Experimental chronic granulomatous inflammatory process in fish: a morphological, ultrastructural and immunocytochemical study

Eliana Reiko MATUSHIMA[1]
Adhemar LONGATTO FILHO[2]
Cristina Takami KANAMURA[2]
Idércio Luiz SINHORINI [1]

Correspodence to:
ELIANA REIKO MATUSHIMA
Departamento de Patologia
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo
Avenida Prof. Dr. Orlando Marques de Paiva, 87 - Cidade Universitária
05508-900 - São Paulo - SP
ermatush@usp.br

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1 - Departamento de Patologia da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo – SP
2 - Divisão de Patologia do Instituto Adolfo Lutz, São Paulo - SP

Abstract

The purpose of the present study was to evaluate the chronic granulomatous inflammatory process experimentally induced in *Oreochromis niloticus* by the inoculation of BCG and to clarify aspects of the inflammatory reaction in fish for a better understanding of the phylogeny of the process. Results obtained by light microscopy and ultrastructural analysis showed the participation of macrophages, thrombocytes, lymphocytes, eosinophils, plasma cells and foreign type giant cells in the inflammatory process. Besides these cell types, a granulomatous reaction mainly consisting of epithelioid cells was also observed ultrastructurally. These epithelioid cells developed desmosomes throughout the experiment, and also began to express receptors for cytokeratin. Both findings are immunocytochemically characteristic of epithelial cells. Pigment cells (melanomacrophages) could also be seen surrounding all the granulomatoid formation in a crescent fashion and participating in the chronic granulomatous inflammatory reaction.

Key-words:
Inflammation.
Fish.
Nile tilapia.

Introduction

Comparative pathology has been accepted as an important parameter to evaluate the natural history of diseases and the mechanisms of pathophysiologic host behavior. The inflammatory process has been studied for this purpose since Metchnikoff's phagocytosis investigations with different phyla, classes and orders of metazoa in order to evaluate the phylogenetic expression of inflammation[1,2,3].

Metchnikoff was also the first to study the inflammatory response in fishes; he observed in vivo phagocytosis after inoculating guinea pig erythrocytes into the coelomic cavity of *Carassius auratus*.

Phagocytosis in fishes was also observed by many investigators using different types of substances or biological agents[4,5,6,7,8,9,10,11,12,13,14,15]. The cellular kinetics of the inflammatory response was also extensively studied[7,11,12,14,15,16,17,18,19,20,21].

Materials and Methods

Animals:

Male and female fish (n=100) of the species Oreochromis niloticus (Nile tilapia), weighing 80 - 100 grams were used. The animals were kept in 100 litre water tanks. The water was constantly aerated with compressor pumps connected to internal filters, and maintained at 22º ± 1º C. The fishes were fed commercial balanced feed ad libitum throughout the whole experimental period.

Anesthesia:

The animals were anesthetized by immersion in a benzocain solution (1 : 10000)[22,23].

Induction of the inflammatory response: 0.05 ml of BCG Vaccine (Bacillus Calmette-Guerin, Moreaux strain) concentrated to 100 mg/ml, and containing 10^8 bacilli/ml was inoculated intramuscularly at: 03, 07, 14, 21, 33 and 45 days.

Muscle Tissue Histology:

At various times after BCG inoculation into the muscle tissue, the animals were anesthetized and bled. The fragments were fixed in Bouin's liquid for 12 hours and then processed for paraffin embedding. 5 ?m sections were stained by the hematoxilin-eosin method (H.E.), Ziehl-Neelsen method (Z.N.), picrosirius red (P.P.)[9] and melanin method, Lillie Variant.

Immunocytochemistry:

The immunochemical assay was perfomed on paraffin sections according to Hsu, Raine and Fanger. 24 employing monoclonal antibodies to cytokeratins AE 1/ AE3 cocktail, Boehringer-Mannheim, USA) and polyclonal antibodies to BCG (DAKO A/S, Denmark). The amplifications step was perfomed with biotinylated anti-mouse IgG and anti-rabbit IgG (both from Vector Labs., USA), for monoclonal and polyclonal primary antibodies, respectively, and the avidin-biotin-peroxidase complex (Vector Labs., USA). The color end product was obtained using diamonobenzidine (Sigma, USA) plus hydrogen peroxide as chromogenic substrate.

Electron Microscopy:

Muscle tissue fragments of approximately 1,0 mm were fixed in 2% glutaraldehyde solution diluted with Milloning buffer solution[25] and post-fixed in a 2% solution of osmium tetroxide. The fragments were embedded in Araldite[25] and ultrafine sections were obtained and stained with uranil acetate[27] and lead citrate[28] Sections were examined under an EM 201 Philips-Holland transmission electron microscope.

