THE DISTRIBUTION OF PLASMA AND OTHER CELLS CONTAINING IMMUNOGLOBULIN IN THE RESPIRATORY TRACT OF NORMAL MAN, AND THE ALTERATIONS FOUND IN SUBJECTS WITH CHRONIC BRONCHITIS

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ABSTRACT

The anatomical distribution of plasma cells and other cells containing immunoglobulin in the respiratory tract, and the relative proportions of the immunoglobulin classes have been estimated on necropsy tissues from nine adult human subjects without respiratory disease and eleven subjects suffering from chronic bronchitis. Cell counts on multiple sections stained by immunofluorescent methods for the presence of immunoglobulin were carried out on upper trachea, main bronchus and lower lobe bronchus, and the general appearances in peripheral lung were noted.

The subjects without respiratory disease consisted of five non-smokers and four smokers, none of whom had cough or sputum. Cells containing immunoglobulin were found mostly in the submucous gland but were also present in the lamina propria of the tracheal and bronchial epithelium. These cells were present in the greatest concentration in the main bronchus and were always present in the lobar bronchus, and in most subjects in the upper trachea. These cells were not always present round small bronchi and bronchioles and were virtually absent from alveolar walls.

Cells containing IgA were much more numerous than those containing other immunoglobulin classes in all subjects except one, in whom IgG and IgE cells were equally numerous. Two subjects appeared to be significantly different from the rest. One non-smoking subject had a marked deficiency of IgA cells at all sampling sites, and one smoker had a marked excess of IgA cells. In spite of these two sub-

jects there was no significant difference between smokers and non-smokers except in lobar bronchus where the smokers had significantly more IgA cells than the non-smokers.

The chronic bronchitic subjects consisted of six subjects dying from complications of severe chronic obstructive bronchitis and five subjects with 'incidental' chronic bronchitis who died from unrelated disease. There was a deficiency of IgA cells in all six subjects with fatal chronic bronchitis when compared with normal subjects. The IgA cell counts in the subjects with 'incidental' chronic bronchitis corresponded to normal values for healthy non-smokers and did not share the increased cell counts in lower lobe bronchus occurring in healthy smokers.

This work suggests that the subjects dying from chronic obstructive bronchitis were deficient in plasma and other cells containing IgA in the respiratory tract, and that subjects with 'incidental' chronic bronchitis were normal in this respect.

These important findings have been further investigated by an immunofluorescent study of the carinal lymph nodes of the same individual normal and bronchitic subjects.

Cells containing immunoglobulin were seen scattered in the medullary cords and cortico-medullary junctions, and also as conglomerates within active germinal centres. Sampling methods on multiple sections were used to count the numbers of single cells containing immunoglobulin (excluding those in germinal centres) and also the numbers of germinal centres containing immunoglobulin.

This work has shown that the numbers of active germinal centres containing immunoglobulin (mostly IgM) were greater in normal smokers, 'incidental' bronchitics and 'fatal' bronchitics than in normal non-smokers ($p < 0.001$, < 0.05, < 0.05 respectively).

There were, however, fewer plasma cells containing IgA and IgM in 'fatal' bronchitics than in normal non-smokers, normal smokers and 'incidental' bronchitics (IgA, $p < 0.01$, < 0.05, < 0.01 respectively; IgM, $p < 0.01$, NS, < 0.005 respectively). These results indicate that the depletion of plasma cells found in the airways in the 'fatal' bronchitic subjects was accompanied by a similar depletion in the regional lymph nodes. Cigarette smoking appeared to increase germinal centre activity even in subjects without chronic cough.

Further evidence of a defect of bronchial IgA secretion in severe chronic bronchitis was sought by estimation of IgA in sputum. Fifty-seven sputum samples were collected at random intervals from fourteen subjects suffering from long-standing chronic bronchitis who had severe airways obstruction and who had been admitted to hospital for an exacerbation of their symptoms. Estimation of immunoglobulin and albumin in serum and processed sputum permitted calculation of the local bronchial secretion of IgA and IgG. Bronchial IgA secretion was low during acute infections in five of the fourteen patients.

These findings are consistent with the hypothesis that bronchial IgA secretion is impaired in a proportion of patients with severe chronic bronchitis. This defect may predispose to bronchial infection and rapid progression of lung damage.

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CHAPTER 1

INTRODUCTION

The ability of the normal human respiratory tract to defend itself from frequent or chronic infection might be regarded as almost miraculous, and contrasts sharply with the frequent infections and chronic bacterial colonisation of the bronchial tree occurring in such diseases as chronic bronchitis.

Defence mechanisms such as ciliary clearance of micro-organisms from the bronchial tree and phagocytic cellular activity have been known for some time, but the description by Chodirker and Tomasi (1963) of a characteristic secretory immunoglobulin in external secretions, including bronchial mucus, and the demonstration by Tourville et al (1968) of accumulations of plasma cells containing IgA in exocrine glands suggested that secretory IgA and other immunoglobulins might play an important part in the defence of the bronchial and gastro-intestinal tracts.

There have been several reports of plasma cells found in varying quantities in the bronchial wall (discussed in Chapter 2) and it became apparent that there was a need for a systematic study of the distribution of plasma cells in the bronchial wall and the class of immunoglobulin contained in them. Studies on bronchial secretions of normal subjects are difficult, for the secretions are too scanty to be obtained except by bronchial washings (which have usually been performed during bronchoscopy in individuals who are not necessarily normal), and interpretation of the results in normals and in patients with respiratory disease is difficult.

For this reason, a more quantitative approach, that of counting plasma cells in necropsy bronchial tissues, has been undertaken, and this thesis relates the development and systematic application of such a method to tissues from nine normal subjects and eleven subjects with chronic bronchitis.

While acknowledging the difficulties in interpretation and technique of measuring immunoglobulins in sputum, a pilot study of sputum immunoglobulins in fourteen living subjects with severe chronic bronchitis has also been attempted, and provides some confirmatory evidence of the abnormalities suggested by the tissue studies.

Chronic bronchitis was chosen for this investigation because of its importance as a cause of ill-health and death, and its associated susceptibility to chronic respiratory infection and chronic bronchial colonisation, and certain other factors suggestive of an immunological abnormality.

This thesis contains review chapters on the normal defence of the respiratory tract against infection (Chapter 2), and on the syndrome of chronic bronchitis (Chapter 3). Chapter 4 consists of a detailed description of the experimental methods used to prepare and examine over 1,800 sections from necropsy tissues from normal subjects and chronic bronchitics. Chapters 5 and 6 are the results of immunofluorescent studies on the airways in normals and in

chronic bronchitics respectively, Chapter 7 is the results of examination of the regional lymph nodes in both groups, and Chapter 8 contains the results of a pilot study of sputum immunoglobulins in chronic bronchitis undertaken to confirm the abnormalities suggested by the tissue findings. In Chapter 9, the findings are discussed, and the implications considered.

CHAPTER 2

NORMAL MECHANISMS OF RESISTANCE TO PULMONARY INFECTION, WITH SPECIAL REFERENCE TO SECRETORY IMMUNOGLOBULINS

Sections:

1. Inhalation of Particles and Sites of Deposition in the Respiratory Tract.
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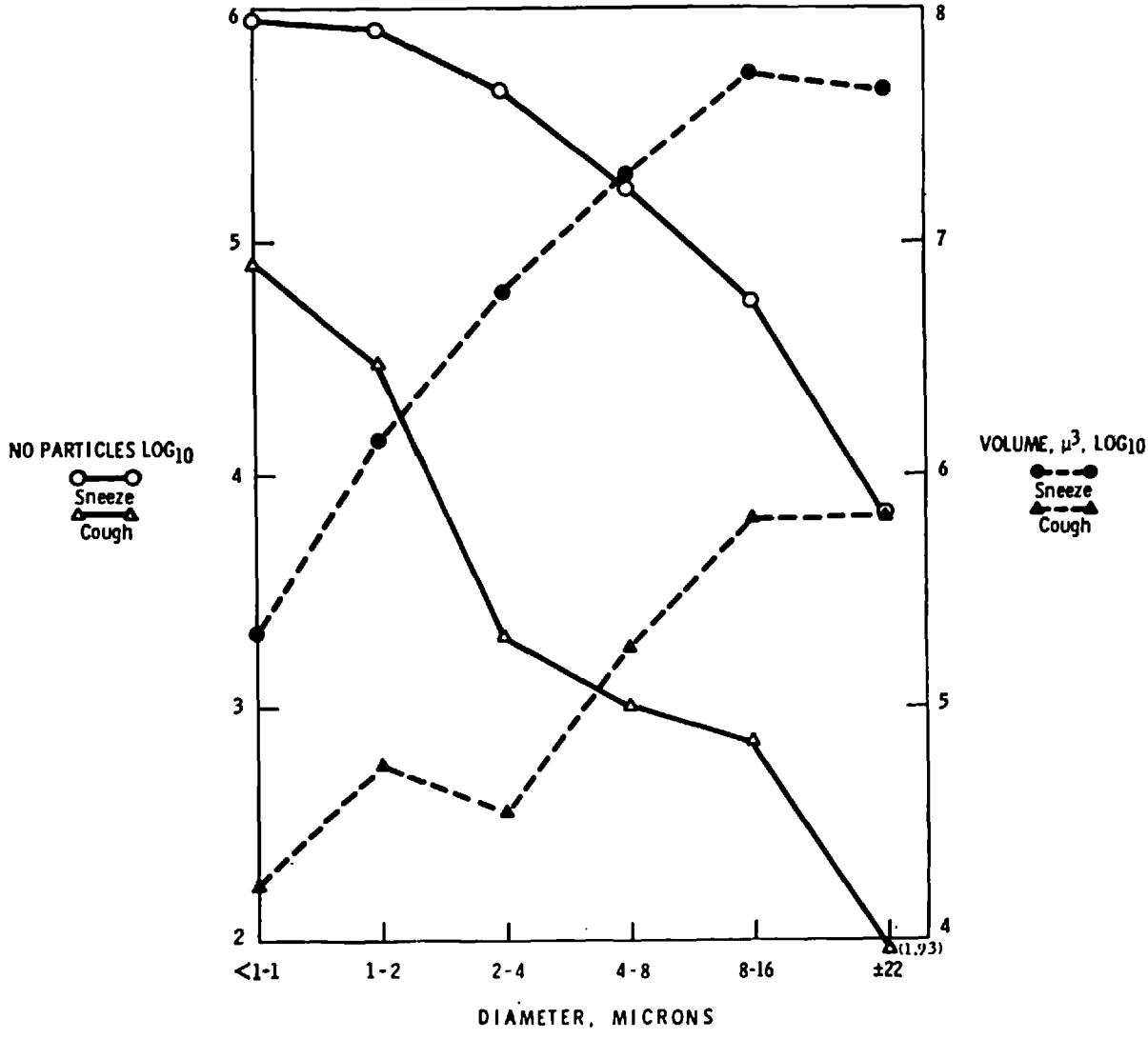
1. Inhalation of Particles and Sites of Deposition in the Respiratory Tract

The air we breathe contains many particles of dust and droplets of moisture. Large particles (more than 20 μ diameter) do not remain airborne for more than a few minutes in still air, so inhalation of these particles is relatively unlikely unless the person is in close proximity to the source of the particle at the time of generation; for example in direct line with a cough or sneeze from another person, or working close to an industrial process generating dust. However, particles smaller than 20 μ have settling velocities slow enough to be relatively stable in still air (Parkes, 1975). Dispersions of these small particles are called aerosols, and when the air is kept in movement, may remain airborne for long periods. Even when they have settled out, particles may become airborne again; for example when floors are swept or beds made (Knight 1973).

Droplet aerosols containing viral and bacterial particles may be released by infected persons during coughing or sneezing. During both these events, the largest number of droplets released are of 1-2 μ size or less; the numbers of particles falling with increasing size (Duguid, 1946, Gerone et al, 1966). The numbers of particles and total volume of particles related to size in coughs and sneezes is set out in Fig. 1 (after Knight, 1973). Even though the total number of large particles is small, their total volume is great. This is an important finding since the concentration of virus has been shown to be more clearly related to the volume than the numbers of particles (Couch, 1965).

Fig. 1

Distribution of particles by size in coughs and sneezes (after Knight 1973)


NO PARTICLES LOG10
Sneeze
Cough
VOLUME, μ3, LOG10
Sneeze
Cough
6
5
4
3
2
8
7
6
5
4
(1.93)
<1-1
1-2
2-4
4-8
8-16
±22
DIAMETER, MICRONS

In addition to micro-organisms, the air may contain mineral dust in sufficiently fine a form to remain aerodynamically stable, and this may be a great problem in industries where the dust is potentially harmful, as in innumerable occupational exposures. Organic materials may also be suspended and inhaled in industry and at home. The highly allergenic house dust mite, animal danders and moulds are released into the air every time domestic dust is raised.

The following remarks on deposition of particles are based on the work of H. D. Landahl, recently reviewed by himself (Landahl, 1972). The site of deposition of aerosol particles after inhalation is a function of their size. The larger the particle, the more likely it is to be filtered out in the nose. Over 90% of particles of 20 μ diameter or more are filtered out by the hairs of the nose and the directional changes in the nasopharynx, but this proportion lessens as the particle decreases in size, so that only about 30% of 2 μ particles are prevented from entering the lower respiratory tract, and an even smaller proportion of finer particles. Most particles deposited in the lower respiratory tract are in the range 0.6 - 6 μ diameter. Particles penetrating thus far are likely to be deposited in small airways, including segmental bronchi, respiratory bronchioles and alveolar ducts. Droplets derived from the respiratory tract behave differently from dusts in this respect; they are hygroscopic and will change in size according to the water saturation of the air around them. When these are inhaled, they take

up moisture and become larger. The effect of this is to increase retention of these particles in the tertiary bronchioles and alveolar ducts. The estimated deposition of 1.5 μ hygroscopic particles within the respiratory tract is described in Table 1.

The respiratory tract presents to the air a large warm moist surface which is bombarded with particles, many of which contain viable micro-organisms. It is also liable to receive mucus and other material, sometimes infected, from the nasopharynx. Yet in spite of this, the lower respiratory tract is normally sterile and overt chest infections are relatively infrequent events in normal man. The local defence mechanisms must be remarkably efficient to maintain this state of affairs.

2. Mechanical Elimination of Inhaled Particles

Many animals studies on the clearance of particles from the lung have shown that between 40 and 70% of inhaled particles are removed from the lung in the first five hours after deposition (Palm et al, 1956; Harper and Morton, 1953; Rylander, 1968). This mechanical clearance is brought about by the action of the cilia of the bronchial epithelium in moving particles trapped in bronchial mucus upwards and out through the larynx. Estimates of flow rates of particles in the trachea range from 14 mm/min. for single particles (Dalhamn, 1955) to about 1-2 mm/min. for overall flow rates in the whole tracheal tube (in the guinea pig) (Rylander, 1968; Carson et al, 1966).

TABLE 1

Deposition of 1.5-Micron Hygroscopic Particles within the Respiratory Tract (from Knight 1973).

	% of Total Air	% Deposition Hygroscopic Particles	% Deposition Nonhygroscopic Particles
Nose	±6	36*	25
Pharynx to secondary bronchi	10	1/	0
Tertiary bronchi to respiratory bronchioles	21	25/	10
Alveolar ducts	63	21/	13
Total retained		83	48

* 24% of 2-μ particles retained on inhalation; 12% of total inhaled particles, 4μ in diameter due to accretion of water, retained on exhalation.

/ Retention as 4-μ particles.

Mechanical clearance of particles declines after the rapid clearance in the first few hours. Harper and Morton (1962) found that only half the particles still present in guinea pig lungs the day after deposition had been eliminated eleven days later. It seems likely that the early, rapid clearance of particles is from the large and medium sized airways by ciliary action on particles trapped in mucus, and the slower later clearance is from small airways and alveoli by ciliary action and other mechanisms. These other mechanisms involve ingestion of particles by macrophages, and transport of these macrophages either upwards in the airways or into the lymphatics or blood stream. Particles probably pass into the lymphatics even without being phagocytosed. Inorganic particles may dissolve or be carried in macrophages to the regional lymph nodes (Gross and Hatch, 1963).

Ciliary clearance has been shown to be reduced by cigarette smoke (Carson et al, 1966; Rylander, 1968) and ethanol (Laurenzi and Guarneri, 1966; Rylander, 1968).

3. Killing of Inhaled Micro-organisms
Macrophage Activity

Animal experiments in which the clearance of bacterial particles from the lungs has been compared with the reduction in counts of viable bacteria remaining in the lungs have shown that reduction in viability is more rapid than the physical clearance of bacterial

particles, implying that an active process of bacterial killing occurs. (Kass and Green, 1964; Laurenzi et al, 1965; Rylander, 1968). It is beyond the scope of this thesis to discuss macrophage function in detail. However, in brief, it appears that most bacteria deposited in alveoli are ingested by alveolar macrophages (Kass and Green, 1964) and it is thought that the macrophage carries out the killing, though the possibility that the bacteria have already been killed before ingestion has not been ruled out. This killing is inhibited by cold, hypoxia, prior ingestion of coal dust and viral infection (Kass and Green, 1964; Laurenzi et al, 1965).

In the blood, macrophages and nuetrophil polymorph leucocytes are capable of ingesting bacterial and virus particles, and digesting them with a battery of intracellular enzymes, including lysosyme (Metchnikoff, 1905; Huff, 1940 (review)).

The process of ingestion is facilitated by the opsonic activity of serum (opsonins and bacteriotropins). Opsonins (Wright and Douglas, 1903, 1904) are thermolabile, relatively non specific substances occurring in normal serum, acting on a variety of bacteria and rendering them liable to phagocytosis. They have many similarities which complement and may be identical with some of its components (Reviewed by Miles, 1964). Bacteriotropins (Neufeld and Rimpau, 1904, 1905) are antibodies, relatively thermostable, which unite specifically with an antigen carried by the bacterial cell. The opsonising activity of these antibodies is mediated through

specific receptors on the macrophage surface which have a high affinity for IgG and for the third component of complement when present in immune complexes (Oakley, 1968; Wright, 1968). In the lung there are many macrophages in the alveoli (Miller, 1950) but many fewer macrophages or neutrophil leucocytes on the surface of the bronchial and bronchiolar epithelium (personal observation). In spite of this, bacteria deposited on the walls of these airways appear to be inhibited, for the normal bronchial tree is sterile (Brumfitt and Willoughby, 1958; Lees and McNaught, 1959). Dold (1943) showed that bacteria inoculated on to rabbit trachea failed to grow unless the tissue was heat treated beforehand, indicating antibacterial activity on the surface of the epithelium. Bronchial mucus has anti-bacterial properties which may account for this. May and Roberts (1969) showed that neat sputum inhibits the growth of bacteria, even though when diluted it is an excellent growth medium.

Bronchial Mucus

Bronchial mucus is composed of water, glycoprotein macro-molecules, small protein molecules and electrolyte ions (Boat and Matthews, 1973 (Review)). The glycoproteins impart to sputum its gel-like structure. Fucomucins are of neutral pH, sialomucins and sulphomucins are acid. Sialomucins bind virus particles (Burnet, 1951). The smaller proteins are either secreted actively into the bronchial mucus, such as immunoglobulin (discussed in the next section), lysozyme, lactoferrin, salivary alpha-2-globulin, and salivary B_1-globulin, or derived from serum by transudation. Albumen is the most prominent of the serum components, although serum immuno-

globulins and many other serum proteins are found (Masson et al, 1965). Electrolyte ions include sodium, chloride, calcium and phosphates. pH estimates range from 6.3 to 8.2 (Boat and Matthews, 1973). Pharmacological mediators such as histamine and serotonin may also be found (Thomas and Simmons, 1969; Levy et al, 1961).

The bronchial mucus is produced by goblet cells of the bronchial surface epithelium, and by the specialised system of mucous and serous cells composing the bronchial mucous glands, which lie in the bronchial walls and drain to the lumen of the airways. The goblet cells are found in large airways and are sparse in normal small airways. The mucous glands are found only in the trachea and bronchi, that is, airways with cartilage in their walls (Miller, 1950).

The components of bronchial mucus known to have antibacterial activity are immunoglobulins (discussed in the next section), lysozyme, able to lyse certain bacteria (Fleming, 1922), and lactoferrin, which inhibits the growth of certain organisms by binding free iron, thus making it unavailable to the organism. (Masson et al, 1966). The importance of these two substances is not known.

Secretory Immunoglobulins

Evidence for a Separate Secretory Immunological System

Besredka, in 1919, as a result of studies on experimentally

induced oral infections by enterobacteria, postulated that local immunity at mucosal surfaces could be established independently of systemic immunity. Davies in 1922 demonstrated that specific antibodies could be found in the faeces (coproantibody) of patients suffering from dysentery, and similar findings on experimentally induced infections in animals suggested an important role for coproantibody in gastrointestinal infections. This was confirmed by the work of Burrows (Burrows and Haven, 1948), studying the effects of cholera vaccine in guinea pigs. He and his colleagues were able to show a good correlation between coproantibody and protection against oral infection, but little relationship between serum antibody titres and protection.

Bull and McKee (1929) called attention to the possible participation of local antibody in the resistance of the respiratory tract to infection by reporting that rabbits could be rendered resistant to pneumococcal respiratory infections by prior intranasal immunisation with the same strain. Resistance occurred in some animals in whom serum antibody could not be detected.

The work of Fazekas de St. Groth and his colleagues (1950) on experimentally induced influenza virus infections in mice showed that, following intranasal instillation of the virus, there was a simultaneous appearance of antibody in both serum and mucus, and that the proportion of muco-antibody to serum antibody was over ten times that produced by subcutaneous immunisation.

In the last fifteen years, interest in secretory antibodies has increased greatly, given impetus by the isolation of the IgA immunoglobulin class by Heremans and colleagues (1959). Gugler et al (1958) first described the presence of IgA in the external secretion colostrum. Hanson (1961) demonstrated the quantitative predominance of IgA over other immunoglobulin classes in colostrum and Chodirker and Tomasi (1963) confirmed this predominance in many external secretions, including saliva, bronchial mucus, conjunctival secretiona, gastro-intestinal secretions and urine. Large accumulations of plasma cells containing IgA have been observed in external secretory glands in many sites including the respiratory tract (Tourville et al 1969; Martinez-Tello et al, 1968) and these are presumed to be the site of manufacture of secretory IgA. Finally IgA in secretions has been shown to be structurally and antigenically different from serum IgA (Tomasi et al, 1965).

Structure of Serum Immunoglobulins

The following general remarks are taken from Roitt, 1971 and Tomasi and Grey, 1972. The specific immune activity of serum is due to the presence of antibodies (immunoglobulins). Analysis of the various types of antibodies present in normal and immune sera, as well as the para-proteins found in myeloma and macroglobulinaemic sera, has led to the definition of five major classes of immunoglobulin, IgG, IgA, IgM, IgE and IgD. Each class of immunoglobulin consists of 'heavy' polypeptide chains (Gamma, alpha, mu, epsilon

and delta chains respectively) which are specific for each immunoglobulin class, and 'light' chains of two varieties, Kappa and Lambda, which are shared by all of the major classes.

IgG (previously gamma-G) is the most plentiful immunoglobulin in serum. It is able to agglutinate, precipitate, and in the presence of complement, to lyse cells or bacteria.

IgA (previously gamma-A or B_2 globulin) is less plentiful in the serum than IgG. It can agglutinate and precipitate but its special function in serum is not very clear, since its response in serum to challenge is inconstant and small in amount. It does not fix complement (Ishizake et al, 1967).

IgM (previously gamma-M) is less plentiful than IgA, but it is a powerful agglutinator, fixes complement, and increases in the serum early in response to challenge.

IgD is present in very small amounts, and its function is not known. IgE is also present in serum in very small amounts, but is important in the mediation of immediate hypersensitivity. It attaches to the surface of mast cells, and when challenged by specific antigen, causes degranulation of the mast cell, with release of chemical mediators, causing oedema and contraction of smooth muscle.

All these immunoglobulin classes are believed to be manufactured by plasma cells in the bone marrow, lymphoid tissue, sites of in-

flammation, and in the case of IgA, in the gut and respiratory tract. Plasma cells contain large amounts of ribo-nucleic acid, and it is this that stains pinky-mauve when methyl green pyronine stains are applied to tissue sections. Electron-micrographs demonstrate in plasma cells a mass of endoplasmic reticulin. The origin of plasma cells has been demonstrated by Miller and Mitchell (1968) to be lymphocytes derived from bone marrow (B cells).

Structure of Secretory Immunoglobulins

Whilst the IgG, IgM and IgE found in external secretions are indistinguishable from that found in serum, secretory IgA is both chemically and immunologically distinct. Serum IgA is predominantly in the form of a monomer, of ultra-centrifuge sedimentation coefficient approximately 7S. Polymeric forms are also fairly common, up to 18S. However, secretory IgA is predominantly in the form of a dimeric molecule of sedimentation coefficient 11A (Tomasi et al, 1965). The sedimentation coefficient of pure IgA dimer is 10S. These dimers are probably formed within the plasma cells of their origin, for human and animal experiments on excised or isolated segments of bowel have shown a high proportion of 10S IgA dimer in the venous return, (Tomasi et al, 1971) and the light chains in each molecule tend to be either Kappa or Lambda chains, but not both (Curry and Small, 1970). The difference in properties between the 10S IgA dimer and the 11S secretory IgA dimer is thought to be accounted for by two additional components, the secretory piece and the 'J' chain. The secretory piece is a short polypeptide chain which is thought to

be manufactured by the epithelial cells of the gland and to be attached to the IgA either within the epithelial cells or on their surface (Tourville et al, 1969). The 'J' chain has only recently been described (Halpern and Koshland, 1970). It is also found in association with IgA polymers in serum and its significance is unknown.

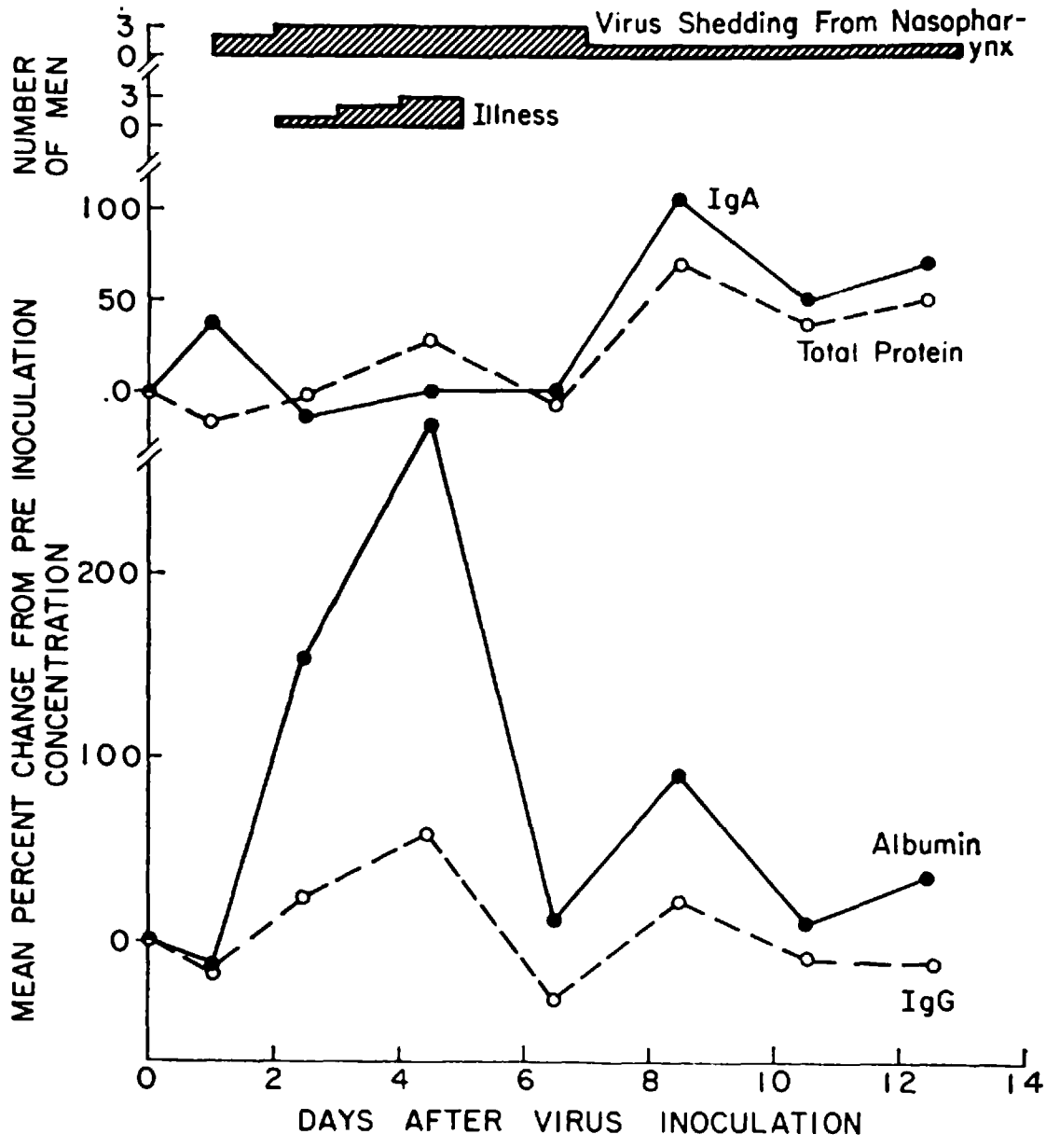
There is some evidence that the secretory IgA molecule is more resistant than serum IgA or IgG to digestion by proteolytic enzymes (Tomasi and Czerwinski, 1968; Brown et al, 1970), although other studies have failed to demonstrate any protective effect of the secretory conformation (Wilson and Williams, 1969). If the secretory IgA molecule is relatively resistant to proteolysis, this may well be useful in protecting the immunoglobulin from degradation by endogenous or bacterial enzymes in the gut or bronchial tree.

Response of Secretory Antibody to Infection

The time of appearance and rate of increase of secretory immunoglobulin has been studied after live vaccines and experimental infections, mostly in the nose and nasal secretions. Three or four days after inoculation there is a rise in albumen and IgG concentration (fig. 2), probably from serum as a result of increased capillary permeability. There is also a slight rise in IgA secretion at this time, possibly a release of IgA stored in epithelial cells. By about eight days, this has subsided, but the IgA increases further,

Fig. 2

Mean percentage change from pre-inoculation concentrations of IgA, IgG, albumin and total protein in nasal secretions from three volunteers after inoculation with rhinovirus (Butler, W.T., 1970)


Virus Shedding From Nasopharynx
Illness
NUMBER OF MEN
3
0
3
0
IgA
Total Protein
100
50
0
MEAN PERCENT CHANGE FROM PRE INOCULATION CONCENTRATION
200
100
0
Albumin
IgG
0
2
4
6
8
10
12
14
DAYS AFTER VIRUS INOCULATION

continuing to rise for several weeks (Butler et al, 1970). Specific antibody does not begin to appear until two or three weeks after inoculation (Cate, 1966; Rossen, 1966). The peak antibody activity is at 4-6 weeks, and thereafter falls gradually, although specific antibody activity may still be detectable as much as a year later (Buscho, 1972; Waldman et al, 1971).

Mechanism of Action of Secretory IgA

IgA polymers are nearly as good agglutinators as IgM, and ten times more efficient that IgA monomer. Secretory IgA exhibits more efficient agglutinating activity than serum 7S igA (Newcombe and Devald, 1969). Salivary IgA does combine with oral bacteria in vivo (Brandtzaeg, 1970) and IgA antibodies coating E.Coli have been described in serum, saliva, gastrointestinal fluids and urine (Tourville et al, 1968).

However, the mechanism by which these antibodies are protective, if indeed they are, is unknown. Lysis and killing of bacteria is generally thought to involve the fixation of complement. Adinolphi et al (1966) have suggested that secretory IgA in the presence of lysozyme and complement may have a lytic capacity for certain types of E.Coli, and this is confirmed by Burdon (1973), although Eddie (Eddie et al, 1971), failed to show any potentiation by lysozyme of lysis of Salmonella Typhimurium by rabbit IgA antibody. Freter (1970) has shown that antibody against cholera may inhibit the growth of this organism on the surface of intestinal mucosa by acting in concert with some additional factor(s) produced by mucosal cells.

There is no good evidence that IgA antibody is involved in opsonisation. Although parotid secretions do promote the ingestion of streptococci by leucocytes in vitro (Shklair et al, 1969), there is no reason to suppose that this activity is due to bacterial coating with IgA. In a recent study on IgA and IgG in rabbit intestinal secretions, the IgG was found to have opsonising properties for Salmonella Typhimurium, but IgA did not (Eddie et al, 1971). Studies in the mouse suggest that macrophages have specific receptors for IgG and IgM but not IgA (Unarue et al, 1974).

Another possible biological function of secretory IgA may be in limiting absorption and/or inactivating non-viable antigens which are inhaled or ingested. The mucosal plasma cells in the gastrointestinal tract do produce antibodies against ingested food antigens. Consistent with this possible function is the finding that the sera of patients with isolated IgA deficiency have a strikingly increased incidence of antibodies to milk protein (Buckley et al, 1968; Tomasi and Katz, 1971).

Activity Against Viral Infections

Studies by Fazekas de St. Groth (1950) on experimental influenza virus infections in mice suggested that the antibodies in secretions bathing the respiratory tract were more directly related to resistance to infection than those in the serum. In 1966 studies by Smith and his colleagues clearly demonstrated that resistance to

challenge with para-influenza type 1 virus was more closely related to the antibody levels in nasal secretions than to serum antibody levels.

In view of these and other similar findings, much work has been directed towards studying immunity to viral infections induced by vaccination by different routes. Parenteral immunisation with inactivated influenza or parainfluenza vaccines produces a serum response, predominantly IgG, and inconstantly produces mucosal antibody, which even when present, is of low titre (Mann et al, 1968; Smith et al, 1966). The nasopharyngeal antibody so produced is primarily IgA, suggesting that antigen or a processed form of it, reaches the secretory lymphoid tissues. Immunisation by the aerosol route elicits higher levels of secretory antibody, predominantly or solely IgA, as well as IgG type serum antibody. (Waldman et al, 1969; Wigley et al, 1970).

When vaccine is given by aerosol, the particle size has an effect on the antibody response. Small particle aerosol (1.5 u diameter) is not retained in the nasal passages, and does not give rise to nasal antibody, but is very effective in eliciting the production of serum antibody, presumably because these small particles are likely to have been deposited in small airways and alveoli, and this to be absorbed quickly into the blood stream. On the other hand, larger particles of approximately 100 u, deposited primarily

in the upper respiratory passages, are more effective in stimulating nasal and sputum antibodies with a minimum of serum antibody (Waldman et al, 1970). With myxoviruses such as influenza and para-influenza, respiratory syncytial virus, rhinovirus and certain types of adenoviruses, after initial implantation the infection remains localised superficially in the mucosa. With other viruses such as polio, ECHO and measles, following the initial mucous membrane phase, systemic dissemination occurs. It might be expected that these differences are relevant to the different effects of immunisation by the parenteral or aerosol routes.

Subjects immunised parenterally with inactivated rhino-virus vaccine develop serum IgG and IgM antibodies but no demonstrable antibodies in nasal secretions. This offers no protection against infection by live virus. Subjects immunised intranasally develop secretory 11S IgA and IgG serum antibody. This does offer significant protection against infection (Perkins et al, 1969).

Resistance to para-influenza virus has also been shown to relate more to secretory antibody than to serum antibody (Smith et al, 1966) and vaccination against influenza is more effective by the aerosol route than the parenteral (Waldmann et al, 1969).

On the other hand, with viruses in which systemic infection occurs after the mucosal infection, serum antibodies are effective in protecting against the systemic infection. Comparing immunisation against polio virus with live oral (Sabin) and killed parenteral

(Salk) vaccines, Ogra and his colleagues (1968, 1969) found that similar titres of serum antibody resulted from either route of administration. Secretory antibody, however, was only found after the oral vaccination. Both vaccines protect against systemic infection with the polio virus, although the presence of antibodies in serum but not in secretions, may allow a symptomless carrier state.

The peculiar type of measles which occasionally occurs in children previously immunised parenterally with inactivated measles virus vaccine is often accompanied by pneumonia. Studies of the local lesions in these cases has revealed deposits of measles virus, IgG antibody and complement in the walls of vessels (Bellanti et al, 1969). These authors postulate that in these cases, absence of secretory antibody permits the virus to multiply in the lung, and on reaching the systemic circulation, it meets plentiful IgG antibodies induced by the previous vaccination and produces an Arthus type hypersensitivity reaction. This process may also occur in infants who have been immunised systemically with killed respiratory syncitial virus and subsequently develop infection with the live virus. Systemic vaccination does not protect against this infection, and in addition may actually be harmful, making the illness worse than it would otherwise have been.

Bacterial Infections

Antibodies reactive with bacteria exist in normal respiratory and gastro-intestinal secretions (Tourville et al, 1968; Tomasi and Grey, 1972). The role of these antibodies is not well defined. Immunisation by the parenteral route often induces both serum and secretory antibacterial antibodies. Bellanti and his colleagues (1967) administered live attenuated Francisella Tularensis vaccine, and detected both serum and nasal antibodies, whether the route of immunisation was parenteral or by aerosol. The nasal antibodies were almost entirely of the IgA type. Immunised subjects were resistant to subsequent challenge with live organism. Burrows (Burrows and Havens, 1948) showed that resistance to experimental cholera in guinea pigs is better correlated with the presence of faecal antibodies than with serum levels of anti-cholera antibodies. Oral administration of killed cholera vaccine will induce a local immune response but minimal serum antibody (Freter and Gangarosa, 1963), while parenteral administration induces both serum and faecal antibody. The intravenous administration of Salmonellae typhosa endotoxin induces in the nasal secretions agglutinating antibodies of IgA and IgG classes.

In 1929, Bull and McKee showed that intranasal inoculation of rabbits with pneumococci produces resistance to subsequent challenge with the live organism in the absence of detectable serum antibody,

and in 1936 Walsh and Cannon showed that, following nasal administration of killed pneumococci, the rabbits were also resistant to an intravenous challenge with the live organism, suggesting that with this organism, as with viruses, local immunisation may also induce a systemic immunity. Conversely, McLeod (1945) showed that parenteral immunisation with pneumococcal antigens reduced the nasopharyngeal carrier rate of this organism among military recruits, and a similar reduction of nasal carriers of meningococcus has been reported following systemic immunisation (Artenstein, 1971). It would seem, therefore, that specific immunity to bacteria in secretions and serum is not so dependent on the route of immunisation as with viruses. The cause of this is not known, but one might speculate that it is related to the sharing of common antigenic determinants between bacteria, to some of which there may be pre-existing immunity.

Response to Inorganic Dusts

Secretory immunoglobulin is now known to be protective against inhaled inorganic dusts, but serum IgA autoantibodies directed against lung tissue have been described in coal pneumoconiosis (Burrell, 1967), and serum IgA levels are raised in complicated coal pneumoconiosis (Soutar et al, 1974).

Deficiency of IgA

Patients with either the congenital or acquired forms of hypogammaglobulinaemia frequently have a deficiency of IgA (Soothill et

al, 1968) and show increased susceptibility to infections, particularly with pyogenic bacteria (MRC Working Party, 1971). Most of these patients have a deficiency in both serum and secretory immunoglobulins (South et al, 1967), and are particularly susceptible to respiratory infections and diarrhoea and malabsorption syndrome.

An isolated deficiency of IgA has been reported to occur in approximately 1 of 500 to 1 of 700 individuals, in normal population surveys (Amman and Hong, 1970, 1971; Bachman, 1968; Cassidy et al, 1969). Many of these subjects are completely assymptomatic (Godfrey et al, 1968; Rockey et al, 1964), but IgA deficient subjects as a group show a high incidence of infections (Amman and Hong, 1973; South et al, 1967), and also of allergic disorders such as urticaria, eczema, allergic rhinitis or asthma (Tomasi and Grey, 1972; Amman and Hong, 1973), and auto immune disorders (Amman and Hong, 1970, 1973).

Selective IgA deficiency is to be found in 50-85% of patients with hereditary talangiectasia. These patients often suffer from recurrent infections and may have an associated deficiency of IgE (Amman et al, 1969).

Pulmonary Lymphoid Tissue

Small aggregations of lymphoid tissue are to be found throughout the lung in association with the arteries, veins and pleura

(Miller, 1950) and in the walls of the bronchi (Bienenstock et al, 1973). These aggregations do not form lymph follicles or nodes.

Lymph nodes are to be found lying outside the walls of the bronchi. The anatomical arrangement has been described by several authors including Sukehnikow, 1903, and Miller, 1950. The largest nodes are to be found in association with the trachea and its division. Although the above authorities do not say so, it is my experience that the largest nodes are usually to be found in the inferior tracheo-bronchial group (carinal nodes) in between left and right main bronchi.

The lung has an extensive system of lymphatic vessels, with many valves permitting flow only towards the hilum and tracheo-bronchial lymph nodes. Efferent vessels from these nodes drain to vessels ascending on the surface of the trachea, and finally form the left and right bronchomediastinal trunks, which in their turn empty either into the right lymphatic duct and thoracic duct respectively, or, more commonly, independently into the junction of the internal jugular and subclavian veins on their own side. (Miller, 1950).

The work of Gowans and Knight (1964) showed that, in the rat, small lymphocytes taken from the thoracic dust, labelled with tritiated adenosine, and returned to the venous blood of other rats, rapidly

disappeared from the blood and were soon found in the thoracic duct lymph of the recipient rats, indicating a continuous circulation from blood to lymph and back again. Examination of rat tissues at various intervals after infusion showed the labelled lymphocytes in the cortex of lymph nodes, the white pulp of the spleen, and Peyers patches of the gut. Labelled cells were seen passing into the nodes from the blood through the endothelial cells lining the post-capillary venules in the nodes. No labelled cells were seen in the lymph follicles in the cortex.

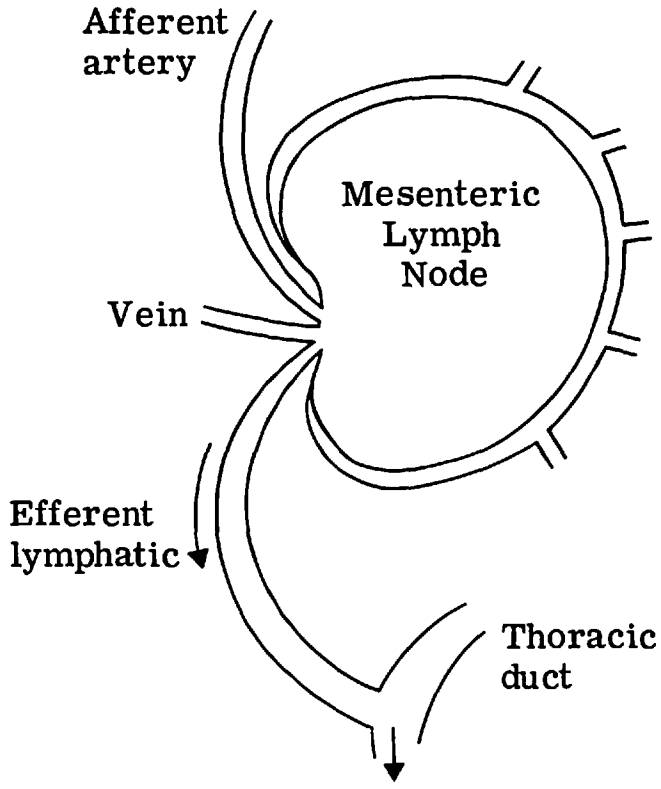
However, the same authors showed that large lymphocytes from the same source did not recirculate from the blood to thoracic duct lymph but lodged in large numbers in the lamina propria of the gut, where they became pyrinophilic, and indistinguishable from young plasma cells. This circulation of small and large lymphocytes is schematically representated in Fig. 3.

Subsequently, Griscelli and his colleagues (1969) showed that large actively dividing lymphocytes from thoracic duct lymph or from mesenteric lymph nodes, localise preferentially in the gut, Peyers patches or mesenteric nodes after infusion, while cells obtained from peripheral lymph nodes return to peripheral nodes.

Similar circulation of lymphocytes in the lungs of man or animals has not yet been described, but it seems likely, because of the common embryonic derivation of the lungs and gut.

Fig. 3

Re-circulation of small and large lymphocytes from thoracic duct to systemic circulation and return to mesenteric lymph nodes and gut wall (after Gowans and Knight, 1964)



SYSTEMIC CIRCULATION

Small Lymphocytes

Return to mesenteric lymph nodes; also pass to Peyers patches, spleen and other lymph nodes.

Large Lymphocytes

Mostly home in gut wall and become plasma cells (a few return to mesenteric lymph nodes)

The Structure of Lymph Nodes (Fig. 4)

Lymph nodes have a hilum at which the afferent and efferent blood vessels and the efferent lymphatic vessels enter and leave. The afferent lymphatics enter at the periphery of the node, and the lymph enters the subcapsular space, subsequently filtering down through the loose reticulum of the gland to the lymph sinuses of the medulla, and thence to the efferent lymphatic vessel.

The cortex of the node is fairly densely packed with lymphocytes, and contains lymphoid follicles, which are more or less isolated masses of lymphocytes. Sometimes these develop germinal centres, when the cells in the middle become large, pale, rapidly dividing lymphoblasts. In the medulla of the node the lymphocytes are more loosely packed and constitute irregularly branching cords between which are the medullary lymph sinuses. Plentiful macrophages are found in the medulla and deeper layers of the cortex, and many plasma cells lie along the medullary cords and in the deep parts of the cortex (para-cortical areas). The post-capillary venules are in the medulla and deeper layers of the cortex (Gray, 1967).

