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Melanomacrophage aggregations in parasite-infected toads, *Bufo regularis*

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ABSTRACT

The amount and distribution of melanomacrophage in *Bufo regularis* was studied during parasite infection by light and electron microscopy. These cells contain massive amounts of melanin, substantial lipofuscin, and some hemosiderin consistent with their phagocytic function. Similar isolated pigmented macrophages are solitary in the liver, spleen, lung and kidney. The number and size of the hepatic aggregations increase almost with progression of the parasite infection. This increase may result from hepatic recruitment of macrophage during parasite infection, and suggests that the size and number of melanomacrophage aggregations may serve as a biological marker for parasitic infection.

Keywords: Toad melanomacrophages, pigment cells, *Bufo regularis,* amphibian parasites.

INTRODUCTION

Melanomacrophage aggregations are discrete groups of large pigmented cells in the hematopoietic and certain other soft tissues of lower vertebrates (Wolke 1992; Meseguer *et al.* 1994; Zuasti *et al*. 1998). They are concentrated in the liver of primitive fishes (Agnatha & Chondrichthyes) and in the kidney and spleen of advanced teleosts, reflecting the lympho-reticular evolutionary relationships (Agius 1980). Ellis *el al*. (1976) found the spleen of teleosts (plaice) to be a center of melanomacrophage activity where resident cells can consume carbon particles. Other macrophage possibly derived from reticulo-endothelial cells, can phagocytize carbon injected into the abdominal cavity, enter the spleen, and join aggregations there (Roberts 1974). Zuasti *et al*. (1990) pointed out that the melanin in these aggregates has historically been believed to have been phagocytized, synthesized elsewhere (Agius 1985; Blazer *et al.* 1987; Buke *et al*. 1992). However, they demonstrate tyrosinase activity in these cells consistent with the presence of melanosomes in the mesentry of one fish species and in the mesentry and spleen of another species. They found that these cells in a third lacked tyrosinase activity, suggesting that the organs in these cells produce or phagocytize melanin which varies among different taxonomic groups. Roberts (1974) and others have demonstrated that these cells can act as scavengers of material of host origin, generating lipofuscin as a byproduct (Spazier *et al.* 1992; Stensvag *et al*. 1999). They have been found to contain hemosiderin consistent with phagocytization of hemoglobin degradation products (Macchi *et al*. 1992; Meinelt *et al*. 1997; Fournie *et al*. 2001).

The function of extracutaneous production of melanin is unknown, but there has been considerable speculation. Zuasti *et al*. (1989) point out that "melanin is a complex polymer that can absorb and neutralize free radicals, cations, and other potentially toxic agents" derived from degradation of phagocytized cellular materials. Wolke *et al*. (1985) suggest that melanin may be important in production of bactericidial compounds, especially hydrogen peroxide, and that their quinone precursors may be bactericidal and of particular benefit to poikilotherms, where enzymatic activity may be severly restricted at low temperatures. They also observed that their numbers increase greatly with disease (Wolke 1992). It has been suggested that increase in melanomacrophage

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centers were related to humoral and inflammatory responses, storage, destruction, detoxification of exogenous and endogenous substances, and iron recycling (Wolke 1992).

The present study is the first examination of tissue pigment cell aggregations of anuran amphibian. The study looks at their distribution among the lymphoid and non-lymphoid organs of parasite-infected *Bufo regularis*. The study examines the relationship between parasite infection, size and number of these aggregations in the different organs of this species. This study also examines the cells morphology and contents to determine how far they are consistent with the inclusions in the melanomacrophages of fish (Agius & Roberts 2003).

MATERIALS AND METHODS

Animals: A total of 50 male and female specimens of the amphibian *Bufo regularis* (family Bufonidae) were used in the present study .All animals were collected from Abo Rawash area, Giza and weighting between 6.8-34.8 g and snout-vent length is 4.3-9.8 cm. Most of animals were used for investigation within a few days of capture. Toads were kept in glass cages with 30 cm deep, with little amount of tap water, grasses and few rocks. Every two days, water and grasses were removed and food (earth worms; *Allolobophra caliginosa*) were given *ad libitum.*

Parasitological examination: The toads were examined for helminth parasites. The body cavity of each animal was opened and their gastrointestinal tract was removed by cutting the oesophagus and rectum. The oesophagus, stomach, small intestine as well as the liver, kidney, spleen and lung were examined separately under a dissecting microscope. Each section was examined individually, and all helminthes were counted and identified according to Barton (1994).

