A study of the cytology of the gastrointestinal tract of some normal and parasitised animals

SUMMARY

of

Dissertation for the degree of Doctor of Philosophy

by

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The gastrointestinal tracts of worm-free sheep and sheep infected with the nematode Ostertagia circumcincta were examined histologically to determine whether a relationship could be demonstrated between parasitic infestation and the presence of globule leucocytes. Globule leucocytes were virtually absent from the mucosa of worm-free sheep, but present in high concentrations in parasitised animals, particularly in the areas which O. circumcincta is found. These results indicated that infection by nematodes is capable of eliciting a globule leucocyte response.

Rats were experimentally infected with the small-intestine nematode Nippostrongylus brasiliensis and killed at intervals over the period of infection. The gastrointestinal tracts were examined for the presence of globule leucocytes, and the results were compared with those from an uninfected control group. A marked globule leucocyte response became apparent in the small intestine on the twelfth day and persisted until the twentieth. The result demonstrated a relationship between globule leucocytes and infestation by nematodes.

A further feature of these results was the apparent relationship between the appearance of globule leucocytes and the onset of the immune response (self-cure), which resulted in the expulsion of the adult worms from the small intestine, commencing about the tenth day.

The possibility that this relationship was fortuitous could not be discounted since the development of globule leucocytes may have been related to some earlier event in the life cycle of the parasite. However, the timing of self-cure can be altered by changing the immunological status of the rat.

Rats were rendered hyperimmune by repeated infections with N. brasiliensis and the timing of the globule leucocyte response to a challenge infection was compared with that previously observed during a primary infestation. The onset of the globule leucocyte response again coincided with self-cure despite the fact that in hyperimmune rats this occurred approximately six days earlier then in a primary infection. This result indicated a direct relationship between globule leucocytes and the immune response.

Pierce and co-workers (1962, 1965) had noted globule leucocytes in the intestinal tracts of fowls during coccidial infections. In view of the possibility that other types of parasite apart from nematodes elicited a globule leucocyte response, suitable material was examined, the results suggesting that globule leucocytes are also associated with cestodes and trematodes.

The possibility that Russell body cells and globule leucocytes are related (White, 1954) was investigated by comparing, with the light microscope, the morphology of experimentally produced Russell body cells and globule leucocytes. At a certain stage of development Russell body cells were found to be morphologically similar to globule leucocytes. The ultrastructure of globule

leucocytes was also described and it was noted that these cells possessed a number of features suggesting a close relationship to the Russell body cell.

The nature of the immune response to N. brasiliensis has been partially resolved into antibody and anaphylactic components by other workers. It therefore became necessary to investigate the role of globule leucocytes in anaphylaxis. Observations indicated that the globule leucocyte granule did not contain the histochemical components characteristic of mast cells and that the cellular distribution within the small intestine was distinct from that of mast cells. Globule leucocytes did not undergo degranulation during experimentally induced anaphylaxis, and it was concluded that globule leucocytes are not related to mast cells nor do they have a role in anaphylaxis.

These findings provided additional indication that the role of the globule leucocyte might be as an antibody producer. The globule leucocyte response was therefore examined in animals which were immunologically impaired. These were neonatally infected rats in which self-cure did not take place, and adult rats which had been neonatally thymectomised, with the consequent abolition of self-cure. A slight globule leucocyte response occurred in neonatal rats after infection with N. brasiliensis, but because of the lack of suitable controls these findings could not be interpreted on a quantitative basis. However, in the neonatally

thymectomised rats comparison with a suitable non-thymectomised control group revealed impairment of the globule leucocyte response.

On the basis of the collected findings it was concluded that the globule leucocyte is a cell of the lymphoreticular series arising from a precursor which is similar or identical to a plasma cell. Such cells are directly related to the immune response towards certain nematodes and are almost certainly involved in antibody production and transport.

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PREFACE

This work was carried out in The University of Glasgow Veterinary School. The work presented in this thesis is original and where assistance was received from the work of others it is acknowledged in the text in the usual way. Personal assistance by colleagues is acknowledged below.

I am indebted to Mr. R.N.C. Aitken and to Professor W. Mulligan, under whose supervision this work was carried out. I am indebted to both for suggesting the original problem as well as for their constant advice and valuable support at all stages of this work. I am grateful to Professor G.M. Wyburn for making available the electron microscope facilities in the Department of Anatomy at this University. I am also indebted to Miss Mary Gracie and the technical staff of the Department of Histology and Embryology, University of Glasgow Veterinary School, for their excellent technical assistance.

I also gratefully acknowledge the following assistance. Miss Mary Gracie gave technical assistance in the development of a technique to recover adult Nippostrongylus brasiliensis from the intestines of unweaned rats, and technical advice in the selection of tests used to determine the histochemical nature of globule leucocyte granules, and assistance in performing these tests. Mr. H.S. Johnston was responsible for the processing , cutting and photography of blocks for electron microscopy.

SECTION I

INTRODUCTION

SECTION II

MATERIALS AND METHODS

SECTION III

EXPERIMENTAL OBSERVATIONS

SECTION 1

INTRODUCTION

Part 1 : The globule leucocyte

The term globule leucocyte (Keasbey, 1923) is translated from the German "Schollenleukozyten", which was the name used in the first comprehensive morphological descriptions of this cell as found in the intestinal mucosa of a number of species, by Weill (1919, 1920). These descriptions formed the basis for their identification by many subsequent authors. While certain cells are therefore readily recognisable as globule leucocytes, a precise definition of the cell is not possible due to the large numbers of reports in the literature of cells which resemble globule leucocytes to a greater or lesser degree, but which do not entirely conform to the original descriptions.

For the purposes of the present investigation the definition of the globule leucocyte has been arbitrarily restricted to those cells which conform fairly closely to the original descriptions of Weill (1919, 1920). Such cells occur inconstantly in the mucosa of the gastrointestinal tract, and possess certain characteristics of morphology and distribution (fig. 1). Round acidophil granules of variable size and number are present in the cytoplasm. These granules are larger than those of blood granulocytes and may reach several microns in diameter. The cytoplasm is refractory to routine histological staining techniques. The nucleus has a chromatin distribution resembling that of a lymphocyte (see Cossel, 1965) or plasma cell (see Sainte-Marie, 1964). The distribution of the cell within the gastro-

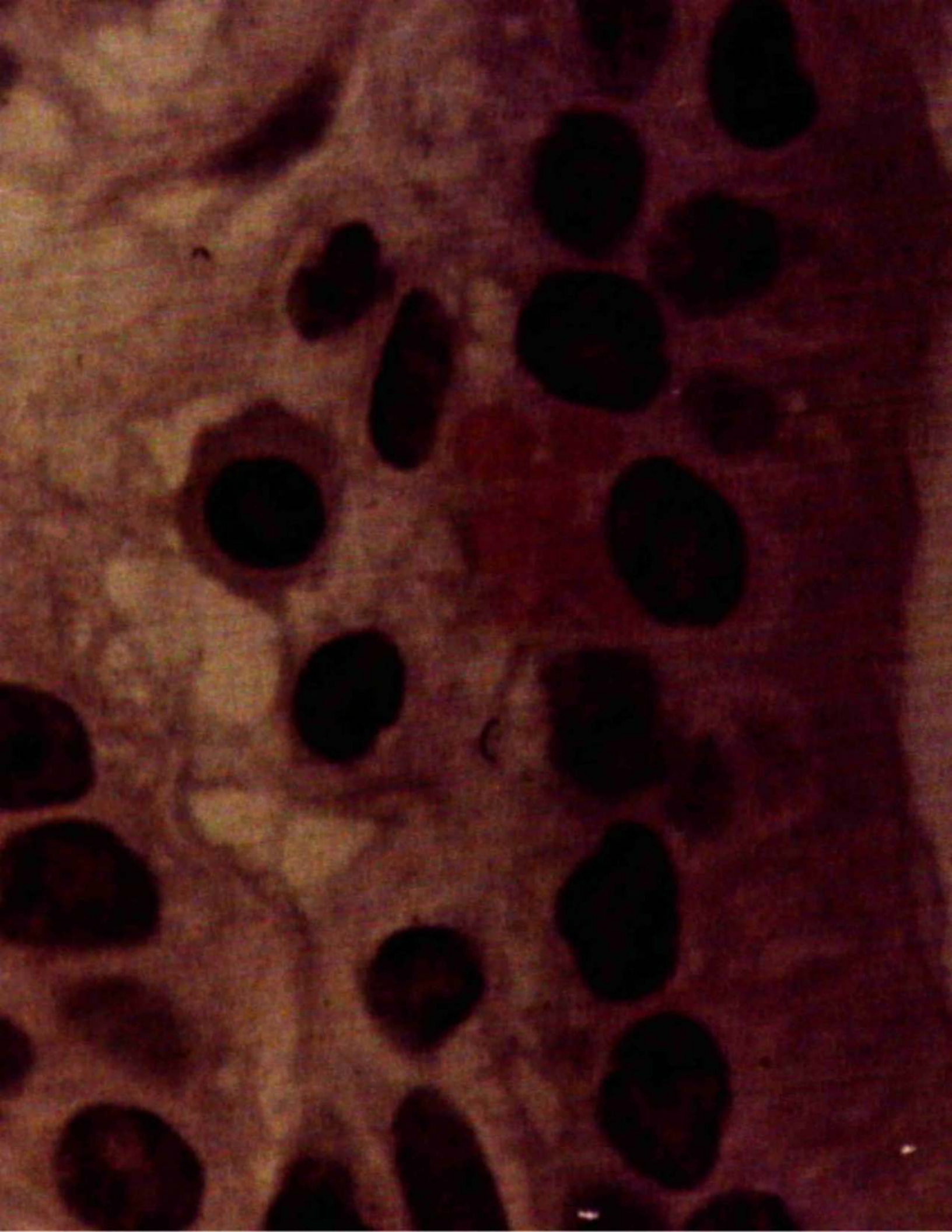

Fig. 1

Globule leucocytes situated in an intraepithelial position, in the abomasum of a sheep infected with Ostertagia spp. The cell on the right illustrates the characteristic nucleus and unstained cytoplasm. The nucleus associated with the cell on the left belongs to an overlying columnar epithelial cell.

Methacrylate section stained with haematoxylin and eosin.

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intestinal mucosa is characteristic in that a large proportion of globule leucocytes are located in an intraepithelial position (Taliaferro and Sanlos, 1939 ; Davletova, 1958). Toner (1965), Whur and Johnston (1966, Appendix 1) and Carr (1966) have studied the ultrastructure of the globule leucocyte in the fowl, rat and mouse respectively, enabling the morphology of the cell to be defined more accurately than was possible with the light microscope. Toner (1965) describes the characteristic globules as homogeneous electron -dense granules which sometimes contain vacuoles. Mitochondria and rough endoplasmic reticulum are scarce but free ribosomes are plentiful. Carr (1966) examined crystalline inclusions in globule leucocytes from the mucosa of the large intestine of the mouse. They were described as rhombohedral units with corner angles measuring 74° or multiples of this number, and had longitudinal striations with a 45 Å interspace. A second order of striation with 23 Å spacing was also apparent, and a lattice structure was produced by cross striations connecting those running longitudinally. Certain amorphous inclusions showed isolated small areas of striation.

Cells conforming to the above description of globule leucocytes have been studied by Heidenhain (1888), Müller (1899), Fenata (1906), Sansonow (1908), Muthmann (1913), Schwarz (1914), Weill (1919, 1920), Corti (1922), Lim (1922), Keasbey (1923), Maximow (1924), Schilling (1925), Claus (1926), Mjassojedoff (1926), Dawson (1927a), Lavrentiev and Lasovskii (1928), Rogosina (1928), Tolwer

(1929), Ubr3 (1929), Plenk (1932), Patzelt (1936), Taliaferro and Sarles (1939), Dawson (1943), Duran-Jorda (1945), Lasovskii (1948), Greulich (1949), Kent (1949, 1952), Hill (1951), Kent et al. (1954, 1956), Sommerville (1956), Davletova (1958), Gordon and Bruckner -Kardoss (1959), Gordon (1960), Rootes (1961), Pierce et al. (1962), Luckey (1963), Anderson et al. (1965), Pierce and Long (1965), Toner (1965), Wakelin (1965), Whur (1965, 1966 a,b,c,d, Appendix 1), Armour et al. (1966), Carr (1966), Copland (1966), Dobson (1966), Mackenzie (1966), Whur and Gracie (1966b, Appendix 1), Whur and Johnston (1966, Appendix 1), and are the subject of the present study.

It is not intended to imply a difference between globule leucocytes as defined above and the many cells which have certain features in common with, but do not entirely conform to the descriptions of Weill (1919, 1920), and which may in fact be identical or related to globule leucocytes. These include cells which have been differentiated solely on the basis that they have been reported in organs other than the gastrointestinal tract. These include cells in the urinary tract (Kirkman, 1947, 1949, 1950 : Ahlqvist and Kohonen, 1959a, b), trachea (Kirkman, 1950), soft palate and gall bladder (Kent, 1952), lung and bile duct (Zipper, 1966), as well as similar cells found in the female genital tract (Asplund and Holmgren, 1947 : Hamperl, 1955 : Wislocki et al., 1957 : Kellas, 1961 : Nellor, 1965). In addition, various types of Russell body cell (Russell, 1890 : Apitz, 1937; Pearse, 1949 : White, 1954 : Thiery, 1958 : Bangle, 1963 :

Munsick and Janovski, 1963 : Yoshida et al., 1964), "grape" cells (Zlotnick et al., 1959) lymph node "globule leucocytes" (Dawson, 1927b), connective tissue basophils (Taliaferro and Sarles, 1939), atypical mast cells occurring in the gastrointestinal tracts of rats (Maximow, 1906 : Hunt and Hunt, 1956), and certain yellow autofluorescent cells (Sainte-Marie, 1965) may be related to the globule leucocyte. The relationship between globule leucocytes and certain of these cells is discussed in the appropriate section.

The description of globule leucocytes has in many cases been the result of observations incidental to a wider survey. Thus short references to globule leucocytes are frequently found in papers which centre around haematological (Ferrata, 1906 : Maximow, 1924) or gastrointestinal (Lim, 1922) studies, and more recently in papers concerned with the pathological aspects of endoparasitic infestation (Taliaferro and Sarles, 1939 : Pierce et al., 1962 : Anderson et al., 1965 : Armour et al., 1966).

Prior to Taliaferro and Sarles (1939), work on the globule leucocyte was restricted to morphological observations with the light microscope. Theories concerning the functional significance or origin of the globule leucocyte based upon morphological criteria have been advanced in a number of papers. Thus, the light microscope observations of Duran-Jorda (1945) upon globule leucocytes, although not recognised as such at the time, led the author to conclude that the cell is a site of erythropoiesis within the gastrointestinal mucosa; Keasbey (1923) suggests that globule leucocytes are linked

in some way to the degeneration of subepithelial capillaries in the area, and that the characteristic globules are derived from erythrocytes, while Kent (1952) suggests that globule leucocytes arise as a reaction to the diffusion of substances of various origins into the mucous membranes.

Baldaferro and Sarles (1939), in an extensive description of the pathology of the rat jejunum during infections with the nematode Nippostrongylus brasiliensis, noted the presence of globule leucocytes at various stages of the infection. However, they do not state that a relationship exists between the presence of globule leucocytes and nematodes. Kirkman (1947) describes cells occurring in an intra-epithelial position in the urinary tracts of rats infested with the roundworm Trichosomoides crassicauda. Although certain differences were noted between these granular cells and globule leucocytes a relationship between the two types of cell, and then to T. crassicauda is tentatively suggested. The differences between the two cells were based upon the absence of iron, haemoglobin and siderophilia, as previously demonstrated in globule leucocytes by histochemical techniques (source not quoted). After further research (Kirkman, 1949, 1950), it seems certain that these cells were in fact globule leucocytes, and that the histochemical differences noted were due to the attempts of earlier authors (probably Keasbey, 1923 and Duran-Jorda, 1945) to demonstrate that globule leucocytes contain erythrocytes or erythrocyte-degradation products. Kirkman's work

was repeated by Ahlqvist and Kohonen (1959a, b), and a relationship between globule leucocytes and T. crassicauda was again suggested. However, no experiment was undertaken in order to test this hypothesis.

Sommerville (1956) made independent observations upon the occurrence of globule leucocytes in association with nematode infestation of the abomasum of sheep. Of 53 abomasa examined, 32 contained no globule leucocytes. With a single exception these organs were from sheep which had either not experienced infestation with helminths , or had been infested for a period of 35 days or less. 21 abomasa from sheep which had been infested with helminths for more than 35 days all contained globule leucocytes. On the basis of these results, together with the observation that the globule leucocyte response was not necessarily located in the area inhabited by the parasite, the author accepts the possibility of an indirect association between globule leucocytes and nematode infestations, but states that the evidence is insufficient to permit any conclusion. Nevertheless, combination of these findings with those of Kirkman (1947, 1949, 1950) and the previous observations of Taliaferro and Sarles (1939) strongly suggests the possibility of a relationship between the presence of nematodes and globule leucocytes.

In addition to the evidence cited above, the work of Pierce et al. (1962) and Pierce and Long (1965) has indicated that infection of domestic fowls with Eimeria tenella also elicits a globule leucocyte response. Long (1965) states that birds in which the cloacal bursa

has been removed , and which are subsequently immunised against E. tenella and exposed to infection produce the same globule leucocyte response as intact fowls which are similarly immunised and infected although, because of bursectomy, these birds are rendered incapable of producing circulating antibodies. The effect of neonatal partial thymectomy (Pierce and Long, 1965) on the globule leucocyte response to E. tenella is not reported.

The origin of the globule leucocyte is unknown. Keasbey (1923) suggests that the globules contain erythrocyte degradation products; thereby implying that the globule leucocyte is a phagocytic cell similar to a macrophage. Maximow (1906) describes the presence of "atypical mast cells" in the small intestine of the rat which may be globule leucocytes, and indicates that they are probably derived from plasma cells. Kirkman (1947) states, on the basis of staining reactions applied to fresh and fixed material, that globule leucocytes are distinct from plasma cells and Russell body cells, but later (1949) suggests a possible relationship to "aberrant plasma cells". In a more extensive study (1950), which included 79 histochemical tests on urinary globule leucocytes this author states that they are distinct from eosinophilic myelocytes, eosinophilic leucocytes, erythroblasts, erythrophages and connective tissue mast cells, but suggests that a close relationship exists between plasma cells with acidophil granules and Russell body cells on the one hand and globule leucocytes on the other, while White (1954) indicates that globule

leucocytes and Russell body cells may be identical. Kent (1949, 1952) describes the development of globule leucocytes from lymphocytes in the lamina propria of the sheep's intestine. He states, on the evidence of histochemical tests, that the globules are not phagocytosed erythrocytes or Russell bodies. Toner (1965), in a study with the electron microscope, supports the theory that globule leucocytes are derived from lymphocytes. However, he apparently did not observe forms intermediate between the two cell types.

A number of experimental studies have been undertaken on the globule leucocyte. Kent et al., (1954) found that treatment with corticotrophin or cortisone reduces the number of globule leucocytes in the small intestines of rats, while Kent et al.(1956) demonstrated that whole-body X-irradiation or selective irradiation of an isolated loop of intestine markedly reduces the number of globule leucocytes present during the next few days. The concentration of cells subsequently returned to the initial level 53 days after irradiation. In addition, the authors demonstrated that a significant reduction in the number of globule leucocytes takes place after hypophysectomy. Gordon and Bruckner-Kardoss (1959) compared the concentrations of globule leucocytes in germfree and conventional fowls, and found them to be significantly lower in the former. Davletova (1958) observed that the gastrointestinal tracts of unweaned lambs contained no globule leucocytes, but that the cells appeared when the lambs were put on to fodder; their appearance at this time was ascribed to physiological

dietary factors.

In a report whose appearance postdated the completion of the experimental work contained in this thesis, Dobson (1966) stimulated a globule leucocyte response in the colon of sheep by infecting them with Oesophagostomum columbianum. Rabbit anti-sheep globulin conjugated with fluorescein isothiocyanate was used to demonstrate that the cytoplasm of globule leucocytes contains condensations of globulin. Attention was drawn to various factors suggesting a relationship between globule leucocytes and plasma cells.

Part 2 : Genus Ostertagia

Ostertagia spp. have a direct life cycle similar to that of other Trichostrongylidae. They occur in the abomasum of ruminants, and more rarely in the small intestine (Lapage, 1962) where the larvae of O. ostertagi penetrate Brunner's duodenal glands (Jubb and Kennedy, 1963). Anderson et al.(1965) classified field cases of parasitic gastroenteritis in calves where O. ostertagi was the predominant parasite into Type 1, pre-Type 2, and Type 2 phases of the disease. Type 1 occurred in calves at grass for the first time , and was accompanied by the classical symptoms of clinical gastritis. Pre-Type 2 ostertagiasis was not apparent clinically, but large populations of O. ostertagi were present in the abomasum, mostly inhibited at the fourth larval stage. The Type 2 syndrome was characterised by loss of weight and diarrhoea. A characteristic part of the pathology of O. ostertagi infections is the presence of raised nodules containing fourth stage larvae. The larvae emerge from the nodules, commencing about two weeks after experimental infection(Ross and Dow, 1965a). Emergence of fourth stage larvae also occurs during the Type 1 phase of field infection, but there are no reports of globule leucocytes being observed (Anderson et al., 1965 : Ross and Dow, 1965b). During the pre-Type 2 phase of inhibition , Anderson et al.(1965) indicate the minimal nature of the pathological reaction to the larvae embedded in the glands. However, in some cases globule leucocytes were observed

around old "emerged" lesions at this stage, together with increased numbers of lymphocytes, plasma cells and eosinophil leucocytes. According to these authors, Type 2 ostertagiasis is characterised pathologically by the sequential development and emergence of the previously inhibited larvae. During this phase of the disease globule leucocytes are present in high concentrations.

Ostertagia spp. infection in sheep has not been similarly classified into phases. Nevertheless, the phenomenon of a "spring rise" in the egg count of ewes occurs (Taylor, 1935) which has been shown by Crofton (1954) to be mainly due to an increased output of eggs by Ostertagia spp. adults. These adults are derived from a larval population ingested much earlier (Dunsmore, 1965). Sommerville (1956) observed globule leucocytes in the abomasa of sheep after experimental infection with O. circumcincta, but they only appeared a minimum of 35 days after infection. They were also found in the intestina mucosa and the duodenal glands, but according to the author there was no correlation between the site of nematode infestation and the region in which the cells were most common.

Part 3 : Nippostrongylus brasiliensis

Nippostrongylus brasiliensis (Travassos, 1914), also called N. muris (Haley, 1961), is a trichostrongyloid worm with a direct life history, whose normal host is the rat. The eggs hatch within 24 hours of being voided in the faeces (Yokogawa, 1922). Maximum hatching of eggs takes place at temperatures between 22°C and 30°C (Luttermoser, 1937). There are three preparasitic larval stages, and two preparasitic moults take place (Haley, 1962). The third stage larva is infective, and remains partially enclosed in its own sheath which is split open at the anterior end (Chandler, 1932). The larvae are capable of vertical and longitudinal migration (Africa, 1931). The normal portal of entry of the infective third stage larvae into the host is via the intact skin (Schwartz and Alicata, 1934). However, for experimental purposes rats may be infected by injecting a larval suspension subcutaneously into the groin region. The larvae reach the lungs via the blood (Yokogawa, 1922) and lymphatic system (Gharib, 1961) approximately 12 to 24 hours later (Taliaferro and Sarles, 1939 : Twohy, 1956), where they grow rapidly (Twohy, 1956) and feed on tissue cells (Taliaferro and Sarles, 1939) and whole blood (Weinstein and Jones, 1956). The third moult takes place in the lungs, and the resulting fourth stage larvae are sexually differentiated (Yokogawa, 1922). These larvae migrate up the trachea, are swallowed, and begin to appear in the small intestine 40 to 60 hours after infection (Sarles

and Taliaferro, 1936 : Trohy, 1956). The final moult occurs in the small intestine about 100 hours after infection (Yokogawa, 1922). The immature adults grow rapidly and attain sexual maturity, so that between 130 and 140 hours after infection nearly all the females contain sperm and fertile eggs (Weinstein and Jones, 1959). Eggs are present in the faeces from the sixth day after infection. The adult worms inhabit the duodenum and jejunum (Haley, 1962), and at a later period small numbers spread into the ileum (Brambell, 1965a). There is one report that the adult worms ingest intestinal contents (Weinstein and Jones, 1956), but it is more generally accepted that the adult worms feed on the host's tissues (Porter, 1935a : Taliaferro and Sarles, 1939 : Rogers and Lazarus, 1949 : Haley, 1962).

The number of eggs voided in the faeces rises rapidly after the sixth day of infection ; egg output then remains steady for between three and five days, and subsequently falls to near zero 15 to 20 days after infection (Africa, 1931 : Graham, 1934). The decline in egg output is closely followed by the rapid expulsion of a large part of the adult worm population (see Neilson, 1965). This is not true however of neonatally infected rats (Jarrett et al., 1966 : Kassai and Aitken, 1966) or where small infections are employed. In the latter case there is a gradual loss of worms extending over the next 30 days (Haley and Parker, 1961). Females are expelled more rapidly than males (Africa, 1931 : Porter, 1935b) and worms remaining from old primary infections are almost exclusively males (Sarles and

Taliaferro, 1936).

The elimination of the adult worm population is known as self -cure, and occurs as a result of an immune response on the part of the host (Mulligan et al., 1965). The mechanism involved in self -cure has been investigated by Mulligan et al.(1965), Urquhart et al.(1965), and Barth et al.(1966). While humoral antibody was shown to be important in interfering with the establishment of an adult worm population, protective serum had no immediately lethal effect upon N. brasiliensis in vitro (Mulligan et al., 1965). Consequently, it was suggested by these authors that antibody may be protective because of its role in anaphylaxis, rather than by a direct action against the worms. Urquhart et al.(1965) investigated the role of local anaphylaxis in N. brasiliensis infections and demonstrated the presence of areas of increased capillary permeability in the part of the jejunum occupied by the worms. It was also demonstrated that intravenous injections of extracts of adult N. brasiliensis into rats previously rendered immune , either by prior experience of N. brasiliensis or by serum transfer, produced anaphylactic reactions in the gut which were demonstrable with Evans blue dye. Barth et al.(1966) studied the effects of non-specific anaphylaxis and passive immunisation on an adult population of N. brasiliensis in vivo. Ovalbumin-induced anaphylaxis or administration of hyperimmune serum alone produced no significant change in the worm population 36 hours later, but hyperimmune serum and anaphylaxis combined caused a partial expulsion

of the worms. This was interpreted as indicating that the physical changes associated with anaphylaxis facilitated the passage of antibody into the subepithelial spaces and intestinal lumen where its effect was specifically directed against the worms. Since a lesion apparently identical to that caused by experimentally induced anaphylaxis is also present in naturally infected rats, it is probable that this lesion plays a similar role in the self-cure reaction (Barth et al, 1966).

Ogilvie (1965b) showed that immunity to N. brasiliensis is stimulated primarily by adult worms. This immunity reacts not only against adult stages, since larvae from a challenge infection are inhibited from developing to maturity (Sarles and Taliaferro, 1936). Brambell (1965a) showed that the percentage of males recovered at post mortem increases from about 50% during the initial 10 days of the infection to nearly 90% after day 20. Ogilvie (1965b) demonstrated that 100 male worms did not consistently stimulate immunity, whereas all rats infected with 10 female worms became immune, and suggested that males survive longer because they produce fewer protective antigens, and are therefore less affected by host immunity. Rats challenged two months after a single infection showed no decline in immunity, whereas at seven months immunity had begun to decline, but was rapidly restimulated by a challenge infection (Ogilvie, 1965b).

N. brasiliensis is generally considered to be a parasite of the upper part of the small intestine of rats (Travassos, 1914 ; Yokogawa, 1920), present in both jejunum and duodenum (Haley, 1961).

It has been reported that at the height of a primary infection the worms are found in the jejunum, but as the infection ages the worms that remain are found only in the duodenum (Africa, 1931 : Chandler, 1935). Brambell (1965a) found that worms were almost completely confined to the anterior part of the jejunum until the first 10 or 12 days of a primary infestation. Worms remaining after self-cure were randomly distributed along the whole of the small intestine, but towards day 20 showed an increasingly marked tendency to be restricted to the duodenum, so that by day 30 no worms were present in the jejunum or ileum. The distribution of N. brasiliensis in immune rats is unlike that of a primary infection. The worms are more evenly distributed throughout the length of the small intestine, and the population mode position is slightly anterior to that of a primary infestation (Brambell, 1965b).

Africa (1931) noted that N. brasiliensis was pathogenic when present in large numbers. Very large doses of infective larvae produced fatal pneumonia two days after injection, but rats surviving the period of pulmonary migration usually survive the intestinal stage (Porter, 1935b). Lesions occur in the skin, lungs and intestinal tract (Porter, 1935b : Sarles and Taliaferro, 1936 : Taliaferro and Sarles, 1937a, b, 1939). The worms are embedded deeply between the villi of the jejunum and cause local tissue damage. During the early stages of an infection they occur in clumps which cause macroscopically visible bright red patches, due to local engorgement of capillaries

(Porter, 1935b).

The cellular reactions to N. brasiliensis have been studied in detail by Sarles and Taliaferro (1936), and Taliaferro and Sarles (1937a, b, 1939). In the normal intestine, sloughing of the epithelium was minimal and very few globule leucocytes were observed. The lamina propria contained small numbers of polyblasts (cells of the lymphocyte-macrophage series), eosinophil leucocytes and connective tissue basophils (believed by the authors to be atypical mast cells). Cellular reactions to a primary infection with N. brasiliensis were divided into four phases :-

1. Between 2.5 and 5.5 days after infection there was little inflammatory response.
2. Between 6 and 9 days, during the egg-laying period, immunity began to develop and inflammation was initiated.
3. Between 10 and 16 days inflammation progressively increased during the period when the worms were being expelled.
4. After the expulsion of the worms inflammation declined.

The cellular reaction during the first phase was confined to small numbers of eosinophil and heterophil leucocytes, with agranulocyt During the second phase, cells of the polyblast series increased markedly in the lamina propria, and cellular infiltration also started in the submucosa, serosa and mesentery. There was general hyperaemia and the jejunum was macroscopically dilated. In the third phase rapid replacement of villus epithelium by increased mitoses at the

crypt level became apparent. Globule leucocytes were numerous in the epithelium, and the lamina propria was heavily infiltrated with cells. Large macrophages were predominant at the tips of the villi, plasma cells at their necks, and connective tissue basophils at crypt level. Eosinophil leucocytes and lymphocytes were also numerous. During the fourth phase globule leucocytes were very plentiful, and the lamina propria was packed with eosinophil leucocytes, connective tissue basophils, plasma cells, and a few agranulocytes.

Reaction to a challenge infection was similar to phase 4, except that globule leucocytes and connective tissue basophils were even more numerous. The inflammatory response was elicited and subsided much more rapidly than in a primary infection, and was more intense. It affected the whole length of the small intestine and not just the anterior part. Reinfection of hyperimmune rats was associated with almost no cellular infiltration. However, globule leucocytes and connective tissue basophils were present in even greater numbers than before.

Mast cell, eosinophil leucocyte, and histamine concentration were studied by Wells (1962) in rats infected with N. brasiliensis. The concentration of histamine did not change significantly, while the number of eosinophil leucocytes more than doubled during the first 20 days of the infection. The concentration of mast cells was reported to increase eightfold after the fifteenth day.

Studies on the effect of a single infection of N. brasiliensis

upon the numbers of goblet cells produced inconclusive results, while no change in numbers was observed following multiple infections (Wells, 1963).

Part 4 : Russell body cells

Russell bodies were initially taken to be associated with cancer, of which they were considered to be the causal agent (Russell, 1890). This suggestion was incorrect, but the presence of Russell bodies in carcinomas, sarcomas, and miscellaneous malignant and benign neoplasms has been confirmed (Rangle, 1963). Russell bodies are also associated with plasma cells. In this case, the bodies develop in plasma cells which have matured beyond the stage of the typically differentiated cell (Marschalko, 1900). It has not been possible to assess the relationship between the Russell bodies associated with neoplasms and those occurring in plasma cells (Fig. 2).

Diverse stimuli have been reported to produce Russell bodies in various tissues. In inflammatory lesions of the central nervous system plasma cells were observed to differentiate into "plasma mast cells", while others underwent "degeneration" with the formation of Russell bodies (Michelsand Globus, 1929). Russell bodies have also been associated with myelomas (Apitz, 1937) and endocervical polyps (Munnick and Janovski, 1963), while Zettergren (1949) observed large cuboidal crystals inside plasma cells from a tumour thought to have been a plasmocytoma, but the relationship of these crystals to Russell bodies could not be stated with certainty. White (1954) produced Russell bodies in lymphoid tissue in response to repeated injections of a number of different antigens, including Proteus vulgaris vaccine.

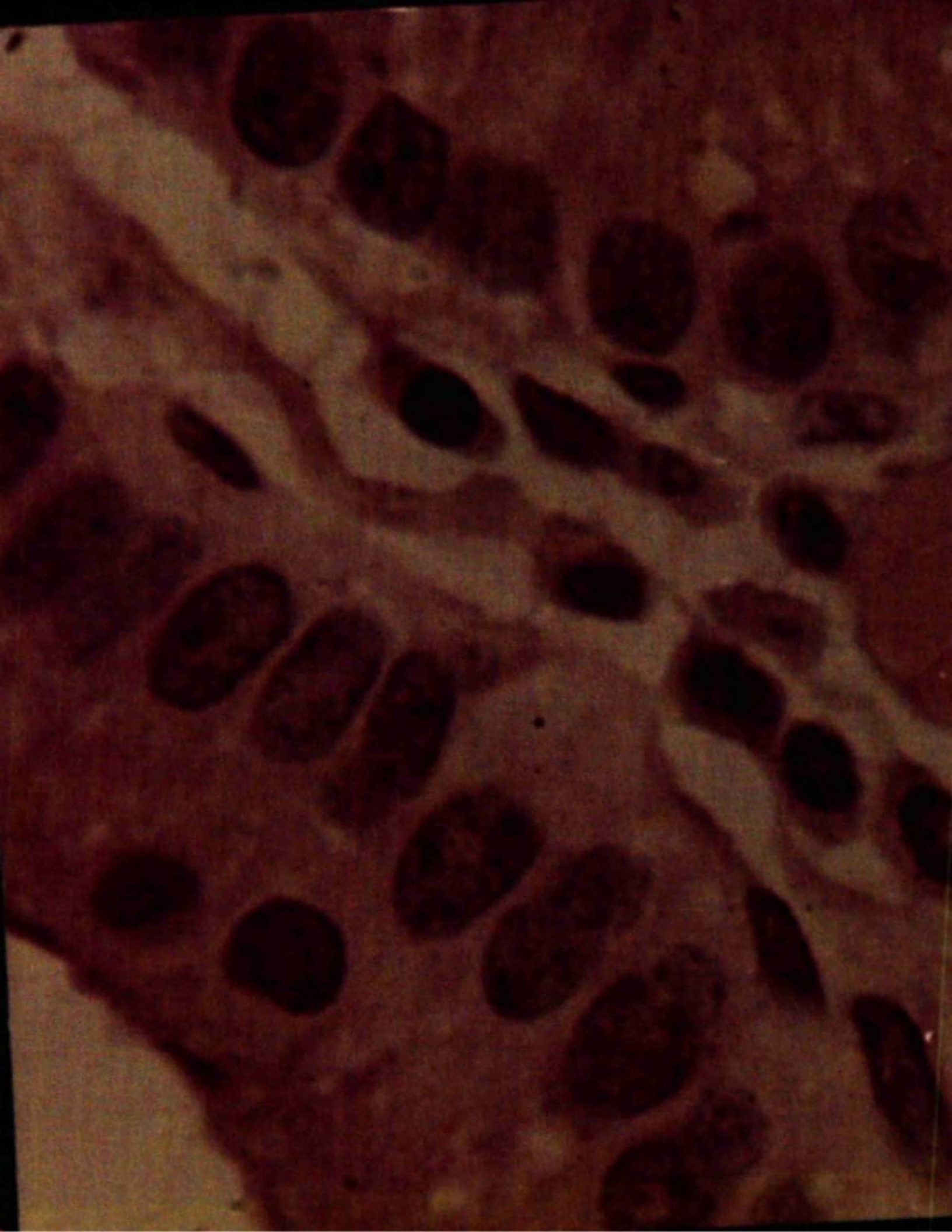


Fig. 2

Russell body cell situated in the lamina propria of sheep abomasum. Methacrylate section stained with haematoxylin and eosin.

(mag : 1300 x)

The ability of plasma cells to develop visible intracytoplasmic inclusions has been known for a considerable time (Krompecher, 1898 ; Marschalko, 1900 ; Maximow, 1906). These inclusions have received the designation of Russell bodies (Dubreuil and Favre, 1921). Earlier suggestions that they consisted of phagocytosed particles (Michels, 1935) or were a sign of degeneration (Michels and Globus, 1929 ; Miller, 1931) have been discarded, and it has since been demonstrated that Russell bodies are an internal product of the cell in which they are located (White, 1954 ; Thiery, 1958 ; Zlotnick et al., 1959). This process has been described from observations on living cells under the phase contrast microscope, and also with the electron microscope (Thiery, 1958). The rough endoplasmic reticulum of the mature plasma cell consists of a well developed network of double membranes. Dilatation takes place so that spaces appear between the membranes, and small dense granules of material accumulate in the cisternae. These granules increase in number and fuse together to form globules of increasing diameter. Thiery (1958) emphasises that during this process the cell remains active and motile, and is not therefore undergoing a degenerative process. The Russell bodies consist of a granular matrix surrounded by a membrane derived from the rough endoplasmic reticulum.