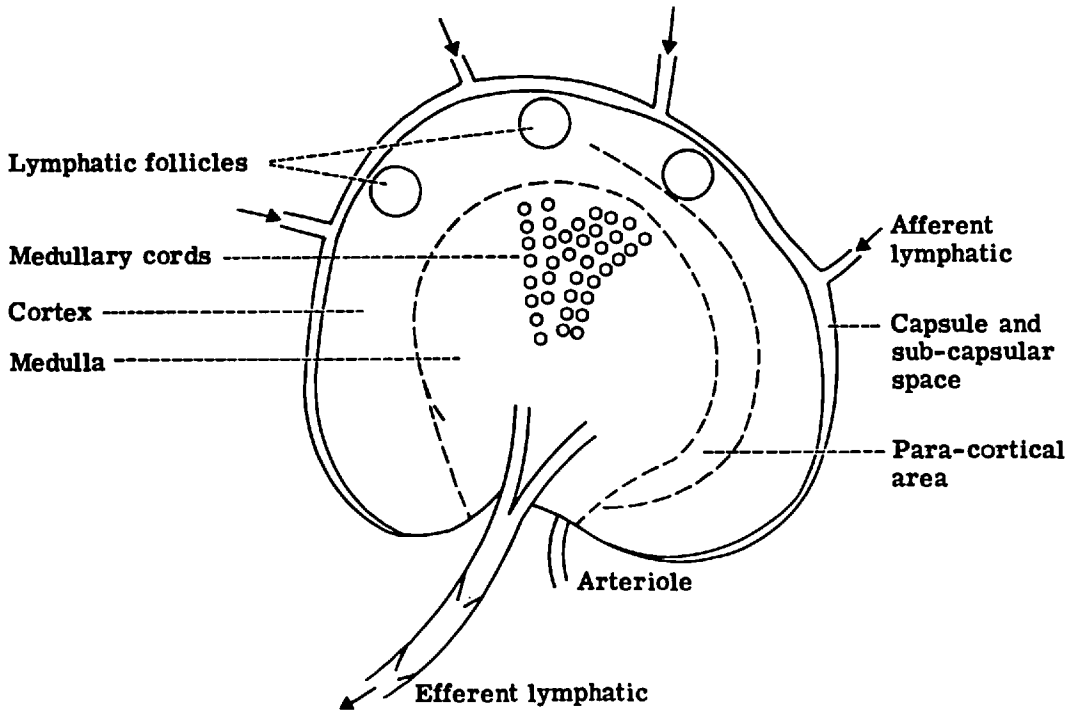
In response to an infection, or application of a sensitising agent to an experimental animal, the regional lymph nodes respond in one or both of two different ways. The humoral response consists of an increase in the number of plasma cells in the medulla,

Fig. 4

Schematic drawing of a lymph node.

(After Gray, 1967).

SCHEMATIC DRAWING OF LYMPH NODE



often, but not always, accompanied by an increase in the number and size of the germinal follicles in the cortex (White, 1960). The cellular response consists of an increase in the number of lymphoblasts and increasing cell division producing small lymphocytes in the middle and deep parts of the cortex (para-cortical areas), (Oort and Turk, 1965). This response is thought to be thymus-dependent because neonatal thymectomy abolishes it, and the cells in the paracortical areas are thought to be thymus-derived because labelled thymus cells home to these areas in all lymph nodes (Parrott et al, 1966).

Immunoglobulin can be identified in lymph nodes by immunofluorescent methods in the plasma cells of the medulla and in the germinal centres of some of the lymphatic follicles. In bronchial and mesenteric lymph nodes, IgA and IgE cells are more frequent than in peripheral nodes (Ishizake et al, 1971).

Chapter 3

CHRONIC BRONCHITIS.

First descriptions of chronic bronchitis, and definitions.
The clinical syndrome of chronic bronchitis.
Role of infections.
Integrity of normal defence mechanisms.
Respiratory and cardiac complications of chronic bronchitis.
Prevalence of chronic bronchitis.
Aetiology: the effect of smoking,
atmospheric pollution,
occupation,
heredity.

FIRST DESCRIPTIONS OF CHRONIC BRONCHITIS AND DEFINITIONS

The term "chronic bronchitis" was first used by Charles Badham in 1808 in his book "Observations on the Inflammatory Affections of the Mucous Membranes of the Bronchiae". Laennec further described the syndrome in his "De l'Auscultation Mediate", though he referred to it as chronic mucous catarrh. Howell (1951) points out that neither of these authors gave prominence to the later cardiac complications of bronchitis, for it was left to Mackintosh in 1931 to note that chronic bronchitis frequently coexisted with diseases of the heart and was a cause of dropsical affections. Watson, in 1948, provided a unified definition of chronic bronchitis as cough, some shortness of breath and expectoration of altered mucus.

It is only in recent years that precise definitions of chronic bronchitis have been formulated. These have been valuable clinically in aiding differential diagnosis, and also in epidemiological studies in distinguishing early cases from the normal population. Scadding (1963) advocated a definition based on symptoms. The Ciba Guest Symposium in 1958 also adopted a symptomatic definition of chronic bronchitis as a chronic or recurrent cough with expectoration on most days for at least three months in the year during at least 2 years.

To ensure accuracy in history-taking, Fletcher et al (1959) investigated the value of a questionnaire on the symptoms of bronchitis

using standard questions and methods of recording. Subsequently, the Medical Research Council Committee on the Aetiology of Chronic Bronchitis issued in 1960 a short and a long questionnaire to many workers, and in 1965 they issued their views on definitions and varieties of chronic bronchitis, based on the questionnaire (M.R.C. 1965). They recommended the use of "Chronic bronchitis" to cover all sufferers and all stages of the disease. "Chronic or recurrent mucopurulent bronchitis" was recommended for those with purulent or mucopurulent sputum, and "chronic obstructive bronchitis" for those with evidence of generalised airways obstruction. "Chronic or recurrent cough with expectoration" implied its occurrence on most days during at least three months for more than two successive years. Localised and specific generalised lung diseases, primary cardiovascular and renal diseases were excluded. "Chronic obstructive bronchitis" was defined as "chronic bronchitis in which there is persistent widespread narrowing of the intrapulmonary airways, at least on expiration, causing increased resistance to airflow.

The questionnaire on which these definitions were based has been revised (M.R.C. 1966) and has been accepted for field work by the American Thoracic Society and the Coal and Steel Community of Western Europe.

THE CLINICAL SYNDROME OF CHRONIC BRONCHITIS

The basic characteristics of patients with chronic bronchitis

were described by Oswald et al (1953) in a series of 1,000 hospital patients, and by Fry (1954) in 127 patients presenting in general practice. Males were more common, although this predominance lessened in the older decades. The highest incidence in general practice was in men aged 60-70 years, and in hospital practice was in those aged 50-60 years. Oswald pointed out the relatively large numbers of middle-aged patients. Their early history included attacks of acute bronchitis or pneumonia in childhood, but the onset of their chronic bronchitic symptoms had been insidious. Even in the early years, the tendency of head colds to go down to the chest was marked. Fry remarked that the characteristic progression of the disease was for a productive cough in the winter to increase until it lasted all the year round, and was accompanied by acute exacerbations, with increase of all symptoms, during the winter months. Breathlessness progressed in severity with age, but nonetheless was found in 75% of those aged less than 50 in Oswald's series. The breathlessness progressed in severity until a state of respiratory and cardiac failure was reached.

The physical signs of chronic bronchitis are the signs accompanying the phase of airways obstruction. They have been fully described by Howell (1951), Barach and Bickerman (1956) and Stuart-Harris and Hanley (1957) and will not be described here.

The pathology of chronic bronchitis has been described by Reid (1954). Study of biopsy specimens and necropsy material showed that the primary changes were hypertrophy of the mucus secreting glands

of the bronchial wall and hyperplasia of goblet cells in the epithelium. Advanced changes included obliteration of bronchioli, with disappearance of alveoli or replacement by fibrous scars, with inflammatory changes including abscesses in small bronchioli. Engel (1958) considered that cylindrical bronchiectasis was usually present, and most authors agree that localised areas of bronchial wall dilatation are a common finding in chronic bronchitis, and squamous metaplasia is frequent (Spain, 1959, Glynn and Michaels 1960), Kourilsky and Hinglais 1961).

Reid (1960) introduced an index of chronic bronchitis based on the ratio of the thickness of the bronchial gland layers to the total width of the wall from cartilage to epithelial surface of main bronchi. The ratio did not exceed 0.36 in the normal and ranged from 0.4 to 0.79 in chronic bronchitis and from 0.33 to 0.7 in chronic bronchiectasis. Thurlbeck et al (1963) and Field et al (1966) used the same method to show a relationship between the size of the index and the history of cigarette smoking in life. The variation in mucous gland hypertrophy in various sites in the bronchial tree has been studied by Thurlbeck and Angus (1967) using the Reid index and by Restropo and Heard (1963) using an alternative method based on the measurement of the area of the bronchial mucous glands in transverse sections of bronchi. The mucous gland hypertrophy extends from trachea to segmental bronchi and is proportionally greater in segmental bronchi.

ROLE OF INFECTION

Clinical studies (Oswald et al 1953, Fry 1954) have emphasised the frequency with which clinical episodes of bronchial infection are associated with chronic bronchitis in its early stages and subsequently, and laboratory studies confirm this.

The bacteria found in the sputum have been described by Stuart-Harris et al (1953) and May (1953), who found that pneumococci and Haemophilus influenzae were the most frequent pathogenic bacteria. Many workers have confirmed these results, although results obtained by cultivation of a single specimen of sputum are open to sampling errors, and may give inconsistent results. This pattern does not alter during exacerbations of the disease, but Cooper et al (1961) have shown that an increase in count of Haemophilus influenzae in sputum occurs during acute exacerbations, and also, to some extent, of Pneumococci. The bronchi in health are sterile (Brumfitt and Willoughby 1958, Lees and McNaught, 1959), but in bronchitics the flora in the bronchi tends to be the same as in the sputum, though often differing from that in the nasopharynx (Brown et al 1954).

Amongst chronic bronchitics there is a high incidence of serum antibodies to H. influenzae antigens (Glynn 1959, Morgan and Wood 1965), May 1965, Burns and May 1967). These are mostly of the IgG and IgM classes, and recently have been shown to be present in increased frequency in smokers without bronchitis (May et al 1974).

Viral infections appear to be associated with many exacerbations of chronic bronchitis. Influenza virus can be recovered from bronchitics during acute illnesses, and fatal illness increases in bronchitics during influenza epidemics (Stuart Harris et al 1953). It is well known that respiratory viruses may cause acute chest infections in patients with chronic bronchitis. Respiratory syncytial virus (Somerville 1963, Carilli et al 1964), parainfluenza and influenza viruses (Stark et al 1965) and rhinoviruses (Eadie et al 1966), have all been shown to cause acute lower respiratory infections in patients with chronic bronchitis.

INTEGRITY OF NORMAL DEFENCE MECHANISMS

The above findings do not necessarily imply that bronchial infection is the cause of the hypersecretion of mucus. The apparently entrenched position of bacteria in the lower respiratory tract could also be the result of impairment of one or more of the various defence mechanisms.

Clearance of inhaled bacteria in animals is inhibited by cold, hypoxia and alcohol (Laurenzi et al 1962, Green and Kass 1965) killing of organisms by macrophages in vitro is inhibited by cigarette smoke (Green and Carolin 1967). Mechanical clearance of inhaled particles may be inhibited by cigarette smoke (Rylander 1968, Carson et al 1966).

Serum immunoglobulin levels in chronic bronchitics tend to be normal or raised (Biegel and Krumholtz 1968, Falk et al 1970), although rarely there may be low serum IgA or IgG values (Falk et al 1972, Seigler and Citron 1974). Falk et al (1972) studied the immunoglobulins in the bronchial washings of a small number of subjects with chronic obstructive bronchitis or emphysema and found that IgA was secreted actively into the bronchial secretions in all cases except one who had a systemic deficiency of IgA. They concluded that bronchial secretion of IgA was not deficient in chronic bronchitis. However, further examination of their figures does show that the IgA/total protein ratios in the sputum of two of the chronic bronchitics were lower than any of six normal subjects.

Medici and Buergi (1971) have made serial measurements of the sputum IgA during infections in three groups of chronic bronchitics distinguished by the duration of their bronchitic symptoms. They found that the resting levels of sputum IgA were lowest in the groups with bronchitis for many years and that, unlike those with bronchitis of short duration, the long-standing bronchitics were unable to increase their sputum IgA in response to natural infections. Further confirmation of this was found by Deuschland Johannsen (1974) who, in the course of measuring local secretion of IgA in the bronchial tree in normal subjects and a variety of diseases, calculated that the IgA in the sputum of two chronic bronchitics was largely derived from serum by exudation through inflamed mucosal surfaces, and that there was abnormally little local secretion of IgA. If these results are confirmed, a local deficiency of IgA secretion in the

bronchi may be one of the mechanisms permitting bronchial infection in chronic bronchitis.

RESPIRATORY AND CARDIAC COMPLICATIONS OF CHRONIC BRONCHITIS

Although many individuals suffering from sufficient cough and sputum to justify a diagnosis of chronic bronchitis have no measurable abnormalities of respiratory function (Bates et al 1962), in those complaining of recurrent chest infections or breathlessness, some abnormalities of respiratory function are almost always detectable.

There has been a vast body of work on the physiological abnormalities to be found in chronic bronchitis (see Bates and Christie 1973, Stewart-Harris 1968). The predominant abnormality is airways obstruction, and measures of this correlate very approximately with the level of dyspnoea on exertion. This airways obstruction is largely irreversible, and is accompanied by a variable amount of hyperinflation and reduced compliance of the lungs.

Arterial hypoxia may be present at rest or only on exertion, and the arterial and venous carbon dioxide may be raised. The diffusing capacity of the lungs is usually normal in the absence of emphysema, except when heart failure supervenes. In advanced cases, abnormalities of blood gas tensions may become severe, and pulmonary

hypertension and cardiac failure complicate the picture. Mortality is related to the degree of impairment of lung function and its rate of progression (Bousby and Cotes 1964, Sutculmalchantra and Williams 1965, Renyetti et al 1966) and death is from respiratory and cardiac failure.

PREVALENCE OF CHRONIC BRONCHITIS

There have been many studies of the prevalence of chronic bronchitis, and these have been reviewed by Stuart-Harris (1968). Estimates of the prevalence of persistent cough and/or sputum in Britain range from 6% to 10% in non-smokers, 13% to 38% in those smoking up to 14 cigarettes a day, and from 26% to 51% in those smoking 15 or more cigarettes a day. The prevalence increases with age, and tends to be higher in men in the unskilled social classes (Higgins et al 1956, Higgins 1957, Clifton 1957, Higgins and Cochran 1958, Fletcher and Tinker 1961, C.G.P. 1961). Studies in the U.S.A. give similar results except that the overall prevalence is lower, and the rising prevalence with increasing age is much less marked.

Mortality from chronic bronchitis was studied by Goodman et al (1953). They pointed out the male predominance, and suggested this might be due to smoking habits. Using the Registrar-General's statistics for 1931, they found a graded relationship between occupational factors and mortality from bronchitis such that Class I

exhibited the lowest, and Classes IV and V the highest. The report of the WHO Epidemiological and Statistical Section (1953) listed England and Wales, Ireland and Scotland at the head of the table of mortality from chronic bronchitis, with the U.S.A. and Scandinavia at the foot. Even though the inaccuracy of death certification as a method of diagnosis casts some doubt on the reliability of these figures, a twenty fold difference in mortality between Finland and England suggests the existence of strong adverse environmental factors in this country. This is supported by evidence that British migrants to the U.S.A. exhibit a lower rate of respiratory symptoms than would be expected if they had stayed in Britain (Reid et al 1966).

AETIOLOGY

The Effect of Smoking

Goodman et al (1953) suggested that the higher mortality from bronchitis in men might be due to smoking habits, and the existence of a strong relationship between smoking and bronchitis mortality has been confirmed by Dean (1966) in Northern Ireland, Crofton and Crofton (1963) in Scotland and by Doll and Hill (1964) in British doctors.

Oswald et al (1953) found among their series of hospital patients a much higher incidence of smokers among bronchitics than controls, and in all the epidemiological studies quoted in the previous section, smoking was found to be strongly associated with persistent cough and sputum.

In the experimental situation, exposure of rats to cigarette smoke has been shown to cause an increase in bronchial mucous glands and goblet cells (Mellors 1958, Lamb and Reid 1969) and to inhibit ciliary action (Hildwig 1956, Dalham 1966, Ballenger 1960).

Bronchoconstrictor responses to cigarette smoke have been demonstrated in guinea pigs (Loomis 1956, Carson et al 1965) and in man (Nadel and Comroe 1961, Samel et al 1963). Airways resistance tends to be higher in smokers than non-smokers (Nadel and Comroe 1961.) The effect on airways resistance of smoking a single cigarette is greater in smokers than non-smokers, and this can be blocked by atropine (Sterling 1967), suggesting that the effect is one of bronchoconstriction in response to irritation.

Many authors have found the changes of airways obstruction among smokers, and these changes are qualitatively indistinguishable from those of chronic bronchitis or emphysema. Even in "college age" students, there is an increased prevalence of cough, morning sputum and wheezing in smokers compared with non-smokers (Peters and Ferris 1967).

Atmospheric Pollution

Goodman et al (1953) found that mortality from bronchitis was higher in large industrial areas than medium or small towns, and

these in turn exceeded the rate for rural areas. These regional differences suggested the importance of the environment in relation to death from bronchitis, and this was reinforced by the London smog of 1952, in which an excess of 4,000 deaths occurred during and after the four day smog, mostly in those already suffering from chronic bronchitis, emphysema or myocardial disorders. This phenomenon has been observed repeated in smogs in London (Logan 1956, Bradley et al 1958, Martin and Bradley 1960, Scott 1963), Sheffield (Clifton et al 1960) and New York City (McCarroll and Bradley 1966).

An analysis of death rates from bronchitis in county boroughs in England and Wales showed a correlation with measures of atmospheric pollution (Stocks 1959, Pemberton and Goldberg 1954).

The prevalence of persistent cough and/or sputum has also been shown to be higher in urban compared with rural populations in Britain (C.G.P. 1961, Higgins et al 1956, Higgins 1957, Higgins and Cochran 1958). The lower prevalence of chronic bronchitis in outside telephone workers in Washington, Baltimore and Westchester, U.S.A. than in three equivalent English towns, Peterborough, Norwich and Gloucester (Holland and Stone 1965) were believed to reflect the influence of atmospheric pollution in Britain as shown by comparative figures for particulate matter and sulphur dioxide in America and Britain (Holland 1966).

There is no proof that the increased concentration of smoke

particles or of sulphur dioxide occurring during smog are the only or the essential cause of clinical effects, but there is evidence that exposure of rats to large concentrations of sulphur dioxide causes an increase in goblet cells in the bronchi (Reid 1963). Exposure of rats to air to which high concentrations of atmospheric pollutants (oxidants) have been added causes an increase in airways resistance, although "ordinary" Los Angeles air does not have this effect (Swann and Balchum 1966). The acute inhalation of sulphur dioxide in man causes no measurable effects even in high doses, except in those with asthma or bronchitis. Inhalation of various inert dusts causes an increase in airways resistance in normal subjects (Dubois and Dautrebande 1958), and Norris and Bishop (1966) showed that inhalation of calcium carbonate dust caused significant changes in the distribution of ventilation in about half of the normal subjects and most of those with chest disease.

Occupation

The influence of occupation on the prevalence of chronic bronchitis is often difficult to separate from the effects of smoking and social class. The Registrar-General's analysis (1958) of the information obtained in the 1951 Census listed occupations such as filers, file cutters and tool grinders in which the mortality from bronchitis was more than twice the national average. Lowe (1968)

compared two groups of occupations all in social class III and pointed out that occupations with a high mortality from bronchitis are in general those with exposure at work to atmospheric pollutants such as smoke, fumes or dust. The wives of such workers also show an excess mortality from bronchitis, and this emphasises the importance of socio-economic circumstances.

Heredity

There does seem to be a familial tendency towards chronic bronchitis. Oswald et al (1953) found that the relatives of hospital patients with bronchitis were three times as likely to have symptoms of bronchitis as those of non-bronchitic patients. Ogilvie and Newell (1957) found that among the citizens of Newcastle upon Tyne, twice as many of the parents and siblings of persons with chronic bronchitis were reported to have the symptoms of bronchitis as the relatives of controls. This has been confirmed in other studies (Clifton, 1957, Layland 1964). A recent study of respiratory symptoms among school-children showed that symptoms were common among children of parents with persistent cough, and were more strongly associated with the parents' chest symptoms than their smoking habits (Colley et al 1974).

However a familial incidence may merely reflect the influence of socio-economic conditions. Efforts to throw light on genetic

factors have been made. An examination of the blood groups of chronic bronchitic patients showed a normal distribution (Stuart-Harris 1965). A comparative study of patients admitted to the Veterans Hospital, Washington (Massaro et al 1965), showed that negro patients had half the prevalence of chronic bronchitis compared with whites, in both smoking and non-smoking categories.

Hereditary deficiency of Alpha, antitrypsin, an inhibitor of proteolytic enzymes, predisposes to emphysema (Laurell and Ericksson 1963, Ericksson 1965, Kneppers et al 1964), but not to chronic bronchitis.

A prospective co-operative study of respiratory function in a population in the U.S.A. has recently been reported by Bates (1974). While the lung function of most of the smokers declined at the rate to be expected by aging, the lung function of a small group (10%) deteriorated at a much more rapid rate, and the only bronchitis mortality was in this group. The only other identifying factor of this group was that they were amongst the heaviest smokers.

CHAPTER 4

MATERIALS AND METHODS

1. Subjects
2. Tissue Samples
3. Immunofluorescent Methods
   - (a) General principles
   - (b) Preparation of specific antisera
   - (c) Fluorescein conjugation
   - (d) Non-specific fluorescence
   - (e) Tests of potency and specificity of the antisera
   - (f) Preparation of tissue sections
   - (g) Staining methods
   - (h) Microscopy and cell counts
   - (i) Precautions to ensure accuracy of cell counts and avoidance of bias
4. Examination of formalin-fixed tissues
5. Statistical analyses
6. Further studies:- sputum immunoglobulin estimations in chronic bronchitic and control subjects.

The anatomical distribution and immunoglobulin class of plasma and other cells containing immunoglobulin in the respiratory tract have been studied by immunofluorescent methods in necropsy material from nine normal subjects and eleven subjects with chronic bronchitis.

1. Subjects

(a) Normal Subjects

Necropsy material was obtained from nine subjects known not to have respiratory disease (and, particularly, not to have had cough or sputum). Medical histories were obtained from the general practitioners who made enquiries of the relatives where appropriate, and from hospital notes where these were available. Five were non-smokers and four did smoke. None were taking anticonvulsants. (Details of age, smoking history, cause of death and interval between death and fixation of tissue are set out in Table 2).

(b) Chronic Bronchitis Subjects

Necropsy tissues were obtained from six subjects with severe chronic obstructive bronchitis who died in hospital after being admitted for an exacerbation of their chronic bronchitis. All had severe airways obstruction, and were known to have had chronic bronchitis for many years (M.R.C. definition, 1969) (Table 3). Tissues were also obtained from five subjects with 'incidental' chronic bronchitis, whose death was not caused by respiratory disease, but who were said by their relatives to have had cough and sputum for many years. They had never complained to their general practitioners

TABLE 2

Clinical details of nine normal subjects.

SUBJECT (SEX)	AGE	SMOKING HISTORY	CAUSE OF DEATH	INTERVAL BETWEEN DEATH AND FIXATION OF TISSUE
1. (F)	58	Never smoked	Barbiturate poisoning	48 hours
2. (F)	65	Never smoked	Ischaemic heart disease	72 hours
3. (M)	60	Never smoked	Pulmonary embolism	24 hours
4. (M)	43	Non-smoker for 10 years; previously smoked 30/day for 10 years	Ischaemic heart disease	16 hours
5. (M)	52	Never smoked	Pulmonary embolism	40 hours
6. (M)	38	10-15 cig/day for 20 years	Head injury, instant-aneous death	24 hours
7. (M)	68	40/day for 40 years but pipe 4oz/ week for last 15 years	Ischaemic heart disease	48 hours
8. (M)	45	20 cig/day for 25 years	Head injury, instant-aneous death	72 hours
9. (F)	26	20 cig/day for 8 years	Barbiturate poisoning	48 hours

TABLE 3

Clinical details of six subjects with 'Fatal' Chronic Bronchitis.

SUBJECT (SEX)	AGE	SMOKING HISTORY	COUGH HISTORY	FEV (ml^1)	CAUSE OF DEATH	INTERVAL BETWEEN DEATH AND FIXATION OF TISSUE
15. (M)	66	40-60/day for 30 years, only snuff for last 8 years	Chronic cough & sputum for 20 years	550	Respiratory and cardiac failure	12 hours
16. (M)	50	20 cig/day for 25 years, 10/day for last 10 years	Chronic cough & sputum for 20 years	600	Respiratory and cardiac failure	12 hours
17. (M)	64	40 cig/day for 30 years, none for last 10 years	Chronic cough & sputum for 20 years	800	Respiratory and cardiac failure	40 hours
18. (F)	51	20 cig/day for 30 years right up to last illness	Chronic cough & sputum for 15 years	450	Respiratory failure	40 hours
19. (M)	54	20 cig/day for 20 years, none for 15 years	Chronic cough & sputum for 30 years	450	Respiratory and cardiac failure	48 hours
20. (M)	48	Never smoked	Chronic cough & sputum for 20 years	400	Respiratory failure	16 hours

TABLE 4

Clinical details of five subjects with 'Incidental' Chronic Bronchitis.

SUBJECT (SEX)	AGE	SMOKING HISTORY	COUGH HISTORY	FEV (ml^1)	CAUSE OF DEATH	INTERVAL BETWEEN DEATH AND FIXATION OF TISSUE
10. (M)	51	35 cig/day for 30 years	Chronic cough & sputum for 15 years	Not known	Ischaemic heart disease	48 hours
11. (M)	62	30-40 cig/day for 40 years	Chronic cough & sputum for 10 years	Not known	Ischaemic heart disease	72 hours
12. (M)	64	30 cig/day for 30 years	Chronic cough & sputum for 20 years	Not known	Ruptured abdominal aneurysm	48 hours
13. (M)	66	60 cig/day for 40 years, 20/ day for last 10 years	Chronic cough & sputum for 20 years	Not known	Head injuries (death instant-aneous)	36 hours
14. (F)	55	30 cig/day for 30 years. Gave up 5/12 before death.	Chronic cough & sputum for 10 years	Not known	Barbiturate poisoning	48 hours

of dyspnoea on exertion or frequent chest infections (except subjects 10 and 11, whose heart disease appeared to be the cause of their dyspnoea). Their respiratory function had not been measured. Clinical details are set out in Table 4. None were taking anticonvulsants.

2. Tissue Samples

At necropsy, an axial bronchial pathway from trachea to right posterior basal bronchus was dissected free, and this was divided and stored. Alternate blocks were snap frozen and stored at -70°C, or fixed in buffered formalin. Blocks were also taken from peripheral lung close to the pleura, and the complete subcarinal lymph node mass was dissected and frozen. Tracheal and bronchial rings too large to be sectioned whole were divided into three or four arcs, and these arcs placed parallel before freezing so that, when sectioned, the whole circumference of the airway would be included in the section. Where present, pharyngeal tonsillar tissue was dissected and frozen, and sometimes it was possible to obtain a blood sample from the common iliac vein.

After some initial assessment (see section on precautions to ensure accuracy of cell count), the sites chosen for examination by immunofluorescence were the upper one third of trachea, right main bronchus close to carina, right lower lobe bronchus, lung close to pleura and carinal lymph nodes. From each of these no less than

sixteen sections were examined by immunofluorescence, making a total of eighty sections per subject. These multiple sections and additional measures to ensure that sampling was reliable, are discussed in the sections on preparation of tissue sections, microscopy and cell counts, and precautions to ensure accuracy of cell counts and avoidance of bias.

3. Immunofluorescent Methods

(a) General Principles

The immunofluorescent technique was first described by A. H. Coons of Harvard Medical School (Coons et al, 1941). Subsequently, he and Kaplan showed that fluorochrome labelling allowed direct observation of the sites of antigen-antibody reactions (Coons and Kaplan, 1950).

The general principles are as follows:-
A fluorochrome such as fluorescein or lissamine rhodamine, which give off bright visible light when excited by ultra-violet light, is linked by chemical means to globulin known to have high antibody activity against the desired antigen. The resulting conjugate is applied to tissue sections, and the conjugated antibody combines with specific antigen present in the section. After washing away all uncombined conjugate, the presence of fluorescent staining indicates where the antibody in the conjugate has combined with specific antigen.

The method described above is the 'direct' method, when the fluorescent conjugate links directly with the antigen. In the 'indirect' methods, a sandwich technique is used, whereby the specific antibody is not itself labelled, but after it has been incubated with the section, is identified by the application of a second, conjugated antiserum directed against the first antiserum. This method is even more sensitive than the 'direct' method, and is also used in the detection of specific antibody in test sera applied to tissues of known antigen content (such as the detection of antinuclear antibody in human sera). In the work reported here, however, the direct method was not used, except in the testing of the specific antisera (described subsequently).

The precautions are related principally to the minimisation of non-specific fluorescence, and the fixation of tissues (Holborow and Johnson, 1973). Details of the method and the precautions taken are given in the sections below.

(b) Preparation of Specific Antisera

The antigens to be identified in this study, human immunoglobulins IgG, IgA, IgM and IgE, are antibodies, but they themselves have specific antigenic properties, and antisera can be raised against them. The antisera were obtained from Hyland laboratories and had been raised in the following manner:-
Purified immunoglobulin of appropriate class was obtained by ammonium sulphate precipitation and ion exchange chromatography of human

myelome serum, and this was digested with papain to separate the Fc and Fab portions of the molecules. The Fc portion contains only heavy chains, and its antigenic properties are specific to the immunoglobulin class. The Fab portion (the portion containing the antibody combining sites) contains light chains, as well as small portions of the heavy chains, and thus has antigenic determinants common to all the immunoglobulin classes. The preparation of Fc fragments was then injected at repeated intervals into goats, whose serum then became hyper-immune to the specific immunoglobulin glass. The use of hyper-immune sera is important in the reduction of non-specific staining. Antisera to human complement (BiC/BiA globulin) were prepared similarly. These sera were conjugated with fluorescein in this laboratory.

(c) Fluorescein Conjugation

Fluorescein is highly suitable for immunofluorescent work, as it gives extremely bright fluorescence when excited. A slight disadvantage is that the colour of the light it gives out (yellow/green) is close to the colour of the light given out by auto-fluorescence of some tissues, but this was not found to be a problem in bronchial tissues. The isothiocyanate is suitable for linking to protein molecules.

The antisera were conjugated with fluorescein isothiocyanate by the standard method described by Holborow and Johnson (1973) and

Nairn (1968), described fully in Appendix 1, but given in abbreviated form below.

Firstly the globulin in the antiserum was separated by simple precipitation with an equal volume of saturated ammonium sulphate solution and separated from the ammonium sulphate by gel filtration on a sephadex column. The globulin was mixed overnight with a measured quantity of fluorescein isothiocyanate, and the conjugated globulin subsequently separated from free fluorescein by further sephadex gel filtration.

The total protein content of the conjugate was estimated by the Lowry method (1951), and the fluorescein content by its spectrophotometric absorption (See Appendix 1.)

(d) Non-specific Fluorescence

Non-specific fluorescence may be caused by the presence of unreacted fluorescein, globulin excessively labelled with fluorescein, or high negative charge on the molecules of the conjugate (Holborow and Johnson, 1973). Purification of conjugates by gel filtration removes most of the unreacted dye, and removal of the remainder of this, and of excessively heavily labelled globulin molecules, and molecules with excess negative charge is achieved by absorption with tissue homogenate as described by Coons and Kaplan (1950). Pig liver powder was used for this, and absorption was carried out on each conjugate before use. The absence of residual free fluorescein

in the conjugate was confirmed by chromatography in thin-layer sephadex (Holborow and Johnson, 1973).

In order to achieve the correct proportions between conjugated globulin and fluorescein, it is important to use the correct amount of fluorescein. Fluorescein:protein molecular ratios (F:P ratio) over 5:1 produce too much non-specific staining to be usable (Holborow and Johnson, 1967). Too low an F:P ratio causes the specific staining to be too weak. The F:P ratios of the conjugates used in this study ranged from 2:1 to 4:1, these values being in the useful range. Undiluted conjugates still give much non-specific staining (caused by the high absolute fluorescein concentration), and require dilution before use (see below).

(e) Tests of Potency and Specificity of Antisera

The antisera were tested for potency and specificity before and after conjugation by immunodiffusion, immunoelectrophoresis and by application on substrates of known antigen content.

Immunodiffusion and Immunoelectrophoresis were used to check the potency and specificity of the antisera against human IgG, IgA and IgM. Diffusion of these antisera against normal human serum showed three distinct lines of precipitation without lines of identity (Fig. 5). Immunoelectrophoresis also showed separate and distinct precipitates in the IgG, IgA and IgM positions (Fig. 6).

Fig. 5

Immunodiffusion of specific antisera against normal human serum. (There is an irrelevant precipitin line between goat anti-IgG and goat anti-IgA.)

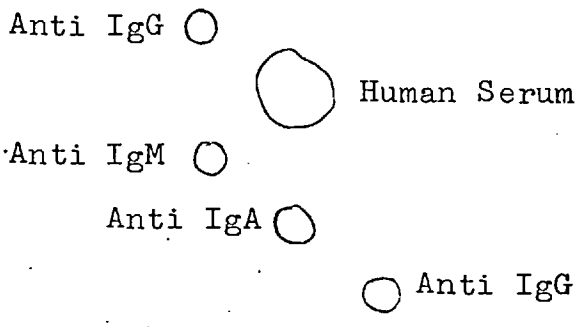
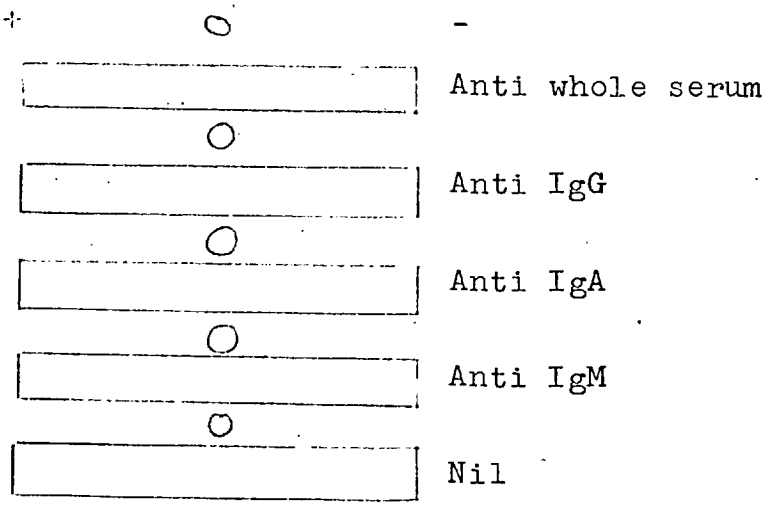
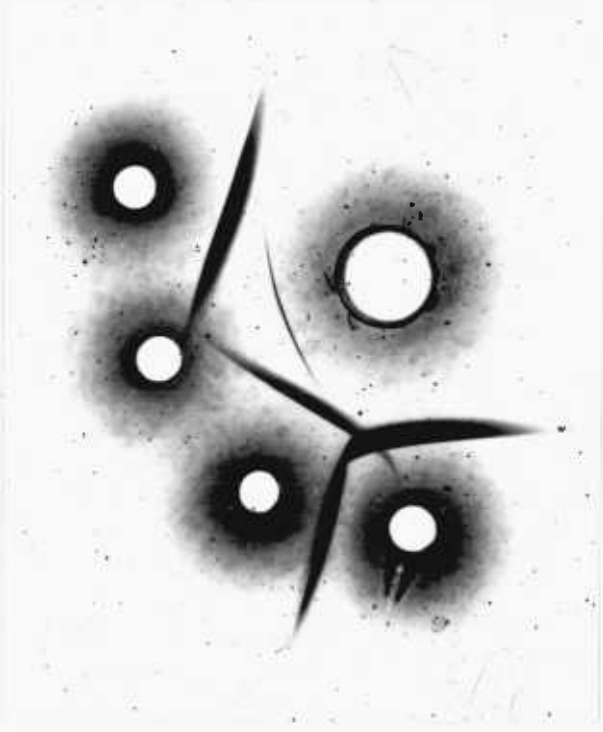
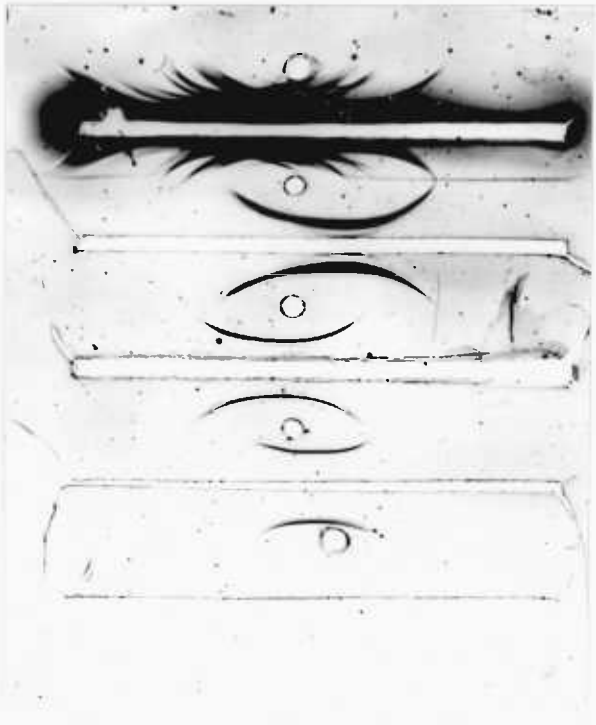


Fig. 6

Immunoelectrophoresis of specific antisera (in troughs) against normal human serum (in wells.)





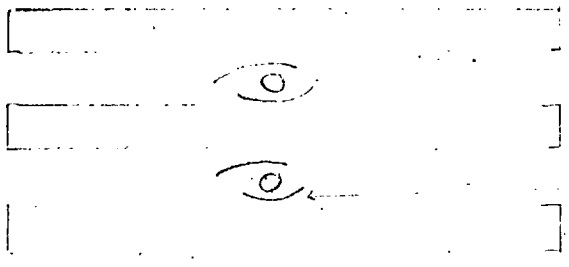


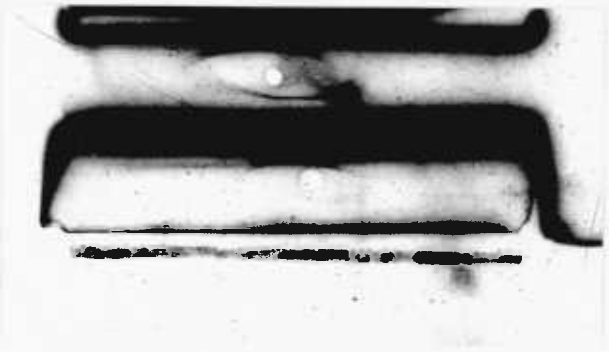
Normal human serum does not contain sufficient IgE for this to be easily demonstrable by conventional immunodiffusion and immunoelectrophoresis, and the application of the anti-IgE serum to a serum known to contain large amounts of IgE failed to produce a visible precipitate in the IgE position by these methods. Therefore the method of Rowe (Rowe, 1969) was used, in which a radiolabelled tracer is used to identify invisible precipitates. Immunoelectrophoresis of neat anti-IgE goat serum against human serum rich in IgE was carried out, and radio-iodine labelled anti-goat immunoglobulins produced in rabbits was added to the troughs. After diffusion over night, the plates were washed carefully and then laid over photographic paper, so that a contact print was obtained, which demonstrated the presence of a precipitation arc between the anti-IgE and the human serum (Fig. 7). No precipitation arcs were seen in the IgG, IgM, or IgA positions.

Further checking of the potency of the conjugated antisera was carried out on frozen sections of human tonsillectomy specimens, which contain easily recognisable germinal follicles, each manufacturing in this case a single class of immunoglobulin. Serial dilutions of the conjugated antisera were applied to these sections, and the highest dilution of each which gave strong specific staining of germinal follicles was chosen as the working dilution. At these dilutions non-specific staining was absent. The working dilutions were:-

Fig. 7

Contact radiograph of radio-immuno-electrophoresis, demonstrating activity and apparent specificity of anti IgE serum. The heavy serum staining is the result of the prolonged staining and exposure times required.





anti-IgG 1/8

anti-IgA 1/5

anti-IgM 1/4

anti-IgE 1/3

In addition, the specificity of the conjugated antisera was checked on frozen sections of rat liver to which human sera known to contain antinuclear antibody of specific immunoglobulin class had been applied. The method was the standard indirect sandwich technique for the detection of antinuclear antibody (Holborow and Johnson, 1973), and the conjugated antisera to be tested were the final layer. This work was done in collaboration with Mrs. P. Haslam of this Department and it showed that all the conjugates were specific except for the anti-IgE, which was never present in isolation, but only when IgG antinuclear antibody was also present (Fig. 8). Even though this activity was not abolished by prior absorption with purified IgG, it is possible that the anti-IgE conjugate also contained some weak activity against IgG. Subsequently conventional immunoelectrophoresis was again performed, using concentrated anti-IgE and concentrated IgE-rich serum, and this showed a precipitation arc in the IgG position (Fig. 9). It was not possible to obtain or purify sufficient purified IgG to absorb out this activity in the antiserum for all subsequent work because of the vast numbers of sections examined. The IgE cell counts may therefore have been augmented by contamination with anti-IgG activity, but they have been included because they represent useful maximum values.

Fig. 8 (a-d)

Testing of specific antisera on antinuclear antibody preparations from one individual. Specific fluorescence demonstrated by anti-IgM serum, but not by anti IgG, IgA or IgE.

Anti-IgG
a

Anti-IgM
b

Anti-IgA
c

Anti-IgE
d

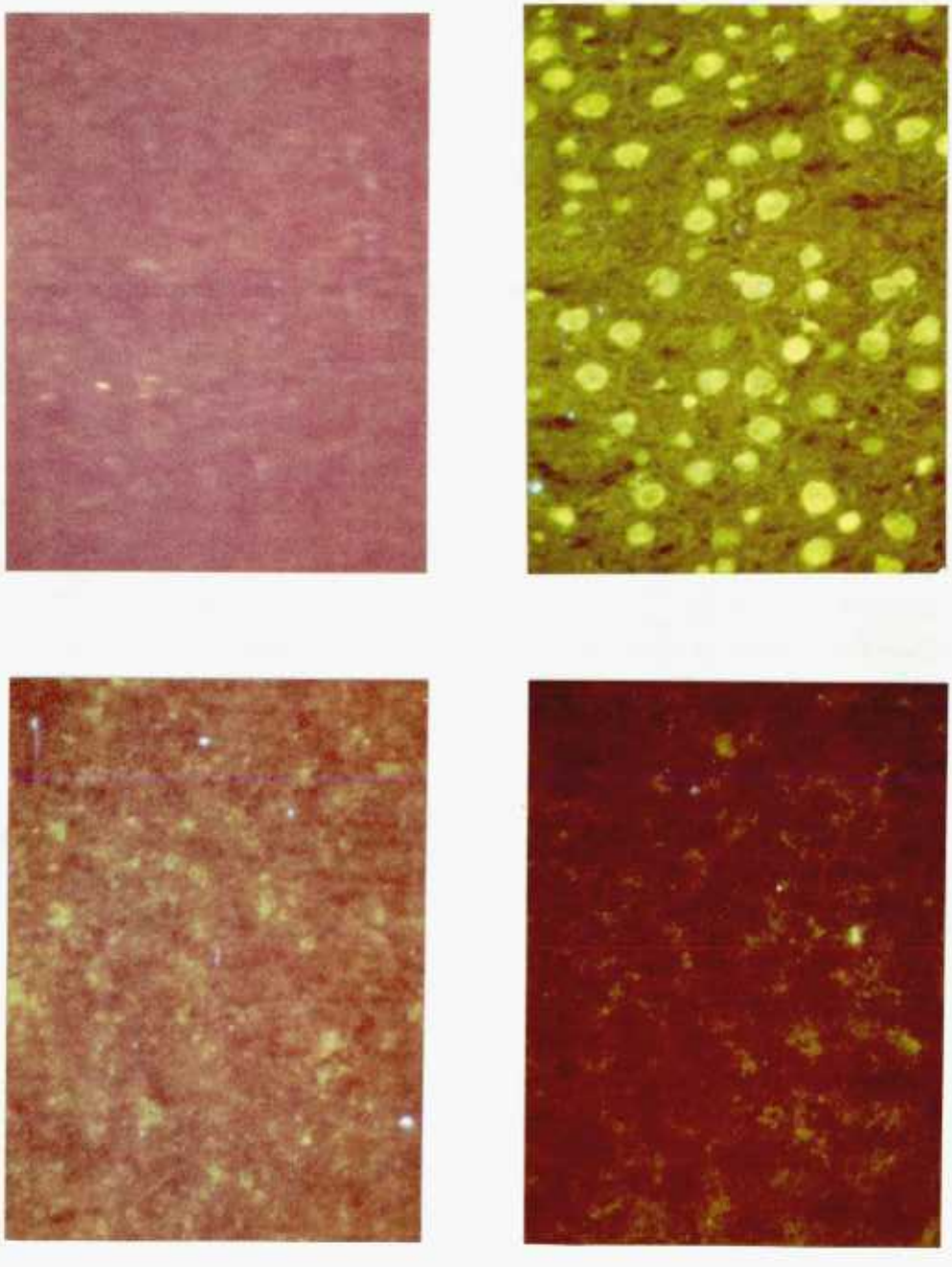


Fig. 8 (e-h)

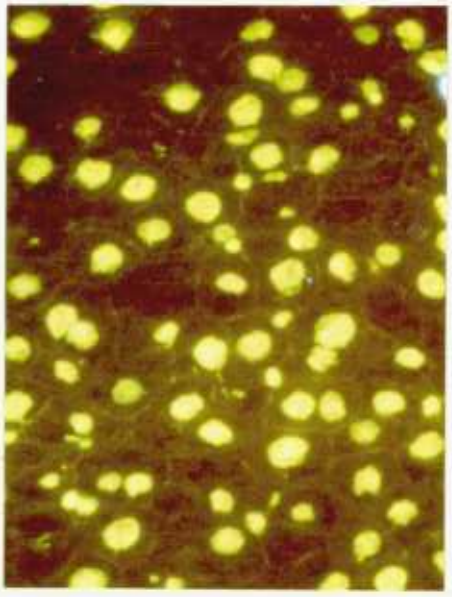
Testing of specific antisera on antinuclear antibody preparations from one individual. Specific fluorescence demonstrated by anti-IgG conjugate but not by anti-IgM or anti-IgA. Positive staining by anti-IgE conjugate may be specific staining or cross-reactivity.

