OVINE BRONCHOPULMONARY GLOBULE LEUCOCYTES

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine, the University of Glasgow.

by

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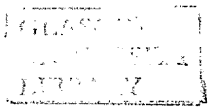
TO

MY WIFE

FAIZA

FOR HER UNFAILING PATIENCE



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ABBREVIATIONS

ABBREVIATIONS

A	Adult stage
AB	Alcian blue
AB/S	Alcian blue/safranin
AO	Acridine orange
Baso	Basophil
Bl	Bronchiole
BNF	Buffered neutral formalin
BO	Bouin's fluid
Br	Bronchus
BS	Biebrich scarlet
CAR	Carnoy's fluid
CC	Carbol chromatrope
CS	Corrosive sublimate
D. L. C.	Differential leucocyte count
Dt	Duct of bronchial gland
Eos	Eosinophil
Gd	Bronchial gland
GL	Globule leucocyte
H & E	Haematoxylin and eosin
HID	High iron diamine
L	Larval stage
Lymph	Lymphocyte
MC	Mast cell
Mono	Monocyte
MSB	Martius scarlet blue
Neut	Neutrophil
PAS	Periodic acid Schiff
P. C. V.	Packed cell volume
RCA	Righ cranial anterior lobe
Rd	Right diaphragmatic lobe
TB	Toluidine blue
T. L. C.	Total leucocyte count
Tr	Trachea

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DECLARATION

DECLARATION

The work described in this thesis is original and has not been submitted in any form to any other University. It was carried out by the author in the Department of Veterinary Pathology, Veterinary School, University of Glasgow, under the supervision of Dr. H.M. Pirie.

SUMMARY

SUMMARY

The main objectives of this thesis were to demonstrate that globule leucocytes can be found in the bronchopulmonary system of sheep and to study the characteristic features of globule leucocytes and mast cells in parasitic bronchopulmonary diseases of sheep. Subsequently a survey on the occurrence of these cells in the tracheobronchial tree of groups of sheep, kept in different management systems. These cells were found in the trachea, bronchi, bronchioles and bronchial glands of sheep which had parasitic pulmonary lesions.

Quantitative studies of the number of globule leucocytes at standard sites in the bronchopulmonary system revealed a close association between these cells and parasitic infection of the respiratory tract, although considerable variation was found in the quantification of globule leucocytes between different lobes of each lung. A similar variation occurred between the standard sites of sampling within the same lobe. This study indicated that the lobes of lungs which harboured more parasitic lesions quantitatively had more globule leucocytes in their epithelial mucosae.

Histochemical methods demonstrated the presence of sulphomucins in the globules of globule leucocytes and granules of mast cells. Both of these cell types were histochemically related but differed from eosinophil leucocytes in lung tissue.

Ultrastructurally, both globule leucocytes and mast cells of the lamina propria contained numerous intracytoplasmic granules, the maxtrixes of which were either completely electron dense and homogeneous or less dense with a fine granular ultrastructure. Globule leucocytes were most numerous in the surface epithelium and were not connected by desmosomes to the epithelial cells. The relationship between the globule leucocyte, mast cell and other cells in the respiratory tract of sheep during the course of a parasitic infection was briefly discussed with reference to recent reports on the presence of these cells in the intestinal

wall of certain animals including sheep.

Globule leucocytes were induced to develop in the tracheobronchial epithelium of lambs experimentally infected with Ascaris suum eggs, Dictyocaulus filaria and Haemonchus contortus larvae. Investigations showed that experimental infection with D. filaria in lambs produced an abundant increase in the number of bronchopulmonary globule leucocytes when compared with other types of experimental infection. Another difference between these experimental infections was revealed in the time required for the appearance and disappearance of these cells in the tracheobronchial tree of sheep.

Exposure of lambs to the dust of mouldy hay and experimental endobronchial inoculation of lambs with Aspergillus fumigatus spores failed to induce any globule leucocytes in their tracheobronchial tree.

The main pathological lesions in all experimental cases were tracheitis, bronchitis and bronchiolitis with peribronchial cellular accumulations and an exudate in the lumen. The majority of the cases had pulmonary eosinophilia, while the remaining lungs had lymphocytic and plasma cell infiltration.

The literature relevant to the study of globule leucocytes and mast cells in sheep was reviewed and discussed.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Bronchopulmonary diseases in sheep are responsible for considerable economic losses throughout the world. Parasitic infections of sheep respiratory tracts represent one of the main groups of these ovine diseases both in Britain and Iraq.

Complications such as viral and bacterial infection of the respiratory tract of sheep could be initiated and enhanced due to the migration and presence of parasites in the pulmonary system. Despite the efficient treatment, the improved husbandry and the immunisation procedures which have been developed to overcome these conditions it is still not possible to control some types of parasitism. This is mainly due to insufficient knowledge of the basic pathogenic and immunological mechanisms involved. However, in recent years there have been many advances in the use of modern techniques in parasitological and immunological research which have helped in the study of these reactions.

In Britain 6 nematode species have been recorded as occurring in the lungs of sheep (Rose, Michel and Harris; 1957 a and b). Dictyocaulus filaria and Protostrongylus rufescens occur in the bronchial tree whereas Muellerius capillaris, Cystocaulus ocreatus, Neostrongylus linearis and Protastrongylus brevispiculum are found in the parenchymal tissue and alveoli. Of these D. filaria and M. capillaris have a world-wide distribution. Clinically significant parasitic bronchitis in sheep is mainly attributed to D. filaria infection.

In Iraq parasitic bronchitis of sheep appears to be widely distributed. It was reported that 9.9 per cent. of ovine fecal samples contained D. filaria larvae; M. capillaris and P. rufescens larvae were also found but at a lower rate (Altaif, 1970).

Heidenhain first described a granular cell within epithelia of mucous surfaces of various mammals in (1888) and these cells were called "Schollenleukocyten" by Weill in (1919), a name that has been translated

into the English literature as "globule leucocyte". These cells were characterized by large acidophilic intractyoplasmic granules or globules with an eccentric nucleus (Kirkman, 1950).

Taliaferro and Sarles (1939) were the first to note an increase in the numbers of globule leucocytes during parasitic infection. Later, studies clearly confirmed that the frequent occurrence of the cell was invariably associated with a parasitic infection (Kirkman, 1950; Kent, 1952). On the other hand, opinions differ on the occurrence of the globule leucocytes in parasite-free animals, although in many reports they are not usually encountered in paraiste-free animals (Kent, 1952; Sommerville, 1956).

The origin of the globule leucocyte has been controversial. It has been attributed to lymphocytes (Kent, 1952; 1966), eosinophilic leucocytes (Casley-Smith, 1968), plasma cells (Whur and Gracie, 1967) and mast cells (Miller et al. 1967; Murray et al. 1968).

The function of the globule leucocyte has also remained uncertain. However, it is generally considered to correspond to the function of its possible precursor cell. Thus many authors suggest that the globule leucocyte is involved in the production and transport of immunoglobulins (Dobson, 1966a; Kent, 1966; Whur and Gracie, 1967), while others consider that biogenic amines are released from the mast cell during its transformation into the globule leucocyte (Jarrett et al. 1967; Miller et al. 1967; Murray et al. 1968).

Globule leucocytes have been recognised by many workers in a variety of species (Kirkman, 1950). These cells have been described in the gastrointestinal tract (Kent, 1952; Dobson, 1966b), the biliary tract (Miller et al. 1967; Murray et al. 1968) and the reproductive tracts of sheep (Kellas, 1961). However, there is virtually no information about the occurrence of globule leucocytes in the ovine bronchopulmonary system. Therefore, the aims of the present investigation were :

1. To study the presence of globule leucocytes in the respiratory tract of different groups of indoor parasite-free and outdoor parasitized sheep.

2. To investigate the induction of globule leucocytes in the respiratory tracts of sheep experimentally infected with A. suum or D. filaria or H. contortus or A. fumigatus or exposure to the dust of mouldy hay.

3. To study the morphology, the ultrastructure and the histochemistry of the globule leucocyte and other cells to which it might be related or confused.

The pathological changes in the respiratory tracts of the sheep naturally and experimentally infected with the above agents were also studied.

CHAPTER ONE

REVIEW OF THE LITERATURE

GLOBULE LEUCOCYTES IN GENERAL

A. HISTORICAL BACKGROUND

From the literature it is apparent that a granular type of cell was seen by many authors in the epithelial mucosae of certain organs in a variety of animal species. This granular cell was later classified as the globule leucocyte (GL) which was defined by a number of authors as an intra-epithelial mononuclear cell with highly refractile, acidophilic, intra-cytoplasmic granules or globules which frequently indented the nucleus (Keasbey, 1923; Dawson, 1943; Greulich, 1949; Kirkman, 1950; Kent, 1952; Kellas, 1961; Toner, 1965; Dobson, 1966b; Whur, 1966a, Whur and Johnston, 1967; Miller, Murray and Jarrett, 1967; Takeuchi, Jervis and Sprinz, 1969; Rahko, 1970b; Cantin and Veilleux, 1972).

The discovery of the GL has been attributed to Heidenhain (1888) who found these cells in the mucosal surfaces of the gut in the dog, rabbit, guinea pig and bat. Later, Weill (1919), in an article on the alimentary mucosa of mammals, published a description of a cell found by him in the alimentary epithelium of the dog, the cat, the mouse, the rabbit and the pig which had all the characteristics of the cell discovered by Heidenhain (1888); Weill (1919) called this granular cell a Schollenleukozyten. In 1920 Weill recorded the presence of a granular intraepithelial cell, situated between the decidual cells in the endometrium of early pregnancy in the human uterus. He regarded these cells as lymphoid cells and considered them to be analogous to Schollenleukozyten of the intestinal tract in other species.

Keasbey (1923) discovered cells like Schollenleukozyten in the ruminant gastric mucosa and translated the name to plaque leucocyte or globule leucocyte in the English and American literature.

The translation of the term Schollenleukozyten to globule leucocyte, by Keasbey, met favour with numerous writers (Clara, 1926; Dawson, 1927; Tehwer, 1929; Toro, 1931; Plenk, 1932; Patzelt, 1936; Dawson, 1943; Kirkman, 1950; Kent, 1952). Many of the characteristics of this cell were described by Kirkman (1950) and Kent (1952) in detail at that time.

Michels (1938) commented that it was still uncertain whether these cells with reddish granules, after staining with haemotoxylin and eosin, were to be regarded as emigrated or locally formed eosinophils, aberrant tissue mast cells, degenerating mast cells, plasma cells with metachromatic granules, plasmaphages (plasma cells with Russell bodies and ingested nuclear material), a variety of leucocytes with ingested erythrocytes, or as secretory leucocytes. This cell was designated as an atypical mast cell (Maximow, 1906), a connective tissue basophil (Talioferro and Sarles, 1939), a granular cell by Ahlqvist and Kohonen (1959) and Kellas (1961), a bundle cell by Hicks (1965) and Monis & Zambrano (1968) and simply as a brilliant cell by Shirai, Kure, Yamada, Kimura, Origasa and Hiramatsu (1976). More recently evidence has accumulated to suggest that GLs are mast cell related (Jarrett, Miller and Murray, 1967; Miller et al., 1967; Murray, Miller and Jarrett, 1968).

B. SPECIES AND ORGANS IN WHICH GLOBULE LEUCOCYTES OCCUR

Globule leucocytes have been reported in most vertebrate classes where they were found exclusively in the mucous surfaces of different organs (Weill, 1919 and 1920; Keasbey, 1923; Dawson, 1943; Kent, 1949; Kirkman, 1950). Kent (1952) reported the occurrence of this cell type within or adjacent to an epithelial cell. Whur (1966b) and Whur & Johnston (1967) noted that GLs in the rat occured in the epithelium and lamina propria of the small intestine. However, Dobson (1966b) considered that mature GLs were restricted to the epithelial mucosa in sheep, while the immature GLs occurred in the lamina propria of the gastrointestinal tract. Although Takeuchi et al. (1969) confirmed that GLs were restricted to the epithelium of the intestinal mucosa of the cat, they never found GLs in the lamina propria or the tissues of the reticulo-endothelial system.

Rahko (1972) found GLs in varying degrees in the livers and bile ducts of goats infected with fascioliasis and dicrocoeliasis; they were seen within the epithelium of the larger intrahepatic bile ducts and the main bile ducts, in addition GLs occurred in the tissues of the lamina propria of the liver in cases where they were particularly numerous.

1. Sheep

Since the discovery of the GLs their distribution in sheep has been described to be in the mucosal surfaces of certain organs examined in the alimentary, biliary and reproductive tracts.

In the alimentary tract GLs were described by Moller (1899), Keasbey (1923), Tehwer (1929), Toro (1931), Duran-Jorda (1945), Kent (1949 and 1952), Sommerville (1956), Davletova (1958), Whur (1966a), Dobson (1966b) and Murray et al. (1968). They occured in the abomasum and the small and large intestine (Kent, 1952; Dobson, 1966b; Whur, 1966a). The distribution of GLs in these organs was highly irregular, even in one animal, either clusters of GLs were distributed randomly throughout the epithelium of the mucous membrane or individual cells were diffusely distributed in the epithelium. Independent of these distributions, variable

numbers of GLs were situated within connective tissues or glands of these organs (Kent, 1952). The distribution of GLs in the abomasum of sheep was considered to be very irregular (Keasbey, 1923; Kent, 1952). Both workers agreed that GLs were rarely present in the cardiac region of the stomach. In the peptic region of the abomasum where the folds were tallest, GLs were commonly found in these areas, almost every gland contained a few of these cells (Keasbey, 1923). These cells were usually situated in the upper half of the mucosa toward the stomach lumen, and were also found in the surface epithelium. Keasbey (1923) found GLs in greatest abundance in areas where the folds became low and began to broaden out. They particularly extended from muscularis mucosae to lumen. In the glandular epithelium they were frequently found in the very fundus of glands pushed between epithelial cells which were crowded with zymogen granules (Keasbey, 1923). Kent (1952) occasionally found a considerable number of these cells in the peptic abomasum, but often no or few cells were found. In the pyloric region of the abomasum GLs were plentiful as described by Keasbey (1923). However, Kent (1952) and Whur (1966a) did not find this abundance of GLs in the pyloric region of the sheep abomasum. This variation in distribution and density of GLs in the tissues might be attributed to whether the sheep were infested and on the nature of that infestation (Dobson, 1966b).

However, Sommerville (1956) found no correlation between the site of the nematode infestation and the region in which these cells were most common. Thus, a sheep infested with the abomasal parasite Haemonchus contortus had very few GLs in that organ, although they were numerous in the intestinal mucosa. In contrast a sheep infested with the intestinal parasite Trichostrongylus colubriformis had numerous GLs in both abomasal and intestinal sections. This phenomenon was confirmed by Rahko (1972) who showed that the intrahepatic bile ducts in goats, which were too small to harbour liver flukes, contained more GLs in dicrocoeliasis than in fascioliasis. In the abomasum GLs were restricted in all parts to the mucosae and this appeared to be the only distribution observed (Kent, 1952).

The small intestine invariably contained GLs throughout its length. In the duodenum they were scattered at comparatively regular intervals, but never appeared to be as plentiful as in the abomasum (Keasbey, 1923; Whur, 1966a). Moller (1899), Kent (1952) and Dobson (1966b) regularly found these cells in large numbers in the duodenum, the jejunum, the ileum and the ileo-caecal valve. Keasbey (1923), on the other hand, found them only in small numbers in the jejunum and below this point in the digestive tract, in only a single sheep where they were present in the ileum.

The large intestine contained considerable numbers of GLs throughout its length; Kent (1952) and Dobson (1966a and b) found these cells to be abundant in the caecum and colon, whereas Keasbey (1923) and Whur (1966a) could show no pattern of distribution of GLs down the small intestine. Dobson (1966b) reported that there was a close correlation between the distribution of GLs and the presence of parasites. In both the small and large intestine the largest peak of numbers of GLs closely followed the peak of larval lesions. Similarly a peak of larval lesions at the ileo-caecal valve was also accompanied by a peak in the number of GLs. The greatest numbers of GLs were found, however, where the adult infestation occurred. Beyond this region in the gut the numbers of GLs decreased until at the anus another much smaller peak in GL numbers occurred, and this coincided with a large peak in the numbers of larval lesions. Globule leucocytes were restricted to the mucosae in the small and large intestine (Keasbey, 1923; Kent, 1952), except in the region of Brunner's glands in which a small concentration usually occurred (Kent, 1952; Whur, 1966a).

In the biliary tracts of sheep GLs were reported to be present intra-epithelially (Kent, 1952; Zipper, 1966; Miller et al., 1967; Murray et al., 1968; Rahko, 1970a) numbers of these cells were often encountered in lining epithelium of the gall bladder (Kent, 1952; Miller et al., 1967).

The occurrence of GLs in the reproductive organs of sheep was also investigated. They were observed during pregnancy in their endometrium (Kent personal communications with Wimsatt 1952).

Kellas (1961) found similar cells in ovine endometrium which he called them the intraepithelial granular cells. These cells had the same morphological and cytochemical properties as GLs and were later considered to be GLs (Toner, 1965; Whur, 1966a; Rahko, 1971).

Kellas described the distribution and density of these GLs in the sheep uteri during different stages, that is an early and late pregnancy and in non-pregnant uteri.

During pregnancy in the intercotyledonary regions GLs were found in the maternal epithelium. The largest numbers occurred in the surface epithelium of the intercotyledonary regions including the outer, or lateral surface of the concave placentomes. They occurred in lesser numbers in the epithelium lining the uterine gland necks, and were usually scarce or absent from the deeper portions of the glands. The cells have not been encountered within the placentomes. Globule leucocyte were basally lying on, or close to, the basement membrane insinuating themselves between the epithelial columnar cells. They could, however, be found at any level within the columnar epithelium. Moreover, they did not appear to be oriented in a constant manner with respect to the basement membrane. Globule leucocytes were commonly concentrated in the uterine epithelium during pregnancy. Although such proportionately large numbers were quite typical, local variations could occur. These cells were not observed in sections taken from the greater and lesser curvatures of the uterus, areas around the os uteri or the anterior extremities of the uterine horns including regions not penetrated by the chorionic sac (Kellas, 1961).

Screening of the epithelia of a small number of uterine tubes and cervices from pregnant sheep indicated that the GLs were rare or absent in these sites (Kellas, 1961). Kellas (1961) reported that the

relative number of GLs in the uterine epithelium in early pregnancy might be great, but certainly not greater than in the later phases of the pregnancy cycle. There could be therefore an enormous increase in the number of these cells as pregnancy proceeded. The GLs examined in early pregnancy were smaller than those found in the later stages of pregnancy.

Following examination of non-pregnant sheep uteri, it was not possible to generalise on the frequency of occurrence of these cells in the uterine epithelium during various stages of the oestrus cycle (Kellas, 1961).

In the lungs of sheep Zipper (1966) described the occurrence of GLs within the epithelium of the bronchi.

2. Cattle

Globule leucocytes have been described in the digestive tract of cattle by many workers (Keasbey, 1923; Tehwer, 1929; Toro, 1931; Duran-Jorda, 1945; Miller et al., 1967; Murray et al., 1968). The first brief description of the distribution of GLs in cattle abomasum was by Keasbey (1923). This was found to be similar to that in sheep. Globule leucocytes occurred in a gland in much greater numbers than did parietal cells (Keasbey, 1923). Globule leucocytes seldom appeared near the cardiac region. Near the mid-region (peptic) of the abomasum they were sometimes present in considerable numbers although often few or none were found (Keaseby, 1923). In the pyloric region GLs were almost uniformly abundant. These cells were restricted to the mucosa in all parts of the abomasum (Keasbey, 1923). In comparison to their occurrence in the pyloric region GLs were scarce in the small intestine; in the duodenum they were scattered at comparatively regular intervals but never appeared to be as plentiful as in the abomasum (Keasbey, 1923). Fewer GLs were found in the jujeunum and they were absent from the ileum and large intestine (Keasbey, 1923).

In the biliary tract of cattle the presence ofGLs was studied during fascioliasis and dicrocoeliasis (Jarrett et al., 1967; Rahko, 1970a & b; 1971; Shirai et al., 1976). These cells were restricted to the epithelial mucosae, no GLs were identified in the subepithelial tissues of the main bile duct walls (Rahko, 1971). They were found in both the surface and glandular epithelium but were most frequent in the epithelium of the crypts (Rahko, 1971). During fascioliasis GLs rarely occurred in bile ducts which were too small to harbour migrating flukes (Rahko, 1971). In contrast, in dicrocoeliasis GLs were frequently found in small bile ducts (Rahko, 1970b). Globule leucocytes were very scarce in the small bile ducts that showed fluke-eggs granuloma (Rahko, 1971).

In the respiratory tract of cattle GLs were reported in the tracheo-bronchial epithelium (Breeze, Pirie and Dawson 1975; Pirie, Breeze, Selman and Wiseman, 1976).

Globule leucocytes were described in the reproductive tract of cattle (Kellas, 1961). These cells were observed in large numbers within the maternal epithelium of the intercotyledonary regions of the placentae, the cells were almost exclusively intraepithelial in position. There was a substantial increase in the number of GLs as pregnancy proceeded (Kellas, 1961).

3. Goat

In the pyloric region of the goat's abomasum GLs were described by Hill (1951). These cells were found between the columnar cells of the surface epithelium and in the glandular epithelium of the gastric glands (Hill, 1951). Rahko (1972) reported that GLs were extremely rare in normal bile ducts. In contrast in dicrocoeliasis and fascioliasis in the goat GLs were present in variable numbers within the epithelium of the larger intrahepatic and the main bile ducts. In liver with very many GLs the cells occurred in the region of the lamina propria also. Intra-hepatic bile ducts which were too small to harbour liver flukes appeared to have more GLs in dicrocoeliasis than in cases of fascioliasis (Rahko, 1972).

Kellas (1961) reported on the occurrence of GLs in the uteri of goats. The largest number of these cells were found in the surface epithelium of the intercotyledonary regions of the placentae. They occurred in smaller numbers in the epithelium lining the uterine gland necks and were usually scarce or absent from the deeper portions of the glands. The GLs found in this organ was almost exclusively intra-epithelial in position. They were not reported in the respiratory system of this species.

4. Deer

The presence of GLs in the respiratory and biliary tract of deer was studied by Zipper (1966). These cells were almost exclusively localised to the epithelium of bronchi and bile ducts.

In similar species GLs were described by Kellas (1961) who investigated their presence in the uteri of duiker (Sylvicapra). In the intercotyledonary regions these cells were found in the maternal epithelium. The largest numbers occurred in the surface epithelium of the intercotyledonary regions including the outer, or lateral, surface of the concave placentomes found in the duiker (Kellas, 1961). They occurred in smaller numbers in the epithelium lining the uterine gland necks and were usually scarce or absent from the deeper portions of the glands (Kellas, 1961).

5. Dog

The presence of GLs lying between the decidual cells in the gastro-intestinal tract of dog was reported by Moller (1899), Ferrata (1906), Weill (1919), Lim (1922) and Toro (1931). These cells were present in small numbers; they occurred mainly near the surface epithelium of the gastric mucosa and occasionally between the cells lining the duct of the gland (Weill, 1919; Toro, 1931). Additionally they were found in the interglandular tissue of the stomach (Lim, 1922).

Dawson (1927) examined lymph nodes from dogs which had previously been irradiated. He found that GLs were uniformly distributed throughout the cortex and medullary cords of both the pyloric and mesenteric lymph nodes. Globule leucocytes were reported in the bronchial epithelium of dogs by Frasca, Auerbach, Parks and Jamieson (1968).

6. Cat

Globule leucocytes were recorded lying between the decidual cells of the alimentary tract (Moller, 1899; Muthman, 1913; Weill, 1919; Lim, 1922; Toro, 1931). More recently, Takeuchi et al., (1969) reported the occurrence of GL in the cat; he found these cells to be most abundant throughout the small and large intestine. In the small intestine they were located usually, but not exclusively close to the crypt - villus junction while in the colon they were more randomly situated. The frequency of these cells along the length of the gut was irregular and varied from animal to animal irrespective of sex. Intestines of cats heavily parasitized with flat and round worms did not contain large numbers of GLs. In the intestinal mucosa GLs were usually nestled between epithelial cells near the basal membrane (Takeuchi et al., 1969). These cells were only seen in the lamina propria in areas where GLs were very numerous in the adjoining epithelium. Takeuchi et al., (1969) examined Peyer's patches but only a few GLs have been found in these lymphoid follicles. In the gastric mucosa GLs were occasionally present in small numbers (Lim, 1922; Takeuchi et al., 1969). In the tracheal and bronchial epithelium, thymus, spleen and mesenteric lymph nodes these cells were occasionally detected (Takeuchi et al., 1969). Dawson (1943) found GLs in the epithelium of the gall bladder wedged between the columnar cells. In a few instances, however, they were observed apparently discharging their granular contents into the lumen of the bladder (Dawson, 1943). A dense population of these cells was found in the gall bladder (Dawson, 1943). Similar studies by Takeuchi et al., (1969) indicated that GLs were more numerous in the gall bladder than any other organ with the exception of the gut. Cats infected with Capillaria feliscati have been reported to have GLs in the epithelium of the urinary bladders (Waddell, 1968). On the nasal

side of the soft palate of the cat GLs were found intraepithelially (Kent, 1952).

7. Pig

Globule leucocytes were reported in the epithelium of the intestinal tract of the pig (Weill, 1919; Lim, 1922; Toro, 1931).

8. Horse

Toro (1931) reported on the occurrence of GLs in the epithelium of the intestinal tract of the horse.

9. Mouse

Globule leucocytes were reported in the intestinal tract by Weill (1919), Carr (1967) and Silva (1967). In the small intestine the intra-epithelial GLs were concentrated in the upper third of the intestinal crypts although there was a more uniform distribution in the lamina propria (Carr & Whur, 1968). In the caecum and colon of mice GLs were frequently seen in the surface epithelium as well as in the crypts (Carr & Whur, 1968). The presence of GLs in the biliary tracts of mice was produced experimentally with a Fasciola hepatica infection (Rahko, 1971). The concentration of these cells in infected bile ducts was many times greater in the chronic stage than in the acute phase. However, Rahko (1971) did not find these cells in the intra-hepatic bile ducts which were free of liver flukes. In the walls of the main bile ducts GLs were numerous between the superficial and glandular epithelial cells (Rahko, 1971). These cells were most frequent in the surface epithelium and GLs were not identified in the subepithelial tissues (Rahko, 1971).

In the mouse placenta GLs were situated intraepithelially as reported by Heine and Schaeg (1977).

10. Rat

In comparison to other species little work has been done to examine the occurrence of GLs in the rat. Taliaferro and Sarles (1939) were the first to report the presence of these cells in the rat. Since then this

rodent has been used for much of the experimental works on GLs.

In the respiratory tract of the conventional laboratory rat the occurrence of GLs has been examined by a number of authors. The concentration of such cells was variable and often were absent. When the cells occured they could be found throughout the respiratory airways (Kent, 1966). In the tracheal epithelium these cells were abundant (Kent, Baker, Ingle and Li, 1954; Kent, 1966; Veilleux and Cantin, 1976), but were sparsely distributed in the bronchi and bronchioles (Taliaferro and Sarles, 1939; Kent, 1966; Jeffrey and Reid, 1975). A few GLs occupied positions within the connective tissue of the mucous membrane or within the epithelium of the tracheal glands, particularly in the ducts and mucus secreting parts (Kent, 1966). Inflammation of the mucous membrane, particularly when accompanied by metaplastic changes in the epithelium, was invariably accompanied by a diminution or disappearance of GL (Kent, Baker, Pliske and Van Dyke, 1956). Kent (1966) examined the larynx which contained many GLs although they were seldom located within or beneath the thin stratified squamous epithelium of the vocal folds. He found that the population of GLs was more likely to be rich above the vocal folds than below them; the laryngeal ventricles and the laryngeal surface of the epiglottis was the most uniformly populated areas. In the glottal area, the infiltration of GLs extended onto the lingual surface of the epiglottis, the roat of the tongue and the lateral and dorsal pharyngeal walls (Kent, 1966). Despite their concentration in the vicinity of the glottis, a few GLs were found in the laryngopharynx and none in the oesophagus (Kent, 1966). Whur (1966a) reported the absence of GLs from the oesophageal or antral part of the stomach, whereas Kent (1966) found occasional cells in this area. The GLs were present in the deeper layers of the stratified squamous epithelium (Kent, 1966). In contrast, the glandular stomach invariably contained GLs in moderate numbers (Kent, 1966; Whur, 1966a). Throughout this area, the majority of the GLs were situated in the epithelium of the depths of the gastric foveoli and the necks of the glands (Kent, 1966).

Peptic, cardiac and pyloric glands contained few GLs in the body and depths of the glands (Kent, 1966).

Initial studies on the rat small intestine suggested that the usual epithelial distribution of GLs was not found in this organ where they were located in the lamina propria (Kent et al., 1956; Kent, 1966). Later workers showed that these cells occurred in the lamina propria and the epithelial mucosa also (Whur, 1966b; Whur and Johnston, 1967; Carr and Whur, 1968). Globule leucocytes were frequently seen in the surface epithelium and also in the crypts (Carr and Whur, 1968).

In the large intestine GLs occurred from the caecum to the anal canal (Kent, 1966; Whur, 1966a; Carr and Whur, 1968). Throughout this area, they were commonly found within the epithelium of the intestinal glands, in the surface epithelium or lamina propria (Kent, 1966; Carr and Whur, 1968).

In the urinary passageways of the rat GLs were frequently found in the transitional epithelia (Kirkman, 1950; Hicks, 1965; Movis and Zambrano, 1968; Cantin & Veilleux, 1972; Veilleux and Cantin, 1976). The vast majority of the cells were distinctly intraepithelial in position, although an occasional cell was found in contact with connective tissue as well as with the epithelium (Kirkman, 1950). Globule leucocytes were distributed throughout the epithelium of the urinary bladder, both ureters and the renal pelvis (Kirkman, 1950; Cantin and Veilleux, 1972). Globule leucocytes were not present in portions of the transitional epithelium of the pelvis which was not separated from adjacent structes by a lamina propria, that is in the epithelium covering the papilla and in the upper regions of the pelvic cavity (Cantin and Veilleux, 1972). They were abundant in all other portions of the urinary pelvis epithelium, particularly at the ureteropelvic junction (Cantin and Veilleux, 1972).

11. Rabbit

Globule leucocytes were reported in the epithelial cells of the intestinal tract of this species by Weill (1919), Lim (1922), Toro (1931).

12. Guinea Pig

Globule leucocytes were abundant in the mucosa of the intestinal tract of the guinea pig according to Toro (1931).

13. Man

Weill (1920) recorded the presence of GLs between the decidual cells of the endometrium in early pregnancy. Later Asplund and Holmgren (1948) found these cells in the stroma and, occasionally, in the uterine gland epithelium of the pregnant and non-pregnant human uterus during the fertile age. In the mucosa of the intestinal tract these cells were described by Toro (1931). The presence of GLs was examined in epidermal tumours of man by Heine and Schaeg (1977).

14. Hedgehog

Corti (1922) recorded the presence of GLs in the mucosa of the intestinal tract of the hedgehog.

15. Bat

Globule leucocytes were found in the intestinal tract of this species by Toro (1931).

16. Birds

In the intestine of various birds (Muthman, 1931) and the chicken (Clara, 1926; Greulich, 1949; Toner, 1965) GLs were described as being common. These cells were found in the basal half of the epithelium sandwiched between its cells (Toner, 1965).

17. Reptiles

Toro (1931) reported the presence of GLs in the intestinal tract of some reptiles.

18. Amphibia

Globule leucocytes were seen in the mucosal surfaces of the intestinal tract in certain amphilian animals such as the tortoise, the crocodile and the frog (Corti, 1922; Toro, 1931).

19. Fish

Corti (1922) and Rogosina (1928) found GLs in the mucosa of the intestinal tract of fish.

C. MORPHOLOGY OF GLOBULE LEUCOCYTES

The morphological similarities amongst GLs from different organs within different species suggested that they could be intimately related (Kirkman, 1950). In all species examined the most prominent feature of these cells was the presence of spherical intra-cytoplasmic inclusions, globules or granules, which varied considerably in size and in number (Kirkman, 1950; Kellas, 1961; Whur and Johnston, 1967). These cells stained with eosin and were metachromatic with toluidine blue (Kirkman, 1950; Kent, 1952; Kellas, 1961). The single nucleus was small, densely stained, eccentrically positioned, indented and exhibited a cartwheel appearance (Kellas, 1961; Toner, 1965; Takeuchi et al., 1969; Rahko, 1972). Globule leucocytes, which were more or less round in outline, were found at various levels within the epithelial mucosa; they had no regular orientation with respect to the basement membrane (Kirkman, 1950; Kent, 1952). Thus GLs could be pleomorphic in some instances depending on their situation in the epithelium (Kent, 1952; Rahko, 1971). Immature cells were small, contained fewer globules and occurred in the lamina propria (Dobson, 1966b), while the mature forms were relatively larger, contained more globules, had distinct nuclei and were often irregularly shaped because of their sandwiched positions between epithelial cells (Kent, 1952; Dobson, 1966b). In a few instances, however, GLs were observed in the lumenal portions of the epithelium apparently discharging their granular contents into the lumen (Dawson, 1943). Morphological differences in GLs of non-pregnant, very early pregnant, and later pregnant uteri were observed by Kellas (1961). He found that in early pregnancy the quantity of granulated material which had accumulated in the cytoplasm was less than in the later phases of pregnancy. Moreover, in the uterine epithelium in early pregnancy an appreciable number of apparently non-granular globule leucocytes (intraepithelial cells) were observed which, except for the lack of granular material appeared to be indistinguishable from neighbouring intraepithelial granule containing cells (Kellas, 1961). In the non-pregnant tracts examined, non-granular intraepithelial cells predominated and the granule containing cells were rare and comparatively small (Kellas, 1961).

In a variety of animal species the morphology of GLs was studied in parasitic infections by many workers (Toner, 1965; Kent, 1966; Miller et al., 1967; Carr, 1967; Whur and Johnstone, 1967; Carr and Whur, 1968; Murray et al., 1968; Takeuchi et al., 1969; Rahko, 1970b; 1971; 1972; Cantin and Veilleux, 1972; Heine and Schaeg, 1977).

1. Light Microscopic Features

In the sheep (Kellas, 1961; Dobson, 1966b), the cat (Dawson, 1943), the rat (Kirkman, 1950; Cantin and Veilleux, 1972) the mouse (Carr, 1967) and the fowl (Toner, 1965) GLs were round to oval in shape becoming irregular in outline as the cell accommodated itself between adjacent epithelial cells. In cattle GLs were pleomorphic and the number and size of the globules varied (Rahko, 1971). Living cells, from sheep uteri, suspended in physiological saline or uterine milk, appeared more or less spherical (Kellas, 1961). Mature GLs occurred in the epithelial layer, wedged between the mucous secreting cells (Dawson, 1943; Dobson, 1966b). The immature forms usually had the same shape but were smaller, contained fewer globules and often occurred in the lamina propria (Dobson, 1966b).

Cells from sheep uterine epithelium were from 8.6 x 7.5μ to 13.3 x 11.2μ in size (Kellas, 1961). Dobson (1966b) found that mature GLs in the digestive tract of sheep averaged 12.27± 0.06μ in diameter, ranging from 6.88 to 17.88μ for 377 cells measured. This agreed with similar studies by Kent (1952) when the young lymphocyte-like stage varied in diameter from 5-6μ while the largest mature cells were 10-12μ in diameter. Kirkman (1950) reported comparable observations on the size of GLs in the urinary tract of the rat, the average diameter of the living cell was about 12.8μ with an approximate range of 9.2μ to 14.2μ.

The cell nucleus was eccentrically placed (Kirkman, 1950; Kent, 1952), while the cytoplasmic granules were grouped as a mass beneath or around one pole of the nucleus more or less filling the cytoplasm at this point of the cell (Keasbey, 1923; Kent, 1952; Rahko, 1970b). While the outline of the cell was never distorted by the contained globules, it was rare to

find a cell with much more cytoplasm than was necessarily comfortable to include the globules (Kellas, 1961). Thus there appeared to be a relationship between cell size and the number of globules (Kellas, 1961).

In the smaller cells the cell membrane was distinct, but as the granular mass increased and the cell became larger, the cell membrane became thinner and more difficult to discern (Dawson, 1943). Its presence in some instances could only be assumed by the retention of the shape of the granular mass (Dawson, 1943).

a. The Nucleus

The nucleus of GLs in the sheep digestive tract, and in most of the other species investigated, were small and round to slightly oval in outline (Dawson, 1943; Kirkman, 1950; Kent, 1952; Dobson, 1966). The nucleus was often indented on one aspect by the intracytoplasmic globules (Kent, 1952; Kellas, 1961; Toner, 1965).

The nuclear membrane was distinct in all cells (Kirkman, 1950). Wheel-like clumping of chromatin (cartwheel arrangement) beneath the nuclear membrane, was frequently noted in GLs (Keasbey, 1923; Dawson, 1943; Kent, 1952; Toner, 1965). The appearance of the nucleus that has been recorded, varies considerably; at one extreme there were relatively deeply staining round nuclei with masses of chromatin distributed in a pattern typical of a plasma cell (Kellas, 1961) on the other hand larger leptochromatic types of nucleus, which were round, or oval or kidney-shaped were found (Kellas, 1961). The nucleus occupied an eccentric position within the cell (Kellas, 1961; Whur and Johnston, 1967; Rahko, 1970b). However Keasbey (1923) reported that the nuclei of these cells were frequently centrally located; although they were generally found to be more or less eccentric, they were seldom in a peripheral location in the sheep.

In the cat Dawson (1943) found that GLs contained small granules, their nuclei were centrally placed and similar in form to the nuclei of small lymphocytes. Other workers, however reported their peripheral

position, dark staining and small appearance with a triangular shape resembling the nuclei of goblet cells.

Mitotic figures were not observed in the globule leucocytes examined (Keasbey, 1923; Dawson, 1943; Kirkman, 1950; Kent, 1952; Kellas, 1961).

Weill (1919) found no evidence of mitosis in these cells in either dog or cat, but mitotic figures were recognised in GLs in the mouse. Pyknotic nuclei and karyorrhexis have been encountered, but not frequently enough to suggest that the GLs represented a degenerating cell form (Kellas, 1961).

b. The Cytoplasm and its Constituents

In cells densely packed with globules, it was difficult to demonstrate the cytoplasmic matrix (Kellas, 1961; Rahko, 1970b) however in cells with fewer and smaller globules the cytoplasm was recognizable (Keasbey, 1923; Kent, 1952). In this case the cytoplasm was either clear and homogenous (Dawson, 1943) or distinctly chromophobic (Keasbey, 1923; Kirkman, 1950; Kent, 1952).

Intense staining demonstrated irregular vacuolation of the cytoplasm (Keasbey, 1923; Kent, 1952). However, the description by Keasbey (1923) indicated that rarely the cytoplasm was faintly acidophilic.

Kellas (1961) studied the staining affinities of the cytoplasm by applying toluidine blue at pH 4.5 to imprints of GLs fixed in Zenker's fluid or Helly's fluid. He found that the cytoplasm of many GLs exhibited a strong basophilia which was similar to the surface epithelial cells of the uterus. Kellas (1961) suggested that this basophilia might be weak or absent in the cytoplasm of other cells. It was not possible to satisfy and correlate the degree of cytoplasmic basophilia with the quantity of globular material present in the cells.

Globules (Granules)

The globules were more or less spherical, acidophilic refractile inclusions which were subject to great variation in size (Keasbey, 1923; Dawson, 1943; Kirkman, 1950; Kent, 1952). The globules had

characteristic features and possessed distinctive staining properties which made them conspicuous in the homogeneous cytoplasm of the cell (Dawson, 1943; Kirkman, 1950). They stained metachromatically with toluidine blue and showed a strong reaction with alcian blue in certain organs (Rahko, 1970b). In fixed tissue the globules varied in size from large spheres with a diameter of about 4μ to minute particles (Kirkman, 1950; Kellas, 1961). In the sheep digestive tract the globules varied in size also, averaging 0.641±0.004μ with a range of 0.23 to 1.28μ for 371 globules measured (Dobson, 1966b). It appeared that the number of globules in a single cell normally ranged from 5-20 (Rahko, 1970b), although as many as 28 have been counted in one cell (Kellas, 1961). However, since the smallest of the globules were too minute to count at the magnifications used, and since globules overlying or underlying a deeply stained nucleus were difficult to detect, the number per cell might be greater than these figures suggested (Kellas, 1961). In the same article Kellas (1961) showed that the size of the globules was not related to the number of globules within the cell. Most globules were homogeneous (Keasbey, 1923), although irregularly shaped globules were found in the sheep digestive tract by Kent (1952); he never observed the cup-shaped, biconcave and crenated forms which Keasbey (1923) described. A few or all of the globules in a cell could have a dark-staining outer zone which blended into a chromophobic inner portion (Kent, 1952). Kellas (1961), however, recorded that when the globules were stained with alcian blue their peripheral rim was surprisingly prominent. Moreover, this brilliant blue outline appeared to show minute irregularities or breaks giving the impression of a border which was not quite entire. He suggested from these findings that there might be an outer shell differing in structure from the material composing the interior of the globules. Alternatively this might reflect a low permeability of the globules to the stain, such that the periphery became more deeply coloured (Kellas, 1961).

Mitochondria

Mitochondria were demonstrated in the cytoplasm of the GLs in preparations supra-vitally stained with methylene blue (Kirkman, 1950; Kent, 1952; Kellas, 1961). They were present in all cells examined as

granules or more commonly as short curved rods; in mature cells they could be found either in close association with the globules or free in the cytoplasm (Kent, 1952).

Golgi Material

Golgi material was demonstrated in GLs in the fowl (Greulich, 1949) and in the sheep (Kent, 1952) by supra-vital staining. Kirkman (1950) also used supra-vital preparations, but failed to detect Golgi material in rat GLs. Golgi material has been found in the GLs of the sheep intestine as strands scattered throughout the cytoplasm (Kent, 1952).

2. Electron Microscopic Features of Globule Leucocytes

Previous literature showed that there was much confusion about the origin, function and relation of GLs to other similar cells when examined with the light microscope. Histologically the GL, plasma cell, and Russel body cell had similar reactions (Dobson, 1966).

In earlier studies the GL of the lamina propria of the rat jejunum was described as an atypical mast cell (Maximow, 1906). Later, this cell, in the same position in the rat, was described as a connective tissue basophil (Taliaferro and Sarles, 1939). In the rat urinary tract it was designated as a granular cell (Ahlqvist and Kohonen, 1959), and finally as a bundle cell (Hicks, 1965; Monis and Zambrano, 1968).

The introduction of electron microscopy and its application to morphological studies of GLs have enabled detailed descriptions to be made on the nature of this cell.

The electron microscopic features of the GL were studied in sheep (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968), goats (Rahko, 1972), cattle (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968; Rahko, 1970b; 1971; 1973a), rats (Hicks, 1965; Kent, 1966; Whur and Johnston, 1967; Miller et al., 1967; Murray et al., 1968, Monis and Zambrano, 1968), mice (Carr, 1967; Silva, 1967; Carr and Whur, 1968; Rahko, 1971; Heine and Schaeg, 1977), fowl (Toner, 1965; Holman, 1970), cats (Takeuchi et al., 1969; Finn and Schwartz, 1972)

and man (Heine and Schaeg, 1977).

Ultrastructural studies of the GLs in the intestinal wall indicated certain differences between species of animals (Carr and Whur, 1968). Moreover the intracytoplasmic membrane bound globules showed various internal structures within a single cell (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968).

In most species examined at the ultrastructural level , GLs were easily recognized by their characteristic large, round, intracytoplasmic inclusions (Carr, 1967; Whur and Johnston, 1967; Takeuchi et al., 1969). The position of the cells in the epithelium corresponded to that observed by light microscopical examination (Tower, 1965; Takeuchi et al., 1969). Globule leucocytes in most species had a similar outline depending on their position within the tissue examined; they were generally round or oval, with minor irregularities (Toner, 1965; Carr, 1967; Monis and Zambrano, 1968; Takeuchi et al., 1969). In the rat ureters, they occasionally appeared as flat branched elements, interspersed among the basal, intermediate or superficial cell layers (Monis and Zambrano, 1968). In the small intestine of the fowl and the cat, GLs sandwiched between the epithelial cells were often elongated (Toner, 1965; Takeuchi et al., 1969). In this situation GLs could become surrounded and enveloped by the cytoplasm of the epithelial cells (Toner, 1965). Other GLs were extremely irregular in outline, with long pseudopod-like processes pushing between adjacent epithelial cells and indenting them; occasinally these structures invaginated and penetrated deeply into their cytoplasm (Toner, 1965; Whur and Johnston, 1967). Thus, in one section there might be no apparent continuity between the parent cell and one of its pseudopodia, which would be presented as an island of cytoplasm within the substance of an epithelial cell (Toner, 1965). In all species investigated when GLs were closely apposed to the neighbouring epithelial cells, the pseudopodia of GLs interlocked with their plasmelema without forming, however, any cellular junction (Toner, 1965; Miller et al., 1967; Whur and Johnston, 1967; Takeuchi et al., 1968).

a. Nucleus

In the cat (Takeuchi et al., 1969), in the fowl (Toner, 1965), in the rat(Whur and Johnston, 1967; Carr, 1967; Carr and Whur, 1968; Monis and Zambrano, 1968), and in mice (Carr and Whur, 1968), the nucleus of GLs were somewhat variable in shape, ranging from large and approximately spherical to compressed and irregular in outline. In the latter type marked identation of the nucleus by the intracytoplasmic globules were observed in GLs of fowl (Toner, 1965), the cat (Takeuchi et al., 1969), the rat (Whur and Johnston, 1967), mice (Carr, 1967) and cattle (Miller et al., 1967; Murray et al., 1968; Rahko, 1973a). The nuclei were frequently eccentric in most species (Kent, 1966; Whur and Johnston, 1967; Rahko, 1970b, 1971).

The cartwheel pattern of chromatin was relatively uncommon in fowl GLs (Toner, 1965) but in the cat (Takeuchi et al., 1969), the rat (Whur and Johnson, 1967), the goat (Rahko, 1972), and cattle (Rahko, 1973a), the nucleus showed a rather concentrated chromatin distributed uniformly throughout the nucleoplasm.

In the cat GLs, the nuclear membrane was not remarkable, and the nucleus might show a prominent nucleolus (Takeuchi et al., 1969). Mitotic figures were not observed in the GLs examined (Kent, 1966; Takeuchi et al., 1969).

b. The Cytoplasm and its Constituents

The cytoplasm of the GLs in fowls (Toner, 1965) and the rat (Carr, 1967) was pale with few distinctive structural features, irrespective of the number of globules, although Kent (1952) reported that in sheep the cytoplasm of GLs became progressively more vacuolated in proportion to the number of globules. These vacuoles were rounded and devoid of demonstrable substance (Kent, 1966). The cytoplasmic stroma contained rather sparse rough endoplasmic reticulum in GLs of rats (Kent, 1966) and mice (Carr, 1967) but in the cat smooth endoplasmic reticulum was slightly more abundant than rough endoplasmic reticulum (Takeuchi et al., 1969). In the fowl there was diffuse rough endoplasmic reticulum

with a few short cisternae (Toner, 1965). Whur and Johnston (1967) in rat GLs mentioned that endoplasmic reticulum was abundant throughout the cytoplasm of GLs containing a few globules, but there was little evidence of it in cells densely packed with these globules, and it was frequently seen to be continuous with the outer nuclear membrane or with the bounding membrane of a globule (Toner, 1965).

Ribosomes were a constant feature and were free and numerous in the cytoplasm of GLs in various species examined (Toner, 1965; Kent, 1966; Carr, 1967; Takeuchi, 1969). In many of the GLs in the rat, rows of pinocytic vesicles were seen adjacent to the plasma membrane (Whur and Johnston, 1967). Delicate microtubules having a diameter of 75-125A occurred in the cytoplasmic stroma of many GLs in the rat (Kent, 1966). The centrioles were surrounded by well developed Golgi complex and their appearance was in agreement with descriptions of centrioles in various cell types reported by other investigators (Kent, 1966; Takeuchi et al., 1969).

Globules

The characteristic feature of the GL is the presence of large numbers of spherical globules in the cytoplasm, except for the Golgi region in which they tend to be absent (Kent, 1966; Whur and Johnston, 1967; Takeuchi et al., 1969). They were varied in amount and appearance in different GLs (Toner, 1965). The globules were bounded by a typical unit membrane (Toner, 1965; Kent, 1966; Takeuchi et al., 1969; Contin and Veilleux, 1972). This membrane occasionally appeared continuous with the endoplasmic reticulum (Toner, 1965; Kent, 1966; Carr, 1967), or the Golgi membranes in rat GLs (Kent, 1966).

The globules in most species examined showed considerable internal variation not only from species to species but frequently within a single cell (Carr and Whur, 1968).

In the fowl the globules were finely granular electron dense homogeneous bodies, occasionally contained one or more vacuoles (Toner, 1965). In sheep (Murray et al., 1968), cats (Takeuchi et al., 1969),

cattle (Miller et al., 1967; Murray et al., 1968; Rahko, 1973a) and in rat (Kent, 1966; Miller et al., 1967; Whur and Johnston, 1967; Murray et al., 1968); the following type of globules were recognized : (i) globules which had homogeneous matrices of moderate electron density and surrounded by unit membrane, (ii) globules with small area of electron-dense and more granular matrix separated the denser homogeneous material from the surrounding membrane. Small vesicles and myelin-like configurations were sometimes found in the periphery of these globules, (iii) globules in which the matrix was partially lost, leaving paracrystalline structures with electron density similar to the original matrix. Longitudinal striations with a periodicity of approximately 70Å were present within the paracrystalline bodies. Often remnants of less dense but coarsely granular matrix were found within these granules. Small vesicles and myelin-like figures were occasionally seen within the globules. Frequently the periglobular membranes were discontinuous at various points, (iv) globules in which the periglobular membranes were lost, leaving paracrystalline structures free within the cytoplasm.

In the mouse, the globules might be either homogeneous or contained crystalline bodies (Carr, 1967; Rahko, 1971).

Mitochondria

The illustrations in the literature often demonstrated mitochondria within GLs but only a few authors made specific reference to them. In the rat (Whur and Johnston, 1967), mice (Carr, 1967) and the fowl (Toner, 1965) a few rod-shaped mitochondria containing loosely packed cristae were present in each GL of these species.

Golgi Material

In the cytoplasmic matrix of the GLs in the rat (Kent, 1966; Whur and Johnston, 1967), the cat (Takeuchi et al., 1969) and the fowl (Toner, 1965), the Golgi apparatus was small and situated near the nucleus. There was no evidence of the formation of globules in this site, and no structural change associated with the accumulation of globules in the cell (Toner, 1965). It consisted of stocks of smooth-

surfaced cisternae slightly curved around a single or a pair of centrally located centrioles (Takeuchi et al., 1969).

During the course of magnesium deficiency in rat Cantin and Veilleux (1972) reported on several changes in the cytoplasm of GLs. In the early stage, the cytoplasm contained relatively few globules, while the rough endoplasmic reticulum and the Golgi complex were prominent. The rough endoplasmic reticulum was sometimes slightly dilated and contained electron-lucent flocculent material. Free ribosomes were abundant and mitochondria were numerous. The smooth endoplasmic reticulum was ordinarily scarce. While in later stages of magnesium deficiency the GLs of the urinary tract, contained very few organelles, their cytoplasm being filled with globules. Mitochondria, rough and smooth endoplasmic reticulum, as well as free ribosomes were extremely rare.

D. ORIGIN AND FATE OF GLOBULE LEUCOCYTES

Although the GL has been studied by many authors, there has been considerable disagreement concerning its origin, function and fate. A few workers thought that the cell originated from certain mononuclear leucocytes (Moller, 1899), wandering cells (Toro, 1929; 1931) and micromyelocytes (Mjdassojedoff, 1926). The majority of investigators considered that GLs originated from the lymphocytes of the lamina propria becoming immature GLs and when they matured they migrated into the epithelium (Weill, 1919; Keasbey, 1923; Dawson, 1927; Michels, 1935; Dawson, 1943; Kent, 1952; 1966).

On the basis of light microscopic observations Jordan (1938) and Kirkman (1950) suggested that the GLs may have originated from plasma cells. However, Toner (1965), who described the GLs in the small intestine of the fowl, believed that there was a certain degree of similarity between GLs and fowl lymphocytes as described by Ackerman (1962), in respect to the diffuse endoplasmic reticulum, mitochondria, Golgi apparatus, and vacuole-containing bodies.

The plasma cells, on the other hand, with its elaborate granular reticulum and large Golgi apparatus, and its larger and more elaborate mitochondria, has little resemblance to the GLs, although by the light microscope they both showed cytoplasmic basophilia and similar nuclear characteristics (Toner, 1965). Therefore Toner (1965) supported the belief that the GLs originated from the lymphocytes and not the plasma cells. Whur and Johnston (1967) suggested that there were many morphological similarities between Russell body cells and GLs indicating a common origin. The local origin of GLs from lymphocytes through a series of transitional form as described by Kent (1952) and Toner (1965), was not confirmed by the investigations of Whur and Johnston (1967). These workers postulated that the GLs observed in their investigation were modified plasma cells migrating to the site of antigenic stimulus. They suggested that the plasma cells transversed the lamina propria of the intestine to the characteristic intra-epithelial position where they remained for a fairly short period before being released into the lumen

of the intestine. This investigation by Whur and Johnston (1967) in the rat was in complete agreement with the observations in the sheep by Dobson (1966b) who concluded that the origin of the GL was related to the plasma cell.

Carr (1967) proposed the plasma cells as a possible precursor of the GLs of the mouse. He supported the immunofluorescent study on the sheep GLs by Dobson (1966b) who showed that it contained condensations of globulin and resembled the plasma cell in its staining reactions.

On the other hand Takeuchi et al. (1969) work in the cat was in agreement with Kent (1966) who had already pointed out the sharp difference between Russell bodies and GL globules. The Russell bodies were limited by rough endoplasmic reticulum while the globules of GLs were enclosed by smooth membranes. Miller et al. (1967) and Murray et al. (1968) studied the relationship between GLs and mast cells in the rat, sheep and cattle. On the basis of histochemical reactions and the ultrastructural appearances they claimed that these two types of cells were derived from the subepithelial mast cells.

In contrast Takeuchi et al. (1969) working in the cat considered that GLs might be easily distinguished from mast cells by the ultrastructural and histochemical characteristics of their granules. They attributed the origin of the GL in the cat to a stem or mesenchymal cell.

The origin of the GLs was attributed to eosinophilic leucocytes by Clara (1926), Rogosina (1928) and Casley-Smith (1968) but this was refuted by Carr (1967) who studied GLs in mouse intestine. He suggested that GLs differed in several points of fine structure from the eosinophils observed in the same experimental animals. Carr (1967) found that the globules of GLs were larger than the specific granules of eosinophils. In addition the crystals in globules of GLs had a variety of shapes while those of eosinophils were rectangular and lay in the long axis of the granules. On high resolution microscopy, eosinophil crystals showed a striation in only one case out of many cells examined. In this isolated case the

striation was at an angle to the long axis of the crystal. While purely morphological criteria cannot rule out a functional relationship between the eosinophil and the GL, the obvious structural differences suggested that they were not closely related, and Carr (1967) supported the belief that it was derived from the plasma cell.

Rahko (1972) agreed with Murray et al. (1968); Miller et al. (1967) and (1971) that the GL originated from the subepithelial mast cell. This opinion rested upon certain cytochemical and ultrastructural evidence. There was a quantitative relationship between the kinetics of mast cell and GL populations in rat infested with Nippostrongylus brasiliensis (Miller, 1971). Subepithelial mast cells and GLs shared several cytochemical properties and they had a comparable cytologic ultrastructure in infested animals Murray et al., (1968).

Little is known about the fate of GLs in many species. Globule leucocytes have been thought to revert to the parent cell type since they are scarce in tissues outside the mucous membranes and they do not commonly show degenerative changes (Moller, 1899; Clara, 1926; Rogosina, 1928).

On the other hand it was suggested that the GLs passed through the epithelium into the lumen of the organs in which they occurred (Corti, 1922; Michels, 1934; Kirkman, 1950; Kent, 1952). However no one observed there. Previous investigations by Keasbey (1923) indicated that there was no evidence for GLs ever approaching the lumen.

Globule leucocytes often lie well above the level of the nuclei of the epithelium (Dawson, 1943; Kirkman, 1950). However they were usually seen within epithelia of mucous surfaces (Dobson, 1966b; Whur and Johnston, 1967; Miller et al., 1967; Murray et al., 1968; Rahko, 1972). In rare instances they have been reported to be in the act of entering the lumen (Kent, 1952). Occasionally they have been observed apparently discharging their granular contents into the lumen (Dawson, 1943).

Whur and Johnston (1967) found, in the rat, that the GLs traversed the lamina propria of the intestine to the characteristic intraepithelial position. They remained there for a short period and then the cells, together with the contents of the globules which was postulated to be an antibody were finally released into the lumen of the intestine. Whur and Johnston (1967), found no evidence of degeneration in situ. Anaphylaxis failed to disrupt the GL globules, or to produce any visible changes in these cells (Whur, 1966b).

E. FUNCTION AND BEHAVIOUR OF GLOBULE LEUCOCYTES

Various functions have been suggested for GLs. A role in the assimilation of food-stuffs was ascribed to these cells (Moller, 1899; Weill, 1919; Clara, 1926; Rogosina, 1928; Toro, 1931). Keasbey (1923) correlated the occurrence of GLs with the degeneration of gastric glands near developing lymphatic nodules. On the basis of cytochemical tests and staining affinities, Keasbey (1923) concluded that the globules contained haemoglobin, but hesitated to call these cells erythrophages. Dawson (1927) believed that the globules formed within GLs were not phagocytosed particles or erythrocytic debris. Michels (1935) however, regarded both the GL and Russell body cell as erythrophagocytes, while Duran-Jorda (1945) thought that the globules were erythrocytes being synthesized within the GL.

In Russia, Davletova (1958) suggested that the function of the GL in lambs was to aid in the assimilation of foreign proteins introduced with plant fodder. The appearance of GLs occurred when the lambs were weaned from milk whose proteins were more easily assimilated than vegetative fodder. This phenomenon was in agreement with the previous work by Toro (1931), when he experimentally produced an increase in the number of the GLs in the digestive tract of various animal species by feeding them with different concentrations of protein.

Another group of workers were investigating the appearance of GLs in association with parasitic infestation, and Taliaffero and Sarles (1939) were the first to find that GLs increased in number during parasitic infection in rat. Kirkman (1947, 1949, 1950) demonstrated that the appearance of such cells in the urinary passageways of the rat was related to round worm infestation. Kent (1952) suggested that GLs do not aid in the assimilation of food-stuffs, nor do they phagocytose erythrocytes. There does not appear to be any relationship between developing lymphatic nodules and the occurrence of GLs as mentioned by Keasbey (1923). Kent (1952) suggested that GLs arose due to a reaction to diffusion of substances of various origins into the mucous membranes. However, he did not determine whether or not parasites

were one source of these substances in the sheep which he examined. Sommerville (1956) and Soulsby (1965) agreed with Kirkman' s (1950) investigations that related the presence of GLs with helminth infestation. Dobson (1966b) found an association between GLs and Oesophagostomum columbianum infestation in sheep, but also noted that worm-free sheep had a small number of these cells within tissues of the small intestine and caecum. Whur (1966a) noted that there was a relationship between the appearance of GLs in the intestinal mucosa and the onset of expulsion of the worm burden in rats infected with N. brasiliensis. This could have indicated that the GL has some functional role to play in the "self-cure" phenomenon. Whur (1966b) disagreed with Sommerville's investigation (1956), who had examined sections removed at the time of "self-cure" and suggested that the presence of the GLs were not associated with self-cure phenomenon. Dobson (1966a) concluded that the cytoplasm of the GL in the digestive tract, contains condensations of immunoglobulins, which indicated that these cells played a role in the immune mechanisms of the gut.

Dobson (1966b) and Whur and Johnston (1967) in their studies on the nature and fate of the GL postulated that there were many similarities betwen GLs and immunoglobulin and the function of the GL was to transport antibody across the epithelium to the target sites in the lumen of the organ. Whur and Gracie (1967) confirmed (i) the demonstration of globulin in the granules of sheep GLs by Dobson (1966a) and (ii) the relationship between GLs and the immune response to N. brasiliensis. Whur (1966b) and Whur and Gracie (1967) indicated that GLs could be involved in the manufacture or transport of antibodies.

Jarrett et al. (1967) suggested that the mast cell to GL transformation might be associated with the effector mechanism involved in the transport of immunoglobulins produced in other cells into the lumen of the gastro-intestinal tract. Murray et al. (1968) found that N. brasiliensis infestation induced two peaks of GLs in the intestinal mucosa. N. brasiliensis produced a substance which caused degranulation of mast cells almost

immediately after the worms reached the intestine (Jarrett, Jarrett, Miller and Urquhart, 1968). At that time, a local vascular leak was established and the connective tissue of the villi and the subepithelial spaces became markedly oedematous. During this degranulation, there was the first peak production of GLs (Jarrett et al., 1968). Later in the infection, the mast cell population regenerated. A sudden episode of mast cell activity associated with the second peak of production of GLs occurred at the commencement of expulsion of the worm burden (Jarrett et al., 1968). It was generally considered that this corresponds to the function of GL; either the cells produced and transported immunoglobulins (Dobson, 1966a; Kent, 1966; Whur and Gracie, 1967) or biogenic amines were released from the mast cells during their transformation into GLs (Jarrett et al., 1967; 1968; Miller et al., 1967; Murray et al., 1968).

Different opinions on the occurrence of the GLs in parasite-free animals still exist in the literature. Kent (1966) and Jeffery and Reid (1975) found numerous GLs in the tracheobronchial epithelium of adult axenic and specific pathogen free rats. It has been suggested that the appearance of the GL within the epithelial mucosa was associated with many other factors rather than parasitic infections.

