens, personal and family allergic history and skin prick testing with common allergens and avian antigens, was not associated with disease. The incidence of rheumatoid factor (as one index of autoimmunity) was not raised in alveolitis, and the HLA and P1 phenotypes appeared to have no bearing on disease. A further constitutional factor of age also appeared to have no bearing on the ability to mount an antibody response nor the development of disease.

The genetic factors which do predispose to antibody responsiveness and to disease susceptibility are still obscure.

Some environmental factors on the other hand have a profound effect on the antibody response. Cigarette smoking appears to inhibit the ability to mount an antibody response to the inhaled antigens, and also the disease was less common among smokers. The mechanism for this inhibition is not understood, but it is thought to be part of a general depression of the immune response which is more evident at the pulmonary level. This inhibitory effect is reversed in some ex-smokers. The extent of avian exposure also affects the antibody response. In general, the greater the exposure (in terms of hours per day, number of birds and years of keeping pigeons) then the greater the incidence of antibody responders. However, paradoxically, these antibody positive subjects had progressive lower mean titres of antibody with increasing exposure. The explanation for this is not clear, there may be a desensitization procedure allied to increasing exposure, or there may be some self-regulation of exposure in the symptomatic (high antibody) subjects, which results in decreased exposure, for example by wearing a mask or reducing the hours per day in avian contact. Some follow-up studies in individuals identified in this thesis will clarify these details.

9. The mechanism for disease proposed and supported by data in this thesis involves humoral immunity. However, this position was taken up at the outset in order to re-evaluate the position of humoral immunity in the disease using more sensitive methods, and stricter clinical selection of subjects than in previous studies. The data collected here should form the basis from which further studies can continue. For example, it is highly probable that cell-mediated immunity is also involved in the disease process,

and work on this particular arm of the immune response would benefit from similar quantitative, antigen-specific analysis.

The antibody profile of individuals could then be compared with their cellular profile at different phases of exposure, and during different stages of disease. Of particular interest would be the elucidation of cellular events during an inhalation provocation test, in association with the other measurable physiological and seriological parameters.

10. In conclusion, it has been demonstrated that the quantitative antibody measurement is of value in the investigation and clinical management of patients with extrinsic allergic alveolitis. One further useful clinical adjunct was intradermal skin testing with antigen, where an immediate weal and flare reaction was indicative of antibody, and strong weal reactions were selective for alveolitis patients.

The clinical relevance of antigen inhalation provocation testing on the other hand was debatable. However, the serological fluctuations associated with a positive acute reaction, and the therapeutic effects of drugs, along with the mechanism of the anaphylactic IgG antibody activity demonstrated on skin testing should be of interest in the investigation of the disease mechanism and the general antigen-handling and immunoresponsiveness of the lung.

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APPENDIX A.1. STUDY GROUPS.

The subjects on whom the work of this thesis was based were predominantly active pigeon breeders. They were contacted during several large-scale surveys at conventions in Central Scotland and Blackpool, by referrals from chest physicians throughout the UK and by visiting various local pigeon racing clubs.

The surveys undertaken in Scotland provided information on 277 individuals (1978/1979 survey) and 102 individuals (1979/1980 survey). All the subjects completed a questionnaire (Table 38) and were interviewed by chest physicians having some experience with alveolitis. A 10ml blood sample was taken, and in the latter survey a full range of skin tests was performed using common allergens and avian-specific antigens. The information obtained formed the basis for Chapter 6 which discussed skin testing and alveolitis. The serological investigation of these survey subjects for whom there was also data concerning symptoms, avian exposure, smoking history and other parameters listed on Table 38, formed the basis for Chapters 3 and 4 of this thesis.

Twenty individuals from a local club at Larbert kindly agreed to donate monthly blood samples for one year and this provided the information for Chapter 5, section 4a. The challenge subjects on whom the rest of Chapter 5 is based were all subjects from Central Scotland who were well known to us and who gave informed consent for investigation. The three patients who were followed during convalescence (Chapter 5, Section 4) were all under the care of Dr. Boyd at Belvidere Hospital, Glasgow.

Sera were also obtained from 30 subjects belonging to a local club at Kirkintilloch, and a further 30 were obtained from subjects attending a convention at Blackpool. These sera were used to measure resting serum complement levels in pigeon breeders and were handled accordingly (Chapter 4, Section 6). These subjects were also tuberculin (Mantoux) skin tested to complement the data in Chapter 6.

A further 98 subjects were referrals from chest physicians over a 5 year period.

2. QUESTIONNAIRE.

This was based on the MRC questionnaire for chronic bronchitis, and was expanded to include details of avian exposure, avian specific respiratory symptoms, general respiratory symptoms and details of occupation, smoking and allergies. The questionnaire was completed under medical supervision for accuracy (Table 38).

3. CLINICAL ASPECTS OF EXTRINSIC ALLERGIC ALVEOLITIS.

The symptoms associated with acute extrinsic allergic alveolitis described in Chapter 1 and listed in questions 8 and 13 of the questionnaire (Table 38) were individually non-specific. For this reason it may be difficult for subjects with minimal avian contact to recognise symptoms. They may put such an episode down to "a touch of flu", or they may imagine that progressive dyspnoea was due to their age. These reasons are thought to explain the insidious nature of chronic alveolitis, especially among subjects exposed to budgerigars, where continuous exposure to just one bird is common. Among pigeon fanciers it is common to spend a few hours per day in contact with copious amounts of antigenic dust during handling large numbers of pigeons and daily cleaning out the loft. This heavier periodic exposure results in more prominent symptoms in sensitized subjects and acute episodes could be recognised.

The assessment of pigeon breeders disease based on the questionnaire and personal interview required the presence of at least one respiratory and one systemic symptom occurring together 4 - 6 hours after avian exposure on at least three occasions. Subjects were considered to have prominent asthmatic symptoms if they had the regular occurrence of at least 3 immediate symptoms on contact with birds. Subjects who described no symptoms formed a clear group, whereas a number with vague symptom pattern: predominantly hay fever were considered as a separate indeterminate group (see Table 39).

TABLE 38. QUESTIONNAIRE:

Pigeon Exposure Data:

1. Do you keep pigeons at present?
If NOT when did you stop and why?
2. How long have you kept pigeons? ____ Years ____ Months
3. Have you kept pigeons continuously or periodically?
4. How many pigeons do you generally keep?
5. Have you ever been in regular contact with other birds?
6. How many hours, on average, do you spend in contact with the pigeons each week during the season?
7. Do you ever wear a mask when in contact with pigeons?

Symptom Data:

8. When in contact with your pigeons have you EVER noticed:
 - a. shortness of breath
 - b. wheezing in your chest
 - c. a dry cough
 - d. sneezing, blocked or running nose
 - e. itchy/running eyes
 - f. tightness in chest
9. Have the above symptoms EVER been severe enough to make you come away from the pigeons?
10. How long after coming into contact with pigeons do these symptoms occur?

less than ten minutes; ten to thirty minutes; more than thirty minutes.
11. Do these symptoms occur:

Occasionally (once/3mths); sometimes (once/2wks); usually (at least twice/wk).
12. How long have you had them?

13. Several hours (4 to 8) AFTER being in contact with pigeons have you EVER experienced attacks of, or persistent:
- shortness of breath
 - wheeze
 - shivering and/or feeling cold
 - sweating
 - fever or temperature
 - dry cough
 - tiredness or aching muscles
 - tightness in chest
14. How long is it before you feel well again?
15. Have you ever noticed "flu-like" feelings in the evening and at night which ve gone by the following morning?
16. How often do these symptoms occur?
- Occasionally (once/3mth); sometimes (once/2wk); usually (at least twice/wk)
17. How long ave you had them?
18. Are these symptoms worse, better or the same -
- at the weekend
 - after attending a pigeon show
 - after a hóliday away from home
 - after cleaning out the loft

Respiratory Data:

19. Do you USUALLY cough first thing in the morning in winter?
20. Do you USUALLY cough during the day in the winter?
21. Do you cough like this on most days for as much as three months each year?
22. Do you USUALLY bring up any spit or phlegm from your chest first thing in the morning in the winter?
23. Do you USUALLY bring up any spit or phlegm from your chest during the day in the winter?
24. Do you bring up spit like this on most days for as much as three months each year?
25. Do you ever get short of breath when walking with others of your own age, on level ground?

