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Reticuloendothelial system of defense in *Oreochromis mossambicus* (Peters)

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ABSTRACT

The reticuloendothelial system (RES) was studied *in vivo* in *Oreochromis mossambicus* by injecting colloidal carbon particles. Histological and cytological examination of the organs from 2 hours to day 10 after injection revealed that the major organs of particle localization in *O. mossambicus* were spleen, kidney, atrium of heart and dermis. Among these organs, spleen showed high antigen trapping ability in its ellipsoids followed by kidney. In the heart the ventricular endocardial cells were more phagocytic than the atrial endocardial cells.

Introduction

Reticuloendothelial system (RES) is the system of phagocytic cells dispersed throughout the body to remove dead cells and particulate matter from circulation. In fishes the reticuloendothelial system is also the most efficient clearing system during a microbial attack and is an important means of defense during the early stages of development before immunocompetence is attained (Tatner and Manning, 1985). In teleost fish, RES consists of pro-monocytes of the haemopoietic organs, monocytes of the blood, macrophages of the loose connective tissue, free and fixed macrophages of spleen, kidney and fixed macrophages of the atrial lining of heart. (Mackmull and Michels, 1932; Ellis *et al*., 1976; Ellis, 1978, 1980, 1982, 1989; Mori, 1980; Secombes and Manning, 1980; Ferguson *et al*., 1982). Dalmo *et al*. (1997) is of the view that other organs like liver, intestine and skin may also be included in the reticuloendothelial network. The objective of the present study was to find out the clearing mechanism in a tropical fish and to assess whether there is any difference in their reticuloendothelial defense network with other fishes.

Materials and methods

Experimental protocol

Healthy *Oreochromis mossambicus* (mean body weight 200 ± 5g) obtained from a commercial fish farm was used for this study. Seventy fish were maintained in a 10 m^3 circular fibre glass tank. The fishes were fed with a formulated pelleted diet @ 2 % body weight twice daily. Seventy five per cent of the water was changed daily. During the two week acclimatization period, the fishes were observed daily for any unusual behaviour, morphological changes and mortality. The water quality parameters were also

checked on alternate days. The mean water quality parameters like temperature, pH and dissolved oxygen were 28 ± 0.5º C, 8.2, and 6 ppm respectively.

Intraperitoneal injection of colloidal carbon

Indian ink (Camlin Ltd.) was diluted in normal saline (0.85 % NaCl) in the ratio 1:10. The thoroughly mixed suspension was centrifuged and then autoclaved. 0.5 ml of this mixture was injected intraperitoneally to sixty fish. Five fish were sacrificed at intervals of 2, 8, 16, 24, 48 h and 4, 8 and 10 days after the injection.

Histological and cytological studies

Kidney, spleen, dermis and heart were dissected out and fixed in 10% buffered formalin for 24 hours. The tissues were dehydrated and embedded in Paraffin wax. Sections of 6µm thickness were cut using a rotary microtome. Deparaffinized sections were bleached in 20 % hydrogen peroxide for 36-48 h. to decolorize the melanin pigment (Ellis *et al*., 1976). The sections were stained with haematoxylin and eosin. The histological sectioning and staining were done following the standard procedure of Weesner (1960). The prepared slides were examined under a compound microscope and photomicrographs taken.

Blood and peritoneal impression smears were prepared on clean, grease free sterilized glass slides. The slides were stained by Pappenheim method (Combined May-Grunwald's staining) (Schaperclaus, 1986) for obtaining the differential cell count of leucocytes. The slides were then thoroughly washed with de-ionized distilled water, air dried and mounted in DPX (Ranbaxy Chemicals). The mounted slides were observed under the microscope (Olympus, 100 X with oil immersion) and photomicrographs taken.

Results

Histology

Spleen **:** Two hours after intraperitoneal injection of colloidal carbon, the ellipsoids of spleen contained occasional carbon laden cells. By 8 hours moderate numbers of carbon containing cells were seen and mostly restricted to the ellipsoids and the ellipsoidal sheath. At 16 hours, large numbers of carbon containing cells were observed in the ellipsoidal sheath. Occasionally, a few cells were seen migrating towards the parenchyma. Numerous carbon containing cells were still present in the ellipsoids at 24 hours and the parenchyma was observed to harbour large number of carbon containing cells at this stage (Fig. 1). By 48 hours, there was an increase in the presence of carbon containing cells in the parenchyma around the ellipsoids (Fig.2). By day 4, a decrease in the number of cells containing carbon was noticed in the ellipsoids. Carbon containing cells were found forming aggregates in the parenchyma and some cells were found to accumulate in the melanomacrophage centres.