Cantin and Veilleux (1972) reported that magnesium deficiency in rat caused an increase in the population of the GLs in the transitional epithelium of the urinary tract, while the subepithelial and the submucosal mast cells degranulated and decreased in number. On the other hand Kent et al. (1954) found that the daily administration of low doses of corticotropin and cortisone acetate caused a marked decline in the population of GLs in the tracheal mucosa of rats; large doses of cortocotropin or cortisone acetate daily caused these cells to disappear completely. Kent et al. (1956) noted that hypophysectomy alone significantly reduced the number of intestinal GLs in the rat intestine.

The effect of X-irradiation on GLs in rat intestine was studied by Kent et al. (1956). They reported that there was a significant reduction in the number of these cells within 12 hours after exposure to irradiation.

There was a 99 per cent. disappearance of GLs (4-5) days after irradiation. These cells began to increase in number about the sixth day, and continued at a 30th day after irradiation. The effect of radiation on GLs was chiefly a local one since shielding the intestine with lead during irradiation prevented their loss. Radiation may act directly on GLs themselves or indirectly as a result of stress induced by the irradiation, since the effect was prevented by hypophysectomy. Dawson (1927) showed that the GLs were present in the lymph nodes of dogs with a previous history of irradiation.

During pregnancy, it was shown that GLs appeared in the epithelium of the human uteri Weill (1920). Kent (1952) reported the occurrence of the GLs in the sheep uteri during pregnancy, although during the oestrus cycle these cells occurred in fewer numbers and were smaller in size than during pregnancy in sheep (Kellas, 1961); they increased in number and size as pregnancy advanced (Kellas, 1961).

F. DISCUSSION

Many conflicting views have been expressed in the literature on the nature species distribution, origin, function and fate of the GL. Inadequate descriptions and illustrations, the variety of techniques which have been used in preparing the materials studied and the resemblance sometimes found between these and other cells, have all contributed to the probability that several types of cells have at one time or another been classified as GLs. In the pig, rabbit, guinea pig and bat, it is possible that the cells Heidenhain (1888) and Weill (1919) described were GLs even though they were unique in some of their morphological features. The descriptions of GLs in the digestive tract of sheep (Keasbey, 1923), in the gall bladder of cat (Dawson, 1943) and in the urinary passageways of the rat (Kirkman, 1950) left no doubt concerning their identity. The reports of these cells in man, guinea pig, bat, turtle, crocodile, frog and salamander were supported by adequate illustrations and descriptions (Toro, 1931).

Reports of GLs in other organs in other species can only be accepted with reservation until further work has been done. However the GLs which Toro (1931) described in the horse resembled eosinophilic leucocytes in many ways and were possibly confused with them because of their relatively enormous inclusions in this species. The cells which Zibordi (1920) described in the hemopoietic organs of the dog and which Corti (1922) believed to be GLs were obviously eosinophilic leucocytes since, in this animal, the granules were irregular and often large.

The intra-epithelial granular cells in the uterine epithelium of sheep and cattle described by Kellas (1961), were considered to be GLs. They were found typically in an intra-epithelial position and contained variable numbers of cytoplasmic granules of assorted sizes. These granules were acidophilic, metachromatic with toluidine blue and displayed other specific staining reactions.

Kirkman (1950) studied GLs in the urinary tract of rat and he discussed the similarities and differences between GLs, plasma cells with acidophilic inclusions and Russell body cells. He suggested that the differences might be no greater than those recorded between GLs from different species and different organs. However, he did not consider that the evidence was sufficient to establish a clear relationship between these cell types. Pearse (1949) and White (1954), on the other hand, appeared to regard Russell body cells and GLs as being the same cell type, although neither author provided evidence for his belief.

On the contrary Kent (1952), believed that the morphology and histochemistry of the GLs proved that it was a cell type distinct from the Russell body, eosinophilic leucocyte and plasma cell with acidophilic inclusions.

On the basis of the morphology, ultrastructure and histochemistry of GLs, it was shown that the cell is mast cell related (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968). The granules of both cell types were bound by a single smooth-surfaced trilaminar membrane, unlike the rough-surfaced membrane bounding the distended cisternae of plasma cells containing Russell bodies (Miller et al., 1967). The rough-surfaced endoplasmic reticulum of the GL was similar to that of the mast cell and quite different from that of plasma cells with Ruseell bodies (Jarrett et al., 1967). Globule leucocytes contained granules identical to those of the mast cell. The granules in both GLs and mast cells have an acid mucopolysaccharide which was metachromatic with toluidine blue at pH 4.0, stained with astra blue at pH 0.3 and fluoresced orange with acridine orange (Miller et al., 1967; Murray et al., 1967). Russell body cells however, failed to stain metachromatically with toluidine blue (Kent, 1952).

Similar cells were described in the rat urinary tract as granular cells (Ahlqvist and Kohonen, 1959) and bundle cells (Hicks, 1965; Monis and Zambrano, 1968). However, the morphology, histochemistry and fine structure of these cells were in agreement with those of the GLs described in the rat urinary tract (Kirkman, 1950; Cantin and

Veilleux, 1972).

The brilliant cells which were described in the biliary tract of cattle (Shirai et al., 1976) have all the characteristics of the GLs described previously in the same species (Rahko, 1970 a & b; 1971; 1973a).

Kent (1952) described a series of transitional stages between lymphocytes and GLs in the intestinal mucosa, but this has not been confirmed by other investigations. Toner (1965) supported a lymphocytic origin for GL on the basis of his ultrastructural observations, placing particular emphasis on the poorly developed endoplasmic reticulum in the GLs. However Kent (1966) was in agreement with this observation, and Takeuchi et al., (1969) suggested that in the cat the lymphocyte granules showed the same heterogenity as the globules in GLs. However, Takeuchi et al., (1969) described the granules of the lymphocyte, as being much smaller than the average globules of the GL in the cat. In addition, the GLs in the cat were not stained metachromatically with Giemsa or toluidine blue (Takeuchi et al., 1969; Finn and Schwartz, 1972), a reaction characteristic of mast cell granules (Selye, 1965). This should not be considered as sufficient evidence to suggest that in the cat the GLs are not mast cell related. Most of the fixatives Takeuchi employed contained formalin or mercuric chloride, and it has been shown that the metachromasia of the mast cell in the rat small intestine was not seen properly with these fixatives (Enerback, 1966b).

The fate of the GL is still unknown. The belief that GL reverts to its unspecified parent cell type (Moller, 1899; Clara, 1926; Rogosina, 1928) is not supported by any evidence. Where as the suggestion that these cells passed through the epithelium into the lumen of the organ (Corti, 1922; Michels, 1934; Kirkman, 1950; Kent, 1952) has not been confirmed by any worker. However, occasionally they have been observed discharging their granular contents into the lumen (Dawson, 1943). Whur and Johnston (1967) believed that the GLs which had an intra-epithelial position, remained there for a short period and then the cells, together with the contents of the globules, were

finally released into the lumen of the intestine.

The function of the GL is also uncertain. Globule leucocytes have been suggested as transporters of proteinaceous food-stuffs from the intestinal lumen into the mucous membrane (Moller, 1899; Weill, 1919; Clara, 1926; Rogosina, 1928; Toro, 1931; Davletova, 1958). This would be the reverse of what would be expected if they played the postulated role of migrating through the epithelium into the lumen. In addition, their occurrence in the urinary tract (Kirkman, 1950; Cantin and Veilleux, 1972), respiratory tract (Kent, 1966), biliary tract (Miller et al., 1967, Murray et al., 1968) and the uterus (Kellas, 1961) could be hardly explained on this basis.

The theory that GLs secrete erythrocytes (Duran-Jorda, 1950) was successfully contradicted by Kirkman (1950). The possibility that the globules of GL were phagocytized erythrocytes (Michels, 1935) has been opposed by the cytochemical findings already discussed by Kent (1952).

It has been suggested that GLs played an important role in dealing with parasitic infestation (Whur, 1966a). However this may not reflect any function since these cells appear in a variety of tissue animals under conditions other than parasitic infestation. They were found in the uterus during the oestrus cycle and pregnancy (Kellas, 1961), the urinary tract of rat with magnesium deficiency (Cantin and Veilleux, 1972), the digestive tract of certain animals fed with different concentrations of protein (Toro, 1931) and the lymph nodes of dogs with a previous history of irradiation (Dawson, 1927).

The occurrence of GLs in the rat urinary tract maintained on a magnesium deficient diet (Cantin and Vielleux, 1972) could be explained by the fact that such a diet induced an endogenous histamine release with mast cell degranulation (Bois, Gascon and Beaulnes, 1963; Bois, 1968). This resembled the effect of N. brasiliensis in the digestive tract of the rat in which degranulation of the subepithelial mast cells was induced (Murray et al., 1967; Miller et al., 1971). During degranulation of the mast cells, there was a peak production of GLs in the mucosa of the

alimentary tract (Jarrett et al., 1968).

The presence of GLs in the endometrium during pregnancy and oestrus cycle of certain ruminants (Kellas, 1961) may be due to the fact that the mast cells were subjected to hormonal influences (Selye, 1965). There seemed to be a significant increase in the number of mast cells during the secretory phase of the menstrual cycle in the human (Iversen, 1960). However during pregnancy there was a significant decrease in mast cells when compared to the number in the secretory phase; vacuolated cells and extra-cellular granules were also seen (Iversen, 1960). On the other hand in the bitch uterus the mast cell count was considerably increased during pregnancy (Nepriakhin, 1956).

The administration of corticotropin and cortisone caused a marked decline in the population of GLs in the tracheal mucosa of rats (Kent et al., 1954). These changes could be attributed to the effect of ACTH or cortisone inducing an alteration in shape, vacuolation and degranulation of mast cells with an eventual decrease in their population (Asboe-Hansen, 1952).

Globule leucocytes were observed in the lymph nodes of dogs with a previous history of irradiation (Dawson, 1927). This could be explained since a single dose of irradiation has been shown to induce a conspic ious infiltration of mast cells into the lung of the rat (Watanobe et al., 1974). According to Sylven (1945) exposure to roentgen ray was followed by degranulation of mast cells and later by an increase in their number. Hill and Praslicka (1958) described discharge or lysis of the mast cell granules after total body irradiation of mice. This was followed by massive differentiation of new mast cells from the reticulum cells of the spleen and resulted in an increase in the total number of mast cells. The reduction in number of GLs in rat intestine after irradiation (Kent et al., 1956) could be explained on the same basis. The number of mast cells in the skin of the rat tended to fall in proportion to the dose of ionizing radiation and to the time which had elapsed since irradiation (Bruni and Massa, 1962).

Toro (1931) experimentally produced an increase in the number of GLs in the digestive tract of various animal species by feeding them different concentrations of protein. This would suggest that pure meat or sugar diet, when fed to the rat, would induce an increase in the number of mast cells in the small intestine of this animal (Vessini, 1933). The same author found that the number of disintegrating mast cells rose in the small intestine of the rat kept on the meat diet and also during fasting when compared with the control animals fed normal diet.

From the above mentioned factors (magnesium deficiency, pregnancy, cortisone and corticotropin, radiation and different concentration of protein in the food), it is possible to suggest that such conditions could be able to induce similar alterations and changes in the mucosal mast cells as parasitic infestation. Murray et al. (1968) suggested that the GLs observed during parasitic infestation were the result of the mast cells discharging amines during the course of migration and of an accompanying alteration in the association between the acid mucopolysaccharide and the basic protein of the granules. The end product of this process was a cell with the staining affinities of the classic GL.

In conclusion the present information reviewed here might indicate that similar mast cells degranulate and release of vasoactive amines might occur from other stimuli induce a marked proliferation of intra-epithelial mast cells or GLs.

Although there is some information about GLs in the alimentary and biliary tracts in sheep. These cells do not appear to have been described in the bronchopulmonary system of sheep even in reports dealing with ovine parasitic lung infestations.

CHAPTER TWO

STUDY OF SHEEP LUNGS FOR THE OCCURRENCE AND DISTRIBUTION OF GLOBULE LEUCOCYTES

A. INTRODUCTION

While studying bronchopulmonary diseases in many groups of sheep during all seasons of the year, a granular type of cell was encountered in large numbers in the epithelium of the respiratory tract. The most prominent feature of these cells was the presence of spherical intracytoplasmic acidophilic granules. These granules varied considerably in size and number, stained with eosin and were metachromatic with toluidine blue. These cells were more or less round in outline and were found at various levels within the epithelium of the respiratory tract.

In those lungs examined during the course of parasitic infection, these cells occurred in large numbers and with such regularity that an account of their distribution, morphology and a few of their staining affinities were recorded. In contrast, the lungs of sheep which did not have a pulmonary parasitic infection had fewer or no such granular cells in their epithelial mucosae.

Previous reports indicated that globule leucocytes which had been described in different animal species (Heidenhain, 1888; Weill, 1919), possibly had the same characteristics as the granular cell encountered in the sheep respiratory tract.

The GL is an intraepithelial mononuclear cell with highly refractile, acidophilic, intracytoplasmic granules, which frequently indent the nucleus (Keasbey, 1923; Dawson, 1943). Taliaferro and Sarles (1930) were the first to demonstrate that GLs increased in number during parasitic infections. Later studies clearly showed that the frequent occurrence of GLs was invariably associated with a parasitic infection (Kirkman, 1950; Kent, 1952).

On the other hand there are reports in the literature cited in the previous chapter, that GLs were found in animal tissues when parasites could not be involved as an etiological stimulus that is magnesium deficiency in rat (Contin and Veilleux, 1972) and radiation in rat (Kent et al., 1956).

In sheep GLs have been described in the mucosa of the digestive tract (Moller, 1899; Keasbey, 1923; Kent, 1952; Sommerville, 1956; Davletova, 1958; Whur, 1966a; Dobson, 1966b; Miller et al., 1967; Murray et al., 1968), and in the biliary tract they were seen intra-epithelially and in the lamina propria too (Kent, 1952; Zipper, 1966; Miller et al., 1967; Murray et al., 1968; Rahko, 1970a). Kellas (1961) found similar cells in ovine endometrium and he called them intra-epithelial granular cells. These cells had the same morphological and cytochemical properties of GLs and were later considered to be GLs (Toner, 1965; Whur, 1966a; Rahko, 1971). Globule leucocytes however have not been described in the bronchopulmonary system of sheep even in reports dealing with parasitic lung infection.

These cells have been recorded in the respiratory tract of certain other animal species. In the rat respiratory tract the GL was described by Kent (1966), Jeffrey and Reid (1975) and Veilleux and Contin (1976). In the respiratory tract of cattle these cells were observed by Breeze et al. (1975) and Pirie et al. (1976).

Thus the present histological and histochemical investigation was undertaken to show that the unknown cell type in the epithelium of the respiratory tract of sheep with parasitic nodules could be considered to be a GL. The relationship of these cells to the subepithelial mast cells and the appearance of these cells in parasitic pulmonary infection was also investigated. Worm-free sheep at various ages were also investigated in an attempt to resolve the question of the occurrence of the GLs in situations not associated with parasitism.

B. MATERIALS AND METHODS

The animals studied and the techniques employed are described below.

1. Animals

The groups of animals used are listed in table (1).

Group (1)

This group was less than one month old and consisted of 11 lambs, reared indoors and maintained parasite free.

Group (2)

This group consisted of 18 lambs, 2-3 months old, reared indoors and kept parasite free.

Group (3)

The 15 lambs in this group were 4-6 months old, reared indoors and kept parasite free.

Group (4)

This group consisted of 25 indoor adult sheep which kept were parasite free.

Group (5)

This group consisted of 25 adult outdoor sheep. The lungs of these sheep were non-pneumonic.

Group (6)

Sixty one adult outdoor sheep made up this group. The lungs of all animals had macroscopic parasitic lesions and the distribution of the lesions on their surface are shown in Fig. 3, 9, 10 and 11.

Those groups of indoor sheep were reared worm-free in clean boxes with no access to pasture to prevent any natural infection with parasite. Clinical and haematological examinations were carried out twice a week for each sheep. Blood samples were drained from jugular vein to examine

for peripheral blood eosinophilia. Fecal samples were checked for respiratory and gastrointestinal parasites also. Material for outdoor sheep was collected at the slaughter house in Glasgow during the years 1976-1978. All the sheep were killed by exsanguination after electrical stunning. The carcases were dressed in the normal manner. The respiratory tract of each animal was stripped from the attached organs. Histological samples were taken from eight standard sites in the bronchi of the right lung of each sheep as shown in Fig. 1. Adjacent pulmonary tissue was also collected.

2. Fixation

The tissue samples were about 3 mm in thickness, and were collected in a variety of fixatives. These were : buffered neutral formalin, sublimate formol, Carnoy's fluid and Bouin's fluid. The details of these fixatives are in Appendix I. All tissues were fixed for 24 hours, dehydrated and cleared in a double embedding series. Finally tissue blocks were embedded in paraffin wax and cut at 6-8μ in a rotary microtome. These sections from material fixed in sublimate formal were treated with Lugol's iodine and sodium hyposulphate to remove the mercuric pigments. Blocks fixed in Bouin's fluid were washed in alcohol to remove the picric acid.

3. Staining Procedures

Haematoxylin and eosin (H & E) stain was used routinely for initial detection of GLs. Various histochemical techniques were also used to confirm that the cells observed with H & E were GLs. These were toluidine blue (TB), alcian blue and astra blue/safranine (AB/S), biebrich scarlet (BS), carbol chromotrope (CC) and acridine orange (AO). The preparation and use of these stains are described in Appendix I.

4. Parasitic Identification

Parasitic identification was carried out by :

(a) Examination of the lungworms under a stereoscopic microscope after extraction from the lung tissue.

(b) Bearmann extraction technique and (c) Histological examination of sections stained with H & E which were prepared from samples of lung lesions. These included samples from the standard sites of the respiratory tract in addition to another six sites from the lung parenchyma; three sites from each of the right cranial anterior lobe (RCA) and the right diaphragmatic lobe (RD).

5. Globule Leucocytes Identification

The main method used to identify GL in tissue samples taken from the sheep was the appearance of cells in sections stained with H & E from blocks fixed in sublimate formol. The reasons for using this method are described in the next section.

C. THE PRESENCE OF GLOBULE LEUCOCYTES IN DIFFERENT GROUPS OF SHEEP

Globule leucocytes were readily identified in the epithelial mucosa of sheep in sections stained by H & E. The cells were characterised by their refractile, acidophilic, intracytoplasmic globules, which showed variation in number and size. The globules frequently coalesced, forming large aggregates in one side of the cell. The nuclei were eccentric and showed a chromatin arrangement similar to that of the plasma cell nucleus.

Mature GLs were usually oval to round in outline and situated in an intraepithelial position. The immature forms were located near the basement membrane in the lamina propria; they were usually smaller in size and their globules were less refractile. These cells were identical with those previously described and illustrated in the digestive tract (Kent, 1952) and in the biliary tract (Rahko, 1970a) of sheep.

Globule leucocytes in the sheep respiratory tract were readily differentiated from eosinophil leucocytes, which were sometimes present in the epithelium but these cells displayed only a small amount of granular material in the cytoplasm. In the GL the individual globule was readily identified by its large appearance and size, which in certain instances, could be about the same size as the nucleus of that cell.

It is not within the scope of this chapter to discuss the whole cytochemistry of GLs. However, certain staining techniques were used (Table 2) to confirm that the cells observed by H & E were GLs identical to those described in other species (Miller et al., 1967; Murray et al., 1968).

The granules of both GLs and eosinophil leucocytes displayed red staining reaction with carbol chromotrope, while the mast cells showed no staining reaction. All three cell types bind biebrich scarlet at pH 8 and up to pH 10. The globules of GLs and mast cells showed violet metachromasia with toluidine blue pH 0.3, and the intensity of this reaction was greater at pH 4.0. This metachromatic reaction was lacking in the granules of

the eosinophil leucocytes. Alcian blue and astra blue/safranin pH 0.3 induced blue staining reactions in the granules of GLs and mast cells. Both these chemical tests showed no staining reaction in the granules of the eosinophil leucocytes. The granules of GLs and mast cells fluoresced red-orange with acridine orange. This reaction was lacking in the eosinophil leucocytes.

Globule leucocytes were absent in the respiratory tract of all groups of indoor sheep examined (group 1, 2, 3 and 4) as in Table 3. The lambs in group 1, examined at one day old or at the time of birth showed no GLs in their respiratory tract. Furthermore the adult indoor sheep showed no evidence of GLs in the epithelial mucosa of their respiratory tract.

In the respiratory tract of the outdoor groups (5 and 6) considerable variation in the occurrence of GLs was observed. Group 5 consisted of sheep with no macroscopic evidence for parasitic pulmonary lesions. However, GLs were present in lungs of 4 of the 25 sheep examined Table 3.

On microscopic examination the lungs of most sheep investigated showed moderate to slight interstitial reaction. Thickening of the alveolar septa with eosinophilic infiltration was the most prominant feature of this group (Fig. 2). Parasitic lesions, which were undetectable macroscopically, were identified microscopically by the presence of disintegrated larvae surrounded by disintegrated eosinophil leucocytes. The parasitic lesions were small in size, few in number and scanty in distribution. The GLs in the lungs of some sheep in this group were restricted to the epithelium of their bronchioles only. No GLs were detected in the tracheal or bronchial epithelia.

Globule leucocytes were abundant in the respiratory tract of most sheep examined in group (6, Table 3). They occurred intra-epithelially and along the length of the trachea, bronchi and bronchioles in the 47 sheep investigated, although considerable variation in their distribution was found. This variation related to differences in the macroscopical and microscopical appearance of the parasitic lesions in their lungs. However the correlation

between the distribution of the GLs and the type of parasitic lesions will be discussed in Chapter 2, D. On the other hand, the remaining 14 sheep in this group did not show the presence of GLs in their lungs despite the presence of parasitic lesions on their lung surfaces. Thus 6 different types of lesions were found, which are described below.

Type 1 parasitic lesion

Areas of dark red consolidation formed diffuse lesions which were confined to the cranial or diaphragmatic lobes of the lung (Fig. 3).

On microscopic examination the consolidated areas were heavily infiltrated with inflammatory cells. These were a mixture of polymorphonuclear leucocytes, macrophages, lymphocytes, plasma cells and patches of eosinophils scattered throughout the tissue. The lumena contained, in addition to adult worms, plugs of mucus, numerous leucocytes, eggs and larvae (Fig. 4 and 5). There was tracheitis and bronchitis with infiltration of the epithelium by polymorphonuclear leucocytes, which plugged the lumen of the bronchi. In the small and respiratory bronchioles an obliterative bronchiolitis was frequently seen. The majority of these lesions were associated with Dictyocaulus filaria.

Type 2 parasitic lesion

Diffuse pulmonary lesions consisting of yellow to green elevated patches projecting above the pleura of the lung were seen irregularly in the dorsal surface of the lungs.

The microscopic picture revealed eggs, larvae and adult lungworms in these lesions. In some cases the larvae could be identified. There were intense foci of infiltrated eosinophils around the larvae, the alveolar spaces became crowded with macrophages and the stretched alveolar walls were thickened by fibrous tissue. The larvae which escaped into the small bronchioles were enclosed in plugs of mucus and cellular debris. There was hyperplasia of the bronchiolar epithelium and thickening of the muscularis. The parasites appeared

in focal accumulation (Fig. 6 and 7). These lesions may be associated with Muellerius capillaris and Protostrongylus rufescens.

Type 3 parasitic lesion

Diffuse lesions of yellowish to grey patches measuring 3-6 cm. in diameter distributed on the dorsal surface of the lung were seen. Sometimes they contained calcified or caseated materials.

In this lesion the microscopic appearance consisted of disintegrated polymorphonuclear leucocytes intermixed with macrophages and plasma cells which, in some cases, surrounded the foci of larvae. D. filaria or P. rufescens larvae were found in this type of lesion. In the calcified or caseated form of the lesion, a central zone of adult Cystocaulus ocreatus or M. capillaris were found undergoing necrosis showing the breakdown of cellular material. The surrounding zone consisted of a ring of fibroblasts intermixed with macrophages, eosinophils and a few plasma cells (Fig. 8). The interlobular septa contained lymphocytes only and were dilated. Alveolar walls were thickened and infiltrated with lymphocytes and macrophages.

Type 4 parasitic lesion

These pulmonary lesions were nodular, reddish to purple and ranged in size from 1 to 3 mm. in diameter. They were soft and haemorrhagic in the early stage of infestation and projected above the pleural surface. This type of lesion could occur anywhere in the lung, but the great majority of them were located beneath the pleura of diaphragmatic lobes. A variable number of these nodules were calcified (Fig. 9 and 10).

The histology of these lesions depends upon the stage of the parasite present and resistance of the host. In some cases it consisted of a small focus of macrophages and lymphocytes, surrounded by a zone of eosinophils and lymphocytes. In other cases the lesions consisted of diffuse, scattered foci of irregularly concentrated eosinophils inside alveoli which contained a parasite. There was fibrous thickening of the alveolar walls with infiltrated lymphocytes

in the septa and around the blood vessels and bronchioles. The parasite found in this lesion was usually a larval stage of M. capillaris.

Type 5 parasitic lesion

Deep brown circumscribed nodules measuring 3-5 mm. in diameter projected above the pleural surface of the diaphragmatic lobe (Fig. 11).

Microscopically, this lesion consisted of a central parasite surrounded by a zone of disintegrated eosinophils with an outer ring of macrophages, lymphocytes and eosinophils. Severe eosinophil infiltration of the alveolar walls, subpleural zone and interlobule septa was seen. The parasites found in this type of lesion could be C. ocreatus.

Type 6 parasitic lesion

Yellow circumscribed nodules measuring 3-5 mm. in diameter were seen. These nodules were found either on the pleura or in the parenchyma.

Microscopically, this lesion was circumscribed with a central parasite surrounded by eosinophils and an outer zone consisting of lymphocytes and macrophages intermixed with the formation of epithelioid and giant cells forming a granuloma (Fig. 12 and 13). Lungworms causing this type of lesion could be adult parasites of M. capillaris.

D. DISTRIBUTION OF GLOBULE LEUCOCYTES IN THE LUNGS

The distribution of GLs in the respiratory tract of sheep were investigated in most lobes of the lung, including the upper and lower trachea, bronchi and bronchioles of different size as shown in (Fig. 1) which illustrates the standard site sampled from the respiratory tract.

Globule leucocytes were extremely rare in the normal respiratory tract of sheep, whereas in lungworm infection the cells were frequently within the epithelial mucosa.

The mature GLs were found in different sites within the epithelial mucosa, from the basement membrane to the lumenal surface (Fig. 14). While the immature cells occurred in the lamina propria, either underneath or on the basement membrane.

The occurrence and distribution of GLs throughout the length of the respiratory tract of sheep were found to be related to the presence and abundance of the parasitic lesions. This clearly indicated the relationship between this cell and certain parasitic infections. Furthermore, considerable variation occurred in the distribution of the GLs within the parasite infested lungs. This dissimilarity in the distribution is related to the type of the parasitic lesions which were classified in chapter two, C. This classification showed variations in the pathology, location and larval stage in each type of lesion which may have induced the same variation in the distribution of the GLs. However parasitic lesions induced by M. capillaris, located on tissue parenchyma or subpleural position, may not show GLs in the bronchi or trachea of the same lung. While infection with adult parasites of D. filaria present in the bronchi may show considerable numbers of GLs in the bronchi or the trachea (at patient phase) due to the laid eggs from the same parasite. On the same basis the distribution of GLs in the sheep respiratory tract are shown in Table 4 correlating the results and the type of lesion and stage of the parasite or eggs laid.

In animals with GLs in their respiratory tract, the trachea almost invariably contained GLs throughout its length. They were regularly present in large numbers in the upper and lower trachea in the pseudostratified ciliated columnar epithelium (Fig. 15). No GLs occurred in the lamina propria. A few GLs were occupying positions within the epithelium of the tracheal glands, especially in the ducts and mucous secreting parts.

The abundance of GLs throughout the tracheal mucosa were found to be related to the chronicity, extension of the parasitic lesions and the laid eggs of D. filaria. Therefore lungs with few parasitic lesions on their subpleural surfaces had little or no GLs in their tracheal mucosa.

In inflammatory conditions of the mucous membrane, particularly when accompanied by metaplastic changes in the epithelium, a diminution or disappearance of the GLs was often found.

Globule leucocytes, in addition to their occurrence in the trachea itself, were seen in large numbers in the bronchi of various diameter in different lobes of the lung (Fig. 16). These cells occupied the same sites and had the same distribution in the bronchial mucosa as in the tracheal mucosa. They were situated within the pseudostratified columnar ciliated epithelium of the bronchi.

In some instances the GLs were found at the lumenal end of the epithelial layer suggesting that they were entering the lumen of the bronchi (Fig. 17). In other instances they were clearly found in the lumen of some bronchi and bronchioles (Fig. 14 & 17).

Although GLs were not found in the connective tissue of the mucous membranes, a moderate number were occupying positions within the epithelium of the bronchial glands, especially in their ducts and mucous secreting parts (Fig. 18 & 19).

Some secondary bronchi were surrounded by parasitic lesions although no parasites were identified in their lumen, but they showed

many GLs in their mucous membrane.

The greatest numbers of the GLs were found in the bronchioles. In the low pseudostratified columnar ciliated epithelium the GLs were markedly abundant (Fig.20). The GLs in the columnar ciliated epithelium of the terminal bronchioles, occupied the same sites and had the same density as in the large bronchioles in the sections examined. The bronchioles were always surrounded by the tissue parenchyma of the lung, but the GLs were never seen in these situations. The respiratory bronchioles which directly connect with the alveolar ducts and alveoli, have low columnar or cuboidal epithelium; it may be ciliated in the proximal portion. The GLs were also detected in these respiratory bronchioles but only in heavily parasitized lung (Fig. 21).

Between the alveoli and the distal portion of the respiratory bronchioles is the alveolar duct, which opens into a group or cluster of alveolar sacs. In these sites, the GLs were not seen, and they were completely absent in the alveoli.

The alveolar walls (interalveolar septa),which separate the alveoli,contained no GLs in any case, although fibroblasts and mast cells were present in normal cases. These cells increased in number together with the eosinophil leucocytes in parasitic infestation.

The visceral pleura which is composed of a thin layer of connective tissue and a layer of mesothelium showed no evidence of the presence of any GLs. The pleura and subpleural layer are rich in mast cells and other cells but no GLs were found in these sites.

The presence of GLs in considerable numbers in the epithelium of the mucous secreting glands and ducts, of both trachea and bronchi was uncommon. They were detectable in these sites only when the lungs had extensive and profuse parasitic lesions. However, lungs with mild or little parasitic lesions did not necessarily have such cells in the epithelium of their glands and ducts.

E. QUANTIFICATION OF THE GLOBULE LEUCOCYTES IN THE LUNGS

In order to compare the response of GLs to various stimuli in sheep more accurately than by subjective impression, the following method was devised to quantify their number objectively. With this technique it would be possible to compare GL numbers in different groups of sheep.

1. Method of Quantification

Globule leucocytes were identified by their morphological and staining characteristics in sections stained with H & E (Chapter Two C). They were counted in the mucous membrane of a transverse section of bronchus from each standard site of sampling (Fig. 1). Since GLs were abundant in the respiratory tracts of the outdoor sheep which had macroscopic parasitic lesions in their lungs, group 6 animals were used for this quantification. Other groups of sheep were excluded because the lungs of these animal groups (1, 2, 3 and 4) lacked GLs (Table 3).

The GLs were quantified by calculating their number per unit length of bronchial epithelium. The standard unit of length was taken to be one centimetre (CM) and the number of GLs per CM of bronchial epithelium was calculated as follows: all the GLs in the epithelium of a transverse section of bronchus (from the standard sites) were counted by examining the epithelium with the high power objective (x 400). The circumference of the epithelium around the lumen of the bronchus was measured by projecting the slide, using a Leitz projector with objective (x 2.5) and eyepiece (x 10), onto a screen. The distance from the projector to the screen was constant in each slide examined. The basement membrane around the lumen of the bronchus was measured on the screen using a map measure. The circumference of the epithelium was converted into (CM) and the number of GLs per CM could then be calculated. Finally the number of GLs counted were expressed as (GL/CM).

2. Results

The results for the quantification of GLs in the bronchial mucosa at the six sample sites in each sheep (group 6) together with a mean and standard error value for the six sites in each lung are set out in Table 5 respectively. The outdoor sheep in group 6 had a GL/CM value which was not only considerably increased compared to the GL/CM value in sheep of indoor groups, but also had a wider range from (0.61) to (123.75) with a mean of (13.80). While the indoor sheep in groups 1, 2, 3 and 4 showed no significant number of GLs in their lungs. This confirmed the observation that the GLs became present in lungs of sheep during parasitic infection.

F. DISCUSSION

Detailed information on the morphology and histochemistry of GLs in mucous membranes of various organs in parasitized sheep is available in several publications (Keasbey, 1923; Kent, 1952; Sommerville, 1956; Whur, 1966a). The most striking feature, by light microscopic examination, of these cells is the prominant acidophilic, intracytoplasmic, refractile globules and the rounded eccentric nuclei (Dobson, 1966b; Miller et al., 1967; Murray et al., 1968). These publications present a relevant basis for the identification of the GL; using the criteria outlined in these papers, the unknown cell type observed in the bronchopulmonary system of outdoor sheep, with parasitic lesions, has been demonstrated to be the same type of cell as the GLs described in the digestive tract (Kent, 1952; Dobson, 1966b) and biliary tract of sheep (Zipper, 1966; Miller et al., 1967; Murray et al., 1968; Rahko, 1970a).

Studies in sheep, cattle and the rat showed that the identification of GLs and their differentiation from other cells could be carried out by certain histochemical staining techniques (Jarrett et al., 1967). Later these findings were confirmed and extended to emphasize the relationship between GLs seen during parasitic infections and the mast cells of these species (Miller et al., 1967; Murray et al., 1968). The present investigation revealed that the histochemical reactions of GLs and sub-epithelial mast cells in the ovine bronchopulmonary system were similar. The one exception was the carbol chromotrope reaction gave negative staining with the mast cells, but produced red staining with GLs and eosinophil leucocytes. The granules of the three cell types stained red with biebrichscarlet at pH 8, 9 and 10. Using toluidine blue at pH 0.3, the granules of mast cells and GLs stained violet in colour and at pH 4.0 an intense metachromasia was seen in both cells; no staining was produced in the eosinophil leucocytes at any pH used. With alcian blue/ safranin (pH 0.3), the staining reaction of the three cell types was the same as that with toluidine blue. The granules of eosinophil leucocytes gave no staining reaction while the granules of both GLs and mast cells

showed a blue staining reaction. The granules of GLs and mast cells had a red-orange fluorescence in the acridine orange staining reaction but this was not seen in the granules of the eosinophil leucocytes.

According to the present histochemical studies, the cytochemical properties of the GLs and the mast cells seen in natural parasitic infection of sheep respiratory tracts are identical to those of the corresponding cells investigated in sheep digestive tracts (Jarrett et al., 1967). Furthermore, this investigation revealed a significant cytochemical relationship between bronchopulmonary GLs and mast cells of the lower respiratory tract. Thus the granules of both types of cells appeared to contain a sulphated acid mucopolysaccharide associated with a highly basic protein. The observed findings were also in agreement with Schiller and Dorfman (1959) and Benditt and Lagunoff (1964) who reported that rat mast cells contained a sulphated mucopolysaccharide in association with a highly basic protein (Spicer, 1963; Benditt and Lagunoff, 1964).

This investigation indicated that the GLs were absent from the respiratory tract of parasite-free sheep, while in parasitic infections these cells were readily identified in the epithelial mucosa of the respiratory tracts. Thus the restriction of the GLs to the mucosal surfaces in this system was compatible to that in the digestive tract (Kent, 1952) and biliary tract of sheep (Miller et al., 1967; Murray et al., 1968; Rahko, 1970a).

The indoor worm-free sheep (group 1, 2, 3 and 4) had no GLs in their respiratory tract when examined at various ages. Lambs in group 1 were examined at 1 day old and even at the time of birth, they had no GLs in their respiratory tracts. This was in agreement with Takeuchi et al. (1969) who stated that in the cat GLs were lacking at birth but were present in the intestinal mucosa ten days later. However, he did not mention whether these cats were parasite free or not. GLs were absent from the respiratory tract of the worm-free sheep examined in this study and this is similar to the findings of Sommerville (1956) and Whur (1966a). On the contrary

GLs were demonstrated in the respiratory tracts of most outdoor sheep (groups 5 and 6) which had parasitic lesions recognised by either macroscopic or microscopic examination of their pulmonary tissues. This agrees well with the occurrence of intestinal GLs (Kent, 1952; Sommerville, 1956; Dobson, 1966b; Whur, 1966a) and biliary tract GLs in parasite infected sheep (Miller et al., 1967; Murray et al., 1968; Rahko, 1970a). Despite the presence of massive parasitic lesions in the lungs of all the outdoor sheep in group 6, GLs were completely absent from the lungs of 14 of the 61 sheep examined. This could be explained on the basis that the sheep were examined after GLs had transversed the epithelium. Whur and Johnson (1967) considered that GLs migrate into the lumen of the intestine after remaining in an intraepithelial position for a period of time after which the cells, together with the contents of the globules, which may be antibody, are finally released into the lumen where they may be effective against the worm population.

The distribution of the GLs in the bronchopulmonary system of outdoor sheep (groups 5 and 6) was dependent on the type or species of parasite which infected the lung and the larval stage of this parasite.

In British sheep, 6 lungworm parasites have been described by Rose, Michel and Harris (1957a and b) namely, Dictyocaulus filaria, Muellerius capillaris, Protostrongylus rufescens, Cystocaulus ocreatus, Neostrongylus linearis and Protostrongylus brevispiculum. D. filaria and P. rufescens occurred in the bronchial tree of the lungs whereas the other species of lungworms were found in the parenchymal tissue and alveoli.

From the above data the parasitic lesions encountered during this study were classified on the basis of the reaction of the lung tissue of the host and the developmental stage of the parasite contained. On the other hand the occurrence of parasitic lesions in any lung does not always result in the appearance of GLs in that location or that lung. This was clear from the studies on the lungs of 14 outdoor sheep which lacked GLs despite the occurrence of parasitic lesions on their pulmonary surfaces (group 6).

The reverse of these observations was found when GLs were identified in 4 outdoor sheep (group 5) with normal looking lungs. Microscopic examination of these lungs showed that many bronchi or bronchioles, which harboured the parasite in their lumen, did not show any GLs in their epithelial mucosae, while, in other sections several bronchi and bronchioles with no parasites in their lumen had many GLs in their mucous membranes. In the respiratory tract of sheep it was difficult to correlate a direct association between GLs and the local presence of nematode infestation. Some secondary bronchi were surrounded by parasitic lesions, although no parasites were identified in their lumen, but they showed many GLs in their mucous membranes. This suggested that these cells appeared intraepithelially either as a result of a reaction to the nearest parasitic lesions which contained disintegrated parasites in their centres or during an expulsion of the parasite from their lumen into the main bronchi as suggested in rat with N. brasiliensis (Jarrett et al., 1968).

The influence of species and larval stages of any parasite on the distribution of the GLs in ovine bronchopulmonary system could be explained in that the 4th stage larvae of D. filaria produced a few GLs in the epithelium of the bronchioles but not in the trachea or the bronchi. Jarrett, McIntyre and Urquhart (1957) stated that it was the 4th stage larvae which left the lymph nodes to migrate to the lungs in cattle and they noticed pulmonary lesions 5 days after initial infection; these lesions were histologically similar to the findings in type 1 and type 3 parasitic lesions in these sheep. However, the young adult stage of the parasite is attained by the time the larger air passages are reached and here growth occurs, followed by mating and subsequent egg production and patent phase (Paynter and Selway, 1966). During the patent phase numerous GLs were seen when there were also adult parasites in the bronchi and even in the trachea possibly due to the effect of the laid eggs. On the other hand no GLs were seen during this phase in the epithelium of the bronchioles which were free from parasites.

The remaining 4 species of lungworms which were found in the parenchymal tissues and alveoli, induced a few GLs in the epithelium of

the bronchioles only but not in the trachea or bronchi. This could be explained in that the adult worm lives in the alveoli and pulmonary parenchyma, especially in the subpleural tissue and it may also live for a short time in the pulmonary arteries (Poynter and Selway, 1966).

Quantification of the GLs in various groups of indoor and outdoor sheep indicated an increase in the numbers of these cells which occurred in the epithelial mucosa of the bronchopulmonary system of outdoor sheep (group 6) only. Lungs of sheep in this group had a GL/CM value which was not only considerably increased compared to the GL/CM value in sheep of indoor groups, but also had a wider range (from 0.61 to 123.75) with a mean of 13.80. The quantification of GLs also revealed that these cells occurred in larger numbers in the right diaphragmatic lobes than the right cranial lobes. This would suggest that the diaphragmatic lobes of sheep lungs harbour more lungworms than other lobes during the course of a parasitic infection. The result obtained in this quantification study indicated that GLs were not always present in each sample studied from the standard sites of sampling in sheep lungs. Although a high population of GLs could be found at any one site. This indicates that the distribution of these cells is not homogeneous throughout the one lobe investigated. On the other hand the indoor parasite free sheep in groups 1, 2, 3 and 4 had no significant numbers of GLs in their lungs. This confirmed the observation that the GLs became present in lungs of sheep during parasitic infection.

Group	Number of sheep	Age	Description
1	11	1 month	Indoor worm-free
2	18	2-3 month	"
3	15	4-6 month	"
4	25	Adult	"
5	25	Adult	Outdoor non-pneumonic lungs
6	61	Adult	Outdoor with pulmonary parasitic lesions

Table 1 : The number, age and description of indoor worm-free and parasitized sheep in group 1-6 are shown.

Staining Method	GL	MC	Eos
H & E	R +++	R ±	R +++
CC	R +++	-	R +++
BS pH 8.9.10	R +++	R +++	R +++
TB pH 0.3	V +	V +	-
TB pH 4.0	V +++	V +++	-
AB/S pH 0.3	B +++	B +++	-
AO	RO	RO	-

Table 2: Differential staining of GLs from mast cells (MC) and eosinophil leucocytes.(Eos).

H & E : Haematoxylin and eosin
CC : Carbol chromotrope
BS : Biebrich scarlet
TB : Toluidine blue
AB/S : Alcian blue/safranin
AO : Acridine organe
- : negative reaction
+ : weak reaction
++ : moderate reaction
+++ : strong reaction
B : Blue V : Violet
R : Red RO : Red orange

Group	Description	No. sheep examined	No. lung with GL
1	Indoor worm-free	11	-
2	"	18	-
3	"	15	-
4	"	25	-
5	Outdoor non-pneumonic lungs	25	4
6	Outdoor with pulmonary parasitic lesions	61	47

Table 3 : Occurrence of GLs in different groups of sheep examined.

Sheep Number	Tr	Br	Bl	Gd	Dt	Type of Lesion
SM51	-	-	-	-	-	L4
SM52	-	-	-	-	-	L4, L6
SM53	-	-	-	-	-	L4, L6
SM54	-	-	-	-	-	L4
SM55	-	-	-	-	-	L2
SM56	+++	+++	+++	+	+	A1, L1
SM57	-	-	-	-	-	L2
SM59	-	-	-	-	-	L4
SM60	-	-	-	-	-	L6
SM61	-	-	-	-	-	L2
SM71	++	++	++	-	-	A1, L1
SM72	++	++	++	+++	+++	A1
SM73	-	-	-	-	-	L1
SM74	-	-	-	-	-	L2
SM75	-	-	-	-	-	L4
SM76	-	++	++	-	-	A1
SM77	-	+++	+++	-	-	A1
SM78	+	+++	-	-	-	A1
SM79	++	+++	-	-	-	A1
SM80	++	+++	-	-	-	A1
SM81	++	++	-	-	-	A1

Table 4 : The relationship between the distribution of GLs and the type of parasitic lesions in sheep lungs.

Sheep Number	Tr	Br	Bl	Gd	Dt	Type of lesion
SM82	+++	+++	-	+++	+++	A1, L1, L4
SM83	-	+++	-	-	-	A1
SM84	-	-	-	-	-	L2
SM85	-	-	-	-	-	L5
SM86	-	+	++	-	-	L1
SM87	-	+	-	-	-	L1
SM88	+++	+++	+++	-	-	A1, A6
SM89	-	+	-	-	-	L4, L5
SM90	++	+++	-	++	++	A1, A6, A4
SM91	-	+	-	-	-	A2, L3
SM92	+++	+++	+++	+++	+++	A1, A6
SM93	++	+++	-	-	-	L1
SM94	-	+	-	-	-	L1
SM95	-	+	-	-	-	A4
SM96	+++	+++	+++	+++	+++	A1, L1
SM97	-	++	-	-	-	A1
SM98	-	+	-	-	-	L1
SM99	-	++	-	-	-	L1, L4
SM100	-	++	-	-	-	A1, A2
SM101	+	++	-	-	-	A1
SM102	-	+	-	-	-	L1

Table 4 : (contd.)

Sheep Number	Tr	Br	Bl	Gd	Dt	Type of Lesion
SM103	-	++	+	-	-	A2
SM104	+	++	-	-	-	A1
SM105	-	++	+	-	-	A1
SM106	+	+	+	+	-	A1, A4, A6
SM107	+	++	-	+	+	A1
SM108	-	++	-	-	-	A3
SM109	-	++	-	-	-	A3
SM110	-	++	-	+	+	A1
SM111	-	+	-	-	-	L1
SM112	-	++	-	-	-	A1
SM113	-	+	+	-	-	A6, A4
SM114	+	+	-	-	-	A1
SM115	+	+	-	-	-	A1, L1
SM116	++	++	+	+	-	A1, L4, L6
SM117	++	++	+	-	-	A1, L4
SM118	++	+	+	-	-	A1, L1
SM119	-	+++	-	-	-	L3
SM120	-	+	+	+	-	A4
SM121	-	++	-	+	-	A1, L1

Table 4 : (contd.)

Tr : trachea
Br : bronchi
Bl : bronchiole
Gd : gland
Dt : duct
L : larval stage
A : Adult stage

ND : not done
- : no globule leucocytes
+ : few globule leucocytes
++ : many globule leucocytes
+++ : large number of globule leucocytes

Sheep Number	Sample Sites						Mean ± SE
	RCA			RD			
	i	ii	iii	i.	ii	iii	
SM56	93.22	7.66	-	32.85	36.16		28.31±12.56
SM71	11.08	-	-	-	3.15	45.00	9.87± 6.53
SM72	-	-	-	-	8.08	12.08	3.72± 1.73
SM76	-	-	9.16	14.20	13.70	-	6.17± 2.08
SM77	-	-	16.09	-	-	14.91	5.16± 2.67
SM78	-	-	-	2.02	6.14	-	1.36± 0.88
SM79	-	-	-	8.18	25.17	70.33	17.28± 9.96
SM80	-	12.46	-	-	1.65	-	2.35± 1.84
SM81	4.95	-	-	1.16	4.16	9.23	3.24± 1.21
SM82	-	26.93	-	-	2.90	23.87	8.95± 4.40
SM83	-	15.51	-	-	8.89	-	4.66± 2.12
SM86	-	6.30	-	-	11.10	3.20	3.43± 1.50
SM87	2.40	-	3.60	-	-	-	1.00± 0.53
SM88	104.36	38.25	34.92	19.68	7.41	27.55	38.69± 7.97
SM89	-	-	-	-	6.15	4.15	1.71± 0.92
SM90	14.63	21.68	-	-	13.11	-	8.23± 2.86
SM91	-	-	-	-	6.40	9.30	2.61± 1.40

Table 5 : Globule leucocyte per centimetre length of bronchial lumen (GL/CM) at six sites in 47 lungs of outdoor sheep group (6). An overall mean GL/CM and standard error value are included for each sheep.

Sheep Number	Sample Sites						
	RCA			RD			Mean ± SE
	i	ii	iii	i	ii	iii	
SM92	-	93.82	101.92	30.29	64.68	123.75	69.07±14.37
SM93	0.61	6.00	-	-	2.16	-	1.46± 0.85
SM94	-	-	-	4.17	-	6.11	1.71± 0.92
SM95	-	-	-	4.73	-	8.03	2.12± 0.63
SM96	-	23.14	-	-	9.32	-	5.41± 3.31
SM97	-	-	10.58	-	6.40	-	2.83± 1.56
SM98	-	-	7.13	-	9.13	-	2.71± 1.42
SM99	-	-	4.46	-	8.32	-	2.13± 1.64
SM100	-	-	15.30	14.11	-	-	4.92± 0.88
SM101	-	5.92	-	-	3.46	-	1.56± 0.71
SM102	-	4.70	18.04	-	11.97	-	5.78± 2.51
SM103	-	-	-	2.52	11.13	-	2.27± 1.67
SM104	18.60	-	14.20	-	-	-	5.46± 2.88
SM105	-	-	-	9.28	14.24	-	3.92± 2.12
SM106	-	23.16	-	-	31.10	-	9.04± 4.78
SM107	-	15.14	-	-	-	6.18	3.55± 2.16
SM108	-	-	3.10	-	7.40	-	1.75± 1.06

Table 5 : (Contd.)

Sheep Number	Sample Sites						Mean ± SE
	RCA			RD			
	i	ii	iii	i	ii	iii	
SM109	-	7.40	-	2.31	7.30	-	2.83± 1.17
SM110	-	-	19.40	7.18	9.40	-	5.99± 2.53
SM111	-	31.70	-	60.12	-	-	15.30± 8.69
SM112	-	-	-	-	2.42	2.96	0.89± 0.46
SM113	-	2.00	4.18	-	3.20	-	1.56± 0.56
SM114	-	-	8.02	6.30	-	-	2.38± 1.25
SM115	-	9.14	-	-	4.70	-	2.37± 1.30
SM116	-	7.16	-	4.30	-	3.08	2.42± 0.93
SM117	-	3.03	-	-	5.21	8.90	2.85± 1.18
SM118	-	9.14	2.49	-	-	8.91	3.45± 1.44
SM119	-	7.60	4.50	-	7.20	-	3.21± 1.10
SM120	-	-	3.11	-	-	4.41	1.25± 0.66
SM121	-	-	7.20	-	8.30	-	2.58± 1.34

Table 5 : (Contd.)

RCA : Right cranial lobe

RD : Right diaphragmatic lobe

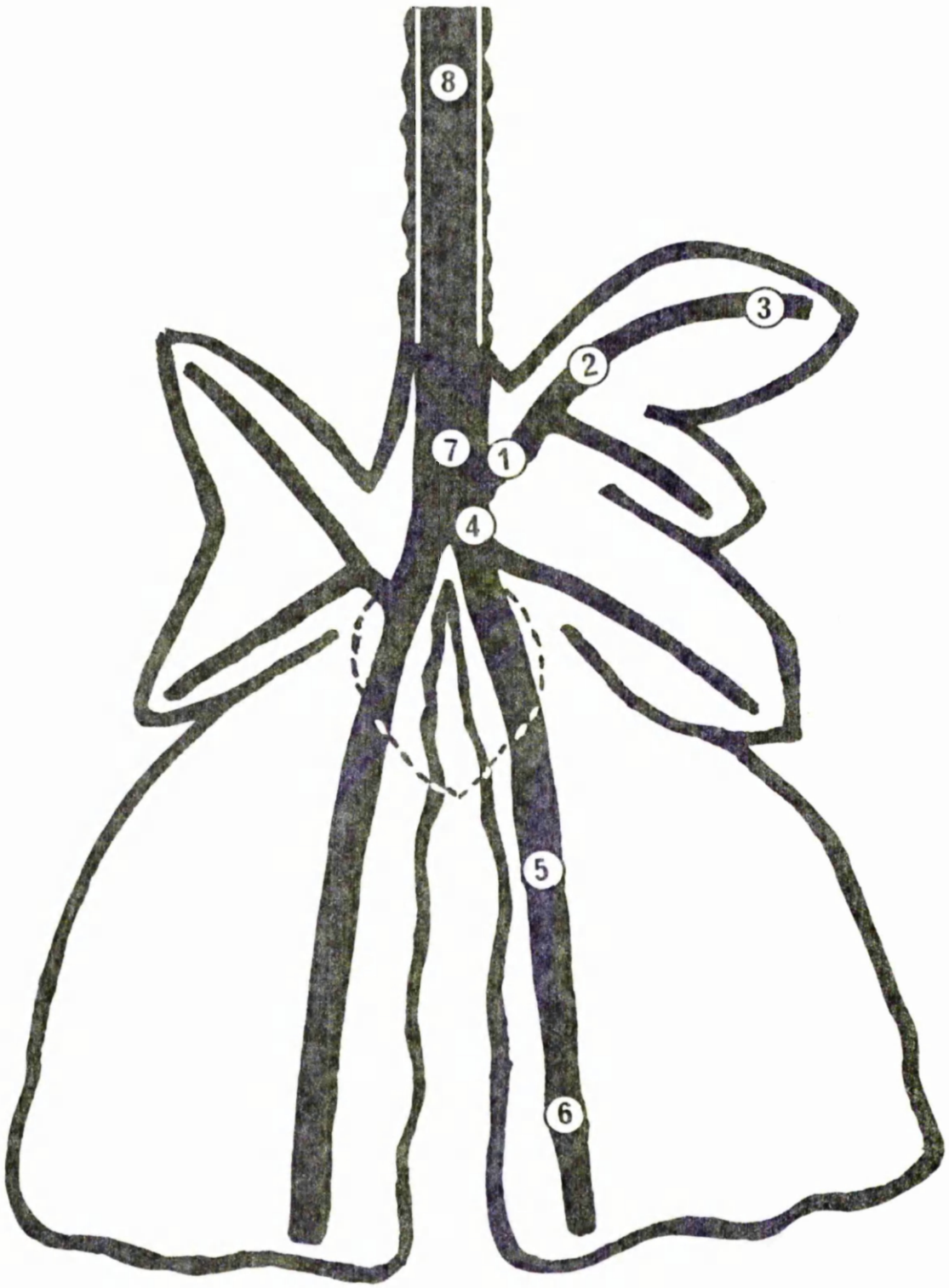


Fig. 1 : Diagram of the tracheobronchial tree of the sheep respiratory tract indicating the standard sites for sampling.

Fig. 2 : Interstitial pneumonia in group 5 of outdoor sheep; thickening of alveolar walls and eosinophil infiltration.
H & E staining, X 250.

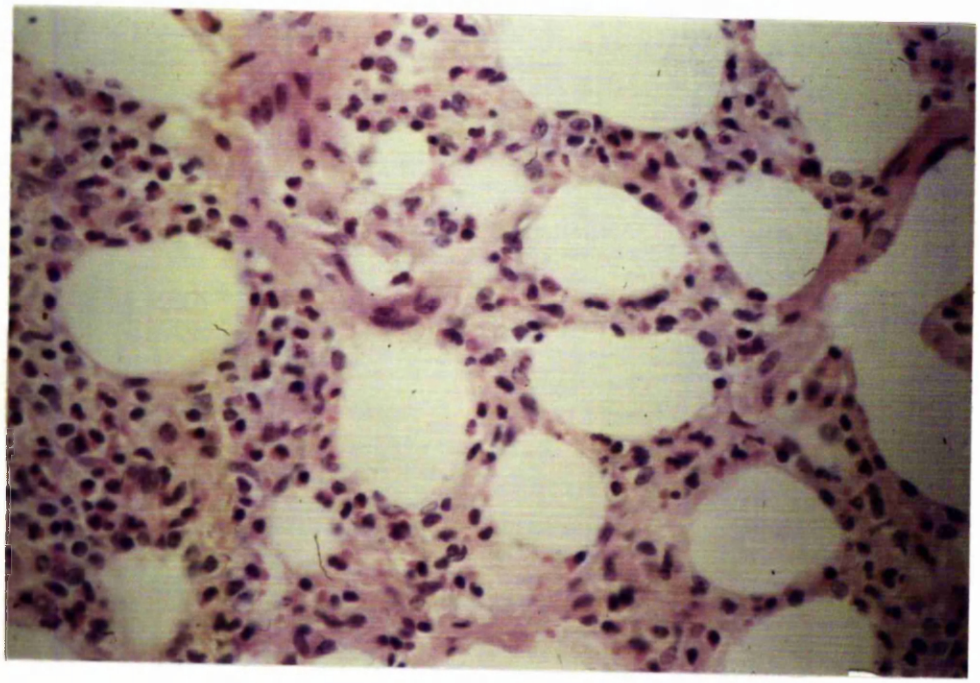


Fig. 3 : Type 1 parasitic lesion consisted of a dark purple consolidated area on the diaphragmatic lobes of outdoor parasitized sheep mainly associated with *D. filaria*.

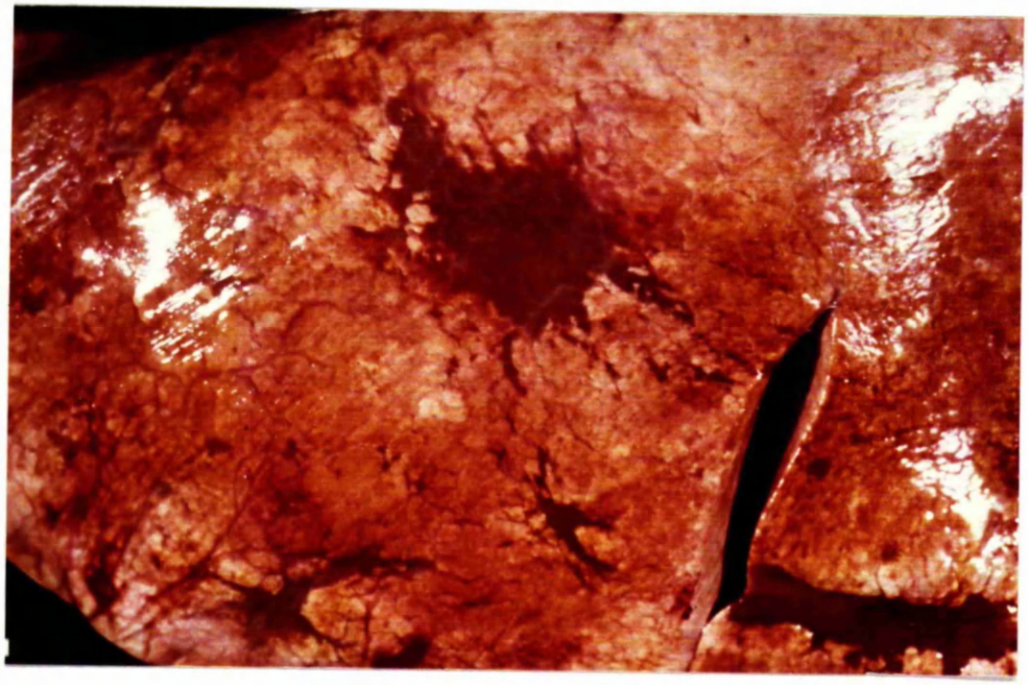
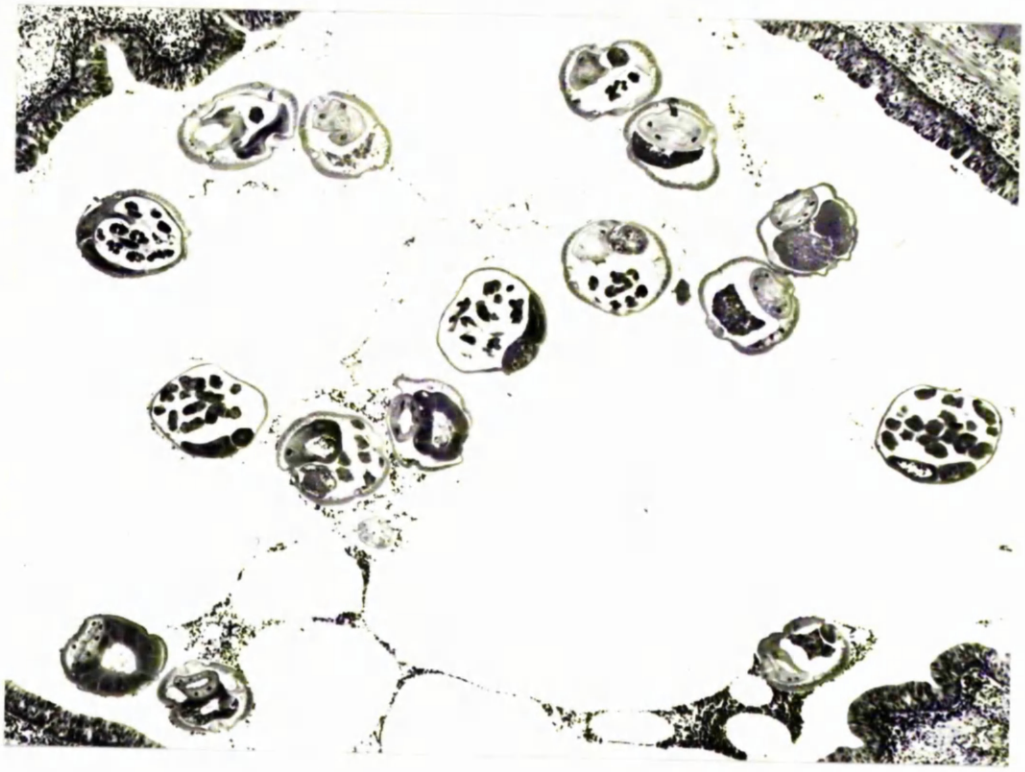


Fig. 4 : Type 1 parasitic lesion. This shows a large bronchus containing several cross sections of the lungworm D. filaria.
H & E staining, X 40.

Fig. 5 : High power view of the above bronchus showing intense cellular infiltration of the epithelium and lamina propria, mostly eosinophils, lymphocytes and plasma cells.
H & E staining, X 250.



