

Histopathological, Immunohistochemical and Ultrastructural Evaluation of Inflammatory Response in *Arius* genus Fish under Experimental Inoculation of BCG

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ABSTRACT

The aim of this study was to evaluate the inflammatory response kinetics after experimental inoculation with BCG in the primitive Arius sp. fish. The BCG was applied through the intramuscular injection in the caudal peduncular region, and the samples were collected for the analyses at days 1, 3, 7, 14, 21, and 33 post-injection. Acute phase inflammatory infiltrate was characterized by the predominant mononuclear cells, interstitial edema, and muscular tissue necrosis. As the inflammatory response evolved, a large number of multinuclear giant cells were formed containing the BCG. These giant cells were positive for the S100 protein at the histochemical analysis, which demonstrate the macrofage activity, confirmed by the ultra-structural analysis showing the lack of the cytoplasmic membrane enveloping the many nuclei within the giant cell. These results led to the conclusion that Arius sp. fish injected with the BCG showed a diffuse inflammatory response characterized by a large number of mononuclear cells, absence of granuloma formation, and predominant giant cells.

Key words: Fish, *Arius*, phylogeny, BCG, inflammation, giant cells

INTRODUCTION

Teleostean fish may be phylogenetically divided into three groups: primitive, intermediary, and modern fish. *Arius* sp. fish are considered primitive, and belong to the Cypriniform order, Siluroidei sub-order (Gosline, 1971). Based on Darwin's evolutionary theory, Metchnikoff (1968) studied the inflammatory response in species from different metazoan phyla, classes, and orders, and proposed that the complexity of this response accompany the evolution. In fishes this complexity may also be observed when studying the chronic response to the inflammatory stimuli in

various species belonging to different groups (primitive, intermediary, and modern). In most primitive teleostean fish, a large number of the giant cells form around the pathogenic agent as the response develops (Albernethy and Lund, 1978, Goodwin and Grizzle, 1991, Xavier and Matushima, 1993, Gómez, 1998). Multinuclear giant cell formation in the chronic inflammatory response in the primitive fish is similar to that observed in the superior vertebrates (Gómez, 1998). Although the inflammatory response has been extensively studied among the exothermic vertebrates, huge gaps of the knowledge are still present. Therefore, this study aimed to

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characterize the inflammatory response in the primitive *Arius* sp. fish after the experimental induction of inflammation in the histopathological, immunohistochemical, and ultrastructural levels.

MATERIALS AND METHODS

Animals

A total of 52 *Arius* sp. fishes with an average length of 22.31cm were collected using line and hook at the Bertioiga canal in SP, Brazil, and divided into six study groups and one control group. The animals were kept at the ACQUA MUNDO – Aquário do Guarujá, Brazil, in the round water tanks with a total capacity of 1000L under constant aeration and water exchange every alternate day.

Experimental intervention

The animals were anesthetized through the immersion in a 1:10000 benzocaine solution in the alcohol (Ferreira et al., 1979). Following the anesthesia, 0.05mL of the 8mg/mL BCG (Calmette bacillus – Guerin) vaccine, obtained from the Butantã Institute (SP, Brazil) was injected intramuscularly in the caudal peduncular region, above the lateral line in the right side. Following the injection, the animals were returned to the tank, where normal behaviour was recovered after 5 minutes. The muscular fragments were collected at days 1, 3, 7, 14, 21, and 33 post-inoculation as described by Matushima (1994). Briefly, the animals were sacrificed in a supersaturated benzocaine solution and the muscular tissue fragments were collected from the inoculation site using a scalpel blade and immediately fixed in a 10% formaldehyde solution for subsequent histopathological and immunohistochemical analysis.

Histopathology

After the 24h fixation period in 10% formaldehyde, the samples were processed and stained by the Hematoxylin-Eosin (HE) technique and mounted in a microscope slide under a coverslip (Luna, 1992). The slides were viewed under the optic microscopy at 40, 100, 200, 400, and 1,000X magnification.

