THE EFFECTS OF SYMPATHOMIMETIC DRUGS ON THE GOBLET CELL POPULATION IN THE PROXIMAL AIRWAYS OF THE RAT

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DECLARATION

The composition of this Thesis and the investigations described were designed and performed by the author except where otherwise acknowledged.

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ABSTRACT

Goblet cell hyperplasia is found in many hypersecretory disease states. Despite this the origin, synthetic and secretory control, and population regulation of goblet cells remain unknown.

The aim of this study was to establish a single-dose model of goblet cell hyperplasia in the rat lung which was free from respiratory infection and a superimposed inflammatory reaction. The agent used was isoprenaline, multiple injections of which had been shown to induce goblet cell hyperplasia in the rat lung.

The effects of a single systemic dose of isoprenaline were investigated. The isoprenaline response was then studied in order to ascertain the mechanism whereby isoprenaline induced goblet cell hyperplasia.

It was found that a single 40 mg/kg dose of isoprenaline induced a reproducible 3 - 4 fold increase in goblet cells in the rat lung. The response could be detected at 12 hours, reached a maximum between 24 - 48 hours and then gradually declined, regaining control values 8 - 16 days later. The response was dose-dependent and a shallow dose-response curve was obtained which could be antagonised by propranolol. Salbutamol and methoxamine were also shown to induce goblet cell hyperplasia in the rat lung. Isoprenaline induced goblet cell hyperplasia

when applied directly to the respiratory epithelium. The goblet cell hyperplasia was stereospecific for (-) isoprenaline at low doses but not at high doses. The response was the same in male and female rats.

It was concluded that the goblet cell response was due either to its actions on β-adrenoceptors or some indirect metabolic effect.

CONTENTS

SYNOPSIS

The original intentions of this project were as follows:-

Firstly, to establish a single dose model of isoprenaline-induced goblet cell hyperplasia in the rat lung. This model was then to be used to study the goblet, about which very little is known, in detail. In particular, it was intended to use the model in conjunction with cell kinetic studies using tritiated thymidine, to ascertain the role of mitosis in the origin of the new goblet cells and also to study the turnover of the goblet cell population in the lung. It was envisaged that this work would be carried out using both light and electron microscopes. In addition, it was proposed to carry out differential cell counts of the respiratory epithelium, such that the same area used for the assessment of goblet cells under the light microscope would be used for the differential cell counts under the electron microscope. It was also intended that the model would be used in conjunction with radioactive labelled mucin precursors to study the effects of isoprenaline on the pattern of mucin synthesis and discharge from goblet cells. This work would also have employed both light and electron microscopes.

To facilitate combined light and electron microscopic studies it was necessary to develop new histological techniques for the fixation, processing, cutting and

staining of large plastic embedded blocks of lung tissue. The requirement for the light microscopic quantitation of goblet cells was for thin sections 1 - 1.5 μm thick encompassing the entire length of the main airway of the left lung of the rat along with all of its side branches. From the remaining block a map was to be made and areas of interest, i.e. those studied at the light microscope level, would be sawn out and sections cut for the electron microscope. It follows that the embedding medium chosen had to be compatible with both the light and the electron microscope. One such medium is araldite and when the project commenced there had just been produced a new design of microtome which, it was claimed, would cut large thin sections of araldite embedded material. However, subsequent experience proved that it was impossible to achieve adequate quality of sections, using this machine, for the light microscopic assessment of goblet cells (see Appendix v). Eventually a compromise solution was reached whereby the left lungs from experimental rats were embedded in glycolmethacrylate (GMA) for the light microscopic assessment of goblet cells and selected right lungs were post-osmicated and embedded in araldite for future study under the light and electron microscopes.

The development and rejection of the various histological techniques tested took up the whole of the first year of the study. However, by the beginning of the second year of the study the histological side of the

project had been decided upon. There was then a further delay of 6 months caused by an outbreak of Sendei virus at the I.C.I Alderley Park breeding unit, during which time no suitable alternative source of animals was found (see Chapter 3 section 3.1). This incident necessitated the repeat of at least one experiment and a complete reassessment of the criteria for the acceptability of lungs into the study. Thus, it was deemed necessary to examine every single lung for the presence of disease before they were used for the quantification of goblet cells. This required the cutting and staining of additional sections and thus lengthened the time between dosing the animals and quantifying goblet cells.

As it was not possible to pursue the development of new techniques for autoradiography in GMA through lack of time, and also because GMA is not electron stable and cannot therefore be used satisfactorily in the electron microscope, both the proposed cell kinetic and differential cell count studies had to be abandoned. It was therefore decided to investigate the isoprenaline response in greater detail and attempt to ascertain a possible mechanism whereby isoprenaline induces an increase in goblet cells. Thus, experiments were designed to establish:

a) the existence or not of a dose-response relationship

b) the details of the timing of the response

c) whether there were male/female differences in the response

d) the stereospecificity of the response using the optically active isomers of isoprenaline

e) whether any other sympathomimetic or para-sympathomimetic drugs were capable of eliciting a similar response

f) if it was possible to antagonise the isoprenaline induced goblet cell hyperplasia pharmacologically with propranolol, a β-adrenoceptor antagonist

g) if isoprenaline, when applied directly to the respiratory epithelium either as an aerosol or as a bolus intratracheal injection, was capable of inducing the same goblet cell response.

Finally, preliminary studies were made to develop a novel fast screening technique for the assessment of tracheal goblet cells. This technique was called the tracheal dab technique and is described in Appendix i. The aim was to develop a very fast method for the quantification of goblet cells which would in turn make feasible the screening of a wide range of drugs for an effect on respiratory goblet cells.

This in essence is the project as it was originally conceived and subsequently developed.

CHAPTER 1

INTRODUCTION

1:1 GENERAL INTRODUCTION

The respiratory epithelium, which forms the lining of the airway passages, contains many specialised cells. One of these is the goblet cell which is responsible for the production of mucus. Mucus production provides a means of trapping and removal, via the mucocilliary transport mechanism, of particulate matter, including bacteria, as it enters the lung.

Very little is known about the factors which control goblet cell numbers within the lung, their origin within the respiratory epithelium or about the mechanisms which control the synthesis and secretion of mucus. In certain hypersecretory disease states the number of goblet cells is known to increase. The mechanism causing this increase is however unknown.

Many attempts have been made to find an animal model of goblet cell hyperplasia. Most of these models have involved direct multiple exposures of the respiratory epithelium to irritant gases. However, a more recent model has been demonstrated using multiple systemic doses of isoprenaline, a β-adrenoceptor agonist. This is the first model of goblet cell hyperplasia in the lung not

complicated by the superimposed inflammatory reaction caused by direct damage to the epithelium by an irritant.

The study reported here was undertaken to investigate the isoprenaline-induced goblet cell hyperplasia in greater detail. Experiments were designed to establish a model of respiratory goblet cell hyperplasia using a single intraperitoneal dose of isoprenaline which could be used to ascertain when the increase in goblet cells occurred and how long it lasted. A series of studies were undertaken in order to find out if the response was dose-dependent. Attempts were made to antagonise the effects of isoprenaline using the β-adrenoreceptor antagonist propranolol. A number of sympathomimetic and parasympathomimetic drugs were tested for a similar effect on respiratory goblet cells. Differing routes of administration were tried to see if they had any effect on the isoprenaline-induced goblet cell hyperplasia. Various novel histological techniques were evaluated in an attempt to develop an improved procedure to make quantification of the goblet cells easier and quicker.

The first section of the introduction will contain descriptions of the lung and the respiratory epithelium with particular reference to the rat proximal airways. This is followed by the historical background associated with the goblet cell, and a detailed description of the goblet cell structure and function. Next there is a

section on the factors known to influence respiratory goblet cells and a brief review of the possible origins of the goblet cell. At the end of this section there is a paragraph on the innervation in the lung and its role in the control of goblet cell synthesis and secretion. Finally, there is a brief review of the pharmacology of isoprenaline with details of both its direct pharmacological actions and its indirect metabolic and biochemical actions.

1:2 STRUCTURE AND FUNCTION OF THE LUNG

Anatomy: The mammalian lung exists primarily to effect gaseous exchange and to perform this function it possesses multiple thin-walled distensible air sacs connected by a series of passages with the external atmosphere. The detailed pattern of airtubes and aerating tissues varies from species to species; all are nevertheless constructed on a similar plan (Spencer, 1977). The nasal passages communicate directly with the atmosphere; these lead into the trachea and this in turn divides at the carina to form the right and left main bronchi.

In the rat the right bronchus further divides outside the lung parenchyma and each of these branches enters one of the four separate lobes of the right lung (see Figure 1). The left lung in the rat, on the other hand, is a single lobe and the left main bronchus divides into two

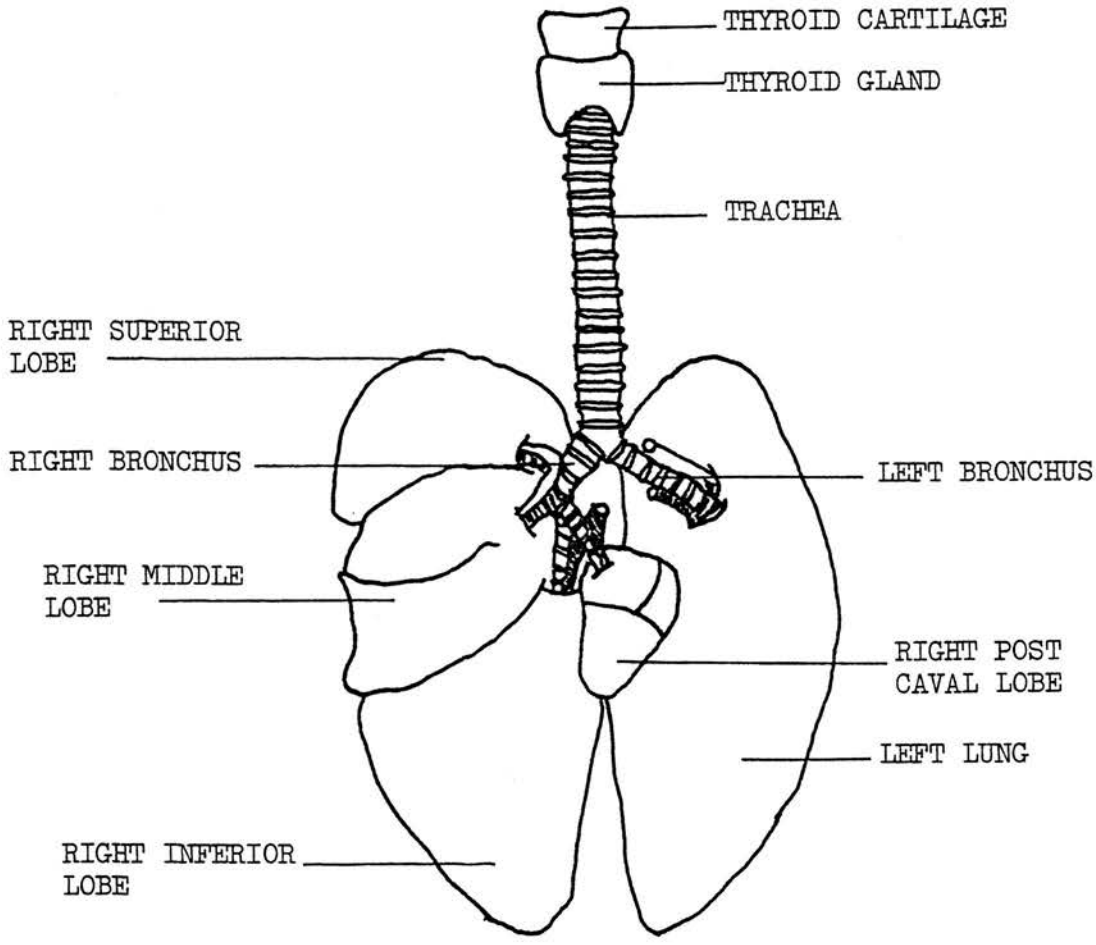


FIGURE 1

Ventral view of the respiratory organs of the rat (after Rowett, 1977).

branches immediately upon entering the lung. In man there are normally three lobes on the right and two on the left.

Upon entering the lung the bronchi divide repeatedly, ultimately ending in terminal bronchioles. In man these give rise to a further one to three generations of respiratory bronchioles. In the rat, however, there are very few respiratory bronchioles (Castleman et al., 1975), and it is the terminal bronchioles and not the respiratory bronchioles which end in alveolar ducts. The alveolar ducts terminate in two or more air sacs and from these arise the alveoli which increase the surface area and constitute the aerating surface.

A series of cartilage rings lend support to the trachea and main bronchi. In man they extend along the intrapulmonary airways as far as the end of the small bronchi; in the rat cartilage rings only occur in the extrapulmonary airways and terminate at the hilum (Jeffrey and Reid, 1977.

The manner in which the airways divide to form a branching network is not the same in all species - in man it is a mixture of monopodal, polypodal and dichotomous branching. In the rat, because the left lung is a single lobe, the manner in which the airways branch is very different (see Figure 2). This simple branching pattern allows division of the lung into set anatomical areas, zones 1 - 5, using the side branches leading off from the

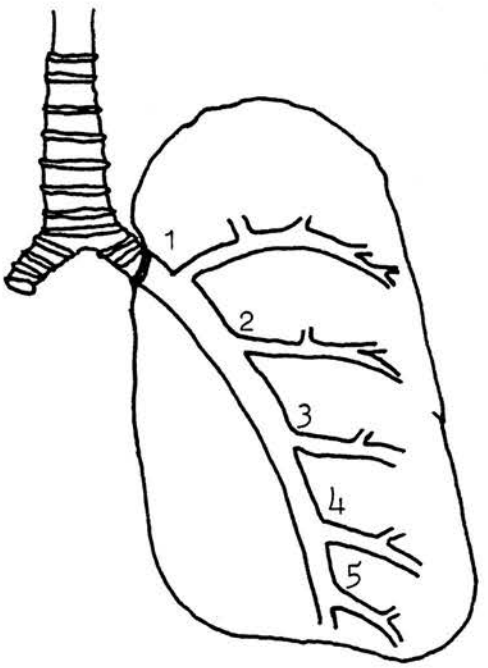


FIGURE 2

Schematic diagram to show the nature of the branching of the main bronchus in the left lung of the rat. (The figures 1 - 5 represent the zones referred to in the text)

main bronchus (Figure 2). All these branches travel in the same plane and by careful orientation of the fixed, inflated lung during embedding and cutting, it is possible to obtain histological sections showing the main bronchus and each of its side branches (see Figure 3). This has important implications for reasons which will be discussed in section 1:33, as it allows study of the respiratory epithelium at the same anatomical site in different rats.

Airways and Respiratory Epithelium: In mammals the airways are lined by a respiratory epithelium which extends from the nasal pharynx right out to the terminal and respiratory bronchioles. Since this thesis is concerned with intrapulmonary respiratory epithelium, most of the following will relate to the ciliated epithelium of the lower respiratory tract.

The basic structure of the airway epithelium was determined by the late nineteenth century and descriptions of various cell types were published by the following: Ciliated and goblet cells (Henle, 1837); goblet, chalice and beaker cells (Knauff, 1857; Shulze 1871; and Frey, 1874); basal and intermediate cells (Frankenhauser, 1879; Waller and Björkman, 1882).

With the advent of the electron microscope ultrastructural details became apparent which had not been seen at the light microscope level and descriptions of increasing numbers of different cell types in the

FIGURE 3

Low power photomicrograph (x1) showing the left full main airway and its branches obtained in 1.5 µm GMA sections (Bullard's haematoxylin/eosin staining).

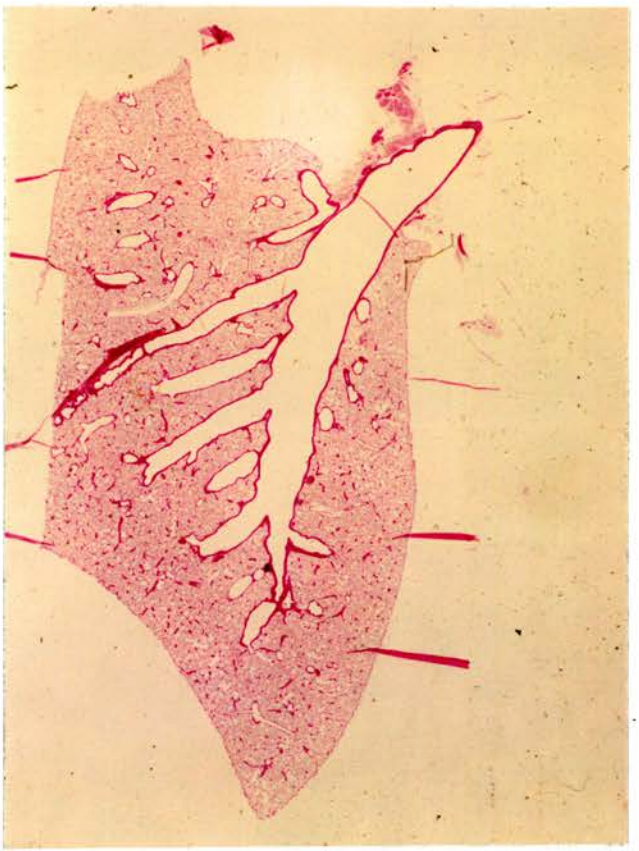


FIGURE 3.

respiratory epithelium were published. There are now several light microscope and electron microscope studies giving detailed descriptions of the cell types present in the rat (Breeze and Wheeldon, 1977; Kuhn, 1976; Jeffrey and Reid, 1975 and 1973a; Rhodin and Dalhamn, 1956; Rhodin, 1959; Kent, 1966). Currently, ten cell types, eight epithelial and two mesenchymal, are recognised within rat airway epithelium (see Table 1).

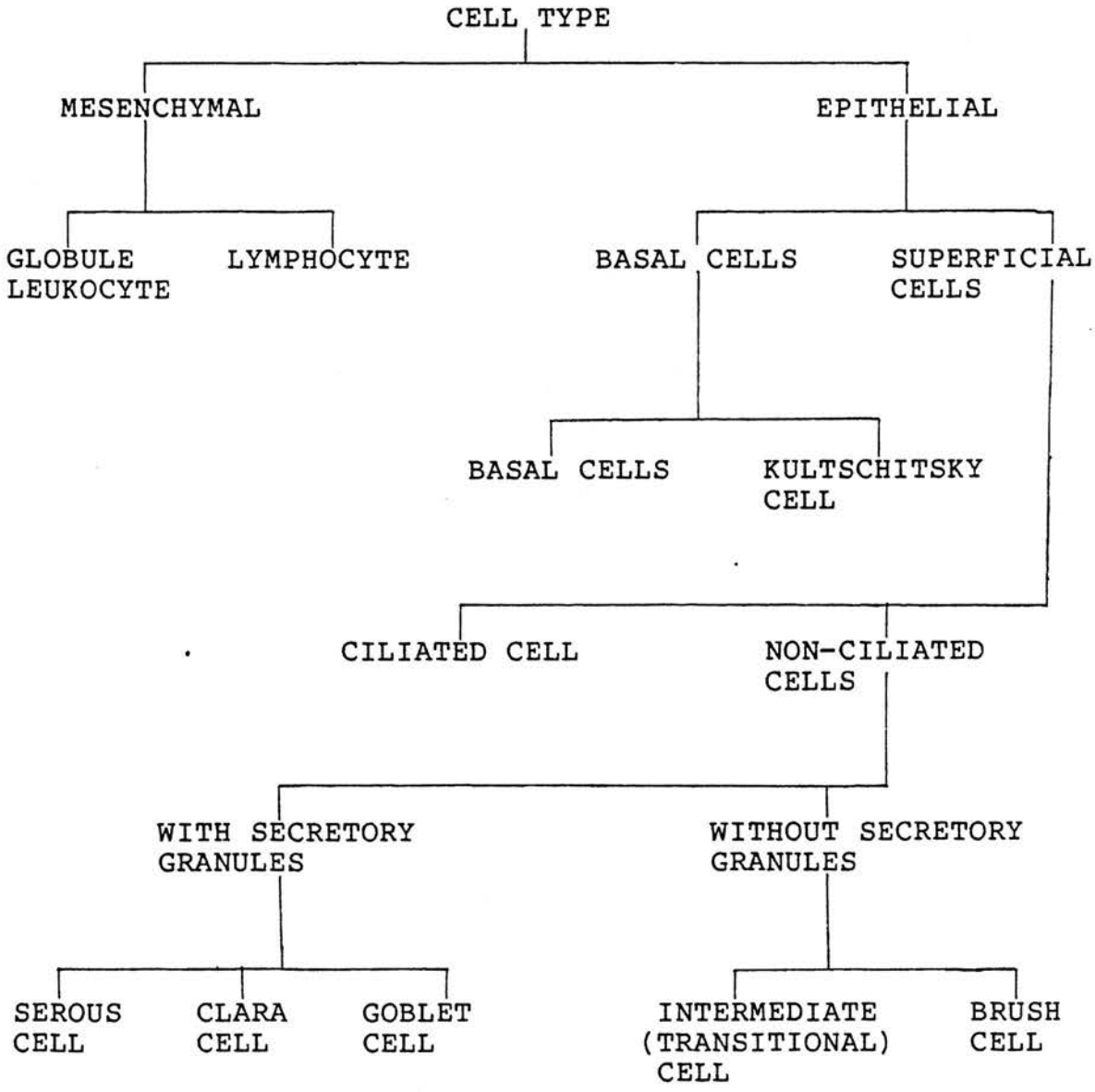
The number and distribution of each cell type within the epithelium are not constant throughout the length of the respiratory tree. Thus a cell may only be found in proximal airways whilst another will show a mainly peripheral distribution. In the rat the distribution of epithelial and migratory cells is as follows:-

Proximal intrapulmonary airways: Ciliated cells, goblet cells, basal cells, intermediate cells, very rarely serous cells and K-cells, occasional brush cells and Clara cells, lymphocytes and globule leukocytes.

Peripheral intrapulmonary airways: Ciliated cells, Clara cells, brush cells, intermediate cells, very rarely K-cells, lymphocytes, and globule leukocytes.

Most can only be identified ultrastructurally or using sophisticated histochemistry. At the light microscope level in proximal airways the following can be reliably identified: ciliated cells (haematoxylin and eosin stain), goblet cells (mucin stains), K-cells (fluorescence and

TABLE 1



The 10 epithelial cell types classified on the basis of position, presence of cilia, and type of secretory granule, if any.

(R. Jones, 1976).

histochemistry), lymphocytes, globule leukocytes, non-ciliated non-secretory cells and non-ciliated secretory cells. The non-ciliated secretory cells include both serous and Clara cells whilst the non-ciliated non-secretory cells include brush cells and intermediate cells.

1:3 THE GOBLET CELL

1:31 The Historical Background to the Goblet Cell

The earliest description of the goblet cell was given by Henle in 1837. Later, various authors also described a large cup-shaped cell in the respiratory epithelium. They were as follows: Bowman, 1847; Knauff, 1857; Schulze, 1871 and Frey, 1847. The term goblet cell was used as a descriptive term because of the shape of the mature form of the cell. They have also been called chalice, beaker and cup-shaped cells.

1:32 The Structure and Function of the Goblet Cell

Structure: Mature goblet cells have an expanded cup-shaped cytoplasm, called the theca, which is filled with secretory droplets. The cells are connected to the epithelial basement membrane by a thin stem into which the cytoplasmic organelles and nucleus are compressed (see Figure 4). Thus, it is often difficult to see the structural details of the organelles even at the ultrastructural level. The nucleus, which is situated at the base of the cell appears

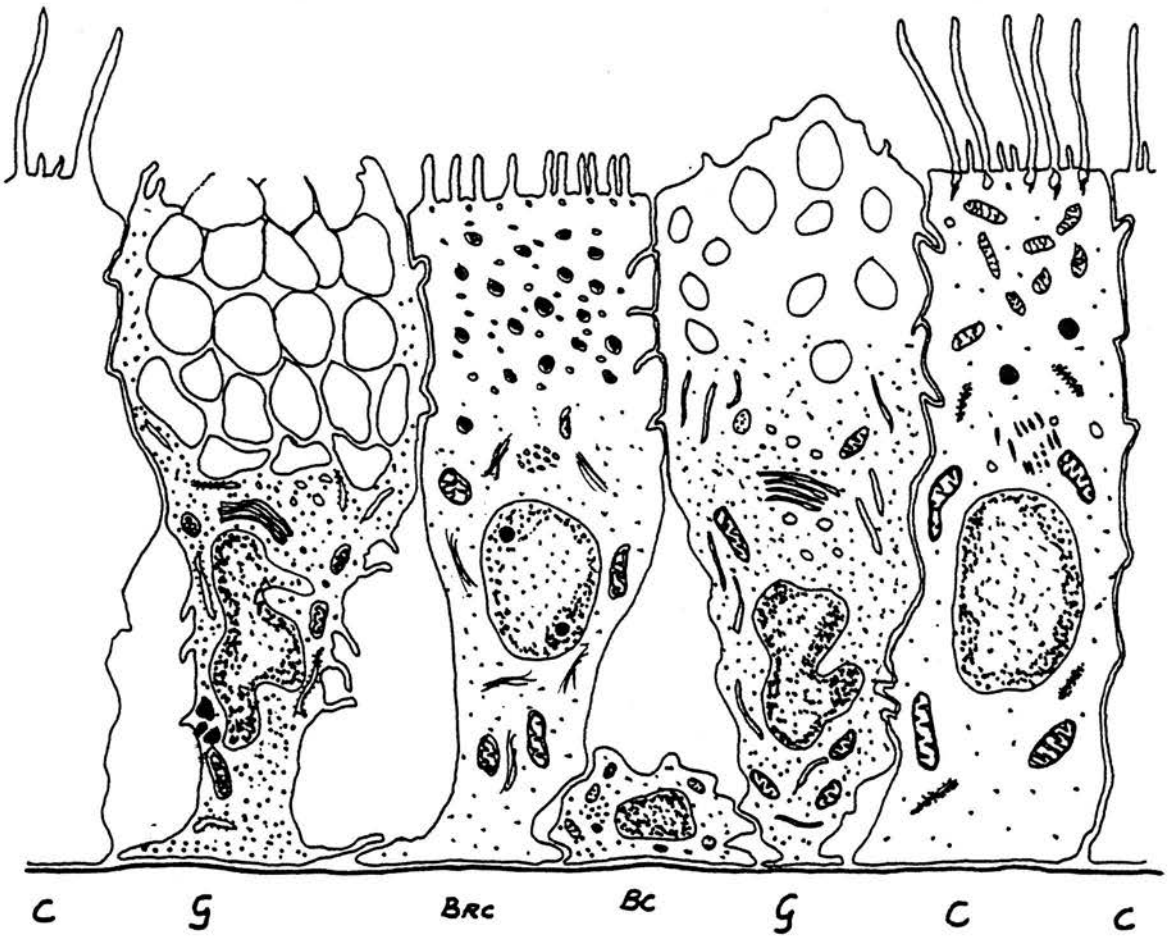


FIGURE 4

A schematic presentation of the tracheal epithelium of the rat. C = Ciliated cells; G = Goblet cells in various stages of secretion; BRC = Brush cell; BC = Basal cell (after Rhodin and Dalhamn, 1956).

very densely stained at the light microscope level. It is irregular in shape and contains a small nucleolus. Immediately above the nucleus there is a prominent Golgi apparatus. It is here that the mucin is synthesised. The supranuclear area also contains extensive profiles of rough endoplasmic reticulum and a few mitochondria and tonofilaments.

The cytoplasm of the goblet cell is relatively electron opaque with many free ribosomes giving it a marked basophilia. At the apex of the cell the cytoplasm contains numerous mucin granules, these are of differing size and are bound by a thin, often discontinuous membrane. The contents of the granules can be either a fibrillar or a homogenous low density material, and in the rat they contain an electron-dense core (Jeffrey, 1975). The luminal surface of the goblet cell is covered by a number of short microvilli which project into the airway lumen.

Function: The function of goblet cells is to synthesise and secrete mucin. The mucin so produced varies from species to species, from cell to cell, from tissue to tissue and with disease, and some cells may even produce two or more types of mucin at the same time. Generally, goblet cells are capable of producing 4 types of mucin, a neutral mucin which stains with PAS (Periodic Acid/Schiff) but not AB (Alcian Blue), and three acid mucins; a sialidase-sensitive sialomucin, a sialidase-resistant sialomucin, both of which stain with combined AB/PAS stains

to give red/blue and blue/red colours, and a highly acid sulphomucin which reacts with AB, Hales Colloidal Iron and High Iron Diamine stains. It appears that under normal conditions a goblet cell population secretes a particular range of glycoproteins but that in response to irritation the product may be modified as in the case of respiratory infection when there is a shift towards a predominance of acid sulphomucin in the rat (Reid, 1970; Jones and Reid, 1978).

Synthesis: Goblet cell synthesis of glycoproteins occurs at 3 sub-cellular sites; the peptide backbone is assembled on membrane-bound ribosomes within the rough endoplasmic reticulum (RER). A series of glycosyltransferases (the so-called multi-glycosyltransferase system), then add the different carbohydrate moieties to the protein backbone as the growing glycoprotein transverses from the RER to the SER (smooth endoplasmic reticulum) and then to the Golgi apparatus where sulphation takes place. Glycoproteins destined for secretion are packaged into secretory vesicles in the Golgi apparatus and these, once released into the cytoplasm, mature into storage vesicles which gradually accumulate at the apex of the cell. Secretion of mucin granules takes place either singly with the granules remaining intact (reverse pinocytosis) or occasionally the contents at the apex of the cell may coalesce and be extruded from the cell as a stream of mucin (Schachter, 1978).

1:33 The Quantification of Respiratory Goblet Cells

Problems of Identification: One of the difficulties encountered when attempting to quantify goblet cells is that not all goblet cells exhibit the classic 'goblet' shape. Immature or recently emptied goblet cells appear as columnar, slender cells often with only a few mucin granules in the cytoplasm. At the light microscope level such cells may not be identified as goblet cells, particularly when mucin specific stains have been used, as insufficient mucin content of the cells makes them appear as non-ciliated secretory cells with a few apical granules - a classification which can include serous, Clara and possibly intermediate cells.

In order to make this source of error as small as possible, it is necessary to decide at the outset the criteria that are to be applied when identifying goblet cells and to adhere to them rigidly throughout any study. In this study only frank goblet cells, i.e. those with a distended mucin-filled theca, were counted as goblet cells.

Problems of Distribution: Another problem which is encountered when counting respiratory goblet cells is their uneven distribution within the respiratory epithelium. Their numbers increase toward the caudal part of the trachea, there is also a greater density of goblet cells in the cartilagenous wall of the trachea than there is on the membranous wall. In intrapulmonary airways goblet cells

occur infrequently either singly or in clusters, with conspicuous clusters at points of bifurcation. Their numbers decrease rapidly towards the periphery, and in the normal, healthy animal there are no goblet cells present on small bronchi and bronchioles. Nevertheless they can be induced at these peripheral sites by disease and irritation.

Because of the wide variation in distribution it is difficult to quantify goblet cells accurately. In a study such as this it is essential therefore that when assessing goblet cells and making a comparison between different groups of rats that the same anatomical area is studied in each case. Sections therefore have to be large enough to make this possible, preferably encompassing the entire length of the main airway in the left lung. Lamb et al. (1969) and Jones (1976) have shown that the distribution of goblet cells in zones 1 and 2 of the intrapulmonary airway of the rat (Figure 2) is fairly constant thus making it a good area for studying changes in the goblet cell population. The advantageous branching system of the airways in the left lung is therefore of great help in defining the area to be counted (Figure 3).

1:34 The Factors Affecting Goblet Cell Function

Introduction: An increase in epithelial goblet cells is a characteristic feature of chronic inflammatory changes in

mucosal membranes (Florey, 1970). Thus, respiratory goblet cells respond to a variety of adverse stimuli by both increased mucus production and an increase in numbers; this is a defensive mechanism used to protect the delicate tissues of the lung.

Amongst the external factors which have been shown to affect the goblet cell population are irritant gases and liquids, particulate matter, infections caused by inhaled bacteria and viruses, and pharmacological agents (Florey, 1932; Reid, 1963; Lamb and Reid, 1968; Mawdesley-Thomas and Healey, 1969; Mawdesley-Thomas *et al.*, 1971; Elmes and Bell, 1963; Freeman, 1964; Freeman *et al.*, 1972; Mellors, 1958; Lamb and Reid, 1969; Cash *et al.*, 1979; Jones *et al.*, 1975; Sturgess and Reid, 1973; Baskerville, 1975; Jones and Reid, 1979a and 1979b; Kleinerman, 1976). The nature of the internal, i.e. physiological, factors which exert a controlling influence on the goblet cell population are not known. Goblet cell secretion does not appear to respond to either parasympathetic or sympathetic nerve stimulation, although both types of nerve endings play a role in controlling submucosal gland secretion. Recently, a substance P-like material has been shown to be present in nerves in the *lamina propria* and basement membrane of the respiratory epithelium, but its relationship to goblet cell activity is not known (Wharton *et al.*, 1979).

Experimental Models of Respiratory Goblet Cell Hyperplasia Using Irritant Liquids and Gases:

FORMALIN LIQUID: Florey (1932) described experiments in which he studied the effects of daily injections of dilute formalin on the airway mucosa. The formalin was injected into the airway via a glass tube passed through the glottis. Ciliated, columnar epithelial cells were replaced by cuboidal shaped cells resembling the transitional epithelium of the bladder; goblet cells were greatly increased in number and the glands showed evidence of exhaustion of secretion.

MUSTARD OIL: In 1932, Florey studied the effects of the direct application of mustard oil on cat respiratory epithelium. The results on goblet cells were inconclusive since there was a superimposed desquamation of the epithelium. However, in areas least affected there was a suggestion of goblet cell increase.

SULPHUR DIOXIDE: In 1963, Reid reported an increase in rat respiratory goblet cells following exposure to SO_2 gas at 200-300 ppm for 5 hours a day for 6 weeks. The aim of the study was to produce an animal model of the changes in chronic bronchitis without a superimposed respiratory infection.

Lamb and Reid in 1968 studied the SO_2 effect in greater detail and found that the goblet cell increase need not directly follow mitosis and that newly formed cells

changed their pattern of glycoprotein production to a predominance of acid glyco-protein. Reid in 1963, and Lamb and Reid in 1968, also studied the reversal of the changes produced within the airway epithelium. They were found to persist up to 3 months after the cessation of exposure.

Subsequent studies have shown that the response to the irritant is dose-dependent (Mawdesley-Thomas and Healey, 1969; Mawdesley-Thomas et al., 1971). In the same studies they reported a similar change induced by SO_2 in the respiratory airways of the lamb. On the other hand, studies in the dog have shown different responses to SO_2. Proximal segmental airways showed a reduction in goblet cell numbers whilst distal airways and bronchioli showed an increase in goblet cells. The trachea and lobar bronchi exhibited squamous metaplasia with a loss of both ciliated and goblet cells (Chakrin and Saunders, 1974). In a later publication they reported that SO_2 exposure had produced a decrease in the number of acidic groups within the goblet cells of the airway surface (Spicer, Chakrin and Wardell, 1974).

There is evidence that in the presence of infection the effects of SO_2 exposure, namely the increase in goblet cells and proliferative changes, are not seen (Goldring et al., 1967; Lightowler and Williams, 1969).

CHLORINE: Exposure of rats to 40 ppm chlorine gas produces goblet cell hyperlasia both in proximal and peripheral airways (Elmes and Bell, 1963).

NITROGEN DIOXIDE: In the absence of disease, rats exposed to 25 ppm of NO_2 for 40 days exhibit respiratory goblet cell hyperplasia (Freeman, 1964). Long term exposure to lower doses of NO_2 (15 ppm) also produces similar changes (Freeman et al., 1972).

Experimental Models of Respiratory Goblet Cell Hyperplasia Using Tobacco Smoke: Mellors (1958) first reported an increase in respiratory goblet cells in the rat following exposure to tobacco smoke. Lamb and Reid in 1969 showed that the increase in goblet cells was dose-dependent and that at some doses there was a shift from neutral to acid mucin production. Studies using lambs (Mawdesley-Thomas and Healey, 1973) failed to demonstrate a statistically significant increase in respiratory goblet cells after exposure to tobacco smoke, but they did show a significant and dose-related increase in gland size.

Expermental Models of Respiratory Goblet Cell Hyperplasia Using Bacteria and Viruses: Cash et al. in 1979 reported a rat model of chronic respiratory infection using Pseudomonas aeruginosa, an organism persistently found in the lungs of patients with cystic fibrosis. In the infected rats they found increased epithelial goblet cells

surrounding areas involved by the infecting organism. Interestingly goblet cell numbers were increased in areas where there are normally none, i.e. peripheral airways, as well as in their usual sites in the proximal airways.

Conversely, in pigs suffering from enzootic pneumonia caused by Mycoplasma hyorhinis, Jones et al.(1975) reported an increase in the height of the bronchial epithelium and a depletion of epithelial goblet cells associated with a marked reduction of both theca size and intracellular glycoprotein.

The essential difference between these two models of respiratory infection is that the former was a chronic infection and the latter an acute infection. This may account for the difference in the goblet cell response.

Experimental Models of Respiratory Goblet Cell Hyperplasia Using Pharmacological Agents: As stated in the opening paragraph of this section, both sympathetic and parasympathetic nerves have been observed within the sub-mucosal bronchial glands of man, the rat and the cat and therefore they may play a role in the control of the secretion of mucus by the glands. However, epithelial goblet cell secretion does not appear to respond to either parasympathetic or sympathetic nerve stimulation and neither type of nerve ending has been observed in close proximity with epithelial goblet cells. The physiological control over goblet cell activity is therefore unknown.

Whilst attempting to find a solution to this question a number of experiments have been reported in which a variety of pharmacological agents have been tested to see if any of them are capable of influencing respiratory epithelial goblet cells.

ISOPRENALINE: Isoprenaline, a β-adrenoceptor agonist, was first reported to induce an increase in respiratory epithelial goblet cells in rats by Sturgess and Reid in 1973. They found that 6 and 12 daily injections of isoprenaline sub-cutaneously, in doses of 10 and 25 mg, produced an increase in both epithelial goblet cells and sub-mucosal gland size. They also demonstrated that the newly formed goblet cells were producing predominantly acid mucin and less neutral mucin. Using organ culture techniques they also studied the effects of isoprenaline in vitro on human bronchial explants; isoprenaline administration was combined with ^{3}H-glucose administration and the secretion of mucin macro-molecules was followed. They found isoprenalne in vitro had no effect on the secretory index of sub-mucosal bronchial glands or on epithelial goblet cells (Sturgess and Reid, 1972).

In a similar study, this time in pigs, Baskerville (1975) reported an increase in gland size and epithelial goblet cell numbers following 6 daily injections of 75 mg to pigs weighing between 32-40 lbs. The new goblet cells were shown to produce acid sialomucin. The increase

reached significant levels 24 hours after the last injection and persisted for 16 days and then gradually declined, taking as long as 84 days to reach control values once more. Baskerville concluded that, as there were no hyperplastic changes within the epithelium and the mitotic acitivity showed no change, the drug was altering cellular differentiation in order to produce the increase in goblet cells.

In a later study, Jones and Reid (1979a) studied the regression in goblet cell numbers in more detail following systemic administration of isoprenaline in rats. Rats were given cumulative daily doses of isoprenalne (10 mg/100 g body weight) for up to 6 days. Rats were killed at different times during the injection schedule; at 24 hours following a single injection they found a 3-fold increase in goblet cells, and this increased to a 4-fold increase following 2 daily injections. This was a maximum increase since following 6 daily injections the increase was still 4-fold. After withdrawal of the drug they found that the goblet cell number fell at different rates in different sites in the lung, e.g. proximal trachea took one week to regain control values, the main bronchus, 8 weeks and the peripheral airways were still slightly above control values at 12 weeks.

SALBUTAMOL: This is a selective β_2-adrenoceptor agonist used to reverse broncho-constriction in asthma and chronic bronchitis. In 1979, Reid and Jones briefly reported a

study in which they compared salbutamol with isoprenaline on the goblet cell population in rat lung. They found that there were regional differences between the two drugs on goblet cell numbers although both drugs altered the type of mucin produced by the same amount to acid mucin. The results, they suggested, indicated differences in β_1- and β_2-adrenoceptor distribution. Further details of this study were reported later in the year (Jones and Reid, 1979b). The doses of the drugs used were 10 mg/100 g body weight sub-cutaneously as 6 daily injections. Isoprenaline increased goblet cells in proximal and distal tracheal epithelium but not in the central portion, and increased goblet cells in all zones of the bronchial epithelium in the lung. Salbutamol, however, only produced significant increases in goblet cells in proximal tracheal epithelium and in proximal bronchial epithelium and had no effect in other zones. Isoprenaline had a much greater effect than salbutamol despite the fact that salbutamol has a longer half-life and therefore persists longer in the body once injected.

PILOCARPINE: Pilocarpine is a para-sympathetic agonist acting on muscarinic receptors. In 1973, Sturgess and Reid reported increases in respiratory epithelial goblet cells in the rat following 10 mg pilocarpine sub-cutaneously for 6 and 12 days. Pilocarpine, unlike isoprenaline, induced an increase in both neutral-mucin-producing and acid-mucin-producing goblet cells. <u>In vitro</u> studies by Sturgess and

Reid (1972) failed to show any influence on epithelial goblet cells by pilocarpine but they did find that parasympathomimetic drugs, among them pilocarpine, increased sub-mucosal gland secretory index and that parasympatholytic drugs, e.g. atropine, decreased gland secretory index - evidence which supports the parasympathetic nervous control of glands in the bronchial epithelium.

METHACHOLINE: Methacholine is a para-sympathetic agonist and its effects on epithelial goblet cells and bronchial glands in the cat were reported by Kleinerman in 1976. 3 mg/kg in 3 doses given daily over a period of 90 days produced both goblet cell 'metaplasia' and bronchial gland hypertrophy. The descriptions were brief however; the glycoprotein of the gland was unchanged and mainly neutral, whilst the goblet cells showed a loss of acid groups.