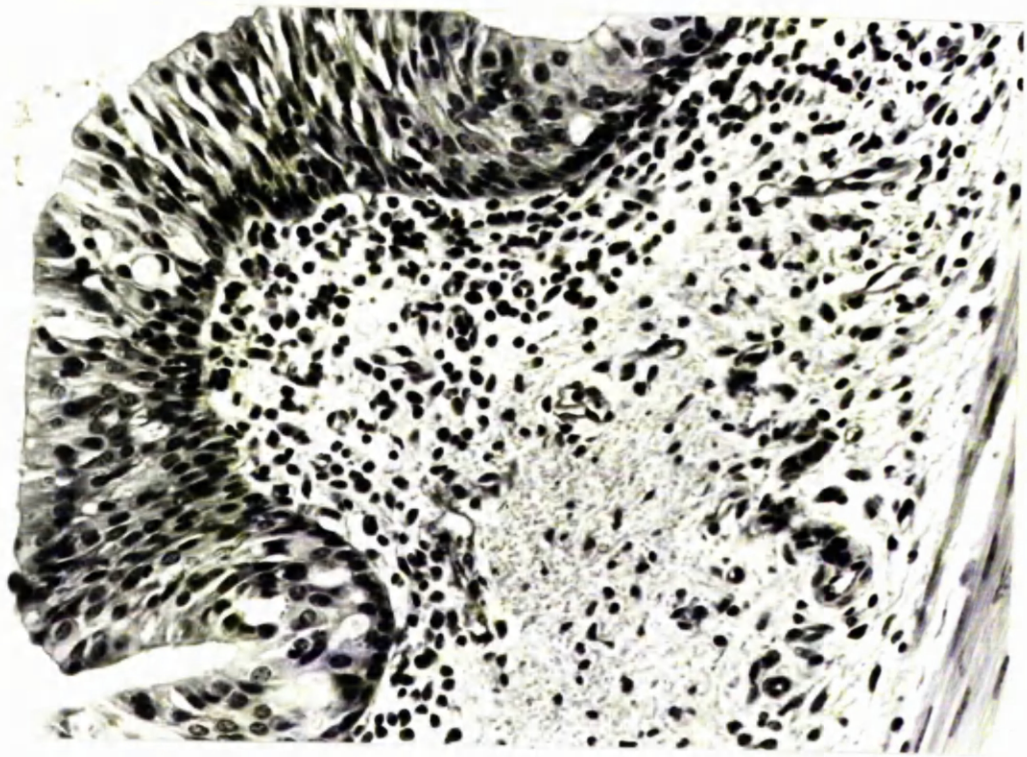
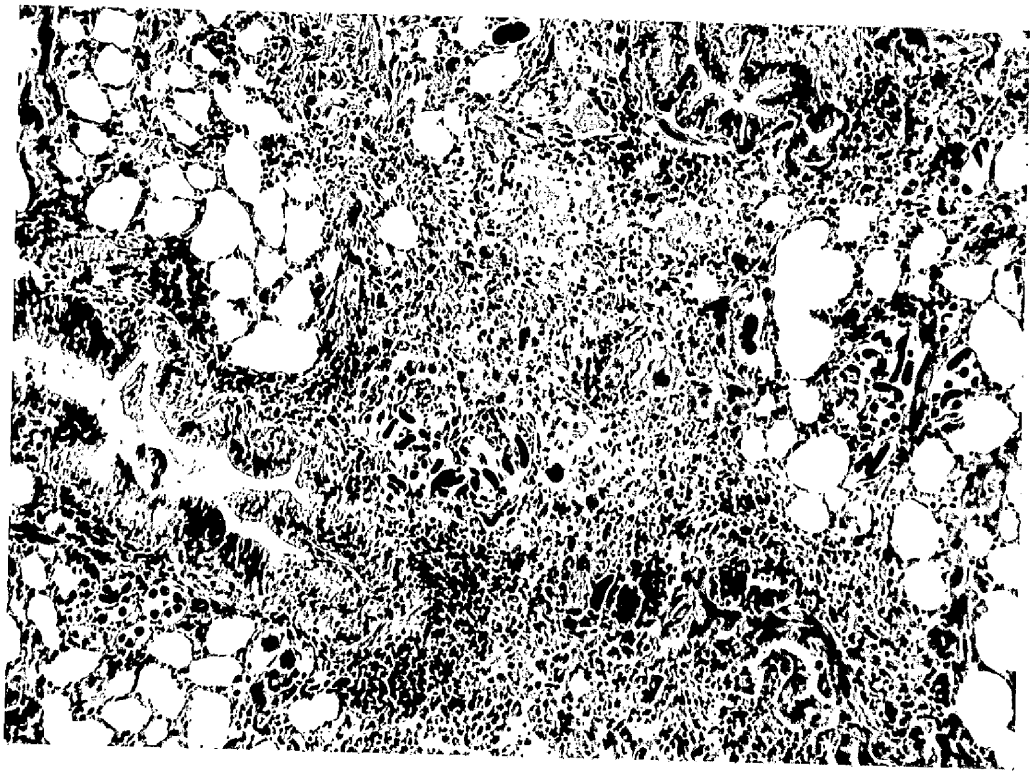


Fig. 6 : Type 2 parasitic lesion showing accumulation of larvae in ruptured alveoli. There are a few scattered eosinophils, lymphocytes and polymorphonuclear leucocytes.
H & E staining, X 40.

Fig. 7 : Detail of a part of the pulmonary diffuse lesion shown in the preceding figure.
H & E staining, X 250.



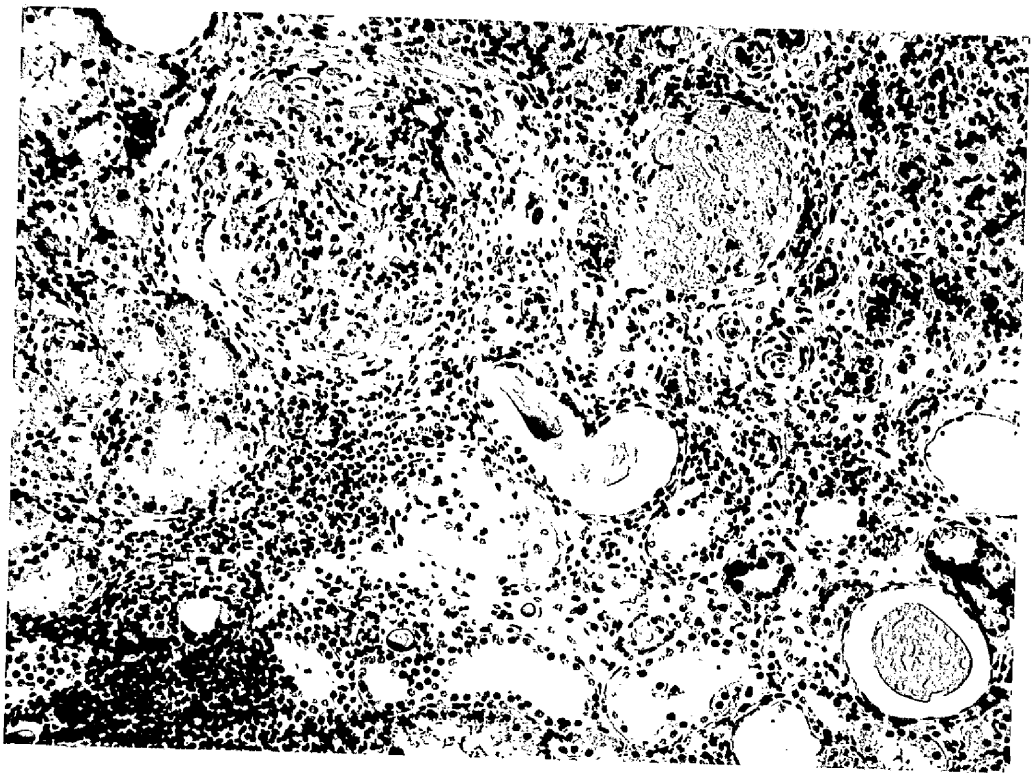


Fig. 8 : Type 3 parasitic lesion. Microscopically the caseated material consisted of a central zone which is a parasite undergoing necrosis surrounded by a zone of fibroblasts, macrophages, eosinophils and plasma cells.
H & E staining, X 250.

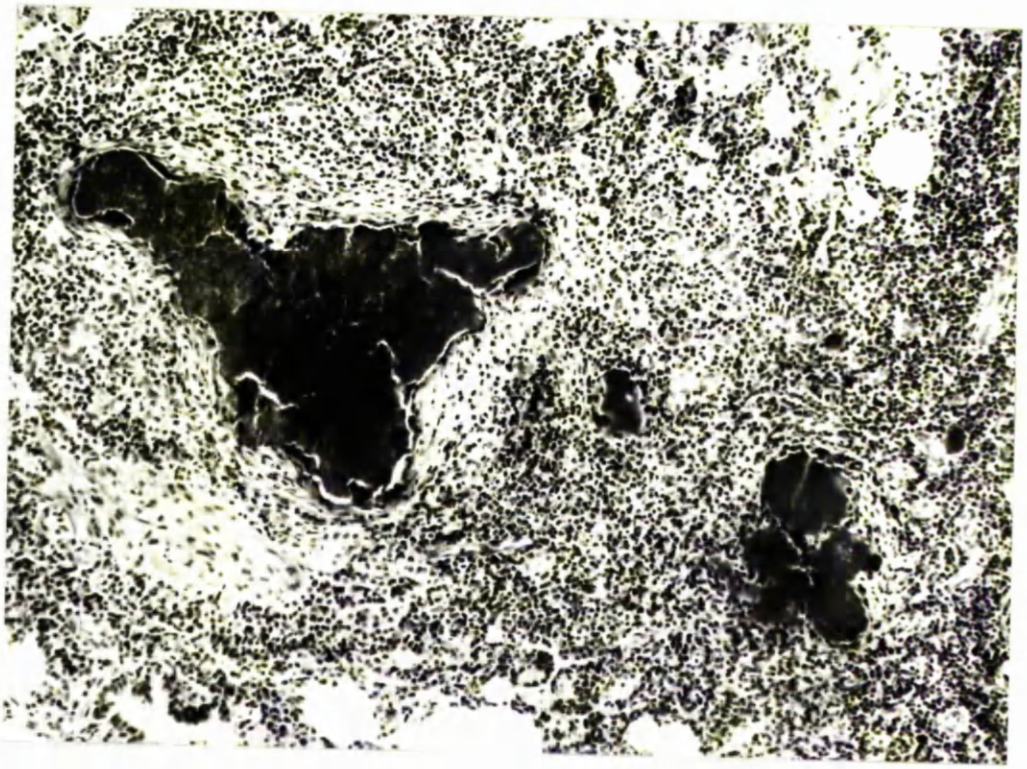
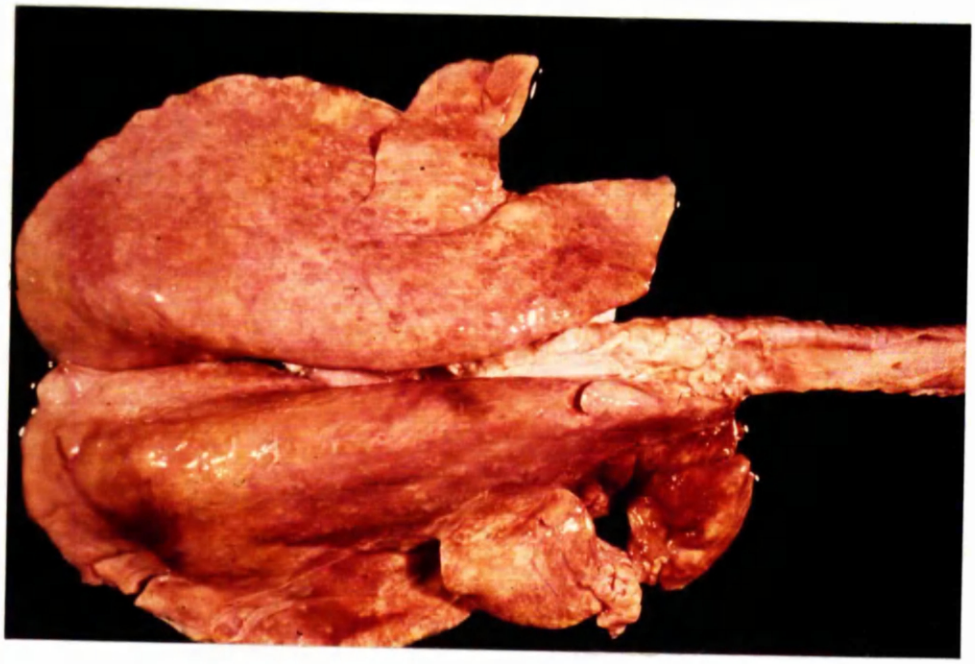


Fig. 9 : Type 4 parasitic lesion. Reddish to purple, circumscribed subpleural nodules caused by M. capillaris in the lungs of sheep.

Fig. 10 : Detail of the above picture.



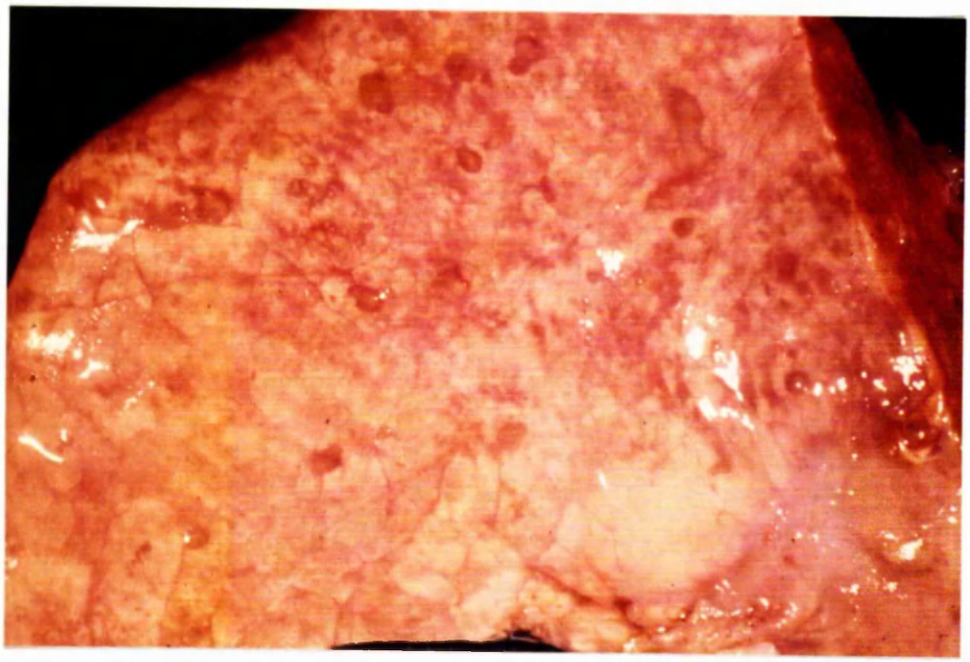


Fig. 11 : Type 5 parasitic lesion. Dark purple circumscribed subpleural nodules in the lungs of outdoor parasitised sheep.

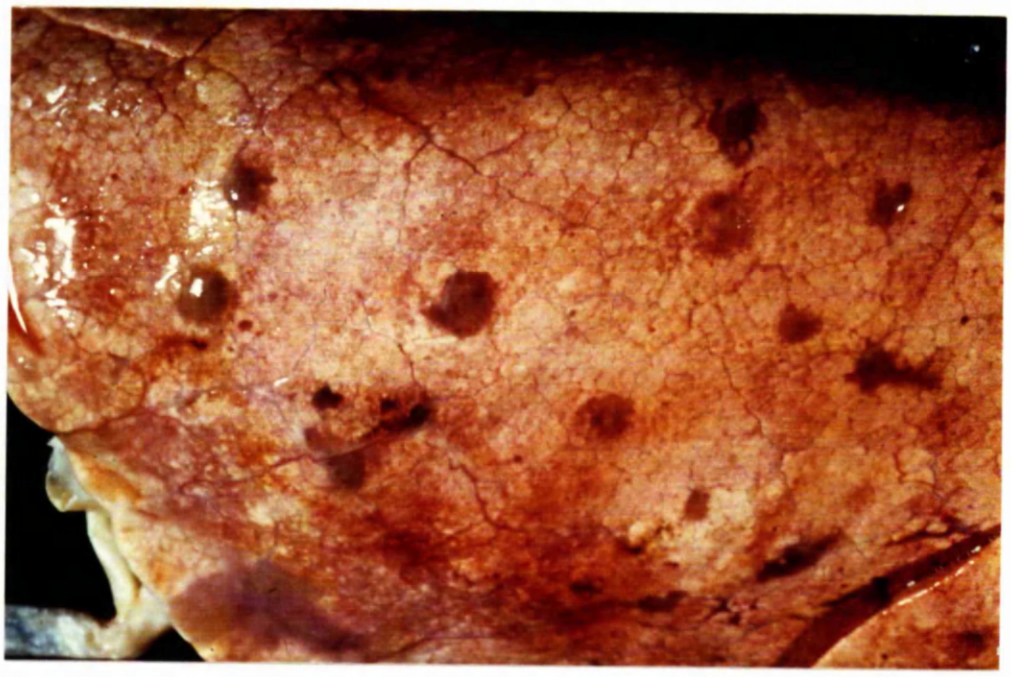
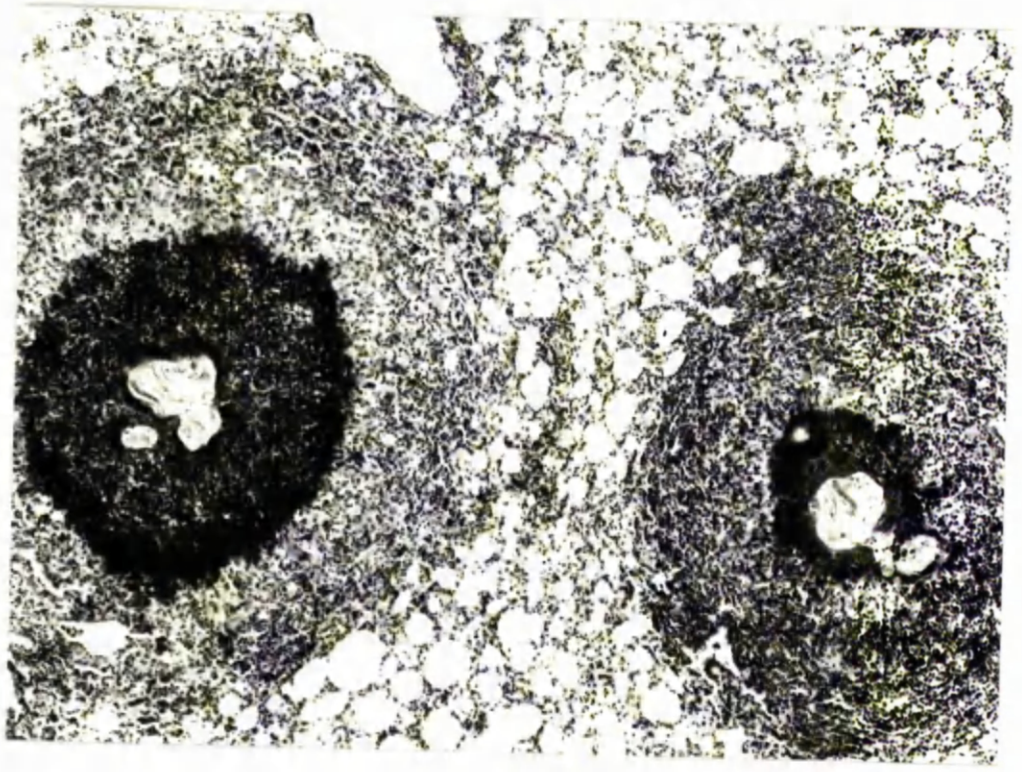


Fig. 12 : Microscopic appearance of type 6 parasitic lesion which is circumscribed, containing a central parasite surrounded by a necrotic zone and disintegrated eosinophils.
H & E staining, X 40.

Fig. 13 : High magnification of the above lesion showing giant cells and macrophages surrounding the disintegrated eosinophils.
H & E staining, X 250.



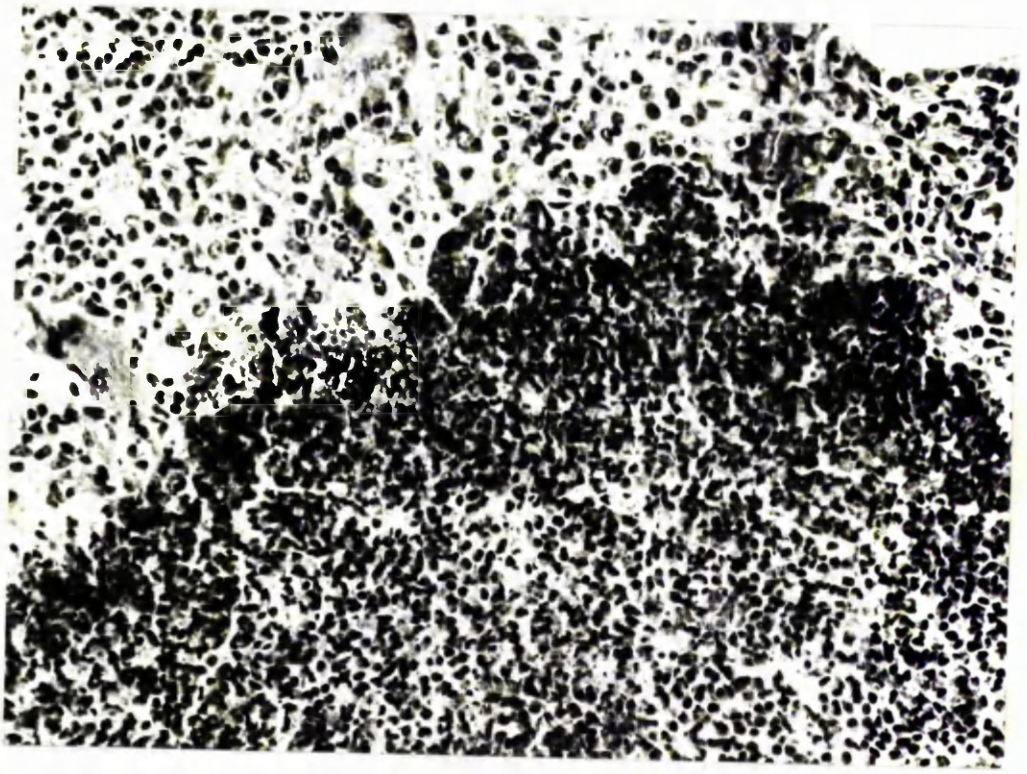
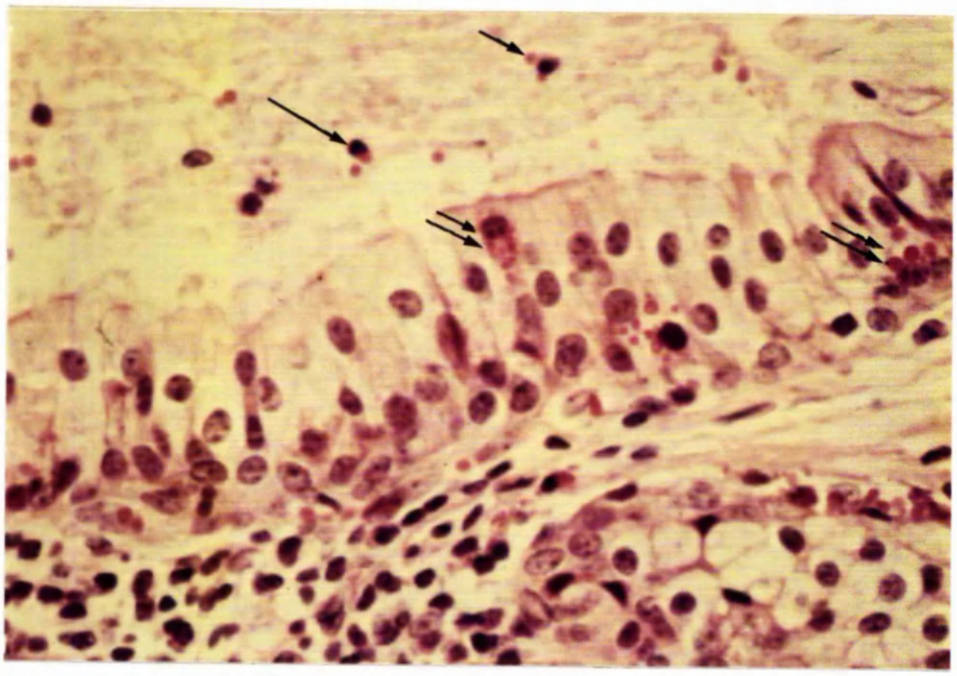


Fig. 14 : Mature globule leucocytes (⟹) found in different sites within the epithelial mucosa of parasitised sheep bronchus, from the basement membrane to the lumenal surface. Other globule leucocytes occurred in the bronchial lumen (⟶).
Carbol chromotrope staining, X 250.

Fig. 15 : Globule leuoocytes (⟶) situated between the pseudostratified ciliated columnar epithelium of the trachea in parasitised sheep.
H & E staining, X 250.



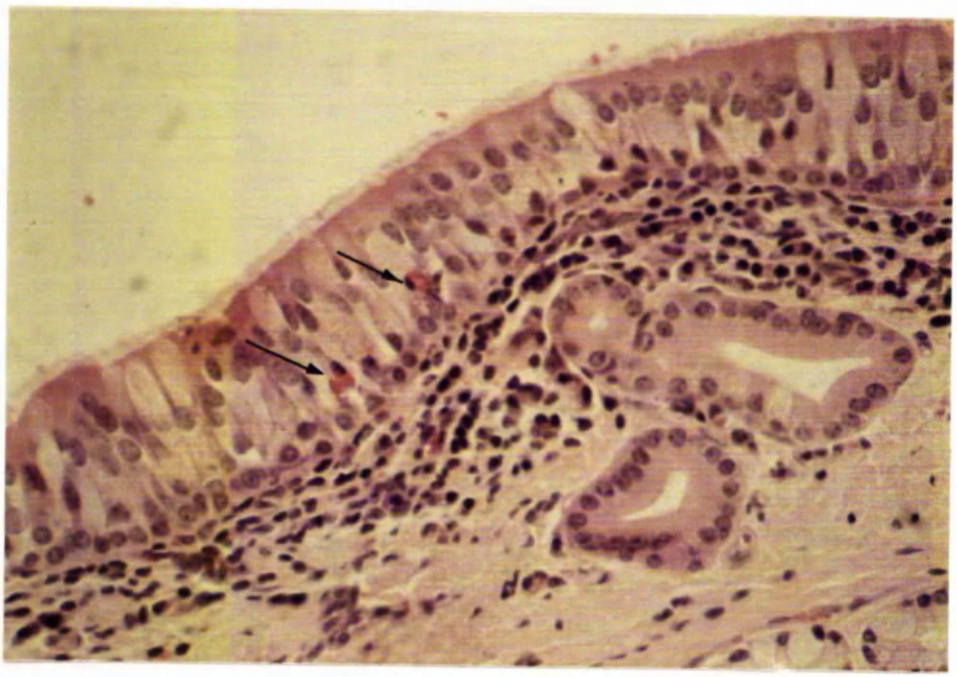
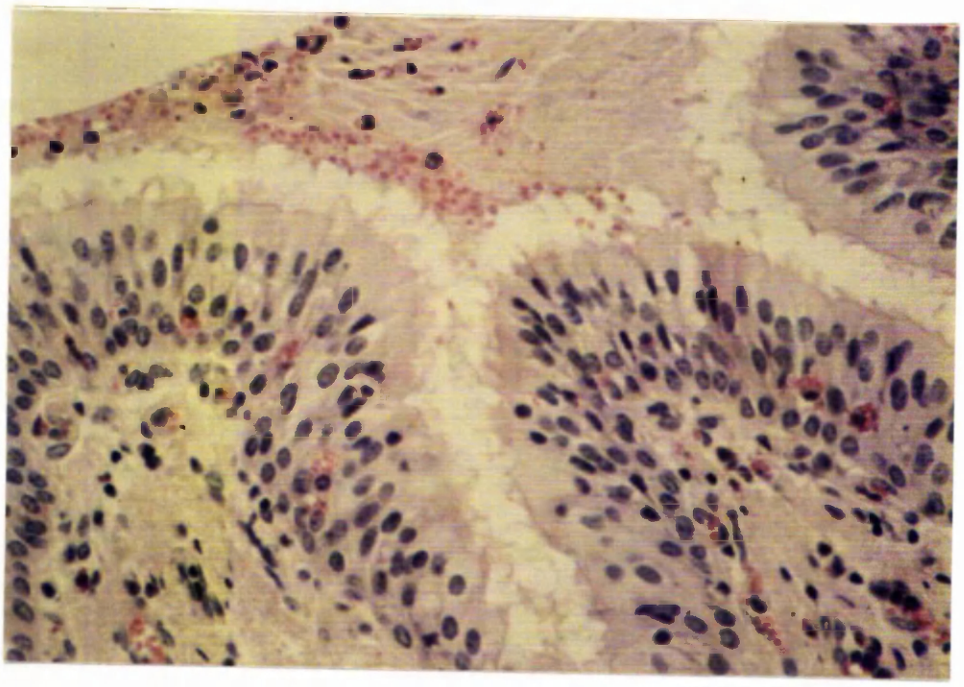


Fig. 16 : Many globule leucocytes found in the bronchial epithelium of parasitised sheep.
H & E staining, X 250.

Fig. 17 : Globule leucocytes at the end of the lumenal cells of the bronchial epithelium before entering the lumen of the bronchi.
Biebrich Scarlet staining, X 100.



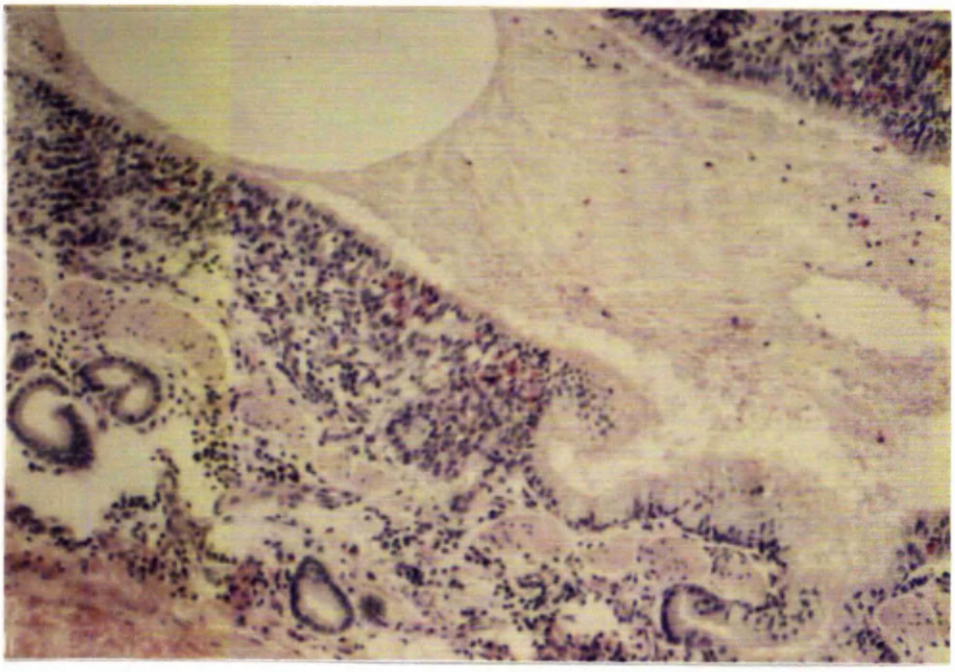
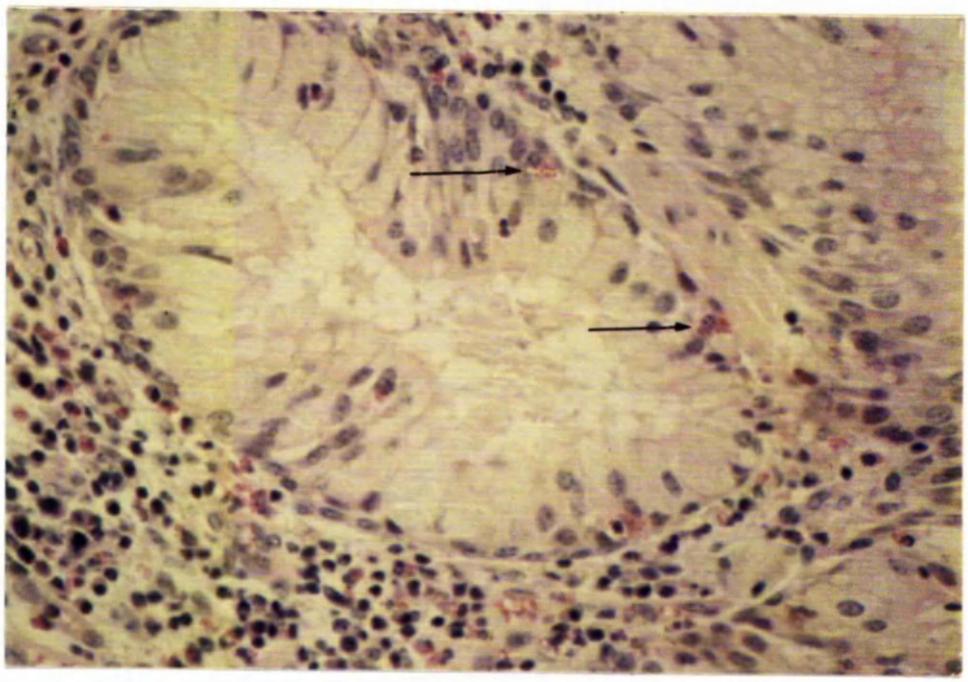


Fig. 18 : Globule leucocytes (⟶) in the epithelium of a bronchial gland in parasitised sheep.
H & E staningin, X 250.

Fig. 19 : Globule leucocytes (⟶) in the epithelium of a bronchial duct in parasitised sheep.
H & E staining, X 250.



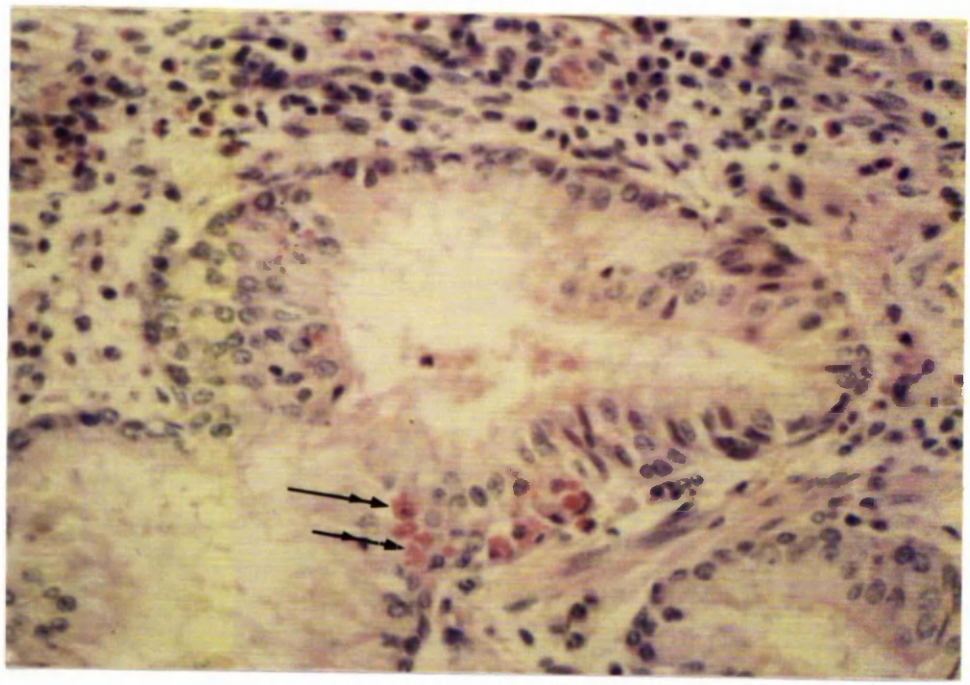
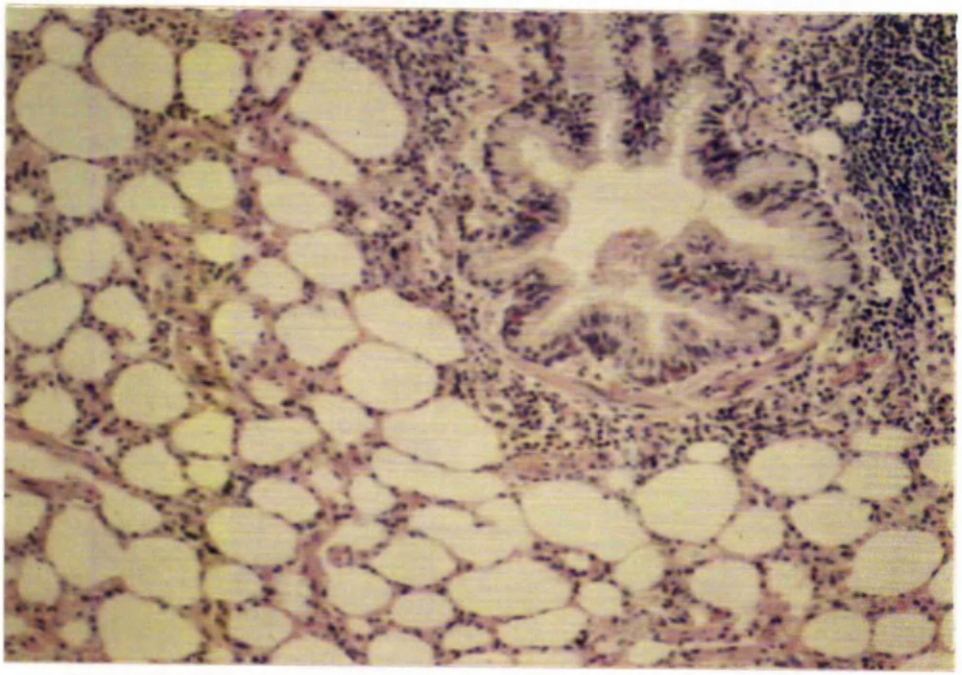
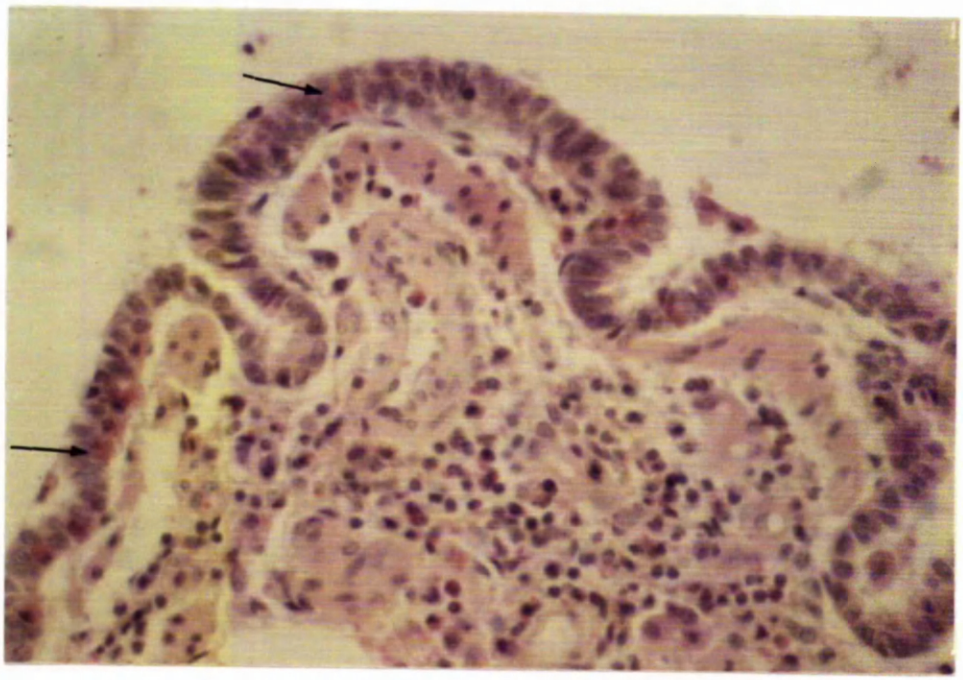


Fig. 20 : Showing globule leucocytes in the epithelial mucosa of the terminal bronchiole in parasitised sheep lungs.
H & E staining, X 100.

Fig. 21 : Respiratory bronchiole of sheep lungs, showing many globule leucocytes (——→) in their epithelia during the course of parasitic infection.
H & E staining, X 400.





CHAPTER THREE

MORPHOLOGICAL, HISTOCHEMICAL AND ULTRASTRUCTURAL STUDIES OF GLOBULE LEUCOCYTES IN SHEEP LUNGS

A. INTRODUCTION

Globule leucocytes were identified in the epithelial mucosa of the sheep respiratory tract during parasitic infection.

The detailed microscopical appearance of these cells in the bronchopulmonary system of sheep has not been described.

The constituents of the globules in the GLs in other organs of cattle and sheep were outlined by Jarrett et al. (1967) who reported that they contained an acidmucopolysaccharide which was metachromatic with toluidine blue at pH 4.0, but not at pH 0.3, stained blue with astra blue at pH 0.3, fluoresced orange with acridine orange and fluoresced green in the catecholamine as shown by the method of Falck, Hillorp, Thieme and Torp (1962). The globules also contained a highly basic protein associated with an acidmucopolysaccharide (Jarrett et al., 1967). This basic protein bound biebrich scarlet at pH 9 especially after mercuric chloride fixation (Jarrett et al., 1967).

Certain differences in GLs in animals have been found. The globules of the GLs in the cat (Takeuchi et al., 1968) and goat (Rahko, 1972) did not show metachromatic staining with toluidine blue at any pH used. This indicated that the globules in these species contained mucosubstances which differed from some other species. In the GLs of the biliary tract of mice, cattle (Rahko, 1971) and goats (Rahko, 1972) the histochemical properties of the globules were well investigated.

However the chemical nature of the globules in the GLs of the sheep respiratory tract has not been investigated. Thus the present chapter will describe some of the histochemical properties of the carbohydrate rich compounds and other constituents in the globules of the GLs in the sheep respiratory tract during parasitic infection. The light microscopic appearance of this cell will be reported to compare its morphological features with those described in other organs of sheep.

The ultrastructural appearance of the GLs has been described in the sheep digestive and biliary tract (Miller et al., 1967; Murray et al., 1968), the fowl (Toner, 1965; Holman, 1970), the rat (Kent, 1966; Whur and Johnston, 1967; Carr and Whur, 1968; Murray et al., 1968; Contin and Veilleux, 1972), the mouse (Carr, 1967; Rahko, 1971), cattle (Jarrett et al., 1967; Rahko, 1970a; 1971; 1973) and the cat (Takeuchi et al., 1968; Finn and Schwartz, 1972).

In each case, the conspicuous feature of these cells was the presence of large, intracytoplasmic, membrane bound globules whose internal structure varied considerably not only from species to species but frequently within a single cell. There are no reports on the ultrastructural features of GLs in the sheep respiratory tract. Thus the fine structure of the GLs will be investigated; in addition, the ultrastructure of other granular cells in the mucosa of the respiratory tract of sheep during the course of parasitic infections will be described.

B. MATERIALS AND METHODS

1. Histological Methods

a. Tissue samples

Blocks of tissue for histological examination were taken from the pulmonary tissue of all the animals in this study. The number and sites for sampling were described in section B of chapter two.

b. Fixation

Blocks of fresh tissue about 3 mm thick were fixed immediately in several fixatives as described in section B of chapter two. After fixation for 24 hours tissues were dehydrated and cleared in a double embedding series. Finally tissue blocks were embedded in paraffin wax, cut at 4-6μ in a rotary microtome and mounted on glass slides.

c. Staining

Paraffin sections were stained with haematoxylin and eosin. When particular morphological changes in the GLs were to be demonstrated more clearly, special stains were employed. These were toluidine blue, alcian blue and astra blue/safranin, biebrich scarlet, Martius scarlet blue and the carbol chromotrope method. Preparation of reagents and staining techniques are given in Appendix I.

2. Measurement of GLs and their Constituents

The lungs of six sheep (Case No. SM1, SM3, SM4, SM9, SM10 and SM30) which were shown to contain GLs were chosen for this study. Tissues were sampled from the standard sites, fixed in corrosive formol and stained with haematoxylin and eosin. Quantitative measurements were made on 10-89 cells in each lung. The cell size was assessed by two measurements : the maximum diameter of each cell and the dimension taken at the right angle to that. The diameter of the longest and smallest globule in each cell was assessed. All the measurements were done using a Leitz micrometer eyepiece calibrated on a standard slide using x 40 objective.

3. Histochemical Techniques

The histochemical techniques and reagents utilised in this study have been listed in Appendix I. Iron was studied by Pearls Prussian blue method and calcium by Von Kossa's silver nitrate staining method according to Carleton's Histological Technique (Drury and Wallington, 1967). Haemaglobin was identified with Lison's method (McManus and Mowry, 1960). Stein's iodine test for bile pigments was performed according to Carleton's Histological Technique (Drury and Wallington, 1967).

The histochemical staining methods for the characterization of carbohydrate-rich compounds were employed on material fixed with the fixatives mentioned above. The terminology proposed by Spicer, Leppi and Stoward (1965) for carbohydrate-rich compounds were employed in the present work.

1,2 hydroxy groups (vicinal glycols) were revealed by periodic acid-Schiff technique. This method was carried out with and without previous diastase digestion (d-PAS, PAS) according to McManus and Mowry (1960). Some sections were counterstained with haematoxylin. PAS-reactivity of the mucosubstances was also investigated after acetylation (acetylation-PAS) and its reversal deacetylation (deacetylation-PAS) of the tissue sections according to Culling (1963). The negative reaction following acetylation was regarded as evidence that vicinal glycols were responsible for the periodate-reactivity observed in sections stained with PAS without previous acetylation (Pearse, 1961).

Toluidine blue was used in a 0.5 per cent. aqueous solution at pH 4-4.5 (TB pH 4.0) and as 0.1 per cent aqueous solution at pH 0.3 (TB pH 0.3) according to Enerback (1966b).

Acid mucosubstances were investigated by alcian blue (8 G X E. Gurr Ltd., London, England) staining at pH 2.5 (AB pH 2.5). Sections were counterstained with 0.5 per cent. safranin O (Hopkin and Williams Ltd.) according to Enerback (1966b). Other sections were stained with

astra blue (G.T. Gurr Ltd., London, England) at pH 2.5 and counter-stained with the same method described above. The system recommended by Jones & Reid (1978) was used to differentiate acid (blue or blue-purple) and neutral (red) mucosubstances, the sequence of alcian blue at pH 2.5 followed by PAS staining was employed (AB pH 2.5 - PAS).

Sulphated mucosubstances were investigated with alcian blue staining at pH 1.0 (AB pH 1.0). To differentiate sulphated (blue or blue-purple) and nonsulphated (red) mucosubstances, the sequence of alcian blue at pH 1.0 followed by PAS staining was used (AB pH 1.0-PAS). In addition alcian blue staining was employed at pH 2.5 or pH 1.0 on sections treated with strong HCI-methylation (methylation-AB pH 2.5 or pH 1.0) followed by saponification (methylation-saponification - AB pH 2.5 or pH 1.0) according to Lillie (1954).

The acid mucosubstances were further investigated by staining selected sections with alcian blue at pH 1.0 or 2.5 after previous digestion with sialidase (sialidase - AB) or hyaluronidase (hyaluronidase - AB).

Basic protein was revealed by using biebrich scarlet stain (BS) at pH 8, 9 and 10 according to Spicer and Lillie (1961).

The fluorescence of acid mucosubstances in the cells were studied using acridine orange (AO), (Fisher Scientific Company, Fair Lawn, New Jersey) according to Jagatic and Weiskopf (1966).

Sections were examined on a Leitz Ortholux microscope filled with a fluorescence vertical illuminator. The light source was a mercury Osram HB0200 watt burner with a BG38 red suppression filter. A BG12 exciter filter was used in conjunction with position three of the filter turret which contained dichromic beam splitting mirrors and built in suppression filters TK510 and K515 respectively.

Photomicrographs were taken with a Leitz Orthomat camera system, mounted on a monocular tube with a X10 eyepiece.

4. Electron Microscopical Techniques

Tissue for electron microscopy was taken from all experimental animals. Those examined for the presence of parasitic lesions on their lung surfaces, were sampled from a main and a small bronchus in each case, for ultrastructural studies.

a. Fixation

Small blocks of tissue were excised, as soon as possible after death, from the mucous membrane of the bronchial tree and from the lung tissue. The specimens were placed in drops of chilled fixative on blocks of dental wax, chopped into pieces about 1 mm in thickness using a grease-free razor blade and then transferred to small vials containing chilled fixative at 4^{o}C. The blocks of tissue were fixed in paraformaldehyde/ glutaraldehyde for four to six hours at 4^{o}C, rinsed in 0.1 M cacodylate buffer containing 0.1 M sucrose, in which they were left overnight. They were then post-fixed in one per cent. osmium tetroxide for one hour. Some samples were fixed in one per cent. osmium tetroxide in Millonig's phosphate buffer for $1\frac{1}{2}$ hours. The details of these fixatives and rinsing buffer solutions are in Appendix I.

b. Embedding

Dehydration was through an ascending series of 70 per cent., 90 per cent. and absolute alcohol. The tissue blocks were then rinsed in propylene oxide before being embedded in araldite in gelatin capsules.

The embedding reagents used were :

Equal parts of araldite resin CY212 and araldite hardener HY964 mixed by stirring overnight and stored at 4^{o}C. Before use, 0.6 ml of accelerator HY960 (CIBA-Geigy) and 2.0 ml of di-n-butylphthalate (B. D. H.) were added to 58 ml of the resin/hardener mixture, and the whole stirred well for 30 minutes. Tissue embedded in this preparation was left to polymerise at 57^{o}C for 48 hours.

c. Sections and staining

Thick sections, approximately 1μ in thickness, were cut on an L.K.B. Mark III Ultratome using glass knives. Sections were mounted on glass slides and stained with borax buffered methylene blue/Azure II (Trump, Smuckler and Benditt, 1961). These sections were then examined to locate specific areas of tracheal or bronchial epithelium and orientate specimens for electron microscopy.

Ultrathin sections were then cut on the ultramicrotome, mounted on copper mesh grids and double stained with 20 per cent. uranyl acetate in methanol (Watson, 1958), followed by lead citrate (Reynolds, 1963). S tained sections were examined with an AEI 6B electron microscope. Preparations and description of stains for thick and ultrathin sections are given in Appendix I.

C. LIGHT MICROSCOPICAL FINDINGS

The morphology of the GLs in the sheep respiratory tract was related to the position of these cells within the tissue. Globule leucocytes in the lamina propria near or on the basement membrane were considered to be immature cells which were in a lymphocyte-like stage in appearance and outline. The immature GLs contained fewer and smaller globules than the mature GLs. Immature cells varied in size from (3.20μ x 4.80μ) to(8.0μ x 8.8μ) in the 300 cells measured.

The intraepithelial GLs were considered to be mature cells and were usually round or oval, although in some instances they were elongated or triangular in appearance because of their position between the adjacent epithelial cells (Fig.22).

Mature GLs varied in size from (4.80μ x 5.60μ) to (16.40μ x 26.80μ) in the 300 cells measured as shown in Table (6) which illustrates the maximum diameter of the 300 GLs. The size of 600 globules measured in the lungs of (6) sheep are contained in Tables (7 and 8).

The cell nucleus was eccentrically placed, while the globules were grouped in a mass beneath or around one pole of the nucleus, more or less filling the corresponding pole of the cell (Fig. 23). While the outline of the cell was never distorted by the globules, a cell with much empty cytoplasm was never found.

The globules generally appeared spherical or very nearly so (Fig. 23) and quite separated from each other, sometimes they appeared to have coalesced (Fig.24). These appearances were related to the method of fixation and staining.

In mature GLs, the characteristic globules varied in number, and were predominantly homogenous, acidophilic, refractile spherules with H & E stain.

In well fixed material the globules varied in size. The largest globules averaged (3.038± 0.108μ) in diameter, ranging from (1.12μ) to (9.00μ), while the smallest globules averaged (1.416± 0.082μ), ranging from (0.24μ) to (6.40μ). Minute particles were also seen when the granular nature of the material could be only just discerned.

The number of globules detected in a single cell usually ranged from (6) to (22), although as many as (32) globules were counted in one cell. The size of the globules was not necessarily related to the size of the cell, nor was it related to the number of globules within the cell. In most preparations the globules, with a few exceptions, appeared as homogeneous bodies. On occasion, a deeply staining rim could be detected around the globules. However, it may be appropriate to record that when the globules were stained with alcian blue, this peripheral rim was surprisingly prominent. Moreover, this brilliant blue outline showed minute irregularities or breaks suggesting the presence of a border which was not quite entire. These two phenomena were observed in isolated globules and in globules within the intact cell, and in mast sizes of globules except the smallest which appeared as mere points of blue stain.

These findings may indicate the presence of an outer shell differing in composition from the material constituting the interior of the globule.

The nuclei were small, round to slightly oval in outline. Usually the nuclei were slightly indented toward the centre of the cell due to the presence of the globules. The nuclei of the immature GLs closely resembled small lymphocyte nuclei, while in mature GLs there was a range in the appearance of the nuclei. They usually occupied an eccentric position within the cell (Fig.23). Some cells had relatively deeply staining round nuclei with masses of chromatin distributed in such a way as to recall the nuclear pattern typical of a plasma cell, or clockwise appearance. In other instances the nucleus appeared as round, oval to kidney shaped. Irregularly shaped nuclei were found in

various forms, but they were not common. However, nuclei which showed curved, longitudinal pyknotic and karyorrhexis forms were encountered in some instances.

Mitotic figures were not observed in these cells. Pyknotic nuclei and karyorrhexis were encountered, but not frequently.

D. HISTOCHEMICAL OBSERVATIONS

1. Fixation

The fixatives used to study the staining properties of GLs and mast cells and the reactions of these cells to the stains used are summarised in Table (9).

a. Corrosive sublimate

Globule leucocytes and mast cells were well stained with most acid dyes employed after corrosive sublimate fixation, although they did not stain metachromatically with toluidine blue nor as brightly with alcian/astra blue as tissue fixed in Carnoy's fluid.

Blocks of tissues were fixed for 12-24 hours, but contrary to the widely held view, much longer treatment does not result in a consistency of tissue that makes sectioning difficult, and improved staining.

b. Carnoy's fluid

Carnoy's fluid when compared with corrosive sublimate, Bouin's fluid and buffered neutral formalin was not satisfactory because the cytological detail was less clearly defined. Globule leucocyte globules and mast cell granules were poorly detected with acidic dyes after fixation with Carnoy's fluid.

The metachromasia and the alcianophilia of the mucosubstances in these cells were more brightly stained after Carnoy than other fixatives.

c. Bouin's fluid

Bouin's fluid gives brilliant staining with cytoplasmic stains. The metachromasia and alcianophilia in the globules of GLs and granules of mast cells were more brightly stained in Bouin than in corrosive sublimate, but were not as good as in Carnoy's fluid.

d. Buffered neutral formalin (ten per cent).

Formalin fixed blocks stained metachromatically well with toluidine blue, but the alcianophilia in the GLs globules and mast cells

granules were partially blocked.

2. Histochemistry of Globule Leucocytes and Mast Cells

The histochemical studies were carried out to investigate the chemical properties of the globules of GLs and the granules of mast cells in sheep bronchopulmonary system since the (2) cells were thought to be related. The mast cells were divided into subepithelial and submucosal populations since there are differences in their staining reactions.

The results of the present study are illustrated in Table (10).

The staining reactions of the GLs and mast cells were sometimes inconsistent resulting in the presence of some uncoloured globules or granules in many cells after certain staining techniques. Hence the result of some staining methods were classified according to whether a positive reaction was evident in some (+), many (++) or most (+++) globules or granules of the cells in the examined sections.

Globule leucocytes were distinctly stained with the haematoxylin and eosin method due to the acidophilic properties of the intracytoplasmic globules particularly after fixation with corrosive sublimate (Fig.25). The subepithelial mast cells were sometimes demonstrated with haematoxylin and eosin in which case they had rounded contours and their acidophilic granules were large but not numerous. In contrast, the submucosal mast cells were spindle-shaped and possessed numerous small intracytoplasmic granules which were revealed only by certain histochemical staining methods.

The methods for staining glycogen, haemoglobin, bile pigment, iron and calcium produced no reaction in the GLs and mast cells.

a. Mucosubstances

The staining techniques for the study and classification of the mucosubstances were carried out on Carnoy's fluid fixed blocks since Carnoy gave the best result compared with other fixatives (Table 9).

The mucosubstances in the globules of GLs and the granules of mast cells were not periodate-reactive after ordinary PAS nor after the removal of acetyl and acidic radicals (Table 10). The location of neutral mucosubstances and their differentiation from the acidmucosubstances were identified by the use of the combined alcian blue-periodic acid-Schiff (AB pH 2.5 - PAS) technique. Neutral mucosubstances did not comprise any proportion of the mucosubstances staining in the GLs and mast cells. All the globules of GLs and granules of mast cells were strongly alcianophilic (Fig. 26 and 27). This appearance could represent the presence of only acid mucosubstances in the globules of GLs and the granules of mast cells.

The acidmucosubstances were investigated with alcian blue and astra blue/safranin technique. The globules of GLs and granules of mast cells stained blue in colour (Fig. 28). The alcianophilia in both CLs and mast cells stained brightly in Carnoy fixed blocks, while in formalin fixed material it was partially blocked in both cell types.

With toluidine blue pH 0.3 the acid mucosubstances in the GLs and mast cells displayed a violet metachromasia (Fig. 29). This metachromasia was deep blue violet in colour when toluidine blue at pH 4.0 was used (Fig. 30).

Acridine orange stained unsatisfactorily when used with faintly corrosive sublimate fixed tissue. The GLs and mast cells stained faintly and could not be differentiated from the goblet cells and other mucous cells. When fixed in Carnoy the acidmucosubstances in the globules of GLs and granules of mast cells fluoresced red to orange with acridine orange (Fig. 31).

Alcian blue pH 1.0 staining followed by the periodic acid-Schiff technique differentiated blue stained sulphated mucosubstances from sialomucins and neutral mucins which coloured red. The result of this staining reaction can be seen in (Fig. 32). In the GLs and mast cells of the sheep respiratory tracts there were many blue staining sulphomucin containing globules and granules.

Alcian blue pH 1.0 and high iron diamine (HID) staining for sulphate groups indicate a similar regular distribution of sulphomucins in both GL globules and mast cell granules (Fig. 33-38).

Methylation-alcian blue pH 1.0 and 2.5 staining revealed blue substances in these cells, while methylation-saponification-alcian blue pH 1.0 and 2.5 methods revealed no blue staining reaction in the GLs and mast cells. Moreover the latter method resulted in an overall "wash out" appearance, representing a weakening of the alcian blue-reactivity. However the alcianophilic substances of the GLs and mast cells were sialidase and hyaluronidase resistant.

The present histochemical studies indicated that the mucosubstances of GL globules and mast cell granules in the sheep respiratory tracts were sulphomucins.

b. Basic protein

Globule leucocyte globules and mast cell granules displayed a red staining reaction when stained with biebrich scarlet (BS) at pH 8, 9 and 10 (Fig. 39). This was due to the basic protein at the sites of carbohydrate rich compounds in the GLs and mast cells in material fixed with corrosive sublimate or Bouin's solution.

When tissues were fixed in Carnoy the acidophilia of the basic protein in these cells were less intense and faintly stained. However in most fixatives employed the eosinophil leucocytes in the same sections stained with biebrich scarlet displayed red-staining reactions in their granules, which could not be differentiated from the mast cell granules in the subepithelial and submucosal layers. Thus the exact identification of mast cells in these sections was impossible. But the sequence of alcian blue pH 2.5- biebrich scarlet pH 9.5 (AB pH 2.5-BS pH 9.5) produced only alcianophilia in mast cell granules and not in the eosinophil leucocyte granules. The same sequence AB pH 2.5 - BS pH 9.5 stained the cortex of the globules blue but the core red in colour.

E. ELECTRON MICROSCOPICAL FINDINGS

Tissue from the ovine bronchopulmonary system was examined with an electron microscope to compare and contrast the fine structural morphology of GL, mast cell, plasma cell and eosinophil leucocyte.

1. Globule Leucocytes

At the ultrastructural level, GLs were easily recognised by their characteristic large, round cytoplasmic inclusions (Fig. 40). Their position in the mucosa of the respiratory tract corresponded to that observed in paraffin sections. Most of the GLs were found in the basal half of the epithelium near the basement membrane, although a few GLs were present in the lamina propria.

Globule leucocytes sandwiched between the epithelial cells were often elongated, although they retained some regularity in their outline. Globule leucocytes in this situation were sometimes surrounded and enveloped by the cytoplasm of the columnar cells. Other GLs were extremely irregular, with long pseudopodia-like processes pushing between adjacent columnar cells and indenting them, at times invaginating and penetrating deeply into their cytoplasm. Thus in any one section there might be no apparent continuity between a cell and one of its pseudopodia, which could be presented as an island of leucocyte cytoplasm within the substance of an epithelial cell. There were no desmosomes between the GLs and the adjacent columnar cells. Other GLs appeared free in the intercellular spaces.

The nucleus of the GL was somewhat variable in appearance, ranging from large and approximately spherical to compressed and irregular in outline. It was located at one side of the cell and was frequently indented by the cytoplasmic globules (Fig. 40). It consisted of condensed chromatin distributed uniformly throughout the nucleoplasm and sometimes had a prominent nucleolus. Mitotic figures were not observed. The nuclear membrane in the GL was well demarcated.

The globules, which gave this cell its name, were the main feature of the cytoplasm. These globules varied in number and appearance in different leucocytes examined. They ranged in size from 0.5 to 4.5μ in diameter and were evenly distributed throughout the cytoplasm with the exception of the Golgi region in which none were present (Fig. 41). The typical globule was spherical, with a regular outline and usually surrounded by a single trilaminar bounding membrane which occasionally appeared to be continuous with a cisternum of the rough surfaced endoplasmic reticulum. Structural variations in the globules did occur and the following types were recognized :

(a) Globules with an electron dense homogenous matrix surrounded by a smooth surfaced membrane (Fig. 42 and 43).

(b) Globules with a dense central core surrounded by a light rim. In some instances the light area was located on one side of the dense matrix giving the globules a ring shape and leaving a halo between the surrounding membrane and the dense central material (Fig. 44, 45 and 46).