Light microscopy: Parts from the liver, spleen, kidney and lung from some animals (non-infected and infected) were fixed in Bouin's fluid and routinely processed for light microscopical examination. Serial sections of paraffin blocks were stained with hematoxylin and eosin.

Transmission electron microscope: Fragments of different tissues were removed from freshly killed specimens and fixed in 5% glutaraldehyde, postfixed in 1% osmium tetraoxide, and embedded in epoxy resin. Ultrathin sections of 60 nm thickness were stained with lead citrate and examined on TEM at X 8650. Some tissues were embedded in epoxy resin and sectioned at 1-1.5mm for light microscopic examination at X 1000.

RESULTS

Parasitology: Throughout the present study, 99% of the animals were parasitic–infected. Although there were considerable variations in the infection pattern among population of *B.regularis*, the study showed that 99% of the collected toads were significantly infected only with nematodes (i.e., single infection). However, 63% and 3% of the collected toads were significantly infected with nematodes and cestodes and /or nematodes and trematodes (i.e., double infection). Prevalence and site of infection for each species of helminth are given in table (1).

Table 1: The incidence of helminthes species in adult *Bufo regularis* in the present

Helminth species	Prevalence %	Site of infection
Nematoda: *Falacaustra cianaeatesbe*	42%	Small and large intestine
Cosmoceroides varabilis	99%	Small and large intestine
Trematoda: *Cephalogonimus americanus*	3%	Small intestine
Cestoda: Ophiotaenia *magna*	63%	Small intestine

Light microscopy: Light microscopical examination revealed aggregations of melanomacrophages (MM) in both liver and spleen (Figs. 1&2). The liver pigment cells (Also named melanin-containing cells) were localized both in the parenchymal area, at the sinusoidal level, and in the hematopoietic component of the liver (Fig.1a). Differences in the amount and distribution of the pigments were found between the specimen collected from single-or double-infected animals (Figs. 1b&c). A dramatic increase in the degree of MM deposition was observed in double-infected animals, but never observed in non-infected animals (Fig.1c). The aggregations were clearly composed of macrophages and were much larger and more abundant in the liver than those in the spleen (Fig.2). The spleen aggregations were most often in the vascular red pulp, usually near the edge of the white pulp. The aggregations from double-infected animals were much larger; consisted of more cells and larger cells than seen in single-infected animals (Fig. 2a, b&c). The aggregations in the liver were located in the small portal veins and the adjaunt sinusoids. Even the largest aggregations were not encapsulated and connective tissues staining showed no trace of collagen or elastin around the masses in either liver or spleen (Figs. 1&2). Individual cells of the hepatic masses were separated from the hepatocytes by thinly stretched endothelial cells. The nucleus of the endothelial cell is often fitting between adjacent macrophages. In large aggregations, the centrally located macrophages seemed not to be in contact with any part of the vessel wall (Figs. 1&2). Individual (non- aggregated) as well as grouped melanin-bearing macrophages are present in the lung, but the kidney had more individual pigmented macrophages than aggregations (Figs. 3&4). Aggregations were not found in the stomach, duodenum and colon of the parasite-infected toads, but individual pigmented macrophages were seen rarely in the mucosa of the digestive tract of double-infected toads. These cells were similar in size and shape to those found in the liver and spleen of parasite-infected toads. Very dense, more stellate, darkly pigmented cells were abundant around the lymphatic vessels of the lungs and kidneys (Figs. 3&4). These appeared superficially more similar to integmentary melanocytes than to the pigmented macrophages (data not shown).

Transmission electron microscopy: Ultrastructural observations revealed that during parasitic infection, MM contained melanosomes that were less homogenous in size and more scattered, with large amorphous cytoplasmic areas (Fig.5a). Conversely, in single-infected animals numerous phagosomes, also containing melanosomes were found (Fig. 5a). The cytoplasm contained some mitochondria, scarce cisternae of endoplasmic reticulum, and vesicles of a small Golgi apparatus. The most common organelle in the cell body was uniformaly electron-dense melanosomes (Fig. 6a). The splenic melanocytes were numerous, had long dendritic processes, and were surrounded by connective tissues. The nucleus possessed many indentations and was peripherally

located. The cytoplasm contained several mitochondria, abundant cisternae of reticulum, numerous clear vesicles, and melanosomes similar to those found in the liver (Fig. 6b). In parasite-infected animals, melanocytes were scanty both in the kidney and in the lung (Fig. 7).