26. During the past three years hve you ever had any chest illnesses have kept you from your usual activities for more than a week?
27. Have you ever had -
 - a. heart trouble
 - b. bronchitis
 - c. pneumonia/pleurisy
 - d. tuberculosis
 - e. asthma
 - f. other chest illnesses
28. Have you ever had any illness requiring a period in hospital?
29. Do you require regular medicines for any condition?
30. Have you ever worked in:
 - a. a coal mine
 - b. any other mine
 - c. a quarry
 - d. a foundry
 - e. a pottery
 - f. a cotton, flax or hemp mill
 - g. with asbestos
31. Do you smoke?
32. How many cigarettes/cigars do you smoke per day?
33. How long have you smoked?
34. If you have ever smoked:
 - a. How long did you smoke for?
 - b. How many did you smoke per day?
 - c. When did you stop?

Allergies:

35. Have you ever suffered from:
 - a. Hay fever
 - b. Asthma
 - c. Eczema/Dermatitis
 - d. Were you "chesty" as a child?
36. What do you suspect causes the/these condition/s in your case?
37. Does any relative have any of the conditions listed above?

	ALL n=102	SYMPTOM GROUP			
		ALVEOLITIS 33	INDETERMINATE 32	ASTHMA 8	NIL 29
<u>Immediate Symptoms:</u>					
Dyspnoea	27	17(52%)	4(12)	6(75)	0
Wheeze	21	13(39)	3(9)	5(62)	0
Dry cough	27	16(48)	7(22)	4(50)	0
Rhinitis	26	10(30)	11(34)	5(62)	0
Conjunctivitis	17	4(12)	9(28)	4(50)	0
Chest tightness	23	13(39)	4(12)	6(75)	0
<u>Late Symptoms:</u>					
Dyspnoea	20	16(48)	2(6)	2(25)	0
Wheeze	8	6(18)	1(3)	1(12)	0
Shivering/Cold	17	16(48)	0(0)	1(12)	0
Sweating	21	19(58)	1(3)	1(12)	0
Fever/Temperature	14	14(42)	0(0)	0(0)	0
Dry cough	24	18(55)	5(16)	1(12)	0
Malaise	25	21(64)	2(6)	1(12)	0
Chest tightness	17	11(38)	4(12)	2(25)	0
'Flu-like'	36	24(73)	9(28)	3(37)	0

TABLE 39. The positive response rate to questions concerning immediate and late symptoms specifically related to avian exposure in 102 pigeon fanciers. The symptomatic groupings are described in the text and the percentages are in parenthesis.

These symptoms are most readily demonstrated by a controlled bronchial provocation test by inhalation of the appropriate antigen. This is often performed to confirm a diagnosis of allergic alveolitis, and is sometimes even necessary in order to convince some pigeon fanciers that the birds are indeed the cause of their disability.

The results of inhalation challenges in alveolitis have been the subject of several reports (see Appendix C), and the most common symptomatic patterns from these reports were general malaise and chest tightness (Harries et al, 1980); malaise, fever with widespread aches and pains (Hendrick et al, 1980) which was summarised in most subjects by describing a 'flu-like illness' in the evening which had resolved by the next day.

These are in general agreement with symptoms described by subjects during positive inhalation challenge tests (see Chapter 5), and also with the symptom score from survey subjects (Table 39).

APPENDIX B.SEROLOGICAL MEASUREMENTS1. RADIOIMMUNOASSAY OF ANTIBODY.

The potential use of a method for accurately measuring IgG antibody in extrinsic allergic alveolitis patients was recognised by Nielsen and co-workers (1974), and this group developed a quantitative radioimmunoassay, analogous to the RAST test, where the soluble antigens relevant to the disease syndrome under investigation were bound to a solid phase. The theory and many practical aspects of this assay are discussed at length in a recent publication (Parratt, McKenzie, Nielsen & Cobb, 1982). Details not covered, however, included the preparation of the antigen and quality control of the assay.

The specific antigen in pigeon breeders disease is the serum gamma-globulin (Faux, Hendrick & Anand, 1978). This was obtained from fresh pigeon blood, following cardiac puncture of ether anaesthetised birds, by ammonium sulphate precipitation of the serum globulin (Hebert, 1973) and column chromatography of the reconstituted globulin through Sephadex G200. The second and largest of the four protein peak elution profile contained the specific antigen (Figure 1). This antigen was then bound to the surface of cyanogen bromide activated Sepharose 4B according to the manufacturer's instructions (Pharmacia UK Ltd), thus providing a solid phase antigen for the assay.

Duplicate amounts of this antigen were incubated in excess amounts, previously established by chequerboard titration with aliquots of patient's serum. After at least 30 minutes the Sepharose antigen-bound antibody complex was washed by 3 cycles of resuspension in phosphate buffered saline, 0.01M, pH 7.2 (PBS) and centrifugation at 2000rpm for 5 min, after each wash the supernatant was aspirated. After the 3rd centrifugation step the Sepharose antigen-antibody pellet was resuspended in a solution of radiolabelled anti-human IgG in excess, previously determined by chequerboard titration.

The radiolabelled anti-human IgG was prepared from sheep anti-human IgG, gamma-chain specific, obtained from the Scottish Antibody Production Unit (SAPU, Law Hospital Carlisle). The

globulin fraction from this antiserum was radiolabelled with Iodine-125 by the method of McConachey and Dixon (1966). The average efficiency of radiolabelling in eight consecutive experiments in this laboratory was 58%. Following a further 30 minutes incubation, the washing procedure was repeated, and the bound radio-activity was measured on a gamma-counter.

The quality control of the assay was observed in the first instance by estimating the upper limit of normal of sera from subjects with no avian exposure, usually 3 or 4 sera from control negative subjects were included in each assay. When the assay worked optimally these values were always below 4 ug/ml. A high titre serum which had been previously measured by quantitative precipitation against pigeon gamma-globulin antigen was diluted serially with normal human serum to generate a standard curve of counts obtained from serial antibody levels, previous work having shown a linear relationship between the bound radioactivity and antibody content. The counts obtained from the unknown sera tested could then be interpolated on to the standard curve and therefore quantified.

The precision of the assay was measured at the most sensitive part of the standard curve; the 50% point. The measurement of 100 replicate samples generated a standard deviation of 4.8%.

The between-assay variability and the accuracy of each measurement was controlled by incorporating negative, low, medium and high standard sera of known value. These samples when measured can form a second internal standard curve by which the values of replicate samples in different assays could be regularised. This was done using a simple mathematical formula:-

$$\text{True Value} = \frac{\text{Measured Value} - \text{Intercept}}{\text{Slope}}$$

Using this, the between assay variation was always less than 10%.

2. ENZYME LINKED IMMUNOASSAY (ELISA) OF ANTIBODY

The indirect microplate method of Voller, Bidwell & Bartlett (1976) was employed using polystyrene EIA microtitration

plates (Flow Laboratories, Irvine) as a carrier surface for antigen. Optimal proportions were established by checkerboard titration; for the binding of the pigeon gamma-globulin antigen on to the plate, this was 60 ug/ml. All the test sera were diluted 1 in 50 with PBS, and the alkaline phosphatase conjugated anti-human IgG was used at 1 in 1000 dilution (Miles Laboratories, Batch Number S725).