(c) Globules with a reticulated matrix (Fig. 42). Their surrounding membrane was still intact and well defined.

(d) Globules with a dense matrix replaced in part by fine granular substances of various coarseness (Fig. 42 and 43).

Some globules had either deformed or undefined surrounding membranes (Fig. 42, 43 and 44). Other globules seemed to have lost much of their fine granular contents leaving vacuoles in their substances. Less frequently globules consisting of loosely packed fine granules, small rods, large particles and vesicular bodies were seen (Fig. 44, 45 and 46). Discrete rounded vacuoles were occasionally present within a globule. A few of these globules were mottled in appearance with irregular dense and loose patches of small rods. From time to time vacuoles approximately the size of the globules were seen and appeared to be empty except for small clumps of material resembling that found in globules. Although vacuoles contained coarse myelin figures were seen. Moreover

some globules had fine granulation suggesting that the globules were undergoing degenerative changes (Fig. 43). In this form of globule no unit membranes were detected and the material of the globules could not be differentiated from the cytoplasmic matrix of the cell. Globules with paracrystalline structures were not seen in any cell examined.

The cytoplasm of the GL was pale with a few distinctive structural features. A few short lengths of rough surfaced endoplasmic reticulum were found throughout the cytoplasm.

The Golgi complex was relatively large with no evidence of globule formation within it (Fig. 41). It was situated near the nucleus at the site of indentation. The Golgi complex consisted of a cluster of vacuoles, vesicles and flattened sacs (Fig. 41).

The centriole was also located near the nucleus adjacent to the Golgi complex. It appeared as short rod-like cylinders. Each cylinder consisted of many fine tubules (Fig. 41).

The mitochondria were short, thick and relatively few in number. Their cristae were parallel and well defined and they had a pale matrix.

Vacuole-containing bodies were plentiful in GLs, the vacuoles being peripheral in position. In many of the GLs pinocytotic vesciles were seen adjacent to the plasma membrane.

2. Mast Cells

These cells were found at various levels in the subepithelial, submucosal, interalveolar septa and subpleural layers of the sheep respiratory system in parasite free conditions. In the lamina propria the mast cells were commonly situated close to blood vessels and along the basement membrane of the epithelium. During the course of parasitic infection the numbers of mast cells increased markedly in the sub-epithelial layer of the sheep respiratory tract.

Mast cells at the basement membrane or in the lamina propria were oval, polygonal, spindle-shaped or irregular in outline (Fig. 50). They were characterised by the presence of intracytoplasmic round or oval granules. Most of these granules had an electron-dense matrix and were closely enveloped by smooth surfaced unit membranes.

In parasite-free sheep the mast cell granule matrices were usually homogeneous without any easily recognized substructures (Fig.51). While in parasitized sheep there was a great variation in size, structure and electron density of the granules (Fig. 52). which was similar to that found for the globules of the GLs (Fig. 40). The Golgi complex and mitochondria were more prominent and well developed in the mast cells of parasitized than in non-parasitized sheep. The number of granules varied from cell to cell, and when they were densely packed, the cytoplasm was largely filled by the mass of granules. In addition many small membrane-bound vacuoles were scattered throughout the cytoplasm of the mast cell (Fig. 52).

Small cytoplasmic projections were found at the border of the cells. These were shorter than those in GLs. The mast cell cytoplasm was finely granular and contained variable numbers of free ribosomes and rough-surfaced endoplasmic reticulum, although the latter was sparse in cells densely packed with granules.

In addition to typical mast cells other cells, with cytoplasmic granules similar to mast cell granules, were found. These cells were circular to quadrangular in outline (Fig. 53). They contained deeply invaginated multilobed nuclei. Typically their nuclei contained 2-3 lobes and were eccentrically placed. The lobes were often, distinctly separated and their chromatin was coarse. No nucleoli were evident. The cytoplasmic granules were the dominant feature of these cells. They were variable in size and shape but generally were large, rounded and membrane bound. These granules had the same structural variation as the granules of the mast cells and the GLs. These cells had a well developed Golgi complex which was identical to that in the mast

cell and GL. Since these cells had cytoplasmic constituents like mast cells, but irregular or lobed nuclei, it was considered that they might be related to basophil leucocytes. Examination of many sheep lungs during parasitic infection indicated that; sheep with many mast cells had few basophil-like leucocytes and those with few mast cells had many basophil-like leucocytes.

3. Plasma Cells

The plasma cells (Fig. 54) were also studied to provide ultra-structural information to enable differentiation to be made between them and the GLs in sheep respiratory tract. These cells present a characteristic picture with their highly developed, sometimes distended rough surfaced endoplasmic reticulum (Fig. 55). The cisternae of the system were filled with a fine granular material. The Golgi complex was large and prominent. Small non-specific intracytoplasmic granules were sometimes present. Both the indented nucleus and the various forms of the intracytoplasmic granules seen in GLs were not detected in the plasma cells.

4. Eosinophil Leucocytes

The eosinophil leucocytes which were abundant in the epithelium and in the lamina propria during the course of parasitic infections, were easily distinguished from GLs. The nucleus was usually reniform or had two lobes connected by a narrow isthmus (Fig.56). The cytoplasm contained spherical granules which were surrounded by smooth unit membranes (Fig. 57). The dense band seen in the numerous granules was the chief distinguishing feature from the globules of the GLs (Fig. 57).

F. DISCUSSION

On the basis of the present light microscopic studies it was established that the frequent occurrence of GLs in the ovine bronchopulmonary system was associated with lungworm infestation. Morphologically, the GLs in this situation were similar to those described in the digestive tract (Kent, 1952; Whur, 1966a; Dobson, 1966b), and biliary tract of sheep (Miller et al. 1967; Murray et al., 1968). Globule leucocytes were large cells and, owing to their striking morphology and frequent occurrence, were readily distinguishable from other cells in the respiratory tract epithelium. The cytoplasm was packed with numerous globules of fairly uniform appearance which often obscured the nucleus. These globules were acidophilic, refractile and rounded giving the cell a characteristic appearance. Therefore, the ultrastructural and light microscopic studies indicated that the GLs in the ovine bronchopulmonary system had a uniform appearance and were similar to the GLs described in the biliary tracts and digestive tracts described by other workers (Miller et al. 1967; Murray et al., 1968, Rahko, 1970a).

In the larger GLs of the bronchopulmonary system the globules frequently appeared as complex structures of varying composition. This feature was demonstrated in many cells stained with alcian or astra blue and toluidine blue. Some of the globules appeared to contain numbers of small intensely blue-positive globules set in a more lightly stained matrix which, in turn, was bounded by a brilliantly stained peripheral rim. Within the same cell there were globules with a deeply blue positive core and a pale periphery, while others appeared as homogeneous blue positive spheres which were deeply stained. There was some uncertainty in the literature as to whether or not the heterogeneous appearance of some of the globules was a fixation artefact. In the alimentary tract in sheep (Kent, 1952), rat (Murray et al., 1968) and the biliary tract of cattle and mice (Rahko, 1971), GLs stained with toluidine blue were found in which some of the globules were markedly metachromatic while others in the same cell remained unstained or partially stained. This phenomenon has been observed in bronchopulmonary GLs too. Kent (1952) recorded the presence of GLs

in the digestive tracts of sheep with irregularly vacuolated cytoplasm which stained poorly or was occasionally acidophilic. However, these features were not observed in the GLs of the bronchopulmonary system of sheep.

The present histochemical studies indicated that corrosive sublimate and to some extent Bouin's fluid were the best fixatives to use on ovine tissue to preserve both the globules of the GLs and the detailed cytological features of lung tissue. Globule leucocytes and mast cells did not stain as metachromatically with toluidine blue or as brightly with alcian or astra blue as tissue fixed in Carnoy's fluid.

Using a variety of histochemical tests, GLs and mast cells in both sites, the subepithelial and submucosal layer, of ovine bronchopulmonary system were shown to contain both a highly sulphated mucopolysaccharide and a basic protein as shown in other organs of sheep investigated (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968; Rahko, 1970a). This agreed well with the histochemical studies on GLs and mast cells in rat (Jarrett et al., 1697), mice (Rahko, 1971) and cattle (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968; Rahko, 1970 a & b; 1971). However, similar histochemical studies in goat biliary tracts indicated that the mucosubstances of the globules of GLs consisted of both neutral mucins and carboxymucins, whereas those in the mast cell granules were sulphomucins (Rahko, 1972).

The occurrence of 2 types of mast cells in the bronchopulmonary system of sheep could not be demonstrated by using alcian or astra blue/safranin pH 0.3, although there were differences between the submucosal and subepithelial mast cells using toluidine blue pH 0.3. The occurrence of 2 different mast cell populations in the intestinal wall of certain animals and in the bile duct wall of sheep has been recorded in sections stained with alcian or astra blue/safranin and toluidine blue pH 0.3 (Enerback, 1966 a & d; Jarrett et al., 1967). Globule leucocytes in the bronchopulmonary system of sheep were cytochemically very similar to the subepithelial mast cells.

On the basis of alcian blue/safranin staining mast cells in the rat were divided into 3 groups (Csaba and Kovacs, 1975). These were the lung

and the heart muscle which contained only alcian blue positive cells; the thyroid, lymph nodes, subcutaneous connective tissue, mesentery and the peripheral nerves contained only safranin positive cells. The peritoneal fluid and the thymus contained both alcian blue and safranin positive mast cells. In addition, the differences between the subepithelial and submucosal mast cells in the rat were revealed by the histochemical methods (Combs, Lagunoff and Benditt, 1965). According to these authors the submucosal mast cells in rat were considered as mature cells. These cells contained vasoactive amines i.e. histamine, heparin and 5-hydroxytryptamine (Benditt and Lagunoff, 1964), as well as a sulphated mucopolysaccharide associated with a highly basic protein (Spicer, 1963; Benditt and Lagunoff, 1964). The submucosal mast cells were stained more easily by safranin than by alcian blue; they bound toluidine blue and stained metachromatically at pH 0.3. The granules of the subepithelial mast cells in the rat contained an acid mucopolysaccharide probably with a lower degree of sulphation than heparin (Enerback, 1966b; Miller and Walshaw, 1972). Only a minute quantity of 5-hydroxytryptamine (Enerback, 1966d and 1970, Miller and Walshaw, 1972; Veilleux and Cantin, 1976) and a relatively small amount of histamine were detected (Miller and Walshaw, 1972; Veilleux and Cantin, 1976). On the other hand, by decarboxylation of administered precursor-amino acids, it was found that the subepithelial mast cells were able to synthesise and store considerable amounts of monoamines (Enerback, 1966b and 1970). The lack of sensitivity of these mast cells towards compound 48/80 has been shown to be another characteristic difference between the subepithelial mast cells and those from other sources in the rat (Enerback, 1966c; 1974; Veilleux, 1973).

The question now was why different mast cells bound various amounts of alcian blue to their granules. Tas (1977) interpreted this phenomenon in that the protein part of mast cell granules could possibly be regarded as a barrier for alcian blue molecules. The chemical characteristics and the the amount of granule protein has been shown to vary greatly between mast cells from different species (Chiu and Lagunoff, 1972; Hodinka and Csaba, 1974), and also between mast cells from the same source or even

within the granule population of one cell (Combs et al., 1965; Combs, 1966). These differences may cause dissimilarities in the rate of penetration of alcian blue into the granules. The chemical configuration of the granule-protein or of the protein-heparin complex as a whole could also be of great importance in the functioning of a barrier. Besides, it was possible that fixation procedures could change the protein configuration and with it the rate of penetration of dye into the granules (Tas, 1977). Fixative effects have been reported previously (Spicer, 1963; Burton, 1964).

The presence of a barrier for the penetration of alcian blue in mast cell granules has been demonstrated (Goldstein and Horobin, 1974; Tas, 1977). Among the possible candidates of components in the mast cell granule which could exert a barrier function, the basic protein should be mentioned first (Tas, 1977). Together with heparin, basic protein of the chymotrypsin or trypsin-like type in vertebrate mast cells was the main component of mast cell granules (Chiu and Lagunoff, 1972). In the rat mast cell the heparin to protein ratio was from 1:1 to 1:2.5 w/w (Tas and Geenen, 1975). Another candidate was histamine, being present to a much lesser extent in vertebrate mast cells; however, the heparin to histamine ratio was from 2:1 to 10:1 w/w. (Tas and Geenen, 1975). Both histamine and heparin were linked electrostatically to the basic protein (Uvnas, Aborg and Bergendorff, 1970). The result of the O-phthaldialdehyde staining showed that the presence of histamine was drastically diminished after all types of fixation (Tas, 1977). Consequently the possibility that histamine might influence alcian blue staining could be excluded.

The present findings on the histochemistry of the mucosubstances within the GLs and mast cells in bronchopulmonary system of sheep were generally in agreement with those previously outlined in the sheep digestive tracts and in certain other species (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968). For a satisfactory analysis of the mucosubstances at a given site it was important to follow the techniques previously employed in the identification of mucosubstances in a variety of animal and human tissues (Spicer, 1960, Reid, 1965; Lamb and Reid; 1969; 1970; Spicer, Chakrin, Wardell and Kendrick, 1971; Fletcher, Jones and Reid, 1976 and

1978). Most of the histochemical studies which were employed previously were based on the proposed classification of the connective and epithelial tissue mucosubstances which were divided into 3 categories, neutral, sulphated acidic and nonsulphated acidic (Spicer, Leppi and Stoward, 1965). Neutral mucosubstances included neutral glycoproteins and fucomucins, all of these being periodate reactive. The remainder of the mucosubstances were acidic and were classified as either sulphated or nonsulphated. Sulphated mucosubstances included substances such as chondroitin sulphate, a major constituent of cartilage, and could be visualised by appropriate histochemical stains (basic dyes at controlled pH's, colloidal iron stain and aldehyde fuchsin). The non-sulphated mucosubstances were divided into hexuronic acid mucopolysaccharides such as hyaluronic acid which did not seem to occur in epithelial tissue (Spicer et al., 1971) and sialic acid rich mucopolysaccharides. The 2 types of acidic mucosubstances found in epithelial tissue were therefore known as sulphomucins and sialomucins. Sialomucins could then be further classified dependent upon their susceptibility to digestion by the enzyme sialidase which was derived from Vibrio cholerae. The sialomucins were either highly susceptible to digestion, slowly digested or completely resistant to digestion. All sialic acid groups were released by acid hydrolysis which was used to differentiate them (Lamb and Reid, 1969).

Since the publication of these findings, investigations into the type of glycoprotein present at a site have been much simplified. A sequence of 3 staining combinations will identify 4 types of glycoprotein-neutral, sulphated, sialidase sensitive and sialidase resistant. The stains used were alcian blue pH 2.6-PAS and alcian blue pH 1.0 - PAS. On the same basis of staining combinations Jones and Reid (1978) identified 4 major groups of intracellular glycoproteins. These were (1) neutral glycoprotein, (2) sialylated glycoprotein in which the sialic acid was sensitive to the enzyme sialidase, (3) sialylated glycoprotein in which the sialic acid was resistant to the enzyme, (4) sulphated glycoprotein.

Our present investigation revealed that the cytochemistry of the subepithelial mast cells was somewhat different from that of the submucosal mast cells both in the control and experimental animals. As in the gastro-intestinal tract (Murray et al., 1968) and biliary tract of sheep (Rahko, 1970a) the GLs of the bronchopulmonary system of sheep were cytochemically very similar to the subepithelial mast cells. The granules of the GL, contained a sulphated glycoprotein which was less reactive than that of the submucosal mast cell to toluidine blue metachromasia.

The application of PAS stain to identify the neutral glycoprotein (group 1) in the proposed classification of Spicer et al., (1965) revealed that the glycoprotein in the granules of GLs and mast cells of the sheep broncho-pulmonary system were not periodate reactive after PAS. While the GLs of the alimentary tract of sheep (Dobson, 1966b) and biliary tract of goat (Rahko, 1972) were strongly periodate reactive. These periodate-reactive substances in the GLs of the goat were diastase-resistant which did not increase after the removal of acetyl radicals and became weakened after the removal of the acidic or the sulphate radicals (Rahko, 1972).

The acid glycoproteins of group 2 of the proposed classification of Spicer et al., (1965) were identified by alcian blue pH 2.6/PAS (Mowry and Winkler, 1956). In goat (Rahko, 1972), cattle and mice (Rahko, 1971) most of the globules of GLs consisted of both neutral and acid glycoproteins. However in the sheep respiratory tract the granules of both the GLs and mast cells contained largely acid glycoproteins.

The application of alcian blue at pH 1.0 and pH 2.5, alcian blue pH 1.0/PAS (Spicer and Henson, 1967), high-iron diamine (Spicer and Henson, 1967) and high iron diamine-alcian blue (Spicer, 1965), indicated that the glycoproteins in the granules of both the GL and the mast cell in the sheep respiratory tract were sulphomucins. This agreed well with the work of Miller et al. (1967), Murray et al. (1968) and Rahko (1970a) on the GLs and mast cells in biliary tracts of sheep. The alcianophilic substances of these cells were resistant to hyaluronidase and sialidase digestion. These phenomena indicated that the observed alcianophilia was

not attributable to hyaluronic acid, chondroitin-4 or -6-sulphates or sialic acid (Pearse, 1961; Gibbons, 1963).

The granules of both the GL and mast cell contained highly basic proteins, showing affinity to biebrich scarlet at high alkalinity. However, this method did not produce red-staining in the mast cell granules in goat. According to Selye (1965) basic proteins were present in the granules, too, and the absence of a staining reaction was probably due to the fact that they were blocked by formalin (Spicer, Staley and Wetzel, 1967). Staining of formalin fixed material had also failed to reveal their presence in cells in cattle, sheep and mice (Rahko, 1970 a, b, 1971).

The present observation showed that the application of alcian blue/safranin pH 0.3 did not produce staining differences between the subepithelial and submucosal mast cells as in the gastrointestinal tract of sheep. This agreed well with Csaba and Kovacs (1975) in that the mast cells in the lung of rat contained only alcian blue positive granules when stained with alcian blue/safranin. The same results were obtained with acridine orange, in that no staining differences were seen between the subepithelial and submucosal mast cells.

The ultrastructural appearance of GLs and mast cells in the bronchopulmonary system did not differ from the description in previous electron microscopic studies on the GLs and mast cells of the gastrointestinal and biliary tracts of sheep (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968). The absence of intercellular junctional complexes alone excluded the possibility that the GL was an epithelial cell. The presence of pseudopodia and the ability to separate the desmosome between epithelial cells attested to their motility. The migration of GLs toward the luminal surface raised the interesting possibility of a discharge of the whole cell. or of its globules into the lumen as previously suggested for the GL of the intestinal tract in rat (Whur and Johnson, 1967). Furthermore the gradual changes in the variation of granular structure of submucosal through subepithelial mast cells to the final stage of the intraepithelial GLs emphasized the strong ultrastructural evidence that GLs were derived from

subepithelial mast cells. Moreover the ultrastructural variation found in the granules or globules of any mast cell or GL did not reflect a morphological basis for typing and classification of the granules. Previously it was shown that in cattle and sheep, histochemical data pointed to a loss of amine and acid glycoprotein from the granules (Miller et al., 1967; Murray et al., 1968), the ultrastructural changes in the granules possibly reflecting this loss. Thus globule type 1 could be considered as an intact globule which after releasing its amine due to antigenic stimulation would transform into type 2, 3 and 4 globules or could even result in an intra-globular vacuole. These phenomena in other cells could occur without serial transformation in the structure of the globules, and when this feature was observed it did not reveal any ultrastructural variation in the GL from one organ to another or between different species. These observations also illustrated a light microscopic feature of variation in the composition of the globules when stained with alcian blue or toluidine blue as described by Kellas (1961).

Globule leucocytes in the epithelial mucosa of the bronchopulmonary system of sheep were found mostly in the intraepithelial position while the lamina propria contained many mast cells or basophil-like cells. The latter cells showed typical granule variation similar to that seen in mast cells and GLs. Their lobulated and highly indented nuclei were in complete agreement with the basophil-like cell of rat intestine described by Miller (1969).

Whur and Johnston (1967) as well as other investigators (Dobson, 1966a, Carr, 1967) have proposed the plasma cell as a possible precursor of the GL in the rat and mouse, the Russell bodies evolving into globules. However, Kent (1966) had already pointed out the sharp difference between Russell bodies and GL globules. Russell bodies were limited by rough endoplasmic reticulum, while the globules of the GL were enclosed by smooth membranes. This observation was in complete agreement with the differences observed between the plasma cell and the GL in the sheep respiratory tract. On the other hand Casley-Smith (1968) suggested that the eosinophil leucocyte might be such a precursor. In the sheep respiratory

tract, there was marked differences between the GL globules and eosinophil leucocyte granules. Although the granules in both cells had a single membrane, the granules of the latter cell had a dense band which was its chief distinguishing feature from other cells. More recently Holman (1970) considered that the GL originated from lymphocytes, while Finn and Schwartz (1972) suggested that it could be a specific cell type, as it can become neoplastic.

Murray et al. (1968) have studied the relationship between GL and mast cells in the rat, cow and sheep. On the basis of histochemical reactions and their appearance ultrastructurally they claimed that these 2 types of cells were similar and often identical, and concluded that in these species GLs were derived from mast cells. The observations described here on GLs and mast cells in the bronchopulmonary system of sheep would also suggest that the 2 cells were related and distinct from plasma cells and eosinophil leucocytes.

CELL SIZE (μ) / SHEEP NO.		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
SM 1	No. of Cells	-	3	2	3	3	3	3	1	2	2	4	-	1	-	-	-	2	-	-	-	-	-	-	-
SM 3		-	-	4	5	6	6	1	4	6	-	6	1	4	1	3	1	3	-	-	-	2	-	1	1
SM 4		-	-	7	5	7	6	15	5	14	-	13	1	11	1	-	-	-	-	-	-	-	-	-	-
SM 9		-	-	3	3	6	4	19	7	16	2	3	1	1	3	-	-	-	-	-	-	-	-	-	-
SM10		-	2	11	6	8	10	9	2	8	1	-	-	1	1	-	-	-	-	-	-	-	-	-	-
SM30		-	-	1	2	1	3	1	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-

Table 6 : Distribution of the size of intraepithelial bronchopulmonary GLs in six sheep.

Size of Smallest Globules Maximum diameter (μ)	Number of Globules measured
<1.0	98
1.0	96
2.0	85
3.0	17
4.0	2
5.0	-
6.0	2
7.0	-
8.0	-
9.0	-
10.0	-

Table 7 : Maximum diameter (μ) for the smallest globules in the GLs of sheep respiratory tracts.

Size of Largest Globules Maximum diameter (μ)	Number of Globules measured
<1.0	7
1.0	9
2.0	95
3.0	102
4.0	62
5.0	14
6.0	6
7.0	3
8.0	1
9.0	1
10.0	-

Table 8 : Maximum diameter (μ) for the largest globules in the GLs of sheep respiratory tracts.

Staining Methods	CS		CAR		BO		BNF	
	GL	MC	GL	MC	GL	MC	GL	MC
Haematoxylin and eosin	R+++	R±	R±	-	R++	-	R±	-
Toluidine blue	B+	B+	B+++	B+++	B++	B++	B++	B++
Alcian blue	B+	B+	B+++	B+++	B++	B++	B±	B±
Astra blue	B+	B+	B+++	B+++	B++	B++	B±	B±
Biebrich scarlet	R+++	R+++	R±	R±	R+	R+	R+	R+
Acridine orange	OY+	OY+	OR+++	OR+++	ND	ND	ND	ND

Table 9 : The staining properties of GLs and MCs in different fixatives employed.

GL : Globule leucocytes

MC : Mast cells

CS : Corrosive sublimate

CAR : Carnoy's fluid

BO : Bouin's fluid

BNF : Buffered neutral formalin

R : Red

B : Blue

OY : Orange yellow

OR : Orange red

ND : Not done

\- : None

\+ : Weak

++ : Moderate

+++ : Strong

Staining Methods	GL	Subepithelial MC	Submucosal MC
Iron	-	-	-
Calcium	-	-	-
Bile pigment	-	-	-
Haemaglobin	-	-	-
H & E	R+++	R±	-
CC	R+++	-	-
MSB	R+++	-	-
BS pH 8, 9 and 10	R+++	R+++	R+++
TB pH 0.3	V+	V+	V+++
TB pH 4.0	BV+++	BV+++	BV+++
AO	RO+++	RO+++	RO+++
PAS	-	-	-
Diastase - PAS	-	-	-
Acetylation - PAS	-	-	-
Deacetylation - PAS	-	-	-
Methylation - PAS	-	-	-

Table 10 : Histochemical characteristics in the granules of mast cells and the globules of the globule leucocyte in sheep respiratory tract.

Staining Methods	GL	Subepithelial MC	Submucosal MC
Methylation-Saponification-PAS	-	-	-
AB pH 1.0.	B+++	B+++	B+++
Methylation-AB pH 1.0	B+++	B+++	B+++
Methylation-Saponification-AB pH 1.0	-	-	-
AB pH 2.5	B+++	B+++	B+++
Methylation-AB pH 2.5	B+++	B+++	B+++
Methylation-Saponification-AB pH 2.5	-	-	-
Hyaluronidase-AB pH 2.5	B++	B++	B++
Sialidase - AB pH 2.5	B++	B++	B++
AB pH 2.5-PAS	B+++	B+++	B+++
AB pH 1.0-PAS	B+++	B+++	B+++
AB-S pH 0.3	B+++	B+++	B+++
AB pH 2.5-BS pH 9.5	Core R++ Cortex B++	Core R++ Cortex B++	Core R++ Cortex B++

Table 10 : (Contd.)

Staining Methods	GL	Subepithelial MC	Submucosal MC
HID	BrBl+++	BrBl+++	BrBl+++
HID-AB pH 2.5	BrBl+++	BrBl+++	BrBl+++

Table 10 : (Contd.)

GL : Globule leucocyte
MC : Mast cell
H & E : Haematoxylin & eosin
CC : Carbol Chromotrope
MSB : Martius scarlet blue
BS : Biebrich scarlet
TB : Toluidine blue
R : Red
V : Violet
B : Blue
BV : Blue violet
RO : Red orange
BrBl : Brown black

AO : Acridine Orange
PAS : Periodic acid-Schiff
AB : Alcian blue
AB-S : Alcian blue - safranin
HID : High iron diamine
HID-AB: High iron diamine alcian blue

\- negative reaction
\+ weak reaction
++ moderate reaction
+++ strong reaction

Fig. 22 : Globule leucocytes in bronchial epithelium of parasitised sheep, illustrating the typical nucleus (⟶) and the large, rounded acidophilic globules.
Carbol chromotrope staining, X 250.

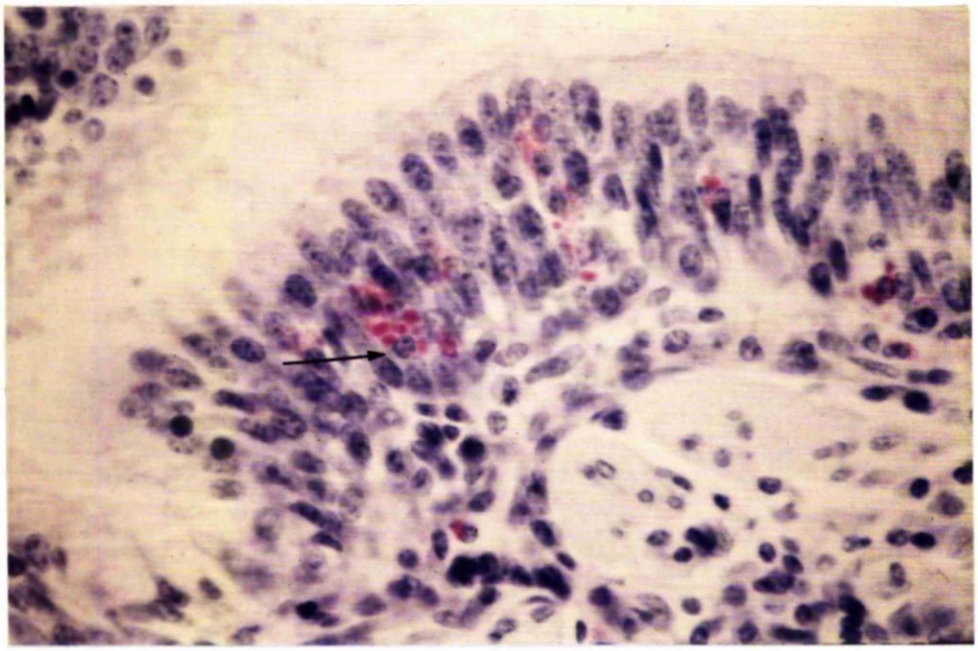
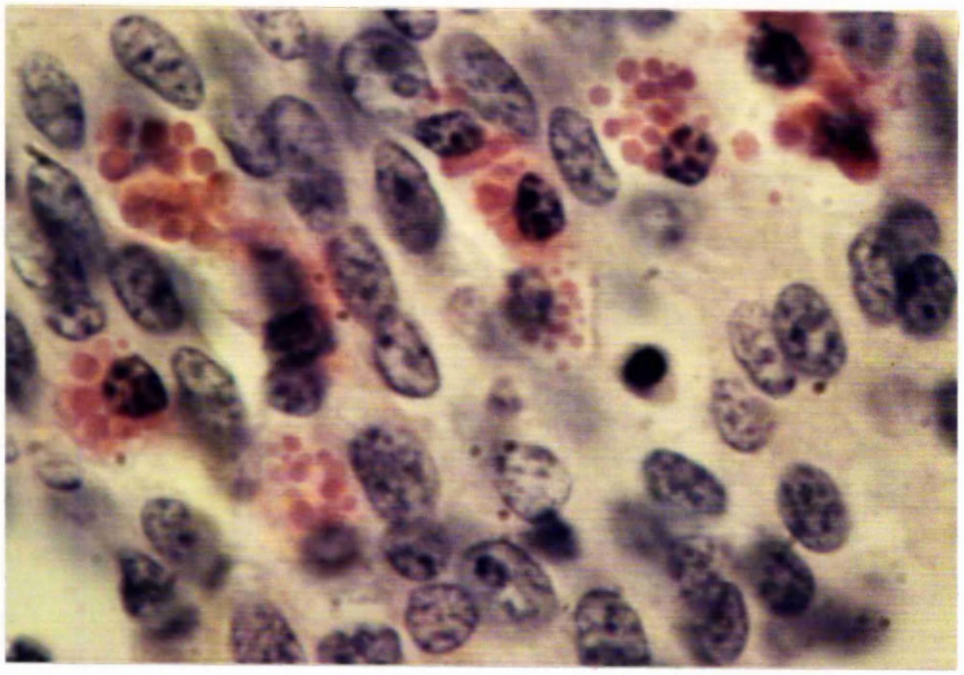


Fig. 23 : High power view of Fig. 22. The globule leucocytes in the bronchial epithelium of parasitised sheep, showing eccentric nuclei which were similar to plasma cell nuclei. The globules were large, rounded, acidophilic and grouped in a mass around one pole of the nuclei. Carbol chromotrope staining, X 1,000.

Fig. 24 : Two globule leucocytes in the bronchial epithelium of parasitised sheep. The cell to the left had prominent globules, quite separated from each other, while the cell to the right had coalesced globules (⟶). A few mast cells were seen in the subepithelial position with small granules.
Carbol chromotrope staining, X 1,000.



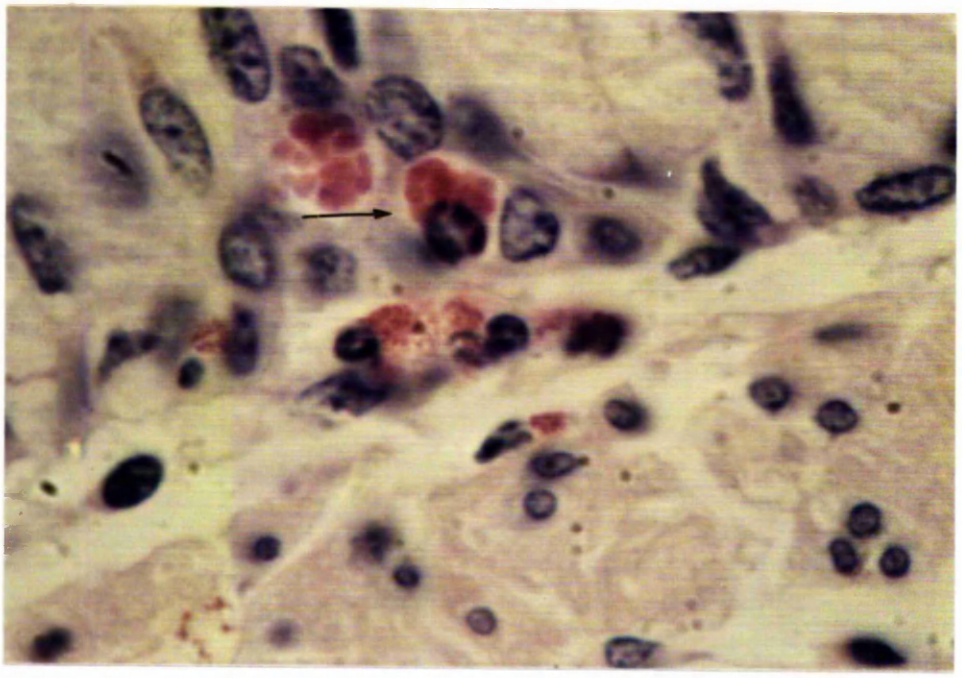


Fig. 25 : Globule leucocytes in the bronchial epithelium of parasitised sheep showing acidophilic properties of the intracytoplasmic globules with the haematoxylin and eosin stain particularly after fixation with corrosive sublimate. X 250.

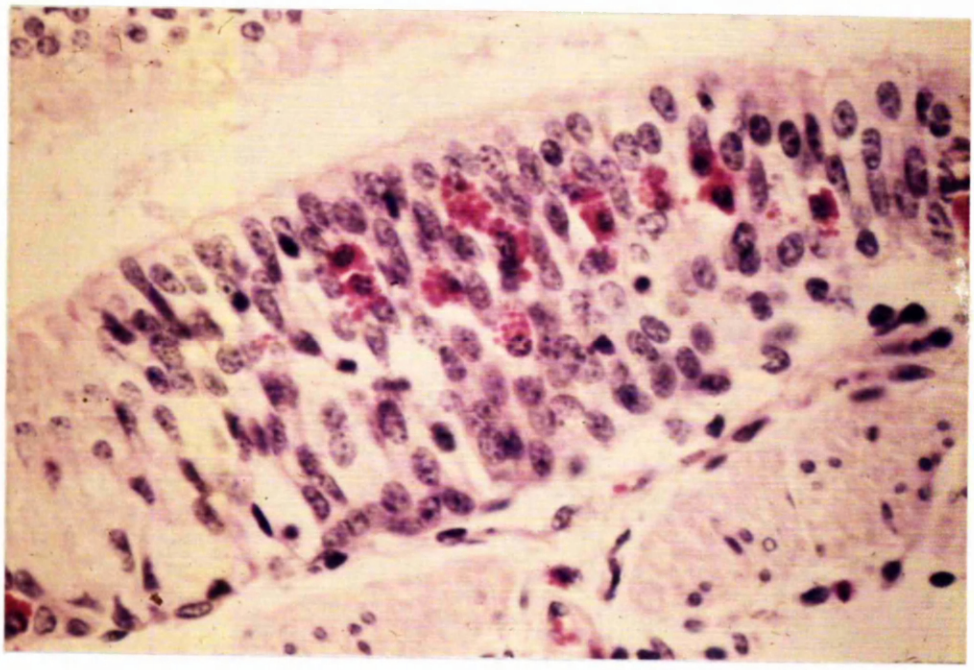
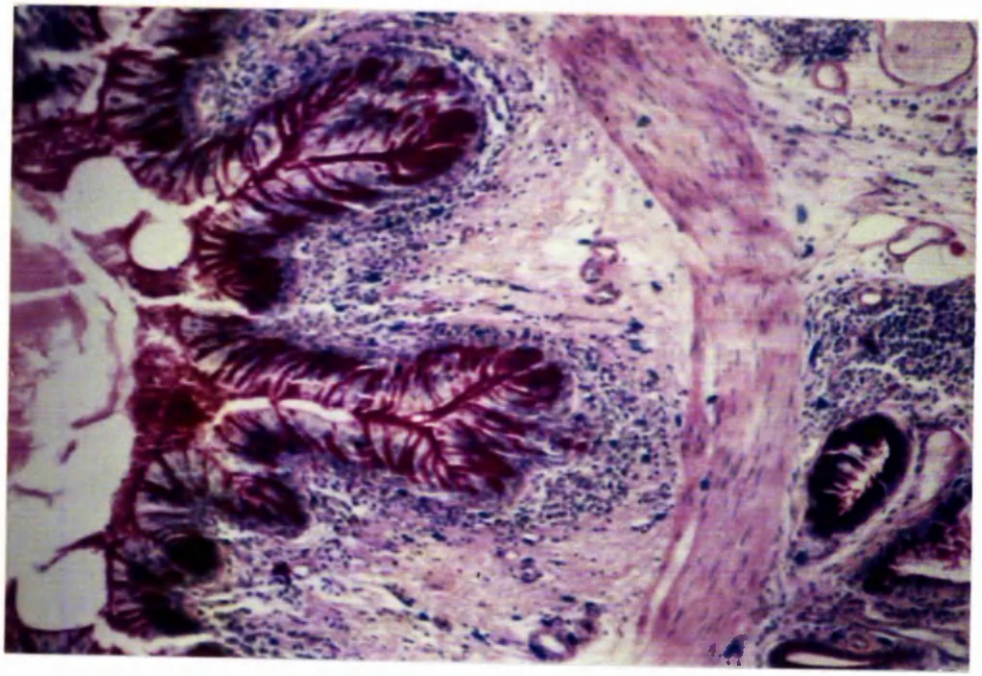


Fig. 26 : Alcian blue pH 2.5/periodic acid-Schiff technique (AB/PAS). All the globules of globule leucocytes and granules of subepithelial and submucosal mast cells stained blue in colour which represented the presence of acid muco-substances in these cells.
X 100.

Fig. 27 : High power view of the above picture, showing similar staining properties of both globule leucocytes and subepithelial mast cells. Alcian blue pH 2.5/ PAS,
X 250.



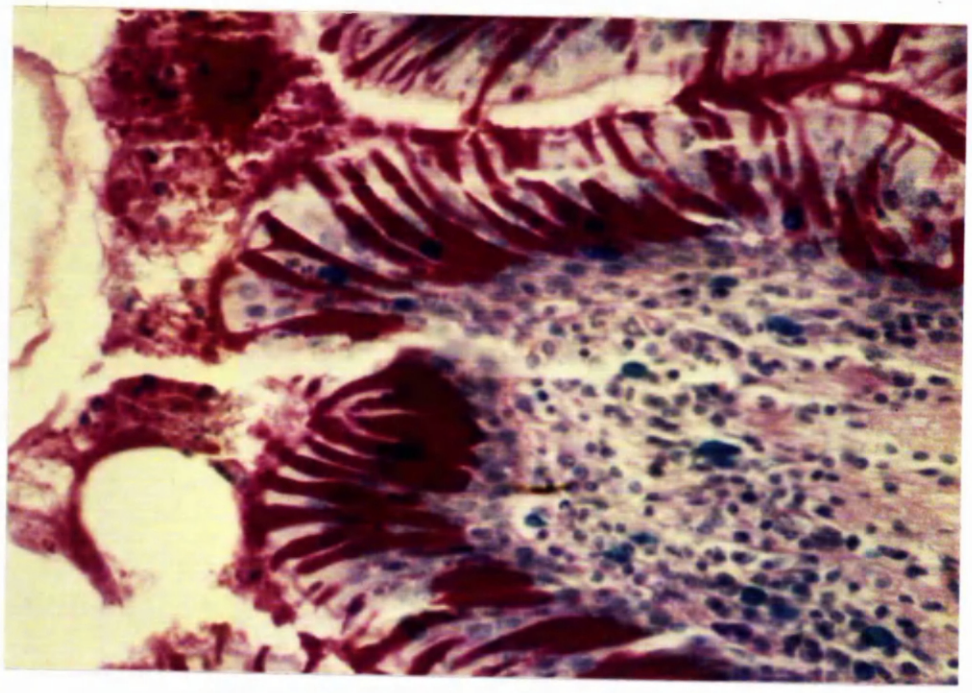


Fig. 28 : Alcian blue/safranin pH 0.3 (AB/S) stained the globules of globule leucocytes and the granules of mast cells blue in colour. No safranophilic substances were detected in the granules of either cell type in the tracheobronchial tree of sheep.
X 400

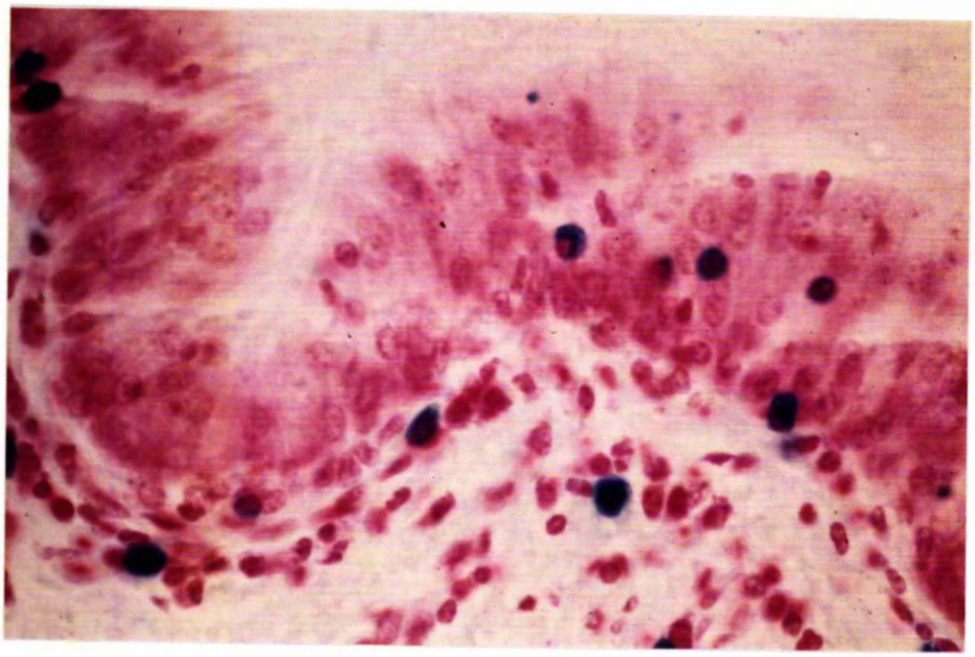
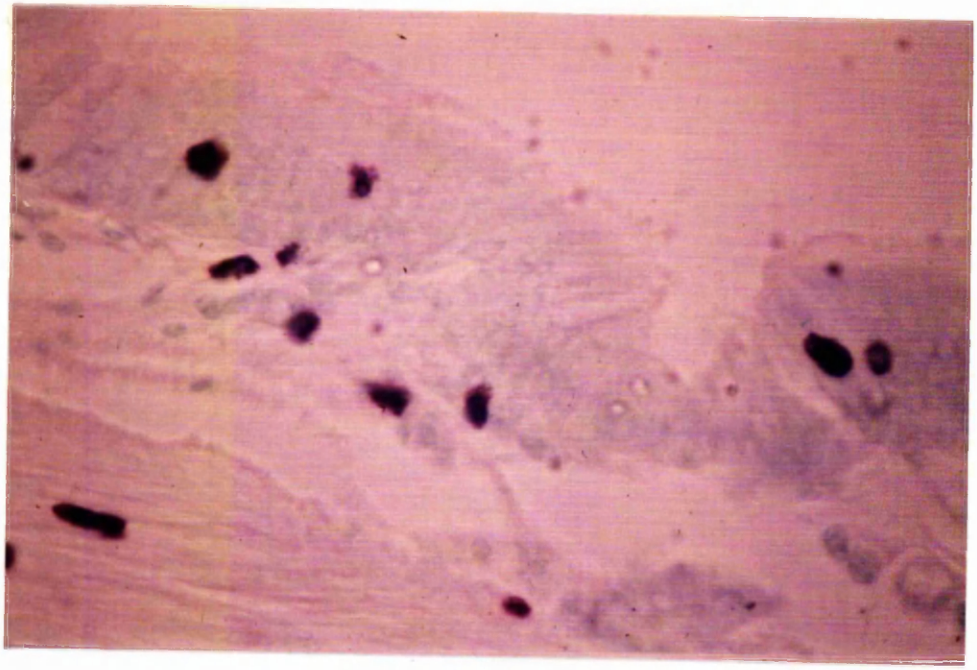


Fig. 29 : Toluidine blue (TB) pH 0.3. The acidmucosubstances in the globules of globule leucocytes and granules of mast cells of sheep tracheobronchial tree displayed a violet metachromasia.
X 400.

Fig. 30 : Toluidine blue (TB) pH 4.0. The globules of globule leucocytes and granules of mast cells in sheep tracheobronchial tree showed deep blue violet metachromasia at this pH. when compared with those stained at pH 0.3 (Fig. 29). X 400.



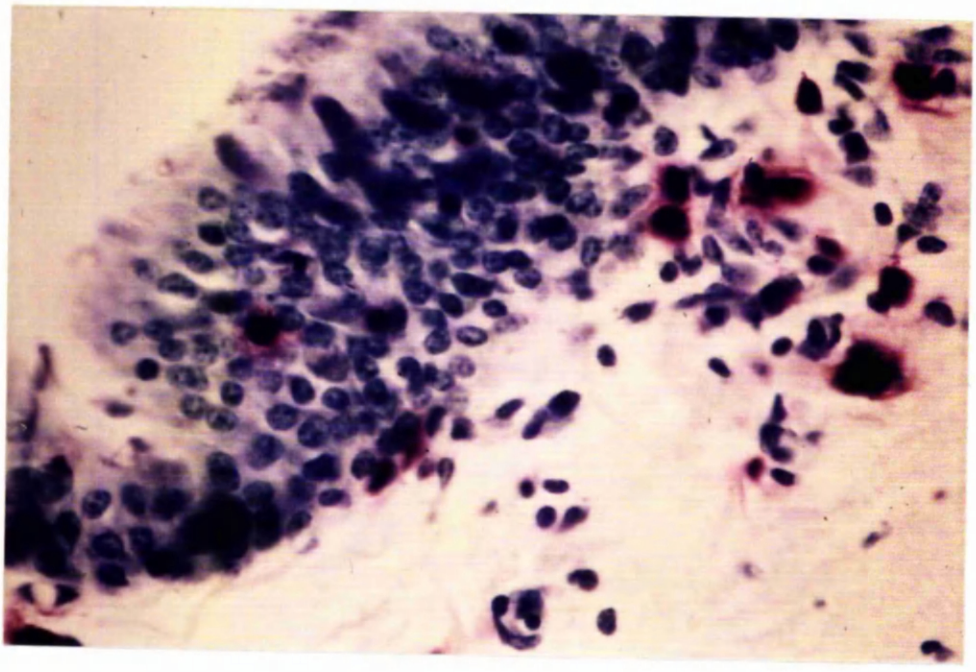
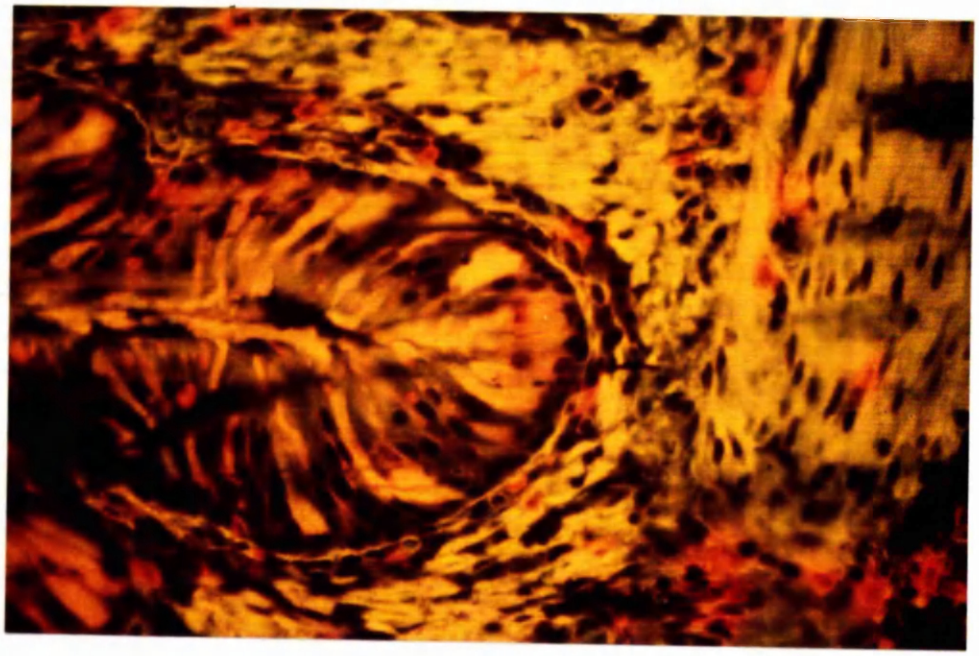


Fig. 31 : Acridine orange stain (AO). The mucosubstances in the globules of globule leucocytes and granules of mast cells in sheep tracheobronchial tree fluoresced red to orange.
X 400.

Fig. 32 : Alcian blue pH 1.0/PAS technique (AB/PAS). Differentiated the blue staining of sulphated mucosubstances from sialomucins and neutral mucins which coloured red. The globules of globule leucocytes and the granules of mast cells in sheep tracheobronchial tree had sulphated mucosubstances.
X 400.



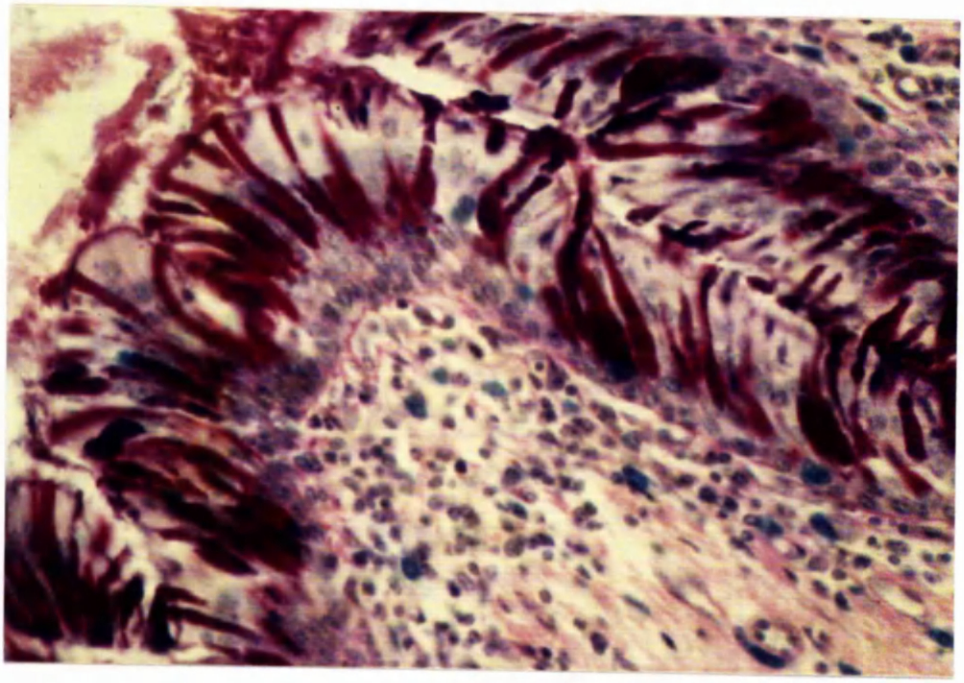
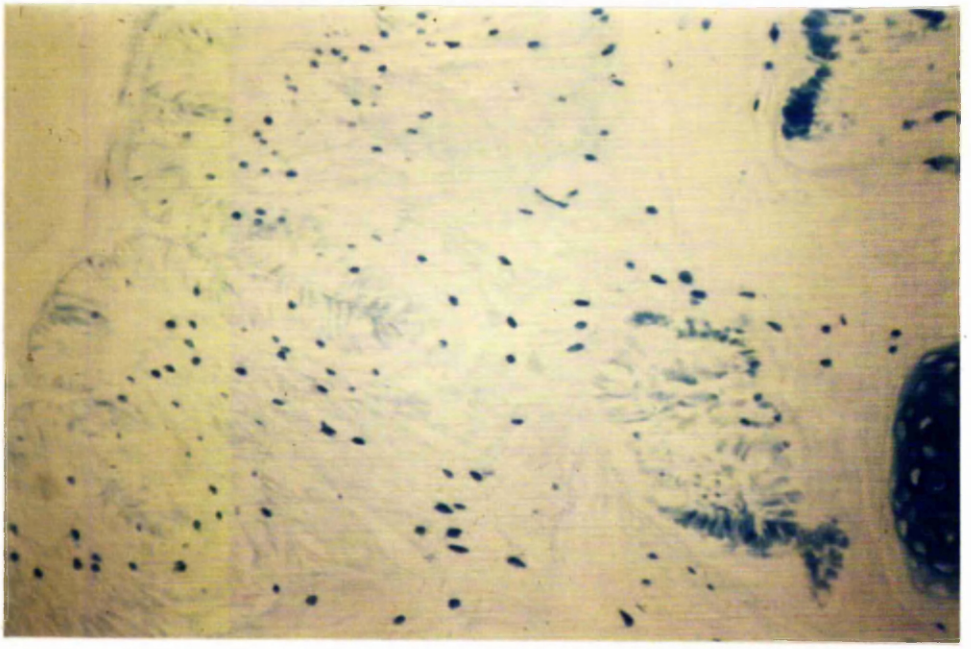


Fig. 33 : Alcian blue pH 1.0 (AB) stain induced blue staining reaction in the globules of globule leucocytes and granules of mast cells in sheep tracheobronchial tree.
X 100.

Fig. 34 : High power view of Fig. 33, represented a similar histochemical staining property in the globules of globule leucocytes and granules of mast cells of the tracheobronchial tree in sheep. AB pH 1.0 staining,
X 400.



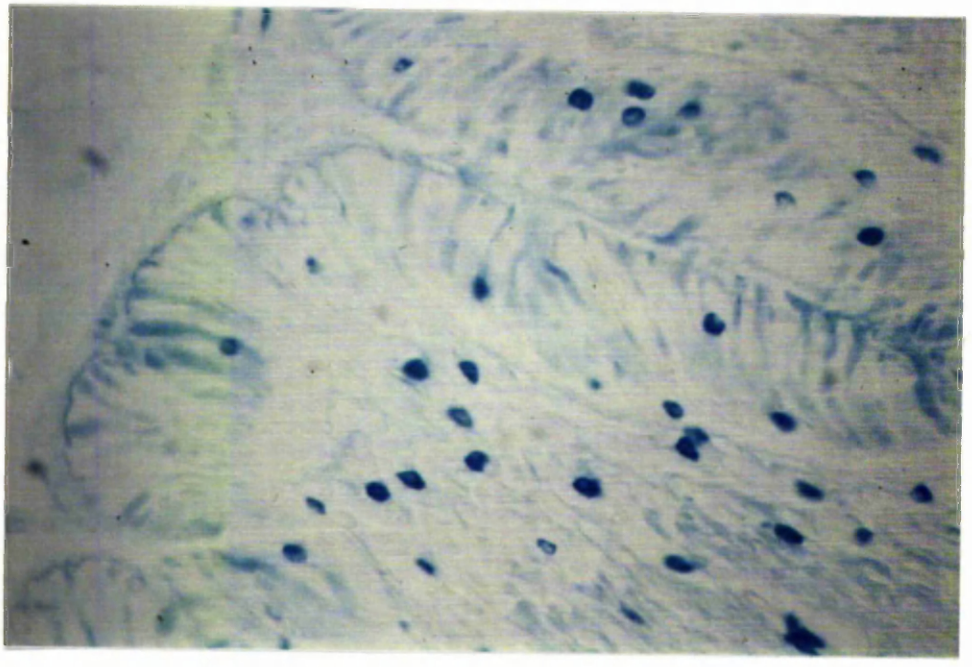


Fig. 35 : Alcian blue (AB) pH 2.5 stain induced a blue staining reaction in the globules of globule leucocytes and granules of mast cells in the tracheobronchial tree of sheep suggesting the presence of sulphomucin in both cell type.
X 250.

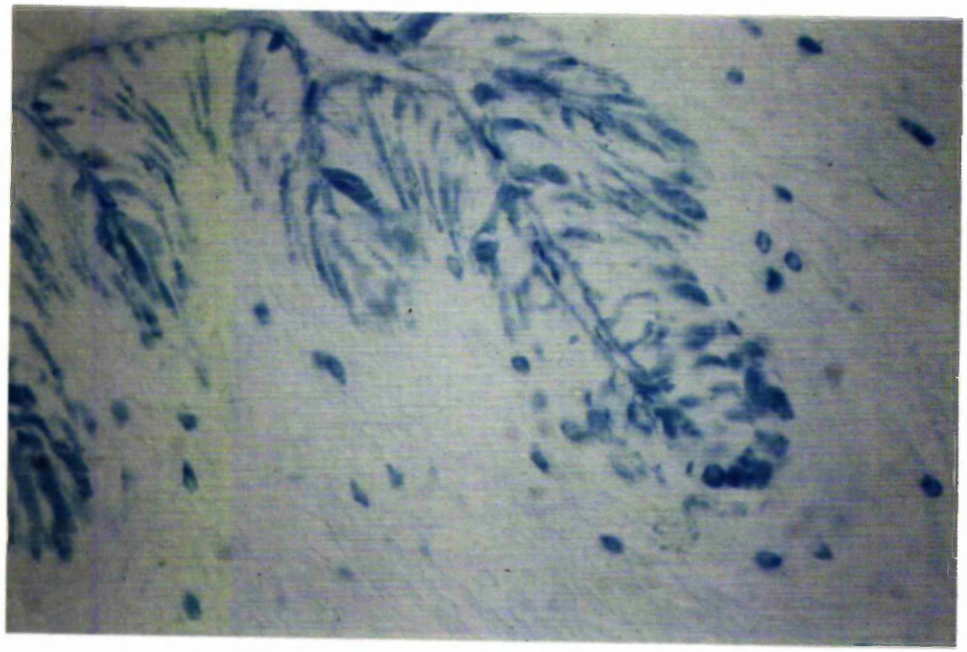
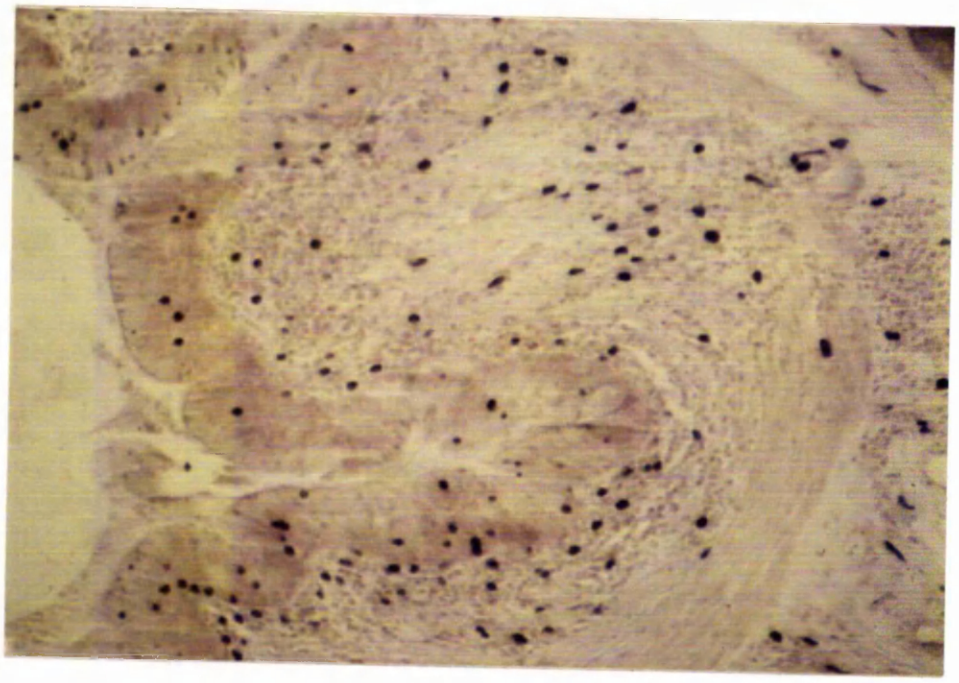


Fig. 36 : High iron diamine (HID) stain indicated the presence of sulphomucins in the globules of globule leucocytes and the granules of both the subepithelial and submucosal mast cells.
X 100.

Fig. 37 : High power view of Fig. 36. The dark blue staining reaction in the globule leucocytes and subepithelial mast cells indicated the presence of sulphomucins in both types of cell. HID,
X 400.