Crystals within plasma cells have been observed by a number of authors (Glaus, 1917 ; Apitz, 1940 ; White, 1954 ; Wellensiek, 1957 ; Thiery, 1958 ; Bessis, 1961 ; Movat and Fernando, 1962).

Thiery (1958) described two types of crystal ; the first being small, and so numerous as to frequently pack the cell, the second very large (up to 30μ long), cross striated, and arising in the same way as, and often in the same cell as Russell bodies. The crystals have also been described as cylindrical and cross striated, with a periodicity of approximately 120 Å (Movat and Fernando, 1962). The relationship of these crystals to the homogeneous form of Russell body is undecided (Zettergren, 1949 ; Movat and Fernando, 1962), but the two structures appear to develop by a very similar process(Thiery, 1958 ; Bessis, 1961).

On the basis of histochemical tests Pearse (1949) suggested that the Russell body consisted of a mucopolysaccharide globule surrounded by a basophilic ribonucleoprotein membrane, while White (1954) indicated that they may consist wholly or partly of mucoprotein or glycoprotein. The intracytoplasmic crystals of plasma cells give uniformly similar staining reactions and are probably composed of similar material (White, 1954). In addition to the histochemical evidence for the presence of protein, the fact that Russell bodies arise from the rough endoplasmic reticulum strongly suggests that they are at least partly protein in nature (Movat and Fernando, 1962).

Fluorescent antibody becomes attached to the surface of Russell bodies (Ortega and Mellors, 1957), presumably on the surrounding membrane (Thiery, 1958). White (1954), who also demonstrated the presence of specific antibodies in Russell bodies, indicates that

the fluorescent antibody technique does not allow a distinction to be made as to whether the Russell body is a solid mass of antibody or whether the antibody is restricted to the surface layer. The presence of polysaccharide as well as protein in Russell bodies does not necessarily preclude the possibility of this antibody appearing in crystalline form (White, 1954). Nossal (1964) has indicated that certain plasma cells contain antibody in a visible crystalline form, while according to Munsick and Janovski (1963) and Thiery (1960) there is considerable indirect evidence to suggest that the protein fraction of Russell bodies, as well as the intracytoplasmic crystals of plasma cells, consists of globulin which is an integral part of the immune mechanism.

Part 5 : The mast cell

There are conflicting reports as to whether or not mast cells (Fig. 3) occur in the small intestine of the rat. Although their presence in this organ has been reported on a number of occasions (Hardy and Westbrook, 1895 : Hunt and Hunt, 1956 : Lindholm, 1960 : Wells, 1962, 1966), Mota et al. (1956b) found that mast cells were practically absent from the area. Furthermore, when mast cells are reported to be present in the small intestine of the rat they are often described as being morphologically atypical (Maximow, 1906 : Weill, 1920 : Taliaferro and Sarles, 1939 : Hunt and Hunt, 1956). This has led to the suggestion that mast cells in the mucosa of the small intestine of the rat are essentially different from those occurring in other tissues of the same species (Weill, 1920). In addition to the morphological peculiarities which have been observed, Nagayo (1928) found that during inanition numerous mast cell granules are eliminated through the intestinal epithelium of the rat, while Cambel et al.(1952) noted the presence of mast cells both in the stomach wall and in the gastric secretion of rats, and assumed that in this species mast cells move out into the gastric secretion by amoeboid motion.

The presence of mast cells in the jejunum of rats during infection with N. brasiliensis has been reported (Taliaferro and Sarles, 1939 : Wells, 1962). Taliaferro and Sarles (1939) indicated that

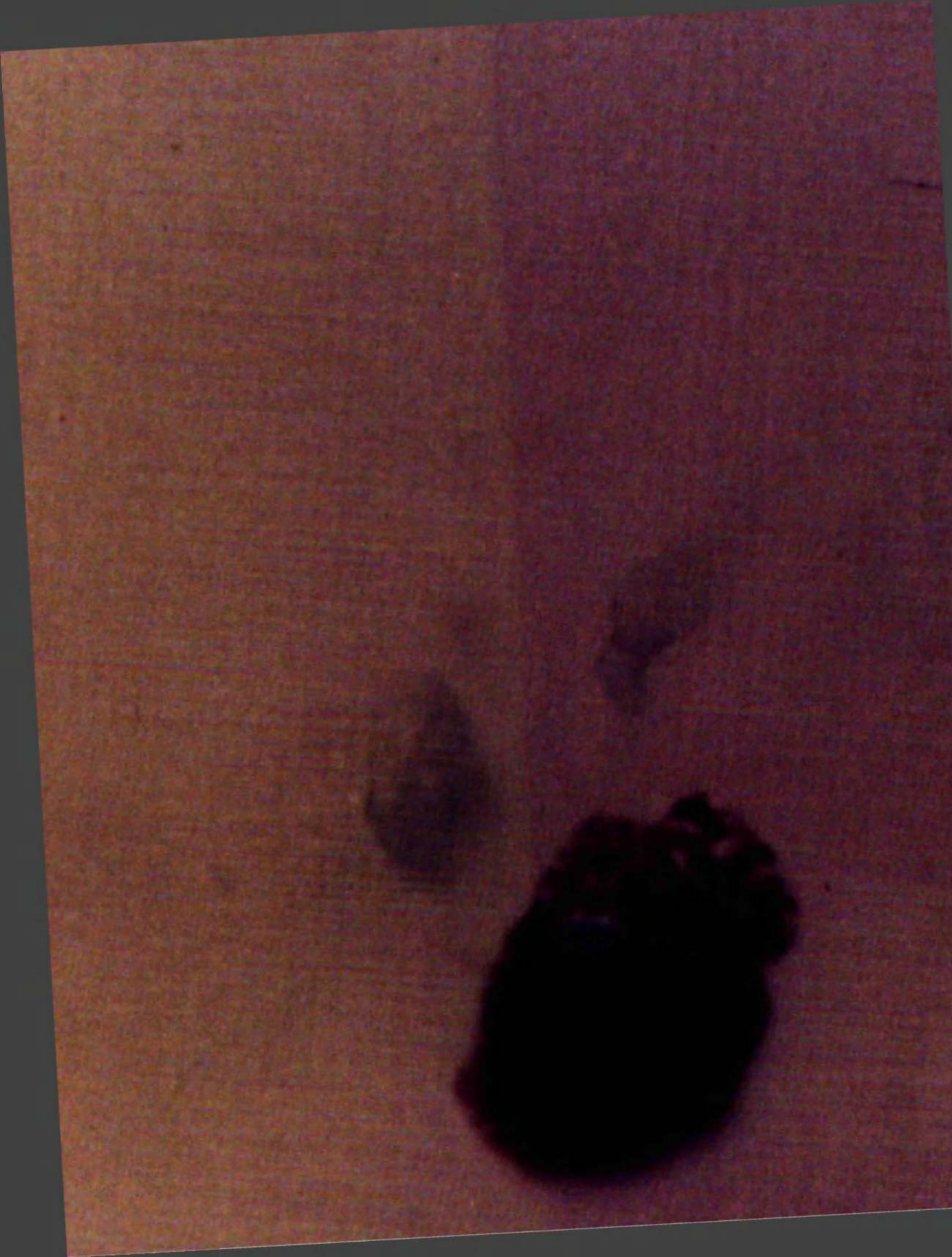


Fig. 3

Mast cells in loose subcutaneous connective tissue from the back region of the rat.

Section stained with toluidine blue at pH 4.2.

(mag : 1300 x)

these cells were morphologically atypical, and designated them connective tissue basophils. Wells (1962) noted a very great increase in the concentration of mast cells on day 15 of a primary infection with N. brasiliensis, but did not report any peculiarities of behaviour or morphology. The possibility that certain of the reports of atypical mast cells in the small intestine of the rat refer to globule leucocytes has not been investigated.

Under the electron microscope the nucleus of the mast cell may be round or indented, and there are usually one or two nucleoli (Bloom et al., 1955). The Golgi complex and rough endoplasmic reticulum are poorly developed and the mitochondria are inconspicuous. Small cytoplasmic processes described as microvilli are frequently visible at the periphery of the cell (Gusek, 1961). The granules of mast cells are either homogeneous electron-dense bodies (Policard et al., 1960 ; Smith and Lewis, 1957), filamentous (Rogers, 1956), striated (Policard et al., 1962), or have a characteristic internal structure consisting of lamellae arranged in the form of cylinders or scrolls (Stoeckenius, 1956 ; Gusek, 1960). These structures are believed to consist of lipoprotein.

The most important components of mast cells are heparin, histamine and 5-hydroxytryptamine (5-HT), together with a great variety of enzymes (Selye, 1965). Heparin is located within the granules and imparts the property of metachromasia. Mast cells are probably the only source of heparin (Jorpes, 1963). On the other hand, while

histamine is present within mast cell granules, there may be little or no correlation between histamine and mast cell concentration in a particular tissue, and this is especially marked in the small intestine of the rat, where very low numbers of mast cells are coupled with relatively high concentrations of histamine (Mota et al., 1956b). In the normal tissues of the rat there exists a good correlation between mast cell counts and 5-HT ; however, even in this species only part of it is located in mast cells (Selye, 1965).

Mast cells may be identified in tissues by a number of techniques of varying degrees of specificity. Stains which can be used for identification purposes include Bismark brown, Giemsa tissue stain (Lillie, 1954) and chrysoidin (Harada, 1957), while other techniques which have high, but generally not absolute specificity can be used to locate heparin, histamine or 5-HT within the granules. The presence of heparin imparts a strong metachromatic reaction with toluidine blue, and according to Jorpes et al.(1948) it may also be detected by a strongly positive reaction with Schiff reagent after treatment with periodic acid, indicating the presence of heparin monosulphuric acid or some other form of heparin containing two adjacent hydroxyl groups. Histamine may be located by precipitation with Reinecke salt and subsequent visualisation under dark ground illumination, or with diazotised sulphanilic acid (Schauer and Werle, 1959) ; also with parabromobenzenediazonium chloride after freeze-drying

and fixation in gaseous formaldehyde (Lagunoff et al., 1960). 5-HT causes gold-yellow fluorescence of unstained mast cell granules in ultraviolet light from a high intensity (1000 watt) source (Rice and Mitchener, 1961), and is also detectable after treatment with Schmorl's ferricyanide solution (Coupland and Riley, 1960).

There is evidence that the mast cell is involved in various hypersensitivity reactions, including anaphylaxis. This manifests itself in a discharge of the mast cell granules. However, the relationship between anaphylaxis and mast cells is not obligatory. Mast cell degranulation may occur without the induction of anaphylactic shock (Veil and Gabe, 1950), while anaphylaxis may occur under certain circumstances without an accompanying mast cell discharge (Lima, 1966). Anaphylaxis can be induced in the small intestines of immune rats injected with antigen prepared from N. brasiliensis (Urquhart et al., 1965), although the mast cell concentration may be very low in this organ (Mota et al., 1956b). The anaphylactic release of histamine in rats is produced by a particular type of antibody, different from precipitating antibody, and designated mast cell lytic antibody (Selye, 1965). The impaired immune response of neonatal rats, which manifests itself in such ways as absence of self-cure during N. brasiliensis infections (Jarrett et al., 1966 ; Kassai and Aitken, 1966) does not extend to mast cell lytic antibody, since anaphylaxis can be induced in neonatal rats after suitable sensitisation at an age when other immune responses, dependent upon precipitating antibody, are impaired or lacking.

Part 6 : Immune responses in young rats

In normal circumstances the body responds only to foreign antigens (Miller, 1966). Burnet and Fenner (1949) postulated that all antigens present during a certain period of embryogenesis are recognised as self antigens and are unable to stimulate an immune response thereafter. In fact, a state of immunological tolerance towards certain non-self antigens can also be induced postnatally as well as in immunologically mature animals (Battisto and Bloom, 1966). It is postulated that tolerance may be due to a suppression of the immune response through a low grade reaction to an antigen (Rowley and Fitch, 1965), or alternatively by cellular destruction (Burnet, 1957) or exhaustion produced by simultaneous maturation of all the stem cells (Sterzl, 1966).

The period during which tolerance can be induced depends on the species of animal and the particular antigen employed (Ingram and Smith, 1965). In the case of the rat , adult capacity is not achieved until some weeks after birth (Miller, 1966), so that neonates of this particular species may be suitable for study under conditions of immunological incompetence. The possible effect of passively derived maternal antibody, which in the rat is transferred via the yolk sac placenta and in the colostrum (Miller, 1966), may need to be considered, because while such antibody has a protective role in young animals, it also causes suppression of active antibody

production (Ingram and Smith, 1965 ; Smith and Ingram, 1965).

Kassai and Aitken (1966) successfully induced immunological unresponsiveness to a primary infection of N. brasiliensis administered to rats under four weeks of age. Such infections failed, in 60 out of 81 cases, to terminate by 10 weeks of age, and when the rats were challenged it was apparent that no resistance had developed. However, suppression of the egg output of primary infections in neonatal rats occurred even in animals harbouring stable female worm populations. This may have been due to some non-immunological mechanism (Kassai and Aitken, 1966). These findings are confirmed in the recently published experiments of Jarrett et al. (1966), where it is also reported that the course of subsequent challenge infections is not modified by primary infection during the period of immunological unresponsiveness.

The newborn of a number of species have an immature lymphoid system devoid of antibody-producing plasma cells. Animals reared in a germfree environment retain an immature lymphoid system with few plasma cells (Thorbecke, 1959), unlike conventional animals. It would therefore appear that antigenic stimulation is necessary for maturation to occur (Miller, 1966). Gordon (1960) reported significantly lower concentrations of globule leucocytes in germfree fowls when compared to conventionally reared birds, and indicated that this was part of a generalised reduction in numbers of cells

belonging to the reticular-lymphoid group. The possibility that the response of cells in the lymphoid series is diminished in rats which are tolerant to N. brasiliensis has not been investigated.

Part 7 : Neonatal thymectomy

The thymus is outside the path of recirculation of immunologically competent cells (small lymphocytes), does not show antibody production, germinal centres or plasmocytopoiesis in an immunised animal, and thus differs markedly from other lymphoid tissues (Miller, 1965). Thymectomy at birth results in a number of physical abnormalities including extramedullary haemopoiesis, reticuloendothelial cell proliferation, and severe depletion of lymphocytes in blood and lymphoid tissues (Miller, 1961, 1962, 1963).

Neonatal thymectomy is also associated with significant impairment of immunological capacity. For example, neonatally thymectomised rodents fail to reject foreign skin grafts (Miller, 1963), and circulating antibody levels are depressed after antigenic stimulation (Roosa et al., 1965). Lymphoreticular cells derived from neonatally thymectomised animals are defective with respect to their capacity to produce graft-versus-host reactions (Yunis et al., 1964), while Parrott and East (1965) state that the thymus is necessary in very early life for the correct establishment of an immunological system to combat the growth of transplanted malignant cells. Impairment of homograft reactivity persists to some extent in animals thymectomised up to seven days after birth. Even in the adult animal the thymus retains part of its potential to regulate the development and responses of the lymphoid tissue (Sherman, 1966).

Thymic lymphocytopoiesis is not affected by antigenic stimulation, suggesting that the proliferative stimulus is derived from within the organ itself (Miller, 1965). Cells from the spleen or lymph nodes of normal adult mice are capable of restoring the immune mechanism in neonatally thymectomised recipients, while thymocytes are reported to be deficient in this respect (Miller, 1965). Subcutaneous implantation into neonatally thymectomised mice, of thymus isolated in a millipore chamber from which the cells could not escape, imparted immunological capacity to these animals, suggesting that the action of the thymus in establishing normal immune capacity is probably mediated by a humoral mechanism (Levey, 1964). On the other hand, Yunis et al. (1964) state that immunological reconstitution of neonatally thymectomised mice can be achieved by injections of isolated thymocytes. However, these observations are not mutually exclusive since humoral stimulation, and thymus passage and peripheralisation of lymphoid precursor cells may both be operative (Balner and Dersjant, 1964).

Sinclair (1965) has emphasised the importance of multiple testing of immunological responses of thymectomised animals, since false results may be obtained by taking a single sample. It was demonstrated that haemolysin titres were identical in thymectomised and sham thymectomised mice nine days after immunisation, but were significantly reduced in the thymectomised group by 20 days. Sex differences have also been reported. A lower incidence of runting and immune depression have been reported in neonatally thymectomised

female mice as compared to males; such a difference may be due to the influence of sex hormones on immune reactivity (Balner and Dersjant, 1966).

Some weeks after neonatal thymectomy a number of animals develop a runt disease syndrome characterised by wasting and death (Miller, 1965). In one series of observations about 10% of neonatally thymectomised hamsters developed runt disease (Roosa et al., 1965). However, there appears to be no correlation between the results of different laboratories in this respect, and McIntyre et al.(1964) have postulated on the basis of experimental evidence that the pathogenesis of the wasting syndrome is due to an infectious agent which affects neonatally thymectomised animals because of their diminished immunological capacity.

The effect of neonatal thymectomy in rats not suffering from the wasting syndrome at the time of examination is to decrease the proportion of small lymphocytes in the blood and lymphoid tissues. Reticuloendothelial cell hyperplasia is marked in liver, spleen and lymph nodes (Fachet et al., 1965a). In similar rats suffering from runt disease these changes were again noted, accompanied by a decrease in the total white cell count of the blood and disseminated necrosis of the liver parenchyma (Fachet et al., 1965b). Aschkenasy (1965) showed that the decrease in total white cell count after neonatal thymectomy in the rat was due entirely to a reduction in the total number of circulating lymphocytes; the numbers of erythrocytes, heterophils and eosinophils were not altered. A sex difference

was noted; in male rats mainly large and small lymphocytes were diminished, whereas in females medium and small lymphocytes were markedly reduced and large lymphocytes were present in only slightly fewer numbers. Depression of serum γ-globulin levels was noted in the majority of thymectomised males, but not in females.

There are no reports concerning the effect of neonatal thymectomy upon the gastrointestinal tract, apart from its lymphoid tissue component, in specifically non-wasting animals. However Hard et al. (1964) have studied the pathology of intestinal crypt lesions in neonatally thymectomised hamsters, and indicated that there was no apparent correlation between clinical condition and enteropathology. The changes observed were loss of Paneth cell granules, disorientation of the columnar epithelium, with cytoplasmic swelling and loss of staining affinity. Increased crypt mitoses suggested excessive epithelial cell proliferation. In other cases there was inflammation at the bases of the crypts accompanied by flattening of the cells lining the crypts together with minute erosions and micro-abscess formation. This was accompanied by inflammatory cell invasion of the adjacent lamina propria. Lesions were not observed in animals under two weeks of age.

No reports have been located which describe the effect of neonatal thymectomy upon the course of N. brasiliensis infections in rats. From the information presented above it is possible that the immune response to a primary infection would be abolished or

impaired. There is some indirect evidence to support this view. Thus, injection of prednisolone into newborn rats causes massive thymic involution accompanied by a number of the effects generally associated with neonatal thymectomy (Branceni and Arnason, 1966), while treatment of rats with prednisolone during a primary infection with N. brasiliensis supressed the initiation of acquired resistance either completely, or at an early stage (Ogilvie, 1965a).

SECTION II

MATERIALS AND METHODS

Part 1 : Experimental animals

Sources

Material from blackface sheep was obtained from the Department of Experimental Parasitology, University of Glasgow Veterinary School. Colleagues in this department were responsible for the worm-free rearing, infection with *Ostertagia* spp. larvae, killing and *post mortem* examination of these animals.

Rats used in experiments were either of the hooded Lister or albino Wistar strains. All livestock was obtained as young adults from Animal Suppliers Ltd., London. These animals were generally free from intestinal helminth parasites, but some batches were infected with the small intestine tapeworm *Hymenolepis nana*. In such cases the rats were discarded. Nevertheless, *H.nana* was occasionally encountered at autopsy. The large intestine nematode *Syphacia obvelata* was also found in stocks maintained in the animal house for any length of time. Newborn rats, used for neonatal infection with *N. brasiliensis* or for neonatal thymectomy, were bred in the department from stock obtained from the above suppliers.

Management

Rats were kept in an animal house in maximum groups of 20. They were housed in wire cages with raised mesh floors over trays. The cages were set on racks and separate racks were used for non-

infected stock and animals infected with N. brasiliensis. Proper maintenance precluded the possibility of cross-infection or reinfection from faeces. Mated pairs, unweaned litters, neonatally thymectomised rats and controls from the same litter were kept separately in small plastic cages with raised metal grid floors. All rats were fed pelleted Diet 41 (from W. Primrose and Son, Glasgow) and water ad libitum, except that females nursing litters of neonatally thymectomised rats and unoperated controls were supplied with water to which Tetracycline syrup ("Achromycin", Cyanamid of Great Britain Ltd.) containing 125 mg tetracycline/5 ml, was added at the rate of 1 ml to 1.25 litres of water. After weaning these litters the neonatally thymectomised rats and their control litter mates were housed individually and tetracycline syrup was continued until the time of killing.

Killing and Anaesthesia

Initially animals were killed by a blow on the head, because of a report that anaesthetics affect the histology of the small intestine (Wells, 1962). Overdose of chloroform was substituted and no differences in gut histology were detected. For single or infrequent inductions of anaesthesia ether was used, but where repeated or prolonged doses of anaesthetics were required trichloroethylene ("Trilene", I.C.I.) was substituted because of the reduced degree of pulmonary irritation and the more readily controlled level of anaesthesia with this drug.

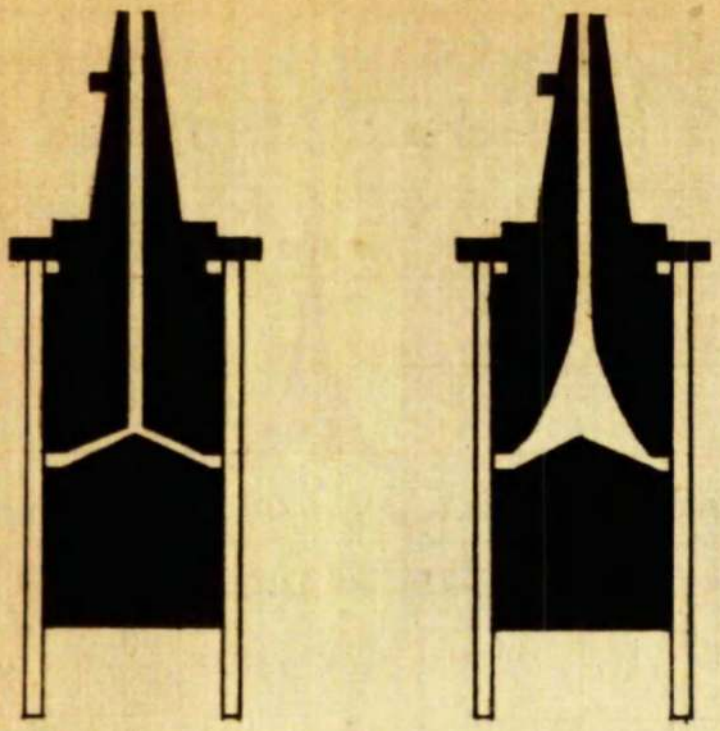


Fig.4

Section through nozzle unit of a Hautmann Automat syringe (A), modified by drilling out (B) in order to increase the dose accuracy when injecting suspensions of *N. brasiliensis* larvae.

Injections

Injections into the tail vein of rats were performed using a tuberculin 1 ml syringe fitted with a no. 19 needle. Prior to injecti the tail was immersed in warm water for one minute and cleaned with cotton wool.

A 2 ml capacity Rautmann Automat tuberculin syringe (Holborn Surgical Instrument Co., London) delivering multiple doses of 0.1 ml was used for the infection of neonatal rats with N. brasiliensis larvae. Previous tests on a syringe of similar type had shown it to be unsuitable for this purpose (Aitken, 1965) because larvae tended to become clumped at the entrance to the nozzle. Consequently some doses contained very few larvae while occasional doses contained very high concentrations. This was overcome by drilling out the nozzle into a tapering cone shape (Fig. 4). Tests on the modified syringe showed that this alteration reduced the error to acceptable proportions (Table 1).

Neonatal thymectomy

Immediately after the birth of a litter, tetracycline syrup was added to the drinking water. Thymectomy was always performed within 24 hours of birth, and most frequently within 12 hours. The number of thymectomies carried out depended upon the size of the litter, and varied between three and six. Operating upon more than this number greatly increased the chance of animals being

NO. OF LARVAE / 0.1 ML				
19	30	29	37	26
33	23	20	17	31
22	25	28	21	24
28	28	28	36	42

MEAN	S.D.
27.35	6.4

Table 1

Test series on modified Rautmann Automat 2 ml capacity syringe delivering 0.1 ml doses of larval suspension with a calculated concentration of 250 larvae/ml.

cannibalised.

Sterile techniques were employed. The newborn rat was anaesthetised by cooling in a refrigerator, placed in a beaker contained within a larger one; the space between the two being filled with ice. It was removed after 20 minutes, and the forelimbs secured with the ventral surface uppermost, on to a contoured foam rubber operating platform (Fig. 5) using 7 mm Weka suture clips (Holborn Surgical Instrument Co., London). The skin was swabbed with methyl alcohol and a median longitudinal incision, cutting the sternum at the level of the first three ribs, was made through the thorax with a no. 11 scalpel. The incision was enlarged with corneal scissors and small forceps were inserted to act as retractors. The thymus was located visually and removed under suction through a glass pipette (Fig. 5). Negative pressure was maintained by a vacuum pump fitted to a 5 litre deadspace.

After the removal of the thymus the wound was dried and sprayed with Achromycin powder (Cyanamid of Great Britain Ltd.) Two braided silk sutures were inserted using a flat bodied half-curved no. 19 needle. After closure of the wound it was covered with "New-Skin" (Harwoods Laboratories, Lancs.) wound dressing, and when this had hardened the thymectomised animal was returned to the litter.

Mortality using the method described above was approximately 98%. It was impossible to assess how many animals died as a direct result of the surgical interference because many live neonates

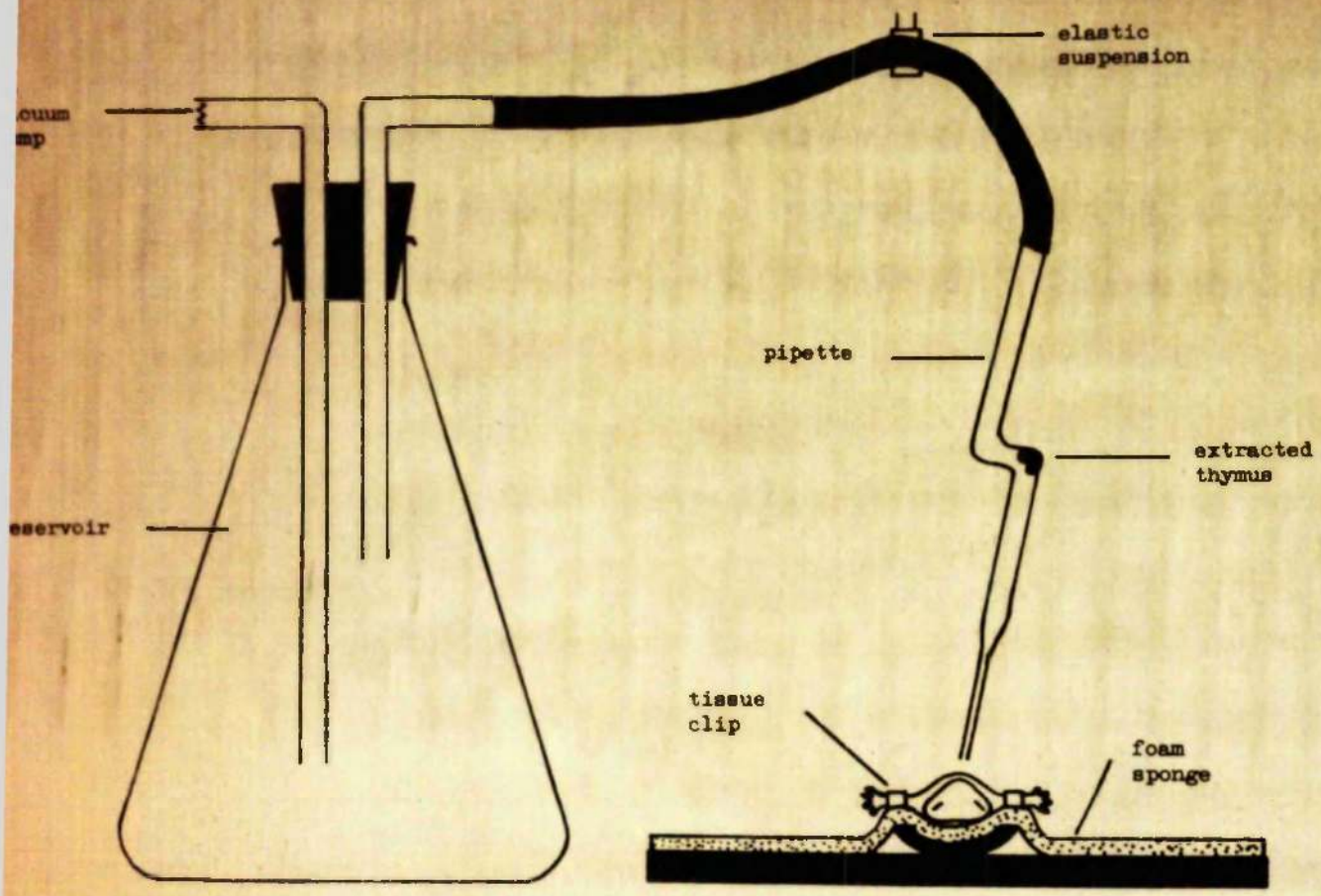


Fig. 5

Apparatus used for neonatal thymectomy of rats.

and frequently whole litters were eaten by the mother after thymectomised rats were returned to the cage. The survival of thymectomised animals depended to a large extent upon careful handling and the temperament of the female. Most of the mortalities occurred within three days of operation; about 10% of animals surviving this period died before the completion of experimentation.

Part 2 : Nippostrongylus brasiliensis

The strain of *N. brasiliensis* used in all experiments was obtained from Dr. J.T.M. Neilson of the Department of Veterinary Physiology, University of Glasgow, and was maintained by regular passage through hooded Lister and albino Wistar rats.

Maintenance of culture

Infective larvae were counted by a dilution technique. 1 ml was removed from a homogeneous larval suspension and diluted to 100 ml. 10 samples of 0.1 ml each were transferred to a Petri dish using an auto-zero pipette. The larvae in each sample were counted under a binocular microscope, and the original larval suspension was then diluted with water to give the required number of larvae per ml, which in the case of rats for culture maintenance was 2500/ml.

Rats were lightly anaesthetised with ether, and the larval suspension was injected subcutaneously into the groin region using a 1 ml syringe fitted with a 20 G x 1½" needle. No antibiotics were added prior to injection. 24 hour faeces collections were made between the seventh and tenth days after infection, by placing a sheet of newspaper beneath the wire grid floor of the cage. Faecal pellets were sieved free of contaminants using a B.S. 3/16" mesh sieve. The clean faeces were placed in a 100 ml homogeniser tube which was then filled to the neck with water, and mixed to a smooth

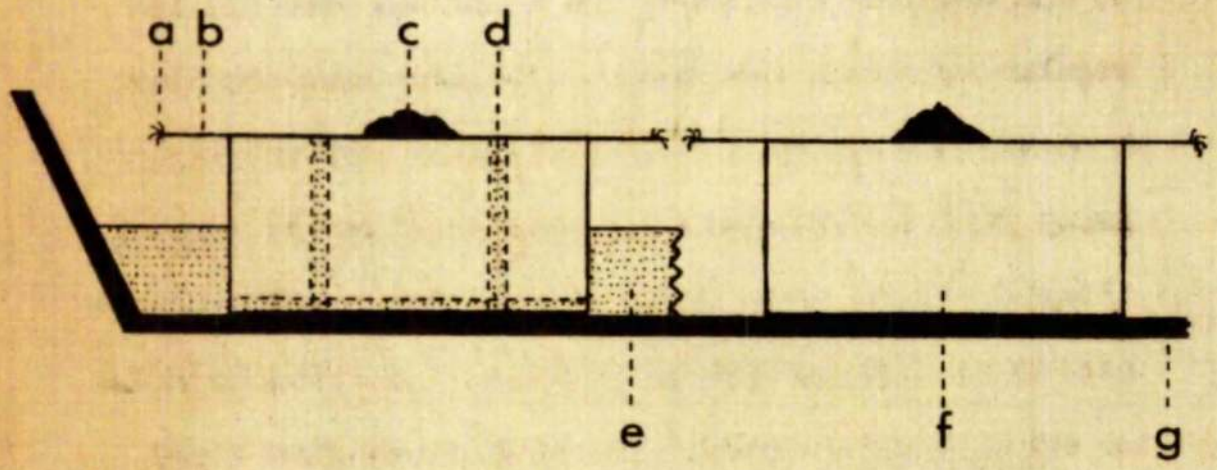


Fig. 6

Cross section through rigid polythene tray to illustrate the wet (left) and dry (right) methods of faeces incubation for the production of *N. brasiliensis* larvae. a : partially ensheathed larvae b: chromatography paper c: faeces d: capillary hole e: water f: Perspex platform g: rigid polythene tray

semifluid consistency using an M.S.E. homogeniser at maximum revolutions for five minutes. This method replaced that described by Neilson (1965) where the faeces were mixed to a paste by hand.

Specially constructed trays were used for the spreading of faeces (Fig. 6). These were made of rigid polythene and measured 42x22x1.6 cm (A. Gallenkamp and Co., London). Three equidistant platforms of "Perspex" were glued longitudinally along the bottoms of the trays, and measured 12$\frac{1}{8}$x1$\frac{1}{2}$x$\frac{1}{2}$". The platforms were drilled through at regular intervals with two rows of 1/8" bore capillary holes, which communicated with a slit at the base. Chromatography papers measuring 5x36 cm (Griffin and George Co., London) were wetted and placed along the slats so that the edge of the paper projected at either side. The semifluid faeces was poured in a line down the centre of the chromatography paper. One day's faeces from eight to ten rats could be spread on six such trays. Water was poured into the trays to a depth of $\frac{1}{4}$" and was carried by capillary action to the chromatography papers, which remained damp. The trays were stacked without lids in an incubator at 24°C. This method replaced that described by Neilson (1965), where the faeces were plated out in small amounts at the centre of a damp filter paper supported on a wet foam sponge pad in a covered Petri dish.

More recently the tray method described above was modified by not filling the trays with water. A high oven humidity was essential for this method; this was obtained by bubbling air through a beaker

PETRI DISH	WET TRAY	DRY TRAY
520,800	788,400	579,800

Table 2

Comparative larval yields from three identical faeces samples incubated in Petri dishes, wet trays and dry trays. Ten rats were infected with 2,500 infective larvae each and the faeces were collected on days 8, 9 and 10. Incubation was at 24°C in a humid oven for 5 to 7 days, depending upon day of collection.

of water with the aid of an aquarium pump.

A test was carried out to determine whether these modifications, which were designed primarily to reduce the number of hours necessary for culture maintenance, were seriously affecting the number of larvae produced. 10 albino female Wistar rats were each injected with 2500 infective larvae, and faeces were collected in the usual way. Faecal samples were divided into three parts of equal weight which were homogenised separately. The first sample was plated out on Petri dishes, the second and third were spread on trays using, respectively, the wet and dry methods described above. Three 24 hour faeces collections were treated in this way and the dishes and trays were incubated at 24°C. The infective larvae were harvested in three groups, using identical procedures. The total numbers of larvae produced by each method from identical weights and samples of faeces was calculated by the dilution technique previously described. The results for each group are shown in Table 2. Most larvae were produced by the wet tray method, while Petri dish and dry tray methods produced uniformly lower yields of larvae. Of the two tray methods, the dry tray system is preferable for routine use, but the wet tray system may be substituted at any time should higher larval yields be required.

The method of harvesting larvae altered with the introduction of new incubation techniques. Harvesting from Petri dishes required the setting out of between 50 and 150 dishes, removal of lids,

filling each dish to filter paper height with water at 37°C, agitating each dish several times, removal, separation and squeezing out of filter papers and sponges, and the pouring of the contents of each dish into a common receptacle for filtration. With the wet tray method, water at 37°C was poured into the trays which were then rocked intermittently. The chromatography papers were removed and the contents of the trays poured into a common receptacle for filtration. Using the dry trays, the originally semifluid faeces became hardened on to the paper strips, enabling them to be dropped into a bucket containing water at 37°C, from which they were removed 10 minutes later, prior to filtration.

Larvae were normally harvested after an incubation period of five to eight days. The warm water containing the larvae was filtered through Green's Hyduro 904 filter paper, 18.5 cm in diameter, using a Büchner funnel attached to a vacuum pump. The filter paper was inverted into an Endecott sieve (mesh 400) over a Baermann apparatus containing water at 37°C. The larvae penetrated the sieve and collected at the bottom of the funnel, from where they were run off into a measuring cylinder for counting and dilution.

Larvae for stock culture were normally injected into rats on the day of harvesting. Where larvae were required at a later date they were placed in "½ RLA +" solution (Wilson and Dick, 1964) with the following formula :-

NaCl.....2.3 g.

KCl.....0.01 g.

$CaCl_2$.....0.44 g.

$NaHCO_3$.....0.04 g.

Streptomycin sulphate.....0.05 g.

Na benzyl penicillin.....0.05 g.

"Actidione" (Koch-Light, Colnbrook).....0.02 g.

Water.....1 litre

The concentration of larvae in this solution was reduced to less than 1000/ml, and the larval suspension was stored at 24°C. in a flask through which air was constantly bubbled. Larvae were kept in this way for a maximum period of two weeks.

