OCCURRENCE OF LYMPHOID CELLS IN THE INTESTINE OF THE GOLDFISH

by

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ABSTRACT

The Goldfish intestine normally contains a large number of lymphocytes, many of them being present in the epithelial layer. After stimulation with antigen, the number of lymphoid cells does not increase, but the proportion of large pyroninophilic cells and plasma cells does. It seems therefore that transformation of B-lymphocytes into plasma cells occurs within the gut epithelium. The Goldfish intestine might be considered a primitive bursa equivalent.

I. INTRODUCTION

Animals have evolved diverse defence mechanisms against foreign matter penetrating their bodies, the most primitive form being non-specific phagocytosis, followed by intracellular digestion or encapsulation. The other form is adaptive immunity, where the threatened organism reacts on the penetrating antigen with the elaboration of a specific antibody or antibodies.

As far as is known, adaptive immunity is characteristic of the vertebrates only. It seems that the evolution of adaptive immunity took place parallel to the evolution of fishes: whereas some primitive fishes like the California Hagfish *(Eptatetrus stoutii)* do not possess any of the criteria of adaptive immunity, amphibians and reptiles show them all.

The Goldfish has long been known to possess two types of immunity, a more primitive cellular one and a humoral one. The first type is illustrated by the experiments of Goodrich and Nichols in 1933 (see Cushing, 1970). They removed scales from their pockets, and exchanged them for others, either from the same Goldfish or from another one. In the first case (autotransplant) the exchange succeeded, whereas in the second case (homotransplant) incompatibility occurred, with reactions varying from inflammation to tissue rejection.

On the other hand, the Goldfish has been proven to be capable of synthesizing specific antibodies, as shown by the work of Uhr et al. (1962), Trump (1970), Trump & Hildemann (1970), Marchalonis (1971) and Everhart (1972). Although immunologically competent cells have not been described in the Goldfish so far, it may be deduced from its ability to form antibodies that it possesses cells of the lymphoid series.

These cells have been found to be present in other fishes. Specific antibody forming cells like plasma cells have been found in the Rainbow Trout (Chiller et al., 1969). These typical cells were found in relation with the anterior kidney (pronephros) or the spleen. Good et al. (1969) found no plasma cells to be present in the lamina propria of fish intestine, and Fletcher & White (1973) suggested that antibody producing cells in the alimentary tract of fish have not yet been recognized.

Fichtelius (1967, 1968, 1969), however, suggested that the intestinal epithelium of all vertebrates (including Teleostei) might be a lymphoid organ, and more precisely an equivalent to the bursa Fabricii of birds. He claims that the lymphocytes homing into the gut epithelium, called from then on theliolymphocytes, are not antigen reactive, but that the cells leaving are.

In the present work, the author therefore undertook to investigate if lymphoid cells (and which types) are present in the epithelium of the Goldfish intestine.

II. MATERIAL AND METHODS

Male and female specimens of the Goldfish, *Carassius auratus* (Linnaeus, 1758), were used, their size ranging from 8-12 cm. In the first stage

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of the present research, the author has tried to draw up an inventory of the lymphoid cells present in normal, healthy fishes. One of the fishes, however, appeared to have a severe liver disease, this organ having a green, granular appearance. This animal will further be referred to as the "hepatitis"-fish.

In the second stage, the author wanted to study the possible development of cells of the B-line after stimulation with an antigen. From the work of Trump (1970), Trump & Hildemann (1970) and Everhart (1972) it was known that the Goldfish shows a good reaction to bovine serum albumin (BSA). At room temperature, antibody production is detected between 7-17 days following stimulation. Development of large pyroninophilic cells and plasma cells (see § III) is therefore to be expected at an earlier stage. Fishes thus stimulated were killed on the 2nd, 4th, 6th, 8th and 10th day.

Stimulation occurred as follows. Six fishes were anesthetized by letting them swim in 1 : 1000 MS222 (Tricaine methanesulfonate, Sandoz S.A.). The moment anesthesia started (indicated by the fishes turning on their sides and falling towards the bottom of the aquarium) they were removed from the narcotic, and laid on a cloth soaked with water, in order not to damage the mucus coating of their scales. While they were wetted regularly, a total dose of 1 mg BSA, diluted in 1 ml bicarbonate saline was injected intramuscularly, on either side of the dorsal fin. The fishes were then transferred into an aquarium with fresh, running water, allowing them to recover from the anesthesia within 1-5 minutes. Five fishes underwent this treatment. The sixth, the "control", was injected with 1 ml saline containing no BSA. The fishes were killed according to the chronological scheme presented before, by incision of the spine just posterior of the gills. The control fish was killed on the last (10th) day of the experiment.