Results

Histology:

During the first week after BCG inoculation, muscle tissue exhibited marked signs of a degenerative process associated with the inflammatory response, including muscle fiber necrosis.

An inflammatory process involving mononuclear cells of large cytoplasm with cellular debris and/or phagocyted BCG bacilli was observed by Ziehl-Nielsen and anti-BCG antibody positivity, together with nuclear features suggestive of macrophage cells. These cells were distributed around the lesion.

As the inflammatory process progressed, the macrophages were found organized in the granuloma and the cellular features changed to a more pronunced epithelial aspect. Cytoplasmic and nuclear areas were enhanced, and the nucleoli were prominent. These characteristics are suggestive of epithelioid cells (Figure 1a).

In the region of the epithelioid cells around the granuloma, we also found a large number of Ziehl-Nielsen positive BCG bacilli in the middle of granulomatous debris, or in the cytoplasmic area of the epithelioid-macrophage cells (Figure 1b).

Immunoreactions:

The bacilli were also demostrated by the use of anti-BCG polyclonal antibody as shown by cytochemical Ziehl-Nielsen staining (Figure 1c).

The cells identified as macrophages in the initial observations of the experiment such as the epithelioid cells in the granuloma were positives to the anti-cytokeratin reaction (Figures 1d).

Picrosirius-Polarization:

Picrosirius showed birefringent green-yellowish ou reddish stains in fibers with fragile aspect, suggesting type III and I colageus, respectivaly.

Ultrastructural evaluation:

Ultrastructural analysis of the BCG-induced lesion at 7, 14, 21 and 33 days after inoculation showed a constant pattern of cellular infiltration that was not time dependent. We observed macrophages, thrombocytes, lymphocytes, eosinophils, plasma cells and melanomacrophage pigment cells.