Faecal egg counts

Faecal egg counts were performed to determine the suitability of faeces for incubation. A minimum count of 20000 eggs/g. of faeces was considered desirable for this purpose. Egg counts were also performed for experimental purposes. 1 g. of faecal pellets was taken from a sample and homogenised in 10 ml of water. The suspension was gently rubbed through a sieve (mesh no. 50) and the filtrate centrifuged for five minutes at 1000 r.p.m. The supernatant was poured off and the precipitate was resuspended in saturated salt solution. A sample was transferred by Pasteur pipette to a McMaster slide, and the total number of eggs in both chambers (0.3 ml) was determined

by examination under a binocular microscope. The result was multiplied by 100 to give the number of eggs/g. of faeces.

Isolation, counting and sexing of adult worms

Methods for the recovery and counting of adult N. brasiliensis from the intestines of adult rats have been described by Jennings et al. (1963) and Ogilvie (1963). Such methods need to be accurate, particularly where estimations of the degree of immunity of the host are to be made on the basis of such counts. The techniques described by these authors have been used routinely during experiments on adult rats and have proved quite satisfactory. The method generally used for adult rats in the earlier experiments consisted of dividing the small intestine into suitable lengths which were opened with bowel scissors. The pieces were then suspended in gauze in a beaker of saline, and incubated for one hour in a water bath at 37°C. Worms were collected with a Pasteur pipette from the base of the beaker and counted in a Petri dish with the aid of a binocular microscope. On application to the recovery of worms from the intestines of neonatal or unweaned rats a number of difficulties were encountered.

Because of their small size and delicate structure, opening the intestines of very young rats with scissors was an extremely difficult procedure and often resulted in a significant loss of worms. The consequent spread of intestinal contents around the site and on instruments required repeated rinsing for the recovery of

worms, so that they were eventually collected together for counting in an excessive quantity of fluid. Such methods were particularly unsatisfactory during the early stages of an infection when immature forms, not visible to the naked eye, were present. These difficulties were eliminated by the development of the following method.

The abdomen was opened and the posterior oesophagus cut; the stomach was then loosened from its surrounding attachments and the small intestine gently unravelled, particular care being taken in the region of the pancreas and caecum, where the intestine is most likely to break. It was then threaded on to a glass rod of 5 mm diameter, one end of which had been drawn out and the tip heated to a ball of such size that it easily entered the lumen of the intestine. The whole small intestine split open during this procedure, and it was then transferred to a 1 litre beaker containing N saline at 37°C. The gloved fingers of the operator and the glass rod were rinsed with saline into the same beaker until no traces of intestinal contents remained. The contents of the beaker were then drained through a layer of surgical gauze spread over the top of a second beaker so that the intestine was retained in the gauze. The first beaker was repeatedly rinsed into the second. The gauze was then caught up on a glass rod so that the intestine was suspended in the saline. The beaker was left in a water bath at 37°C for one hour, by which time the worms had penetrated the gauze and were lying at the bottom of the beaker in 500 to 1000 ml of saline. The contents

of the beaker were filtered through a Büchner funnel using Green's Hyduro 904, 18.5 cm diameter filter paper, which had been previously marked off in 8 mm squares on a duplicating machine, with repeated rinsing to ensure that all the worms were transferred from the beaker to the filter paper. By adjusting the flow from the beaker the worms were spread evenly over all but the periphery of the filter paper.

The filter paper was removed and, if the worms were required only for counting, was allowed to dry completely. The worms were easily visible as red coils adhering closely to the paper, and were counted at a magnification of 12.5 x using a binocular microscope. The 8 mm squares on the filter paper acted as convenient guidelines. If the worms were required alive the filter paper, while still wet, was laid for counting on a sheet of "Perspex" 20 cm in diameter. Afterwards the filter paper was inverted over a 1/16" mesh sieve on top of a filter funnel containing N saline at 37°C. and fitted with a tap. The worms descended through the saline and collected in the narrow stem above the tap, from where they were run off into a stoppered measuring cylinder to the required dilution.

The ratio of males to females in an adult population of N. brasiliensis was calculated by sexing the total worm burden or 200 worms selected at random where the worm burden was higher than this figure. These were transferred by Pasteur pipette in small groups to a Petri dish, and examined under a binocular microscope.

The criteria used for differentiating males were small size, pale colour, presence of bursa, and for females larger size, redder colour, and the presence of eggs in the uterus.

SECTION III

EXPERIMENTAL OBSERVATIONS

Part 1 : Comparison of globule leucocyte concentration in the abomasum and intestine of nematode-infected and worm-free sheep.

INTRODUCTION

Some authors consider, or assume that the globule leucocyte is a constant feature of the gastrointestinal tract (Weill, 1920 : Keasbey, 1923 : Kent, 1952). On the other hand, Kirkman (1947, 1949, 1950) provided evidence that infection with Trichosomoides crassicauda is associated with large numbers of globule leucocytes in rat urinary tract. This finding was supported by Ahlqvist and Kohonen (1959a, b) but without reference to a suitable control group. In addition, Sommerville (1956) examined abomasa from infected and worm-free sheep, and found a good correlation between the presence of nematodes and globule leucocytes.

Contrary to the above evidence, Rootes (1961) states that globule leucocytes occur normally in the walls of the alimentary tract in a number of species, while Davletova (1958), who observed the development of gastrointestinal globule leucocytes in weaned lambs put on to fodder, interprets their appearance in terms of changes in physiological requirements during digestion. Also, Bloom and Fawcett (1962) do not mention any relationship between globule leucocytes and nematode parasites.

On the basis of these observations it was decided that the

relationship of the globule leucocyte to nematode infection should be investigated, and for this purpose gastrointestinal tracts from four worm-free and four parasitised sheep were subjected to histological examination.

MATERIALS AND METHODS

Worm-free sheep (SU 1-4)

This group consisted of Blackface lambs reared worm-free at Glasgow University Veterinary Field Station. SU/1 was a six monthhold castrate male in apparent good health at the time of death. Nine blocks were examined from different parts of abomasum, small intestine and caecum. SU/2 was an eight month old male which had been operated on two weeks previously for urolithiasis. 14 blocks were examined from abomasum and small intestine. SU/3, an entire male of five months , was suffering from marked cystitis, ruptured bladder with urine in the abdominal cavity, and patches of acute enteritis in the distal half of the small intestine. These findings indicated indicated obstructive urolithiasis of the urethra. 15 blocks were examined from abomasum, small intestine, caecum and rectum. SU/4 was an entire eight month old male which had been injected with tritiated thymidine prior to death. 22 blocks were taken from abomasum, small intestine, caecum, colon and rectum.

Parasitised sheep (SP 1-4)

SP/1-2 were nine month old Blackface sheep reared under the

same conditions as the worm-free group, and infected 63 days prior to slaughter with 28000 larvae each of Ostertagia circumcincta. Eight blocks were examined in each case from abomasum and duodenum. SP/3-4 were eight month old farm reared Blackfaces and clinical cases of ostertagiasis, for which they had been dosed with thiabendazole two weeks prior to slaughter. In addition to Ostertagia spp., both sheep harboured Muellerius capillaris, while SU/4 was also infected with Dictyocaulus filaria and Fasciola hepatica. 14 blocks were examined in each case, from abomasum and small intestine.

Preparation and examination of sections

All sheep were killed by captive bolt and exsanguination. Blocks were fixed in buffered neutral formalin, and the time between death and fixation varied between 10 and 90 minutes. The blocks were embedded in paraffin wax, and routine sections were cut at 5μ and stained with Massons trichrome method. Adequate differentiation against the Ponceau-acid fuchsin component of this stain ensured that the globules stood out well against a green background as bright red spherules and were readily counted (Fig. 7). 100 fields were examined under oil using a squared eyepiece disc, to give a total area of 2.1 mm^2; starting at the muscularis mucosae and working vertically towards the surface of the mucosa. The number of globule leucocytes counted was then expressed as the number of cells per square millimetre of tissue section (GL/mm^2).

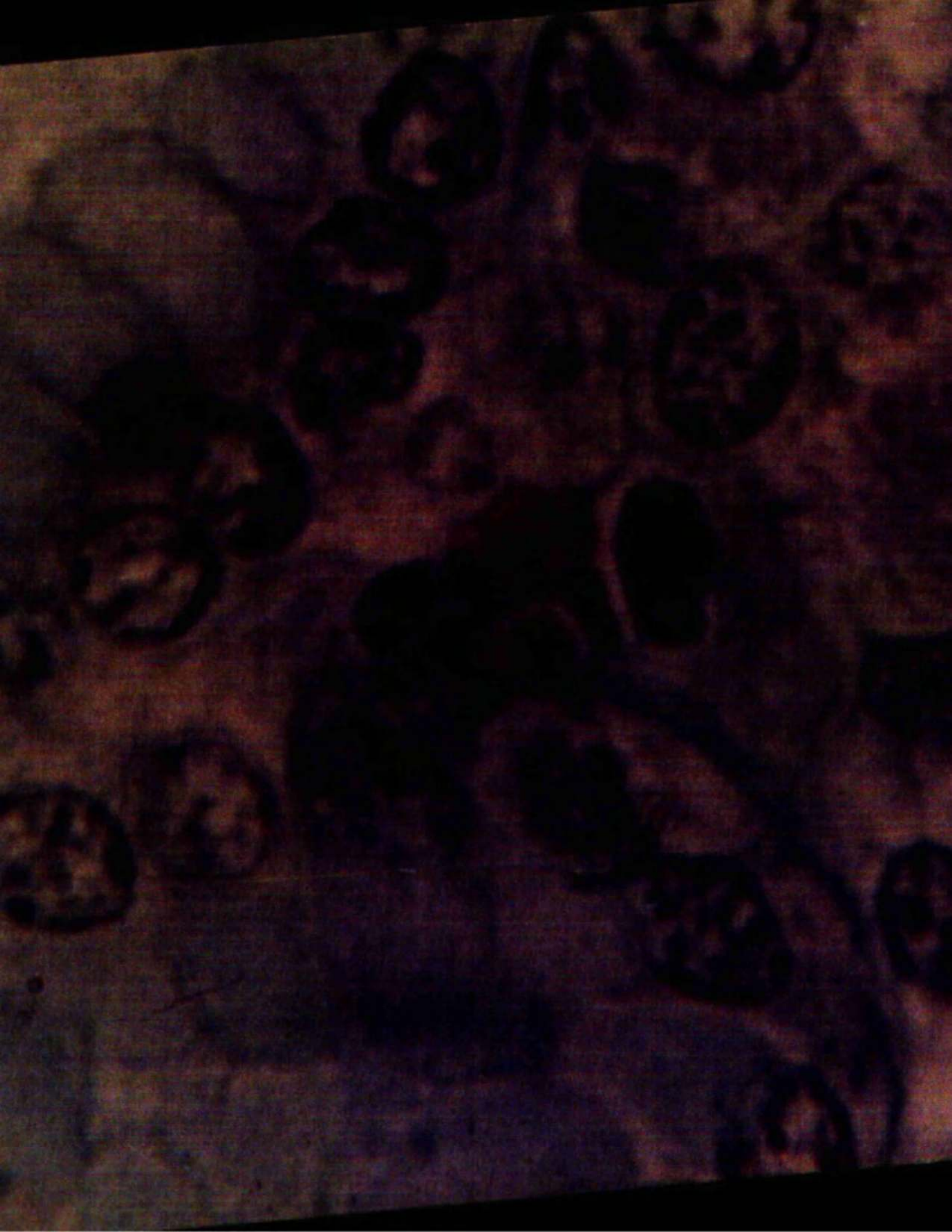


Fig. 7

Globule leucocyte in the mucosa of sheep abomasum, illustrating the appearance of these cells in sections used for counting.

Section stained with Masson's trichrome.

(mag : 1300 x)

Globule leucocytes form only a proportion of the cells found in an intraepithelial position in the gastrointestinal tract. According to Teir et al.,(1963) and Gibbs (1964) these other cells are mainly granulocytes of haematogenous origin migrating into the lumen of the digestive tract. In addition, lymphocytes are frequently observed (Andrew, 1965). However, no difficulty was encountered in distinguishing globule leucocytes from other cells.

Only globule leucocytes in an intraepithelial position were counted, and possibly related cells in the lamina propria were ignored. Neither cells found in Brunners glands, which contained globule leucocytes in infected sheep, nor Peyers patches, which did not, were included in counts.

RESULTS

Worm-free sheep (SU/1-4)

Of the total 60 blocks examined, 58 had a concentration of 0 GL/mm^2, and two of 1 GL/mm^2, corrected to the nearest whole number. The total number of globule leucocytes seen in the 60 blocks within the areas counted was eight cells (Table 3).

Parasitised sheep (SP/1-4)

42 blocks were examined, of which 38 contained globule leucocytes (Fig. 8) in concentrations of between 1/mm^2 and 311/mm^2. Four of

AREA	SU/1	SU/2	SU/3	SU/4	SP/1	SP/2	SP/3	SP/4
ABOMASUM :								
omasal junction	0	0	0	0	*	4	14	6
10 cm from junction	0	-	-	-	-	-	-	-
20 cm from junction	0	-	-	-	-	-	-	-
mid lesser curvature	-	0	0	0	153	41	18	7
mid greater curvature	-	0	0	0	311	8	18	105
pylorus	-	0	0	-	22	*	18	8
duodenal junction	0	-	-	0	-	-	168	123
SMALL INTESTINE :								
abomasal junction	-	0	0	0	103	55	-	-
5 cm from junction	-	-	-	1	36	60	-	-
10 cm from junction	0	0	0	0	23	5	6	6
15 cm from junction	-	-	-	0	0	1	-	-
20 cm from junction	0	0	0	0	-	-	1	2
30 cm from junction	-	0	-	0	-	-	-	-
40 cm from junction	0	0	-	0	-	-	-	-
50 cm from junction	-	0	0	0	-	-	0	4
70 cm from junction	-	1	-	0	-	-	-	-
100 cm from junction	-	-	0	0	-	-	0	*
100 cm from caecum	-	-	0	0	-	-	66	8
50 cm from caecum	-	-	0	0	-	-	4	12
20 cm from caecum	-	0	0	0	-	-	1	1
10 cm from caecum	-	0	0	0	-	-	-	-
caecal junction	0	0	-	0	-	-	0	*
LARGE INTESTINE :								
mid caecum	0	-	0	0	-	-	-	-
mid colon	-	-	-	0	-	-	-	-
mid rectum	-	-	0	0	-	-	-	-
TOTAL BLOCKS EXAMINED	9	14	15	22	8	8	13	13
AVERAGE GL/mm^2	0	0	0	0	93	25	24	26

Table 3

Concentrations of globule leucocytes in the gastrointestinal tract of four worm-free (SU/1-4) and four parasitised sheep (SP/1-4).

* Globule leucocytes present, but concentration not determined.

these sections were not counted (Table 3) because of poor fixation, obliqueness, or large areas of lymphoid tissue. Four sections contained no globule leucocytes. In general the highest counts occurred in the posterior abomasum and anterior duodenum. Globule leucocytes were invariably present in Brunners glands in rather low concentrations, but were absent from lymphoid tissue.

DISCUSSION

The average value for GL/mm^2 in the two groups of sheep (Table 3) clearly indicates that a relationship exists between the globule leucocyte and nematode infection of the gastrointestinal tract, and that all but an insignificant number of globule leucocytes can be attributed to this infection.

The hypothesis of Davletova (1958), relating the presence of globule leucocytes in lambs to the assimilation of foreign proteins contained in plant fodder at the time of weaning, as well as a similar suggestion by Lavrentiev and Lazovskii (1928), is not supported, since the worm-free sheep in the present experiment were also reared on a diet of hay and concentrates but were free from globule leucocytes. The appearance of globule leucocytes in Davletova's lambs is equally well explained by parasitic infection, due to the ingestion of larvae on the fodder.

While the significance of the small numbers of globule leucocytes

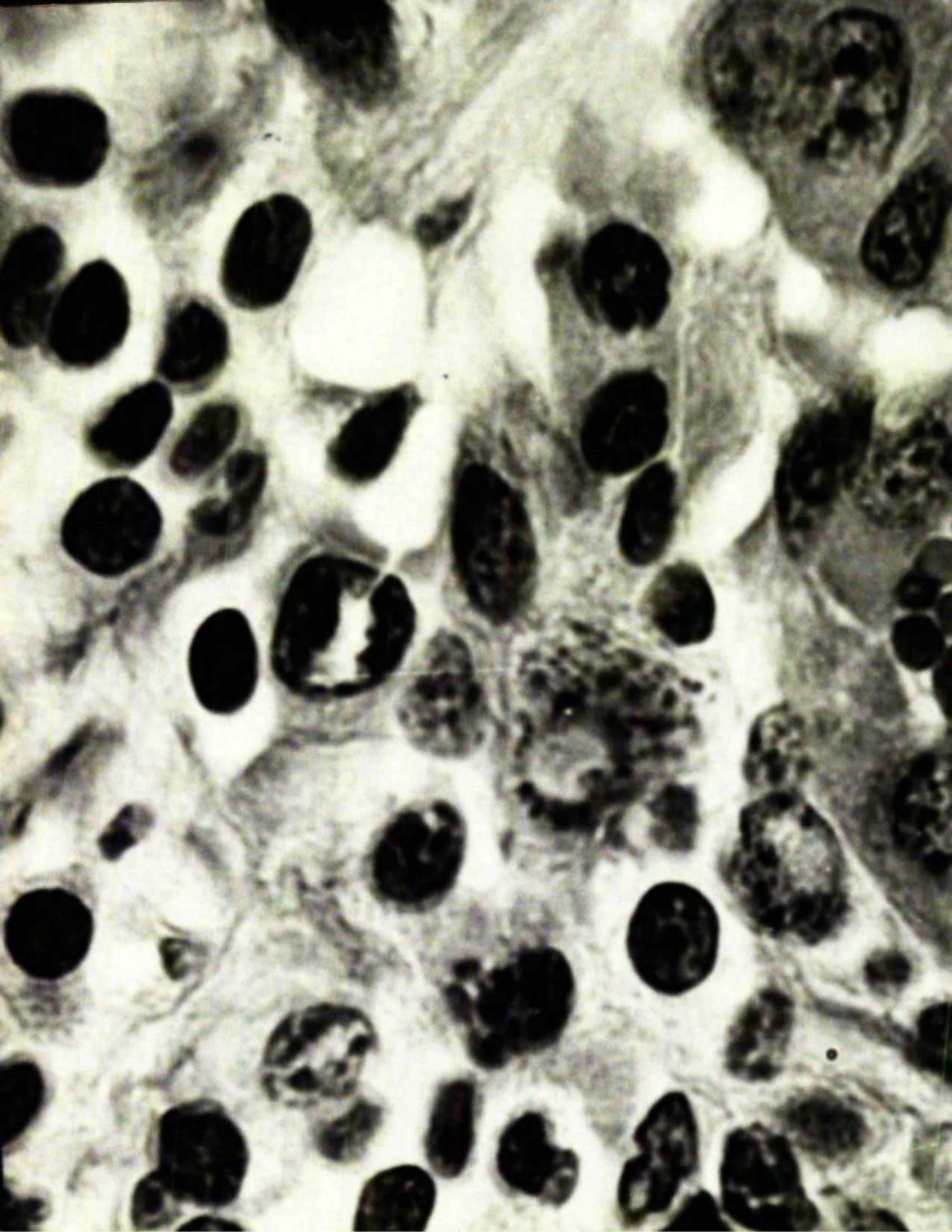


Fig. 8

Globule leucocyte from the intestine of a parasitised sheep, lying in an intraepithelial position.

Methacrylate section stained with haematoxylin and eosin.

(mag : 1400 x)

found in some of the worm-free sheep remains unknown, they may only indicate infection by some nematode with a direct life cycle and therefore difficult to eliminate, such as Strongyloides papillosus (Lapage, 1962). Alternatively, Rootes (1961) may be correct in his suggestion that these cells occur normally in a number of species but that their numbers are increased by a variety of disease processes. This seems unlikely, however, in view of the large numbers of investigations carried out on normal and pathological gastrointestinal mucosa where globule leucocytes have not apparently been observed (Wood and Taft, 1958 : Fresh et al., 1963 : Creamer, 1964a, b, c : Gould et al., 1964 : Ragins et al., 1964 : Reid and Brunser, 1964 : Trier, 1964 : Wurth and Musacchia, 1964 : Andrew, 1965 : Leblond, 1965).

The fact that the highest counts were, in general, in parasitised sheep in the posterior abomasum and at the abomaso-duodenal junction, indicates that the globule leucocytes occurred mainly at the site of the parasite, since this is the area in which Ostertagia circumcincta are found. The presence of the cell in Brunners glands would also be expected, since larvae of this species penetrate the mucosa of the small intestine and become embedded in the glands (Jubb and Kennedy, 1963).

It is therefore concluded that the occurrence of intraepithelial globule leucocytes observed in the present investigation was due to the presence of gastrointestinal nematodes, and that the highest

concentrations are found in the immediate vicinity of the worms.

RAT NO.	PRIMARY INFECTION	CHALLENGE DAY 20	DAY KILLED
1R/1	+	-	2
1R/2	+	-	4
1R/3	+	-	6
1R/4	+	-	8
1R/5	+	-	10
1R/6	+	-	12
1R/7	+	-	14
1R/8	+	-	16
1R/9	+	-	18
1R/10	+	-	20
1R/11	+	+	22
1R/12	+	+	24
1R/13	+	+	26
1R/14	+	+	28
1R/15	+	+	30
1RC/1-2	-	-	10
1RC/3-4	-	-	20
1RC/5-6	+	-	30

Table 4

Details of experimental *N. brasiliensis* infections given to rats for the purpose of studying the globule leucocyte response.

Part 2 : The relationship between globule leucocytes and infection with Nippostrongylus brasiliensis in the gastrointestinal tracts of non-immune and partially immune rats.

INTRODUCTION

The results of the investigation into globule leucocyte concentration in the gastrointestinal tracts of nematode-infected and worm-free sheep described in Section III (1), together with the previous observations of Kirkman (1947, 1949, 1950), Ahlqvist and Kohonen (1959a) and Sommerville (1956), indicate that a relationship exists between these cells and the presence of nematodes. In order to test this hypothesis it was considered necessary to produce a globule leucocyte response to nematode infection under controlled experimental conditions.

Taliaferro and Sarles (1939) observed many globule leucocytes in the lungs and small intestines of rats after infection with Nippostrongylus brasiliensis, while normal small intestines contained only a few cells. This observation suggests, although the authors do not state it directly, that N. brasiliensis elicits a globule leucocyte response. It was therefore decided to investigate the globule leucocyte response in the gastrointestinal tract to a single infection in non-immune worm-free rats, and to reinfection after the expulsion of the primary worm population at self-cure.

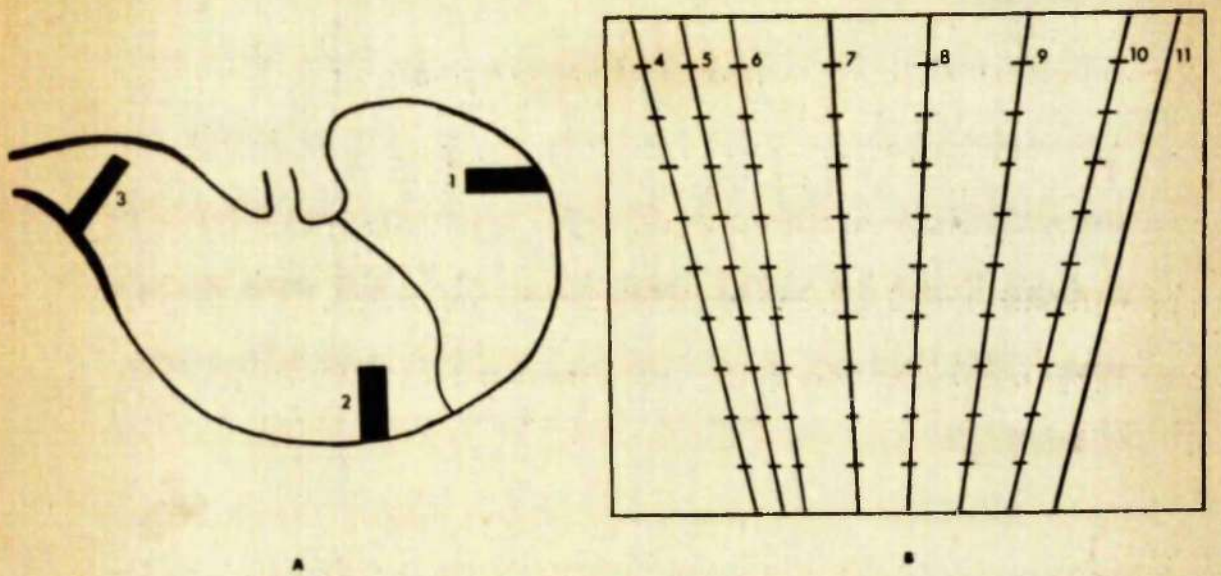


Fig. 9

A: Blocks from stomach were taken from oesophageal (1), fundic (2) and pyloric (3) areas.

B: Gauge used for obtaining intestinal blocks. Area 11 is 5 cm from area 10 across the whole width of the gauge.

MATERIALS AND METHODS

Infected rats (1R/1-15)

Adult hooded Lister females were used. They had an average weight of 120 g. and were obtained from nematode-free stocks. 15 rats were infected with with 3500 larvae of *N. brasiliensis* by subcutaneous injection (Table 4). 1R/1-10 were killed every other day between days 2 and 20 after infection. 1R/11-15 were reinfected with a further 3500 larvae on day 20 and killed each alternate day for the next 10 days.

Control rats (1RC/1-6)

Two uninfected controls were killed on day 10 and two on day 20 (1RC/1-2 and 1RC/3-4). Two further controls (1RC/5-6) were given a single dose of 3500 larvae on the first day, and killed on day 30. These procedures are summarised in Table 4.

Preparation and examination of sections

Rats were killed by a blow on the head. Stomach and intestines were carefully uncoiled and removed from the abdomen. Sections of stomach were taken from three areas which corresponded to the pars cardiaca (area 1), pars angularis (area 2) and pars pylorica (area 3) of Oehmke (1963) (Fig. 9). The small intestine was placed on a gauge (Fig. 9), and six equidistant blocks (areas 4, 6-10)

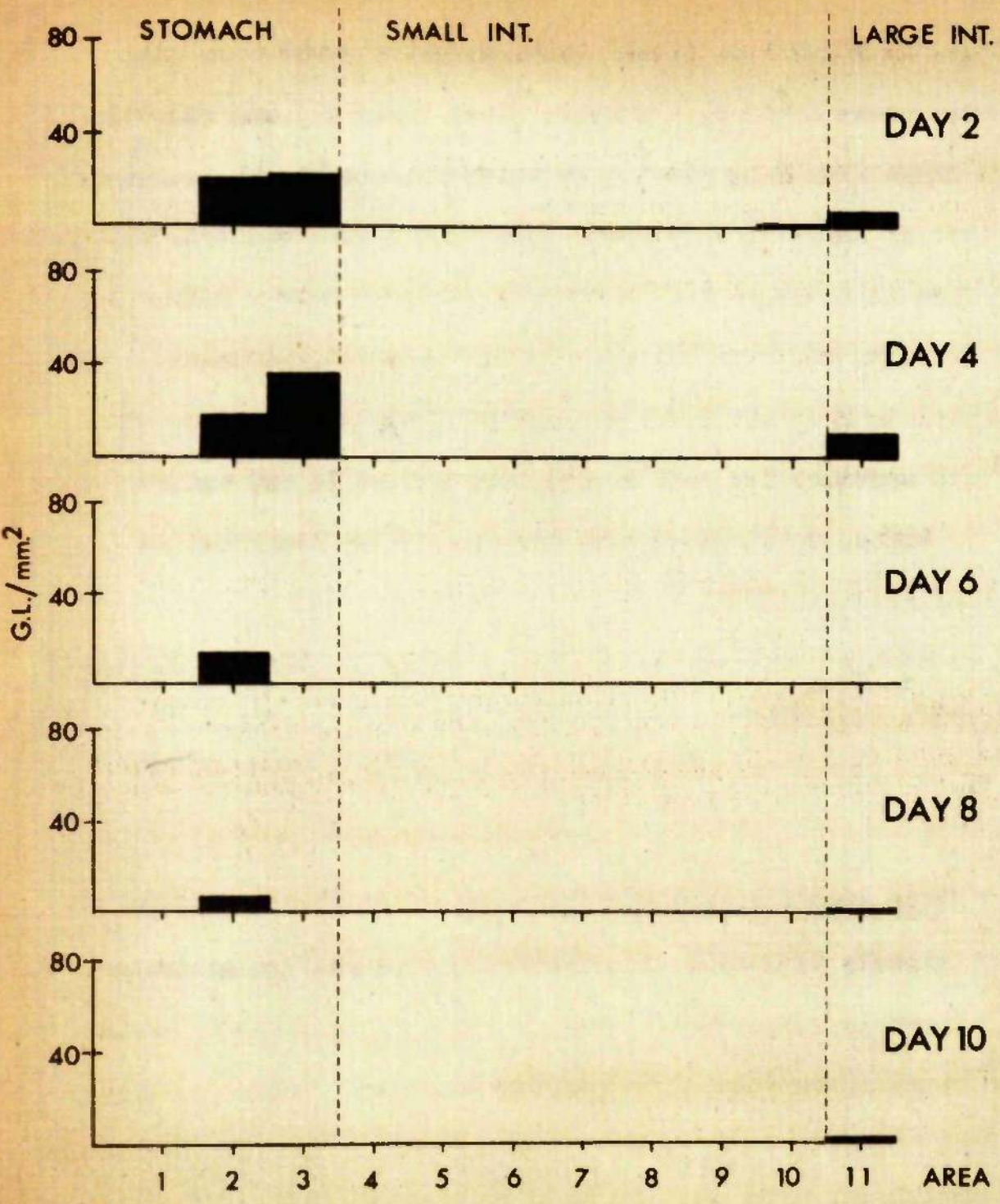


Fig. 10

Globule leucocyte response between days 2 and 10 of a primary infection with *N. brasiliensis*.

were taken. An additional block (area 5) was removed from half way between areas 4 and 6. A further block (area 11) was removed from the large intestine about 5 cm below the ileocaecal junction. In the case of 1R/11-15 only areas 4,6,7 and 8 were removed. All tissues were in fixative within three minutes of death. Buffered neutral formalin was used throughout, and tissues were embedded in methacrylate by a modification of the method described by Conkie (1965). All sections for cell counts were cut at 2μ and stained with amido black. Cell counts were carried out as described in Section III (1).

Worm counts were carried out as described in Section II (2).

RESULTS

Rats receiving single infection (1R/1-10)

The globule leucocyte distribution throughout the gastrointestinal tract is shown in Figs. 10-12. During the first 10 days of the infection (1R/1-5) small numbers of globule leucocytes were present in the stomach and large intestine, but there was a virtually complete absence of these cells from the small intestine (Fig. 10), although worms were present there in considerable numbers after the initial three or four days. This picture changed radically on day 12 (1R/6) (Fig. 11). There were now large numbers of globule leucocytes in the duodenum and jejunum at the site of the nematode, and the stomach

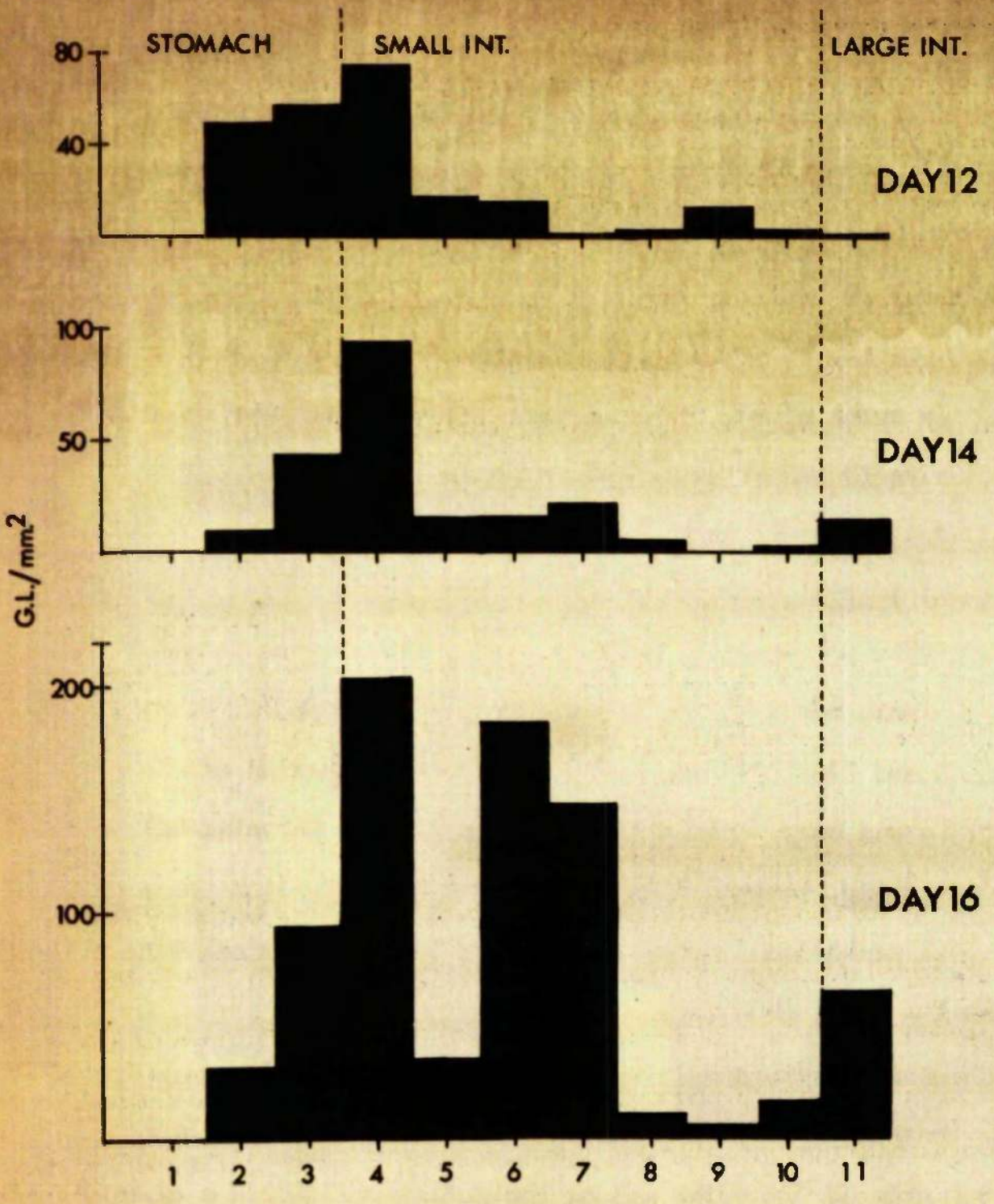


Fig. 11

Globule leucocyte response between days 12 and 16 of a primary infection with *N. brasiliensis*.

response had also increased. The concentration of globule leucocytes continued to rise in the anterior small intestine until day 18 (1R/9), with secondary peaks arising further down the jejunum on days 16 and 18 (Figs. 11 and 12). Concentrations in stomach and large intestine were also higher. By day 20 (1R/10) the high concentrations had declined markedly. At no time were globule leucocytes seen in the pars cardiaca (area 1) (Figs. 10-12). Worm counts showed that all but a small number of worms were expelled from the intestine between days 10 and 12 (Table 5).

Uninfected control rats (1RC/1-4)

Globule leucocyte distribution in these animals is shown in Fig. 13. There was no significant difference between those killed on day 10 and those killed on day 20. The distribution of globule leucocytes was very similar to that seen in rats infected with N. brasiliensis, between days 2 and 10 of the infection (1R/1-5), i.e. small concentrations in stomach and large intestine, with virtually none in the small intestine.

Because of the increased spread of N. brasiliensis along the small intestine during a challenge infection (Brambell, 1965b) only the site of the worms during the earlier stages of a primary infection (areas 4,6,7 and 8) were examined from 1R/11-15, to avoid examining areas which might in practice be experiencing worms for the first time. An average value for GL/mm^2 was obtained for these areas (Fig. 14).

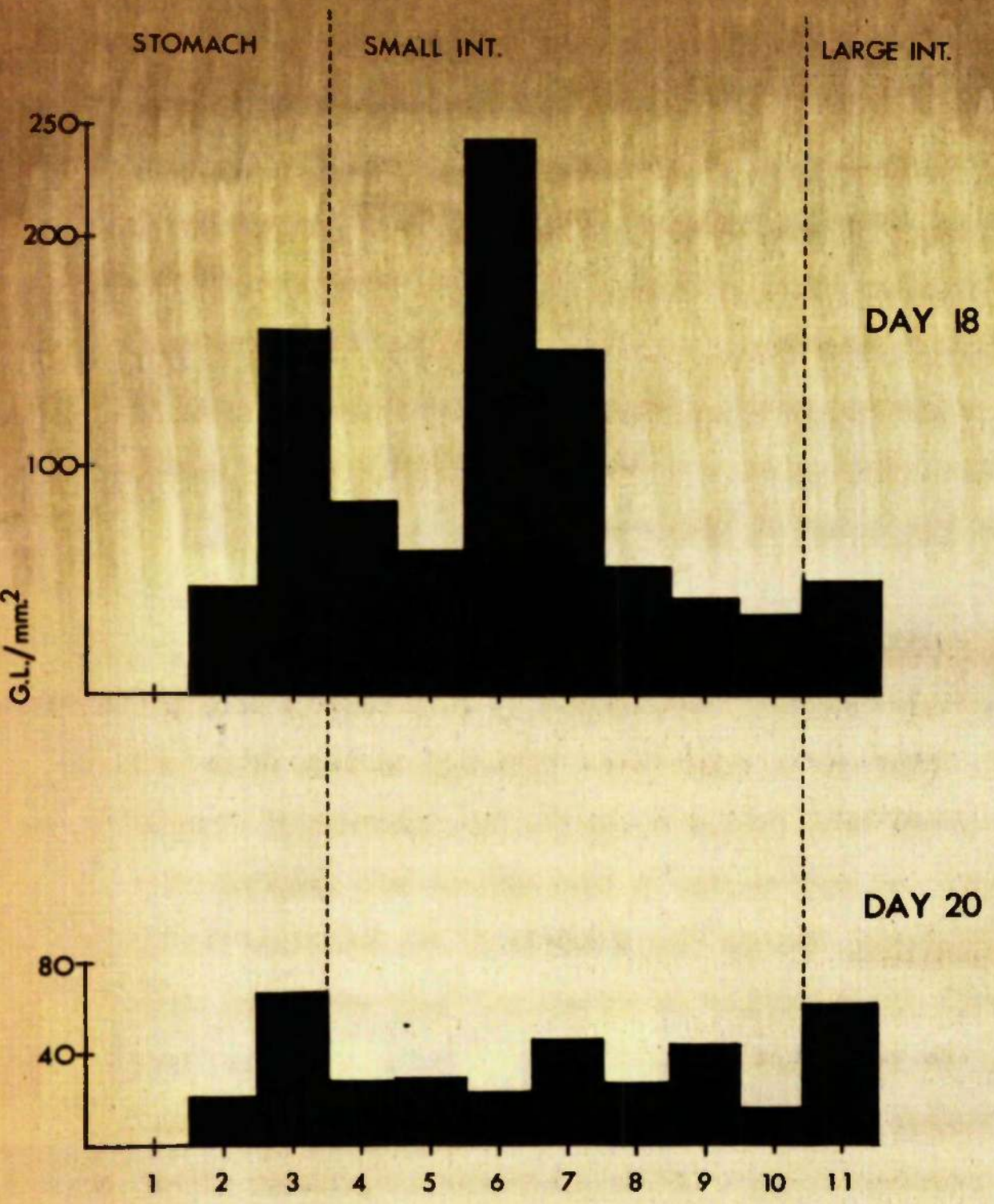


Fig.12

Globule leucocyte response on days 18 and 20 of a primary infection with *N. brasiliensis*.