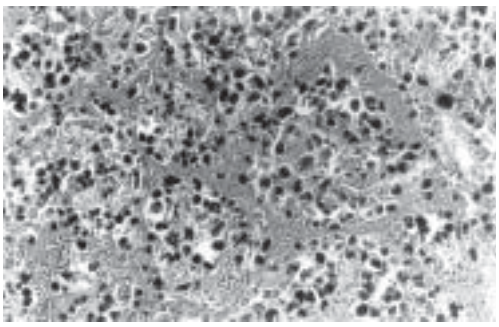

Fig. 1. Section of the spleen at 24 hours showing macrophages with ingested carbon in the ellipsoids . (H & E x 200)

On day 8, carbon aggregates were seen mostly in association with melanomacrophage centres and the number of carbon containing cells in the ellipsoids and ellipsoidal walls dropped

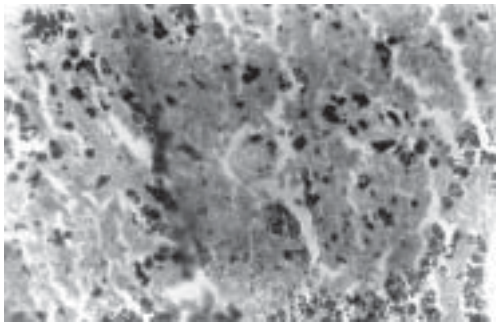

Fig. 2. 48 hour section of the spleen showing carbon particles in the parenchyma. (H & E x 200)

(Fig.3). On day 10, the numbers of carbon containing cells were very much reduced even in the melanomacrophage centres and only a few carbon containing cells observed in the ellipsoids.

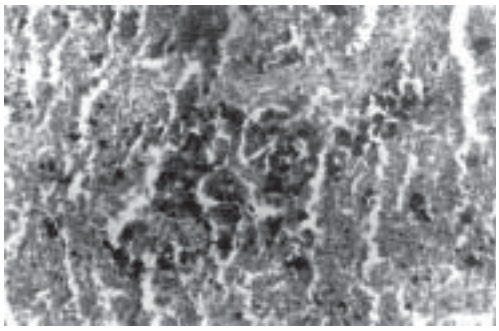

Fig. 3. Spleenic section on day 8 showing carbon aggregation in the melanomacrophage centres (H & E x 400)

***Kidney*:** Colloidal carbon particles were seen in the kidney 2 hours after intraperitoneal injection of carbon particles. After 8 hours, moderate number of cells containing carbon particles was observed in the kidney haemopeitic tissues. In 16 and 24 hour post-injection kidney sections, the carbon containing cells continued to increase and appeared as small aggregates in some areas. Carbon containing cells continued to aggregate and localize in some areas of the haemopoietic tissue after 48 hours. During this time small numbers of carbon containing cells started appearing in the glomerular capillaries and intertubular blood vessels (Fig.4). By day 4, the number and size of aggregates increased and reached a peak level on day 8. On day 10, the aggregations were still seen in the interstitial tissue, but the overall carbon content in the kidney decreased.

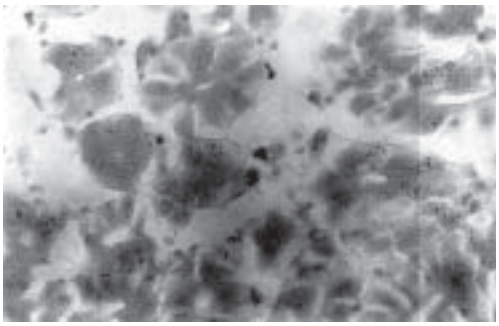

Fig. 4. Section of the kidney showing carbon bearing macrophages in the interstitial tissue after 48 hours. (H & E x 400)