SUMMARY: A pharmacological model of goblet cell hyperplasia is the first not to be complicated by inflammation and infection, and therefore it is easier to study the goblet cell response after the drug has been administered. Until now the drugs have been administered in multiple doses over a period of days; however, should it be possible to establish a single dose model of goblet cell hyperplasia then detailed studies of the goblet cell using cell kinetics and autoradiography will be possible. Also the administration of the drugs is much easier and more

reproducible than the inhalation and inoculation techniques previously required and this means that comprehensive studies using large numbers of animals become feasible.

1:35 The Origin of Goblet Cells

Cell turnover in the rat lung is very low: less than 1% of the total epithelium population being labelled one hour after injection with ^{3}H-thymidine (Hackett, 1979). Consequently the question of whether goblet cells in the respiratory tract arise directly from the stem cell population or via some other indirect route is a vexed one, and many conflicting theories have been proposed.

Donnelly et al. (1980) concluded that in the rat tracheal epithelium, all cell types with the exception of ciliated cells were proliferative and that the intermediate cell was the precursor cell for the ciliated cell. Conversely, also in the rat, Bindreiter et al. (1968) concluded that ciliated and goblet cells were not proliferative and that the basal cells were the stem cell population with one basal cell division giving rise to one basal cell and one differentiating cell, the latter having one subsequent division giving rise to differentiated functional cells, e.g. ciliated and goblet cells. Another theory has been proposed by Boren and Paradise (1978) in which they put forward a model scheme consisting of three kinetic compartments in which self-maintaining basal cells

feed into a dividing mucus cell compartment from which ciliated cells arise.

In peripheral airways, where there are no basal cells, the Clara cell is believed to be the progenitor cell for the bronchiolar epithelium (Evans et al., 1978). This proliferative role is in addition to their normal secretory function. When damage is inflicted on small airways by agents such as nitrogen dioxide (Evans et al., 1978) and ozone (Lum et al., 1978), Clara cells cease their secretory activity and undergo mitosis giving rise to new ciliated and goblet cells and thus effect repair to the bronchiolar epithelium.

The respiratory epithelium forms a flat and continuous sheet of cells. There are therefore no clearly defined compartments of stem cells, differentiating cells and mature functional cells. In the small intestine, however, the linear progress of cells from their origin at the base of the crypt to maturity and eventual loss at the tip of the villus can be studied. This makes it possible to trace the origin and development of the different cell types in the villus epithelium. As a consequence several studies on the origin of goblet cells have been carried out using intestinal villus epithelium. The following theory for the origin of villus goblet cells has been proposed: a self-maintaining stem cell population at the base of the crypt feeds into a mixed proliferative precursor cell population consisting of proliferative pre-columnar and proliferative

pre-goblet or oligomucus cells. These proliferating precursor cells occupy the lower half of the crypt and in turn give rise to the differentiating non-proliferating functional cells which occupy the top half of the crypt (Cairnie, 1970; Merzel and Leblond, 1969). Additional villus goblet cells also arise on the side of the villus by transformation of undifferentiated columnar cells (Porcella et al., 1979).

This scheme for the development of villus goblet cells has some similarities with the schemes for the development of respiratory goblet cells outlined earlier. It is possible therefore, that the pre-goblet or proliferative oligomucus cell of the small intestine epithelium has a counterpart in the respiratory epithelium which has yet to be identified and which may play a role in the development of respiratory goblet cells.

1:4 EXPERIMENTAL TECHNIQUES AVAILABLE FOR GOBLET CELL STUDY

1:41 Animal Models of Goblet Cell Hyperplasia

For various reasons it is necessary to resort to whole animal experiments in order to study the goblet cell in detail. Firstly, human material is scarce and cannot easily be obtained, and it is virtually impossible to obtain 'normal' specimens. Secondly, attempts at organ

culture using strips of tracheal or bronchial epithelium are complicated by the fact that the respiratory epithelium rapidly de-differentiates *in vitro* losing all its specialised structures including goblet cells (Heckman *et al.*, 1978).

An important consideration when choosing the species of animal for use as a model is the similarity between the animals respiratory mucosa structure and that of human respiratory mucosa structure. For example, human chronic bronchitis is characterised by increased sub-mucosal gland size and secretory activity and increased numbers of epithelial goblet cells. Since the hamster, mouse, rabbit, and guinea pig have very few sub-mucosal glands they are not as suitable for studies into the aetiology and pathogenesis of chronic bronchitis as animals which do contain sub-mucosal glands such as the rat, pig, lamb and dog.

The majority of studies in this field have used the rat as it provides a convenient model of hypersecretory disease states particularly as groups can be fairly large. In addition, due to its size, the whole of the left lung of the rat can be mounted in cross section in such a way as to reveal the entire length of the main airway and part or all of its side branches (Figure 3). It is therefore possible to ensure that the same anatomical area is used for quantification of the goblet cells in each rat used for the study. When larger animals are used, such as pigs, then the problem of sampling and orientation, which was

discussed in section 1:33, becomes much greater and calls for detailed dissection and careful handling of the tissue to ensure that the results from each animal are truly comparable.

1:42 Present Histological Techniques for Studies on Goblet Cells in the Respiratory Tract

Many previous quantitative studies on the lung goblet cell population have taken place using 5 µm thick paraffin sections stained with a mucin specific stain such as the combined Alcian Blue/Periodic Acid Schiff (AB/PAS) stain. The specificity of this stain precludes identification of other cell types as only those containing mucin show up. Application of counterstains is not helpful as they often mask the mucin staining itself. Other disadvantages of paraffin sections are the poor preservation of the lung tissue, with shrinkage being a particular problem, and the thickness of sections which often means that nuclei in the field of view are in different planes and do not always corresond to the layer of cells under study. Since in a study such as this it is necessary to be able to identify goblet cells quickly and reliably and to quantify them in a linear epithelium, these are serious drawbacks.

With the advent of plastic embedding of tissue and the resultant thinner sections, i.e. 1 - 1.5 µm thick, improved histology is now possible. There are, however, special

problems encountered with these techniques, particularly when dealing with the lung, and their effects on this study are discussed in detail later in the thesis (see Appendix v).

An automated counting system can be utilised to count goblet cells (Mawdesly-Thomas and Healey, 1969a), but such a technique was not available in this study. Accordingly goblet cells could only be counted by manual methods using the light microscope.

1:43 The Problem of Pulmonary Infection in Expermental Animals

The use of animals in experiments necessitates the implementation of careful controls in order to minimise the variables inherent in such experiments. In pulmonary studies one such variable is the presence of respiratory infection in the animal stock.

In healthy rats the lungs contain very small amounts of lymphoid tissue (Reid, 1970; Lamb, 1975). In the presence of chronic respiratory infection the amount of lymphoid tissue in the lungs increases and aggregates of lymphocytes around major airways ('cuffing') with invasion and destruction of the overlying epithelium in more severe cases, can be observed (Lamb, 1975). At this stage of the infective process the animals appear otherwise healthy and

routine microbiological screening for mycoplasma and Pasturella pneumotropica is usually negative.

A progressive clinically obvious decline in the colony may take several more months to develop and the lung pathology of animals from such a colony will show extension of the lymphocytes to involve the tissue around small blood vessels and airways in the periphery of the lung. This peripheral involvement may progress to frank pneumonitis and true inflammation of the alveolar portion of the lung (Lamb, 1975). Such changes are often more severe in older animals and the use of young animals in pulmonary studies can reduce the incidence of lung pathology.

Due to the effect on the bronchial epithelium, namely damage to the epithelium and the destruction of its normal structure, animals with moderate or severe lymphocyte cuffing around airways are unsuitable for a whole range of investigations into the structure of the respiratory epithelium.

Since histology is the most sensitive method of identifying the early stages of chronic respiratory disease in rats (Lindsey et al., 1971) every lung used for quantification in this study was checked for lymphocyte cuffing and any seriously affected animals were rejected from the study. Nevertheless, problems were encountered with respiratory infection during the course of this study and these are reported in detail in section 1 of Chapter 3.

1:5 INNERVATION OF THE RESPIRATORY EPITHELIUM

The innervation of the human lung consists of preganglionic para-sympathetic nerves arising from the vagus and post-ganglionic sympathetic nerves. The former relay in ganglia close to the tissue they innervate, whilst the latter synapse in the second, third, and fourth thoracic sympathetic ganglia. Sensory nerves which initiate most if not all respiratory reflexes travel in the vagus nerve (Spencer, 1977).

Jeffrey in 1973 demonstrated that both myelinated and non-myelinated sub-epithelial nerves were commonly found in the lamina propria of both the extra- and the intra-pulmonary airways in the rat. At no site did they cross the basement membrane and enter the epithelium. He did however find a mixed population of intra-epithelial axons; these were confined to extra-pulmonary airways and none were found in intra-pulmonary airways. These axons were associated with all epithelial cell types particularly basal cells. Approximately 33% of axons were efferent terminal motor adrenergic endings, 17% of axons were efferent cholinergic endings and 50% of axons were afferent sensory nerve endings. There are three main afferent pathways: from the pulmonary stretch receptors situated in airway smooth muscle; from lung irritant receptors situated in the mucosa of cartilagenous airways in particular the trachea; and from type-J receptors which

lie close to pulmonary capillaries in the interstitium of the inter-alveolar septa (Breeze and Wheeldon, 1977).

Substance P-like immunoreactive nerves have also been demonstrated in mammalian lung (Wharton et al., 1979). They were found in all parts of the lungs of guinea pigs, rats, mice and pigs, but none were found to be in the epithelium of airways. It is thought that they may form part of a complex system of regulation of lung function.

Parasympathetic nerves have been shown to enter sub-mucosal glands (Widdicombe, 1978; Bensch et al., 1965). The role of sympathetic nerves in the control of gland secretion is not yet understood although Meyrick et al. (1970) reported axons containing dense cored vesicles in human bronchial sub-mucosal glands. Both parasympathetic and sympathetic nerve stimulation cause an increase in mucin production in vivo in dogs and cats (Ueki et al., 1980; Peatfield et al., 1980; Gallagher et al., 1975; Davis et al., 1979). Intra-epithelial goblet cells have, however, been shown not to respond to either parasympathetic or sympathetic nerve stimulation (Gallagher et al., 1975; Last et al., 1980).

1:6 THE PHARMACOLOGY OF ISOPRENALINE

Isoprenaline is the most active of the sympathomimetic amines that act almost exclusively on β-adrenoceptors by virtue of their N-alkyl substitution (Innes, 1970).

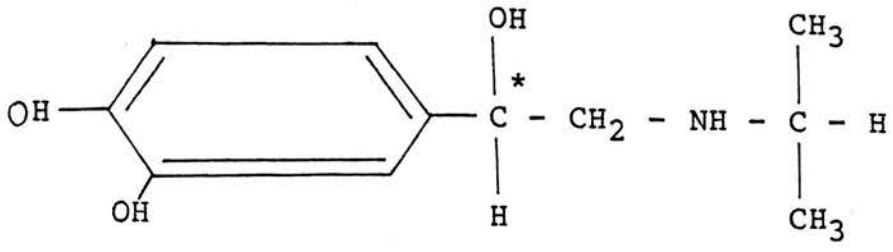


Figure 5. Isoprenaline Structure

1:61 The Structure of Isoprenaline

The asterisk denotes an asymmetric carbon atom, and because of this, like nor-adrenaline and adrenaline, isoprenaline has two optically active isomers. These were first resolved and investigated by Kerschbaum and Benedikt in 1952, and Beccari et al. in 1953. In dogs the levo-rotatory form of isoprenaline known as the (-) isomer has been shown to be approximately 90 times more potent as a β_2-adrenoceptor agonist decreasing arterial blood pressure than the (+) isomer (dextro-rotatory), and approximately twice as active as the racemic mixture (Beccari et al., 1953). In man, the difference in potency of the (-) isomer and (+) isomer is 50 times. This difference is probably due to different rates of racemisation in body fluids after administration. Virtually all pharmacological studies are performed using the racemic mixture, the usual form of the drug. It is both costly and difficult to isolate the pure forms of the two isomers and

as they are subject to spontaneous racemization they are only rarely used.

1:62 The Metabolism and Duration of Action of Isoprenaline

The duration of action and the mode of metabolism is very similar to that of adrenaline. In rats, isoprenaline given intravenously is rapidly O-methylated by catechol-O-methyl transferase (COMT) and removed from the blood stream by the liver where further conjugations may occur (Nilsson, 1974). In the liver, glucoronyl transferase acts on 3-O-methyl isoprenaline to produce isoprenaline glucoronide. Isoprenaline can also be conjugated with sulphate by various sulphatases, particularly in the gut wall and therefore it is largely inactive when given orally. Excretion occurs in the urine, the major metabolite in the dog and man being conjugated

COMT is not stereoselective with respect to the (-) and (+) isomers of isoprenaline (Garg et al., 1971; Guldberg and Marsden, 1975).

1:63 The Direct Pharmacological Actions of Isoprenaline

Isoprenaline has a powerful action on β-adrenoceptors and almost no action on ∝-adrenoceptors. Its main actions, therefore, are on the heart, the smooth muscles of

bronchi, skeletal muscle vasculature, and the alimentary tract.

Cardiovascular Effects of Isoprenaline: Intravenous infusion of isoprenaline in man lowers peripheral vascular resistance and diastolic pressure falls. It raises cardiac output by positive inotropic and chronotropic effects; in conventional doses the increase in cardiac output is sufficient to maintain or raise systolic pressure, but in higher doses its administration is followed by a striking fall in blood pressure.

Effects of Isoprenaline on Smooth Muscle: Isoprenaline relaxes almost all varieties of smooth muscle. When the resting tone is high this action is most pronounced on bronchial and gastrointestinal smooth muscle causing marked bronchodilation and decreased tone and motility of intestine musculature. On some muscles it has an additional α-adrenoceptor activity seen only with high doses, and it causes blanching of the skin when injected intradermally in man.

Effects of Isoprenaline on Metabolism and the Central Nervous System: Isoprenaline causes hyperglycaemia in man; this is not as pronounced as that which follows adrenaline and results from the stimulation of glycogenolysis. Isoprenaline also stimulates lipolysis and this results in increased levels of circulating free fatty acids. Like

adrenaline it can cause central excitation, but this has not been extensively studied.

1:64 The Indirect Actions of Isoprenaline

Reed (1978) discusses the physiology and pharmacology of β_2-adrenergic agents and lists all the known events occurring after either β_1- or β_2-adrenoceptor stimulation (see Table 2).

β-adrenoceptor stimulation is followed by a large number of wide ranging effects, biochemical, metabolic and mitotic. Those of particular interest to this project are the stimulation of mitosis, the increase in protein content of saliva, the increase in intra-cellular adenosine 3',5'-cyclic monophosphate and the calorigenic actions.

Isoprenaline has been shown to stimulate both hyperplasia and hypertrophy in salivary glands of rats (Barka, 1968; Selye et al., 1961) and mice (Baserga, 1966). This is a two-fold effect as in addition to stimulating mitosis in salivary glands it also increases the protein content of their secretions by altering the synthesis. An increase in cell proliferation has also been demonstrated in the mouse kidney by Malamud and Malt (1971) and in the rat lung by Bolduc and Reid (1978). In the lung, Bolduc and Reid showed that isoprenaline increased the mitotic index of the respiratory epithelium, they also found a smaller increase following pilocarpine administration. The subsequent

Table 2 The Physiological and Pharmacological Actions of Sympathomimetic Amines

Tissue	α	β
Heart	? weak inotropic	Inotropic and chronotropic
Smooth Muscle	Vasoconstriction, bronchoconstriction, uterine contraction, bladder sphincter constriction, GI tract inhibition	Vasodilation, bronchodilation, uterine relaxation, detrusor relaxation, GI tract inhibition
Skeletal Muscle	-	Facilitates neuromuscular transmission (tremor), increased strength of contraction
Glands	Increased renal tubular reabsorption of Na^+	Inhibits gastric acid secretion, increased protein concentration of saliva, increased mitosis of salivary glands, decreased tubular reabsorption of Na^+, renin production
Blood and Blood Cells	Eosinophilia, platelet aggregation (possibly more dopaminergic?)	Eosinopenia, lymphopenia and granulocytosis. Increased clotting factor VIII and fibrinogen. Kininase activation, decreased mediator release, decreased lysosomal enzyme release, decreased lymphokine production.
CNS	-	Arousal, insomnia, nervousness
Metabolism	Efflux of K^+; Glucagon release; growth hormone release; inhibition of insulin release; inhibition of lipolysis	Muscle glycogenolysis; hepatic glycogenolysis; inhibition of growth hormone release; insulin release; gluconeogenesis; lipolysis.
Fundamental Biochemical	Influx of C^{2+}, efflux of K^+, membrane depolarisation	Activation of adenylate cyclase leading to increased intracellular c-AMP. Membrane stabilisation
Mitosis	-	Inhibits epidermic thymocytes, stimulates salivary glands, stimulates mouse kidney (Malamud et al., 1971) No effect on hair follicle. Effects on the small intesine are equivocal (Shering et al., 1972)

increase in goblet cells was due, they felt, to increased cell division with a change in the pattern of differentiation rather than increase in secretory activity leading to an increase in cell division. The main reason for this conclusion is the evidence of Sturgess and Reid (1972) where they demonstrated that isoprenaline has no effect on the secretion of goblet cells in vitro.

CHAPTER 2

MATERIALS AND METHODS

2:1 MATERIALS USED IN THE STUDY

2:11 Animals

All the animals used were supplied by Imperial Chemical Industries P.L.C. and were Alderley Park Strain I rats, weight range 200 - 250 g. They were bred in strict SPF conditions and were free from overt respiratory disease. The animals were kept in a 12 hour light and dark cycle and fed ad libitum with standard rat diet and water. Experiments were carried out both in Edinburgh and at Alderley Park, the exact location being indicated in the experimental design section. Batches of rats were transported up to Edinburgh by road, the journey taking roughly 5 - 6 hours; they were then housed in the animal house and allowed one full week to acclimatise to their new surroundings before being used in an experiment.

2:12 Precautions Taken to Prevent Cross-Infection of Experimental Animals

The room in which the animals were housed was sprayed with disinfectant twice between each batch of animals, once immediately after the room was emptied and once immediately prior to reoccupation with a new batch of animals. Gowns

and masks were kept in the room and laboratory coats worn elsewhere in the building were removed outside the room and a gown and mask put on immediately upon entering the room. The animals were housed in plastic cages in which the sawdust bedding was changed daily to keep soiling to a minimum. During the acclimitisation period the rats were inspected daily and checked for signs of respiratory disease such as coughing, sneezing and nasal discharge. The number of people allowed to enter the room was strictly limited and the animals were taken from the room only for post-mortem purposes or for exposure to sulphur dioxide gas. Animals which had been dosed with drugs or exposed to sulphur dioxide gas were housed in racks separated from the control rats and those yet to be dosed. Surgical gloves were worn whenever it was necessary to handle the animals. Food and water were dispensed from a bin and taps in the room itself and not brought in daily from the animal house store.

2:13 Drugs Used in the Study

dl-isoprenaline sulphate (rac emic). Sigma, London.

Levo (-) - isoprenaline d-bitartate. Aldrich Chemicals, Dorset.

Dextro (+) - isoprenaline d-bitartate. Aldrich Chemicals, Dorset.

Salbutamol sulphate. Glaxo, Herts.

Methoxamine hydrochloride. Wellcome, London.

Phenylephrine. Sigma, London.

Pilocarpine. Sigma, London.

Nicotine alkaloid. Sigma, London.

Terbutaline. Astra.

Clenbuterol. ICI, Cheshire.

Carbachol. Koch Light, Bucks.

Propranolol. ICI, Cheshire

SO_2 cylinders. BDH.

Pentobarbitone. May and Baker, Dagenham.

2:14 Fixatives and Reagents Used in the Study

All reagents used were of analar quality.

(a) Fixation for Light Microscopy

37 - 40% Formaldehyde (Tech. grade)

Sodium dihydrogenorthophosphate

Sodium hydroxide

(b) Fixation for Electron Microscopy

EM grade glutaraldehyde

Sodium cacodylate

Hydrochloric acid

Osmium tetroxide

2:15 Embedding Media and Reagents

(a) Reagents for Glycolmethacrylate Embedding

2-hydroxyethyl methacrylate

2-butoxyethanol

Benzoyl peroxide

Polyethylene glycol 400

N.N. dimethylaniline

(b) Reagents for Araldite Embedding

Araldite resin Cy212

DDSA

Dibutyl phthalate

HY960

2:2 METHODS - EXPERIMENTAL TECHNIQUES EMPLOYED IN THE STUDY

2:21 Isoprenaline Formulation

In all cases, with the exception of the intratracheal and nebuliser routes of administration (see section 2:24), the isoprenaline rac emate and isomers were administered intraperitoneally (IP) in 0.5 ml sterile physiological saline. The drug solutions were prepared immediately prior to use and consequently did not require the addition of preservatives or anti-oxidants. Control animals in each case received 0.5 ml sterile physiological saline IP.

2:22 Other Drug Formulations

Salbutamol, terbutaline, methoxamine, phenylephrine, pilocarpine, carbachol, nicotine, and propranolol were all administered IP in 0.5 ml sterile physiological saline.

Clenbuterol was administered IP in 0.5 ml of polyethylene glycol 400 (PEG400) diluted 3:1 with distilled water. Where necessary solutions were warmed under the hot tap to facilitate solution of the compound.

2:23 Technique Employed for the Exposure of Groups of Rats to Sulphur Dioxide Gas

The technique employed was the same as that first described by Reid in 1963. The apparatus used was in fact similar to that which had been used by Reid (1963), Lamb and Reid (1968) and Lamb (1968) at St George's Hospital.

The apparatus consisted of three perspex chambers into which a mixture of sulphur dioxide gas and air was pumped. The chambers were vented to the outside, the used air and gas mixture being first scrubbed through a water column to dissolve out most of the sulphur dioxide gas. A vacuum cleaner working in reverse was used to supply the flow of air and the sulphur dioxide gas was added to this at the rate of 15 to 20 cc/min. This flow rate of sulphur dioxide was sufficient to give a concentration of the gas of approximately 400 - 500 ppm. During an exposure run the concentration in each chamber was checked at hourly intervals using Dreger tubes and the flow of sulphur dioxide gas was altered whenever necessary to maintain the concentration at 500 ppm. Up to 5 rats could be housed in

each chamber and thus a total of 15 adult rats could be exposed to sulphur dioxide simultaneously.

2:24 Nebulised and Intratracheal Routes

Intratracheal administration of drugs was done under anaesthesia. The animal was placed on its back with the neck extended over a support, the tongue was held up away from the back of the mouth and a long curved wide-bore blunt needle was inserted into the trachea. A change in the breathing pattern usually accompanied entrance into the trachea. The needle was sufficiently long to travel down as far as the carina. The dose administered was calculated thus:

Volume administered was 0.4 µl/g body weight, and therefore a 250 g rat received 100 ul of drug solution containing a total of 20 mg of drug. Once injected the animals sometimes coughed or gasped and were then placed on their left side to recover from the anaesthetic. The anaesthetic used was Sagatal (sodium phenobarbitrate, 60 mg/ml), this was diluted 1:10 in 10% ethanol and administered IP in a dose of 0.75 ml/100 g body weight.

For the nebulised route of administration, the aerosol was produced using a Wright's Nebuliser and generated by saturated compressed air flowing at 6 l/min. The aerosol mist so produced passed directly into a perspex chamber at a rate of 10.5 l/min. Rats were held in position along the

sides of the chamber (5 at a time), by means of perspex cylinders which screwed into holes in the chamber. The head of each animal was thus forced through a thin rubber seal and in this way snout only exposure was achieved. The apparatus used was set up at the ICI Central Toxicology Laboratories at Alderley Park and had been in constant use for some time and was therefore a tried and tested system.

The atmosphere generated in the chamber was sampled in two ways whilst each drug solution was being tested. The first method of sampling was the total atmosphere sampler (TAS); this consisted of a holder containing a single preweighed Metricel 1.2 μm pore 25 mm diameter filter connected to a vacuum pump which drew air from the chamber through the filter at a constant rate of 2 l/min for up to 15 mins. The wet weight of the filter was quickly recorded and the filter was then dried in a dessicator under a slight vacuum overnight and reweighed. The amount of solid deposited on the filter could then be calculated and by extrapolation it was possible to obtain an indication of amount of drug present in the chamber at any one time.

The second method of sampling was rather more sophisticated and gave a more reliable indication of how much respirable compound was being generated. The Anderson 4 stage impactor was made of aluminium and consisted of 4 impactor plates with pores of known size and a single Metricel 2.5 μm pore 25 mm diameter filter. The aluminium plates were designed to allow particles of <6 μm in

diameter through into the sampler. Plate 1 trapped particles >5.5 μm diameter, plate 2 trapped particles 5.5 - 3.5 μm diameter, plate 3 trapped, particles 3.5 - 2 μm diameter, plate 4 trapped particles 2 - 0.3 μm diameter and finally the filter trapped particles less than 0.3 μm. The atmosphere was sampled at the rate of 1.4 l/min.

The deposition characteristics of aerodynamic particles is very complex and has only been roughly worked out; however, it is generally believed that, for particles >5 μm diameter, 50% is deposited in the nose and the rest in the upper respiratory tract, i.e. trachea, pharynx and larynx. For particles <5 μm diameter approximately 20% is deposited in the nose, up to 50% in the upper respiratory tract and the remaining 30% in the lower respiratory tract - bronchi and bronchioles. The maximum deposition in the lung possible appears to be about 70% and occurs with particles about 0.5 μm diameter. By weighing the various plates before and after exposure it was possible to calculate approximately the amount of drug administered to the animals in respirable form and the most likely site of maximum deposition.

2:3 METHODS - HISTOLOGICAL TECHNIQUES EMPLOYED IN THE STUDY

2:31 Fixation for Light Microscopy Using Glycol-methacrylate (GMA)

Millonig's phosphate buffered formaldehyde (modified by Carson et al., 1972) was used and this was made up as follows:

40 ml 37.40% technical grade formaldehyde
360 ml distilled water
7.44 g NaH_2PO_4
1.65 g NaOH

The NaOH and NaH_2PO_4 were dissolved in the water and the solution allowed to cool. The formaldehyde was then added and the fixative stored in an airtight container until required. A fresh batch was made up for each experiment.

2:32 Fixation for Light and Electron Microscopy Using Araldite

The materials and fixative used were exactly the same as those used for GMA. However, once the material had been fixed for a minimum period of 24 hours it was post-fixed in osmium tetroxide as follows:

Processing: Solution composition.

Cacodylate Buffer 0.2M pH 7.4

42.8 g sodium cacodylate

6.9 ml N HCl

993.1 ml distilled water

0.5% Osmium Tetroxide

0.25 g OsO_4

25 ml distilled water

25 ml 0.2M cacodylate buffer

Method:

i) Place trimmed, fixed tissue in 0.2M cacodylate buffer and leave for 1 hour at 4°C.

ii) Replace the cacodylate buffer with 0.5% OsO_4 and leave for 3 hours at 4°C.

iii) Wash tissue twice in 10% alcohol, each time for 30 mins at 4°C, the tissue is then ready for processing through graded alcohols.

2:33 Technique for Inflation Fixation of Intact Lungs

The apparatus used is shown overleaf. This was adapted from that used by Fawell and Lewis (1971). The apparatus was designed to deliver the fixative at a constant pressure to the lungs at all times. It did this by maintaining a constant head of fixative 20 cms above the lungs. As soon as the Spencer wells were removed fixative

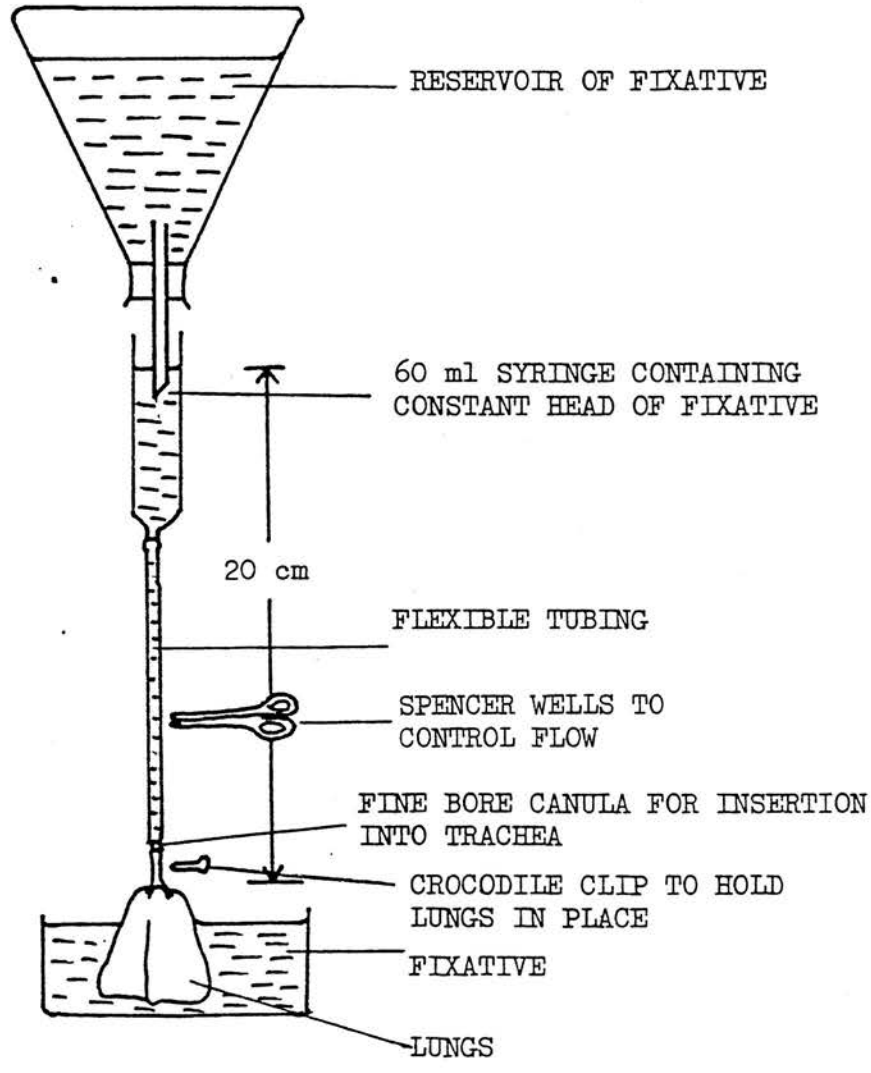


FIGURE 6

Apparatus used for the inflation-fixation of rat lungs (Adapted from Fawell and Lewis, 1971).

flowed down into the lungs from the syringe, simultaneously fixative flowed from the reservoir at the same rate thus maintaining a constant 20 cm level in the syringe. When the lungs were fully inflated back pressure stopped the flow of fixative and the Spencer wells were replaced. The trachea was then tied securely to prevent leakage and the lungs removed and placed in a histology jar filled with fresh fixative. The use of this apparatus meant that each time it was possible to reproduce the same conditions for inflation fixation and prevented the lungs from being over-inflated. It also speeded up the dissection time by allowing the experimentor to start on the next animal whilst the previous lungs were being inflated.

2:34 Technique Employed to Trim Blocks of Lung Ready for Embedding in Plastic

The left lung was used for the assessment of goblet cells using the light microscope in all but a few experiments. The left lung was separated from the trachea at the carina and placed on a board with the ventral surface uppermost. The tissue had previously been fixed for a minimum period of 24 hours. Using a dermatome knife the top 2 - 3 mm of the lung tissue were sliced off using a single horizontal stroke of the knife. With practice it was possible to judge the depth of this cut such that the top surface of the main airway and its branches can just be seen in the lung. A sawing action of the blade was avoided

as this leads to the cut surface being uneven and which in turn leads to problems when trimming the block ready for section cutting. Care was taken not to cut too deeply because the further in the tissue is trimmed at this stage, the less material is available for trimming-in and orientation of the block when cutting the section.

The technique employed for trimming the tissue for both glycolmethacrylate and araldite embedding was basically the same. However, lungs destined for araldite were trimmed to give a narrow block of tissue consisting of the entire length of the main airway and just the start of each of its side branches. Blocks destined for glycol-methacrylate consisted of the entire width of the lung and thus incorporated the main airway and all of its side branches right out to the periphery of the lung. To facilitate penetration of the resin the pleural surface on the dorsal surface of the lung was also sliced off. The trimmed block was then placed in a perforated metal cassette along with its label ready for processing in graded alcohols.

2:35 Processing of Fixed Tissue for Light Microscopic Studies in Glycolmethacrylate and Araldite

The fixed, trimmed material was dehydrated in graded alcohols over a period of 24 hours at room temperature. The sequence of changes was 2 x 10% alcohol, 2 x 30% alcohol, 2 x 50% alcohol, 1 x 70% alcohol, 2 x 90% alcohol

and 2 x absolute alcohol. This procedure was followed for material destined for either glycolmethacrylate or araldite embedding.

2:36 Impregnation and Embedding of Tissue in Resin

(a) Methodology for GMA Embedded Tissue - Adapted from Sims (1974)

Impregnation and embedding: solution composition.

GMA solution A (infiltrating solution)

80 ml 2-hydroxyethyl methacrylate (monomers)
8 ml 2-butoxyethanol
0.5 g benzoyl peroxide
(Prepare just before use)

GMA solution B (promotor solution)

8 ml polyethylene glycol 400
1 ml N.N. dimethylaniline
(Prepare just before use)

GMA working solution (polymerising solution)

42 parts solution A
1 part solution B
(Mix thoroughly and use immediately)

Impregnation: method.

i) Remove tissue from last change of absolute alcohol. Place in the first change of GMA solution A. No link reagent required. Leave for 24 hours at room temperature under 27 ins Hg vacuum.

ii) Replace tissue in fresh GMA solution A. Leave for 12 hours at room temperature under vacuum, then change GMA solution A once more and leave for a further 12 hours under vacuum.

iii) Before carrying out polymerisation make test working solution, i.e. 43 ml and check that polymerisation occurs controllably.

iv) Make up sufficient polymerisation solution for whole batch to be processed.

v) Place tissue in polymerisation solution and agitate thoroughly using a roller-mix. Usually 10 - 15 mins is possible before the solution starts to thicken.

vi) Immediately the polymerising solution shows signs of thickening transfer the tissue blocks, cutting face down, to fresh polymerising solution in disposable flat bottomed moulds.

vii) Once the reaction is under way and heat is being generated place the moulds on ice to dissipate the heat and prevent gas bubbles forming in the blocks.

viii) Once blocks are hard, remove from the moulds. Trim away any excess soft resin and place any soft blocks in an incubator at 60°C to harden.

ix) Trim blocks to a convenient size for cutting using a hacksaw and a surform plane.

(b) Methodology for Araldite Embedded Material

Impregnation and embedding: solution composition.

Araldite Solution A

200 ml resin (CY212)
200 ml hardener (DDA)
(Mix well and store at 4°C)

Araldite Solution B

20 ml dituty1 phthalate (plasticizer)
1 ml accelerator (HY960)
(Mix well and store at 4°C)

Araldite Working Solution

19 ml Solution A

1 ml Solution B

(Mix well and store at 4°C)

Impregnation: method.

i) Remove tissue from last change of absolute alcohol and place in epoxypropane. Leave for 1 hour at room temperature (epoxypropane is used as a link reagent between the alcohol and araldite).

ii) Place tissue in 10 - 15 ml of araldite working solution, taking care not to desiccate the tissue. Put under 27 ins Hg vacuum for 24 hours at room temperature.

iii) Change the resin and leave under 27 ins Hg vacuum for a further 6 - 12 hours.

iv) Put 7 - 10 ml of fresh araldite working solution in the bottom of a rigid ice cube mould tray and place the tissue, cutting face down, in the araldite.

v) Cure blocks at 60°C for one week or until hard. During this time the blocks can be sliced to a convenient size and shape (approximately 3 days)

whilst they are still soft, otherwise they require sawing.

2:37 Technique Employed for Cutting Large, Thin, Plastic Sections of Lung

All the blocks, i.e. both araldite and glycolmethacrylate were trimmed-in and orientated using a Reichart-Jung Autocut Microtome. The knife used for this had a tungsten-carbide coated cutting edge and was capable of trimming both araldite and glycolmethacrylate blocks at 15 - 20 μm thickness. Once the block had been faced-up and the airway began to appear in the sections the knife was changed. For glycolmethacrylate blocks a standard sharp D-profile steel knife was used and 1.5 μm sections were obtained in which the entire left lung of the rat was present. For araldite blocks a glass knife was used. This had a blade width of 12.5 mm and was therefore capable of cutting blocks up to a maximum width of 10 mm, hence the additional trimming required prior to processing and embedding the tissue. Using this knife the autocut was capable of cutting 1.5 μm araldite sections approximately 1 cm wide and 1.5 cm long. The quality of sections thus obtained was poor and of very limited use. Hence the use of glycolmethacrylate as the embedding resin for the light microscopic assessment of goblet cells throughout the study.

2:38 Staining Methods for Glycolmethacrylate and Araldite Sections for Light Microscopy

(a) Staining Methods for GMA Sections

i) Alcian Blue/Periodic Acid Schiff (AB/PAS)

1% alcian blue in 3.0% acetic acid, pH 2.5

1% periodic acid Schiff reagent prepared using "Lillie Cold Method" (Lillie, 1965), prepared as follows:

For 500 mls:

5 g aldehyde fuchsin

9.5 g sodium metabisulphite

500 ml 0.15 N HCl

(Shake solution thoroughly until straw coloured; can be left overnight. Filter through activated charcoal until decolourised. Store at 4°C or freeze until required)

Procedure:

Stain sections with Alcian Blue for 15 - 20 mins at room temperature. Rinse and dry in air. Treat sections for 5 mins with 1% periodic acid. Rinse in tap water, then distilled water. Dry in air. Place sections in Schiff reagent for 20 - 25 mins at room temperature. Wash sections for

5 mins in running tap water to bring up staining. Dry sections in air.

ii) Solochromecyanin/Eosin (Hogg and Simpson, 1975).

90 ml of distilled water containing 10 ml 4% ammonium ferric sulphate
0.5 ml concentrated sulphuric acid
0.2 g solochrome cyanine R.S.

Dissolve the dye in the concentrated sulphuric acid, add the distilled water and then the ferric ammonium sulphate. Mix thoroughly then filter, store at room temperature.

The eosin is made up in distilled water as a 1% solution to which is added:

1 ml of 2.5% calcium chloride
1 ml of 2% phloxine

The fluid volume is approximately 500 ml. Filter and store at room temperature.

Stain sections with solochromecyanin for 3 - 4 mins at room temperature. Rinse quickly in tap water and differentiate briefly in 1% acid alcohol put sections into Scott's tap water for 30 - 60 secs: (1 litre of tap water containing 20 g $MgSO_4$, 3.5 g $Na_2(CO_3)$ and 1 crystal of

thymol). Counterstain sections in eosin for 20 - 30 secs. Rinse briefly in water and dehydrate sections through graded alcohols to xylene prior to mounting.

iii) Bullard's Haematoxylin and Eosin.

Bullard's haematoxylin:

8 g haematoxylin
16 ml glacial acetic acid
144 ml 50% ethanol
20 g aluminium ammonium sulphate
250 ml distilled water
8 g red mercuric oxide
275 ml 95% ethanol
330 ml glycerol
18 ml glacial acetic acid
40 g aluminium ammonium sulphate

Preparation:

Dissolve haematoxylin in 144 ml 50% ethanol. Add 16 ml glacial acetic acid. Add to this a heated solution of 20 g ammonia alum dissolved in 250 ml H_2O. Heat mixture to boiling and add slowly (to prevent frothing) 8 g red mercuric oxide. Cool rapidly and filter. Add 275 ml of 95% ethanol, 330 ml of glycerol, 18 ml glacial acetic acid, and finally 40 g of ammonia

alum. Mix thoroughly and store at room temperature.

Method:

Stain sections in Bullard's haematoxylin at room temperature for 5 mins. Wash briefly in water. Differentiate sections in 1% acid alcohol and blue in Scott's tap water (see solochrome cyanin method) for 30 - 60 secs. Counterstain sections in 1% eosin for 20 - 30 secs (see also solochromecyanin method). Rinse briefly in water and dehydrate sections through graded alcohols to cylene prior to mounting.

(b) Staining Methods for Araldite Sections

i) Toluidine Blue.

1% solution in 1% Borax or 1% solution in 1% Na_2CO_3. Staining carried out at 60°C for 30 - 60 secs on the slide using a hot plate or 2 - 3 mins in a water bath at 60°C.

2:39 Mounting Stained Sections

(a) GMA sections.

All GMA slides were mounted in DePeX.

(b) Araldite sections.

All Araldite setions were mounted in Harleco coverbond, (xylene based).

2:4 QUANTITATION OF GOBLET CELLS

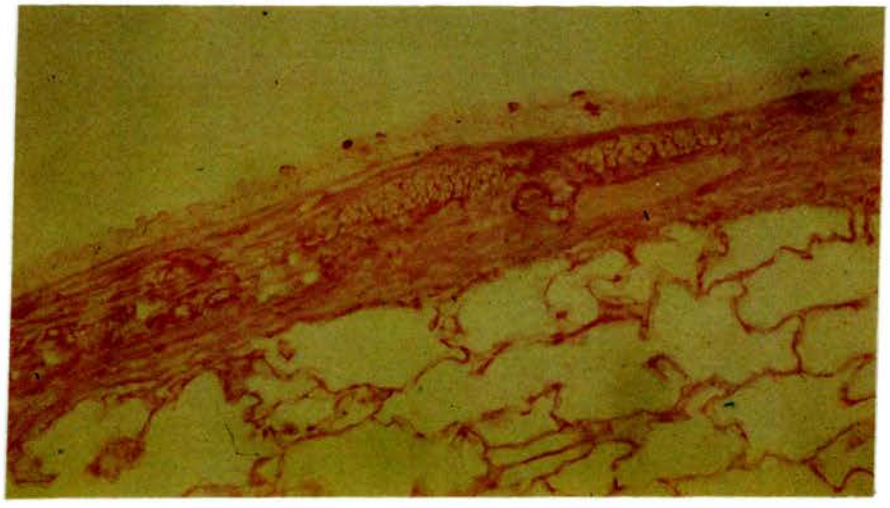
2:41 Criteria for Identification and Quantitation

It has been stated previously that the distribution of goblet cells in the lung varies continuously from proximal to peripheral airways. In addition increased numbers of goblet cells occur at the points of bifurcations of airways. It is therefore essential in any quantitative study on respiratory epithelial goblet cels that the same area is selected for assessment each time in every rat included in the study. In order to do this histological sections must encompass the whole of the longitudinal section of the main airway and part or all of the side branches arising from it. The side branches enable one to divide the different regions of the main airway into zones (Figure 3). In addition to this requirement it is essential that the goblet cells stand out from the rest of the epithelial cells, and therefore, the sections have to be stained selectively for mucin glycoproteins with stains such as AB/PAS.