Concentrations of globule leucocytes were higher than during the post self-cure phase of a single infection, and on day 10 of the second infection reached almost double the highest concentration reached during the course of a single infection. Worm counts were as follows :-

1R/11	194
1R/12	44
1R/13	50
1R/14	1
1R/15	5

Single infection controls (1RC/5-6)

Only the worm site was examined, as indicated above. No globule leucocytes were seen in any of the blocks (Fig. 14). No worms were present in either rat at autopsy.

DISCUSSION

By comparing the distribution of globule leucocytes (Fig. 15) in 1R/1-5 with that seen in the uninfected control group 1RC/1-4 (Figs. 10, 13) it becomes evident that no significance can be attached to the stomach and large intestine responses during this period. Further investigation showed that the response in the large intestine might be attributable to the nematode Syphacia obvelata which was infecting bought-in worm-free rats within a few days of arrival.

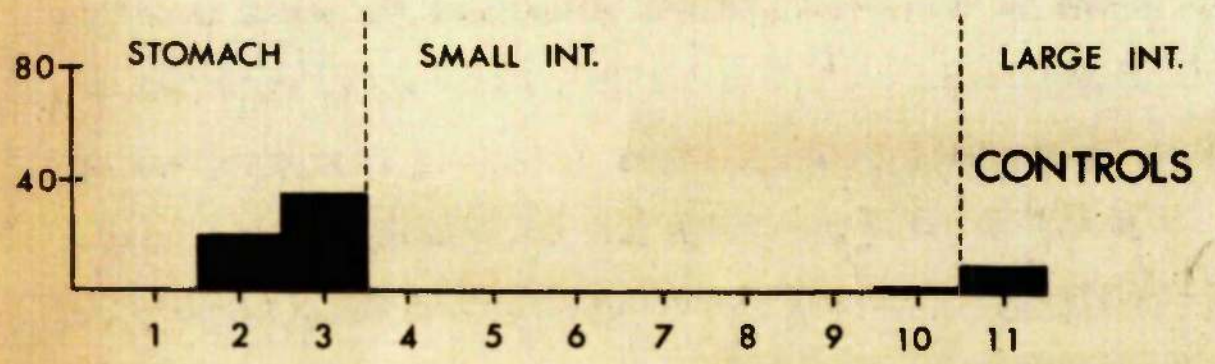


Fig. 13

Concentration of globule leucocytes in the gastrointestinal tracts of control rats which were not infected with N. brasiliensis.

It is probable that the stomach response can be explained similarly. However, the increased concentrations of globule leucocytes in the stomach and large intestine from day 12 onwards probably do represent to some extent the overlap of a response to N. brasiliensis. The site of greatest globule leucocyte response after self-cure (Figs. 11 and 12) coincided closely to that of the nematode before it was expelled. The cell reaction became more widespread later on, with high concentrations throughout the small intestine. This appears to correspond to the redistribution throughout the small intestine of the residual worm population surviving self-cure, which has been described by Brambell (1965a). The globule leucocyte distribution over the whole of this period corresponded closely to the distribution of N. brasiliensis adults during a primary infection. Nevertheless there was a fairly marked response to either side of the actual site of the parasite, and in the case of the stomach and large intestine this occurred outside the area usually associated with a pathological response to N. brasiliensis.

The timing of the globule leucocyte response during a primary infection is striking, in that it coincides closely with self-cure (Table 5). While this provides no definite indication of a direct link between globule leucocytes and the immune response, as the apparent relationship may be fortuitous, such a relationship should be investigated further. The demonstration of antibody in Russell body cells, which may be similar to globule leucocytes (White, 1954),

DAY	GL/mm^2	WORM COUNT
2	0	36
4	0	892
6	0	207
8	0	1225
10	0	266
12	29	15
14	39	15
16	146	1
18	150	0
20	38	0

Table 5

Globule leucocyte response at the worm site (areas 4,6,7,8), showing a close correlation to self-cure, as indicated by worm counts. These results appear in graphical form in Fig. 16.

is added reason for doing so.

The nature of the globule leucocyte response in 1R/11-15 appears to support the suggestion that the immune response, rather than the actual duration of the infection, is the related stimulus. Reinfection on the twentieth day produced an immediate and massive globule leucocyte response, without the time lag noted in a primary infection. These differences in magnitude and timing between the responses in primary and challenge infections may be due to the immune state of the animal after being exposed to the initial infection, and demonstrated by the characteristically rapid expulsion of the second worm population.

Comparison between reinfected rats 1R/11-15 and their single infection control group 1RC/5-6 (Fig. 14) shows that the globule leucocytes resulting from the primary infection had completely disappeared, and that on day 30 all globule leucocytes at the worm site were the consequence of the challenge infection.

It is concluded that all intraepithelial globule leucocytes present at the worm site in this experiment were produced in response to N. brasiliensis and that these cells only appear when a state of immunity exists, and probably only during the period of an active immune response, that is at self-cure or during subsequent infection. The experiment also showed that the globule leucocyte response extends beyond the location of the parasite, and that it has subsided completely within 30 days of a single infection. It also suggests that

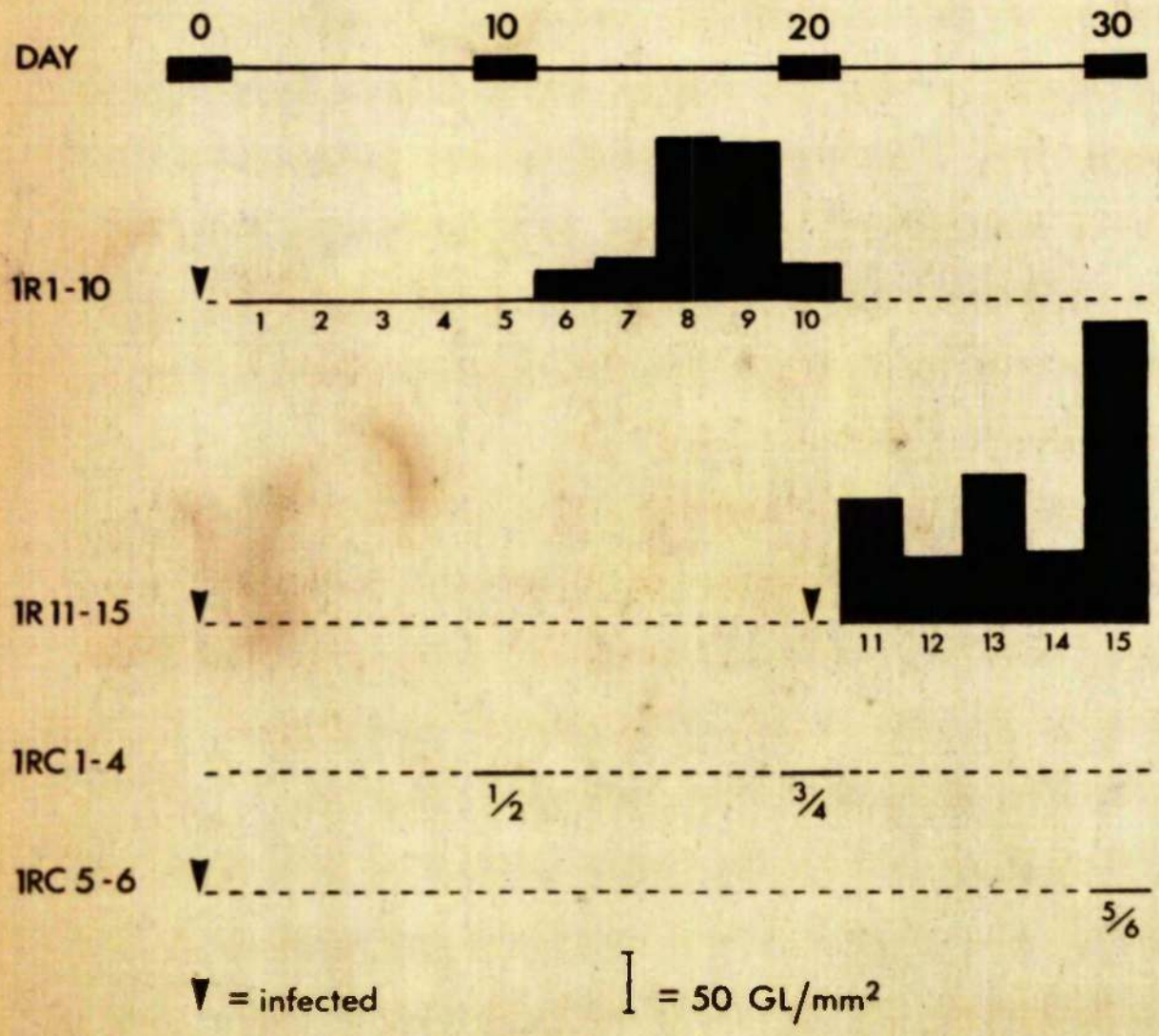


Fig. 14

Globule leucocyte response in the anterior small intestine during a primary infection (1R 1-10), and after reinfection on day 20 (1R 11-15)..

the greater the degree of immunity the larger is the globule leucocyte response, but a direct relationship between globule leucocytes and the immune response remains to be demonstrated.

Fig. 15

Globule leucocytes in the jejunal crypt epithelium, produced in response to a single infection of *N. brasiliensis*.

Methacrylate section stained with amido black.

(mag : 1300 x)

Part 3 : Observations relating globule leucocytes to infection by cestodes and trematodes.

INTRODUCTION

The results reported in Section III (1,2) clearly establish a relationship between certain nematode infections and the presence of globule leucocytes in the gastrointestinal mucosa, and indicate that these cells are almost completely absent from parasite-free animals. In addition Pierce et al.(1962) and Pierce and Long (1965) have reported that coccidial infection in fowls produces a globule leucocyte response.

Because of the possibility that other types of parasite also elicit a globule leucocyte response, available cestode- and trematode-infected material has been examined for the presence of this cell.

MATERIALS AND METHODS

Fasciola hepatica

Blocks were obtained from the liver of a nematode-free Blackface sheep which was suffering from acute fascioliasis at the time of killing. This animal had previously been fed copper at high levels for other experimental purposes. Blocks were fixed in buffered neutral formalin and stained with haematoxylin and eosin.

Hymenolepis nana

The gastrointestinal tracts of two rats infected with the ileum tapeworm H. nana were examined for the presence of globule leucocytes. Blocks were taken from the same areas as before (Section III (2)), one set being fixed in Susa and the other in ethylene glycol monomethyl ether. They were embedded in methacrylate, sectioned at 2μ and stained with amido black. The presence of H. nana was confirmed at autopsy.

RESULTS

Fasciola hepatica

Globule leucocytes, which appeared to be morphologically identical to those previously observed in the gastrointestinal tracts of sheep infected with Ostertagia spp.(Fig. 8) were present in large numbers in the bile duct epithelium. In some areas they were so numerous that a layer three cells thick was formed.

Hymenolepis nana

Blocks from stomach, anterior small intestine and large intestine contained no globule leucocytes, but in the posterior small intestine a single isolated block, area 8 in each case , contained high concentrations of the cell, while adjacent areas (7 and 9) contained very low or zero concentrations. The location of this response

corresponds to the site of H. nana in the rat.

DISCUSSION

The presence of globule leucocytes in close association with cestodes and trematodes suggests that these classes of parasite can cause a globule leucocyte response in the same way as nematodes and coccidia. However, these observations must be interpreted with caution because of the small numbers of animals examined and the absence of controls.

Part 4 : Globule leucocyte response in hyperimmune rats infected with Nippostrongylus brasiliensis.

INTRODUCTION

In Section III (1,2) experimental evidence was presented which demonstrated that certain nematode infections elicit a globule leucocyte response in the gastrointestinal tract, and confirmed the observations of previous authors on this subject (Taliaferro and Sarles, 1939 : Kirkman, 1950 : Sommerville, 1956). It was noted during the course of N. brasiliensis infection in rats that the appearance of globule leucocytes in the mucosa of the small intestine coincided with the onset of the immune response.

Non-immune rats were used in this experiment, and in such animals infective larvae of N. brasiliensis become mature adults in the small intestine about 5½ days after injection (Weinstein and Jones, 1959). Five to seven days later expulsion of this adult worm population from the small intestine commences. The globule leucocyte response in such rats coincided with the commencement of self-cure and continued during the period when expulsion of the worms was taking place.

The coincidence between the appearance of globule leucocytes and the onset of the immune response may, however, have been accidental, for it is equally possible that their occurrence was related to

the arrival of the fourth stage larvae in the small intestine a week earlier (Sarles and Taliaferro, 1936), or to some other event in the life cycle of the parasite.

The pattern of self-cure is altered in rats which have had previous experience of N. brasiliensis and are thereby rendered immune. The immune response in the small intestines of such rats commences at the time when fourth stage larvae reach the intestine ; that is about six days earlier than in non-immune rats which are similarly infected. This difference in the timing of self-cure in non-immune and immune rats may be used to determine whether the occurrence of globule leucocytes is related to the immune response, or if it has a fixed relationship to some other feature of the life cycle of N. brasiliensis, the timing of which is not altered by changing the immunological status of the rat.

The importance of establishing the precise nature of the relationship between globule leucocytes and the immune response lies in the possibility that these cells play an active part in self-cure. The purpose of the present investigation was to examine the globule leucocyte response occurring to a challenge infection with N. brasiliensis in a group of rats rendered hyperimmune by repeated previous infections, with the aim of determining the timing of the response, and its relationship to the self-cure phenomenon.

MATERIALS AND METHODS

MATERIALS AND METHODS

Adult female albino Wistar rats from nematode-free stocks were used, with a terminal weight in the range 200-210 g. They were each infected initially with 2000 infective larvae of N. brasiliensis which were injected under ether anaesthesia into the groin region. The infection was repeated at intervals of two weeks; the dose of infective larvae being increased by 2000 on each occasion so that the final immunising dose was 8000 larvae per rat. Six weeks were then allowed to elapse in order that any globule leucocyte response provoked by the immunising infections would subside.

30 of these hyperimmune rats (R/1-30) were then infected with 3500 larvae, administered as previously described, while a further eight rats (C/1-8) were retained as controls. Challenged rats were sacrificed in pairs at two day intervals over the next 30 days, while the unchallenged controls were killed in pairs on days 0, 10, 20 and 30. Faecal egg counts were carried out each day from a randomly selected 1 g. sample obtained by mixing faeces from the cages housing R/1-30.

Rats were killed by an overdose of chloroform and the small intestine unravelled and placed on a gauge. Two lengths of unopened jejunum were removed (areas 1 and 2) at a point calculated by Brambell (1965b) as being the mode of the distribution of a secondary infection of N. brasiliensis at less than six days duration. This area is

DAY	EGGS/GM. FAECES	RAT NO.	GL/mm^2 AREA 1	GL/mm^2 AREA 2	AVERAGE	WORM COUNT
0	-	C 1 C 2	13 5	5 4	7	9 0
1	0					
2	0	R 1 R 2	11 6	8 16	10	36 7
3	66					
4	133	R 3 R 4	4 25	5 31	16	39 91
5	0					
6	66	R 5 R 6	62 87	86 112	87	2 14
7	0					
8	200	R 7 R 8	127 112	110 199	137	11 17
9	0					
10	200	C 3 C 4 R 9 R 10	19 11 305 73	19 7 231 87	14 174	4 9 9 6
11	0					
12	0	R 11 R 12	77 83	80 86	82	5 8
13	0					
14	0	R 13 R 14	67 120	136 198	130	0 17
15	0					
16	66	R 15 R 16	71 104	87 180	111	6. 5
17	0					
18	0	R 17 R 18	27 49	29 58	40	9 7
19	0					
20	0	C 5 C 6 R 19 R 20	7 15 13 55	5 5 12 44	8 31	8 5 1 0
21	0					
22	0	R 21 R 22	50 5	39 7	25	3 0
23	0					
24	0	R 23 R 24	14 78	24 64	45	7 4
25	0					
26	0	R 25 R 26	11 2	14 3	8	2 4
27	0					
28	0	R 27 R 28	1 10	4 31	12	7 2
29	0					
30	0	C 7 C 8 R 29 R 30	3 6 6 0	20 4 8 3	8 4	1 20 0 3

Table 6

Experimental results of a challenge infection with 3,500 larvae of *N. brasiliensis* in hyperimmune rats (R1-30) and in a similar group of uninfected hyperimmune controls (C1-8).

approximately 5 cm further forward than in a primary infection. Blocks were fixed in buffered neutral formalin, embedded in methacrylate and sectioned at 2μ. The number of intraepithelial globule leucocytes per square millimetre of tissue section (GL/mm^2) was calculated as described previously. The four values of GL/mm^2 for each pair of rats were pooled and an average obtained, which was used as an expression of globule leucocyte concentration on that particular day. A worm count was carried out on each rat at autopsy, using the method described in Section II (2). No differentiation was made between immature and fully adult forms.

RESULTS

Numerical results are set out in Table 6.

Worm counts

Small numbers of worms were found in the majority of control rats (C/1-8). These worms represented the remnants of the immunising infections remaining after self-cure. Considerable numbers of immature forms were found in the small intestines of the infected rats killed on day 4 (R/3-4), indicating that a proportion of the infective larvae had escaped the inhibition of larval stages associated with challenge infection (Ogilvie, 1965b) and had succeeded in reaching the small intestine. However, on day 6 the worm counts immediately

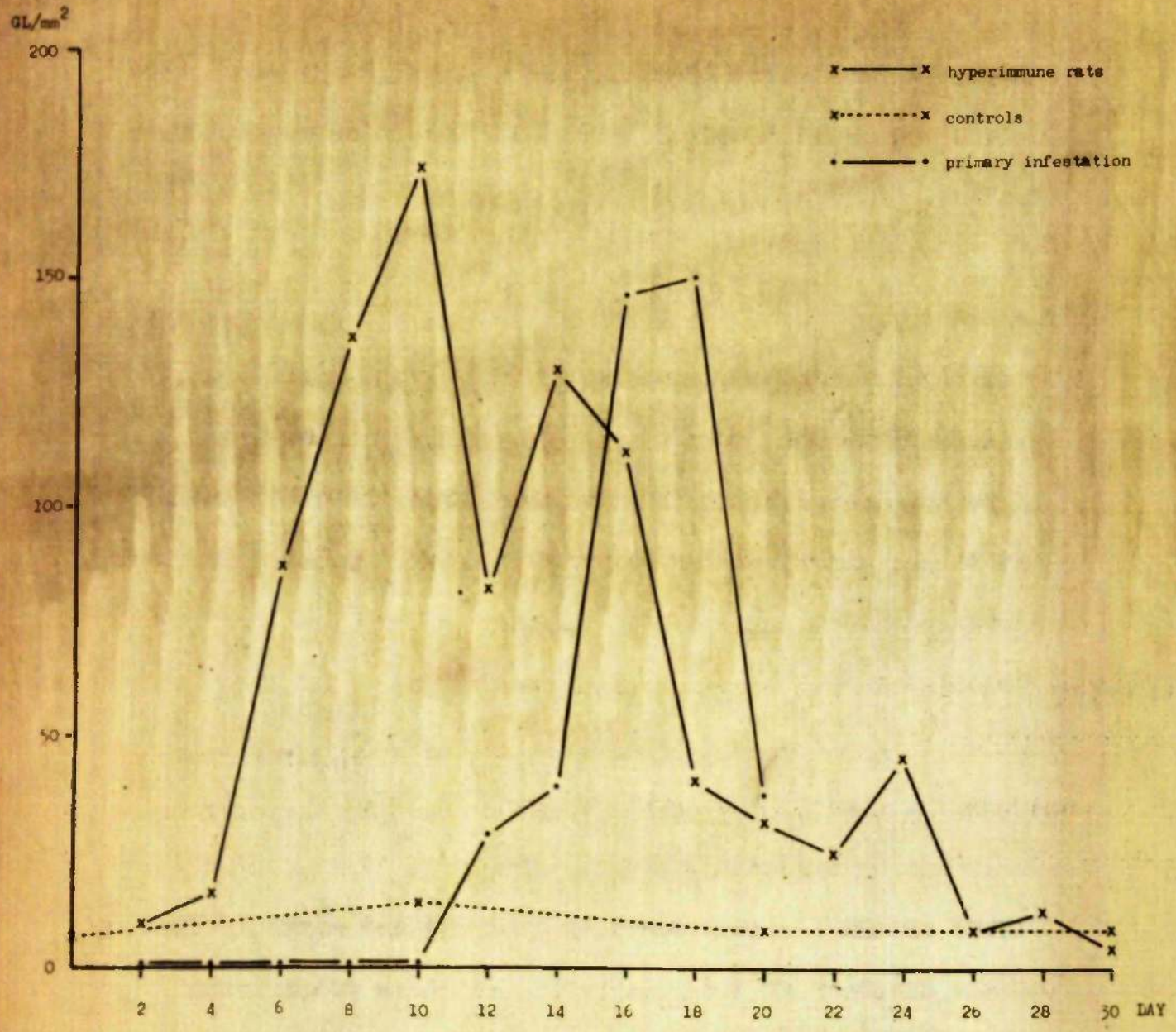


Fig. 16

Globule leucocyte response in the jejunum of hyperimmune rats infected with 3,500 larvae of *N. brasiliensis*, as compared to uninfected hyperimmune controls, and the globule leucocyte response occurring during the course of a primary infestation (graphical representation of figures given in Table 5).

declined again to the levels found in the control group, confirming that self-cure commenced immediately the new infection reached the small intestine.

Egg counts

The results of daily egg counts confirmed that the repeated immunising infections had rendered the rats solidly immune. No egg-laying period ensued after the adults reached the small intestine, in contrast to the normal pattern observed during a primary infection, and the concentration of eggs in the faeces remained low throughout the experiment.

Globule leucocytes in control rats (C/1-8)

Globule leucocytes were present in low concentrations in all the blocks examined. Their concentration did not markedly fluctuate throughout the duration of the experiment, enabling comparisons with the infected group to be made (Fig. 16). The presence in control rats of globule leucocytes correlates with the finding of small numbers of adult worms noted above.

Globule leucocytes in challenged rats (R/1-30)

The numerical value obtained for GL/mm^2 in each area examined is shown in Table 6, and the bi-daily averages are plotted in Fig. 16. A marked globule leucocyte response (Fig. 17) commenced between

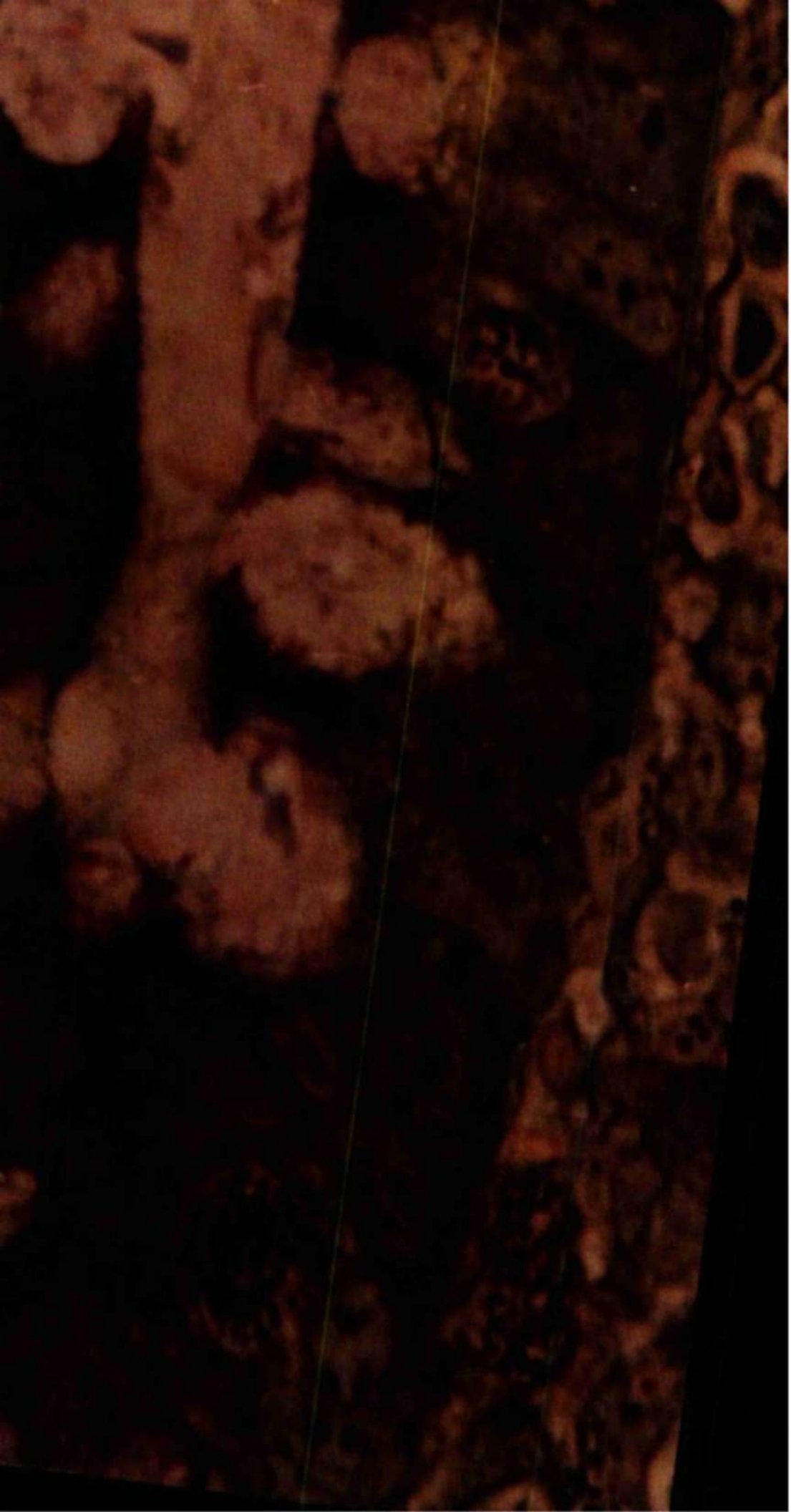


Fig. 17

Globule leucocytes in the jejunal crypt epithelium and lamina propria of a hyperimmune rat after a challenge infection of N. brasiliensis. Methacrylate section stained with amido black and photographed under phase contrast.

(mag : 1300 x)

days 4 and 6, in the period immediately following the entrance of the worms into the small intestine, and coinciding with the onset of self-cure. The average concentration of globule leucocytes reached a maximum on day 10, and thereafter declined so that by day 26 the number of globule leucocytes in the infected group was similar to that of the control group.

DISCUSSION

The globule leucocyte response in the small intestines of hyperimmune rats subjected to further experimental infection with N. brasiliensis is compared to similar uninfected control rats in Fig. 16. In order to compare this response to that occurring in non-immune rats the concentration of globule leucocytes produced in response to a primary infection, and previously reported in Section III (2), is inserted in the same figure.

The course of infection by N. brasiliensis in hyperimmune rats differs in a number of well defined ways from a similar infection of non-immune rats. These changes are the result of the development of partial or complete immunity to reinfection, stimulated primarily by the presence of adult worms in the small intestine (Ogilvie, 1965b). In a primary infection the arrival of adult worms in the small intestine is followed by an egg-laying period which terminates as immunity develops, and which is closely followed by the rapid

expulsion of a large part of the adult worm population (see Neilson, 1965). Once immunity has developed, the ability of adult worms to establish themselves in the small intestine is impaired, resulting in the partial or complete abolition of the egg-laying phase and the immediate onset of self-cure. These effects were manifested in the present case and resulted in the negligable concentrations of eggs in the faeces and very low post mortem worm counts noted in the infected group R/1-30. Since immunity is not stage specific and inhibits the development of larvae to maturity (Ogilvie, 1965b), it is probable that the number of fourth stage larvae reaching the small intestine was considerably lower than would have occurred in a similar primary infection.

The globule leucocyte response in the reinfected hyperimmune group R/1-30 commenced between days 4 and 6 (Fig. 16); about six days earlier than that observed previously when non-immune rats were used. Consequently self-cure and the globule leucocyte response coincide in hyperimmune rats as well as in primary infections, despite the different timing of the immune response resulting from previous experience to N. brasiliensis. This indicates that the occurrence of globule leucocytes is related to the onset of an immune reaction against the worms in the small intestine , and not to some other event in the life cycle of N. brasiliensis such as the appearance of worms in the intestine, the timing of which is not altered by changing the immunological status of the rat.

Part 5 : Morphological comparison with the light microscope of globule leucocytes and experimentally produced Russell body cells

INTRODUCTION

A high incidence of Russell bodies at different sites has been associated with degenerative change (Bangle, 1963), chronic inflammation (Yoshida *et al.*, 1964) and immunological processes (Munsick and Janovski, 1963). White (1954) observed Russell bodies in cells of rabbit spleen after repeated injections of *Proteus vulgaris* live vaccine, which were probably similar to certain large "dead" eosinophilic cells observed by Congdon (1964) in splenic white pulp after antigenic stimulation. Using fluorescent antibody and cytochemical techniques White (1954) demonstrated that specific antibody was present in Russell bodies or on their surfaces. Zlotnick *et al.*(1959), in similar experiments on the rabbit, related the development of splenic "grape cells" (i.e. plasma cells containing Russell bodies) to immunisation, hyperglobulinaemia and plasma cell hyperplasia. Pearse (1949) observed increased numbers of plasma cells and Russell bodies around malignant tumours, and it has been suggested that this is indicative of an autoimmune reaction to abnormal proteins produced by malignant tumour cells (Zlotnick *et al.*, 1959). Consequently there is a considerable amount of evidence to relate

Russell body cells to immune processes.

Globule leucocyte inclusions bear some resemblance to Russell bodies; in fact both White (1954) and Kirkman (1950) indicate that they are very closely related. Kent (1952), on the other hand, states that the two types of cell are readily distinguishable using morphological and cytochemical criteria. In order to compare the two types of cell morphologically, intestinal globule leucocytes produced in response to two infections of N. brasiliensis and Russell body cells , produced in spleen and submandibular lymph node in response to repeated injections of Proteus vulgaris vaccine, were examined with the light microscope.

MATERIALS AND METHODS

Female hooded Lister rats, average weight 120 g. were used. Material containing globule leucocytes was obtained from the jejunum of rats infected twice with 3000 N. brasiliensis larvae at an interval of 20 days, and killed 10 days after the second infection.

P. vulgaris vaccine was manufactured from the National Type Culture collection catalogue no. 4175/batch 4. The organism was grown in tryptic digest broth for 18 hours at 37°C to give a concentration approximately equal to a no. 1 Wellcome opacity tube; equivalent to 2×10^8 organisms/ml. 0.25 ml of the live vaccine was injected intravenously into the tail vein at three day intervals, under

trichloroethylene anaesthesia. 10 injections were given over 30 days, and the animals killed six days later. This method was similar to that described by White (1954). Four rats were given vaccine injections and two additional non-vaccinated animals were killed at the same time.

Animals were killed by a blow on the head. All tissues were fixed in buffered neutral formalin and embedded in methacrylate. Sections were cut at 2μ and stained with haematoxylin and eosin. Cell measurements were carried out using a Watson micrometer eyepiece.

RESULTS

Russell body-containing cells were present in the spleen and submendibular lymph nodes of vaccinated and non-vaccinated rats. However, they were much more plentiful in vaccinated animals, and the Russell bodies were in some cases very much larger. The highest concentration of such cells occurred in the lymphatic medullary cords, where they were interspersed with plasma cells. The plasma cells and Russell body cells occurred in groups, so that many fields contained few or no cells of this type.

In Fig. 18 the cells are about equally divided between mature plasma cells and metaplasmocytes under going Russell body formation. Fig. 19 illustrates a Russell body cell of slightly more mature type, which is morphologically similar to the globule leucocyte

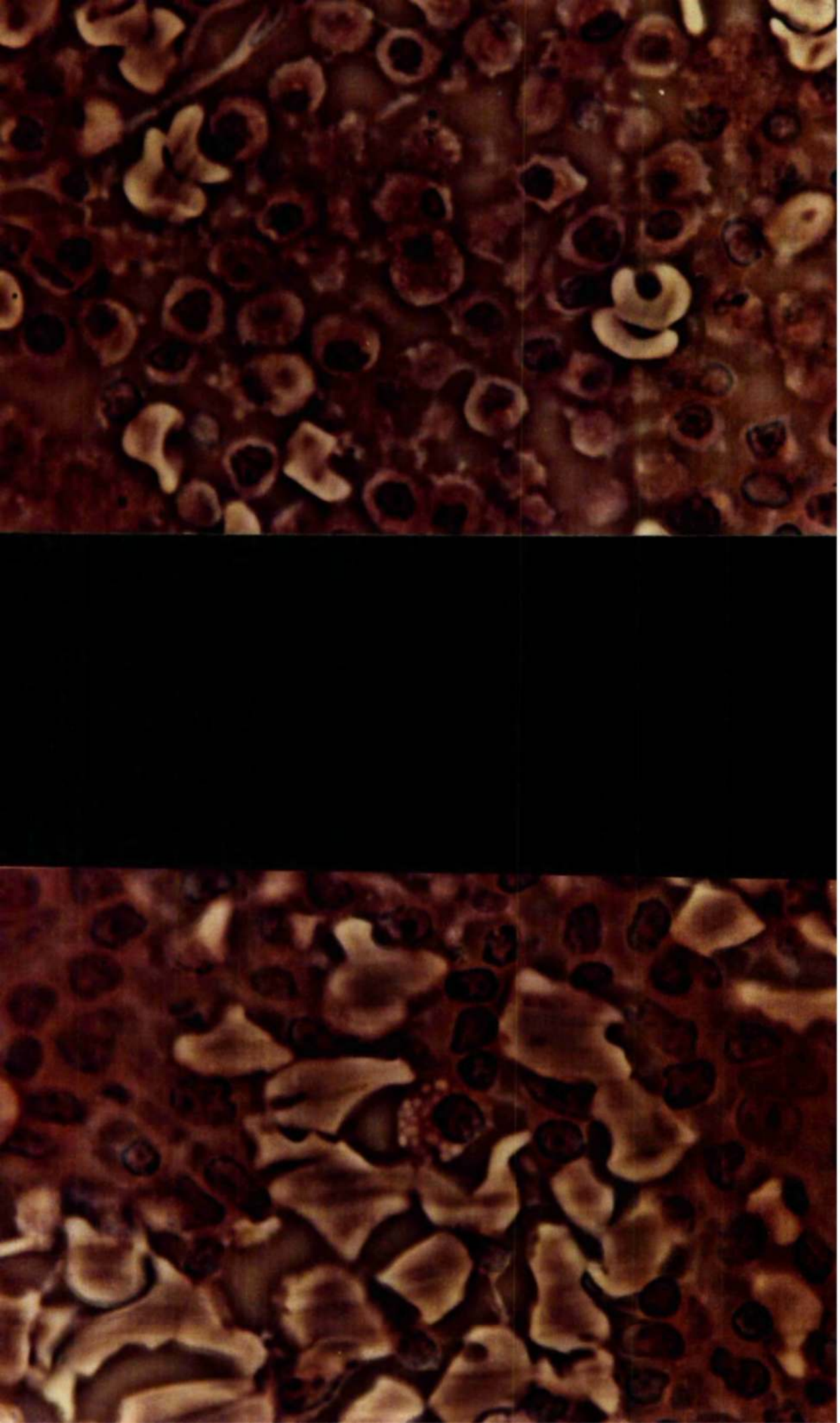


Fig. 18

Medullary cord of a lymph node from a rat immunised with repeated injections of P. vulgaris vaccine, showing a number of mature plasma cells interspersed with cells at various stages of Russell body formation.

Methacrylate section stained with haematoxylin and eosin and photographed under phase contrast.

(mag : 1300 x)

Fig. 19

Metaplasmacyte containing relatively small Russell bodies. Such cells have a morphology which is similar to that of the globule leucocyte.