***Heart* :** Carbon was mostly seen in the epicardial lining cells and also in free cells lying in the lumen of the heart after 2 hours of intraperitoneal injection. No carbon was present in the endocardial lining cells of atrial trabeculae. Eight hours after injection, the endothelial lining of ventral aorta revealed the presence of carbon. Endocardial cells lining the atrium also had carbon (Fig.5). After 16 hours, the entire valvular lining and ventricular endocardial lining contained large amount of carbon. During this time the endocardial linings of the atrial trabeculae showed the presence of carbon and this was more than what was seen in the sections taken after 8 hours. Many of the endocardial carbon containing cells got detached from the trabeculae, appeared round in shape and found lying free in the lumen of heart. The cardiac sections taken after 24 hours showed further increase in accumulation of car-

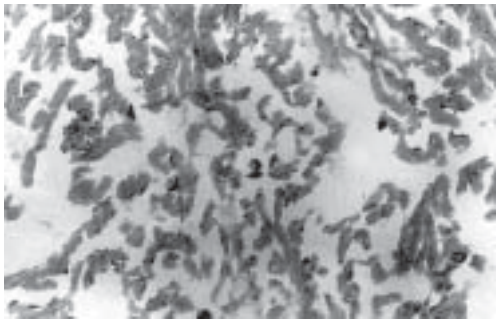

Fig. 5. Atrial sections showing endocardial cells with carbon after 8 hours. (H & E x 200)

bon in the endocardial lining cells of atrium. The section taken after 48 hours indicated even more accumulation of carbon in the endocardial cells of the atrium. These cells were filled with colloidal carbon. The 4th day sections of the heart showed a mild reduction in the carbon present in the endocardial cells of the atrium. Sections of day 8 post injection showed further reduction of carbon in the atrial lining cells. By day 10, most of the endocardial cells did not contain any carbon. No aggregation of carbon containing cells was seen in the atrium.

Dermis **:** Very few carbon containing cells were observed after 2 hours of intraperitoneal injection of colloidal carbon particles. After 8 hours, carbon accumulation was seen. There was an increase in the carbon containing cells after 16 hours. No further change was observed after 24 hours. Sub-cutaneous carbon accumulation was seen in the epidermis as well as melanomacrophage centres in the dermis after 48 hours. Histological sections on the 4th day showed more carbon accumulation in the melanomacrophage centres in the dermis. Mild aggregation of carbon containing cells was seen in the subcutaneous tissue. On day 8 also accumulation of carbon in the epidermis and subcutaneous tissue was seen. The aggregation of carbon containing cells showed an increase on day 10.

Cytology

Impression smears prepared from peritoneum indicated presence of carbon containing cells up to 48 hours. The touch smears did not show any carbon containing cells on 4th, 8th and 10th days. Blood smears prepared in fishes injected with colloidal carbon had carbon containing macrophages and granulocytes until 48 hours. Mild uptake of carbon was also observed in some erythrocytes and thrombocytes. Blood smears prepared after 4th, 8th and 10th days did not have any carbon particles in the blood cells.

Discussion

The study revealed that the major organs of antigen localization were spleen, kidney, atrium and peritoneum. Among the three organs studied, carbon localization first occurred in spleen followed by kidney. Appearance of carbon occurred late in atrium. Massive amount of carbon appeared in spleen. In teleost fishes the antigenic materials are trapped in three locations namely spleenic ellipsoids, sinusoidal blood vessels, reticular phagocytic cells of kidney, atrial endocardial cells and ventricular endothelial cells of heart and to some extent liver and skin (Zapata and Cooper, 1990; Dannevig *et al*., 1990, 1994; Press *et al*., 1994; Nakamura and Shimozawa, 1994; Zapata *et al*., 1996; Dalmo *et al*., 1997).

It is believed that the colloidal carbon particles injected into the peritoneal cavity are taken up by the circulating blood phagocytes and transported to various reticuloendothelial tissues. This is substantiated by the fact that carbon containing particles were first seen in blood cells and cells of the peritoneal smears after 2 hours and during this period the reticuloendothelial organs contained very little carbon. Subsequently, an increase in the carbon content was noticed in the reticuloendothelial organs especially spleen which showed the maximum amount of carbon containing cells. A simi-

lar observation was made by Ellis *et al.* (1976) in the plaice *Pleuronectes platessa* (L.). They stated that since fish did not have a diaphragm, they are capable of absorbing particles from the peritoneal fluid transport them to the reticuloendothelial tissues *via* the vascular system.