Immunohistochemistry

Fixed samples were suspended in the paraffin and 5 micron cuts were mounted onto the slides previously treated with 4% sylvane in acetone. The slides were then incubated in primary antibody buffer with either *M. bovis* – BCG (1:10,000 – Dako A/S, Denmark), AE1/AE3 cytokeratin (1:800, Dako A/S, Denmark), or S100 (1:15000, Dako A/S, Denmark) antibodies.

Electron Microscopy

The muscular tissue fragments collected at days 14, 21, and 33 were fixed in a 2% glutaraldehyde in Milloning buffer solution for subsequent processing and viewing under the transmission electron microscopy.

RESULTS

Histopathology

At day 1 post-inoculation, a diffuse inflammatory response comprised of mainly the mononuclear infiltrates, interstitial edema, and muscular necrosis was observed. As days post-inoculation increased, a large number of the phagocytic mononuclear cells were observed, as well as a progressive increase in the multinuclear giant cells characterized by a large cytoplasm containing the agent (Fig. 1). After 21 days post-inoculation, no reaction changes were observed, maintaining a diffuse inflammatory response at the site of the lesion, with a mononuclear infiltrate and a large number of multinuclear giant cells. Although the lesion did not organize into a granuloma, a tendency towards its retreat was observed, because the interstitial edema, hemorrhagic sites, and tissue necrosis decreased, besides the presence of many giant cells around the BCG deposits (Fig. 2).

Immunohistochemistry

All the slides were positive for the BCG at all the experimental times, initially diffused, then defined to the interior of the multinuclear giant cells (Fig. 3). S100 was also found in all the slides, which demonstrate the presence of the macrophage cells in the site of the lesion (Fig. 4). On the other hand, all the slides were negative for the AE1/AE3 cytokeratin, which in turn demonstrated that the macrophages did not differentiate into cytokeratin excreting epithelioid cells (Fig. 5).

Ultrastructure

The multinuclear giant cell ultrastructure analysis demonstrated the presence of many nuclei without a cytoplasmic membrane enveloping each one,

which showed that all belonged to a same cell (Fig. 6).

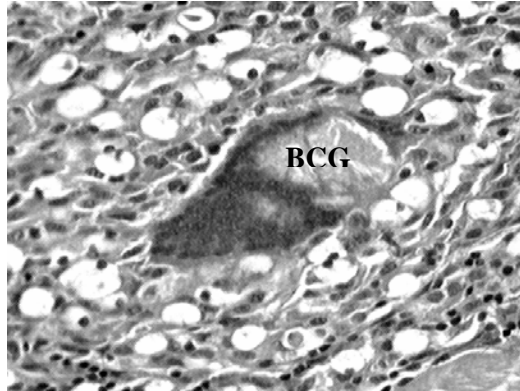


Figure 1 - Photomicrography of *Arius* sp. muscle tissue at day 7 post-inoculation. Multinuclear giant cell with BCG in its interior. HE X 400.

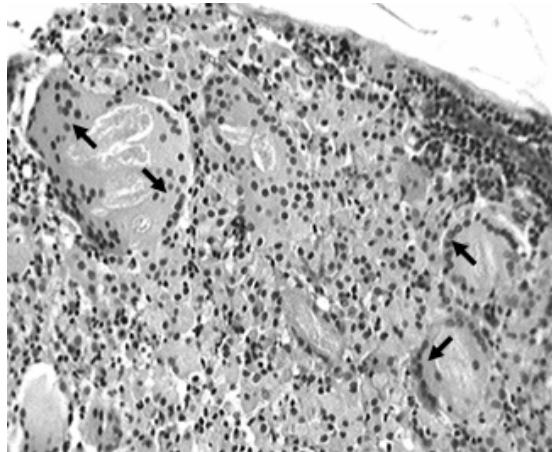


Figure 2 - Photomicrography of *Arius* sp. muscle tissue at day 33 post-inoculation. Many giant cells (arrows) containing BCG. HE X 200.