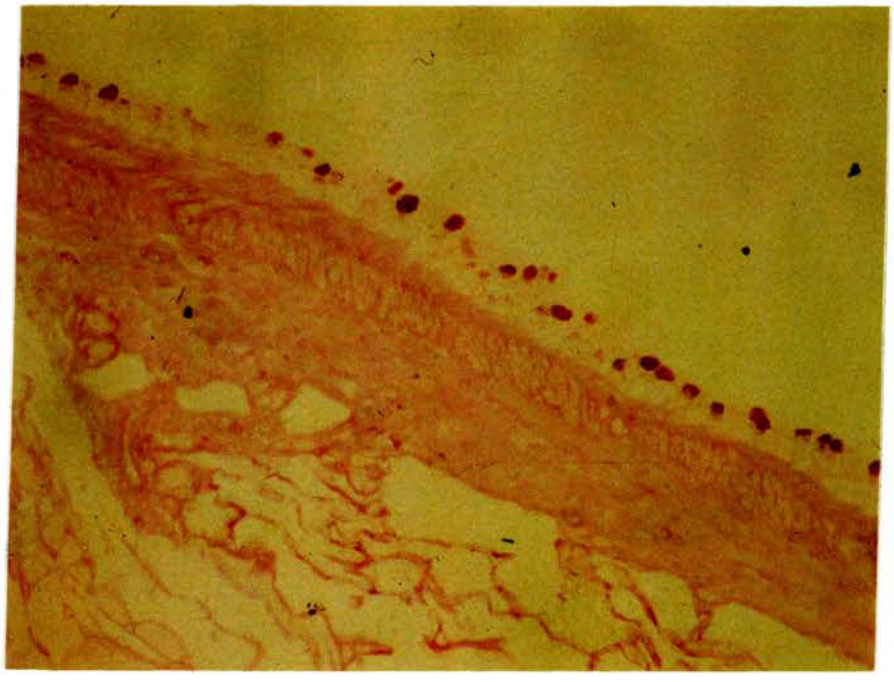
Examples of different staining methods on rat resiratory epithelium are shown in Figures 7, 8 and 9. In

FIGURE 7

1.5 µm sections of rat lung embedded in GMA. AB/PAS staining. x25 magnification. a) Control lung, b) after 40 mg/kg isoprenaline showing an increase in PAS positive intra-epithelial goblet cells.



(a)

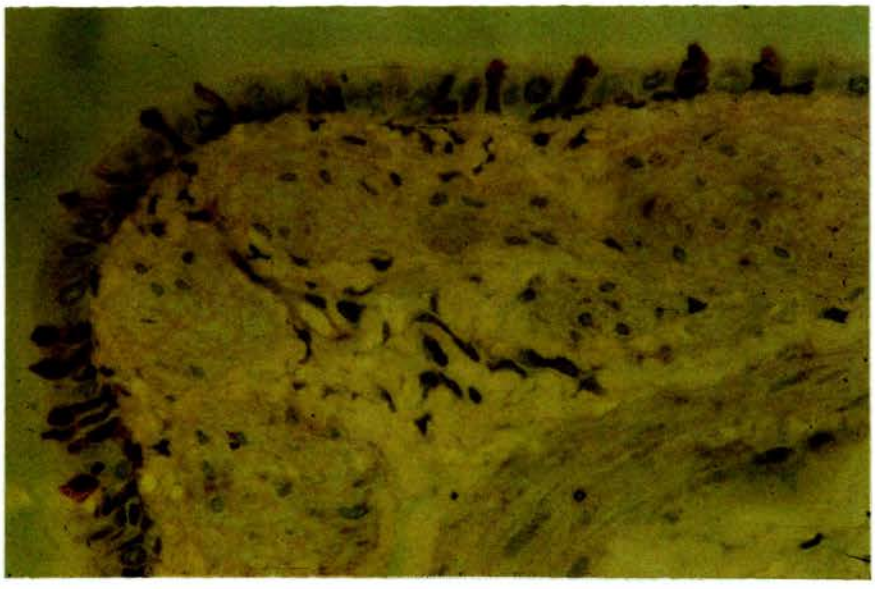


(b)

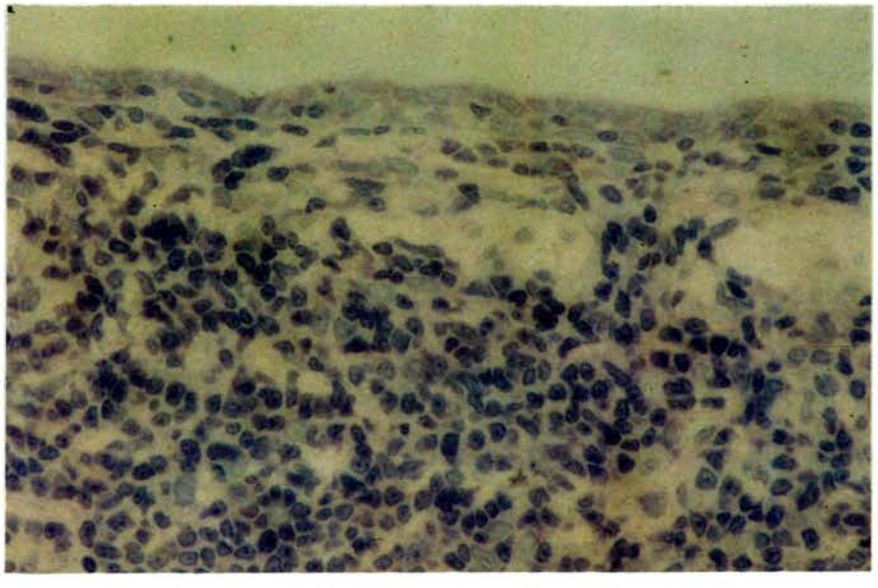
FIGURE 7.

FIGURE 8

As for Figure 7 but stain used was PAS/TB and magnification = x40. a) shows goblet cell staining, note high background staining due to the GMA taking up the Toluidine Blue, b) shows the effect on the epithelium of sub-epithelial lymphocytes: loss of goblet cells and flattening of epithelium.



(a)



(b)

FIGURE 8.

FIGURE 9

As for Figure 7 but stain used was Solochrome cyanin/ eosin. This was the stain routinely used to screen the lung pathology checking for evidence of respiratory infection.

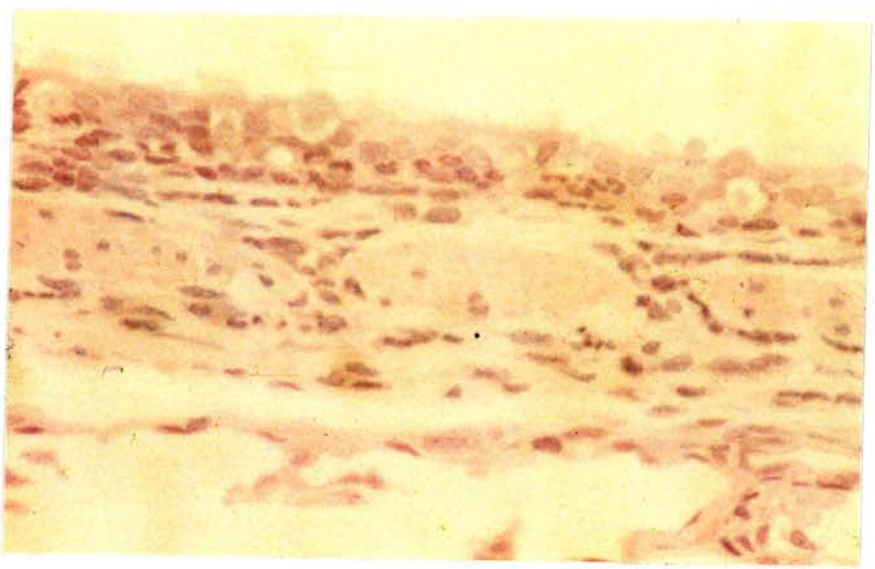


FIGURE 9.

sections stained with AB/PAS goblet cells are easily distinguished by their prominent AB/PAS positive theca.

Occasionally small clusters of PAS-positive granules were observed at the apex of cells. These were not included as goblet cells in this study, neither were cells with a few supranuclear PAS-positive granules as they may have contained glycogen or may have been lysozomes. Thus, only obvious mucous filled cells were counted as goblet cells and no attempt was made to distinguish immature sparsely filled goblet cells at the light microscope level.

2:42 Methodology for Counting Goblet Cells in the Lung

As the respiratory epithelium of the rat appears as a linear single layer of cells in 1.5 μm sections, goblet cells can be quantified as the mean number of goblet cells per unit length of epithelium which for convenience is generally one Higher Power Field (HPF) using a x40 objective. In this study this was equivalent to 0.35 mm of epithelium, and all the results are expressed as the mean number of goblet cells per 0.35 mm epithelium. As a general rule 10 consecutive HPF's were counted on each side of the airway in approximately the same position in zones 1 and 2.

The two results for each slide expressed as the number of goblet cells per HPF were then averaged to give a single result. This was then used to calculate the group

mean number of goblet cells per HPF. Thus the results obtained looked like this:-

SLIDE NO.	Goblets/10 HPF Straight	Goblets/10 HPF Branched	Average Goblets/HPF	Group Mean Goblets/HPF
Rat 1	40	44	$\frac{4.0 + 4.4}{2} = 4.2$	$\frac{4.2 + 4.0 + \ldots 4.1}{10}$ (no in group)
Rat 2	38	42	$\frac{3.8 + 4.2}{2} = 4.0$	
.	.	.	.	
.	.	.	.	
.	.	.	.	
Rat 10	36	46	$\frac{3.6 + 4.6}{2} = 4.1$	

As stated previously there are pronounced clusters of goblet cells at the points of bifurcation and in order to keep the two sides of the epithelium as comparable as possible counting on the branched side of the airway omitted the extreme points of bifurcation. Sections in which the epithelium in question had been cut tangentially were also excluded from the results. In any study such as this it is important to eliminate observer bias from the results as much as is possible. It was therefore necessary to randomise all the slides before counting and not break the code until counting had been completed. In this way it was not known to which treatment group any slide belonged to until counting was complete, and thus observer bias was eliminated from the results.

2:43 Assessment of Reproducibility

Having chosen the criteria for identifying goblet cells it was necessary to test them and see if they could be applied consistently enough to give reproducible results using 1.5 um GMA sections stained with AB/PAS.

Ten slides were selected at random for counting from a batch that had previously been randomised and counted; it was not therefore known to which groups the slides belonged, i.e. treated or control. Each slide was counted 5 times on separate occasions with either 2 or 3 days between each counting session. The results of the previous counts were put away and not referred to again until all 5 counts had been made, thus previous results were not allowed to influence the count in progress at any time. The results are shown in Chapter 3 section 2.

2:5 METHODS - STATISTICAL

In most experiments, group means were calculated together with standard deviations and errors. Occasionally the groups were so small that only a mean was obtained. In larger experiments, e.g. 5 or more animals to a group, it was possible to apply statisical analysis in order to test the significance of the results obtained. In these cases the Student's t-test was applied to find out whether the difference between the treated group and the control group was real or apparent. Only those results giving a P value

of less than 0.05 were considered significantly different from controls. All results which were significant are marked with an asterisk on the graph and the relevant P value is written alongside or given in the legend. Further details of the statisical methods employed are given in the Appendix vi at the end of the thesis.

CHAPTER 3

VALIDATION OF TECHNIQUES

3:1 QUALITY CONTROL OF ANIMALS

When the present study started the lungs from the ICI rats on gross examination were disease-free. On histological section this was confirmed by the lack of lymphocytic nodules, and very consistent control goblet cell numbers from one batch of rats to the next (Table 3). However, during the mid-part of the second year of the study there was an outbreak of Sendei virus within the colony at Alderley Park. This is a respiratory virus which can prove fatal and causes chronic inflammatory changes in the lungs of affected rats. The most noticeable change is a marked increase in both the size and numbers of lymphocyte aggregates many of which surround the airway to form a cuff of lymphocytes. In fact, the incidence of lymphocyte cuffing is a particularly good pointer towards the presence of a viral infection in the lung (Lamb, 1975).

It was at this time that the first investigation into the timing of the response (experiment 3, section 4:3) was carried out. The marked increase in control goblet cell numbers in this batch of rats was pointed out to ICI and shortly after this Sendei was confirmed in the colony and the breeding unit was shut down for six months. As a

result, no ICI rats were available for experiments. Enquiries to other animal suppliers revealed that Sendei was endemic in nearly all their colonies, and the few examples looked at were rejected on the grounds of too high an incidence of lymphocyte cuffing.

After this experience a check was made on control goblet cell numbers in all the batches of animals used and prior to goblet cell quantitation in the left lung H and E sections were screened and any diseased rats rejected from the study. Animals were considered to be diseased if more than 10% of the main airways was involved with lymphocyte infiltration. In addition, the presence of lymphocyte cuffing in zones 1 and 2, the areas to be counted, was also considered grounds for rejection even if the total area involved constituted less than 10% of the main airway. Another sign of possible infection was unusually large numbers of free macrophages within the alveolar spaces; whenever this occurred the animal was also rejected from the study even though the proximal airway epithelium appeared to be normal. Finally, increased numbers of intraepithelial globule leucocytes and sub-epithelial mast cells were considered to be indicative of abnormality and animals showing this were also rejected from the study.

It was essential to look at all the lungs in each experiment, otherwise isolated phenomena were missed. Occasionally, it was necessary to reject one whole cage of

animals, although usually it was only necessary to reject one or two out of approximately 100 animals.

As stated earlier in section 1:43, the presence of lymphocyte cuffing has a profound effect on the structure of the overlying respiratory epithelium. The cells become cuboidal in shape and there is a loss of ciliated and goblet cells. In areas of epithelium adjacent to these thus affected, there is often a marked increase in the number of goblet cells. Thus, not only is the area of epithelium lying directly over a lymphocyte aggregate affected but the area adjacent to it is also affected and shows an abnormal structure. Obviously animals thus affected cannot be used in a quantitative study such as this and hence the criteria for rejection which were applied.

Table 3 shows a series of observations made throughout the study. Column one shows the date on which the batch of animals was received. Column two gives the number of lungs examined for the presence of lymphocyte cuffing. Column three shows the degree of lymphocyte cuffing graded as follows:- $\pm$ = <10%, + = 10-20%, ++ = 20-30% and +++ = >30%. Column four gives the mean number of control goblet cells per 0.35 mm epithelium, and the mean number of goblet cels per 0.35 mm epithelium observed 24 hours after a single IP dose of 40 mg/kg isoprenaline. The final column gives the percentage change from control induced by 40mg/kg isoprenaline IP. It can be seen that there are no

TABLE 3 THE VARIATION IN CONTROL AIRWAY GOBLET CELLS 1977-1980

Date of Animal Batch	No of Lungs Examined	Degree of Lymphocyte Cuffing	Mean Cell Count (mean ± SE) Control	Mean Cell Count (mean ± SE) 40 mg/kg isop (24 hrs)	% Increase from Control
Sept '77	20	±	2.3 ± 0.3	6.6 ± 1.0	287#
Feb '78	18	±	2.5 ± 0.4	6.0 ± 0.65	236
July '78	17	±	4.4 ± 0.5	-	-
Oct '78 }	26	+	5.8 ± 0.5	-	-
Oct '78 }			4.4 ± 0.5	-	-
Sendei confirmed in breeding colony.					
Post-Sendei					
Feb '79	14	+	2.1 ± 0.3	6.1 ± 1.0	291*
March '79	18	±	1.2 ± 0.3	4.9 ± 0.6	408*
April '79	12	±	2.5 ± 0.3	8.8 ± 1.9	352
May '79	26	+	2.2 ± 0.3	5.7 ± 0.8	259**
May '79	8	+++	3.0 ± 0.5	-	-
May '79	7	+++	3.5 ± 0.9	Nebuliser	-
July '79	13	±	3.8 ± 0.7	8.5 ± 0.7	224
March '80 }	20	±	2.5 ± 0.3	7.4 ± 0.6	296
March '80 }			4.1 ± 0.7	Transtracheal	
April '80 }	10	±	2.9 ± 0.5	8.4 ± 0.7	290##
April '80 }			2.2 ± 0.3	-	-

\# = 3.7 mg/kg isoprenaline daily for 10 days
= 10 mg/rat isoprenaline intratracheally
* = 22 hours
** = 21 hours

obvious relationships between the incidence of lymphocyte cuffing, the magnitude of the response to 40 mg/kg isoprenaline nor the control goblet cell number. What is striking, however, is the wide variation in all three parameters in the period immediately after the Sendei infection. It was not until a year later that the control goblet cell numbers and the isoprenaline response resumed their pre-Sendei values.

To summarise, the effects of respiratory infection on the present study were as follows:-

a) Increased incidence of lymphocyte cuffing.

b) Increased control goblet cell numbers

c) Increased within-group variability as reflected in larger standard errors.

d) Diminution of the isoprenaline response.

To overcome the increased within-group variability and also to allow for what, at times, became a high rejection rate of animals from the study, groups were kept large, i.e. 10 treated and 20 controls, and thus some experiments involved over 100 animals. The large numbers of rats and the need to check each lung prior to quantification meant a great deal of extra work and this prolonged the amount of practical work required, particularly as the experiment immediately prior to the Sendei outbreak had had to be

repeated once the virus had been eliminated from the colony at Alderley Park.

3:2 ASSESSMENT OF REPRODUCIBILITY OF GOBLET CELL COUNTING

The results of the reproducibility study described in section 2:43 are shown overleaf in Table 4 and Figures 10 and 11. Table 4 shows the individual results of each count with means, standard deviations and standard errors for each slide. In all cases the mean of the five counts for each slide had a standard error of less than 1 and only one slide had a standard deviation of greater than 1. Thus, it was possible to count the same slide 5 times and reach a goblet cell count which generally varied by less than 1 goblet cell from the mean.

Figures 10 and 11 show the same results in a slightly different way. Using the data generated the upper and lower limits of the mean at the 95% confidence level were calculated, thus in the graphs the horizontal bars through the mean show the 95% upper and lower confidence limits. Whereas the standard error of the mean indicates how good an estimate of the mean of the whole population the calculated mean actually is, the 95% confidence limits indicate that there is a 95% certainty of the population mean being within the range shown (Ridgman, 1975). Thus, with one exception all the means assessed were within $\pm$ one goblet cell or less of the 95% confidence range. This is very encouraging as it shows that within the area chosen to

TABLE 4

RESULTS OF REPRODUCIBILITY COUNTS OF 10 GMA SLIDES

COUNTED 5 TIMES

Slide	Results for Straight Side			Results for Branched Side		
	Mean	SD	SE	Mean	SD	SE
88	2.5	0.25	0.11	4.6	0.27	0.12
12629	1.8	0.24	0.11	2.9	0.17	0.17
14314	1.2	0.37	0.17	2.1	0.20	0.09
22297	6.4	0.98	0.44	7.9	0.86	0.38
33058	4.1	0.34	0.15	3.4	0.46	0.21
36969	6.4	0.33	0.15	9.6	0.96	0.43
42162	3.8	0.75	0.34	7.7	0.47	0.47
52746	5.1	0.42	0.17	3.8	0.46	0.46
67790	9.6	0.71	0.32	6.0	1.78	0.79
95148	4.1	0.65	0.29	4.8	0.83	0.37

Fig 10 RESULTS OF REPRODUCIBILITY COUNTS OF 10 GMA SLIDES COUNTED 5 TIMES

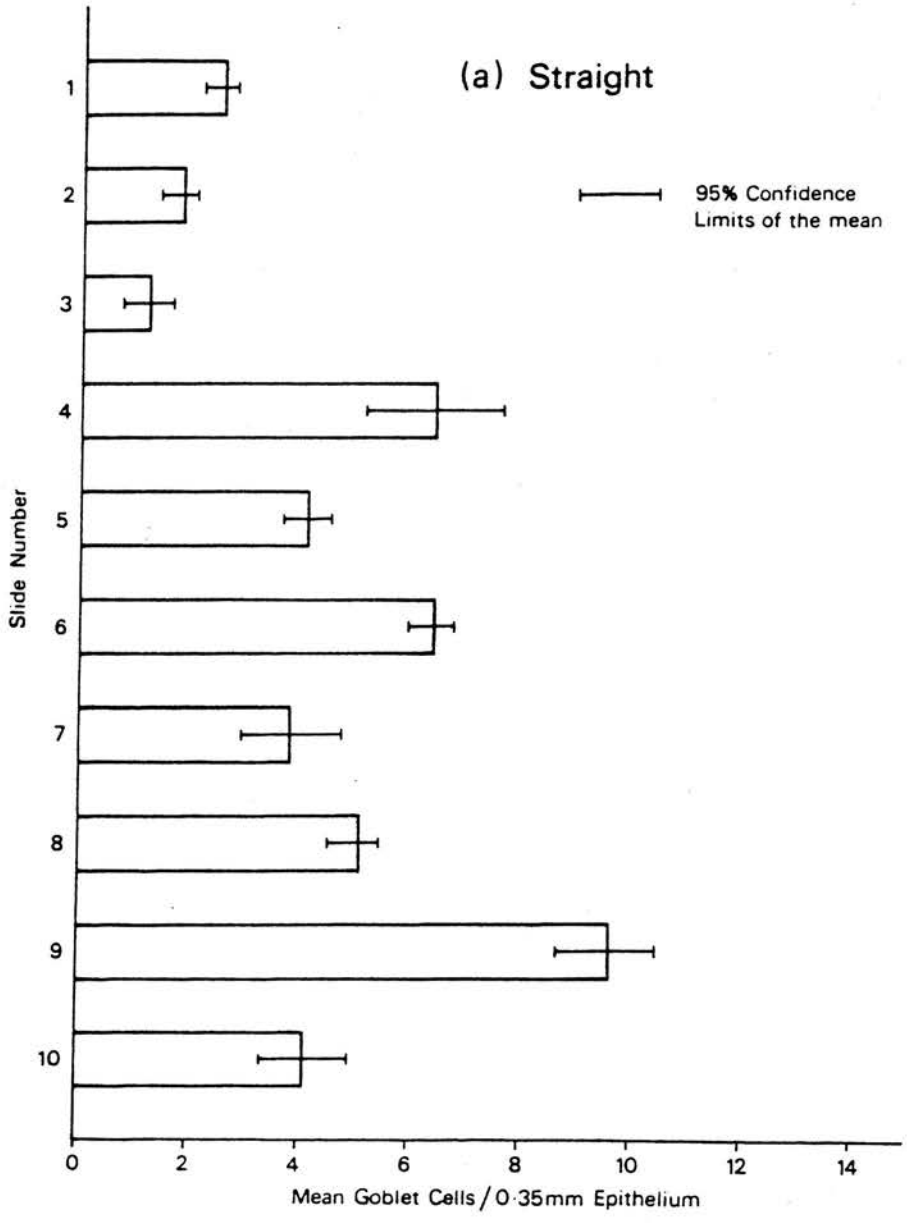


FIGURE 10

Showing the results of counting goblet cells in zones 1 and 2 on the straight side of the main bronchus in 10 GMA slides on 5 separate occasions. The columns give the mean of the 5 counts for each slide and the horizontal bars show the upper and lower limits or the 95% level confidence limits.

Fig 11 RESULTS OF REPRODUCIBILITY COUNTS OF 10 GMA SLIDES COUNTED 5 TIMES

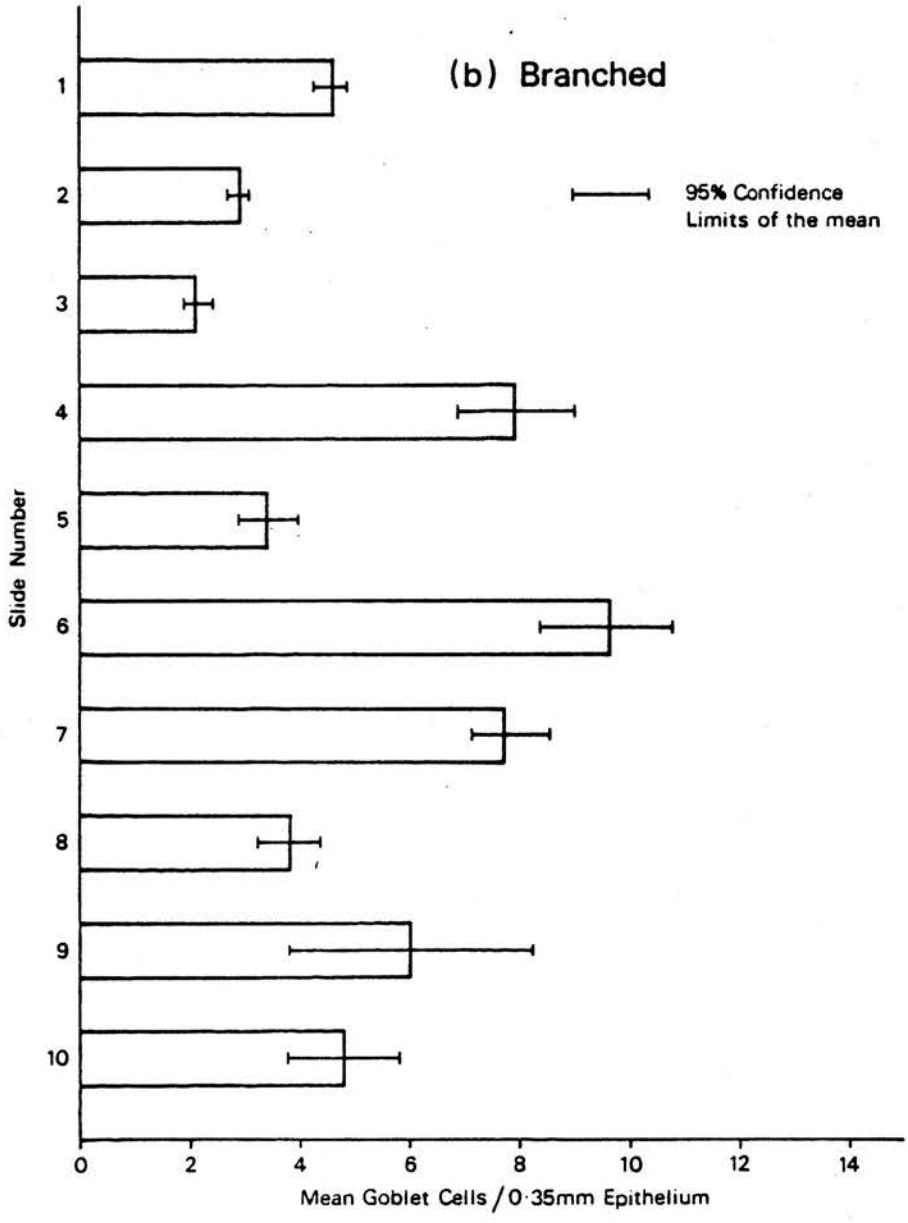


FIGURE 11

As for Figure 10 but showing the results for the branched side of the main bronchus in zones 1 and 2.

be used for quantification of goblet cells the population is relatively evenly distributed, and also that they can be reliably and repeated recognised. In no case was it possible to ensure that the same 10 HPF's were counted each time, and the variations obtained in this study probably reflect clusters of goblet cells in the different HPF's used for counting. In the case of slide 9 with the high standard deviation and large confidence limits there were two noticeable clusters of goblet cells in zone 2. The clusters were situated on the branched side of the airway near to the junction with zone 3. As a result, their inclusion in the counts depended on whether the count started very high in zone 1 or slightly lower down. Nevertheless, in 1.5 μm thick GMA slides stained with AB/PAS it is possible to select and identify goblet cells consistently and reliably for quantitative studies.

CHAPTER 4

EXPERIMENTAL DESIGN AND RESULTS

4:1 EXPERIMENT 1

INVESTIGATION OF THE GOBLET CELL RESPONSE OVER ELEVEN DAYS FOLLOWING A SINGLE EXPOSURE TO SULPHUR DIOXIDE GAS

4:11 Objectives

The aims of this experiments were a) to establish whether or not a single exposure to SO_2 induced goblet cell hyperplasia in the rat lung, and b) to study the timing of that response.

4:12 Experimental Details

A total of 15 male rats approximately 390 g body weight were used. On day zero 12 rats were exposed to SO_2 gas simultaneously in 3 chambers for a total period of 4 hours 15 minutes. The concentration used was approximately 400 - 500 ppm. The atmosphere in the chambers was sampled at hourly intervals throughout the exposure period using Dreger tubes, and any appropriate adjustments were made to the SO_2 flow-rate in order to maintain the above concentration.

At the end of the exposure period the treated rats were rehoused in standard cages, returned to the stock room and killed in groups of 2 on days 1, 2, 4, 6, 8 and 11 following the SO_2 exposure. Four rats served as controls; they were not exposed to SO_2 and were kept separate from the SO_2 exposure room at all times. They were killed in groups of 2 on days 1 and 11.

4:13 Tissues and Processing

The lungs were inflation fixed in Millonig's fixative and the right lungs subsequently processed into GMA for goblet cell assessment.

4:14 Location

Edinburgh.

4:15 Results

The single exposure to SO_2 gas resulted in a respiratory goblet cell hyperplasia in the rat (Figure 12). The increase was first detected on day 2 after the exposure and reached a maximum of 11.7 goblet cells/0.35 mm epithelium on day 6, thereafter the goblet cell numbers fell slightly and on day 11 at 9.5 goblet cells/0.35 mm epithelium were still much greater than the control value of 4 goblet cells/0.35 mm epithelium. These results

Fig 12 GOBLET CELL RESPONSE IN THE LUNG FOLLOWING A SINGLE EXPOSURE TO SO_2 GAS AT 450ppm

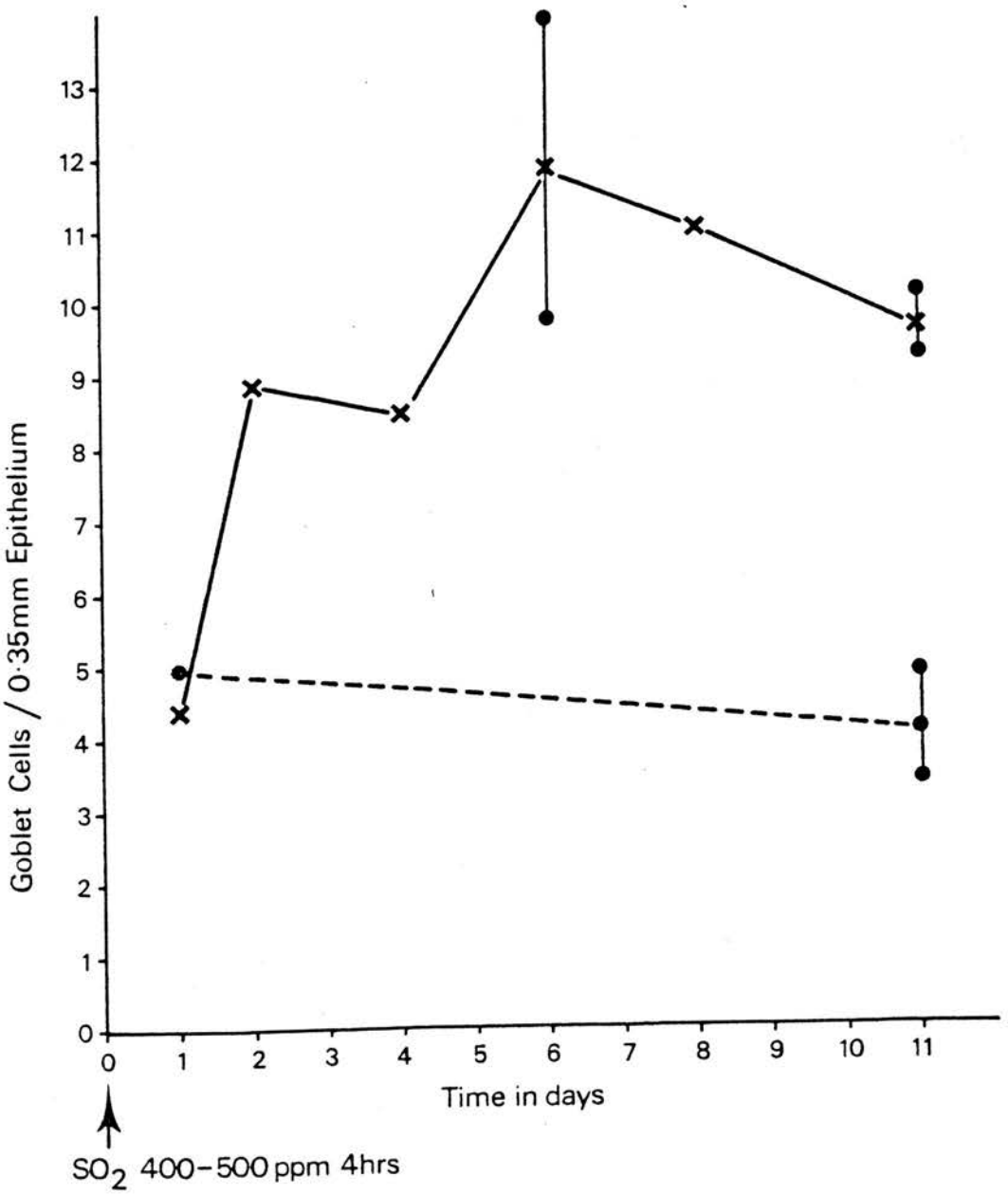


FIGURE 12

Showing the timing of the response of the goblet cell population in zones 1 and 2 following a single 4 hour exposure to 400 - 500 ppm sulphur dioxide gas. The vertical bars show the range of results obtained from 2 animals with the average indicated at the mid-way point. Other results are single observations.

contrast sharply with those obtained in experiments 3 and 4 for the isoprenaline timecourse.

4:2 EXPERIMENT 2

A COMPARISON OF 10 DAILY DOSES WITH A SINGLE DOSE OF RACEMIC ISOPRENALINE OR ITS ISOMERS

4:21 Objectives

The aims of this experiment were a) to repeat the earlier work of Sturgess and Reid using Alderley Park rats, b) to determine which of the two isomers of isoprenaline was more effective in inducing an increase in respiratory goblet cell numbers, and c) to establish whether or not the goblet cells of the lung responded to a single dose of isoprenaline.

4:22 Experimental Details

Groups of 10 rats were used consisting of 5 males and 5 females. All the animals were killed 24 hours after the last injection.

Group 1 received 3.7 mg/kg ($\pm$) isoprenaline every day for 10 days.

Group 2 received 2.4 mg/kg (-) isoprenalne every day for 10 days.

Group 3 received 2.4 mg/kg (+) isoprenaline every day for 10 days.

Group 4 received 0.5 ml saline every day for 10 days.

Group 5 received a single 3.7 mg/kg dose of (±) isoprenaline.

Group 6 received a single 2.4 mg/kg dose of (-) isoprenaline.

Group 7 received a single 2.4 mg/kg dose of (+) isoprenaline.

Group 8 received a single 0.5 ml dose of saline.

4:23 Tissues and Processing

The lungs were inflation fixed in Millonig's fixative and the left lung embedded in paraffin wax. Goblet cells were assessed in 5 μm paraffin sections stained with AB/PAS and counterstained with haematoxylin.

4:24 Location

Alderley Park.

4:25 Results

Figure 13 shows the results obtained following both 10 daily injections and a single dose of either racemic isoprenaline or each of its two isomers. Ten daily doses of 3.7 mg/kg racemic isoprenaline resulted in an increase in respiratory goblet cells from a control value of 2.3 goblets/HPF to 6.64 goblets/HPF. Similarly 10 daily doses of 2.4 mg/kg (-) isoprenaline resulted in an increase of

FIGURE 13

Comparing the results obtained after 10 daily doses of racemic isoprenaline and its isomers with those obtained after a single dose of the racemate and its isomers. The columns show the mean obtained after each treatment and the vertical bars show the standard error of the mean.

Control n = 18
3.7 mg/kg x 10 (±) isoprenaline n = 8
3.7 mg/kg x 1 (±) isoprenaline n = 9
2.4 mg/kg x 10 (-) isoprenaline n = 7
2.4 mg/kg x 1 (-) isoprenaline n = 10
2.4 mg/kg x 10 (+) isoprenaline n = 10
2.4 mg/kg x 1 (+) isoprenaline n = 8

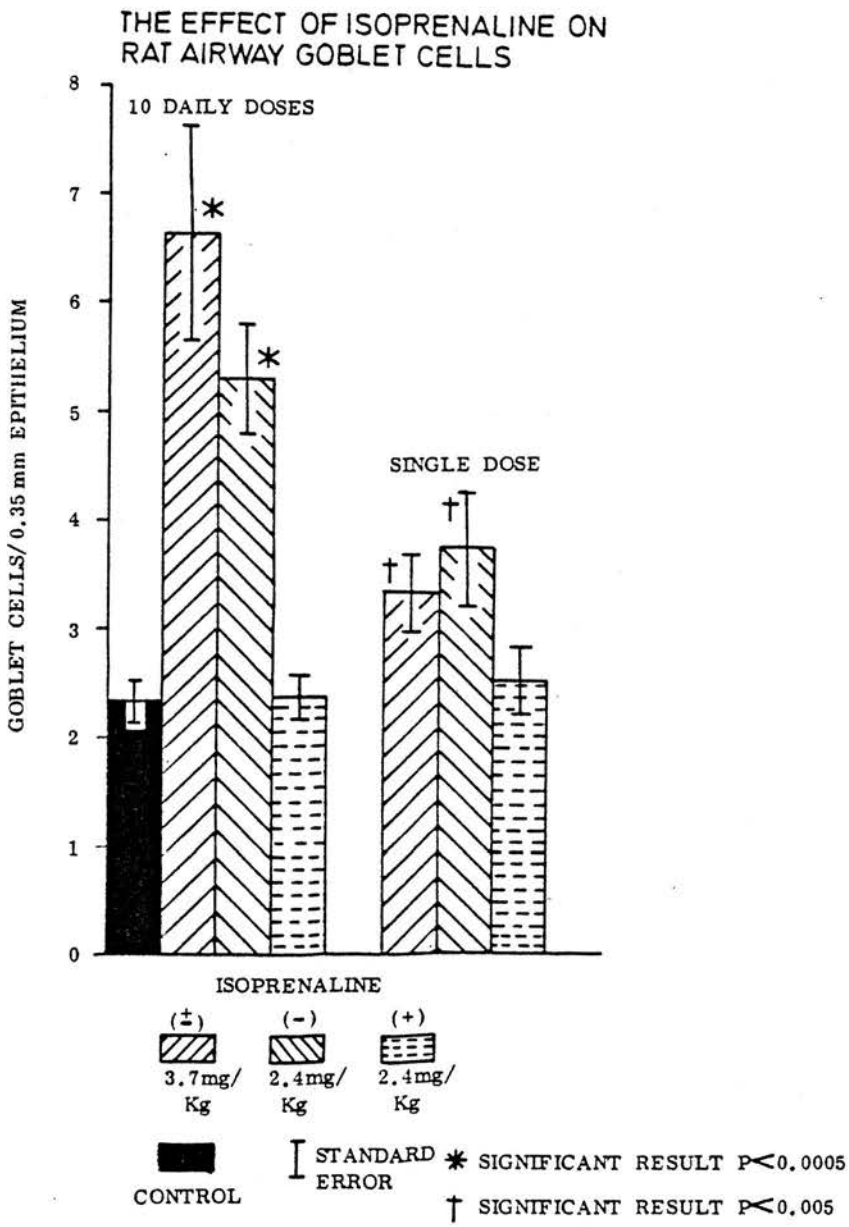

THE EFFECT OF ISOPRENALINE ON
RAT AIRWAY GOBLET CELLS
10 DAILY DOSES
SINGLE DOSE
GOBLET CELLS/0.35 mm EPITHELIUM
0
1
2
3
4
5
6
7
8
ISOPRENALINE
(±)
(-)
(+)
3.7mg/ Kg
2.4mg/ Kg
2.4mg/ Kg
CONTROL
STANDARD ERROR
* SIGNIFICANT RESULT P<0.0005
† SIGNIFICANT RESULT P<0.005

FIGURE 13.

respiratory goblet cells from a control value of 2.3 goblets/HPF to 5.3 goblets/HPF. Both these results were significantly different from the control with P = <0.0005. A single 3.7 mg/kg dose of racemic isoprenaline induced a much smaller increase in respiratory goblet cells to 3.33 goblets/HPF whilst a single 2.4 mg/kg dose of (-) isoprenaline induced an increase to 3.75 goblets/HPF; both these results were significantly different from the control value of 2.3 goblets/HPF with P = <0.005. The (+) isomer of isoprenaline failed to produce any effect on respiratory goblet cells when administered as either 10 daily doses or a single dose of 2.4 mg/kg. The use of the Student t-test showed that in none of the groups were there any significant differences between the responses of the two sexes. Their results have therefore been pooled and each column in Figure 13 represents their combined mean.

4:3 EXPERIMENT 3

INVESTIGATION OF THE ISOPRENALINE RESPONSE OVER 16 DAYS: TIMECOURSE 1, BEFORE SENDEI

4:31 Objectives

Having established that a single dose of isoprenaline could induce goblet cell hyperplasia in the lung it was important to know when that increase occurred and how long

it lasted before attempting further study of the nature of the response.

4:32 Expermental Details

Groups of 6 rats, all male, received 40 mg/kg racemic isoprenaline on day 0 and were killed in groups on days 2, 4, 8 and 16. Saline controls in groups of 3 were injected with 0.5 ml saline on day 0 and killed in groups on days 2, 4, 8 and 16.

4:33 Tissues and Processing

The lungs were inflation fixed in Millonig's fixative and the left lung embedded in paraffin. Goblet cells were assessed in 5 μm paraffin sections stained with AB/PAS.

4:34 Location

Edinburgh.