Immediately after killing, the gut was excised, and brought in a bicarbonate saline containing 27.8 mM glucose (Krebs & Henseleit, 1932). The gut then underwent preparation for either light microscopy or electron microscopy.

Light microscopy: for the first stage of the research the guts were fixed in Zenker-formaldehyde-trichloracetic acid fixative (Veldman, 1970). Subsequently, the gut was divided in 16 segments of equal length, which were embedded in paraffin. From these numbered blocks, longitudinal sections were cut with a thickness of 4 μm. These sections were mounted in series and stained with methyl green and pyronine, modified according to Brachet (Veldman, 1970). In this way a survey of the whole gut was ensured.

Electron microscopy: for the first as well as the second stage of the project, guts were fixed in a formaldehyde-glutaraldehyde fixative (Karnovsky, 1965). The fixative was injected with a syringe through the gut, leaving the organ soaked in fixative for 30 minutes at room temperature. Thereafter, the gut was cut into pieces which were put into small jars according to their origin: intestinal bulb, midgut or rectum (Weinberg, 1976), and fresh formaldehyde-glutaraldehyde fixative was added, the fixation being pursued during 3 hours at 20°C. Washing occurred in 0.1 M phosphate buffer during 8 hours, and postfixation was carried out with 1.33% S-collidine buffered OsO_4 during 2 hours. Both washing and postfixation were done at 4°C. After a second postfixation in 1% aqueous uranyl-acetate for 30 minutes at 20°C, the fragments of gut were dehydrated in ethanol, carried through propylene oxide, and embedded in Epon 812 (Luft, 1961). Sections with a thickness of 400-700 Å were cut with glass knives on a LKB Ultratome. Some sections were stained with uranyl-acetate and/or lead-citrate. The sections were examined with Philips EM200 and EM300 electron microscopes, with an accelerating voltage of 60 kV.

III. THE LYMPHOID CELL SYSTEM

The lymphoid cells exist in two distinct populations, called B- and T-cells. This nomenclature finds its origin in the situation as it is found in birds, where stem cells originating from the bone-marrow migrate either to the thymus or to the bursa Fabricii. The cells undergo further differentiation in either of these organs, giving rise to T-cells (thymus-dependent) and B-cells (bursa-dependent), respectively. Most non-avian vertebrates possess B-cells (capable of humoral reactions), though they lack a bursa Fabricii. One or more other organs may play a role as a bursa equivalent in these animals.

Fig. 1 schematizes origin and fate of lymphoid cells. In the bursa (or equivalent) and thymus, the stem cells develop into small lymphocytes. These cells, which cannot be distinguished by morphological data [2]), possess different immunocompetent

[2]) It has recently become possible to distinguish these cells by means of scanning electron microscopy and of freeze-etching techniques. However, both techniques have only been applied to isolated cells from the peripheral blood, and not to cells lying within a tissue.

properties. Hence, we call them B-lymphocytes and T-lymphocytes, respectively. When the organism is now stimulated with an antigen, B- and/or T-cells alike develop into "blast"-like cells. These "blasts", which contain many polyribosomes (RNA) stain with pyronine, and are therefore called "large pyroninophilic cells". These "blasts" undergo further differentation into plasma cells (= stimulated B-cells) or stimulated T-cells. This last type of cell, morphologically speaking a small lymphocyte, is responsible for the cellular immune response, where the antigen (virus

