Evaluation of the inflammatory response experimentally induced by a foreign body in fresh water turtles (*Trachemys dorbignyi*)

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Abstract: This report describes the inflammatory response induced by a foreign body in fresh water turtles, *Trachemys dorbignyi*. The cellular infiltrate was composed of two types of cells, which were classified according to the presence or absence of citoplasmic granules as granulocytic or agranulocytic cells. The transmission electron microscopy study characterized the granulocytic cells as heterophils and the agranulocytic ones as macrophages and trombocytes. On the 14 th day, a stratified squamous epithelium was observed around the foreign body. A keratinized pattern was verified on the 21 st day, suggesting a host attempt to return the foreign body to the external environment, even though a transepithelial migration of inflammatory cells towards the suture thread was present until last day of observation. This finding could be explained by the presence of necrotic debris close to the foreign body that could act as an amplificating factor of chemotaxis.
Keywords: Fresh water turtle; Trachemys dorbignyi; Inflammation; Macrophage; Heterophil

Resumen: Respuesta inflamatoria inducida experimentalmente mediante un cuerpo extraño en extremidades del galápago (*Trachemys dorbignyi*).- Se describe la respuesta inflamatoria inducida por un cuerpo extraño en el galápago *Trachemys dorbignyi*. El infiltrado celular estuvo constituido por dos tipos de células, que fueron clasificados de acuerdo con la presencia o ausencia de gránulos citoplasmáticos, como células granulocíticas o agranulocíticas. El estudio mediante microscopia electrónica de transmisión caracterizó las células granulocíticas como heterófilos y las agranulocíticas como macrófagos y trombicitos. En el decimocuarto día, se observó un epitelio escamoso estratificado alrededor del cuerpo extraño. Se verificó un patrón queratinizado el vigesimoprimer día, sugiriendo por parte del huésped el intento de expulsar el cuerpo extraño al exterior. La migración transepitelial de células inflamatorias en dirección al hilo se sutura estuvo presente hasta el último día de observación. Este hallazgo pudo ser explicado por la presencia de restos necróticos cerca del cuerpo extraño, que actuó como un factor amplificador de la quimiotaxis.
Palabras clave: Fresh water turtle; *Trachemys dorbignyi*; Inflammation; Macrophage; Heterophil

INTRODUCTION

According to METHCNIKOFF (1968) and later comparative studies, it was established that the complexity of inflammation increases along with phylogenesis. ZWART (1963), wrote "the higher the phylum, the more the whole organism is involved in the inflammatory process and its types in several phyla of animals was seen a progression from a simple local reaction in Spongia to highly differentiated inflammatory reactions in Mammals".

In reptiles, studies of the inflammatory process begin with RYERSON's works (1943), that studying comparative hematology, verified evident heterophilic response in the peripheric blood of chelonians, after subcutaneous injections of turpentine.

GRANT *et al*., (1981), studied the pulmonary inflammatory response in snakes *Boa constrictor* and verified that in the acute phase there was a acidophilic granulocytic infiltration and in the chronic phase macrophages cells were seen.

In alligators maintained at 25 ºC and stimulated subcutaneously with turpentine, a heap of heterophils was observed 4 hours P.I., and the presence of monocytes was evident at 24 hours P.I. After one week, granuloma-like lesions were observed, where vacuolated multinucleated giant cells in palisades were seen involving the inoculo (MATEO *et al.*, 1984).

In a comparative review of the inflammatory process, MONTALI (1988), asserts that classical types of inflammation like catarrhal, fibrinous, purulent as abcedant and phlegmonous, besides granulomatous and proliferative, were described in reptiles, occurring under natural or experimental conditions.

SMITH and BARKER (1988), observed heterophil and macrophage participation in the inflammatory exudate surgically induced in snakes *Thamnophis sirtalis*.