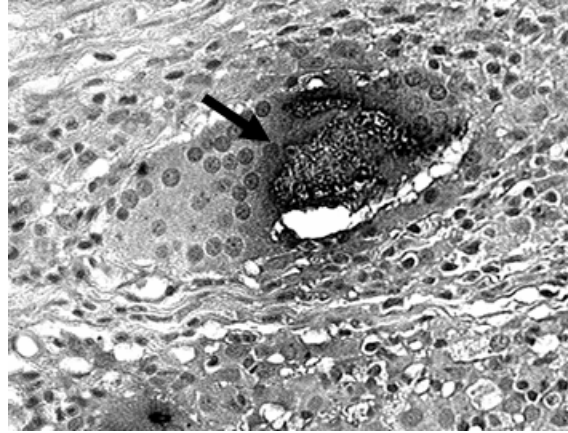


Figure 3 - Photomicrography of *Arius* sp. muscle tissue. The arrow indicates immunohistochemical positivity for BCG inside a giant cell. ABC X 400.

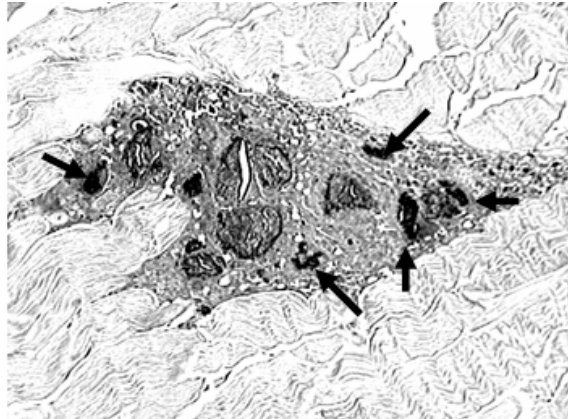


Figure 4 - Photomicrography of *Arius* sp. muscle tissue. The arrows indicate immunohistochemical positivity for S100. ABC X 100.



Figure 5 - Photomicrography of *Arius* sp. muscle tissue. The absent of darkness demonstrate immunohistochemical negativity for AE1/AE3 cyokeratin. ABC X 100.

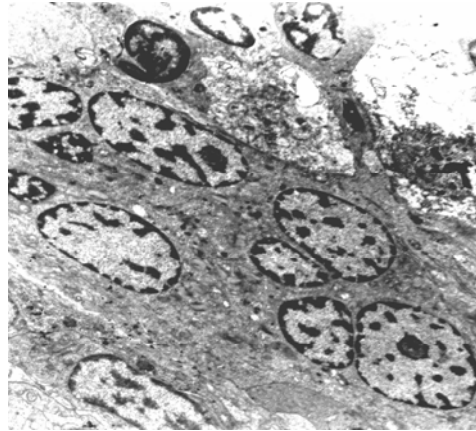


Figure 6 - Ultrastructural photomicrography of *Arius* sp. muscle tissue. No cytoplasmic membrane enveloping each nucleus. X 11.200.

DISCUSSION

In the primitive fish species, a diffuse inflammatory response comprised mainly of the giant cells, without granuloma formation and without differentiation of the macrophages into the epithelioid cells (Goodwin and Grizzle, 1991; Xavier and Matushima, 1993; Gómez, 1998; Talaat et al., 1999; Howard and Byrd, 2000) - negative for the cytokeratin expression - is observed. In this study, both the morphological and cellular differences were observed during inflammatory response. At the day 1 post-inoculation of BCG, the process was characterized as a diffuse inflammatory response with the mononuclear infiltrates, interstitial edema and the sites of tissue necrosis, which agree with the study by Finn and Nielsen (1971).