The lung contained isolated MM in the central core of all alveolar trabeculae. These alveolar trabeculae comprised loose connective tissue. They contained various types of cells such as fibroblasts, smooth muscle cells, melanocytes, occasional granulocytes, macrophages and mast cells (Fig. 7a). The intracellular matrix was rich in the collagen and elastic fibers. The melanocytes were represented by only few isolated cells with a central body containing a nucleus and numerous long, thin, and branched cytoplasmic processes. The cytoplasm contained scarce organelles and numerous, oval, highly electron-dense melanosomes of different sizes (Fig. 7b). These melancytes were irregular, with many cytoplasmic processes containing melanosomes. Melanosomes also occurred in the hematopoietic fan of the kidney, thus suggesting an active transfer of melanosomes from the cytoplasmic processes of the melanocytes to their surrounding kidney cells (Fig. 7c)

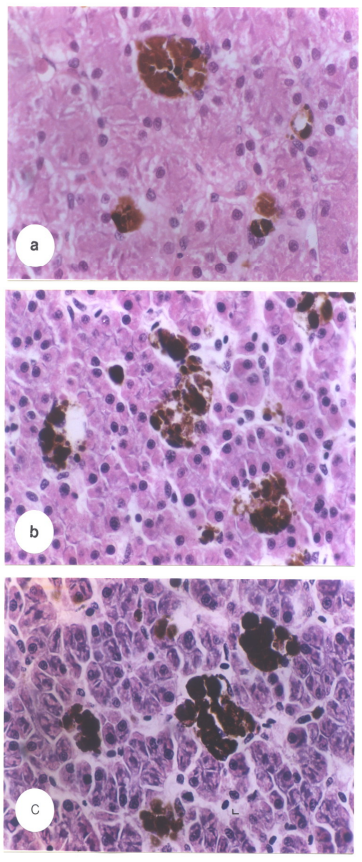

Fig. 1: Section in the liver of *B .regularis* showing pigment cell aggregations. (a) Non infected control . (b) Single pigment cells infected parasites. (c) Double infected parasites. Note all photograph are from near the margin of the liver where pigment cell aggregations are more concentrated. X 800

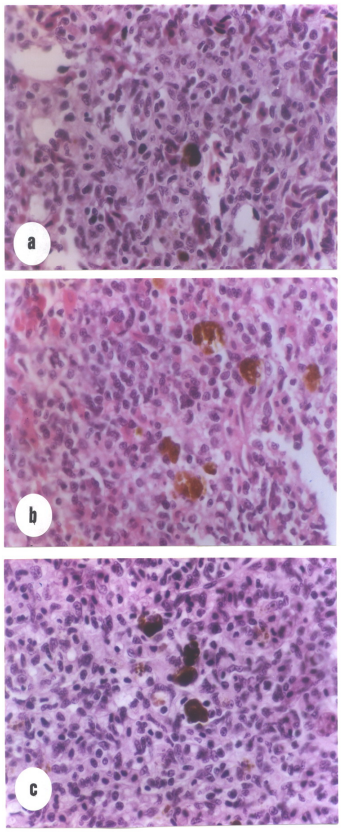

Fig. 2: Pigment cell aggregation in the spleen of *B.regularis.* (a) Non infected control. (b) Single infected parasites. (c) Double infected parasites. Aggregations in the spleen are rarely more than three cells and individual pigmented macrophages are more common than aggregation. **W**: white pulp; **R**: red pulp. X 600.

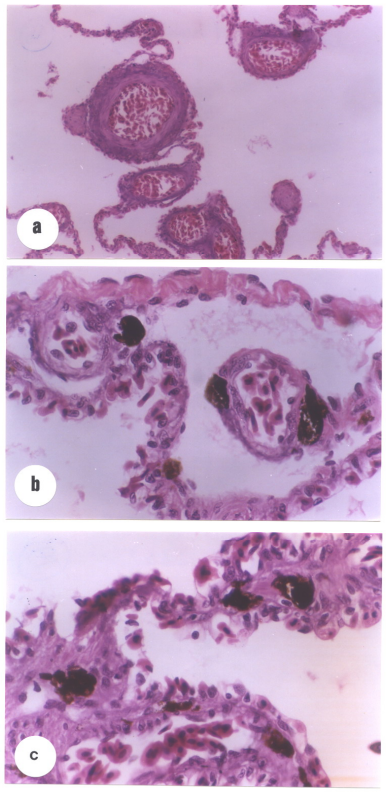

Fig.3: Pigment cell aggregation in the lung of *B.regularis.* (a) Non infected control. (b) Single infected parasites. (c) Double infected parasites. Note all individual pigmented macrophages are widely