The assay procedure involved coating the wells of the plate with antigen diluted in carbonate buffer 0.1M, pH 9.6, at 150 ul per well, overnight at 4°C. The plate was washed three times by immersion in wash buffer PBS 0.1M, pH 7.2 containing 0.5% Tween-20. The test sera were added in duplicate at 100 ul per well and incubated for 3 hours at room temperature. The plates were washed three times as before and 100 ul of enzyme conjugate was added to each well and incubated at room temperature for a further three hours. After a further three washes, a substrate solution of p-nitrophenyl phosphate (104 phosphatase substrate - Sigma) at 1 mg/ml in 10% diethanolamine, pH 9.8 was added at 100 ul per well. After approximately 30 minutes, when sufficient colour had developed in positive control samples, the reaction was halted by adding 50 ul of 3M sodium hydroxide. The absorbance of each well at 405 nm was read using a spectrophotometer (Multiskan, Flow Laboratories).

The results of a comparison between this ELISA method and the standard RIA for 102 samples are shown on Figure 36. The results correlated highly significantly ($r=0.93$).

This assay procedure was used for other antigen preparations, e.g. pigeon IgA at 50 ug/ml and feather extract (bloom) at 100 ug/ml, and for other antibody classes eg, anti-human IgA (batch S873) and anti-human IgM (batch S874) both from Miles-Yeda, each used at 1 in 200 dilution.

An amplified ELISA system was used to measure the IgG subclass activity against avian antigens. This method followed the standard ELISA procedure except that antisera against the subclasses of IgG were used after the incubation with patients' serum. Anti-human IgG subclass 1-4 (M1175) were obtained from the Dutch Red Cross, and from Seward Laboratories Ltd. Both polyclonal (BA235 - BA238) and monoclonal (BAM 8-11) were

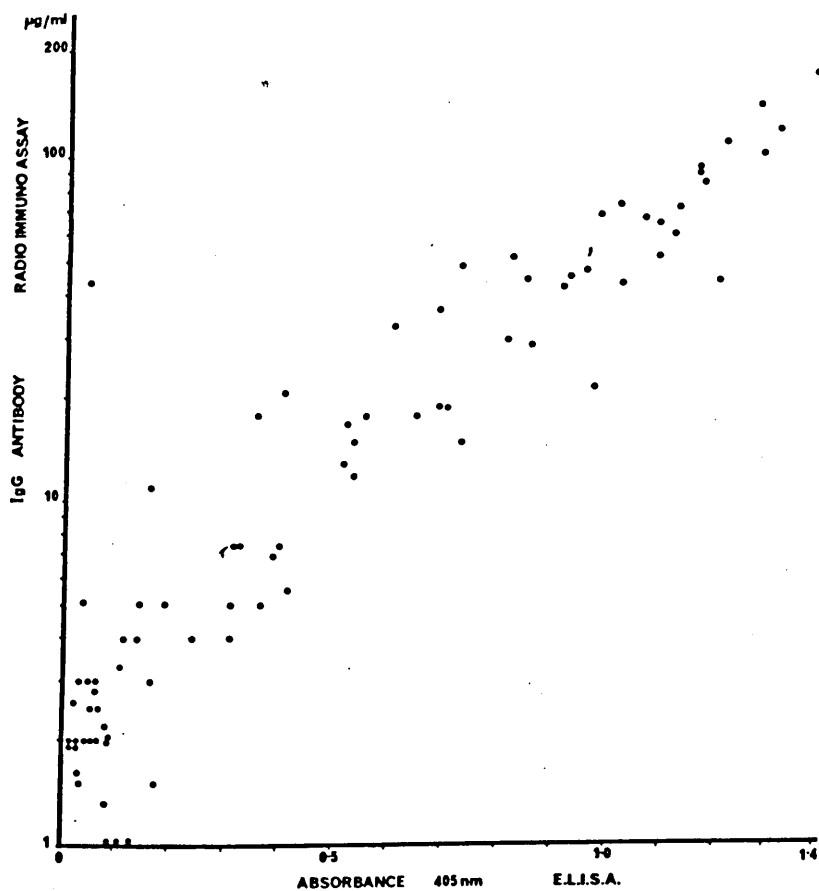


FIGURE 36. Comparison of individual values of serum IgG antibody against avian antigens in 101 pigeon breeders measured by radio-immunoassay and enzyme-linked immunosorbent assay (ELISA). The correlation coefficient was $r = 0.93$.

obtained. The polyclonal sera were raised in sheep and, therefore, the next incubation step in these cases was with alkaline phosphatase labelled anti-sheep IgG (Miles-Yeda, S836). The monoclonal sera were from mouse ascitic fluid, therefore, the next step was an overlay with alkaline phosphatase labelled anti-mouse IgG (Miles-Yeda, S148). The washing procedure, and application of substrate were as before.

3. PRECIPITIN TESTS FOR ANTIBODY.

(i) Double diffusion in agar.

Precipitating antibody against pigeon serum (N and N/10 dilution) in the sera of 102 pigeon breeders was measured using Onchterlony double diffusion in agar. The results plotted on Figure 37, show an overlap of antibody values between the precipitin positive and negative, although in general this technique could detect antibody greater than 40 ug/ul which is in general agreement with other workers (Parratt, 1975; Gill, 1970).

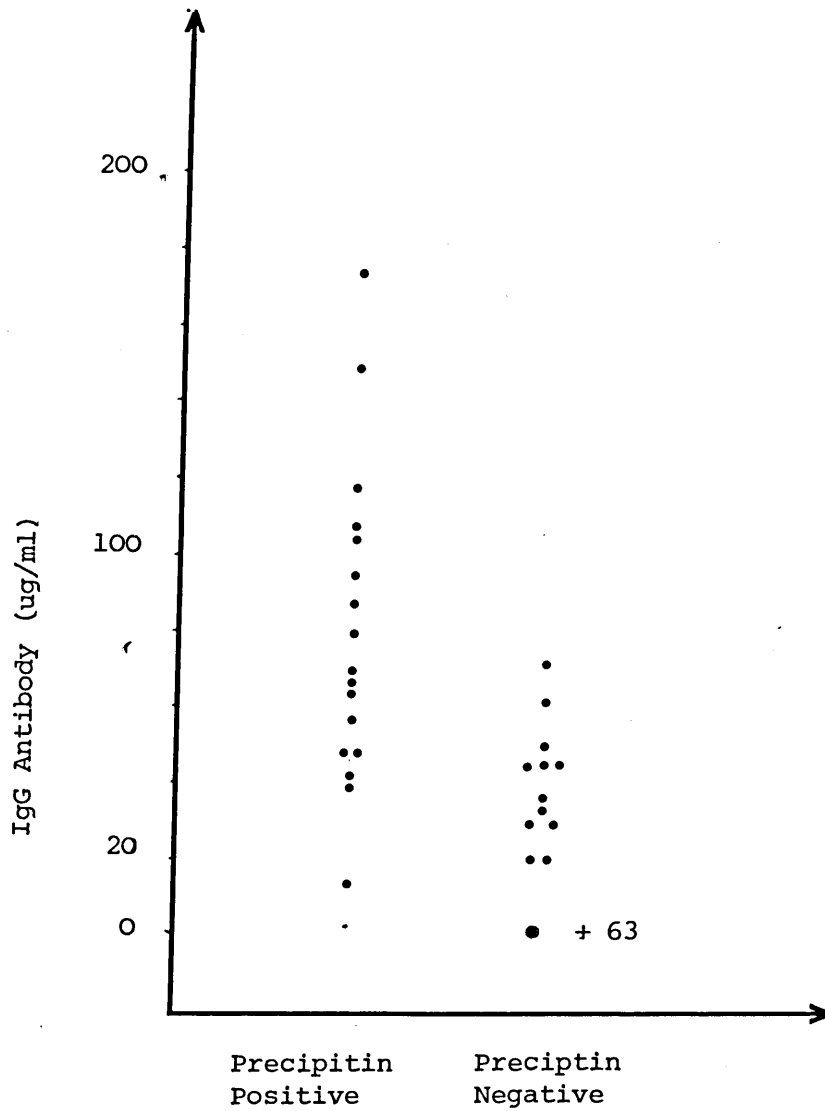
(ii) Counter current immuno-electrophoresis. CCIEP.