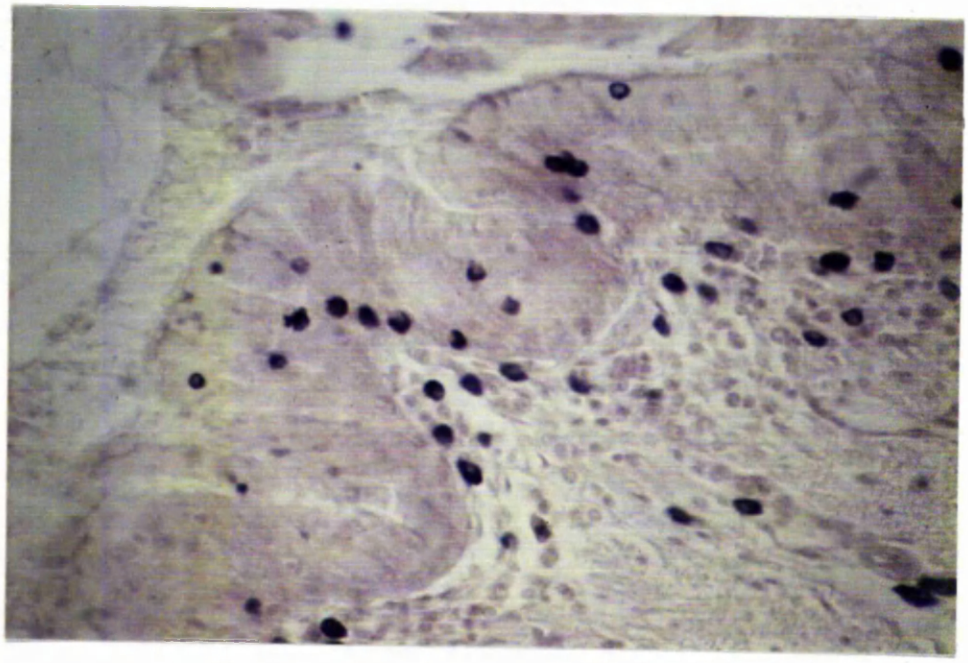
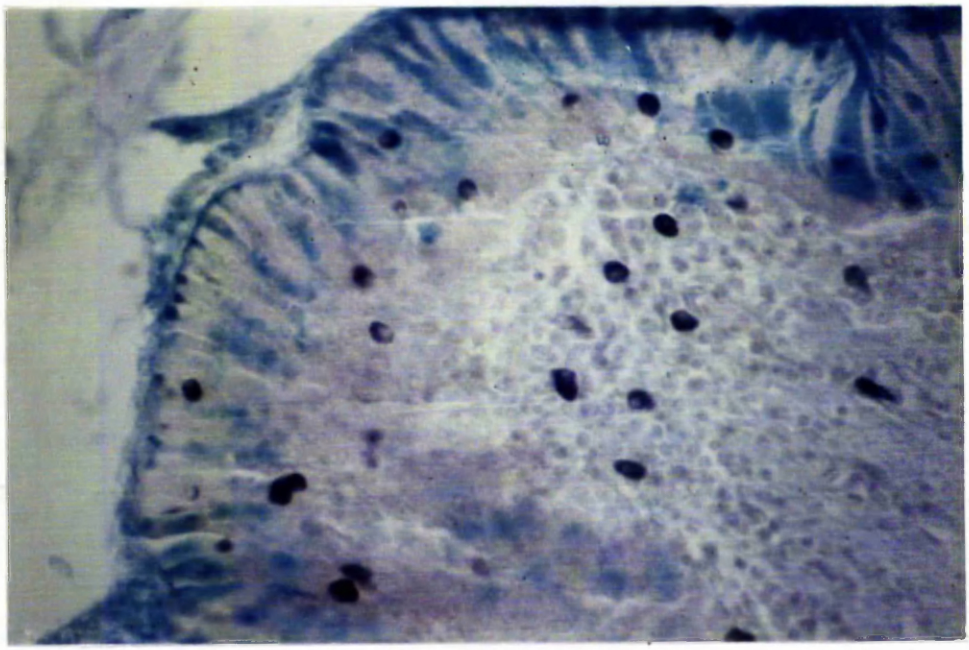


Fig. 38 : High iron diamine/Alcian blue (HID/AB) technique. The presence of dark blue staining reaction in the globules of globule leucocytes and granules of mast cells in the tracheobronchial tree of sheep suggested the presence of sulphomucins in the granules of both cell types. X 400.

Fig. 39 : Biebrich scarlet (BS) pH. 8, 9 and 10 showed a red staining reaction in the globules of globule leucocytes suggesting the presence of considerable amounts of basic protein in these cells of the sheep tracheobronchial tree. X 400.



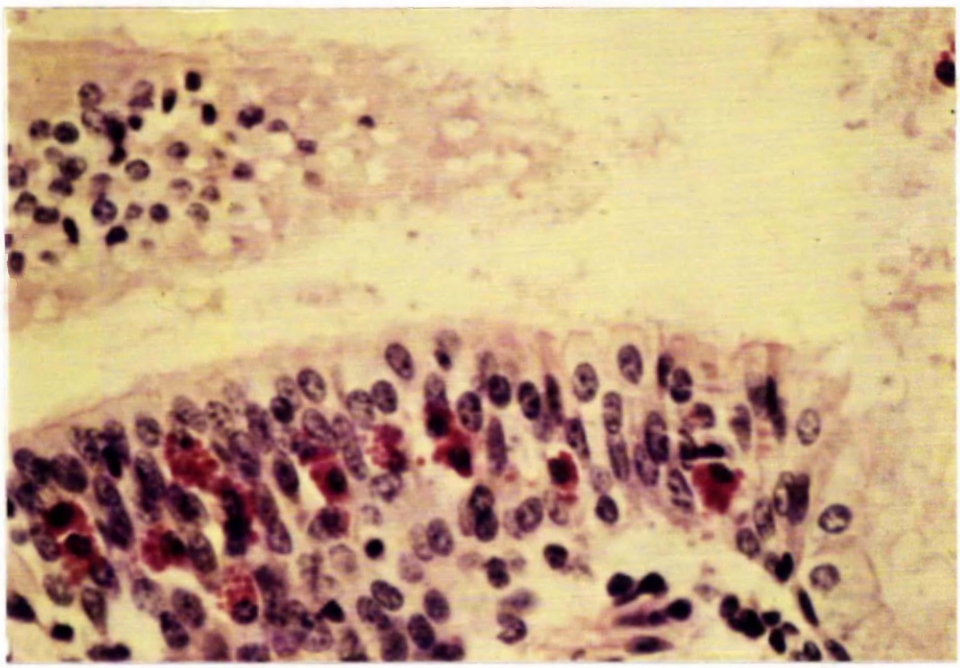
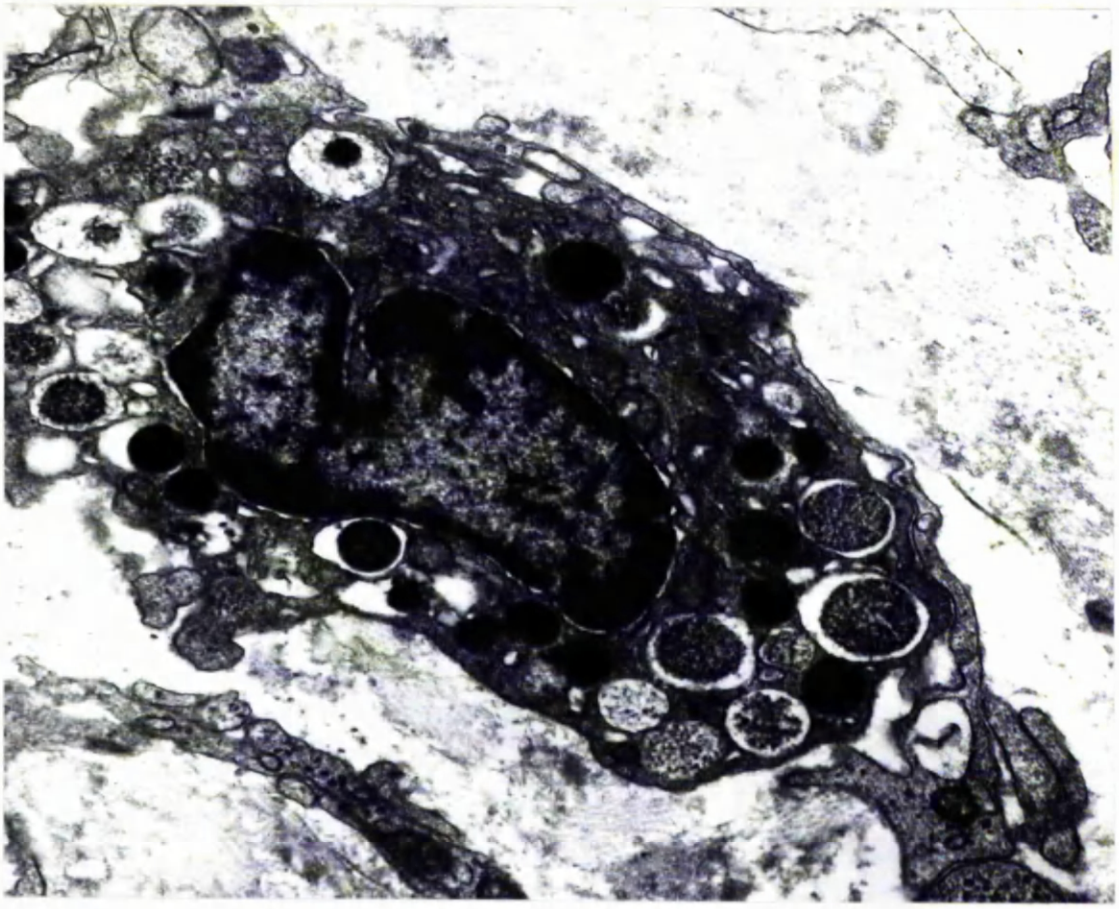


Fig. 40 : Globule leucocytes within the respiratory tract mucosa of parasitised sheep showing various types of characteristic large, round intracytoplasmic globules.
X 20,000.

Fig. 41 : A globule leucocyte in the respiratory tract epithelium of parasitised sheep showing a centriole (C) and Golgi complex (gc).
X 20,000.



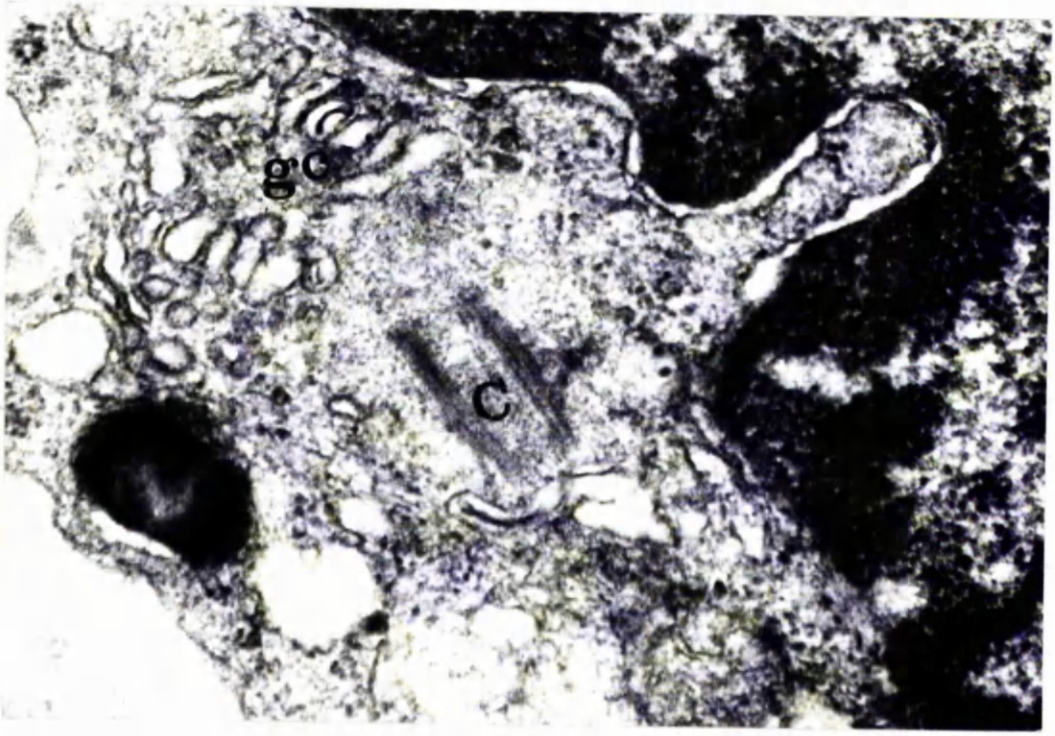

gc
C

Fig. 42 : A globule leucocyte within the respiratory tract epithelium of parasitised sheep showing details of globule types. Type (a) globule showed a unit membrane (⟶) bounding homogeneous electron dense material. Type (b) globules showed small or large rims of less electron-dense matrix. Type (c) globules showed reticulated matrix. Type (d) globules showed uniformly distributed fine granular substance.
X 60,000.

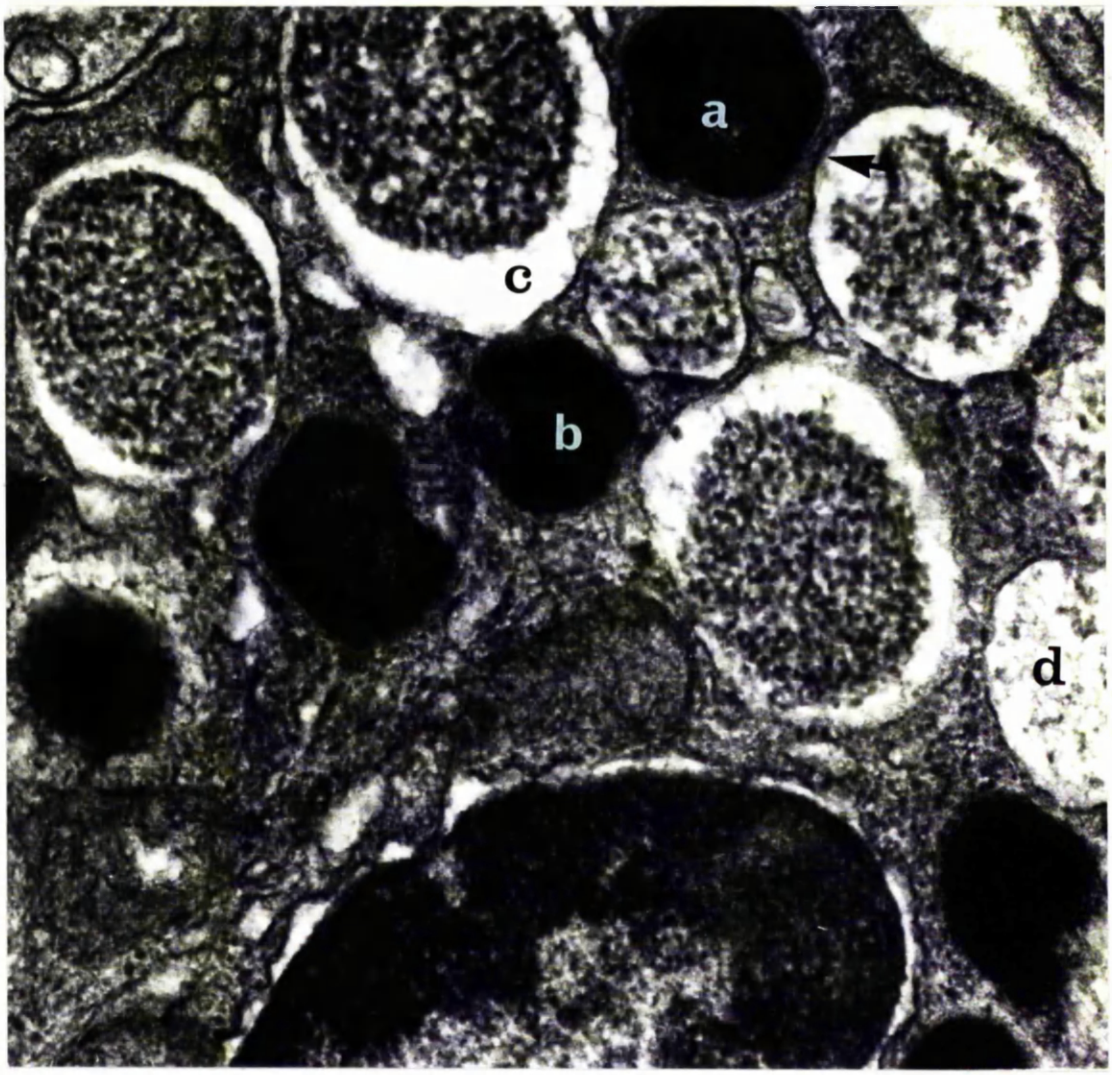
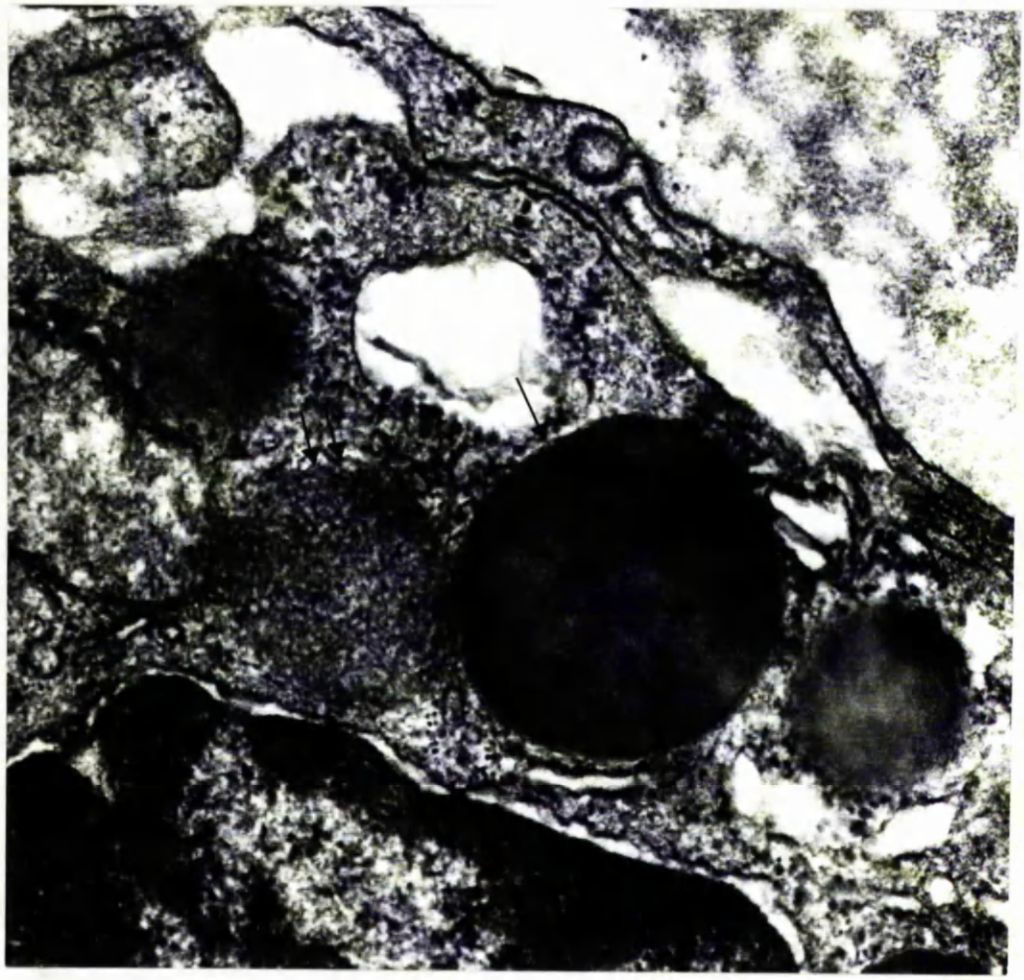

a
c
b
d

Fig. 43 : A globule leucocyte in the respiratory tract epithelium of parasitised sheep showed a globule with homogeneous electron dense matrix (⟶) and a non-membrane-bound globule with fine granulation (⟹). X 60,000.

Fig. 44 : Globules with a well-defined membrane which have lost much of their fine granular content leaving vacuoles in their substances (⟶). Membrane-bound globules were seen with dense, finely granular centres surrounded by a less dense peripheral zone containing fine granules and a vesicle (ve) (⟹). A coarse textured globule showing a vescile (ve) and separated from the surrounding membrane by a vacucle (va) (⟹). X 60,000.



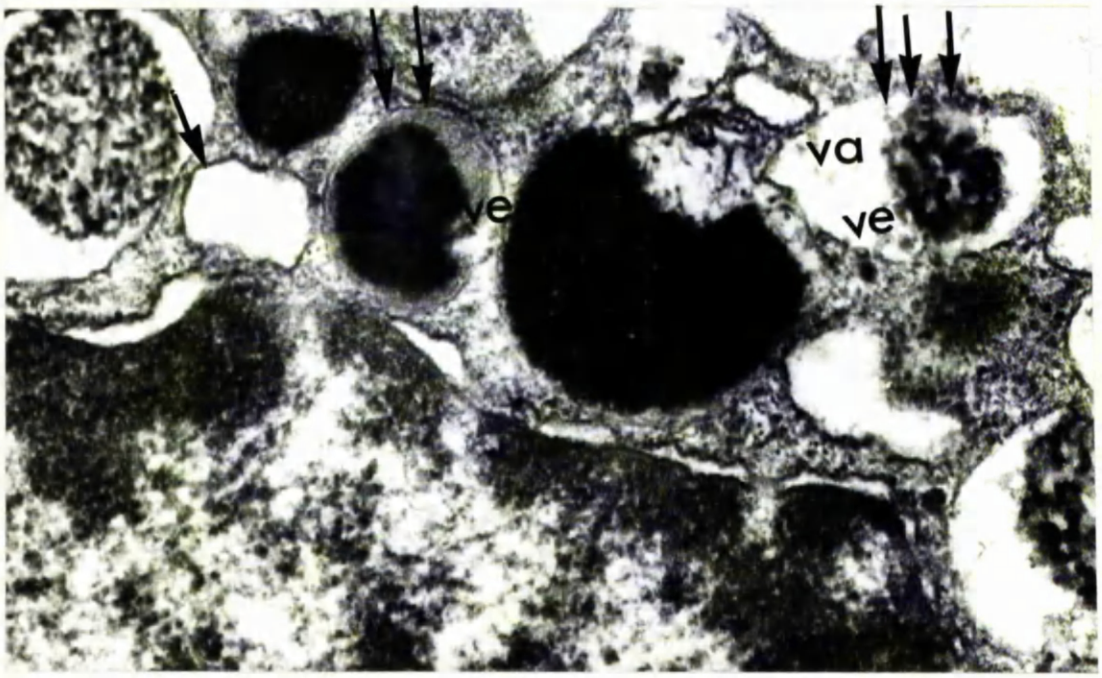
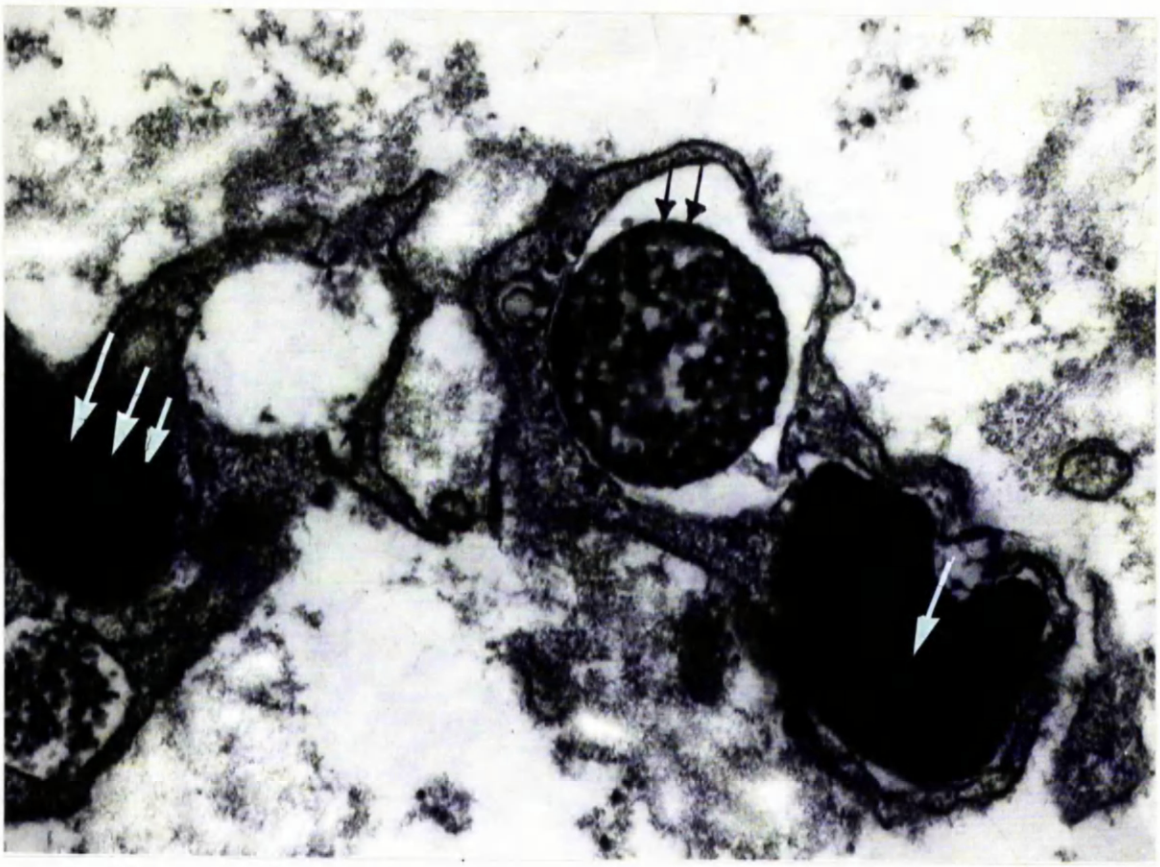

va
ve

Fig. 45 : Atypical type (b) globule with pronounced horse-shoe shaped form and peripheral vacuolation (⟶). Typical type (c) globule with pronounced peripheral vacuolation (⟹). A non-membrane-bound globule with dense homogeneous matrix was seen (⟹) X 60,000.

Fig. 46 : A membrane-bound globule with horse-shoe shaped dense matrix invaginated by a light area which contained a vesicle (ve) (⟶).
X 60,000.



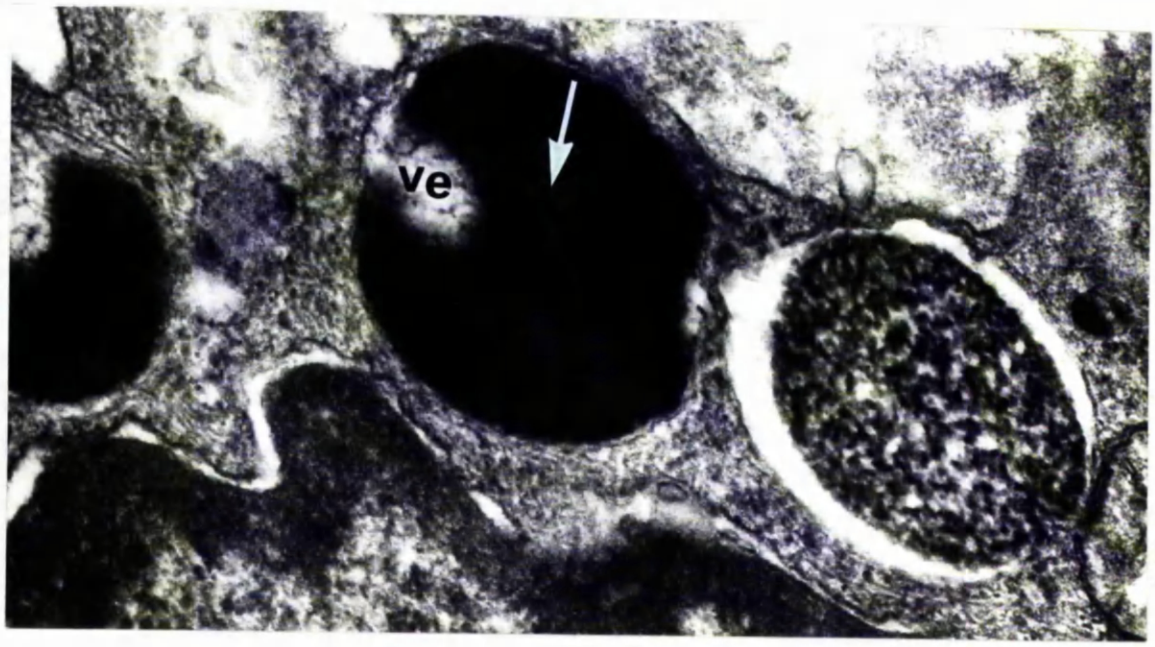
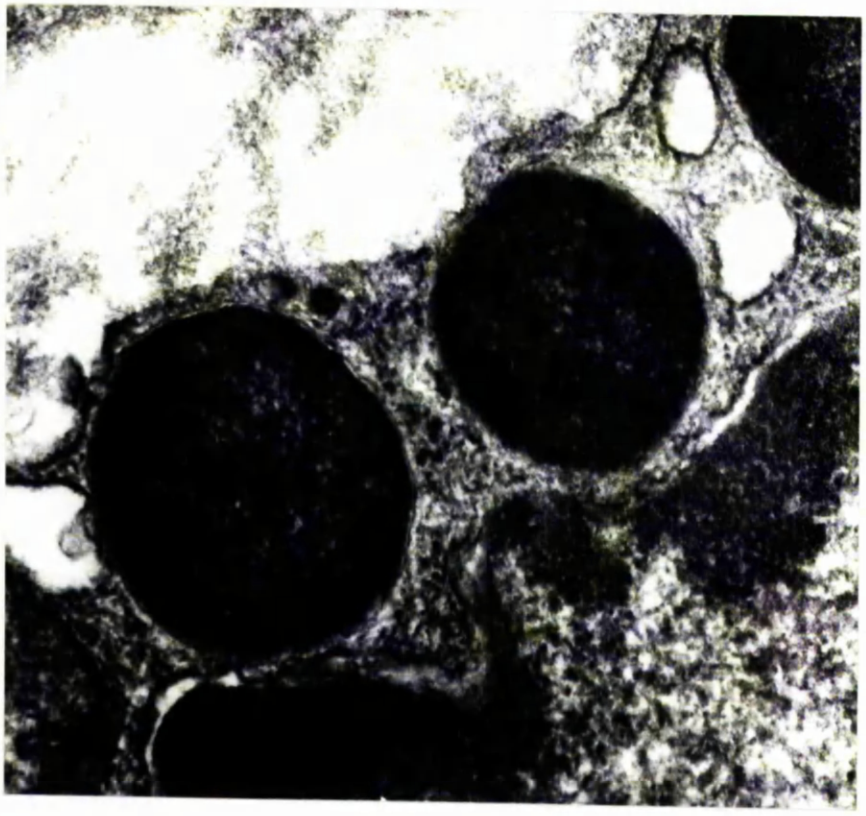

ve

Fig. 47 : Membrane-bound, electron dense globules of the globule leucocyte in the respiratory tract of sheep showing an early stage of reticulation.
X 40,000.

Fig. 48 : A globule leucocyte showed two membrane-bound structures; the larger one (⟹) contained fine granular material and the smaller one (⟶) was completely electron lucent.



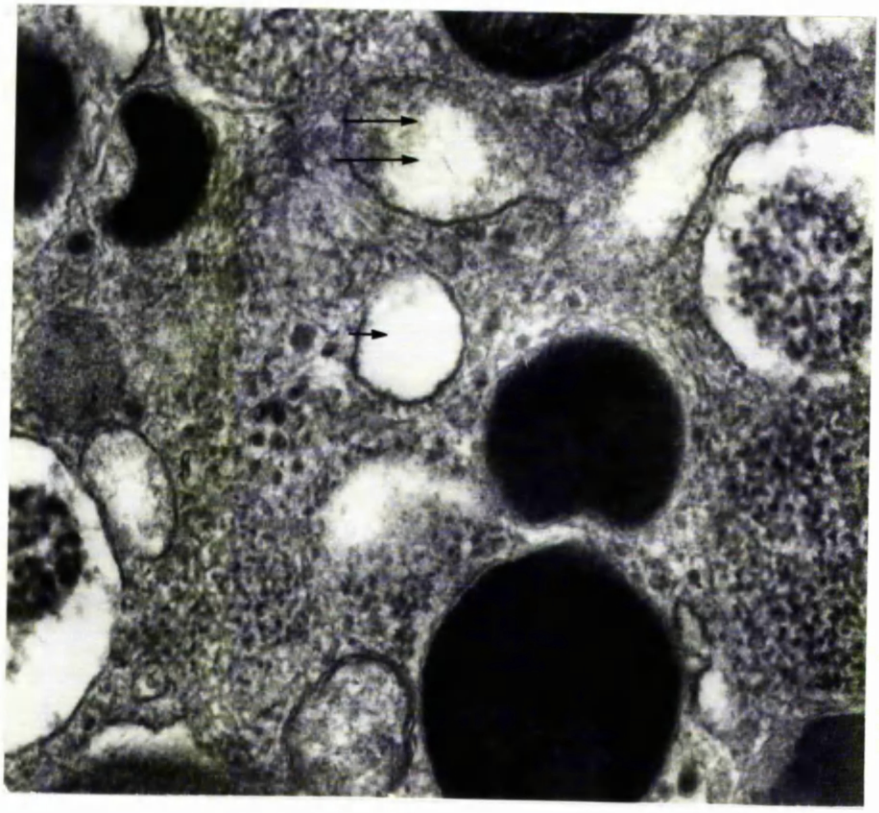


Fig. 49 : A globule leucocyte of the sheep respiratory tract showed an intraglobular vacuole (⟶) as well as intracytoplasmic vacuoles (⟹).
X 60,000.

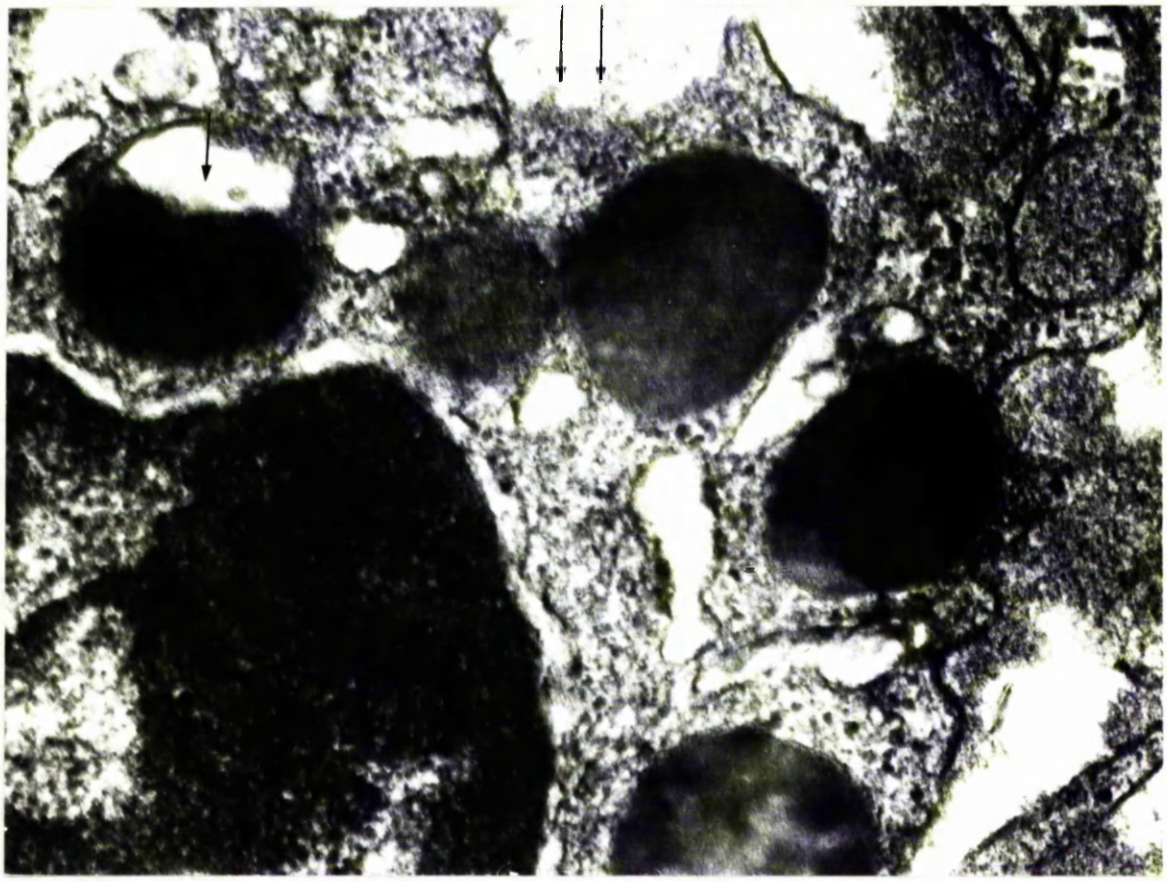
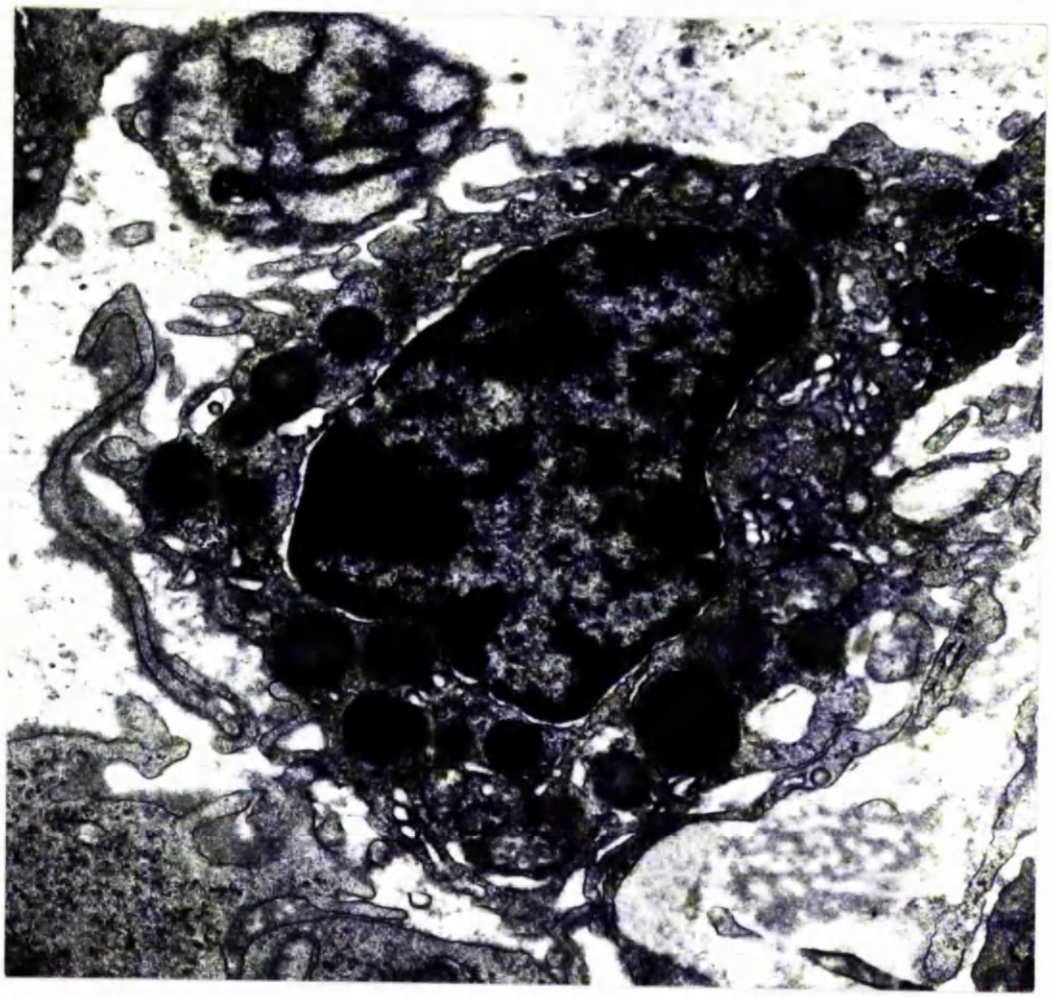


Fig. 50 : A characteristic subepithelial mast cell in the respiratory tract of parasite free sheep showed typical membrane-bound granules.
X 20,000.

Fig. 51 : High power view of mast cell granule (G) showing dense homogeneous matrix.
X 60,000.



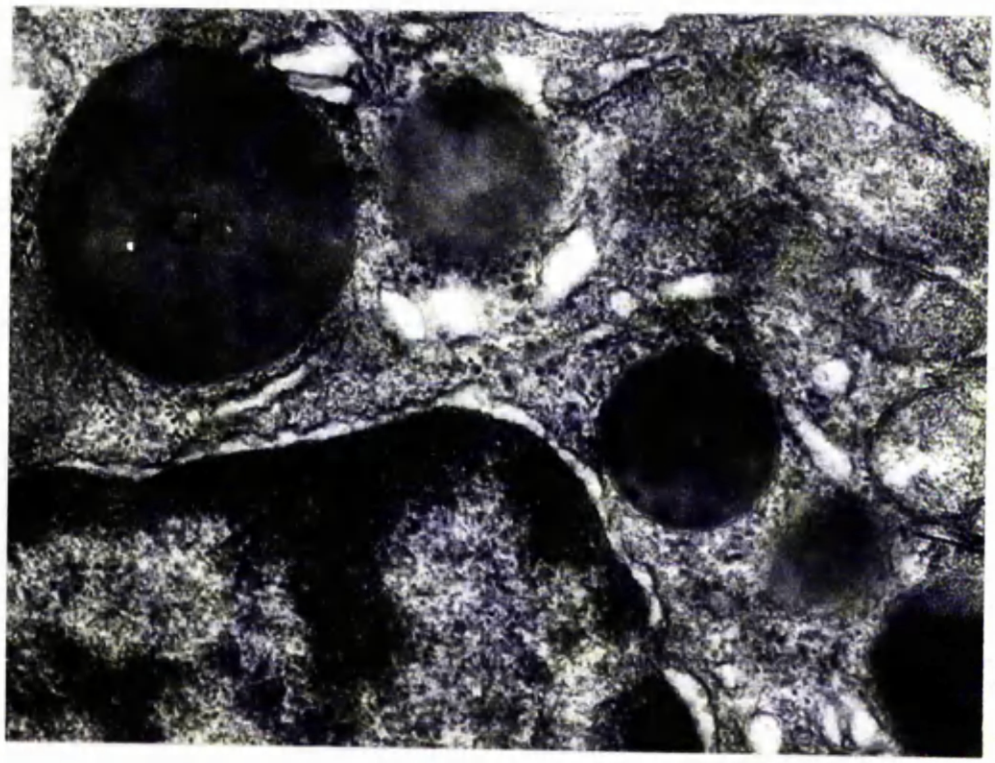
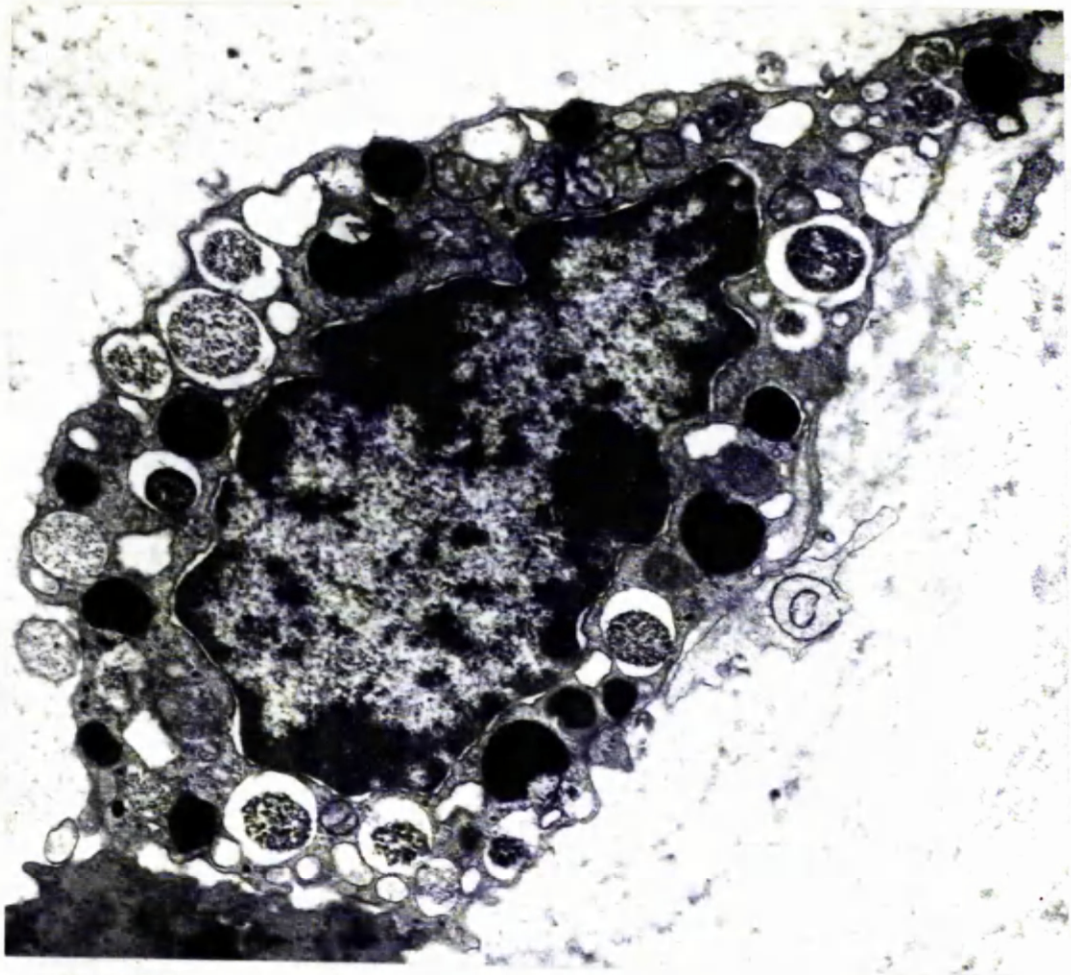


Fig. 52 : -A characteristic subepithelial mast cell in the respiratory tract of parasitised sheep showing various forms of granules similar in appearance to the globules of the globule leucocyte.
X 20,000.

Fig. 53 : Basophil-like cell in the lamina propria of the respiratory tract of parasitised sheep showing various forms of granule indistinguishable from the globules seen in the globule leucocyte and the granules seen in the subepithelial mast cells of parasitised sheep.
X 20,000.



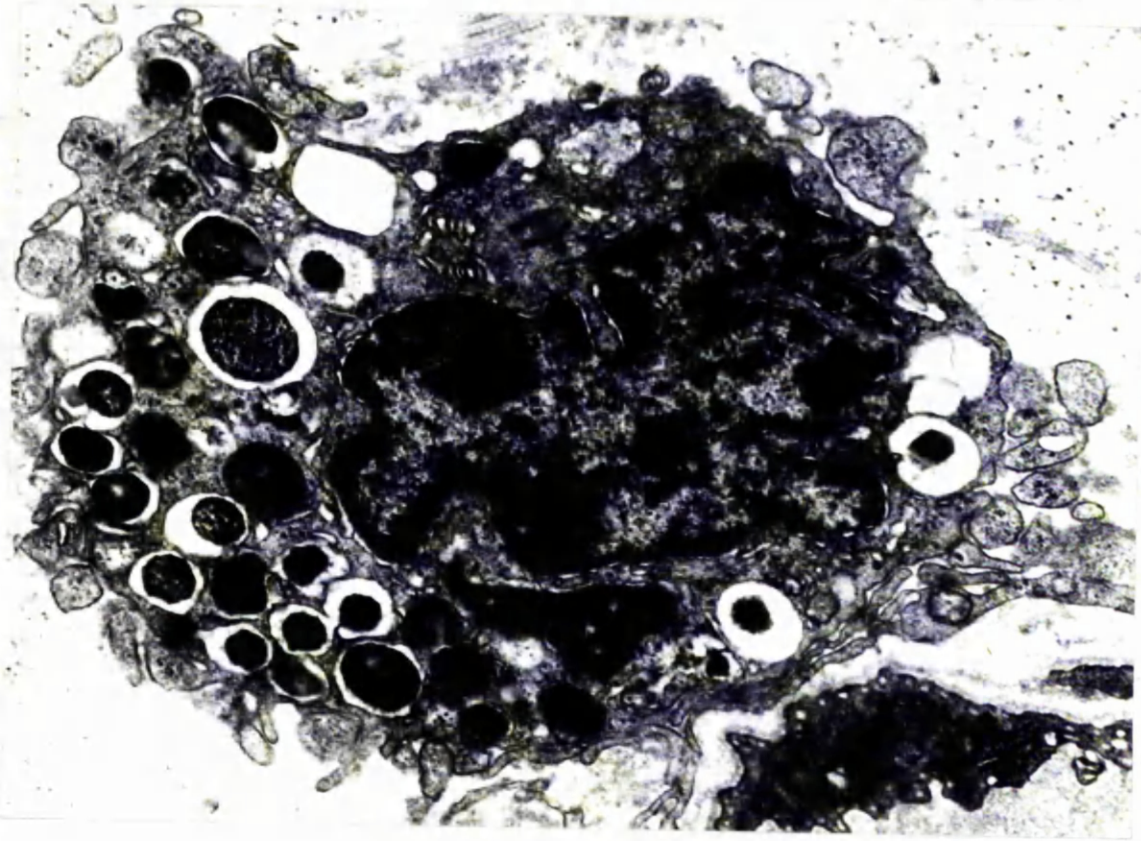
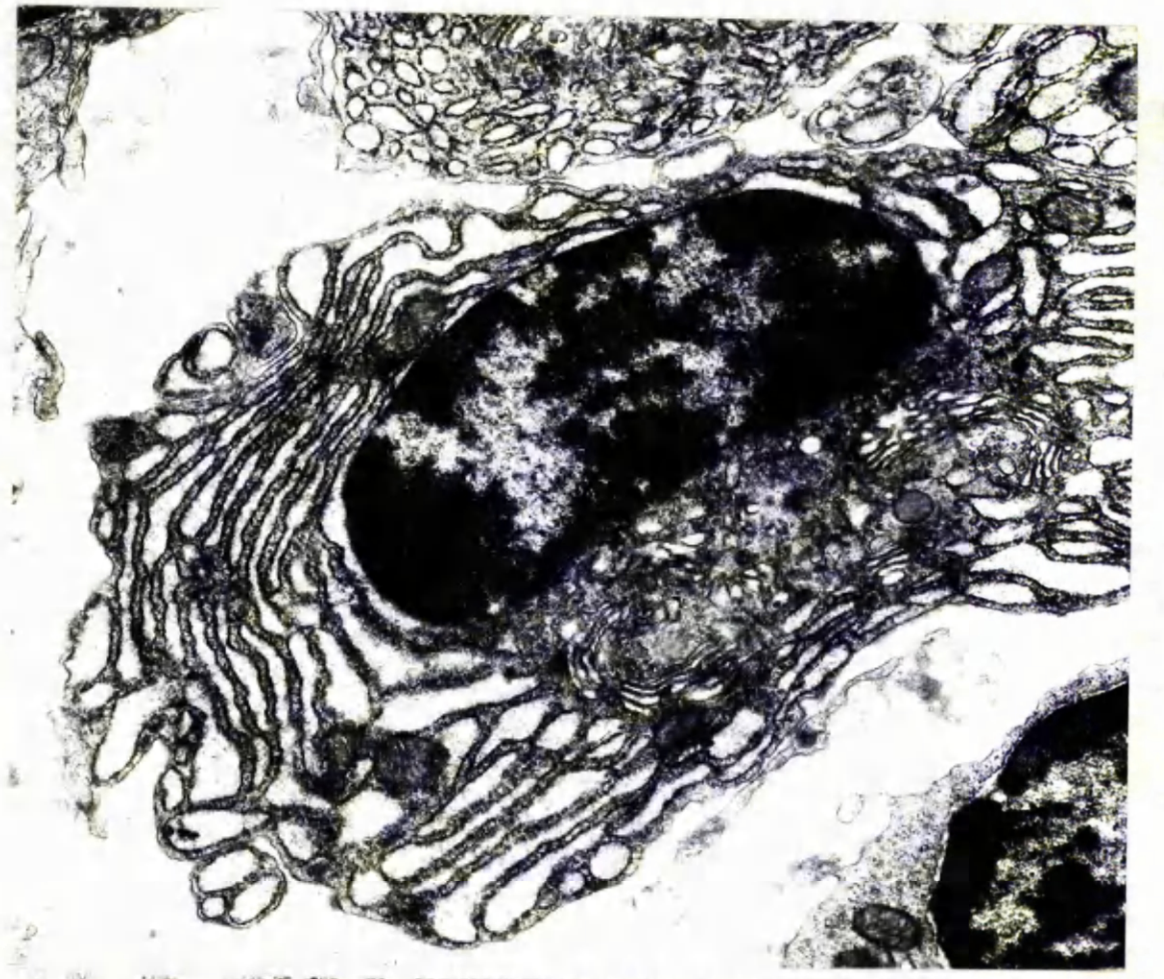


Fig. 54 : - A plasma cell in the lamina propria of the respiratory tract of parasitised sheep.
X 20,000.

Fig. 55 : High power view of part of a plasma cell showing distended rough surface endoplasmic reticulum.
X 60,000.



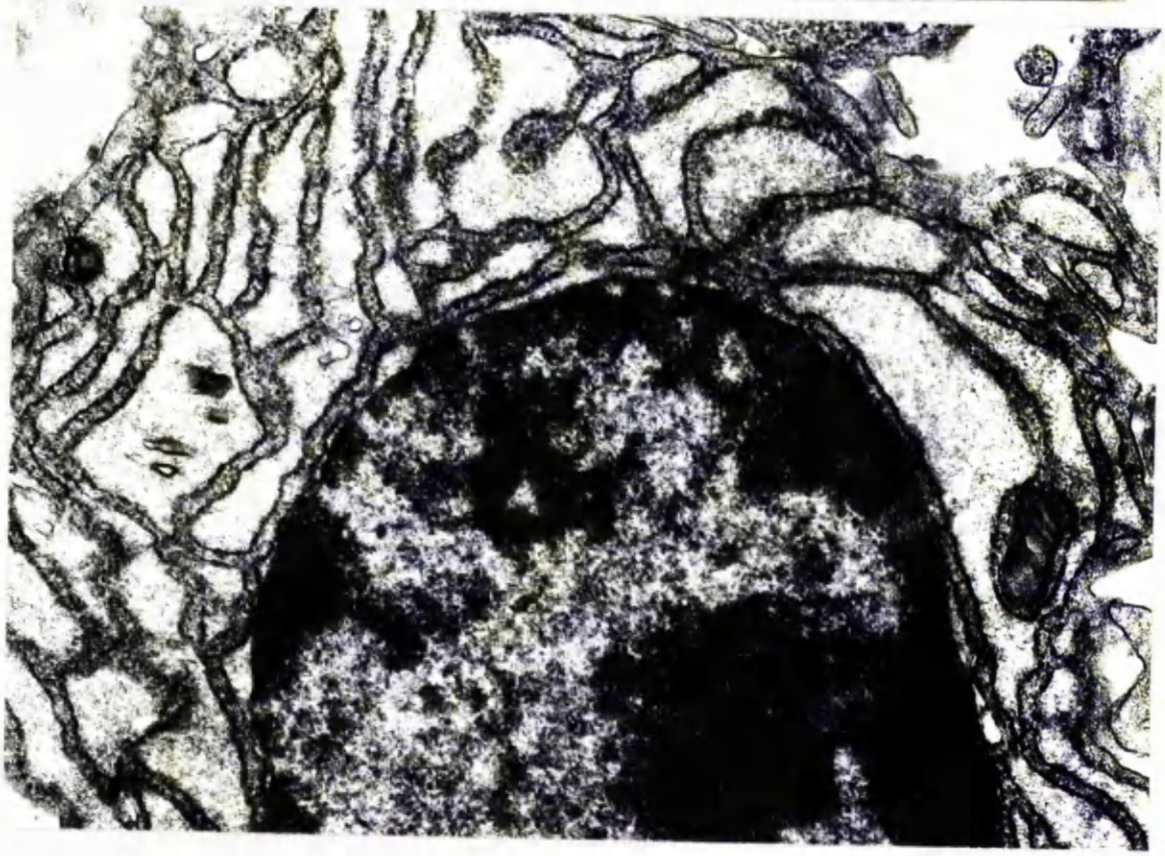
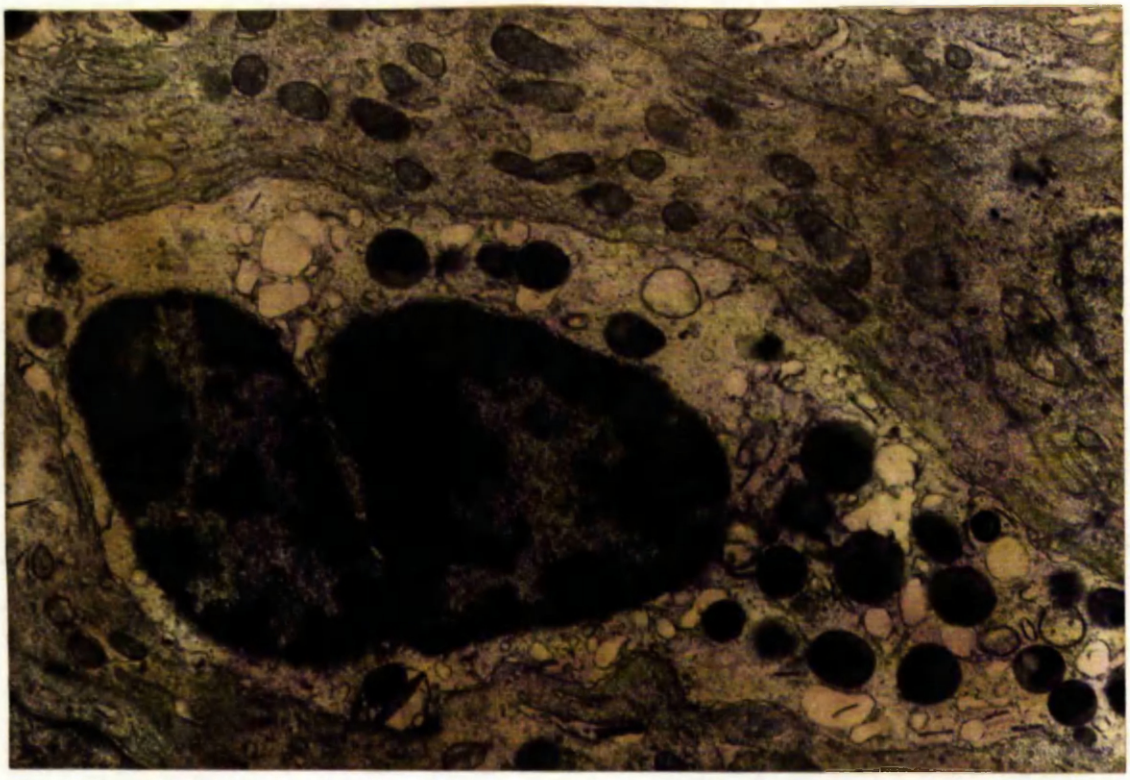
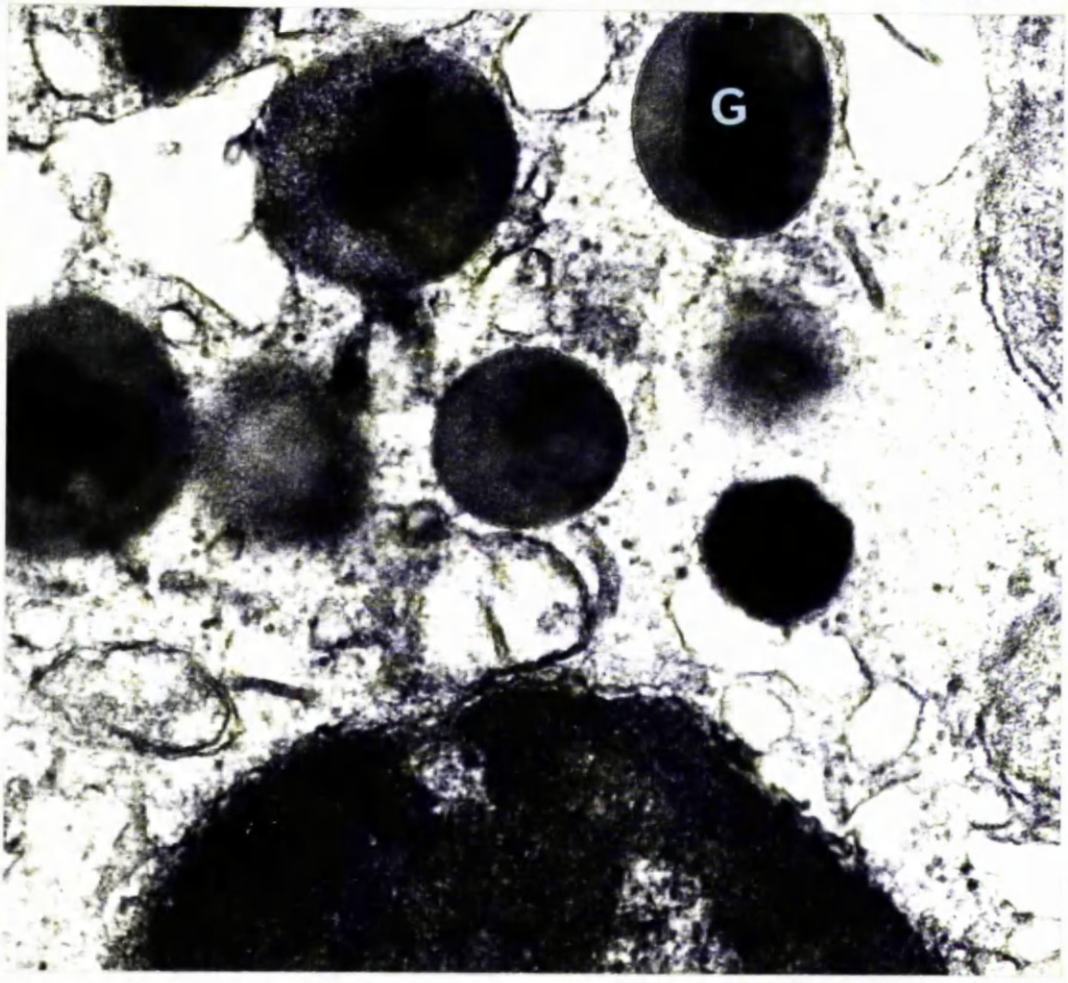


Fig. 56 : Eosinophil in the epithelial mucosa of a parasitised sheep showing the bonded granules typical of this cell type in sheep. X 20,000.

Fig. 57 : High power view of eosinophil granules (G) showing bounding membranes and internal banding.