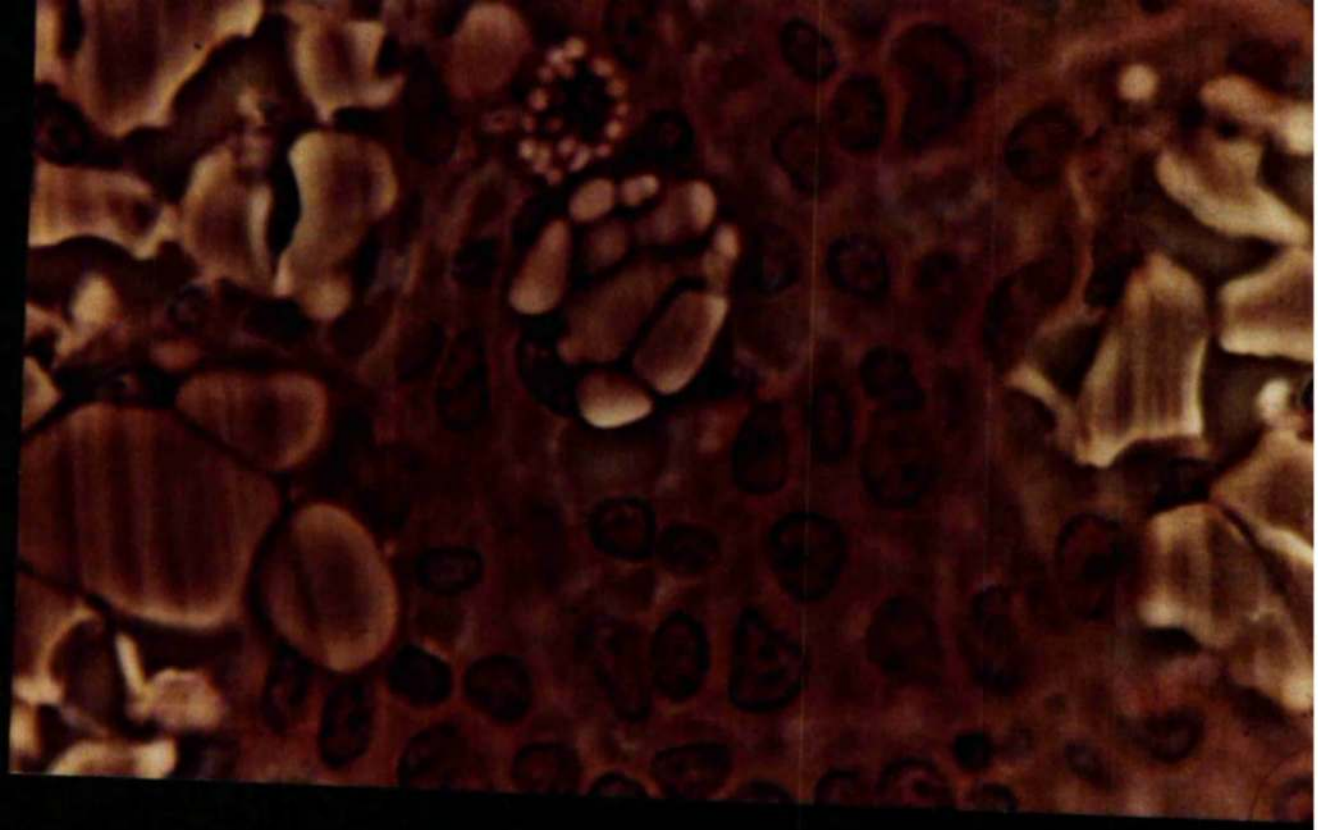
Methacrylate section stained with haematoxylin and eosin and photographed under phase contrast.

(mag : 1300 x)

except that the latter type of cell is frequently elongated, perhaps because it is undergoing active migration. The changes which take place as the Russell bodies enlarge are illustrated in Fig. 20. The number of Russell bodies within each cell appears to decrease. This may be due to coalescence or, alternatively, to the fact that with increasing size fewer of them appear in a single section. The bodies are at first rounded; later they become contoured against one another and eventually against the surrounding tissue, so that the cell finally ceases to be spherical. The nucleus becomes flattened peripherally, but does not show any signs of degeneration. No Russell bodies were definitely identified as being extracellular.

The smallest Russell bodies could not be accurately measured. The diameter of the largest inclusion observed was 22μ, while the largest Russell body cell located had a diameter of 30μ. Cells of comparable size and morphology to globule leucocytes measured about 10μ across, while their globules, which were also of similar size, measured approximately 1μ across.

In contrast to the Russell body cells, globule leucocytes were of markedly uniform morphology. The only variable noted was the shape of the cell, which tended to be elongated in the lamina propria and rounded in the intraepithelial position. Inclusions were of fairly uniform size from cell to cell and within any single cell, and were approximately 1μ in diameter. They were almost spherical in shape and did not appear to indent the nucleus, which was frequently



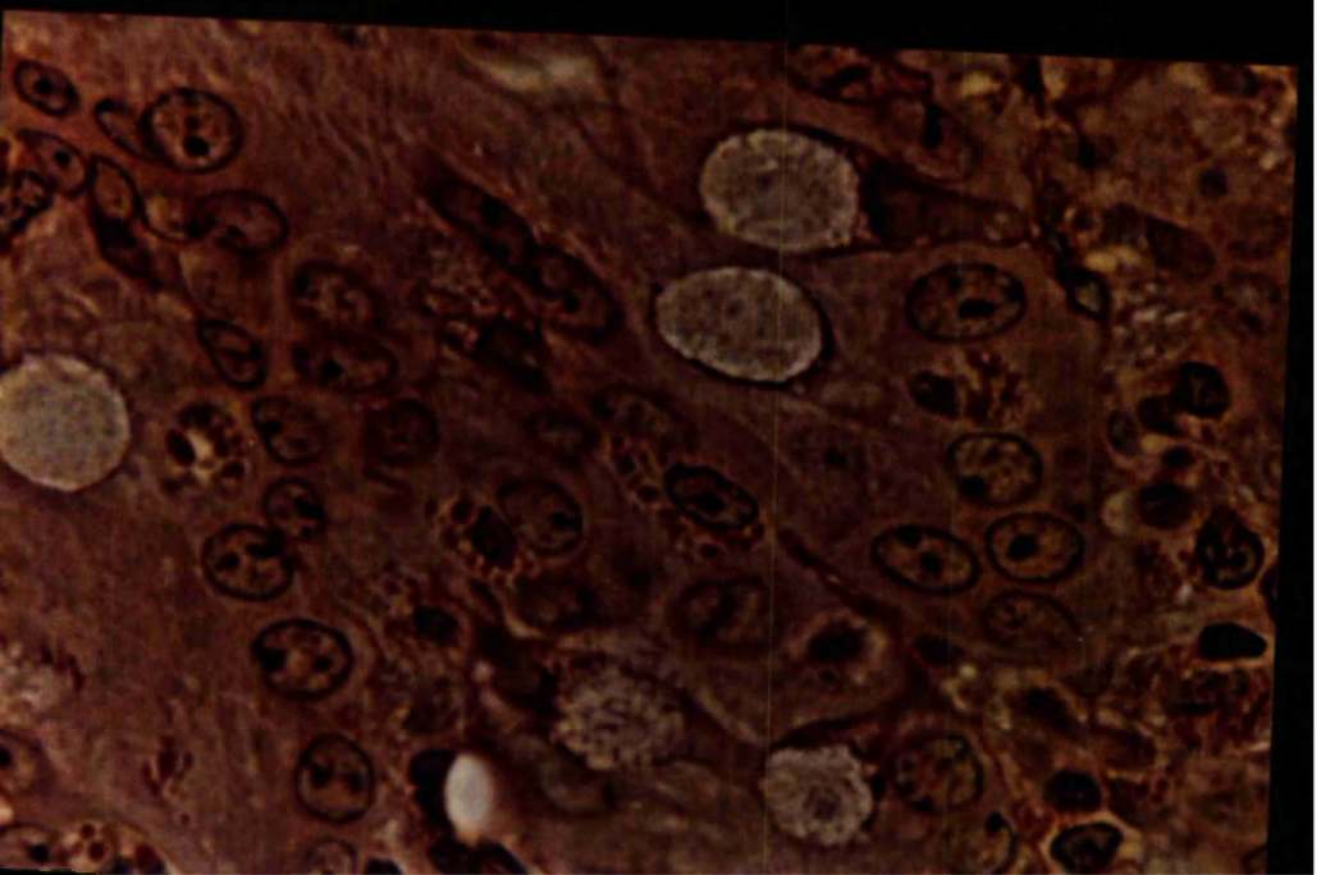


Fig. 20

Russell body cells at various stages of development. One cell is spherical and contains small round inclusions. A second cell retains its spherical shape but the inclusions have become contoured against each other. The third cell shows three large inclusions. The nucleus is flattened peripherally, and the cell has lost its spherical shape. Methacrylate section stained with haematoxylin and eosin, and photographed under phase contrast.

(mag : 1300 x)

Fig. 21

Globule leucocytes in the jejunum of an adult rat, produced in response to *N. brasiliensis* infection.

Methacrylate section stained with haematoxylin and eosin, and photographed under phase contrast.

(mag : 1300 x)

centrally placed within the cell. As has been previously noted, the chromatin distribution of the nucleus resembled that of a plasma cell. The globule leucocytes observed corresponded very closely in their morphology, within the limits of light microscopy, to the Russell body cells of the type illustrated in Fig. 19, except that when viewed under phase contrast Russell bodies appeared whitish while globule leucocyte inclusions were red in colour (Figs, 19 and 21).

DISCUSSION

The process by which plasma cells change into Russell body -containing cells after antigenic stimulation is described by Bessis (1961), from observations with the electron microscope. It is apparent from this investigation that a large number of small Russell bodies make a simultaneous appearance in the cytoplasm. This is in accordance with the results of the present investigation. The releasing of large inclusions found in the most mature Russell body cells, although not observed during the present study, may result in the extracellular Russell bodies observed in different organs in a variety of pathological conditions, so that Russell bodies are sometimes described as being intracellular (Apitz, 1937), and sometimes extracellular (Yoshida et al., 1964).

In contrast to the Russell body cells, globule leucocytes

were of quite uniform morphology. Their structure conformed very closely to the type of Russell body cell occurring at a relatively early stage of Russell body development. This observation can be explained on the basis that while the development of the Russell body cell through various stages is occurring at a single site within a lymphoid organ, the globule leucocyte matured to its observed stage of development outside the organ in which it was being observed; this is based on the assumption that the globule leucocyte does not have a local origin. If the globule leucocyte is a migratory cell this fact would account for the change in shape of cells between the lamina propria and the epithelium. Russell body cells are known to be motile, from observations made on living cells (Thiery, 1958). However, the antigenic stimulus produced by intravenous injections of P. vulgaris vaccine is presumably not directional like that of adult N. brasiliensis, i.e. is not restricted to one area (the gut lumen) towards which the cells might migrate. Consequently Russell body cells might be expected to undergo their complete development in the one area without undergoing migration, thus enabling all stages of Russell body formation to be observed.

In contrast, the local antigenic stimulus of N. brasiliensis, situated in the lumen of the gut, would appear to be directional. Consequently, cells migrating towards it would tend, at any one level, to be at the same stage of development. The intense immune response to N. brasiliensis may result in their rapid migration

into the lumen of the small intestine, and preclude any further development. Observations reported in Section III (6) also support the theory that the globule leucocyte is a migratory cell.

The significance of the different colouration of Russell bodies and globule leucocyte inclusions when viewed under phase contrast is difficult to assess. It appears from observations reported in Section III (9) that certain globule leucocytes do not exhibit this difference under phase (Fig. 38), and these findings are discussed in the appropriate section.

It is concluded that while the majority of Russell body cells are easily distinguishable from globule leucocytes on a morphological basis, certain early forms of the cell cannot be readily distinguished. Objections to a relationship between globule leucocytes and Russell body cells based upon morphological criteria do not therefore appear to be valid.

Part 6 : Ultrastructure of globule leucocytes in the mucosa of the small intestine of the rat, produced in response to Nippostrongylus brasiliensis infection.

INTRODUCTION

Weill (1919, 1920) has described, in a number of species, the morphology of the globule leucocyte under the light microscope. It is a large cell with a nucleus similar in appearance to that of a lymphocyte or plasma cell. The extensive cytoplasm is refractory to routine staining techniques, but contains a considerable number of spherical eosinophilic granules which make identification relatively simple. The ultrastructure of fowl globule leucocytes has been studied by Toner (1965), who showed that their inclusions are homogeneous granules, some of which contain vacuoles. More recently Carr (1966) has examined the structure of apparently crystalline inclusions in globule leucocytes from the mucosa of the large intestine of the mouse, and has tentatively suggested that they might be protein in nature.

Kent (1952) has described a series of transitional stages between lymphocytes and globule leucocytes in the intestinal mucosa. Toner (1965) has supported a lymphocytic origin on the basis of ultrastructural observations, placing particular emphasis on the poorly developed rough endoplasmic reticulum of the globule leucocyte.

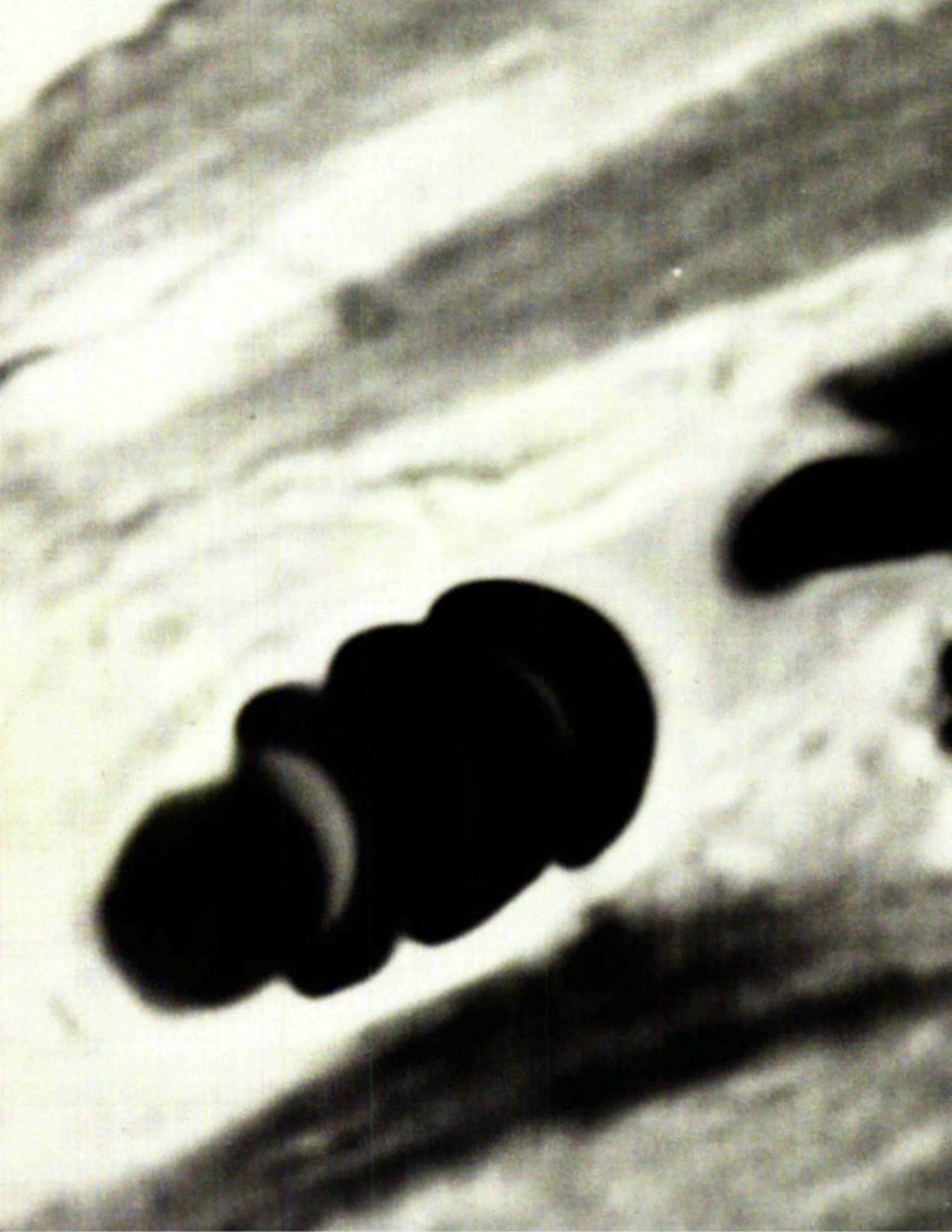


Fig. 22

A small blood vessel in the muscularis externa from the jejunum of a rat infected with N. brasiliensis, showing a globule leucocyte situated in a perivascular position.

Methacrylate section stained with amido black.

(mag : 3000 x)

On the other hand, Kirkman (1950) has suggested a close relationship between the globule leucocyte and the Russell body cell, thus implying a plasma cell origin. In addition to these morphological observations, a relationship has been shown to exist between the globule leucocyte and the immune response which occurs in rats infected with the small intestine nematode N. brasiliensis (Section III (2 and 4)).

The examination of globule leucocytes with the electron microscope is undertaken with particular reference to the role of this cell in the immune response and to the possibility of a relationship to the lymphocyte, plasma cell or mast cell. In order to obtain maximum concentrations of globule leucocytes in the tissues to be examined, all material was obtained on a day of maximal cell response to a challenge infection, from the small intestine worm site of rats previously immunised by a single infection of N. brasiliensis larvae.

MATERIALS AND METHODS

Worm-free adult hooded Lister rats, average weight 120 g, were injected subcutaneously with 4000 infective larvae of N. brasiliensis. On the twentieth day of the infection it was repeated and samples were collected 10 days later. Rats were anaesthetised with trichloroethylene and tissues obtained from the jejunum worm site, which was located on the gauge described previously (Fig. 9). Tissues

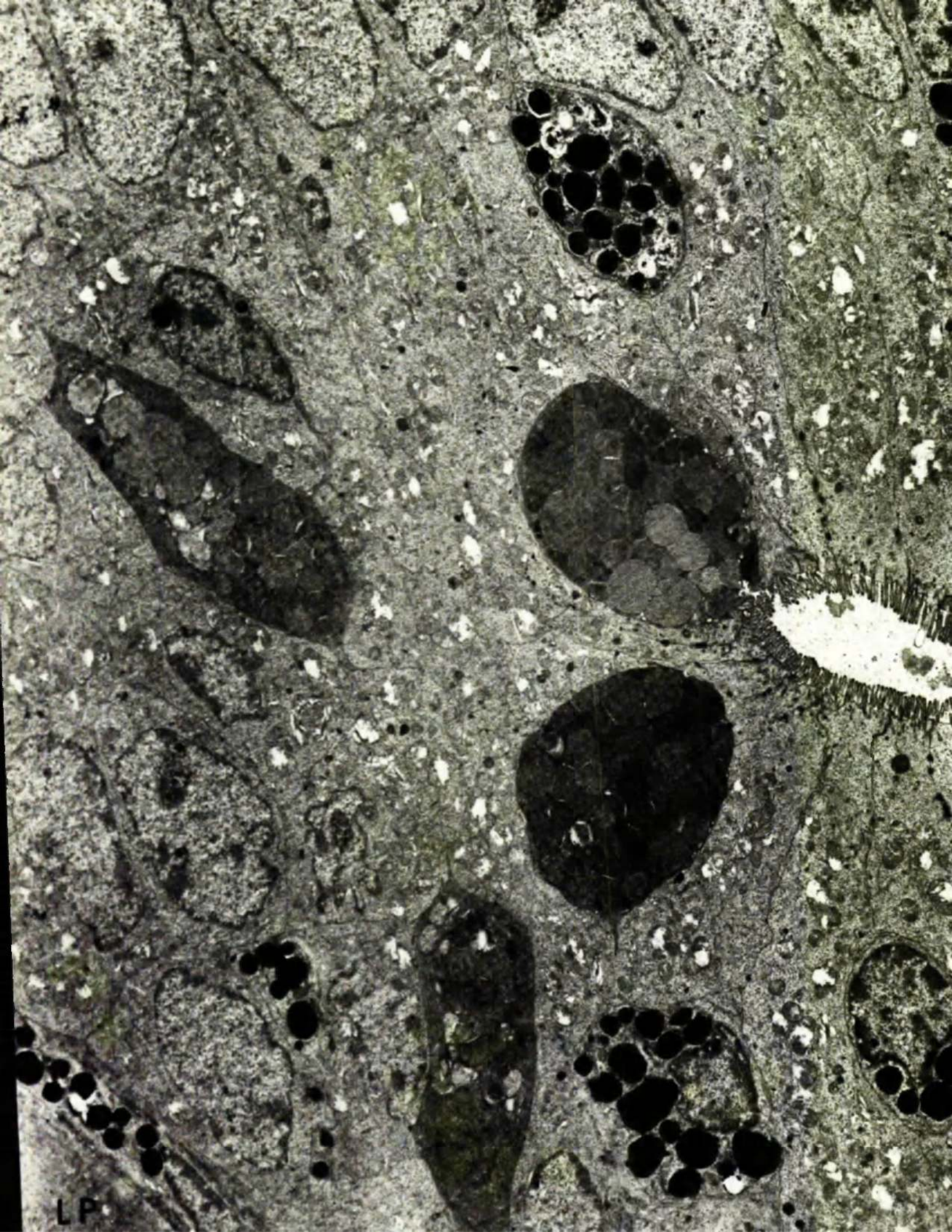

LP

Fig. 23

Low power electron micrograph of the columnar epithelium at crypt level, from the jejunum of a rat harbouring N. brasiliensis. The lumen of the crypt, lined with microvilli, is situated to the right and a number of goblet cells are present. Six globule leucocytes are present between the columnar epithelial cells , and a seventh, visible in the bottom left hand corner, is situated in the adjacent lamina propria (L P).

(mag : 5200 x)

were fixed in chilled 1% buffered isotonic osmic acid pH 7.4 (Zetterqvist, 1956) or in 5% buffered glutaraldehyde pH 7.4 (Sabatini et al., 1964). They were dehydrated in ascending grades of ethanol and cleared in two changes of propylene oxide. The araldite embedding method of Luft (1961) was used. Silver-grey sections were cut on an LKB microtome and mounted on uncoated or carbon/collodion -coated grids. They were stained in uranyl acetate in 50% ethanol and screened on a Phillips EM 200 electron microscope at 60 kv. Sections for light microscopy were fixed in buffered neutral formalin, embedded in methacrylate, sectioned at 1μ and stained with amido black.

RESULTS

In 1μ sections of parasitised material globule leucocytes were readily identifiable as large cells with unstained cytoplasm, containing the characteristic clock face-type of nucleus and cytoplasmic inclusions which stained intensely with amido black. These cells were identical to those previously described and illustrated by Taliaferro and Sarles (1939) in rats infected with N. brasiliensis. They were present in very high concentrations in the mucosa of the proximal jejunum, and were also frequently observed in a perivascular position in both the submucosa and the external muscular coat (Fig. 22). In the mucosa they were about equally distributed between the lamina propria and epithelium of the crypts, while occasional cells

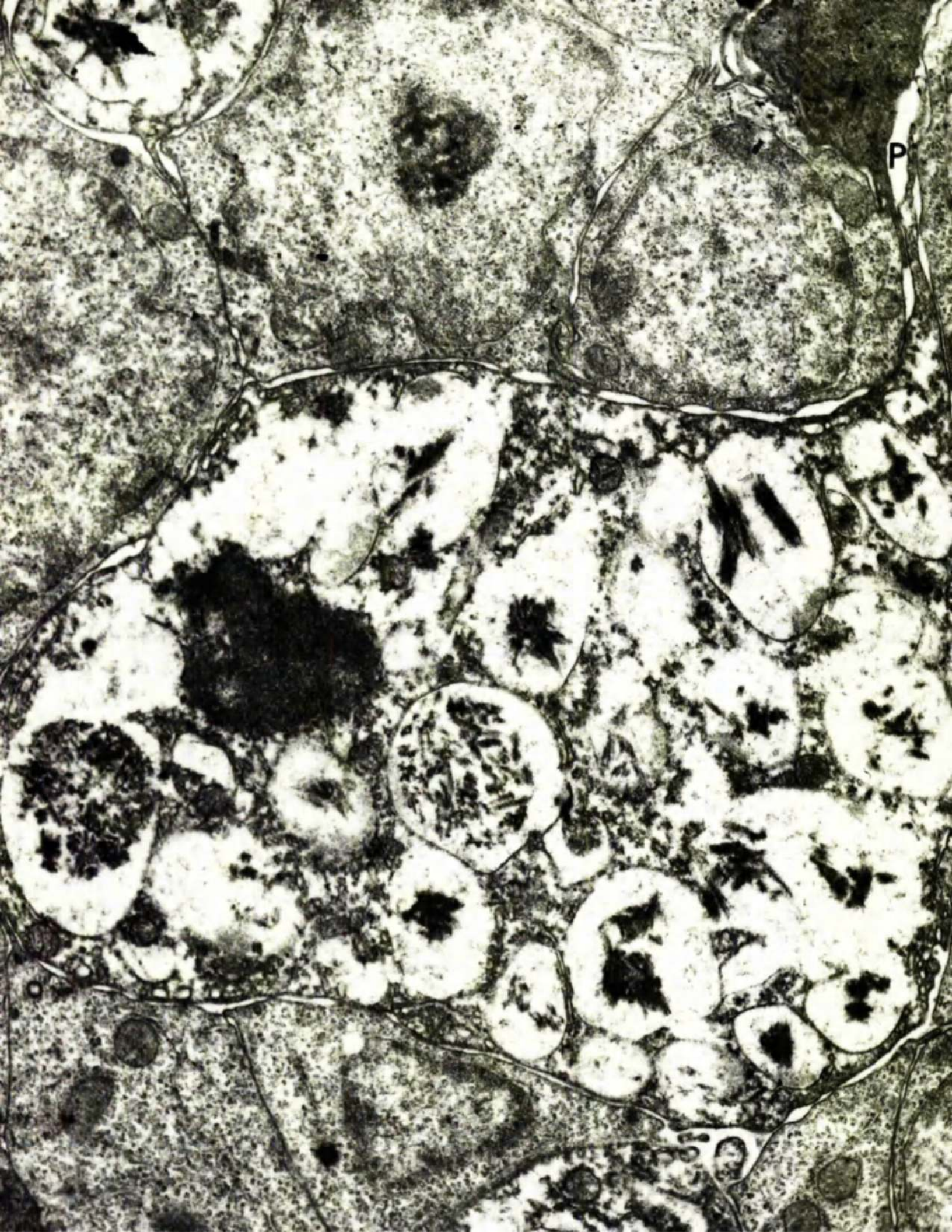

P

Fig. 24

Intraepithelial globule leucocyte showing the variable width of the intercellular space and illustrating at the top right a long cytoplasmic process (P) interdigitating between adjacent epithelial cells.

(mag : 17400 x)

were present in the villus core and epithelium. Some variation in the general form of the cells was observed, depending upon their location in the mucosa. Thus, globule leucocytes in the lamina propria were often elongated in the direction of the long axis of the crypt, while those in an intraepithelial position tended to be more spherical (Fig. 23).

Observations with the electron microscope showed that intraepithelial globule leucocytes were separated from neighbouring cells by an intercellular space of variable dimensions. Small irregular protrusions of the cytoplasm were sometimes present in this space (Figs. 24 and 26), while occasional longer processes penetrated between the surrounding epithelial cells (Fig. 24). The nucleus of the globule leucocyte was somewhat variable in shape, ranging from large and approximately spherical to compressed and irregular in outline. In the latter type marked indentation of the nucleus was frequently observed (Fig. 26). The Golgi complex was situated near the nucleus and was visible as a cluster of vacuoles, vesicles and flattened sacs (Fig. 26). Rough endoplasmic reticulum was abundant throughout the cytoplasm of globule leucocytes containing fewer inclusions, but there was less evidence of it in cells densely packed with these bodies. Polysomes were quite numerous in the cytoplasm (Fig. 28) and a few spherical or short rod-shaped mitochondria containing loosely packed cristae were present in each cell (Fig. 28). In many of the globule leucocytes rows of vesicles, thought to be

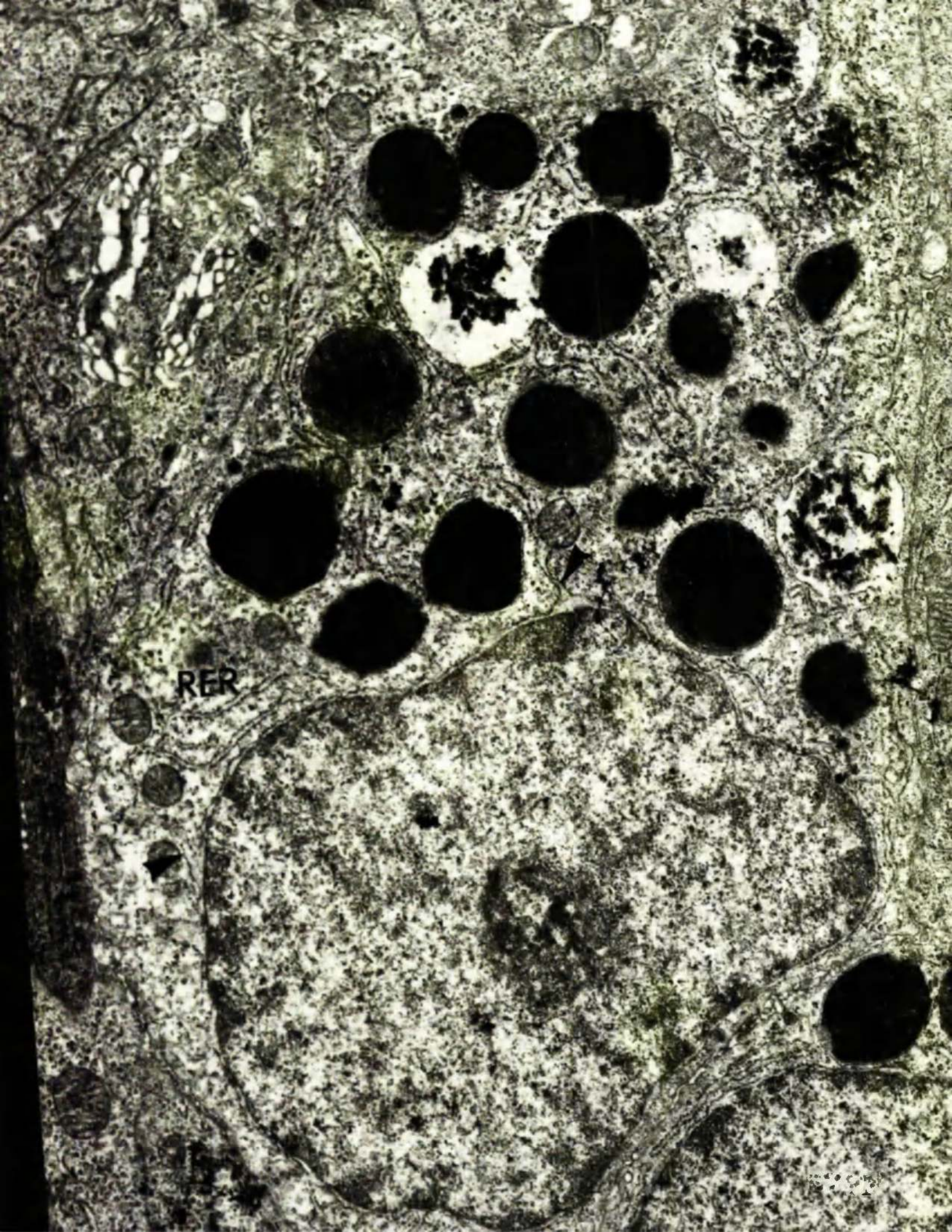

RER

Fig. 25

Crystalline, homogeneous and intermediate types of inclusion are illustrated. These are surrounded by a clearly defined membrane which may be continuous with the rough endoplasmic reticulum (RER). This is extensive, and continuity with the outer nuclear membrane is visible at a number of points (arrows). In the nucleus there is a central nucleolus and part of the chromatin is arranged in clumps around the periphery. Groups of ribosomes are plentiful in the cytoplasm.

(mag : 14600 x)

pinocytotic, were seen adjacent to the plasma membrane (Fig. 27).

The most characteristic feature of the globule leucocyte was the presence of large numbers of spherical inclusions in the cytoplasm. These varied in structure from homogeneous , dense membrane -bound structures to vesicles containing rod-shaped crystalloid bodies, which appeared very similar to the crystalline form of Russell bodies and certain other crystalline inclusions of plasma cells (Thiery, 1958, 1960) (Fig. 25). The number of these apparently crystalline structures was roughly inversely proportional to their size, and at medium magnification they appeared longitudinally striated ; consisting of electron-dense lines embedded in or bordering a less dense substance (Fig. 24), and at higher magnifications regular longitudinal striations were visible (Fig. 29). Some globule leucocytes contained one or other type of inclusion, while in other cells both types were present, together with intermediate forms containing both the homogeneous matrix and crystalline rods in differing proportions.

DISCUSSION

During the courseof the present investigation globule leucocytes were observed in considerable numbers in the external muscle layer and submucosa in a perivascular position, indicating a source outside

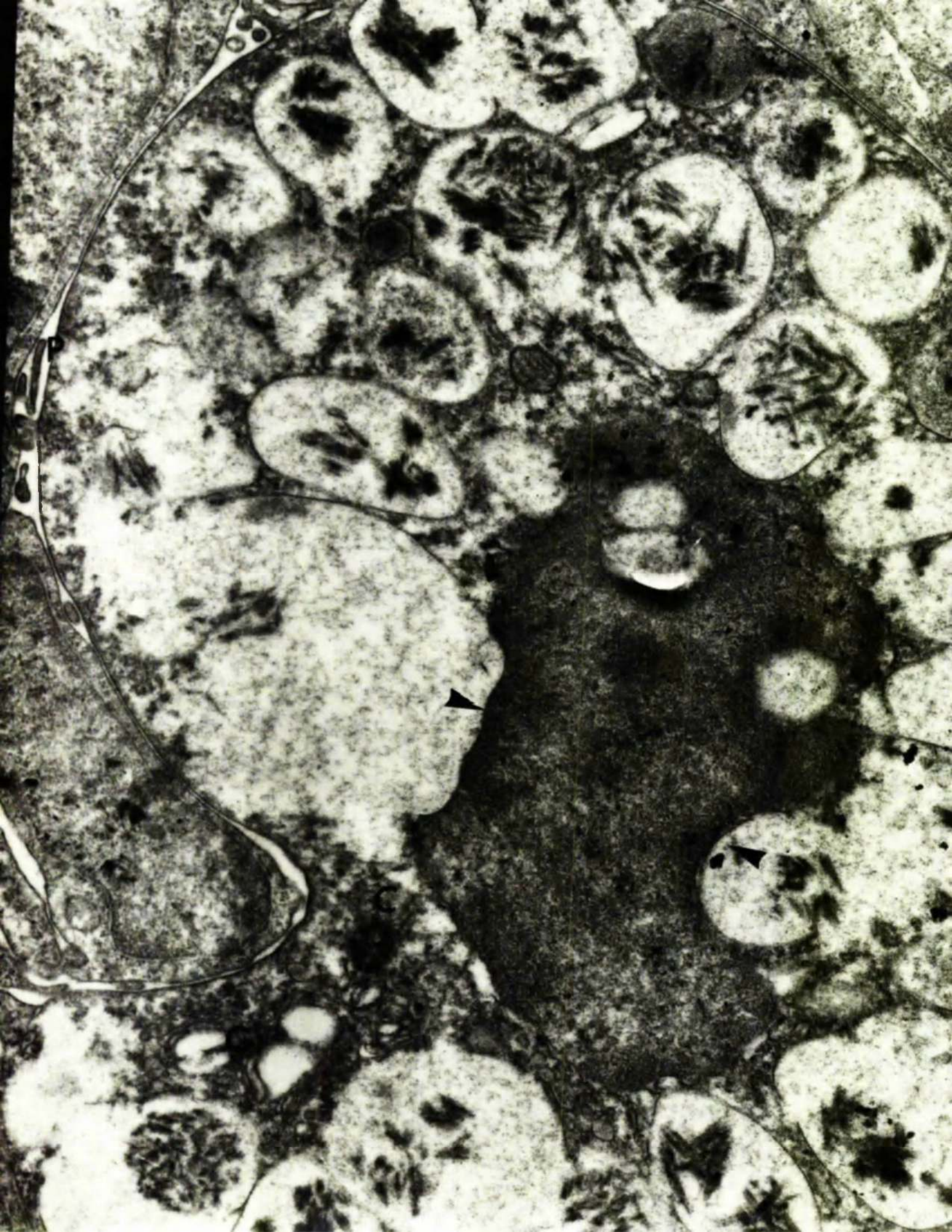

P

Fig.26

Nucleus of an intraepithelial globule leucocyte showing marked indentation by adjacent vesicles (arrows). Three apparent vacuoles within the nucleus may be portions of such vesicles since they are of similar electron density. The Golgi complex (G) and centrosome (C) are visible at the bottom left. Small irregular cytoplasmic processes (P) project into the intercellular space around the periphery of the cell.

(mag : 19700 x)

the small intestine for at least a proportion of intraepithelial globule leucocytes. The local origin of globule leucocytes from lymphocytes, through an observable series of transitions (Kent, 1952), could not be confirmed. A lymphocytic origin is also contra-indicated because intraepithelial globule leucocytes in the small intestines of rodents and man, as described by Andrew and Andrew (1945), Andrew and Collings (1946), Andrew and Sosa (1947) and Andrew (1965) in studies with the light microscope and electron microscope, differ markedly from the globule leucocyte in a number of respects. Thus, according to the observations of these authors lymphocytes in the lamina propria differ morphologically from those in the epithelium by a marked reduction of rough endoplasmic reticulum and mitochondria in the latter situation. Secondly, lymphocytes increase in size upon entering the epithelium. Thirdly, lymphocytes degenerate in situ, frequently within the cytoplasm of the epithelial cells. No similar phenomena were observed in connection with globule leucocytes. Finally, the constant presence of granules in globule leucocytes contrasts markedly with the completely agranular lymphocytes invariably observed in the same location by these authors.