To demonstrate precipitating antibody against pigeon feather (bloom) extract antigens, which have been shown to be negatively charged at pH 8.6, the sera of 97 pigeon breeders were tested by CCIEP on an 8 x 8 cm agar slab at 10 v/cm for 2 hours against this antigenic extract and the number of precipitin bands which resolved was counted. This number was correlated with the IgG antibody titre as measured by RIA (Figure 8).

4. HAEMAGGLUTINATION.

The pigeon red blood cells are thought to have surface bound immuno-globulin light chain which is a major antigenic fragment in PBD, and since these cells are nucleated and settle quickly they are very convenient for measuring agglutinating antibody directly (mainly IgM class antibody) or following reduction by dithiothreitol treatment (Olson, Weiblen, O'Leary, Moscovitz & McCulloch, 1976) to measure IgG class antibody. The latter agglutinating titres were compared with the specific IgG antibody levels as measured by RIA in 100 subjects (Figure 9).

FIGURE 37: The serum IgG antibody levels against pigeon gamma globulin antigen in 102 pigeon breeders with and without precipitins against pigeon serum.



5. STAPHYLOCOCCAL PROTEIN A BINDING ASSAY FOR IMMUNE COMPLEXES

The cell wall of Staphylococcus aureus contains a protein (Protein-A, M. Wt = 42,000 D) which binds non-specifically to the Fc portion of human IgG (Forsgren & Sjoquist, 1966), except IgG3, and several other mammalian species. Taking advantage of the strong affinity of this binding, protein-A bearing staphylococci have been used as solid phase immunabsorbents for localisation of IgG-antigen complexes (Brunda et al, 1977) and radiolabelled protein A conjugates have been used to measure antibody (Marier, Jansen & Andriole, 1979) and immune complexes (Stevens & Bridts, 1981; Langone, 1982). The method used in the present study utilised the immunabsorbent properties of a protein A rich staphylococcal immunabsorbent and radiolabelled protein A to detect circulating IgG immune complexes in the serum of subjects with extrinsic allergic alveolitis. The method used was that of Barkas (1981) without any modifications.

Staphylococcus aureus, Cowan strain 1, NTCC 8530, head-killed and formalin-treated, was obtained from the Microbiological Research Establishment, Porton Down, and used as a 1% (v/v) suspension in PBS containing 0.5% sodium azide pH 7.2.

Protein A was obtained from Sigma and iodinated by the method of McConachy & Dixon (1966) to a specific activity of between 9 and 13 mCi/mg in different experiments. 50 ug (50 ul) of protein A was mixed with 1 mCi (10 ul) of I-125 and 4 ug (5 ul) chloramine T at room temperature for 1 min. 5 ug (5 ul) of sodium metabisulphite was added. All reagents were dissolved in PBS. 500 ul of PBS containing 1% BSA was added and the mixture fractionated on a 20 x 1cm column of Sephadex G25 equilibrated with PBS/BSA. The eluted protein was stored at 4°C. The binding efficiency was generally greater than 80%. Test sera were prepared by adding 100 ul to an LP3 tube containing 8 ul of 500mM EDTA pH 7.5, followed by 300 ul PBS, pH 7.2. 2 ml of 6% (W/V) polyethylene glycol 6,000, PEG (BDH) in 0.1M boric acid, 0.025M sodium tetraborate, 0.075M sodium chloride pH 8.3, was added and the samples incubated at 4°C overnight. The tubes were then centrifuged at 1000g for 20 minutes at room temperature. The supernatants were aspirated and the pellets were washed with

1ml of 5% PEG prepared by diluting 5 parts of the 6% solution above with 1 part of distilled water. The final pellets were re-dissolved in 400 ul PBS at 100 temperature for 15 min.

Duplicate 100 ul samples were incubated with 100 ul of the 1% S. aureus suspension at 37°C for 1 hour. 500 ul of PBS + 1% BSA was added to each pellet followed by 50 ul of radiolabelled protein A; between 14 and 20 ng in different experiments, the pellets were re-suspended and incubated at 4°C for 30 min. 500 ul of PBS + 1% BSA was added and the pellets washed as before twice. Precipitated radiolabel was measured on an EMI Nuclear Enterprises Gamma Counter NE1612. The results were calculated as the percentage of the counts bound over the counts added.

To assess the validity of the assay, IgG was purified from normal human serum by salt fractionation and column chromatography. The IgG was adjusted to 20 mg/ml and heated at 63°C for 30 min to induce aggregation, (Soltis et al, 1979). The high molecular weight aggregates were separated from the monomeric IgG by column chromatography using ACA-22. The elution fractions were assayed for IgG by protein A binding (x---x) and for IgG aggregates by the above method (o---o) (Figure 38). This demonstrated that monomeric IgG did not interfere with the assay.

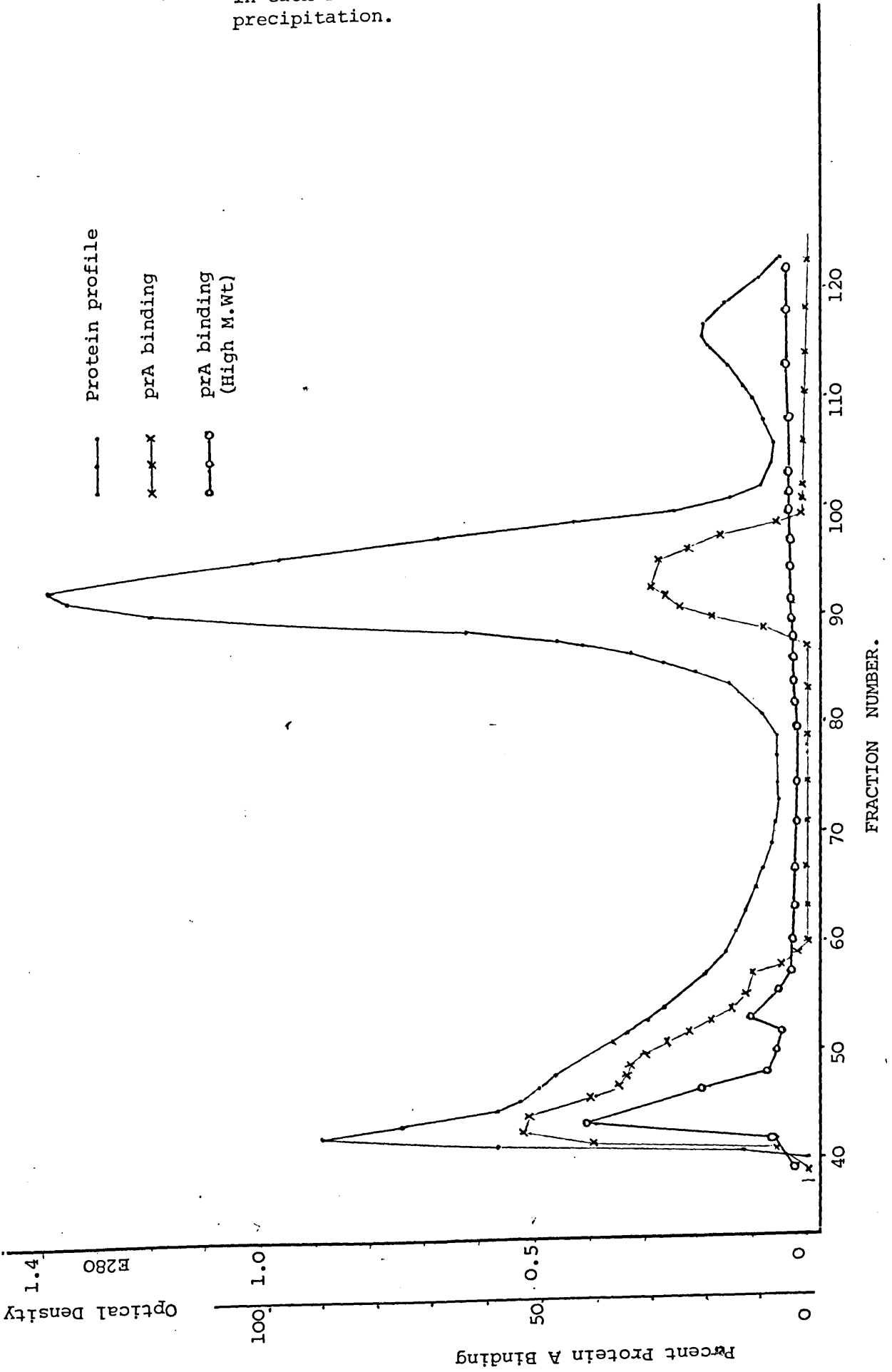
For quality control of the assay, various control positive and negative sera were used for calibration (Table 20).