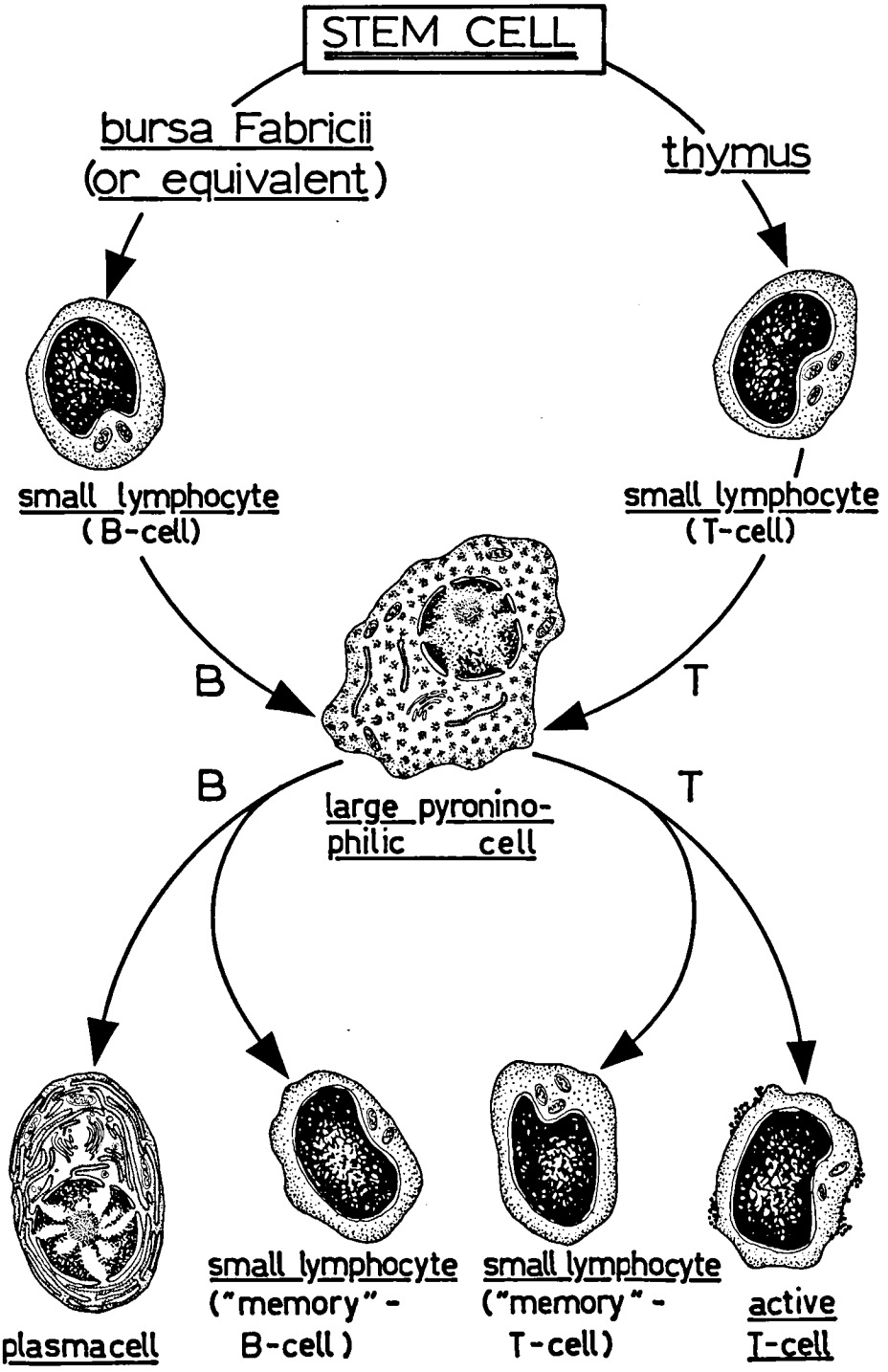

Fig. 1. Origin and fate of lymphoid cells. Differentiation of B-cells and T-cells.

or bacterium for instance) is immobilized, and then chemically rendered inoffensive.

The plasma cell combats the antigen by way of the humoral immune response, i.e. synthesis of specific antibodies (immunoglobulins). These antibodies are released into the bloodstream, and neutralize or destroy the antigen.

In the same time, "memory"-B-cells and "memory"-T-cells are formed, which are small lymphocytes, capable of quick reaction during a following contact with the same antigen (immunological memory). On a morphological basis, these are also small lymphocytes, and it is therefore impossible, most of the time, to distinguish between B- and T-cells, except for the plasma cell which is typical of the B-line.

An excellent review on the cells and tissues of the immune system is given by Weiss (1972).

IV. RESULTS

1. Normally present lymphoid cells

In the epithelium of the intestinal folds of the Goldfish numerous motile cells are encountered (McVay & Kaan, 1940; Yamamoto, 1966; Weinberg, 1976) with small, dark-staining nuclei and a very small rim of cytoplasm. The same cell type is found in the lamina propria. Fig. 2a shows a detail of an intestinal fold. The dark bands in the epithelial cells are due to RNA in the cytoplasm (ribosomes), being stained by pyronine. The clear zones correspond to the terminal web (Yamamoto, 1966; Gauthier & Landis, 1972; Weinberg, 1976), containing no organelles, and the supranuclear cytoplasm, containing mainly mitochondria. The nucleoli of the epithelial cells are also clearly visible. The small cells appear to be lymphocytes and their number amounts to an average of some 40% of the total number of cells within the epithelial layer. No appreciable difference was to be encountered between any of the parts of the gut in this respect. The lymphocytes are nearly all restrained to the basal part of the epithelium.