The question of the possibility of a relationship between the globule leucocyte and the mast cell has not yet been considered. Jarrett (1965) has stated that observations on globule leucocytes during experimental infection of sheep with Ostertagia spp. have indicated certain similarities between the two cells. It is difficult

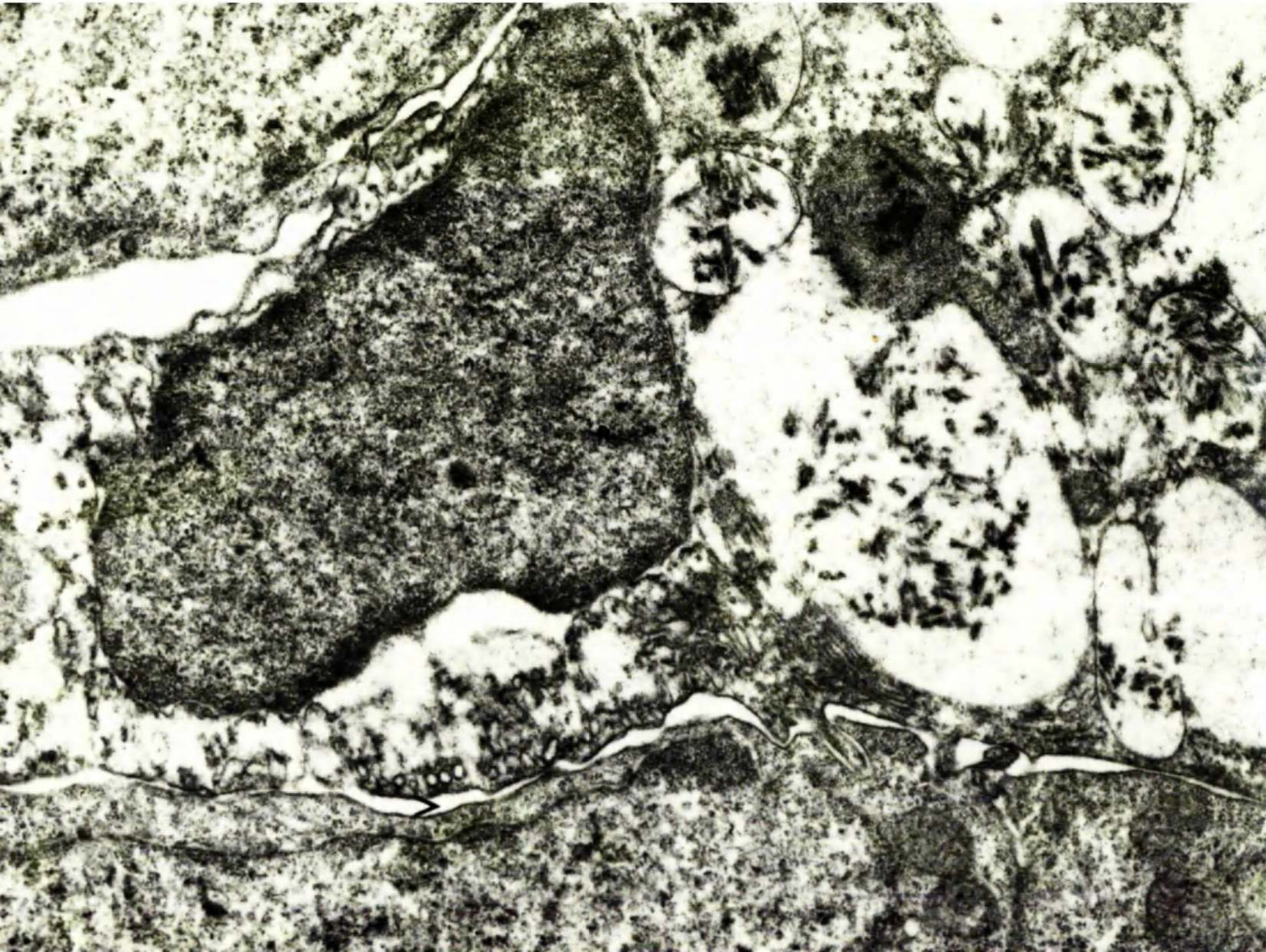


Fig. 27

A row of pinocytotic vesicles (V) are visible adjacent to the cell membrane on the left side of the globule leucocyte. A number of the crystals present within the inclusions have the double-line appearance which was frequently observed at medium power magnifications.

(mag : 22900 x)

to define precisely the ultrastructural characteristics of the mast cell because of structural variations which appear to exist when the findings of different authors are compared (Köksal, 1953 : Asboe-Hansen, 1954 : Bloom et al., 1955 : Stoeckenius, 1956 : Rogers, 1956 : Smith and Lewis, 1957 : Gusek, 1960 : Policard et al., 1960 : Thiery, 1963). However, the mature granule of the mast cell, as frequently illustrated, has a very characteristic appearance which bears no resemblance to the globule leucocyte granule as seen in the present study or reported by Toner (1965) and Carr (1966). This internal structure is illustrated by Gusek (1960) and Thiery (1963), and consists of granular membranes arranged in whorls or scroll formations. Such formations are in contrast to the apparently crystalline or homogeneous forms of inclusion observed in globule leucocytes.

The significance of perivascular channels has been discussed by Sainte-Marie (1964), who suggested that plasma cells migrate along them to sites of antigenic stimulus, where they release their antibodies. In this connection, it has been shown that plasma cells containing Russell bodies are extremely motile (Thiery, 1958). The apparent similarity of the globule leucocyte migration through the various layers of the intestine, where they were observed in a perivascular position, suggests a relationship to the plasma cell rather than to the lymphocyte, since the latter cell is not associated with migration in a perivascular position.

Most of the cells observed were concentrated in the lamina

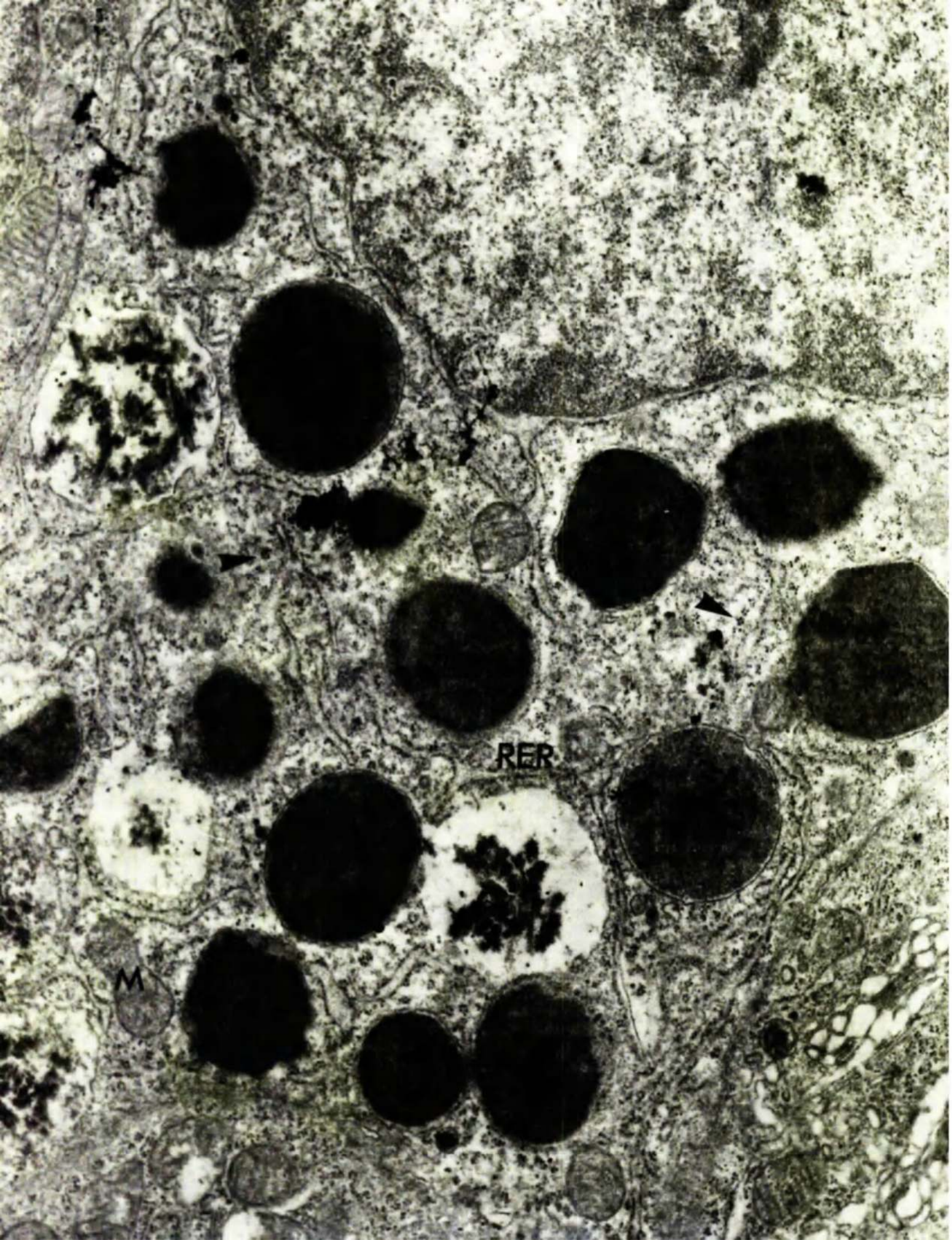

RER
M

Fig. 28

Detail from Fig. 25. The homogeneous inclusions have a fine granular structure. The darker areas within these inclusions (*) may represent areas of crystal formation, resulting in the development of the intermediate type which can be seen to the right of the lower crystalline inclusion. The presence of polysomes can be distinguished (arrows) in addition to membrane-associated (RER) ribosomes. The mitochondria (M) are small, spherical or short rod-shaped structures, and are not numerous.

(mag : 29700 x)

propria and epithelium of the crypts, while only a few were seen in the cores of the villi, indicating that the majority of them migrated from the lamina propria into the epithelium at the crypt level. Because of the constant movement of epithelial cells over the surface of crypts and villi (Leblond and Stevens, 1948 : Leblond and Messier, 1958 : Leblond, 1965) the almost total absence of globule leucocytes from the epithelium of the villi suggests that the cells actively migrated into the lumen of the intestine after remaining in an intraepithelial position for a few hours at the most. There was no evidence of degeneration in situ.

The morphological similarity of the globule leucocyte inclusion at a particular phase of Russell body formation in plasma cells (Section III (5)) suggests a similar development for the two types of cell. The homogeneous inclusions, and those containing crystalline material were both similar to the respective types of Russell body, while the crystals showed striations at high magnification which appeared to resemble that of paracrystalline structures in plasma cells described by Wellensiek (1957), Thiery (1960) and Movat and Fernando (1962).

Indentation of the nucleus by vesicles was sometimes observed (Fig. 26). This phenomenon is similar to that observed when antiserum was added to fibroblasts in tissue culture (Iatta, 1959). Part of the reaction consisted of the development of " large spherical vesicles lying in contact with many nuclei and even indenting them", together

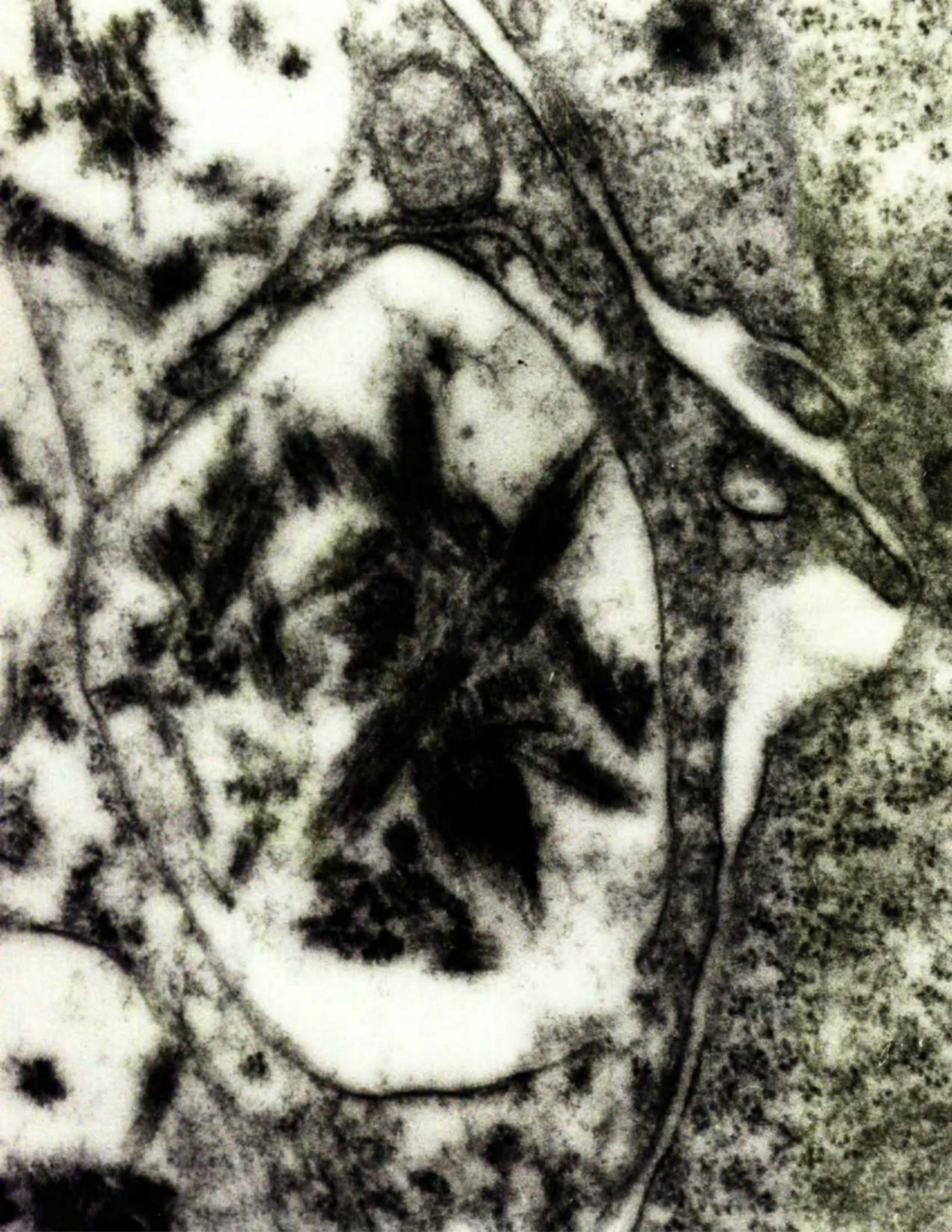


Fig. 29

A crystalline inclusion from a globule leucocyte showing crystals contained within an otherwise almost empty vesicle. They are irregularly -shaped fragments, but are clearly and regularly longitudinally striated.

(mag : 69800 x)

with a reduction in nuclear size. Examination of such vesicles with the electron microscope showed that their limiting membranes were derived from the outer nuclear membrane and were continuous with the endoplasmic reticulum (Latta, 1959). It is therefore possible that nuclear indentation observed in certain globule leucocytes, which also was associated with shrinkage of the nucleus, may have been the result of interaction between antibody and antigen.

Wellensiek and Coons (1964) have reported the uptake of antigen by pinocytosis, while Movat and Fernando (1962) state that the finger-like processes of plasma cells, mast cells and macrophages are regarded as being concerned with the uptake of antigen by pinocytosis. Such cytoplasmic processes were a constant feature of globule leucocytes and may have been related to the production of pinocytotic vesicles. While it is therefore possible that the pinocytotic vesicles observed were involved in the uptake of M. brasiliensis antigens, there is a need for further experimental investigation of this point.

A number of morphological and functional similarities therefore seem to exist between Russell body cells and globule leucocytes, which suggests a similar origin. On the basis of these observations it appears reasonable to postulate that the globule leucocytes observed in the present investigation were similar in origin and function to Russell body cells, and that they were migrating to the site of antigenic stimulus via the perivascular channels. They then traverse the lamina propria of the intestine to the characteristic intraepithelial

position where they remain for a fairly short period, after which the cells, together with the contents of the globules which may be antibody, are finally released into the lumen of the intestine where they may be effective against the worm population.

Part 7 : Mast cell and globule leucocyte response to *Nippostrongylus brasiliensis* infection and to induced anaphylaxis.

INTRODUCTION

Infection of rats with a suitable dose of larvae of *N. brasiliensis* leads to a self-cure which commences about day 10 of a primary infection. The expulsion of worms at the time of self-cure has been shown to be an immune phenomenon (Mulligan *et al.*, 1965). An anaphylactic reaction is manifest in the small intestine of immune rats when injected with antigen prepared from whole adult worms, and there is some evidence that a similar anaphylaxis may also occur during self-cure (Urquhart *et al.*, 1965). It has been suggested that self-cure is the result of increased capillary permeability and associated antibody release into the gut, since heterologous intestinal anaphylaxis alone cannot initiate self-cure, but accelerates the action of hyperimmune serum (Barth *et al.*, 1966).

Mast cells play an important role in anaphylaxis, during which they degranulate and probably release histamine and other active substances (Selye, 1965). Degranulation is detectable histologically and should therefore be observable in the small intestines of rats which are immune to *N. brasiliensis* and which are shocked by the intravenous injection of whole worm antigen. Unfortunately, the

precise identification of mast cells in the small intestine of the rat is complicated by reports that mast cells in this area differ morphologically from those in other tissues (Maximow, 1906 : Hunt and Hunt, 1956 : Taliaferro and Sarles, 1939). It is even possible that these cells are not true mast cells, since true mast cells are reported to be practically absent from the small intestine of the rat (Mota et al., 1956b). There is in fact an obvious similarity between some of the descriptions of morphologically atypical mast cells and the globule leucocyte. Kent et al.(1956) demonstrated that whole body X-irradiation causes a virtual disappearance of globule leucocytes from the small intestine of the rat, while Eisen et al.(1956) showed that the histamine level in the gastrointestinal tract of the rat is also affected by this procedure. While this histamine might be bound to cells other than globule leucocytes, it is possible that these two findings are linked. Consequently, no clear differentiation can at present be made between certain atypical mast cells reported in the literature and globule leucocytes.

As previously shown (Section III (2 and 4)) there is a relationship between the appearance of globule leucocytes in the jejunum and the onset of self-cure in rats infected with N. brasiliensis. This may indicate that the globule leucocyte has some functional role to play in self-cure. The present experiment, therefore, seeks to elucidate the relationship of mast cells, so-called atypical mast cells and globule leucocytes to one feature of self-cure, namely

anaphylaxis. A necessary prerequisite for such an investigation was the identification of these three types of cell and some evaluation of their relationship to each other.

In an attempt to obtain this information small intestines from worm-free and N. brasiliensis-infected rats were examined histologically after staining by a number of techniques known to give characteristic, though not necessarily specific reactions for mast cells and globule leucocytes. Secondly, immune rats were subjected to anaphylaxis induced by whole worm antigen, and the small intestines were examined histologically to determine the effects of the intestinal anaphylaxis upon mast cell and globule leucocyte granulation.

MATERIALS AND METHODS

Adult hooded Lister female rats weighing between 80-130 g were used. Tissues were taken, in infected and non-infected animals alike, from the position in the jejunum where the worms are found in highest concentrations (Brambell, 1965a). All tissues were fixed in buffered neutral formalin, embedded in methacrylate and sectioned at 2μ. Sections were stained with Bismark brown, toluidine blue, Giemsa tissue stain (Lillie, 1954) and chrysoidin (Harada, 1957) for mast cells, and with amido black (Puchtler and Leblond, 1958) for globule leucocytes.

Whole worm antigen was prepared from adult N. brasiliensis

GROUP	DAY 0	DAY 20	DAY 30
1	-	killed	
2	3500 larvae	killed	
3	3500 larvae	3500 larvae	killed
4	4000 larvae	6000 larvae	I.V. antigen killed
5	4000 larvae	6000 larvae	I.V. saline killed

Table 7

Experimental infections with N. brasiliensis given to each group of rats before histological examination of the jejunum. Group 4 received intravenous whole worm antigen to induce anaphylaxis before killing.

concentrated in buffered N. saline at 1000/ml and homogenised. This suspension was then transferred to an ice-cooled ultrasonic disintegrator and given 6 separate bursts of 1 minute, after which it was centrifuged at 2000 r.p.m. for 30 minutes. The resulting supernatant whole worm antigen was used on the day of preparation.

The rats were divided into five groups and the experimental procedures applied to each were as follows (Table 7) :-

Group 1 consisted of non-infected rats.

Group 2 were killed on day 20 of a primary infection with 3500 N. brasiliensis larvae, since it has been reported that at this time mast cells are present in high concentrations in the small intestine (Wells, 1962).

Group 3 were initially infected with 3500 larvae and then reinfected with a futher 3500 larvae on day 20. They were killed 10 days later on day 30, at a time when globule leucocytes are present in high concentrations.

Group 4 received an initial infection of 4000 larvae and a second infection of 6000 larvae on day 20. 500 worm-equivalents of whole worm antigen were injected intravenously under trichloroethylene anaesthesia 10 days later on day 30. The six animals in this group were killed in pairs at three different periods- ½ hour, 4 hours and 24 hours after receiving the injection of antigen.

Group 5 were treated as Group 4 but received only buffered saline on day 30.

GROUP	MAST CELLS	ATYPICAL MAST CELLS	GLOBULE LEUCOCYTES	DEGRANULATION
1	-	-	-	
2	-	-	+	
3	-	-	+	
4	-	-	+	-
5	-	-	+	-

Table 8

Occurrence of cells in the jejunal mucosa , and effect of anaphylaxis upon globule leucocyte granules.

RESULTS

Groups 1-3

Mast cells were absent from the mucosa of all the sections of jejunum examined (Table 8). However, morphologically typical mast cells were present in the adjacent mesentery, and these were used to check morphology and staining reactions (Fig. 30). The granules of these cells reacted very distinctly to all the stains used except amido black (Table 9). No atypical forms of mast cell were observed in the mucosa.

Globule leucocytes were absent in Group 1, but present in high concentrations in both lamina propria and epithelium of rats in Groups 2 and 3 (Table 8). The cells stained distinctly with amido black and Giemsa, but not with any of the other stains listed (Table 9). No morphological irregularities, or intermediate forms between mast cells and globule leucocytes were present. Mast cells and globule leucocytes were therefore distinguishable on the basis of their staining reactions, tissue distribution, and presence or absence in the various groups.

Groups 4-5

Group 4 rats killed ½ hour after the injection of whole worm antigen showed macroscopic gut changes similar to those previously outlined (Urquhart *et al.*, 1965), and indicative of anaphylaxis, namely hyperaemia with an increased amount and fluidity of mucus.

	CHRYSOIDIN	BISMARK BROWN	TOLUIDINE BLUE	GIEMSA	AMIDO BLACK
MAST CELL	+	+	metachromatic	+	-
GLOBULE LEUCOCYTE	-	-	not metachromatic	+	+

Table 9

Staining reactions of mast cell and globule leucocyte granules.

These changes are similar to those observed during N. brasiliensis infections (Symons, 1957). Group 5 rats, and rats in Group 4 killed at 4 and 24 hours showed no gross changes. Microscopic examination of the jejunum of both groups showed a complete absence of mast cells , including atypical forms, from the mucosa and a high concentration of intact globule leucocytes. Anaphylaxis had failed to disrupt the globule leucocyte granules (Fig. 31) or to produce any visible change in these cells.

DISCUSSION

The absence of mast cells from the jejunal mucosa of the rats examined, which included normal as well as parasitised animals, confirms the observations of Mota et al. (1956b). The presence of intact mast cells in adjacent mesentery indicates that lysis of mast cell granules did not take place accidentally during fixation or processing. The absence of mast cells from the mucosa of rats in Group 1 is of particular significance in this connection, since any mast cells in Groups 2-5 may have undergone degranulation during anaphylaxis associated with N. brasiliensis infection.

Contrary to the present findings, a previous investigation into the mast cell population of the rat jejunum during N. brasiliensis infection has indicated that mast cells are present throughout a primary infection and reach their highest concentration on the

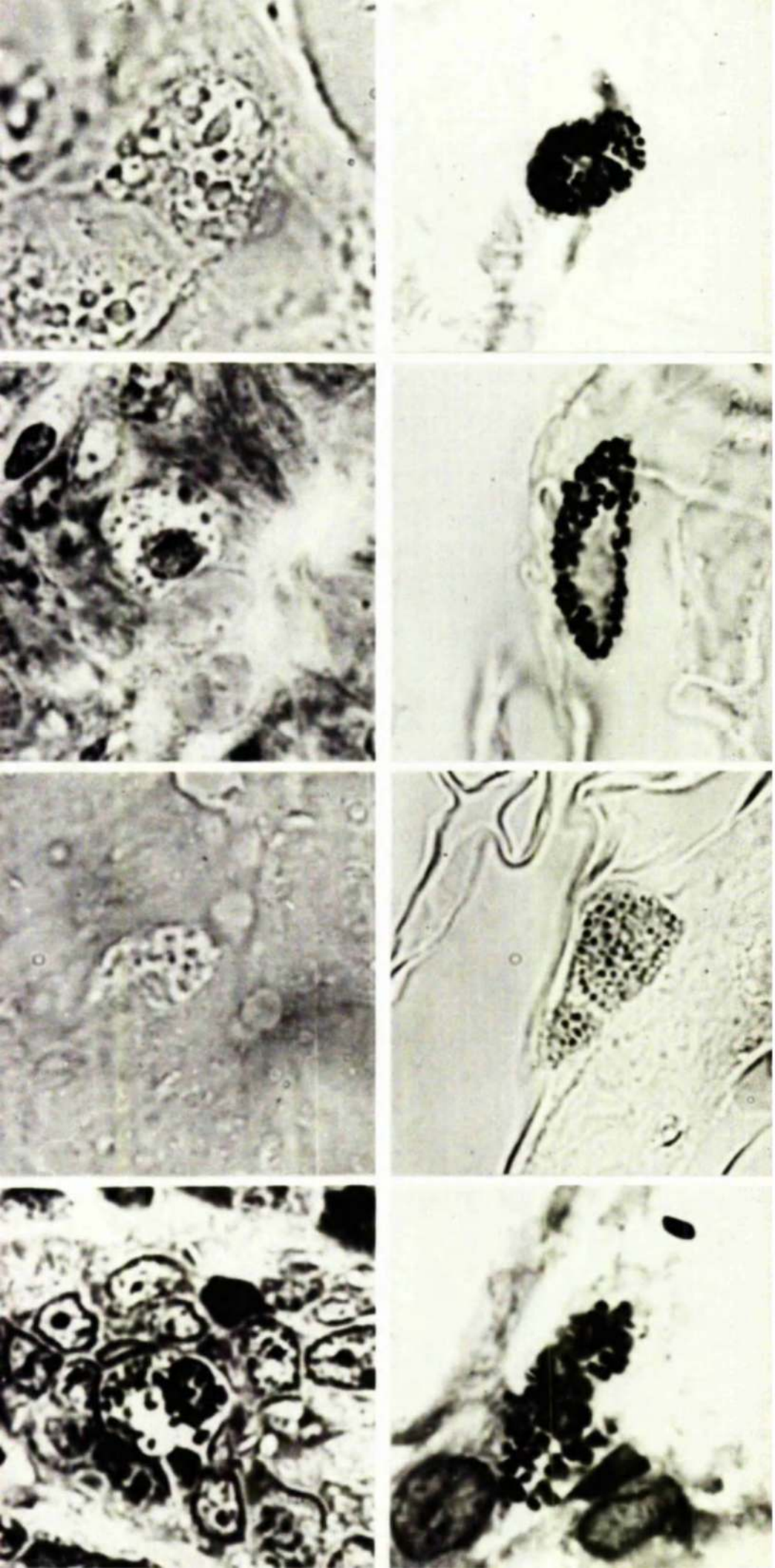


Fig. 30

Series of photographs comparing the staining reactions of globule leucocytes (left) with mast cells (right). Mast cells show a positive reaction to each of the stains which are, from the top, chrysoidin, toluidine blue, Bismark brown and Giemsa tissue stain. Globule leucocytes give a positive reaction with Giemsa, but are negative to the other stains used.

(mag : 1000 x)

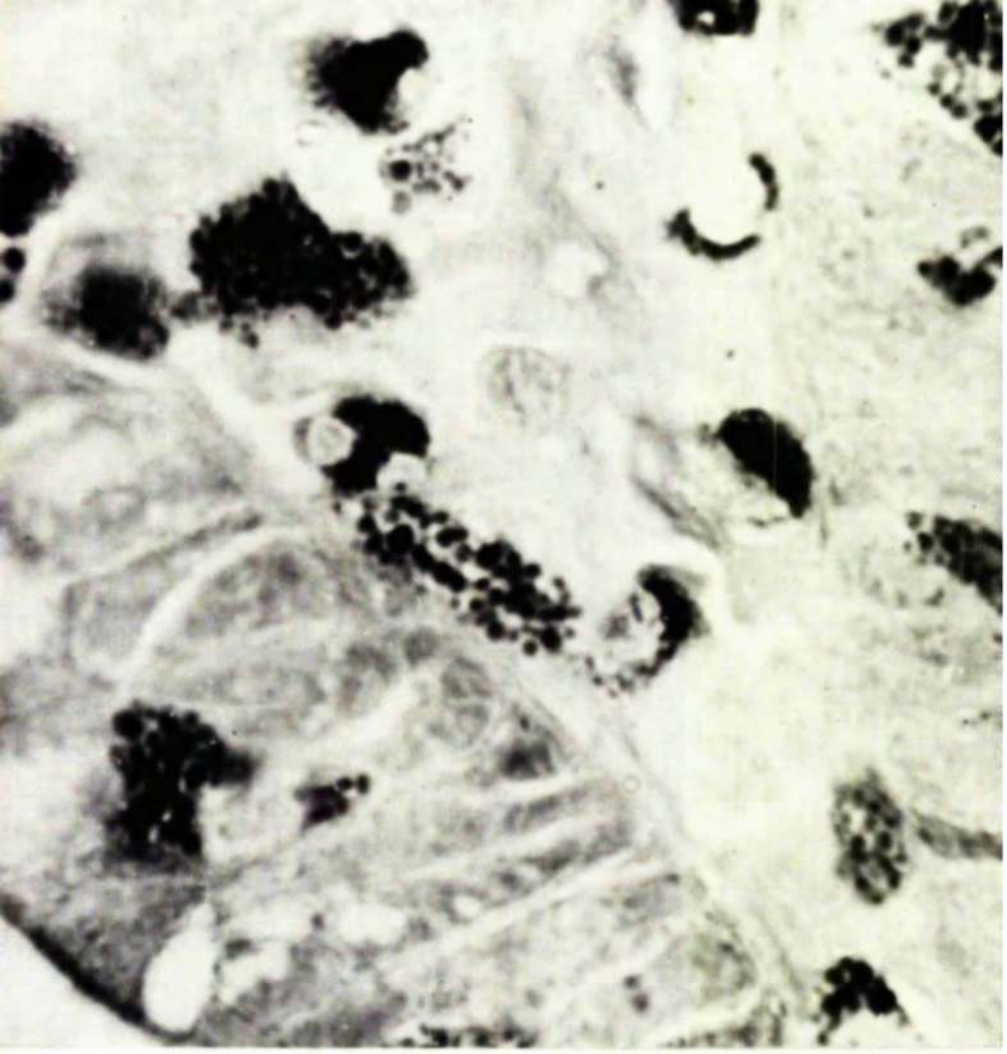


Fig. 31

In the top photograph normal globule leucocytes from a Group 5 control rat are shown. The globule leucocytes in the bottom picture are from a Group 4 animal killed ½ hr. after the induction of anaphylaxis. No disruption of granules has taken place and the cells in the two illustrations are identical in appearance.

Methacrylate section stained with amido black.

(mag : 1300 x)

twenty third day (Wells, 1962). The apparent contradiction may be dependant on the failure of this author to employ stains other than Giemsa, which stains both globule leucocytes and mast cells (Fig. 30).

The absence of mast cells, including atypical forms, in the jejunum of the rats examined is inconsistent with a number of previous reports (Maximow, 1906 : Hunt and Hunt, 1956 : Taliaferro and Sarles, 1939). This may be explained on the basis that the necessary conditions for the presence of atypical mast cells did not exist in the rats examined ; alternatively the atypical mast cells described in this region by previous authors may have been globule leucocytes. There is some evidence to support the latter suggestion. Thus, Taliaferro and Sarles (1939) described and illustrated cells which occurred in the lamina propria of the jejunum during N. brasiliensis infections, which they believed to be atypical mast cells and which they designated connective tissue basophils. It appears from ultrastructural observations (Section III (6)) that these cells are globule leucocytes identical to those in the overlying epithelium. In addition the atypical mast cells described by Maximow (1906) and Sansonow (1900) appear to resemble globule leucocytes very closely. Parasitic infection is one factor stimulating the appearance of large numbers of globule leucocytes in the rat's small intestine. The frequency with which rats harbour intestinal parasites makes it likely that many of the reports of atypical mast cells in the small intestines of rats

refer to globule leucocytes.

Mast cells and atypical mast cells were absent from the small intestines examined and it was not therefore possible to determine the effects of whole worm antigen-induced anaphylaxis on these types of cell. The view that the cells identified as globule leucocytes are not a form of mast cell is reinforced by their failure to degranulate after the injection of whole worm antigen. This suggests that the granules of mast cells and globule leucocytes differ markedly in composition. Together with the differences of staining and distribution already noted, this experimental observation strongly suggests that globule leucocytes and mast cells are not closely related to one another.

The absence of mast cells would suggest that the chemical mediators of anaphylaxis are either from a local non-mast cell source (Mota et al., 1956a) or are extrinsic (Dale, 1950), while the failure of globule leucocytes to degranulate suggests that they are not involved in anaphylaxis. This is in general agreement with previous observations which suggest a relationship to the plasma cell and antibody production.

Part 8 : Histochemical comparison of mast cell and globule leucocyte granules in the rat.

INTRODUCTION

As previously noted, investigations into the cell population of the mucosa of the small intestine of the rat have resulted in reports indicating the presence of mast cells, atypical mast cells and globule leucocytes. The apparently contradictory results of a number of these investigations has suggested that some or all of these cells may be identical or at least closely related to one another. In an attempt to resolve the relationship between these cells, small intestines from worm-free rats and from rats at various stages of N. brasiliensis infection were examined for the presence of atypical and normal mast cells and globule leucocytes, which were identified by suitable staining techniques (Section III (7)), and anaphylaxis was induced in the small intestines of rats which were hyperimmune to N. brasiliensis. The results obtained suggested that mast cells and globule leucocytes were distinct and unrelated cells.

The evidence that globule leucocytes do not degranulate during anaphylaxis cannot be regarded as entirely conclusive, since anaphylaxis may occur under certain conditions without affecting mast cells (Lima, 1966). On this basis, a histochemical investigation of the

globule leucocyte granule has been undertaken which is designed to show whether or not it contains three of the substances associated with the granules of mast cells, namely heparin, 5-HT and histamine.

MATERIALS AND METHODS

Material for histological examination was obtained from adult albino Wistar rats infected with 3000 larvae of N. brasiliensis. The rats were infected again with a similar dose of larvae 20 days later and sacrificed after a further 10 days, at a time when globule leucocytes are present in very high concentrations in the jejunal mucosa. An unopened segment of jejunum approximately 1 cm long was taken from each animal for the examination of globule leucocytes. Mast cells in subcutaneous connective tissue from the back region served as controls. Both tissues were subjected to identical procedures throughout. Two tests each were undertaken for the detection of heparin, 5-HT and histamine.

Heparin

(1) Tissue from each area was fixed in 4% basic lead acetate for 24 hours, followed by 10% neutral formalin for a further 24 hours, embedded in paraffin and cut at 5μ. Sections were stained with 0.1% toluidine blue at pH 4.2 for 1 minute (Gomori, 1952), dehydrated in acetone, cleared in xylol and the granules of both

cells examined for metachromasia.

(2) Tissues were fixed in buffered neutral formalin, embedded in paraffin and sectioned at 5μ. They were stained with periodic acid Schiff (PAS) and the granules examined.

5-HT

(1) Frozen sections were cut at 5μ on a cryostat and fixed for 10 minutes in acid formalin at pH 5.5. A second set of tissues was fixed in buffered neutral formalin, embedded in paraffin and cut at 5μ. Both sets of sections were then examined for gold-yellow fluorescence under ultraviolet light (Rice and Mitchener, 1961) using a Wild fluorescence microscope fitted with an Osram 200 watt mercury vapour lamp and dark field illumination.

(2) Tissues were fixed in calcium formol at pH 6. Frozen sections were cut at 10μ, stained with Schmorl's ferricyanide solution and mounted in glycerine (Coupland and Riley, 1960). The granules of mast cells and globule leucocytes were examined for a positive blue reaction.