6. Clq BINDING ASSAY FOR IMMUNE COMPLEXES.

This assay method depended on the binding of human Clq onto the surface of plastic tubes, and the subsequent binding of immune complexes via the Fc region which has affinity for the solid phase Clq. The method followed was based on that of Hay, Nineham & Roitt (1976) using human Clq purified from serum by the method of Yonemasu & Stroud (1971). One ml volumes of Clq solution 10 mg/l in PBS were incubated in polystyrene tubes (LP3, Luckham Ltd., Sussex) for between 1 and 3 days at 4°C. After three washes with PBS the tubes were filled with 2ml of 0.01% gelatine solution in PBS and incubated at room temperature for 2 hours. After three more washes with PBS the tubes were ready for use or could be stored frozen with the last wash buffer.

Test sera were prepared by mixing 50 ul with 100 ul EDTA

FIGURE 38. Elution profile of aggregated and monomeric IgG through ACA-22. The protein A binding activity in each fraction with and without previous PEG precipitation.



(0.2M, pH 7.5) and incubating for 30 min at 37°C, after which the sera are kept on ice. A duplicate 50 ul samples were aliquoted into the Clq - tubes along with 950 ul PBS + 0.05% V/V Tween 20. The tubes were incubated at 37°C for one hour and a further 30 min at 4°C. After three washes with cold PBS the tubes were incubated with 1 ug anti-IgG, iodine-125 labelled, in one ml PBS-Tween for 1 hour at 37°C and 30 min at 4°C. The tubes were counted empty after three washes with PBS.

7. RADIAL IMMUNODIFFUSION FOR PROTEIN MEASUREMENT.

Serum proteins including alpha 1-antitrypsin, IgG, IgG subclasses 1-4, IgM, IgA, Clq, C4, C3, Factor B, C-reactive protein were measured by radialimmunodiffusion (Mancini, Carbonara & Heremans, 1965) using commercially available plates (Seward Laboratories Ltd) or by incorporating appropriate antisera into gel. This latter method was used for measuring C3d, using antisera supplied by the Dutch Red Cross, and C3d standards supplied by Prof. K. Whaley, Pathology Dept., University of Glasgow. Serum Clq was measured similarly, using commercial anti-Clq and pooled normal human serum as a standard for Clq, namely 100%.

The total IgG subclass levels were measured similarly using the antisera described in section 2. Subclass standards were supplied by Seward Laboratories (BR99). The precipitin rings were often too faint to be read therefore the gel was washed in saline for 48 hours and immersed in a dilution of anti-sheep (or anti-mouse if appropriate) antiserum usually at 1 in 10 in PBS (both antisera supplied by SAPU, Law Hospital, Carlisle). Following 24 hours incubation, the gels were again washed for 48 hours with saline, dried and stained for protein. The diameter of the precipitin rings could then be read.

If necessary, a similar enhancing procedure was applied to the measurement of C3d.

8. LATEX AGGLUTINATION.

Human serum rheumatoid factor was measured by the agglutination of latex particles coated with gamma-globulin (Ortho R.A. Test). This system is calibrated using WHO rheumatoid

arthritis reference sera.

9. TOTAL AND SPECIFIC IgE MEASUREMENT.

Total and specific IgE was measured by the Phadebas PRIST and RAST methods respectively (PHARMACIA, U.K. Ltd), according to the manufacturers instructions.

APPENDIX CInhalation Provocation Tests in Extrinsic Allergic Alveolitis

The demonstration of a positive reaction to antigen challenge by exposure to the dusts normally encountered at the workplace, or at home, or by inhalation of a nebulised solution of antigen in a controlled environment is of value in establishing a causal role for these antigens in disease.

These studies were predominantly undertaken to clarify obscure causes of respiratory and systemic problems and also as an experimental model for following the pathology of the disease during the acute phase. The main literature reports on inhalation provocation testing with reference to alveolitis are listed below.

Table 40 Inhalation Provocation Testing: Literature.

1. Reference: Williams (1963)

Subjects : 14 patients with farmers lung, 44 controls.

Antigens : 1% w/v saline extract of mouldy hay nebulised for 6 mins.

Results

One immediate reaction, 9 developed symptoms of malaise and fatigue, feeling hot and shivery, gradually between 3 and 8.5 hours, lasting from 4 to 24 hours, 6 developed pyrexia 5 to 6 hours post-challenge and in 3 it was 10 to 13 hours. The fever subsided gradually over 3 to 4 hours. The pulse and respiratory rates rose with the fever. ECG showed a sinus tachycardia. Basal crepitations heard in 6 about the same time as the fever and persisted for up to 24 hours. Rhonchi were not heard. Leucocytosis occurred in 6 with no increase in eosinophils. No radiological changes were seen.

The mean FEV₁ decrease at the height of the reaction was 19%, (the inhalation of 1% isoprenaline had little effect on this), the mean FVC decrease by -19%, the MVV by -27%, the PEF_R by -21%, the static compliance by -46%, the D_{Lco} by -31%. The FEV₁ - FVC ratio did not change and the mean MV increased by 42%.

Challenges with equivalent amounts of 12 common allergen extracts were negative, as were all control challenges.

2. Reference: Reed et al (1965)

Subjects : 3 pigeon breeders, 3 control subjects.

Antigens : Dust from pigeon feathers.

100 mg pigeon droppings extract aerosolised.

Sterile pigeon serum, 3 ml.

Results

Feather dust provoked a dramatic febrile episode with rales and a decreased VC. Two controls showed no effects. Pigeon droppings extract provoked a fever of 39.2°C and a decrease of 24% in diffusing capacity. Three controls had a pyrexia up to 38.2°C with no fall in diffusing capacity. Pigeon serum provoked a pyrexia of 39.2°C, malaise and cough. The diffusing capacity fell by 14%. Exertional dyspnoea persisted for several weeks. Control subjects developed temperatures up to 37.5°C, no symptoms and a 4% drop in diffusing capacity.

3. Reference: Hargreaves et al (1966)

Subjects : 10 bird breeders, 7 controls

Antigens : Avian serum at 1 in 100, and 1 in 10 if necessary, then droppings extract at 10 mg/ml.

Results

The most consistent reaction was a rise in temperature at about 7 hours, accompanied in 5 subjects by malaise and shivering, with a leucocytosis at 8 hours, which returned to normal at 24 hours in 8 cases and 48 hours in 2. Eosinophilia occurred in 4 subjects after 24 hours, the highest occurring in one asthmatic subject. Basal crepitations were heard in the 3 most sensitive subjects who developed sharp systemic reactions to relatively low doses (1.0 - 2.5 ml of 1 in 100 pigeon serum).

One patient who had had typical attacks of alveolitis until he was desensitised did not react to 1.75 ml of pigeon serum at 1 in 10.

Two subjects had falls in FVC below 85% of pre-test values at 8 - 10 hours which was not reversed by isoprenaline. There was a slight fall in TLCO between 8 and 48 hours in 7 of 9 reactors.

Control subject did not react to similar challenges.