The main purpose of the methyl green and pyronine staining method was to discover the existence of large pyroninophilic cells. Although they were encountered, their number was extremely low. Fig. 2b shows half an intestinal ridge, in the lamina propria of which only one pyroninophilic cell was encountered (arrow). This situation is normal for an unstimulated fish. Fig. 2c shows the detail of a large pyroninophilic cell, with its typical dark staining cytoplasm (containing many ribosomes) and large nucleolus. This type of cell was encountered mainly in the lamina propria, in or near capillaries, but they were sometimes found in the basal part of the epithelium.

On account of the extreme scarceness of pyroninophilic "blasts" and owing to the fact that light microscopy did not allow the identification of one single plasma cell, these types of cells were not expected to occur in the (much smaller) sections which were prepared for electron microscopy. This assumption proved to be correct, but electron microscopy allowed the small cells to be identified as typical small lymphocytes (fig. 2d).

These cells, lying in the basal part of the epithelium, have nuclei containing dense heterochromatin which are surrounded by a narrow rim of organelle-less cytoplasm.

Luck helped the author a little further. In one of the fishes examined, there seemed to be a local viral infection. The particles lying between the epithelial cells were polyhedral, and had a diameter of about 900 Å. One of the epithelial lymphocytes (fig. 2e) was found to have bound a great number of these particles to its surface, seemingly engaged in some immune reaction. The cell was therefore identified as a T-lymphocyte, which seemed to indicate that at least this type of lymphoid cell is to be encountered in the epithelium of the Goldfish intestine.

2. Lymphoid cells after stimulation

The fish that were used in the second stage of this research yielded some interesting results. The "control"-fish (injected with saline only) showed the same pattern as the one previously described for normal fishes.

Stimulated fishes, however, possessed epithelial large pyroninophilic cells and plasma cells, in addition to the normally present small lymphocytes. The total number of epithelial lymphoid cells did not seem to have increased. The new cell types were encountered in fishes killed 2, 4 and 6 days after injection with BSA, and in the "hepatitis"-fish. Because stimulation with antigen seemed to induce a complete cellular transformation of the B-cell types within two days, the fishes killed on day 8 and 10 were not examined.

Fig. 3a shows a motile lymphocyte between two epithelial cells. The cell, coming from the "hepatitis"-fish, is visibly more active than the usually encountered lymphocytes (cf. fig. 2d). Its organelles lie on one side of the nucleus, a feature typical for active lymphocytes. Mitochondria, rough en-