Histamine

(1) Tissues were fixed in Reinecke salt solution (Schauer and Werle, 1959) for 24 hours. Frozen sections were cut at 10μ. Unstained sections were mounted in glycerine and examined under dark ground illumination. Sections were also examined for light

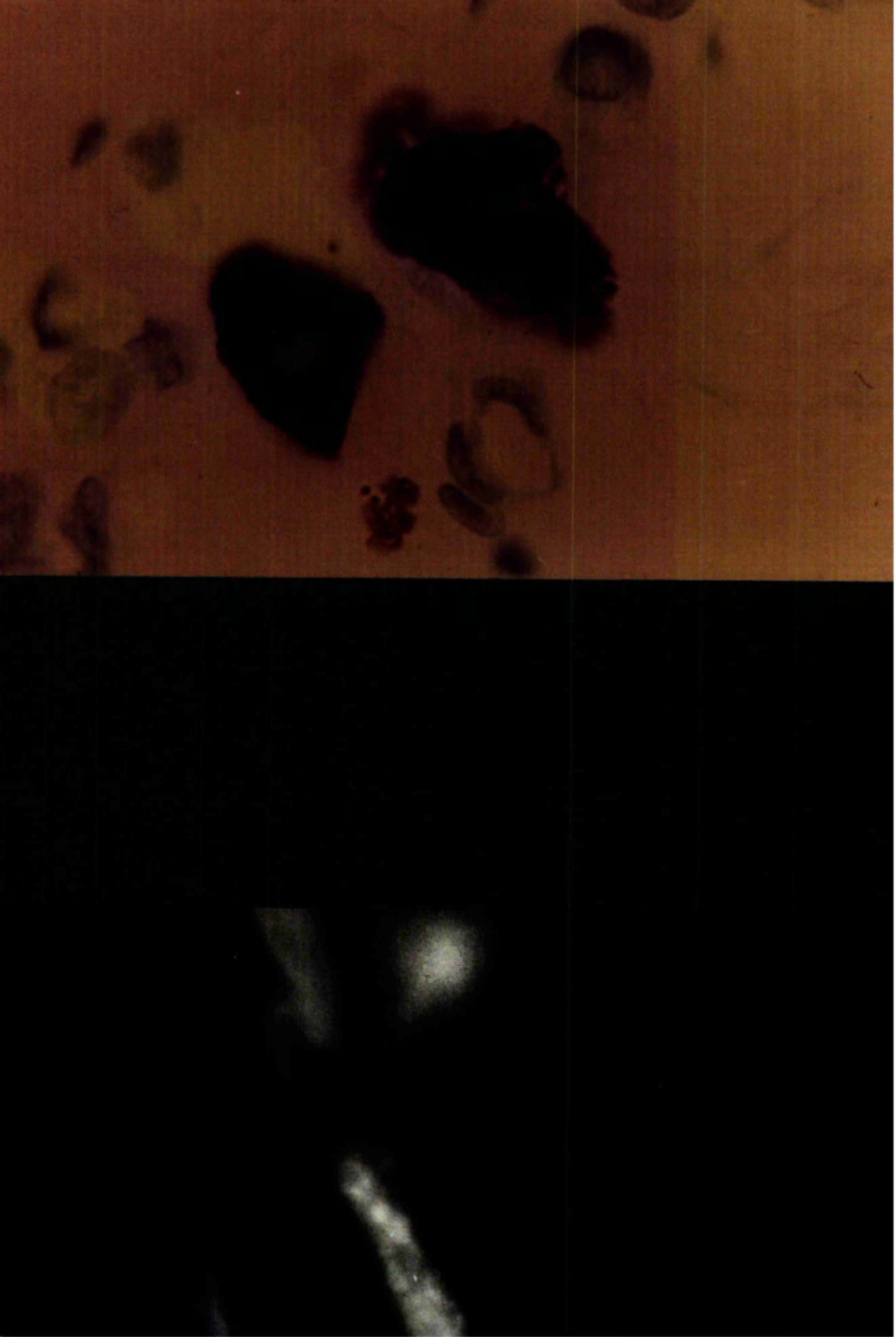


Fig. 32

Mast cell of rat subcutaneous tissue exhibiting metachromasia with toluidine blue at pH 4.2, after fixation in basic lead acetate and buffered neutral formalin.

(mag : 1300 x)

Fig. 33

Mast cell of rat subcutaneous tissue from an acid-formalin fixed preparation exhibiting gold-yellow fluorescence under ultraviolet light.

(mag : 1300 x)

red staining of the granules after diazo coupling (Schauer and Werle, 1959).

(2) Tissues were immersed in isopentane which had been precooled in liquid nitrogen, and were frozen-dried and embedded in an Edwards tissue drier TD 2. They were then cut at 5μ, fixed in formaldehyde vapour, and stained with diazotised parabromoaniline (Lagunoff et al., 1961). The granules were examined for a light orange colour.

RESULTS AND DISCUSSION

Intense metachromasia was given by mast cell granules after staining with toluidine blue (Fig. 32), but globule leucocyte granules failed to react. Although metachromasia is not specific for heparin, it can be assumed that the positive reaction given by mast cell granules is due to heparin, and that the negative result for globule leucocyte granules therefore indicates an absence of this substance. Neither mast cell nor globule leucocyte granules were PAS-positive. Inconsistency in PAS staining of mast cell granules has been noted previously (Compton, 1952) and may be due to the fact that only one form of heparin, the monosulphuric acid, has the necessary two adjacent hydroxyl groups for reaction with periodic acid to occur (Jorpes et al., 1948).

Mast cell granules in control sections viewed unstained in ultraviolet light with the Wild fluorescence microscope exhibited

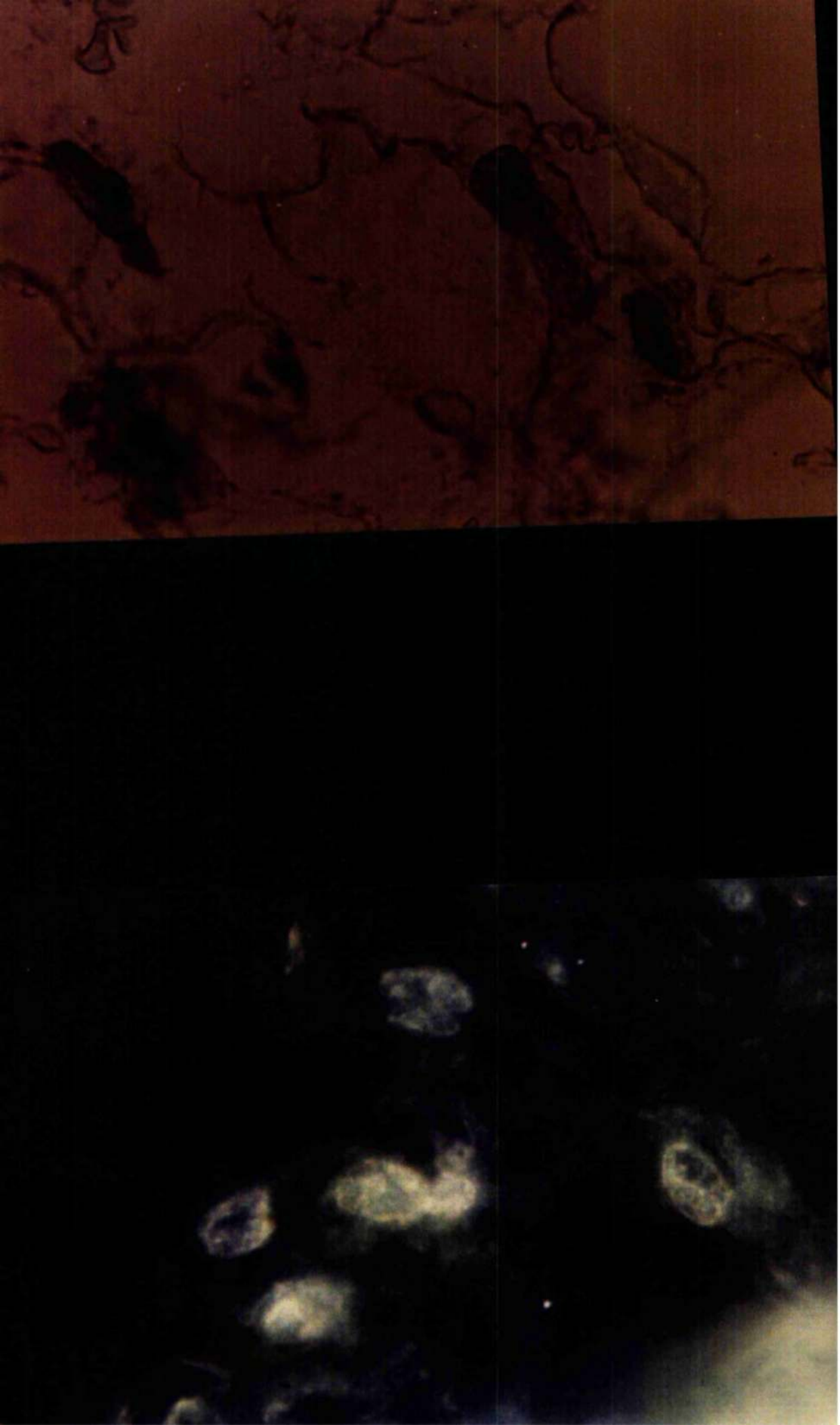


Fig. 34

Mast cells from a frozen section of rat subcutaneous tissue fixed in calcium formol at pH 6, and stained with Schmorl's ferricyanide solution.

(mag : 1300 x)

Fig. 35

Mast cells of rat subcutaneous tissue under dark field illumination in a frozen section fixed with Reinecke salt solution.

(mag : 1300 x)

a brilliant gold-yellow fluorescence against a bluish background (Fig. 33). Globule leucocytes in either type of section failed to fluoresce. Since gold-yellow fluorescence is reported to be characteristic of 5-HT (Benditt and Wong, 1957) it is concluded that while mast cell granules contained 5-HT the granules of globule leucocytes did not. After treatment with Schmorl's ferricyanide solution the granules of mast cells were stained a greenish-blue shade (Fig. 34); globule leucocyte granules failed to give a positive reaction. Although this reaction is not entirely specific for 5-HT (Coupland and Riley, 1960) it is taken to indicate the presence of this substance in the mast cell granules examined and the absence of 5-HT from globule leucocyte granules.

Examination of mast cells and globule leucocytes after Reinecke salt fixation showed that while mast cell granules shone brilliantly under dark field illumination (Fig. 35), globule leucocytes were not visible. This suggests that Reinecke salt had precipitated the histamine in the mast cells (Schauer and Werle, 1959) and that this substance was absent from globule leucocytes. However, after diazo coupling no colour reaction was observed in either cell. The significance of this result is not known. Staining with diazotised parabromoaniline after freeze-drying also produced a negative result in both cells.

On the basis of these results it is concluded that the granules of globule leucocytes from the small intestines of rats infected

Fig. 36

Globule leucocytes in the lamina propria and crypt epithelium of rat jejunum, produced in response to two infections of N. brasiliensis. Methacrylate section stained with azure eosin at pH 3.8.

(mag : 1300 x)

with N. brasiliensis (Fig. 36) do not contain heparin or 5-HT. Due to the difficulty of demonstrating histamine in mast cell granules the results of tests on globule leucocytes are less conclusive for this substance. This is due to the failure of mast cells to give positive reactions for histamine in two cases, and not because of any indication that histamine was present in globule leucocyte granules. The absence of heparin, 5-HT, and possibly histamine from the granules of globule leucocytes clearly indicates that mast cells and globule leucocytes are distinct and unrelated types of cell, and confirms earlier observations on this question (Section III (7)).

These findings also indicate that mast cells, including atypical forms, are absent from the small intestinal mucosa of apparently normal rats. However, mast cells probably occur at this site in low concentrations as indicated by Mota et al. (1956b), and under specific stimuli their numbers may increase to a sufficient level for them to be readily detected.

Part 9 : Globule leucocyte response in neonatal rats infected with Nippostrongylus brasiliensis and in uninfected neonatal rats.

INTRODUCTION

A relationship between the presence of globule leucocytes in the small intestines of rats and infection with N. brasiliensis has been demonstrated experimentally (Section III (2 and 4)). It was noted that globule leucocytes were absent during the pre-immune phase of a primary infection, but that high concentrations were present at the time of, and in the period following the immune response in primary and challenge infections. One possibility suggested by these results is that the globule leucocyte plays an active role in self-cure. Mulligan et al.(1965), Urquhart et al.(1965) and Barth et al.(1966) have shown that at least two factors are operative during self-cure. Evidence is presented in Section III (7 and 8) that the globule leucocyte is not involved in the anaphylactic reaction believed to be associated with self-cure, but the possibility that the globule leucocyte is a carrier of antibody against N. brasiliensis requires further investigation.

If the globule leucocyte was involved in antibody production or transport it is probable that the globule leucocyte response would change significantly in rats whose immunological status to

N. brasiliensis was altered. It is well known that the immunological responses of very young animals of certain species are impaired (Ingram and Smith, 1965 : Smith and Ingram, 1965), and preliminary work on infection of neonatal rats with N. brasiliensis showed that young rats tolerated for more than a month infections large enough to be capable of inducing self-cure in adult rats. Jarrett et al. (1966), and Kassai and Aitken (1966) have performed more extensive experiments which indicate that self-cure does not occur in rats infected under the age of four weeks, and that such infections persist for many months. The inability of such rats to respond in the normal way to a challenge infection in later life indicates that a state of tolerance has been induced towards the primary infection.

Since anaphylaxis can be produced in suitably sensitised rats within the period when tolerance to N. brasiliensis primary infections may be induced, it is likely that self-cure does not take place because of the absence or significant reduction of precipitating antibodies. Consequently, demonstrable impairment of the globule leucocyte response in neonatal rats infected with N. brasiliensis would constitute indirect evidence that the globule leucocyte was involved in the manufacture or transport of antibody against N. brasiliensis.

It was necessary to infect neonatal rats at different ages in order to determine whether any globule leucocyte response observed

RAT NO.	AGE INFECTED	AGE KILLED	NO. OF DAYS INFECTED	WORM COUNT	GL/mm^2
1R/1	0	5	5	41	5
1R/2	0	10	10	11	6
1R/3	0	20	20	17	19
1R/4	5	10	5	97	2
1R/5	5	15	10	75	6
1R/6	5	25	20	71	27
1R/7	10	15	5	62	0
1R/8	10	20	10	110	2
1R/9	10	30	20	112	23
1RC/1	-	5	-	0	5
1RC/2	-	10	-	0	1
1RC/3	-	20	-	0	2
2RC/1	-	0	-	-	+
2RC/2	-	0	-	-	+
2RC/3	-	5	-	-	+
2RC/4	-	5	-	-	+
2RC/5	-	10	-	-	+
2RC/6	-	10	-	-	+
2RC/7	-	20	-	-	+
2RC/8	-	20	-	-	+

Table 10

Details of experimental *N. brasiliensis* infections in neonatal rats showing *post mortem* worm burden and globule leucocyte concentration in the jejunum.

was due to the age of the rat or, alternatively, whether it occurred at a fixed number of days after infection, irrespective of age.

MATERIALS AND METHODS

Two litters of albino Wistar rats were used. Of the first litter, three rats (1R/1-3) were infected on the day of birth (Table 10), three at five days of age (1R/4-6) and three at ten days old (1R/7-9). Rats were killed on the fifth, tenth and twentieth days after infection in order to obtain specimens at the periods corresponding to the pre-immune phase, initiation of self-cure, and immune phase. Three uninfected rats from the same litter (1RC/1-3) were killed at five, ten and twenty days of age. Because the results indicated the possibility of cross-infection from infected litter mates to controls in the same cage, additional rats from a second and uninfected litter (2RC/1-6) were killed in pairs at birth and at five, ten and twenty days of age, and examined for the presence or absence of globule leucocytes.

Animals for infection were anaesthetised lightly with ether and given subcutaneously a calculated dose of 400 infective larvae of N. brasiliensis in 0.1 ml of water using a modified Rautmann Automat syringe (Section II (1)). The rats were killed by an overdose of chloroform and the intestine carefully unravelled. Jejunum area 6 was removed using the gauge described previously, and a worm count

was carried out. Blocks were embedded in methacrylate and sections were stained with amido black for counting globule leucocytes, and with haematoxylin and eosin for general observation.

RESULTS

The worm counts in the three groups of rats infected at 0, 5 and 10 days of age showed no difference whether killed 5, 10 or 20 days after infection with N. brasiliensis (Table 10). The average percentage take ($\frac{\text{no. of adult worms recovered at autopsy}}{\text{no. of infective larvae injected}} \times 100$) in the first of the three groups was considerably lower than in the other two :-

1R/1-3 : 6%

1R/4-6 : 20%

1R/7-9 : 24%

No worms were found in the intestines of uninfected control rats from the same litter (1RC/1-3).

Globule leucocytes were found in all the rats examined including uninfected control rats. Because of this a second, completely uninfected litter (2RC/1-8) was examined for globule leucocytes, in order to determine whether their presence in uninfected controls from litter 1R was due to cross-infection from infected litter mates. Small numbers of globule leucocytes were found in all rats from this litter (2RC/1-8). The concentration of globule leucocytes was uniformly

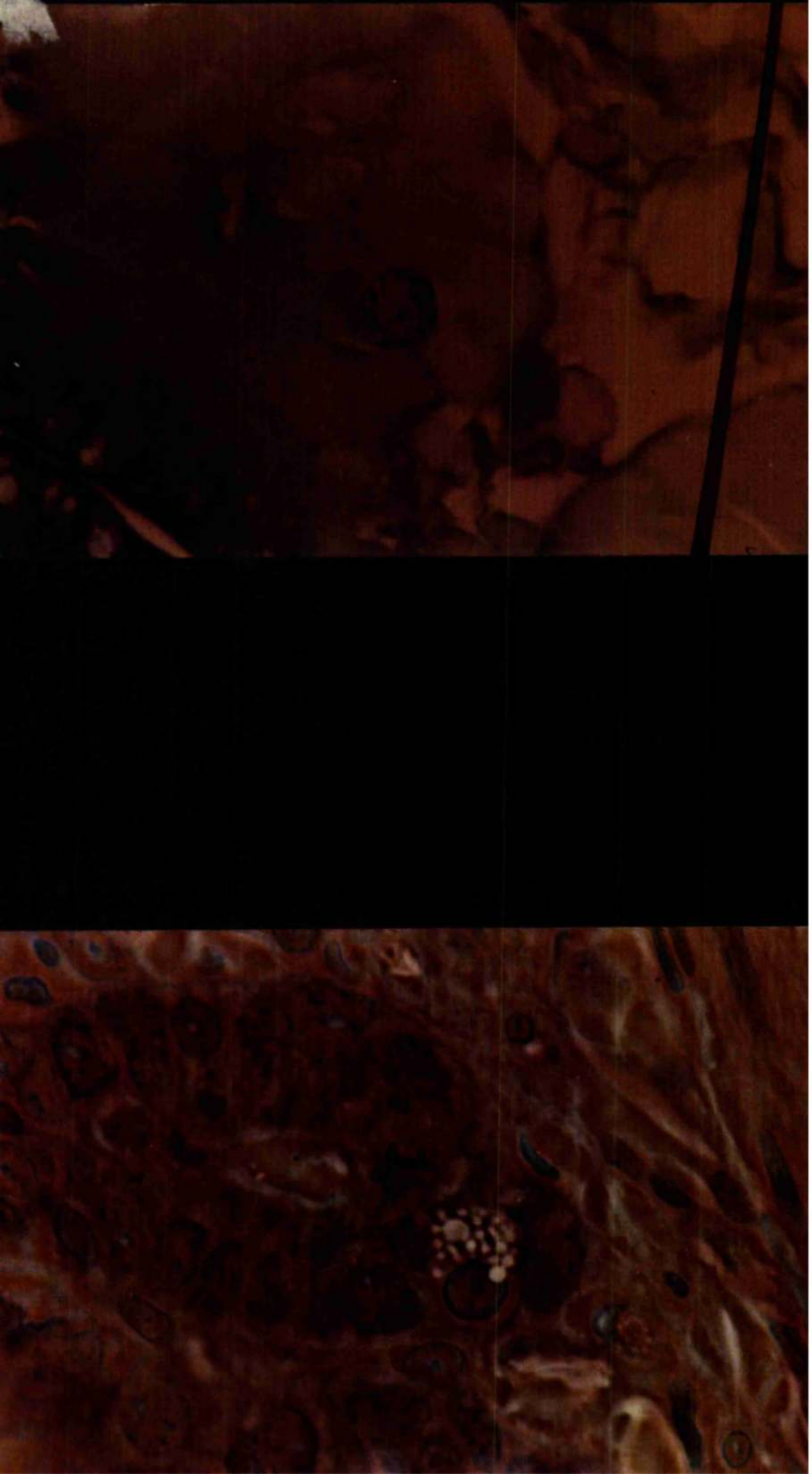


Fig. 37

Globule leucocyte situated in the epithelium of the jejunum of a 5-day-old worm-free rat.

Methacrylate section stained with amido black.

(mag : 1300 x)

Fig. 38

Globule leucocyte in the epithelium of a jejunal crypt of a newborn rat. The appearance of this cell coincides closely with the Russell body cell illustrated in fig. 19, but is not typical of the globule leucocytes seen in adult rats (fig. 21).

Methacrylate section stained with haematoxylin and eosin and photographed under phase contrast.

(mag : 1300 x)

low in all rats examined, with the exception of 1R/3, 6 and 9, where they were present in higher concentrations (Fig. 39). These rats were of different ages when killed, but had all been infected with N. brasiliensis 20 days previously (Table 10).

In amido black-stained sections the globule leucocytes appeared to be similar to those observed in previous experiments (Fig. 37), but when examined in sections stained with haematoxylin and eosin under phase contrast the globules were whitish (Fig. 38), in contrast to those observed previously, which had a pronounced red colouration (Fig.21).

DISCUSSION

No expulsion of worms took place during the period of the experiment (Table 10). This finding agrees with the results of Jarrett et al.(1966) and Kassai and Aitken (1966) and indicates that the rats were immunologically incompetent towards the infection. It is suggested that the lower percentage take noted in rats infected on the first day of life may have been due to the intestine being too small at this age to accomodate the larger numbers of worms seen in 1R/4-9.

A globule leucocyte response occurred between the tenth and twentieth days of the infection (Fig. 39), irrespective of the age of the animal at the time of infection. In consequence these results

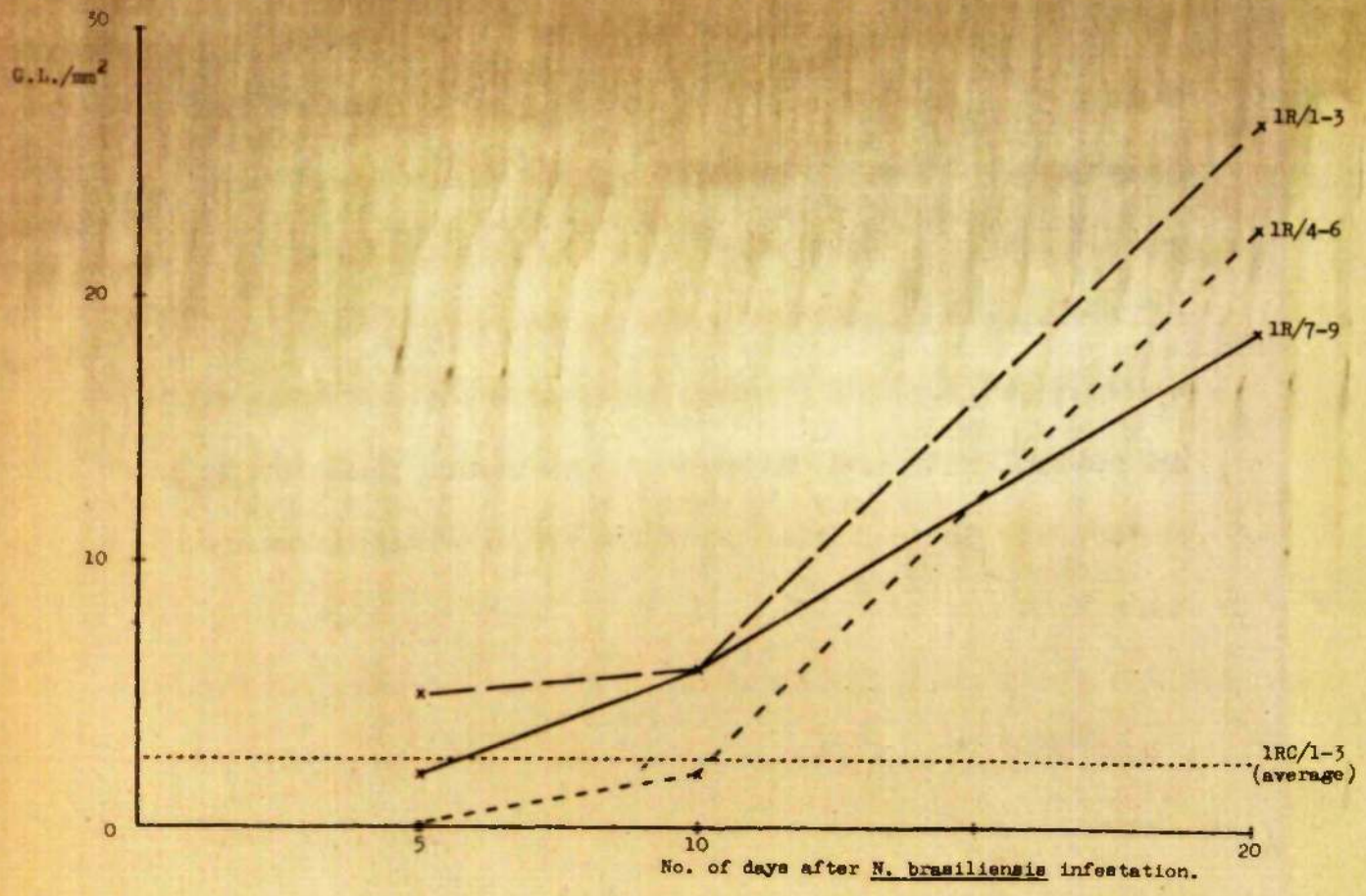


Fig. 39

Globule leucocyte response in the jejunum of neonatal rats (1R 1-9) infected with N. brasiliensis, and in similar uninfected control rats (1RC 1-3).

fail to show conclusively that impairment of the immune response also leads to recognisable impairment of the globule leucocyte response. However, in view of the very low concentrations of cells observed it may be considered doubtful that they were present in sufficient numbers to have instigated an immune response in an immunologically competent rat.

The constant presence of globule leucocytes in low concentrations in uninfested control rats and during the pre-immune phase of N. brasiliensi infection is contrary to previous observations. The significance of these findings is not clear, but the very small numbers which were observed suggest that the causative stimulus is very slight compared to that of N. brasiliensis infection. It is possible that a very mild immune reaction occurs to proteins contained in the colostrum which are present in the small intestine during this period (Miller, 1965), but the subject requires further investigation.

Under phase contrast, the globule leucocytes (Fig. 38) differed from those previously observed, and were apparently identical to the type of Russell body cell illustrated in Fig.19. The alteration in colour under phase contrast may indicate a change in the composition or density of the globule contents, and it may be related to the immunological unresponsiveness of the neonatal rat towards N. brasiliensis infection.

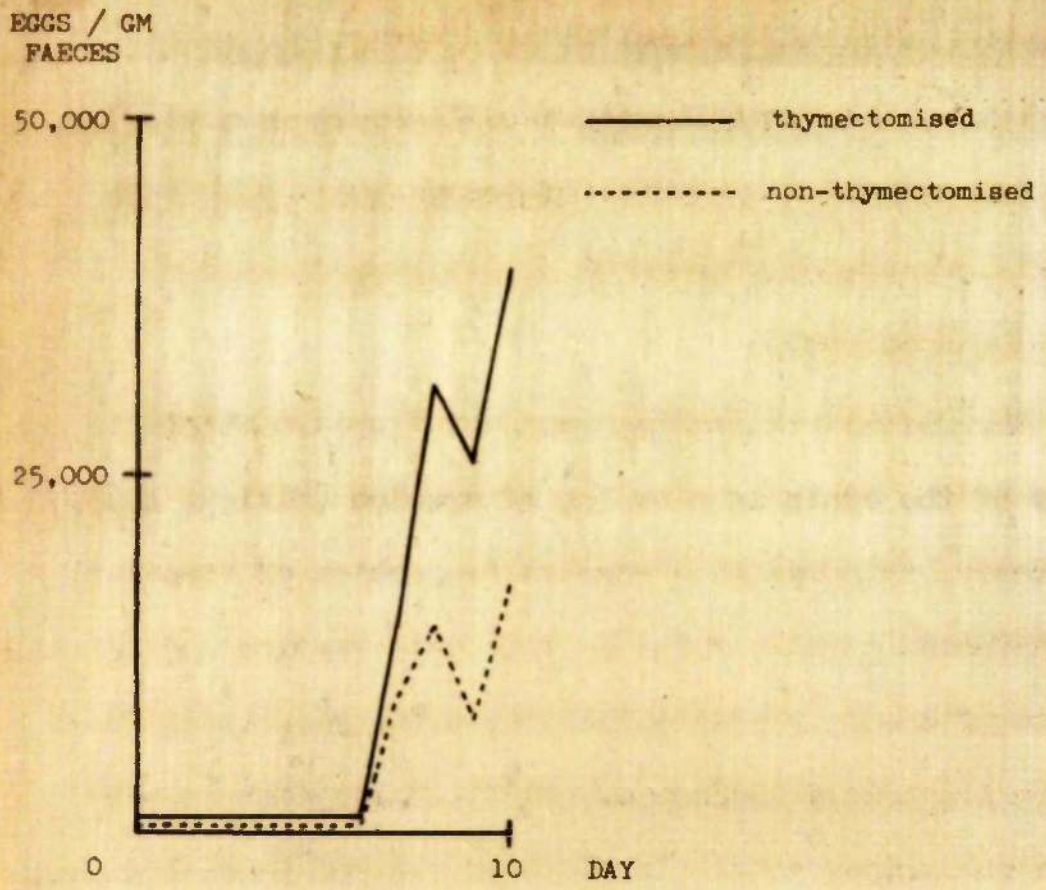


Fig. 40

Faecal egg counts of thymectomised rat R1 and control rat C1, during the course of a primary infection with *N. brasiliensis*.

Part 10 : Effect of neonatal thymectomy on the globule leucocyte response in the jejunum of rats infected with Nippostrongylus brasiliensis : preliminary results.

INTRODUCTION

Neonatal thymectomy results in measurable impairment of the immune response of the adult in a number of species (Miller, 1963). Among other effects, this may give rise to depression of circulating antibody levels (Roosa et al., 1965) as well as depletion and immunological defects of cells of the lymphoreticular series (Miller, 1961, 1962, 1963 : Yunis et al., 1964 : Aschkenasy, 1965). These factors are associated with partial or total inability to respond immunologically to antigenic stimulus. It appears likely, therefore, that the normal course of N. brasiliensis infection would be modified in rats which had been neonatally thymectomised, with the further possibility that this would be reflected in impairment of the globule leucocyte response. Alterations in the immune response to N. brasiliensis are readily detectable by appropriate measurements, such as faecal egg output , post mortem worm counts and worm population sex ratios.

A previously reported experiment (Section III (9)) using rats which were immunologically tolerant towards a primary infection of N. brasiliensis failed to demonstrate conclusively that impairment of the immune response led to demonstrable reduction in the globule

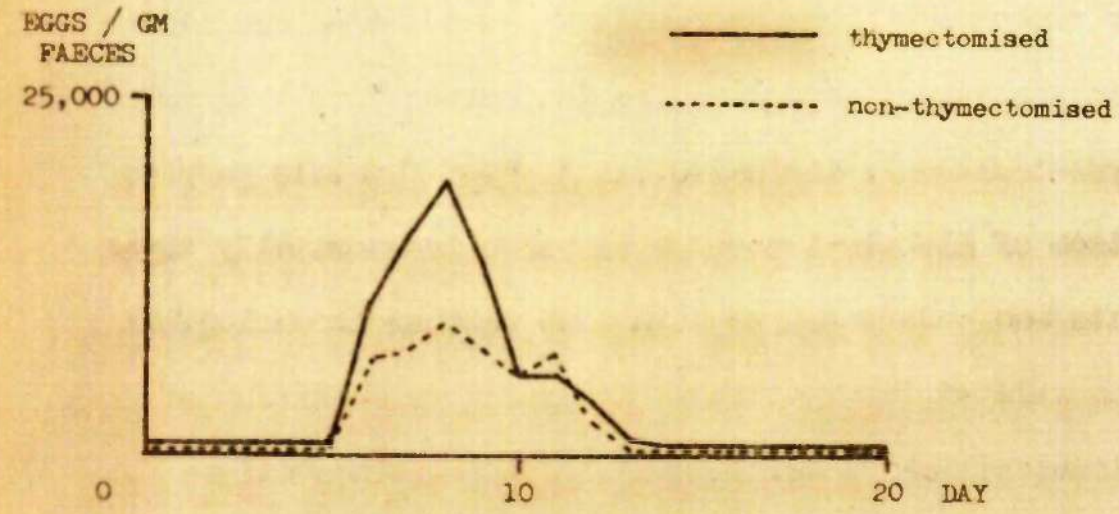


Fig. 41

Faecal egg counts of thymectomised rat R2 and control rat C2, during the course of a primary infection with N. brasiliensis.

leucocyte response. This was due to the absence of a suitable control group of similar weight and age which were immunologically competent towards N. brasiliensis, against which the globule leucocyte response in tolerant rats could be quantitatively compared. Neonatal thymectomy as a means of studying globule leucocyte response under conditions of impaired immunological capacity does not suffer from this defect, since non-thymectomised litter mates can be used for this purpose.

Comparison of globule leucocyte response in neonatally thymectomised and intact rats was undertaken to determine whether immunological impairment was related to significant reduction or abolition of the globule leucocyte response. A number of other observations were made with the aim of determining that total thymectomy and measurable immunological impairment had been achieved.

MATERIALS AND METHODS

Neonatal thymectomy and the care of thymectomised animals is described in Section II (1). Neonatally thymectomised rats and non-thymectomised litter mates were weaned at four weeks of age and kept in isolation. Because infection of rats under four to six weeks of age with N. brasiliensis induces a state of immunological tolerance which persists into adult life (Jarrett et al., 1966 : Kassai and Aitken, 1966) no animals were infected before they were 40 days old. A single dose of 1500 infective larvae of N. brasiliensis

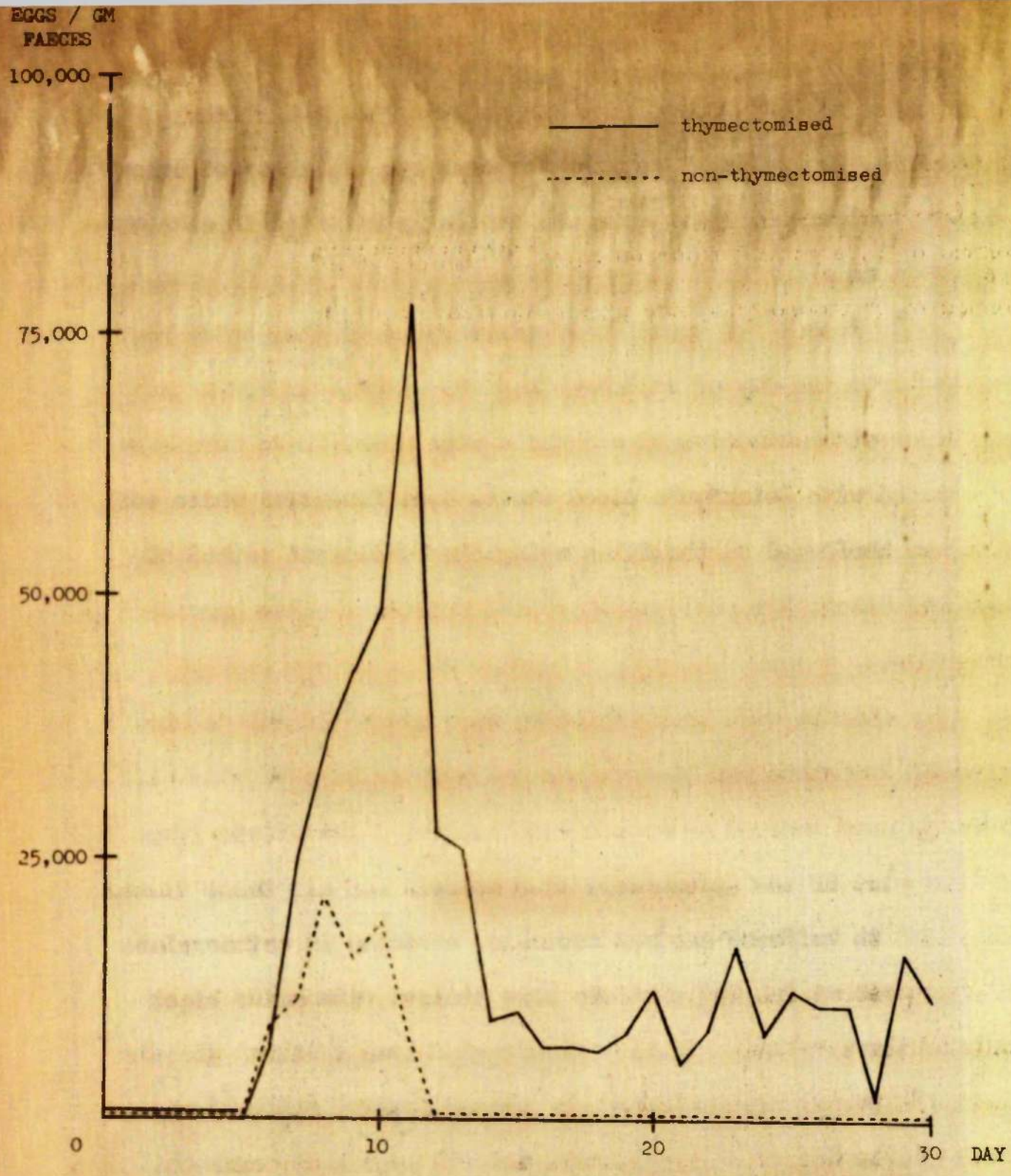


Fig. 42

Faecal egg counts of thymectomised rat R3 and control rat C3, during the course of a primary infection with *N. brasiliensis*.

was injected subcutaneously into the groin region under ether anaesthesia. The relevent details for each rat are recorded below. A non-thymectomised litter mate was similarly infected in each case and served as a control.