4. Reference: Pepys et al (1968)

Subjects : 3 bird breeders, 2 allergic aspergillosis

Antigens : 1% w/v Aspergillus fumigatus solution, avian serum

Results

The early and late reactions associated with inhalation of Aspergillus fumigatus and the late reactions associated with inhalation of avian serum were inhibited by pre-treatment with disodium cromoglycate, however, subsequent challenges with the same allergens provoked milder reactions, considered to be due to a loss of sensitivity caused by repeat challenging.

5. Reference: Hargreave and Pepys (1972)

Subjects : 81 subjects

Antigens : Nebulised avian serum initially at concentrations which caused a weal of no more than 3 mm on prick testing. If skin testing was negative then the starting dilution was 1 in 100 for 5 minutes in 3 divided doses of 1 then 2 then 2 minutes, with 10 minutes between, to look for immediate reactions. If negative, repeat the next day with 1 in 10 for 5 minutes, and if necessary for 10 minutes on the following day. If this was negative, try similarly with dropping extract up to 10 mg/ml for 20 minutes.

Results

Nine immediate reaction, 27 late reactions and 4 dual (early and late) reactions. The immediate reaction was associated with a history of asthma, immediate skin reactivity (Type 1 reaction). Late reactions included pyrexia and polymorphonuclear leucocytosis, with feature of a reaction in the peripheral respiratory tissues and asthma, often unassociated with wheezing, not reversed by isoproterenol.

Fever tended to be the first sign and usually cleared by 24 hours, although in 5 it persisted for between 30 and 72 hours. The highest temperature recorded was 39.9°C. Systemic symptoms usually preceded or accompanied the fever, these most commonly consisted of

chills, shivering, feeling hot or sweating (22 subjects), malaise (16 subjects) and headache (10 subjects). Leucocytosis occurred in 67% of febrile late reactions, was seen between 6 and 14 hours, and tended to fall, but was still raised in 35%, by 24 hours. In 16% of the tests the WBC count was normal at 6 - 14 hours but raised at 24 hours. All returned to normal by 48 hours. The increase was due to a neutrophilia with a mild increase in eosinophils, usually at 24 hours, the monocytes and basophils remained consistent, with a decrease in lymphocyte number.

Respiratory signs were mild. Nine symptomatic responders had no measurable change in ventilatory capacity. There were no changes in TL_{co} .

6. Reference: Moore et al (1974)

Subjects : 10 pigeon breeders; 5 with and 5 without alveolitis

Antigens : Nebulised 2ml pigeon serum

Results

No clinical details. Airflow obstruction, febrile response and leucocytosis only in symptomatic subjects. Prechallenge CH-50 levels were normal, or slightly raised in symptomatic subjects, but tended to be low in asymptomatic breeders. At 4 hours post-challenge the CH-50 dropped in 4 asymptomatic subjects, in one subject this was noticed at one hour and persisted to 12 hours. In the responders the CH-50 did not change at 4 hours.

7. Reference: Pepys & Hutchcroft (1975)

Subjects : One pigeon breeder

Antigens : Pigeon serum

Results

Pre-treatment with disodium cromoglycate prevent subsequent reaction.

8. Reference: Schatz et al (1976)

Subjects : Family of pigeon breeders, one with and 5 without alveolitis

Antigens : Pigeon serum

Results

No clinical details. One reacted, 5 did not. The

responder had an early (one hour) increase in CH-50 of 10% and the other 3 did not vary uniformly. By 8 hours all sera had 10% less CH-50 than pretest levels and at 24 hours all sera had higher CH-50 (between approx. 10 - 20%).

9. Reference: Warren et al (1977)

Subjects : 4 bird breeders

Antigens : 2ml nebulised avian serum 1 in 10; 2ml feather extract at 40 mg/ml.

Results

No symptoms nor reduction in exercise tolerance for 2 hours, then they developed dry cough, dyspnoea, fever, malaise, headache, and a restrictive ventilatory defect developed at the same time.

There were no changes in lung volumes, VC, FEV₁, V max and N₂ washout curves in the first hour, therefore there was no evidence of an immediate pulmonary reaction to inhalation of avian antigen in extrinsic allergic alveolitis.

10. Reference: Slavin (1978)

Subjects : 29 subjects, 2 controls

Antigens : Serum and dander from laboratory animals and domestic pets.

Results

Diagnostic significance was established when inhalation challenges with animal serum and dander extracts provoked 6 hours reaction of fever, dyspnoea and reduction of DLCO. Minimal contact with gerbils (transporting 3 in the back of a car) was enough to provoke a response.

11. Reference: Schuyler et al (1978)

Subjects : 1 pigeon breeder

Antigens : 2ml sterile pigeon serum nebulised.

Results

Five hours post challenge the patient developed myalgia, fever and malaise. Vital capacity dropped from 101% to 91% predicted. Leucocyte count rose from 7,000 to 12,000 per mm, oral temperature rose from 36°C to 38.5°C, arterial PO₂ dropped

from 79 to 69mmHg, and single breath diffusing capacity from 125% to 86% predicted. All symptoms and signs had resolved by 24 hours.

12. Reference: Voisin et al (1979)

Subjects : 6 bird breeders

Antigens : Pigeon serum diluted 1 in 1000, nebulised at 0.5 ml/min for 0.5, 1, 1.5, and 2 mins. separated by 10 mins. Then repeated at 10 fold concentration on subsequent days.

Results

	Symptoms	Pyrexia	Leucocytosis	Pulmonary function
1	+	+	+++	not significant
2	+++	++	++	+++
3	+++	-	++	++
4	-	-	+	+
5	+/-	-	nd	+
6	-	+	++	+

The complement CH-50 decreased in one subject only, No.2.

13. Reference: Baur et al (1980)

Subjects : 1 pigeon breeder, 3 farmers

Antigens : Nebulised pigeon serum 0.1 ml. Hay dust (strewing hay, 15-30 l, in a small cabin for 30-60 mins.)

Results

Typical reactions occurred between 3 and 7 hours.

Pyrexia and leucocytosis associated with symptoms of cough, fatigue and mild dyspnoea. DLCO, VC and FEV₁ decreased, the DLCO proved to be the most sensitive method for detecting the pulmonary disturbances, whereas VC and FEV₁ did not always show significant changes. No significant change in airways resistance was found in any of these subjects.

Before challenge, all four patients had decreased CH-50, two also had decreased C3, and C5 was increased in two. After challenge there was a significant increase in CH-50 in all four

cases (by 28%, 32%, 93% and 117%), in C3 in one, C4 in two and Factor B and C5 in one. In most cases these elevations were highest 5 to 7 hours or 24 hours after challenge. C3 split products could not be detected in the two sera tested.

14. Reference: Hendrick et al (1980)

Subjects : 29 bird breeders, 2 controls

Antigens : Avian serum, 1 in 100-1 in 5 in saline for 5 - 30 mins. and extracts of avian droppings, 3-40 mg/ml in saline for 5-30 mins.

Natural exposure occurred in a budgerigar aviary and in a pigeon loft for 1, 1-5, or more than 5 hours.

Results

Twenty-three positive reactions, 21 developed malaise, fever and headache after 2-9 hours and these symptoms were delayed for 12-18 hours in 2 subjects. Respiratory symptoms were less striking; few were troubled by undue breathlessness at rest although all noted this on exertion. None felt wheezy, several developed a dry cough and difficulty taking a deep satisfying breath.

"Facial appearance" proved to be the most informative clinical sign and the observers soon learned to recognise the onset of positive responses from the loss of normal cheerful and active expressions. At this stage the subjects themselves rarely admitted to any change in well-being. As the response worsened, the unwell appearance was generally readily apparent. Auscultatory signs were uncommon. Body temperature exceeding 37.2°C identified 78% of positive reactors and excluded 95% of negatives. Leucocytosis fully attributable to neutrophils was a feature of reactors, but there was no significant changes in the number of circulating eosinophils. The lymphocytes decreased in reactors, but not consistently.

The mean ratio of $\text{FEV}_1 - \text{FVC}$ was 81% before and at maximum fall in FVC. Vital capacity was reduced, mean -8%. The single breath diffusing capacity for carbon monoxide was only significant (greater than 15% fall) in 2 reactors. There were no radiological changes in these subjects.