The macrophages, granulocytes and erythrocytes from the peritoneal exudate and blood smears were found to contain carbon particles from 2 to 48 hours. Our observations were in contrast to that of Ellis *et al.* (1976) who noticed carbon containing cells only after 48 hours in the plaice *Pleuronectes platessa*. Erythrocytes and thrombocytes are weakly phagocytic and there is still no information about their capacity to kill and digest intracellularly (Secombes, 1996). Page and Rowley (1984) observed carbon localisation from 3 hours to 12 days of post injection in the granulocytes circulating freely in the blood in adult lamprey, *Lampetra fluviatilis* (L.). The authors further noted that the percentage of phagocytic granulocytes was only 0.8 – 2 %, rarely lymphoblasts also contained carbon. They also reported changes in the morphological appearance of leukocytes containing carbon in the blood or peritoneum. In our study such morphological changes were not noticed.

In the kidney also carbon containing cells in the blood vessels and sinuses migrated to the interstitial tissues and formed aggregates. Dannevig *et al.* (1990) reported that tissue endothelial cells of kidney are phagocytic (endocytic also) in a receptor dependent manner. The presence of carbon containing cells in the kidney after 48 hours observed in this study strengthens the views of Ellis *et al.* (1976) that kidney renal portal system might provide a filter bed to trap blood borne phagocytes containing carbon.

Peleteiro and Richards (1990) described phagocytic cells in the epidermis and melanomacrophage centres in the dermis which could trap antigenic materials. They also described migration of macrophages from dermis to epidermis in rainbow trout *Salmo gairdneri* (R.). The present study has also recorded carbon accumulation in the melanomacrophage centres of both epidermis and dermis but the extent of accumulation was mild when compared to spleen or kidney indicating that it plays only a limited role in antigen trapping.

Initially carbon was seen in the epicardial cells of heart. It was only later that the atrial endocardial cells were filled with carbon particles. By 48 hours there was a peak of carbon accumulation in the atrium. There was no aggregation or storage of carbon in the atrium. The atrial endocardial lining cells of *O. mossambicus* were phagocytic for carbon particles. This was also reported by a number of authors in tilapia and other fishes (Mackmull and Michels, 1932; Ellis *et al.*, 1976; Ellis, 1980; Mori, 1980; Nakamura *et al.*, 1992; Nakamura and Shimozawa, 1994; Dalmo *et al.*, 1997). Dalmo *et al.* (1995) obtained a time dependent uptake of injected immunomodulator laminarian in the endothelial cells of atrium and ventricle of the cod, *Gadus morhua* (L.), but got no response in a similar study in the Atlantic salmon *Salmo salar* (L.) (Dalmo *et al.*, 1995). Another feature found in the present study was that ventricular endocardial cells were phagocytic (endocytic). This is the first report in *O. mossambicus*. Similar observations were made in Amazon molly (*Poecilia formosa* (P.) and in the medaka, *Oryzias latipes* (Temminck and Schlegel) by Woodhead (1981) and Nakamura and Shimozawa (1994) respectively. The latter also reported that there are species differences of phagocytic activity in fish endocardial cells. The reason for this selective phagocytic nature of the ventricular lining macrophages is to be further studied as it is not known whether it is a feature

restricted only to highly evolved fishes. There was no aggregation or storage of carbon in the atrium but, free and rounded carbon containing cells were found in the lumen of heart. This observation did not mean that the atrial endocardial cells were less phagocytic when compared to those of spleen and kidney. This fully agreed with the views of Mackmull and Michels (1932) , Ferguson (1975) and Ellis *et al*. (1976) who described that the atrial carbon containing cells rounded up and became free phagocytes (as observed in this study also) which might migrate to other organs.

In the present study, it was noticed that the major organ of antigen localisation was spleen, where the cells containing carbon particles were first seen in the ellipsoids, migrated to the parenchymal tissues and finally aggregated in melanomacrophage centres. This differed from the views of Ellis *et al*. (1976) and Ferguson *et al*. (1982) who observed that kidney was the major organ of antigen localization. MacArthur *et al*. (1983) studying the fate of injected ^{51}Cr-TRBC in plaice *P. platessa* reported that kidney had higher counts due to its larger size.