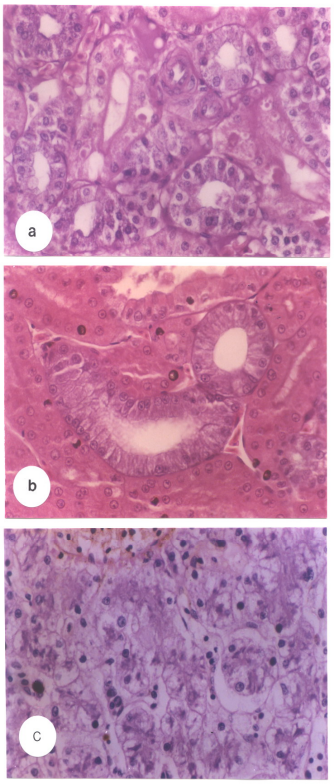

Fig.4: Pigment cell aggregation in the kidney of *B.regularis.* (a) Non infected control. (b) Single infected parasites. (c) Double infected parasites. Note individual pale coloured focal

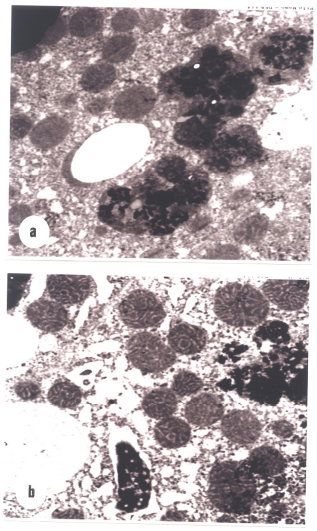

Fig.5: Electron micrograph of the liver of *B.regularis.* Pigment cells in the parenchymal (sinusoid) (a) and hemopoietic (sub capsular space) (b) areas of parasite infected animals (a). Melanomacrophage centre with melanocytes are grouped together and surrounded by a thin connective capsule. X 10000 (b). They are formed by dense aggregates of melanin containing cells. X 9300.

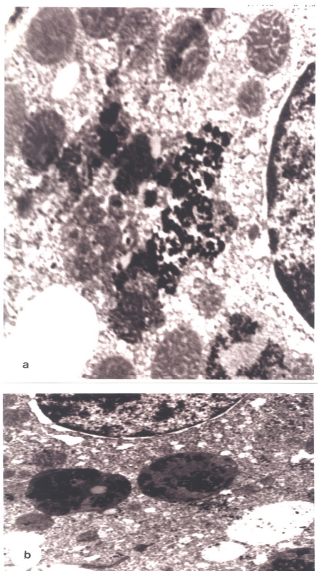

Fig. 6: Electron micrograph of the spleen of *B.regularis.* Note the different distribution of melanosomes in the white (a) and red (b) pulp. The arrows indicate the presence of numerous phagocytic bodies in the spleen. Melanocytes are included in the tissue. In their cytoplasm, there are melanosomes of different shapes, size and degree of melanization. X 9300.

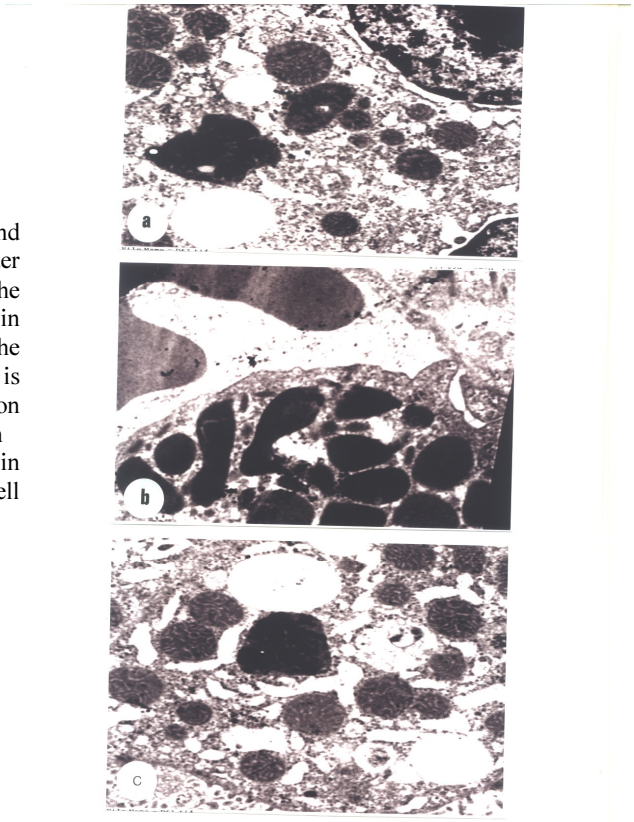

Fig.7: Electron micrograph of the lung (a and b) and kidney (c) of *B.regularis.* The lighter colored amorphous material beneath the black melanin granules includes lipofuscin and small amounts of hemosiderin (a). In the connective tissue of the alveolar trabeulae is an isolated melanocyte containing electron dense melanosomes surrounded by collagen Fibers (b). The cytoplasm of melanocytes in the kidney shows melanosomes and cell debris (c). X 9, 3000.