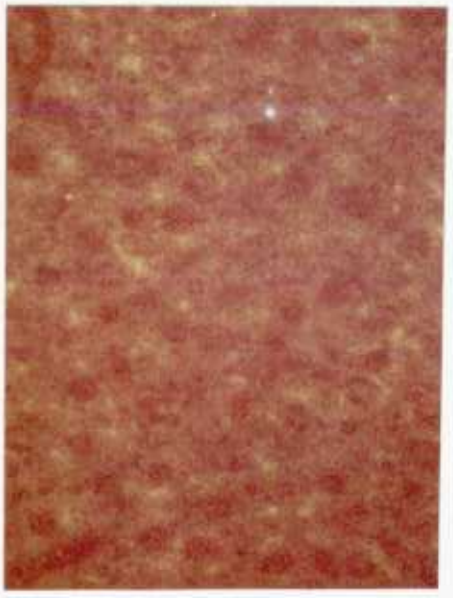
Anti-IgG
e

Anti-IgM
f

Anti-IgA
g

Anti-IgE
h





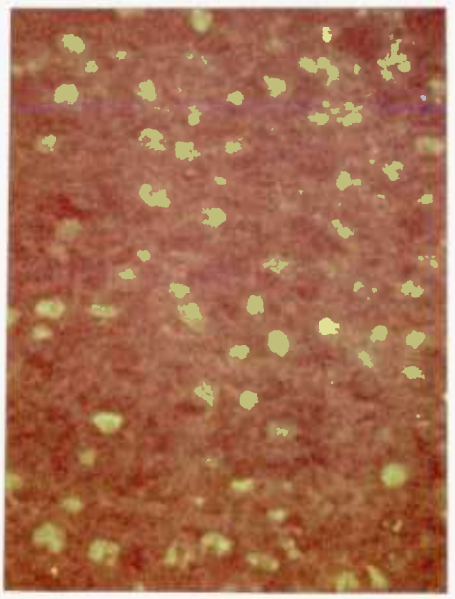
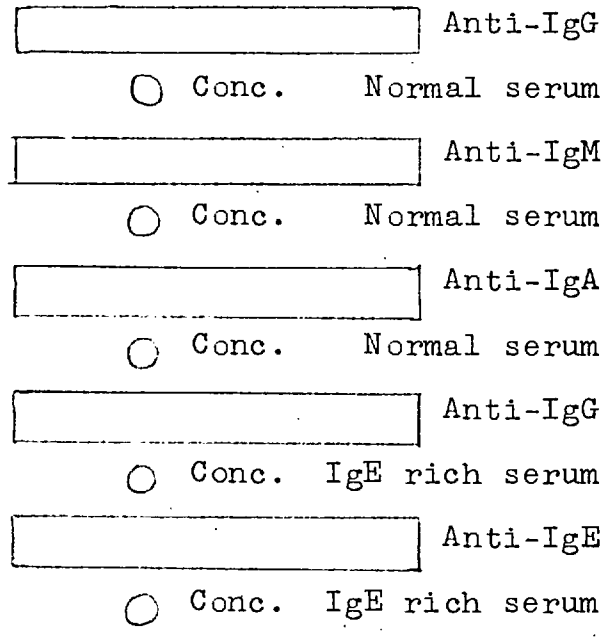
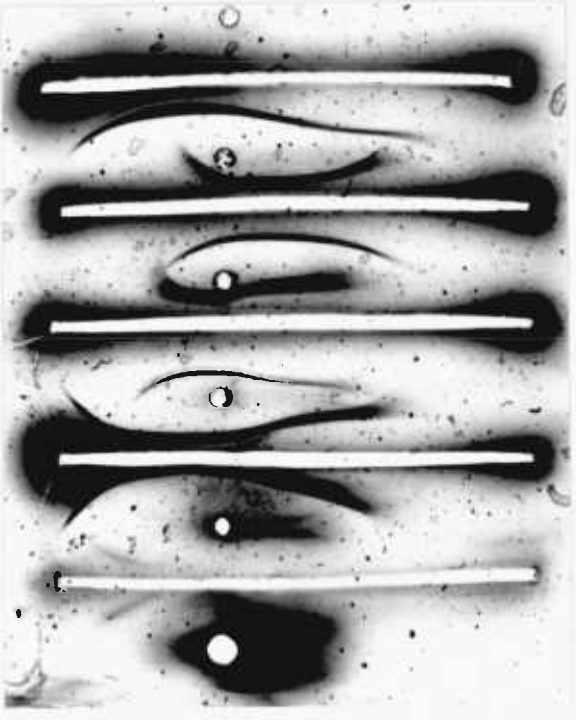


Fig. 9

Immunoelectrophoresis of concentrated anti-IgE serum against concentrated normal human serum, demonstrates a precipitate in the IgG position. The heavy serum staining and broad precipitin lines are the result of the concentrations of serum and prolonged diffusion times used.





The potency and specificity of the antiserum against human B_1C/B_1A component of complement was confirmed by application on positive anti-nuclear antibody preparations as above, but in which the serum containing antinuclear antibody had been mixed with fresh normal serum and buffer in the proportions 1:4:5 (instead of the usual 1:9 dilution in buffer), thus providing a source of complement. Application of the conjugated anti BiC/BiA as the final layer caused bright nuclear staining, whereas control sections in which the fresh serum had been incubated at 56°F for 30 mins. to destroy the complement were negative. The chosen working dilution was 1/4. Application to frozen sections of tonsil showed no specific or non-specific staining.

Positive staining on the sections was confirmed as specific immunological staining by a blocking procedure in which the sections were incubated with unconjugated specific antiserum and this prevented or substantially reduced subsequent staining by the conjugated antiserum by occupying the antibody combining sites (Fig. 10). Control sections used an inappropriate antiserum as the blocking layer; dilutions of antisera used for this procedure are given in Appendix II.

(f) Preparation of Tissue Sections

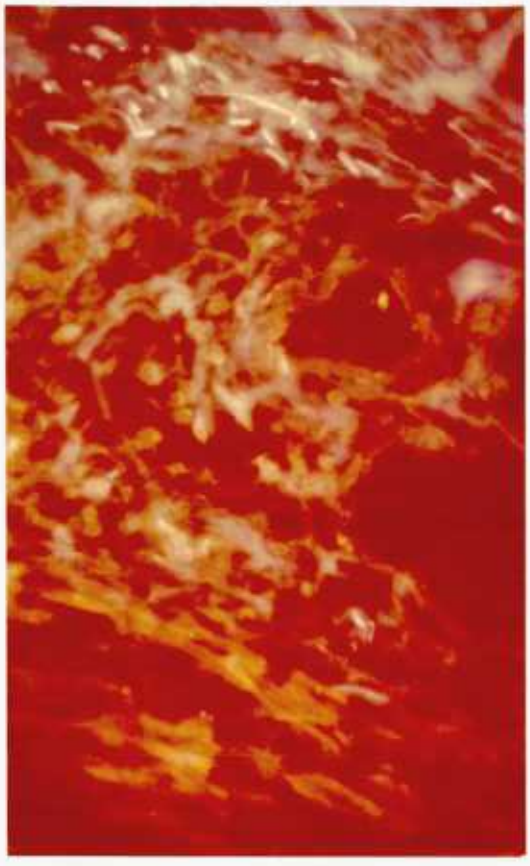
5 u sections of frozen tissues were cut on a rocking microtome in a refrigerated cabinet. No fixative was used except in one instance described below. Transverse sections including the whole circumference of the airway were cut. The presence of cartilage

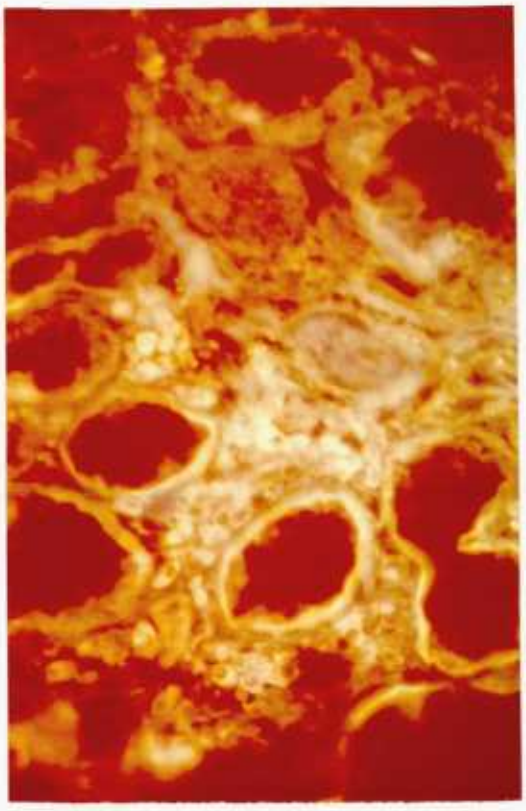
Fig. 10 (a-d)

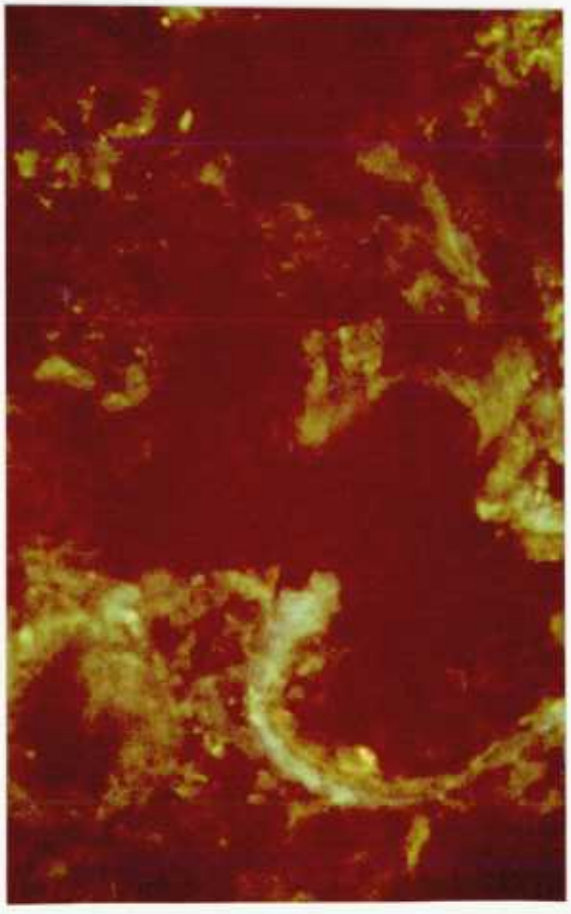
Testing of specificity of immunofluorescent staining by blocking procedure on bronchial sections. Reduction of brightness by blocking confirms specificity of conjugated antiserum. a and b are adjacent sections, c and d likewise. The staining on a and c has been reduced by the blocking procedure.

a		b	
First layer.	Unconjugated. Anti-IgA	First layer.	Unconjugated Anti-IgG
2nd layer.	Conjugated. Anti-IgA.	2nd layer.	Conjugated Anti-IgA.

c	d
as above	as above







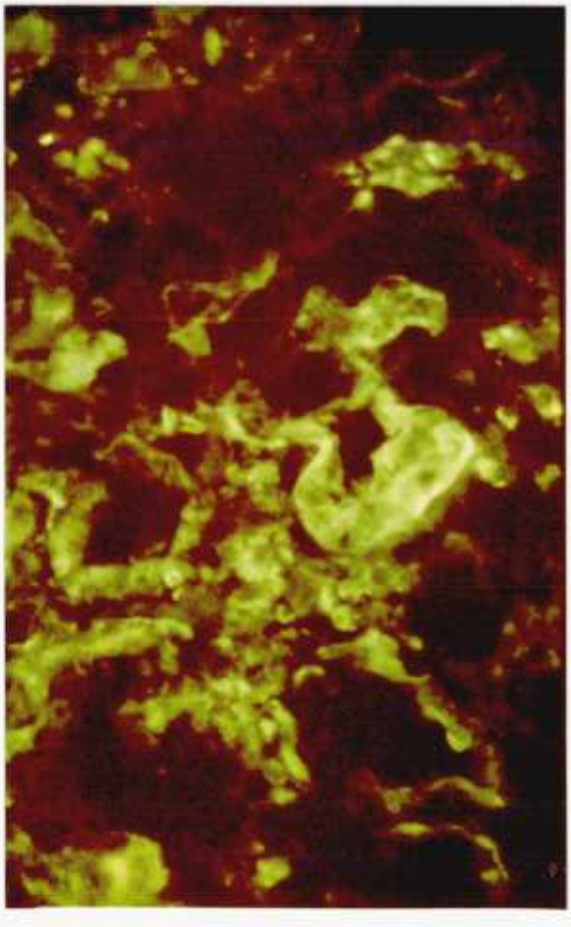


Fig. 10 (e-h)

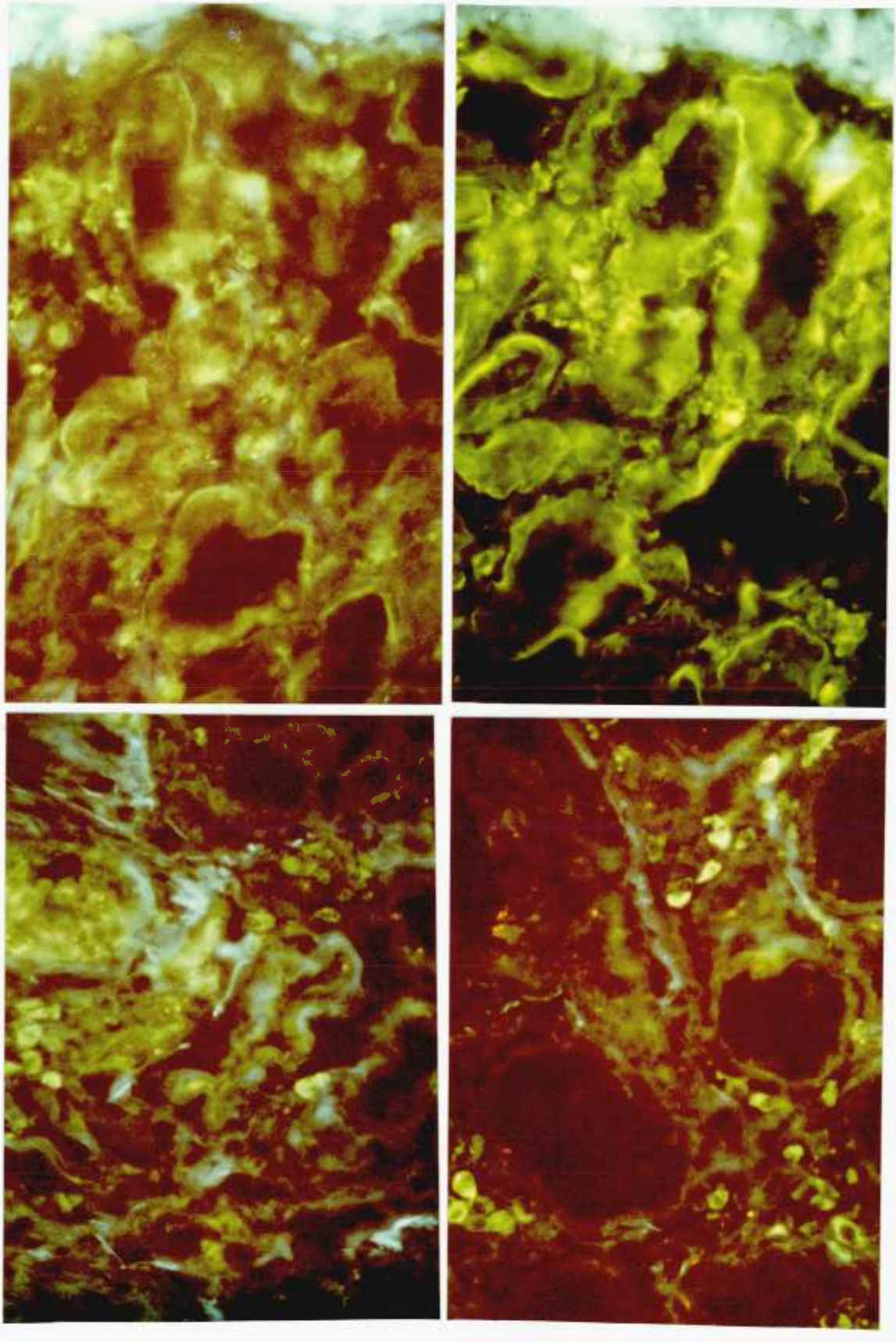
Testing of specificity of immunofluorescent staining by blocking procedure on bronchial sections.

e and f are adjacent sections, g and h likewise.

Staining on e and g has been reduced by the blocking procedure.

e		f	
First layer.	Unconjugated. Anti-IgA	First layer.	Unconjugated. Anti-IgG
2nd layer.	Conjugated. Anti-IgA	2nd layer.	Conjugated. Anti-IgA

g		h	
First layer.	Unconjugated. Anti-IgE	First layer.	Unconjugated. Anti-IgM
2nd layer.	Conjugated. Anti-IgE	2nd layer.	Conjugated. IgE



makes bronchus and trachea difficult to section, but this was made easier by cooling the tissue, knife and anti-roll device with solid carbon dioxide, and satisfactory sections containing the whole circumference of the airway were obtained from all tissues.

After some initial assessment, the sites chosen for examination by immunofluorescence were upper one third of trachea, right main bronchus close to carina, right lower lobe bronchus, lung close to pleura, and carinal lymph nodes.

The sections were transferred to glass slides at room temperature, and then fixed by drying in room air under a fan for an hour. As is described in the next two sections, multiple sections of each tissue were examined. It was not usually possible to cut serial sections of these frozen tissues, but one satisfactory section in every two or three was usually possible. These sections are referred to as near-serial. Carinal lymph nodes, often multiple, were bisected at their widest point, and sections taken from the cut surfaces of the two largest pieces. The nodes were weighed before sectioning.

(g) Staining Methods

The dried sections were first washed in Coons buffer (Coons and Kaplan, 1950) for ½ hour. This tends to wash away much of the serum present in the section. Irrigation during this procedure and subsequently was kept to a minimum to avoid washing away sections containing cartilage. This tendency of sections containing cartilage to wash away was also minimised by scoring the cartilage transversely with the point of a needle while drying. When washing, occasional

stirring of the buffer by sucking with a Pasteur pipette was found to be sufficient. The diluted immunofluorescent conjugate was applied over the sections and staining was for three-quarters of an hour, then the sections were washed as before, but for one hour. The sections were mounted in a 50% solution of glycerol in the buffer, and were examined within twenty four hours.

(h) Microscopy and Cell Counts

A Reichert-Zetopan microscope was used, with transmitted ultra-violet light. The lamp was the high pressure mercury vapour type. The exciter filter Schott BG 12/6 mm. was used with a dark ground condenser, the secondary filter being Wratten 2B/3 mm. Objective lenses were 1/20, 1/40, 1/60 and eyepieces were X8. Photographs were taken on high speed Ectachrome, and exposure times of between two and three minutes were required. Some of the photographs of carinal lymph nodes were taken on a Leitz Ortholux II using incident light (exciter filter 2XKP490, suppression filter TK445 and S525).

Bronchial sections were first examined generally to see the tissue morphology and check that the section was complete and correctly stained. Incomplete sections were discarded. Then the number of recognisable cells whose cytoplasm was stained by the fluorescent conjugate (except epithelial cells) were counted; the section was scanned systematically with the aid of a square graticule in the eyepiece of the microscope until the whole surface of the section had been covered. Thus all fluorescent cells in sixteen transverse sections of airway in each tissue block were

counted, there being blocks of trachea, main bronchus and lower lobe bronchus from each subject. Peripheral lung sections were examined to see the general appearances, though no cell counts were performed. The total number of sections examined exceeded 1,800 (see statistical analysis).

Examination of sections of carinal lymph node revealed that immunoglobulin was present either in the cytoplasm of single cells scattered through the substance of the node or as massive conglomerations in germinal follicles, either within or outside cells. Both these appearances were counted; the germinal follicles as the total number per section; the single cells by the following sampling technique:-

The section was scanned systematically until the whole section had been covered, the square graticule previously mentioned overlying consecutive square fields of the section; the number of stained cells in every fourth field was counted and recorded, excluding cells forming part of germinal follicles, so that by the time the whole section had been scanned, the cells in a quarter of the area had been counted. Where the fourth field fell on a fissure or on connective tissue, the next field was counted. Covering the whole section in this way usually involved counting in many more than twenty fields (up to a hundred), but where the node was so small that less than twenty fields were counted, the procedure was repeated, starting from a different part of the section. The projected area of the square graticule was .067 sq. mm. Twenty sections from

carinal lymph nodes in each subject were examined.

(i) Precautions to ensure accuracy of cell counts and avoidance of bias

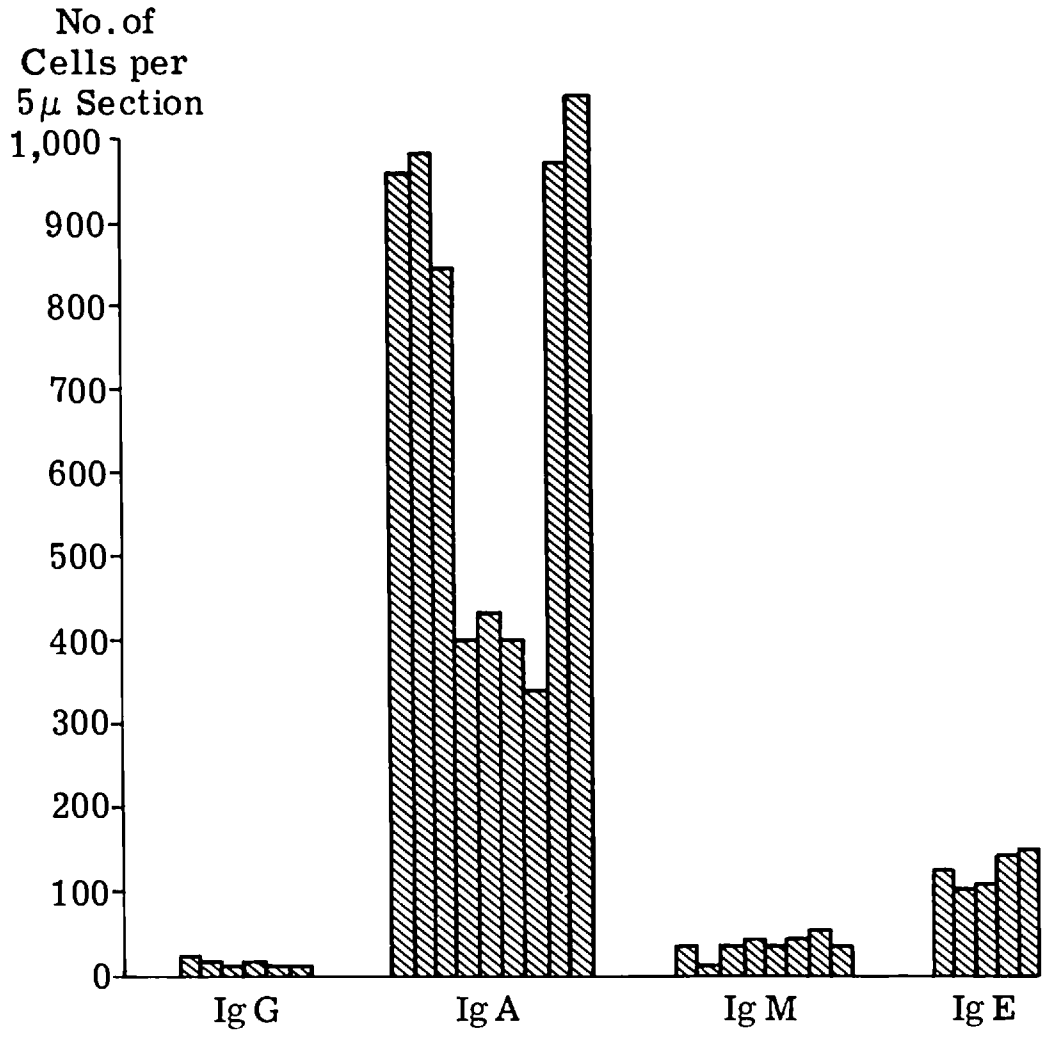
Staining of all sections for IgA, IgG, IgM and IgE was carried out in strict rotation, so that sections stained with identical conjugates were never immediately adjacent. In addition, sectioning and staining of each tissue was divided into two parts, and carried out on different days, thus lessening errors induced by slight differences in technique.

The slides were coded and read out of order. Three or more tissues were sectioned on each day, all from the same site, but from different subjects, to avoid recognition of the tissue by the observer. Cell counts were later examined for consistency, and the mean taken.

As a preliminary exercise to assess reproducibility of cell counts, thirty two near serial sections were taken from the trachea, and the same number from the main bronchus of a normal subject not included in the rest of the study (because of incomplete collection of tissues), and tissue stains and cell counts carried out as described above. The results set out in Figure 11 indicated that cell counting was quite practical and reproducability was within reasonable limits.

Fig. 11a

Cell counts on near-serial sections from a 2mm. length of trachea.

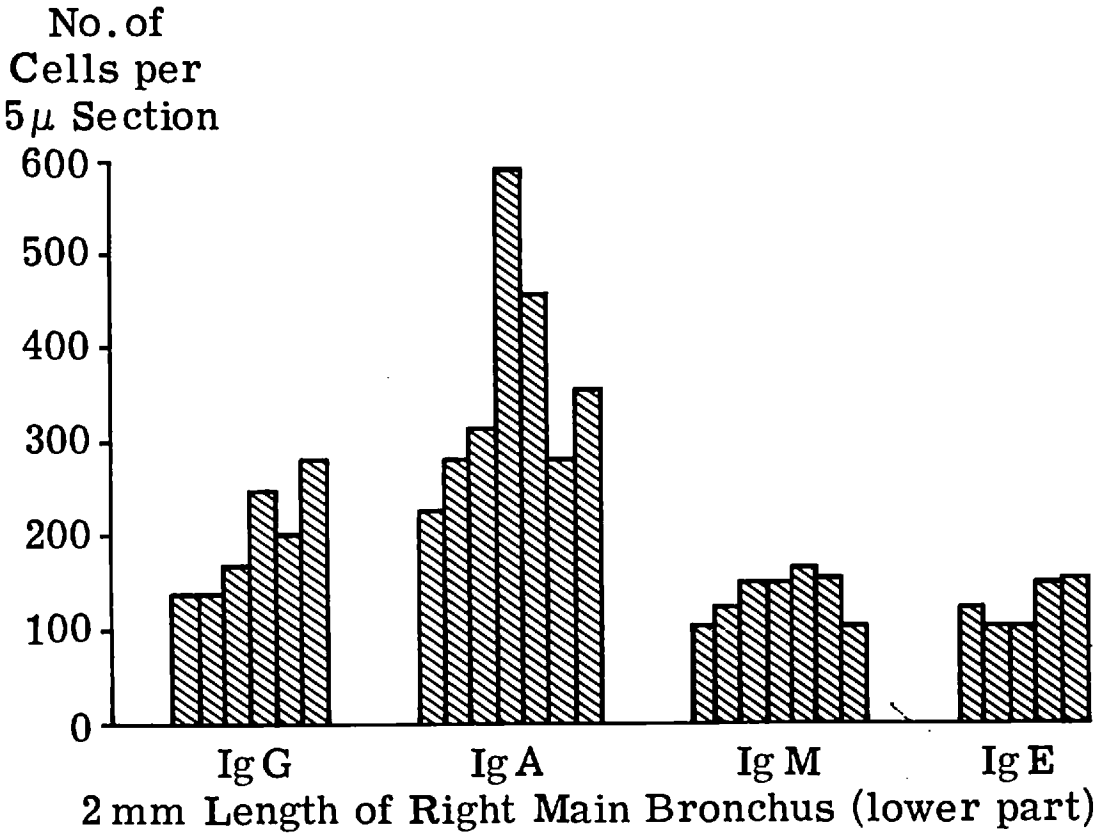


2 mm Length of Trachea

Significance of difference between Ig A & Ig E, $p << .005$,
between Ig E & Ig G, $p << .005$.

Fig. 11b

Cell counts on near serial sections from a 2mm. length of right main bronchus.



2 mm Length of Right Main Bronchus (lower part)

Significance of difference between Ig A & Ig G, $p < .01$, between Ig G & Ig E, NS.

Positive staining with the anti-C_3 complement conjugate was not seen in sections of airway, except for very occasional cells with granular cytoplasm, presumed to be eosinophils (which attract non-specific staining by fluorescein conjugates by virtue of their charge). Staining with this conjugate was therefore performed on only one section from each tissue. Further discussion of eosinophils in sections of airways is to be found in the paragraph on staining of paraffin sections.

Among the cells in carinal lymph nodes showing specific fluorescence with the anti-immunoglobulin conjugates, there were variable numbers of fluorescent cells whose cytoplasm was granular. Some of these had bilobed nuclei, though many appeared to have round nuclei, similar to those of plasma cells. However, all these cells were shown to be eosiniphils, for the non-specific nature of the staining on these cells was confirmed by double staining with bovine serum albumen conjugated with Lissamine Rhodamine (Nordic Laboratories) as well as fluorescein-conjugated anti-immunoglobulin. When these sections were viewed by incident light with filters suitable for visualising the fluorescein (yellow/green) light wavelengths (2 x KP490, TK455 and S525), staining was seen on both plasma cells and eosinophil-like cells. When the filters were changed to those suitable for visualising the rhodamine stain (red) (S546 + BG36, TK580 and K610), in the same fields, only the eosinophils were stained, confirming the non-specificity of the staining on these cells, (Fig. 12). The anti-C_3 complement conjugate was used

Fig. 12 a and b

Counterstaining of lymph nodes to show non-specific staining of eosinophils. Sections stained with fluorescein anti-IgA and rhodamine-borine serum albumin.

a.

Light filters, adjusted to visualise fluorescein staining demonstrate both IgA plasma cells and eosinophils.

b.

Identical field, filters adjusted to visualise rhodamine. IgA plasma cells no longer visible, but presence of non-specific staining of eosinophils confirmed.

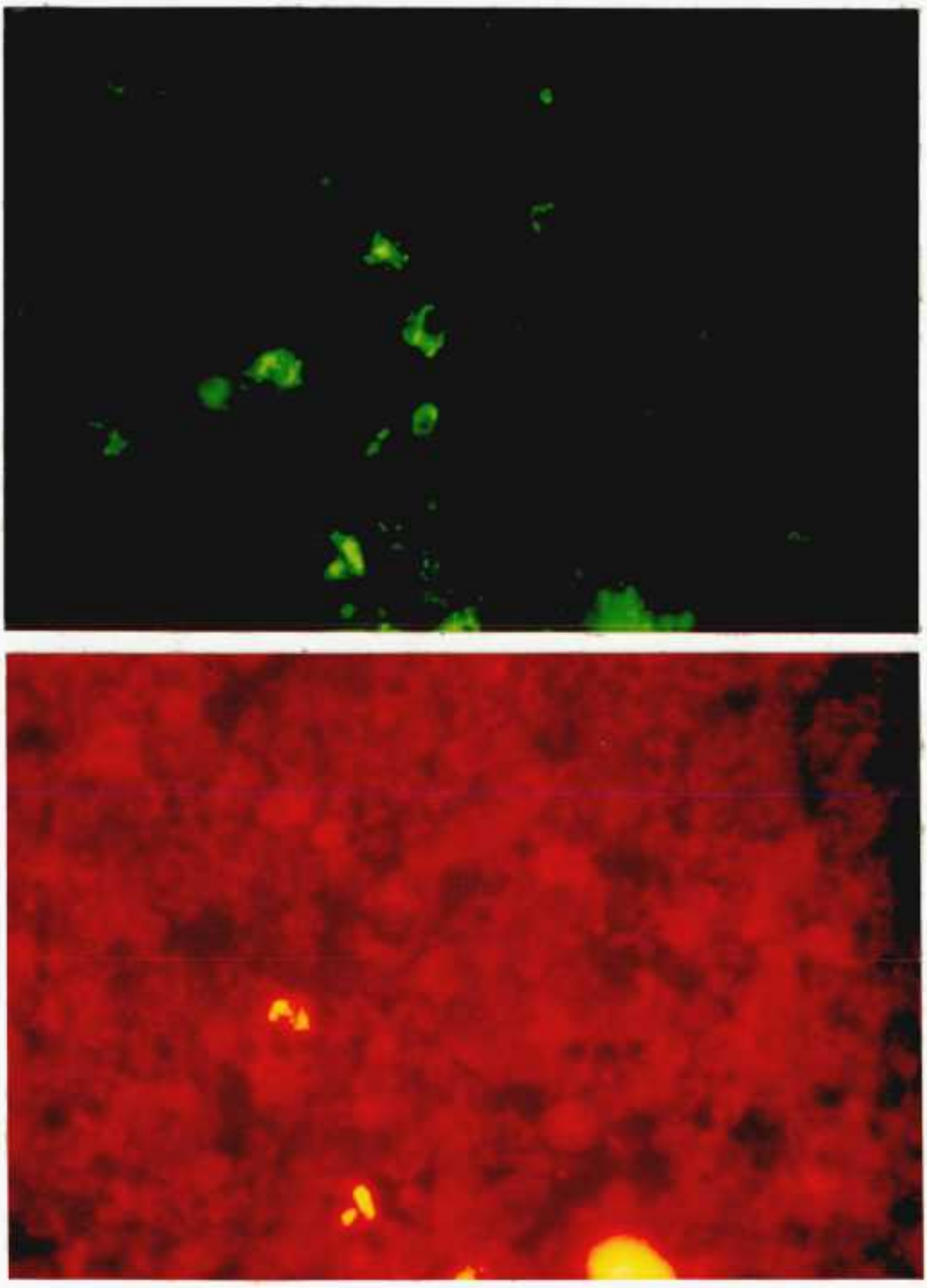


Fig. 12 c and d

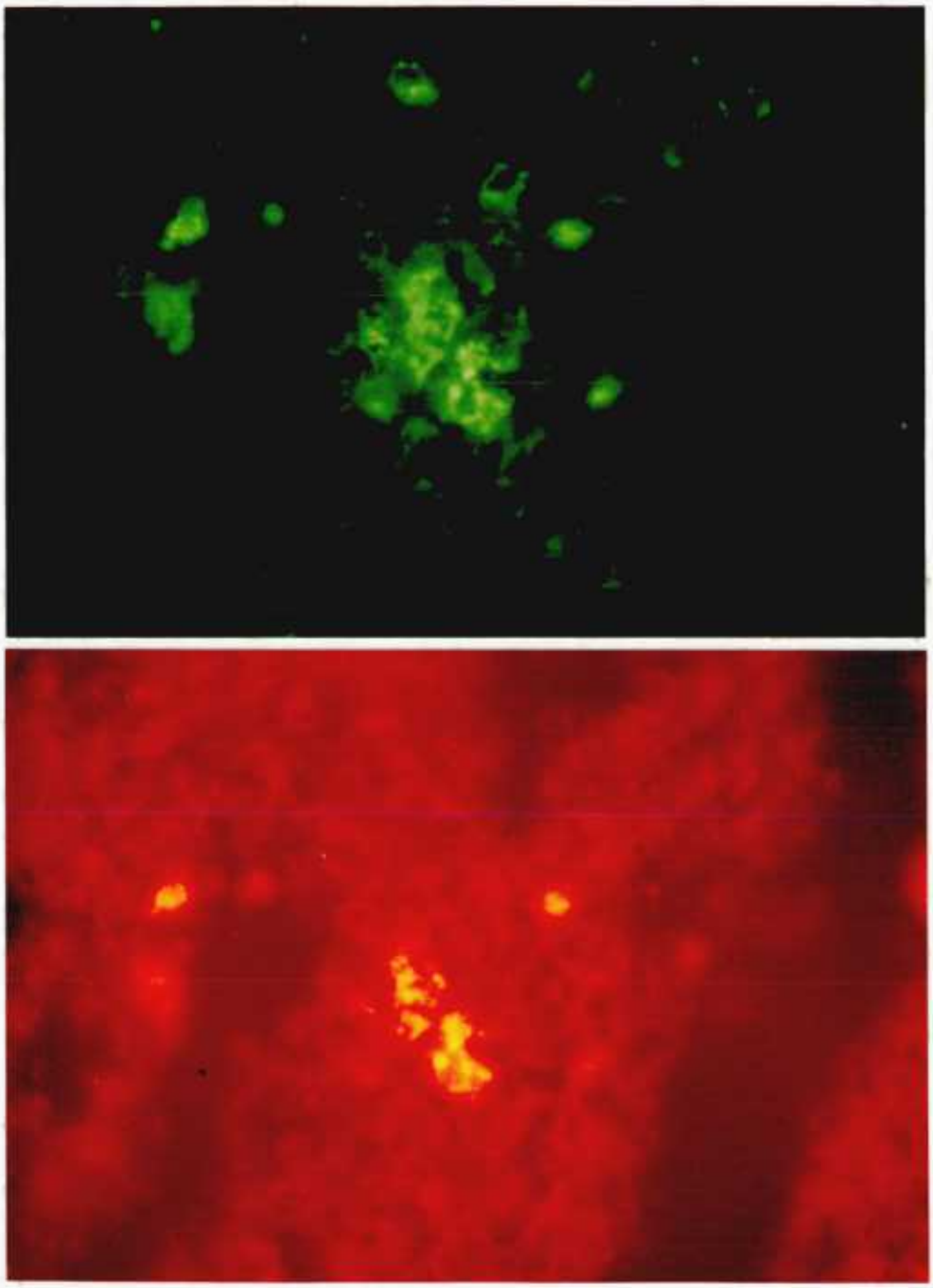
Counterstaining of lymph node to show non-specific staining of eosinophils.

c.

Filters show fluorescein staining. IgA plasma cells and eosinophils visible.

d.

Identical field. Filters show rhodamine staining. Only eosinophils visualised.



to gauge the extent to which non-specific fluorescence on eosinophils was influencing to plasma cell counts. To this end, two sections from each lymph node were stained with the anti-C_3 conjugate and the number of fluorescent cells counted in the same way as with the other stains. Cells stained by this conjugate were all granular and appeared to be eosinophils. Sections stained in this way were processed at the same time as other stains, and the same precautions observed. No attempt was made, during cell counts, to distinguish between cells having the appearance of plasma cells, and those like eosinophils, since this would not have been possible to carry out with accuracy in many cases.

4. Examination of Formalin-Fixed Tissues

Paraffin sections of formalin-fixed tissues adjacent to those sections of airway examined by immunofluorescence were stained by haematoxylin and eosin to examine general tissue morphology and the presence of eosinophils, and by methyl green pyronine stains to visualise the plasma cells. Gland/wall ratios were measured using an eyepiece graticule as described by Reid (1960).

5. Statistical Analyses

Each group of cell counts on four sections from each site of trachea or bronchus stained with one antiserum were examined for consistency and meaned. In the tables and figures these means are given for ready appreciation. For statistical purposes, these means were

converted to square roots to normalise the distribution, and analysis of variance and paired 't' tests used to calculate the significance of the observed differences (Fig. 13).

Cell counts on each lymph node section were meaned, and divided by the projected area of the graticule, thus being expressed as cells/sq. mm. The counts for each of the four corresponding sections were examined for consistency and then meaned. The counts were then treated in a similar way to those in the bronchi (Fig. 14).

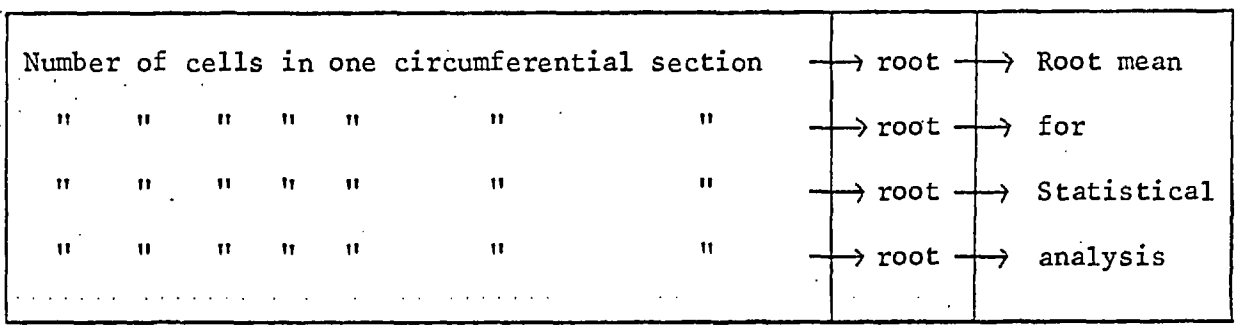
Thus in each of the twenty subjects (except for the five with incidental bronchitis), the minimum numbers of sections examined were twenty sections for IgA, twenty for IgG, twenty for IgM, twenty for IgE, six for complement, ten for haematoxylin and eosin, ten for methyl green pyronine, and extra sections for double-checking and blocking, making a total of over one hundred sections per subject. In the "incidental" bronchitic subjects, the immunoglobulin staining was confined to IgA only. The total number of sections examined in the whole study was over 1,800.

6. Further Studies:- Sputum immunoglobulin measurements in chronic bronchitic and control subjects

As will be discussed in subsequent chapters, plasma cell counts showed a marked deficiency of IgA plasma cells in the lungs of subjects dying from chronic bronchitis. Subsequently, while acknowledging the technical and interpretive difficulties of measuring immuno-

Fig. 13

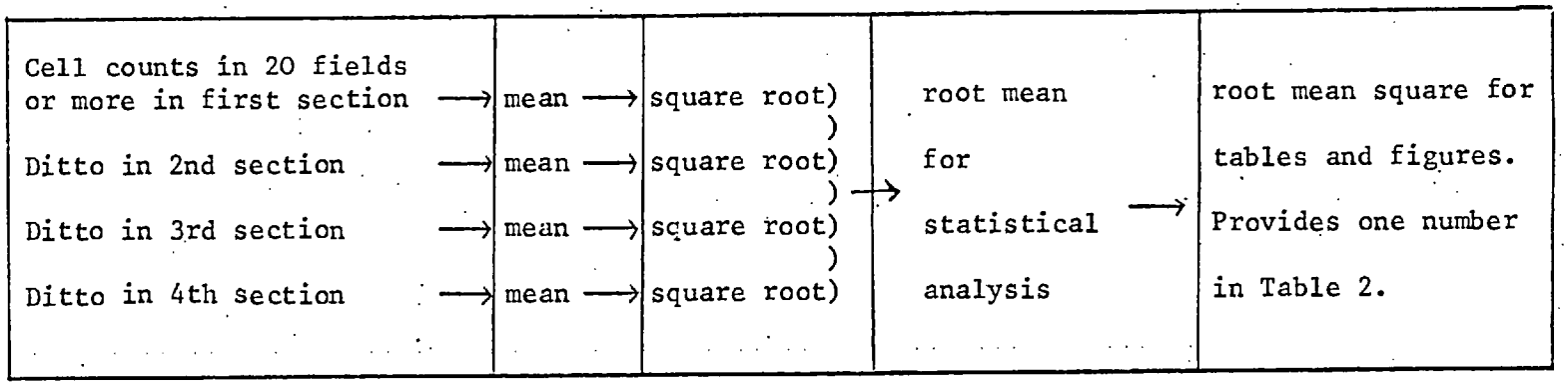
Arithmetic processing of cell counts for each immunoglobulin class at each tissue site (trachea, main bronchus and lobar bronchus).



(Arithmetic mean for illustrations and tables)

Fig. 14

Arithmetic processing of cell counts in eighty fields or more in four sections to produce one mean cell count for one immunoglobulin class in one subject.



globulins in sputum, a pilot study of immunoglobulins in the sputum of living subjects with severe chronic bronchitis was undertaken to seek further evidence of a bronchial IgA deficiency in severe chronic bronchitis.

Subjects

Chronic Bronchitis

Fourteen consecutive subjects admitted to hospital during one winter season suffering from exacerbations of their chronic bronchitis were studied. All had smoked more than twenty cigarettes a day for most of their lives, and had had daily productive cough for fifteen years or more. All had severe airways obstruction (FEV1 less than 800ml) without reversibility of more than 15% and none had blood or sputum eosinophlia. Some had radiographic evidence of severe emphysema in addition to their chronic bronchitis, and in all their exacerbations were characterized by an increase in cough, sputum or dyspnoea. Three died during that admission to hospital, and four subsequently during the same winter (many during an epidemic of Influenza A.) Three received artificial ventilation for between two and seven days, and the opportunity was taken to obtain bronchial aspirate.