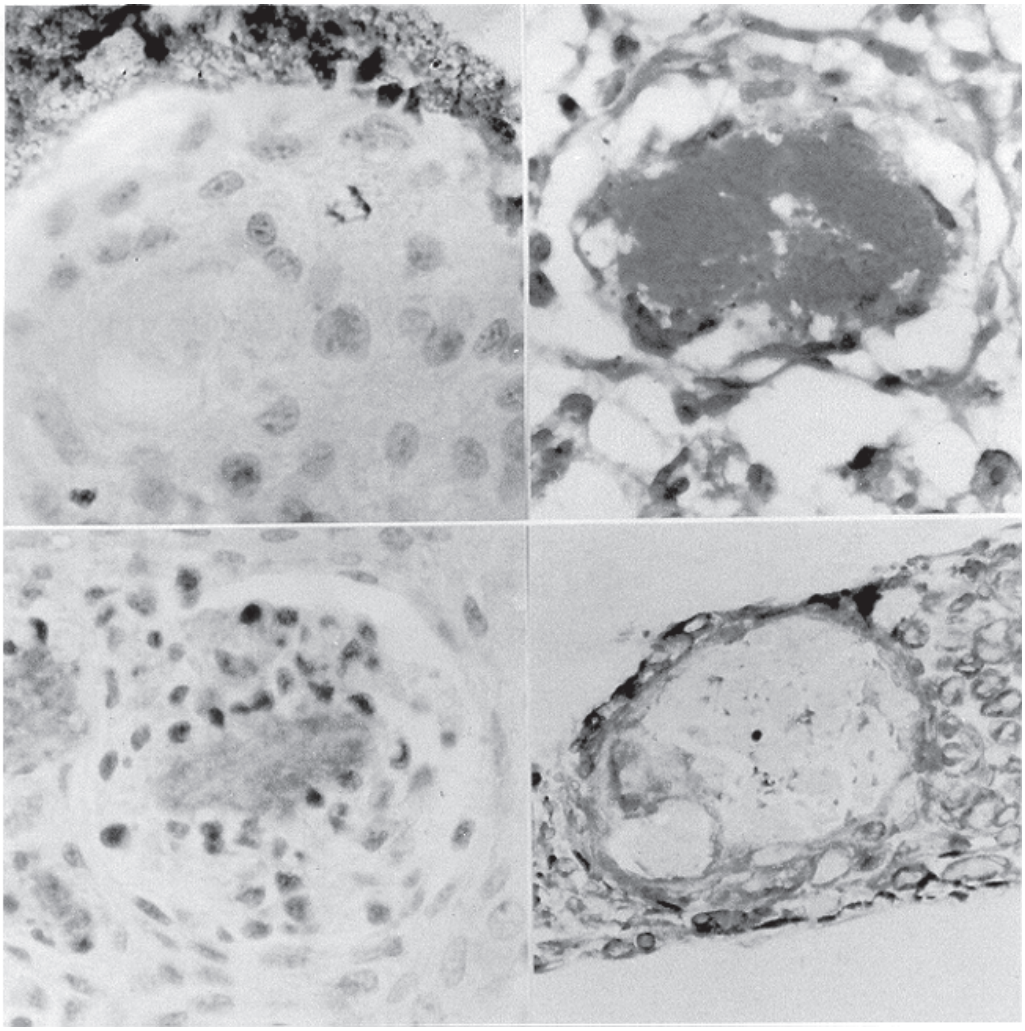

Figure 1 - Photomicrograph of muscle tissue inflammed of O. niloticus. The macrophages in the granuloma with pronunced epithelial aspect (a) H. E. stain, x 1650; in the middle of granulomatous debris and in the cytoplasmic area of the epithelioid-macrophages cells observed a large number of Ziehl-Nielsen positive BCG bacilli (b) Ziehl-Nielsen, x 1650; the bacilli were demostrated by use of anti-BCG polyclonal antibody (c) Avidin biotin peroxidase, x 660; the epithelioid cells in the granuloma were positives to the anti-cytokeratin reaction (d) Avidin biotin peroxidase, x 660

The granulomatous reaction area exhibited multiple areas of inflammatory cells. In the outer border regions we also observed fibroblasts, pigmented cells and macrophages, all of them linked by desmosome bridges, which appeared as electron-dense structures(Figure 2).

Discussion

Although the basis of the inflammatory response has been long known many questions still need to be answered. A particular point concerns the different patterns of granulomatous reaction in various species.

Many authors have been employed experimental protocols in order to obtain scientific information about this subject[11,29,30,31,31,32,33,34,35], and the findings reported are very similar to those observed in the present study.

We observed that the macrophages usually presented, a high concentration of alcohol acid resistant bacilli as demonstrated