15. Reference: Edwards & Cockcroft (1981)
Subjects : 6 hospital theatre workers, 2 controls
Antigens : Nebulised contaminated humidifier water,
concentrated x 100. 2ml was the equivalent of 8
hours the exposure.

Results

Four reacted, including 2 who had no previous symptoms (one with and one without precipitins). The reactions were mainly systemic with fever, chills, headache, arthralgia, malaise and a feeling of discomfort on deep inspiration, maximal in 3 between 10 and 12 hours and 18 hours in the other. All subjects felt better the next day but in retrospect said they felt somewhat unwell for up to a week after the challenge.

Vital capacity fell but there was no fall in gas transfer factor. No x-ray changes were observed. The WBC count increased in all reactors, and also by 180% in one precipitin-positive non-reactor. The ESR did not produce any significant changes and complement levels showed no significant increase or decrease in either group.

16. Reference: Edwards & Davies (1981)
Subjects : 16 farmers
Antigens : 2ml of soluble M. faeni antigens at 1 mg/ml for 10
mins.

Results

Three positive reactors had a pyrexial response starting at 6 hours, peaking at 15 hours and gradually resolving at 20 hours. Each described a feeling of malaise, and there was a fall in VC and FEV₁ maximal at 12 hours. One further subject described cough and dyspnoea alone. There were no significant increases or decreases in CH-50 levels over the first 6 hour period after challenge for either responders (2 tested) or non-responders (12 tested; 7 with precipitins).

17. Reference: Lozano et al (1982)
Subjects : 37 bagasse workers; 5 bagassosis patients
Antigens : Daily work exposure.

Results

Before work there were no significant differences in pulmonary function between patients and workers. After work there were no differences in pulmonary functions in the workers since the morning, however, the patients had a significant mean reduction in FVC at 8 hours of -28%. There were differences between the groups in leucocyte count and serum IgG. There were no differences in complement components C3c or C4 nor in CH-100

Other literature reports which discuss inhalation challenge but do not give full details include:

18. Rodey et al (1979). Inhalation challenge on 51 symptomatic and 100 asymptomatic bird breeders.
19. Muller et al (1976). 6 cases of bird fanciers lung. Natural exposure at home resulted in 5 acute reactions.
20. Sennekamp et al (1975). 8 bird breeders - natural exposure.

APPENDIX DAnimal Models of Extrinsic Allergic Alveolitis

A recent review has drawn attention to the need for animal models for a better understanding of the common diseases of the respiratory tract (Reid, 1981). The models that exist for extrinsic allergic alveolitis are firstly, where the disease occurs naturally (Slauson & Hahn, 1981), and secondly where a similar disease profile can be induced. A summary of these models, and the major literature reports are listed on Table 41.

There occurs a natural disease in horses and cattle which is clinically, aetiologically, immunologically and morphologically similar to EAA in man (Table 41: 8, 12 - 18), and the bovine form of farmer's lung was first described in cattle from the same district in Britain where the first human case was described (16,17). These observations on the natural history of the disease are of interest, but the experimental use of these animals is limited by the comparative rarity of the disease and the expense of the animals. However, some interesting data on the seasonal sensitivity of the peripheral blood lymphocytes to M. faeni antigen (18), and seasonal differences in antibody to M. faeni (17) has been reported, correlating with the seasonal pattern of disease, and this is in keeping with observations in pigeon breeders disease (McSharry et al, 1983). Also there may be some economic benefit from work which shows that progressive desensitisation of cattle is possible with repeated antigen injections (15).

Studies using animal models of EAA started in the late sixties, and the main workers include Fink and colleagues in Milwaukee studying pigeon breeders disease (1-7), Salvaggio in New Orleans (21-29) and Wilkie in Texas (11-15) studying Farmer's lung, and Richerson in Iowa City (33-39), and Willoughby in Baltimore (44-47) looking at miscellaneous pulmonary hypersensitivities.

One early observation made during attempts to induce lung disease in animals by insufflation of nebulised soluble antigens such as ovalbumin, pigeon serum or horseradish peroxidase, was the

relative non-responsiveness of the animals (1,4,30,32). However, experiments using animals primed with antigens emulsified in Freund's complete adjuvant (FCA) demonstrated that this extra stimulation was required to render most of them responsive to subsequent aerosol challenge with nebulised antigen (4,5,32-34,41,45), although some animal models were still unresponsive despite this vigorous procedure (1,6a). The adjuvant effect of carrageenan, alum, concanavalin A, BCG and muramyl dipeptide have also been used to prime animals (4,7,38,45,46). Administration of some of these agents alone without antigen can cause pulmonary inflammation (4,33,48) and although most workers have been careful to control for this, it must still obscure the interpretation of lung histology following antigen challenge.

Some workers could induce an immune response by antigen inhalation without the preceding adjuvant stimulus (4,5,40,42) but this response was unpredictable and usually required longterm antigen insufflation. The response also differed with the same antigen in different animal species, for example bovine serum albumin (BSA) was a poor antigen in rats (1) but strongly immunogenic in rabbits (23,45), and pigeon serum was a poor antigen in guinea pigs (6a) but strongly immunogenic in monkeys (2,10). Also within the same species, different proteins could have different immunogenicities, for example in rats BSA was a poor antigen whereas pigeon droppings extract gave a good response (1), also, in guinea pigs, ovalbumin induced a severe pneumonitis but pigeon serum failed to produce significant disease (6a). In another laboratory, however, pigeon serum was used successfully to initiate disease in guinea pigs (6b).

One model of alveolitis in rabbits has been carefully established by Salvaggio (23) using antigens from *Micropolyspora faeni*, the causative agent in farmer's lung. The disease can be reproduced without using prior adjuvant stimulation, but intrinsic adjuvant properties of the antigens have been described (23-25).

The particulate nature of the antigen has been suggested to cause a local inflammatory response (23) but soluble antigens can also result in disease (1, Reed, 1972), and inhalation challenges with soluble antigens can reproduce symptoms. It is

possible that the particulate nature of these antigens may help to initiate disease, and also these particles may act as a vehicle to carry the soluble antigens to the peripheral airways where the disease is evident. In this respect, the pigeon bloom may be of similar importance in pigeon breeders disease, as discussed in Chapter 2.

Another important factor which needs standardising in animal experiments is the dose and route of antigen administration. Passive breathing of nebulised antigen in animals results in only 0.2% (33) and 0.34% (45) of the delivered dose being immediately retained in the lung, corresponding to a total dose of a few micrograms of antigen, whereas by direct instillation, up to 50 milligrams of antigen delivered via the trachea has been reported (23). This is clearly a different situation, and indeed the extent of responsiveness to antigen, irrespective of adjuvant priming, has been suggested to be related to the antigen dose (45).

Animal models of pigeon breeders disease have been produced in rats (1,5) guinea pigs (6a, 6b) rabbits (4,7a,7b) mice (9a,9b) and monkeys, (2,3,10). Animal models of farmer's lung disease using antigens of *Micropolyspora faeni* have been produced in rats (21) guinea pigs (11,20) and rabbits (19, 22-30). This range of models of disease suggests that no one model is entirely satisfactory.

The mechanism for disease is considered to be either humoral, immune complex mediated (6a, 6b, 34,44-47) based on histology, immuno-fluorescence, passive transfer with serum, protection by cobra venom factor (40,43,46 Olenchock et al, 1976) superoxide dismutase (McCormick et al, 1980) and steroids (42), or by cell-mediated hyper-sensitivity based on histology and passive transfer of disease with lymphoid cells. However, the consensus of opinion is that hyper-sensitivity involving both arms of the immune response is necessary for the full disease profile of alveolitis (2,11, Roberts & Moore, 1977).