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CHAPTER FOUR

EXPERIMENTAL INDUCTION OF GLOBULE LEUCOCYTES IN SHEEP LUNGS WITH ASCARIS SUUM

A. INTRODUCTION

Chapter two of this thesis was an attempt to show the relationship of GLs to a natural infection with parasites in the lungs of sheep. It was done by comparing the lungs in different groups of worm-free and parasitized sheep. Because such a relationship was shown to exist, this chapter reports on an investigation to produce GLs in the lungs of parasite-free sheep by experimental infection with a parasite. The parasite chosen was Ascaris suum.

Ascaris suum is one of the most common and important parasites which frequently occurs in the intestine of the pig (Ransom and Foster, 1919). The life cycle of A. suum was first marked out by Stewart (1916, 1918) in rabbits and guinea pigs during experimental infection. The infected eggs, after being swallowed by pigs or other species of animals, hatch and the infective larvae move through the intestinal wall and enter the blood stream; in this they are carried to the liver, heart, and then to the lungs. (Lapage, 1965). In the lungs of these animals the migrating larvae cause cell degeneration, haemorrhage and exudate into the alveoli producing areas of consolidation (Kennedy, 1954). Larvae, if not destroyed in the lungs, gradually move up the airways eventually reaching the trachea from where they are coughed up into the larynx and then swallowed to re-enter the gastrointestinal tract. Patency is reached in the small intestine, the whole life-cycle taking 54-62 days (Lapage and Muncey, 1952).

In an abnormal host the migration of the larval stage of A. suum has been recognized for many years. Kaino (1922) showed that man is one of the mammals in which the larvae of A. suum will migrate. He swallowed infected eggs and produced, in himself respiratory symptoms due to the effects of the larvae on his lungs. A few cases have been described in which A. suum have developed to maturity in the intestine of heterologous hosts such as lambs (Andrew, 1932; Johnson, 1963; Mcdonald and Chevis, 1965) and cattle (Raveus and Christensson, 1977).

In lambs it was concluded that after experimental infection with A. suum eggs, the larvae followed the same pattern of migration as in other species (Fitzgerald, 1962; Hayat, Rehman and Ahmed, 1973).

Clinical signs of dyspnoea, expiratory grunt, elevated respiratory rate and a frequent cough have been seen in calves and attributed to the effect of A. suum larvae migrating through the lungs (Allen, 1962). Experimental infection of calves has shown a correlation between the occurrence of these clinical signs and the presence of larvae in the bronchopulmonary tissues (Greenway and McCraw, 1970).

The mast cells are normal connective tissue elements, while GLs are absent from the tissues of parasite-free animals (Sommerville, 1956). In parasitic infections GLs occurred frequently within the epithelium of different mucous membranes and several studies have shown that the mast cells increased in number too (Wells, 1962; Fernex, 1968; Rahko, 1971).

The experimental induction of GLs in the mucous membrane of the rat small intestine has been studied in N. brasiliensis infection (Whur, 1966b; Miller et al., 1967; Murray et al., 1968), in the wall of bile ducts in fascioliasis of mice (Rahko, 1971) cattle (Rahko, 1970b; 1971) goat (Rahko, 1972) and sheep (Murray et al., 1968; Rahko, 1970a) and in dicrocoeliasis of cattle (Rahko, 1970b) and sheep (Rahko, 1970a). In the abomasum of sheep GLs were induced by experimental infections with H. contortus, T. colubriformis and Ostertiagia circumcincta (Sommerville, 1956). While in the small and large intestine of sheep these cells were induced by experimental infection with O. columbrianum (Dobson, 1966b). On the other hand, the literature reviewed did not include information on the induction of GLs in the respiratory tracts of sheep in association with parasitic infection.

The present experiments were undertaken therefore to study the effects of a parasite that would migrate through the pulmonary tissues of sheep on the induction of GLs in their respiratory tracts. Accordingly sheep were infected, in several different experimental procedures, with A. suum

and their lungs were examined for the presence of GLs and the presence of pulmonary lesions produced by this parasite :

1. The effect of sensitizing and challenge doses of A. suum larvae given by the intrapulmonary route.

2. The effect of multiple sensitizing oral doses of A. suum eggs.

3. The effect of low repeated oral doses of A. suum eggs given to a group of lambs over prolonged periods.

4. The effect of a sensitizing and challenge oral dose of A. suum eggs on 4 groups of lambs to determine the accurate time required for the appearance and disappearance of GLs in sheep respiratory tracts.

5. A comparison between the population and distribution of these cells in sheep respiratory tracts, experimentally infected with A. suum eggs, and those lungs of sheep which showed natural parasitic lesions (Chapter 2, C) were carried out.

6. The gross and microscopic lesions in the respiratory tracts of sheep induced by A. suum infection were also studied.

B. MATERIALS AND METHODS

The experimental animals and the techniques employed are described below.

1. Experimental Animals

Nine groups of lambs were designated as group A1, A2, A3, A4, A5, A6, A7, A8 and A9. Each group consisted of 2 lambs. The details of the experimental design have been given in Table (11). A total of 18 lambs were purchased from the Veterinary School, Cochno Field Station. They were obtained when they were less than 4 weeks old. To avoid the problem of adventitious helminth infections, all the animals were reared indoors and were kept in large concrete floored pens which were cleaned, disinfected, dried and bedded down with wood shavings 2-3 times a week. Special clothing, utensils and other equipment were provided for use in the animal house. Access was strictly limited to authorised persons in order to ensure that there was no contact between this house and any other animal accommodation. All animals were clearly identified with numbered ear tags.

All groups of lambs except group A1 were infected orally, using a 20 ml plastic syringe to which was attached a 75 mm. length of polyvinyl chloride tubing. A.suum eggs were suspended in 10 ml of distilled water and the whole volume was expressed into the throat of the animal followed by flushing the syringe and tube with water. All indoor experimental lambs were starved for 24 hours before infection. Lambs in group A1 were infected by intrapulmonary inoculation with A. suum larvae by an endo-bronchial catheter passed while the animals were anaesthetised.

Details of the experimental groups have been given below.

Group A1

Two lambs, SM133 and SM134, were used to study the effect of intrapulmonary inoculation of infective second stage larvae of A. suum Table (12). These lambs received a sensitizing dose of 5,000 larvae by the intrapulmonary route into the right diaphragmatic bronchus. The dose

was administered as a 20 ml solution, using a sterile plastic syringe and catheter. The animals were anaesthetised by intravenous injection of methohexitane sodium (Brietal Sodium, Elanco Product Ltd.) at the rate of 6 mg per kg. body weight. The plastic catheter was inserted into the bronchus while watching the screen of a Fluoroscope (9" x 5") Philips Immage Intensifire; this was coupled to a 200 Kv. 1000 m A. (Elema Schonander) X-ray generator. These lambs were challenged with 200,000 second stage larvae, given by the intrapulmonary route, 4 weeks after the sensitizing doses. They were killed 4 weeks after the challenge dose.

Group A 2

This group was used to study the effect of 2 sensitizing doses and a challenge dose of A. suum eggs given orally Table (13). Two lambs, SM139 and SM140 were used. The sensitizing doses were 200,000 eggs followed by 100,000 eggs orally. The challenge dose was 1,000,000 eggs administered orally. The interval between the doses was 3 weeks. This group was killed 4 weeks after the challenge dose.

Group A3

This group was used to study the effect of repeated low oral doses of A. suum eggs over prolonged periods. The two lambs in this group, SM135 and SM136, received an oral dose of 50,000 eggs weekly for at least 7 weeks and were killed 3 weeks after the last dose Table (14).

Group A4

Lambs SM137 and SM138 in this group were used to study the effect of a low oral sensitizing dose and a high oral challenge dose of A. suum eggs. They received a dose of 100,000 eggs and after 3 weeks they were challenged with 10 million A. suum eggs. These lambs were killed 6 weeks after the challenge dose Table (15).

To determine an accurate time for the appearance and disappearance of GLs in the respiratory tract of sheep the following groups received the same programme of sensitizing and challenge doses of A. suum eggs

orally. These groups were killed at serial intervals 2, 4, 6 and 8 weeks after the challenge dose.

Group A5

This group consisted of 2 lambs SM425 and SM429. They received a sensitizing dose of 50,000 A. suum eggs orally, and a challenge dose of 3.5 million A. suum eggs after 3 weeks. They were killed 2 weeks after the challenge dose Table (16).

Group A6

This group consisted of 2 lambs SM424 and SM426; they received the same programme of doses as group A5 but they were killed 4 weeks after the challenge dose Table (16).

Group A7

The 2 lambs in this group, SM432 and SM435 received the same programme of doses as in group A5 but they were killed 6 weeks after the challenge dose Table (16).

Group A8

This group received the same doses as in group A5 but were killed 8 weeks after the challenge dose. This group consisted of 2 lambs SM439 and SM440 Table (16).

Group A9

Two lambs, SM145 and SM146, at the same age as the above groups were used as a control group for this experiment.

After infection of the experimental lambs, routine clinical tests were carried out twice a week. These included rectal temperature, respiratory rate per minute, pulse rate per minute and fecal examination; blood was collected from the jugular vein in vacuum vials containing (EDTA). These tests were carried out twice weekly. Smears for differential white cell counts were made directly from the blood and they were stained with Giemsa. Packed cell volumes were determined with a Clay-Adams microhematocrit centrifuge (Archer and Hirsch, 1963). Total white cell counts were made

using the Coulter counter system.

2. Laboratory Culture of Ascaris suum

a. Extraction and culture of eggs.

Mature female A. suum worms were obtained from the small intestines of newly slaughtered pigs. These worms were washed with normal saline 3-4 times. The vagina and proximal 15-20 mm. of the uteri (Fig. 58 and 59) were removed from each worm and the eggs were expressed from these structures by gentle crushing with a mortar.

The thick outer albuminous layer of the egg (Fig. 60) was removed by a method similar to that described by Fairbairn (1955). The eggs were placed in a homogenizer with sodium hypochlorite for 10 minutes (10 ml. of sodium hypochlorite to each 100 ml. of egg suspension). The mixture was allowed to stand for 30 minutes at room temperature until no outer albuminous layer was seen on the eggs (Fig. 61). The eggs were washed twice and spread thinly on wide plastic petri dishes (10-14 cm) containing 1 per cent. formalin to a depth of 3 mm. and covered. These eggs were incubated at 26°C. Generally, at least 46 days incubation was required for infectivity to be attained.

b. Harvesting of eggs

Incubated eggs were examined, under the microscope, periodically to check the viability of the larvae which could attain their infectivity at the end of 46 days (Fig. 62). Eggs were collected in a 100 ml beaker washed twice, concentrated and counted.

c. Counting of eggs

Infective eggs were not administered to animals, unless accurate counting was carried out. This was done by serial dilution of the stock bottle of the egg suspension. A volume (0.025 ml.) from the final dilution of egg suspension was spread on a slide using a standard pipette. This process was repeated at least 12 times. All the eggs in each 0.025 ml. were counted on each slide with X25 magnification using a stereoscopic microscope. The average number in these 12 slides was calculated and

then multiplied by the number of dilutions and this gave the total number of eggs/ml. in the stock storage bottles.

d. Determination of the incubation period required for eggs to attain their infective state.

In order to determine the accurate period of incubation which the eggs required to become infective a group of 34 guinea pigs was inoculated orally with eggs incubated for different periods Table (17). Each guinea pig was killed 7 days after infection and Baermann preparations were made of liver and lung. The rate of larval recovery was obtained for the lungs and liver of each animal in this group. The peak of larval infectivity of the A. suum eggs were recorded when the highest percentage of larval recovery was obtained Table (17).

c. Testing infectivity of eggs

Following determination of the incubation period for the cultures of infective A. suum eggs, eggs were not used for dosing any sheep in these experiments until the eggs were tested for infectivity by recovering larvae from laboratory animals (Fig. 63). Test animals, guinea pigs or rabbits, were starved for 24 hours and dosed with 3,000-5,000 eggs in a 1 ml. suspension. The guinea pigs or rabbits were killed after 7 days and Baermann preparations made of their livers and lungs. Most larvae were found in the lungs by this time after infection.

f. Hatching of eggs

Ascaris suum eggs were decoated with 0.5 per cent. pepsin, washed and concentrated in preparation for hatching. To liberate the second stage larvae from the eggs a number of methods were used as follows:

i. Digestion by pancreatin

A method similar to that described by Jaskoski and Colucci (1964) was used. The decoated eggs were placed in Erlenmeyer flasks containing a 1 per cent. solution of freshly prepared pancreatin. The flasks were placed in a shaker - bath incubator at 37^{9}C. (Grant Instruments Cambridge Ltd.)

The addition of 20-30 glass beads to each flask assisted in the mechanical rupture of the egg shells. A solution of 2 per cent. pancreatin was also tested.

ii. Digestion by pepsin

Decoated eggs were treated with 1 per cent pepsin using the same method as described above for pancreatin.

iii. Digestion by trypsin

One per cent trypsin was used to hatch the decoated eggs as described by Jaskoski and Colucci (1964).

iv. Bile hatching

Decoated eggs were treated by bile procedure for hatching as described by Jaskoski and Colucci (1964).

v. Carbon dioxide hatching

Jaskoski and Colucci (1964) used carbon dioxide to hatch eggs by bubbling it through flasks containing decoated eggs, at a rate of 1 cubic foot per hour, while the flasks were in a shaker-bath incubator at 37°C.

The hatched eggs in any method described above were immediately washed in 1 litre of normal saline at 37°C. The liberated larvae were Baermannized, washed 3-4 times with sterile PBS and concentrated. For sterilization of the larvae 100μ of penicillin G and 100 mg. of streptomycin per ml. larval suspension was used. Larval counts were carried out as on page (149).

3. Pathology

The respiratory tracts were examined for (a) GLs and mast cells and (b) pulmonary lesions associated with the experimental infection; the other internal organs were also examined for lesions. For histological examination eight pieces of tissue representing the standard sites of lung and bronchial tree (Fig. 1) were collected along with routine samples of tissues from lung around the bronchi and from other organs also. Tissues

were fixed, embedded and stained as in Chapter 2B. for the detection of GLs and mast cells. Haematoxylin and eosin stained sections from corrosive formol fixed blocks were used for the routine detection of GLs. When particular morphological changes in the GLs were to be demonstrated more clearly, special stains were employed. These were toluidine blue, alcian blue and astra blue/safranin, biebrich scarlet, Martius scarlet blue and the carbol chromotrope method. Preparation of reagents and staining techniques are given in Appendix I.

C. RESULTS

1. Determination of the Incubation Periods of Eggs

The minimum time required for A. suum eggs to attain infectivity was 40-45 days when incubated in 1 per cent formalin at 26°C. The highest percentage of larval recovery from animals infected with eggs kept for this period was 15.33 per cent Table (17).

2. Hatching of the Eggs

Digestion with 1 per cent pancreatin gave the best overall result for hatching of A. suum eggs. Within the first 3 hours of digestion with this enzyme, 70 per cent of the decoated eggs were hatched Table (18). Although digestion was terminated after 3 hours most of the larvae died after this time if they were not washed with distilled water.

Digestion with 1 per cent pepsin or trypsin failed to give any successful results. Digestion with bile was employed as described by Jaskoski and Colucci (1964), but no hatching was obtained. The carbon dioxide method described by the above authors was used and most of the eggs gave viable larvae but they died very shortly after hatching.

3. Haematology

The result of sheep SM137, SM138, SM139 and SM140 which were considered to be typical of the response to A. suum infection are given in Appendix II. The peripheral blood eosinophilia showed a characteristic response in lambs infected with a sensitizing and challenge dose of A. suum eggs. Peripheral blood eosinophilia reached 6-8 per cent on day 8-9 after infection with the first sensitizing dose and 14 per cent on day 11 after infection with the second sensitizing dose as seen in lambs SM139 and SM140. There was a high peak of eosinophilia which reached 20-35 per cent on day 9-11 after the challenge dose.

4. Globule Leucocytes and Mast Cells

Experimental group

Globule leucocytes were not detected in the respiratory tract of lamb SM133 in group A1, Table (19) which received a single intrapulmonary infection of A. suum larvae. On the other hand lamb SM134 in the same group had GLs in the bronchial epithelium of its lung after the sensitizing and challenge intrapulmonary doses of A. suum larvae. In group A2 which received two sensitizing doses and a challenge dose, only lamb SM139 had GLs in its lungs, when examined 5 weeks after the challenge dose Table (19). .

Group A3 was infected with 7 repeated doses of A. suum eggs. Both lambs in this group had GLs in their lungs when examined 3 weeks after the last dose. Globule leucocytes occurred in the lungs of lamb SM137 of group A4. This group received single low sensitizing doses and a high challenge dose. They were killed 6 weeks after the challenge dose.

Group A5-A8 dosed with the same programme of sensitizing and challenge doses Table (20), were killed at different time intervals to check the possible time for the appearance and disappearance of Gls in their respiratory tracts. Only the lambs in group A6 which were killed 4 weeks after the challenge dose had GLs in their respiratory tracts. No GLs were seen in group A5, A7 and A8 which were killed 2, 6 and 8 weeks post-infection respectively.

Typical GLs in A. suum infection were present in varying positions within the epithelium of the respiratory tracts of lambs. Although few immature cells were found in the lamina propria of the bronchi. The distribution of these cells in lambs infected with A. suum infected eggs were concentrated in the bronchial epithelium of their respiratory tracts Table (19 & 20). A few GLs were found in the tracheal and bronchiolar epithelium. Although no cells were seen in the glands or their duct epithelium.

Few GLs in A. suum infection showed striking differences in the structure of the intracytoplasmic globules (Fig.64 & 65). The globules were usually liable to coalesce, forming more or less large aggregations in the cytoplasm of the cells in most fixatives employed. An attempt using stains other than H & E (TB, AB/S, BS, CC) showed that neither the cells, nor the contained globules, attained as great a size as those found in the later stages of natural parasitic infection. In many cases a X100 oil immersion objective was necessary to confirm the presence of the globules. The nucleus of the GLs appeared eccentric in position and elongated. Globule leucocytes were readily differentiated from eosinophilic leucocytes, which were sometimes present in the epithelium but displayed only small granules in the cytoplasm.

To confirm that the cells observed by H & E stain were GLs identical to those described in other species (Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968), certain techniques were used Table (2). The reaction in the globule of GLs and the granules of both mast cells and eosinophil leucocytes obtained here was compatible to that described in Chapter 2B which distinguished the three cell types.

In those lungs of the lamb in group A1 (SM133) which received a single infection with A. suum larvae, a minor increase in the number of mast cells were observed. Multiple or repeated infection with A. suum eggs produced a manifold increase in the quantity of pulmonary mast cells, particularly in the lamina propria of the infected sites of the airpassages. However, mast cells were more abundant in the connective tissues and alveolar septa of infected groups than in control group. Mast cells in the lamina propria of parasitized sheep were revealed distinctly by staining methods for acidmucosubstances Table (2). The mast cells were more or less spindle-shaped, and presented numerous small intracytoplasmic granules, which showed a tendency to coalesce in the cytoplasm. The nucleus of the cells showed variation in size, form and chromatin density.

Control group

Globule leucocytes were not seen in the respiratory tracts of the indoor worm-free control sheep. In contrast to the GLs, mast cells occurred frequently in the lamina propria and the fibro-muscular layer. These cells were found in the outer layers of the walls of blood vessels. Mast cells were normally present in the interalveolar septa and in very high numbers in the pleural and subpleural tissues.

5. Pathology

Group A1

Lambs SM133 which died under anaesthesia before inoculation with the challenge dose, had no evidence of any gross changes in its lungs.

Microscopic examination revealed thickening of the alveolar septa, infiltration with neutrophils, lymphocytes and plasma cells, some erythrocytes were also seen. A few pulmonary lesions were seen in which the alveolar sacs were filled with oedema fluid and fibrin. Lymphocytic aggregations were observed in peribronchial and peribronchiolar positions.

Lamb SM134 which was killed 4 weeks after challenge dose, had areas of consolidation on the distal and peripheral portions of the diaphragmatic lobes. The remaining portions of the diaphragmatic lobes were moderately enlarged and had patches of overinflation. A few subpleural nodules and haemorrhages were observed on the lung surfaces. Major bronchi and the trachea were filled with a frothy red exudate.

Pronounced microscopic changes were observed in the lung of this lamb. Areas of consolidation and parasitic granuloma were found in diaphragmatic lobes, while the cranial lobes had overinflation. The subpleural nodules consisted of a mass of lymphocytes surrounded by eosinophils and erythrocytes. Bronchitis and bronchiolitis with infiltration of the bronchial and bronchiolar epithelium by neutrophils and lymphocytes were detected in this lamb.

Group A2

Both lambs exhibited similar gross changes in the lungs. These consisted of scattered areas of consolidation mainly in the ventral portions of the diaphragmatic lobes, interspersed with many small subpleural nodules. These nodules were yellow-brown having translucent centres measuring 2-3 mm. in diameter. The lungs were rubbery on palpation. The cut surface through the lung showed extensive bronchitis and oedematous fluid mixed with blood were oozing from such sections. The liver showed white milky spots distributed throughout its surfaces. These spots were 2-3 mm. in diameter, surrounded by an area of fibrosis, which underwent calcification.

On microscopic examination, the interstitial reaction and the subpleural nodules showed the same picture as in the previous lamb. There was bronchitis which was haemorrhagic in nature and the bronchial lumen contained exudate mixed with blood. Parasitic granulomas were numerous and they sometimes contained disintegrated larvae at their centres.

Group A3

The lambs in this group had minute nodules less than 1.0 mm in size, distributed on the subpleural surfaces of their lungs. These nodules were numerous and covered all the lung lobes. The lungs failed to collapse and were grey to red in colour. The ventral surfaces of the lobes were firm and consolidated. The liver had several white foci about 2.0 mm in diameter. No gross lesions were seen in other organs.

Microscopical examination of the lung detected diffuse interstitial pneumonia with focal lesions consisting of lymphocytic and eosinophilic alveolitis. The interlobular septa were thickened, dilated and contained eosinophils. The nodules, found at necropsy, consisted of a mass of lymphocytes surrounded by a thin fibrous capsule and a layer of eosinophils.

Group A4

Two lambs in this group were necropsied 6 weeks after the challenge dose. The predominant changes were large subpleural white to grey nodules on the whole surface of the lung lobes; the lobes themselves were rubbery in consistency and pale in colour. The cut surface revealed mild bronchitis and the bronchi were filled with foamy mucus. No other gross changes were observed. The liver showed many large calcified areas surrounded by fibrosis.

Microscopically the large nodules found in this group consisted of lymphocytic aggregations with necrotic debris and eosinophils in their centres. Usually these nodules were encapsulated by a fibrous layer. The interlobular septa were dilated with oedema fluid and fibrin and were infiltrated with lymphocytes. In addition to the diffuse interstitial pneumonia, lymphocytic foci and peribronchial lymphocytic infiltration were observed.

Group A5

The lambs in this group were killed 2 weeks after the challenge dose and both exhibited similar gross changes in their lungs. They were firm in consistency and deep red in colour. Oedema fluid was present in the interlobular septa of all lobes, while the cut surface was uniformly dark red and of a meaty texture. A frothy red exudate was present in the trachea and bronchi. There was widespread distribution of subpleural miliary nodules throughout the lung surfaces (Fig. 66). No lesions of consolidation or overinflation were seen in the lungs of this group. The liver had numerous subcapsular white foci about 1-2 mm. in diameter.

Pronounced microscopic changes were observed in the lungs of these lambs. The main lesion was that of pulmonary eosinophilia but small patches of exudative pneumonia were also found. The interlobular septa contained eosinophils, lymphocytes and neutrophils, in great numbers. In this area they accumulated along the swollen lymphatics where they

had a tendency to form streaming patterns. Alveolar walls of other lobules were diffusely thickened and many alveoli were filled with a proteinaceous exudate. Mononuclear cells were present in this exudate, but eosinophils were seldom observed in alveoli. Bronchi and bronchioles, near areas of alveolar exudation, usually contained mucus admixed with polymorphonuclear leucocytes (Fig. 67). The lamina propria and the epithelium of both the bronchi and the bronchioles were infiltrated with neutrophils and lymphocytes. Subpleural nodules were observed, and while the majority of these contained mostly mononuclear cells, others had a mixture of eosinophils, mononuclear cells and giant cells. A few disintegrated larvae were also seen in the centre of some nodules.

Group A6

Both lambs of this group were necropsied 4 weeks after the challenge dose. Their lungs had marked consolidated patches on the ventral surface of the diaphragmatic lobe. The miliary subpleural nodules found in previous groups were not present in this group, although a few larger nodules were present in the diaphragmatic lobes of these lungs. Abundant subpleural haemorrhages were observed and the cut surfaces were mottled with red and grey spots. A thin yellow exudate oozed from the cut surface. The remaining parts of the diaphragmatic lobes were moderately enlarged by alveolar overinflation. The trachea and bronchi were filled with a frothy exudate. The liver had a massive distribution of "white milk spots" surrounded by areas of fibrosis and in addition there were haemorrhagic spots.

Microscopic examination revealed similar pathologic changes to those seen in previous groups. The subpleural nodules however, were very scarce and consisted of focal lymphocytic aggregations (Fig. 68 & 69). The consolidated lobules had more fibrin deposit within the alveoli. The exudate in alveoli consisted mainly of lymphocytes and macrophages with slight numbers of eosinophils, and the exudative lesions were undergoing resolution. The interlobular septa were more thickened, distended and filled with fibrin and lymphocytes, but no eosinophils, when compared

with the lungs of the animals in group A5 (Fig. 70).

Group A7

Lambs in this group received the same programme of doses as in the last 2 groups, but they were necropsied 6 weeks post-challenge infection. Both lungs were pale in colour, rubbery in consistency and collapsed. Six raised, spherical, white to grey subpleural nodules, 4-6 mm. in diameter, were found scattered over the lung surface. Scattered areas of collapse, mainly in the ventral portions of the diaphragmatic lobes, interspersed with a few darker consolidated lobules were observed. The interlobular septa were thickened. Cranial lobes showed consolidated patches surrounded by an area of overinflation. In the liver of these lambs there was pronounced fibrosis surrounding a few parasitic nodules. Haemorrhagic spots in the subcapsular position occurred as well.

Microscopically there was alveolar collapse and rupture of the alveolar septa in most lobules examined. The bronchopulmonary exudate was undergoing resolution and organization.

Group A8

The dosing programme was similar to that in group A5, but the lambs were killed 8 weeks after challenge dose. Both lambs exhibited similar gross changes. The lungs were pale to grey in colour with scattered areas of atelactasis throughout the dorsal surfaces of the diaphragmatic lobes. Five elevated, spherical translucent subpleural nodules, 4-7 mm. in diameter were found scattered over the lung surface (Fig.71). The cranial lobes had areas of consolidation and over-inflation. The livers of both lambs had fibrotic lesions but the "milk spots" were not seen.

Microscopically there was thickening of the alveolar septa with lymphocytic and macrophage infiltration. The alveolar airspaces and the lumen of the bronchi and bronchioles contained no exudate. There was alveolar collapse with shrinking of the lobules. The interlobular septa

were slightly thick, and contained lymphocytes, macrophages and fibroblasts. There were no subpleural lymphocytic aggregates, while the nodules consisted of a mass of lymphocytes surrounded by a delicate fibrous capsule. Pasteurella haemolytica was isolated from pneumonic lesions in the cranial lobe.

D. DISCUSSION

The development of GLs in the bronchopulmonary epithelium of sheep following experimental infection with a parasite that migrates through the lungs, was demonstrated in the present study. It was also shown that GLs were not normally present in the bronchopulmonary system of parasite-free sheep. They were seen frequently following A. suum infection. This agrees well with previous studies on the occurrence of GLs in the intestinal and biliary tracts of sheep after experimental parasitic infection (Dobson, 1966b; Whur, 1966a; Murray et al., 1968; Rahko, 1970b).

The present investigation revealed that, in most cases, no differences existed between the structural and histochemical reactions of ovine GLs in ascariasis and natural lungworm infections of sheep as studied in Chapter 2 of this thesis. In a few instances, however, the GLs and their contained globules showed marked differences in structure and size. The globules were often liable to coalesce, forming large aggregations in the cytoplasm of the cell which partially or completely covered the nucleus. The failure of these cells and their globules to attain as great a size as those found in the later stages of natural parasitic infection might be attributed to the migrating larvae of A. suum which remained in the lungs of sheep for only 9 days (2-11) post-infection or due to a variation in the ability of A. suum larvae to degranulate mast cells compared with the adult worms. This was observed in our morphological studies on the GLs in Chapter 2 (Table 4) in which the GLs in the sheep lungs infected with adult worms had larger and more rounded globules than the cells of sheep infected with larval stages which were smaller in size and showed coalesced globules which partially masked the cell nucleus.

Globule leucocytes were not seen in the lungs of lamb SM133 which had received a single intrapulmonary infection of A. suum second stage larvae. On the other hand lamb SM134 had GLs in the bronchial epithelium of its lung after the administration of sensitizing and challenge intra-pulmonary doses of A. suum larvae.

In group A2 which received 2 sensitizing doses and a challenge dose, only lamb SM139 had GLs in its lungs when examined 5 weeks after the challenge dose. Lamb SM140 in the same group had no GLs in its bronchopulmonary system. This might be due to the development in the lamb of sufficient immunity from the sensitizing doses to prevent the larvae reaching the lungs on challenge dose. In addition the number of GLs may have been so small that the technique used failed to detect them. It was interesting that examination of the intestine and liver from lamb SM140 revealed numerous GLs in these situations. On the other hand, lamb SM139 which had GLs in its bronchopulmonary system had no GL throughout the whole route of larval migration (intestine and liver).

Group A3 was infected with 7 repeated low doses of A. suum eggs. Both lambs in this group had GLs in their lungs when examined 3 weeks after the last dose. It thus seemed that the administration of low and repeated doses over a prolonged period was a useful method of inducing GLs in the lungs of lambs. Since only one lamb in group A4 had Gls after only one sensitizing dose, it was interesting that the lamb lacking GLs in its lung had numerous GLs in its intestine and liver. This was similar to the findings of lambs in group A2.

It may be concluded, however, that the time of examination after the last dose was different, since lambs in group A2 and A4 were examined 5 and 6 weeks respectively after challenge dose, while lambs in group A3 were examined 3 weeks after the challenge dose. To investigate this aspect of GL development groups A5-A8 were studied. From these animals, it appeared that the optimum period required to observe the maximum numbers of GLs after one sensitizing dose and one challenge dose was 4 weeks.

Low repeated doses probably most resembles a natural infection. Dobson (1966b), however, associated the appearance and the increase in number of GLs in tissues of the sheep gut with multiple infestations which were related to the duration of the first infection. In animals slaughtered at 1 and 2 weeks post-infection the numbers of GLs were low, while in

animals slaughtered at 10 weeks after infestation large numbers of these cells were found. This relationship was not so evident in sheep with multiple infestations, because there were a large number of GLs already in the tissues as a result of a previous infestation (Dobson, 1966b).

It was likely that during A. suum migration in the lungs of sheep, which may last about 9 days, the GLs appeared in the epithelial mucosa for a certain time after which, the cells, together with the contents of the globules, which may be antibody (Dobson, 1966a; Whur and Gracie, 1967) or vasoactive amine (Murray et al., 1968), were finally released into the lumen of the organ where they may be effective against the worm population.

In A. suum experimental infection the distribution of the GLs was mostly concentrated in the bronchial epithelium and few GLs were found in the tracheal epithelium. This could be explained in that most of the larvae were destroyed during their migratory route before reaching the trachea.

The present investigation revealed an enormous increase in the number of mast cells in the lamin propria and submucosal layers. These findings were supported by previous works on the appearance of the intestinal mast cells (Wells, 1962; Jarrett et al., 1967; Miller et al., 1967; Murray et al., 1968). Similar studies have also shown that in experimental fascioliasis of cattle and mice the mast cells in the biliary tracts increased significantly in number (Rahko, 1971).

The present experiment indicated that A. suum infected eggs hatched in the intestine of lambs and that the larvae migrated from the intestine through the liver to the lungs where they became third stage larvae on 2-11 days.

The predominant cellular response of a host to helminth infection has been frequently characterized by the presence of a marked eosinophilia (Soulsby, 1961). Results of the present study indicated that circulating eosinophilia was increased in sheep experimentally infected with A. suum eggs. This agreed well with many investigators who have reported an

increment in number of circulating eosinophils in A. suum infection of sheep (Fitzgerald, 1962; Hayat et al., 1973), cattle (Kennedy, 1954; Greenway and McCrow, 1970) and pigs (Litt, 1964; Moncol and Batte, 1967). The increment in the number of circulating eosinophils in most species examined occurred between 8-16 days after infection with A. suum eggs (Fitzgerald, 1962; Hayat et al., 1973; Moncol and Batte, 1967) and this increase was generally greater after challenge infection (Kennedy, 1954; Greenway and McCrow, 1970).

In the present study, eosinophils were found in abundance in the lungs of lambs which were killed 2 weeks after challenge dose. They frequently formed streaming patterns along lymphatics in the interlobular septa. The eosinophil was a prominent leucocyte in sheep (Fitzgerald, 1962) and cattle infected with A. suum (Kennedy, 1954; McCrow and Greenway, 1970; McLennan, Humphris and Rac, 1974). This effect was also noticed by Vaughn (1953) who gave A. suum extracts to non-sensitized guinea pigs and observed these leucocytes in "tiny processions".

It was surprising to note that the eosinophilia rapidly resolved and by 4 weeks after the challenge dose it had been completely replaced by a lymphocytic reaction. By this time miliary subpleural nodules had subsided while a few larger nodules were present in the lungs of this group. Subpleural lymphocytic nodules have been reported in lambs experimentally infected with A. suum egg (Fitzgerald, 1962). Similar nodules were seen in Metastrongylus elongatus infection of pigs (Mackenzie, 1958) and Dictyocaulus viviparus infection of cattle (Jarrett and Sharp, 1963). In pigs the nodules were said to be more numerous in long-standing infections and were a feature of post-patent disease (Mackenzie, 1958). In cattle they were more likely to be found following vaccination with X-irradiated larvae (Jarrett and Sharp, 1963), treatment with anthelmintics (Jarrett, McIntyre and Sharp, 1962) or reinfection (Jarrett et al., 1960). It was shown that the presence of such lymphocytic nodules in the lungs of cattle was an indication of an immune response to D. viviparus (Pirie et al., 1971).

In lambs killed 6 and 8 weeks after the challenge infection few subpleural lymphocytic nodules were seen. These nodules became larger in size and diminished in number as the infection progressed.

In calves the subpleural nodules persist for at least 6 months following a double dose of X-irradiated larvae (Pirie et al., 1971). They were not seen following a single experimental infection. Unfortunately our experimental design did not include a single infection of A. suum in lambs to compare with that data.

Group	Number of animals	Method of infection	Type of infection	Week killed after last dose
A1	2	1 Sensitizing and 1 Challenge dose intrapulmonary	A. suum larvae	4
A2	2	2 Sensitizing and 1 Challenge dose orally	A. suum eggs	5
A3	2	7 Repeated doses orally	"	3
A4	2	1 Sensitizing and 1 Challenge dose orally	"	6
A5	2	" "	"	2
A6	2	" "	"	4
A7	2	" "	"	6
A8	2	" "	"	8
A9	2	control		

Table 11 : General outline of A. suum experiments in lambs.

Animal number	Week of Experiment		
	0	4	9
SM133	5,000	Killed*	
SM134	5,000	200,000	Killed

Table 12 : Group A1 lambs which were dosed with second stage larvae of A. suum by the intrapulmonary route

*This lamb died under anaesthesia before receiving the challenge dose of A. suum larvae.

Animal number	Week of Experiment			
	0	3	6	11
SM139	200,000	100,000	1,000,000	Killed
SM140	"	"	"	"

Table 13 : Group A2 lambs which received double oral sensitizing doses and one oral challenge dose of A. suum eggs.

Animal number	Week of Experiment							
	0	1	2	3	4	5	6	9
SM135	50,000	50,000	50,000	50,000	50,000	50,000	50,000	Killed
SM136	"	"	"	"	"	"	"	"

Table 14 : Group A3 lambs which received repeated low oral doses of A. suum eggs.

Animal number	Week of Experiment			
	0	3	9	11
SM137	100,000	10×10^6	Killed	
SM138	"	"	"	
SM145	Control	-	-	Killed
SM146	"	-	-	"

Table 15 : Group A4 lambs which were sensitized with low dose of A. suum eggs orally and challenge with high dose of A. suum eggs orally.

Group number	Animal number	Week of Experiment		
		0	3	Killed
A5	SM429	50,000	3.5×10^6	5
	SM425	"	"	"
A6	SM424	50,000	3.5×10^6	7
	SM426	"	"	"
A7	SM432	50,000	3.5×10^6	9
	SM435	"	"	"
A8	SM439	50,000	3.5×10^6	11
	SM440	"	"	"

Table 16 : Lambs sensitized and challenged with A. suum eggs to determine time of appearance and disappearance of GLs.

Guinea Pig number	Incubation days at 26°C	Dose	Number of larvae recovered after 8 days		% of recovered larvae
			Lung	Liver	
1	20	3,000	0	0	0
2	"	"	"	"	"
3	"	"	"	"	"
4	"	"	"	"	"
5	"	"	"	"	"
6	"	"	"	"	"
7	25	4,200	0	0	0
8	"	"	"	"	"
9	"	"	"	"	"
10	"	"	"	"	"
11	"	"	"	"	"
12	"	"	"	"	"
13	30	3,000	0	0	0
14	"	"	"	"	"
15	"	"	"	"	"
16	"	"	"	"	"
17	"	"	"	"	"
18	"	"	"	"	"

Table 17 : Determination of the incubation period required for A. suum eggs to reach infectivity in guinea pigs.

Guinea Pig number	Incubation days at 26°C	Dose	Number of larvae recovered after 8 days		% of recovered larvae
			Lung	Liver	
19	35	4,000	0	0	0
20	"	"	"	"	"
21	"	"	"	"	"
22	"	"	"	"	"
23	"	"	"	"	"
24	"	"	"	"	"
25	40	8,000	340	"	4.25
26	"	"	220	"	2.75
27	"	"	160	"	2.00
28	"	"	117	"	1.46
29	"	"	180	"	2.25
30	45	6,000	920	"	15.33
31	"	"	432	"	7.20
32	"	"	140	"	2.33
33	"	"	620	"	12.00
34	"	"	870	"	14.50
35	control	0	0	0	0
36	control	"	"	"	"

Table 17 : (contd.)

Time per hour	Percentage hatched
1.00	10
1.30	30
2.00	50
2.30	60
3.00	70
4.00	Most larvae were dead by this time

Table 18 : Percentage of hatched eggs of A. suum which digested with 1.0 per cent. pancreatin.

Group number	Animal number	Type of infection	Weeks examined post infection	Presence of GLs in the respiratory tract			
				Tracheal epithelium	Bronchial epithelium	Bronchiolar epithelium	Glands and ducts
A1	SM133	Single i/P	4	-	-	-	-
	SM134	double i/P	"	+	+	-	-
A2	SM139	repeated oral	5	+	+	+	-
	SM140	"	"	-	-	-	-
A3	SM135	repeated oral	3	+	+	-	-
	SM136	"	"	-	+	-	-
A4	SM137	double oral	6	-	+	+	-
	SM138	"	"	-	-	-	-

Table 19 : The induction of GLs in the bronchopulmonary system of different groups of sheep infected with A. suum eggs or larvae.

i/P : Intrapulmonary - : No globule leucocytes + : Few globule leucocytes

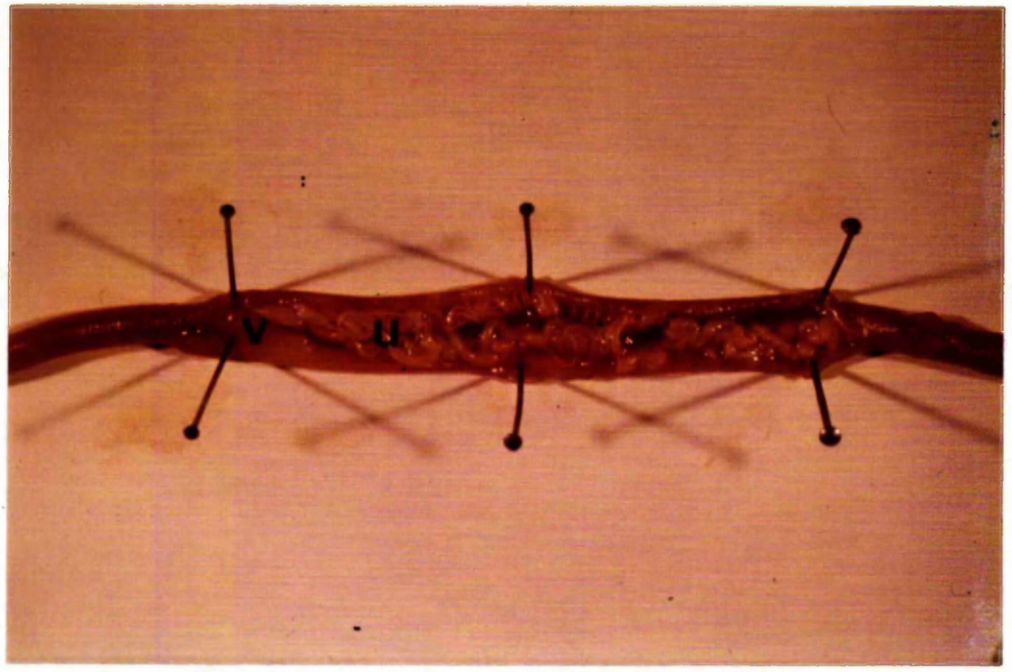
Group number	Animal number	Type of infection	Weeks examined post infection	Presence of GLs in the respiratory tract			
				Tracheal epithelium	Bronchial epithelium	Bronchiolar epithelium	Glands and ducts
A5	SM429	double oral	2	-	-	-	-
	SM425	"	"	-	-	-	-
A6	SM424	double oral	4	+	+	-	-
	SM426	"	"	-	+	-	-
A7	SM432	double oral	6	-	-	-	-
	SM435	"	"	-	-	-	-
A8	SM439	double oral	8	-	-	-	-
	SM440	"	"	-	-	-	-
A9	SM145	control	0	-	-	-	-
	SM146	"	0	-	-	-	-

Table 20 : Time required for the appearance and disappearance of GLs in sheep respiratory tracts infected with A. suum eggs.

- : No globule leucocytes + : Few globule leucocytes

Fig. 58 : Longitudinal section through an adult female of Ascaris suum to show the vaginal region (v) and the two coiled uteri. (u).

Fig. 59 : The vagina and proximal 20-25 mm. of uteri which were removed from each worm to obtain the eggs of A. suum.


V
II

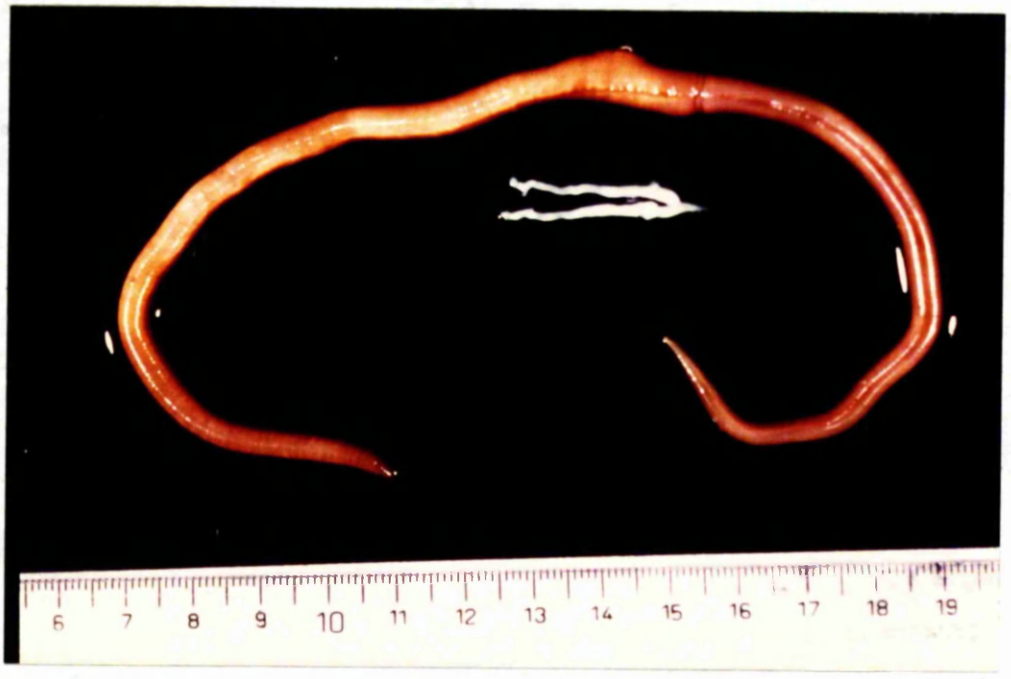

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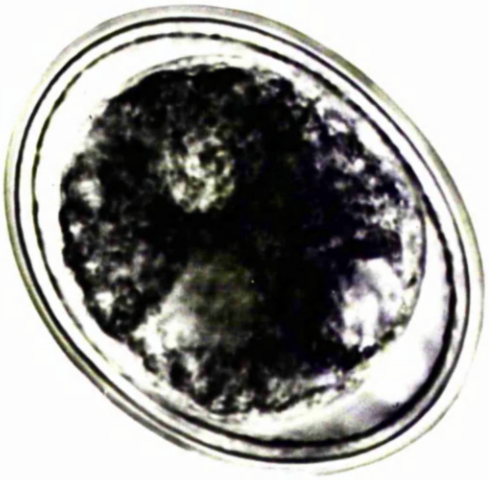
Fig. 60 : Ascaris suum egg with its rugase albuminous coat, covering the thick smooth shell.
X 400.

Fig. 61 : Decorticated eggs of A. suum after treatment with sodium hypochlorite to remove the outer albuminous layer.
X 400.



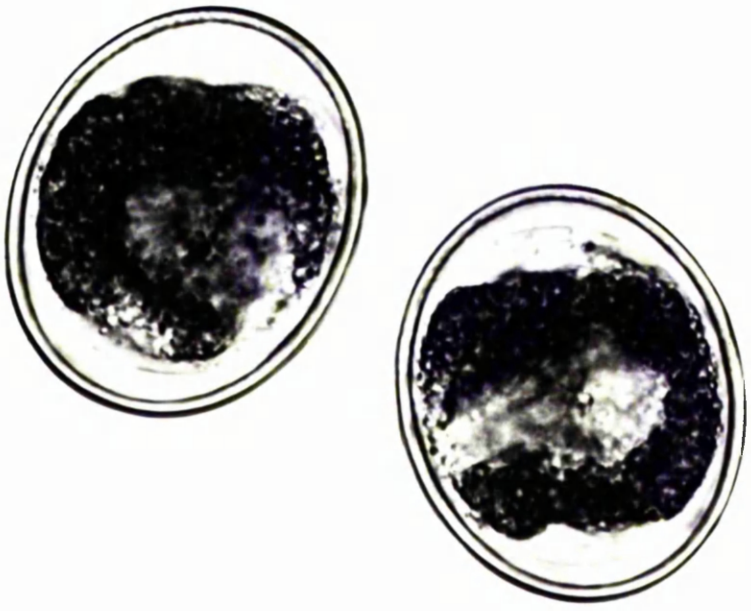
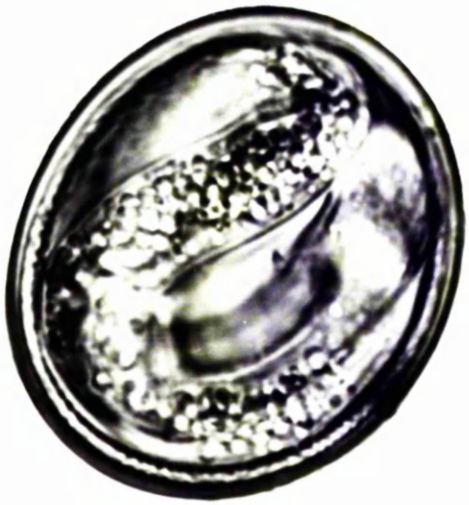


Fig. 62 : Larvated infective egg of A. suum after incubation at 26°C for at least 46 days.
X 400.

Fig. 63 : Third stage larva of A. suum recovered from lungs of laboratory animals 7 days after infection.
X 400.



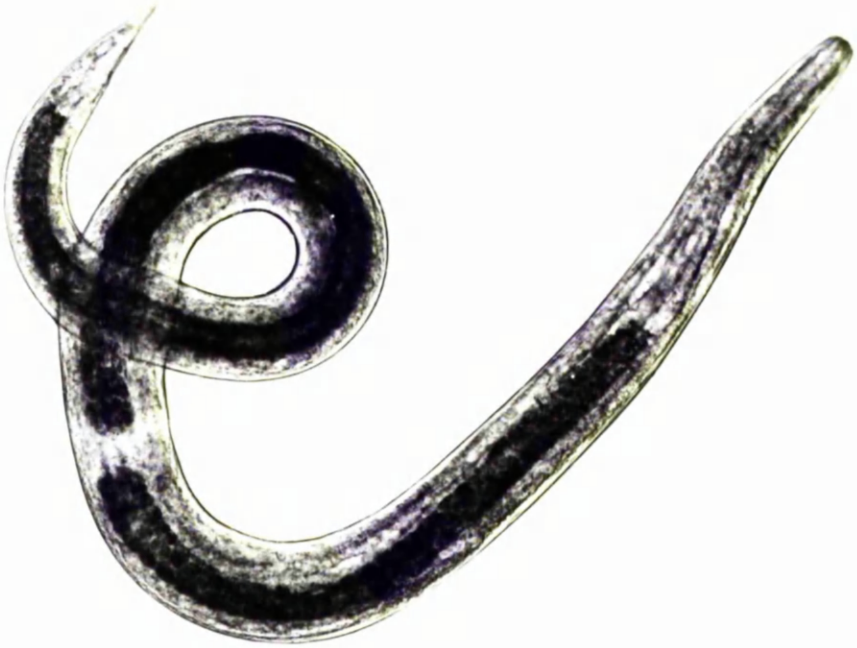
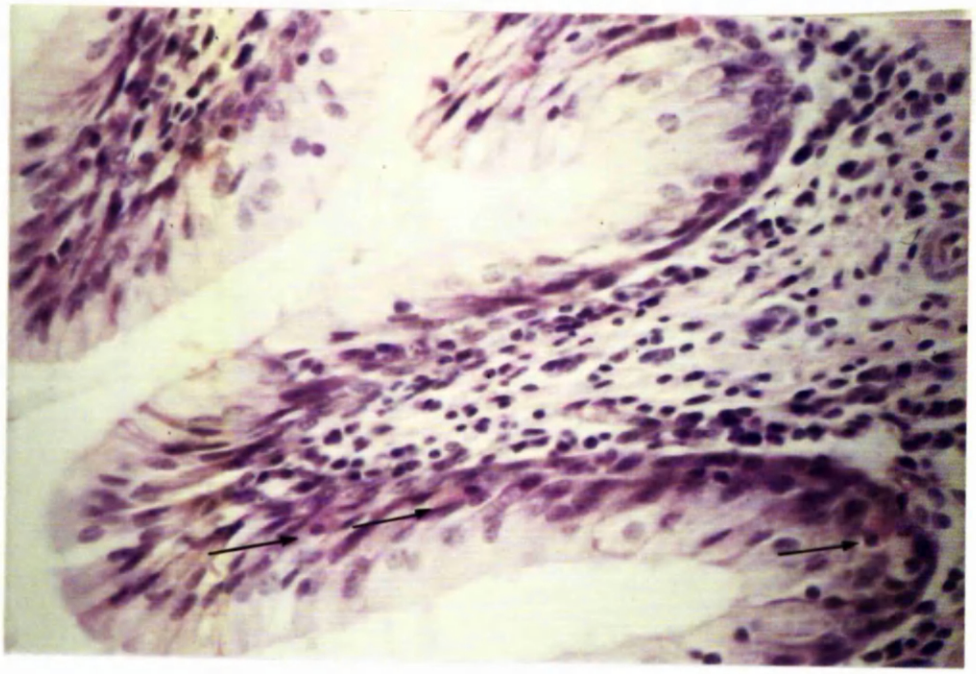


Fig. 64 : A few globule leucocytes induced in the epithelium of the bronchi (⟶) of sheep lungs with A. suum infection. The globules were usually coalesced forming an aggregation in the cytoplasm of the cells.
H & E staining, X 250.

Fig. 65 : High power view of Fig. 64. The globules of the globule leucocyte were not well formed as in the typical globule leucocyte (Fig. 23).
H & E staining, X 1000.



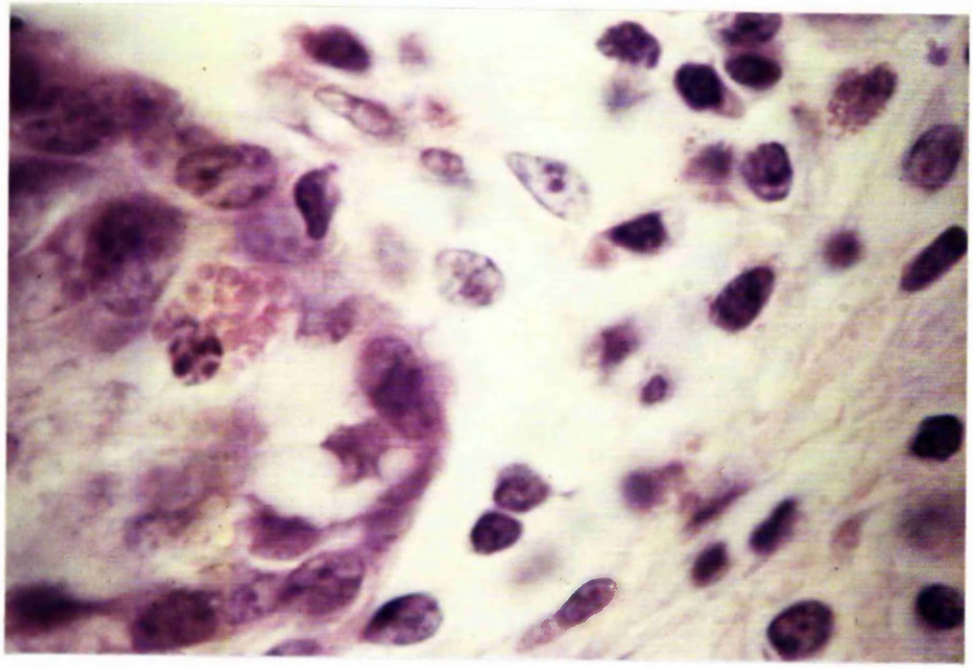
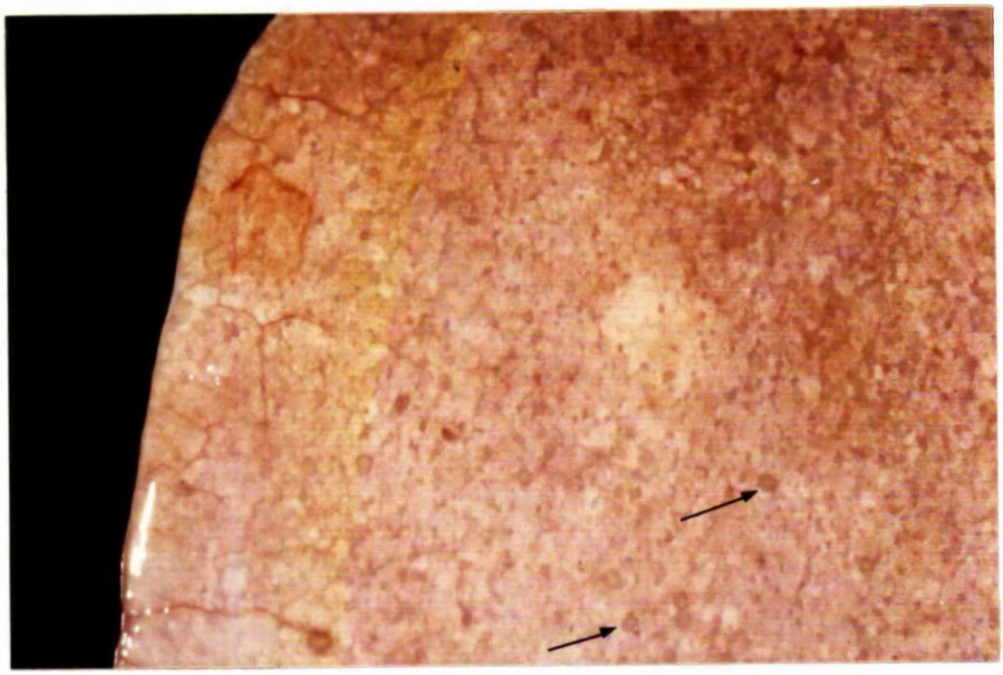


Fig. 66 : Group A5 pulmonary lesions showed typical subpleural miliary nodules (⟶) due to A. suum infection. This lesion was restricted to the lungs of lambs of this group which were killed 2 weeks postinfection.

Fig. 67 : Exudative pneumonia in a lamb of group A5 which was killed 2 weeks postinfection. The bronchiolar lumen was filled with polymorphonuclear leuocyte exudate.
H & E staining, X 100.



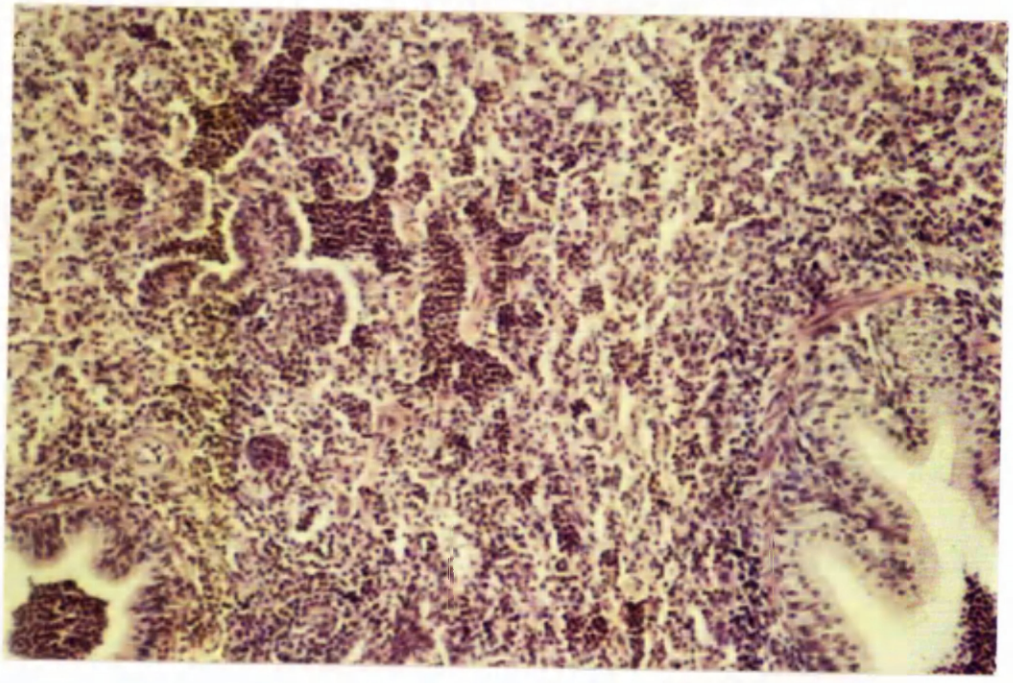
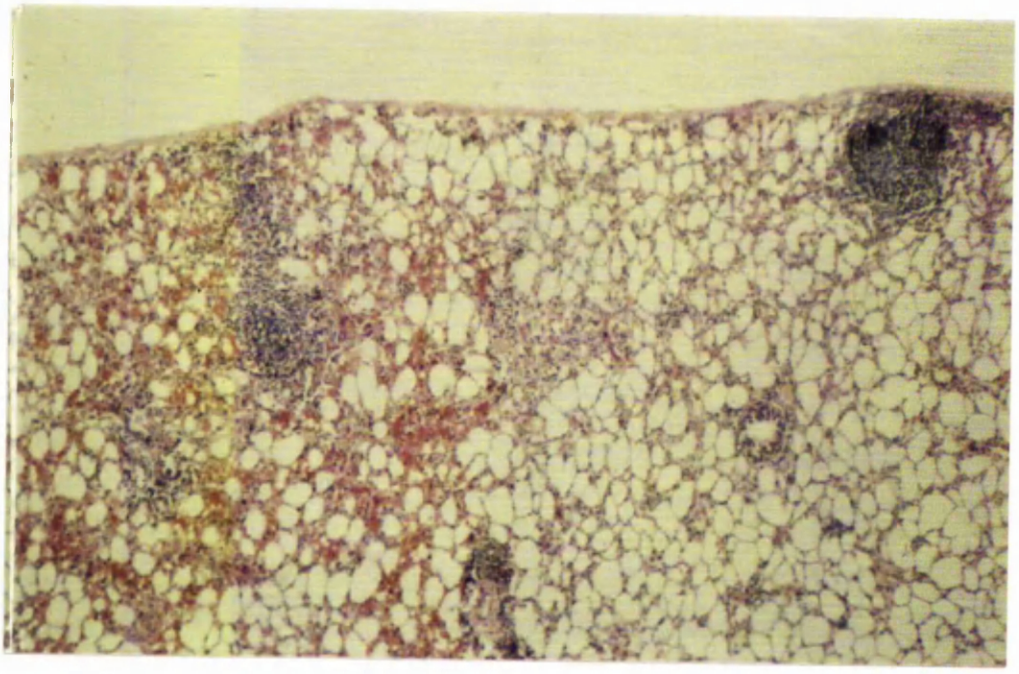


Fig. 68 : Lambs killed 4 weeks postinfection with A. suum showed subpleural parasitic nodules in their lungs.
H & E staining, X 40.

Fig. 69 : Lambs killed 4 weeks postinfection with A. suum, showing a cross section of a nodule similar to that shown in the above picture. Microscopically it consists of a mass of lymphoid cells.
H & E staining, X 400.