The first observation of carbon particles in the spleenic ellipsoids, then in the parenchyma and finally in the melanomacrophage centres were consistent with the observation of Ferguson (1976), Ellis *et al*. (1976) and Zapata *et al*. (1996). The diffusion of carbon particles from the ellipsoids into the parenchyma observed in this study may be due to the open blood circulation of spleen (Zapata, 1983). Bach *et al*. (1978) emphasised the importance of spleenic ellipsoids and suggested that ellipsoids were specialised areas of phagocytosis where few bacteria were seen passing to the pulp spaces beyond. The reticular cells of the pulp yield much greater surface area over which the blood can percolate so that antigens were more dispersed and not visibly concentrated. A low response obtained in the spleen by several authors after an intraperitoneal injection of antigenic material might be due to this reason.

Though it was observed that spleen was the major organ of antigen localisation, other workers have presented different views. Press *et al*. (1994) and Haaparanta *et al*. (1996) had observed great differences in the morphology of spleen between individuals of the same species. Quesada *et al*. (1990) is of the opinion that differences in the antigen trapping capability of spleen are proportional to the ratio of its red and white pulp contained in it. They observed a difference in immunological capability of spleen in roach and perch and held the view that this could be the case for other fish species also. Agius (1979) using different stains emphasized the role of melanomacrophage centres of spleen in the metabolism of iron in normal and starved *Seratherodon mossambicus* (P.) (= *O. mossambicus*) and indicated that spleen could be the major organ of defense in fishes. However, Sailendri and Muthukaruppan (1975) stated that *O. mossambicus* spleen also contained a well defined red and white pulp of which the latter is poorly developed. In the present study not much difference was noticed in the antigen trapping capacity of spleen between individual fishes in normal haematoxylin and eosin staining. Herraez and Zapta (1986) observed that after administering SRBC, erythrocytes first appeared in cytoplasm of the spleenic ellipsoid macrophages and then only in the phagocytic reticular cells / macrophages of kidney in the gold fish *Carassius auratus* (L.). The findings of the above authors give credence to our view that spleen was more important than kidney as an antigenic trapping organ. *In vitro* studies using isolated and adhering phagocytes from the reticuloendothelial

organs revealed that spleenic phagocytes showed maximum phagocytic activity (Nair, 1996).

In our study aggregation of carbon particles in the melanomacrophage centres was observed. The principal locations of melanomacrophage centres in fish are epidermis, dermis, hypodermis, kidney, spleen and liver (Agius and Roberts, 1981). The precise function of these melanomacrophage centres is still doubtful (Wolke, 1992). Several authors describe the centres as depots for metabolic wastes, centres for antigen trapping, processing and as a place to centralize exogenous and endogenous material for destruction and detoxification or recycling (Roberts, 1975; Agius, 1979, 1980, 1981, 1985; Ellis, 1978, 1982, 1989; Secombes and Manning, 1980; Vogelbein *et al*., 1987). Zapata *et al*. (1996) were of the opinion that doubts still persisted on the antigen containing macrophages, whether they moved to pre-existing melanomacrophage centres as proposed by Mori (1980) or form aggregates as observed by Ellis *et al*. (1976). Another factor which influenced the above phenomenon was the amount of engulfed materials in the macrophages and number of melanomacrophages present (Herraez and Zapata, 1986). In teleost kidney, melanomacrophage centres are distributed randomly throughout the lymphohaemopoietic tissues with those containing melanin being more abundant in the pronephros than in mesonephros (Zapata *et al*., 1996). We observed carbon containing cells forming aggregates in the kidney and being deposited in the melanomacrophage centres of spleen. The reason for this varied response of the injected particles in the spleen and kidney in our study is not known.

Thus the non specific mechanism of reticuloendothelial system is very important to fish. Though there is some agreement in the current knowledge on the tissues and organs which constituted the reticuloendothelial system, significant differences still exist between various fish species and sometimes even among fishes of the same species. Thus more studies are still needed to understand the major organs of clearance of antigenic materials in different teleost fishes.

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