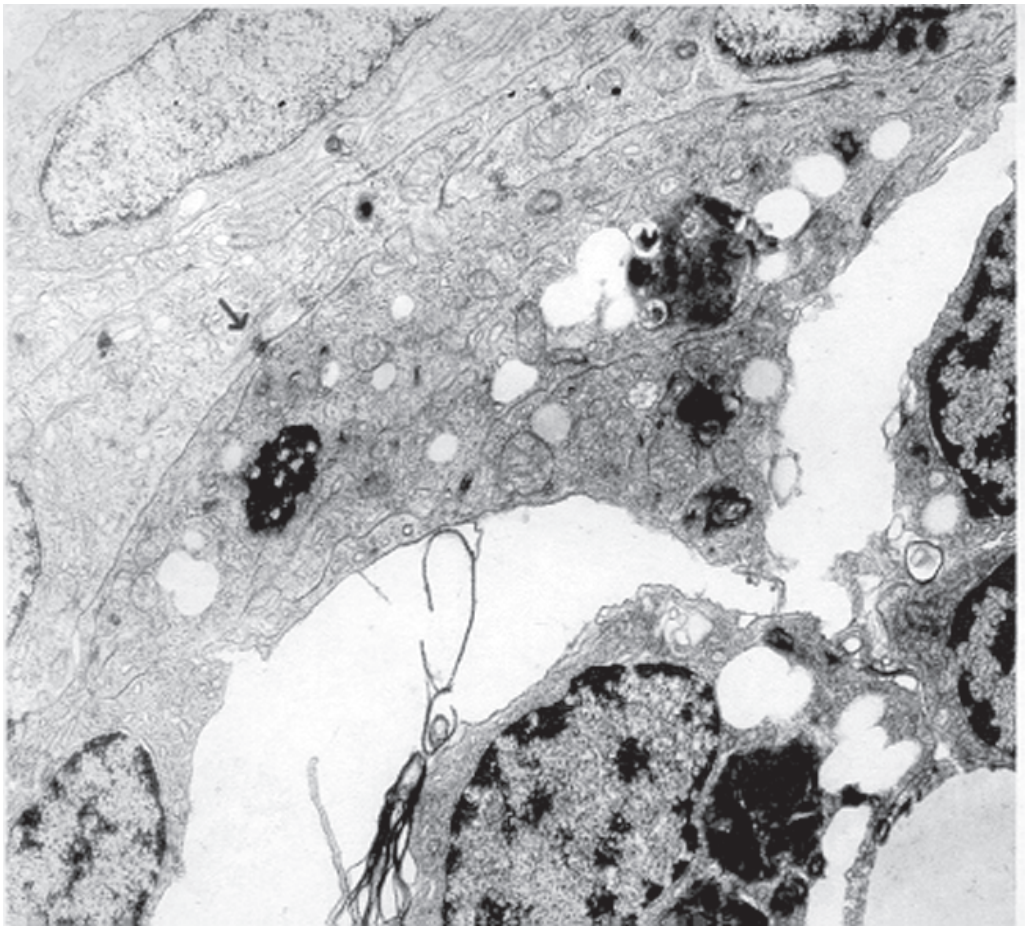

Figure 2 - Electron micrograph of muscle tissue inflammed of *O. niloticus* showing the granulomatous reaction area exhibited inflammatory cells linked by desmosome bridge, which appeared as electron-dense structures. x 10,528

by Ziehl-Nielsen staining and by the anti-BCG immunoreaction. Epithelioid macrophages were also observed in the granulomatous reaction. These findings have been previously reported in other studies of this kind of inflammatory reaction[11,29,30,31,32,34,36,37].

The main characteristics of epithelioid cells, such as desmosome projections and interdigital plasmalemma, can be partially observed by ultra-structural examination. Epithelioid cells also demonstrated positive reactivity to anti-human cytokeratin. Noga, Dykstra and Wright[19] have studied mycotic granulomatous reactions in different teleost fishes and also reported desmosome figures associated with tonofilaments. On the other hand, Timur,Roberts and Mc Queen[38] studying the carrageenin-induced granulomatous reaction in *Pleuronectes platessa*, only observed the cytoplasmic interdigital structures.

Pigmented cells or melanomacrophages were found surrounding the granulomatous reaction. Melanomacrophages have been occasionally reported in a few studies, without a clear discussion of their controversial origin and function[34,35].

Melanomacrophages have been frequently observed surrounding blood vessels and granulomatous lesions in Nile tilapia. Morphologically, these cells exhibit a fusiform and central nucleus, and melanic cytoplasmic pigments. According to Roberts[39] these cells may probably be melanin-phagocyted macrophages.

Melanomacrophages may also be cells with hematopoietic-like structures observed in the liver, cephalic kidney and spleen of the teleost fishes[39]. According to Roberts[39], these cells may be similar to a germinative center of superior animals and part of a phagocytic mononuclear system of the fish. Thorpe[40] has observed a migration of these cells to the spleen and kidney vessels after a experimentally induced infection with *Aeromonas liquefaciens* in *Salmo trutta*. This fact suggests the participation of melanomacrophages in the inflammatory response of fishes.

Melanin is also well known to have a bactericidal function. Edelstein[41] suggested that melanin has a free-radical characteristic, producing hydrogen peroxide and acting like a bactericidal substance. Therefore, we may speculate that the possible function of melanomacrophage cells in the BCG-induced chronic-inflammatory granulomatous process is to eliminate the etiologic agent. Epithelioid cells also participate in this granulomatous lesion, presenting cytoplasmic interdigitation and desmosome structures. These positive cytokeratin cells mimic epithelial characteristics, with efficient links between the cells, isolating in the central area of the granuloma the bacillar component of the lesion.

Further studies using an experimental model of inflammatory response are needed to explain the functions of melanomacrophage and of other cells and substances involved in the granulomatous process, with emphasis on phylogenetic behavior.

Processo inflamatório crônico granulomatoso experimental em peixes: um estudo morfológico, ultraestrutural e imunohistoquímico

Resumo

O objetivo do presente estudo foi avaliar o processo inflamatório crônico granulomatoso induzido experimentalmente em *Oreochromis niloticus* através da inoculação de BCG e elucidar aspectos da reação inflamatória em peixes para uma melhor compreensão da filogenia do processo. Os resultados obtidos por microscopia de luz comum e ultra-estrutural demonstraram a participação de macrófagos, trombócitos, linfócitos, eosinófilos, células plasmáticas e células gigante tipo corpo estranho no processo inflamatório. Além desses tipos celulares, uma reação granulomatosa constituída predominantemente de células epitelióides também foram observadas ultraestruturalmente. Essas células epitelióides desenvolveram desmossomos ao longo do experimento, e também passaram a expressar receptores para citoqueratina, características estas de células epiteliais. Células pigmentares (melanomacrófagos), envolvendo de maneira crescente toda a formação granulomatóide e, participando ativamente da reação inflamatória crônica granulomatosa.

Palavras-chave:
Inflamação.
Peixes.
Tilápia-do-Nilo.

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