Control Subjects

Sputum and serum samples were collected from thirty control subjects. This group included: eleven patients with bronchiectasis characterised by long-standing chronic cough producing purulent

sputum, associated with radiographic abnormalities consistent with bronchiectasis; six young adult patients suffering from recurrent bronchial infections of unknown cause, not associated with evidence of bronchiectasis on a plain chest radiograph, or associated with chronic cough; five patients with a history of variable asthma and with demonstrably reversible airways obstruction; and seven patients with asthma as above and also evidence of past or present allergic broncho-pulmonary aspergillosis (that is, the presence of typical radiographic appearances (McCarthy et al, 1970) accompanied by a positive immediate prick test response to aspergillus fumigatus and the presence of precipitating antibody to aspergillus fumigatus in the serum). All were selected for their ability to produce sputum and none had low serum immunoglobulin values.

Sputum expectorated between waking and midday was collected from the subjects. Intervals between samples were varied to obtain an estimate of variability from day to day and week to week. Patients were encouraged to swallow or spit their saliva before expectorating. Serum samples were taken at the time of the first sputum sample and subsequently only at times when venesection was justified on clinical grounds. Sputum was immediately frozen at -20 degrees Centigrade until processing.

Processing of Sputum

The method of Biserte (Biserte et al, 1963) was used for con-

verting the sputum to a form suitable for immunodiffusion studies. This provides a solution from which the gel has been separated, for this gel tends to prevent diffusion by blocking the pores in the agar. Because of losses during processing, it is unsuitable for obtaining an absolute measure of immunoglobulins in sputum, but has been used by other workers for detailed biochemical investigation of sputum (Masson et al 1965), and is entirely suitable for comparing ratios of immunoglobulin and albumen; these ratios are used to calculate the proportions of immunoglobulins in sputum which were actively locally secreted or passively derived from plasma. The calculation has been shown by Deuschl and Johannson (1974) to provide consistent estimates of local secretion. Their demonstration of low bronchial IgA secretion in two chronic bronchitic subjects by this method has already been discussed.

The sputum was processed as follows:- the frozen specimen was thawed gently and mixed with four parts of distilled water. This was then dialysed against distilled water at +4°C for 4 days, with frequent changes of water. The gel was then separated by centrifuging at 17,000 rpm for 30 minutes and the solution was lyophilised and stored. Lyophilised material was reconstituted with water at a concentration of 2.5 mg/ml for measurement of immunoglobulins, though a concentration of 5 mg/ml was occasionally required.

Immunoglobulin measurement

IgA, IgG and albumen were measured by immunodiffusion (Mancini

et al 1965) on low-level plates (Berhingwerke), using a serum standard. The use of a serum IgA standard in estimating IgA in secretions underestimates the concentration of secretory IgA because of its larger molecular size, but this error is systematic. Because of the difficulties in obtaining a secretory IgA standard, most workers have accepted this error and used a serum IgA standard.

Serum immunoglobulins were measured by a similar technique, and albumen by a bromocresyl green binding technique (Northam and Widdowson, 1967). These serum measurements were carried out by the Brompton Hospital routine laboratory.

Calculation of local bronchial immunoglobulin secretion

The formula of Deuschl and Johannson (1974) was used to calculate active bronchial secretion of immunoglobulins into sputum. It is based on the assumption that immunoglobulins in serum which pass passively into the bronchial tree through inflamed mucosal surfaces do so at the same rate as the serum albumen. This assumption has been considered justified by other authors (Soothill, 1967), and provides an estimation of the clearance of serum into the bronchi on which an estimate of the serum contribution to the immunoglobulins in sputum can be calculated. The difference between this figure and the actual immunoglobulin content of sputum gives the local bronchial secretion, and is expressed as a percentage of the total, i.e.

local bronchial secretion of immunoglobulin (%)

$$= \frac{\text{Total sputum Ig} - \left(\frac{\text{Serum Ig X Sputum albumen}}{\text{Serum albumen}}\right)}{\text{Total sputum Ig}} \times 100.$$

Technical assistance

The author collected all tissue samples and prepared them for storing, and performed all laboratory technical work for the first eighteen months of the study, except for the paraffin sections. All microscope work and every cell count was carried out by him. The sputum study was also personal labour. Assistance was given latterly by individual colleagues thanked at the end of this thesis.

CHAPTER 5

RESULTS:

THE DISTRIBUTION AND CLASS OF IMMUNOGLOBULIN WITHIN PLASMA AND OTHER CELLS IN THE RESPIRATORY TRACT OF NORMAL MAN

Sections: (a) General Appearances

Cells containing immunoglobulin

(b) Cell counts

Consistency

IgA

IgG, IgM, IgE

(c) Paraffin sections

(d) Reid Indices

(a) General Appearances - Cells Containing Immunoglobulin

Cells containing immunoglobulin were present in all sections lying between the submucous gland acini and ducts (Fig. 15) and in the lamina propria of the bronchial epithelium, often applied closely to the bronchial basement membrane (Fig. 16). The cells in submucous gland were vastly in the majority. Many cells were large rounded cells with eccentric nuclei and plentiful cytoplasm resembling mature plasma cells (Fig. 17). Others had only scanty cytoplasm and were presumably immature plasma cells or lymphocytes (Fig. 18). Cells containing IgA were present in larger numbers than those containing other immunoglobulin classes and in some subjects this was obvious at a glance (Fig. 19). In each subject cells containing IgA tended to be either almost uniformly large, mature plasma cells at all sampling sites, or were smaller immature plasma cells with scanty cytoplasm, at all sites. Three out of five of the non-smokers had small IgA cells and one out of four of the smokers had small IgA cells (Table 5). This difference is not significant.

In sections of peripheral lung, cells were often, but not invariably seen around small bronchi and bronchioles (Fig. 20) but almost never in alveolar walls or terminal bronchiole (only one plasma cell was seen in this situation in all of the subjects examined (Fig. 21).

IgA was also demonstrated in the cytoplasm of the epithelial

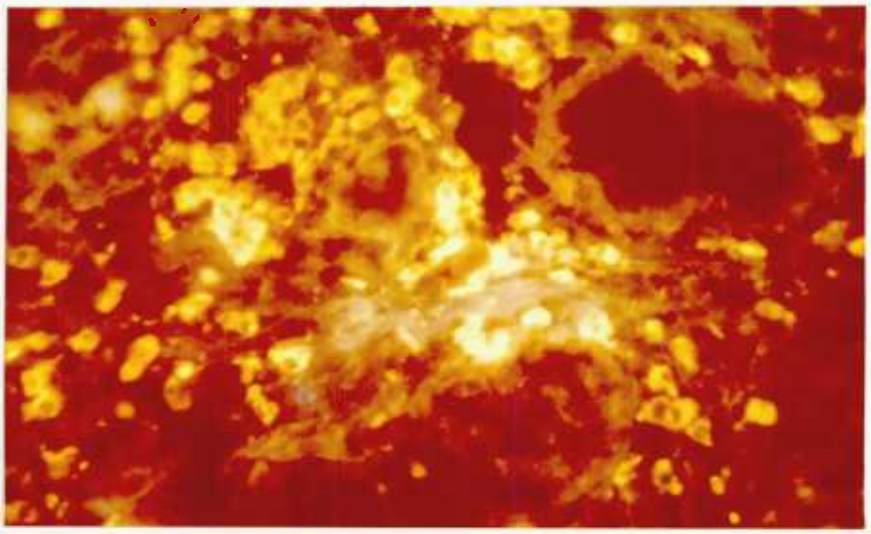
Fig. 15

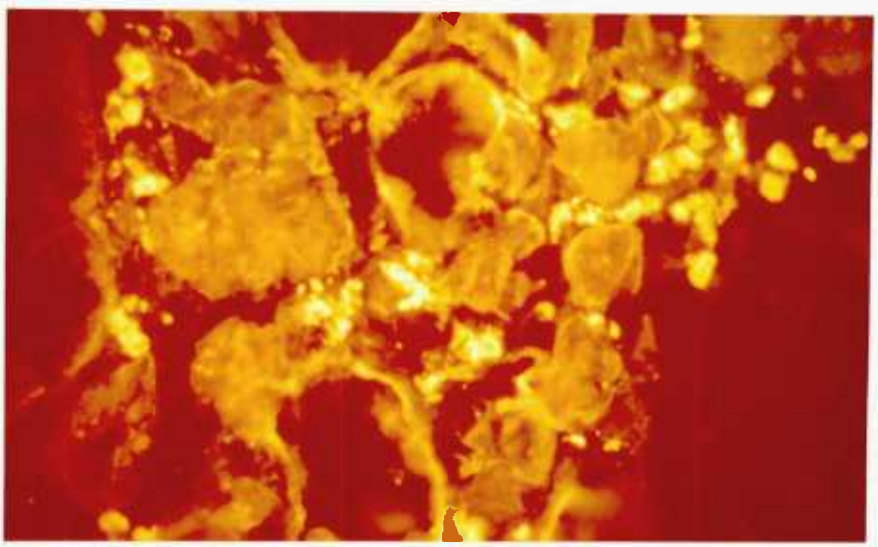
IgA plasma cells in submucous gland.

a. x 200

b. x 200

c. x 300





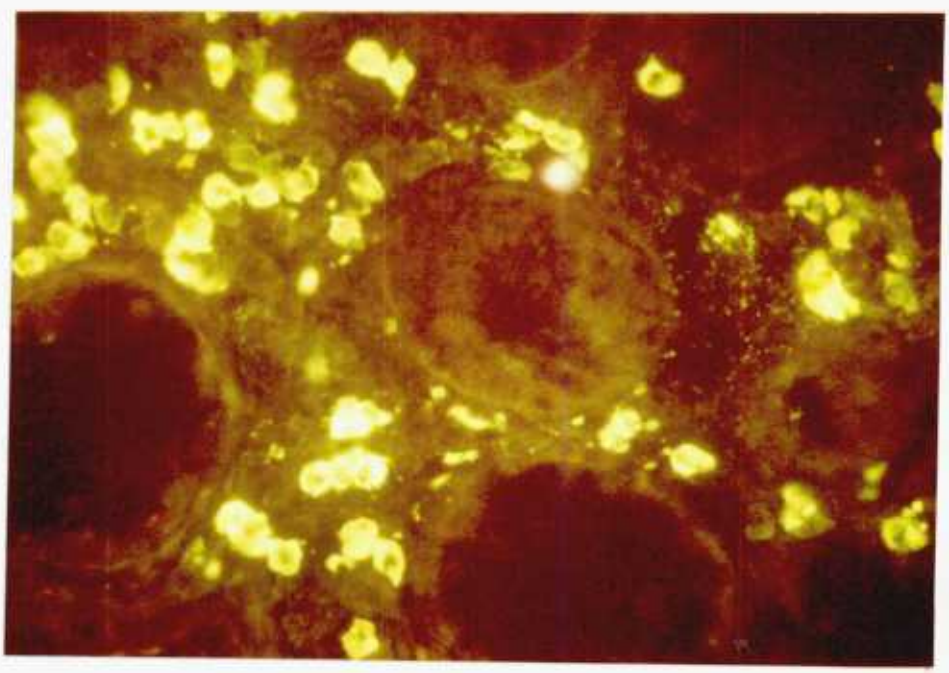


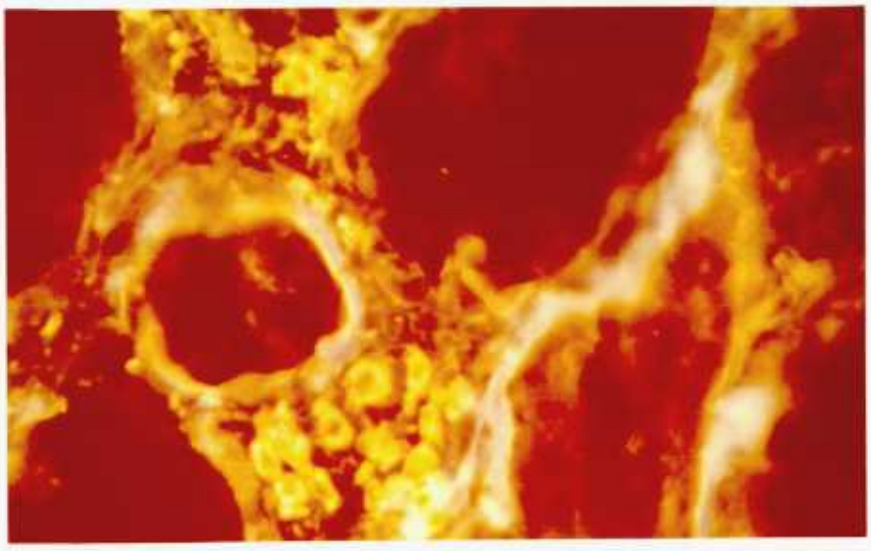
Fig. 15 (continued)

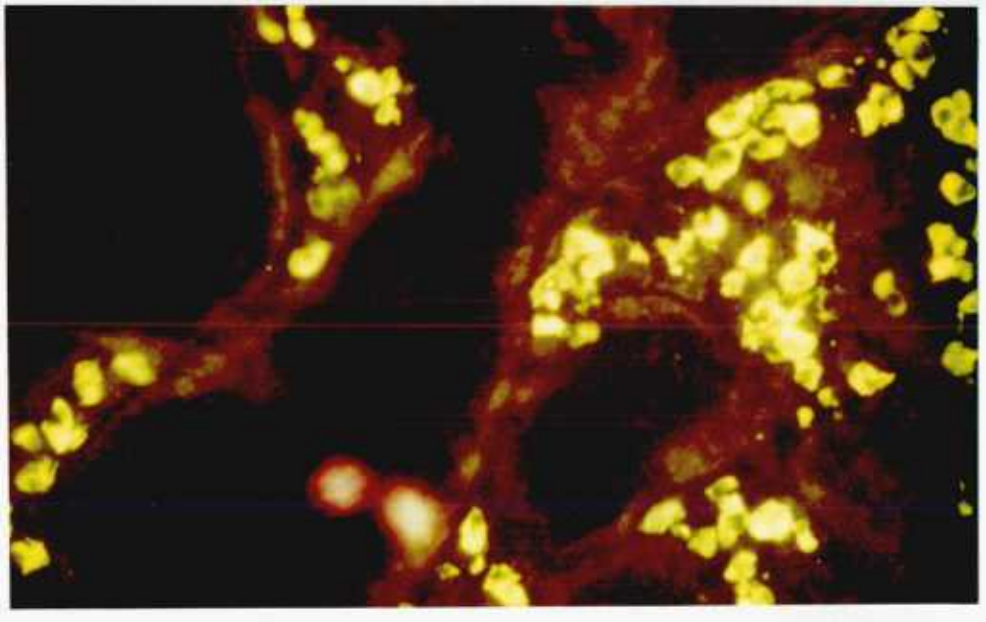
IgA cells in submucous gland

d. x 300

e. x 300

f. x 400





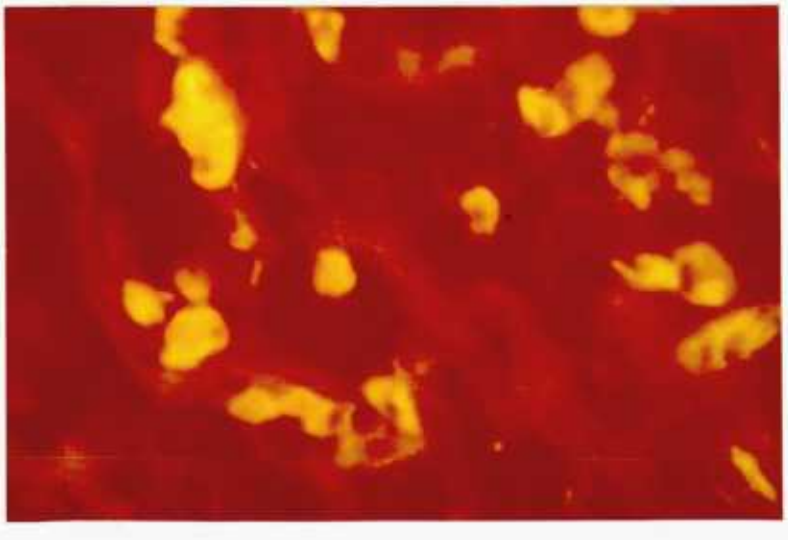
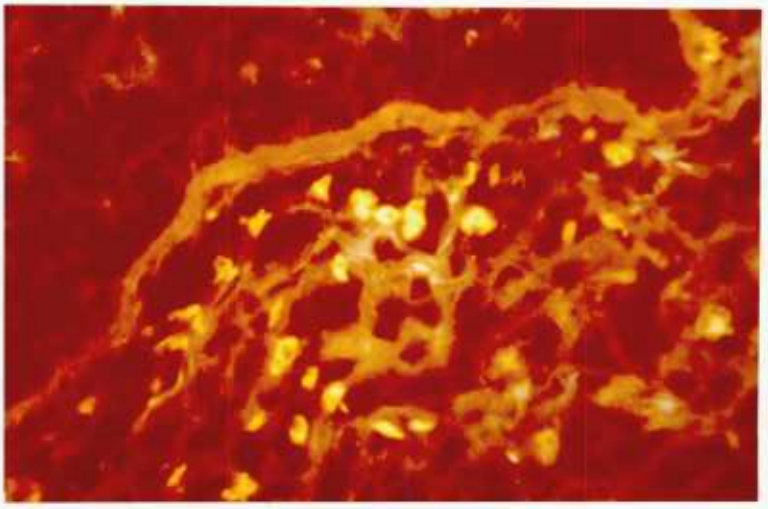


Fig. 16

IgA plasma cells in lamina propria.

a. x 200. The bronchial basement membrane is seen passing diagonally across the frame.

b. x 400. IgA plasma cells closely applied to bronchial basement membrane. The bronchial epithelial cells have been wiped off.



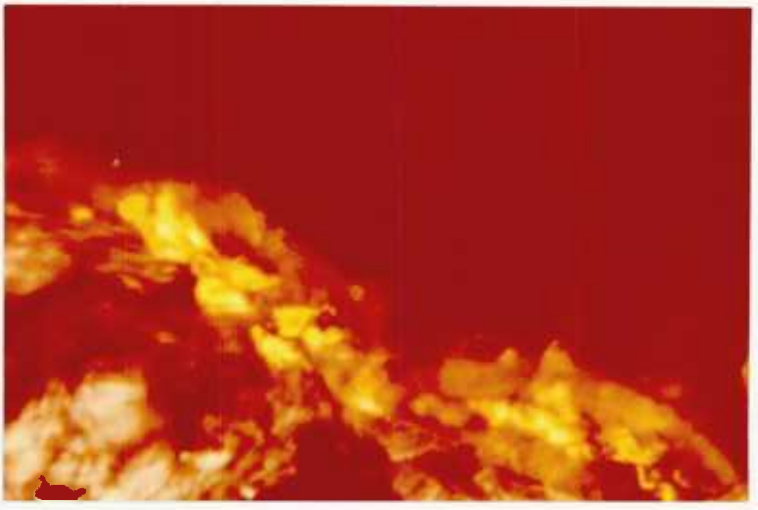
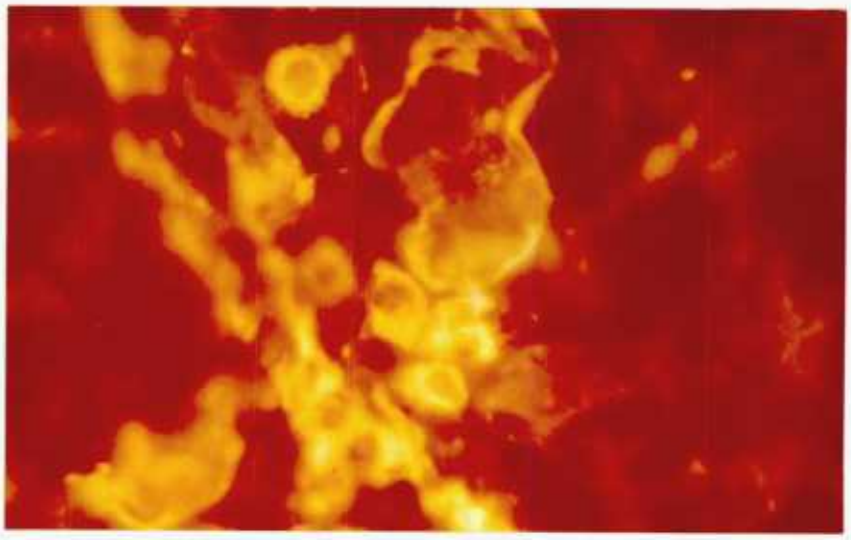


Fig. 17 (a and b)

Large IgA plasma cells in submucous gland.

a. x 400

b. x 440



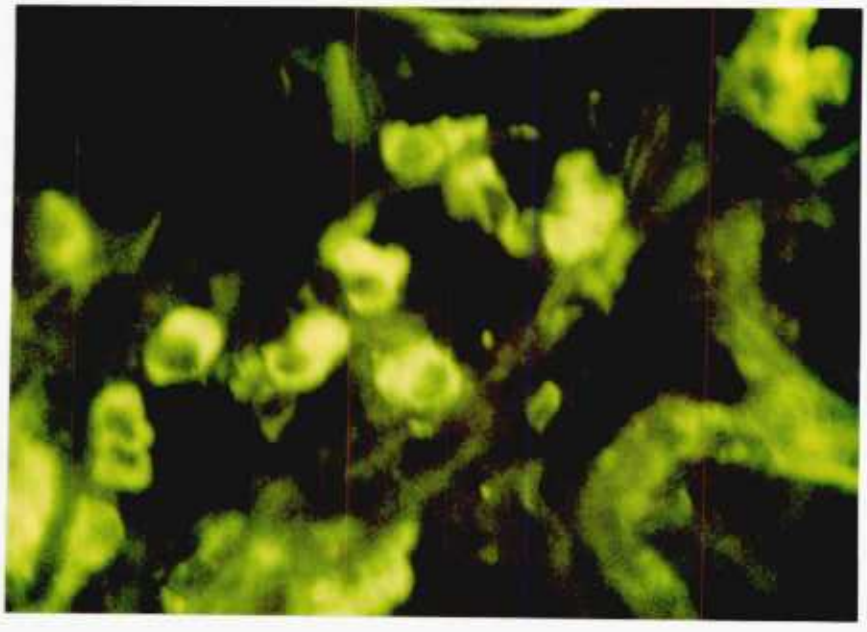
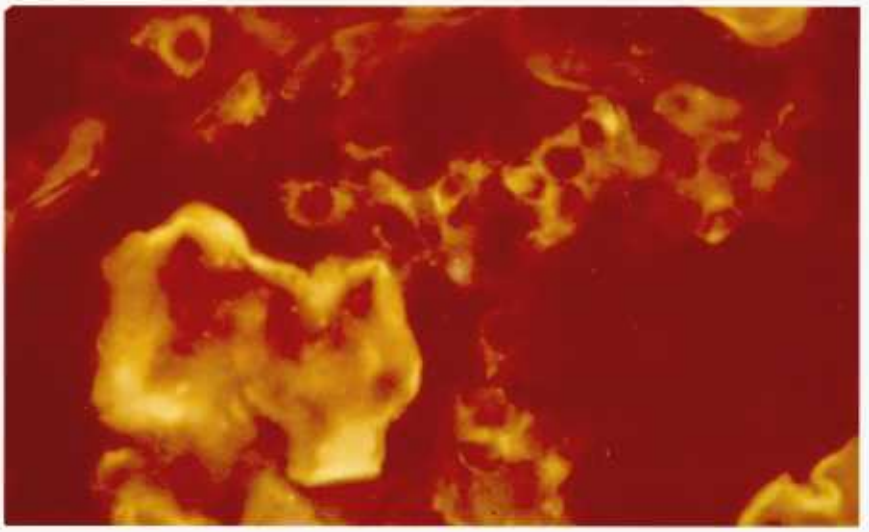


Fig. 18

Small IgA cells or lymphocytes in submucous gland. IgA staining is also seen on the epithelial cells of the submucous gland acini.

a. x 400

b. x 440



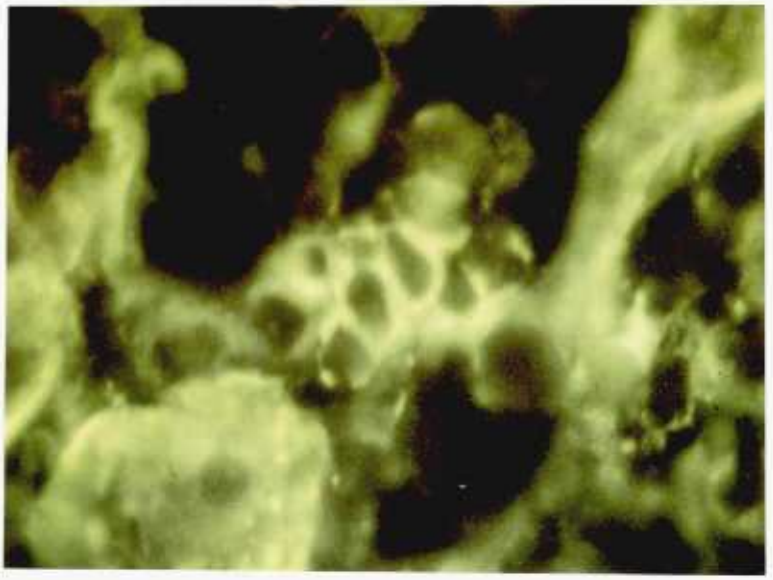


Fig. 19

IgM plasma cells in submucous gland.

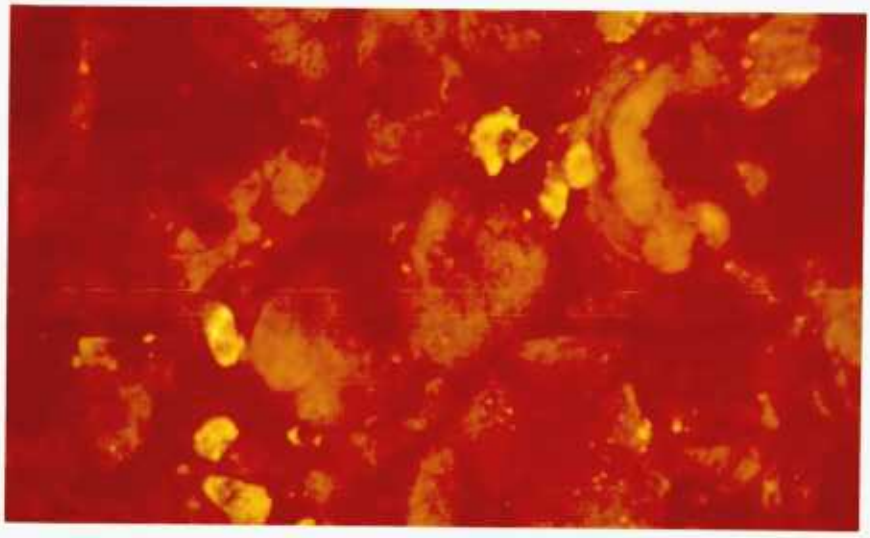
x 400

Fig. 20a

IgA plasma cells round bronchiole

x 400

(definition has been lost during reproduction)



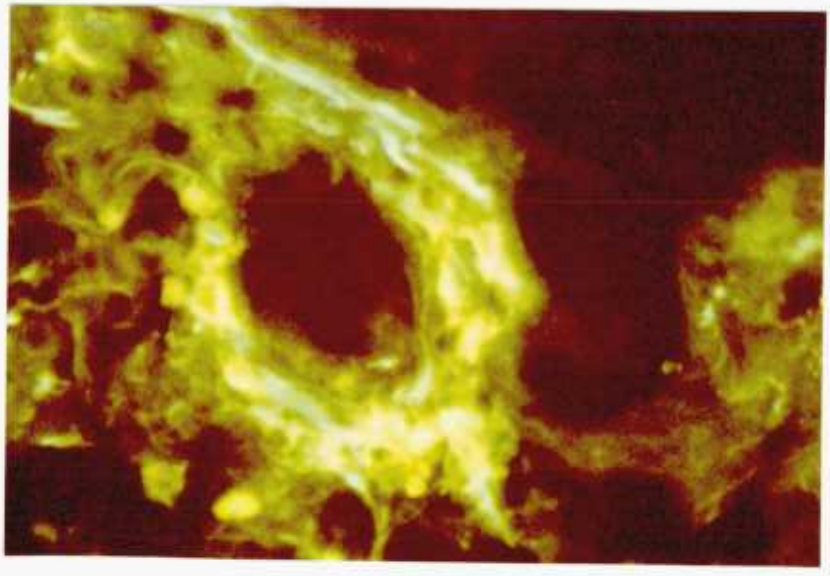


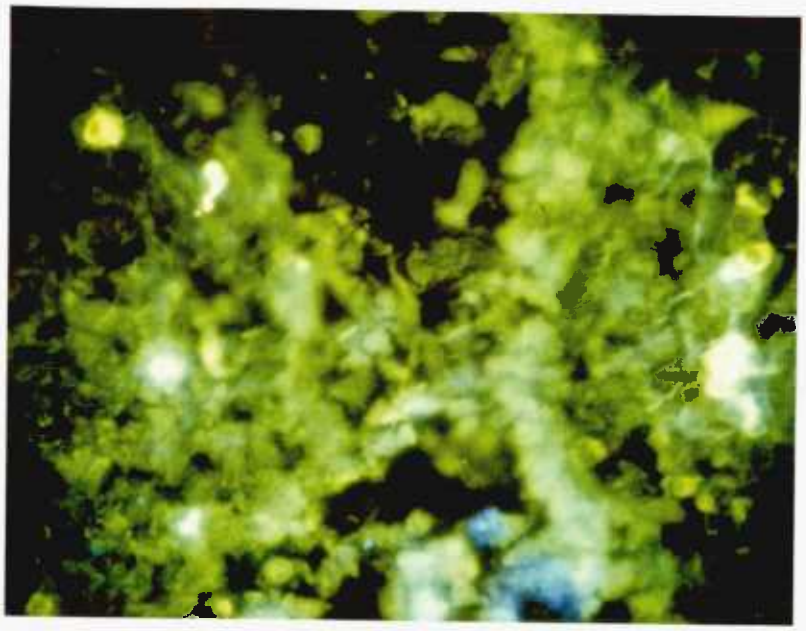
Fig. 20b

IgA cells round bronchiole

x 300

Fig. 21

The only plasma cell (IgA) in an alveolar wall, seen in the whole study



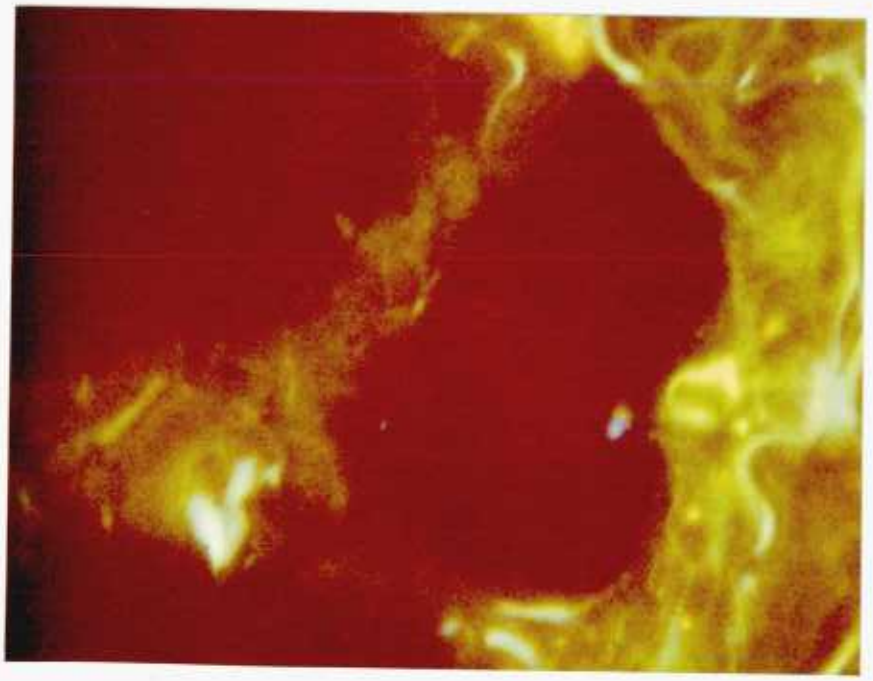


TABLE 5

Observed size of plasma cells in nine normal subjects.

	Subjects	Size of Plasma Cells	
		Large	Small
Five	Normal Non-smokers	2	3
Four	Normal Smokers	3	1

cells of the submucous glands (Fig. 22). This staining was sometimes very intense but not present in every subject. IgA in this site was often adjacent to large IgA plasma cells but not invariably; occasionally dense IgA staining on epithelial cells was adjacent to small plasma cells or even not adjacent to any recognisable cells containing immunoglobulin, (Fig. 23). Secretions in the submucous gland ducts also often stained positive with IgA conjugates.

Rarely, IgM or IgG deposits were seen on the basement membrane of the bronchial epithelium, (Fig. 24), often granular in appearance.

In one subject (subject 2), who had pulmonary oedema of cardiac cause, had many dilated vessels in the bronchial mucosa, presumably veins and lymphatics. These contained many cells, which stained with all conjugates. Close examination revealed these to be eosinophil leucocytes, (Fig. 25) and this was confirmed by haematoxylin and eosin stains. These cells were not included in the cell counts, and eosinophils were not apparent outside the vessels.

(b) Cell counts

(i) Consistency

There was naturally considerable variation within each group of four cell counts (on four sections from the same tissue block, each stained with the same antiserum) but at all tissue sites the range of cell counts was within $\pm$ 50% of means greater than 100 cells per section, and within $\pm$ 40 cells of mean counts less than

Fig. 22a, b

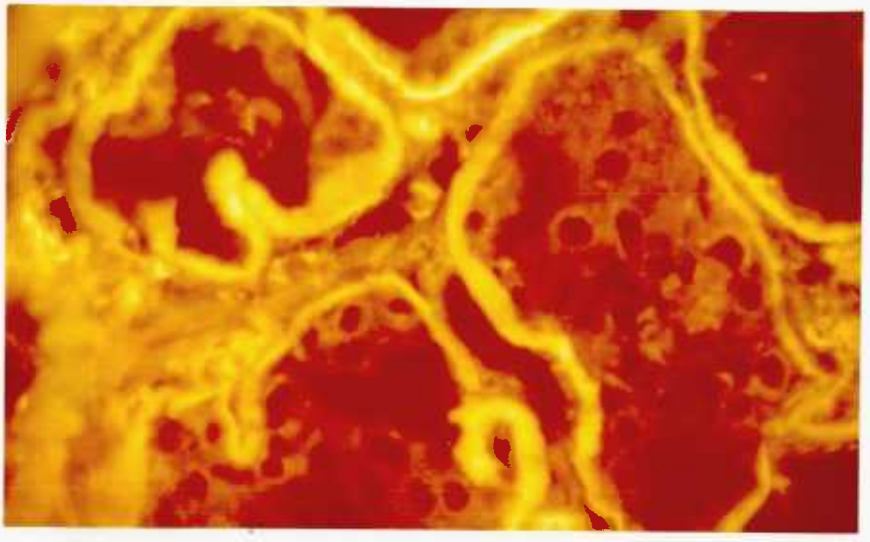
Staining by anti-IgA conjugate of epithelial cells of bronchial submucous gland.

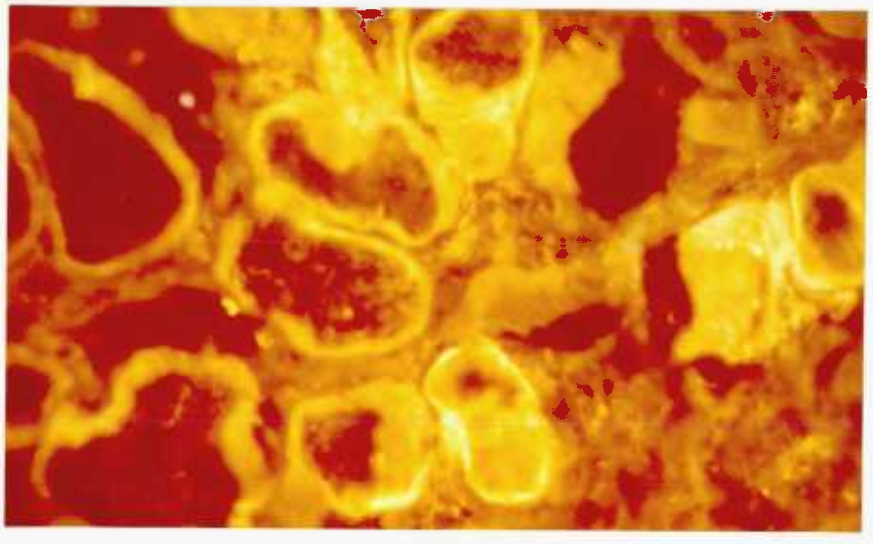
a. x 200

b. x 200

Fig. 23

IgA in epithelial cells of bronchial submucous gland without demonstrable plasma cells nearby





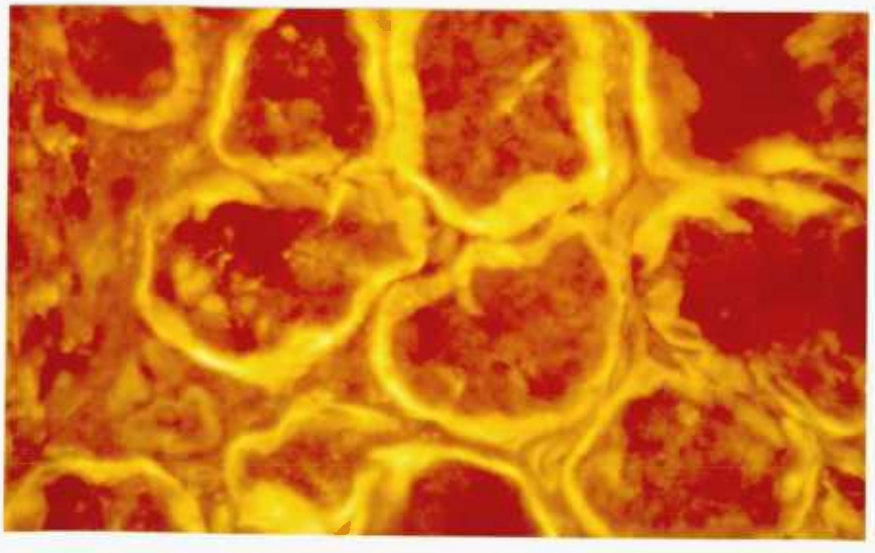
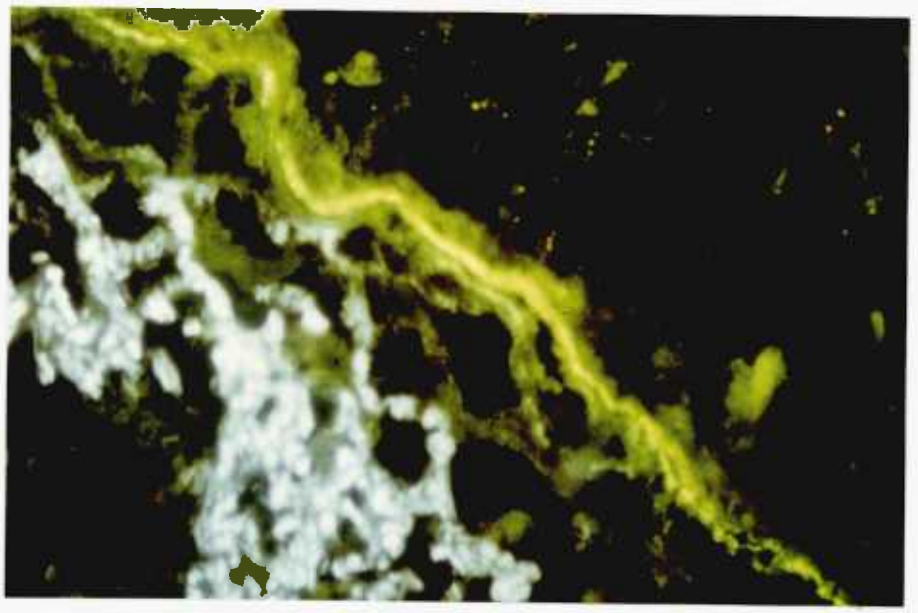


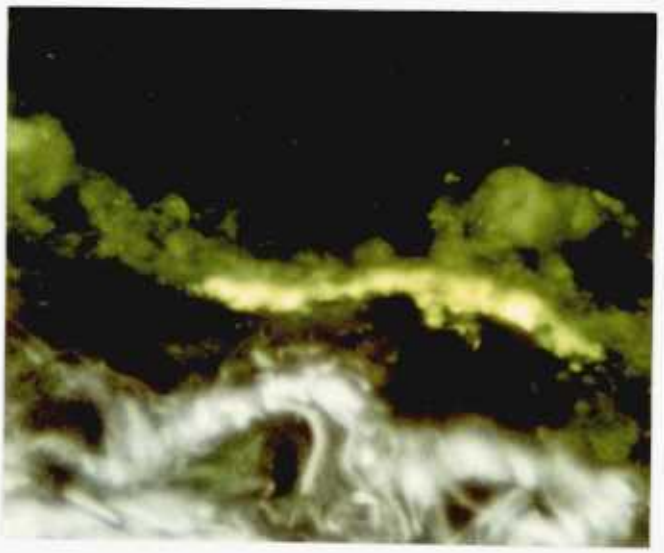
Fig. 24

a. IgM on basement membrane of bronchial epithelium. Blue/white appearance is auto-fluorescence of collagen. x 200

b. IgG deposits. x 300

c. IgG deposits. x 400





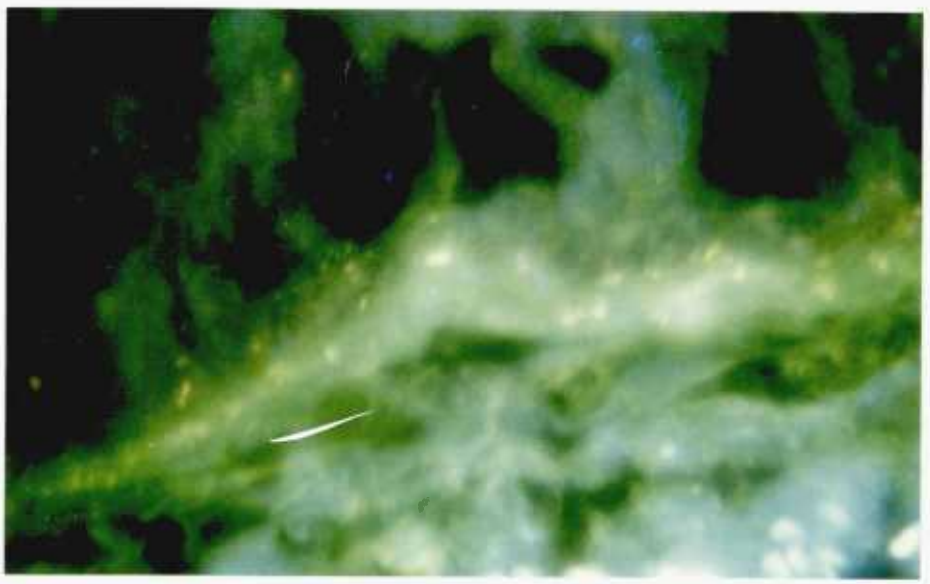
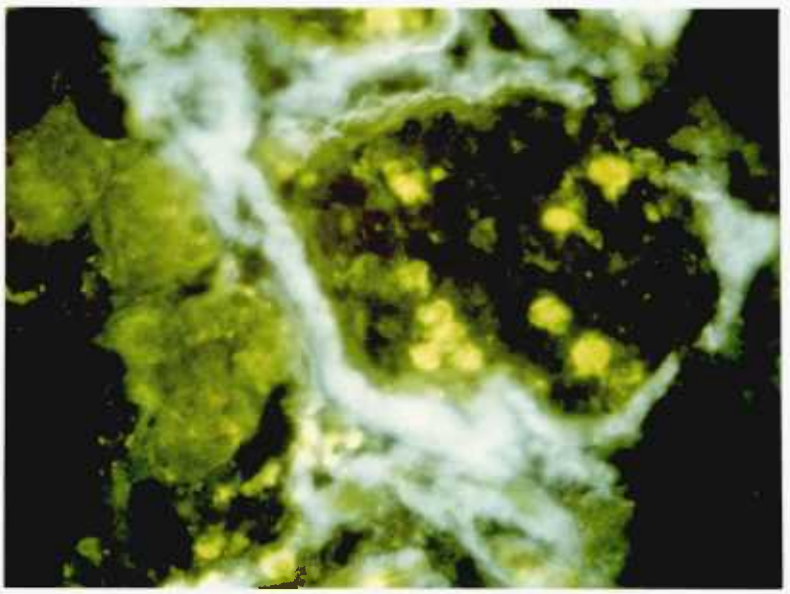


Fig. 25

Non-specific fluorescence of eosinophils in dilated vessel in bronchial mucosa. x 300



100 cells per section.

Statistical analysis of variance was carried out on root transformed cell counts, and this demonstrated good consistency of cell counts, the variance within each group of four cell counts (between slides, within subjects) was much smaller than the variance between subjects. The differences between subjects at all tissue sites and for all immunoglobulin classes were highly significant ($p < 0.001$ in all cases except for the IgM cell counts in upper trachea, where $p < 0.05$).