RAMOS (1997), investigated the chronic inflammatory process experimentally induced by injection of live and heated *Mycobacterium marinum*, and demonstrated that granulomatous response reached a maximum level on the 28th day.

This work intends to contribute to the comprehension of the inflammatory phenomena in reptiles, evaluating the inflammatory process induced by a foreign body in fresh water turtles *Trachemys dorbignyi*.

MATERIAL AND METHODS

Twenty-five, young or subadult fresh water turtles (*Trachemys dorbignyi*, Duméril and Bibron, 1835), obtained from the São Paulo Zoological Park Foundation, were used. The environmental temperature was kept at 24 ºC, with a confidence interval of 1 ºC, during the experimental period.

The animals were maintained in slanted plastic boxes with 3.0 cm of water covering 2/3 of the area. Animals were fed with ground beef, fish and lettuce, daily. These foods were accepted by all animals.

The inflammatory response was induced by the transfixation of the muscular tissue of the rear limb with a 4.0 (Supramid, Cirumédica) nylon suture thread.

The turtles were divided into 5 groups of 5 animals which corresponded to 1, 3, 7, 14 and 21 days following transfixation.

After these periods the animals were anesthetized with tiletamine and zolazepam (Zoletil, Virbac) at 80 mg/kg, i.m., when biopsies of the region inflammated by the foreign body were performed.

The fragments were fixed in Bouin's liquid at room temperature during 24 hours, embedded in paraffin and from these materials 5 µm slices were obtained and stained by hematoxilin-eosin and Mallory techniques.

The qualitative evaluation model of the histological pattern of lesion was as proposed by FINN & NIELSEN (1971), who consider the phenomenon examined positive when it is present in (n/2) + 1 of the animals studied. In this evaluation was considered: necrosis, inflammatory cellular infiltration, fibroplasia and lesion edge epithelization.

A 400 point II Zeiss morphometric ocular was utilized to quantity the cellular composition of the reaction area, corresponding to 1.64 mm^2 per animal. The inflammatory cellular kinetics was analyzed with the above described method, but the punctual density utilized was 100, corresponding to 160 μm^2 per animal. Data were statistically analyzed through variance followed by Duncan's multiple range test, with the level of significance set at $p < 0.05$.

For the transmission electron microscopy analysis, fragments of inflammated tissue were fixed in 0.1 M phosphate buffered 2% glutaraldehyde (2 hr) at environmental temperature and subsequently fixed in 1% osmium tetroxide (1 hr) at 4 ºC; kept in 0.5% uranyl acetate overnight at 4 ºC (JUNQUEIRA & SALLES, 1975), dehydrated in acetone and embedded in Araldite Epoxi resin 502 (LUFT, 1961). Semithin sections were obtained with a glass knife in a MT Sorwall ultramicrotome

and stained with toluidin blue. Ultrathin sections (60-70 nm) were stained by uranyl acetate (WATSON, 1958), and lead citrate (REYNOLDS, 1963) and examined in an EM 201 Philips transmission electron microscope, at 80 kv.

RESULTS

Two distinct groups of inflammatory cells were morphologically characterized during the histological observation under immersion: granulocytic cells with pleomorphic eosinophilic granules and polar nuclei; and agranulocytic cells, showing agranular basophilic cytoplasm when present and condensed nuclear chromatin, some cells had intracytoplasmatic contents.

Ultrastructurally, heterophils were characterized as the major granulocytic cellular type (Figure 1); these contained electrondense pleomorphic granules of various sizes and large peripherical nuclei. Among the agranulocytic cells, macrophages showed a great number of phagossomes (Figure 2), besides characteristic nuclei and cytoplasmatic membrane extensions. Thrombocytes were also seen, and contained open canalicular system and nuclei showing longitudinal clefts.

On the first experimental day, necrotic areas were observed characterized by abundant cellular debris, haemorrhages surrounding the foreign body and predominant heterophilic cells.