4:35 Results

The results obtained are shown in Figure 14. There was a rapid increase in goblet cell numbers which in this experiment reached a maximum level of 15.8 goblets/HPF on day 2, the first day sampled. Thereafter there was a gradual decrease in the goblet cell numbers up to day 8, where the level reached was 9 goblets/HPF. Between days 8 and 16 the decrease levelled off and the final value

FIGURE 14

Showing the timing of the response following a single dose of isoprenaline. Control rats were killed in groups of 3 on days 2, 4, 8 and 16. Their results were combined to give a mean (the open circle) and the hatching shows the range of the standard error of that mean. The X's represent the mean result obtained on the different days after isoprenaline treatment and the vertical bars show the standard error of each mean. All the isoprenaline results were significantly different from control with P = <0.001, <0.005, <0.05, and <0.05 on days 2, 4, 8 and 16 respectively.

Control n = 9
Isoprenaline day 2 n = 4
Isoprenaline day 4 n = 4
Isoprenaline day 8 n = 5
Isoprenaline day 16 n = 5

(This experiment was conducted just prior to the Sendei virus outbreak)

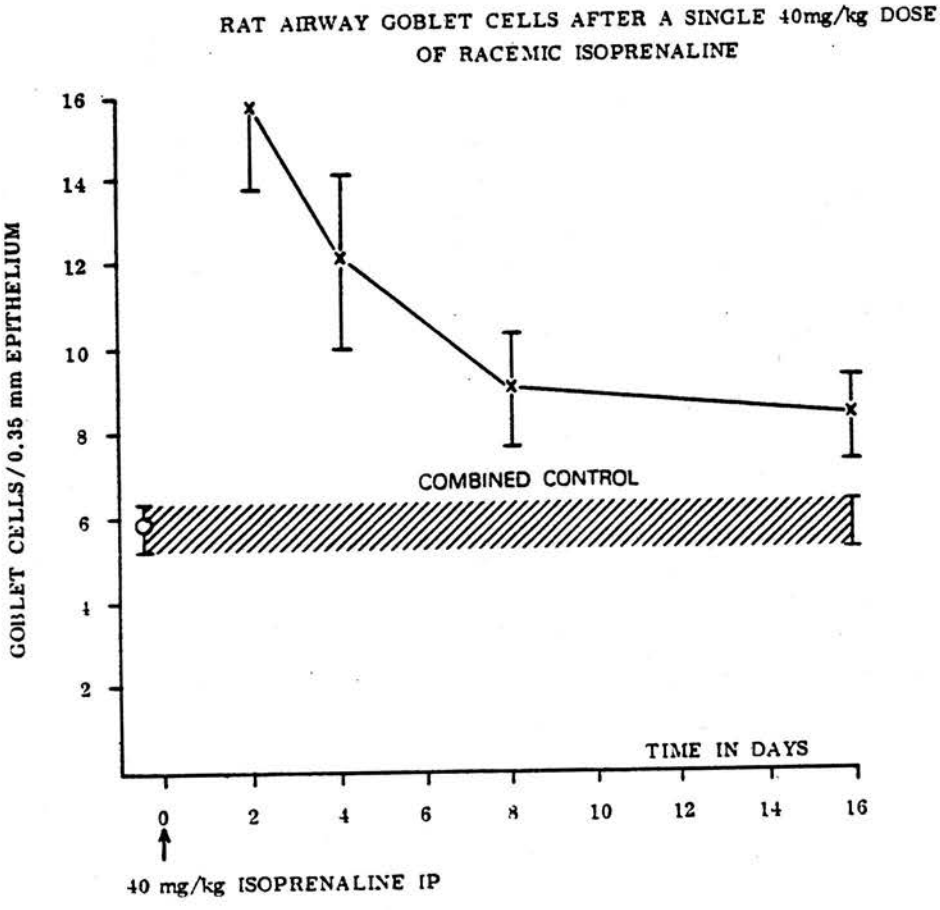

RAT AIRWAY GOBLET CELLS AFTER A SINGLE 40mg/kg DOSE OF RACEMIC ISOPRENALINE
GOBLET CELLS/0.35 mm EPITHELIUM
16
14
12
10
8
6
4
2
COMBINED CONTROL
TIME IN DAYS
0
2
4
6
8
10
12
14
16
40 mg/kg ISOPRENALINE IP

FIGURE 14.

obtained was 8 goblets/HPF on day 16. This was still significantly above the combined control value of 5.8 goblets/HPF and in fact all the responses obtained were significantly different from the control with P = <0.001 on day 2 and P = <0.05 on day 16.

4:4 EXPERIMENT 4

INVESTIGATION OF THE ISOPRENALINE RESPONSE OVER 16 DAYS: TIMECOURSE 2, AFTER SENDEI

4:41 Objectives

These were two-fold, a) to confirm the results of the previous timecourse in the absence of Sendei infection, and b) to look at the pattern of response in more detail with the aid of additional time-points.

4:41 Experimental Details

Groups of 5 male rats received 40 mg/kg racemic isoprenaline on day 0 and were killed in groups on days 1, 2, 4, 6, 8, 10, 12, 14 and 16. Saline controls were injected with 0.5 ml saline on day 0 and were killed in groups of 5 on days 1, 2, 4, 6, 8, 10, 12, 14 and 16.

4:43 Tissues and Processing

The lungs were inflation fixed with Millonig's fixative and the left lung embedded in GMA. Goblet cells were assessed in 1.5 μm GMA sections stained with AB/PAS.

4:44 Location

Edinburgh.

4:45 Results

The pattern of goblet cell increase and decline following isoprenaline as seen in Figure 15 was very similar to that obtained in experiment 3, thus confirming the previous results. Once again the increase in goblet cells rapidly reached maximum values of 8.8 goblets/HPF on day 1 and 9.3 goblets/HPF on day 2; thereafter the goblet cell numbes gradually fell reaching control values of 2.2 goblets/HPF by day 8. Significant results were only obtained on days 1, 2 and 4, the response was therefore both rapid and short-lived.

FIGURE 15

As for Figure 14 but with additional time points. The results marked with an asterisk are significantly different from control with P = <0.0005 on days 1, 2 and 4, and P = <0.005 on day 6.

Control n = 16
Isoprenaline day 1 n = 4
Isoprenaline day 2 n = 4
Isoprenaline day 4 n = 5
Isoprenaline day 6 n = 5
Isoprenaline day 8 n = 5
Isoprenaline day 10 n = 2
Isoprenaline day 12 n = 3
Isoprenaline day 14 n = 4
Isoprenaline day 16 n = 5

(This experiment was conducted once the Sendei virus had been eradicated from the breeding colony)

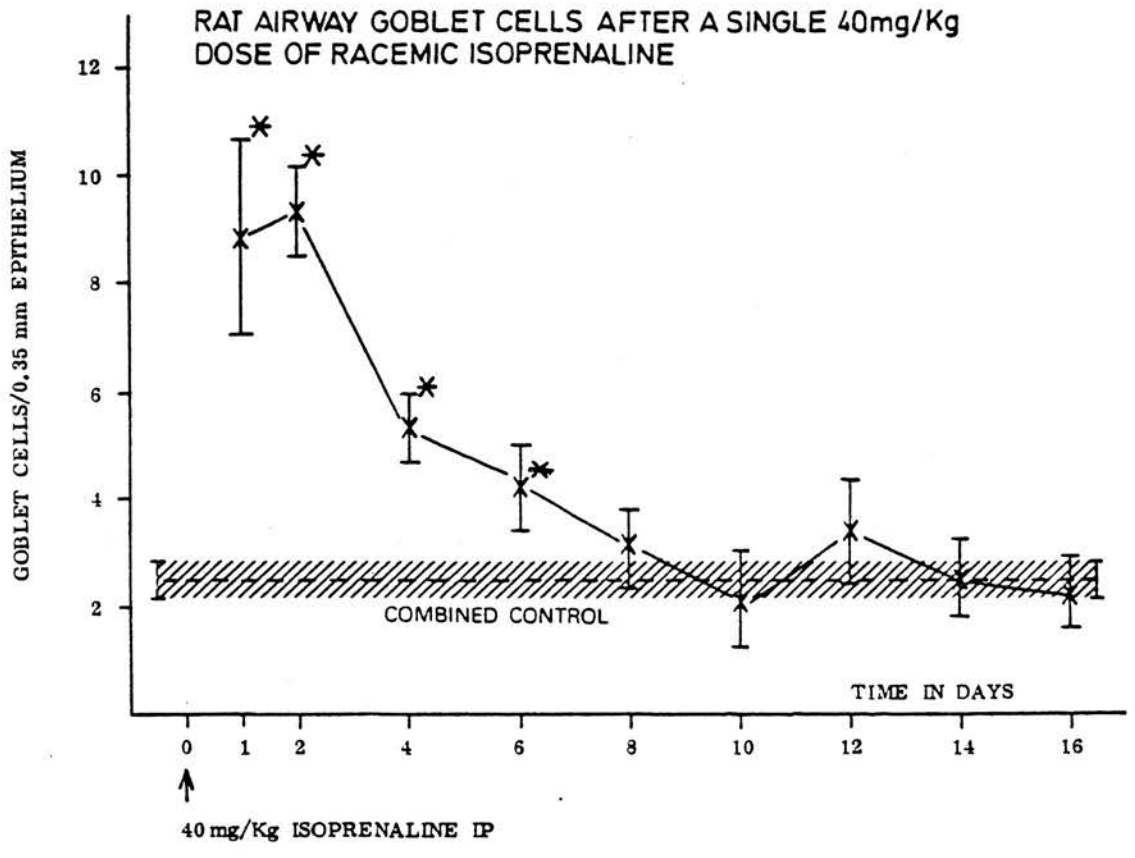

RAT AIRWAY GOBLET CELLS AFTER A SINGLE 40mg/Kg DOSE OF RACEMIC ISOPRENALINE
GOBLET CELLS/0.35 mm EPITHELIUM
12
10
8
6
4
2
COMBINED CONTROL
TIME IN DAYS
0
1
2
4
6
8
10
12
14
16
40 mg/Kg ISOPRENALINE IP

FIGURE 15.

4:5 EXPERIMENT 5

INVESTIGATION OF THE ISOPRENALINE RESPONSE OVER THE FIRST 21 HOURS AFTER ISOPRENALINE

4:51 Objectives

The previous timecourse studies, experiments 3 and 4, showed that the first 24 hours after the administration of isoprenaline was the period of maximum change within the respiratory epithelium. This experiment was designed to look at this important period in detail.

4:52 Experimental Details

Groups of 6 male rats received 40 mg/kg racemic isoprenaline at time 0 and were killed in groups 6, 9, 12, 15, 18 and 21 hours later. Control animals received 0.5 ml saline at time 0 and were killed in groups of six 9, 15 and 21 hours later.

4:53 Tissues and Processing

The lungs were inflation fixed in Millonig's fixative and the left lung embedded in GMA for goblet cell assessment. The right lungs were post-osmicated and embedded in araldite.

4:54 Location

Edinburgh.

4:55 Results

Figure 16 shows the pattern of the early response obtained in the lung following isoprenaline. At 6 hours there was a slight drop in goblet cell numbers; this was just significant. At 12 hours there was a small increase in goblet cell numbers, likewise at 15 hours; these increases were not significant. At 18 hours, however, a significant increase in goblet cells was found ($P = <0.0025$), the numbers being 5.11 goblets/HPF for the treated group and 2.2 goblets/HPF for the control group. A further increase was found at 21 hours to a value of 5.7 goblets/HPF and in this instance $P = <0.005$. These results complement those obtained in experiment 4 and thus it is possible to surmise that goblet cell numbers steadily increase during the first 24 hours after isoprenaline, reach a maximum between 24 and 48 hours and thereafter steadily decline, regaining control values between days 8 and 16.

FIGURE 16

As for Figure 14 but showing the response during the first 24 hours after the isoprenaline administration. The results marked with an asterisk are significantly different from control with P = <0.01 at 6 hours, P = <0.0025 at 18 hours, and P = <0.005 at 21 hours.

Control n = 11
Isoprenaline 6 hours n = 5
Isoprenaline 9 hours n = 5
Isoprenaline 12 hours n = 6
Isoprenaline 15 hours n = 6
Isoprenaline 18 hours n = 5
Isoprenaline 21 hours n = 5

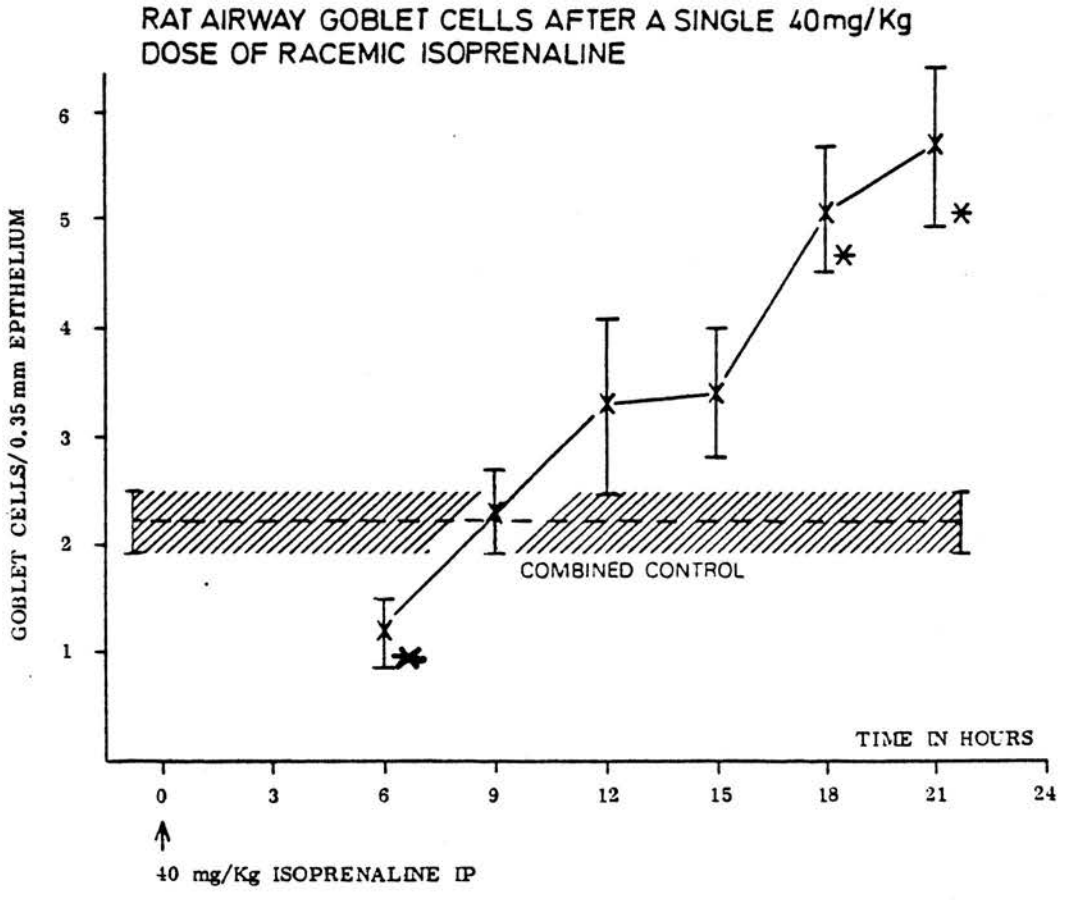

RAT AIRWAY GOBLET CELLS AFTER A SINGLE 40mg/Kg
DOSE OF RACEMIC ISOPRENALINE
GOBLET CELLS/0.35 mm EPITHELIUM
6
5
4
3
2
1
COMBINED CONTROL
TIME IN HOURS
0
3
6
9
12
15
18
21
24
40 mg/Kg ISOPRENALINE IP

FIGURE 16.

4:6 EXPERIMENT 6

INFLUENCE OF CIRCADIAN RHYTHM ON THE GOBLET CELL RESPONSE TO ISOPRENALINE IN THE LUNG

4:61 Objectives

Schering et al. (1972) demonstrated that in mice the time of injection of isoprenaline and the subsequent time of death alters the effect seen on mitotic activity in the small intestine. Thus at times there are significant decreases in mitotic activity following isoprenaline and at other times the results following isoprenaline are the same as the controls. In this experiment, the influence of the time of death on the isoprenaline response in the lung was investigted, and with the aid of the control animals any circadian variation was looked for in the goblet cell population at this site.

4:62 Experimental Details

A total of 90 male rats were used, 12 of which were controls. The animals were injected in various groups with either 40 mg/kg isoprenaline or saline at 4 hourly intervals from 8 pm to 4 pm the following day. Groups of 3 treated and 2 controls were then killed at various times later according to the time of injection of the isoprenaline (see Table 5). As the schedules for killing and injections overlapped during the first night it was decided

TABLE 5

SCHEDULE FOR INJECTIONS AND TIME OF DEATH FOR EXPERIMENT 6

Time of injection	No. injected	No. killed and hours after injection					
		12hr	22hr	30hr	38hr	50hr	96hr
8 pm	18 + 2 controls	3	3	3	3	3	3 + 2 controls
12 midnight	12 + 2 controls	-	3	-	3	3	3 + 2 controls
4 am	12 + 2 controls	-	3	-	3	3	3 + 2 controls
8 am	12 + 2 controls	-	3	-	3	3	3 + 2 controls
12 mid-day	12 + 2 controls	-	3	-	3	3	3 + 2 controls
4 pm	12 + 2 controls	-	3	-	3	3	3 + 2 controls

to kill the animals throughout the experiment by the quicker method of CO_2 gas overdose instead of using Sagatal.

4:63 Tissues and Processing

The lungs were inflation fixed in Millonig's fixative and the left lung embedded in GMA for goblet cell assessment.

4:64 Location

Edinburgh.

4:65 Results

In the lung there was a very slight variation in control goblet cells over a 24 hour period (Table 6). Both groups at 4pm and 8 am had very small variations within the group and were both in the region of 0.5 goblets/HPF $\pm$ 0.01, whilst at 8 pm and 12 midnight (12 pm) the numbers were higher and the within-group variation was much greater, ranging from 1 to 3 goblets/HPF. In the absence of larger groups it is not possible to say whether these differences are significant. When the mean of all the control values was taken an overall control value of 1.2 $\pm$ 0.26 goblets/HPF was obtained and this is used in Figures 17 and 18 which show the timecourse and responses obtained at 22 hours and 38 hours after isoprenaline. The

TABLE 6

VARIATION IN CONTROL AIRWAY GOBLET CELLS IN THE RAT IN A SINGLE 24 HOUR PERIOD

Time of Death		Goblets/HPF	Mean Goblets/HPF	Group Mean Goblets/HPF
8 pm	Rat 1	0.95	1.65	
	Rat 2	2.35		
12 midnight	Rat 1	1.1	2.10	
	Rat 2	3.1		
4 am	Rat 1	0.65	0.67	1.2 SD = 1.0 SE = 0.26
	Rat 2	0.7		
8 am	Rat 1	0.4	0.43	
	Rat 2	0.45		
12 noon	Rat 1	2.0	2.0	
	Rat 2	not counted		

FIGURE 17

Showing the timing of the response following a single 40 mg/kg dose of isoprenaline at 8 pm. The control is represented as explained in Figure 14. The treated results are shown as the individual responses (the closed circles) and the mean is indicated by an X. The figures given below each mean are the total in each treated group.

Control n = 9

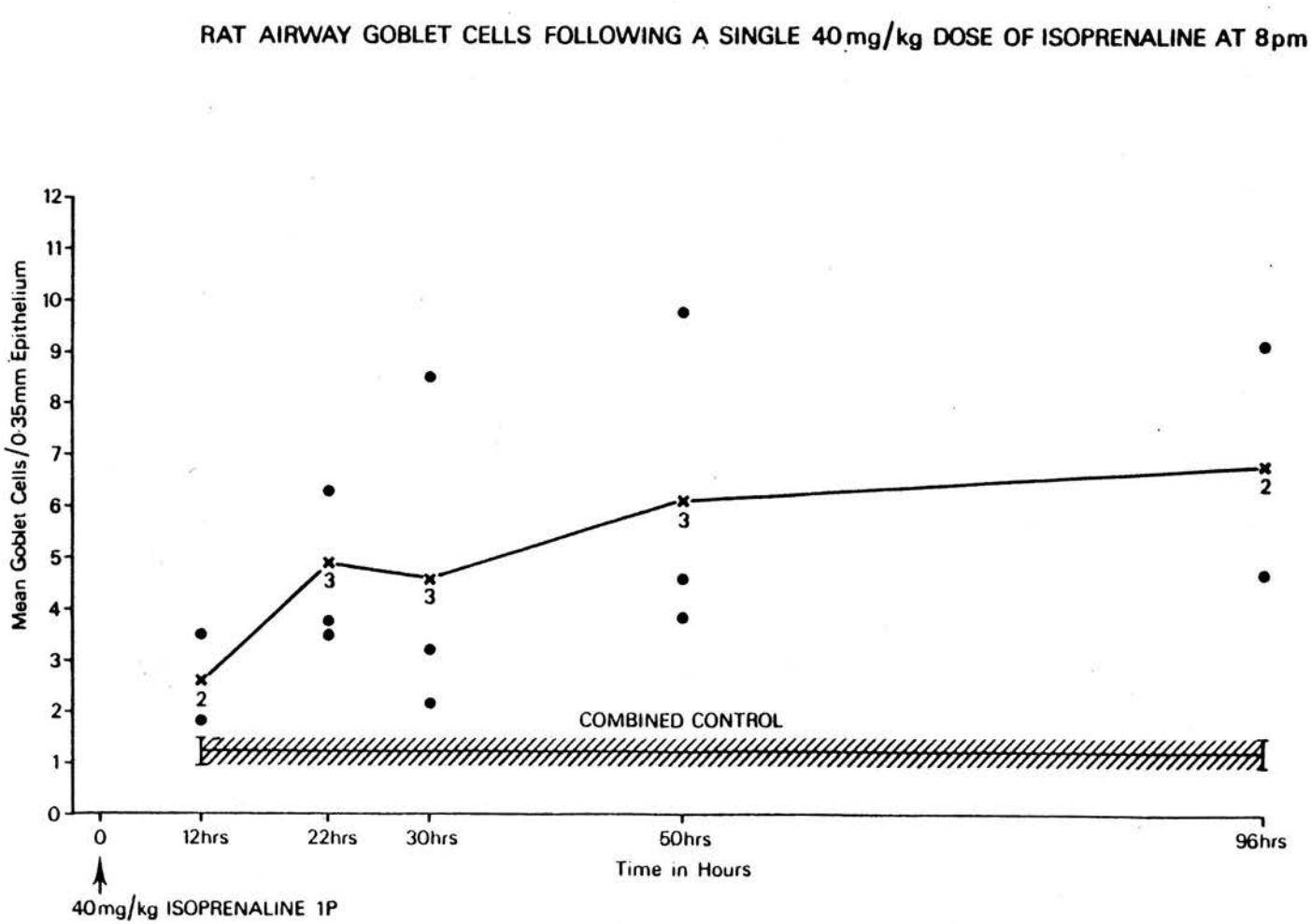

RAT AIRWAY GOBLET CELLS FOLLOWING A SINGLE 40mg/kg DOSE OF ISOPRENALINE AT 8pm
Mean Goblet Cells/0·35mm Epithelium
0
1
2
3
4
5
6
7
8
9
10
11
12
COMBINED CONTROL
2
3
3
3
2
0
12hrs
22hrs
30hrs
50hrs
96hrs
Time in Hours
40mg/kg ISOPRENALINE IP

FIGURE 17.

timecourse was obtained from rats all injected at 8 pm and killed at 12, 22, 30, 50 and 96 hours later in groups of 3. In Figure 17 the individual results for each group have been shown as solid black circles and the group mean as a cross. As in experiment 5, a detectable increase was observed as early as 12 hours after the isoprenaline injection, and by 48 hours there was a threefold increase in goblet cells to 6.1 goblets/HPF. Unlike experiment 4 the number of goblet cells had not fallen by day 4 (96 hours) but had increased slightly to 6.8 goblets/HPF.

Because of the staggered injections it was possible to investigate whether or not the time of death had any effect on the goblet cell response in the lung. Figure 18 shows the goblet cell response to isoprenaline in the lung throughout two 24-hour periods, the first 22 hours after injection and the second 38 hours after injection. It can be seen that in both instances, regardless of the time of death, all the lungs exhibited an increase in goblet cells after the administration of isoprenaline. The increase at 38 hours was slightly larger than that at 22 hours, thus the overall group mean for 38 hours was 7.5 goblets/HPF and that for 22 hours was 5.4 goblets/HPF; these were significantly different.

The time of death had no significant effect on any of the results obtained in this experiment. Control goblet cells were consistently low throughout a single 24-hour period and the response to a single dose of isoprenaline

FIGURE 18

Showing the results obtained during two 24 hour periods, a) 22 hours and b) 38 hours after a single 40 mg/kg dose of isoprenaline. The control is as for Figure 17. The treated results are the mean of a number of observations as indicated next to each point.

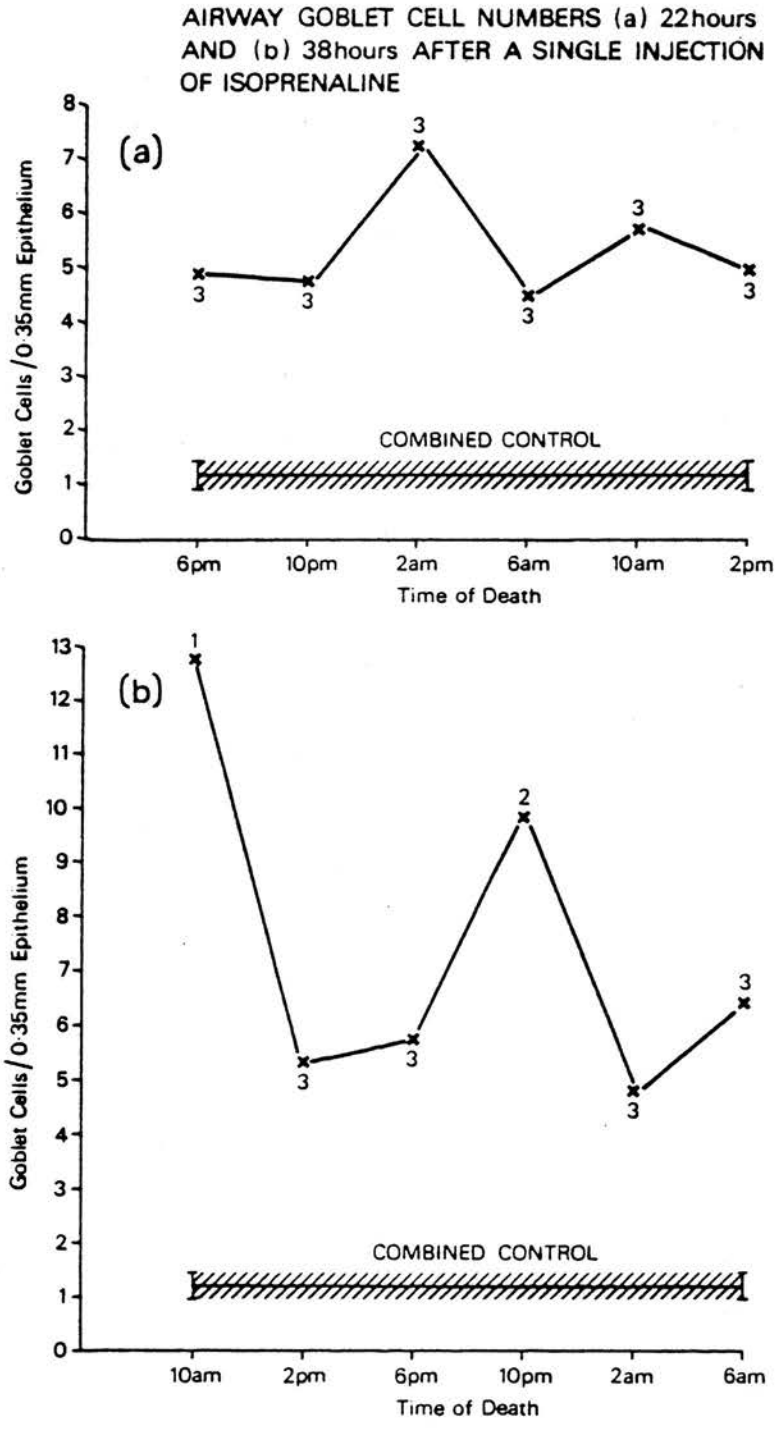

AIRWAY GOBLET CELL NUMBERS (a) 22hours AND (b) 38hours AFTER A SINGLE INJECTION OF ISOPRENALINE
(a)
Goblet Cells/0·35mm Epithelium
COMBINED CONTROL
6pm
10pm
2am
6am
10am
2pm
Time of Death
(b)
Goblet Cells/0·35mm Epithelium
COMBINED CONTROL
10am
2pm
6pm
10pm
2am
6am
Time of Death

FIGURE 18.

was also consistent throughout 2 separate 24-hour periods. The isoprenaline resonse is therefore uninfluenced by any circadian rhythm effect in the lung.

4:7 EXPERIMENT 7

DOSE-RESPONSE INVESTIGATION USING RACEMIC ISOPRENALINE. DOSE-RESPONSE CURVE 1: PILOT STUDY USING FEMALE RATS

4:71 Objectives

Experiment 2 suggested that there appears to be a relationship between the dose of isoprenaline and the degree of goblet cell hyperplasia in the lung. In this experiment a series of IP doses of isoprenaline were tried and, in an attempt to see if higher doses had a longer lasting effect, rats were killed 22 hours, 41 hours and 6 days after receiving the different doses of isoprenaline.

4:72 Experimental Details

Animals in groups of 4 were dosed with either 0.5 ml saline or the following doses of isoprenaline: 600 ug/kg and 1.25, 2.5, 5, 10, 20, 40, 80 and 120 mg/kg isoprenaline. Owing to limited availability of rats from Alderley Park, 80 female rats were used for this experiment instead of males.

4:73 Location

Edinburgh.

4:74 Tissues and Processing

The lungs were inflation fixed with Millonig's fixative and the left lungs embedded in GMA for goblet cell assessment.

4:75 Results

The response to isoprenaline was dose-dependent (Figure 19). Shallow dose-response curves were obtained at 22 hours (a) and 41 hours (b) after the isoprenaline injections but at 6 days (c) the results were less conclusive and no clear-cut dose-response relationship was obtained. At 22 hours and 41 hours maximum responses were found after 40 mg/kg with lesser increases to 20, 10 and 5 mg/kg the responses to 80 and 120 mg/kg at both times were sub-maximal.

The dose-response curve obtained at 41 hours after the injection of the isoprenaline showed an overall higher response than the curve obtained at 22 hours at all dose-levels tested. The responses at 22 hours were therefore sub-maximal and insufficient time had elapsed for the full response to develop.

FIGURE 19

Showing a series of dose-response curves obtained in female rats, a) 22 hours, b) 41 hours and c) 6 days after the administration of the isoprenaline. The vertical bars represent the standard errors of the mean. The control in each graph is a combined control from groups killed at 22 hours and 6 days, n = 8, in the treated groups n = 3-4.

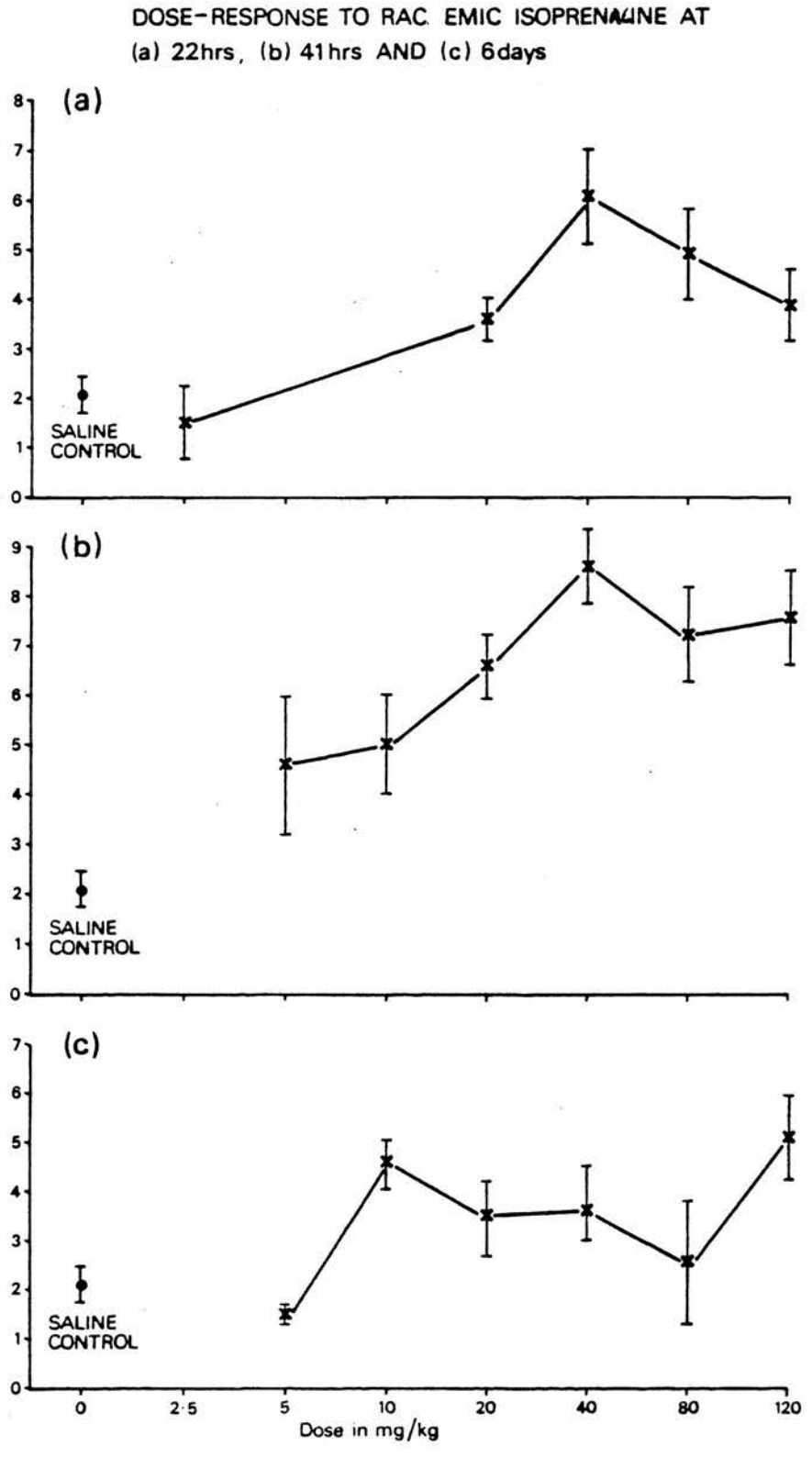

DOSE-RESPONSE TO RACEMIC ISOPRENALINE AT
(a) 22hrs, (b) 41hrs AND (c) 6days
(a)
(b)
(c)
SALINE CONTROL
SALINE CONTROL
SALINE CONTROL
0
2·5
5
10
20
40
80
120
Dose in mg/kg

FIGURE 19.

The maximum response obtained at 6 days was to 120 mg/kg with lesser increases obtained with 40, 20 and 10 mg/kg. There was a suggestion, therefore, that higher doses had a longer lasting effect on goblet cells in the lung.

4:8 EXPERIMENT 8

DOSE-RESPONSE INVESTIGATION USING RACEMIC ISOPRENALINE. DOSE-RESPONSE CURVE 2: DETAILED STUDY USING MALE RATS

4:81 Objectives

The results of the female dose-response curves indicated that there was a very shallow dose-response relationship. This experiment was designed to look at this relationship in greater detail using larger groups of male rats and additional dose-levels. In addition two extra groups of rats were included in a preliminary study to see if the isoprenaline response could be antagonised pharmacologically using a β-adrenoceptor antagonist.

4:82 Experimental Details

Groups of 10 male rats were given isoprenaline in doses of 5, 10, 20, 30, 40, 60 and 80 mg/kg IP; an additional 20 rats received 0.5 ml saline and these were the controls. Also included in this experiment were 2

extra groups of 10 rats, one group receiving 50 mg/kg propranolol IP alone whereas the other received 50 mg/kg propranolol IP 35 minutes before receiving 40 mg/kg isoprenaline IP. All the animals were killed 24 hours after treatment.

4:83 Tissues and Processing

The lungs were inflation fixed wih Millonig's fixative and the left lung embedded in GMA for goblet cell assessment.

4:84 Location

Edinburgh.

4:85 Results

As in the previous experiment a shallow dose-response relationship was found to exist between the degree of goblet cell hyperplasia and the dose of isoprenaline employed (Figure 20). The saline controls gave a mean of 2.5 goblets/HPF, after 5 mg/kg isoprenaline respiratory goblet cells had increased to a mean of 4.1 goblets/HPF. This was further increased to 5.1 goblets/HPF after 10 mg/kg, the response then levelled off between 6.5 and 7.5 goblets/HPF following doses of 20, 30, 40, 60 and 80 mg/kg. Unlike experiment 7 a decrease in response at the higher doses was not seen.

FIGURE 20

Showing a dose-response curve obtained in male rats 24 hours after the administration of the isoprenaline. The vertical bars represent the standard error of the mean. All doses of isoprenaline produced significant increases in goblet cells with $P = <0.01$ for 5 mg/kg and $P = <0.0005$ for all other doses. The reduced response to 40 mg/kg isoprenaline in the presence of propranolol (propranolol blockade), was significantly different to the response obtained with 40 mg/kg isoprenaline alone $P = <0.0025$ and was also significantly different to the saline control, $P = <0.025$.

Saline control n = 19
Propranolol control n = 9
Propranolol Blockade n = 10
Isoprenaline 5 mg/kg n = 10
Isoprenaline 10 mg/kg n = 9
Isoprenaline 20 mg/kg n = 9
Isoprenaline 30 mg/kg n = 8
Isoprenaline 40 mg/kg n = 10
Isoprenaline 60 mg/kg n = 9
Isoprenaline 80 mg/kg n = 7

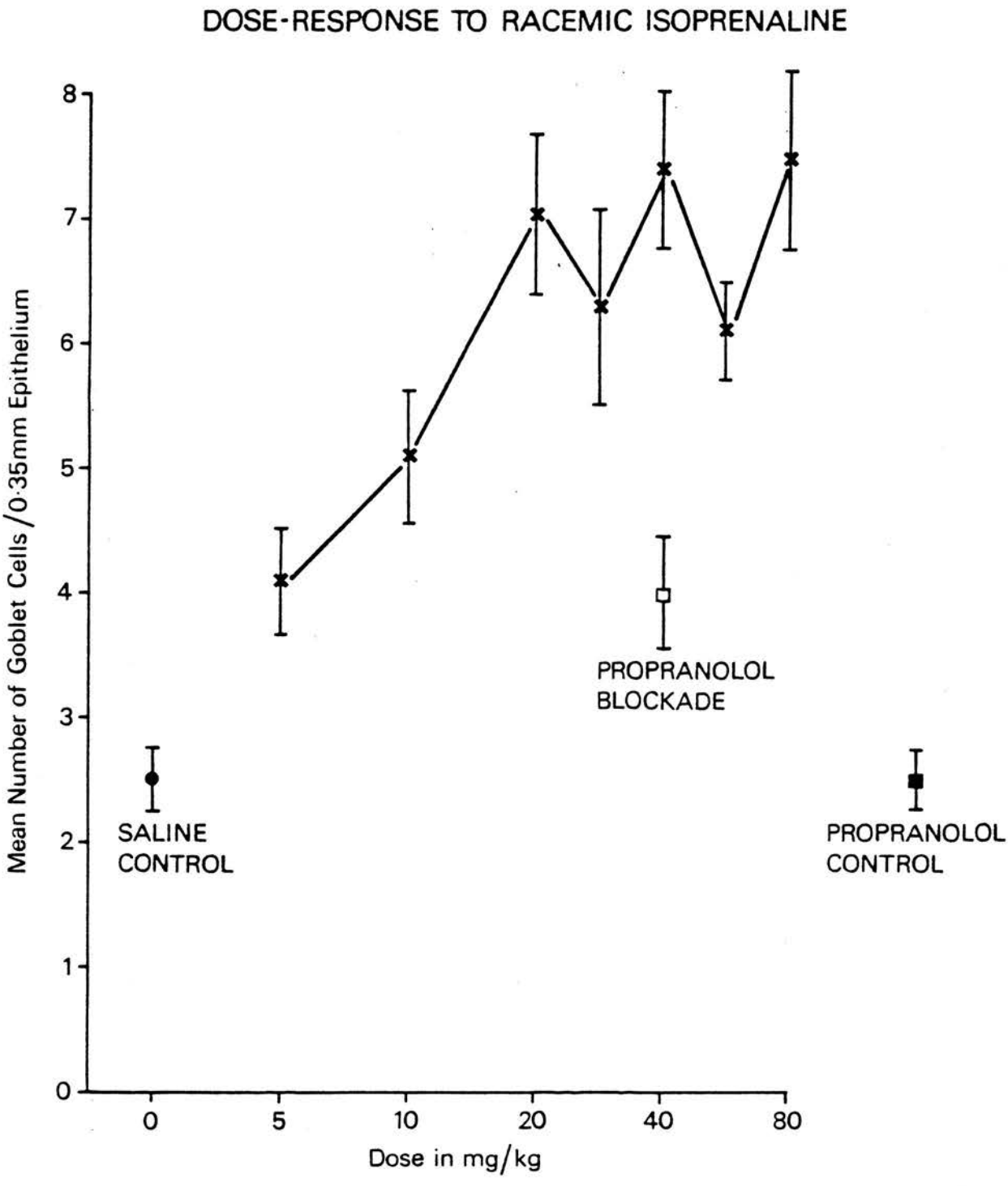

DOSE-RESPONSE TO RACEMIC ISOPRENALINE
Mean Number of Goblet Cells /0·35mm Epithelium
0
1
2
3
4
5
6
7
8
0
5
10
20
40
80
Dose in mg/kg
SALINE CONTROL
PROPRANOLOL BLOCKADE
PROPRANOLOL CONTROL

FIGURE 20.

Propranolol (50 mg/kg) on its own gave a mean of 2.5 goblets/HPF the solid square in Figure 20, identical to that of the saline controls. It therefore has no effect on respiratory goblet cells.