(ii) IgA

The cell counts are set out in Appendix III, and a small sample is set out in Table 6. This sample represents one fortieth of the total number of sections examined, not including the sections of carinal lymph node reported in chapter seven.

IgA cells were more frequent than cells containing the other immunoglobulin classes in all subjects and in all sites (Tables 7, 8, 9, 10. Fig. 26), except in subject 6 in whom IgG and IgE cells were equally common. The mean IgA cell count was highest at the main bronchus (mean 578, SD 299) and less at lower lobe bronchus (mean 376, SD 407) and upper trachea (mean 283, SD 209) (Table 7). In all subjects IgA cells were present in main and lobar bronchus but some subjects had very few cells in upper trachea (Fig. 27). Sections of peripheral lung demonstrated that IgA cells were to be found round small bronchi and bronchioles in the lamina propria (5-20 cells per circumferential section) in five subjects but absent in the other four (two non-smokers and two smokers). The cells

TABLE 6

Sample of arithmetic processing of cell counts:- IgA cell counts in five normal non-smokers in right main bronchus. The 5 figures on the right, derived from twenty cell counts, form part of the centre column in Table 7.

Subject	IgA cell counts Cells/section	Root mean for Statistical Analysis	Arithmetic means for Tables and Figures
1	130 143 83 132	10.99	122
2	920 810 694 780	28.27	801
3	478 710 622 817	25.51	657
4	444 498 450 620	22.38	503
5	395 410 520 470	21.15	449

TABLE 7

Mean IgA Cell counts on five normal non-smoking subjects (1-5) and four normal smokers (6-9). Each figure represents the mean of four cell counts.

SUBJECT	MEAN IgA CELL COUNTS (CELL PER 5μ SECTION)		
	UPPER TRACHEA	MAIN BRONCHUS	LOWER LOBE BRONCHUS
1	70	122	70
2	296	801	160
3	234	657	206
4	430	503	107
5	399	449	272
GRAND MEAN	286	506	162
6	17	1142	1391
7	134	574	383
8	710	533	573
9	264	421	222
GRAND MEAN	286	668	643
SIGNIFICANCE OF DIFFERENCE BETWEEN NON-SMOKERS AND SMOKERS	NS	NS	$p < 0.05$

TABLE 8

Mean IgG Cell counts on five normal non-smoking subjects (1-5) and four normal smokers (6-9). Each figure represents the mean of four cell counts.

SUBJECT	MEAN IgG CELL COUNTS (CELL PER 5μ SECTION		
	UPPER TRACHEA	MAIN BRONCHUS	LOWER LOBE BRONCHUS
1	19	41	26
2	18	31	20
3	66	115	28
4	27	45	23
5	61	39	25
GRAND MEAN	38	54	24
6	76	943	695
7	55	86	15
8	79	69	78
9	32	26	19
GRAND MEAN	61	281	202
SIGNIFICANCE OF DIFFERENCE BETWEEN NON-SMOKERS AND SMOKERS	NS	NS	NS

TABLE 9

Mean IgM Cell counts on five normal non-smoking subjects (1-5) and four normal smokers (6-9). Each figure represents the mean of four cell counts.

SUBJECT	MEAN IgM CELL COUNTS (CELLS PER 5μ SECTION)		
	UPPER TRACHEA	MAIN BRONCHUS	LOWER LOBE BRONCHUS
1	35	35	24
2	41	53	55
3	23	38	13
4	17	20	23
5	19	36	17
GRAND MEAN	27	37	26
6	65	232	477
7	20	126	20
8	32	38	31
9	16	37	20
GRAND MEAN	33	108	137
SIGNIFICANCE OF DIFFERENCE BETWEEN NON-SMOKERS AND SMOKERS	NS	NS	NS

TABLE 10

Mean IgE Cell counts on five normal non-smoking subjects (1-5) and four normal smokers (6-9). Each figure represents the mean of four cell counts.

SUBJECT	MEAN IgE CELL COUNTS (CELL PER 5μ SECTION)		
	UPPER TRACHEA	MAIN BRONCHUS	LOWER LOBE BRONCHUS
1	17	11	9
2	46	183	92
3	25	26	18
4	24	16	14
5	87	43	41
GRAND MEAN	40	56	35
6	144	1373	771
7	41	57	13
8	71	36	41
9	65	31	21
GRAND MEAN	80	374	212
SIGNIFICANCE OF DIFFERENCE BETWEEN NON-SMOKERS AND SMOKERS	NS	NS	NS

Fig. 26

Median cell counts for each immunoglobulin class in three sites in nine normal subjects.

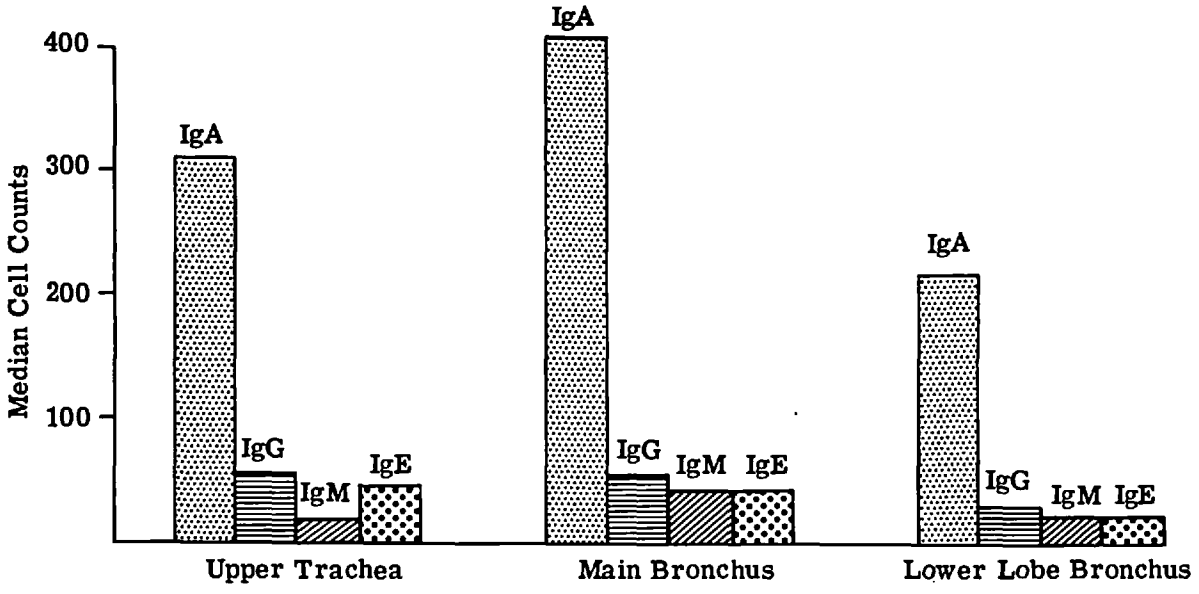
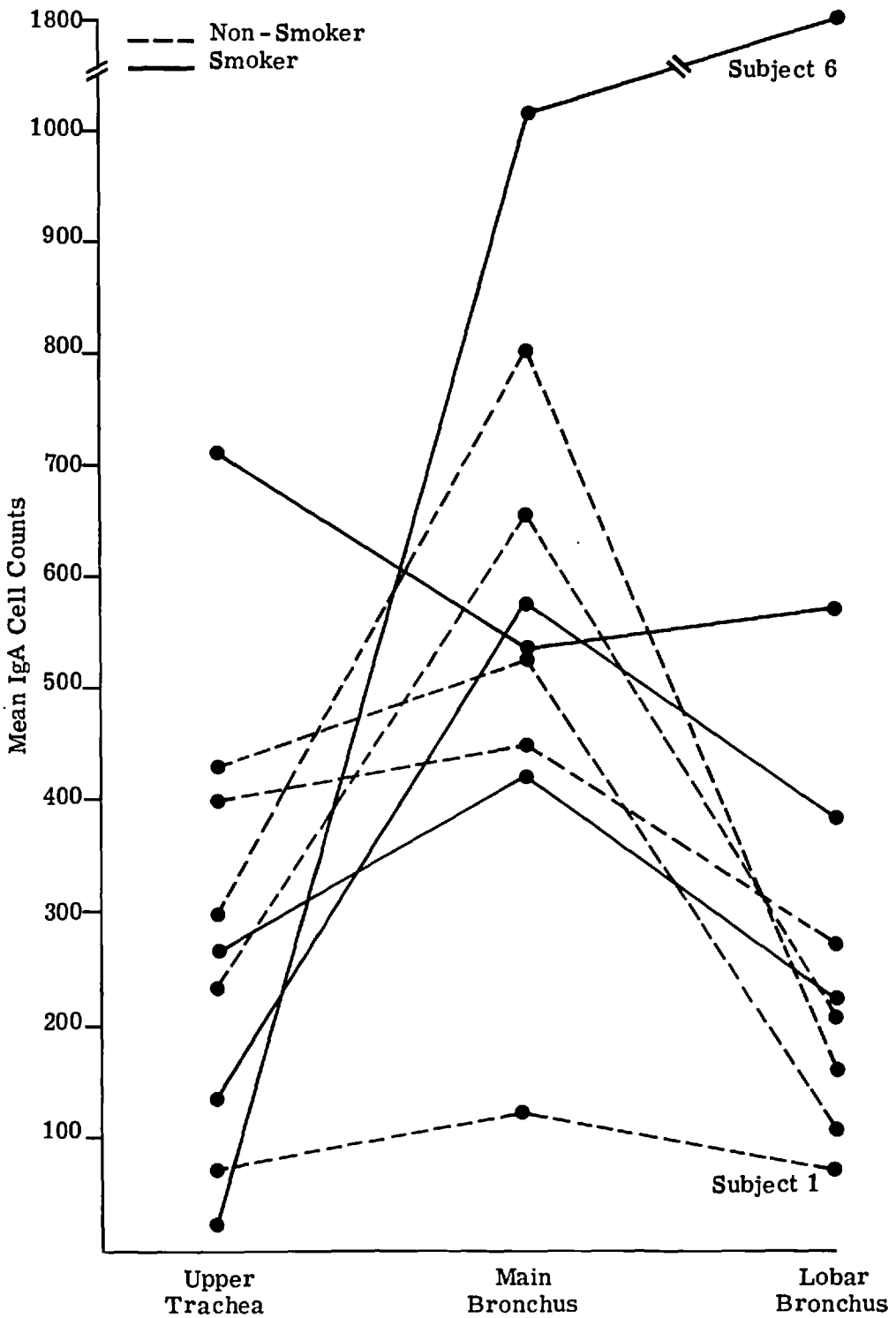

Median Cell Counts
400
300
200
100
IgA
IgG
IgM
IgE
IgA
IgG
IgM
IgE
IgA
IgG
IgM
IgE
Upper Trachea
Main Bronchus
Lower Lobe Bronchus

Fig. 27

Mean IgA cell counts at three sites in nine normal subjects.


1800
1000
900
800
700
600
500
400
300
200
100
Mean IgA Cell Counts
Non-Smoker
Smoker
Subject 6
Subject 1
Upper Trachea
Main Bronchus
Lobar Bronchus

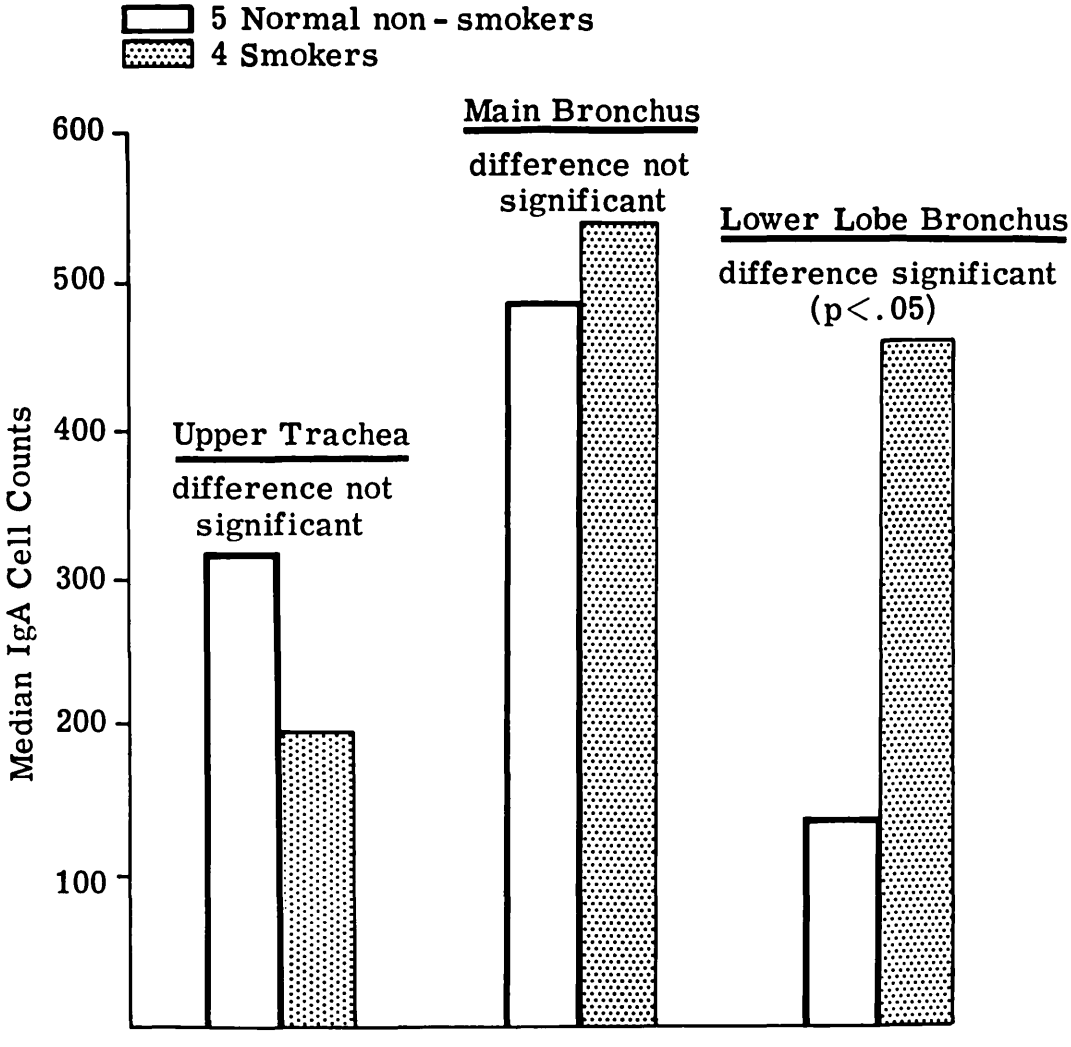
were virtually absent from respiratory bronchioles and alveoli. Free IgA was not demonstrated lining the walls of respiratory bronchioles or alveoli, either in washed sections or after fixation.

The IgA cell counts of two subjects lie outside two standard deviations from those of the other subjects (Fig. 27). Subject one had much lower cell counts at all sampling sites. This subject had never smoked, the IgA cells were of the small variety and were not demonstrated in small bronchi or bronchioles. Examination of a post-mortem sample of serum from this subject showed IgA to be present in normal amounts (170 mg%). By contrast, subject 6 had strikingly high IgA cell counts in main and lower lobe bronchus and strikingly low cell counts in upper trachea. This subject, who was also unusual in having very high IgG and IgE cell counts (Tables 8, 10), was a smoker without history of atopy), and died as a result of shooting himself in the head. The IgA cells were of the large variety and they extended to the small bronchi and bronchioles in large numbers.

In spite of the counts from these two atypical subjects, the counts for the non-smokers did not differ significantly from the smokers in main bronchus, but in lobar bronchus the smokers had significantly higher IgA cell counts than the non-smokers ($p < 0.05$) (Fig. 28).

Fig. 28

Median IgA cell counts, non-smokers and smokers compared.


5 Normal non - smokers
4 Smokers
Median IgA Cell Counts
600
500
400
300
200
100
Upper Trachea
difference not significant
Main Bronchus
difference not significant
Lower Lobe Bronchus
difference significant (p<.05)

(iii) IgG, IgM, IgE

The mean IgG, IgM and IgE cell counts are set out in tables 8, 9, 10 and Figure 26. These means are derived from cell counts on three hundred and twenty four sections (set out in Appendix III). The IgG, IgM and IgE cell counts were much lower than the IgA cells, although one subject had as many IgG and IgE cells as IgA (subject 6). This subject had no history of atopy, and the total IgE in a post-mortem sample of serum was 1,000 iu/ml which is normal. The distribution of these cells followed that of the IgA cells but in much smaller numbers. Neither the age of the subjects nor the interval between death and fixation of tissues appeared to have any influence on the cell counts; comparison of IgA cell counts in right main bronchus with the ages of the subjects and with the interval before fixation did not demonstrate any significant trend (Figs. 29, 30).

(c) Paraffin Sections

Examination of paraffin sections of tissue adjacent to those examined by immunofluorescent methods demonstrated normal morphology. Eosinophils were very scanty so that the likelihood of non-specific staining of these cells affecting the plasma cell counts was small. Plasma cells were demonstrated in numbers consistent with the cell counts on sections stained by immunofluorescent methods, although the quality of methyl green pyronine stains on these necropsy tissues was poor, (Fig. 31).

Fig. 29

Comparison between IgA counts in main bronchus and age of nine normal subjects.

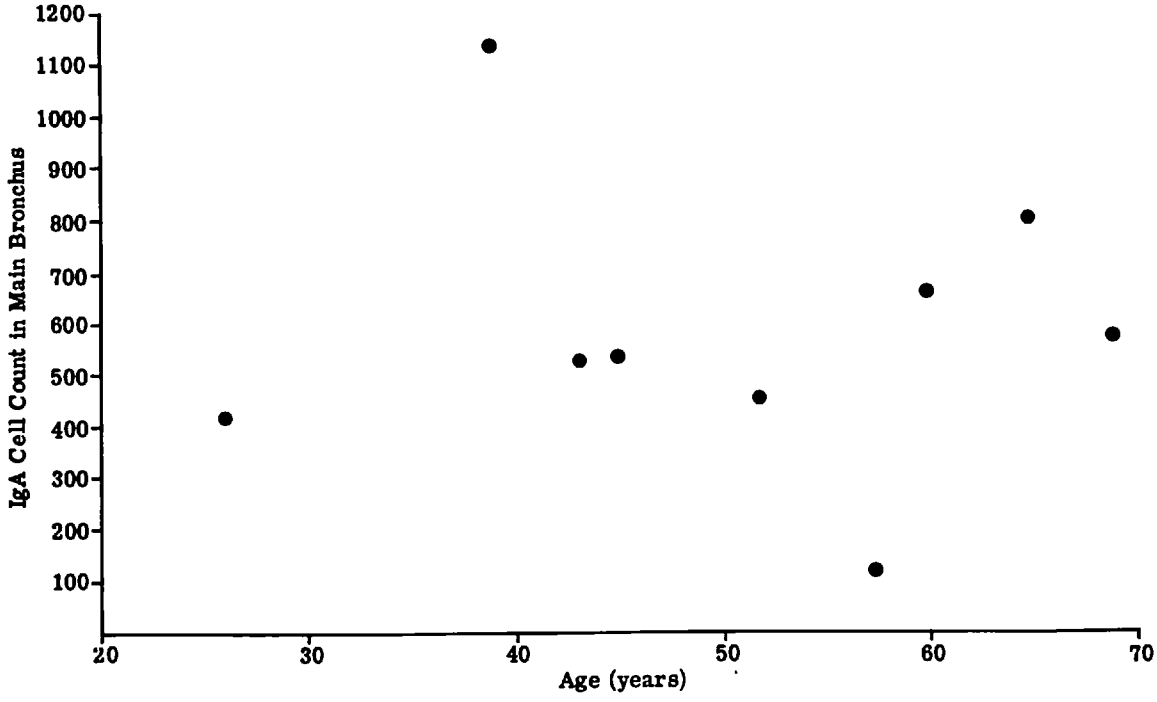

IgA Cell Count in Main Bronchus
1200
1100
1000
900
800
700
600
500
400
300
200
100
20
30
40
50
60
70
Age (years)

Fig. 30

Comparison between IgA cell counts in main bronchus and interval between death and fixation of tissue.

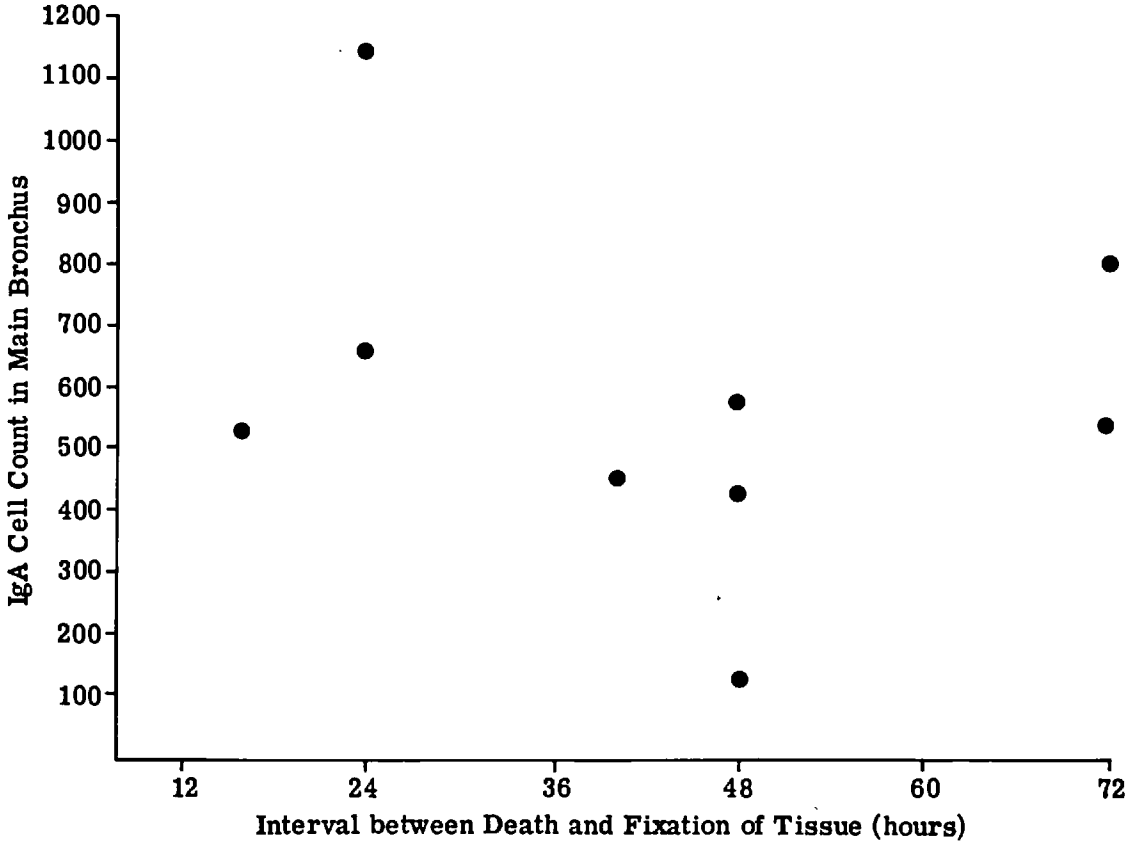
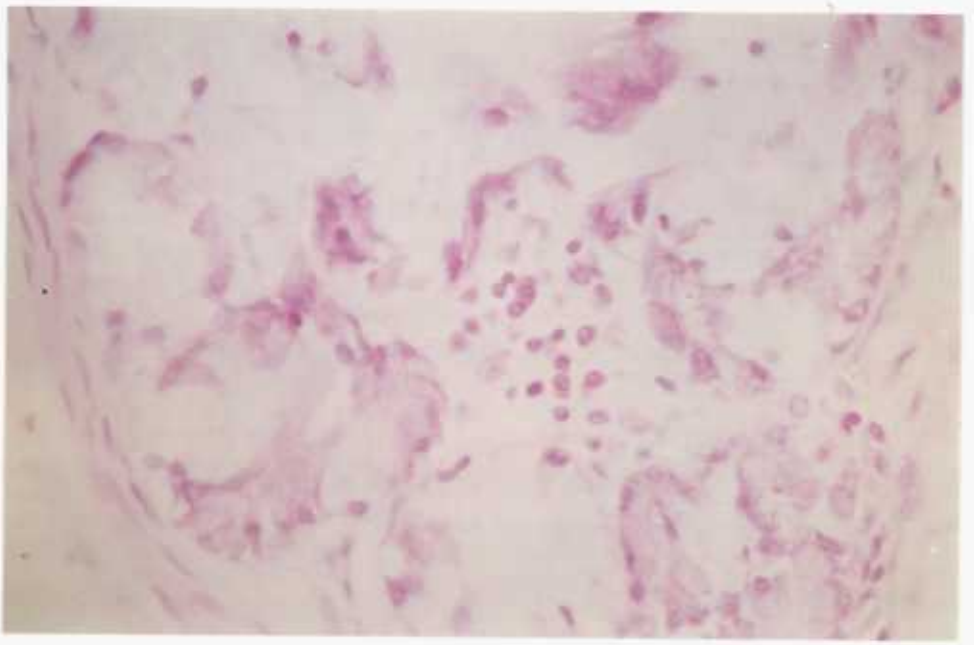

1200
1100
1000
900
800
700
600
500
400
300
200
100
IgA Cell Count in Main Bronchus
12
24
36
48
60
72
Interval between Death and Fixation of Tissue (hours)

Fig. 31

Methyl green pyronine stain of bronchial mucous gland showing plasma cells (pink cytoplasm) between acini. (These stains were of poor quality on these necropsy tissues).



Reid Indices

The gland/wall ratios (Reid Indices) of the main bronchi of all the non-smokers and two of the smokers were in the normal range described by Reid (1960) (up to 0.36). However, in two of the smokers, the index was higher, and closer to the values described by Reid in twenty chronic bronchitic subjects (0.40-0.79). The mean values of the smokers are significantly higher than the non-smokers ($p < 0.001$) Table 11).

TABLE 11

Gland/wall ratios (Reid indices) of nine apparently normal subjects (normal up to .36 - Reid 1960).

SUBJECT		GLAND/WALL RATIO	
Normal non-smokers	1	0.30	Mean .27
	2	0.16	
	3	0.24	
	4	0.30	
	5	0.33	
Normal smokers	6	0.32	Mean .34 (p .001)
	7	0.37	
	8	0.40	
	9	0.28	

CHAPTER 6

RESULTS:

THE DISTRIBUTION AND CLASS OF IMMUNOGLOBULIN WITHIN PLASMA AND OTHER CELLS IN THE RESPIRATORY TRACT OF SUBJECTS WITH CHRONIC BRONCHITIS

Sections: (a) General Appearances

(b) Cell Counts

Consistency

IgA cell counts

IgG, IgM, IgE cell counts

Effect on cell counts of age and interval between death and fixation of tissues.
Paraffin sections and gland/wall ratios.
Serum IgA.

(a) General Appearances

Plasma cells and other cells containing immunoglobulin were most plentiful in bronchial or tracheal submucous gland, but were also present in the lamina propria of the bronchial epithelium, this being a similar distribution to that found in normal subjects. However, in the subjects with "fatal" chronic bronchitis, there was a striking lack of IgA cells in all sites in the bronchial tree (Fig. 32), so that the usual predominance of IgA over other immunoglobulin classes was much reduced. The cells were almost universally small with scanty cytoplasm. Plasma cells were also rarely seen in the wall of bronchioles and alveoli.

By contrast, the five subjects with "incidental" chronic bronchitis had plentiful IgA cells in trachea, main bronchus and lower lobe bronchus, although these cells appeared to be scantier than normal round small bronchi and bronchioles in peripheral lung sections. In upper trachea, the cells were of the small variety with scanty cytoplasm, but in main bronchus and lobar bronchus three of these subjects had large cells with plentiful cytoplasma, while the other two subjects had small cells at all sites, (Table 12).

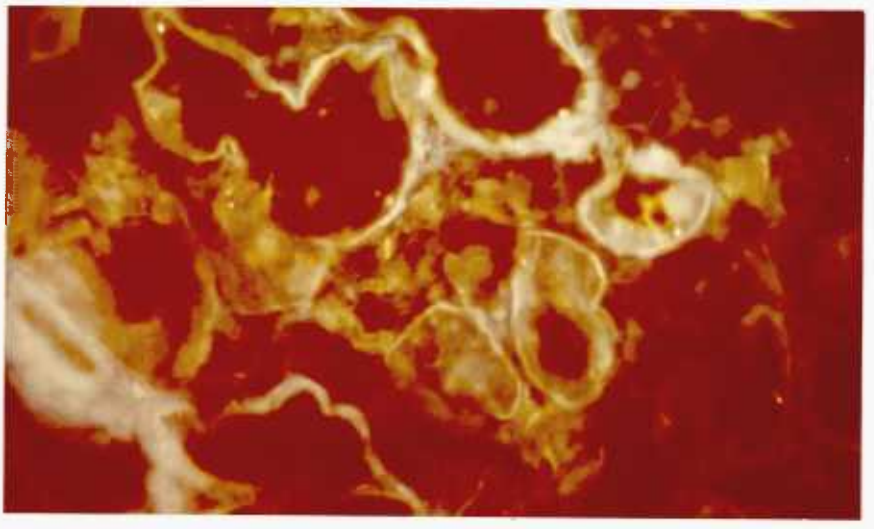
Fluorescent deposits on the basement membrane of the bronchial epithelium were seen occasionally, but no more frequently than in normals. It was usually granular in appearance.

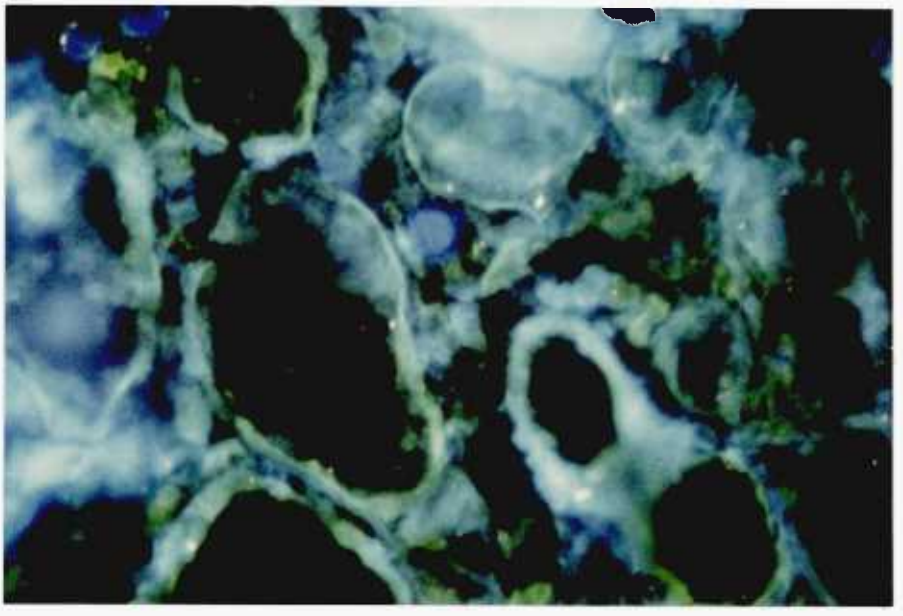
Fig. 32 a, b, c

a. Lobar bronchial submucous gland in fatal bronchitics; lack of IgA plasma cells. x 200

b. Tracheal submucous gland in fatal bronchitics; lack of IgM. (Blue colour is auto-fluorescence of collagen).

c. Main bronchial submucous gland in fatal bronchitics. IgA cells scanty and small in this field. (Definition has been lost in photographic processing).





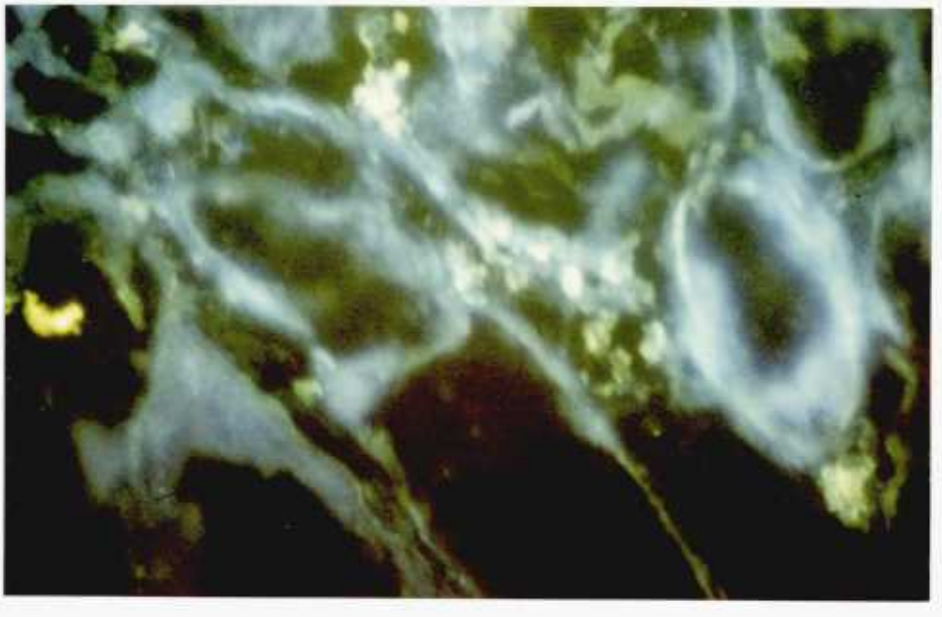
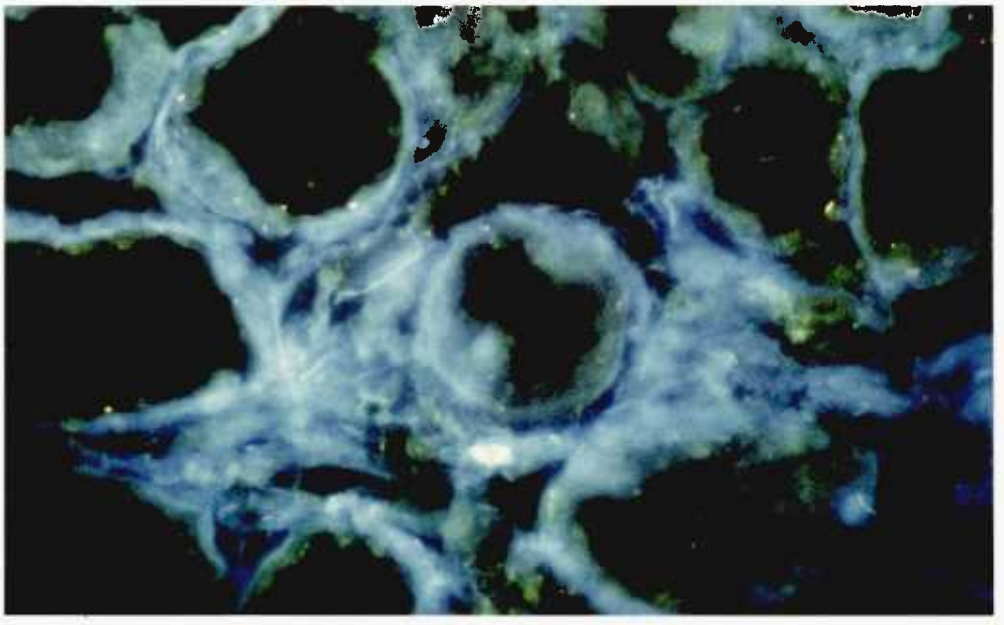


Fig. 32, d and e

d. Tracheal submucous gland in fatal bronchitis. IgM cells absent from this field. (IgM conjugates caused very little background staining).

e. Lobar bronchial submucous gland. IgA cells scanty. (Incident light, green barrier filter).



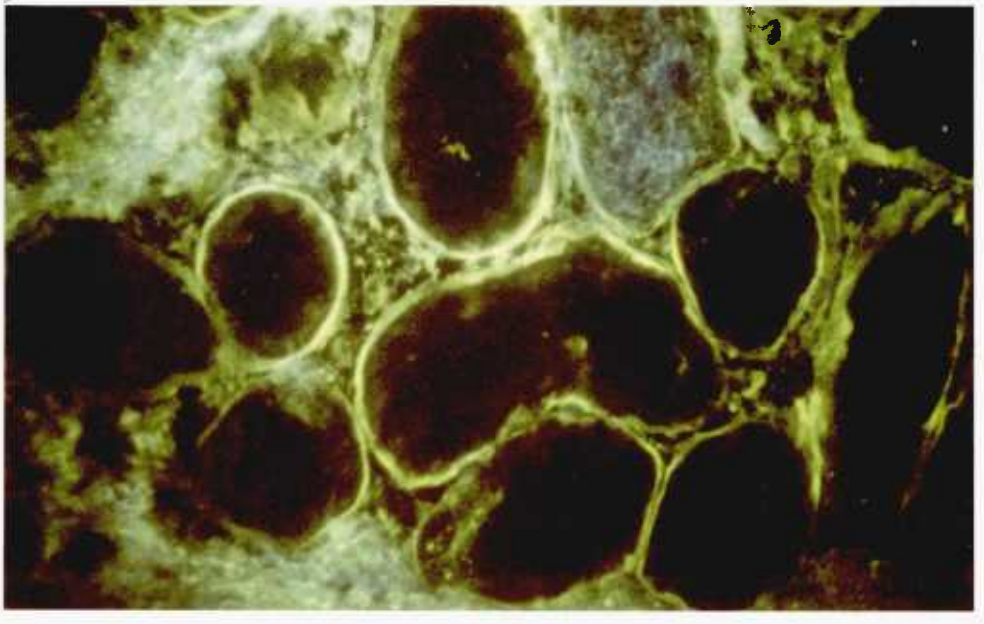


TABLE 12

Predominant size of plasma cell population in bronchial tree sampled in upper trachea, main bronchus and lobar bronchus.

	SMALL CELLS (SUBJECTS)	LARGE CELLS (SUBJECTS)
5 Normal non-smokers	3 (1, 3, 4)	2 (2, 5)
4 Normal smokers	1 (8)	3 (6, 7, 9)
5 Incidental bronchitis	3 (11, 12, 13)	2 (Small in trachea, see text) (10, 14)
6 Fatal bronchitics	6 (15-20)	0
All	13	7

(b) Cell Counts

Unprocessed cell counts are set out in Appendix 4.

(i) Consistency

Repeated cell counts were consistent, the range of cell counts within each group of four sections (from the same tissue block and stained with the same anti-serum) was within $\pm$ 50% from means greater than 100 cells per section and up to $\pm$ 50 cells from mean counts less than 100. Statistical analysis of variance on root transformed cell counts showed that counts within each group of four sections were consistent, the variance between slides within subjects being much less than the variance between subjects ($p < 0.001$ at all sampling sites).

(ii) IgA Cell Counts

Mean IgA cell counts in upper trachea, right main bronchus and right lower lobe bronchus in six subjects with "fatal" chronic bronchitis and five subjects with "incidental" chronic bronchitis are set out in Table 13 and illustrated in Figure 33. The "fatal" bronchitics have much lower IgA counts than the "incidental" chronic bronchitics in all three sites ($p < .005$ in trachea and main bronchus, $p < .05$ in lobar bronchus).

Comparison with the normal subjects reported in the preceding chapter show that "fatal" bronchitics have significantly lower cell counts at all sites than normals and "incidental" bronchitics

TABLE 13

Mean IgA cell counts in five subjects with 'incidental' chronic bronchitis (10-14) and six subjects with 'fatal' chronic bronchitis (15-20).

Unprocessed cell counts are set out in Appendix 4.

SUBJECT	IgA COUNTS (MEANS OF COUNTS ON FOUR SECTIONS)		
	UPPER TRACHEA	MAIN BRONCHUS	LOWER LOBE BRONCHUS
10	310	622	296
11	555	341	209
12	225	406	118
13	218	526	183
14	234	608	209
GRAND MEAN	308	501	203
15	14	57	29
16	42	170	157
17	35	105	139
18	392	313	59
19	28	276	23
20	5	94	70
GRAND MEAN	86	174	79.4
	$p < 0.05$	$P < 0.01$	$P < 0.01$

taken together, significantly lower counts than normal smokers in main bronchus and lobar bronchus and significantly lower counts than normal non-smokers in trachea and main bronchus, (Fig. 33, 34).

It should be noted that one normal non-smoker (subject 1) had cell counts in the same range as the subjects with severe chronic bronchitis (Fig. 33).

(iii) IgG, IgM, IgE cell counts

The unprocessed cell counts are set out in Appendix 4.

The numbers of cells containing IgG, IgM and IgE in subjects with severe bronchitis were not significantly different from the normal subjects reported in the preceding chapter. The cell counts for the "fatal" bronchitics are set out in Tables 14, 15, 16 and Figure 35. IgA cells do not predominate to the same extent as in normal subjects, and there appears not to be a compensatory increase of IgM cells.

(iv) Effect of age and interval between death and fixation of tissues

There did not appear to be a relationship between age and the IgA cell counts, nor between the cell counts and interval between death and fixation of tissue. Comparison of IgA cell counts in main bronchus in normals and bronchitics did not show any clear relationship between the cell counts and these two factors, (Figs. 36, 37).

Fig. 33

Mean IgA cell counts at three sites in five 'incidental' chronic bronchitics and six 'fatal' chronic bronchitics. Normal subjects and included for comparison. (Each point represents the mean of cell counts on four sections.)

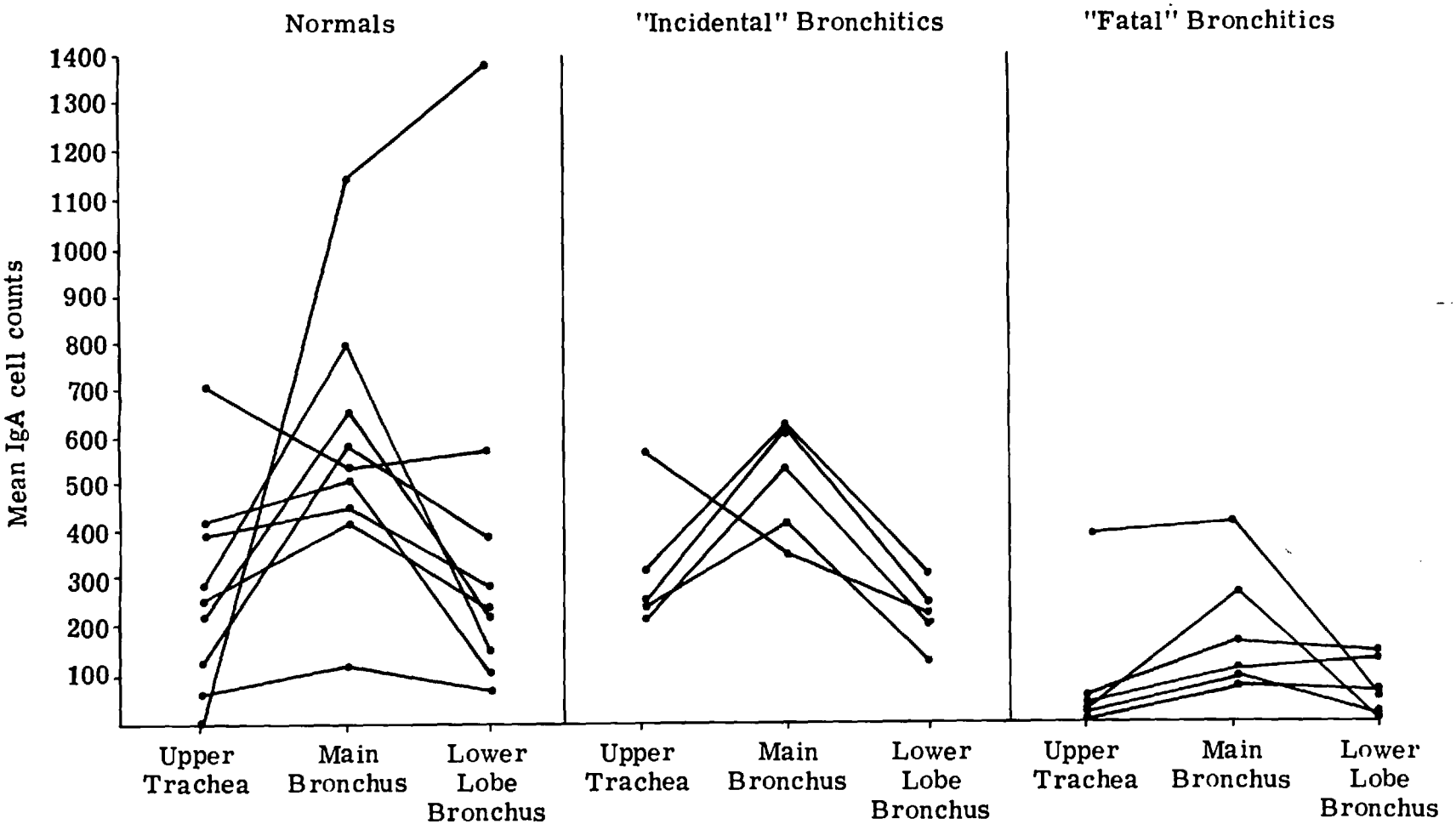

Normals
"Incidental" Bronchitics
"Fatal" Bronchitics
Mean IgA cell counts
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
Upper Trachea
Main Bronchus
Lower Lobe Bronchus
Upper Trachea
Main Bronchus
Lower Lobe Bronchus
Upper Trachea
Main Bronchus
Lower Lobe Bronchus

Fig. 34

Overall mean IgA cell counts at three sites in five normal non-smokers (NNS), four normal smokers (NS), five 'incidental' bronchitics (IB) and six 'fatal' bronchitics (FB).