On the third day, a tendency of organization of the necrotic area was verified, with large amounts of inflammatory cells and the disappearance of the haemorrhagic component.

It was also observed that migrated heterophils and macrophages formed a circumscribed region surrounding the foreign body, showing an attempt to reorganize the process. By the seventh day, foreign body giant cells were seen.

On the fourteenth day, the foreign body was still circled by necrotic debris corresponding to the migration of a large amount of heterophils, which seemed to be better preserved on the borders. These tightly arrange cells were involved by a stratified squamous epithelial tissue supported by a connective tissue (Figure 3).

On the last experimental day studied (twenty-first day), a more evident epithelial specialization was observed that showed keratin and exhibited granulocytic and agranulocytic leukocyte transepithelial migration. Newly formed blood vessels remained present and the subepithelial connective tissue became more compact. In some cases there was the presence of giant cells arranged in palisade around the necrotic debris (Figure 4). It was also verified that the epithelium surrounding the foreign body was derived from the revestiment epithelium.

The parameters analyzed according FINN & NIELSEN (1971) method are shown in table one.

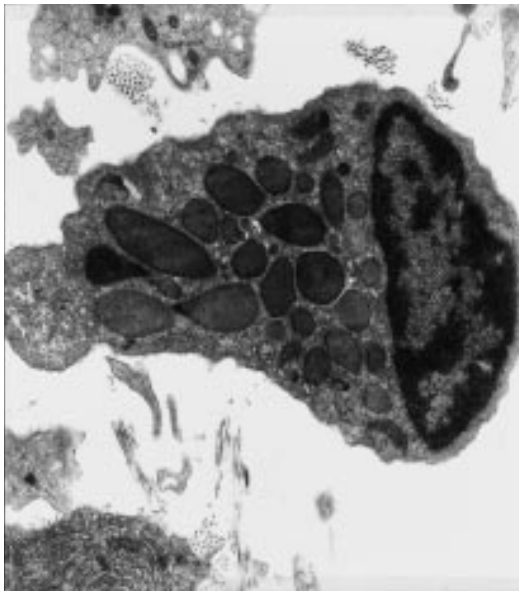

Figure 1.- Heterophil. Note the electrondense pleomorphic granules and large peripherical nuclei, x 15296.

Figura 1.- Heterófilo. Observense los gránulos pleomórficos densos a los electrones y el nuclo periférico largo.

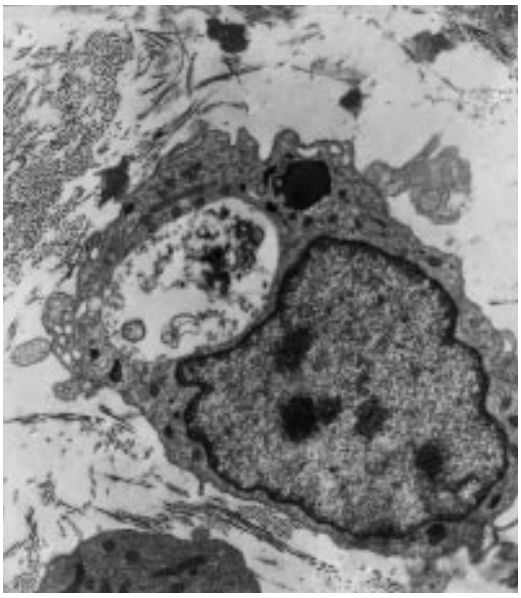

Figure 2.- Macrophage. Note the great number of phagossomes and the pseudopodia, x 10953.
Figura 2.- Macrófago. Observese el gran número de fagosomas y de pseudopodia.

Quantitatively, the wound resection area exhibits a rising profile during the experiment and on the twenty-first day the values obtained were significantly higher than those obtained during the second week (Figure 5), probably due to the reduction of necrosis and degeneration of muscular tissue and an increase in fibroplasia and epithelization (Table 1).