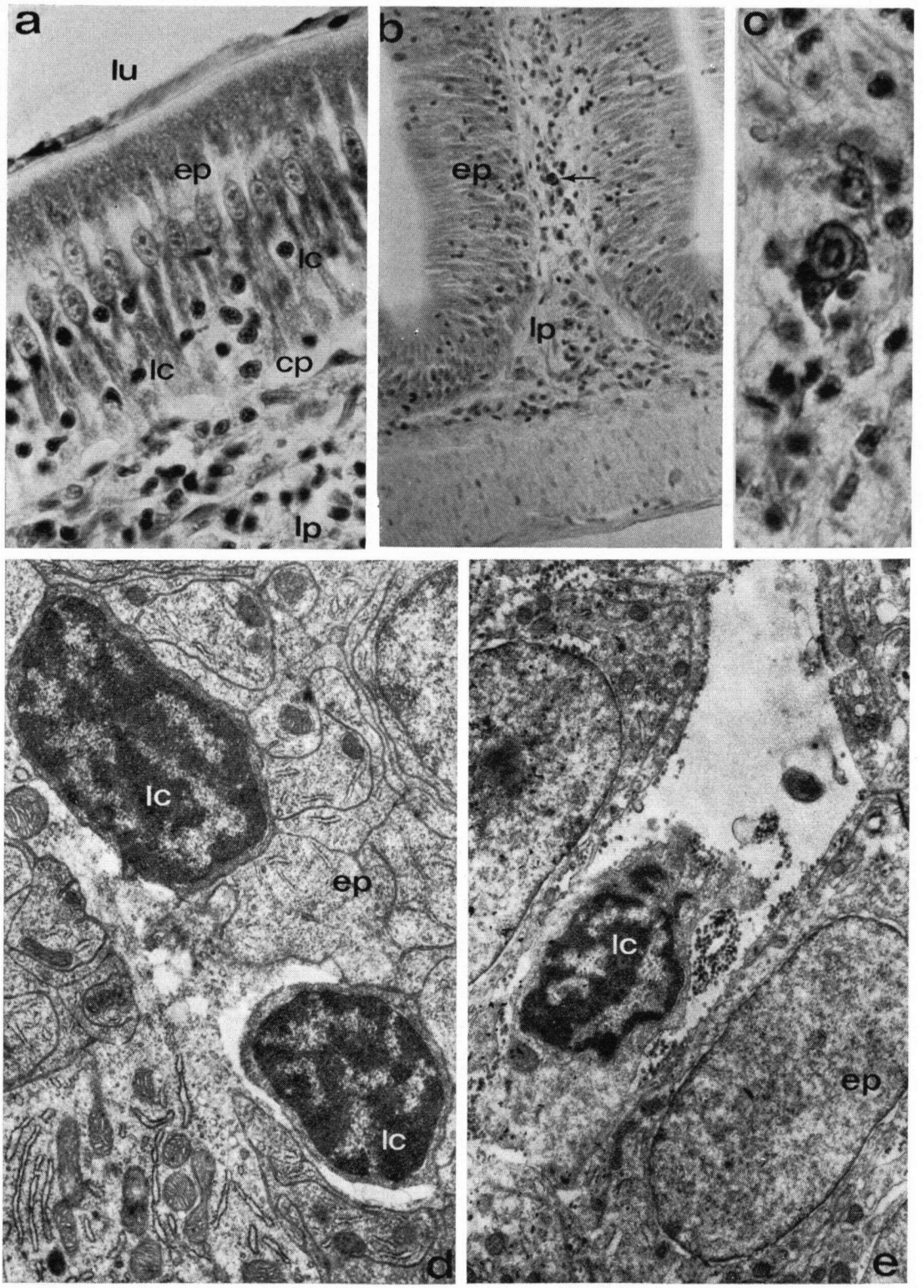

Fig. 2. (a) Detail of epithelium and lamina propria of an intestinal fold containing a large number of theliolymphocytes with heavily stained nuclei; 865 ×. (b) Detail of an intestinal fold showing one solitary pyroninophilic "blast" in the lamina propria; 340 ×. (c) Large pyroninophilic cell, the cytoplasm containing many ribosomes and the nucleolus are stained with pyronine; 1 280 ×. (d) Two small lymphocytes lying in the basal epithelium; 15 000 ×. (e) T-lymphocyte with viral particles bound to its surface; 10 200 ×. cp = capillary; ep = epithelial cells; lc = lymphocyte; lp = lamina propria; lu = lumen.