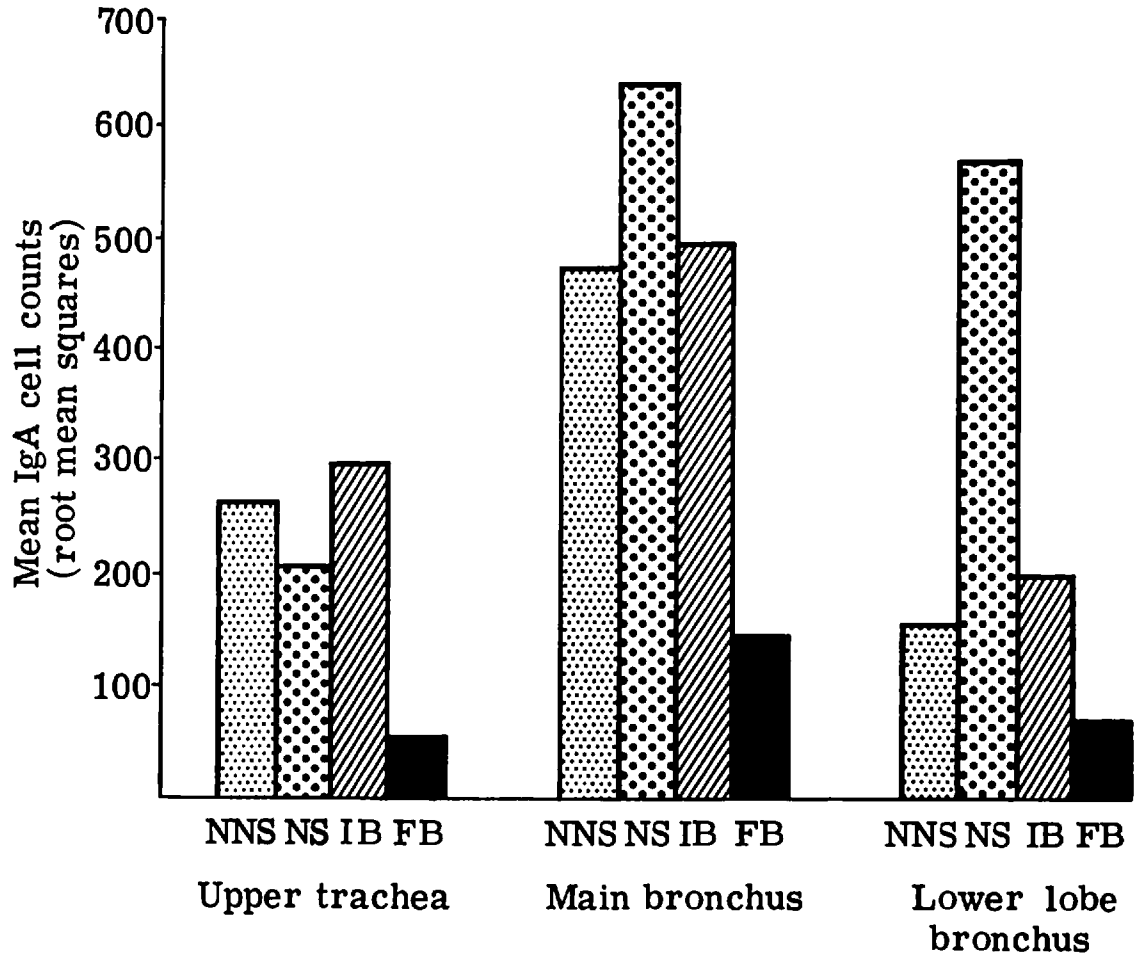

Mean IgA cell counts
(root mean squares)
700
600
500
400
300
200
100
NNS NS IB FB
Upper trachea
NNS NS IB FB
Main bronchus
NNS NS IB FB
Lower lobe
bronchus

TABLE 14

IgG counts in six subjects with 'fatal' bronchitis.

Unprocessed cell counts are set out in Appendix 4.

SUBJECT	IgG CELL COUNTS (MEANS OF COUNTS ON 4 SECTIONS)		
	UPPER TRACHEA	MAIN BRONCHUS	LOWER LOBE BRONCHUS
15	37	51	10
16	28	69	59
17	32	128	81
18	175	52	4
19	21	21	4
20	8	235	55
OVERALL MEAN	50	93	35

TABLE 15

IgM cell counts in six subjects with 'fatal' bronchitis.

Unprocessed cell counts are set out in Appendix 4.

SUBJECT	IgM CELL COUNTS (MEANS OF COUNTS ON 4 SECTIONS)		
	UPPER TRACHEA	MAIN BRONCHUS	LOWER LOBE BRONCHUS
15	21	26	18
16	12	64	40
17	28	62	17
18	134	192	75
19	7	18	2
20	29	46	48
OVERALL MEAN	35	68	33

TABLE 16

Mean IgE cell counts in six subjects with 'fatal' bronchitis.

Unprocessed cell counts are set out in Appendix 4.

SUBJECT	IgE CELL COUNTS (MEAN OF COUNTS ON 4 SECTIONS)		
	UPPER TRACHEA	MAIN BRONCHUS	LOWER LOBE BRONCHUS
15	16	9	25
16	44	28	12
17	27	54	13
18	2	1	1
19	2	3	2
20	44	25	13
OVERALL MEAN	22	20	11

Fig. 35

Mean cell counts for each immunoglobulin class in trachea, main bronchus and lobar bronchus in six 'fatal' bronchitics. The predominance of IgA cells is less striking than for the normal subjects.

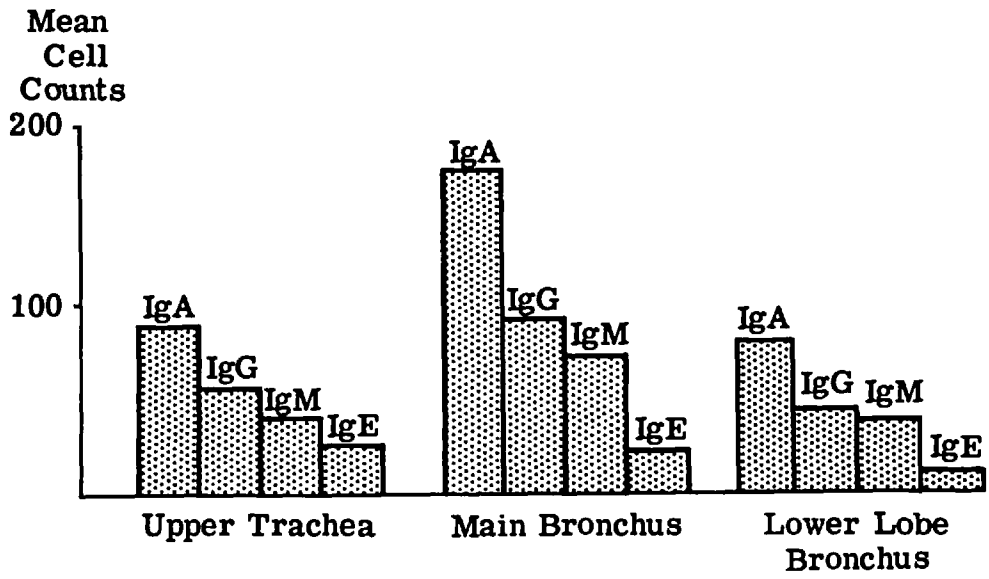

Mean
Cell
Counts
200
100
IgA
IgG
IgM
IgE
IgA
IgG
IgM
IgE
IgA
IgG
IgM
IgE
Upper Trachea
Main Bronchus
Lower Lobe
Bronchus

Fig. 36

Comparison between IgA cell counts at main bronchus with the age of the subject (nine normals and eleven chronic bronchitics).

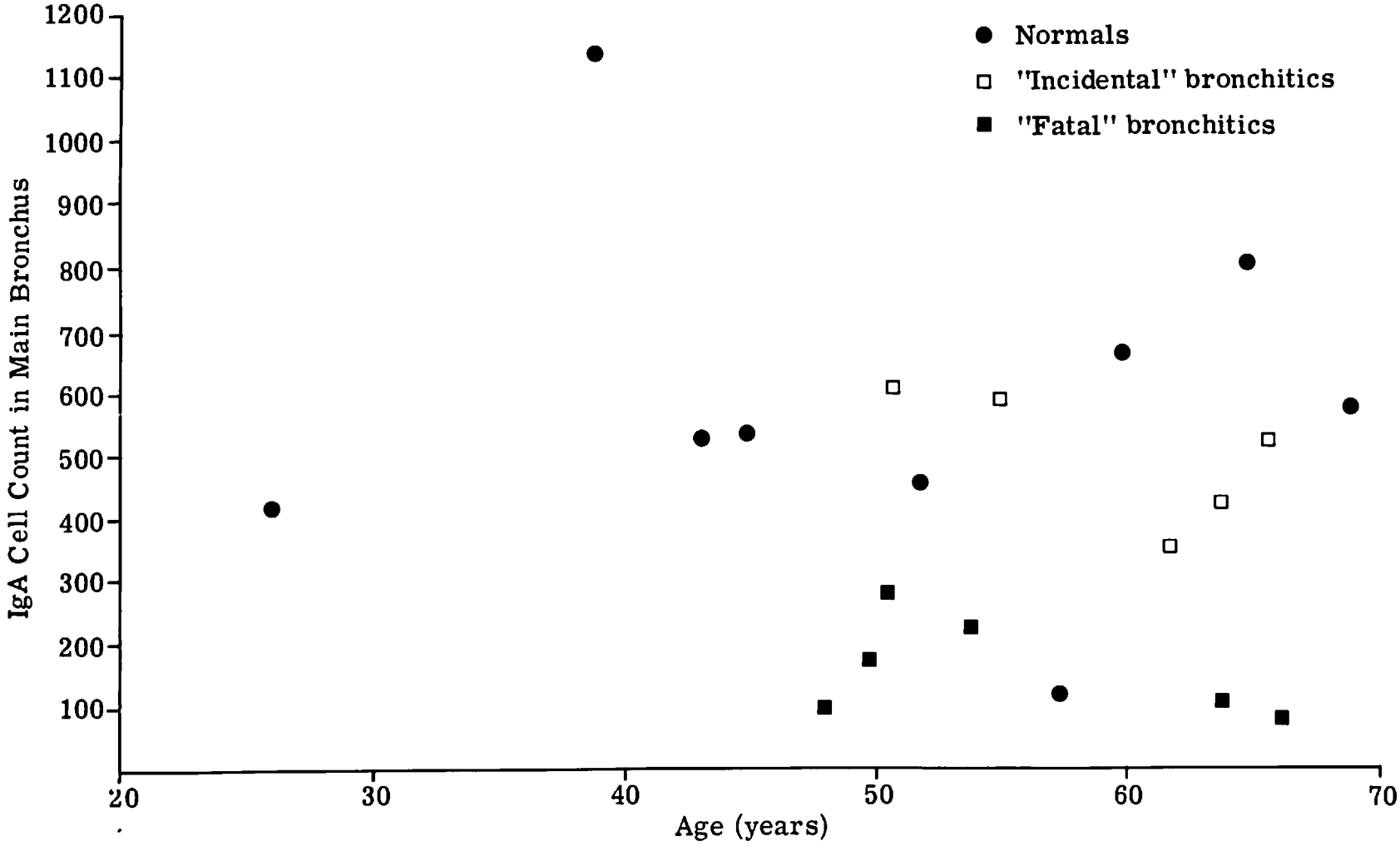
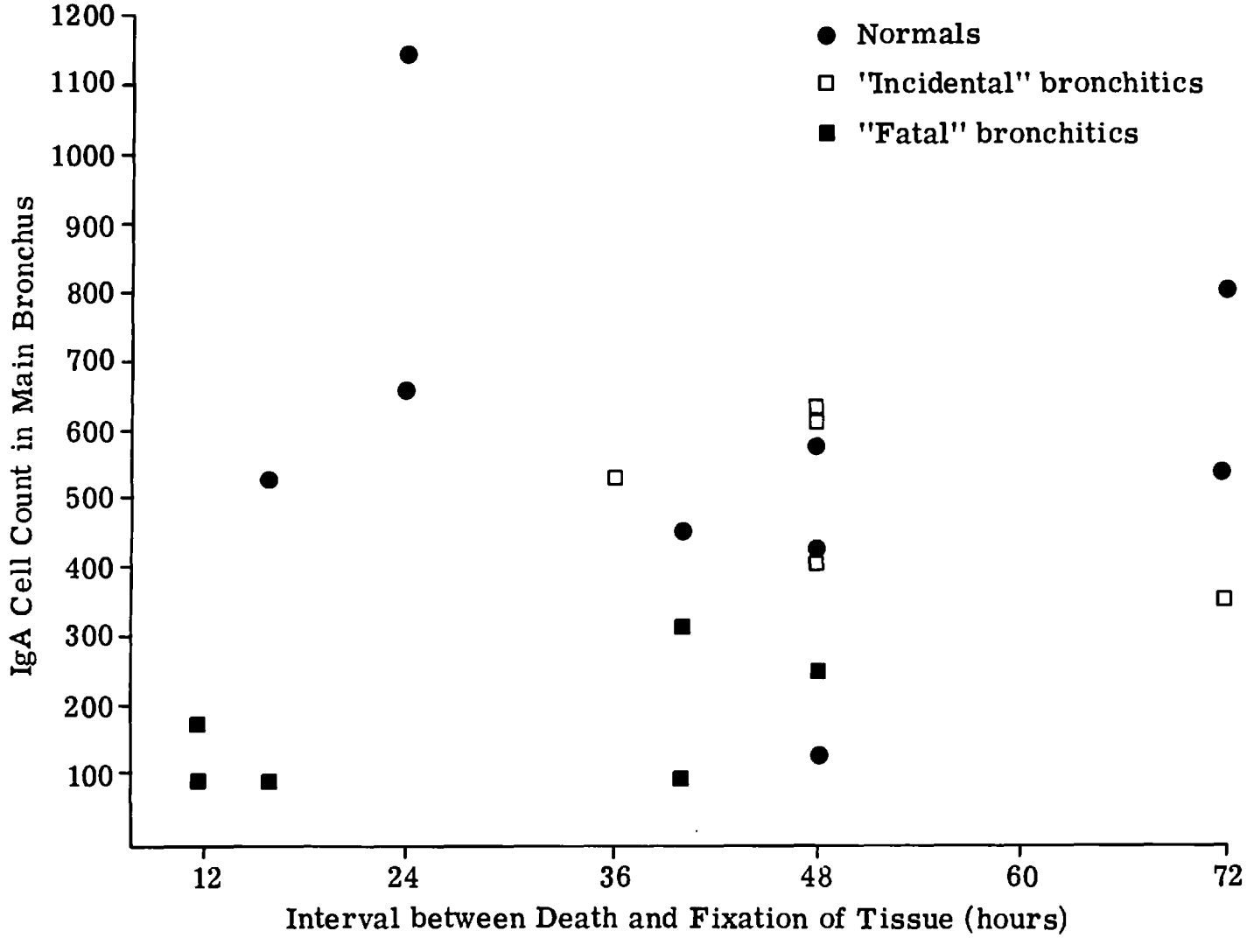

Normals
"Incidental" bronchitics
"Fatal" bronchitics
IgA Cell Count in Main Bronchus
1200
1100
1000
900
800
700
600
500
400
300
200
100
20
30
40
50
60
70
Age (years)

Fig. 37

Comparison between IgA cell counts at main bronchus with the interval between death and fixation of tissue (nine normals and eleven chronic bronchitics).


Normals
"Incidental" bronchitics
"Fatal" bronchitics
IgA Cell Count in Main Bronchus
1200
1100
1000
900
800
700
600
500
400
300
200
100
12
24
36
48
60
72
Interval between Death and Fixation of Tissue (hours)

(c) Paraffin sections and gland/wall ratios

Examination of paraffin sections by haematoxylin and eosin stains confirmed the mucous gland hypertrophy to be expected in chronic bronchitic subjects. Gland/wall ratios (Reid Indices) are set out in Table 17. The quality of pyronine stains on these post-mortem tissues were poor and, in any case, very few plasma cells were seen.

Eosinophils were scanty.

(d) Serum IgA

Serum samples were not taken from these subjects but in order to estimate whether the non-smoking subject with severe chronic bronchitis (subject 20) had a systemic deficiency of IgA, IgA and albumen were estimated by immunodiffusion (against a serum IgA standard) in an extract from homogeonised tracheal tissue. The IgA: albumen ratio was 1:16. This estimate, though admittedly very crude, suggests that the serum IgA was not reduced, and may have been raised.

TABLE 17

Gland/wall ratios (Reid indices) of eleven chronic bronchitic subjects (normal up to 0.36, chronic bronchitis 0.36-0.79).
(Reid 1960).

	SUBJECT	GLAND/WALL RATIO
'Incidental Bronchitics'	10	0.52
	11	0.61
	12	0.63
	13	0.70
	14	0.50
'Fatal' Bronchitics	15	0.39
	16	0.56
	17	0.58
	18	0.57
	19	0.69
	20	0.53

CHAPTER 7

RESULTS:

THE FREQUENCY OF PLASMA AND OTHER CELLS CONTAINING IMMUNOGLOBULIN IN THE CARINAL LYMPH NODES OF NORMAL SUBJECTS AND SUBJECTS WITH CHRONIC BRONCHITIS

Sections: Weight and general appearances

Immunofluorescent stains

Cell counts

Counts of germinal centres

Relationship between cell counts and lymph node mass

Weight and general appearances

The weights of the inferior tracheobronchial lymph node masses varied widely between subjects (range 0.6 to 7.2 gms) (Table 18) and there were no significant differences in weight between the clinical groups, even though the two heaviest gland masses were found in 'fatal' bronchitics. Examination of sections stained by haematoxylin and eosin showed that parts of the nodes were replaced by areas of fat or fibrous tissue, and this was more marked among the 'fatal' bronchitics. The nodes appeared anatomically normal, and some lymphatic follicles appeared to have active germinal centres.

Immunofluorescent stains

Plasma cells and other cells containing immunoglobulin were clearly seen lying in the medullary cords and the cortico-medullary junction and around vessels and sinusoids in all lymph nodes (Fig. 38) and were infrequent in the cortex. The germinal centres of active lymphatic follicles also contained immunoglobulin either within the cell cytoplasm or on the surface of the cells (Fig. 39). The surrounding cuff of lymphocytes never showed specific staining.

The appearances on staining with anti-IgG conjugates were less distinct than for the other immunoglobulins because of the high background of specific fluorescence due to the presence of IgG in between the cells. This probably represented serum IgG, although it may have been secreted by the cells of the node. It was absent from the tightly packed lymphocytes in the peripheral cuff of the lymphatic follicles. In spite of this high background staining, individual plasma cells and germinal centres could still

TABLE 18

Weights of carinal lymph node masses - * only part of the lymph node mass was obtained from this subject.
$\neq$ means are actually root mean squares.
Differences between groups not significant.

	SUBJECT	WEIGHT OF CARINAL NODES (gms)	
Normal non-smokers	1	1.82	
	2	2.93	
	3	2.59	mean 2.31^{+}
	4	1.07	$\neq$
	5	3.88	
Normal smokers	6	4.34	
	7	4.24	
	8	1.08	mean 2.19^{+}
	9	0.6	
'Incidental' bronchitics	10	3.87	
	11	2.09	
	12	2.99	mean 2.14^{+}
	13	-*	
	14	1.20	
'Fatal' bronchitics	15	3.65	
	16	2.55	
	17	7.24	mean 3.89^{+}
	18	3.96	
	19	6.62	
	20	1.29	

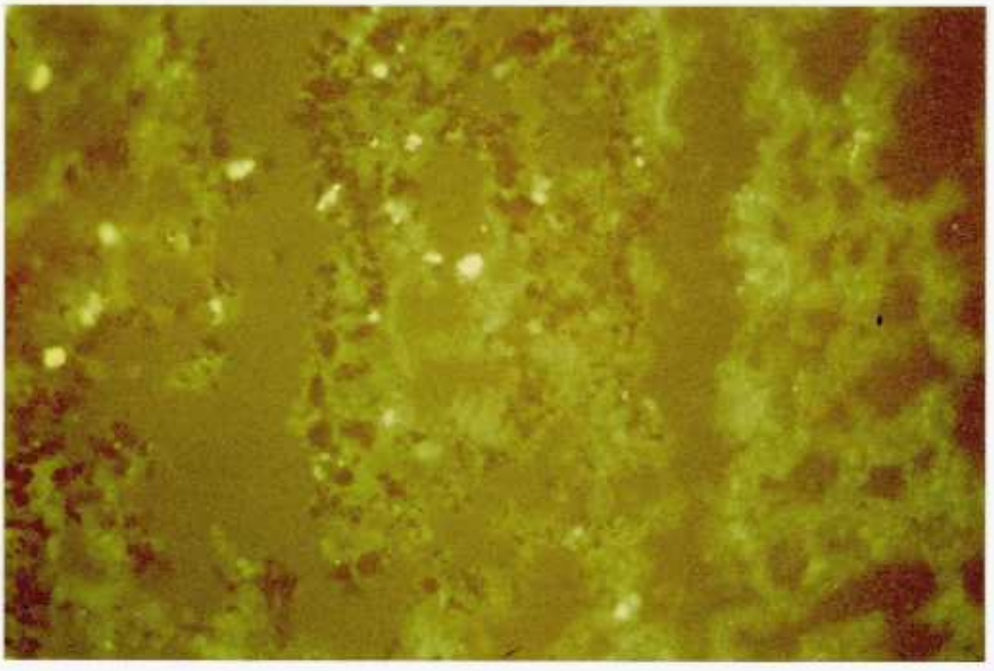
Fig. 38, a, b, c.

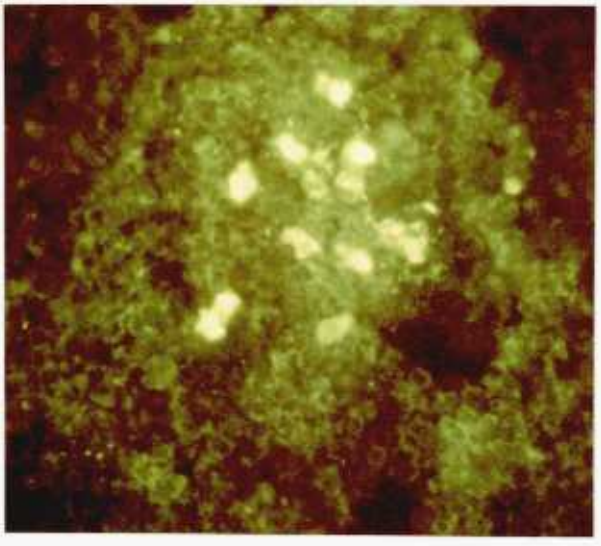
IgA cells in medulla and cortico-medullary junction of carinal lymph nodes.

a. x 200

b. x 300

c. x 300





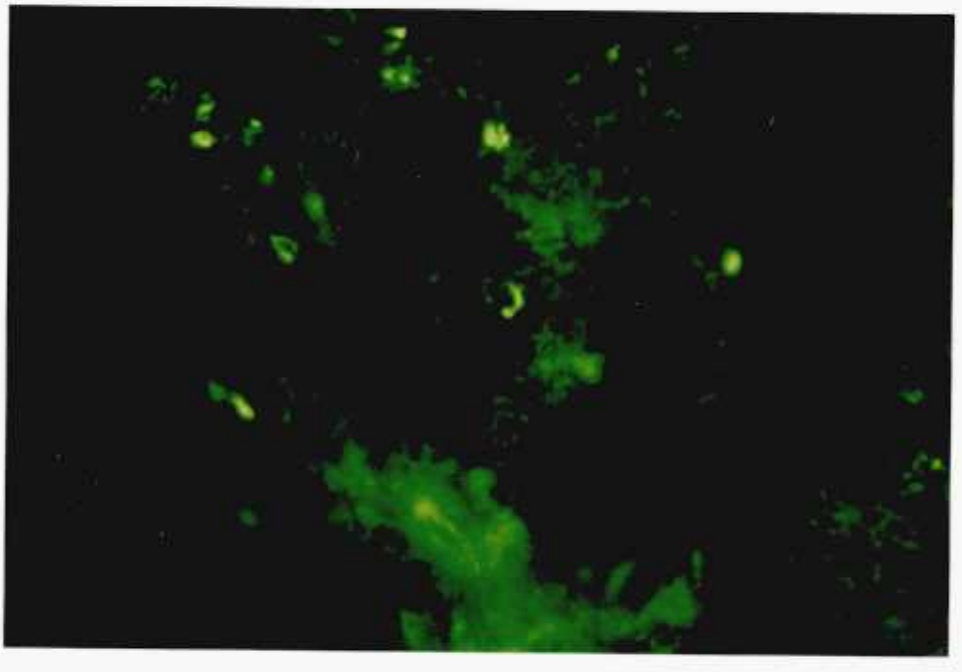


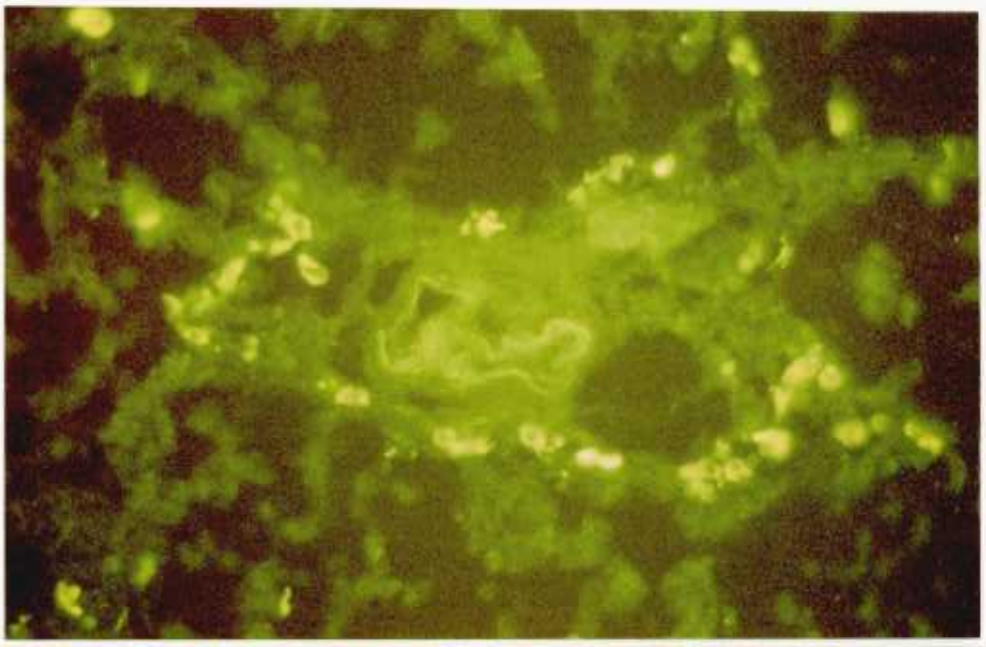
Fig. 38, d, e, f.

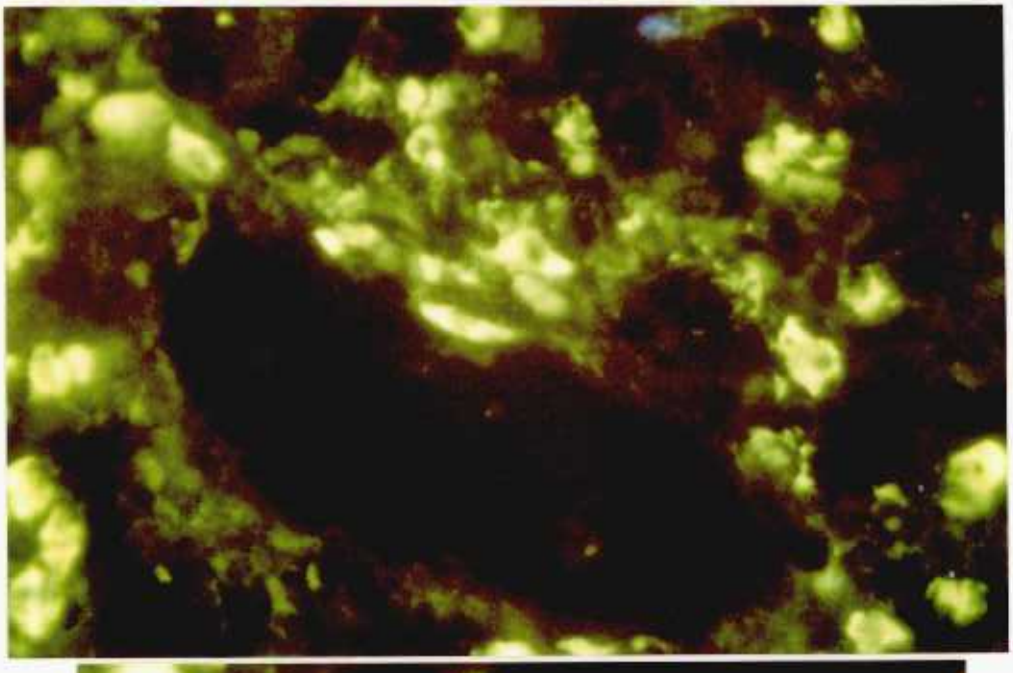
d and e IgA cells round vessel in medulla of carinal lymph node.

d. x 200

e. x 300

f. IgM cells round vessel. An eosinophil showing non-specific fluorescence is passing through the vessel perimeter. x 400





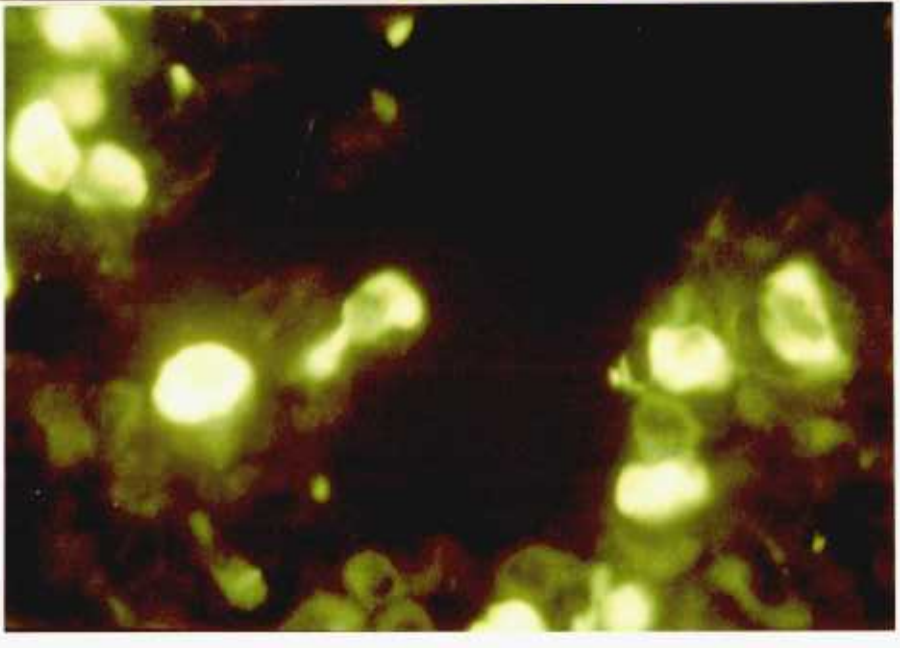
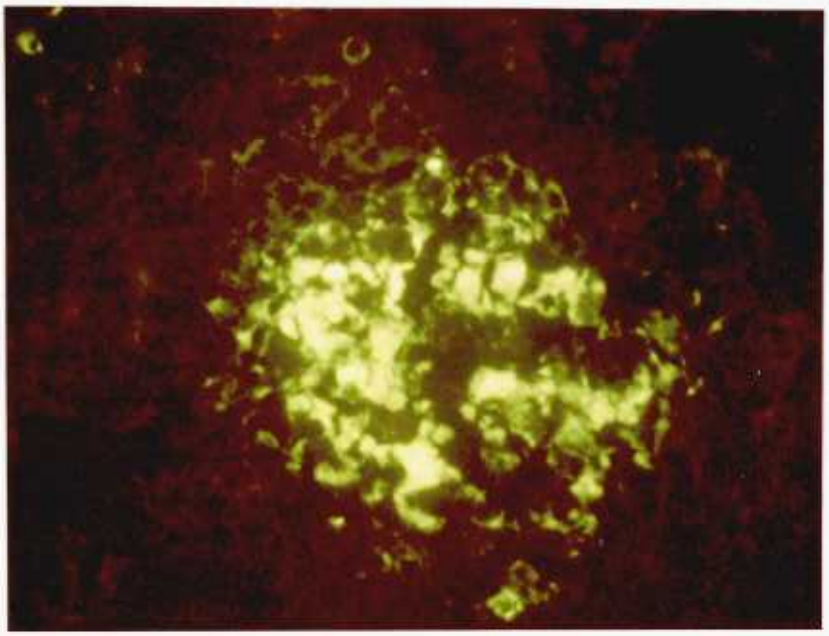


Fig. 39 a and b

Germinal centres containing IgM in:

a. Normal subject, x 200

b. 'Fatal' bronchitic, x 200



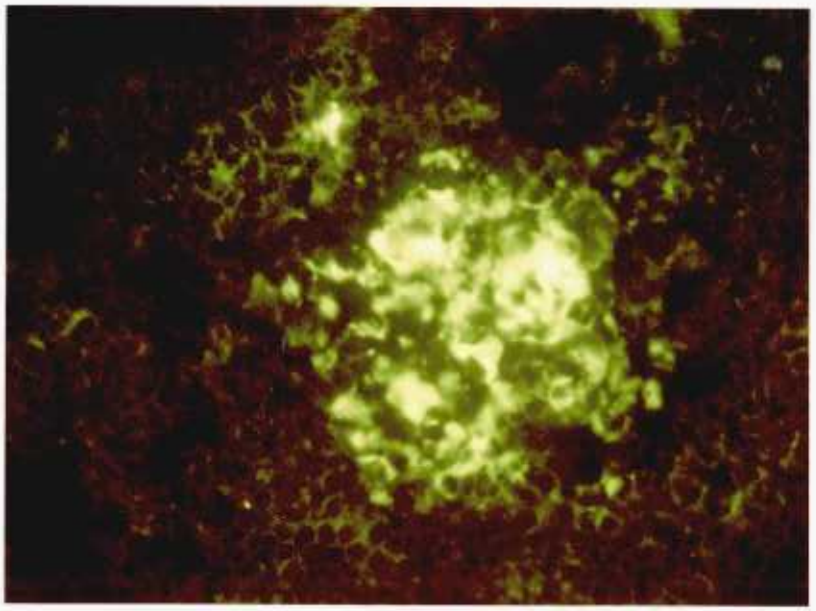
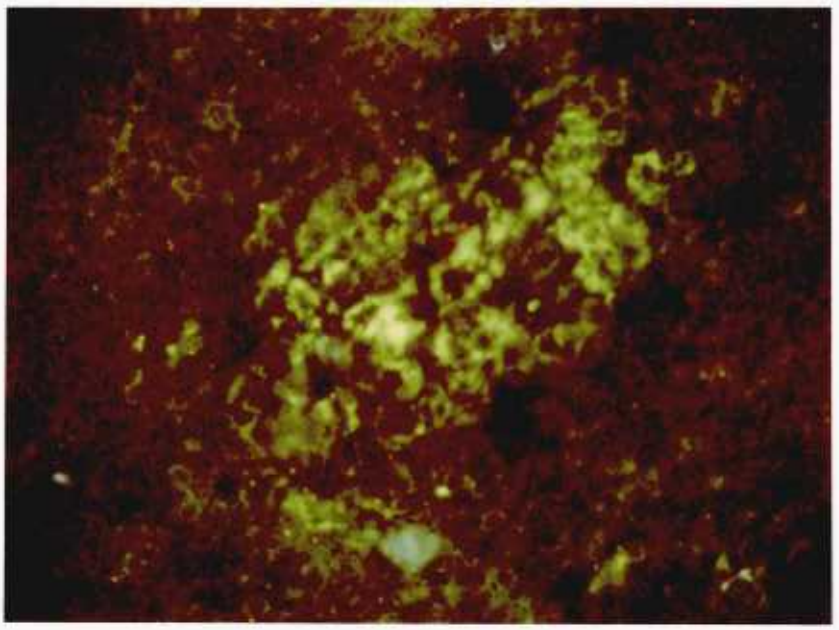
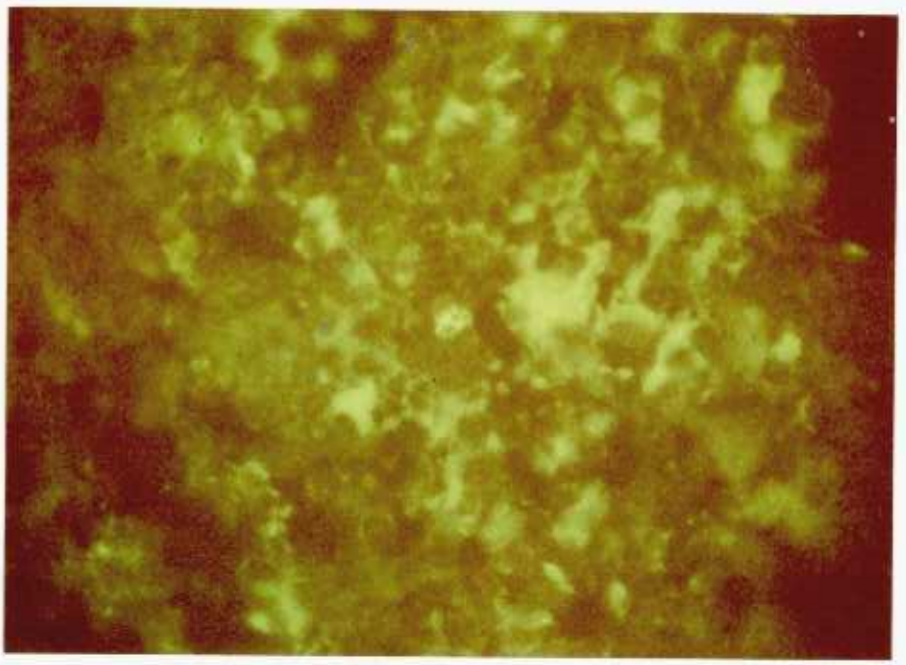


Fig. 39, c and d

c. Germinal follicle containing IgA in normal subject. x 200

d. Germinal follicle containing IgA in 'fatal' bronchitic. x 400





be recognised though with less precision than for the other immunoglobulin classes.

The immunoglobulin-containing cells in the medullary cords mostly had the appearances of mature plasma cells, although some had scanty cytoplasm and resembled immature plasma cells or lymphocytes. The cells also congregated around thin walled vessels in the medulla. A small number of cells had the appearance of eosinophils, recognised by their granular cytoplasm and bilobed nuclei. All the cells stained by anti-complement conjugates had the appearance of eosinophils.

Cell Counts

Counts of cells containing immunoglobulin showed that IgA and IgM cells were most common in all subjects (Table 19) (Fig. 40) although cells containing IgG and IgE were also numerous. An example of the arithmetic derivation of one figure in Table 19 from the original cell counts per unit area of section is set out in Table 20. All other basic data are set out in Appendix 5. There were no significant differences in cell counts between normal non-smokers, normal smokers and 'incidental' bronchitics in any of the immunoglobulin classes, but the 'fatal' bronchitics had significantly lower IgA and IgM cell counts than normal non-smokers (IgA, $p < 0.01$; IgM, $p < 0.01$), lower IgA cell counts than normal smokers ($p < 0.05$) and lower IgA and IgM cell counts than 'incidental' bronchitics (IgA $p < 0.01$; IgM, $p < .005$) IgG and IgE cell counts

TABLE 19

Mean Cell Counts in the carinal lymph nodes of nine normal subjects and eleven bronchitics.

	SUBJECT	MEAN CELL COUNTS (CELL/mm^2 IN 4 SECTIONS)				
		IgA	IgM	IgG	IgE	Eosinophils
Normal non-smokers	1	110	144	69	34	10
	2	164	161	114	128	19
	3	76	53	50	50	42
	4	99	93	59	59	37
	5	144	45	96	91	36
	MEAN*	116	93	69	68	27
Normal smokers	6	140	150	121	198	10
	7	185	70	185	142	28
	8	78	29	86	31	18
	9	69	55	19	67	54
	MEAN*	114	70	91	98	25
'Incidental' bronchitics	10	82	58	24	17	82
	11	99	83	33	40	16
	12	248	112	236	143	7
	13	178	147	167	115	36
	14	134	109	122	140	24
	MEAN*	142	99	99	81	28
'Fatal' bronchitics	15	70	42	55	31	21
	16	64	24	111	44	17
	17	82	34	103	88	8
	18	39	52	31	25	25
	19	36	34	84	9	2
	20	82	21	60	19	27
	MEAN*	61 +	37 +	73	24	14

* Means are actually root mean squares.

\+ Indicates cell counts significantly different from other clinical groups.

Fig. 40

Mean cell counts for each immunoglubulin class in the carinal lymph nodes of five normal non-smokers, four normal smokers, five 'incidental' bronchitics, and six 'fatal' bronchitics. Each column represents the root mean square of cell counts on four sections. The IgA and IgM cell counts are significantly lower in the 'fatal' bronchitics than in the other groups (see text).

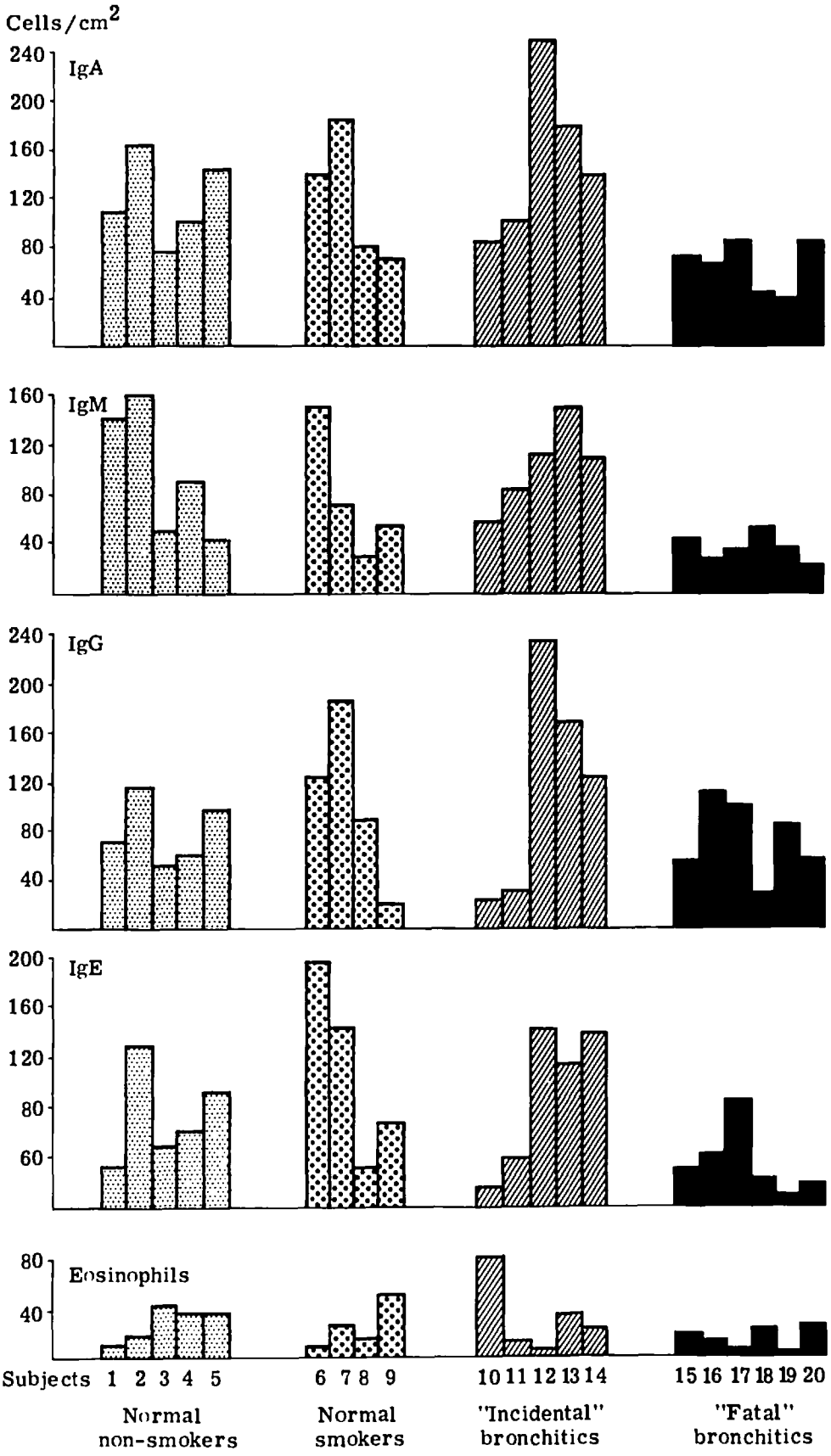

Cells/cm2
IgA
240
200
160
120
80
40
IgM
160
120
80
40
IgG
240
200
160
120
80
40
IgE
200
160
120
80
60
Eosinophils
80
40
Subjects 1 2 3 4 5
6 7 8 9
10 11 12 13 14
15 16 17 18 19 20
Normal non-smokers
Normal smokers
"Incidental" bronchitics
"Fatal" bronchitics

TABLE 20

Derivation of the mean IgA cell count from four sections of carinal lymph node from Subject 1. (This mass of data provides only one of the 100 numbers contained in Table 19).