The total inflammatory cellular infiltrate reached a maximum on the seventh day, which decreased on the fourteenth day and then remained stable until the twenty-first day. When separately analyzed, the agranulocytic and granulocytic kinetics showed outlines similar to the total inflammatory kinetics. The number of heterophils was significantly higher than the agranulocytic cells only on day one; on the third, seventh and twenty-first day no statistical difference between the number of cells was seen (Figure 6).

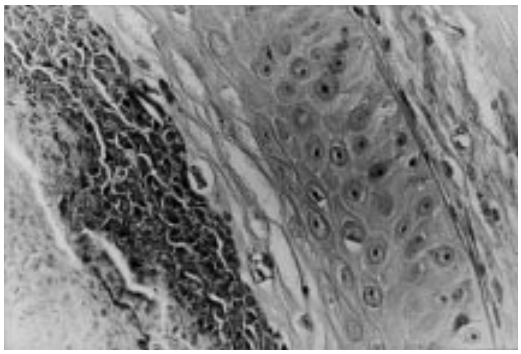

Figure 3.- Necrotic debris surrounded by granulocytic cells better preserved on the borders and stratified squamous epithelial tissue, 14 th day, HE x 140.
Figura 3.- Residuo necrótico rodeado de células granulocíticas, mejor conservadas en los bordes y en el tejido epitelial escamoso estratificado.

DISCUSSION AND CONCLUSIONS

The transfixation of the epithelial and muscular tissues of the rear limb with a nylon suture thread utilized in this work appears to be an appropriate model to study the inflammatory process in these ectothermic vertebrates. The animals don't need to be sacrificied and yet a conspicous inflammatory infiltration was obtained to study cellular kinetics.

The inflammatory reaction induced by the foreign body had the permanent participation

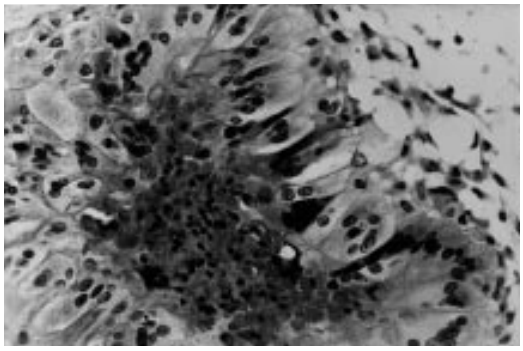

Figure 4.- Necrotic debris surrounded by inflammatory giant cells arranged in palisade, 21 st day, HE x 360.
Figura 4.- Residuo necrótico rodeado de celulas gigantes inflamatorias ordenadas en empalizada.

Table 1: Number of animals positive during the qualitative histological evaluation of the inflammatory response phenomena induced by a forign body in fresh water turtle paws.
Tabla 1: Número de animales que dieron positivo en la evaluación histológica cualitativa de la respuesta inflamatoria afectada inducida experimentalmente mediante un cuerpo extraño en la extremidad de un galápago.

Phenomena	N	EVALUATION DAY				
		1	3	7	14	21
NECROSIS	5	5	5	5	0	0
I.C.I. *	5	5	5	5	5	5
FIBROPLASIA	5	0	0	5	5	5
EPITHELIZATION	5	0	0	0	5	5

* I.C.I. = Inflammatory Cellular Infiltration.