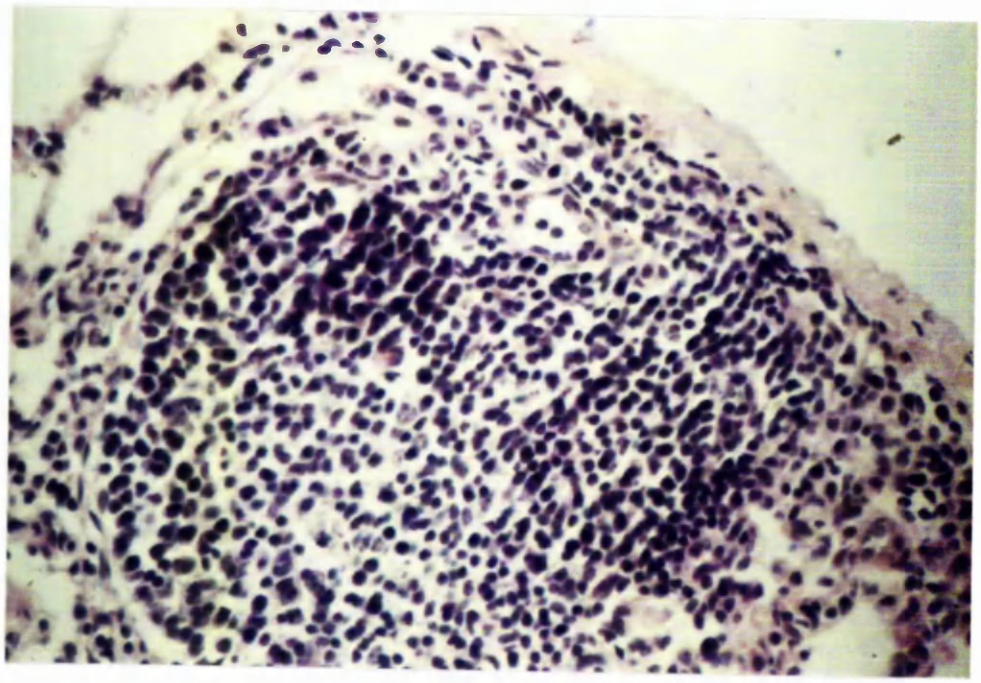
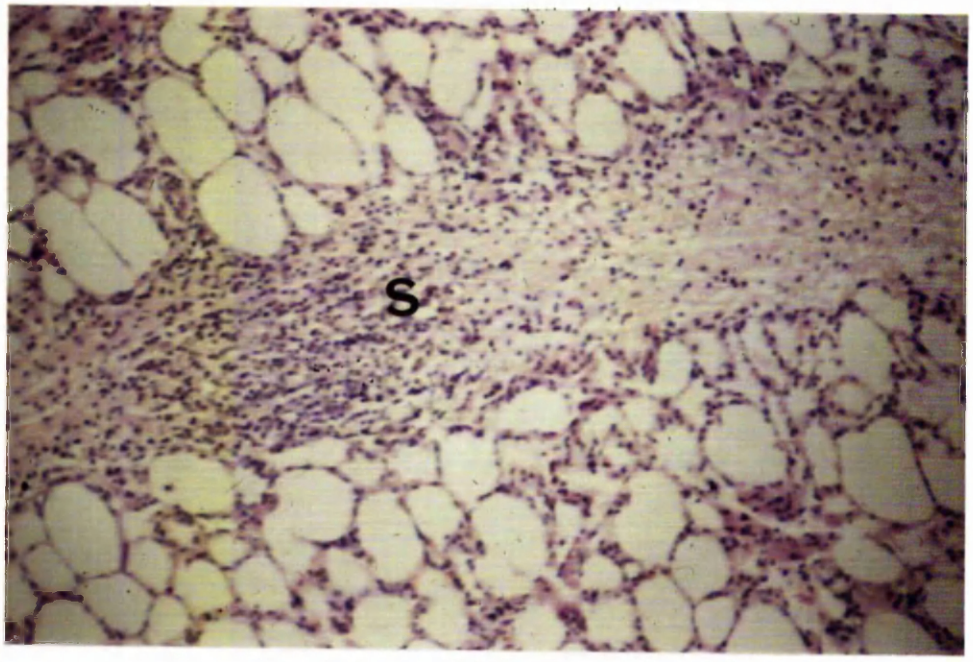
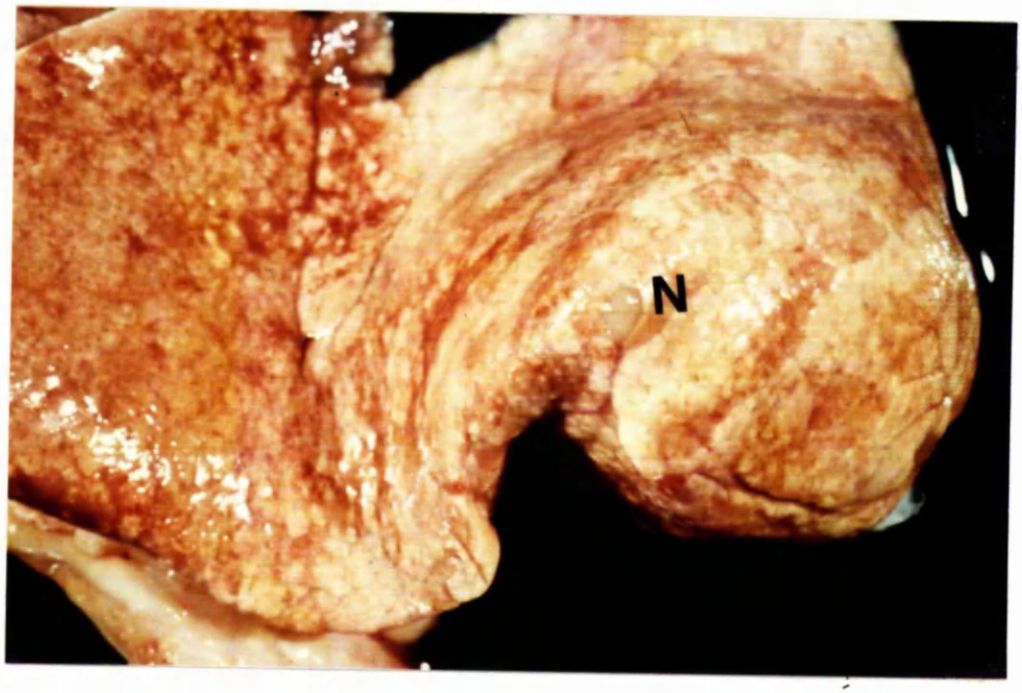


Fig. 70 : Lambs killed 4 weeks postinfection. The interlobular septa (S) were thickened by oedema and lymphocytic infiltration.

Fig. 71 : Lambs killed 8 weeks postinfection. A subpleural parasitic nodule (N) was seen in the cranial lobe of the right lung.


S


N

CHAPTER FIVE

EXPERIMENTAL INDUCTION OF GLOBULE LEUCOCYTES IN SHEEP LUNGS WITH DICTYOCAULUS FILARIA

A. INTRODUCTION

Parasitic bronchitis in sheep is mainly attributed to Dictyocaulus filaria infection, which has a world-wide distribution in sheep, goats and some wild ruminants. The clinical and pathological features of the disease in sheep have not been described in detail, but reports by Jarrett, McIntyre and Urquhart (1957) and Jarrett, Jennings, McIntyre, Mulligan, Sharp and Urquhart (1960) have provided much information on the clinical and pathological aspects of a similar disease in cattle caused by Dictyocaulus viviparus. These workers divided the disease into 4 stages according to the time interval from initial infection. There are the penetration phase (0-7 days) which is of little clinical significance; the pre-patent phase (7-25 days) during which the small bronchi and bronchioles are blocked by the eosinophilic exudate produced in response to the developing larvae migrating up the bronchi; the patent phase (25-55 days) which is associated with the presence of adults in the bronchi and during which the major clinical effect is caused by the pneumonia arising from the aspiration of larvae and exudate into the alveoli, and the post-patent phase (55-90 days) in which the majority of animals gradually recover. However, in about one quarter of the animals which survive to this phase, disease recurs and is characterised by the development of an alveolar epithelialisation of which the aetiology is unclear. Although the pre-patent period of this parasitic infection in cattle is shorter than that of the ovine species (Dunn 1969), it is probable, but not certain, that similar phases occur in D. filaria infection in sheep. Moreover, Dunn (1969) suggested that the pathogenesis D. filaria infection in sheep appears to be similar to that of D. viviparus infection in cattle, with epithelialisation of the alveoli as well as peribronchial cuffing and interstitial and pulmonary emphysema. The pneumonia in sheep is often complicated by bacterial infection and accordingly the postmortem appearance of the lungs may be complex, with occasional whitish or yellow purulent areas (Dunn, 1969).

Wilson (1970) reported consolidation in varying degrees in the lungs of all sheep after repeated infection with D. filaria despite no worms being recovered at autopsy. He noticed that many leucocytes were present

in the pulmonary fluid. Up to 90 per cent. of the leucocytes were eosinophils while other leucocytic components did not appear to be markedly increased.

A previous Chapter(4)of this thesis confirmed the association between the migration of A. suum larvae and the induction of GLs in sheep respiratory tracts. On the other hand, the literature reviewed did not include information on the induction of GLs in the sheep respiratory tract in relation to D. filaria infection. There are however no adequate reports concerning the pathological studies of parasitic bronchitis in sheep. Thus the following attempts were made to investigate the appearance of GLs in the ovine respiratory tract following experimental infection with D. filaria. This study was also carried out to compare the population of the GLs and their distribution in sheep respiratory tracts in relation to D. filaria and A. suum experimental infection. The macroscopic and microscopic apperance of the bronchopulmonary lesions in response to experimental infection with D. filaria in sheep were studied also.

B. MATERIALS AND METHODS

The experimental animals and the techniques employed are described below.

1. Experimental Animals

Seven groups of lambs were designated as groups D1, D2, D3, D4, D5, D6 and D7. Each group consisted of 2 lambs. The details of the experimental design have been given in Table (21). A total of 14 lambs were purchased from the Veterinary School Field Cochno Station. They were purchased when they were less than 4 weeks old. These lambs were reared indoors and kept parasite free as described in Chapter 4B. The experimental lambs were infected by oral or intravenous routes. Oral infection was carried out by using a 20 ml. plastic syringe to which was attached a 75 mm. length of polyvinyl chloride tubing. The larvae were suspended in 10 ml. of tap water and the whole volume was expressed into the throat of the animal. Intravenous infection was carried out by using a 10 ml. plastic syringe. The larvae were suspended in 5 ml. of distilled water and the whole volume was injected into the jugular vein of the animal. After infection of the experimental lambs, routine clinical tests and bleeding from the jugular vein were carried out twice a week as described in Chapter 4B. Fecal examination was done 2 weeks post-infection. The experimental groups were designated as follow :

Group D1

Two lambs; SM413 and SM415, were used to study the GL response to a single oral dose of 1,000 third stage larvae of D. filaria Table (21). They were killed 10 weeks postinfection.

Group D2

The 2 lambs in this group, SM412 and SM421, were sensitized with a dose of 1,000 third stage larvae orally and challenged with the same dose intravenously after exsheathing the third stage larvae. The interval between first and second doses was 10 weeks and they were killed 6 weeks after the challenge dose Table (22).

Group D3

Lambs SM427 and SM434 were sensitized with 2,000 third stage larvae intravenously and challenged with 1,000 third stage larvae intravenously after an interval of 8 weeks. They were killed 6 weeks after the challenge dose Table (23).

To determine an accurate time for the appearance and disappearance of the GLs in the respiratory tract of sheep the following groups received the same programme of doses but were killed at different periods after the challenge dose.

Group D4

This group consisted of 2 lambs, SM428 and SM431, which received a sensitizing dose of 1,000 third stage larvae intravenously and a challenge dose of 2,000 third stage larvae intravenously. The interval between the doses was 4 weeks and the lambs were killed 4 weeks after the challenge dose Table (24).

Group D5

Two lambs making up this group, SM430 and SM433, received the same sensitizing and challenge doses as group D4. They were killed 6 weeks after the challenge dose Table (24).

Group D6

This group received the same sensitizing and challenge doses as group D4. The two lambs, numbered SM437 and SM438, were killed 8 weeks after the challenge dose Table (24).

Group D7

Two lambs, SM410 and SM411, were used as a control group for this experiment and they were killed together with the last group of the experimental animals.

2. Culturing of D. filaria

Infective third stage larvae of D. filaria for culture purposes were obtained from Dr. N.N. Sewell of the Royal (Dick) School of Veterinary Studies, Edinburgh. These larvae were administered orally into 2 lambs at a dose of 2,000 larvae for each lamb. The lambs were housed indoors and kept parasite free as described in Chapter 4B. Four weeks post-infection, both lambs were coughing and first stage larvae of D. filaria were detected in their feces.

a. Extraction of larvae

When cultures were to be set up, all the feces passed overnight from the culture lambs were collected by means of a bag. The first stage larvae of D. filaria (Fig. 72) were then extracted from the feces using a Baermann apparatus (Soulsby, 1965). For this purpose the feces were broken up and transferred into 150 mm. diameter, 60 micron aperture, 250 mesh sieves (Endecatt Test Sieves Ltd., London), until each sieve was approximately half full. The feces were covered with a single 190 mm. diameter milk filter.

The sieves were then placed in 200 mm. diameter polythene funnels containing water at a temperature of 40°C. Water at 40°C was then poured over the feces until it just covered the top of the filter. The apparatus and its content were left for 16 to 24 hours during which time most of the first-stage larvae had passed into the lower part of the funnel.

About 200 ml of fluid was then withdrawn from each funnel. The samples were pooled and stored overnight at 4°C.

b. Larval cleaning and storage

A method similar to that described by Purvis (1971) was used. The chilled, inactivated larvae and fecal debris were allowed to settle to the bottom of the storage vessels while they were in the refrigerator. Most of the fluid was then carefully decanted and the sediment, together with a little fluid was centrifuged in conical tubes for 21 minutes at 1,500 r.p.m.

The supernatant was syphoned off and the sediment suspended in about 1.0 ml. of fluid in each tube, poured on a layer of "Kleenex" tissue which was stretched by means of a rubber band over the mouth of an inverted, cut-off plastic bottle (4 ounce wide mouth pattern). The plastic bottle was set in a 100 mm. diameter glass funnel, half-filled with warm water. The "Kleenex" tissue held back most of the dead larvae and fecal debris. After 5-6 hours, during which time most of the first-stage larvae had passed into the lower part of the funnel, the larval suspension was collected from the stem into clean labelled 8 ounce medical-flat bottles.

Small samples were taken for microscopic examination to observe the viability of the larvae and to estimate the number present. The suspension of larvae was kept at room temperature. The water was changed every second day and by the end of one week the larvae had developed to the infective third stage (Fig. 73 & 74). They were then stored in Universal bottles at $4^{o}C$.

c. Counting larvae

To prepare third stage larvae for inoculation, larvae were transferred from the storage bottles into 2 ml. conical tubes and centrifuged for 2 minutes at 1,500 r.p.m. Most of the supernatant was symphoned off and the suspended larval sediment transferred drop by drop into an embryo block using a Pasteur pipette. The larvae in each drop were counted under X 2.5 magnification with a stereoscopic microscope. The larvae were then carefully washed into clean conical tubes until the required number was reached. The larvae were then quantitatively transferred to a syringe for administration.

d. Exsheathing larvae

Exsheathing of third stage larvae was necessary when the inoculum was to be administered intravenously. This was done by treating the larval suspension with an equal volume of 5 per cent. Milton solution (sodium hypochlorite) for 5 to 10 minutes, in small petri dishes. The exsheathing process was carefully checked under the stereoscopic microscope. Following exsheathing of the larval double sheath (Fig. 75), the larvae

were washed several times in distilled water, concentrated and quantitatively transferred to a syringe for inoculation.

3. Pathology

The respiratory tracts and other internal organs of all groups were examined for lesions caused by D. filaria experimental infection. For histochemical examination 8 pieces of tissue representing the standard sites of lung and bronchial tree (Fig. 1) were collected along with routine samples of tissues from lung around the bronchi and from other organs also. Tissues were fixed, embedded and stained as in Chapter 2B. Haematoxylin and eosin stained sections from corrosive formol fixed blocks were used for the routine detection of GLs. When particular morphological findings in the GLs were to be demonstrated more clearly, special stains were employed. These were toluidine blue, alcian and astra blue/safranin, biebrich scarlet, Martius scarlet blue and the carbol chromotrope method. Preparation of reagents and staining techniques are given in Appendix I.

C. RESULT

1. Parasitological and Haematological Observations

The first stage larvae of D. filaria reached the infective third stage larvae at approximately 8 days when incubated at room temperature. Those lambs which were infected with a single oral dose of D. filaria became patent from 30 to 36 days post-infection. Although most other lambs which were infected by the intravenous route became patent from 20 to 23 days post-infection.

The peripheral blood eosinophilia showed a characteristic response in lambs infected with a single oral dose of D. filaria (group D1 and D2). The result of sheep SM413, SM415, SM421 and SM427 which were considered to be typical of the response to D. filaria are given in Appendix II. There were 2 peaks of peripheral blood eosinophilia; the first peak reached 13 per cent. on 13-20 days post-infection and the second peak reached 14 per cent. on 37-40 days post-infection.

Lambs in group D3, D4, D5 and D6 were inoculated with D. filaria by the intravenous route. They showed peripheral blood eosinophilia which reached 12 per cent. on day 10 post-infection. On challenge dose the infected animals in these groups showed similar massive secondary eosinophil reactions that was in accordance with those animals in first infection.

2. Globule Leucocytes and Mast Cells

A pronounced induction of a considerable number of GLs was noted in the epithelium of the bronchopulmonary systems of most lambs infected with D. filaria (Table 25). These cells were present in 5 of the 6 experimental groups of lambs infected. Globule leucocytes were numerous in the tracheal and bronchial epithelium of group D1 which was killed 10 weeks post-infection with a single dose of D. filaria orally. Lambs in group D2 which received sensitizing and challenge dose of D. filaria, had GLs in their tracheal and bronchial epithelium. The lambs of group D3, which had received the same programme of doses as those in group D2, had many GLs in their bronchial epithelium, but no GLs in their tracheas.

Group D4, D5 and D6 received the same sensitization and challenge doses of D. filaria, to determine the appearance and disappearance of the GLs in their bronchopulmonary systems. Globule leucocytes were not found in the bronchopulmonary systems of lambs in group D4 which were killed 4 weeks after the challenge infection but they were detected in groups D5 and D6 which had been killed 6 and 8 weeks respectively post-infection (Table 25).

Globule leucocytes occurring in the lungs of these groups, were found in an intra-epithelial position. No GLs were identified in the subepithelial tissue of the respiratory tracts. The globule leucocytes were round to oval in appearance when stained with H & E stain and contained the characteristic acidophilic intracytoplasmic globules. These sometimes, appeared to coalesce, forming large aggregations in the cytoplasm. The nuclei of the GLs appeared similar to that of the lymphocyte, and occupied an eccentric position within these cells (Fig. 76).

To confirm that the cell observed by H & E stain were GL and not other cell type, sections were also stained with the stains employed in Chapter 2B. Although eosinophil leucocytes were abundant in the lamina propria and the epithelial mucosae of all the experimental groups the histochemical characteristics of the cells, given in Table 2, are a clear indication of the different nature of both cells.

Globule leucocytes were not detected in the bronchioles, the bronchial glands or their ducts in any group of sheep examined after infection with D. filaria.

3. Pathology

Group D1

Lambs of this group were killed 10 weeks after a single oral dose. Their lungs had different gross pictures. Lamb SM415 exhibited an area of consolidation in the diaphragmatic lobes. While lamb SM413 had petechial and ecchymotic haemorrhages scattered over the entire subpleural region of the lungs (Fig. 77). The cut surface through these lungs, which were oedematous and spongy in consistency, were mottled with red haemorrhagic

spots. A thick haemorrhagic exudate oozed from the cut surface. The trachea and diaphragmatic bronchi were filled with a frothy red exudate. The liver had no gross changes.

Microscopically lamb SM415 had marked pulmonary consolidation, while the lungs of lamb SM413 had only focal areas of intra-alveolar and subpleural haemorrhages. The general pulmonary lesions were of a diffuse interstitial pneumonia, in which the alveolar wall was mainly infiltrated with lymphocytes. No pulmonary eosinophilia was seen. There was bronchitis and bronchiolitis in which the main cells involved were eosinophils and lymphocytes. Large numbers of eosinophils had accumulated around the pulmonary arteries and veins. No parasitic granuloma were seen.

Group D2

This group were sensitized with 1000 larvae orally prior to a similar challenge intravenous dose. Both lambs were necropsied 6 weeks after the challenge dose. Grossly there were brown to red nodules, 3-5 mm. in diameter, over the lung surfaces. These nodules were elevated and surrounded by an area of hyperaemia (Fig. 78). The interlobular septa were very distinct, being filled with blood and oedema fluid. Abundant subpleural haemorrhages were seen. The cut surface was uniformly dark red and of a meaty texture. A frothy red exudate was present in the trachea and bronchi of these lungs, which also had tracheitis and bronchitis.

Microscopically there was tracheitis and bronchitis, with infiltration of the epithelium by polymorphonuclear leucocytes. The lamina propria in both the trachea and the bronchi were infiltrated with lymphocytes and plasma cells only. There were lobules with patches of exudative pneumonia. The brown-red nodules seen macroscopically were found to be parasitic granulomata. Such foci were common in the immune lambs but were only seen occasionally in the susceptible lambs. These nodules consisted of a mass of disintegrated eosinophils with disintegrated larvae in their centres. This mass was surrounded by epitheloid and giant cells and

an outer zone of lymphocytes, plasma cells and eosinophils. The interlobular septa contained lymphocytes only and were dilated with fibrin and oedema fluid. There was peribronchial lymphocytic aggregation. Alveolar walls of other lobules were thickened and infiltrated with lymphocytes and macrophages. Perivascular areas were filled with eosinophils. There was hypertrophy of the smooth muscle of the bronchi and of the pulmonary arteries.

Group D3

The lambs of this group were necropsied 6 weeks after a challenge dose of 1000 larvae intravenously. Grossly the lungs of both lambs had bronchitis and tracheitis. The peripheral parts and ventral surfaces of the diaphragmatic lobes had extensive areas of consolidation. The remaining parts of the lobes had areas of collapse interspersed with many parasitic nodules (Fig. 79). These nodules were small 1-2 mm. in diameter with translucent centres surrounded by a zone of hyperaemia. Interlobular septa were thickened and the subpleural surfaces had haemorrhagic and ecchymotic spots. The cut surface had a meaty texture. The lungs were firm and oedematous. The liver had no gross changes.

Microscopic examination revealed that the main change was a marked pulmonary eosinophilia. The interlobular and subpleural connective tissues were thickened by oedema fluid and by large numbers of eosinophils (Fig. 80), but no lymphocytes as in previous groups. In some lobules there was alveolar collapse with clear alveolar airspaces but other lobules had eosinophilic alveolitis. There was a widespread tracheitis, bronchitis and bronchiolitis in which the main cell involved was the eosinophil. The smooth muscle of the bronchi and pulmonary arteries was not hypertrophied as in the previous group. The pulmonary nodules seen grossly consisted of masses of eosinophils.

Group D4

This group was sensitized with 1000 larvae and after 4 weeks they were challenged with 2000 larvae intravenously. The lambs were necropsied 4 weeks after the challenge dose. Their lungs had consolidation

and subpleural haemorrhage mostly in the diaphragmatic lobes (Fig. 81 & 82). Tracheitis and bronchitis were present and adult worms were found in the bronchi of this group. The interlobular and subpleural spaces were dilated and filled with blood. The liver was normal.

Microscopically the lesions consisted of exudative pneumonia . The consolidated area had infiltrates of eosinophils which blocked the small bronchi and bronchioles causing alveolar collapse distal to the block. The subpleural and interstitial connective tissues were not dilated or thickened but were infiltrated by large numbers of eosinophils and contained free erythrocytes.

Group D5

This group received the same programme of doses as in group D4 but it was necropsied 6 weeks after the challenge dose. Grossly the lesions were similar to those in groups D2 and D3, but these lungs had large white-yellow nodules 3-6 mm. in diameter which were distributed on the sub-pleural surfaces. The liver was normal.

On microscopic examination no pulmonary eosinophils were seen compared with group D4. The exudate which filled the airways consisted mainly of polymorphonuclear leucocytes. Parasitic larvae were found in the alveoli and adult worms were found in the bronchial lumen (Fig.83). The nodules which were seen grossly consisted of large subpleural lymphocytic mass with many germinal centres (Fig.84 & 85). Peribronchial lymphocytic aggregations were also present. Tracheitis and bronchitis were common in the lungs of this group.

Group D6

Lambs in this group received the same programme of doses as in group D, but they were killed 8 weeks after challenge dose. On macroscopic examination the lungs were pale grey in colour and rubbery in consistency. There was pneumonic consolidation of the cranial lobes from which Pasteurella haemolytica-like organisms were isolated. The diaphragmatic lobes had small subpleural nodules, 1-2 mm. in diameter. These nodules

were white-grey in colour and slightly elevated from the lung surface.

Microscopically there was tracheitis and bronchitis in which the main cells involved were polymorphonuclear leucocytes. The alveolar walls were thickened and infiltrated with lymphocytes and macrophages but no eosinophils. The interlobular and subpleural connective tissues were also thickened and infiltrated with lymphocytes, eosinophils and macrophages. The nodules seen macroscopically consisted of disintegrated larvae surrounded by lymphocytes or focal aggregations of the latter cell type only.

D. DISCUSSION

The present investigation demonstrated that GLs could be induced to develop in the bronchopulmonary system of sheep by infecting them with D. filaria. The structural and histochemical results of these GLs were similar to those of outdoor sheep with natural parasitic infection and to those in A. suum infection. The result of this study was in agreement with Rahko (1970b) who suggested that there was no difference in the structure and histochemical reaction of bovine GLs in fascioliasis and dicrocoeliasis. However, differences seem to occur in the distribution of GLs between ascariasis and D. filaria infection in sheep. Moreover the required time for the appearance and disappearance of the GLs in sheep experimentally infected with D. filaria was markedly different from that in A. suum infection.

In all lambs examined 6 weeks or more after challenge dose, GLs were found in the lung. The response of sheep to D. filaria was seen to be more consistent than with A. suum, since many lambs did not have bronchopulmonary GLs after infection with A. suum eggs. Possibly the main point of difference was the presence of adult worms in the lungs of lambs for a long period of time during D. filaria infection whereas A. suum larvae were present for 9 (2-11) days only.

Most lambs infected with D. filaria became patent on day 30-36 post-infection. The patent phase persisted until day 80 postinfection. Thus the prepatent and patent period of D. filaria infection in sheep was longer than that of D. viviparus infection in cattle. This agrees well with the finding of Al-Samarrae (1975) in D. filaria infection in sheep. It was interesting to note that prepatent and patent phases in sheep given D. filaria by the intravenous route were different; patency was advanced with the oral route and terminated sooner.

The failure of infections with D. filaria to become patent in sheep have been reported by many workers (Kauzal, 1934; Michel, 1968). This was largely attributed to an early reduction in the number of worms present, which was likely to occur if the number of infective larvae

administered was small, if the host was young or if it was so severely affected that it subsequently succumbed to the infection (Michel, 1968).

Unlike lambs infected with A. suum, these studies showed that a single dose of D. filaria was sufficient to produce GLs in the broncho-pulmonary system of lambs (group D1), either due to the presence of the worms in the bronchi or when the infection became patent.

Globule leucocytes were demonstrated in the tracheal and bronchial epithelium of group D2, while in group D3 the GLs were present in the bronchi only. This variation in distribution of these cells might be explained by fewer worms developing in the lungs of the latter group due to an immunity from the initial higher sensitising dose. Thus the induction of GLs in tracheal and bronchial epithelium of group D2 may be due to the laid eggs or larvae from the adult worms of the initial infection.

Group D4, D5 and D6 received the same sensitising and challenge doses of D. filaria, in order to determine the appearance and disappearance of the GLs in the bronchopulmonary system of sheep. Globule leucocytes were not found in the lungs of the sheep in group D4 which were killed 4 weeks after the challenge infection but they were detected in groups D5 and D6 which had been killed 6 and 8 weeks respectively following the challenge dose. Therefore, at the present time it is not known how quickly the GLs appeared after primary infection. It was interesting that group D5 was examined 10 weeks after primary infection at a time when GLs were seen after a single infection. Whereas group D4 was examined only 8 weeks after the initial infection. Therefore the appearance of GLs in the lungs of these groups was attributed either to the presence of the adult worms in the bronchi or to the released first stage larvae which also induced enormous numbers of GLs along the length of the trachea.

The experiment in lambs infected with D. filaria revealed 2 peaks of peripheral eosinophilia. The first peak was 13-20 days postinfection and the second peak 37-40 days postinfection. Previous work on sheep infected with D. filaria also revealed 2 peaks of peripheral eosinophilia, although they were at different times (Al-Samarrae 1975). In calves infected

with a single dose of D. viviparus the peripheral eosinophil response also revealed 2 peaks, the first at 9 to 15 days and a higher one at about 40 days postinfection (Cornwell, 1962). The second peak of eosinophilia in these lungworm infections was usually attributed to the release of larvae from the adult worms.

The results of the gross and microscopic changes in the lungs of lambs infected by oral and/or intravenous routes with D. filaria revealed no differences in the pathological picture between the routes.

The lambs in group D1 which were killed 10 weeks after a single oral dose had different pulmonary lesions. Lamb SM413 had petechial and ecchymotic haemorrhages scattered over the entire subpleural region of the lungs. This lesion together with the microscopic findings could be attributed to inhalation of the laid eggs or hatched larvae, although this lamb was killed at the same time as lamb SM415 which was patent. In this connection it was relevant to note that Nickel (1962), working with D. filaria in sheep, observed that the larval count was not related to the severity of clinical signs while Poynter and Selway (1966) attributed the severity of the latter to the number of eggs being aspirated. A similar observation was made by Jarrett et al, (1960) and Kassai and Hollo (1962) in D. viviparus infection in cattle. In addition the other lamb (SM415) in the same group had moderate consolidation in the diaphragmatic lobes, although Wilson (1970) found moderate to heavy consolidation in the lungs of sheep killed during the patent period of infection. Therefore a similar mechanism could be proposed to explain the presence of considerable pulmonary consolidation in lambs infected with D. filaria as in those infected with A. suum (Chapter 4, Discussion).

Lambs in group D2 and D3 had numerous subpleural parasitic nodules. In cattle, identical nodules were seen in immune cases (Jarrett and Sharp, 1963). Michel and Mackenzie (1965) agreed that the presence of such parasitic granulomata may be associated with an allergic or immune response in calves. Severe eosinophil infiltration of the connective tissue, particularly of the subpleural zone and interlobular septa, was seen in

immune calves after their initial treatment and this corresponded to the peripheral eosinophil counts (Michel and Mackenzie, 1965). Similar changes were seen in our experimental lambs in groups D2 and D3. Muscular hypertrophy of the tunica media of small pulmonary arteries occurred with eosinophil invasion and proliferation of the intima. This reaction was similar to that seen in lungworm infection of cattle (Michel and Mackenzie, 1965) and cats (Mackenzie, 1960).

All lambs in group D4, D5 and D6 were sensitised with 1,000 larvae and after 4 weeks they were challenged with 2,000 larvae intravenously. Lambs in group D4 were killed 4 weeks after challenge dose. However, despite the presence of numerous adult worms in the bronchial tree, neither lamb had GLs in its lungs. This could be due to the exudative type lesion and severe polymorphonuclear leucocyte infiltration of the bronchopulmonary epithelium which may have obscured the detection of GLs even when sections were stained specially for GLs and mast cells (Kent et al., 1956). The predominant reaction provoked by the presence of adult worms in the bronchial tree of lambs in this group was pulmonary eosinophilia. This was specially marked in the interlobular and subpleural areas of the lungs where there was also considerable oedema and haemorrhage. This was seen in calves infected with D. viviparus larvae (Jarrett et al., 1957). At this time an eosinophilic bronchitis and bronchiolitis and often tracheitis affected most of the pulmonary airways so it would be reasonable to conclude that there was a delayed response to the presence of larvae in the airways.

Despite the presence of certain larval stages and adult worms in the bronchial tree of lambs in group D5, it was interesting to note that the pulmonary eosinophilia, which was a characteristic feature of the lungs in group D4, had rapidly resolved in this group which was necropsied 5 weeks after the challenge dose. In this group the pulmonary eosinophilia had been completely replaced by a lymphocytic infiltration of the lung tissue. In addition, subpleural lymphocytic nodules still existed in this group and also in group D6 which was killed 8 weeks after the challenge dose. These nodules were identical to those seen in previous groups of

lambs infected with A. suum and D. filaria (Chapter 4) and in D. viviparus of cattle (Jarrett and Sharp, 1963). Thus, the presence of GLs and the subpleural lymphocytic nodules in lambs of group D5 and D6 were an indication of an immune response to D. filaria infection in the lambs studied in this chapter.

	number	Dose & Route	Dose & Route		after last dose
D1	SM413 SM415	1,000 (O)	-	-	10
D2	SM412 SM421	1,000 (O)	1,000 (I/V)	10	6
D3	SM427 SM434	2,000 (I/V)	1,000 (I/V)	8	6
D4	SM428 SM431	1,000 (I/V)	2,000 (I/V)	4	4
D5	SM430 SM433	"	"	4	6
D6	SM437 SM438	"	"	4	8
D7	SM410 SM411	Control			16

Table (21) : Description of experimental lambs infected with D. filaria

O : oral dose
I/V : intravenous dose

Animal number	0	Week of experiment 10	16
SM412	1000	2000	Killed
SM421	"	"	"

Table (22) : Group D2 lambs sensitized orally and challenged with intravenous injection of exsheathed third stage larvae of D. filaria

Animal number	Week of experiment 0	8	14
SM427	2000	1000	Killed
SM437	"	"	"

Table (23) : Group D3 lambs sensitized and challenged with intravenous injection of exsheathed third stage larvae of D. filaria.

Group number	Animal number	Week of experiment				
		0	3	8	10	12
D4	SM428	1,000	2,000	Killed	-	-
	SM432	"	"	"	-	-
D5	SM430	1,000	2,000	-	Killed	-
	SM433	"	"	-	"	-
D6	SM437	1,000	2,000	-	-	Killed
	SM438	"	"	-	-	"

Table (24) : Groups and numbers of lambs sensitized and challenged with intravenous injections of exsheathed third stage larvae of D. filaria.

Group number	Animal number	Type of infection	Week examined post infection	Presence of GL in respiratory tract epithelium			
				Trachea	Bronchi	Bronchioles	Glands & duct
D1	SM413	Single	10	+	+	-	-
	SM415	"	"	-	+	-	-
D2	SM412	Double	6	+	+	-	-
	SM421	"	"	+	+	-	-
D3	SM427	Double	6	-	+	-	-
	SM434	"	"	-	+	-	-
D4	SM428	Double	4	-	-	-	-
	SM431	"	"	-	-	-	-
D5	SM430	Double	6	+	+	-	-
	SM433	"	"	+	+	-	-
D6	SM437	Double	8	-	+	-	-
	SM438	"	"	+	+	-	-

Table (25) : The induction of GLs in different groups of lambs infected with infective stage of D. filaria larvae

+ : Globule leucocytes present.
- : Globule leucocytes absent.

Fig. 72 : First stage larva of D. filaria extracted from the feces of infected sheep by Bearman technique.
X 250.

Fig. 73 : Third stage larvae of D. filaria developed from the first stage larva after one week of incubation at 26°C.
X 250.

Fig. 74 : Detail of the above picture showing the double sheath of third stage larvae of D. filaria.
X 750.

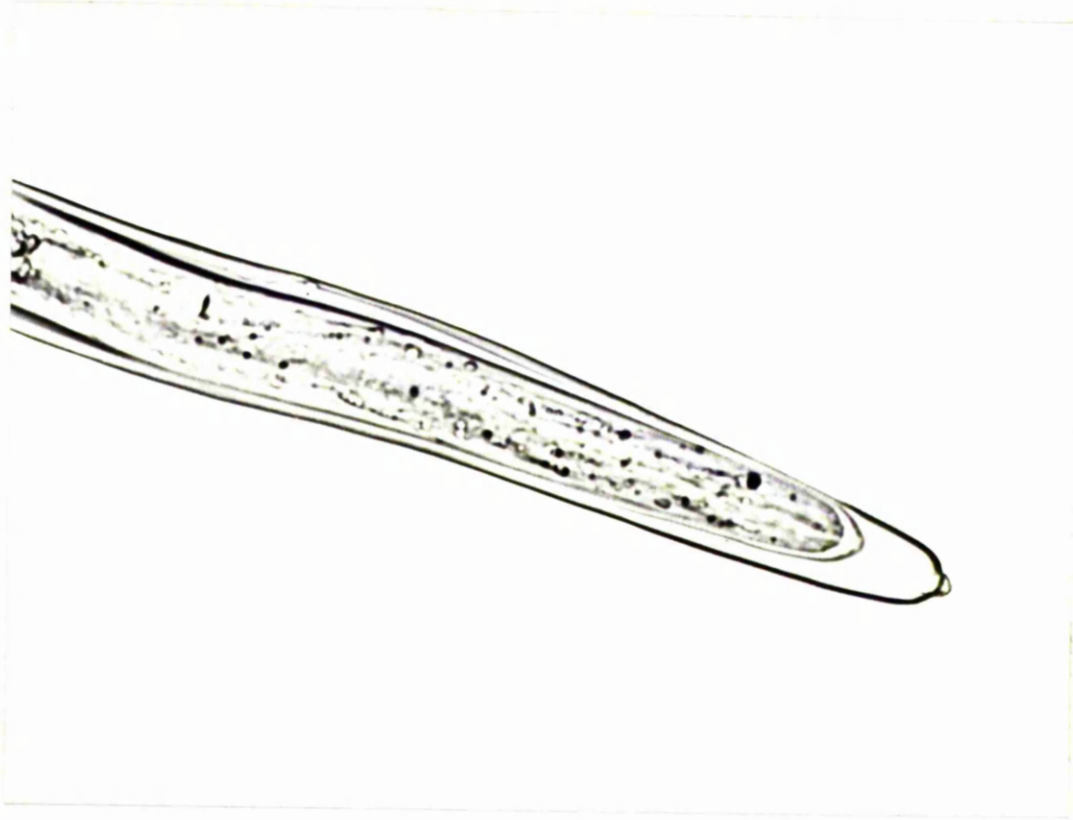


Fig. 75 : Third stage larvae of D. filaria after exsheathing of the double sheath with 5 per cent. sodium hypochlorite. X 250.

Fig. 76 : Typical globule leucocytes seen in the bronchiol epithelium of sheep experimentally infected with D. filaria.
X 250.

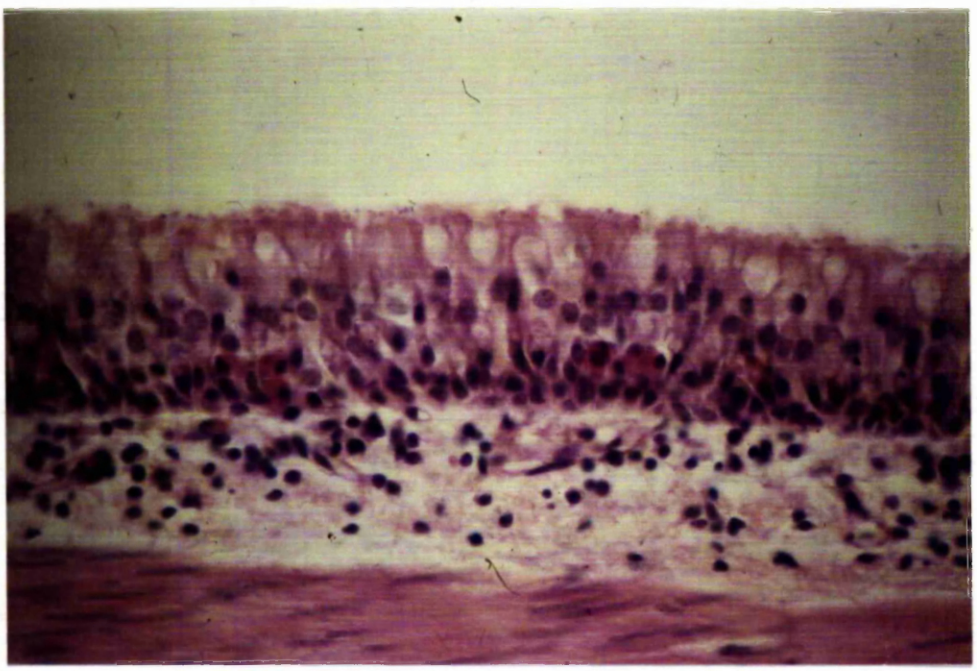
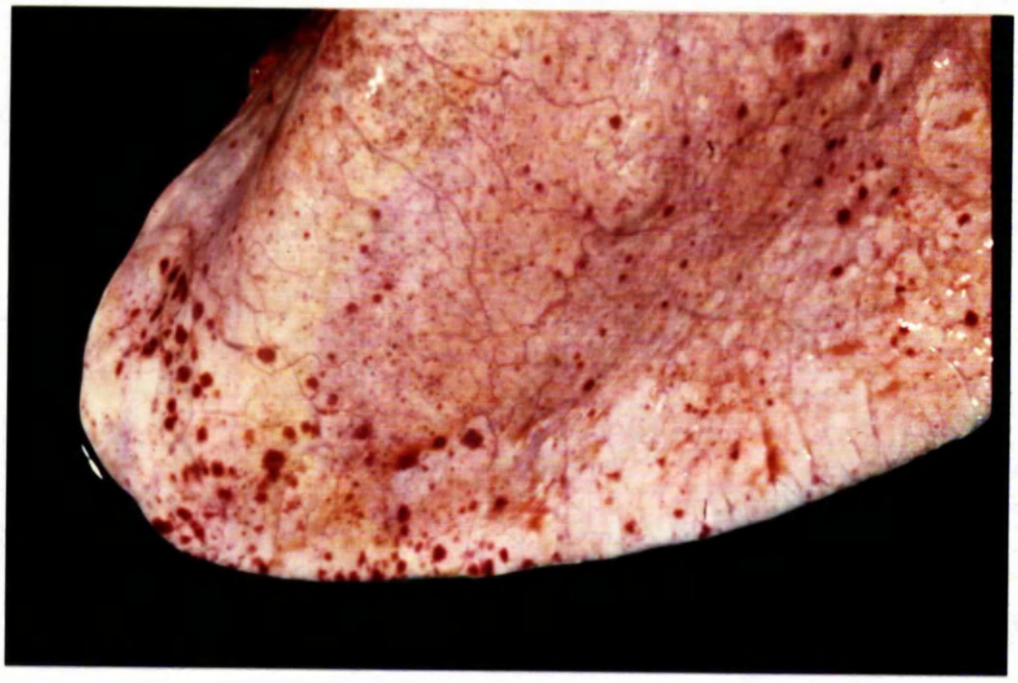


Fig. 77 : Right lung from lamb in group D1 killed at 10 weeks postinfection. Petechial and ecchymotic haemorrhages are scattered over the entire lung surface.

Fig. 78 : Right lung from lamb in group D2 which was killed 6 weeks after the challenge dose. Subpleural parasitic nodules are seen in the diaphragmatic lobe.



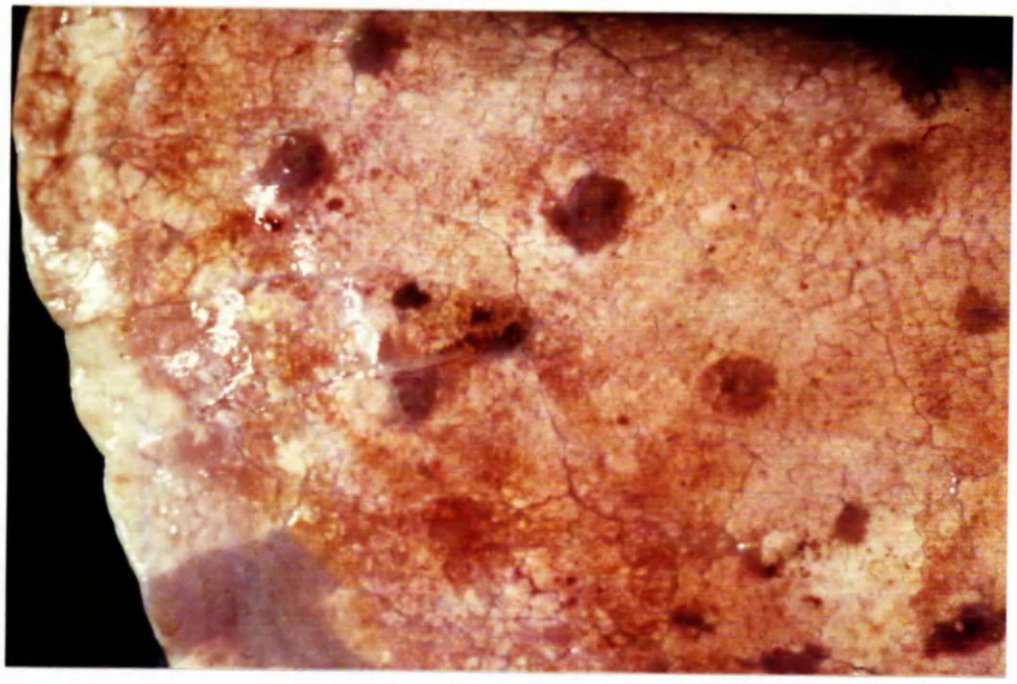
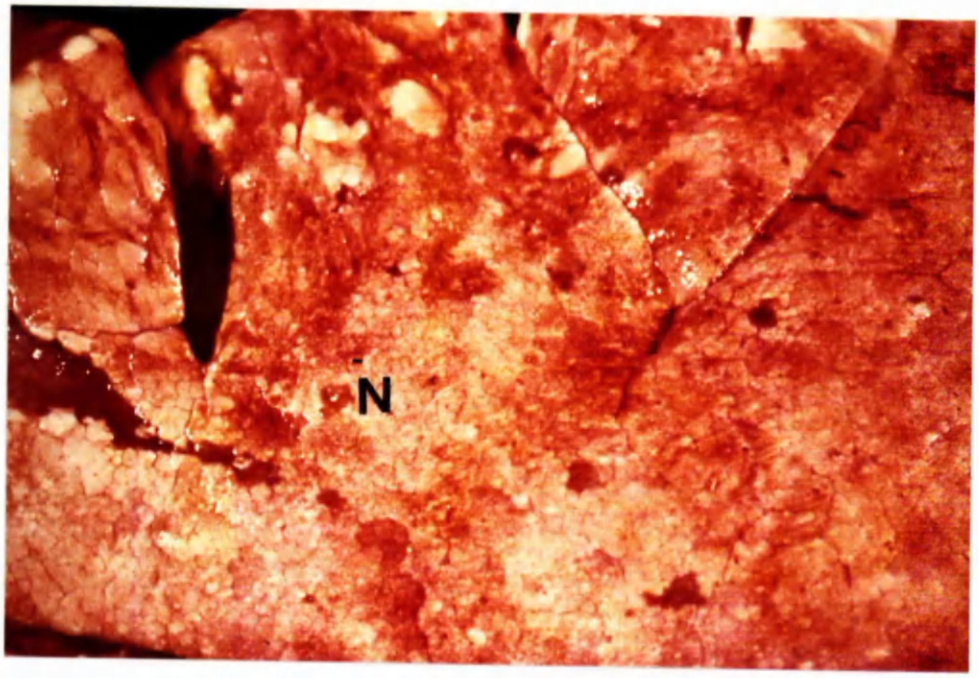


Fig. 79 : Lung of lamb in group D3 which was killed 6 weeks after the challenge dose. An area of collapse interspersed with many parasitic nodules (N) is seen.

Fig. 80 : Lambs in group D3 which were killed 6 weeks after the challenge dose. Microscopically the interlobular septae (S) are widened by oedema and eosinophilic infiltration. An area of alveolar collapse and haemorrhage (C) is also present.
H & E staining, X 40.


N

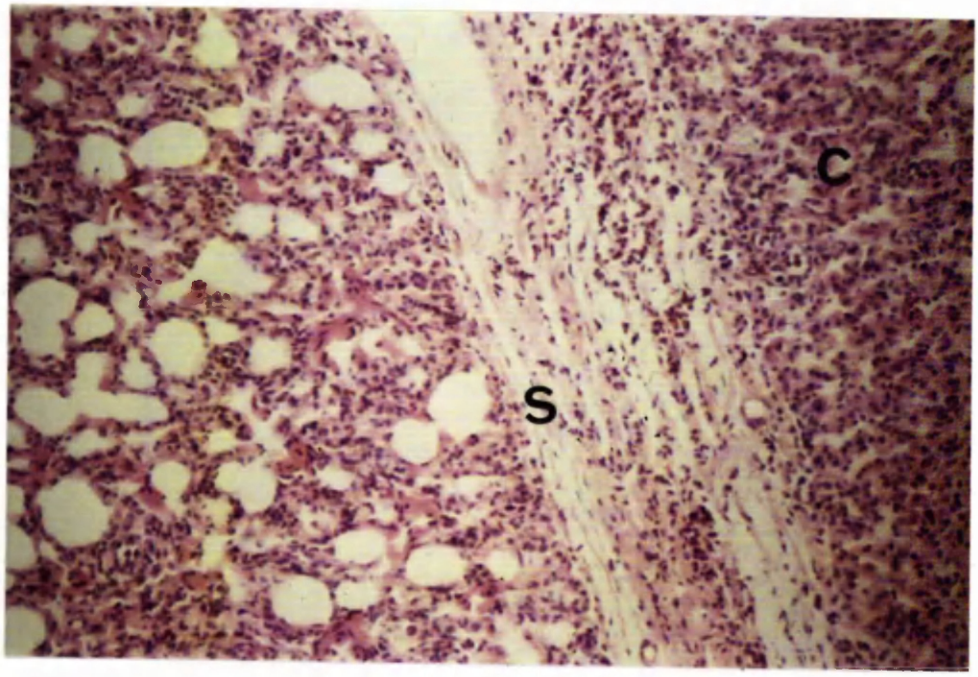
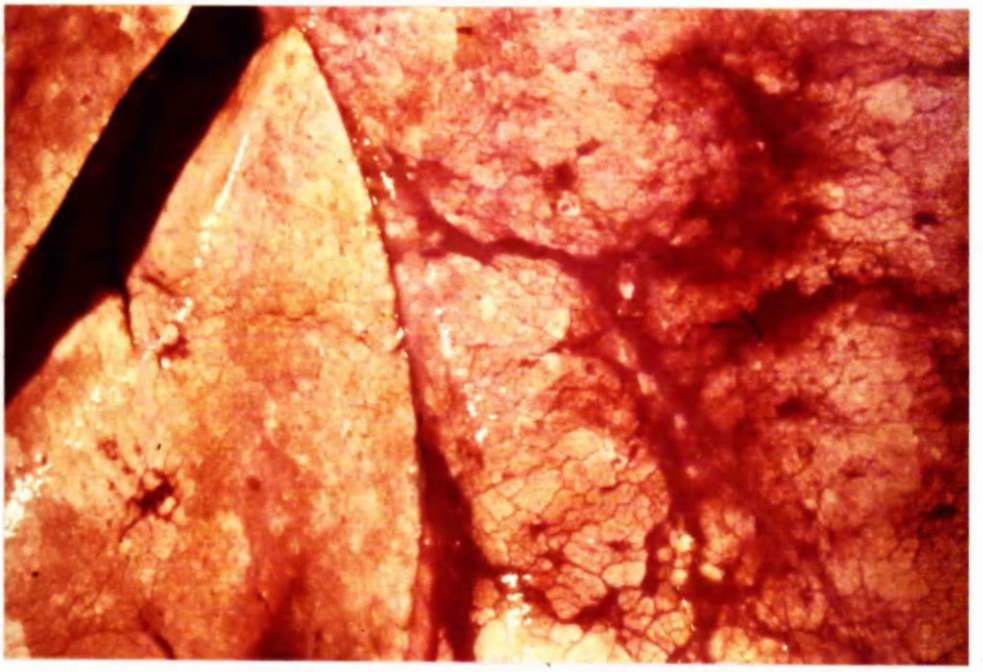

C
S

Fig. 81 : Lambs of group D4 which were killed 4 weeks after the challenge dose. Grossly their lungs had consolidation and subpleural haemorrhage mostly in the diaphragmatic lobes.

Fig. 82 : Cross section through the consolidated area of the above picture, showing an adult worm in the bronchi of this lung.



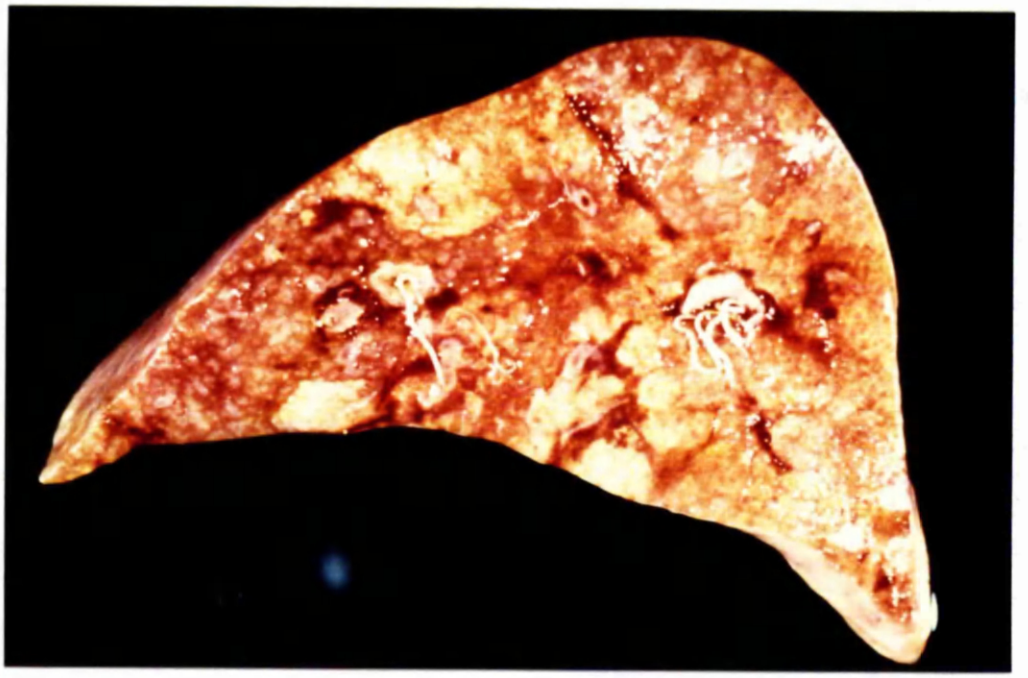


Fig. 83 : Lung of lamb in group D5 which was killed 6 weeks after the challenge dose. Microscopically a large bronchus containing 6 cross-sections of parasite is seen. H & E staining, X 250.

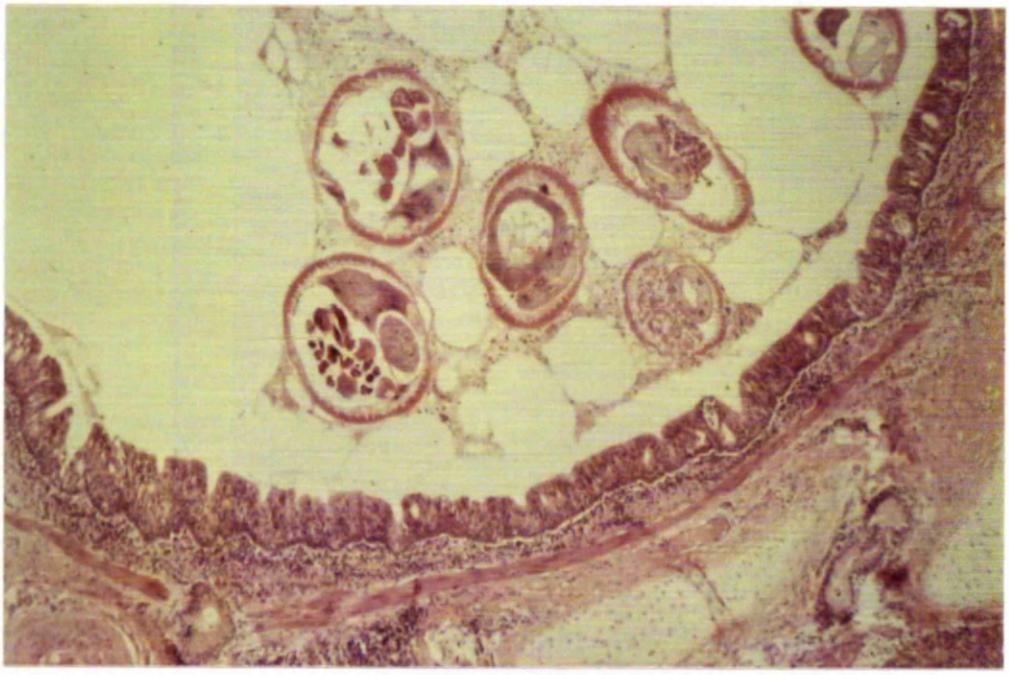
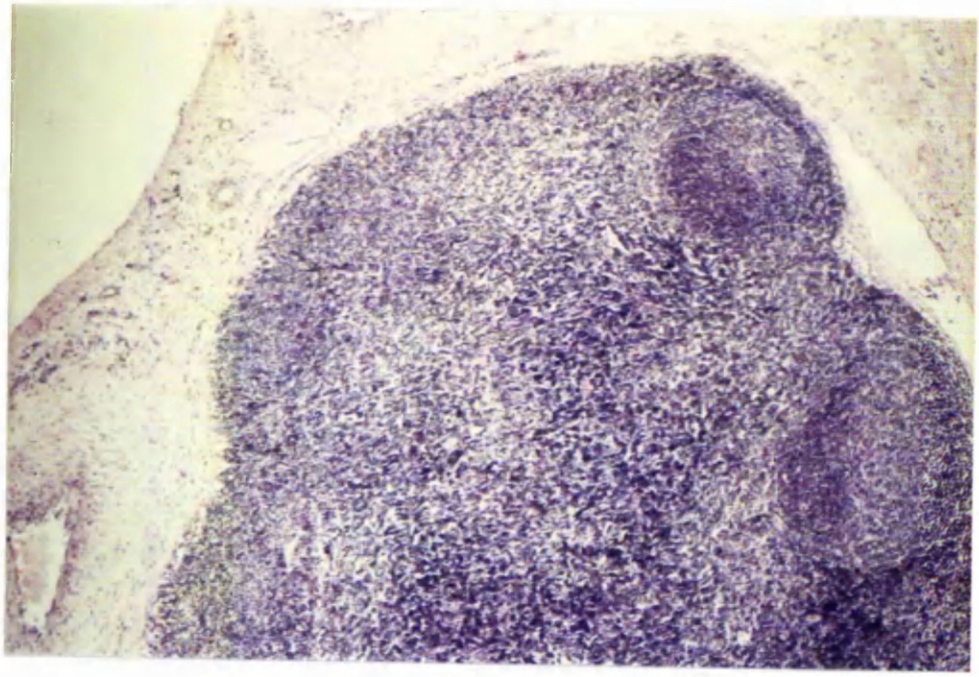
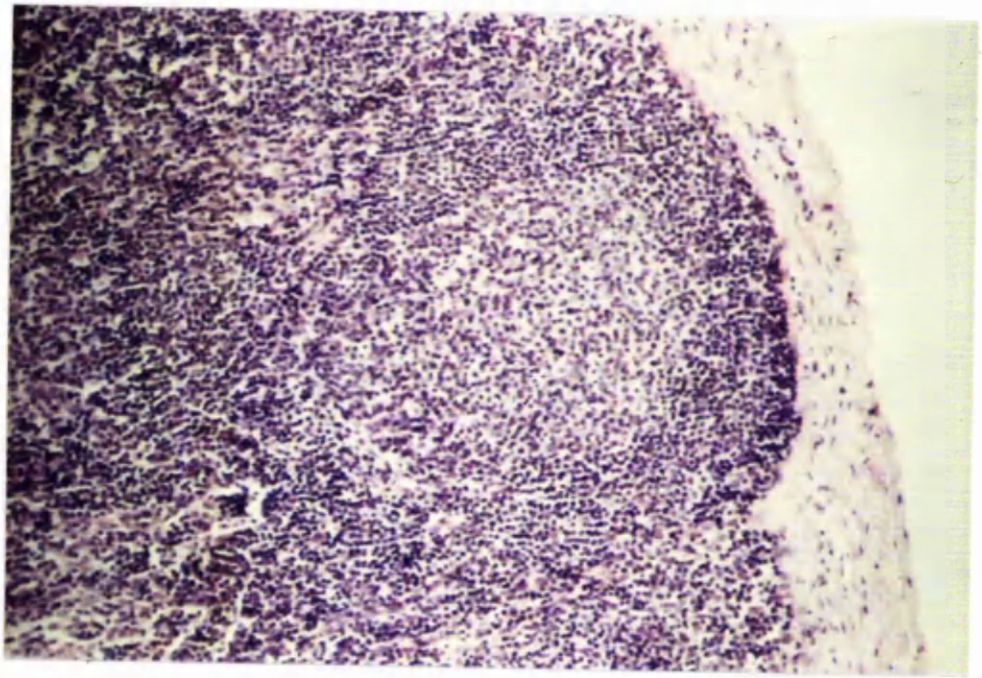


Fig. 84 : Lung of lamb in group D5 killed 6 weeks after the challenge dose. Microscopically this shows a cross section through a large subpleural nodule which consisted of a lymphocytic mass with many germinal centres. They were arranged concentrically around the periphery.
H & E staining, X 100.

Fig. 85 : Detail of a part of the subpleural nodule shown in the above picture.
H & E staining, X 400.