Daily faecal egg counts were carried out by the method previously described. On the day of killing blood was withdrawn from a tail vein under ether anaesthesia and films were made. These were air-dried and stained with Leishman's blood stain. A differential white cell count was performed on the films using the battlement method of field selection. 200 cells were counted and the results expressed as percentages.

The animals were then killed by an overdose of chloroform. The small intestine was placed on a gauge and area 6 (Section III (2)) of the jejunum removed as an unopened length. A mesenteric lymph node and part of the spleen were also removed and all these tissues were fixed in buffered neutral formalin, embedded in methacrylate and sectioned at 2μ. Gut sections were stained with amido black and haematoxylin and eosin. The number of intraepithelial globule leucocytes per square millimetre of tissue section (GL/mm^2) was calculated as described previously, and the number of eosinophil leucocytes in the lamina propria of the same area was also calculated in order to determine the effect of neonatal thymectomy upon a cell definitely not in the lymphoreticular series but nevertheless part of the cellular response to N. brasiliensis. Sections of spleen

	R1	C1	R3	C3
TOTAL LYMPHOCYTES	56	75	49	84
HETEROPHILS	35	23	50.5	12
EOSINOPHILS	7	0	0	1.5
MONOCYTES	2	2	0.5	2.5
BASOPHILS	0	0	0	0

Table 11

Differential white cell counts of two neonatally thymectomised rats (R1, R3) compared to non-thymectomised litter mate controls (C1, C3). All rats were infected with *N. brasiliensis*.

and lymph node were stained with amido black, haematoxylin and eosin and azure eosin at pH 3.8, and they were examined for changes which might be associated with neonatal thymectomy.

The thorax was removed entire from thymectomised rats and fixed in buffered neutral formalin. It was decalcified in Zero-Carb 225 (Permutit Co., London) for two weeks and embedded in paraffin. Serial sections were cut at 5μ and sections at intervals of 100μ were examined microscopically for thymic remnants after staining with haematoxylin and eosin. Results from rats found to contain thymic remnants were discarded.

Post mortem worm counts were carried out by the method described previously.

Rat R1 and control C1

Both were male albino Wistars. R1 was thymectomised on the day of birth (day 0). Both received 1500 infective larvae of N. brasiliensis on day 42 and were killed 10 days later (day 52).

Rat R2 and control C2

R2 was a male albino Wistar thymectomised on the day of birth. The non-thymectomised litter mate C2 was a female. Each received 1500 larvae of N. brasiliensis on day 43 and was killed 20 days later on day 63.

Fig. 43

Cortex of a mesenteric lymph node from a neonatally thymectomised rat. A nodule is shown and it is apparent that the peripheral layer of small lymphocytes is absent although the germinal centre is of relatively normal appearance.

Methacrylate section stained with haematoxylin and eosin.

(mag : 300 x)

Fig. 44

Similar field to that shown in Fig. 43, from a non-thymectomised rat, illustrating the normal appearance of the area.

Methacrylate section stained with haematoxylin and eosin.

(mag : 300 x)

Rat R3 and control C3

R3 was a male and C3 a female albino Wistar rat. R3 was thymectomised on day 0 and both rats received 1500 larvae of N. brasiliensis on day 41. They were killed 30 days later on day 71.

RESULTS

Deaths before completion of experimentation together with rejects due to incomplete thymectomy resulted in a high rate of loss. Consequently results were obtained for only three animals and are reported as preliminary findings.

Rat R1 and control C1

Faecal egg counts are shown in Fig. 40. Eggs were present in the faeces in small numbers on day 6, and the concentration rose steadily until the rats were killed on day 10. At this time the number of eggs in the faeces of the thymectomised rat was approximately double that of the control.

The post mortem worm burden of R1 was 378 (25%) with a male to female ratio of 1.4♂ : 1♀ . In the control rat C1 the number of worms recovered at autopsy was 336 (22%) and the sex ratio was similar to that of R1.

The results of the differential white cell count are shown in Table 11. The percentage of circulating lymphocytes was lower

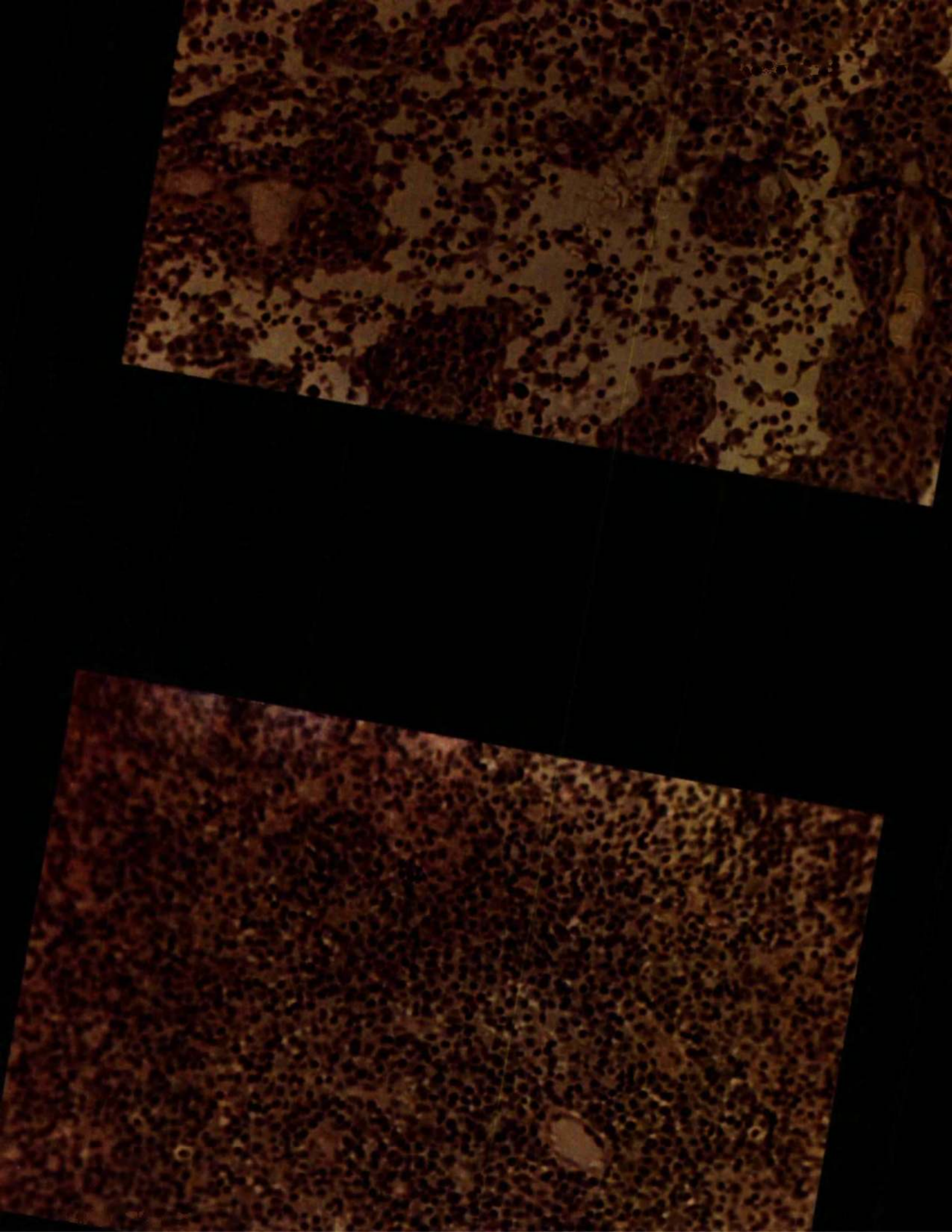


Fig. 45

Medulla of a mesenteric lymph node from a neonatally thymectomised rat. There is a depletion of cells of the lymphocyte series together with hyperplasia of the reticuloendothelial elements.

Methacrylate section stained with haematoxylin and eosin.

(mag : 300 x)

Fig. 46

Similar area to that shown in Fig. 45, from a non-thymectomised rat, illustrating the normal appearance of the region.

Methacrylate section stained with haematoxylin and eosin.

(mag : 300 x)

in the thymectomised rat than in the control. Eosinophil leucocytes were present in a concentration of 79/mm^2 in R1 and 236/mm^2 in C1. Intraepithelial globule leucocytes were absent from R1 and present in a concentration of 4/mm^2 in C1.

The mesenteric lymph node of the thymectomised rat R1 was depleted of small lymphocytes (Figs. 43-48) and the nodules in the cortex were not surrounded by a peripheral layer of these cells (Fig. 43). Reticuloendothelial hyperplasia was apparent in the medulla (Figs. 45 and 47) and also in the splenic red pulp. The white pulp of the spleen contained increased numbers of degenerating cells and mitotic figures (Fig.49). Russell body cells were not located in the spleen or lymph node of either rat.

Rat R2 and control C2

Faecal egg counts are plotted in Fig. 41. Apart from a somewhat higher peak of egg production in the thymectomised animal both rats followed a similar pattern with the highest concentration of eggs on day 8 declining to near zero on day 13.

The worm burden at post mortem was 333 (22%) in R2, compared to 6 (0%) in the control C2. The worm sex ratio of R2 was 2.38 : 1♀.

Blood smears were not obtained from this pair of rats. The concentration of eosinophil leucocytes in the jejunal mucosa of R2 was 386/mm^2 compared to 331/mm^2 in C2. Intraepithelial globule leucocytes at the same site were found in a concentration of 155/mm^2

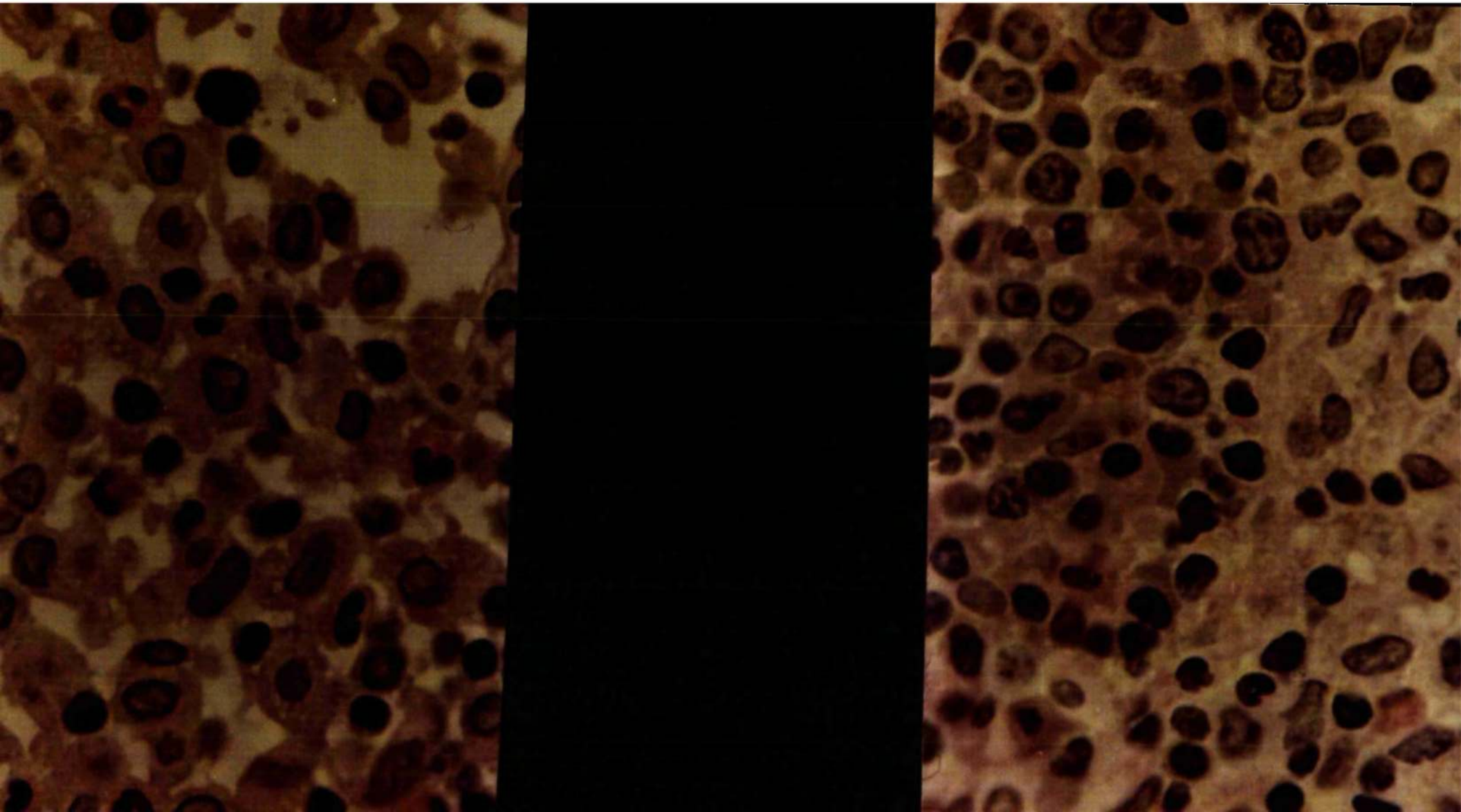


Fig. 47.

Higher magnification of area shown in Fig. 45. The high proportion of reticuloendothelial elements is apparent.

Methacrylate section stained with haematoxylin and eosin.

(mag : 1300 x)

Fig. 48

Higher magnification of area shown in Fig. 46. Lymphocytes and plasma cells are more numerous than in similar areas from thymectomised rats (Fig. 47).

Methacrylate section stained with haematoxylin and eosin.

(mag : 1300 x)

in R2 and 406/mm^2 in C2.

The appearance of the spleen and lymph node of R2 were similar to those of R1.

Rat R3 and control C3

The egg count rose to a peak around day 10 in the control and to a considerably higher level in the thymectomised rat on day 11. Egg counts dropped to near zero on day 12 in the control, but despite a considerable decline in numbers in the thymectomised rat levels around 10000/g faeces were maintained until killing on day 30 (Fig. 42).

The post mortem worm count in the thymectomised rat R3 was 438 (29%) with a sex ratio of 0.93 : 19. A total of 39 worms (2%) were recovered from the control C3.

Differential white cell counts are detailed in Table 11. There was a marked decline in the percentage of circulating lymphocytes in R3 compared to C3. Eosinophil leucocytes were present in a concentration of 210/mm^2 in R3, and 263/mm^2 in C3. In R3 intraepithelial globule leucocytes were present in a concentration of 4/mm^2 as compared to 130/mm^2 in the control C3.

The appearance of the spleen and lymph node of the thymectomised rat was again similar to that of R1 (Figs. 43-49).

DISCUSSION

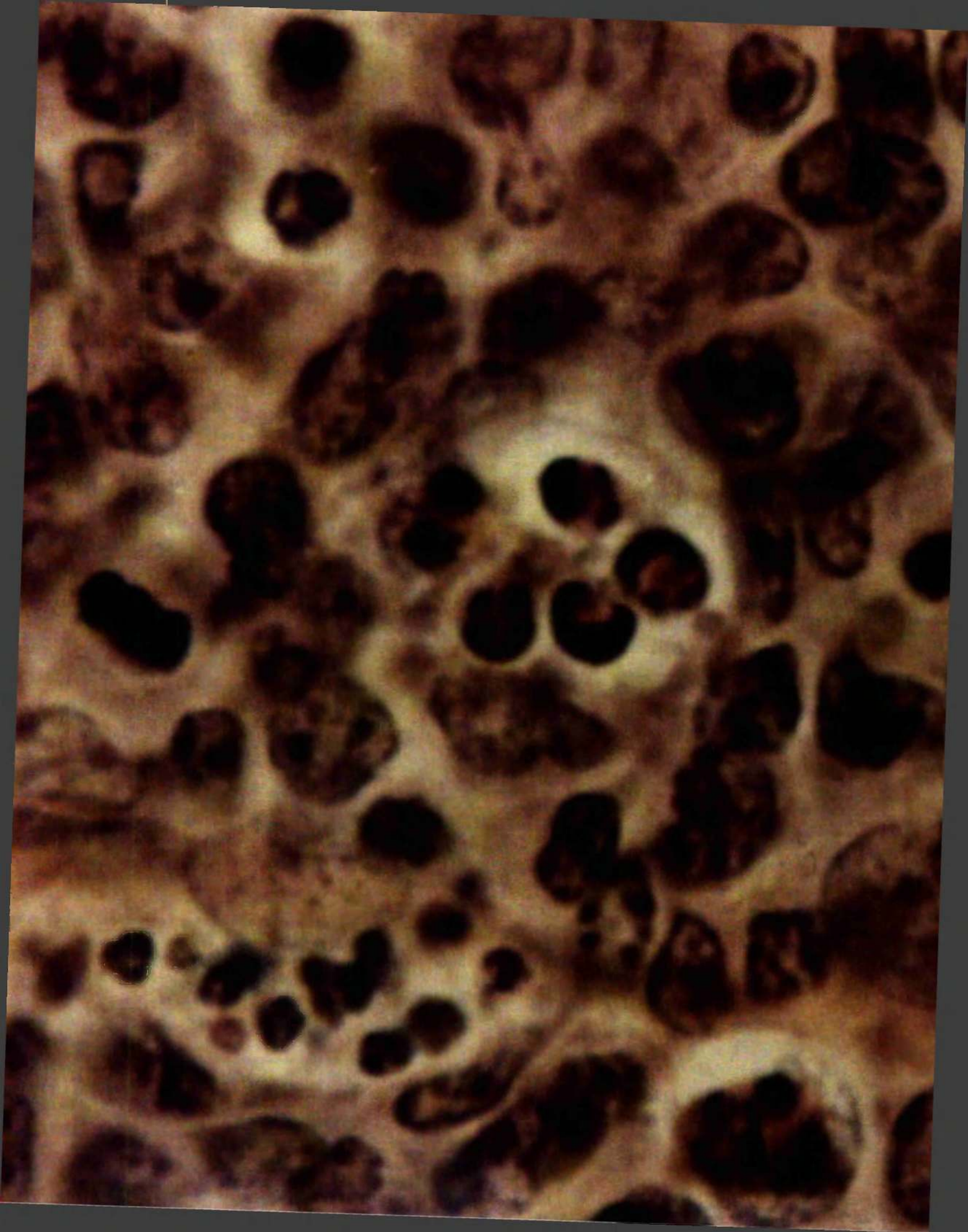


Fig. 49

Part of the splenic white pulp of a neonatally thymectomised rat. Groups of degenerating lymphoid cells coupled with increased numbers of mitotic figures are apparent.

Methacrylate section stained with haematoxylin and eosin.

(mag ; 1300 x)

DISCUSSION

The success of neonatal thymectomy was readily established by reference to parameters which have been described by previous authors, namely reduction in the percentage of circulating lymphocytes, and reticuloendothelial hyperplasia and depletion of small lymphocytes in lymphoid organs. Such findings confirmed the negative results of a thorax search for thymic remnants. A further indication of successful thymectomy was the absence of self-cure in thymectomised animals. This was apparent in R2 and R3 whose worm burdens (22% and 29%) were in the same range as the preimmune rats R1 and C1 (25% and 22%).

In view of this finding the pattern of faecal egg counts is not readily explained since although during the egg-laying period the concentration of eggs in the faeces was invariably higher in the thymectomised rat than in the control, the egg output of such rats declined at the same time as or soon after that of the control. This pattern is similar to that observed by Kassai and Aitken (1966) in neonatally infected rats, but whether it is a non-immune phenomenon or is indicative of an immune response is not known.

No significant difference in the globule leucocyte response was apparent between the thymectomised rat R1 and the control C1. This would be expected in view of the fact that these animals were

killed before the time at which self-cure would normally occur. The fact that self-cure has not taken place was confirmed by the post mortem worm counts, and the almost total absence of globule leucocytes from both rats would be expected from the results reported in Section III (2). The rather low concentration of eosinophil leucocytes in the jejunal lamina propria of the thymectomised rat ($79/mm^2$) is not considered to be a significant finding.

In R2 and R3 , both of whose non-thymectomised litter mate controls had undergone self-cure, there was marked impairment of the globule leucocyte response associated with the failure of self-cure to take place. Since in neither of these rats was there any reduction in the concentration of eosinophil leucocytes in the thymectomised animals, although these cells also represent a response to N. brasiliensis, it is concluded that impairment of the globule leucocyte response is directly related to impairment of the immune response against N. brasiliensis resulting from neonatal thymectomy.

Since these conclusions are derived from preliminary results they must be regarded as tentative. However the results provide evidence that the globule leucocyte is a cell of lymphoreticular origin concerned with the production of antibody against N. brasiliensis.

SECTION IV

GENERAL DISCUSSION AND SUMMARY

Previous work on the globule leucocyte indicated the possibility that a relationship existed between the appearance of this cell and infection by nematodes. Taliaferro and Sarles (1939) had noted the occurrence of globule leucocytes in the epithelium of the small intestine of rats during infection with Nippostrongylus brasiliensis, while Sommerville (1956) investigated the specific question of a relationship by examining abomasa from worm-free sheep and sheep infected with Ostertagia circumcincta. Kirkman (1947, 1949, 1950) related Trichosomoides crassicauda infections in the urinary tracts of rats to the appearance of certain intraepithelial granule-containing cells which bore a close resemblance to globule leucocytes.

The gastrointestinal tracts of four worm-free and four sheep infected with O. circumcincta were examined for intraepithelial globule leucocytes. High concentrations were found in the parasitised sheep, and only very minute numbers in the worm-free sheep, indicating that a relationship exists between the presence of the nematodes and the occurrence of globule leucocytes. Sommerville (1956) had been unable to demonstrate a precise site relationship between the two factors, and had concluded that any relationship between the two must be regarded as tentative and indirect. In the present case, however, high concentrations of globule leucocytes were found almost exclusively in the posterior part of the abomasum and the

first segment of the small intestine; thus there was a reasonable degree of site correlation.

Having found that the hypothesis of a relationship between globule leucocytes and parasitic infection was valid, this was tested by attempting to produce globule leucocytes in response to nematode infection in previously worm-free stocks. Rats were experimentally infected with the small intestine nematode N. brasiliensis. These animals were killed at different stages of the ensuing infection and segments from the small intestine examined for globule leucocytes alongside an uninfected control group. Despite that the fourth stage larvae are present in the small intestine from about two days after infection, globule leucocytes appeared for the first time between days 10 and 12. Concentrations of the cell were highest in the anterior small intestine initially, but the response became more scattered later on. This appeared to demonstrate conclusively that certain nematodes produce a globule leucocyte response, and that it is fairly closely related to the actual site of the parasite, for the globule leucocyte distribution mirrored the distribution of the worms at the various stages of the primary infection fairly closely.

These results were complicated by the presence of globule leucocytes in the gastric and large intestinal mucosa, not only in infected but also in uninfected control rats. This was investigated, and infection by the large intestine nematode Syphacia obvelata was

found to be endemic in the stocks used. However, the occurrence of globule leucocytes in the gastric mucosa has not been satisfactorily explained.

An interesting feature of these results was the apparent relationship which existed between the appearance of globule leucocytes and the immune response to N. brasiliensis, which results in the expulsion of the adult worm population at self-cure, commencing about day 12 of a primary infection. This was investigated in a preliminary way by reinfecting five rats on the twentieth day after the primary infection. There was an increase in the numbers of globule leucocytes present in the jejunal epithelium, reaching the highest level on day 30, as compared to rats which had only received a single infection.

The results of this experiment did not demonstrate that the relationship between the globule leucocyte response and self-cure was direct. In fact, this response may have occurred as a result of any previous event in the development of N. brasiliensis within the host. However, rats which have previously experienced N. brasiliensis infection become immune, and the ability of the adult worms to establish themselves in the small intestine is impaired. This results in interference with or abolition of the egg-laying phase and the immediate onset of self-cure. Timing of prior events in the life cycle is apparently unaffected; consequently determination of the timing of the globule leucocyte response in immune rats would reveal

whether or not there was a relationship to the onset of self-cure.

Rats were rendered hyperimmune to N. brasiliensis by repeated infections with increasing doses of larvae. Six weeks were allowed to elapse between the last immunising infection and the experimental challenge infection to allow any globule leucocyte response to subside. The experimental group were then infected with N. brasiliensis and killed at intervals over the next 30 days. The concentration of globule leucocytes in the anterior jejunum was compared to that of similar hyperimmune uninfected controls. Small concentrations of globule leucocytes were found in the controls and these correlated with the presence of very light worm burdens, which represented the remnants of the immunising infections. In the experimental group a marked increase in globule leucocytes became apparent by day 6, and reached a peak on day 10. The concentration then declined fairly steadily over the next 20 days until it reached the levels found in the control group.

Comparison of the globule leucocyte response in primary infections and in hyperimmune rats showed that in the latter case the response occurred about six days earlier. However, in both cases the globule leucocyte response coincided closely with the onset of the immune response in the small intestine. It was concluded that a direct relationship exists between the appearance of globule leucocytes and the immune response, and suggested that the globule leucocyte may have some role to play in the expulsion of the adult worm population from the

intestine.

The demonstration of a relationship between globule leucocytes and certain nematodes increased speculation concerning the types of stimuli capable of producing a globule leucocyte response. A brief review of the literature on pathological conditions of the intestine indicated that globule leucocytes were not associated with bacterial infection. It seemed likely, however, after reports by Pierce and co-workers (1962, 1965) that globule leucocytes were present in the intestines of chickens during coccidial infections, that other types of parasite might also elicit a globule leucocyte response.

No experimental work was undertaken in this connection, but suitable material which became available was examined. Sections of liver from a sheep suffering from acute Fasciola hepatica infection were examined and found to contain large numbers of globule leucocytes. They were situated in the bile duct epithelium and it appears reasonable to suggest therefore that they represented part of the cellular response to the parasite. The small intestines of rats infected with the tapeworm Hymenolepis nana were also examined and a heavy localised concentration of globule leucocytes was found at the normal site of this parasite in the posterior part of the ileum. In consequence it is tentatively suggested that trematodes and cestodes, in addition to nematodes, are capable of eliciting a globule leucocyte response. The common factor in such infections was not investigated further.

The origin, nature and function of the globule leucocyte has been investigated by a number of authors. Kent and co-workers (1949, 1952, 1954, 1956) have indicated that this cell has a lymphocytic origin and that intermediate gradations between lymphocytes and globule leucocytes can be observed. On the other hand, Kirkman (1950) believed that the cells occurring in the urinary tract epithelium of rats during T. crassicauda infection were closely related to Russell body cells and plasma cells.

Since it is already well established that one type of Russell body cell originates from plasma cells (Thiery, 1960), globule leucocytes and Russell body cells were compared morphologically. For this purpose rats were given repeated injections of a vaccine manufactured from Proteus vulgaris and administered intravenously, using a method adapted from White (1954) and originally used to produce Russell body cells in the lymphoid organs of rabbits. For comparison, globule leucocytes were produced in the small intestines of rats by infecting them with N. brasiliensis. All stages of Russell body cell were present in the spleens and lymph nodes of vaccinated rats, and it was apparent that these cells arose from plasma cells. At one stage of Russell body formation these cells appeared morphologically identical to globule leucocytes, and it was concluded that the two types of cell could not be distinguished at this stage using purely morphological criteria.

Even in the type of Russell body cell which resembled the

globule leucocyte a constant difference between the two was noted. When the two types of cell were observed in stained sections under the phase contrast microscope the Russell bodies resolved as light -coloured bodies while the inclusions of globule leucocytes appeared dark. While this difference might have been considered significant, it was noted during later studies on neonatal rats that otherwise typical globule leucocytes also had granules which appeared light -coloured under phase contrast. The significance of this finding is not clear, but it suggests that the previous difference observed did not preclude a fairly close relationship between the two types of cell.

Toner (1965) published the first account of the ultrastructure of the globule leucocyte. These cells were located in the gastrointestinal tract of domestic fowls and their aetiology was not investigated. The inclusions of these cells were found to be homogeneous bodies, while another notable feature was the poorly developed rough endoplasmic reticulum, the latter suggesting to the author a lymphocytic rather than a plasma cell origin. Carr (1966) has recently reported observations on mouse globule leucocytes, with particular emphasis on the structure of the inclusions, many of which were of a crystalline nature. In this case also the animals had not been subjected to any experimental procedures, and the aetiology of the cells was unknown.

Globule leucocytes in the small intestines of rats, produced in response to a challenge infection with N. brasiliensis, were

examined with the electron microscope. During the examination of thick (1μ) sections it was repeatedly observed that globule leucocytes were not restricted to an intraepithelial location. They were also present in the lamina propria and submucosa and were frequently observed adjacent to the small blood vessels which penetrate the muscularis externa from the surrounding mesentery. These observations indicate that the source of many, if not all globule leucocytes is outside the small intestine, and contradict the findings of Kent (1949, 1952) who reported that globule leucocytes developed from lymphocytes in the lamina propria. In consequence it is suggested that globule leucocytes are derived from precursors in the mesentery or elsewhere and actively migrate into the intestine and then out into it's lumen.

Intraepithelial globule leucocytes were located almost exclusively at crypt level, and only a very occasional cell was noted in the epithelium covering villi. In view of the fact that intestinal epithelial cells move up the sides of the villi from the crypt region and are lost from the extrusion zone at the tip of the villus after two or three days (Leblond, 1965) it would appear that the time spent by the globule leucocyte within the epithelium is relatively restricted. It also provides additional evidence that these cells do in fact migrate out into the lumen of the intestine.

Under the electron microscope the globule leucocytes examined were found to contain three types of inclusion. The first was homogeneous

and electron dense, the second type contained apparently crystalline structures with striations similar to those described by Carr (1966), while the third type was intermediate between these two. There was evidence that these inclusions were associated with the rough endoplasmic reticulum, which in some cells was more extensive than that observed by Toner (1965) in fowl globule leucocytes. While the precise nature of the contents of the inclusions is unknown Carr (1966) indicated that they may be protein in nature. This appears to be confirmed by the recent report of Dobson (1966) who demonstrated by immunofluorescence techniques that they contain globulin. The structure of the granules and their apparent association with the fairly extensive rough endoplasmic reticulum suggests that globule leucocytes are similar to metaplasmocytes containing Russell bodies. Pinocytotic vesicles were an almost constant feature of intraepithelial globule leucocytes, and it is speculated that these may have been associated with the uptake of antigen.

Despite the morphological similarities existing between globule leucocytes and Russell body cells, the nature of these observations precludes definite conclusions concerning a relationship. Since anaphylaxis has been demonstrated to be one factor involved in the immune response of rats against N. brasiliensis (Mulligan et al., 1965 ; Urquhart et al., 1965 ; Barth et al., 1966) it became necessary to clarify the role of the globule leucocyte with reference to anaphylaxis. At the cellular level this problem resolves into the

question of a possible relationship between the globule leucocyte and the mast cell. This question required investigation because Taliaferro and Searles (1939) reported the presence of atypical mast cells (connective tissue basophils) in the lamina propria of the small intestine during N. brasiliensis infection, while Wells (1962) noted a marked increase in the concentration of mast cells under the same conditions.

The jejunal mucosa of normal and N. brasiliensis-infected rats were examined for the presence of mast cells, atypical mast cells and globule leucocytes with the use of suitable staining techniques. Mast cells, including atypical forms, were not found in any of the sections examined, while globule leucocytes were present in sections from infected rats. It became apparent that these observations were contradictory to those of the previous authors cited , but that this contradiction was resolved if it was assumed that a single cell type was being referred to.

The possibility that globule leucocytes, although apparently morphologically distinct from mast cells, possessed similar functions remained to be determined. Anaphylaxis was induced in the small intestines of immune rats infected with N. brasiliensis by the intravenous injection of an antigen prepared from whole adult worms. The intestines exhibited macroscopic signs of anaphylaxis but no degranulation of globule leucocytes occurred, although such a reaction would normally have been expected of mast cells under the same

conditions (Selye, 1965). However, under certain circumstances anaphylaxis may occur without mast cell degranulation (Lina, 1966), so that a histochemical study of the globule leucocyte granule, using subcutaneous mast cells from the same animal as controls, was undertaken for the purpose of demonstrating whether or not these granules contained heparin, 5-HT and histamine. The results clearly demonstrated the absence of heparin and 5-HT from globule leucocyte granules and there was some evidence that histamine was also absent. On the basis of these collected results it is concluded that the globule leucocyte is functionally unrelated to the mast cell and plays no part in anaphylactic reactions during the immune response to N. brasiliensis.

The results reported above, together with the findings of other authors, notably Dobson (1966), provided a strong basis for attempting to determine the role of the globule leucocyte in the immune response to N. brasiliensis with particular reference to the mechanism of production and transport of cell-bound antibody. The possibility of using immunofluorescence techniques for this study did not appear to be immediately practicable because of possible difficulty in obtaining responses of the required degree of specificity against the probably complex antigenic stimuli derived from nematodes.

Jarrett et al.(1966) and Kassai and Aitken (1966) had demonstrated that infection of rats under four to six weeks of age with N. brasiliensis was tolerated indefinitely, with the consequent abolition of self-cure

in such animals. Neonatal rats infected with N. brasiliensis were therefore examined. In order to differentiate between a globule leucocyte response occurring at a particular age as opposed to a particular phase of N. brasiliensis infection three groups of rats were infected at five day intervals and compared to uninfected controls. A small globule leucocyte response occurred in all infected rats within 30 days of infection, despite the fact that post mortem worm counts had indicated that self-cure did not take place. It was not possible to devise a suitable control group i.e. non-tolerant rats of the same age and weight, so that it could not be stated with absolute certainty that immune unresponsiveness resulted in marked impairment of the globule leucocyte response. However, the globule leucocyte response was very much less than that previously observed in adult rats during self-cure.

Contrary to previous findings, small numbers of globule leucocytes were a constant feature in uninfected neonatal rats. This was not investigated further, but it is tentatively suggested that they represent a low grade response to proteins contained in the colostrum present in the small intestine at this time. As previously noted, these cells resembled certain types of Russell body cell rather more closely than the globule leucocytes normally observed in adult rats.

Review of the literature on thymectomy indicated that this operation, when performed immediately after birth, results in detectable

immunological impairment. In view of the failure to demonstrate conclusively that there was impairment of the globule leucocyte response in neonatal rats tolerant to N. brasiliensis rats were neonatally thymectomised, in the hope of diminishing the immune response to N. brasiliensis in the adults to such an extent that self-cure did not take place. Due to the very low survival rate in neonatally thymectomised rats and also to the fact that many such rats were subsequently found to contain thymic remnants the results of this investigation are preliminary in nature.

Rats were thymectomised at birth and allowed to mature beyond four weeks of age, after which time normal rats become immunologically competent towards N. brasiliensis (Jarrett et al., 1966 : Kassai and Aitken, 1966). They were then infected with N. brasiliensis and killed at different stages of infection. Each neonatally thymectomised rat was compared to a non-thymectomised control rat from the same litter. Successful thymectomy was confirmed at autopsy by examination of serial thorax sections, and the occurrence of lowered lymphocyte percentages in differential blood white cell counts, and by small lymphocytopoenia in the spleen and mesenteric lymph nodes.

Faecal egg counts did not show any marked alteration except that there appeared to be a slight increase in the quantity and duration of egg output in thymectomised rats. Post mortem worm counts, however, clearly indicated that self-cure had not taken place in thymectomised rats, although control rats adhered to the

normal pattern. Examination of sections of jejunum showed that although eosinophil leucocytes were present in equal concentrations in thymectomised and control rats, the number of globule leucocytes was markedly reduced in thymectomised animals. These results are interpreted to indicate that globule leucocytes are derived from cells of the lymphoreticular series and that they are directly associated with the immune response to N. brasiliensis.

The experimental observations reported in this thesis advance knowledge of the globule leucocyte in a number of directions. It may now be stated that the globule leucocyte is associated with nematode and probably other types of parasitic infection. In such infections the globule leucocyte is related to the immune response, almost certainly in connection with antibody manufacture and transport. The cell has a lymphoreticular origin and it probably develops from a plasma cell or from a less differentiated cell of the same series, by a process identical or similar to that which Russell body formation occurs. The location of its progenitors is unknown, but undifferentiated cells in the adjacent mesentery must be considered as a likely source.

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APPENDIX 1

The following papers contain material reported in the present thesis:-

1. WHUR, P. (1965a) Relationship of the globule leucocyte to parasitism. Spring meeting of the British Society for Parasitology, Glasgow.

2. WHUR, P. (1965b) Relationship of the globule leucocyte to parasitism. Parasitology 55:2p (abstract)

3. WHUR, P. (1966a) Relationship of globule leucocytes to gastrointestinal nematodes in the sheep, and Nippostrongylus brasiliensis and Hymenolepis nana infections in rats. J. comp. Path. 76:57

4. WHUR, P. (1966b) Mast cells and globule leucocytes in Nippostrongylus brasiliensis infections. Ann. Conf. Association of Veterinary Teachers and Research Workers, Scarborough.

5. WHUR, P. (1966c) Mast cell and globule leucocyte response to Nippostrongylus brasiliensis infection and to induced anaphylaxis. Int. Archs Allergy appl. Immun. 30:351

6. WHUR, P. (1966d) Globule leucocyte response in hyperimmune rats infected with Nippostrongylus brasiliensis. J. comp. Path. (in press)

7. WHUR, P. and GRACIE, M. (1966a) A method of isolating and counting Nippostrongylus brasiliensis from unweaned rats. Nature, Lond. 209:630

8. WHUR, P. and GRACIE, M. (1966b) Histochemical Comparison of mast cell and globule leucocyte granules in the rat. (submitted for publication)

9. WHUR, P. and JOHNSTON, H.S. (1966) Ultrastructure of globule leucocytes in immune rats infected with Nippostrongylus brasiliensis and their possible relationship to the Russell body cell. J. Path. Bact. (in press).

APPENDIX 2

Statistical Methods

The "t" test was used to demonstrate the statistically significant difference (P less than 0.05) between globule leucocyte concentrations in infected and non-infected rats between days 12-20 of a primary infection (Section III (2)) and days 6-24 of a challenge infection with N. brasiliensis (Section III (4)).