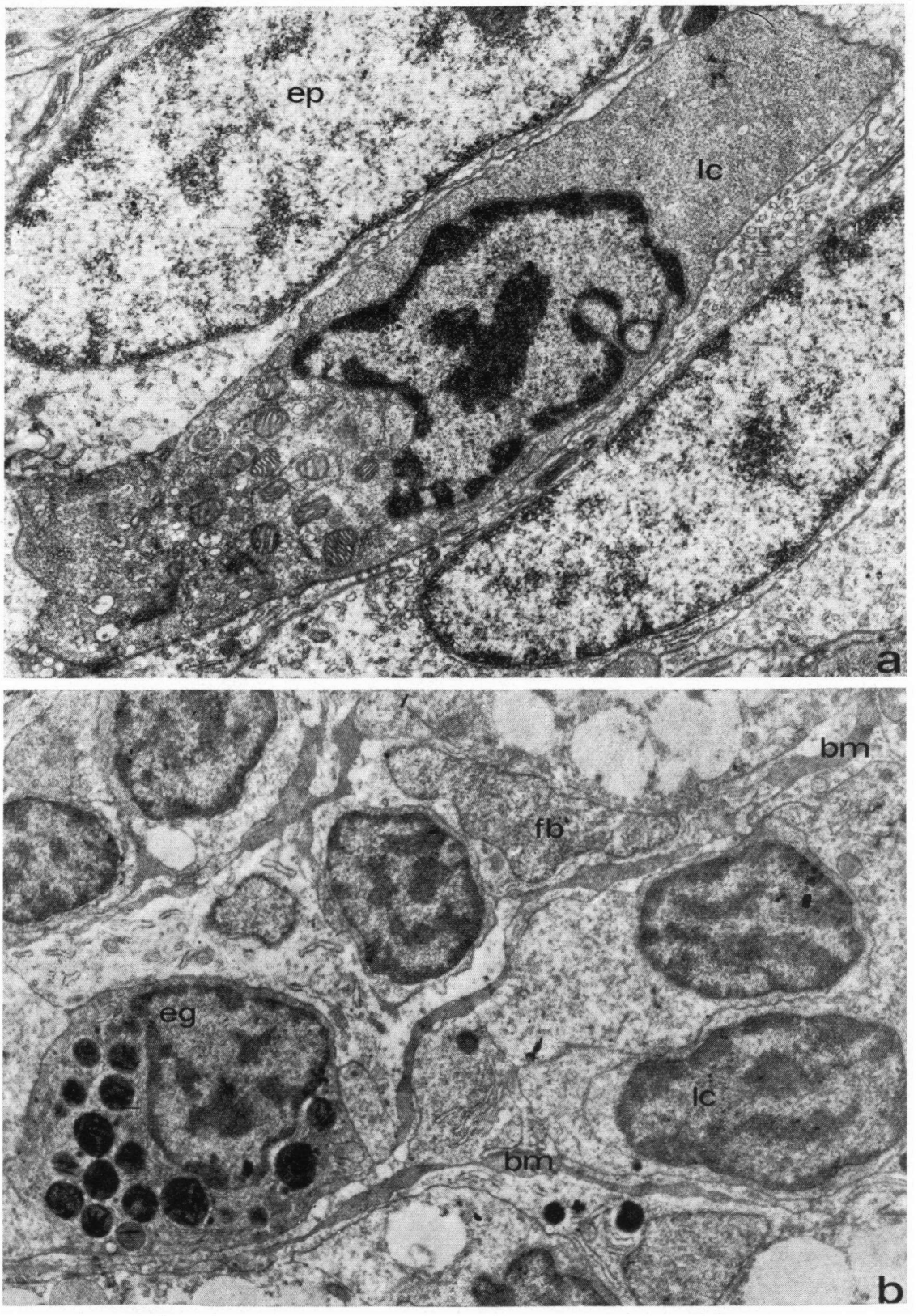

Fig. 3. (a) Motile medium lymphocyte between two epithelial cells, with organelles lying on one side of the cell; 14 850 ×. (b) Eosinophilic granulocyte in the lamina propria; 10 000 ×. bm = basement membrane; eg = eosinophilic granulocyte; ep = epithelial cell; fb = fibroblast; lc = lymphocyte.