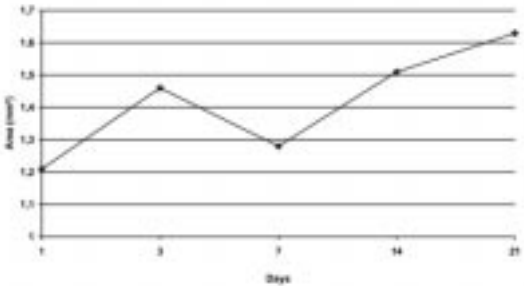

Figure 5.- Wound reaction area experimentaly induced by a foreign body in fresh water turtle limb.
Figura 5.- Área afectada inducida experimentalmente mediante un cuerpo extraño en la extremidad de un galápago.

of two cell kinds throughout the experimental period: granulocytic and agranulocytic. Ultrastructurally, the first were characterized as heterophils, since it is known that differentiation from eosinophils under light microscopy is difficult (MONTALI, 1988 and MONTALI pers. com.). The inflammatory focus upon reptiles includes several studies showing the predominance of heterophils among granulocytic cells (RYERSON, 1943; MATEO *et al.*, 1984; SMITH & BARKER,1988). Heterophils peroxidases have been identified in several species of turtles, snakes and lizards and although biochemical differences exist, reptilian heterophils seen to have the same role of mammalian neutrophils (MONTALI, 1988).

The other cellular type observed was an agranulocyte and the ultrastructural evaluation elucidated that the main cellular types in this group were macrophages and thrombocytes. Lymphocytes were not observed, in contrast with previous experiments conducted with bullfrogs in our laboratory (CATÃO-DIAS & SINHORINI, 1999). However, the kinetics found agree with the literature available for reptiles, where heterophils are seen as the main cellular type in the first hours in experimental studies on comparative haematology, inflammation and scarring process (RYERSON, 1943; MATEO *et al.*, 1984; SMITH & BARKER, 1988). When the granulocytic and agranulocytic components were compared after the first hours, there was no agreement in the literature about the kinetics in this animal class. MATEO et al., 1984, refers prevalence of the mononuclear agranulocytes starting from the seventh until the thirtyth day in alligator (*Alligator mississippiensis).* SMITH & BARKER (1988), verified the number of heterophils to excel the number of macrophages until the tenth day in *Thamnophis sirtalis* serpents. In fresh water turtles, *Trachemys dorbignyi*, the heterophils and the mononuclear agranulocytes migrated in equal number after the first through the twenty-first day, except for the seventh day.

There was early necrotic debris formation, composed mainly by heterophils, surrounding

the foreign body in the inflammatory process induced in *Trachemys dorbignyi*. Next the epithelial cells were observed in the wound site tightly arranged and relatively well-preserved. According to MONTALI *et al*. (1988), the necrotic debris seems to act like a strong attractive to a macrophage response.

On the fourteenth experimental day, the presence of a stratified squamous epithelium was verified surrounding the foreign body. By way of this phenomenon the agent is replaced in the external medium, as a form to eliminate the inflammatory stimulus.

Cellular inflammatory transepithelial migration was observed from the fourteenth through the twenty-first day, showing epithelial participation in this inflammatory process experimentally induced in fresh water turtles. The epithelium, even then it was keratinized, was permeable to inflammatory cells, which migrated towards the necrotic debris surrounding the foreign body. Similar phenomenon was also observed in bullfrog tadpoles (DIAS & SINHORINI, 1991). Agreeing with MONTALI *et al.*, (1988), this probably happened due to an amplification of the irritant power that these debris had when the suture thread first introduced, increasing the diversity of inflammatory agents in the injured site.

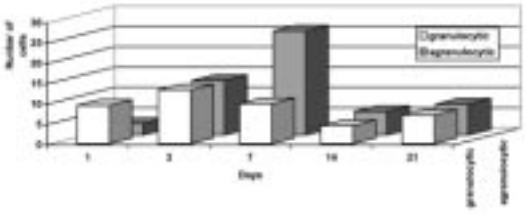

Figure 6.- Number (mean value) of granulocytic and agranulocytic cells observed in 1.64 mm^2 in the wound area experimentaly induced by a foreign body in fresh water limbs.

Figura 6.- Número medio de células granulocíticas y agranulocíticas observadas en 1,64 mm^2 en el área afectada afectada inducida experimentalmente mediante un cuerpo extraño en la extremidad de un galápago.

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