The results of the group of rats pretreated with 50 mg/kg propranolol 35 minutes before receiving 40 mg/kg isoprenaline are shown in Figure 20 as an open square. The mean number of goblet cells obtained was 3.99 goblets/HPF; this response is 54.4% smaller than the response of 7.4 goblets/HPF obtained when 40 mg/kg isoprenaline was given on its own. Propranolol has partially antagonised the isoprenaline response in the lung.

4:9 EXPERIMENT 9

DOSE-RESPONSE INVETIGATION USING RACEMIC ISOPRENALINE. DOSE-RESPONSE CURVE 3: ANTAGONISM OF THE ISOPRENALINE RESPONSE BY PROPRANOLOL

4:91 Objectives

To study in detail the pharmacological antagonism of the isoprenaline response by propranolol. Two isoprenaline dose-response curves were carried out, one with isoprenaline alone and the other in the presence of propranolol. By using sub-maximal doses of isoprenaline it was hoped to see if propranolol was capable of inducing a

shift to the right of the isoprenaline dose-response curve.

4:92 Experiment Design

Ten groups of male rats each consisting of 8 animals were made up as follows:-

1 rat received saline 0.5 ml IP
1 rat received 5 mg/kg isoprenaline IP
1 rat received 10 mg/kg isoprenaline IP
1 rat received 20 mg/kg isoprenaline IP
1 rat received 50 mg/kg propranolol IP
1 rat received 50 mg/kg propranolol 35 mins before receiving 5 mg/kg isoprenaline
1 rat received 50 mg/kg propranolol 35 mins before receiving 10 mg/kg isoprenaline
1 rat received 50 mg/kg propranolol 35 mins before receiving 20 mg/kg isoprenaline

Thus, every group consisted of one rat from each of the 8 different treatment groups. All the animals were killed 24 hours after treatment.

4:93 Tissues and Processing

The lungs were inflation fixed using Millonig's fixative and the left lungs embedded in GMA for goblet cell assessment.

4:94 Location

Edinburgh.

4:95 Results

The doses of 5, 10 and 20 mg/kg isoprenaline, when administered alone, produced a series of dose-related responses (Figure 21).

Thus, after 5 mg/kg isoprenaline there were 5.3 goblets/HPF, after 10 mg/kg there were 6.5 goblets/HPF and after 20 mg/kg there were 8.0 goblets/HPF. These results are all significantly different ($P = <0.0025$) from the saline control value of 2.2 goblets/HPF.

50 mg/kg propranolol alone resulted in a mean number of goblet cells of 2.0 goblets/HPF and this compares favourably with that obtained for the saline controls. As in the previous experiment propranolol has had no effect on respiratory goblet cells. In the presence of 50 mg/kg propranolol the three doses of isoprenaline tested (5, 10 and 20 mg/kg) failed to produce an increase in respiratory goblet cells. Propranolol therefore antagonised the isoprenaline response and abolished the isoprenaline dose-response curve.

FIGURE 21

Showing the effect of propranolol (50 mg/kg) on the isoprenaline dose-response curve. The vertical bars represent the standard error of the mean. The open circles show the responses obtained to 5, 10 and 20 mg/kg isoprenaline. They are all significantly different from control, with $P = <0.0005$ for 5 and 20 mg/kg and $P = <0.0025$ for 10 mg/kg. The closed circles show the responses obtained with 5, 10 and 20 mg/kg isoprenaline in rats pretreated with 50 mg/kg propranolol, of these the results obtained with 10 and 20 mg/kg isoprenaline are significantly different from the corresponding results obtained in the absence of propranolol with $P = <0.025$ and $P = <0.0005$ respectively. The saline control is indicated by X and the propranolol control by a closed square.

Saline n = 10
Propranolol n = 9
Isoprenaline 5 mg/kg n = 4
Isoprenaline 10 mg/kg n = 7
Isoprenaline 20 mg/kg n = 7
Propranolol + 5 mg/kg isoprenaline n = 9
Propranolol + 10 mg/kg isoprenaline n = 9
Propranolol + 20 mg/kg isoprenaline n = 8

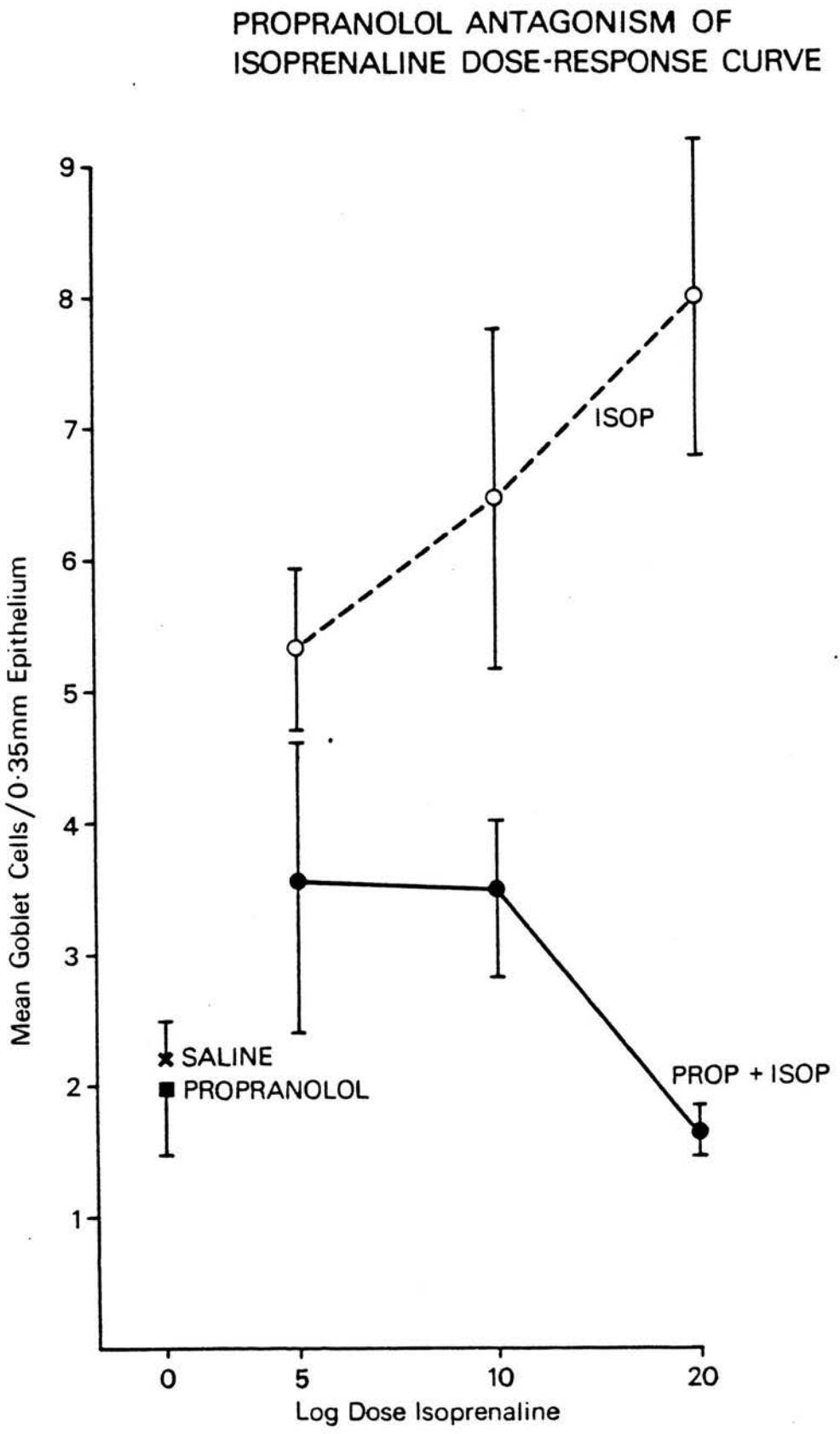

PROPRANOLOL ANTAGONISM OF
ISOPRENALINE DOSE-RESPONSE CURVE
Mean Goblet Cells/0·35mm Epithelium
9
8
7
6
5
4
3
2
1
ISOP
SALINE
PROPRANOLOL
PROP + ISOP
0
5
10
20
Log Dose Isoprenaline

FIGURE 21.

4:10 EXPERIMENT 10

THE EFFECT OF VARIOUS ADRENERGIC AND CHOLINERGIC DRUGS ON RAT AIRWAY EPITHELIUM

4:10.1 Objectives

Isoprenaline is a powerful β-adrenoceptor agonist and it is tempting to think that its effect on respiratory goblet cells is related to its chief pharmacological actions. So far the only evidence obtained in support of that assumption is the very shallow dose-response curves and the propranolol antagonism of the isoprenaline response. Further evidence in support of this hypothesis may be obtained by examining the effects of other drugs of known pharmacological properties. This experiment was designed to test several drugs with known pharmacological properties for an effect on respiratory goblet cells.

4:10.2 Expermental Details

Groups of 5 male rats were given the following drugs and doses:-

				Receptor
Salbutamol	(IP)	40 mg/kg and	4 mg/kg	(β_2)
Terbutaline	(IP)	40 mg/kg and	4 mg/kg	(β_2)
Isoprenaline	(IP)	40 mg/kg and	4 mg/kg	(β_1 and β_2)
Methoxamine	(IP)	20 mg/kg and	2 mg/kg	(α)
Phenylepherine	(IP)	20 mg/kg and	2 mg/kg	(α)

Pilocarpine	(IP)	40 mg/kg and	4 mg/kg	(Muscarinic)
Carbachol	(IP)	60 µg/kg and	6 µg/kg	(Muscarinic)
Nicotine	(IP)	62 µg/kg and	6.2 µg/kg	(Nicotinic)

Ten additional rats were given 0.5 ml saline IP and these served as controls. All the animals were killed 24 hours after treatment.

4:10.3 Tissues and Processing

The lungs were inflation fixed with Millonig's fixative and the left lungs embedded in GMA for goblet cell assessment.

4:10.4 Location

Edinburgh.

4.10.5 Results

Of the drugs tested only the sympathomimetic drugs were found to be capable of producing an increase in respiratory goblet cells (Figures 22 and 23). Control goblet cell numbers for this experiment were 3.84 goblet/HPF; 40 mg/kg racemic isoprenaline increased goblet cell numbers to 8.49 goblets/HPF, 40 mg/kg salbutamol induced a rise to 6.5 goblets/HPF and 20 mg/kg methoxamine induced a rise to 7.8 goblets/HPF. All these increases were significantly different from the control with $P = <0.0005$, <0.025 and <0.005 respectively. None of the other

FIGURE 22

Showing the responses obtained to a variety of sympathomimetic drugs. The columns represent the means obtained for each drug and the vertical bars indicate the standard errors. The responses marked with an asterisk were significantly different from the controls, $P = <0.0005$, $P = <0.005$ and $P = <0.025$ for the isoprenaline, methoxamine and salbutamol respectively.

Saline n = 8
Isoprenaline 40 mg/kg n = 5
Salbutamol 40 mg/kg n = 4
Terbutaline 40 mg/kg n = 4
Methoxamine 20 mg/kg n = 4
Phenylephrine 20 mg/kg n = 2

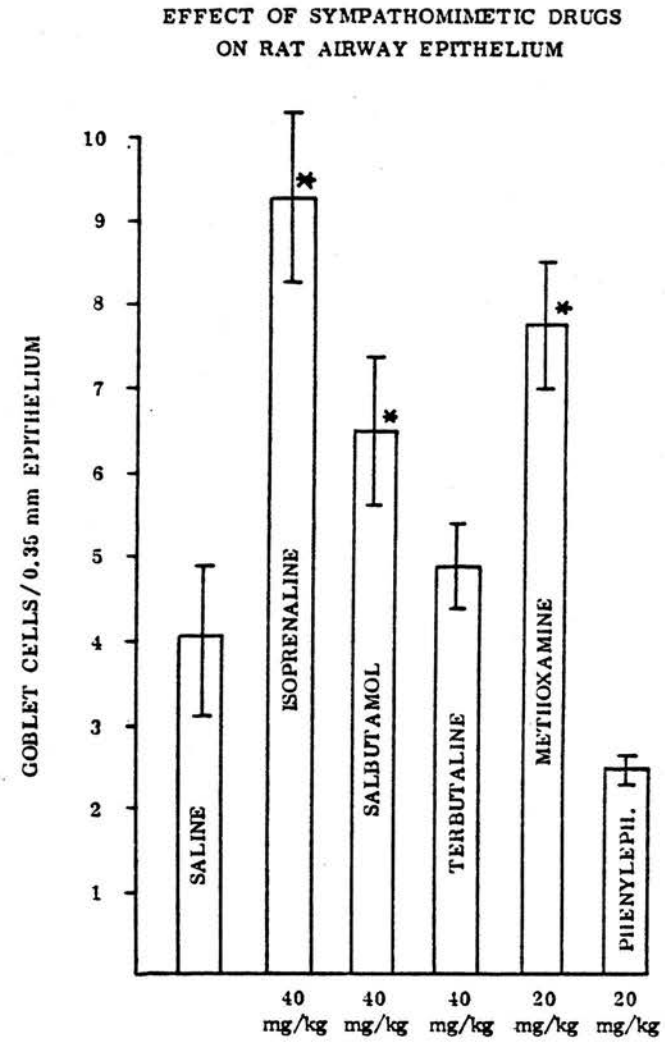

EFFECT OF SYMPATHOMIMETIC DRUGS
ON RAT AIRWAY EPITHELIUM
GOBLET CELLS/0.35 mm EPITHELIUM
10
9
8
7
6
5
4
3
2
1
SALINE
ISOPRENALINE
SALBUTAMOL
TERBUTALINE
METHOXAMINE
PHENYLEPH.
40 mg/kg
40 mg/kg
40 mg/kg
20 mg/kg
20 mg/kg

FIGURE 22.

FIGURE 23

As for Figure 22 but with parasympathomimetic drugs. The response to 4 mg/kg pilocarpine was significantly different from control, $P = <0.05$.

Saline n = 8
Isoprenaline 40 mg/kg n = 5
Pilocarpine 40 mg/kg n = 5
Pilocarpine 4 mg/kg n = 5
Carbachol 60 µg/kg n = 5
Carbachol 6 µg/kg n = 5
Nicotine 61.7 µg/kg n = 3
Nicotine 6.2 µg/kg n = 4

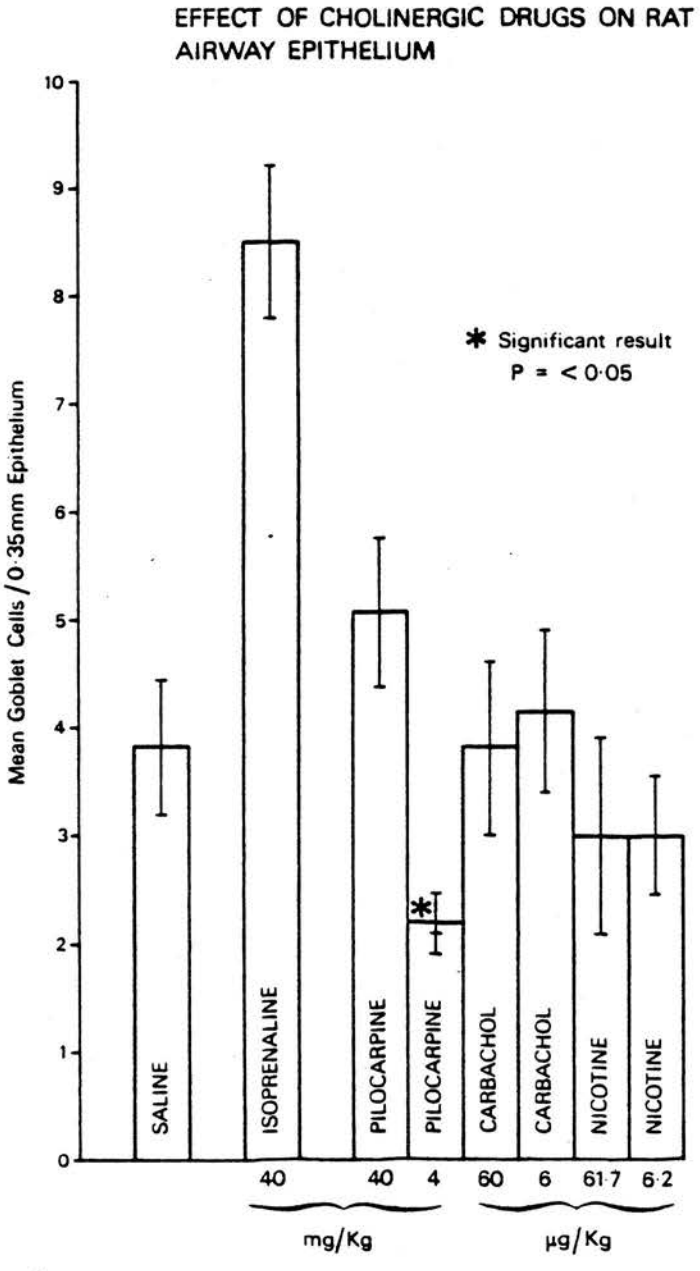

EFFECT OF CHOLINERGIC DRUGS ON RAT AIRWAY EPITHELIUM
Mean Goblet Cells / 0·35mm Epithelium
10
9
8
7
6
5
4
3
2
1
0
* Significant result
P = < 0·05
SALINE
ISOPRENALINE
PILOCARPINE
PILOCARPINE
CARBACHOL
CARBACHOL
NICOTINE
NICOTINE
40
40
4
60
6
61·7
6·2
mg/Kg
µg/Kg

FIGURE 23.

drugs or doses produced significant effects on respiratory goblet cells with the exception of 4 mg/kg pilocarpine which induced a reduction in goblet cell numbers to 2.19 goblets/HPF and this was just significant with $P = <0.05$. However, 40 mg/kg pilocarpine produced a slight but not significant rise in goblet cells. The significance of these conflicting results is not known.

No clear-cut receptor type has emerged as being involved in the goblet cell response in this experiment. Of the drugs inducing goblet cell hyperplasia, isoprenaline acts equaly on β_1 and β_2-adrenoceptors, salbutamol is β_2-adrenoceptor selective, and methoxamine is α-adrenoceptor selective.

4:11 EXPERIMENT 11

NEBULISER ADMINISTRATION: EFFECT OF ISOPRENALINE AEROSOL ON RESPIRATORY GOBLET CELLS

4:11.1 Objectives

All the previous experiments involved the systemic administration of a single high dose of isoprenaline. This experiment was carried out to see if, by applying the isoprenaline directly to the respiratory epithelium, it was possible a) to eliminate non-specific systemic effects and b) to decrease the dose necessary to induce goblet cell hyperplasia.

4:11.2 Experimental Details

Three groups of 5 male rats were exposed to either Nebulised isoprenaline solution (100 mg/ml or 10 mg/ml) or saline for 45 minutes. The aerosol was produced using a Wright's Nebuliser as described in the methods section 2:2.4. All the animals received a 5 minute acclimatisation period with saline aerosol before being exposed to the different drug solutions. The isoprenaline solution contained approximately 10^{-2}M ascorbic acid as antioxidant because it was felt that the continual agitation and mixing with air under pressure would almost certainly lead to breakdown of the isoprenaline. 10^{-2}M ascorbic acid was also added to the saline solution. During each exposure period the atmosphere in the chamber was sampled twice using a total atmosphere sampler and once with an Anderson 4-stage impaction sampler. Each sample took place over 15 minutes and therefore the atmosphere was monitored continuously throughout the run.

At the end of the exposure period the rats were removed from the chamber, rehoused in standard cages and killed 24 hours later using an overdose of CO_2 gas.

4:11.3 Tissues and Processing

The lungs were inflation fixed with Millonig's fixative and the left lungs embedded in GMA for goblet cell assessment.

4:11.4 Location

Alderley Park.

4:11.5 Results

An increase in respiratory goblet cells was observed following aerosol administration of isoprenaline (Figure 24). The increases observed, however, were small. 100 mg/ml isoprenaline solution increased goblet cells from the control level of 3.82 goblets/HPF to 5.72 goblets/HPF, just significantly different from the control with $P = <0.05$. The smaller dose of 10 mg/ml resulted in an increased level of goblet cells of 5.8 goblets/HPF, this was also just significantly different from the control with $P = <0.05$. Thus, the ten-fold difference in drug solutions (100 mg/ml and 10 mg/ml) was not reflected in the response.

Calculations of the atmosphere generated in the chamber and the approximate dose to the rats were made from the data obtained by the various sampling techniques. The calculations are shown in detail in Appendix iii. A summary of the results is given overleaf:-

Solution	Dose by TAS	Dose by Anderson	Majority Particle size	% Absorbed in lung
100 mg/ml	658.3 µg/rat in 45 mins	2.15 mg/rat in 45 mins	< 3 µm	20 - 50%
10 mg/ml	162.4 µg/rat in 45 mins	516 µg/rat in 45 mins	< 3 µm	20 - 50%

FIGURE 24

As for Figure 22 but showing the responses obtained to saline and two solutions of isoprenaline (100 mg/ml and 10 mg/ml) when administered as an aerosol mist for 45 minutes. Both the solutions of isoprenaline tested produced significant increases in goblet cells when compared to the saline control, $P = <0.05$.

Saline n = 5
100 mg/ml isoprenaline n = 3
10 mg/ml isoprenaline n = 2

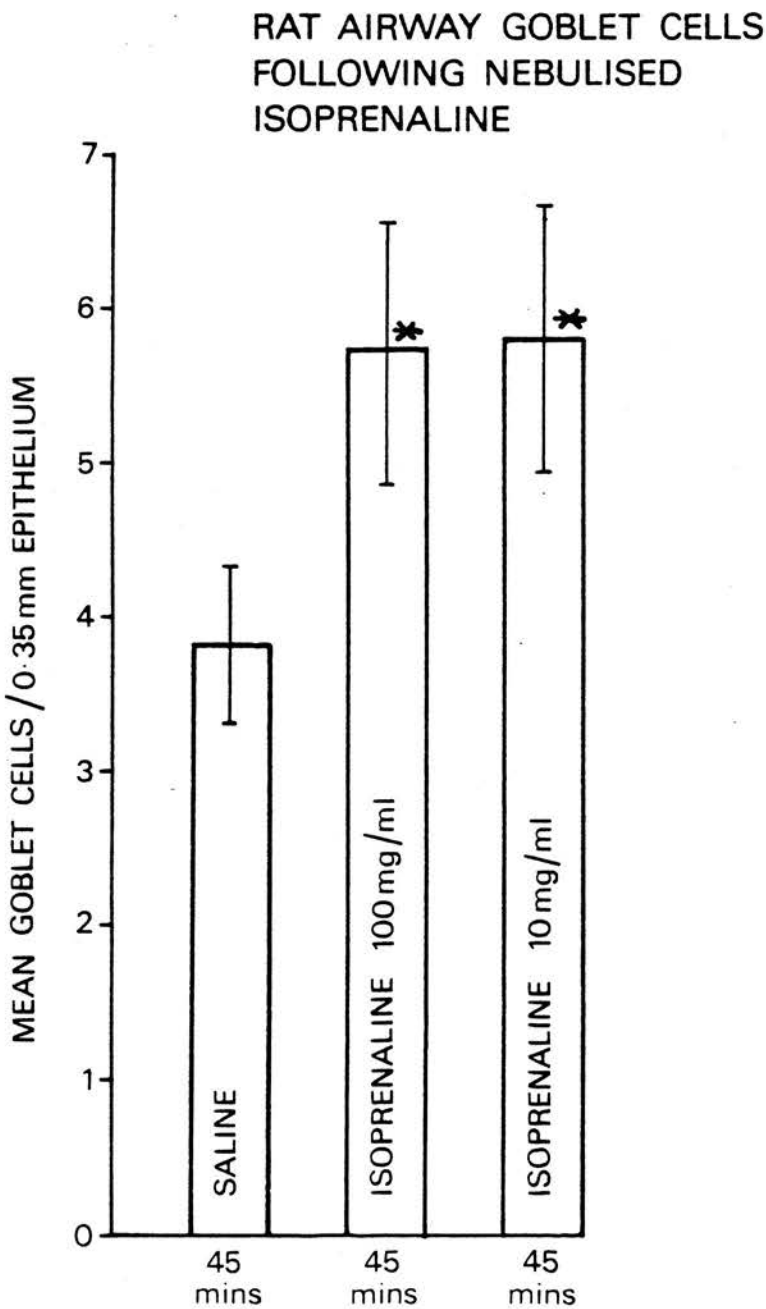

RAT AIRWAY GOBLET CELLS
FOLLOWING NEBULISED
ISOPRENALINE
MEAN GOBLET CELLS /0·35 mm EPITHELIUM
0
1
2
3
4
5
6
7
SALINE
ISOPRENALINE 100 mg/ml
ISOPRENALINE 10 mg/ml
*
*
45
mins
45
mins
45
mins

FIGURE 24.

There is a big discrepancy in the doses calculated using the two methods, with the TAS (total atmosphere sampler) giving a lower reading than the Anderson sampler. It is therefore not possible to say precisely the eventual dose received by each rat in either run, however, it does appear that a goblet cell increase was induced using much lower doses of isoprenaline than had been used systemically.

4:12 EXPERIMENT 12

RAT AIRWAY GOBLET CELLS FOLLOWING INTRATRACHEAL ISOPRENALINE: DOSE-RESPONSE INVESTIGATION I

4:12.1 Objectives

This particular technique has the advantage over aerosol administration in that the actual dose of drug is known. It is, however, time-consuming to carry out. This experiment was designed to investigate the dose-resonse curve obtainable using this route of administration.

4:12.2 Experimental Details

Groups of 8 male rats were anaesthetised with Sagatal, as described in section 2:2.4. Because of the time factor each group was divided in half and two separate lots of

four were dosed on different days. The doses of isoprenaline employed were:-

10 mg/rat IT ≡ 40 mg/kg IP

4 mg/rat IT ≡ 16 mg/kg IP

1.25 mg/rat IT ≡ 6 mg/kg IP

0.625 mg/rat IT ≡ 2.5 mg/kg IP

0.25 mg/rat IT ≡ 0.1 mg/kg IP

0.1 ml saline was injected intratracheally into 8 control rats. All the animals were killed 24 hours later using an overdose of CO_2 gas.

4:12.3 Tissues and Processing

The lungs were inflation fixed with Millonig's fixative and the left lungs embedded in GMA for goblet cell assessment.

4:12.4 Location

Alderley Park.

4:12.5 Results

A very clear dose-response relationship was observed between the degree of goblet cell hyperplasia and the dose of isoprenaline administered (Figure 25). The range of doses used was lower than those used in the systemic dose-response curve (Experiment 8) and as a consequence a

FIGURE 25

Showing the dose-response curve obtained with 0.25, 0.625, 1.25, 4 and 10 mg/rat isoprenaline administered as an intratracheal injection. The vertical bars represent the standard errors of the means.

Saline n = 7
0.25 mg isoprenaline n = 5
0.625 mg isoprenaline n = 8
1.25 mg isoprenaline n = 7
4 mg isoprenaline n = 8
10 mg isoprenaline n = 7

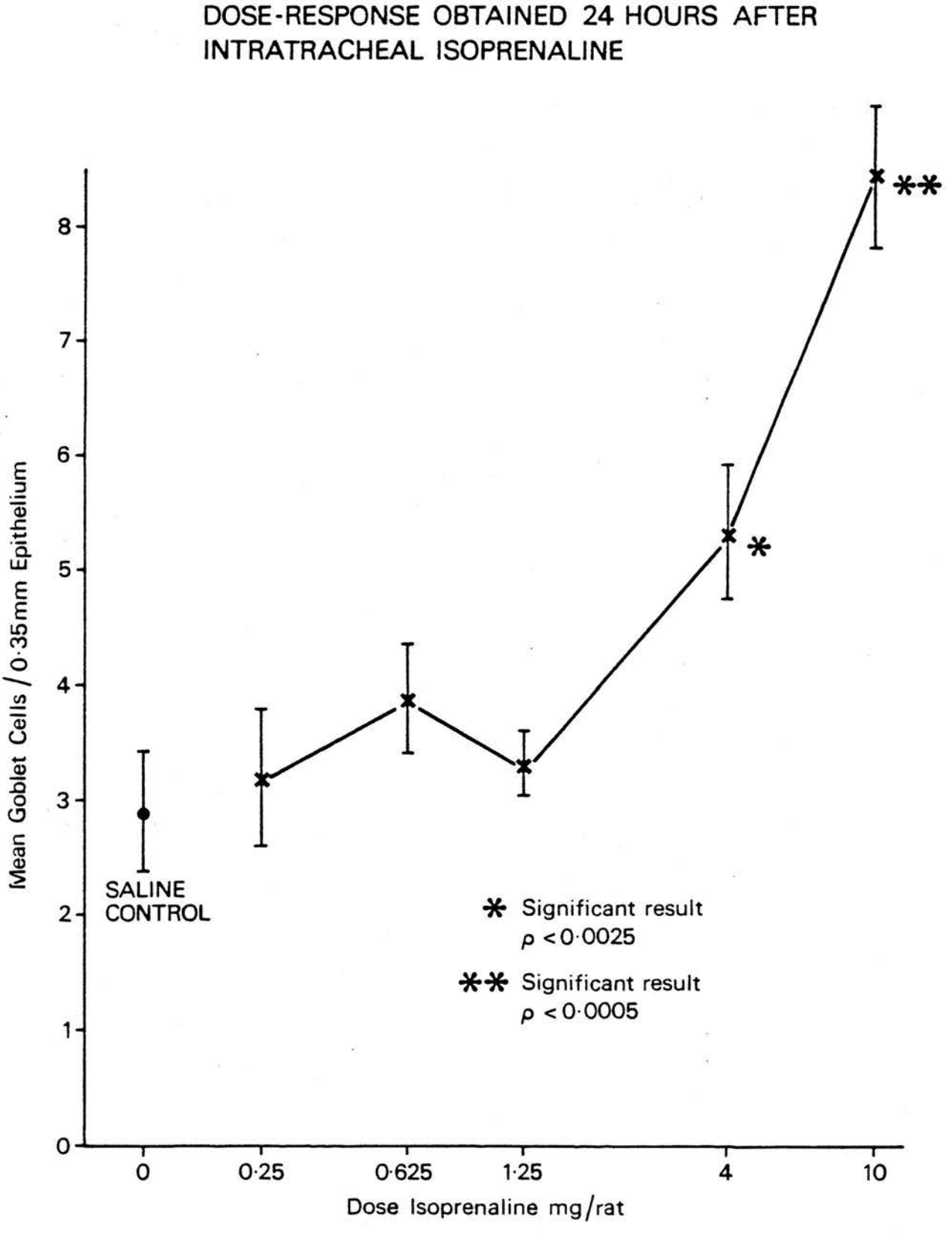

DOSE-RESPONSE OBTAINED 24 HOURS AFTER INTRATRACHEAL ISOPRENALINE
Mean Goblet Cells / 0·35mm Epithelium
0
1
2
3
4
5
6
7
8
SALINE CONTROL
* Significant result ρ < 0·0025
** Significant result ρ < 0·0005
0
0·25
0·625
1·25
4
10
Dose Isoprenaline mg/rat

FIGURE 25.

maximum plateau response was not observed. The maximum dose of 10 mg/rat intratracheally increased respiratory goblet cells from a control value of 2.9 goblets/HPF to 7.9 goblets/HPF, a 192% increase. In experiment 8 the nearest equivalent IP dose to this IT dose which was 40 mg/kg IP gave a 196% increase in respiratory goblet cells. Therefore, isoprenaline given intratracheally induces increased numbers of respiratory goblet cells, the increase so produced being of the same order of magnitude and occurring after similar dose-levels to those which were used to produce the systemic dose-response curve.

The use of the Student's t-test showed that in none of the groups was there any significant difference between the two halves operated on separate days. Figure 25 therefore shows the overall mean for each group calculated using the results of two halves.

4:13 EXPERMENT 13

CONFIRMATION OF THE STEREOSPECIFICITY OF THE RESPONSE FOR THE (-) ISOMER OF ISOPRENALINE

4:13.1 Objectives

In experiment 2 it was observed that the goblet cell response was stereospecific for the (-) isomer of isoprenaline. However, very low doses were used in this experiment. Since the bulk of the work in this study has

involved single large doses of the isoprenaline racemate, the following experiments were designed to reconfirm the stereospecificity of the response using comparable large doses of the isomers.

4:13.2 Experimental Details

Experiment A:

Groups of 5 male rats received 40 mg/kg IP of either racemic isoprenaline, (-) isoprenaline, (+) isoprenaline or 0.5 ml saline. The isomers used in this experiment were the same as those used in Experiment 2.

Experiment B:

Groups of 10 male rats received 40 mg/kg IP of either racemic isoprenaline, (-) isoprenaline, (+) isoprenaline or 0.5 ml saline. The isomers used in this experiment were bought immediately prior to use and their optical rotations were checked by Aldrich and found to be correct.

All the animals were killed 24 hours after dosing.

4:13.3 Tissues and Processing

The lungs were inflation fixed with Millonig's fixative and the left lungs embedded in GMA for goblet cell assessment.

4:13.4 Location

Both in Edinburgh.

FIGURE 26

As for Figure 22 but showing the results of two separate experiments comparing the two isomers of isoprenaline with the racemate. The isomers used in experiment A were the same as those used in experiment 2; the isomers used in experiment B were purchased immediately before the experiment and their optical rotations were checked by the manufacturers, Aldrich Chemicals. In both experiments all 3 drugs produced significant increases in goblet cells with $P = <0.0005$.

Experiment A	Experiment B
Saline n = 8	Saline n = 19
(±) isoprenaline n = 5	(±) isoprenaline n = 10
(-) isoprenaline n = 4	(-) isoprenaline n = 8
(+) isoprenaline n = 5	(+) isoprenaline n = 10

EFFECTS OF THE ISOMERS OF ISOPRENALINE
ON RESPIRATORY GOBLET CELLS

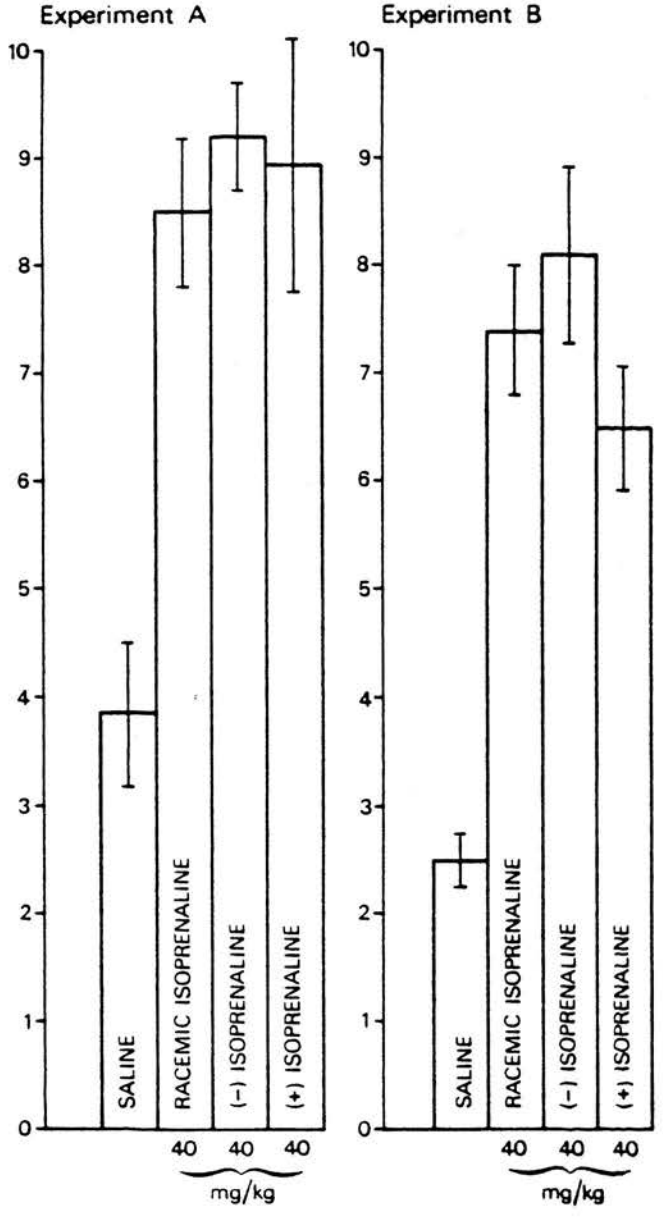


FIGURE 26.

4:13.5 Results

Experiment A:

Both isomers at the dose of 40 mg/kg induced the same degree of goblet cell hyperplasia as 40 mg/kg of the racemate (Figure 26A). There was no significant difference between the increases induced by the three drugs.

Experiment B:

Very similar results were found with the recently purchased isomers (Figure 26B). Thus, at 40 mg/kg there was no significant difference between the responses to the two isomers and the response to the racemate.

In conclusion therefore, at 40 mg/kg the goblet cell response was not stereospecific for either isomer of isoprenaline.

4:14 SUMMARY OF THE EXPERIMENTAL RESULTS

1) A single IP dose of 40 mg/kg isoprenaline racemate was shown to induce a consistent and reproducible three-fold increase in respiratory goblet cells in the rat.

2) Using this single dose model it has been possible to demonstrate a shallow dose-response relationship between the dose of isoprenaline and the degree of goblet cell hyperplasia induced.

3) The single dose model has also been used to study the time-course of the goblet cell response. The earliest detectable increase occurs at 12 hours after the isoprenaline administration and the increase first reaches statistical significance at 18 hours.

4) The maximum increase in goblet cells occurs between 24 and 48 hours after the isoprenaline administration.

5) The isoprenaline induced goblet cell hyperplasia lasted between 8 and 16 days after the administration of a single 40 mg/kg dose of the drug.

6) It was found that the goblet cell response was unaffected by either the time of injection or the time of death.

7) Study of control goblet cell numbers in the rat lung showed that during a 24 hour period there was no circadian fluctuation in the goblet cell population.

8) It was possible to inhibit the isoprenaline-induced goblet cell hyperplasia pharmacologicaly with a β-adrenoceptor antagonist. Thus, in the presence of propranolol the isoprenaline dose-response curve was abolished.

9) It was also possible to mimic the isoprenaline effect on respiratory goblet cells with other systemic sympathomimetic drugs. The β_2-adrenoceptor agonist salbutamol, and the α-adrenoceptor agonist methoxamine were both shown to induce statistically significant increases in respiratory goblet cells when given as a single large dose.

10) Ten small daily doses of isoprenaline (3.7 mg/kg IP) were found to induce a larger increase in respiratory goblet cells than a single 3.7 mg/kg IP dose.

11) The response to ten daily 3.7 mg/kg doses of isoprenaline IP was comparable to that which followed a single 40 mg/kg IP dose of isoprenaline.

12) At lower doses (2.4 mg/kg IP for 10 days, or as a single dose) the goblet cell response was found to be stereospecific for the (-) isomer of isoprenaline.

13) This stereospecificity was lost at higher doses, e.g. both isomers produced similar responses at 40 mg/kg IP (single dose), as a single dose of 40 mg/kg racemic isoprenaline IP.

14) Isoprenaline was capable of inducing goblet cell hyperplasia in the lung when applied directly to the respiratory epithelium as an aerosol mist.

15) Isoprenaline was also capable of inducing goblet cell hyperplasia in the lung when administered as a single intratracheal injection.

16) Isoprenaline applied intratracheally had a dose-dependent effect on respiratory goblet cells.

17) The goblet cell increase induced by intratracheal isoprenaline was the same size as that induced by the comparable systemic dose of isoprenaline.

18) There was no difference between the goblet cell responses of male and female rats.

19) There was a tendency for goblet cells to increase with age/body weight in control rats.

20) A single exposure to a high concentration of SO_2 gas induced goblet cell hyperplasia in the rat lung.

21) The timecourse of the SO_2 gas single dose model was different to the timecourse observed after isoprenaline.

22) Salivary gland and heart weights were recorded at autopsy in most of the isoprenaline experiments. A single IP 40 mg/kg dose of isoprenaline had no effect on the salivary gland or heart weight. However, in experiment 2 the groups receiving multiple doses of isoprenaline or its two isomers had increased salivary gland and heart weights.

CHAPTER 5

DISCUSSION

5:1 GENERAL DISCUSSION

This study has shown that a single intraperitoneal dose of 40 mg/kg of racaemic isoprenaline produces a reproducible three-fold increase in epithelial goblet cells in the proximal respiratory epithelium of the rat. The timing of the response has been investigated and it was found that a slight increase in goblet cells can be detected as early as 12 hours after the isoprenaline administration. A maximum increase occurs between 24 and 48 hours after the isoprenaline administration and thereafter the increase gradually declines taking up to 8 - 16 days to regain control goblet cell numbers. The dose dependency of the response was studied in some detail and a shallow dose-response curve was found. Various sympathomimetic and parasympathomimetic drugs were tested to see if any were capable of mimicking the isoprenaline induced goblet cell hyperplasia. Of the drugs tested only salbutamol, a β_2-selective adrenoceptor agonist and methoxamine, an α-selective adrenoceptor agonist, were able to induce significant increases in epithelial goblet cells in the proximal respiratory epithelium of the rat. Attempts were made to antagonise the isoprenaline response pharmacologically using propranolol, a β-adrenoceptor

antagonist. It was found that propranolol, when given 30 minutes prior to the isoprenaline, was capable of abolishing the isoprenaline dose-response curve. All the above findings were made using the systemic administration of the isoprenaline; however, further experiments were carried out to see if differing routes of administration had any effect on the respiratory goblet cell response. Pilot studies were carried out using intratracheal injections of the isoprenaline and also the aerosol administration of the isoprenaline generated by a Wright's nebuliser. Both routes of administration resulted in an isoprenaline-induced increase in the goblet cell population of the proximal respiratory epithelium. The intratracheal route was further investigated and the dose-dependency of the goblet cell response was studied using this route of administration. A dose-reponse curve was obtained, the range of which was similar to that obtained using systemic injections. Thus, direct application of the isoprenaline to the respiratory epithelium did not result in a reduction in the dose of isoprenaline needed to induced goblet cell hyperplasia. Furthermore, the magnitude of the response thus obtained was very similar to that obtained using the systemic route. In many experiments the heart and salivary gland weights were recorded at autopsy. Multiple injections of isoprenaline were found to increase the weight of both organs, however, a single 40 mg/kg dose of

isoprenaline failed to produce a change in either organ weight 24 hours later.