The intense peri-vascular inflammatory response observed in other studies (Finn and Nielsen, 1971; Nash et al., 1986; Matushima, 1994) was also observed in this study and was indicative of the macrophage migration towards the lesion site. During the experiment, the morphological alterations were observed in the phagocytic cells, such as differentiation into the multinuclear giant cells and this process was time-dependent. The multinuclear giant cell presence was demonstrated in the inflammatory response in the primitive fish in the previous studies also (Albernethy and Lund, 1978; Goodwin and Grizzle, 1991; Pulsford and Matthews, 1991; Xavier and Matushima, 1993; Gómez, 1998; Talaat et al., 1999; Howard and Byrd, 2000). Although some sort of organization

of this lesion was shown, mainly due to the absorption of the BCG into these giant cells, the lesion did not evolve into a granuloma and maintained a diffuse aspect. In *Arius* sp. Fish, this was discordant with other authors (Finn and Nielsen, 1971; Huizinga and Nadakavukaren, 1997; Gómez, 1998; Talaat et al., 1999; Howard and Byrd, 2000) who demonstrated the formation of an organized granuloma formed by the differentiation into the epithelioid cells during inflammatory response in the primitive fish.

The positivity to the anti-BCG antibody found in this study was also demonstrated by Radhakrishnan et al. (1991), Matushima (1994), Carabias et al. (1998) and Rego (1999). In the same manner, positive signals for the anti-S100 antibody, demonstrated in all the experimental times in this study, was also demonstrated by Rego (1999) in *Poecilia* sp. fish, phylogenetically classified as the intermediary (Gosline 1971), and in mammalian granulomas formed by the epithelioid cells (Falk et al., 1988; Muller and Takeshita, 1991; Momotani et al., 1993). The negative response towards the anti AE1/AE3 cytokeratin was also expected, since the histopathological analysis did not show the formation of keratin-secreting epithelioid cells (Noga et al., 1989; Matushima, 1994). The ultrastructure analysis of the multinuclear giant cells showed various nuclei without a cytoplasmic membrane division between them and this was in agreement with Mariano (1972).

The fact that some primitive fish produce the epithelioid cells (Finn and Nielsen, 1971;

Huizinga and Nadakavukaren, 1997; Gómez, 1998; Talaat et al., 1999; Howard and Byrd, 2000) led to speculate that these species were phylogenetically closer to the intermediary fish and were thus beginning to produce similar responses to the inflammatory stimuli, such as the macrophage characteristics, but did not yet possessed sufficient cellular differentiation in order to produce the morphological and functional traits usually found in the epithelioid cells, such as the presence of desmosomes between the adjacent cells and positivity to the anti-AE1/AE3 cytokeratin antibody.

CONCLUSION

Following the intramuscular inoculation with the BCG, *Arius* sp. fish, phylogenetically classified as primitive, produced a diffuse inflammatory response characterized mainly by the mononuclear infiltrated and an increase in the multinuclear giant cells, without, however, organization into an inflammatory granuloma.

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RESUMO

Avaliou-se a cinética da resposta inflamatória induzida experimentalmente com BCG em peixes primitivos pertencentes ao gênero *Arius*. Os animais foram inoculados com BCG por via intramuscular na região do pedúnculo caudal, sendo realizada a coleta do material nos tempos experimentais de 1, 3, 7, 14, 21 e 33 dias pós-inoculação. A fase aguda da resposta inflamatória se mostrou na forma de infiltrado inflamatório composto predominantemente por células mononucleares, edema intersticial e necrose de

tecido muscular. À medida que o processo se desenvolveu, houve formação e aumento no número de células gigantes multinucleadas envolvendo o inóculo. Essas células gigantes, ao exame imunohistoquímico, apresentaram positividade à proteína S100 indicando ação de células macrofágicas, além da ultraestrutura apontar a ausência de membrana citoplasmática entre os inúmeros núcleos presentes nas células. Em vista dos resultados obtidos podemos concluir que em peixes pertencentes ao gênero *Arius* sp. inoculados com BCG, verificou-se durante todo tempo experimental uma resposta inflamatória difusa composta predominantemente por células mononucleares, não havendo a formação granuloma, porém havendo o predomínio de células gigantes.

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