DISCUSSION

The pigmented macrophages of parasite-infected *Bufo regularis* are similar to those reported by Barni *et al*. (2002) in *Rana esculenta,* although they lack the limiting capsule around the aggregations in both liver and spleen that was observed *Xenopus laevis* (Corsaro *et al.* 2000), *Triturus cristatus* (Corsaro *et al.* 2000), *Rana esculenta* (Sichel *et al*. 2002) and *Triturus carnifex* (Barni *et al*. 1999). The hepatic aggregations were surrounded only by the stretched endothelium of the sinusoids somewhat similar to that observed in the smaller aggregations of the pigmented cells of *R. esculenta* liver by Corsaro *et al*. (2000). The lack of encapsulation may permit almost unlimited expansion of aggregation through the exposure of the toad to parasites. The location of the aggregations in the sinusoid positions the macrophages well for removing bacteria and other agents from the blood. The presence of hemosiderin in the hepatic pigment cells is consistent with phagocytosis of hemoglobin metabolites and the presence of lipofuscin, accumulating in increasing amounts in the liver of parasite-infected toads, supports the phagocytic function of these cells in toads .These compounds have been reported by many authors in fish (Zuasti *et al.* 1990), frogs (Sichel *et al.* 2002) and turtles (Christiansen *et al.* 1996). We have demonstrated that the number and size of pigment cells aggregation increase with the progression of the parasite infection in the liver and spleen of *Bufo regularis.* This increase may be due to migration into the liver of macrophages produced elsewhere. Migration of melanin and carbon bearing macrophages to macrophage centers such as the liver is well known in toads and frogs *(*Barni *et al*.1999; Corsaro *et al*. 2000; Sichel *et al*. 2002). In addition to phagocytizing components of dead host cells, these macrophages may function in defense against bacterial and viral infection. Bacteria and viruses are concentrated in these centers in

fish (Lamas *et al*.1995; Falk *et al*.1995; Brattgjerd & Evensen 1996; Palenznela *et al*.1999). In fish the pigment cell aggregations increase with disease (Agius & Roberts 2003), and as with our toads, with parasite infection. Parasite-infected toads have a system of visceral pigment cells that involve aggregations in both the liver and spleen. The presence of aggregated pigmented macrophages in hepatic sinusoids (apparently not in contact with the vessel wall or with hepatic tissue) and the presence of similar, individual (non-aggregated) pigmented macrophages, in the liver, spleen, kidney and lungs, suggests a behavior inconsistent with the concept of the cells of Von Kuppffer (Gallon *et al*. 2002). We agree with Sichel *et al.* (2002) that it is desirable to have a term for these versatile cells with macrophage capabilities and the ability of some to phagocytize and other to synthesize melanin, as well as to have a hierarchy of specific terms for specialized colonies of these and other pigment cells. Much work is required like that of Aguis & Roberts (2003) on the specialization of these cells within each tissue of parasite infected vertebrates. As this paper demonstrates, the amphibians present particularly interesting problems regarding adaptations of pigment cells after parasite infection. Further study will be necessary to gain a further understanding of this cell system.

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الملخص العربى

تجمعات الخلايا الأكولة الحاوية على الميلانين فى الضفدع بوفوريجيولاريس المصاب بالطفيليات

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فى هذا البحث تم تعيين كميات وتوزيع الخلايا الصبغية الأكولة الحاوية على الميلانين فى الضفدع بوفوريجيولاريس باستخدام المجهر العادى و الألكترونى .
وقد وجد أن هذه الخلايا تحتوى على كميات من الصبغيات تتفق وقدرتها على عملية التبلعم , وقد وجدت تكونات أحادية فى الكبد والطحال والرئة والكلية. كما وجد أن عدد التجمعات الخلوية وحجمها فى الكبد يزداد حسب معدل الإصابة حيث أن هذه الزيادة يمكن تعيينها فى الحيوانات المصابة بالطفيليات وأن حجم وعدد هذه التجمعات من الخلايا الصبغية الأكولة يمكن أن يكون مقياس بيولوجى للاصابة بالطفيليات داخل الأنواع المختلفة من هذه الحيوانات