To conclude, this study has established a reproducible single-dose pharmacological animal model of goblet cell hyperplasia in the respiratory epithelium which is free from infection and inflammation. This, therefore, has great potential as a tool for studies on the goblet cell hitherto not possible, such as cell kinetics studies to find their origin within the respiratory epithelium.

5:12 AIMS OF THE DISCUSSION

1) To relate the new findings of this study with previous work. With special reference to:
   (a) Isoprenaline multiple injection studies in both rats and pigs.
   (b) Sulphur dioxide multiple and single exposure studies.
   (c) Tobacco smoke and nicotine exposure.

2) To discuss the problems encountered during the course of the study. These will be divided into three sections:
   (a) Histological problems particularly with araldite embedding and cutting techniques.
   (b) Development of a novel, faster screening technique.
   (c) Animal health and rat lung pathology.

3) To discuss the mechanism by which the isoprenaline induces an increase in goblet cells. This will be divided into two sections:

   (a) Evidence for a direct pharmacological effect.

   (b) Evidence for an indirect biochemical, metabolic or humoral effect.

4) To outline the possible origins of the isoprenaline-induced new goblet cells. There are five possible mechanisms which will be dealt with in turn:

   (a) Isoprenaline increases mitosis within the respiratory epithelium leading either to increased overall cellularity or selectively increased goblet cells.

   (b) Isoprenaline induces altered differentiation in a multipotential precursor cell population such that they 'switch on' mucin synthesis.

   (c) Isoprenaline stimulates a quiescent population of pre-goblet cells into mucin synthesis.

   (d) Isoprenaline induces the transformation of a differentiated mature cell into goblet cells by causing mucin synthesis to occur - 'metaplasia'.

   (e) Isoprenaline blocks mucin secretion with or without increasing mucin synthesis. This would lead to an increase in full goblet cells and a subsequent decrease in empty goblet cells, therefore more would be seen.

This will be followed by a concluding statement on the origin of the new goblet cells.

5) To discuss the clinical implications of the findings.

In the next chapter, Chapter 6, a series of brief statements will be made about the main conclusions of the study. This will be followed, in Chapter 7, by proposals for further work arising from the study.

5:2 COMPARISON OF THE RESULTS OF THIS STUDY WITH THOSE OF PREVIOUS STUDIES ON THE GOBLET CELL

5:21 Studies using Sympathomimetic Drugs in the Rat

In this study a single intraperitoneal dose of 40 mg/kg racemic isoprenaline produced a reproducible 3 to 4-fold increase in goblet cell numbers in the proximal respiratory epithelium of the rat. This compares very favourably with the results of Sturgess and Reid (1973), Bolduc and Reid (1978), and Jones and Reid (1979a and 1979b), all of whom used much higher doses (100 mg/kg subcutaneously) administered daily for either 6 or 12 days. Thus, a 4-fold increase in goblets is the maximum response obtained following isoprenaline administration.

In this study the increase in goblet cells following a single 40 mg/kg dose of isoprenaline was found to persist for up to 8 to 16 days after the isoprenaline

administration. Jones and Reid (1979a) found that in the trachea goblet cells took one week to return to control values, 8 weeks in the main bronchus, and more than 12 weeks in the peripheral airways following 6 daily injections of 100 mg/kg isoprenaline. It therefore appears that the longer the isoprenaline stimulus, the longer the goblet cell response persists, and also that goblet cells in different areas of the respiratory tract return to control values at different rates.

The finding in this study that salbutamol, a β_2-selective adrenoceptor agonist was less potent than isoprenaline as an inducer of goblet cell hyperplasia confirms work carried out by Jones and Reid (1979b). In the present study, the dose used was 40 mg/kg as a single intraperitoneal injection; Jones and Reid gave 100 mg/kg Salbutamol daily for 6 days subcutaneously. They found that isoprenaline at the same dose level had a much greater effect on goblet cell numbers than did Salbutamol. They also found regional differences between the responses to the two drugs. Whereas isoprenaline induced an increase in goblet cells in zones 1 and 3 of the trachea and all zones IV - VII in the lung, salbutamol only increased goblet cells in zones I and V (upper trachea and proximal intra-pulmonary airways). However, both drugs were found to produce the same change in the type of mucin synthesised to predominantly acid glycoprotein. The significances of these differences are not known.

5:22 Studies using Pilocarpine in the Rat

Pilocarpine, a parasympathomimetic drug, did not produce an increase in respiratory goblets in the proximal airways of the rat in this study. The doses administered were 4 mg/kg and 40 mg/kg as a single intraperitoneal injection. However, other workers have found an increase in respiratory goblet cells following pilocarpine administration. Sturgess and Reid (1973) administered 40 mg/kg daily for 6 and 12 days and found an increase in the trachea from 40 goblet cells per 6 mm epithelium to 60 after 6 injections and a further increase to 160 goblet cells per 6 mm epithelium after 12 injections. They also stated that an increase occurred in the bronchial epithelium but gave no details. Bolduc and Reid (1978) on the other hand found that after 40 mg/kg pilocarpine daily for 12 days, a significant increase in goblet cells occurred in the upper trachea only; there was a slight, but not significant, rise in the proximal airway epithelium. The evidence for a cholinergic element in the control of goblet cell numbes is therefore slender and an increase in tracheal goblet cells has only been detected after prolonged administration of high doses of pilocarpine.

5:23 Other Studies on the Goblet Cell Population of the Lung

Baskerville (1975) studied the effect of isoprenaline on respiratory goblet cells in the pig. He showed that after 6 daily injections of 75 mg isoprenaline there was a significant increase in goblet cells in the bronchial epithelium. The increase occurred within 24 hours of the last injection and was maintained for 16 days, thereafter there was a gradual decline in goblet cell numbers until they regained control values 84 days after the last injection of isoprenaline. Although the increase in goblet cells occurring in the rat after a single dose of isoprenaline does not last as long as it does in the pig after multiple doses of isoprenaline, there are important similarities between the responses of the two species. The fact that the response is not limited to the rat implies that, whatever the mechanism is by which goblet cell hyperplasia is induced, it occurs in more than one species of animal and may also occur in man, particularly as the pig respiratory epithelium is very similar to that of man.

Much work has been carried out looking into the effects of tobacco smoke on the cell populations of the bronchial epithelium. Lamb and Reid (1969) demonstrated that cigarette and cigar smoke produced a dose-dependent increase in goblet cells throughout the lung. The effect was, however, greatest in the proximal airways. Tobacco smoke contains a mixture of elements and it is impossible

to say which of its constituents is responsible for the goblet cell hyperplasia. However, Wang et al. (1979) have shown that in the guinea pig exposure to 1% nicotine aerosol twice a day, 5 days a week for 3 weeks produces a goblet cell and Clara cell hyperplasia in the large airways. In the present study a single systemic dose of nicotine intraperitoneally (6.2 µg/kg and 62 µg/kg) failed to have any effect on the goblet cell population of the proximal airways of the rat. This may be because the effect is only seen after prolonged exposure directly to the respiratory epithelium, or alternatively, the doses given systemically may have been sufficiently high to be toxic and thus masked any effect.

5:24 Studies using Sulphur Dioxide Gas

One experiment using sulphur dioxide gas was performed in this study. Rats were exposed to approximately 500 ppm sulphur dioxide gas for a single 4 hour period. They were then killed at various times later. Following exposure to the gas, goblet cells in the proximal airways of the rat steadily increased over a matter of days reaching a maximum value 6 days after the SO_2 exposure. Thereafter there was a very gradual decline in numbes such that at 11 days after the SO_2 exposure numbers were still much greater than the control. A single dose of isoprenaline on the other hand produces a rapid increase in goblet cells which reaches a maximum between 24 and 48 hours after the injection,

thereafter goblet cell numbers gradually decline regaining control values between 8 and 16 days after the administration of the isoprenaline. The timing of the two responses are therefore very different. In addition, the response to sulphur dioxide is much greater, particularly when administered over a prolonged period of time (Lamb and Reid, 1968). Sulphur dioxide gas is an irritant and can cause severe inflammatory reactions within the respiratory epithelium. The delay in the increase in goblet cells is, in fact, due to desquamation of the surface epithelium which is followed by regeneration and an increase in goblet cells (Lamb and Reid, 1968). Thus, sulphur dioxide brings about an increase in goblet cels by means of an inflammatory action whilst isoprenaline does so by some unknown pharmacological, biochemical or humoral effect.

5:25 Summary

Although much of the work of this thesis is new and has not been reported before, it compares very favourably with the limited amount of similar work previously reported, namely, the size and duration of the response. It has been possible to produce an increase in goblet cells of the proximal airways of the rat, using a single 40 mg/kg dose of isoprenaline, equal to that previously produced using 12 and 6 daily injections of 100 mg/kg isoprenaline. This increase has been shown to be reproducible and occurs equally in both male and female rats. The response was not

confined to a single species of animal or a particular strain of rat and as such represents a very useful tool for the study of the goblet cell.

5:3 PROBLEMS ENCOUNTERED DURING THE COURSE OF THE STUDY

5:31 Problems with Histological Techniques

Many previous quantitative studies on the lung goblet cell population have taken place using 5 um thick paraffin sections stained with a mucin specific stain, such as the combined Alcian Blue/Periodic Acid Schiff (AB/PAS) stain. The specificity of this stain precludes identification of other cell types as only those containing mucus show up. Application of counterstains is not helpful as they mask the mucin staining itself. Other disadvantages of paraffin sections are the poor preservation of the lung tissue, with shrinkage being a particular problem, and the thickness of sections which often means that nuclei in the field of view are in different planes and do not always correspond to the layer of cells under study.

Ideally what is required for a study such as this one are large araldite sections, 1 μm thick, of post-osmicated material. These would allow the same area to be studied at both the light and electron microscope level. In practice this was found to be impossible and a compromise was worked out (see Appendix v): The left lung was embedded in GMA

and large 1.5 μm sections cut and then stained with AB/PAS. These were used for the light microscopic assessment of goblet cell numbers. Meanwhile, selected right lungs were post-osmicated and embedded in araldite awaiting development of the technique for using larger glass knives.

All the difficulties encountered in the study serve to emphasise the need for a new embedding medium suitable for both the light and electron microscopic study of histological material. This would ideally be a resin which is water soluble in the unpolymerised state thus facilitating easier staining with a wide range of stains; all the reagents necessary should be less toxic than those presently in use; and whilst permitting easy handling of large thin sections the polymerised resin should be 'soft' enough to cut easily, thus eliminating the use of glass or diamond knives as required for epoxy resins. It would be advantageous if the new material were somewhat cheaper than those at present in use which work out at considerable cost for each block processed and cut.

With regard to cutting technique, the Reichart-Jung autocut was found to be excellent for dealing with large blocks of GMA. The tungsten carbide knives recommended for use with large araldite blocks were hopelessly inadequate and they have been relegated to extremely expensive trimming-in knives. The quality of epoxy resin sections cut using larger glass knives still falls short of the conventional EM 'thick' section, primarily because it is

almost impossible to flatten the sections once cut sufficiently for study at higher powers of magnification. In our hands, at least, this particular technique still requires a lot of work.

5:32 Development of a Fast-Screening Technique for the Quantitation of Tracheal Goblet Cells

Details of this technique along with the various methods of quantitation employed can be found in Appendix i. The actual methodology of the technique is very simple and fast, it being possible to have slides ready for counting the same day as the animals were killed. When compared to the 3 - 5 weeks delay for GMA embedded material, this is an obvious advantage. However, there exists some doubt as to whether the technique is capable of detecting all of the available goblet cells, particularly from the areas between the cartilage rings. When examined under the microscope the impressions obtained showed numerous discrete, highly PAS positive dots interspersed by occasional mucus strands and sheets of epithelial cells (see Figure 29). Proof is still required that these actually are goblet cells. In addition, more work is required on the method of quantitation and its reproducibility.

The preliminary nature of the work carried out using this technique makes it difficult to accurately assess the

technique as a whole. However, when an independent observer counted several slides and their results were compared with mine, there was close agreement between the two sets of results on several occasions. Bearing in mind that this was the first time that they had attempted to count goblet cells using this type of preparation this was very encouraging. It therefore seems likely that the technique has great potential for the fast quantification of tracheal goblet cells and it certainly merits further investigation.

5:33 Problems Encountered with Animal Health

Section 3:1 describes in some detail the effects that an outbreak of Sendei virus had on the project. This is a respiratory infection which has profound effects on the lung pathology of infected animals. Nevertheless, because it is of viral origin, commercial animal breeding units can still qualify for 4-star Specific Pathogen Free accreditation even when it is endemic in their breeding colonies. Lung disease has also been demonstrated in the lungs of rats reared (apparently successfully) under germ-free conditions (Lamb, 1975). It therefore appears that the onus for ensuring that animals used in lung research are free from respiratory disease lies with the purchaser and not with the supplier - a far from satisfactory state.

Histology is accepted as the most sensitive method of identifying early changes of chronic respiratory disease in

rats (Lindsey et al., 1971) and this was demonstrated in this project by the rise in control goblet cells which preceeded the clinical manifestation of Sendei virus infection at the Alderley Park breeding unit. It would be helpful if histological examination of longitudinal sections of airways always formed part of routine quality control in commercial animal breeding units, as this would detect early changes in lung pathology long before the animals became clinically ill. It would then be easier to obtain rats for lung research with reliably 'clean' lungs, thus eliminating one important source of variability.

5:4 POSSIBLE MECHANISM FOR THE ISOPRENALINE REPONSE

There are two distinct possible ways in which isoprenaline may induce respiratory goblet cell hyperplasia. These are firstly, that the effect is a straight forward pharmacological one; that is, it is linked to the drugs activity as a β-adrenoceptor agonist and its interaction with β-adrenergic receptors located in the airway. Secondly, the effect may be due to some indirect, metabolic, biochemical or humoral effect which need not necessarily be instigated within the airway epithelium itself.

The work carried out in this thesis has supplied evidence both for and against the response having a direct

pharmacological basis. This evidence has been summarised below and is followed by a discussion of each item.

Evidence for a pharmacological basis:

(i) The response exhibits dose-dependency

(ii) The response can be antagonised pharmacologically

(iii) The response was limited to sympathomimetic drugs

(iv) The response shows stereo-specificity at low doses

(v) Preliminary studies suggest the response is not limited to goblets cells in the lung

Evidence against a pharmacological basis:

(i) The systemic dose required is very high

(ii) Likewise the dose of β-antagonist required to inhibit the response is very high

(iii) No clear cut receptor type has emerged from studies with other sympathomimetics

(iv) The dose of isoprenaline required to increase respiratory goblet cells was not decreased by applying it directly to the respiratory epithelium

(v) The stereo-specificity of the response was lost at high dose levels

5:41 Discussion of Evidence for a Pharmacological Basis

Dose-Response Relationship: For the goblet cell response to have a pharmacological basis it must involve a drug-

receptor interaction. In this instance, this is an interaction between isoprenaline and β-adrenoceptors (adrenergic receptors). Such interactions obey (in simplistic terms) the law of mass action (Fingl and Woodbury, 1970). Thus, the higher the concentration of a drug at the receptor site the more numerous the interactions and the greater the reponse is likely to be. A study of the dose-reponse relationship for the isoprenaline induced goblet cell hyperplasia revealed that there was indeed a shallow dose-response relationship. This was demonstrated using both the systemic, i.e. intraperitoneal, and the inhalation, i.e. intratracheal routes of administration. The range of doses being from 5 mg/kg - 40 mg/kg by the intraperitoneal route and 1.25 mg - 10 mg by the intratracheal route. Doses higher than 40 mg/kg intraperitoneally resulted in either a response of similar magnitude obtained with 40 mg/kg or in a smaller response, there was therefore a maximal effect. The doses involved are very high when compared with those required to elicit changes in heart rate and blood pressure <u>in vivo</u> in the rate, namely 10 μg/kg - 100 μg/kg. However, other drugs have been shown to have different pharmacological effects at different dose-levels, e.g. aspirin at relatively low dose levels effectively relieves the pain of headache while at much higher dose levels it can relieve arthritic pain.

As stated earlier the dose-reponse curve for the goblet cell hyperplasia is shallow and doses above 40 mg/kg

resulted in a reduced response. These two characteristics probably reflect the toxic nature of such high doses of isoprenaline. It is likely, therefore, that the isoprenaline may no longer be acting as a selective β-adrenoceptor agonist and the existance of a dose-reponse relationship should only be taken as tenuous support for a pharmacological basis to the goblet cell response.

Pharmacological antagonism of the response by propranolol:
Propranolol is a competitive β-adrenoceptor antagonist and as such competes directly with the isoprenaline for the same β-adrenergic receptors. If the isoprenaline is acting through β-adrenoceptors to induce goblet cell hyperplasia then propranolol should, in a sufficient dose, be capable of antagonising the response. This it has been shown to do on two occasions. Firstly 50 mg/kg propranolol given 30 minutes prior to the administration of 40 mg/kg isoprenaline resulted in a goblet cell response which was 50% smaller than that obtained with 40 mg/kg isoprenaline on its own. Secondly, propranolol (50 mg/kg) was shown to abolish the isoprenaline/goblet cell dose-response curve obtained using 5, 10 and 20 mg/kg isoprenaline. As with the dose-reponse evidence, care must be exercised in interpreting these results as evidence in support of a pharmacological basis for the response.

The dose of propranolol used was, with hindsight, too large and further work is required using smaller doses to see if isoprenaline can indeed overcome the propranolol, i.e.

successfully compete with the propranolol for the β-adrenoceptors thus confirming the competitive nature of the blockade.

The Effect of Other Sympathomimetic Drugs on Respiratory Goblet Cells: Isoprenaline belongs to a family of drugs classed as sympathomimetic because their actions *in vivo* mimic that of adrenergic nerve stimulation (the sympathetic nervous system). The drugs can be sub-divided according to the type of adrenergic receptor they are selective for into α- and β-adrenoceptor agonists. The β-adrenoceptor agonists can be further divided into β_1- and β_2-adrenoceptor agonists; isoprenaline is a β-adrenoceptor agonist acting equally on both β_1- and β_2-adrenoceptors. This classification is based on the selectivity of the drugs for a particular receptor type. None of them however, can be said to have specificity for a single receptor type. Thus, although at lower doses they are capable of selectively activating α-, β_1- or β_2-adrenoceptors, at higher doses this selectivity may be lost and they may act on any of the three types of adrenoceptors. Attempts were made in this study to mimic the effect of isoprenaline on respiratory goblet cells using a number of sympathomimetic and parasympathomimetic drugs. Methoxamine, a selective α-adrenoceptor agonist, and salbutamol, a selective β_2-adrenoceptor agonist, were found to be capable of producing significant increases in respiratory goblet cells 24 hours after a single dose of 20

and 40 mg/kg respectively. None of the parasympathomimetic drugs tested produced an increase in resiratory goblet cells following a single intraperitoneal dose. Thus, although the response was limited to sympathomimetic drugs no single adrenergic receptor type has emerged. In fact, drugs acting on all three receptor types have been found to be capable of inducing goblet cell hyperplasia in the respiratory epithelium of the rat. Since β_2-adrenoceptors are found in bronchial smooth muscle and when stimulated give rise to bronchodilatation, and α-adrenoceptors are found in pulmonary vasculature and when stimulated give rise to vasoconstriction and finally, β_1-adrenoceptors occur almost exclusively in cardiac smooth muscle and give rise to positive inotropic and chronotropic effects when stimulated, there is no common site or action associated with the three receptor types so far shown to be involved in goblet cell hyperplasia. This fact along with the failure by terbutaline, another β_2-adrenoceptor agonist, to induce goblet cell hyperplasia lends very little support for the direct involvement of adrenoceptors in the goblet cell response.

The Effect of the Optical Isomers of Isoprenaline on Goblet Cells: Isoprenaline in common with many of the sympathomimetic drugs has within its molecular structure an asymmetric carbon atom. The molecule can therefore be resolved into two optically active isomers, a levo-rotatory isomer and a dextro-rotatory isomer. The levo-rotatory

isomer is the pharmacologically active isomer being approximately 90 times more potent than the dextro-rotatory isomer in reducing blood pressure in the dog (Becarri et al., 1953). Their relative potencies vary from species to species due to differences in the rate of racemisation in body fluids. Nevertheless, in all species the levo-isomer is the pharmacologically more active and is generaly more potent than the racemate. When the two isomers of the drug were tested in this study the goblet cell response was found to be stereo-specific for the levo-isomer at lower doses of the drugs but not at higher doses. The stereo-specificity of the response at low doses lends support to the theory of a pharmacological basis to the goblet cell response. The lack of stereo-specificity at higher doses on the other hand raises serious doubts about this and will be discussed in the next section.

The Effect of Isoprenaline on Goblet Cells Elsewhere in the Body: Goblet cells do not occur exclusively in the respiratory epithelium. They can be found in all mucosal surfaces where mucus is required. Thus, goblet cells can be found throughout the length of the alimentary tract and in the small intestine they occur along the sides of the villi, and as stated in the introduction, much of the work carried out to elucidate the origin of goblet cells using cell kinetics has been performed using the small intestine. The response to isoprenaline by goblet cells may not, therefore, be limited to the lung. Should it occur at

other goblet cell sites around the body then the mechanism of the isoprenaline action may be involved in the normal physiological control mechanism governing the goblet cell population as a whole, and not just in the respiratory epithelium. This is an important area for future study.

5:42 Discussion of the Evidence Against a Pharmacological basis

Dosages required: As pointed out in the previous section the dosages involved are very high. It is therefore impossible to be absolutely certain that the isoprenaline is acting purely as a β-adrenoceptor agonist. In man, high doses of isoprenaline have been shown to cause increased salivation and this has been attributed to α-adrenoceptor stimulation by the isoprenaline. It can also cause blanching of the skin when injected intradermally; this, too, has been attributed to α-adrenoceptor stimulation (Innes and Nickerson, 1970). It is worth remembering that isoprenaline is the most active of the sympathomimetic drugs acting exclusively on β-adrenoceptors, and as such normally requires very low doses to achieve a pharmacological β-adrenergic effect.

However, the high doses required may simply reflect the need for the presence of the isoprenaline over a set length of time before the goblet cell response is seen. To confirm this an experiment is needed in which a low dose of isoprenaline is given as a slow infusion over a number of hours.

High Dosage Levels of β-antagonist Employed: The previous section mentioned the fact that propranolol has in addition to its β-adrenoceptor antagonist activity the ability to cause membrane stabilisation (membrane stabilisation activity - MSA). MSA is in fact a direct local anaesthetic action on cell membranes leading to an increase in electrical threshold. It is prominent only with doses larger than those required to produce significant β-receptor blockade (Nickerson, 1970). In this study the single dose of 50 mg/kg propranolol intraperitoneally was found to inhibit the isoprenaline induced goblet cell hyperplasia. However, observation of the animals receiving the propranolol revealed that there were within the group, two distinct groups of animals. The majority of the rats receiving the propranolol became very depressed and showed loss of muscle co-ordination and in some cases appeared to exhibit a form of catatonia. The rest of the rats, however, appeared to be unaffected by the dose of propranolol and behaved normally.

It was thought probable that the severely depressed rats were exhibiting signs of MSA caused by the high dose of propranolol. If this was indeed the case then those animals would be unreceptive to most pharmacological stimuli by virtue of the raised electrical theshold and local anaesthetic effect on the cell membranes. In other words, the propranolol was no longer acting as a β-adrenoceptor antagonist but as a highly potent local

anaesthetic. To try to ascertain whether or not this was the case a small numbers of rats were fitted with carotid canulas at the I.C.I. laboratories at Alderley Park and continuous monitoring of heart rate and blood pressure was carried out following a variety of procedures, the details of which are given in Appendix ii.

Of the two rats receiving propranolol, one showed signs of MSA and this was confirmed by the severe drop in resting heart rate 35 minutes after the administration of the drug. The rats were then given 40 mg/kg isoprenaline and a continuous recording of heart rate and blood pressure was made. The rat without MSA on receiving the isoprenaline showed a marked increase in heart rate and a fall in blood pressure. The increase in heart rate was smaller than that obtained in a rat receiving isoprenaline alone; it was nevertheless considerably higher than the control heart rate. The fall in the blood pressure observed was, on the other hand, very similar to the fall in blood pressure observed in the isoprenaline control.

The rat with signs of MSA also exhibited the same decrease in blood pressure after isoprenaline as the rat without MSA and the isoprenaline control. The effect on heart rate was, however, different. Prior to receiving the isoprenaline, the rat with signs of MSA had a markedly reduced heart rate, almost half the normal resting value. After the administration of the isoprenaline the heart rate increased sharply to just below the control rate and then

dropped again. Unfortunately it was not possible to obtain readings from this animal longer than 15 minutes after receiving the isoprenaline. This experiment has shown that 50 mg/kg propranolol intraperitoneally may induce MSA in the majority of the rats so dosed. Where MSA has occurred the propranolol does inhibit the effect of isoprenaline on heart rate to some extent but has no effect on the reduction in blood pressure induced by the isoprenaline.

Where propranolol has failed to have any MSA effects the dose of 50 mg/kg intraperitoneally has not antagonised the cardiovascular effects of 40 mg/kg isoprenaline. Thus, although propranolol abolished the isoprenaline dose-response curve for the goblet cell hyperplasia, this might not be due to pharmacological antagonism brought about by competition for the β-adrenoceptors, but may be brought about by the powerful membrane stabilisation effect such a high dose of propranolol has in the rat.

Receptor Type Involved in the Response: As stated earlier, all three types of adrenoceptor have been implicated in the response. In addition, Sturgess and Reid (1973) found a significant increase in respiratory goblet cells following 6 and 12 daily doses of 10 mg pilocarpine. However, a single 40 mg/kg dose of pilocarpine failed to have any effect on respiratory goblet cell numbers in this study.

In section 5:51 it was pointed out that there was no common site of action or effect associated with the

selective adrenergic actions of the three drugs found to be capable of increasing respiratory goblet cells. The classification of adrenergic receptors as α- (peripheral excitatory), β_2- (peripheral inhibitory) and β_1- (cardiac excitatory) also applies to the receptors for the metabolic and the central nervous actions of the drugs (Weiner, 1980). In fact, many of the metabolic and central nervous effects can readily be classified as being due to a particular receptor type. If one or more of these indirect metabolic or central nervous effects were responsible for the goblet cell response then this may account for the variety of sympathomimetic drugs which induced goblet cell hyperplasia. The effects on metabolism associated with sympathomimetic drugs are as follows:-

Efflux of K^+	Associated mainly with α-adrenoceptor agonists
Glucagon release	
Growth hormone release	
Inhibition of insulin release	
Inhibition of lipolysis	
Muscle glycogenolysis	Associated mainly with β-adrenoceptor agonists
Hepatic glycogenolysis	
Inhibition of growth hormone release	
Gluconeogenesis	
Lipolysis	

Thus the order of potency of the various adrenergic agonists enables the metabolic effects to be divided into α-associated and β-associated effects, the effects of adrenergic blocking agents on metabolic processes in man and other species can also be divided into α- and β-adrenoceptor associated effects (Weiner, 1980).

Since the goblet cell response requires large doses of the various drugs, it is very probable that they are no longer selective in their metabolic effects and that a common alteration in metabolism is occurring which may be responsible for triggering the goblet cell increase.

The same considerations apply to the central nervous effects of the drugs, and it is not possible at this point to say which of the varied effects, both metabolic and in the brain, may be responsible for inducing a goblet cell hyperplasia.

The Lack of Stereo-Specificity at High Dose Levels: When the optical isomers of isoprenaline were administered as a single intraperitoneal dose of 40 mg/kg both isomers induced an increase in respiratory goblet cells. The increase so induced was of the same order as that induced by 40 mg/kg of the racemic mixture. Thus, all three drugs proved equipotent at inducing goblet cell hyperplasia when given as a single intraperitoneal dose of 40 mg/kg. One One possible explanation for this may be that the (+) isomer contained a small amount of the active (-) isomer which was

present in sufficient quantity at 40 mg/kg to produce goblet cell hyperplasia but not at 2.4 mg/kg.

. Alternatively, there may be a common metabolic or CNS (central nervous system) effect which all three drugs have at 40 mg/kg and which only the levo-isomer and racemic mixture produce at lower doses because of their greater potency. The loss of stereo-specificity at higher doses lends support to a non pharmacological basis for the response.

Dosage Required by the Intratracheal Route: If the goblet cell response is the result of interaction between isoprenaline and β-adrenoceptors located in or near the respiratory epithelium, then it might be anticipated that applying the isoprenaline directly to the respiratory epithelium would lead to a reduction in the dose of isoprenaline needed or an increase in the response.

Isoprenaline was found to have a direct effect on respiratory goblet cells. In the event, however, it was found that intratracheal injections of isoprenaline resulted in the same size of goblet cell increase as did systemic doses. The amount of isoprenaline administered by the intratracheal route was in the range 1.25 mg - 10 mg per rat; as the rats weighed 250 g the dose of 10 mg was equivalent to 40 mg/kg. Thus, the intratracheal dose was similar to the intraperitoneal dose. It has been shown that there is little airway absorption of isoprenaline but there is rapid absorption in alveolar regions; systemic

effects such as tremor and tachycardia can be detected 30 secs after inhalation (Newhouse and Ruffin, 1978; Dashe et al., 1974). Drugs given by inhalation are therefore rapidly absorbed and dispersed by the blood stream. The high dose of intratracheal isoprenaline required to induce goblet cell hyperplasia argues against a localised effect within the respiratory epithelium and therefore does not lend support to the theory of a pharmacological basis to the response.

5:43 Summary

The findings of this study have not made it possible to say definitely that the goblet cell response is directly related to the pharmacological interaction between isoprenaline and β-adrenergic receptors. However, several of the findings point towards some kind of sympathetic control over the goblet cell population. The nature of that control and its mediation are not known. It may be biochemical, metabolic or humoral and need not necessarily be initiated within the respiratory epithelium itself. The fact that the response is not limited to isoprenaline, or confined to rats, but can occur in other species of animals and strains of rats shows that it is not an idiosyncratic side effect of high doses of isoprenaline. As such it is a useful tool for elucidating much that is not known about goblet cells such as their origin and synthetic and secretory control mechanisms.

5:5 THE EFFECT OF ISOPRENALINE ON THE RESPIRATORY EPITHELIUM WHICH LEADS TO INCREASED EPITHELIAL GOBLET CELLS: THE ORIGIN OF THE NEW GOBLET CELLS

5:51 Introduction

There are several possible ways in which the new goblet cells can arise. These are listed below with brief details, each possibility is then discussed more fully in the following four sections.

Possible Origins of the New Goblet Cells

(i) Isoprenaline stimulates mitosis within the respiratory epithelium. This may either result in an increase in the overall cellularity of the respiratory epithelium, including goblet cells, or the increased mitosis may only give rise to new goblet cells.

(ii) Isoprenaline stimulates differentiation from precursor undifferentiated or differentiating cells into goblet cels by switching on the synthesis of mucin.

(iii) There may a be quiescent population of goblet cells, i.e. committed dormant goblet cells, which are stimulated to synthesise mucin by isoprenaline. Once they contain stored mucin the

cells are identified as goblet cells using the light microscope and mucin specific stains.

(iv) Isoprenaline stimulates the transformation of existing functional differentiated cells into goblet cells. Possibilities being Clara cells in peripheral airways and ciliated cells in proximal airways.

(v) Isoprenaline may block the secretion of mucin with or without stimulation of synthesis. This would result in greater numbers of full goblet cells and very few empty goblet cells. The numbers of goblet cells identified using the light microscope and mucin specific stains would subsequently increase.

5:52 The New Goblet Cells Arise From Isopenaline Stimulated Mitosis

Isoprenaline has been shown to affect mitosis in a variety of tissues and species. Schering et al. (1972) found isoprenaline flattened the mitotic curve in the gastro-intestinal tract of mice. Also in mice, Malamud and Malt (1971) reported that isoprenaline stimulated mitosis in the kidney. In the rat isoprenaline has been shown to induce an increase in mitosis in the salivary gland, resulting in an alteration in the pattern of secretion with increased protein content and output of saliva (Seifert,

1967). Bolduc and Reid (1978) have also reported an increase in mitosis in the epithelium of the rat lung following isoprenaline administration. Conversely, Baskerville (1975) concluded that since the respiratory epithelium was not hyperplastic and there was no change in mitotic activity following isoprenaline administration in pigs, the drug was inducing an alteration in differentiation which was responsible for the increase in epithelial goblet cells.

The fundamental biochemical action of isoprenaline which immediately follows its interaction with β-adrenoceptors is an increase in the intracellular levels of adenosine 3',5'-cyclic monophosphate (c-AMP). c-AMP has been implicated in the regulation of cell growth through its inhibitory action on DNA synthesis (Abell and Monahan, 1973). They have shown that increased levels of c-AMP can inhibit cell division by reducing the synthesis of DNA; this effect was greater on malignant cell growth than on normal cell growth and occurred *in vitro*. Thus, increased intracellular c-AMP has been associated with a decrease in cell division and a shift towards increased cell differentiation. *In vivo*, however, isoprenaline has been shown to increase the synthesis of DNA in the parotid gland of the mouse, and this stimulatory effect was dependent on isoprenaline inducing increased c-AMP levels within the cells (Guidotti *et al.*, 1972). Isoprenaline has also been shown to stimulate DNA synthesis *in vitro* in haemopoietic

stem cells (Byron, 1972). This stimulatory effect was dependent on an intracellular increase in c-AMP and was inhibited by propranolol. These results are at variance with those of Abell and Monahan (1973). The role of c-AMP in cell division is therefore unclear and appears to vary in different tissues and species.

The response of goblet cells to isoprenaline is very fast, increased numbers can be detected 12 hours after the isoprenaline administration and the increase reaches significance by 18 hours and reaches a maximum between 24 and 48 hours. In the mouse kidney the stimulatory effect on DNA synthesis reaches a peak at 34 hours and in the parotid gland at 22 hours (Malamud and Malt, 1971), and in mouse haemopoietic stem cells peak DNA synthesis occurs 28 hours after the isoprenaline administration (Byron, 1972). Thus, the increase in goblet cells in the lung occurs before the peak increase in DNA synthesis in other tissues. Furthermore, the biochemical events, prior to mitosis, which occur in murine or rat salivary glands after a single injection of isoprenaline have been studied by Fujioka et al. (1963). They showed that there were between 30 and 33 hours delay from the injection of isoprenaline and the occurrance of mitosis. In the small intestine it has been estimated that it takes 10 hours for the appearance of immature goblet cells in the crypt, following stem cell division, and 40 hours for 'mature' goblet cells to appear at the base of the villus (Cairnie, 1970; Merzel and

Leblond, 1969; Leblond and Messier, 1958). The small intestine has a much faster cell turnover than the lung, and in the small intestine the process from stem cell division to fully mature goblet cell takes up to 40 hours. It therefore seems unlikely that in the lung, with its much slower cell turnover, that the process of stem cell division to fully mature goblet cell would take as little as 12 - 18 hours. Is 12 - 18 hours sufficient time for a cell in the resting phase of the cell cycle to enter the DNA synthesis phase, prepare for and then undergo mitosis, mature and synthesise a theca full of stored mucin? It seems unlikely. An example of goblet cell hyperplasia induced by increased mitotic activity in the lung is that caused by exposure to sulphur dioxide gas. The irritant damages the respiratory epithelium, this leads to increased mitotic activity and repair of the epithelium with a subsequent increase in the number of goblet cells. This increase reaches a maximum 6 days after the exposure to the sulphur dioxide (experiment 1, chapter 4). In addition to the difference in the timing of the response, there is another important difference between the isoprenaline induced goblet cell hyperplasia and the sulphur dioxide induced hyperplasia, and that is the size of the response.

The response to sulphur dioxide is much greater than that due to isoprenaline. The latter is therefore stimulating a finite population of cells into becoming goblet cells. Now, the effect of isoprenaline on

intracellular c-AMP is dependent on the presence of a β-adrenergic receptor on the exterior surface of the cell, which in turn stimulates the synthesis of c-AMP. It is possible that within the respiratory epithelium only a limited number of cells have such a receptor and that these cells are capable of undergoing mitosis to give rise to new goblet cells. However, this action on c-AMP is a highly selective property of β-adrenergic agonists and requires low concentrations of the drug in vitro for its initiation (Byron, 1972). As stated in the previous section, the response of goblet cells to isoprenaline requires very large doses and is not obviously related to its pharmacological activity as a β-adrenoceptor agonist. It is therefore highly unlikely that the new goblet cells detected after a single dose of isoprenaline arise through its stimulatory effect on DNA synthesis and a subsequent increase in cell proliferation leading to increased numbers of goblet cells.

5:53 The New Goblet Cells Arise From Isoprenaline Induced Altered Differentiation

In the summary of possibilities outlined at the beginning of this sections, points (ii), (iii) and (iv) are all related to alterations in differentiation. Points (ii) and (iii) will be dealt with together in this section and point (iv) will be dealt with separately in the next section. The essential difference between points (ii) and

(iii) is that in (ii) the cells which may be stimulated by the isoprenaline are multipotential and can become any cell type whatsoever within the respiratory epithelium. In (iii), however, the cells in question are already committed to becoming goblet cells and isoprenaline has speeded up the process.

In the larger airways the epithelium has a pseudo-stratified appearance and this is due to the presence of basally situated cells called basal cells. These cells act in a similar way to the germinal layer of the epidermis and represent the stem cell population for the tracheal and large airway epithelium (Blenkinsopp, 1967; Breeze and Wheeldon, 1977). As such, basal cels are multipotential and each division gives rise to one new basal cell and one new undifferentiated cell, the fate of the latter is at present unknown. Section 1:35 of the Introduction gave an outline of the various theories proposed for the origin of respiratory epithelial goblet cells. Donnelly et al. (1980) stated that all tracheal epithelial cells were proliferative with the exception of ciliated cells. Conversely, Bindreiter et al. (1968) concluded that ciliated and goblet cells were non-proliferative and that the proliferative basal cells feed into an undifferentiated proliferative compartment of cells which in turn give rise to differentiated functional cells. Finally, Boren and Paradise (1978) proposed a scheme whereby basal cells feed into a dividing mucus cell compartment from which

functional ciliated cells arise. In the small intestine it is now generally accepted that goblet cells do not undergo mitosis but are derived from a precursor proliferative pre-goblet or oligomucous cell situated in the lower half of the crypt (Cairnie, 1970; Merzel and Leblond, 1969). There have not, so far, been any descriptions of a cell resembling the intestinal oligomucous cell within the respiratory epithelium. However, two poorly differentiated cell types have been described, the intermediate cell and the transitional cell (Jeffrey, 1975; Rhodin and Dalhamn, 1956; Breeze and Wheeldon, 1977). The intermediate cell is thought to be an undifferentiated cell capable of proliferation and differentiation into either ciliated or secretory cells. The transitional cell is a cell showing morphological characteristics of more than one cell type, i.e. mucin granules in the cytoplasm plus evidence of ciliogenesis and basal bodies at the apex (Osada, 1964; Harris et al., 1975; Breeze and Wheeldon, 1977).

It is possible that the intermediate cell, which is multipotential and is capable of differentiating into either goblet or ciliated cells, is being selectively stimulated by isoprenaline to synthesise mucin and differentiate into goblet cells. Jeffrey (1975) reported that under the electron microscope intermediate cells contained either an electron dense cytoplasm or an electron lucent cytoplasm, the former being destined to become goblet cells and the latter ciliated cells. Futher studies

using the electron microscope are required to ascertain whether or not the electron dense intermediate cell resembles the so-called oligomucous pre-goblet cell of the small intestine. Until this has been done it is not possible to clarify more fully whether or not isoprenaline is stimulating a committed pre-goblet precursor cell or if it is selectively altering the differentiation of a multi-potential precursor cell towards mucin synthesis.

5:54 The New Goblet Cells Arise From Isoprenaline Induced Transformation of Existing Functional Differentiated Cells

As stated in the previous section a transitional cell showing the morphological characteristics of more than one cell type has been described in the respiratory epithelium. Precisely what this means is not as yet understood. In the small intestine observations of the villus cell populations have revealed that not all the goblet cells found in the villus epithelium arise within the crypt, some are derived by transformation of undifferentiated columnar cells situated on the villus (Porcella et al., 1979). A similar situation may also arise in the respiratory epithelium (Reid, 1977). Certainly studies of the terminal bronchiolar epithelium have suggested that functional non-ciliated secretory cells, Clara cells, are the progenitor cell for this region (Evans et al., 1978; Lum et al., 1978). Evans et al. in a detailed study using the electron microscope

and tritiated thymidine labelling, also suggested that there was a heterogenous population of Clara cells consisting of 3 types:- Type A cells containing no secretory granules and with or without profiles of smooth endoplasmic reticulum; Type B cells with secretory granules but no smooth endoplasmic reticulum and a dense cytoplasm, and finally functional Clara cells with dense secretory granules and profiles of smooth endoplasmic reticulum. They suggested that Type A cells were Clara cells minus secretory granules and that these were the proliferative form of the Clara cells, and that Type B cells were a transitional cell between Type A and Clara cells, which because of their dense cytoplasm could be confused with the serous cell.

Until a universal systematic form of lung cell nomenclature has been adopted it is impossible to distinguish fully between serous, intermediate, Type A Clara and Type B Clara cells. Likewise, it is not possible to speculate at the present time precisely which of these cells responds to the isoprenaline and differentiates into mucin synthesising goblet cells. This work needs to be carried out using the electron microscope as it is only at the ultrastructural level that subtle differences between cell types can be detected. In addition, autoradiographic studies using labelled mucin precursors could give useful information about alterations in the pattern of mucin synthesis and whether or not mature functional cells, such

as ciliated cells, are able to switch on mucin synthesis following isoprenaline.

5:55 The New Goblet Cells Arise Through Interference by the Isoprenaline with the Synthetic and Secretory Processes of the Goblet Cell

There is no evidence as yet that epithelial goblet cell secretion is influenced by either nerve stimulation or a wide variety of drugs. The sub-mucosal bronchial glands have, however, been shown to be under nervous control (Ueki et al., 1980; Peatfield and Richards, 1980; Davis et al., 1979; Davis et al., 1979a; Ueki et al., 1979; Gallagher et al., 1975; Florey et al., 1932). In addition, a variety of adrenergic agonists and antagonists and cholinergic agonists and antagonists have been shown to influence bronchial gland secretion (Phipps et al., 1980; Borson and Nadel, 1979; Whimster and Reid, 1973). Gallagher et al. (1975) showed that in the cat neither parasympathomimetic drugs, parasympathetic nerve stimulation, sympathomimetic drugs or sympathetic nerve stimulation had any effect on epithelial goblet cell secretion.