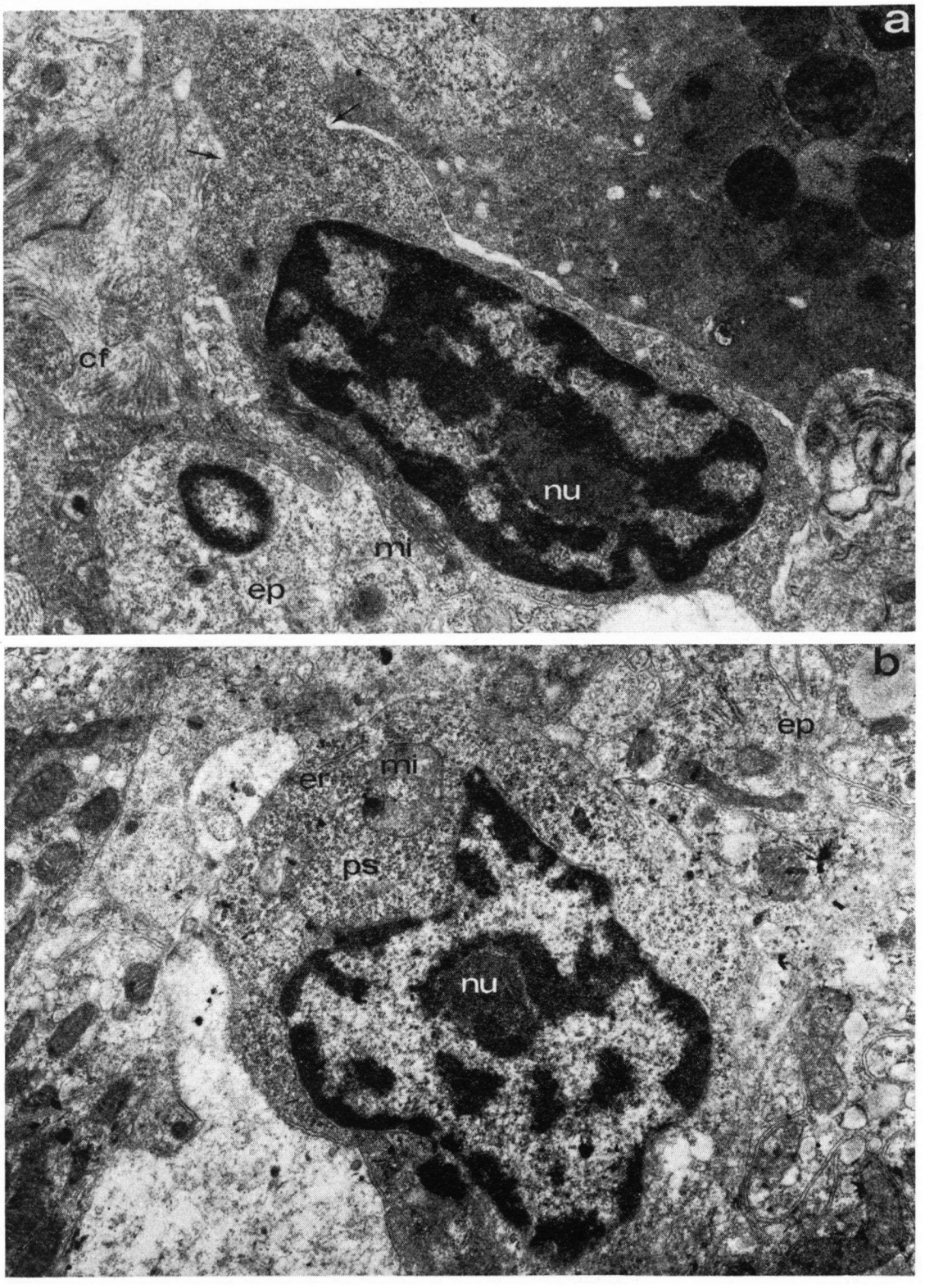

Fig. 4. (a) Large pyroninophilic cell engaged in diapedesis; the cell is leaving the epithelium and penetrating through the basement membrane (arrows); 16 600 ×. (b) Large pyroninophilic cell in the basal part of the epithelium, its cytoplasm containing many polyribosomes, some mitochondria and a few profiles of rough endoplasmic reticulum; 16 600 ×. cf = collagen fibres of the basement membrane; ep = epithelial cell; er = endoplasmic reticulum; mi = mitochondrion; nu = nucleolus; ps = polysomes.