CELL COUNTS IN EVERY FOURTH CONSECUTIVE FIELD COVERED BY A SQUARE GRATICULE .67 SQ. MM. AREA	ARITHMETIC MEAN $\pm$ SD	CONVERSION TO CELLS/CM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE CELLS/CM^2
1st section 7,4,7,4,12,0,12,2,12,10,3,13 4,8,7,1, 6,6, 6,16,4,13,3, 0 6,9,4	6.62 $\pm$ 4.28	98.80	9.94	10.47	110
2nd section 19,15,13,18,8,4,10,15,2,12, 12,12,8,1,17,20,1,8,20,6,6,4, 3,11	10.13 $\pm$ 6.22	151.19	12.29		
3rd section 5,4,14,4,3,3,5,9.6,0,1,2,5, 3,7,2,5,7,2,11,4,6,8,3,2	4.56 $\pm$ 3.38	68.06	8.25		
4th section 16,10,2,3,10,10,8,1,37,11,11, 12,0,4,4,7,3,12,6,12,7,8,10, 7,5,0,4,9,7,0,11,5,9,1,2,8, 3,7	8.73 $\pm$ 9.98	130.29	11.41		

were not significantly altered.

Thus, the 'fatal' bronchitics had significantly lower concentration of plasma and other cells containing IgA and IgM than normals or 'incidental' bronchitics.

Cell counts of cells stained by anti-complement conjugates (apparently all eosinophils) were mostly small compared with the plasma cell counts (Table 19) (Fig. 40) and there were no significant differences between the groups. This staining may all have been non-specific, though cells containing complement in antigen/antibody complexes, which would stain specifically with anti-complement conjugates, would also have been included in the cell counts. While the amount of non-specific staining produced by different conjugates is unlikely to be identical, the cell counts with anti-complement conjugates do suggest that non-specific staining of eosinophils was unlikely to have materially altered the plasma cell counts.

Counts of germinal centres

Counts of active germinal centres containing immunoglobulins (Table 21) showed that these were rare in normal non-smokers (seen in only one subject out of five), but commonly seen in normal smokers (seenin all four subjects), ($p < .001$) and that this difference is mainly accounted for by an increase in the numbers of germinal centres containing IgM ($p < 0.01$) and IgG ($p < .05$).

TABLE 21

Total numbers of germinal centres containing immunoglobulins in sections of carinal lymph nodes of normal and chronic bronchitic subjects.
Each figure (other than totals and means) is the total number of germinal centres containing immunoglobulin in four sections.

	SUBJECT	TOTAL NO. OF GERMINAL CENTRES CONTAINING IMMUNOGLOBULINS IN FOUR SECTIONS				
		IgA	IgM	IgG	IgE	ALL Ig (16 SECTIONS)
Normal non-smokers	1	4	1	0	1	6
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0
	MEAN*	0.16	0.04	0	0.04	0.25
Normal smokers	6	0	9	2	0	11
	7	4	1	0	0	5
	8	0	12	2	8	22
	9	0	4	2	5	11
	MEAN*	0.25	8.2 +	1.1 +	1.6	11.5 +
'Incidental bronchitics	10	32	56	8	11	107
	11	0	0	0	0	0
	12	0	1	1	0	2
	13	0	1	0	0	1
	14	0	0	0	0	0
	MEAN*	1.3	3.6 +	0.6	0.4	6.5
'Fatal' bronchitics	15	0	23	7	10	40
	16	7	28	8	6	49
	17	0	0	0	0	0
	18	0	1	5	0	6
	19	19	41	22	0	82
	20	2	0	2	1	5
	MEAN*	1.9	8.5	5.3	1.2	20.3 +

* Means are actually root mean squares.

\+ Indicateswhere group values are significantly greater than normal non-smokers.

The 'incidental' bronchitics had significantly more germinal centres containing IgM than normal non-smokers ($p < .05$) but the overall numbers of active centres of all immunoglobulin classes was not significantly greater than in normal non-smokers. There were no significant differences between normal smokers and 'incidental' bronchitics. In the 'fatal' bronchitics the number of active centres was also greater than in normal non-smokers ($p < 0.05$) and this was accounted for mainly by an increase in the numbers of centres containing IgM, although the differences for this immunoglobulin alone were not significant. There were no significant differences between normal smokers, 'incidental' bronchitics or 'fatal' bronchitics.

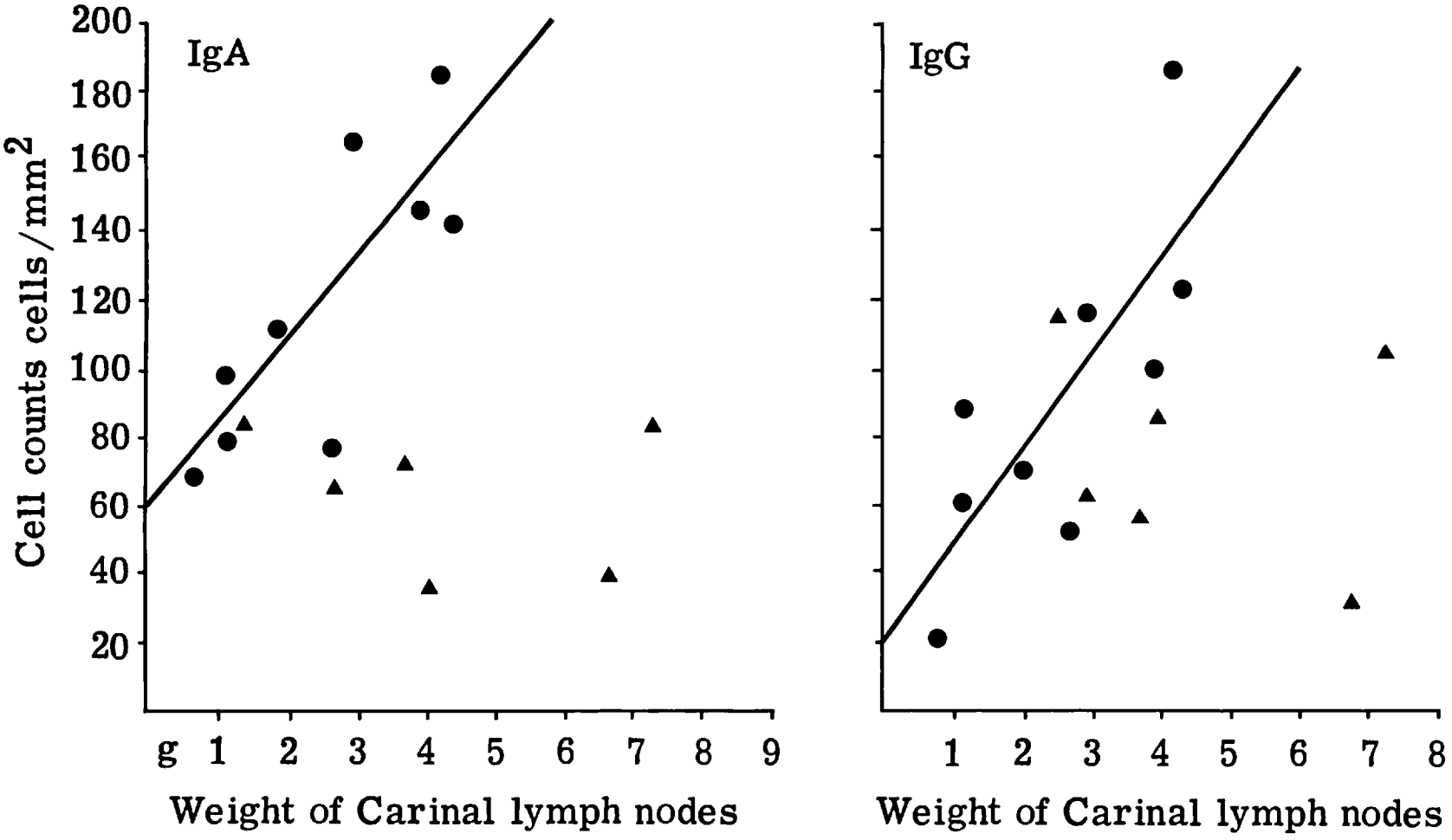
Thus, there was an increase in germinal centre activity not only in those with chronic bronchitis ('incidental' and 'fatal') but also in normal smokers without cough or other respiratory symptoms.

Relationship between cell counts and lymph node mass

There was a rough linear relationship amongst normal subjects between the IgA cell count and the total weight of the carinal gland mass ($p < 0.01$, Fig. 41) and a similar correlation between IgG cell count and gland weight ($p < 0.05$, Fig. 41). The 'fatal' bronchitics had significantly lower IgA cell count/gland weight ratios than the normal, that is, there were fewer cells in relation to the gland mass than in the normals, ($p < 0.01$). The IgG cell count/gland weight ratios were not significantly different from normal.

Fig. 41

Comparison between the IgA and IgG cell counts and weight of gland mass in the carinal lymph nodes of nine normal subjects and six 'fatal' bronchitics. The linear regression for the normals (O) has been drawn in ($p < 0.01$) for IgA, and $p < 0.05$ for IgG. The cell count/gland weight ratios for the 'fatal' bronchitics (A) are significantly different ($p < 0.01$) for IgA only.


IgA
IgG
Cell counts cells/mm2
200
180
160
140
120
100
80
60
40
20
g 1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8
Weight of Carinal lymph nodes
Weight of Carinal lymph nodes

CHAPTER 8

RESULTS:- SPUTUM IMMUNOGLOBULIN MEASUREMENTS

1. Bronchial immunoglobulin secretion in chronic bronchitic subjects.

2. Bronchial immunoglobulin secretion in control subjects

1. Bronchial immunoglobulin secretion in chronic bronchitic subjects

IgA

Estimates of the proportion of the sputum IgA which was locally secreted in fifty seven samples from fourteen subjects with severe chronic bronchitis are set out in Table 22 and Fig. 42. In nine subjects the local IgA secretion was greater than 70% in all samples, or fell below this value on only one occasion (subjects a to i), and in these subjects values in samples taken on different days were consistent. However, in the other five subjects (j to n) the local IgA contribution was less than 70% on three or more occasions.

In this latter group there were striking fluctuations in the percentage of local IgA, which in many cases appeared to show a progressive fall and subsequent rise through the period of illness. In four of the subjects the local IgA percentage actually fell to nil before rising again as the illness resolved.

Bronchial aspirate was only obtained when the patient was being artificially ventilated for clinical reasons. These tended to show low or absent local secretion of IgA, but these results tended to be quite consistent with the trend in the results obtained from sputum samples taken before and after ventilation, and confirm that these low values were not the result of contamination with saliva.

TABLE 22 (First Part)

Local bronchial secretion of immunoglobulin in chronic bronchitic subjects.

Subject	Age	No. of Estimations	Material	Local Secretions	
				IgA (%)	IgG (%)
A	62	2	Sputum	92	0
			"	87	0
B	59	4	"	56	10
			"	78	56
			"	85	61
			"	80	46
C	67	3	"	93	7
			"	91	9
			"	97	18
D	46	2	"	86	0
			"	84	0
E	59	4	"	96	0
			"	96	0
			"	97	0
			"	98	22
F	70	3	"	86	0
			"	87	0
			"	89	0
G	68	3	"	86	0
			"	80	0
			"	82	0
H	68	3	"	92	40
			"	92	38
			"	66	40
I	41	2	"	92	1
			"	93	14

(Continued overleaf)

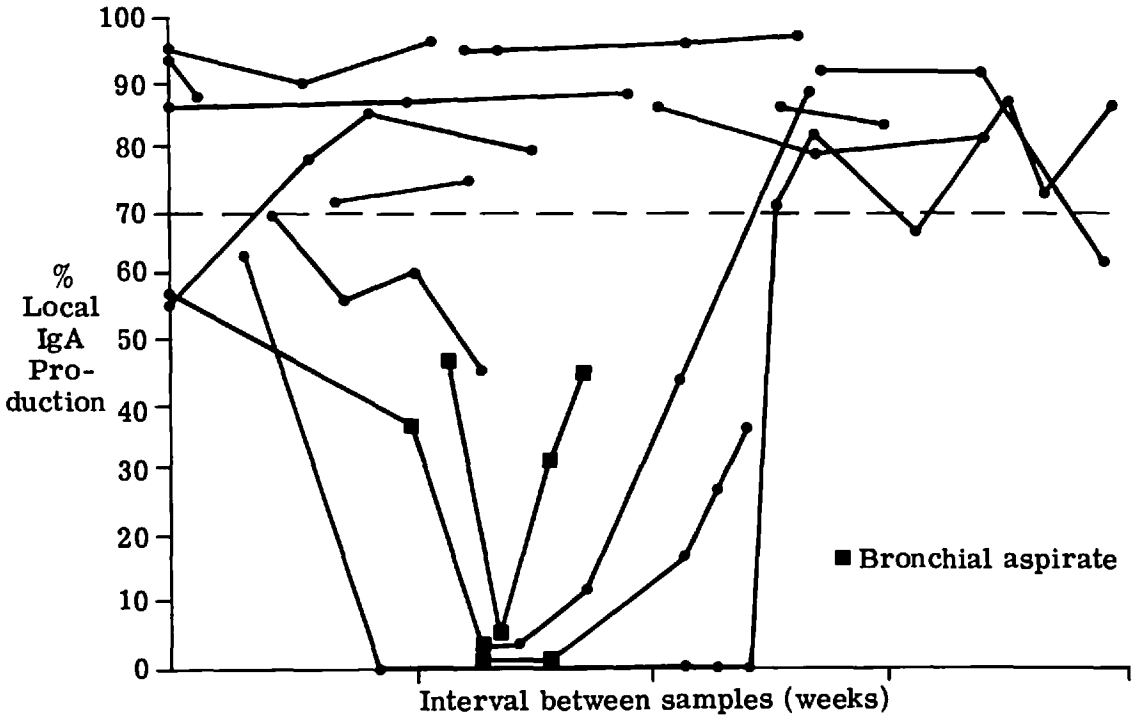
TABLE 22 (Continued)

Local bronchial secretion of immunoglobulin in chronic bronchitic subjects.

Subject	Age	No. of Estimations	Material	Local Secretions	
				IgA (%)	IgG (%)
J	65	5	Bronchial Aspirate	0	0
			"	0	0
			Sputum	16	0
			"	25	0
			"	36	0
K	55	7	"	56	0
			Bronchial Aspirate	37	0
			"	0	0
			Sputum	0	0
			"	11	0
			"	44	0
			"	88	0
L	55	11	"	62	0
			"	0	0
			"	0	0
			"	0	0
			"	0	0
			"	71	0
			"	83	0
			"	66	0
			"	87	0
			"	72	0
			"	87	0
M	75	4	"	69	31
			"	55	29
			"	60	42
			"	44	15
N	46	4	Bronchial Aspirate	46	0
			"	0	0
			"	32	0
			"	44	0

Fig. 42

Random estimations of bronchial IgA secretion in 14 chronic bronchitic subjects. Separation along the X axis is for the purposes of visual clarity. Intervals between samples are correct.


100
90
80
70
60
50
40
30
20
10
0
% Local IgA Pro-duction
Interval between samples (weeks)
Bronchial aspirate

IgG

In only six subjects was local secretion of IgG demonstrated, and in only five was it consistently present. Where active IgG secretion was absent it tended to remain absent in all samples examined, through all phases of the acute illness. IgG was, of course, present in all of the sputum samples, but in the other nine subjects could be accounted for by the serum contribution.

No relationship, positive or negative, was demonstrated between the local secretion of immunoglobulin and sputum purulence.

2. Bronchial immunoglobulin secretion in control subjects

The proportions of IgA and IgG which were locally secreted by the bronchi in 29 control subjects are set out in Table 23. Only one estimation was carried out for each individual. The mean local IgA secretion of these subjects (none of whom were normal, of course) was 82%, consistent with the values found by Deuschl and Johannson (1974) in bronchial washings. Single estimations in small numbers of individuals do not allow satisfactory comparisons between bronchiectatic subjects, those with recurrent infections of unknown cause, and those with asthma with or without allergic broncho-pulmonary aspergillosis, but there are no striking group differences. Five subjects out of twenty nine had local IgA secretion of less than 70%, one subject in each clinical group, but only one value was less than 50%.

TABLE 23

Local Secretion of Immunoglobulin in the sputum of 29 control subjects.

Subject	Condition	Local Secretions	
		IgA (%)	IgG (%)
1	Bronchiectasis	87	11
2	"	61	0
3	"	84	0
4	"	81	10
5	"	83	72
6	"	89	0
7	"	96	53
8	"	97	21
9	"	36	0
10	"	96	42
11	"	83	34
12	Rec. Infections	97	32
13	"	98	13
14	"	91	0
15	"	70	60
16	"	53	0
17	"	77	0
18	Asthma	76	0
19	"	90	0
20	"	83	0
21	"	92	3
22	"	52	12
23	Asthma + Allergic Aspergillosis	81	0
24	"	78	0
25	"	65	0
26	"	98	78
27	"	96	43
28	"	96	27
29	"	89	0
MEAN		81.9 SD ± 15.7	17.6 SD ± 24.0

IgG local secretion was always less than IgA, usually much less (mean 18%), and often absent. There were no obvious differences between the clinical groups.

CHAPTER 9

DISCUSSION

1. Normal Subjects

   (a) Predominance of cells containing IgA in the respiratory tract.

   (b) Distribution of cells containing immunoglobulin in the respiratory tract.

   (c) The effects of smoking on cells containing immunoglobulin in the respiratory tract.

   (d) Differences between individual subjects.

2. Chronic Bronchitic Subjects

   (a) Bronchial IgA deficiency in fatal chronic bronchitis; immunofluorescent studies.

   (b) Bronchial IgA deficiency in severe chronic bronchitis:- sputum studies.

   (c) "Incidental" chronic bronchitis.

3. Immunoglobulin-containing Cell Populations in Carinal Lymph Nodes of Normal Subjects and Chronic Bronchitics.

   (a) Carinal lymph nodes.

   (b) Kinetics of bronchial plasma cells.

   (c) The effects of smoking on bronchial defence mechanisms.

4. General Implications.

1. Normal Subjects

(a) Predominance of cells containing IgA

The finding in normal individuals of a predominance of IgA in plasma and other cells containing immunoglobulin is consistent with the findings of most previous workers. Tourville and his colleagues (Tourville et al 1969) found cells containing IgA to be the predominant type in exocrine glands of all varieties, including the gut, salivary glands, urinary tract and gall bladder, as well as the upper and lower respiratory tracts. By contrast, Martinez-Tello and his colleagues (Martinez-Tello et al 1968), while confirming the large numbers of IgA cells in the bronchial wall, found an almost equally large number of IgG cells. This difference, if not caused by technical factors (IgG cells are difficult to count because of the high background staining from IgG in interstitial fluid) may be explained by differences in the populations studied; for the most part, their group of subjects were children under the age of thirteen, and it is quite possible that children do have a different pattern of immunoglobulins in their bronchial secretions, for it is known the level of serum IgA in children under the age of twelve is rather variable, many being well below adult values (Allansmith et al 1968, West et al 1962).

Quite conceivably, IgG may play a larger part in the defence of the child's respiratory tract than in the adult, and this may have been reflected by the large numbers of IgG cells seen by these workers. An alternative explanation is suggested by the cell counts

of one of the normal subjects reported in this thesis, who also had very large numbers of IgG cells. This subject had no medical history other than his smoking habits to suggest he was anything but normal, and it is possible that a proportion of the normal population have as many IgG as IgA cells in their respiratory tract. Support for this is provided by the studies of immunoglobulins in sputum reported in this thesis, which demonstrated considerable variation in local secretion of IgG between individuals, though much less variation in IgA local secretion. The discrepancy between the findings of Martinez-Tello and colleagues and those reported in this thesis may therefore be the result of random sampling errors in these necessarily small groups.

Rossen and his colleagues (1968) confirmed the presence of many IgA cells in adult parotid gland, but were unable to demonstrate Ig cells in adult human bronchus from the only subject they examined. This finding has been attributed by other workers to the technical difficulty of cutting sections of tissues containing cartilage, although, in the light of the findings reported in this thesis, it may have been a correct observation.

Although measurements of immunoglobulins in sputum and bronchial washings can be confused by contamination with serum exudation from inflamed mucosal surfaces, or by saliva, nevertheless many studies have shown that the IgA/IgG ratio is mugh higher in sputum than in serum, confirming that IgA is actively secreted into bronchial mucus (Masson et al, 1966, Keimowitz, 1964, Falk et al, 1972). Until recent work by Deuschl and Johannsen (1975) it was,

however, not clear how much IgG was actively secreted. These authors used the concentration of albumen in bronchial washings as a marker of the amount of serum present in the secretions, and on the basis of the concentrations of immunoglobulins in serum and secretion, calculated the relative amounts which were locally secreted or derived from serum. The result for IgA confirmed that over 80% was locally secreted, but showed that less than 10% of IgG in bronchial secretion was locally secreted, the rest being derived from serum. This calculation, of course, assumes that albumen is not actively transported into bronchial secretions, and that albumen and immunoglobulin leak from serum to sputum at the same rate, an assumption considered by previous authors to be justified (Soothill, 1967).

These findings, therefore, are consistent with the predominance of IgA in cells in normal bronchial tissues found in the work reported in this thesis.

(b) <u>The distribution of cells containing immunoglobulin in the respiratory tract</u>

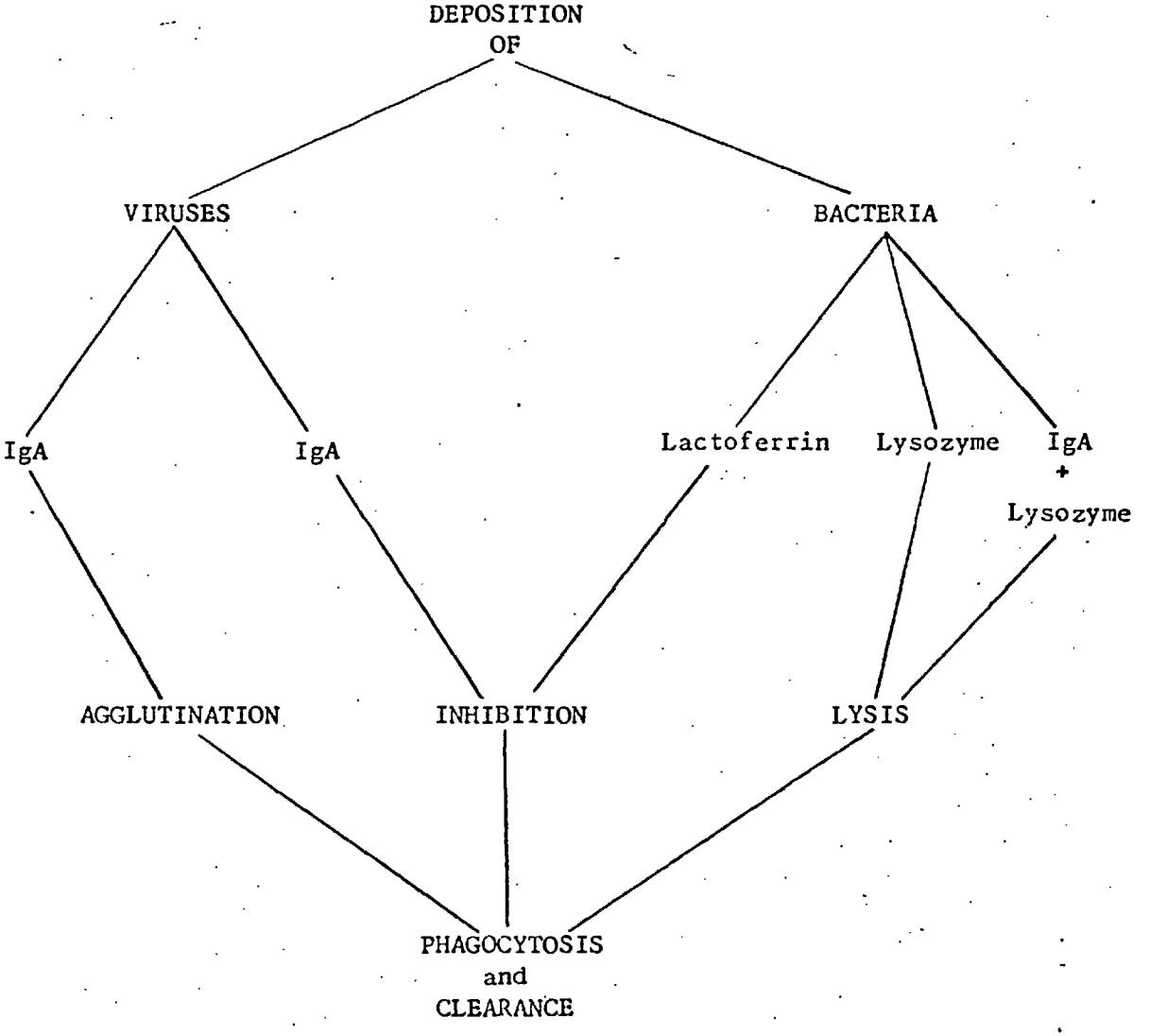
The vast majority of IgA cells in the normal respiratory tract were found in the mucous glands, lying between the Acini and ducts. The remaining small proportion were found in the lamina propria of the bronchial epithelium. If this biased distribution is useful it is presumably because the IgA serves its protective function better when discharged into the lumen of the bronchus than it would within the tissues of the bronchial wall. Actual invasion of the bronchial wall by micro-organisms might be expected to be dealt with by the

usual mechanisms of cellular and humoral immunity within the body, the latter predominantly involving IgG and IgM antibodies, with consequent complement fixation and bacterial lysis. This is quite distinct from the actions of IgA, which although poorly understood, seem not to involve the fixation of complement (Ishizaka et al, 1965), except in the presence of lysozyme (Adinolphi et al, 1966). Possibly IgA coats and agglutinates foreign particles, and provides a barrier between them and the host tissues, allowing time for anti-bacterial substances in the bronchial secretions, such as lactoferrin and lysozyme, to inhibit or destroy the organisms, which may then be engulfed by macrophages before final removal by the flow of mucus up the ciliary escalator, and by coughing. Certainly coating viruses with IgA impairs their ability to penetrate cells and might be expected to be protective.

The sequence of events illustrated in Fig. 43, from deposition of micro-organisms in the bronchial tree to their final killing, might occur. It will be noted that macrophage killing of organisms occurs fairly late in the sequence. This supposition is based on some personal observations of the relative sparseness of macrophages in sputum, and the presumed difficulty that macrophages and polymorph leucocytes might have in moving through the thick mucus, so that, if it were not for the anti-bacterial and antiviral properties of sputum, a bacterium might be able to divide and multiply, or a virus to invade an epithelial cell, before the macrophage could reach it. Of course, some samples of sputum contain

Fig. 43

Scheme of sequence of events in bronchial protective mechanisms.


DEPOSITION
OF
VIRUSES
BACTERIA
IgA
IgA
Lactoferrin
Lysozyme
IgA
+
Lysozyme
AGGLUTINATION
INHIBITION
LYSIS
PHAGOCYTOSIS
and
CLEARANCE

very large numbers of neutrophil polymorphs and macrophages and in this case these cells may be able to mount their attack very quickly. Processing of antigen and production of specific antibody is discussed in a subsequent section.

In the normal individuals, IgA cells were present in the greatest numbers in the mucous glands in the region of the main bronchus. Although many subjects had plentiful IgA cells in the upper trachea, this was not a constant finding.

At first sight it is something of a surprise to find so many plasma cells in the trachea and main bronchi, since those aerosol particles small enough to have passed the nasopharynx and larynx in any quantities are unlikely to be deposited in any numbers until they reach the tertiary bronchi (see Chapter 2). Nevertheless, there may be deposition of important numbers of microorganisms in the trachea and main bronchi, for those larger particles which do pass the larynx and might deposit in these large proximal airways, are able to carry disproportionately large numbers of viral or bacterial particles (see Chapter 2). Furthermore, the trachea and major bronchi are from time to time likely to receive relatively massive quantities of aspirated nasopharyngeal mucus or regurgitated food during sleep or by accident, and the plasma cells in main bronchi may be useful in defending the upper airways from this attack.

To some extent, the larger numbers of plasma cells in main bronchus than in lobar bronchus may merely be a reflection of the

larger surface area of the airway, and, of course, assuming that the right lower lobe bronchus was representative of all lobar bronchi, there are vastly more cells in total at lobar bronchial level than in both main bronchi.

IgA cells were to be found in the bronchi wherever mucous gland was present. In bronchioles, where mucous gland was lacking, IgA cells were often found in quite large numbers in the lamina propria of the bronchial epithelium, although this was not a constant finding, some subjects having many cells in this site and others none at all. In none of the subjects were IgA cells found around respiratory bronchioles or alveoli, nor was IgA demonstrable in fixed sections coating the walls of these airways. Presumably, therefore, a foreign particle deposited in this region would be dealt with by mechanisms independent of secretory IgA. Macrophages are plentiful in the alveoli, (Miller, 1950), and seem likely to be concerned in protection from particles deposited in these sites. They certainly ingest inhaled inorganic dusts such as asbestos fibres, coal and silica, (Parkes, 1974). It is not clear what proportions of these alveolar macrophages migrate up the airways to the bronchial mucus, and eventually up the ciliary escalator to the larynx, and what proportion migrate by lymphatics to the regional lymph nodes. Macrophages packed with organic dust can be seen following both routes.

The studies of Laurenzi and colleagues (1963) in animals have shown that pulmonary alveolar macrophages take up inhaled bacteria, and are of considerable importance in restoring the sterility of the lung.

Particles deposited in the respiratory bronchioles and alveoli are in almost direct contact with the internal environment of the body, without the protection of a thick epithelium or even a layer of mucus. It might be expected, therefore, that immunological reactions would be of the systemic kind. This indeed seems to be the case, for Chanock's work (see Chapter 2) has shown that bacterial aerosols containing only small particles likely to be deposited in small airways tend to cause good serum antibody responses, while those containing only large particles likely to be deposited more proximally cause good secretory antibody, but poor serum responses.

Conceivably the distribution of IgA in cells and in secretions in the respiratory tract may influence the clinical effects of inhalation of an infectious agent. A subject lacking IgA cells in the upper part of the trachea might be especially liable to tracheitis from appropriate organisms, or someone with very few cells in the bronchioles might be especially liable to infections of the small airways. Organisms penetrating through to the alveoli may cause pneumonia, or in the case of foreign antigens such as the Micropolyspora group of moulds, an alveolar allergic reaction (Farmers lung, extrinsic allergic alveolitis).

There seems no obvious cause for these variations in distribution of IgA cells, nor whether these differences would have been permament ones if the subjects had lived. Nor is it clear how

the IgA cells congregate in the mucous glands of the bronchial epithelium. The kinetics of lymphocytes and plasma cells are discussed in a subsequent section, but there presumably must be chemotactic factors attracting plasma cell precursors to the mucous glands. Griscelli and his colleagues (Griscelli et al, 1969) have suggested that circulating sensitized B lymphocytes "home" towards the antigen to which they have been sensitized, but although this may be true, and might explain the presence of IgA cells in the lamina propria of the bronchial epithelium, it does not explain the vast numerical preponderance of IgA cells in mucous gland, for there is no reason to suppose that antigen is concentrated there. Presumably there are one or more chemotactic factors in bronchial gland specific for IgA cell precursors. This factor (or factors) is not known and may be secreted by the mucous gland epithelial cells. To speculate, two well known components of bronchial secretion are secreted by these cells; secretory piece (Tourville et al, 1969) and lactoferrin (Masson et al, 1966). The more likely candidate of these two as a chemotactic agent is secretory piece, which is found in sputum linked to molecules of IgA dimer. It is thought to be linked to the IgA molecules either inside or on the surface of the epithelial cells of the mucous gland. Its function is unknown (see Chapter 2), but conceivably it might be the unknown chemotactic agent. Some support for this idea is provided by a recently reported case of secretory IgA deficiency in the presence of normal serum IgA associated with a deficiency of secretory component. (Strober et al, 1976).

(c) The effects of smoking on cells containing immunoglobulin in the bronchial wall

The subjects who smoked (but had no cough or sputum) had significantly higher IgA cell counts in lobar bronchus than the non-smokers, suggesting that smoking may have an initial stimulatory effect on the bronchial plasma cells. This conclusion is supported by the findings in the carinal lymph nodes, in which there was an increase in germinal follicles containing immunoglobulins (discussed in the next section). This change in the bronchi was apparently confined to the lobar bronchus, though may have been present in the more distal airways, and this localisation is consistent with other estimates of the site of early change in chronic bronchitis, which is believed to affect small airways before larger ones, so that measurements of small airways narrowing such as closing volume, and mean mid-expiratory flow rate may indicate an abnormality in smokers before traditional tests of large airway obstruction become abnormal (McCarthy et al, 1972, McFadden and Linden 1972, Cochrane et al, 1974). Furthermore, the mucous gland hypertrophy of chronic bronchitis has been shown to be proportionately greater in lobar and segmental bronchi than in other more proximal sites. (Restropo and Heard, 1963).

The mechanism by which the bronchial plasma cells in smokers are increased might involve direct chemical stimulation of plasma cells by tobacco smoke components, or the production of specific antibody against antigenic components of the smoke. Alternatively, the plasma cells may be manufacturing specific antibody against bacteria which have invaded the lower respiratory tract as part of the natural history of chronic bronchitis, but preceding the

development of chronic cough and sputum. Relevant to this possibility is the finding of May and colleagues (May et al, 1973) that the presence of serum antibody to Haemophilus influenzae (suggesting bronchial colonisation with this organism) is related strongly to smoking habits. The effects of tobacco smoke are further discussed in the next section.

(d) Differences between individual subjects

Two of these nine apparently normal subjects are seen to be significantly different from the rest, one with many more IgA cells than the whole group, and the other with fewer cells. The subject with an excess of IgA cells was also unusual in having an excess of IgG, IgM and IgE cells, and so appears to be quite different from the others in several respects. He had no history of atopy or of other medical abnormality to account for these differences. He was a moderate smoker, although, as he died by his own hand as a result of conditions of acute social stress, it is conceivable that he had been smoking more heavily for a period before his death.

The other subject, with an apparent deficiency of IgA cells, is perhaps more interesting. This subject, female, was a non-smoker, and appeared to have a relative deficiency of IgA cells at all sites examined. While it is possible that this represented a temporary resting state of the bronchial plasma cell population as a result, say, of a lack of antigenic or infective challenge for a long period, the much higher cell counts in the other non-

smokers, none of whom had had recent chest infections, suggest that the low IgA cell counts in this subject were the result of internal factors, not environmental. The serum IgA of this subject was normal, excluding a systemic IgA deficiency. Presumably, therefore, this subject either represents the extreme lower end of the normal range of IgA cells in bronchial tissues, or had a defect in the normal programming or production of IgA in the bronchi. Whichever of these two suggestions is nearer the truth, the response in the bronchial tree to an infection, inhaled antigen or cigarette smoking might have been completely different, at least in the first instance, from that in a subject with plentiful numbers of bronchial IgA cells.

Except for the case with secretory piece deficiency discussed in the previous section, subjects with a local bronchial IgA deficiency, but normal serum IgA, had not previously been described, although healthy subjects with serum and salivary IgA deficiency have been reported (Rockey et al, 1964) and the incidence of systemic IgA deficiency among the population has been estimated to be 1 in 700 (Bachmann, 1965). Subjects with systemic IgA deficiency have a higher than usual incidence of respiratory infections and autoimmune disease, (Amman and Hong, 1971), the latter possibly stimulated by the easy access afforded to foreign antigens or organisms by the lack of the protective function of secretory IgA. How commonly a local bronchial IgA deficiency is to be found among the population, and whether it does predispose to disease, remains to be determined. Studies of secretory piece were not included in this work.

2. Chronic Bronchitic Subjects

(a) Bronchial IgA deficiency in fatal chronic bronchitis:- immunofluorescent studies

This study of eleven chronic bronchitic subjects has shown that the six dying from complications of long-standing severe chronic obstructive bronchitis had a marked deficiency of cells containing IgA in their respiratory tracts, while the five whose bronchitis had not been sufficiently severe to cause them to consult their doctors, and who died from unrelated causes, had normal numbers of IgA cells. Morphological differences in the airways between these two clinical groups have been demonstrated by Scott and Steven (1975) who used post-mortem tantalum bronchography to demonstrate much greater obliteration of small airways in "fatal" than "incidental" bronchitis.

The apparent defect in IgA plasma cells in fatal chronic bronchitis may have been the result either of an excessive depletion of IgA within cells or a failure of production. These six subjects all suffered from recurrent respiratory infections, and these might be expected to effect changes in the immunological defence mechanisms in the bronchi. Little is known of the morphological changes in bronchial plasma cells in response to acute or chronic infection, nor how immunoglobulin is released from the cell, or whether the appearance of the cell changes when release occurs. Apart from the immunoglobulin within plasma cells, IgA is probably

also stored in or on the surface of the secretory cells of the bronchial and nasal mucous glands, (Tourville et al, 1969), and it is likely that the initial rapid rise in IgA in secretions following acute infections represents the IgA stored in these epithelial glandular cells. It is not known whether the plasma cells in the mucous gland contribute by releasing IgA at the same time, although it is probable that they are the source of the steadily increasing amounts of IgA appearing in the sections some 15 days after the onset of infection. (Rossen and Butler, 1973).

If the release of immunoglobulin from a plasma cell in response to infection leaves behind an empty shell without detectable immunoglobulins in its cytoplasm, this event would certainly make detection of these cells by immunofluorescence difficult, and this is one possible cause of the apparent deficiency of bronchial plasma cells in fatal chronic bronchitis. More knowledge is required of the changes in bronchial plasma cells occurring in response to infection. However, this explanation does seem unlikely, for the infections in these bronchitic subjects were of a recurrent or chronic nature. The normal response of tissues to chronic infection includes an increase in numbers of plasma cells, and the work of Martinez-Tello and his colleagues (1968) confirmed that this may happen in the bronchial tree, for their study of children dying from cystic fibrosis with chronic chest infections, showed an excess of IgA and IgG cells compared with control subject.

It therefore seems likely that the defect is one of manufacture of IgA in the bronchial tissues. This might result from failure of transport of plasma cell precursors to the submucous glands, or a failure of maturation of plasma cell precursors even though present in the bronchial wall. Such precursors would not have been detected by immunofluorescent methods until their cytoplasm contained immunoglobulin. Methyl green pyronine stains might have detected such plasma cell precursors if they had been present, for pyronine stains demonstrate ribonucleic acid, of which plasma cells contain large quantities, not immunoglobulin. However, the quality of pyronine stains in these post-mortem tissues was too poor to provide this information.

An alternative explanation is that the plasma cell precursors failed to arrive in the submucous glands. The kinetics of the lymphocyte circulation to the bronchial tree have been discussed in Chapter 2 and, omitting for the moment possible defects of transmission of specifically sensitised B. lymphocytes from regional lymph nodes into the systemic circulation (discussed in the next section), there might be a defect in the chemotactic factors inducing B lymphocytes capable of producing IgA to colonise the bronchial mucous gland. These chemotactic factors have not yet been demonstrated, but surely must be present to account for the differential distribution of plasma cells between mucous gland and lamina propria in the bronchial wall.

The possible mechanisms and causes of this deficiency are further discussed in the next section.

(b) Bronchial IgA deficiency in severe chronic bronchitis:- sputum studies

The results of the pilot study of immunoglobulins in sputum reported in this thesis indicate a deficiency of local bronchial secretion of IgA in five out of fourteen subjects with severe chronic obstructive bronchitis studied during infective episodes.

Similar measurements in control subjects with other selected respiratory diseases demonstrated that the local bronchial IgA secretion was usually more than 70%. These results are compatible with the values found by Deuschl and Johannson (1974) on bronchial washings:- over 80% of the IgA in normal subjects was locally secreted, less in those with inflammatory lung disease, this being compatible with increased serum contamination of sputum when inflammation of the bronchial mucosa was present.

Nine of the chronic bronchitic subjects whose sputum has been studied had local IgA secretions of 70% or more, and there was good agreement between different samples, confirming the consistency of the method. However, in five subjects thelocal IgA contribution was below 70% on three or more occasions, and in four subjects underwent a progressive fall and subsequent rise through the period of

illness, some values even falling to nil. These findings are consistent with the low local bronchial IgA secretion found by Deuschl and Johannson (1974) in two chronic bronchitic subjects, and with the work of Medici and Buergi (1971), who found that severe chronic bronchitic subjects did not increase their total sputum IgA in response to infections, unlike subjects with mild bronchitis in whom a rise and fall did occur.

While it is possible that this apparent deficiency of IgA secretion was induced by an outpouring of serum exudate, so that the sputum was largely composed of serum, it is at least consistent with the hypothesis that there is a deficiency of bronchial IgA secretion in a proportion of patients with severe chronic bronchitis. In some this appears to be a transient event, but in others it persisted.

The transience of the deficiency seen in some subjects during exacerbations may be the result of exhaustion of the bronchial secretory IgA system, which can secrete small amounts of Iga in the resting state, but fails to increase it in response to infections. The appearance of the bronchial IgA plasma cells in "fatal" bronchitis, which, in addition to being few in numbers, characteristically have only a thin rim of cytoplasm around the nucleus, would be consistent with this.

Not all workers confirm this deficiency. Falk and his colleagues (1972) were unable to demonstrate a significant def-

iciency of IgA in the sputum of chronic bronchitic subjects. They, however, made no attempt to allow for IgA derived from serum, and further examination of their published figures does show that two of their six chronic bronchitic subjects had lower sputum IgA/total protein ratios than any of the control subjects, and it is possible that if they had been able to study a larger group of patients, or allow for serum contamination of sputum, they would have found a significant proportion of chronic bronchitic subjects with reduced bronchial IgA secretion.

(c) "Incidental" chronic bronchitis

The bronchial IgA deficiency demonstrated in the "fatal" bronchitics contrasts with the normal cell counts in the chronic bronchitics whose disease had not caused them to consult their doctors. The difficulty of obtaining clinical information about these subjects denies precise knowledge of their respiratory function or frequency of respiratory infections, but these parameters may be presumed to have been much less abnormal than in the "fatal" bronchitics.

The "incidental" bronchitics were of a similar age range to the "fatal" group, and had smoked as heavily (indeed, they all were still smoking at the time of death) and yet for some unknown reason they had not developed disabling bronchitis, and their bronchial plasma cell populations were virtually normal.

It is easy to imagine that subjects who are deficient in bronchial IgA cells are more likely to suffer progressive lung damage due to infections. Without the protective action of secretory IgA, bacteria can multiply more easily in the bronchial tree and viruses can invade epithelial cells without hindrance. These infections would then be dealt with within the tissues by cellular mechanisms and by serum antibodies of the IgG and IgM classes. These antibodies are likely to fix complement and cause further tissue damage, giving rise to bronchial scarring and obliteration of small airways.

It will be recalled that the healthy smoking subjects (without cough or sputum) had an excess of IgA cells in the lobar bronchus, suggesting that smoking had stimulated plasma cell activity in that site. This excess was not found in the "incidental" bronchitics, even though they smoked even more heavily than the healthy smokers, for their IgA cell counts in lobar bronchus corresponded more closely to those for non-smokers. Compared with smoking controls, therefore, the "incidental" bronchitics had a relative deficiency of IgA cells in lobar bronchus, and it is possible that this represented a minor degree of the same severe IgA cell deficiency seen in the "fatal" bronchitics.

The bronchial IgA cell deficiency in "fatal" bronchitics may have been induced by smoking or other environmental factors, or it

may have been present from an early stage, predisposing to severe bronchitis. The means by which smoking and other environmental factors may interfere with the bronchial immune defence mechanisms are discussed in the next section.

If there were an immune deficiency in "fatal" bronchitics present from an early stage, it would make the deficient subjects especially liable to lung damage from a variety of external insults, including smoking, atmospheric pollutants, inhaled antigenic proteins and the respiratory infections of ordinary life. If this were so, then fatal bronchitis would not be confined only to smokers, and indeed this is the case, for one of these six "fatal" bronchitics was a lifelong non-smoker, and Oswald and his colleagues (1953) found chronic bronchitis among non-smokers, though less commonly. The non-smoking "fatal" bronchitic reported in this thesis had had frequent respiratory infections since he was a young adult, and it seems likely that in his case, the bronchial IgA deficiency preceded the chronic bronchitis. Conceivably the other three "fatal" bronchitics also had pre-existing immune deficiency. It will be remembered that one healthy non-smoker had an apparent bronchial IgA deficiency. Perhaps, if this subject had smoked, she also would have developed rapidly progressive chronic bronchitis leading to disability and death, the smoking habit in this case being an essential secondary factor.

It should not be forgotten, however, that the chronic bron-

chitis syndrome probably has multiple causes, including such factors as cigarette smoking, atmospheric pollution, immune defects and infections with different natural histories, and the final common pathway of severe obstructive chronic bronchitis is probably the result of a summation of these factors. We may therefore not find the same pathological or immunological abnormalities in every case.

It is important to bear this in mind when studying chronic bronchitic subjects, and research on chronic bronchitis should, where possible, define the clinical characteristics carefully, with reference to such easily defined characteristics as their degree of impairment of lung function, smoking history, frequency of infections and sputum eosinophilia.

3. Immunoglobulin-containing Cell Populations in Carinal Lymph Nodes of Normal Subjects and Chronic Bronchitics

(a) Carinal lymph nodes

The tracheo-bronchial lymph nodes, of which the carinal group is the largest (Inferior tracheo-bronchial group), are presumably concerned with the protective immunity of the bronchial tree. The lymphatic drainage from the bronchi is towards the tracheo-bronchial lymph nodes (Miller, 1950), and, by analogy with peripheral lymph nodes, antigen deposited in the bronchi may be carried by macrophages to these nodes (White, 1960). Once there in the germinal centres of lymphatic follicles, antigen may stimulate the production of cells with the capacity to make specific antibody, or

stimulate such cells, if already present, to produce this antibody.