If isoprenaline was simply inducing an increase in mucin synthesis which was faster than the baseline rate of mucin secretion then the number of full goblet cells would increase. Consequently, using the light microscope and mucin specific stains the number of goblet cells detected

would increase. However, the evidence of this study does not support this hypothesis. Firstly, isoprenaline induces a rapid 4-fold increase in respiratory goblet cells. If it is doing this by increasing the mucin synthesis then it follows that at any one time the number of empty or resting goblet cells is three times greater than the number of full goblet cells. That represents a very large reserve population even taking into account the fact that mucus production is a first line of defence within the respiratory tract. In fact, the increase in mucus following irritation is immediate and is more likely to come from the more numerous glands than the scattered populations of goblet cells. Secondly, the timing of the goblet cell response has shown that the maximum increase occurs between 24 hours and 48 hours after the isoprenaline administration and that the increased numbers persist for upwards of 2 to 8 weeks after cessation of the isoprenaline administration. It is highly unlikely that isoprenaline would affect mucin synthesis for upwards of 8 weeks after it was last administered. As it is, the delay of 24 hours before the maximum increase implies that rather more than just mucin synthesis has occurred, indeed Neutra and Leblond (1966) have shown that it takes just 4 hours for goblet cells in the colon of the rat to absorb ^{3}H-glucose, incorporate it into mucin granules within the Golgi complex and secrete the labelled mucin from the apex of the cells.

Further work is required using radioactive mucin precursors and the electron microscope to clear up the question of whether or not isoprenaline has any effect on the synthesis of mucin in goblet cells.

5:56 Summary

Isoprenaline induces a reproducible 4-fold increase in goblet cells in the respiratory epithelium of the rat. The source of the new goblet cells is as yet not known. However, the evidence gained from this study does not support the involvement of increased mitosis or a direct effect on goblet cell mucin synthesis with or without any effect on mucin secretion. Taking into account the size of the response, the timing of the response and the diversiy of cell types within the respiratory epithelium, many of which have no obvious function, it is probable that isoprenaline is inducing an alteration in the differentiation pattern of the respiratory epithelium leading to increased goblet cells. Due to the lack of ultrastructural studies it is not possible to say which cell or cells are being induced to synthesise mucin and become goblet cells. Possible candidates include serous, intermediate, ciliated and Type A and Type B Clara cells.

5:6 THE CLINICAL IMPLICATIONS OF THE FINDINGS

The primary clinical use of isoprenaline is as a bronchodilator to relieve bronchoconstriction in asthma.

Dosage is generally by aerosol as the hydrochloride or sulphate and the recommmended adult dose is 1 - 3 inhalations of 80 µg repeated if necessary after 30 minutes up to a maximum of 8 inhalations in 24 hours. A stronger aerosol is available giving 400 µg and this can be used in a similar manner up to a maximum of 8 inhalations in 24 hours (Martindale, 1977).

Asthma is only rarely fatal in its own right. However, on the few occasions when sudden death has occurred from status asthmaticus it has been possible to study the pathological changes in the lung, due to the asthma, uncomplicated by a superimposed respiratory infection. The microscopic changes observed were that the small bronchi (diameters 0.2 - 1 cm) were filled with plugs of mucus and the bronchial epithelium mostly shed into the lumen. However, where the epithelium remained intact it showed extensive mucous metaplasia, the ciliated epithelium being replaced almost entirely by goblet cells. Mucous glands were also increased in numbers and size and their lumens filled with thick mucus. Similar findings have been found in chronic asthma, where the bronchial epithelium has been shown to undergo almost total mucous metaplasia and the lumens filled with tenacious mucus plugs (Spencer, 1977a).

Martindale (1977) states that sudden death caused by overdosage and indiscriminant use of aerosols has been

reported and self administered doses of up to 100 mg daily have been observed. These deaths have been ascribed to cardiac failure.

Although the isoprenaline-induced goblet cell hyperplasia in the rat lung requires a single large systemic dose of isoprenaline, it has been shown in this study that a much smaller dose, i.e. 3.7 mg/kg administered daily for 10 days can also induce goblet cell hyperplasia. In addition the nebuliser study (Chapter 4, experiment 11) has shown that isoprenaline aerosol can induce goblet cell hyperplasia in the rat lung when given as a single dose over 45 minutes. The various methods used to determine the final dose administered to the rats in this experiment yielded conflicting results, however all the doses calculated were considerably less than the systemic dose (the actual range calculated being 2.15 mg - 658 µg per rat for the higher concentration of 100 mg/ml and 516 µg - 162.4 µg per rat for the lower concentration of 10 mg/ml). These doses, if correct, are well within the dosage range of the metered dose inhalers in routine clinical use. It would be interesting to speculate what effect long term isoprenaline aerosol inhalation has on the goblet cell population in the lungs of asthmatics, particularly those that regularly overdose themselves. A prospective study in which the lung pathology of asthmatics dying in <u>status asthmaticus</u> after the introduction of aerosol therapy was compared with the lung pathology of asthmatics

dying in status asthmaticus before the use of aerosols may yield some interesting information. Likewise, the use of isoprenaline aerosols to relive constriction in chronic bronchitis raises questions as to what effect the drug has on the goblet cell population in their lungs. It is interesting to note that in both asthma and chronic bronchitis despite relieving the bronchoconstriction, isoprenaline does not increase the pO_2.

Could it be that excessive mucous production is to blame for this? These are interesting questions raised by the results of this study and they should be answered before further synpathomimetic-type aerosols are developed for use in asthma and chronic bronchitis therapy.

CHAPTER 6

CONCLUSIONS

(1) A reproducible non-inflammatory and non-infective model of goblet cell hyperplasia in the proximal airways of the rat has been established. The agent employed to induce the model was a single intra-peritoneal dose of 40 mg/kg isoprenaline. This is the first time that a single-dose pharmacological model of goblet cell hyperplasia has been reported. The model has been studied and characterised. Thus, an optimum systemic dose has been established along with the time at which the maximum increase occurs. It has been shown that the response is the same in both male and female rats and that is is not influenced by either the time of injection or the time of death and that it can be antagonised by propranolol. Finally, it has been possible to induce a similar increase in respiratory goblet cells with systemic salbutamol and methoxamine and also with isoprenaline applied directly to the respiratory epithelium.

Since the goblet cell hyperplasia occurs after a single dose of isoprenaline the model can be used in conjunction with cell kinetic studies to study the origin of the new goblet cells. Such studies have hitherto been impossible as the previous animal models of goblet cell hyperplasia have all involved either multiple exposures to

irritants such as sulphur dioxide or tobacco smoke, or multiple doses of isoprenaline. The model can also be used in conjunction with radioactive labelled mucin precursors, administered *in vivo*, to study the pattern of mucin synthesis and secretion in respiratory goblet cells.

(2) The evidence gathered in the course of this study supports both the involvement of a fundamental pharmacological interaction with β-adrenoceptors and also the involvement of some non-specific, metabolic, biochemical or humoral effect. Further work is required with β-and ∝-antagonists before a more definite conclusion can be drawn.

(3) The origin of the new goblet cells is unknown; however, the involvement of mitosis is unlikely due to the rapidity of the response. It is probable that isoprenaline is stimulating the differentiation of a precursor cell population into goblet cells. Future studies, such as differential cell counts using the electron microscope, and autoradiographic studies with radioactively labelled mucin precursors, which would also require the use of the electron microscope, are needed before it can be ascertained from which cell or cell types the new goblet cells are derived.

(4) The difficulties encountered with the histological techniques available at the present moment have highlighted the need for a new embedding medium which is compatible with both light microscopic and electron microscopic studies. One of the most serious drawbacks of the existing embedding media was the length of time required to process the tissue and the labour intensive nature of the section cutting. As a consequence, an attempt was made to develop a fast screening technique which would facilitate the quick assessment of the tracheal goblet cell population. The tracheal dab technique has shown potential for this, however, it requires considerably more work to characterise it more fully, in particular to minimise the inherent variability of the preparation.

CHAPTER 7

PROPOSALS FOR FUTURE WORK

(1) Further studies are required with a wider range of sympathomimetic drugs to see which, if any, are also capable of inducing goblet cell hyperplasia.

(2) As stated earlier, the fundamental biochemical event which follows β-adrenoceptor stimulation is an increase in intracellular c-AMP levels. This is caused by the stimulation of the enzyme adenyl cyclase which is responsible for the synthesis of c-AMP. Intracellular c-AMP can also be increased by the inhibition of phosphodiesterase, the enzyme responsible for the breakdown of c-AMP. It would be interesting to see what effect phosphodiesterase inhibitors had on the goblet cell population and also whether or not they had any effect on the isoprenaline-induced goblet cell hyperplasia.

(3) Further inhalation studies using nebulised isoprenaline are required. However, the system employed will have to be very thoroughly characterised in advance in order that the dose administered can be accurately guaged.

(4) Differential cell counts are required on the early stages of the response during the period of maximum change in the epithelial cell population. This would have to be carried out using the electron microscope and may shed some

light on the origin of the new goblet cells.

(5) The single dose model could be utilised for *in vivo* cell kinetic studies, using ^{3}H-thymidine, to find out what role, if any, mitosis plays in the formation of the new goblet cells.

(6) Likewise, the model could be exploited to study *in vivo*, with the aid of radioactive labelled mucin precursors, the possible effect isoprenaline may have on their pattern of uptake, incorporation and discharge from goblet cells. It may be possible to see changes in uptake using GMA and the light microscope, but use of the electron microscope would yield more detailed information about their incorporation and discharge.

(7) A small study in which radioactive labelled isoprenaline was administered intraperitoneally followed by autoradiography in GMA would show if any actually reaches the respiratory epithelium following this route of administration.

(8) Likewise, administration of labelled isoprenaline by intratracheal injection, followed by autoradiography would yield interesting data about its absorption and fate in the respiratory tract. A similar study with labelled isoprenaline aerosol would yield valuable information about its pattern of deposition and fate in the respiratory tract; however, such a study would require very stringent

safety precautions because of the radioactive mist which would be generated.

(9) Further work is required to develop the histological techniques necessary for many of these future studies. In particular, large, thin, araldite sections of an adequate quality are still not possible using the Autocut and large glass knives. Since in many of the proposed studies it would be imperative to study the same area using first the light microscope to count goblet cells, and then secondly the electron microscope for differential cell counts, an embedding medium compatible with both is required. Araldite is the best that we have available at the moment and this has been shown to have many disadvantages in this study. However, intensive work using the Autocut and large glass knives may yield a workable solution to the problem of obtaining large thin sections.

Quantitative electron microscopic studies of the respiratory epithelium would also require the adaptation of existing techniques to overcome the problems of sampling and orientation.

Finally, further work is required on the tracheal dab technique so that the fast screening of a wide range of drugs can be carried out.

(10) Goblet cells occur in almost all mucosal membranes and are therefore widely distributed throughout the body. In particular, large numbers are found in the alimentary

canal. Further studies to see what effect isoprenaline has on goblet cells elsewhere in the body would be particularly interesting, since, if it can be shown that the isoprenaline effect occurs in several goblet cell populations, it would be indirect evidence that the mechanism involved may be part of the overall physiological control of the goblet cell population.

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APPENDICES

APPENDIX i

DEVELOPMENT OF A SCREENING TECHNIQUE FOR THE FAST ASSESSMENT OF GOBLET CELL NUMBERS

The number of goblet cells in rat and human trachea increases from the caudal to the distal end of the trachea and in addition there are higher numbers of goblet cells present on the anterior (cartilagenous) surface than on the posterior surface with no cartilage. This fact makes quantitation of the goblet cell population tedious although the problem can be overcome by careful sampling. One existing method for studying the distribution of goblet cells is to embed the whole of the trachea opened and flat, and to cut serial sections longitudinally from one side to the other and count the cells. This is too time consuming for a screening test. Another method is to cut the trachea in the coronal plane at predetermined anatomical sites and count the goblet cells occurring in a set number of sections. Dr A. Adams of the Department of Ophthalmology, Edinburgh University has developed a simple technique for studying conjunctival mucus layer patterns and goblet cell distribution by dabbing the conjunctival surface with a Millipore filter (Adams, 1979). The tracheal dab technique is an adaptation of his method.

TRIAL PREPARATION A: EN FACE WHOLEMOUNT PREPARATION OF LUNG BRONCHIAL TREE

Prior to discussions with Dr Adams, attempts were made to develop a screening technique using the whole lung. Preliminary work succeeded in clearing a lung tissue block in which a 'gutter' comprising the bronchial tree, cut in longitudinal section, had been stained using PAS. The lung was fixed and sectioned longitudinally in such a way that almost all the bronchial tree was exposed as a 'gutter'. The epithelium was carefully painted first with periodic acid and then with Schiff reagent with minimal spillage over the rest of the lung tissue. Methyl salicylate was used as a clearing agent. It was then possible to visualise and count discrete PAS stained magenta dots dispersed throughout the bronchial tree using a dissecting microscope with a long working distance. However, this method was not pursued further as the preparation did not keep long before going completely dark and thus became unusable.

Comment

This preparation has potential for studying goblet cell numbers in the lung. However, it will not be possible to count any other cell types, and numbers will have to be large as the size of the goblet cells themselves will be critical to the number detected, thus leading to increased variability in small groups.

TRIAL PREPARATION B: TRACHEAL DAB TECHNIQUE USING MILLIPORE FILTERS

In essence, fresh tracheas were dissected into two halves, a front half with the semi-circular cartilage rings, and a back half consisting of muscle. The length used was the entire distance between the ring immediately below the larynx and the cartilage ring immediately above the carina. A careful note was made of the top and bottom of each half (very important when quantitation is performed) and an imprint was made of the tracheal mucosa from each half on separate pieces of Millipore filter attached to microscope slides. The imprints were fixed in Millonig's fixative for 2 - 3 minutes, dried thoroughly and then stained wih PAS. Each imprint was trimmed, cleared in xylene and mounted in xylene-based coverbond on a conventional microscope slide.

The initial results were very promising (Figure 29). Impressions of the whole of the tracheal sections were obtained in which it was easy to see the placing of the cartilage rings. By varying the pressures used and employing various pretreatments, it was possible to demonstrate numerous discrete highly PAS positive dots interspersed by occasional mucous strands and sheets of epithelial cells. There is still some doubt as to whether the technique is able to demonstrate part or all of the available goblet cell population. Confirmation is also

FIGURE 29

Example of the tracheal dab technique. Stained with PAS and x40 magnification. The PAS positive dots are goblet cell thecae and just discernable are sheets of epithelial cells which have not taken up the stain.

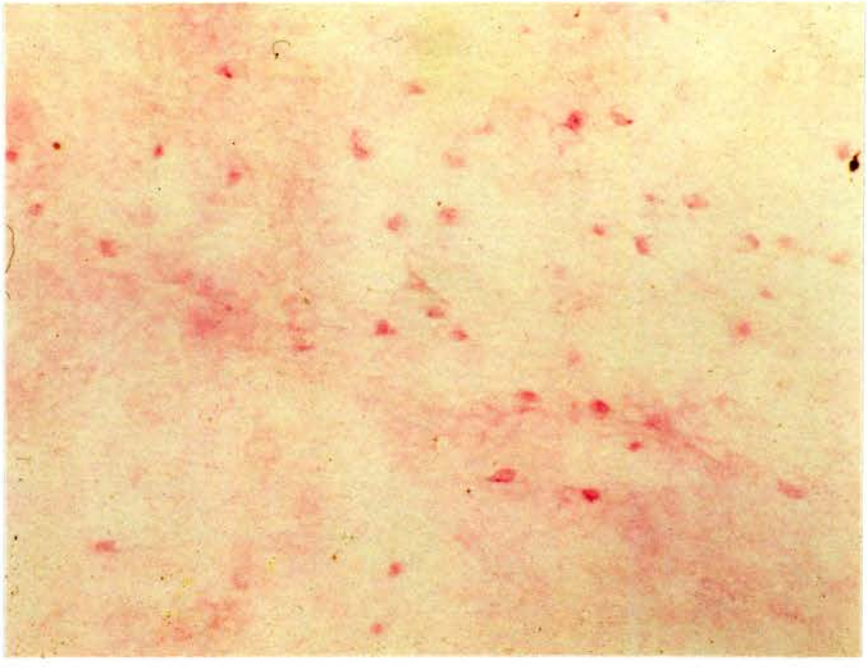


FIGURE 29.

required as to whether the PAS stained dots actually represent the mucin-filled thecae of epithelial goblet cells. There are three possible ways this can be tackled:

(a) By embedding the remains of the trachea after the imprints have been taken. Sections taken longitudinally down the length of the tracheas should indicate how far the epithelium was penetrated. If the epithelium is denuded of goblet cell mucous blebs then the imprints almost certainly show goblet cells.

(b) Using large groups of animals it may be possible to compare results obtained from tracheas sectioned in the usual manner with those obtained from tracheal dabs. In addition, if the groups of animals receive different treatments beforehand, i.e. saline and 40 mg/kg isoprenaline, it may be possible to demonstrate a similar percentage increase in tracheal goblet cells using the two methods. This would lend support to the idea that the tracheal dab technique is capable of detecting changes in the goblet cell population and the difference detected using the two methods would give some idea as to the sensitivity of the dab technique.

Alternatively, a comparison could be made between tracheal goblet cell counts using dabs, with results obtained for bronchial goblet cell counts from plastic lung sections, following both saline and 40 mg/kg

isoprenaline. If both methods were to detect an increase in goblet cells then this would be circumstantial evidence that the dab technique is demonstrating tracheal goblet cells.

(c) By comparing counts obtained using scanning electron microscopy of the tracheal mucosa with those obtained using the dab method. It was felt that this was not worth following up as using the SEM it is only possible to obtain an idea of the ratio of the areas of non-ciliated cells to ciliated cells. It is not yet possible to distinguish the different non-ciliated cell types using the SEM, and therefore the two techniques are not measuring the same thing.

However, specimens of normal trachea and tracheas which had been used for dab preparation were examined using a scanning electron microscope. The latter showed areas of flattened and damaged epithelium denuded of cilia and non-ciliated cells, and also areas of relatively normal epithelium with cilia and non-ciliated cells. It is therefore a rather patchy preparation. However, it is not known whether or not the non-ciliated cells which remained were goblet cells or serous, Clara or intermediate cells. Figure 29 shows a fairly even distribution of goblet cells with no large empty patches and it may be that the Millipore filter removes the mucin-filled thecae

without necessarily removing the rest of the tracheal epithelium.

QUANTITATION OF TRACHEAL DABS

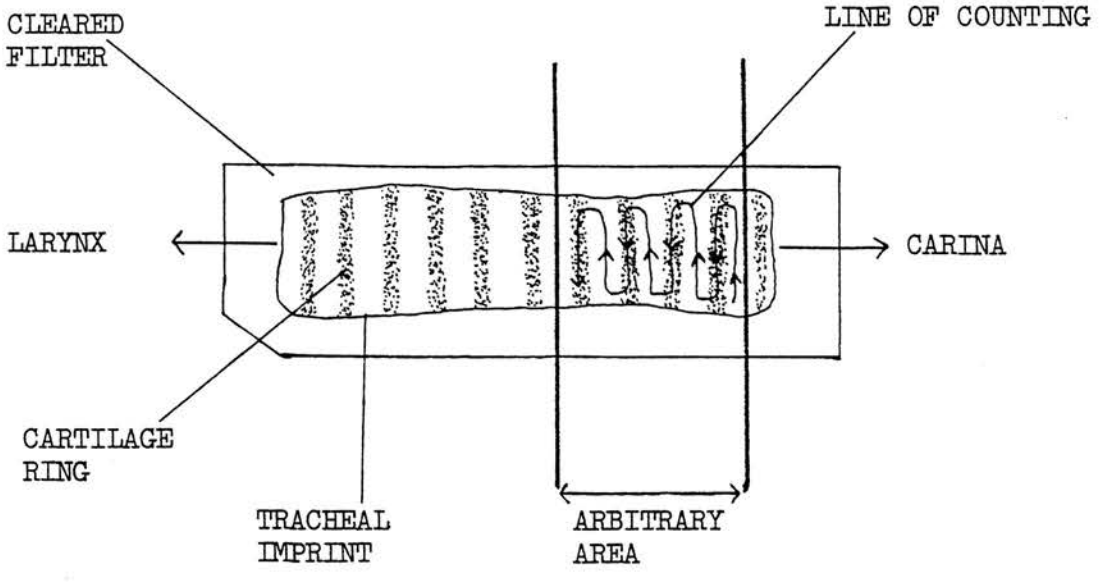
Using a graticule with a small square of known dimensions it was possible to assess goblet cells per unit area. However, this has the disadvantage of only covering a very small area of the whole and so an investigation was carried out to determine the minimum number of squares it was necessary to count and the area from which they would be taken in order to obtain a reliable and representative number of goblet cells.

As the number of goblet cells varies over the surface of the trachea; they tend to be in clusters and occur more frequently over the cartilage rings in the anterior mucosal surface than in the areas between the rings, and as a whole their numbers increase from the larynx to the carina; there are several different methods by which quantitation could be performed. The results of several methods tried so far are shown below, others are still being assessed.

Method (a): An arbitrary area was marked on the slide at the lower 3 - 4 mm of the trachea. It contained a minimum of 4 cartilagenous rings and 4 areas between the cartilage rings. Counting was carried out using a x40 objective and a graticule containing a small square measuring 53.3 x 53.3 μm. This was moved across the trachea in lines

running parallel to the rings; thus the square travelled from one side of the trachea to the other. Having completed one line of counting, the stage was moved to a small distance to the left of the area just counted and the procedure repeated.

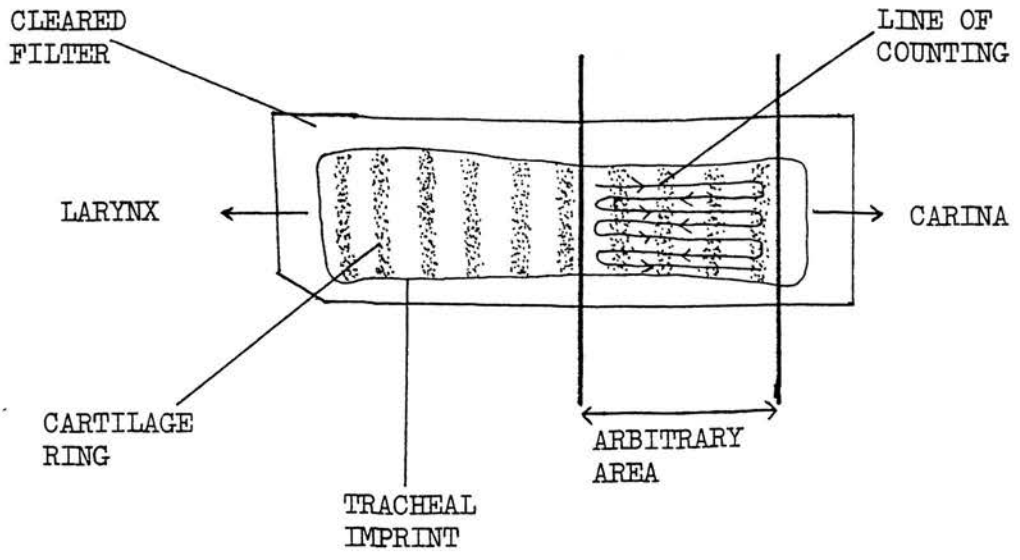
Thus:



In practice it proved very difficult to keep the areas of cartilage and non-cartilage equal. The results can be seen in Table 7.

Method (b): A similar arbitrary area was marked as in the previous method. This time, however, the line of counting travelled at right angles to the cartilage rings. The area covered was as near as possible to the centre of the trachea and four parallel lines were covered.

Thus:



The results can be seen in Table 7.

Results

Method (a)	Mean goblet cells/square	2.48	SD	0.2	SE	0.047
Method (b)	Mean goblet cells/square	2.18	SD	0.026	SE	0.0086

From Table 7 it can be seen that Method (b) gave a reasonably consistent value for goblet cells once 90 - 100 squares had been counted. Method (a), however, gave more variable values and still had not reached consistency after counting 200 squares. Therefore, for consistency and convenience Method (b) is the method of choice.

The two methods give reasonably comparable mean values; however, method (a) gave a slightly higher value than method (b). A combination of factors may account for this. When counting by method (a) it was very difficult to

TABLE 7 COMPARISON OF COUNTING TECHNIQUES FOR THE TRACHEAL DAB PREPARATION

Number of Squares Counted	Average Number of Goblet Cells/Square	
	Method A	Method B
10	2.0	2.7
20	2.8	1.96
30	2.88	1.7
40	2.35	1.7
50	2.26	1.68
60	2.06	1.86
70	2.3	2.06
80	2.64	1.86
90	2.74	2.2
100	2.62	2.2
110	2.5	1.99
120	2.58	1.99
130	2.6	2.2
140	2.56	2.21
150	2.5	2.21
160	2.4	2.18
170	2.42	2.18
180	2.44	2.16
190	2.5	2.2
200	2.52	2.2

keep the areas counted over cartilage rings and areas between them equal. There was also the added problem of the area to be counted extending to the rather ill-defined edges of the preparation. Generally, it was observed that the areas at the edge which corresponded with the cut end of a cartilage ring were better defined and the resulting dab more complete than corresponding areas obtained between the rings. This is partly due to the fact that the trachea is not flat, and the presence of the cartilage underneath the mucosa makes it slightly raised, whilst the areas in between are slightly depressed. The pressure of contact between the filter and the mucosa is therefore probably better over the cartilage rings than elsewhere. Similarly, as the trachea is circular and the cartilage gives the anterior surface a semi-circular shape, even contact over the whole surface is difficult. The dabs were taken with sufficient downward pressure of the filter to flatten the trachea and in so doing the extreme edges become blurred whilst the central areas give very good results. In method (b) it was the better central area which was used for counting, and since this gives nearly equal reproduction of the mucosa both over the cartilage and in between, the areas of each from which goblet cell numbers were determined were more likely to be equal. The slightly higher value obtained from method (a) can be explained by taking into account all of the above factors, the main reason probably being that since the mucosal reproduction

was better and wider over the cartilage rings than over the non-cartilage areas, more was counted from these areas. In Table 7, the periodicity of goblet cell numbers as they rise and fall, depending on whether the area counted was overlying a cartilage ring or not, can be clearly seen.

Obviously, counting 200 squares using a x40 objective is tedious and requires a lot of time. The primary interest was to see if the preparation could be used as a fast screening technique for goblet cell population fluctuations and consequently further methods of quantitation were investigated to see if a shorter method could be found.

Since the imprints were obviously 'patchy' in their effectiveness in lifting off goblet cells, a study was carried out to investigate the result of only counting the better areas and ignoring the poorer areas completely. The best areas were always found in the centre of the cartilage rings and these were used for quantification as follows:

Method (i) Using the x40 objective and the larger square in the graticule counts were made along the centre of each of the four lowest cartilage rings.

Method (ii) Using the x40 objective and the smaller square in the graticule counts were made along the centre of each of the four lowest cartilage rings.

Method (iii) Using the x25 objective and the photographic square in the photography graticule counts were made along the centre of the rings in the lower region of the trachea (i.e. the four lowest cartilage rings.

Method (iv) Using the x25 objective and the photographic square in the photography graticule counts were made from the two 'best' squares present in the lower region of the trachea (i.e. the four lowest cartilage rings).

Methods (iv) and (v) were as in (i) and (ii) but using the x6.5 objective.

Method (vi) An independent observer counted the same two rings as myself using a x25 objective and the photographic square. They also recorded the best two squares from these rings.

The results for methods (i) and (ii) are shown in Tables 8 and 9. It is evident that with a x40 objective and the larger of two graticule squares the results are highly variable. However, with the smaller square at the same magnification the results for each ring reached consistency once 10 - 15 squares had been counted.

TABLE 8 RESULTS OF QUANTITATION METHOD (i)

Ring No.	Number of Squares Counted	Average Number of Goblet Cells per Square	Method (i)
1	1	27	x40 objective
	2	22.5	
	3	24.3	Large
	4	26.8	graticule
	5	26.8	square
	6	26.5	
	7	26.4	
2	1	21	
	2	27.5	
	3	26.7	
	4	27.3	
	5	27.4	
	6	25	
	7	24.4	
3	1	15	
	2	18.5	
	3	16.2	
	4	21.2	
	5	24.2	
	6	24.8	
	7	-	
4	1	15	
	2	15	
	3	15.7	
	4	17.8	
	5	20.2	
	6	19.4	
	7	19.3	

TABLE 9 RESULTS OF QUANTITATION METHOD (ii)

Ring No.	Number of Squares Counted	Average Number of Goblet Cells per Square	Method (ii)
1	5	4	x40 objective
	10	3.5	
	15	3.9	Small
	20	3.9	graticule
	25	4.1	square
	30	4.1	
2	5	5.8	
	10	4.0	
	15	3.8	
	20	4.0	
	25	4.3	
	30	4.2	
3	5	4.2	
	10	4.0	
	15	4.0	
	20	4.1	
	25	4.6	
	30	4.2	
4	5	2.8	
	10	3.3	
	15	3.6	
	20	3.6	
	25	3.6	
	30	3.2	

The results for method (iii) are shown in Table 10. The results show fairly consistent numbers although this is not as good as with method (ii).

Methods (iv) and (v) were totally unsatisfactory as the magnification was too low. Method (iii) was preferred to method (ii) as it was felt that the x25 objective gave a more suitable magnification than did the x40 objective. The latter, it was felt, was too high a magnification, and the ease of picking out the goblet cells was greater with the x25 objective. Accordingly reproducibility by an independent observer was carried out at this power. The results are shown in Table 11 [Method (vi)]. It is evident that in some instances we agreed very closely with each other, in others we differed by so large an amount that it cannot be ignored. Occasionally, this could be explained by either one or other continuing to count areas which the other had rejected as too poor. Also, included in Table 11 are the results of the 'best' two squares by the two observers. None of these correspond very closely and it is obvious that two squares are not sufficient. Further work is required particularly in deciding the criteria for which areas should and should not be counted.

In conclusion, therefore, the tracheal dab technique provides a fairly quick and easy source of material for the assessment of tracheal goblet cell populations. Unfortuntely, owing to the patchy nature of the imprints sometimes obtained, quantitation has to be carried out over

TABLE 10 RESULTS OF QUANTITATION METHOD (iii)

Ring No.	Number of Squares Counted	Average Number of Goblet Cells per Square	Method (iii)
1	1	65	x25 objective
	2	61	
	3	59.5	Photographic square
	4	69	
	5	68.5	
	6	61	
2	1	58	
	2	60	
	3	51.5	
	4	48.5	
	5	49	
	6	-	
3	1	58	
	2	58	
	3	50	
	4	50	
	5	42	
	6	41	
4	1	40.5	
	2	30.5	
	3	35.5	
	4	36	
	5	38.5	
	6	-	

TABLE 11 REPRODUCIBILITY OF GOBLET CELL ASSESSMENT BETWEEN TWO INDEPENDENT OBSERVERS

Slide No.	Mean Goblet Cells/Square (Results of Best 2 Rings Combined)		Best 2 Squares	
	DL	MB	DL	MB
1	37.7	39.1	59 and 46	121 and 126
2	54.3	54.4	69 and 64	95 and 83
3	26.6	26.7	92 and 62	72 and 100
4	35.3	18	67 and 83	85 and 77
5	16	24.5	43 and 25	51 and 51
6	32.9	55.7	42 and 31	52 and 69
7	37.9	22.3	70 and 70	86 and 83
8	47.1	70.5	65 and 61	83 and 67
9	56.6	66.5	76 and 67	74 and 99
10	36.6	18.7	46 and 44	41 and 28
11	47.6	53.8	59 and 59	72 and 73
12	20.5	19.4	23 and 27	20 and 25
13	29.4	23.9	64 and 67	57 and 45

a very wide area of the sample and this, of course, takes time. It also necessitates large groups of animals to combat the subsequent within-group variation. As a technique, however, it definitely has potential, particularly for obtaining information much more quickly than by conventional histology. The problems of quantitation require further study, and unfortunately it has not been possible to do more in the course of this study. The preparation has proved remarkably stable on storage, and the material collected here is still usable despite, in some instances, being two years old, and thus is available for further study.

APPENDIX ii

OBSERVATION OF HEART RATE AND BLOOD PRESSURE:
RESPONSES TO VARIOUS DRUGS IN CONSCIOUS CATHETERISED RATS

Objectives

Owing to the very high doses of isoprenaline employed there was some doubt as to whether the dose of propranolol which antagonised the goblet cell response was also capable of antagonising the systemic pharmacological actions of isoprenaline namely, increased heart rate and decreased blood pressure. This experiment was designed to answer this question using catheterised rats from which heart rate and blood pressure could be continuously recorded.

Experimental details

Rats were fitted with carotid artery catheters, under general anaesthesia using the method of Popovic and Popovic (1960). The animals were allowed to recover from the operation for two full days and were used for the experiment in the third post-operative day.

To make recordings animals were restrained two at a time in perspex tubes, the carotid cannula was then connected to a pressure transducer using polythene tubing. Once the catheter had been flushed out with heparinised saline it was possible to obtain readings for the blood pressure on a Devices 2-channel recorder linked to the

pressure transducer. The pulse thus detected was used to trigger a rate-meter which then gave a reading for heart rate. The animals were left in the tubes, undisturbed, long enough to reach a 'steady' pulse and blood pressure, this was taken as a control resting value. Those rats with high resting heart rates, i.e. greater than 450 beats per minute were not used for the experiment. Having obtained a resting heart rate the animal was disconnected from the pressure transducer and removed from the plastic tube. It was then given one of several possible drugs intraperitoneally and either replaced immediately in the tube and reconnected to the pressure transducer or, as in the case of propranolol, put in a cage for 35 minutes before receiving isoprenaline intraperitoneally and then immediately reconnected to the pressure transducer.

Animals remained in the tube and connected to the pressure transducer throughout the first 30 minutes of recording. They were then removed from the tube and placed in a cage. For subsequent readings animals were taken from the cage, placed in the tube and connected to the pressure transducer and allowed 10 minutes to reacclimatise before taking the next reading. In this way the animals were not too distressed by being confined within a perspex tube for too long a period of time.

The drugs treatments tested were as follows:

Rat 1 40 mg/kg isoprenaline IP

Rat 2 40 mg/kg isoprenaline IP. This animal died.

Rat 3 40 mg/kg isoprenaline IP

Rat 4 40 mg/kg Clenbuterol IP

Rat 5 Saline 0.5 ml IP

Rat 6 Saline 0.5 ml IP

Rat 7 50 mg/kg propranolol IP followed by 40 mg/kg isoprenaline IP 35 minutes later. This rat did not show signs of being greatly depressed after the propranolol and did not exhibit signs of MSA as judged by a normal heart rate 35 minutes after the propranolol had been administered.

Rat 8 50 mg/kg propranolol IP followed by 40 mg/kg isoprenaline IP 35 minutes later. This rat was extremely depressed following the propranolol. It was catatonic and the heart rate was so low that it was impossible to trigger a recording on the Devices recorder even after the administration of isoprenaline.

Rat 9 50 mg/kg propranolol IP followed by 40 mg/kg isoprenaline 35 minutes later. This rat was also catatonic following the propranolol and showed

signs of MSA (membrane stabilisation activity) as judged by a very low heart rat at 35 minutes. However, it was possible to obtain a few readings from this rat after it had received the isoprenaline before the signal became too weak to trigger the Devices recorder.

Location

Alderley Park

Results

These are shown in Figures 27 and 28. The results for the two rats receiving 40 mg/kg isoprenaline were pooled together to give an average result for each reading as were those of the two saline controls. Control heart rate remained reasonably steady throughout the 6 hours of the experiment, the range being from 350 - 450 beats per minute. Likewise control blood pressure showed very little variation, the range being from 100 - 130 mm Hg. 40 mg/kg isoprenaline on its own induced a rapid increase in heart rate from a resting level of 420 beats per minute to 545 beats per minute; during the following 6 hours heart rate gradually fell to 455 beats per minute. Clenbuterol, a selective β_2-adrenoceptor agonist, also induced a rapid increase in heart rate from a resting value of 380 beats per minute to 505 beats per minute. However, unlike isoprenaline which took 6 hours to regain control values, Clenbuterol regained control heart rate by 50 minutes. 40

FIGURE 27

Showing the effects of 40 mg/kg isoprenaline and 40 mg/kg clenbuterol and 0.5 ml saline on heart rate *in vivo* in rats. Also shows are the effects in two rats of 50 mg/kg propranolol on the changes in heart rate produced by 40 mg/kg isoprenaline *in vivo*. In the rat receiving propranolol and isoprenaline and which did not exhibit symptoms of MSA (membrane stabilising activity) propranolol has failed to antagonise the isoprenaline induced increase in heart rate: represented by ▲.....▲ prop + isop (no MSA). In the rat which did exhibit symptoms of MSA, confirmed by the drop in heart rate 35 minutes after receiving the propranolol (P35), propranolol was partially inhibited the isoprenaline induced increase in heart rate: represented by △_·_·_·△ prop + isop (with MSA).

Saline n = 2
Isoprenaline 40 mg/kg n = 2
Clenbuterol 40 mg/kg n = 1
50 mg/kg propranolol + 40 mg/kg isoprenaline (- MSA)
n = 1
50 mg/kg propranolol + 40 mg/kg isoprenaline (+ MSA)
n = 1

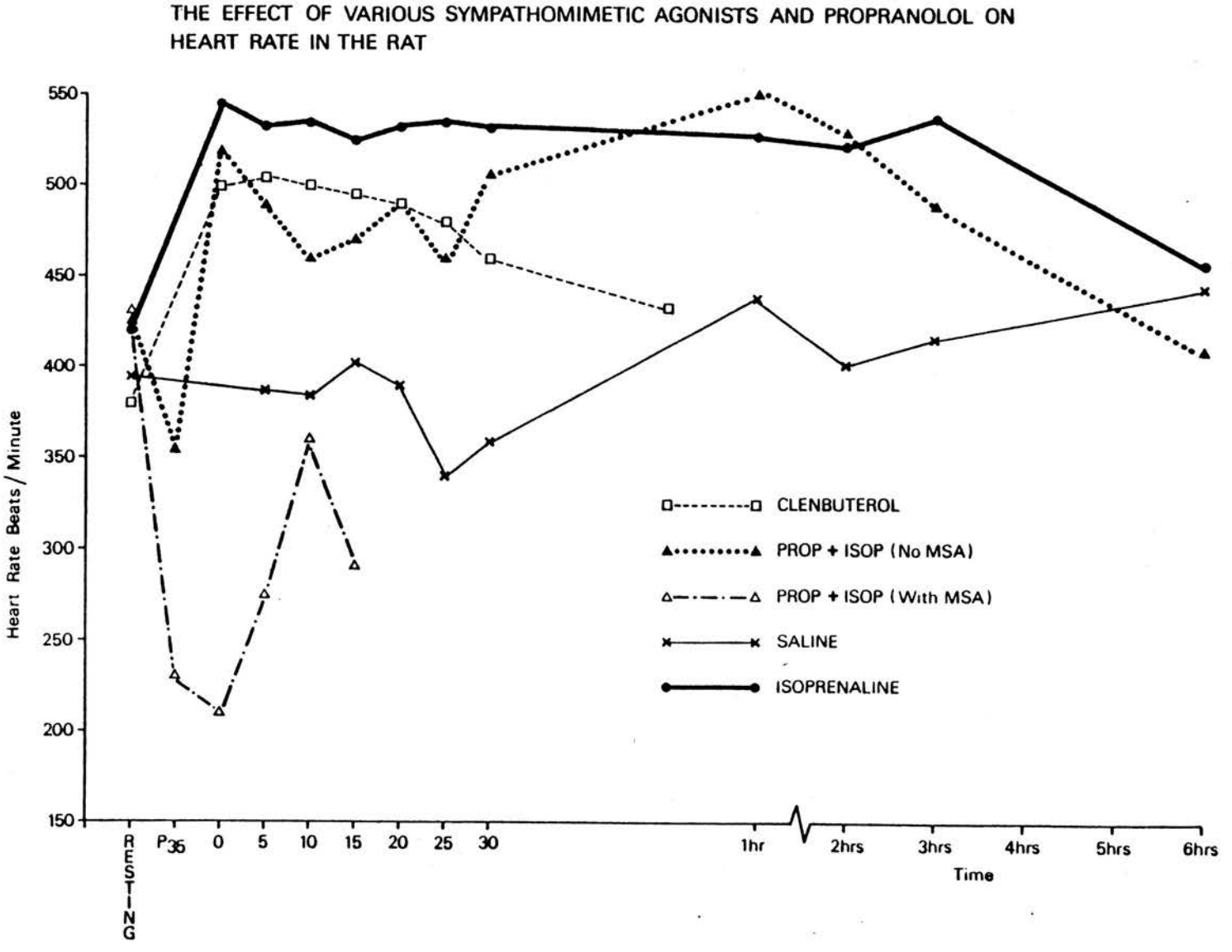

THE EFFECT OF VARIOUS SYMPATHOMIMETIC AGONISTS AND PROPRANOLOL ON HEART RATE IN THE RAT
Heart Rate Beats / Minute
550
500
450
400
350
300
250
200
150
RESTING
P35
0
5
10
15
20
25
30
1hr
2hrs
3hrs
4hrs
5hrs
6hrs
Time
CLENBUTEROL
PROP + ISOP (No MSA)
PROP + ISOP (With MSA)
SALINE
ISOPRENALINE

FIGURE 27.

mg/kg isoprenaline also produced a rapid decrease in blood pressure, from a resting value of 112 mm Hg to 72 mm Hg, thereafter during the following 6 hours the blood pressure remained at the same low level and was still only 77 mm Hg at 6 hours, well below the control value of 120 mm Hg. Clenbuterol produced a slightly slower decrease in blood pressure from 140 mm Hg to 75 mm Hg and at 50 minutes the blood pressure was 75 mm Hg, well below the control level of 110 mm Hg.