The relevance of these animal models to the study of the human condition is based on extrapolating what can be interpreted from the histology and immunology of controlled manipulation of the animal models in ways which would not be possible in humans

with alveolitis. In addition to this, the human condition has a broad clinical and immunological spectrum, the causative antigens are diverse and the extent of each individuals' exposure history is varied. Inhalation challenge on humans are generally unhelpful and can be dangerous in inexperienced hands, and only rarely would lung biopsies be indicated. However, some similarities between the animal models and the human condition can be seen from the available data. Firstly, the predominant state in animal and man is non-responsiveness to inhaled antigens (4, McSharry et al, 1984). However, where an immune response becomes established, both specific antibody and sensitised lymphocytes are found, and the early acute phase of disease is associated with high antibody titres and acute haemorrhagic pneumonitis with positive immunofluorescence for antigen, antibody and complement. This is similar to acute cases investigated in humans (Barrowcliff & Arblaster, 1968; Seal et al 1968; Pepys, 1969; Wenzel et al, 1971; Ghose et al, 1974; Fink, 1974). More chronic antigen exposure results in increasing cellular involvement, with a characteristic mononuclear cell infiltrate on lung histology (Hensley et al, 1969). For the complete disease profile in animals and man both the antibody and cell mediated hypersensitivities seem to be involved (Roberts and Moore, 1977). This is best illustrated in non-human primates (2). This model is the closest to the human condition and some recent studies have shown that the regulation of alveolitis in monkeys is by a lack of T-suppressor cell activity (10). Similar cellular suppressor influences in asymptomatic subjects have been reported, in the human condition, which are absent or non-functional in symptomatic subjects (Keller et al, 1982).

During studies on the active phase of the disease in animals it was overlooked until relatively recently that the disease activity and the immune response could wane during prolonged antigen insufflation (14,37,39,40). The mechanisms involved are unresolved, but whether there is an active tolerance or desensitisation would be of considerable theoretical interest for insight into the disease process, and the antigen handling and immune responsiveness of the lung. The same phenomenon may also exist in humans (McSharry et al, 1984), and it may also be of

economic value for treating alveolitis in cattle (15).

The unpredictability of the response to inhaled antigens in humans reflects individual constitutional differences and the same would apply to animals. However, experiments to establish the disease in different strains of mice has allowed the mapping of the genes involved (9a,9b). This responsiveness was multigenic; the H-2 linked genes mapped within the I-B subregion, and some non H-2 linked genes are also involved, perhaps the same as those controlling the pulmonary inflammatory responsiveness to BCG (Allen et al, 1977). So far there have been no fruitful genetic analyses of the disease in humans (see Chapter 1, 4g), however with greater understanding of the spectrum of clinical presentation of the disease and the immune responsiveness to inhaled antigens, perhaps subpopulations of subjects may be defined having acute or chronic disease, or subjects may be defined as "high" or "low" antibody responders or indeed "non-responders" to inhaled antigens. Only when these groups are defined will there be any progress on HLA analysis.

TABLE 41. Animal Models of EAA

<u>Reference</u>	<u>Species</u>	<u>Antigen</u>	<u>Observation</u>
1.	Rat	PDE BSA	BSA poorly immunogenic.
2.	Monkey	PS	FCA used. Sensitised and antibody
3.	Monkey	PS	the full disease profile.
4.	Rabbit	PDE	Humoral response only. Normal lung histology. CH50 drops on challenge.
		PDE +BCG	Humoral plus cellular response. Abnormal lung histology. No drop of CH50 on challenge.
5.	Rat	PS	Early "immune response", later "allergic" response following `prolonged insufflation.
6a.	Guinea Pig	PS OVA	PS poor immunogen even with CFA OVA plus CFA gives IgE mediated disease.
6b.	Guinea Pig	PS	PS in CFA gives a good response. Immune-complex mediated disease.
7a.	Rabbit	PDE	Additional inflammatory stimulus required for disease.
7b.	Rabbit	PDE	Repeated aerosol challenge gives CMI response and chronic disease.
8.	Horse	CS	Natural disease.
9a.	Mouse	PS	Disease involves both B and T memory cells.
9b.	Mouse	PDE	Multigenic control of lung disease susceptibility.
10.	Monkey	PS	Disease modulated by T-suppressor cells.

B. FARMER'S LUNG DISEASE

11.	Guinea Pig	Mf	CFA used. Sensitised cells and serum both required for passive transfer of disease.
12,13,14.	Cattle	Mf	Natural disease.
15.	Cattle	Mf	Desensitisation by antigen injection.
16,17.	Cattle	Mf	Natural disease.
18.	Cattle	Mf	Cell mediated immune reaction in peripheral blood lymphocytes of cattle.
19.	Rabbit	Mf	Type I reaction.
20.	Guinea Pig	Mf	Minimal effects of steroids.
21.	Rat. Rabbit	Mf	Antigen i.v. gave no DTH skin or peripheral node lymphocyte reactivity. Antibody positive.
22.	Rabbit	Mf	MMIF production by sensitised respiratory tract lymphocytes.
23.	Rabbit	Mf +BSA	M.faeni adjuvant boosts the anti-BSA response.
24,25.	Rabbit	Mf	Local adjuvant effect of spores.
26.	Rabbit	Mf	Passive transfer of sensitivity with lymphoid cells.
27.	Rabbit	Mf	Review of animal models.
28.	Rabbit	Mf	Steroids abrogate disease.
29.	Rabbit	Mf	M. faeni spores activate alveolar macrophages.
30.	Rabbit	Mf	Prolonged antigen insufflation results in waning of disease and antibody levels.

C. EXPERIMENTAL ALVEOLITIS

31.	Monkey	Af	Passive transfer of sensitivity with human serum.
32.	Guinea Pig	OVA	CFA used. Both humoral and cellular hypersensitivity involved in disease.
33.	Rabbit	CFA OVA	Non-specific granuloma formation. Only 0.2% aerosolised dose retained in lung.
34.	Guinea Pig	OVA PPD Haptens	Induction of type I, III and IV hypersensitivity in the lung.
35.	Guinea Pig Rabbit	OVA PPD Haptens	Conditions favouring CMI give EAA; conditions favouring anti-body give haemorrhagic pneumonitis.
36.	Rabbit	Ferritin OVA	Antigen in CFA. Reaction histologically similar to alveolitis.
37.	Rabbit	OVA	Types I, III or IV reactions depending on protocol.
38.	Rabbit	OVA	Adjuvant effect of muramyl dipeptide in initiating disease.
39.	Rabbit	OVA	Repeated intravenous or aerosol challenge caused antigen-specific desensitisation.
40.	Rabbit	HSA	Disease pattern following in-halation challenge did not cor-relate with presence of precipitins.
41.	Rabbit	HRP	CFA used. Pulmonary lesions correlate with CMI.
42.	Rabbit	HRP	No adjuvant used. Reaction suppressed by steroids.

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| 43. | Guinea pig | OVA | Suppression with CoVF and passive transfer with serum. |
| 44. | Rabbits | BSA | Mitogenic stimulation, Con A required to initiate disease. |
| 45. | Rabbits | BSA | Extra stimulus required for disease. Increased exposure resulted in decreased lung response despite maintenance of antibody. |
| 46. | Rabbit | BSA | Stimulation with Con A - suppression with CoVF. |
| 47. | Rabbit | BSA | Lung disease inhibited by cholera toxin. |

Abbreviations for Table 41

PDE = Pigeon droppings extract	HSA = Human serum albumin
BSA = Bovine serum albumin	HRP = Horseradish peroxidase
PS = Pigeon serum	CMI = Cell mediated immunity
FCA = Freund's complete adjuvant	s.c.= subcutaneous administrn.
BCG = Bacille Calmette Guérin	i.v.= intravenous administrn.
OVA = Ovalbumin	MMIF= Macrophage migration inhibition factor
CS = Chicken serum	DTH = Delayed type hypersensitivity
Mf = Micropolyspora faeni	CoVF= Cobra venom factor
Af = Aspergillus fumigatus	ConA= Concanavalin A.
PPD = Purified protein derivative	

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