By analogy with the circulation of lymphocytes from thoracic duct lymph to blood and back to gut and mesenteric lymph nodes demonstrated by the animal studies of Gowans and Knight (1964), the tracheo-bronchial lymph nodes may release B lymphocytes via lymphatic efferents into the systemic circulation which then home to bronchial mucosa and become plasma cells.

The results reported in this thesis therefore reflect changes in three aspects of humoral immunity in the bronchial tree; the number of active germinal centres containing immunoglobulin may reflect the antigenic stimulation of the production of appropriately sensitised B lymphocytes; the numbers of cells containing immunoglobulin in the medulla and cortico-medullary junctions of the lymph node may reflect the activity of B lymphocytes in actually producing specific antibody; and the numbers of cells in the bronchial wall may be an indirect measure of the numbers of sensitised B cells released by the lymph node and passing in the blood stream to the bronchial wall, as well as a reflection of the activity of those cells in producing immunoglobulin in that site.

The number of active germinal follicles containing immunoglobulin appears to be increased by smoking cigarettes, even in

subjects without cough or other respiratory disease. Active germinal follicles are also increased in subjects with chronic bronchitis, whether this was the cause of death or incidental to it. Presumably the bacterial colonisation and frequent infections of the respiratory tract in this disease provide a plentiful supply of antigen which stimulates germinal follicle activity.

When the counts of cells containing IgA, excluding those in germinal follicles, are considered, there were no group differences between normal non-smokers, normal smokers and "incidental" bronchitics, although a rather wide scatter of cell counts among smokers and in "incidental" bronchitics suggests that individuals might have been responding to their smoking in different ways. However, the "fatal" bronchitics had significantly reduced numbers of cells containing IgA and IgM in their carinal lymph nodes, and this result is consistent with the reduced IgA cell counts found in the bronchial wall. There were many fewer IgM cells in the bronchial wall than IgA, and small differences in IgM cell counts in the bronchus might not have been detected.

This apparent deficiency may have been the result of excessive depletion of cells containing immunoglobulin, or of a failure of production. In the experimental situation, the secondary response in peripheral lymph nodes to an appropriate antigenic stimulus is a rise and subsequent fall in numbers of medullary plasma cells. However, even if the "fatal" bronchitics died at the time of this

fall, following a recent respiratory infection, there seems no reason why the cell counts should fall below normal values, and furthermore it is likely that the chronic bacterial colonisation of the bronchial tree in this disease provides a constant antigenic challenge to the draining lymph nodes, quite unlike the short antigen exposure in the experimental situation.

It seems likely, therefore, that the reduced numbers of cells containing IgA are the result of a failure of production, either of appropriately sensitised B lymphocytes, or a failure of manufacture of immunoglobulin by cells even though specifically sensitised B cells are present. The presence of active germinal follicles in five out of our six "fatal" bronchitics suggests that an attempt to produce specifically sensitised lymphocytes was being made, so the defect may have been a failure of maturation of sensitised lymphocytes into plasma cells.

It is curious that the sixth subject appeared not to have active germinal follicles containing immunoglobulins. Possibly this represented a defect at an earlier stage in the production of antibody, perhaps in the transport of antigen by macrophages to the lymph nodes, or its processing by these cells into a form suitable for programming of lymphocytes to produce specific antibody.

The accompanying IgA cell deficiency found in the bronchial and tracheal wall in these "fatal" bronchitics, may therefore be

the result of the deficiency demonstrated in the regional lymph nodes, or it may in addition be the result of a failure of production of IgA by properly programmed B lymphocytes once they have arrived in the bronchial mucous glands.

(b) Kinetics of Bronchial Plasma Cells

A scheme for the primary production of mucosal antibody, from the initial deposition of antigen on bronchial wall, to the final release of specific secretory IgA into the bronchial lumen might be as follows:-

(1) The antigen is taken up by a macrophage, attacked by lysozomal enzymes within the cell, and broken up into smaller components. The macrophage migrates back to the regional lymph nodes. Macrophages in alveoli or bronchial lamina propria have ready access to lymphatics: macrophages in the bronchial lumen however, have no obvious route of re-entry to bronchial tissues. There is no structure in the bronchial tree obviously analagous to the crypts in the pharyngeal tonsil, through which antigen may enter the nostril and permit specific programming of lymphocytes at the periphery of the tonsil (Koburg, 1967). It is possible that none of the antigen-bearing macrophages in the bronchial lumen ever return to the tissues but are coughed up, swallowed and lost, but if they do return, it is presumably by passing through the bronchial epithelium or into the ducts of the bronchial glands. It may be rather far-fetched to imagine that they are gently coughed up to lie on the surface of the pharyngeal tonsil and re-enter through the tonsillar crypts, though this mechanism seems possible.

(2) The processed antigen, transported by macrophages to the germinal centres of the lymphoid follicles, programmes lymphoid cells with the potential to produce specific antibody. These cells in the germinal centre initially containing antibody, divide rapidly to form a surrounding cuff of small B lymphocytes, not manufacturing immunoglobulin but with the potential to do so. Some of them mature to plasma cells and their specific immunoglobulin probably contributes to the plasma pool (White et al, 1967). Others pass into the efferent lymphatics, and via the thoracic duct to the systemic circulation.

(3) Circulating sensitised B lymphocytes home to bronchial wall attracted by specific antigen in the lamina propria, and unknown chemotactic factors in the mucous glands with particular attraction for potential IgA cells.

(4) The B lymphocytes in the bronchial wall mature into plasma cells, producing IgA dimer. This is released and is bound to secretory piece on the surface of or within the glandular epithelial cells.

(5) The secretory IgA is then released, fully mixed with bronchial mucus in response to such factors as irritation, infection, vagal stimulation or cholinergic drugs.

"T" cell function

No attempt was made to measure cortical area in these frozen sections, but the disproportionately low plasma cell count/gland weight ratios in the nodes of the "fatal" chronic bronchitics, suggest that the "T" cell function of the node was maintained even though the B cell function was reduced.

(c) The effects of smoking on bronchial defence mechanisms

Cigarette smoking may interfere with the workings of the bronchial defence mechanisms in several ways. It interferes with ciliary activity (discussed in Chapter 2), and thus impairs the clearance of particles and mucus from the bronchial tree. It impairs the ability of macrophages to kill bacteria (at least in vitro), and might conceivably interfere with the ability of the macrophages to process antigen into the form required for induction of the antibody response. On the other hand, alveolar macrophages are increased in number and activity in smokers (Harris et al, 1970), though this has not been measured in severe chronic bronchitis.

In vitro, tobacco smoke products inhibit the production of specific antibody by lymphoid cells (Roszman and Rogers, 1973), and inhibit T-lymphocyte transformation in response to Phytohaemagglutinin (Desplaces et al, 1971), although only in concentrations much higher than would be expected in the peripheral blood of smokers. However, if tobacco smoke products are concentrated in tracheobronchial lymph nodes, they may reach concentrations sufficiently high to inhibit maturation of B lymphocytes in the lymph nodes, thus causing the reduction in plasma cells found in the carinal lymph nodes of "fatal" chronic bronchitics. Furthermore, the tobacco smoke products might also be expected to reach high concentrations in the bronchial mucosa and may inhibit maturation of B lymphocytes into plasma cells in that site, causing an apparent deficiency of plasma cells in bronchial mucous gland, even though the B cell precursors may be present. Smoking has been shown to cause a reduction in plaque-forming cells in the thoracic lymph

nodes in mice (Thomas et al, 1974).

However, smoking does not inevitably cause these defects, for the "incidental" bronchitics, who all smoked heavily, had normal numbers of plasma cells within their respiratory tracts, presumably because they were relatively resistant to the ill-effects of tobacco smoke. This resistance might take the form of better clearance of tobacco smoke products via the tracheal route, more robust lymphocytes, or more efficient detoxification of tobacco smoke products. The converse of this is that the "fatal" bronchitics may have had deficient detoxifying mechanisms or lymphocytes unusually sensitive to tobacco smoke products. The extreme case would be a pre-existing deficiency of the bronchial humoral defence mechanisms, rendering the bronchial tree unable to defend itself properly against tobacco smoke, atmospheric pollutants and infection.

One of the apparently normal subjects reported in this thesis appeared to be deficient in bronchial IgA cells, and although it is not certain that this state would have been permanent, this would appear to support the theory that bronchial IgA deficiency may occur in the absence of, or before the development of chronic bronchitis. This subject did not smoke, but perhaps if she had she would have been likely to develop rapidly progressive chronic obstructive bronchitis.

The most likely explanation must surely be that a combination of adverse factors gives rise to chronic bronchitis. Isolated IgA deficiency, whether systemic or local bronchial, only gives rise to recurrent respiratory infection when allied with associated immune defects or adverse environmental factors such as heavy smoking or industrial pollution or fortuitous severe infections. Severe obstructive chronic bronchitis is likely to result from varying combinations of individual susceptibility and environmental insult. The spectrum of disease will include those with pre-existing systemic or localised bronchial immune deficiency in whom smoking and atmospheric pollution play a small or secondary part, those with an originally fragile but intact bronchial defence system disrupted by external factors, and those in whom heavy smoking, air pollution or chance severe infections overwhelmed an originally perfectly healthy bronchial defence system.

Summary of Main Findings and General Implications

The preceding findings have shown an apparent immune deficiency in the bronchial tree and its regional lymph nodes in patients dying from chronic bronchitis. Such an immune deficiency in a chronic bronchitic subject might well encourage recurrent or chronic bronchial infection, and the failure of the protective effect of secretory IgA might permit the invasion of tissues by micro-organisms, release of bacterial toxins, and tissue damaging reactions between foreign proteins and serum IgG and IgM antibodies, thus leading to scarring and destruction of the normal lung architecture. It is easy to imagine that a chronic bronchitic suffering

from such an immune deficiency might undergo much more rapidly progressive lung damage and disability than one whose secretory immune system was relatively intact.

This work has shown that some chronic bronchitics, whose disease had not been so severe that they had consulted their doctor about it, did not have the immune deficiency demonstrated in the disabled "fatal" bronchitics. It is not clear whether this deficiency arose early or late in the natural history of the chronic bronchitis. Since many bronchitics never develop airways obstruction and do not suffer from frequent chest infections, it is unlikely that a bronchial immune deficiency is the inevitable end result of long-standing chronic bronchitis. It therefore seems to pick out some individuals and not others. This is confirmed by the co-operative prospective study reported by Bates (Bates, 1974), in which a small group (10%) of chronic bronchitics showed much more rapid deterioration of respiratory function than the rest, who deteriorated at a rate no faster than would be expected by the process of aging. This rapidly worsening group were heavy smokers, though not all heavy smokers were in this group.

One of the subjects reported in this thesis died from chronic bronchitis but had never smoked. He had suffered from frequent respiratory infections for most of his adult life, and it seems likely that his bronchial immune deficiency was of long standing and may have predisposed to his chronic bronchitis. It is

not clear whether the other subjects dying from chronic bronchitis, who had smoked heavily, also had a pre-existing immune deficiency or whether their deficiency was the result of their chronic bronchitis. Their smoking habits were likely to have exacerbated their bronchitis in either case.

Tobacco smoke products in vitro and in mice, (Thomas et al, 1974) may behave as immunosuppressants (Roszman and Rogers, 1974), and it is conceivable that the concentration of tobacco smoke products achieved in the bronchial mucosa and regional lymph nodes of heavy smokers may be sufficient to cause a local immune paresis. Some slight support for this argument is the finding that the IgA plasma cell population in bronchial mucosa, and the germinal centre activity in regional lymph nodes appears to be greater in smokers (without cough) than in non-smokers, suggesting that smoking stimulates the local immune system. It is not difficult to imagine that smoking, causing an early stimulation of the immune system, might later cause a depression as may occur in mice, (Thomas et al, 1974).

If this should be shown to be the case, then by identifying and taking steps to remove the components of tobacco smoke with immunosuppressive potential, it should be possible to alter drastically the mortality from chronic bronchitis, and the widespread ill-health and loss of earnings induced by the recurrent infections and disability of chronic bronchitis. This seems as valid a way of

attacking the public health problem of chronic bronchitis as the attempt to persuade the population not to smoke, which is not proving to be an easy task.

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APPENDIX 1

Method for the conjugation of hyperimmune serum with fluorescein isothiocyanate. (From Holborow and Johnson, 1967; Nairn, 1968).

Materials

Hyperimmune serum (Hyland (Travenol) Laboratories).

Fluorescein isothiocyanate (FITC), pure (Sigma Chemicals).

G-25 Sephadex.

Whatman cellulose powder.

Commercial salt-free protein standard

Saturated ammonium sulphate solution

Buffered physiological saline:- (pH 7.0, 0.15 M)	NcCl	8.5 gms
	$Na_2HPO.12H_2O$	16.8 gms
	$NaH_2PO_42H_2O$	8.65 gms
	Distilled water	2 litres
Unbuffered saline, 0.15 M:-	NaCl	17.53 gms
	Distilled water	3 litres
Carbonate buffer, pH 9.0, 0.5M:-	0.5 M Na_2CO_3	5 ml
	0.5 M $NaHCO_3$	25 ml

Red cell haemolysate (used as visual protein indicator).

Procedure

All solutions and buffers are pre-chilled at 4^oC.

1. Dilute hyperimmune serum (2 or 4 mls. usually) with buffered saline in the proportion 1:9. Add slowly and with mixing an equal volume of saturated, filtered ammonium sulphate solution, and leave at 4^oC for 1 hour to precipitate the globulins.

2. Spin down in refrigerated centrifuge at 17,000 rpm for 15 minutes.
3. Drain precipitate well, and redissolve in buffered saline the same total volume as before.
4. Repeat the precipitation with ammonium sulphate, and the centrifugation.
5. Redissolve the drained precipitate in a very small volume of unbuffered saline (1-2 mls).
6. Add 1-3 drops of haemolysate (diluted 1 in 4) to colour the protein fraction.
7. Pass through a G-25 Sephadex column previously equilibrated with unbuffered saline, with additional unbuffered saline. This removes the ammonium sulphate.
8. The coloured globulin fraction is collected, pooled and kept at $4^{\circ}C$.
9. Determine the protein concentration on an aliquot of the solution by the Lowry method (1951) using the Folin Phenol Reagent.
10. Dilute solution to protein concentration of 10mg/ml. with unbuffered saline and pH 9.0 carbonate buffer, so that the carbonate buffer contributes 10% to the final volume.
11. Mix 0.03 mg. pure FITC per mg. protein with cellulose powder (to disperse FITC), and add to the protein solution and mix well. This provides a slight excess of FITC. Mix continuously on a rotator for 18 hours at $4^{\circ}C$, for conjugation to occur.
12. The next day, spin down and remove supernatant from cellulose.
13. Apply supernatant to a larger G-25 Sephadex column, chilled to $1^{\circ}C$ by iced water circulating through a water-jacket. Pass

through with buffered saline (Buffered physiological saline was used because phosphate buffered saline tended to cause precipitation of the protein on to the sephadex.)

14. Collect bright yellow fraction.

15. Clear column with 1% NaOH to remove fluorescein. Store column in hibitaine solution.

16. Pool most brightly coloured fractions and estimate protein concentration by Folin method. The weaker yellow fractions may also be pooled and kept.

17. Estimate fluorescein concentrations by optical density measurement of serial dilutions of an aliquot of the conjugate. This is discussed by Holborow and Johnson, 1967. The relationship between optical density and fluorescein concentration is linear within the range normally used for spectrophotometric analysis, and the diluted conjugate is read against a buffer blank at 492 mμ. The optical density is also read at 320 mμ, and half the extinction value at this wavelength (E320 mμ) is subtracted from E492 mμ, to correct for irrelevant absorption.

    The fluorescein/protein (F:P) ratio is calculated from the formula $F/P = \frac{2.06 \times E492 - \frac{1}{2}E20}{Cp}$ where the Cp is in the protein concentration, and 2.06 is a constant derived from the molecular weights and extinction coefficient of fluorescein.

18. The conjugate is stored in a glass bottle at $4^{o}C$.

19. This is absorbed with pig liver powder before use.

Chapter 4 Appendix 2

Confirmation of specificity of immunofluorescent staining.

Dilutions for blocking procedure:-

1. For IgA staining

Blocking layer:	unconjugated	IgA	Dilution	1/5
Staining layer:	conjugated	IgA	Dilution	1/5
Control:-				
1st layer:	unconjugated	IgG	Dilution	1/5
Staining layer:	conjugated	IgA	Dilution	1/5

2. For IgG staining

Blocking layer:	unconjugated	IgG	Dilution	1/5
Staining layer:	conjugated	IgG	Dilution	1/8
Control:-				
1st layer:	unconjugated	IgA	Dilution	1/5
Staining layer:	conjugated	IgG	Dilution	1/8

3. For IgM staining

Blocking layer:	unconjugated	anti IgM	Dilution	1/5
Staining layer:	conjugated	anti IgM	Dilution	1/4
Control:-				
1st layer:	unconjugated	anti IgA	Dilution	1/5
Staining layer:	conjugated	anti IgM	Dilution	1/4

4. For IgE Staining

Blocking layer:	Unconjugated	anti	IgE	Dilution 1/3
Staining layer:	Conjugated	anti	IgE	Dilution 1/3
Control:-				
1st layer:	Unconjugated	anti	IgM	Dilution 1/5
Staining layer:	Conjugated	anti	IgE	Dilution 1/3

Staining time 30 minutes, washing time 45 minutes.

APPENDIX 3

UNPROCESSED CELL COUNTS IN NORMAL NON-SMOKERS (SUBJECTS 1-5)

CELL COUNTS IN UPPER TRACHEA

Subject	IgA	IgG	IgM	IgE
1	76	10	11	31
	105	29	68	4
	66	22	14	17
	31	17	47	17
2	287	8	63	47
	337	28	74	27
	245	18	10	49
	316	18	18	63
3	140	52	33	21
	182	70	21	15
	320	80	18	43
	295	62	22	20
4	360	33	6	23
	320	39	15	37
	452	9	19	17
	540	29	29	19
5	440	33	18	93
	389	73	33	84
	331	70	7	101
	415	70	17	69

APPENDIX 3 (CONTINUED)

UNPROCESSED CELL COUNTS IN NORMAL NON-SMOKERS

IN RIGHT MAIN BRONCHUS

Subject	IgA	IgG	IgM	IgE
1	130	57	19	6
	143	35	68	8
	83	27	15	16
	132	47	39	15
2	920	31	64	125
	810	23	60	130
	694	52	54	227
	780	20	34	250
3	478	141	56	45
	710	189	32	31
	622	82	24	12
	817	47	40	18
4	444	47	18	14
	498	49	28	15
	450	50	25	20
	620	63	11	15
5	395	37	64	60
	410	18	26	23
	520	39	23	39
	470	54	32	49

APPENDIX 3 (CONTINUED)

UNPROCESSED CELL COUNTS IN NORMAL NON-SMOKERS IN LOWER LOBE BRONCHUS

Subject	IgA	IgG	IgM	IgE
1	34	39	8	12
	56	28	35	10
	83	16	15	6
	107	22	39	8
2	196	15	43	75
	129	11	64	102
	190	23	44	88
	124	33	71	105
3	274	39	16	10
	140	43	5	32
	180	5	17	12
	228	27	13	18
4	103	43	32	25
	94	18	23	12
	93	17	20	11
	137	13	19	8
5	223	30	17	23
	301	11	21	64
	294	19	17	33
	260	39	13	43

APPENDIX 3 (CONTINUED)

UNPROCESSED CELL COUNTS IN NORMAL SMOKERS (SUBJECTS 6-9) IN UPPER TRACHEA

Subject	IgA	IgG	IgM	IgE
6	8	55	29	163
	7	112	26	72
	7	66	84	168
	45	72	120	172
7	190	37	17	46
	103	60	17	29
	92	49	36	37
	152	78	12	52
8	820	37	43	62
	717	86	18	71
	593	71	29	90
	711	121	39	61
9	205	52	18	53
	340	7	7	79
	240	5	20	42
	270	63	20	87

APPENDIX 3 (CONTINUED)

UNPROCESSED CELL COUNTS IN NORMAL SMOKERS IN RIGHT MAIN BRONCHUS

Subject	IgA	IgG	IgM	IgE
6	877	1110	302	1557
	892	750	228	801
	1380	910	199	1363
	1420	1003	198	1770
7	402	63	196	50
	374	92	121	45
	645	105	117	90
	874	86	70	43
8	550	94	57	31
	435	47	49	47
	656	90	28	24
	491	46	17	43
9	334	32	58	48
	530	30	18	28
	420	33	31	31
	402	9	42	18

APPENDIX 3 (CONTINUED)

UNPROCESSED CELL COUNTS IN NORMAL SMOKERS IN LOWER LOBE BRONCHUS

Subject	IgA	IgG	IgM	IgE
6	1018	798	597	837
	1238	945	470	540
	1620	462	390	981
	1690	574	450	729
7	370	15	23	18
	310	9	10	20
	490	18	18	11
	362	19	30	4
8	427	65	43	69
	610	95	22	33
	537	72	32	22
	720	81	27	41
9	170	6	24	37
	209	24	26	16
	300	13	25	23
	210	33	7	9

APPENDIX 4

UNPROCESSED CELL COUNTS (CELLS/SECTION) IN SIX 'FATAL' BRONCHITICS IN UPPER TRACHEA

Subject	IgA	IgG	IgM	IgE
15	10	20	11	8
	11	54	26	24
	15	31	19	14
	19	42	30	17
16	31	23	11	18
	33	14	10	17
	24	19	15	61
	81	57	14	82
17	40	20	31	23
	47	40	47	34
	19	49	17	28
	35	18	16	25
18	395	164	201	0
	410	97	98	0
	295	270	138	7
	470	170	98	2
19	6	18	16	0
	31	10	5	1
	31	27	6	0
	43	31	1	5
20	3	16	25	43
	2	12	35	56
	11	4	21	36
	6	1	36	43

APPENDIX 4

UNPROCESSED CELL COUNTS (CELLS/SECTION) IN SIX 'FATAL' BRONCHITICS IN MAIN BRONCHUS

Subject	IgA	IgG	IgM	IgE
15	40	52	23	30
	83	69	13	37
	105	26	27	22
	122	56	43	17
16	183	73	62	23
	194	25	42	35
	110	83	89	65
	192	95	64	84
17	145	166	75	153
	34	146	101	90
	91	95	28	52
	150	105	43	29
18	166	23	128	2
	89	23	291	3
	364	118	169	0
	296	43	181	0
19	220	37	11	0
	470	10	19	2
	148	19	35	5
	267	18	7	2
20	148	205	65	73
	74	260	44	25
	102	305	42	17
	54	190	32	42

APPENDIX 4

UNPROCESSED CELL COUNTS (CELLS/SECTION) IN SIX 'FATAL' BRONCHITICS IN LOWER LOBE BRONCHUS

Subjects	IgA	IgG	IgM	IgE
15	20	9	52	37
	62	18	8	27
	18	9	12	20
	17	6	0	76
16	163	67	35	35
	170	58	60	7
	96	40	38	11
	199	70	29	19
17	167	39	21	27
	110	54	18	53
	101	106	10	55
	179	125	20	43
18	45	0	69	0
	116	8	89	2
	47	0	76	0
	31	7	66	4
19	31	4	0	0
	16	3	2	3
	22	0	3	0
	22	11	4	5
20	67	15	77	45
	128	34	53	22
	38	65	29	14
	49	106	34	21

APPENDIX 4

UNPROCESSED IgA CELL COUNTS IN FIVE 'INCIDENTAL' BRONCHITICS (SUBJECTS 10-14)

Subject	IgA Cells per section		
	Upper Trachea	Main Bronchus	Lower Lobe Bronchus
10	327 290 270 352	650 777 491 572	298 285 341 262
11	720 521 508 470	426 235 477 226	250 170 210 205
12	232 170 160 340	449 274 490 410	146 112 97 116
13	290 191 220 170	610 520 510 465	236 143 162 191
14	281 198 217 240	590 530 601 710	230 230 148 230

APPENDIX 5

Counts of single cells containing IgA in sections of carinal lymph nodes from five normal non-smokers.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (0.067 sq.mm. area).

SUBJECT	MEAN CELL COUNTS PER AREA (4 SECTIONS)	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 1	6.62 10.13 4.56 8.73	98.8 151.19 68.06 130.29	9.94 12.29 8.25 11.41	10.47	109.7
Subject 2	9.42 9.76 11.72 13.13	140.59 145.67 174.92 195.97	11.86 12.07 13.23 13.99	12.79	163.52
Subject 3	4.84 5.80 5.45 4.40	72.24 86.57 81.34 65.67	8.49 9.30 9.02 8.10	8.73	76.24
Subject 4	5.0 6.13 6.53 9.34	74.63 91.49 97.46 139.40	8.64 9.57 9.87 11.81	9.97	99.42
Subject 5	8.34 11.46 11.05 8.04	124.48 171.04 164.93 120.00	11.16 13.08 12.84 10.95	12.01	144.19

APPENDIX 5

Counts of single cells containing IgA in the carinal lymph nodes of four normal smokers.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (.067 sq.mm. area).

SUBJECT	MEAN CELL COUNTS PER AREA (4 SECTIONS)	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 6	8.44 9.78 10.86 8.51	125.97 145.97 162.09 127.01	11.22 12.08 12.78 11.27	))) 11.83)	139.87
Subject 7	12.13 11.40 15.83 10.61	181.04 170.15 236.36 158.36	13.43 13.04 15.37 12.58	))) 13.61)	185.33
Subject 8	3.54 6.66 5.55 5.31	52.84 99.40 82.84 79.25	7.27 9.97 9.10 8.90	))) 8.81)	77.63
Subject 9	4.93 4.85 4.53 4.16	73.58 72.39 67.61 62.09	8.57 8.51 8.22 7.88	))) 8.29)	68.84

APPENDIX 5

Counts of single cells containing IgA in the carinal lymph nodes of 'incidental' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (.067 sq.mm. area).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 10	5.00 6.25 4.58 6.24	74.62 93.28 68.35 93.13	8.63 9.65 8.26 9.65	9.04	81.72
Subject 11	3.45 7.75 8.87 7.35	91.49 115.67 132.38 109.70	7.17 10.75 11.50 10.47	9.97	99.40
Subject 12	17.09 13.07 20.37 16.30	255.07 195.37 304.02 243.28	15.97 13.97 17.43 15.59	15.74	247.75
Subject 13	14.60 10.44 10.97 11.96	217.91 155.82 163.73 178.50	14.76 12.48 12.79 13.36	13.34	177.96
Subject 14	8.97 8.54 8.54 9.96	133.88 127.46 127.46 148.65	11.57 11.28 11.28 12.19	11.58	134.09

APPENDIX 5

Counts of single cells containing IgA from the carinal lymph nodes of six 'fatal' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (0.67 sq.mm. area).

SUBJECT	MEAN CELL COUNTS PER AREA	CELL/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 15	4.24	63.28	7.95	)	
	5.59	83.43	9.13	)	
	3.76	56.11	7.49	) — 8.39	70.39
	5.46	81.49	9.02	)	
Subject 16	5.22	77.91	8.82	)	
	3.15	47.01	6.85	)	
	4.61	68.80	8.29	) — 8.02	64.32
	4.42	65.97	8.12	)	
Subject 17	3.72	55.52	7.45	)	
	4.68	69.85	8.35	)	
	7.11	106.11	10.30	) — 9.07	82.26
	6.97	104.02	10.19	)	
Subject 18	3.82	57.01	7.55	)	
	2.34	34.92	5.90	)	
	2.13	31.70	5.63	) — 6.26	39.19
	2.40	35.82	5.98	)	
Subject 19	2.72	40.44	6.35	)	
	2.45	36.56	6.04	)	
	1.90	28.35	5.32	) — 6.01	36.12
	2.69	40.14	6.33	)	
Subject 20	5.32	79.40	8.91	)	
	6.00	89.55	9.46	)	
	6.08	90.74	9.52	) — 9.03	81.54
	4.54	67.76	8.23	)	

APPENDIX 5

Counts of single cells containing IgM in sections of carinal nodes of five normal non-smokers.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area 0.67 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 1	9.02 7.63 11.42 10.94	134.62 113.88 170.44 163.28	11.60 10.67 13.05 12.77	12.02	144.48
Subject 2	8.88 10.96 11.3 12.22	132.53 163.58 168.65 182.38	11.51 12.78 12.98 13.50	12.69	161.04
Subject 3	4.17 2.91 3.78 3.50	62.23 43.43 56.41 52.23	7.88 6.59 7.51 7.23	7.30	53.29
Subject 4	4.95 7.30 6.47 6.42	73.88 108.95 96.56 95.82	8.59 10.43 9.82 9.78	9.65	93.12
Subject 5	1.42 2.38 4.80 4.00	21.19 35.52 71.64 59.70	4.60 5.96 8.46 7.72	6.68	44.62

APPENDIX 5

Counts of single cells containing IgM in sections of carinal nodes of four normal smokers.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area 0.067 sq.mm.)

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 6	9.22 6.77 13.91 10.89	137.61 101.04 207.61 162.53	11.73 10.05 14.40 12.74	12.23	149.57
Subject 7	5.76 3.22 5.12 4.85	85.97 48.05 76.41 72.38	9.27 6.93 8.74 8.50	8.36	69.89
Subject 8	1.95 2.47 2.02 1.42	29.10 36.86 30.14 21.19	5.39 6.07 5.49 4.60	5.38	28.94
Subject 9	4.47 3.56 3.08 3.73	66.71 53.13 46.11 55.67	8.16 7.28 6.79 7.46	7.42	55.06

APPENDIX 5

Counts of single cells containing IgM in sections of carinal nodes of five 'incidental' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 10	3.72 5.04 3.74 3.23	55.52 75.22 55.82 48.20	7.45 8.67 7.47 6.94	7.63	58.22
Subject 11	10.92 5.64 4.93 2.35	162.98 84.17 73.43 35.07	12.76 9.17 8.56 5.92	9.10	82.81
Subject 12	6.46 8.52 8.57 6.64	96.41 127.16 127.91 99.10	9.81 11.27 11.30 9.95	10.58	111.94
Subject 13	10.04 8.15 10.45 10.94	149.85 121.64 155.97 163.28	12.24 11.02 12.48 12.77	12.12	146.89
Subject 14	7.48 6.60 7.08 8.02	111.64 98.50 105.67 119.70	10.56 9.92 10.27 10.94	10.42	108.58

APPENDIX 5

Counts of single cells containing IgM in sections of carinal lymph nodes of six 'fatal' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 15	4.08 2.60 2.97 1.85	60.89 38.80 44.32 27.61	7.80 6.22 6.65 5.25	6.48	41.99
Subject 16	1.24 1.66 1.60 2.11	18.50 24.77 23.88 31.49	4.30 4.97 4.88 5.61	4.94	24.40
Subject 17	2.25 2.82 1.88 2.15	33.58 42.08 28.05 32.08	5.79 6.48 5.29 5.66	5.80	33.64
Subject 18	4.85 2.62 3.90 2.88	72.41 39.10 58.20 42.98	8.50 6.25 7.62 6.55	7.23	52.27
Subject 19	2.94 2.15 2.22 1.99	43.92 31.98 33.13 29.73	6.62 5.65 5.75 5.45	5.86	34.34
Subject 20	2.32 1.23 1.38 0.80	34.62 18.35 20.59 111.94	5.88 4.28 5.53 3.45	4.53	20.52

APPENDIX 5

Counts of single cells containing IgE in sections of carinal lymph nodes from five normal non-smokers.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area 0.067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 1	1.28 1.70 4.39 2.21	19.10 25.37 65.52 32.98	4.37 5.03 8.09 5.74	5.80	33.64
Subject 2	7.95 7.21 9.21 10.11	118.65 107.61 137.46 150.89	10.89 10.37 11.72 12.28	11.31	127.92
Subject 3	3.20 3.88 2.67 3.69	47.76 57.91 39.85 55.07	6.91 7.60 6.31 7.42	7.06	49.84
Subject 4	3.2 2.78 4.79 5.32	47.76 41.49 71.49 79.40	6.91 6.44 8.45 8.91	7.67	58.83
Subject 5	5.44 3.95 8.46 7.16	81.19 58.95 126.26 106.86	9.01 7.6 11.23 10.33	9.56	91.39

APPENDIX 5

Counts of single cells containing IgE in sections of carinal lymph nodes from four normal smokers.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 6	10.58 14.25 12.60 15.90	157.91 212.68 188.05 237.31	12.56 14.58 13.71 15.40	14.06	197.68
Subject 7	10.17 7.96 7.62 13.08	151.79 118.80 113.73 195.22	12.32 10.89 10.66 13.79	11.91	141.85
Subject 8	3.70 3.42 4.00 4.71	55.22 51.04 59.70 70.29	7.43 7.14 7.72 8.38	5.53	30.58
Subject 9	5.00 3.23 4.72 5.12	74.62 48.20 70.44 76.41	8.64 6.94 8.39 8.74	8.17	66.75

APPENDIX 5

Counts of single cells containing IgE in sections of carinal lymph nodes from five 'incidental' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 10	1.17 0.70 1.70 1.64	17.46 10.44 25.37 24.47	4.17 3.23 5.03 4.94	4.16	17.31
Subject 11	0.90 3.72 2.60 4.35	13.43 55.52 38.80 64.92	3.66 7.45 6.22 8.05	6.34	40.19
Subject 12	10.86 9.43 5.50 13.61	162.08 140.74 82.08 203.13	12.73 11.86 9.06 14.25	11.97	143.28
Subject 13	6.12 7.59 8.90 8.30	91.49 113.28 132.83 132.88	9.56 10.64 11.52 11.13	10.71	114.70
Subject 14	10.78 10.23 7.19 9.69	160.89 152.68 107.31 144.62	12.68 12.35 10.35 12.02	11.85	140.42

APPENDIX 5

Counts of single cells containing IgE in sections of carinal lymph nodes from six 'fatal' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (over .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 15	2.89 1.89 1.84 1.80	43.13 28.20 27.46 26.86	6.56 5.31 5.24 5.18	5.57	31.02
Subject 16	2.28 2.10 3.69 3.91	34.02 31.34 58.35 58.35	5.83 5.59 7.63 7.63	6.61	43.69
Subject 17	4.98 6.03 6.38 6.39	74.32 90.00 95.22 95.37	8.62 9.48 9.75 9.76	9.40	88.36
Subject 18	1.16 2.63 1.20 2.04	17.31 39.25 17.91 30.44	4.16 6.26 4.23 5.51	5.04	25.40
Subject 19	0 0 2.8 2.4	0 0 41.79 31.49	0 0 6.46 5.61	3.01	9.06
Subject 20	1.00 1.21 1.27 1.56	14.93 18.06 18.96 23.28	3.86 4.25 4.35 4.82	4.32	18.66

APPENDIX 5

Counts of single cells containing IgG in sections of carinal lymph nodes from five normal non-smokers.

Each figure in the left hand column represents arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 1	2.47 3.40 6.36 7.04	36.86 50.74 94.92 105.07	6.07 7.12 9.74 10.25	8.29	68.72
Subject 2	7.65 7.60 5.43 10.20	114.17 113.43 81.18 152.27	10.68 10.65 9.01 12.34	10.67	113.85
Subject 3	3.21 2.17 3.0 5.06	47.91 32.38 44.77 82.70	6.92 5.69 6.69 9.09	7.09	50.27
Subject 4	4.48 2.69 6.26 2.89	66.86 40.14 93.43 43.17	8.17 6.33 9.66 6.57	7.68	58.98
Subject 5	5.45 6.74 5.18 8.76	81.34 100.59 77.31 130.74	9.01 10.02 8.79 11.43	9.81	96.24

APPENDIX 5

Counts of single cells containing IgG in sections of carinal lymph nodes from four normal smokers.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 6	6.30 8.09 8.78 9.60	94.02 120.74 131.04 143.28	9.69 10.98 11.44 11.97	11.02	121.44
Subject 7	10.62 11.08 16.32 12.11	158.50 165.37 243.58 180.74	12.58 12.85 15.60 13.44	13.61	185.23
Subject 8	4.55 4.65 4.66 10.01	67.91 69.40 69.55 149.50	8.24 8.33 8.33 12.22	9.28	86.12
Subject 9	0.57 1.23 1.61 1.78	8.50 18.35 24.02 26.56	2.91 4.78 4.90 5.15	4.31	18.58

APPENDIX 5

Counts of single cells containing IgG in sections of carinal lymph nodes from five 'incidental' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 10	1.36 1.70 1.81 1.66	20.29 25.37 27.01 24.77	4.50 5.03 5.19 4.97	))) — 4.92)	24.21
Subject 11	4.54 5.26 3.55 4.86	67.76 78.05 52.98 72.53	8.23 8.83 7.27 8.51	))) — 5.37)	32.63
Subject 12	8.50 27.00 23.76 8.50	126.86 402.98 354.62 126.86	11.26 20.07 18.83 11.26	))) — 15.35)	235.62
Subject 13	18.50 10.20 6.75 10.91	276.11 152.23 100.89 162.83	16.61 12.33 10.04 12.76	))) — 12.93)	167.18
Subject 14	6.52 8.76 7.64 10.07	97.31 130.59 114.02 150.29	9.86 11.42 10.67 12.25	))) — 11.05)	122.10

APPENDIX 5

Counts of single cells containing IgG in sections of carinal lymph nodes from six 'fatal' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Subject 15	3.07 4.31 4.94 2.67	45.82 64.32 73.73 39.85	6.76 8.02 8.58 6.31	7.41	54.91
Subject 16	6.91 6.00 9.36 7.81	103.13 89.55 139.70 116.56	10.15 9.46 11.81 10.79	10.55	111.30
Subject 17	6.03 5.57 10.09 6.27	90.00 83.13 150.59 93.58	9.48 9.11 12.27 9.67	10.13	102.62
Subject 18	2.16 2.46 2.10 1.77	32.23 36.71 31.34 26.41	5.67 6.05 5.59 5.13	5.61	31.47
Subject 19	11.0 4.39 3.60 4.81	164.17 65.52 53.73 71.79	12.81 8.09 7.33 8.47	9.17	84.09
Subject 20	3.11 4.25 2.87 6.19	46.41 63.43 42.83 92.38	6.81 7.96 6.54 9.61	7.73	59.75

Appendix 5

Counts of single cells staining with anti-complement conjugates (apparently all eosinophils) in sections of carinal lymph nodes from five normal non-smokers and four normal smokers.

Each figure in the left hand column represents the mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNTS PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
Non-smokers					
Subject 1	0.51 0.87	7.61 12.98	2.75 3.60	) 3.17	10.05
Subject 2	2.0 0.75	29.85 11.19	5.46 3.34	) 4.40	19.36
Subject 3	2.33 3.41	34.77 50.89	5.89 7.13	) 6.51	42.38
Subject 4	2.39 2.56	35.67 38.20	5.97 6.18	) 6.07	36.84
Subject 5	2.2 2.64	32.83 39.40	5.73 6.27	) 6.00	36.00
Smokers					
Subject 6	0.67 0.68	10.00 10.14	3.16 3.18	) 3.17	10.05
Subject 7	2.47 1.38	36.86 20.59	6.07 4.53	) 5.30	28.09
Subject 8	1.59 0.87	23.70 12.98	4.86 3.60	) 4.23	17.89
Subject 9	2.95 4.32	44.02 64.47	6.63 8.02	) 7.32	53.58

APPENDIX 5

Counts of single cells staining with anti-complement conjugates apparently all eosinophils in sections of carinal lymph nodes from five 'incidental' bronchitics and six 'fatal' bronchitics.

Each figure in the left hand column represents the arithmetic mean of cell counts in twenty or more square fields (area .067 sq.mm.).

SUBJECT	MEAN CELL COUNT PER AREA	CELLS/MM^2	SQ. ROOT	ROOT MEAN	ROOT MEAN SQUARE
'Incidental' bronchitics					
Subject 10	6.05 5.02	90.92 74.92	9.50 8.65	) 9.07	82.26
Subject 11	1.37 0.82	20.44 12.23	4.52 3.49	) 4.00	16.00
Subject 12	0.64 0.37	9.55 5.52	3.09 2.34	) 2.71	7.34
Subject 13	2.54 2.36	37.91 35.22	6.15 5.93	) 6.04	36.18
Subject 14	1.33 1.93	19.89 28.80	4.46 5.36	) 4.91	24.11
'Fatal' bronchitics					
Subject 15	1.6 1.19	23.88 17.76	4.88 4.21	) 4.54	20.61
Subject 16	1.75 0.64	26.11 9.55	5.11 3.09	) 4.10	16.81
Subject 17	0.47 0.57	7.01 8.50	2.64 2.91	) 2.77	7.67
Subject 18	1.06 2.38	15.82 35.52	3.97 5.96	) 4.96	24.60
Subject 19	0 0.45	0 6.71	0 2.59	) 1.29	1.66
Subject 20	1.25 2.44	18.65 36.41	4.31 6.03	) 5.17	27.04

APPENDIX 5

Counts of germinal follicles containing immunoglobulin in 16 sections of carinal lymph node from each of five normal non-smokers.

Each number represents the total number of such follicles seen in a whole transverse section of lymph node.

Subject	Immunoglobulin Class	Numbers of Germinal Follicles					
		Section Number				All Sections	Sq. Root
		1	2	3	4		
1	IgA	2	0	0	2	4	2
	IgM	0	1	0	0	1	1
	IgE	1	0	0	0	1	1
	IgG	0	0	0	0	0	0
	ALL	3	1	0	2	6	2.44
2	IgA IgM IgE IgG	NONE					
	ALL						
3	IgA IgM IgE IgG	NONE					
	ALL						
4	IgA IgM IgE IgG	NONE					
	ALL						
5	IgA IgM IgE IgG	NONE					
	ALL						

APPENDIX 5

Counts of germinal follicles containing immunoglobulin in 16 sections of carinal lymph node from each of four normal smokers.

Each number represents the total number of such follicles seen in a whole transverse section of lymph node.

Subject	Immunoglobulin Class	Numbers of Germinal Follicles					
		Section Number				All Sections	Sq. Root
		1	2	3	4		
6	IgA	0	0	0	0	0	0
	IgM	3	5	1	0	9	3
	IgE	0	0	0	0	0	0
	IgG	2	0	0	0	2	1.41
	ALL	5	5	1	0	11	3.32
7	IgA	NONE		3	1	4	2
	IgM			0	1	1	1
	IgE			0	0	0	0
	IgG			0	0	0	0
	ALL			3	2	5	2.24
8	IgA	0	0		0	0	0
	IgM	4	5	NO	2	12	3.46
	IgE	5	3	NE	0	8	2.83
	IgG	1	1		0	2	1.41
	ALL	10	10		2	22	4.69
9	IgA	0	0	0	0	0	0
	IgM	0	0	2	2	4	2
	IgE	0	1	2	2	5	2.24
	IgG	1	0	1	0	2	1.41
	ALL	1	1	5	4	11	3.32

APPENDIX 5

Counts of germinal follicles containing immunoglobulin in 16 sections of carinal lymph nodes from each of five 'incidental' bronchitics.

Each number represents the total number of such follicles seen in a whole transverse section of lymph node.

Subject	Immunoglobulin Class	Numbers of Germinal Follicles					
		Section Number				All Sections	Sq. Root
		1	2	3	4		
10	IgA	10	17	2	3	32	5.66
	IgM	15	18	11	12	56	7.48
	IgE	1	6	1	3	11	3.32
	IgG	3	5	0	0	8	2.83
	ALL	29	46	14	18	107	10.34
11	IgA IgM IgE IgG	NONE					
12	IgA	0	0	0	0	0	0
	IgM	1	0	0	0	1	1.00
	IgE	0	1	1	0	2	1.41
	IgG	0	0	0	1	1	1.41
	ALL	1	1	1	1	4	2.00
13	IgA	0	0	0	0	0	0
	IgM	1	0	0	0	1	1
	IgE	0	0	0	0	0	0
	IgG	0	0	0	0	0	0
	ALL	1	0	0	0	1	1
14	IgA IgM IgE IgG	NONE					

APPENDIX 5

Counts of germinal follicles containing immunoglobulin in 16 sections of carinal lymph node from each of six 'fatal' bronchitics.

Each number represents the total number of such follicles seen in a whole transverse section of lymph node.

Subject	Immunoglobulin Class	Number of Germinal Follicles					
		Section Number				All Sections	Sq. Root
		1	2	3	4		
15	IgA	0	0	0	0	0	0
	IgM	11	7	3	2	23	4.79
	IgE	2	1	6	2	10	3.16
	IgG	2	2	1	2	7	2.65
	ALL	15	10	10	5	40	6.32
16	IgA	1	4	0	2	7	2.65
	IgM	7	10	5	6	28	5.65
	IgE	0	0	2	4	6	2.45
	IgG	6	0	2	0	8	2.83
	ALL	14	14	9	12	49	7.00
17	IgA IgM IgE IgG	NONE					
18	IgA	0	0	0	0	0	0
	IgM	1	0	0	0	1	1.00
	IgE	0	0	0	0	0	0
	IgG	0	3	0	2	5	2.24
	ALL	1	3	0	2	6	2.45
19	IgA	0	0	11	8	19	4.36
	IgM	0	2	21	18	41	6.40
	IgE	0	0	0	0	0	0
	IgG	0	0	18	4	23	2.35
	ALL	0	2	50	30	82	9.06
20	IgA	2	0	0	0	2	1.41
	IgM	0	0	0	0	0	0
	IgE	0	0	1	0	1	1.00
	IgG	0	1	0	1	2	1.41
	ALL	2	1	1	1	5	2.24