Of the two rats receiving 50 mg/kg propranolol prior to receiving 40 mg/kg isoprenaline, from which successful recordings were obtained, one had MSA and one did not. In the rat without MSA propranolol reduced the resting heart rate from 425 beats per minute to 355 beats per minute, whilst in the rat with MSA it fell from 425 beats per minute to 230 beats per minute. In neither rat did propranolol effect the blood pressure. When isoprenaline was administered to the rat without MSA it produced a rapid increase in heart rate which was only slightly lower than that of the isoprenaline controls. During the subsequent 6 hours the heart rate in this rat showed a very similar pattern to that of the isoprenaline controls. The propranolol in the absence of MSA had not therefore antagonised the heart rate effect of the isoprenaline. Similarly, in the rat without MSA, propranolol had no effect on the blood pressure response to isoprenaline.

FIGURE 28

As for Figure 27 but showing the corresponding effects on blood pressure in the same rate. Propranolol wih or without MSA has had no effect on the isoprenaline induced decrease in blood pressure.

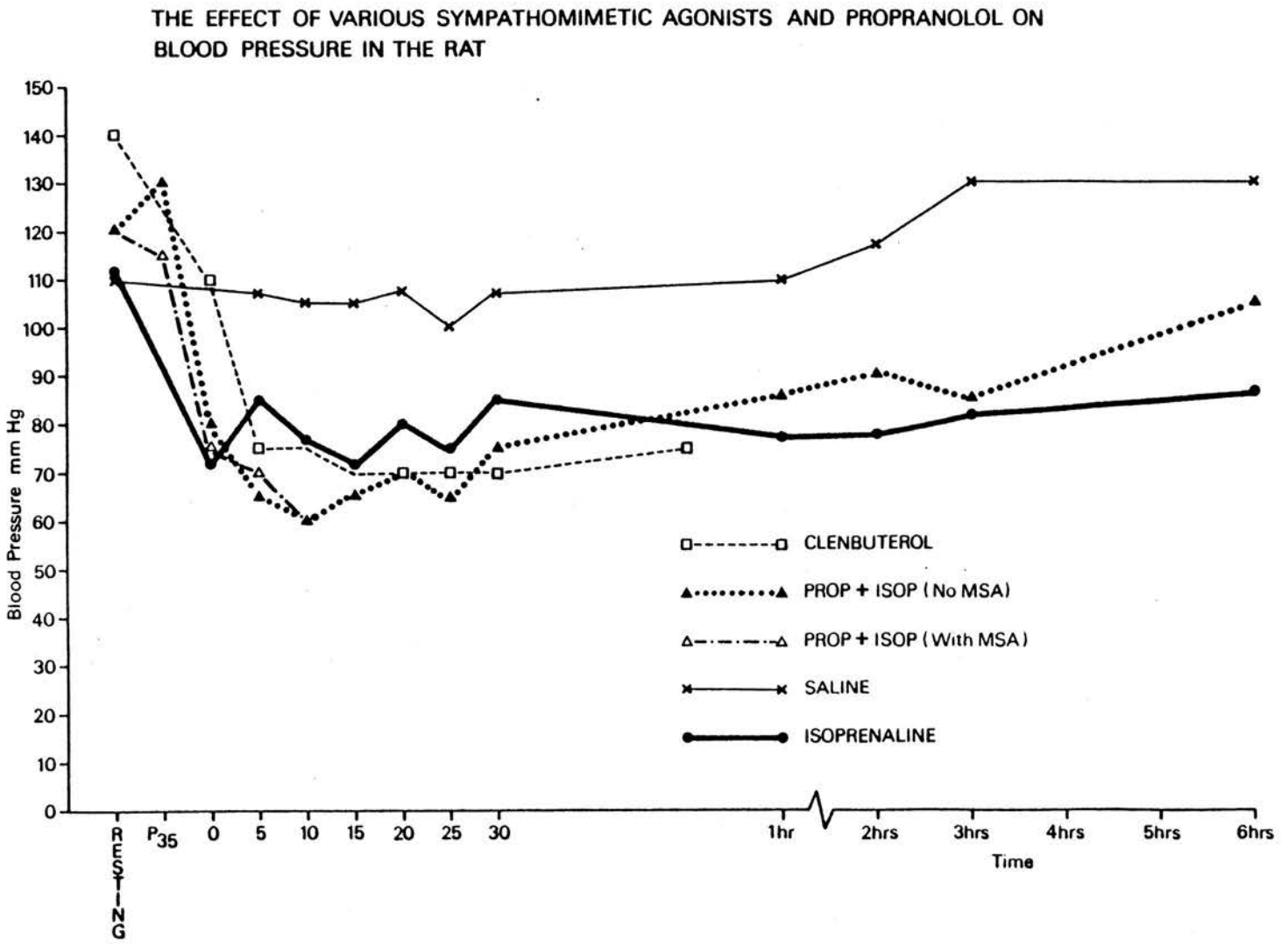

THE EFFECT OF VARIOUS SYMPATHOMIMETIC AGONISTS AND PROPRANOLOL ON BLOOD PRESSURE IN THE RAT
Blood Pressure mm Hg
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
RESTING
P35
0
5
10
15
20
25
30
1hr
2hrs
3hrs
4hrs
5hrs
6hrs
Time
CLENBUTEROL
PROP + ISOP (No MSA)
PROP + ISOP (With MSA)
SALINE
ISOPRENALINE

FIGURE 28.

However, in the rat with MSA isoprenaline appeared to produce only a transient increase in heart rate from 210 beats per minute to 360 beats per minute and at 15 minutes the heart rate had again fallen to 295 beats per minute, at which point recording ceased. The propranolol in conjunction with MSA had therefore inhibited the isoprenaline heart rate response. However, propranolol again had no effect on the blood pressure response to isoprenaline even in the presence of MSA.

Conclusions

This experiment has shown that in some rats 50 mg/kg propranolol IP can produce a marked degree of membrane stabilisation. This means that in addition to its actions as a β-adrenoceptor antagonist, it is acting as a powerful local anaesthetic. It is therefore inhibiting a great deal of the normal nerve impulse traffic and electrophysiological activity throughout the body. It is interesting that in the presence of MSA propranolol appeared to inhibit to some extent the effects of isoprenaline on heart rate whilst not affecting its effects on blood pressure. The former is due to β_1-adrenoceptor stimulation in the heart whilst the latter is due to β_2-adrenoceptor stimulation in the smooth muscle of the peripheral vasculature. The positive chronotropic and inotropic effects on the heart, of isoprenaline, are dependent on an efficient conducting system within the myocardium; in particular the sinu-

atrial node, the Purkinje tissue, the bundle of His and the atrioventricular node (Keele and Neil, 1971). All of these specialised conducting tissues will have been subjected to membrane stabilisation by the propranolol and this in turn will prevent the isoprenaline from having any effect on heart rate. However, β_2-adrenoceptors are not innervated and bring about relaxation of the muscle by means of increased intracellular levels of c-AMP. Thus, the effect of isoprenaline on blood pressure, not being dependent on electrophysiological phenomena, is unaltered by the existance of MSA.

The relationship between the presence of MSA and the antagonism of the isoprenaline-induced goblet cell hyperplasia is not known. Further work is required to study the cardiovascular effects in greater detail and also to see if in the absence of MSA propranolol is capable of antagonising the goblet cell response. In all of the experiments to date, animals exhibiting signs of MSA were assigned to a group receiving isoprenaline whilst those not exhibiting signs of MSA were used as propranolol controls. The propanolol control group therefore generally consisted of mainly depressed (MSA) rats with a few non-MSA rats, whilst the propranolol versus isoprenaline groups generally consisted of only those animals with MSA.

In the light of the evidence so far, it is probable that the propranolol antagonism of the isoprenaline-induced

goblet cell hyperplasia is due to its membrane stabilisation activity and not just competition for the β-adrenoceptors, since the studies on blood pressure have shown that propranolol was unable to block the isoprenaline induced fall in blood pressure.

APPENDIX iii

CALCULATIONS FOR THE ATMOSPHERE CONCENTRATIONS DURING THE NEBULISER STUDY DATED 1.5.80 CTL, ICI: EXPERIMENT II

1st Run

Five rats exposed to solution containing Saline and 10^{-2}M ascorbic acid.

Flow rate of compressed air (saturated) was 6 l/min
Outflow with chamber was 10.5 l/min
Total atmosphere sample taken at 2 l/min for 15 mins
Total exposure time = 45 mins

Wet filter weight	= 0.01891 g	post dry wt.	= 0.01445 g
Pre-dry weight	= 0.01434 g	pre-dry wt.	= 0.01434 g
Gain net weight	= 0.00457 g	Gain in dry solid	= 0.00011 g

2nd Run

Five rats exposed to solution containing 100 mg/ml isoprenaline, 10^{-2}M ascorbic acid in physiological saline.

Solution contained the following solids in 1 ml:

1.98 mg ascorbic acid
9.00 mg NaCl
100.00 mg isoprenaline

Total solids in 1 ml = 110.98 mg

% of total solids = isoprenaline = $\frac{100}{100.98} \times 100 = 90.1\%$

Flow rate of compressed air (saturated) was 6 l/min

Outflow into chamber was 10.5 l/min

Total atmosphere sample 1 taken at 2 l/min for 10 mins

Anderson sampler used at 1.41 l/min for 15 mins

Total atmosphere sample 2 taken at 2 l/min for 5 mins

Total exposure time = 45 mins

Solution used during exposure = 15 - 12.5 ml = 2.5 ml/45 mins

Calculations

(i) Total atmosphere sample 1 collected 0.0005 g total solids (dry wt.)

.·. 0.5 mg were collected in 20 l in 10 mins

.·. Total atmosphere conc. filter 1 = 0.025 mg/l

= 25 µg/l solids

Now 90.1% solids = isoprenaline = 22.53 µg/l isoprenaline conc.

Assuming rat minute volume = 500 ml/min

Then each rat received 11.26 µg/min/rat isoprenaline

.·. In 45 mins exposure each rat received 506.7 µg isoprenaline.

(ii) Total atmosphere sample 2 collected 0.00045 g total solids (dry wt.)

.·. 0.45 mg were collected in 10 l in 5 mins

.·. Total atmosphere conc. filter 2 = 0.045 mg/l

= 45 µg/l solids

Now 90.1% solids = isoprenaline = 40.55 µg/l isoprenaline

Assuming rat minute volume = 500 ml/min

Then each rat received 20.275 µg/rat/min isoprenaline

∴ In 45 mins exposure each rat received 912.4 µg/rat isoprenaline

If we average total atmosphere conc. from filters 1 and 2 we get the following dose:

Filter 1 TA concn. = 25 µg/l

Filter 2 TA concn. = 45 µg/l

Average TA concn. = 35 µg/l (90.1% isoprenaline)

∴ per rat per min $\frac{31.55}{1000}$ x 500 = 15.775 µg/rat/min isoprenaline

∴ In 45 mins exposure each rat received 709.8 µg/rat isoprenaline

(iii) Anderson sampler. 1.4 l/min for 15 mins = 21 l.

plate 1 + 0.00011 g

plate 2 - 0.00009 g

plate 3 + 0.00065 g

plate 4 + 0.00059 g

Filter + 0.00088 g

$\frac{\text{Wt. collected}}{\text{Total volume}}$ = atmos. conc. (mg/l)

$\therefore \frac{0.11}{21}$ = 0.00524 mg/l plate 1

$\frac{0.65}{21}$ = 0.03095 mg/l plate 3

$\frac{0.59}{21}$ = 0.028095 mg/l plate 4

$\frac{0.88}{21}$ = 0.04190 mg/l plate 5

Total solids in 1 litre = 0.1619 mg

However, only 90.1% total solids = isoprenaline

$\therefore$ Isop. collected in 1 litre = 0.09568 mg/l

Assuming rat minute volume of 500 ml/min

Each rat received 0.04783 mg/isop/min/rat

$\therefore$ In 45 mins exposure total dose of isoprenaline received = 2.1523 mg isop/rat.

(iv) Weight loss from bottle

Solution used = 2.5 ml/45 mins

= 3.333 ml/hr

Generation rate into box = 10.5 l/min

= 630 l/hr

Total solids in 1 ml solution = 110.98 mg

$\therefore$ In 3.333 ml solids = 3.69.89 mg

$\therefore$ Atmos. concn. = $\frac{369.98}{630}$ = 0.5871 mg/l

Now 90.1% solids = Isoprenaline

$\therefore$ Total concn. of isoprenaline in atmosphere = 0.529 mg/l

Assuming minute volume of rat = 500 ml/min

Then dose to rat = 0.2645 mg/min/rat

∴ In 45 mins total exposure dose received = 11.90 mg/rat/isop.

3rd Run

Five rats exposed to solution containing 10 mg/ml isoprenaline, 10^{-2}M ascorbic acid and physiological saline.

Solution contained following solids in 1 ml:

10 mg isoprenaline

1.98 mg ascorbic acid

9.0 mg NaCl

Total solids in 1 ml = 20.98 mg

% total solids = isoprenaline = $\frac{10}{20.98}$ .x 100 = 47.61%

Flow rate of compressed air (saturated) = 6 l/min

Outflow into chamber was 10.5 l/min

Total atmosphere sample 4 taken at 2 l/min for 15 mins

Anderson sapler used at 1.4 l/min for 15 mins

Total atmosphere sample 5 taken at 2 l/min for 11 mins

Total exposure time = 45 mins

Solution used during exposure = 15 - 11.2 ml = 3.8 ml/45 mins

Calculations

(i) Total atmosphere sample 4 collected 0.00046 g in 15 mins

.·. 0.46 mg were collected in 30 l in 15 mins

.·. Total atmosphere concn. filter 4 = 15.33 µg/l solids

Now 47.61% solids = isoprenaline = 7.298 µg/l isoprenaline

Assuming rat minute volume = 500 ml/min

Then each rat received 3.649 µg/min isoprenaline

Therefore in 45 mins exposure each rat received 164.21 µg/rat isoprenaline

(ii) Total atmosphere sample 5 collected 0.00033 g in 11 min dry solids

.·. 0.33 mg were collected in 22 l in 11 mins

.·. Total atmosphere conc. filter 5 = 0.015 mg/l

= 15 µg/l solids

Now 47.6% solids = isoprenaline = 7.14 µg/l isoprenaline

Assuming rat minute volume = 500 ml/min

Then each rat received 3.57 µg/min isoprenaline

Therefore in 45 mins exposure each rat received 160.65 µg/rat isoprenaline

If we average Total Atmosphere concn. from filters 4 and 5 we get the following dose:

Filter 1 TA concn. = 15.33 µg/l

Filter 2 TA concn. = 15.00 µg/l

Average TA concn. = 15.165/l (47.6% isoprenaline)

∴ per rat per min $\frac{7.218}{1000}$ x 500 = 3.609 µg/min/rat

∴ In 45 mins exposure each rat received 162.42 µg/rat/isoprenaline

(iii) Anderson sampler. 1.4 l for 15 mins = 21 l total volume.

plate 1 + 0.00024 g

plate 2 + 0.00012 g

plate 3 - 0.00001 g

plate 4 + 0.00019 g

Filter + 0.00037 g

$\frac{\text{Wt. collected}}{\text{Total volume}}$ = atmos. concn. (mg/l)

∴ $\frac{0.24}{21}$ - 0.01143 mg/l plate 1

$\frac{0.21}{21}$ = 0.01 mg/l plate 2

$\frac{0.19}{21}$ = 0.009048 mg/l plate 4

$\frac{0.37}{21}$ = 0.01762 mg/l plate 5

Total solids in 1 litre = 0.048095 mg

However only 47.6% total slids = isoprenalne

∴ Isoprenaline collected in 1 litre = 0.02289 mg/l

Assuming rat minute volume of 500 ml/min

Each rat received 0.01146 mg/min isoprenaline

∴ In 45 mins exposure total dose of isoprenaline received = 0.5157 mg/rat isoprenaline

(iv) Weight loss from bottle

Solution used = 15 - 11.2 ml = 3.8 ml in 45 mins

= 5.066 ml/hr

Generation rate into box = 10.5 l/min

= 630 l/hr

Total solids in 1 ml solution = 20.98 mg

∴ In 5.066 ml solids = 106.285 mg

∴ Atmos. concn. = $\frac{106.285}{630}$ = 0.1687 mg/l solids

Now 47.6% solids = isoprenaline

∴ Concentration of isoprenaline in atmosphere = 0.0801 mg/l isoprenaline

Assuming minute volume of rat = 500 ml/min

Then dose to rat = 0.040203 mg/min/rat

∴ In 45 mins exposure total dose received = 1.809 mg/rat isoprenaline

Summary of Atmosphere Concentrations and Isoprenaline Dosage

100 mg/ml run

	Atmos. concn.	Dose
Total Atmos. Sample 1	22.53 µg/l isop. =	506.7 µg/rat/45 mins
Total Atmos. Sample 2	40.55 µg/l isop. =	912.4 µg/rat/45 mins
Average =	31.54 µg/l isop. =	709.8 µg/rat 45 mins
Anderson Sampler	95.68 µg/l isop. =	2.15 mg/rat/45 mins
Weight loss from bottle	0.529 mg/l isop. =	11.9 mg/rat/45 mins

10 mg/ml run

	Atmos. concn.	Dose
Total Atmos. Sample 1	7.298 µg/l isop. =	164.21 µg/rat/45 mins
Total Atmos. Sample 2	7.14 µg/l isop. =	160.65 µg/rat/45 mins
Average =	7.218 µg/l isop. =	162.42 µg/rat/45 mins
Anderson Sampler	22.92 µg/l isop. =	0.516 mg/rat/45 mins
Weight loss from bottle	80.405 µg/l isop.=	1.809 mg/rat/45 mins

Characteristics of Anderson Impaction Sampler

Plate 1 traps particles > 5.5 μm

Plate 2 traps particles 5.5 - 3.5 μm

Plate 3 traps particles 3.5 - 2.0 μm

Plate 4 traps particles 2.0 - 0.3 μm

Filter 5 traps particles < 0.3 μm

Deposition characteristics of aerosol particles

For particles > 5 μm 50% deposits in nose, the rest in URT. For particles < 5 μm < 20% deposits in nose, up to 50% in the URT and the rest in LRT. Maximum deposition in LRT possible is approximately 70% at about 0.5 μm (see graph below).

Wright's Nebuliser produces particles nominally < 8 μm.

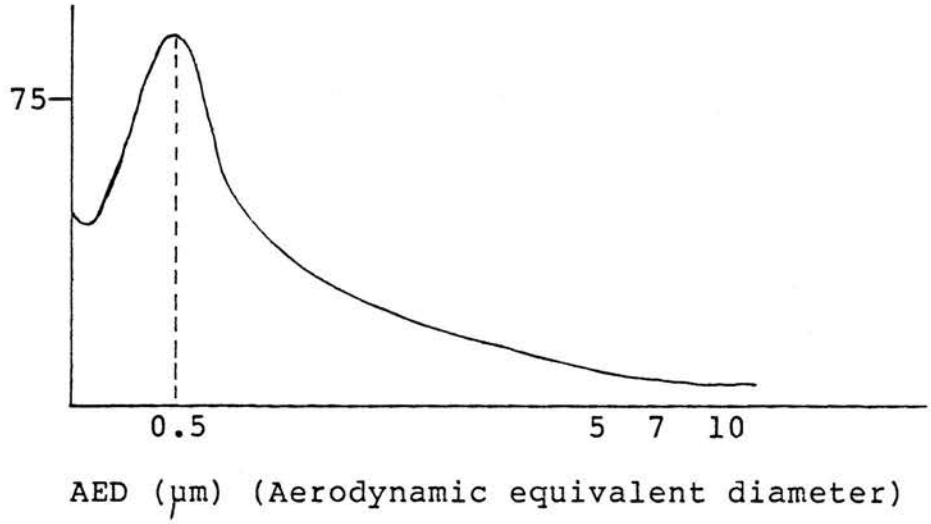


Figure 30. The Effect of Particle Size on the Percentage Deposition in the Lower Respiratory Tract

APPENDIX iv

COMPARISON OF VARIOUS FIXATIVES AND THEIR SUITABILITY FOR THE STUDY

There are several fixatives recommended as being compatible for studies at both the light and electron microscope level (Carson et al., 1972). Some have the disadvantage of being costly and difficult to make up on a large scale, however, there remain several possibilities which it was considered should be tried out to see which was most suitable for this study.

EXPERIMENTAL DETAILS

Fixatives and Formulation

1) Glutaraldehyde in phosphate buffer

32 ml of 25% Glutaraldehyde (EM grade)
368 ml of Millonig's phosphate buffer (170 ml of 2.5% NaOH and 830 ml of 2.26% NaH_2PO_4).

2) Millonig's phosphate buffered formaldehyde (Modified by Carson et al., 1973)

40 ml 37-40% Technical grade formaldehyde
360 ml Distilled water
7.44 g NaH_2PO_4
1.68 g NaOH

3) 4CF-1G (4 parts formaldehyde : 1 part glutaraldehyde)

4.64 g NaH_2PO_4

1.08 g NaOH

344 ml Distilled water

40 ml 37-40% formaldehyde (Technical Grade)

16 ml 25% glutaraldehyde (EM Grade)

4) Buffered 10% Formalin (Departmental routine fixative)

100 ml 37-40% Technical grade formaldehyde

900 ml Distilled water

6.5 g Na_2HPO_4

4.0 g NaH_2PO_4

Tissues

Four rats were used for the study. They were killed using an overdose of sagatal and the following tissues taken for histology:

1 mm slices of liver

Short sections of ileum fixed in rings

Kidneys sliced in half

Intact heart

Intact spleen

Stomach opened and cleaned

Lung inflation fixed. Left lung for light microscopy, right lung for electron microscopy

A piece of each tissue was placed in each of the four fixatives being tested. Initial fixation was carried out at 4°C for 24 hours, after which time storage continued at room temperature. Pieces of tissue were taken for histology at 24 hours, 3 weeks and several months later. For light microscopy the tissues were embedded in GMA, for electron microscopy the tissues were post-fixed in 0.5% osmium tetroxide (OsO_4) in Millonig's phosphate buffer for 3 hours and then processed into araldite using the routine procedure adopted in the EM Suite of the Department of Pathology.

CONCLUSIONS

Of the four fixatives tested Millonig's phosphate buffered formaldehyde was chosen for the following reasons: it gave good results in most tissues at both the light microscope and EM levels, particularly for the lung and small intestine. Penetration was superior to that of glutaraldehyde in the spleen and liver and the heart was adequately fixed when left whole. It is very simple to make up, stable for long periods of time and unlike glutaraldehyde, it is reasonably cheap and relatively non-toxic.

Glutaraldehyde was excellent at the EM level and produced very good EM results. However, penetration was very poor in the liver, spleen and heart. An additional

drawback is that during the fixation process, glutaraldehyde releases a large number of free-aldehyde groups within the tissue and this interferes with the specificity of PAS staining making it very non-selective. This, of course, masks to some extent the mucin staining and makes it much harder to identify goblet cells.

The routine formaldehyde tested gave adequate fixation for light microscopy but there was evidence of shrinkage artefact in the small intestine, and relatively poor fixation for EM study. The mixture of formaldehyde/ glutaraldehyde tested gave least penetration, and produced poor results at both the light and EM levels.

APPENDIX v

DEVELOPMENT OF NEW HISTOLOGICAL TECHNIQUES FOR THE QUANTITATIVE ASSESSMENT OF THE GOBLET CELL AND OTHER EPITHELIAL CELL TYPES IN THE RAT LUNG USING BOTH THE LIGHT AND ELECTRON MICROSCOPE

Bearing in mind the original intentions of the project as given in the synopsis at the beginning of the thesis, the histological methods employed must, if they were to be capable of demonstrating both goblet cells and other cell types within the respiratory epithelium, at both the light and electron microscope levels, satisfy the following requirements:

(1) To be able to identify all the different cell types at the light and electron microscope levels; ideally using one technique, or it may be necessary to use a combination of techniques.

(2) The embedding medium and the fixation technique employed should be compatible with both the light and the electron microscope.

(3) It must be possible to count individual goblet cells quickly and unequivocally.

(4) In order to overcome the problems of sampling caused by the uneven distribution of goblet cells in the respiratory tract, the sections prepared for the light microscope need to encompass the entire longitudinal

section of the main airway in the rat lung and some of its side branches. They therefore need to be quite large, at least 1 x 1.5 cm and ideally 2 x 2 cm.

(5) It must be possible to stain nuclei and cytoplasmic detail sufficiently to allow identification of labelled cells in autoradiographs.

(6) The sections must be thin (1.5 - 2.0 μm) if the nuclei and cells are not to overlap cells underneath, that is, sections must be less than one cell thick.

(7) In order to facilitate rapid assessment of new compounds for goblet cell hyperplasia activity a fast screening procedure needs to be developed: see the tracheal dab technique Appendix i.

SELECTION OF A SUITABLE EMBEDDING MEDIUM

Ideally the embedding medium chosen had to be compatible with both the light and electron microscopes. There are a few resins available which satisfy this requirement. These are the epoxy resins Araldite and Epon 812, Spurr resin, and a resin made up from a combination of Epon 812 and Araldite.

Spurr resin was tested for suitability and quickly rejected after preliminary work revealed that sections stained for the light microscope rapidly faded to such an extent that they were no longer usable. In addition most

of the reagents for this resin are extremely toxic and hazardous and it is therefore particularly unpleasant to work with on a large scale.

Epon 812, like Araldite, is an epoxy resin and as such has many characteristics in common with Araldite, particularly with regards staining properties for the light microscope. Since Araldite was in routine use in the Department's EM suite and was therefore well tested for use in routine EM work, it was decided to concentrate on Araldite as an embedding medium. Likewise, the combination of Epon 812 and Araldite was not pursued beyond the preliminary stage as it exhibited the same staining properties as Araldite when used alone.

PROCESSING LARGE BLOCKS OF TISSUE INTO ARALDITE

The ultimate aim of the histological techniques employed was to achieve large, thin plastic sections of tissue. It follows, therefore, that large blocks of tissue, in this case lung, had to be processed and embedded in Araldite, the chosen embedding medium. This presented a number of problems. Firstly, the processing procedure, i.e. dehydration from fixative to Araldite had to be adapted to allow adequate penetration of the alcohols and other reagents throughout the tissue block. A method was worked out which was an adaptation of that used in the EM suite for very small blocks of tissue and that used for

routine paraffin embedding. This work was carried out with the assistance of Mr J.K. Rae of the Departmental technical staff and the method eventually developed is his adaptation. Details of the processing and embedding procedures for araldite are given in the histology methods sections in Chapter 2, along with details of the polymerisation procedure adopted, and the methods for the trimming-in of blocks and the cutting of sections. Since the cutting of large, thin sections of araldite embedded material presented a number of problems not discussed elsewhere in this thesis a brief outline of these difficulties will be given here.

PROBLEMS ENCOUNTERED WHEN CUTTING LARGE, THIN SECTIONS OF ARALDITE EMBEDDED MATERIAL

The sections obtained lacked definition, were of uneven thickness, were poorly stained and very difficult to focus adequately for study under higher powers on the light microscope. The blame for this inadequacy was first thought to be an inability to achieve crisp and definitive staining of the sections. However, a wide range of stains, 'mordants' and promoters all failed to improve the staining quality sufficiently (see next section).

At the time sections were cut using a tungsten carbide edged knife fitted to a Reichert-Jung Autocut microtome, a new machine which had been specifically developed by the manufacturers to make it possible to cut large plastic

embedded sections, of any thickness from 0.5 μm - 30 μm. Because of the newness of the techniques involved, all stages of microtomy, namely fixation, processing, embedding, cutting, floating out, staining and mounting of sections, had to be developed from the start. Having established that staining was not the problem it was necessary to see which of the preceding events was at fault. The fixative was not thought to be the problem as it produced adequate results both at the EM level and in paraffin (see Appendix iv). The processing technique evolved to cope with larger blocks of tissues was very similar to that used routinely for EM preparation, however, it was more protracted to allow adequate penetration of the tissue by all reagents. In order to see if this had any effect on the section quality, a large araldite block was prepared using the new regime and then sawn up into 3 mm x 3 mm blocks, mounted on beam capsules and 1.5 μm sections cut using a 6.25 mm glass knife on a conventional ultramicrotome. The improvement in staining and histological detail was very pronounced (see Figures 31 - 33). By comparing Figure 31 and Figure 33, it can be seen that the quality obtained was comparable to that achieved using conventional EM techniques. The conclusion drawn from this was that the fault lay with the cutting technique employed, i.e. the tungsten carbide knife. This knife is not exceptionally sharp like a conventional D-profile steel knife or a glass knife and cuts blocks by virtue of the

FIGURE 31

1 µm section of rat lung embedded in araldite. Post-osmicated and stained with 1% Toluidine Blue in 1% borax. Magnification = x40.
Section cut using Reichart-Jung Autocut fitted with a tungsten carbide edged steel knife. Note the apparent loss of cilia, the broken alveolar structure, variable depth of staining and inability to focus the whole field of view. The block cut was approximately 1 in by 1 in.

FIGURE 31.

FIGURE 32

As for Figure 31, except this section was cut with a large glass knife (12.5 mm wide) fitted to the Autocut. It is impossible to focus any part of the section at x40 magnification. The light and dark striations are caused by excessive judder in the block causing 'chatter' at the knife edge and variable thickness of the section along with severe wrinkling. The block cut measured 1 cm wide by 1.5 cm long.

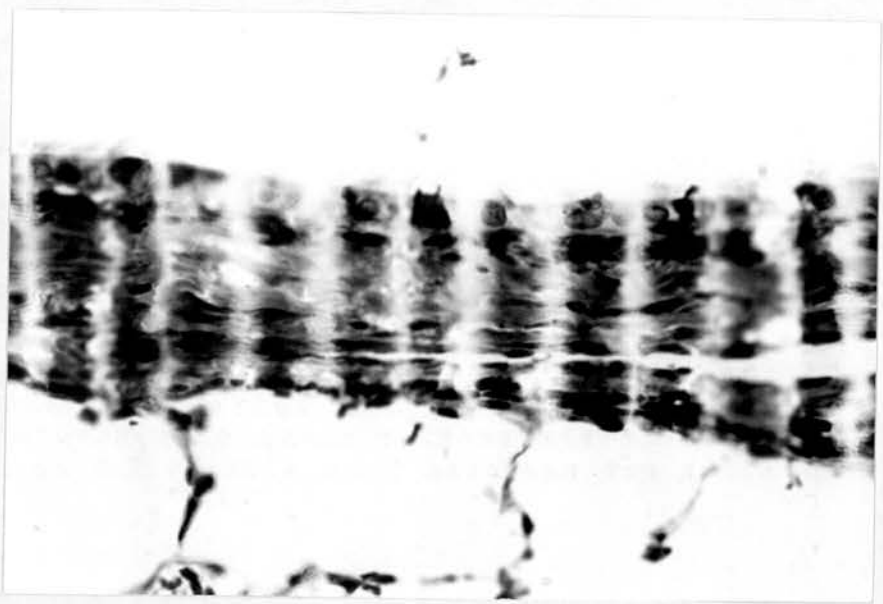


FIGURE 32.

FIGURE 33

As for Figure 31, except this section was cut on a conventional ultramicrotome using a 6.25 mm wide glass knife. In fact the block used to obtain this section was sawn out of the block used for Figure 31 and mounted on a Beem capsule, it measured approximately 5mm x 5 mm. The cytological detail obtained is excellent and compares very favourably with that obtained using conventional EM techniques. It was this section which provided the evidence that the tungsten carbide knife was the cause of the poor quality of sections obtained in the larger blocks.

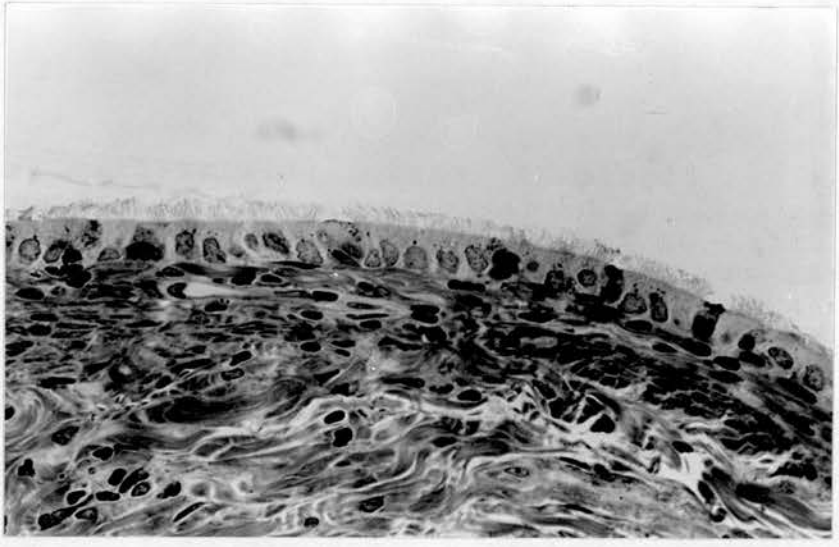


FIGURE 33.

pressure exerted at the knife edge, rather than by shearing off a slice of block as sharper knives do. In some way this adversely affects the quality of the sections obtained and also renders them less permeable to stains.

At this stage it became obvious that if large, thin plastic sections of the quality desired were to be achieved then either a large glass knife had to be fitted to the Autocut or a new 'softer' resin had to be used as an embedding medium.

Reichert-Jung at this time produced an adaptation of the glass knife holder which allowed it to take 12.5 mm thick glass knives. Using one of these it was hoped to be able to cut post-osmicated araldite embedded blocks 1 cm x 1.5 cm large. By careful trimming of the lung prior to embedding, and of the blocks once hardened, it was thought that complete longitudinal sections of the main airway of the left lung of the rat may be obtained. However, there was a delay in the delivery of the glass knife holder and during this time various different embedding media were tried as alternatives.

One of them, glycolmethacrylate (GMA), a water soluble methacrylate resin, proved to be suitable. It had several advantages over araldite, not least being ease of staining. It was also quicker to process, easier to cut requiring a conventional D-profile steel knife only, and this allowed the use of large blocks. Unfortunately, GMA is not

suitable for the EM as it is not electron-stable, and also in our experience it is not compatible with osmium tetroxide and this limits contrast in the sections obtained. Finally, background staining in autoradiographs makes them difficult to study.

All this had taken several months to establish and as time was pressing the decision was taken to embed the left lungs in GMA for goblet cell assessment, post-osmicate selected right lungs, embed them in araldite and cut sections using a 12.5 mm glass knife as and when this technique became available. At the time of writing the use of the 12.5 mm glass knife still does not give sections of an adequate quality (see Figure 32). This is mainly because it has proved impossible to flatten the sections sufficiently when mounted on the microscope slide to allow for their study under the higher powers of magnification on the light microscope.

STAINING METHODS APPLIED TO ARALDITE SECTIONS

The objectives were to achieve crisp, definitive staining of all cell types in the respiratory epithelium and also to be able to distinguish goblet cells unequivocally for easy quantitation.

Toluidine Blue

1% solution in 1% borax or 1% solution in 1% Na_2CO_3.

Staining was carried out at 60°C for 30 - 60 secs on the slide using a hot plate or for 2 - 3 mins in a water bath set at 60°C.

Methylene Blue/Basic Fuchsin (Jones et al., 1977)

1% solution of methylene blue in 1% borax

0.5% solution of basic fuchsin in alcohol

Staining carried out at 60°C for 60 secs in methylene blue using a hot plate followed by 15 - 20 secs at room temperature in basic fuchsin.

Periodic Acid-Schiff (PAS) Toluidine Blue (Jones et al., 1977)

2.5% periodic acid

Schiff reagent, prepared using the 'Lillie Cold Method' (Lillie, 1965)

Preheat slides for 1 hour in xylene at room temperature. Dry sections at 60°C and rinse in water. Treat with 2.5% periodic acid at 60°C for 15 mins. Wash sections then dry. Place in Schiff reagent and leave in the dark overnight. Wash sections and allow colour to develop, approximately 5 mins. Dry sections and counterstain with 1% Toluidine Blue in 1% borax at 60°C for 45 - 60 secs.

Tribasic Stain (Grimley et al., 1965)

100 ml 30% ethanol containing 0.4 g Azure B; 1 g malachite green; 1 g aniline; 1 g phenol crystals.
Filter and store at room temperature.

Prepare sections for staining using following 'mordant' procedure: 10 mins at room temperature in 0.5% $KMnO_4$. Wash. 5 mins at room temperature in 1.0% oxalic acid. Wash. 1 min at room temperature in 1.0% Li_2CO_3. Wash. Cover sections with stain solution and leave for 1 - 2 mins at room temperature. Rinse and mount sections having air dried them. Sections can be counterstained with 4% saturated solution of aqueous basic fuchsin in distilled water: filter and warm basic fuchsin to 50°C, cover section and leave for 1 - 5 mins, checking all the time for depth of staining. Blot sections, rinse and dry in air.

Azure B (Grimley et al., 1965)

100 ml 30% ethanol containing 0.4 g azure B; 1 ml aniline, 1 g phenol crystals.
Filter and store, do not dilute.

Prepare sections using the same mordant procedure as for the tribasic stain. Cover sections with the Azure B solution and stain for 0.5 - 1 min at room temperature. Rinse sections and air dry. Sections can be counterstained with basic fuchsin as in the tribasic stain.

Results

The only really satisfactory stain for araldite sections was 1% Toluidine Blue in 1% borax. Definition and cytoplasmic detail was particularly good in post-osmicated material provided the sections were cut with a glass knife (Figure 33). Staining of sections cut with the tungsten carbide knife was patchy and the overall picture seen with the light microscope was hazy (Figure 31).

The use of methylene blue/basic fuchsin gave very little improvement over Toluidine Blue although it was found that elastic laminae in blood vessels and airway musculature showed up very well indeed.

The Tribasic stain gave interesting results in the kidney and may be quite useful for sections of kidney, however, it offered no improvement over Toluidine Blue in the lung. Likewise for Azure B, which was more difficult to produce than Toluidine Blue and was not as effective.

The PAS staining in araldite was extremely faint despite the long drawn out staining method and it ws considered inadequate for the reliable quantitation of goblet cells using the light microscope.

Many people have claimed to get good results with a variety of stains following de-aralditing procedures (Donald Mayor et al., 1961; Yensen, 1968; Imai et al., 1968). These usually involve exposure of sections to

alcoholic sodium methoxide or bromine gas, neither of which are pleasant to work with. Sodium methoxide treatment was tried on a number of slides; a large number of sections became detached from the slides but those that remained and which were subsequently stained with Toluidine Blue revealed that, along with the araldite, a lot of the tissue itself had disappeared. This was particularly true in the lung, and this method too was abandoned.

To conclude, therefore, the only really satisfactory stain for araldite embedded material is Toluidine Blue. It was not possible to apply any mucin specific stains and this would have made the quantification of goblet cells difficult; particularly as, although human goblet cell mucin readily displays metachromasia with Toluidine Blue in the rat, it does so only rarely. The cytological detail in the sections used for quantification would have therefore needed to be of a very high quality if goblet cells were to be identified easily and accurately.

CONCLUSIONS

Araldite proved unsatisfactory for the purposes of this project for the following reasons:

(1) It was incompatible with most histological stains and it proved impossible to stain selectively for goblet cell mucins.

(2) It was very difficult to cut large, thin sections from araldite blocks using both the tungsten carbide edged knife and a large glass knife.

(3) It was impossible to achieve adequate flattening of the sections when mounted on glass microscope slides. This meant that the sections could not be studied using the higher powers of magnification on the light microscope.

(4) Processing and polymerisation took place over long periods of time. This led to a prolonged period of time between dosing the animals and acquiring the sections for counting.

(5) All the techniques employed were highly labour intensive and this also contributed to the time factor.

(6) Autoradiographs proved very unreliable and the techniques were not pursued beyond the very early stages.

(7) Many of the reagents employed are highly toxic and allergenic and this necessitates the use of a fume cupboard throughout the embedding procedure.

THE COMPROMISE SOLUTION EMPLOYED FOR THE REST OF THE PROJECT

It had taken many months to find that the cause of the poor histology lay with the cutting technique employed, i.e. the tungsten carbide knife. At this point there were three alternative paths which could be followed:

1) Search for a completely new embedding medium compatible with the light and electron microscopes.

2) Wait for the delivery of the new glass knife holder and develop the techniques for cutting large araldite blocks with this.

3) Change to an alternative plastic embedding medium which was compatible with most stains, easily cut with a conventional D-profile steel knife, but could not be used for EM purposes, i.e. glycolmethacrylate (GMA).

GMA has been in use for several years as an alterative to paraffin and therefore a number of stains have been used routinely on material embedded in it. The Autocut has now made it possible to extend its use to the cutting of large, thin (1 - 1.5 μm) sections of GMA embedded tissue. Since there was already some experience within the department of this resin it was decided to investigate its suitability for the study of goblet cell numbers in the rat lung.

It was very quickly found that GMA was amenable to most of the routine histological stains and that large, thin sections could easily be cut on the Autocut using a conventional D-profile steel knife. The combined AB/PAS stain proved easy to achieve and it was also consistent, which meant that goblet cells could easily be identified and quantified in the rat lung (see Figure 7). It was therefore decided that goblet cell assessment would be carried out in the left lung using 1.5 μm GMA sections and that selected right lungs would be post-osmicated and embedded in araldite to await future developments with the glass knife.

As it was no longer possible to study the same area using both the light and electron microscopes the proposed differential cell count studies had to be abandoned. Likewise the autoradiographic studies had to be postponed until the technique had been developd using GMA. The emphasis of the project was therefore changed at this point from a concentrated study of the goblet cell to a detailed investigation of the isoprenaline response designed to identify the mechanism whereby isoprenaline induced goblet cell hyperplasia in the rat lung

APPENDIX vi

STATISTICAL ANALYSIS

The following statistical formulae have been applied to the results obtained in the course of this study.

$$\text{Mean } (\bar{x}) = \frac{\sum x}{n}$$

where x = individual result

n = total number in group

$$\text{Standard Deviation (SD)} = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

$$\text{Standard Error (SE)} = \frac{SD}{\sqrt{n}}$$

Significance of the results was tested using the Student's t-test where

$$t = \frac{(m_t - m_c)}{SD} \sqrt{\frac{n_t n_c}{n_t + n_c}}$$

n_t = total number in treated group

n_c = total number in control group

m_t = treated mean

m_c = control mean

Confidence Limits (95% level) = t x SE

where t is taken at the 5% level for (n - 1) degrees of freedom.