CHAPTER SIX

EXPERIMENTAL INDUCTION OF GLOBULE LEUCOCYTES IN SHEEP LUNGS WITH HAEMONCHUS CONTORTUS, MOULDY HAY AND ASPERGILLUS FUMIGATUS

A. INTRODUCTION

Globule leucocytes were numerous in the respiratory tracts of sheep experimentally infected with A. suum and D. filaria as described in the last two chapters. Both of these parasites migrate through the lungs of sheep and induce GLs to develop. Although conflicting opinions still exist in the literature concerning the appearance of GLs in the mucosa of various organs in association with a "self-cure" phenomenon (Sommerville, 1956). This author suggested that there was no correlation between the site of nematode infestation and the region in which these cells were most common.

To study the correlation between the site of parasitic infestation and the region in which GLs were appearing, certain gastrointestinal parasites of sheep were employed to investigate the appearance of such cells in the respiratory tract of sheep. H. contortus is an abomasal parasite of sheep which has a direct life cycle (Veglia, 1915). Sheep are usually infected by swallowing the infective third stage larvae of H. contortus which develops to the fourth, fifth and adult stages in the abomasum without migrating through the lungs (Lapage, 1965). Thus the first purpose of this chapter was to describe the study of the appearance of GLs in sheep respiratory tracts following experimental infection with H. contortus.

In cattle with farmer's lung numerous GLs were demonstrated in the epithelium of the bronchial tree (Breeze et al., 1975). Farmer's lung or extrinsic allergic alveolitis is an allergic respiratory disease of man which develops after exposure to the dust of mouldy hay containing the spores of Micropolyspora faeni and other thermophilic actinomycetes. The condition was first described by Cadham (1924) in Canada, then in Britain by Campbell (1932). Exposure to M. faeni produces a clinical respiratory disease which is considered to be the result of an Arthus-type hypersensitivity reaction in the lung, and precipitating antibodies to M. faeni are found in the patients sera (Pepys, 1969). The lung lesions are associated with vascular damage by immune complexes : there is haemorrhage, polymorphonuclear leucocyte accumulation and diffuse alveolar septal infiltration by lymphocytes and plasma cells, epithelioid granulomata and bronchiolitis obliterans; pulmonary fibrosis and cystic changes are found in more longstanding cases (Seal, Hapke, Thomas, Meek and Hayes, 1968).

A respiratory disease in cattle which was similar to farmer's lung of man has been described (Pirie, Dawson, Breeze, Selman and Wiseman, 1971). Such adult cattle had precipitating antibodies to M. faeni in their sera and histologically there was diffuse infiltration of alveolar septa by lymphocytes, plasma cells and mononuclear cells, epithelioid granulomata and bronchiolitis obliterans. There is no information available in the literature which describes a disease similar to farmer's lung in sheep exposed to mouldy hay. Thus the following work was carried out to find if GLs would appear in the respiratory tract of sheep exposed to the dust of mouldy hay or to fungi found in mouldy hay such as Aspergillus fumigatus.

To investigate all of these ideas the three groups of sheep detailed below were studied :

1. A group of lambs were experimentally infected with the gastro-intestinal parasite H. contortus.
2. A group of lambs was exposed to mouldy hay containing M. faeni.
3. A group of lambs was experimentally infected with A. fumigatus.

B. MATERIALS AND METHODS

The experimental animals and the technique employed are described below.

1. Experimental Animals

A total of 22 lambs aged 4 weeks were purchased from the Veterinary School Field Station, Cochno. These lambs were housed indoors and kept parasite free as described in Chapter 4B. The lambs were divided into 4 groups of experimental animals as listed in Table (26). The description of the animal groups are as follows :

Group H1

This group consisted of 16 indoor lambs which were used to study the influence of some gastrointestinal parasites on the appearance of GLs in their respiratory tracts. These lambs were infected when they were 8 months old with 20,000 third stage infective larvae of H. contortus orally. Ten lambs of this group were killed 5 weeks postinfection and the remaining 6 lambs were killed 12 weeks postinfection Table (26).

Group H2

This group consisted of 2 lambs, numbered SM143 and SM144. They were exposed to mouldy hay in a small clean pen box, when they were 3 months old. The 2 lambs in this group were not killed until they showed respiratory disorder, high rectal temperature, nasal discharge and precipitins to M. faeni in their sera.

Group H3

This group consisted of 2 lambs SM141 and SM142. They received 2 sensitizing doses of A. fumigatus at an interval of 4 weeks and a challenge dose of A. fumigatus 3 weeks later Table (27). The sensitizing doses contained the following :

1 ml. of a suspension containing 170 million spores of A. fumigatus which had been killed with 1.0 per cent. formalin.

1 ml. of a suspension containing 4×10^9 of Bacillus pertussis (Wellcome Research Laboratories, England). These two suspensions were mixed

and injected intramuscularly near the popliteal lymph nodes.

The challenge dose was 50 ml. of sterile PBS containing 500 million spores of A. fumigatus, to which 100 units of penicillin G and 100 mg of streptomycin per 1.0 ml. of sterile PBS were added. This suspension was administered through a catheter passed into the main bronchus of the right diaphragmatic lobe of each lamb using the same procedure described in Chapter 4B. These 2 lambs were killed 3 weeks after the challenge dose.

Group H4

Two lambs, SM25 and SM26, were used as a control group for this experiment.

2. Laboratory Culture of Haemonchus Contortus

To obtain infective nematode larvae for experimental use, it was necessary to culture feces known to contain ova of H. contortus. The feces were best obtained directly from the animal to minimise contamination by free-living larvae or fungal spores. Feces were examined by the McMaster technique for the presence of H. contortus eggs. The feces were crumbled lightly and placed in 500 ml screw topped glass jars. Moist filter papers were inserted in the lid of the jar to provide necessary humidity. Jars were incubated at 21-24^{o}C for 7 days by which time the eggs had hatched and the larvae had reached the infective third stage.

To harvest the larvae, these jars were filled with warm water and allowed to stand for 2 hours. The resulting suspensions were filtered through a coarse sieve to retain the larger particles of debris. The clear fluid which contained the larvae was then Baermannised. After 24 hours the larvae settled to the bottom of the funnel where they were drawn off, washed, concentrated and counted as described in Chapter 4B.

3. Culturing of Aspergillus Fumigatus

Aspergillus fumigatus was grown on Czapek-Dox Agar (Oxoid code No. CM97) incubated at 37^{o}C for 7 days. Spores were harvested in 20 ml. of saline plus few drops of tween 80 (Wetting agent) and mixed on rotamixer.

Spores were counted by the Coulter counter system.

4. Antigen Preparation

Antigens of A. fumigatus were prepared by culture of the organisms grown in 1.0 per cent. glucose-peptone broth at 28°C for 6 weeks. The culture filtrate was then dialysed against running tap water, filtered, concentrated 4 times and used as antigen.

Antigen of M. faeni (No. 151) was prepared from cultures of the organism grown in nutrient broth at 55°C for 3 days. Cultures were dialysed against running tap water for 36 hours, concentrated 4 times in carbowax then millipore filtered and used as antigen.

Antigen of M. faeni (No. 153) was prepared from cultures of the organisms grown on Sabourand Dextrose agar at 55°C for 2 weeks, and freeze-thawed 4 times. Fluid was decanted, Seitz-filtered and dialysed against running tap water for 36 hours. It was then concentrated 4 times, millipore filtered and used as antigen.

5. Clinical Observations and Bleeding

After infection of the experimental animals, routine clinical tests were carried out i.e. twice a week. These included; rectal temperature, respiratory rate per minute, pulse rate per minute and fecal examination. Bleeding was done twice a week from the jugular vein and the blood was collected in vacuum vials containing EDTA. Smears for differential white cell counts were made directly from the blood and stained with Giemsa. Packed cell volumes were determined with a Clay-Adams microhematocrit centrifuge (Archer and Hirsch, 1963). Total white cell counts were made using the Coulter counter system. Lambs in groups H2 and H3 were bled twice a week into vacutainers containing no additive and stored at 4°C overnight. These samples were spun at 1500 r.p.m. for 10 minutes and the serum was tested against the antigens derived from M. faeni and A. fumigatus.

6. Double Diffusion Test

The sera were tested against each antigen by double diffusion in 1.5 per cent. ionagar (No. 2 Oxoid) prepared with MacIlvaine's citric acid buffer. The A. fumigatus antigens were tested by double diffusion in borate buffered agar.

7. Pathology

The methods used were similar to those described in Chapter 4 and 5.

C. RESULTS

1. Parasitological and Haematological Observations

First stage larvae of H. contortus reached the third infective stage 7 days after incubation at 21-24°C. Those lambs in group H1, infected with H. contortus, had eosinophilia on day 14 postinfection and reached 17 per cent. Lambs in group H2 had precipitins to M. faeni in their sera 4 weeks after exposure to mouldy hay. During this time there was a high rectal temperature in both lambs reaching 105.8°F. Pulse rate and respiratory rate were also increased. Nasal discharge and abdominal respiration were the main clinical signs. Group H3 which was inoculated with A. fumigatus by the intrapulmonary route had antibodies in their sera 7 weeks after the first sensitizing dose.

2. Globule Leucocytes

Globule leucocytes were detected in the lungs of 4 sheep which had been infected with H. contortus (Group H1) and killed 5 weeks post-infection (Table 28). These cells were not seen in the lungs of the remaining sheep even those which were killed 12 weeks postinfection with H. contortus.

Globule leucocytes were not seen in the lungs of the sheep in group H2 and H3 which were exposed and inoculated with mouldy hay and A. fumigatus respectively. The GLs which were found in the lungs of the sheep in group H1, were detected in the bronchial epithelium. No such cells were found elsewhere, and examination of many sections from each lung revealed no parasitic lesions. The GLs in these lungs had the characteristic acidophilic intracytoplasmic globules with H & E stain and eccentric nuclei. Alcian blue, astra blue/safranin, toluidine blue and biebrich scarlet stains were used as described in Chapter 2B to confirm and differentiate the GLs from other cells (Table 2). No GLs were seen in the lungs of the control group.

3. Pathology

Group H1

No gross changes were seen in the lungs of the lambs in this group.

Microscopic examination revealed an interstitial pneumonia. There was thickening of the alveolar septa by fibrinous exudate. There was infiltration of the alveolar wall by lymphocytes, macrophages and a few free erythrocytes were seen. The bronchial epithelium had slight hyperplastic changes with a few lymphocytic infiltrations.

Group H2

Small dark to grey subpleural nodules, less than 1.0 mm in diameter, were distributed over the lung surfaces, mostly in the diaphragmatic lobes. There was thickening of the interlobular septa. The cranial lobes had an overinflated area. The cut surface of the lung lobes revealed a normal appearance with only slight oedematous fluid oozing from the bronchial lumen.

Microscopical examination revealed various pulmonary lesions. The most characteristic of these was an interstitial pneumonia with infiltration of the alveolar septa by lymphocytes, plasma cells and interstitial reaction (Fig.86). There were intra-septal aggregates of lymphocytes which showed no germinal centre. Diffuse lesions were found where the alveoli contained oedema fluid, red blood cells, neutrophils and macrophages. In the alveolar septa neutrophils were frequently found along with the plasma cells and lymphocytes (Fig.87). Bronchiolitis obliterans (Fig. 88) occurred and there was a diffuse peribronchial lymphocyt infiltration in most lobes examined. In the sheep which were exposed to mouldy hay, bronchitis and bronchiolitis were the main features of their lungs. The lamina propria of their airpassages were infiltrated with numerous plasma cells, lymphocytes, neutrophils and eosinophils. The lumena of some bronchi and bronchioles were filled with mucus which contained red blood cells and neutrophils. In some lobes, however, there was overinflation with bronchiolar stenosis, dilated alveolar ducts and disrupted alveolar septa.

Group H3

The lungs of this group had pleurisy and there were adhesions between the lungs and the thoracic wall. The lesions occurred on the

subpleural surface of the lung and consisted of diffuse nodules, 1-5 mm in diameter. These small nodules were grey - white in colour surrounded by a narrow rim of hyperaemia. In addition to these nodules pulmonary abscesses were found in the dorsal surface of the diaphragmatic lobes. The periphery of the diaphragmatic lobes had marked consolidation. The cut surfaces were mottled red and grey. A yellow mucopurulent exudate oozed from the cut surface. The remaining part of the diaphragmatic lobes were congested. There was moderate overinflation of the cranial lobes at their apices, while the remaining parts of the lobes underwent complete consolidation. The opened respiratory tracts revealed a haemorrhagic tracheo-bronchitis, the airways being filled with a frothy red exudate.

On microscopic examination the pulmonary lesions of this group were similar to those found in group H2 which were experimentally exposed to mouldy hay. The same widespread lesions of bronchitis, bronchiolitis, bronchiolitis obliterans, cellular infiltration of alveolar septa and epithelioid granulomata were seen. However, a generalized interstitial reaction was found. There was infiltration of alveolar septa and the walls of alveolar ducts by lymphocytes, macrophages and plasma cells. In some lobules, however, there was focal alveolar septal fibrosis. The inter-lobular septa were thick, distended, and filled with fibrin and oedematous fluid. There was peribronchial and peribronchiolar lymphocytic aggregation. The lamina propria was infiltrated with lymphocytes, plasma cells and macrophages which usually obliterated the lumen of the bronchioles. The lumena of the bronchi and bronchioles were plugged with a mucus containing neutrophils, lymphocytes and erythrocytes. The white to grey foci in the subpleural surfaces were astroid bodies. These were epithelioid granulomata containing fungal elements ringed by eosinophilic material and surrounded by multinucleated giant cells (Fig. 89).

D. DISCUSSION

This work provides the first description of an association between bronchopulmonary GLs and H. contortus infection in sheep. Globule leucocytes were commonly found in the respiratory tract of animals infected 5 weeks previously; at which time a patent phase of the infection had developed (Lapage, 1965). However, previous investigations indicated the presence of GLs only in organs in which the parasite or its eggs occurred (Kirkman, 1950; Kent, 1952; Rahko, 1972).

This study agrees well with recent work on bovine schistosomiasis which revealed numerous GLs in the stomach, intestine, liver, gall bladder, kidney and lungs (Lawrence, 1977). Although this author concluded that the presence of GLs in the lungs of cattle could be associated with a response to migrating schistosomulae. However, in the present case it is not applicable since H. contortus does not have any migratory route to the lungs of sheep.

The possible explanation of the induction of GLs in the broncho-pulmonary system of sheep infected with H. contortus is that; as there is a quantitative relationship between the kinetics of the mast cell and GL population in rats infested with N. brasiliensis (Miller, 1971), then it must be accepted that any induction of GLs must be preceded by an increase in the population of mast cells. This agrees well with an examination of the liver of mice experimentally infected with F. hepatica which showed that the proliferation of hepatic mast cells preceded the appearance of the first GL in the bile duct epithelium (Rahko, 1973a). Moreover, Solonitsyna (1973) studied the reaction and population of mast cells in experimental trichocephalosis in sheep. He found an increase in the population of mast cells in the small and large intestine, liver and lungs of sheep when infected with this parasite. Thus it could be concluded from our result here that H. contortus induced a similar systemic increase in the population of mast cells which could be transformed to GLs in the epithelial mucosae of sheep lungs. In addition, it is well known that H. contortus produces an abundance of GLs in the

mucosae of the abomasum (site of parasitic infection) of sheep as described by Keasbey (1923) and Sommerville (1956).

The absence of GLs in ovine lungs following exposure to the dust of mouldy hay and experimental Aspergillosis could be due either to an unsuitable necropsy time of these sheep or that the infection with such a microorganism does not stimulate the induction of GLs. The appearance of GLs in the bronchial epithelium of cattle with farmer's lung (Breeze, 1975) may not be related to the effect of M. faeni or other thermophilic actinomycetes, but due to the presence of a gastro-intestinal parasite in the cattle which may then result in the appearance of GLs in their lungs. Therefore the present studies indicated that an exposure to the dust of mouldy hay in sheep induced a disease condition similar to that of farmer's lung in man and cattle, but evidence of GLs in the bronchopulmonary system of such animals was not found.

Group	Animal number	Type of infection	Week killed after last infection
H1	16	H. contortus oral	7-12
H2	2	Mouldy hay	10
H3	2	A. fumigatus	3
H4	2	Control group	12

Table (26) : Groups and numbers of lambs infected with H. contortus; A. fumigatus and exposed to mouldy hay dust.

Animal number	Weeks of experiment			
	0	4	7	10
SM141	170×10^6	170×10^6	500×10^6	Killed
SM142	"	"	"	"

Table (27) : Group H3 lambs inoculated intrapulmonary with A. fumigatus after sensitization with 2 intramuscular injections of A. fumigatus and B. pertussis.

Group	Animal number	Week killed post infection	Presence of GLs			
			Trachea	Bronchi	Bronchioles	Gland & duct
H1	SM1	7	-	+	-	-
	SM2	"	-	+	-	-
	SM11	"	-	+	-	-
	SM12	"	-	-	-	-
	SM13	"	-	-	-	-
	SM14	"	-	-	-	-
	SM15	"	-	-	-	-
	SM16	"	-	+	-	-
	SM17	"	-	-	-	-
	SM18	12	-	-	-	-
	SM20	"	-	-	-	-
	SM21	"	-	-	-	-
	SM22	"	-	-	-	-
	SM23	"	-	-	-	-
	SM24	"	-	-	-	-
H2	SM143	10	-	-	-	-
	SM144	"	-	-	-	-
H3	SM141	3	-	-	-	-
	SM142	"	-	-	-	-
H4	SM25	12	-	-	-	-
	SM26	"	-	-	-	-

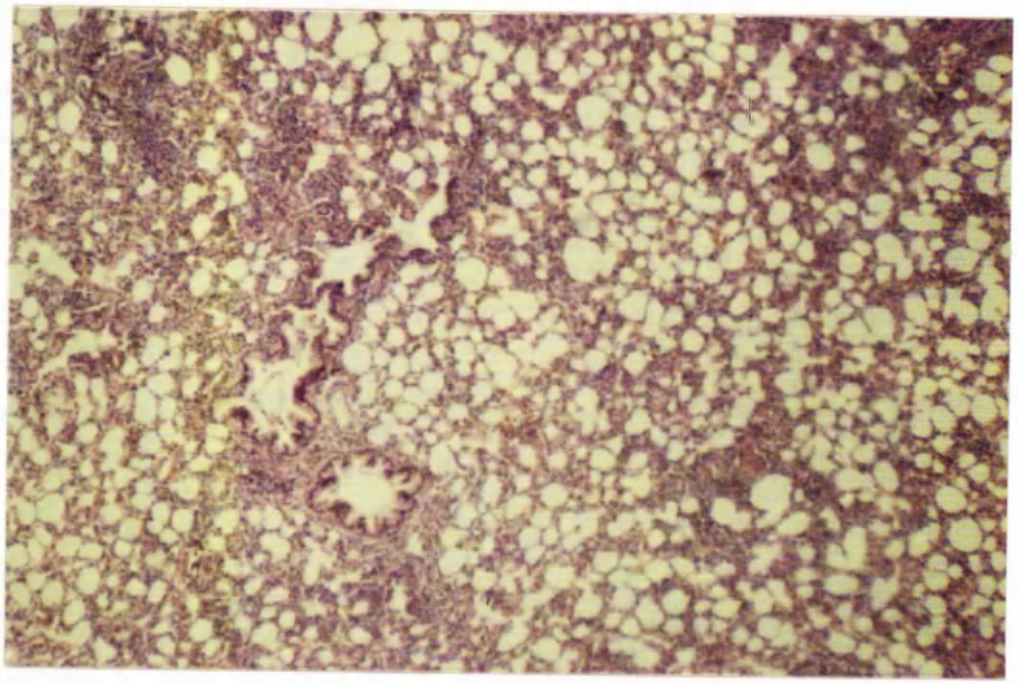
Table (28) : The presence of GLs in respiratory tracts of sheep infected with H. contortus, A. fumigatus and exposed to mouldy hay dust.

- : no globule leucocytes

+ : few globule leucocytes

Fig. 86 : Lung of lamb in group H2 killed 10 weeks after the exposure to the dust of mouldy hay. Microscopically it shows interstitial pneumoniae.
H & E staining, X 100.

Fig. 87 : Detail of a part of a section from the above lesion. Microscopically it shows thickening of the alveolar septa and infiltration with neutrophils, lymphocytes and plasma cells.
H & E staining, X 400.



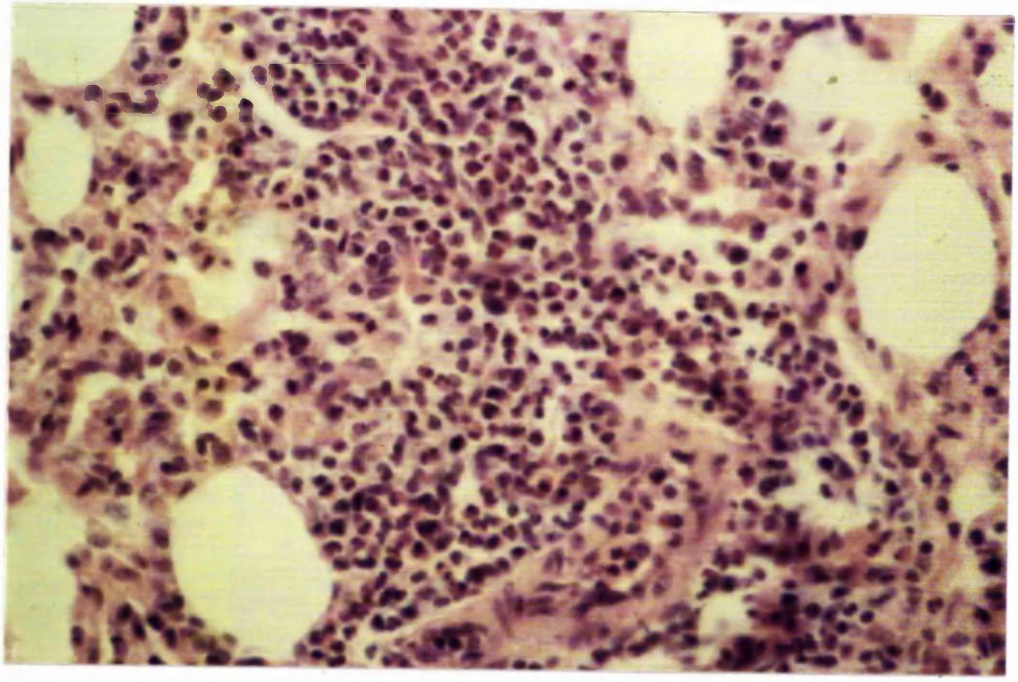
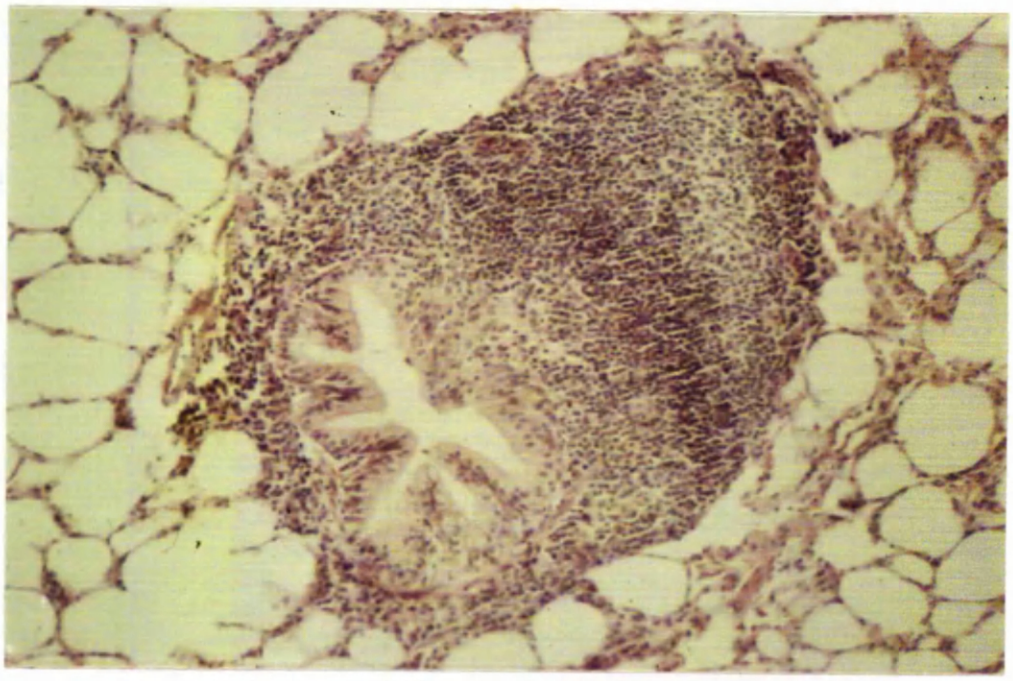
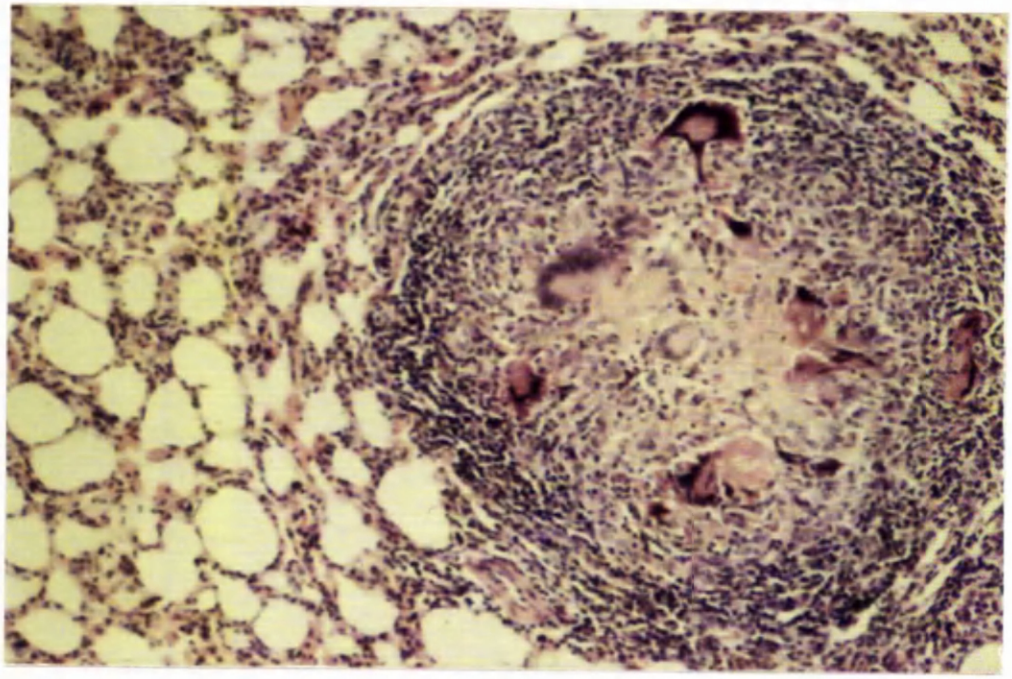


Fig. 88 : Lung of lamb in the same group as in Fig. 86 & 87. Microscopically it shows bronchiolitis obliterans. H & E staining, X 250.

Fig. 89 : Lung of lamb experimentally inoculated with A. fumigatus. Microscopically it shows an astroid or epithelioid granuloma arranged in clubs and ringed by multinucleated giant cells. H & E staining, X 400.





CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

In the first chapter of this thesis, a considerable number of references to GLs in man and many animal species were reviewed. The GL was recognised as a cell with specific morphological characters which appeared in epithelia of mucous membranes under the influence of certain factors such as; parasitic infection (Kirkman, 1950), magnesium deficiency (Contin and Vielleux, 1972), radiation (Kent et al., 1956), oestrus cycle and pregnancy (Kellas, 1961) and certain neoplastic changes (Finn and Schwartz, 1972).

Because GLs were numerous in the mucous membranes of ovine organs during the course of parasitic infections, the present investigation was carried out to provide detailed information on the nature of the cells seen in the bronchopulmonary system of sheep during parasitic infection.

It was shown that cells identical to GLs occurred in the ovine bronchopulmonary epithelium. A survey on the lungs of indoor worm-free and outdoor parasitised sheep indicated that GLs were absent from the lungs of indoor parasite-free sheep, while they were abundant in the lungs of outdoor parasitised sheep. The density and distribution of such cells was related to the type of lesion and species of parasite involved.

Globule leucocytes and mast cells of the sheep tracheobronchial tree had histochemical, morphological and ultrastructural properties similar to those described previously for GLs and mast cells in the alimentary and biliary tracts of sheep (Miller et al., 1967; Murray et al., 1968). However, both the GL and mast cell of the bronchopulmonary system of sheep presented certain histochemical differences from their counterparts in other species.

The histochemical studies carried out here indicated that the mucosubstances in both GL and mast cell granules were sulphomucins, while in the goat the globules of GL consisted of both neutral mucins and carboxymucins, whereas the mast cell granules contained sulphomucins

(Rahko, 1972). Moreover, in the sheep bronchopulmonary system no histochemical differences were revealed between the connective tissue and subepithelial mast cells on the basis of alcian blue/safranin sequence.

Previous studies have shown differences between the ultrastructure of GLs in different species of animals (Carr and Whur, 1968; Rahko, 1972). Recent investigations have also reported electron microscopical differences between granules in mast cells in various animal species (Weinstock and Albright, 1967; Vollarth and Wahlin, 1970; Murata and Spicer, 1974). The findings reported here suggested that the fine structure of the GL in the ovine tracheobronchial tree was similar to that of the GL in the intestinal tract of sheep, cattle and rat (Miller et al., 1967; Murray et al., 1968). Moreover, the fine structure of the GL closely resembled that of the mast cell and basophil in the lamina propria. These 3 cells increased markedly during parasitic infection of the respiratory tract suggesting a possible association between these cells.

Release of histamine from basophil granulocytes induced ultrastructural changes in their granules as shown in infection with T. colubriformis in the small intestinal lamina propria of guinea pigs (Huxtable and Rothwell, 1975). These changes were similar to the findings in the granules of GLs, mast cells and basophil granulocytes of sheep respiratory tracts during parasitic infection.

Experimental infection of lambs with A. suum eggs and D. filaria induced enormous numbers of GLs in their bronchopulmonary system. This agrees well with the previous explanation that mast cells play a role in immunological responsiveness as effector cells mediating an inflammatory reaction via the release of vasoactive amines (Mongar and Schild, 1962; Mota, 1963). Thus the interaction of IgE and worm allergen at the mast cell surface, release of the vasoactive amines during parasite expulsion will induce the appearance of GLs. Murray (1972) postulated that expulsion was achieved by the summation of several immunological events at the mucosal surfaces and in particular that IgE potentiated the release and passage of local-antiworm antibody (IgA) or

systematically produced antiworm (IgG).

Another explanation for the induction of GLs in the mucosa of organs during parasitic infection was the potent mast cell degranulator found in the ascarid larvae cuticle (Uvnas and Wold, 1967). If all helminths had the ability to degranulate mast cells this might explain the presence of GLs in helminth infections (Kirkman, 1950; Kent, 1952).

Experimental infection of lambs with A. suum, D. filaria and H. contortus often resulted in increased numbers of circulating and tissue eosinophils. This is comparable to other experimental infections in animals with various nematodes (Zolov and Levine, 1969; Dobson, 1972; Jarrett, 1973). The eosinophils examined in the ovine broncho-pulmonary system were found to be quite distinct from GLs by histo-chemical and ultrastructural methods.

One of the components of the mast cell granules was the eosinophilic chemotactic factor of anaphylaxis which attracts eosinophils to an area of anaphylaxis (Warren, 1976). Since helminths have the ability to degranulate mast cells then this may confirm the association of an eosinophilia in helminth infections. The functional role of the eosinophil was not yet fully resolved. Eosinophils may modulate the allergic process by regulating the release of histamine from mast cells (Warren, 1976). Evidence has also accumulated to suggest that the eosinophil may be involved in immunological processes. Eosinophils appear to be the effector cells in acquired resistance to Schistosomal infections (Butterworth, Sturrock and Houba, 1975; Mahmoud, Warren and Peter, 1975).

In conclusion it has been shown that GLs developed in the epithelium of the ovine bronchopulmonary system in association with parasitic infections. Parasitic pulmonary infections, in which the parasite remain for a considerable time in the lungs for example D. filaria are more effective on inducing GL development than parasites present in the lung transiently as part of their migratory route. In addition it was found that parasitic invasion of other organs such as the abomasum with H. contortus appear to be associated with the appearance of small numbers of GLs in the

bronchopulmonary system of the sheep.

Dictyocaulus filaria infection was the best method of inducing GLs in the bronchopulmonary system. Having established the time of appearance of GLs in the lungs during infection with this parasite a useful system for further studies on the behaviour of bronchopulmonary mast cells and GLs in the sheep has been developed.

APPENDIX I

HISTOPATHOLOGICAL, HISTOCHEMICAL AND ELECTRON MICROSCOPIC TECHNIQUES

FIXATIVES

1. Carnoy's fluid (Culling, 1963)

Absolute alcohol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

2. Bouin's fluid (Lillie, 1954)

Picric acid, saturated aqueous	750 ml
Formaldehyde (40 per cent)	250 ml
Glacial acetic acid	50 ml

3. Sublimate formol (Drury and Wallington, 1967)

Saturated aqueous solution of mercuic chloride	900 ml
Formaldehyde (40 per cent)	100 ml

4. Buffered neutral formalin ten per cent. solution (Luna, 1968)

Formaldehyde (40 per cent)	100 ml
Distilled water	900 ml
Sodium phosphate monobasic	4 gm
Sodium phosphate dibasic amhydrasis	6.5 gm

STAINING TECHNIQUES

1. Haematoxylin and eosin

1. Take sections to water.
2. Lugals iodine for two minutes.
3. Rinse in water.
4. Five per cent sodium thiasulphate.
5. Rinse in water.
6. Haemolum for five minutes.
7. Rinse in water.
8. Blue in Scotts Tap Water Substitute.
9. Rinse with eosin for two to three minutes.
10. Dehydrate through alcohols to xylene and mount.

Histochemical result :

Nuclei - blue

Cytoplasm - pink

2. Martius Scarlet Blue

1. Take sections to water.
2. Stain with celestine blue for two minutes.
3. Rinse in water.
4. Stain in Mayer's Haemalum for two minutes.
5. Rinse in water.
6. Blue in Scotts Tap Water Substitute.
7. Bring sections up to 90 per cent. alcohol.
8. Stain in Martius Yellow for two minutes.
9. Bring sections down to water.
10. Stain in Brilliant Crystal Scarlet for ten minutes.
11. Rinse and differentiate in one per cent. phosphotungstic acid controlling under the microscope.
12. Wash in water.
13. Stain in soluble blue for two minutes.
14. Rinse in water.

15. Blot, dehydrate in absolute alcohol.

16. Clear in xylene and mount.

Histochemical result :

Nuclei	- blue to black
Fibrin	- red
Collagen	- blue
R.B.C.	- yellow
Muscle	- red

3. Carbol Chromatrope

1. Take sections to water
2. Stain in haemalum for five minutes
3. Rinse in water
4. Blue in Scotts Tap Water Substitute.
5. Wash in water
6. Stain in carbol chromatrope for 30 minutes
7. Rinse in water
8. Dehydrate through alcohol to xylene and mount

Histochemical result :

Nuclei	- blue
Eosinophil granule	- red
R.B.C.	- orange red

4. Perl's Prussian Blue for Iron

1. Take sections to water
2. Transfer to a mixture of equal parts of two per cent. potassium ferrocyanide and two per cent. hydrochloric acid in distilled water for 30 minutes.
3. Wash well in distilled water
4. Counterstain in one per cent. neutral red for two minutes.
5. Rinse rapidly
6. Blot, rinse in absolute alcohol.
7. Clear in xylene and mount

Histochemical result :

Haemosiderin pigment - blue

Nuclei - red

5. Von Kossa's Method For Calcium

1. Take sections to water
2. Stain sections in freshly prepared solution of 1.5 per cent. silver nitrate in the dark for 20 minutes.
3. Wash sections very thoroughly in running water
4. Reduce in freshly prepared 0.5 per cent. hydroquinone for five minutes.
5. Wash sections in water
6. Counterstain in one per cent neutral red for two minutes
7. Rinse rapidly
8. Blot, rinse in absolute alcohol.
9. Clear in xylene and mount.

Histochemical result :

Calcium deposits - black

Nuclei - red

6. The Leuco-Dye Method For Haemoglobin (Lison, 1938, Dunn and Thompson, 1946)

1. Take sections to water
2. Stain in the leuco-blue-peroxide reagent for three to five minutes
3. Rinse in water
4. Counterstain in one per cent. neutral red for two minutes.
5. Rinse in water
6. Dehydrate, clear and mount.

Histochemical result :

Haemaglobin - dark blue

Nuclei - red

Preparation

To 100 ml. of one per cent aqueous Potent Blue add 10g. of powdered zinc and two ml. glacial acetic acid. Boil until colourless. Cool

filter. This is stable in a tightly stoppered bottle. Immediately before use take ten ml. of stock solution, add two ml. of glacial acetic acid and one ml. of three per cent. hydrogen peroxide.

7. Stein's Iodine For Bile Pigments (Stein, 1935)

1. Take sections to water
2. Treat with a mixture of three parts lugal's iodine and one part of alcoholic tincture of iodine.
3. Wash in water
4. Decolorize with five per cent. aqueous sodium thiasulphate for 30 seconds.
5. Rinse in water
6. Counterstain with one per cent. neutral red for five minutes
7. Rinse in water
8. Blot, rinse in absolute alcohol
9. Clear in xylene and mount.

Histochemical result :

Bile pigment - green

Nuclei - red

8. Stains For Neutral Mucosubstances (McManus and Mowry, 1960)

(i) Periodic acid-Schiff (PAS) Technique

1. Bring sections to water
2. Rinse in 70 per cent alcohol for two minutes
3. Place in solution A for seven minutes
4. Rinse in 70 per cent alcohol for two minutes
5. Place in solution B for two minutes
6. Rinse in 70 per cent alcohol for two minutes
7. Wash in water until free of alcohol
8. Place in solution C for 30 minutes
9. Wash in water to intesify for five minutes
10. Stain in Haemalum for one to two minutes
11. Wash and blue in Scotts Tap Water Subsitute
12. Dehydrate, clean and mount

Histochemical result :

PAS positive substances stained deep red (magenta)

Solution A : Alcohol Periodic acid

Periodic acid	0.4g
Distilled water	10 ml
M/5 sodium acetate buffer	5 ml
Absolute ethanol	35 ml

Solution B : Acid Reducing Rinse

Potassium idoine	1 g
Sodium thiosulphate	1 g
Distilled water	20 ml
Absolute ethanol	30 ml
2 N HCL	0.5 ml

Solution C : Fuchsin sulphite (Schiff's Reagent)

Dissolve 2 g basic fuchsin in 400 ml of boiling water. Cool to 50°C and filter. Add to the filtrate 10 ml of 2NHCL and 4 g. potassium metabisulphite. Stopper and leave in a cool place overnight. Add 1g. of decolourising charcoal and filter promptly. Add up to 10 ml or more of 2NHCL in small amounts until the mixture, when allowed to dry in a thin film on a slide, does not become pink. This solution should be kept in a dark well-stoppered bottle in a dark cupboard. It will keep for two months.

All three solutions are kept in a refrigerator in dark bottle.

(ii) Diastase digestion

1. Bring two serial sections to water
2. Rinse in distilled water
3. Digest one section in preheated diastase solution at 37°C for 30 minutes.
4. Wash in water for five minutes
5. Stain both sections with periodic acid-Schiff

Histochemical result :

Diastase digestion selectively eliminates PAS staining attributableto glycogen.

Enzyme solution

A 0.1 per cent solution of malt (mixed α and β amylase, B.D.H.) is prepared in distilled water and used immediately.

9. Blocking Staining Techniques

(i) Acetylation/Periodic acid Schiff Technique (Acetylation/PAS) (Culling, 1963)

1. Bring two serial sections to water
2. Rinse in distilled water
3. Treat one section in a mixture solution of 13 ml. acetic amhydride and 20 ml. of pyridine for 24 hours at room temperature
4. Wash in water
5. Stain both sections in Periodic acid-Schiff Technique

Histochemical result :

A PAS positive substance which after acetylation, gives a negative PAS reaction, indicates that the original reaction was due to a 1:2 glycal group.

(ii) Deacetylation/Periodic acid Schiff Technique (Deacetylation/PAS) (Culling, 1963)

1. Acetylate with acetic amhydride as above
2. Wash in water
3. Treat with 0.1 N potassium hydroxide for 45 minutes at room temperature.
4. Wash in water
5. Stain with Periodic acid-Schiff Technique

Histochemical result :

A positive result in a given structure indicates that the reaction was due to a 1:2 glycal group.

(iii) Methylation Technique (Lillie, 1954)

1. Bring sections to water
2. Leave in one per cent. hydrochloric acid in absolute methyl alcohol for four hours at $37^{o}C$
3. Rinse in alcohol and stain with the appropriate technique (Periodic acid-Schiff Technique or Alcian blue).

Histochemical result :

Acid and sulphated mucopolysaccharide no longer stain after methylation.

(iv) Methylation and Saponification Technique (Lillie, 1954)

1. Methylate with one per cent. hydrochloric acid in methyl alcohol, as above.
2. Saponify with 0.1N potassium hydroxide in water at room temperature for 20-30 minutes
3. Wash gently in water and stain with the appropriate stain.

Histochemical result :

Subsequent demethylation or saponification will restore the staining of carboxyl group (COOH), leaving sulphate groups still blocked.

10. Stains for Acid and Neutral Mucosubstances

(i) Alcian blue-periodic acid-Schiff Technique (AB pH 2.5/PAS) (Mowry, 1956)

1. Bring sections to water
2. Stain in filtered Alcian blue pH 2.5 (one per cent Alcian blue in three per cent acetic acid) for 30 minutes.
3. Wash in tap water for two minutes
4. Rinse in distilled water
5. Place in solution A (page) for seven minutes
6. Wash in tap water for five minutes
7. Rinse in 70 per cent alcohol
8. Stain in solution B (page) for two minutes
9. Rinse in 70 per cent alcohol
10. Wash in water until clear of alcohol

11. Stain in solution C (page) for 30 minutes
12. Wash in water to intensify for five minutes
13. Stain in Haemolum for one to two minutes
14. Wash in Scotts Tap Water Substitute for one to three minutes
15. Wash in water
16. Dehydrate, clear through graded alcohols, and mount

Histochemical result :

Acid mucosubstances - blue

Neutral mucosubstances - red

11. Stains for Sialomucins

(i) Neuraminidase digestion (McCarthy and Reid, 1964)

1. Bring two serial sections to water and dry
2. Flood one section with neuraminidase enzyme at 37°C overnight. Flood control section with four per cent calcium chloride, cover and incubate overnight.
3. Wash carefully in distilled water.
4. Stain both sections by the combined AB/PAS method.

Histochemical result :

Neuraminidase reacts with neuraminidase - sensitive sialomucins to eliminate metachromasia and alcian blue affinity.

Comparison of control and test sections revealed the removal of sensitive sialomucins indicated by a colour change from blue to red with the AB/PAS stain.

Enzyme solution

Neuraminidase (Wellcome Research Laboratories, Beckenham, Kent) is a filtrate from Vibrio cholerae which is stored in 25 ml. bottles at 4°C until required. The marking solution is composed of eight parts of enzyme to one part of four per cent. calcium chloride solution.

12. Stains for Sulphomucins

(i) Alcian blue pH 1.0 - periodic acid-Schiff (AB pH 1.0/PAS) (Spicer & Henson, 1967)

1. Take sections to water

2. Stain in freshly filtered one per cent. Alcian blue in 0.1 normal hydrochloric acid (pH 1.0) for 30 minutes.
3. Wash in water for two minutes
4. Oxidise for five to ten minutes in one per cent. of aqueous periodic acid (solution A page).
5. Wash in running water for five minutes.
6. Rinse in 70 per cent alcohol.
7. Stain in solution B (page) for two minutes
8. Rinse in 70 per cent alcohol
9. Wash in water until clear of alcohol
10. Stain in solution C (page) for 30 minutes
11. Wash in water to intensify for five minutes
12. Stain in Haemolum for one to two minutes
13. Wash in Scotts Tap Water Substitute for one to three minutes
14. Wash in water
15. Dehydrate, clear through graded alcohols, and mount

Histochemical result :

Sulphomucins - blue

Sialomucin and neutral mucins - red

(ii) Alcian blue pH 2.5 AB pH 2.5 (Spicer, Horn and Leppi, 1966)

1. Bring sections to water
2. Stain in Alcian blue (one per cent. Alcian blue in three per cent. acetic acid) for 30 minutes
3. Wash in running water for five minutes
4. Dehydrate in alcohol, clear in xylene and mount

Histochemical result :

Sialomucins, hyaluramic acid and weakly acid sulphate mucosubstances stain dark blue.

(iii) Alcian blue pH 1.0 (AB pH 1.0) (Spicer, Horn and Leppi, 1966)

1. Bring section to water
2. Stain in Alcian blue (one per Alcian blue in 0.1 N hydrochloric acid, pH 1.0) for 30 minutes.
3. Blot dry with filter paper without rinsing

4. Dehydrate in two changes of absolute alcohol and one of equal parts absolute alcohol and xylene, clear in xylene and mount.

Histochemical result :

Sulphated mucosubstances are selectively stained deep blue.

(iv) High-iron diamine (HID) (Spicer, Horn and Leppi, 1966).

1. Bring section to water
2. Stain in HID stock solution at room temperature for 24 hours
3. Rinse quickly in water
4. Dehydrate, clear and mount.

Histochemical result :

sulphated mucosubstances are selectively stained brown-black

Diamine solution

Dissolve 120 mg. of N, N-dimethyl-m-phenylenediamine-dichloride (Eastman Kodak Co., Rochester, New York) and 20 mg of N, N-dimethyl-p-phenylenediamine-monochloride (Sigma Chemicals, London) simultaneously in 50 ml of distilled water (pH 7.0). When the reagents are dissolved, pour this solution immediately into a Coplin jar containing 0.9 ml of standard ferric chloride solution (60 per cent w/v) and 0.5 ml of concentrated hydrochloric acid. Thus 50 ml of staining solution contains 180 mg of $Fe^{(3+)}$ ions. The pH of the HID solution is approximately 1.7.

(v) High-iron diamine-alcian blue (HID/AB) (Spicer, 1965).

1. Bring section to water
2. Stain in fresh diamine solution at room temperature for 24 hours
3. Rinse quickly in water
4. Stain in 1 per cent alcian blue in 3 per cent acetic acid (pH 2.5) for 30 minutes
5. Dehydrate through 95 per cent and absolute alchol, clear and mount

Histochemical result :

most sulphated mucosubstances are purple-black; acid mucosubstances lacking sulphate esters (i.e., hyaluronic acid and sialomucins) are unstained. The post-staining for 30 minutes in one per cent alcian blue in three per cent acetic acid colours sialomucins and hyaluronic acid blue.

13. Stains for Hyaluronic Acid

(i) Hyaluranidase digestion (Hyaluranidase/AB pH 2.5) (Spicer, Leppi and Stoward, 1965)

1. Bring two serial sections to water
2. Rinse in distilled water
3. Incubate one section with hyaluranidase at room temperature for 24 hours. Incubate the other section in buffer solution at room temperature for 24 hours.
4. Wash in running water for five minutes
5. Stain one per cent Alcian blue in three per cent acetic acid (pH 2.5) for 30 minutes
6. Wash, dehydrate, clear and mount

Histochemical result :

Basophilia is eliminated by testicular hyaluranidase indicating the presence of hyaluronic acid.

Enzyme solution

Dissolve 1,000 units of hyalase (Testicular hyaluranidase, Sigma Chemicals, London) in 100 ml of phosphate - buffered saline (pH 6.9) (Difco).

14. Stains For Acid Mucosubstances

(i) Alcian blue or Astra-blue/safranin pH 0.3 (AB/S pH 0.3) (Enerback, 1966b)

1. Take sections to water
2. Stain with 0.1 per cent. astra blue or Alcian blue in 0.7 normal hydrochloric acid for 30 minutes
3. Rinse in 0.7 normal hydrochloric acid for 30 minutes

4. Wash in water
5. Counterstain in 0.5 per cent. safranin in 0.125 normal hydrochloric acid for ten minutes
6. Rinse in water
7. Dehydrate through alcohols to xylene and mount

Histochemical result :

Mast cell nuclei - red

Mast cell nuclei - red

Background tissue - pink

(ii) Toluidine blue pH 4.0 (TB pH 4.0) (Enerbakc, 1966b)

1. Take section to water
2. Stain in 0.5 per cent. toluidine blue in McIlvane's buffer for 45 seconds
3. Rinse in water
4. Blot dry
5. Dehydrate through alcohols to xylene and mount

Histochemical result :

Mast cell granules and mucin - purple red

Background tissue - blue

(iii) Toludine blue pH 0.3 (TB pH 0.3) (Enerback, 1966b)

1. Take sections to water
2. Stain in 0.1 per cent toluidine blue in 0.7 normal hydrochloric acid for ten minutes
3. Rinse in 0.7 normal hydrochloric acid for ten minutes
4. Rinse in distilled water
5. Dehydrate rapidly to xylene and mount

Histochemical result :

Mast cell granules and mucin - purple

15. Fluorescent Staining For Acid Mucosubstances

(i) Acridine orange (AO) (Jagatic and Weiskopt, 1966)

1. Take sections to water
2. Stain in Weigert's hematoxylin for five minutes

3. Wash in tap water three to five minutes
4. Stain in acridine orange, 1:1000 aqueous solution for five to six minutes
5. Rinse in tap or distilled water for one minute
6. Dehydrate in 95 per cent. alcohol three changes for one minute each.
7. Dehydrate in carbol xylal for one minute
8. Clear in two-three changes of xylol
9. Mount with DPX or some other nonfluorescing mounting medium

Histochemical result :

Mast cell granules fluoresce a bright red-orange

16. Stains for The Basic Protein

(i) Biebrich scarlet (BS) (Lillie, 1954).

1. Take sections to water
2. Stain in a solution of one per cent. Biebrich scarlet in glycine buffer at appropriate pH for twenty minutes
3. Wash in water or blot and dehydrate in absolute alcohol
4. Clear in xylene and mount

Histochemical result :

Mast cell granules	- red
Eosinophil granules	- red
Background stains	- pale orange

ELECTRON MICROSCOPIC TECHNIQUES

1. FIXATIVES

Paraformaldehyde/Glutaraldehyde Fixation (Karnovsky, 1965)

2 gm. of paraformaldehyde is dissolved in 25 ml. of distilled water. The solution is heated to 60°-70°C shaking continuously. Add 1-3 drops of 1N NaOH still shaking until the solution is clear or slightly turbid. Allow the solution to cool then add 5 ml. of 50% glutaraldehyde solution (or 10 ml. of 25% solution) and make up to 50 ml. with 0.1M cacodylate buffer - pH 7.4 - 7.6. Final pH should be 7.2. 25 mg. of anhydrous calcium chloride are added. Dilute this solution with another 100 ml. of buffer.

The above solution is a mixture of 1.3% paraformaldehyde and 1.6% glutaraldehyde.

Method :-

1. Fix for $4\frac{1}{2}$ - 6 hours at 4°C
2. Rinse for 2 minutes in 0.1M cacodylate rinsing solution and then leave overnight in fresh rinse at 4°C

Osmium Tetroxide (in Millonig's phosphate buffer).

Stock acid solution-monosodium phosphate 2.26%

Stock alkali solution - sodium hydroxide 2.52%

Buffer :-		
	NaH_2 PO4	83 ml.
	NaOH	17 ml.
	Dist. H_2O	10 ml.
	Sucrose	0.54 gm.

Adjust to pH 7.2 - 7.4

1 gm. of Osmic Acid is added to this buffer.

Method : - 1. As a primary fixative fix for $1\frac{1}{2}$ hours at 4°C.

2. As a postfixative, fix for 1 hour at 4°C.

2. BUFFERS

Cacodylate with Sucrose Rinse

0.1M solution of sodium cacodylate (21.4 gm/l) adjusted to pH 7.4-7.6 by addition of a few drops of concentrated hydrochloric acid.

Add 0.1M sucrose (34.2 gm/l) and adjust pH to 7.2-7.4.

Millonigs Rinsing Solution

NaH_2PO4	83 ml.
NaOH	17 ml.
Dist. H_2O	10 ml.
Sucrose	0.54 gm.

Adjust to pH 7.2 - 7.4

3. EMBEDDING RESINS

Araldite

Stock mixture :- Equal parts of Araldite Resin (CY212) and Araldite Hardener (HY 964).

Heat both to 55°C and mix by hand or leave at room temperature and mix overnight on the mixer. This mixture can be left indefinitely.

Before use :-

Mix - Stock mixture	58 ml.
Accelerator (HY960)	0.6 ml.
Di-n-Butyl-phthalate	2.0 ml.

This mixture should be mixed for at least $\frac{1}{2}$ an hour before use.

Curing :- 48 hours at 57°C

4. THICK SECTION STAINING

Methylene Blue/Azur II

1. Rinse briefly in water
2. Stain for 5-15 minutes (or as necessary) in a freshly prepared solution of equal parts of:-
   - 1% Azur II
   - 1% methylene blue
   - 1% borax
3. Heat-but do not allow to dry
4. Rinse in water
5. Dry and mount

5. THIN SECTION STAINING

Uranyl Acetate (Watson, 1958)

20 per cent. solution in absolute methanol. Store in a dark bottle in the refrigerator.

Lead Citrate (Reynolds, 1963)

Lead nitrate Pb (NO_3P_2)	1.33 g.
Sodium citrate Na_3 ($C_6H_5O_7$) $2H_2O$	1.76 g.
Distilled water	30 ml.

Each salt is dissolved in 15 ml. of distilled water and when dissolved completely mixed together in a 50 ml. volumetric flask. The resultant precipitate is shaken for about 1 minute and then left to stand for 30 mins. with intermediate shakings to ensure complete conversion of lead nitrate to lead citrate.

8.0 ml. of N sodium hydroxide is added and the suspension is diluted to 50 ml. with distilled water and mixed by inversion. The lead citrate dissolves and the staining solution is ready for use. pH 12.0 ± 0.1. Store in refrigerator.

Allow to heat to room temperature before use.

Method :-

1. Spin the uranyl acetate for 5 minutes
2. Float grids on blobs of the stain for 20 minutes
3. Wash the grids in a) Conc. methanol
   b) 50% methanol-twice
   c) Dist. water - twice
4. Blot dry
5. Float the grids on bolbs of lead citrate for 10-15 minutes
6. Wash the grids in a) 0.02N sodium hydroxide
   b) Dist. water - twice
7. Blot dry

APPENDIX II

BLOOD PICTURES OF SHEEP EXPERIMENTALLY INFECTED WITH ASCARIS SUUM AND DICTYOCAULUS FILARIA

PCV	:	Packed cell volume
TLC	:	Total leucocyte count
DLC	:	Differential leucocyte count
Neut	:	Neutrophil
Lymph	:	Lymphocyte
Eos	:	Eosinophil
Baso	:	Basophil
Mono	:	Monocyte

Day	PCV	TLC	D. L. C.				
			Neut	Lymph	Eosin	Baso	Mono
*0	23	7600	34	66	0	0	0
2	28	6800	28	72	0	0	0
5	30	7400	12	86	2	0	0
9	27	11400	21	80	0	0	0
12	28	9700	24	70	6	0	0
16	29	8000	20	78	2	0	0
*21	30	7000	18	82	0	0	0
23	34	8400	30	62	8	0	0
30	31	14100	29	62	9	0	0
33	32	12100	20	72	8	0	0
37	28	11300	14	69	9	8	0
40	26	17600	10	87	2	1	0
44	30	8700	17	77	6	0	0
54	30	9600	18	82	0	0	0

Sheep SM137 : Received a sensitizing dose of 100,000 A. suum eggs on Day 0* and a challenge dose of 10,000,000 A. suum eggs on Day 21*

Day	PCV	TLC	D.L.C.				
			Neut	Lymph	Eosin	Baso	Mono
*0	26	6000	38	62	0	0	0
2	29	7700	42	58	0	0	0
5	27	8200	10	90	0	0	0
9	24	12400	18	76	6	0	0
12	27	11500	36	58	6	0	0
16	27	8500	26	72	2	0	0
*21	30	9700	24	76	0	0	0
23	30	6100	36	64	0	0	0
26	33	11000	32	64	4	0	0
30	32	16600	26	68	6	0	0
33	30	13400	12	53	35	0	0
37	29	12600	23	50	16	11	0
40	29	12200	23	65	6	4	2
44	30	9500	20	78	2	0	0
54	32	10600	40	60	0	0	0

SM138 : Received a sensitizing dose of 100,000 A. suum eggs on Day 0* and a challenge dose of 10,000,000 A. suum eggs on Day 21*

Day	PCV	TLC	D. L. C.				
			Neut	Lymph	Eosin	Baso	Mono
*0	35	5800	30	70	0	0	0
4	34	3600	22	78	0	0	0
8	35	6200	40	54	6	0	0
11	33	7500	10	88	2	0	0
15	29	11300	28	66	6	0	0
*21	30	9800	50	50	0	0	0
25	27	10800	36	60	4	0	0
29	28	7200	28	68	4	0	0
32	30	10400	46	40	14	0	0
39	32	7800	38	54	8	0	0
*43	32	5900	38	54	8	0	0
46	33	6700	24	72	4	0	0
50	27	18000	18	58	24	0	0
53	29	13400	28	48	24	0	0
57	29	11400	22	66	12	0	0
60	27	8400	20	72	8	0	0
64	32	8500	38	60	2	0	0
67	28	27600	27	73	0	0	0

Sheep SM139 : Received a first sensitizing dose of 200,000 A. suum eggs on Day 0* and second sensitizing dose of 100,000 A. suum eggs on Day 21*. It was challenged on Day 43* with 1,000,000 of A. suum eggs.

Day	PCV	TLC	D.L.C.				
			Neut	Lymph	Eosin	Baso	Mono
*0	40	8800	46	54	0	0	0
4	39	9200	40	56	4	0	0
8	33	8400	22	70	8	0	0
11	35	9900	44	50	6	0	0
15	30	7600	22	70	8	0	0
*21	33	8600	32	66	2	0	0
25	34	9800	25	73	2	0	0
29	30	7900	34	66	0	0	0
32	33	9800	32	66	2	0	0
39	36	8000	18	82	0	0	0
*43	38	7100	32	64	4	0	0
46	35	6200	20	68	12	0	0
50	37	11400	32	48	20	0	0
53	32	8400	37	59	4	0	0
57	34	9700	40	42	18	0	0
60	33	8200	26	68	6	0	0
64	33	7100	34	66	0	0	0
67	35	9800	24	76	0	0	0

Sheep SM140 : Received a first sensitizing dose of 200,000 A. suum eggs on Day 0* and second sensitizing dose of 100,000 A. suum eggs on Day 21*. It was challenged on Day 43* with 1,000,000 of A. suum eggs.

Day	PCV	TLC	D.L.C.				
			Neut	Lymph	Eosin	Baso	Mono
*0	40	12800	35	65	0	0	0
4	38	9800	30	69	1	0	0
10	40	7500	30	70	0	0	0
20	36	12300	27	71	1	0	1
27	35	8500	21	76	0	0	3
34	32	9100	31	65	4	0	0
41	34	8100	17	76	6	0	1
48	33	8700	43	54	3	0	0
55	33	8900	35	63	2	0	0
62	35	7700	35	63	2	0	0
69	38	9400	34	66	0	0	0

Sheep SM413 : Received an oral dose of 1,000 larvae of D. filaria on Day 0*

Day	PCV	TLC	D. L. C.				
			Neut	Lymph	Eosin	Baso	Mono
*0	38	11200	40	60	0	0	0
4	40	10400	47	52	1	0	0
10	39	10900	41	57	1	0	0
20	32	10600	35	52	13	0	0
27	34	9900	51	40	9	0	0
34	32	7200	34	52	14	0	0
41	30	8600	44	44	12	0	0
48	31	9700	41	59	0	0	0
55	27	12100	54	44	2	0	0
62	29	11000	54	43	1	0	0
69	35	10800	43	57	0	0	0

Sheep SM415 : Received an oral dose of 1,000 larvae of D. filaria on Day 0*.

Day	PCV	TLC	D. L. C.				
			Neut	Lymph	Eosin	Baso	Mono
*0	38	11100	38	62	0	0	0
4	37	10500	43	57	0	0	0
10	33	6400	39	63	0	0	0
20	25	7000	32	55	11	0	0
27	29	4800	34	65	1	0	0
34	30	5000	42	58	0	0	0
41	27	6400	26	71	3	0	0
48	30	8400	35	60	5	0	0
55	32	7000	34	64	2	0	0
62	30	8600	48	52	0	0	0
69	27	6200	30	66	3	0	1
*74	28	7200	34	64	2	0	0
81	26	7200	33	60	6	0	1
86	30	8000	40	47	13	0	0
94	29	8400	28	69	3	0	0
101	27	7000	32	63	5	0	0
108	28	8800	22	70	8	0	0
115	30	6500	30	68	2	0	1

Sheep SM421 : Received a sensitizing dose of 1,000 larvae of *D. filaria* orally on Day 0* and a challenge dose of 1,000 larve of *D. filaria* intravenously on Day 74*.

Day	PCV	TLC	D. L. C.				
			Neut	Lymph	Eosin	Baso	Mono
*0	40	9800	36	64	0	0	0
4	40	10700	38	60	2	0	0
10	41	10800	42	47	12	0	0
20	43	12500	43	53	4	0	0
27	39	12800	31	65	3	0	1
34	39	12300	34	65	1	0	0
41	42	14200	33	66	1	0	0
48	33	15200	44	52	0	0	4
55	32	11800	23	74	2	0	1
62	33	12200	28	70	1	0	1
69	30	11400	32	66	2	0	0
*74	33	12500	39	58	3	0	0
81	31	14400	43	54	14	0	0
86	29	15000	41	55	4	0	0
94	30	14000	38	57	2	0	3
101	28	12200	29	60	9	0	2
108	30	10100	32	62	6	0	0
115	29	9200	32	65	3	0	0

Sheep SM427 : Received a sensitizing dose of 2,000 larvae of D. filaria intravenously on Day 0* and a challenge dose of 1,000 larvae of D. filaria intravenously on Day 74*.

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