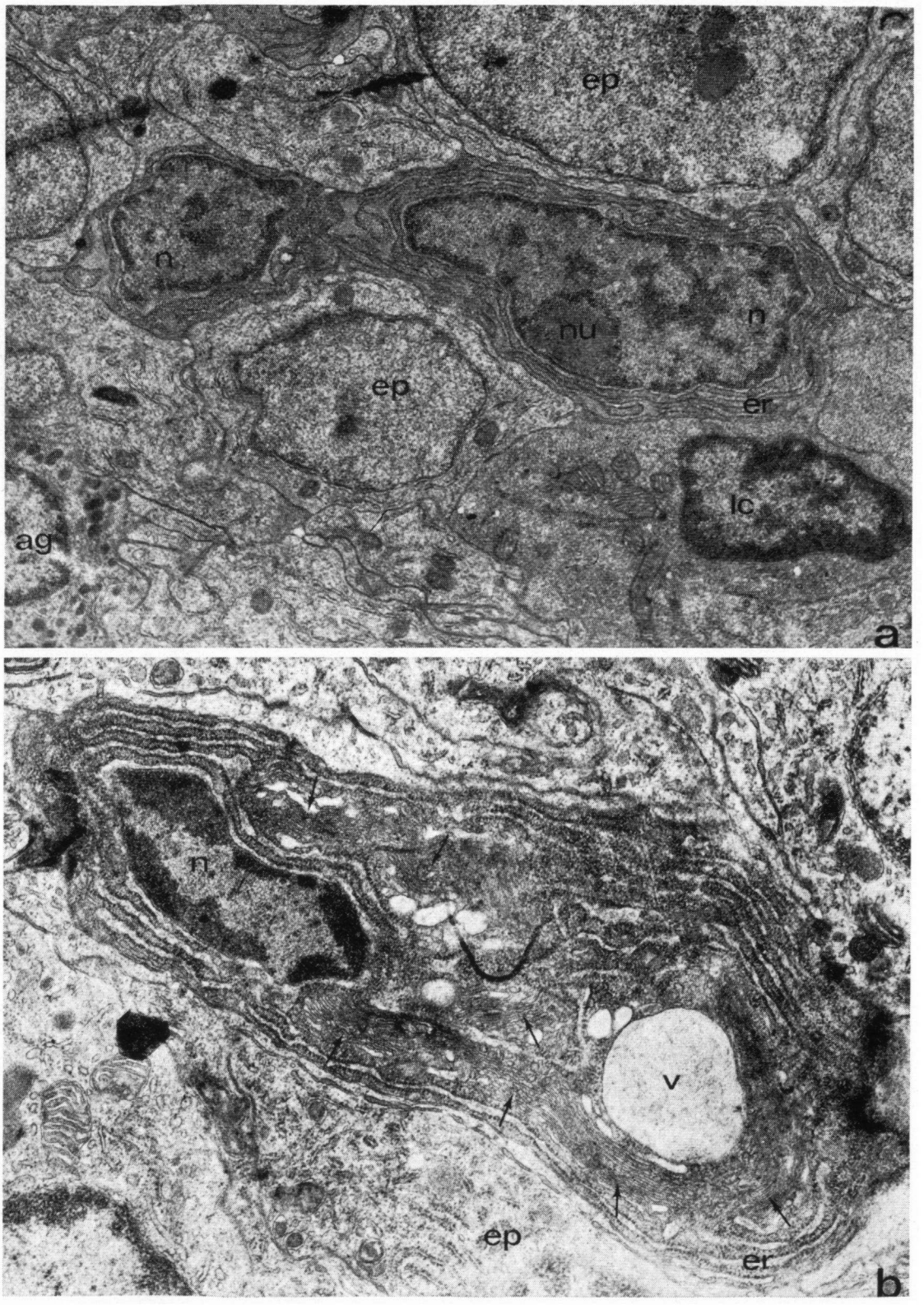

Fig. 5. (a) Plasma cell between epithelial cells, with endoplasmic reticulum presumably containing antibody; the lobated nucleus has been cut twice; 14 600 ×. (b) Plasma cell; the numerous Golgi complexes (arrows) seem to indicate that the cell is engaged in active protein synthesis; 23 000 ×. ag = argentaffin cell; ep = epithelial cell; er = endoplasmic reticulum; n = nucleus; nu = nucleolus; lc = lymphocyte; v = vacuole.

doplasmic reticulum and a Golgi apparatus are to be found in the granular cytoplasm. According to its size (3 × 11.5 μm) this is a medium lymphocyte.

Four and six days after stimulation, large amounts of granulocytes were found to have infiltrated the lamina propria. According to Weiss (1972) this type of cell may accompany an immunological reaction. Fig. 3b shows such a cell, lying amidst some small lymphocytes. This cell seems to be an eosinophilic granulocyte, to judge by the needle-like crystals contained by the granules.

Fig. 4 shows two large pyroninophilic cells. Both cells were encountered on day 4, and show a distinct nucleolus. The cell of fig. 4a is engaged in diapedesis (arrows) and its cytoplasm contains many ribosomes. The other cell (fig. 4b) shows typical marginal heterochromatin, and the cytoplasm contains polyribosomes, some mitochondria and a few profiles of rough endoplasmic reticulum.

Fig. 5a shows a medium lymphocyte and a plasma cell encountered on day 2. The plasma cell closely resembles the homologous cell type encountered in mammals, with a conspicuous rough endoplasmic reticulum of flattened appearance, being dilated in some spots. It presumably contains antibody. The nucleus is probably lobated, and was cut twice in this plane of section, giving the cell a double-nucleated appearance. Fig. 5b, finally, shows a similar cell, found in the "hepatitis"-fish. The many Golgi complexes (arrows) suggest that this cell is engaged in active protein synthesis.

V. CONCLUSION

Lymphocytes are common in the gut epithelium of fishes. Poor antibody producers seem to have low numbers of theliolymphocytes, whereas fishes with good antibody production possess higher numbers of these cells (Fichtelius, 1969). The present study confirms these facts. The Goldfish is a good antibody producer (Uhr et al., 1962; Trump, 1970; Trump & Hildemann, 1970; Marchalonis, 1971; Everhart, 1972), and the number of theliolymphocytes we encountered (some 40%) equals the percentage quoted by Fichtelius (1969) as typical for good antibody producers.

However, as far as we know, the typical transformation of lymphocyte into large pyroninophilic "blast" and plasma cell has not yet been demonstrated in the teleost gut. Even their presence was questioned (Good et al., 1966; Fletcher & White, 1973). The present study may therefore contribute to the idea that the gut epithelium in lower vertebrates acts as a diffuse bursa Fabricii, a thought that was first brought forward by Fichtelius (1969). Further investigation, especially regarding the homing of lymphoid precursor cells into the intestinal epithelium during embryonic development, should confirm this hypothesis.

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