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False Positive Reaction of the Immunohistochemistry Technique Using Anti-BCG Polyclonal Antibodies to Identify *Mycobacterium leprae* in Wild Nine-Banded Armadillos[1]

ABSTRACT

The authors studied 66 wild nine-banded armadillos from Brazil. The ear samples were collected and Ziehl-Neelsen or Fite-Faraco stains were performed, as well as immunostaining using polyclonal BCG antibody, to avaluate the presence of the *Mycobacterium leprae*. The AFB were not detected by the Ziehl-Neelsen or Fite-Faraco staining, neither immunoexpression of the BCG marker. However, many normal structures from the ears of the nine-banded armadillos, such as condrocytes, condroblasts, fibroblasts and endothelial cells, and Gram positive bacteria cocci, showed false positive reaction by the BCG marker. The authors discuss the use of the immunohistochemical studies with the polyclonal BCG antibody to identify *M. leprae* antigens in wild armadillos.

RÉSUMÉ

Les auteurs ont étudié 66 tatous à neuf bandes sauvages du Brésil. Les échantillons d'oreille furent prélevés et des colorations de Ziehl-Neelsen et de Fite-Faraco, ainsi qu'un immunomarquage utilisant un sérum polyclonal dirigé contre le bacille de Calmette et Guérin (BCG), afin d'évaluer la présence de *Mycobacterium leprae*. Des bacilles acido-résistants (AAR) ne furent pas détectés par le Ziehl-Neelsen ou le Fite-Faraco et une immuno-expression du marqueur BCG ne fut pas observée non plus. Cependant, de nombreuses structures normales des oreilles de tatous à neuf bandes, telles que des chondrocytes, des chondroblastes, des fibroblastes et des cellules endothéliales, ainsi que des coques bactériennes positives à la coloration de Gram, ont montré une réaction faussement positive au marqueur BCG. Les auteurs discutent de l'utilité des études immunohistochimiques avec un anticorps polyclonal dirigé contre le BCG pour identifier des antigènes de *M. leprae* chez les tatous sauvages.

RESUMEN

Se estudiaron 66 armadillos silvestres de 9 bandas de Brasil. Se colectaron muestras de las orejas, se seccionaron y se tiñeron por Ziehl-Neelsen o Fite-Faraco, y se evaluó la presencia de *Mycobacterium leprae* utilizando un anticuerpo policlonal anti-BCG. No se detectaron bacilos ácido-resistentes por las tinciones de Ziehl-Nelseen/Fite-Faraco y tampoco se encontraron bacilos con el suero anti-BCG. Sin embargo, se observó que muchas estructuras normales de las orejas de los armadillos, tales como condrocitos, condroblastos, fibroblastos y células endoteliales, y bacterias Gram negativas, dieron reacciones falsas-negativas con el reactivo anti-BCG. Los autores discuten el uso de los estudios inmunohistoquímicos con el suero policlonal anti-BCG para identificar antígenos de *M. leprae* en los armadillos silvestres.

TO THE EDITOR:

Current identification of *Mycobacterium leprae* is done by Fite-Faraco or Ziehl-Neelsen stains, but low numbers of bacillus in the tissue could make identification difficult. Some authors have reported that immunohistochemistry techniques demonstrate antigens of *M. leprae* instead of the whole bacillus, increasing the sensibility of the histopathology diagnose mainly in paucibacillary forms ([1, 5, 7]). Takahashi, *et al.* ([7]) identified more then 20% of the AFB from of the skin biopsy of the indeterminate leprosy (paucibacillary form) using immunostaining with anti-BCG (bacilli of Calmette-

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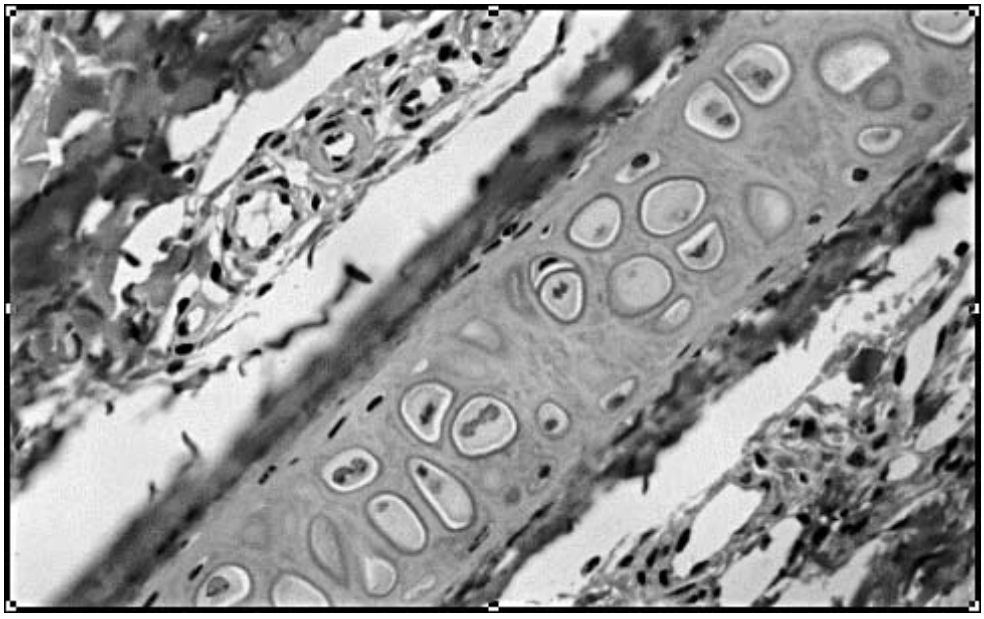

FIG. 1. Immunohistochemistry findings in ear fragment. Nucleous of the condrocytes, condroblasts, fibroblasts, endothelial cells presenting immunoexpression to the anti-BCG marker. (Objective 40×).

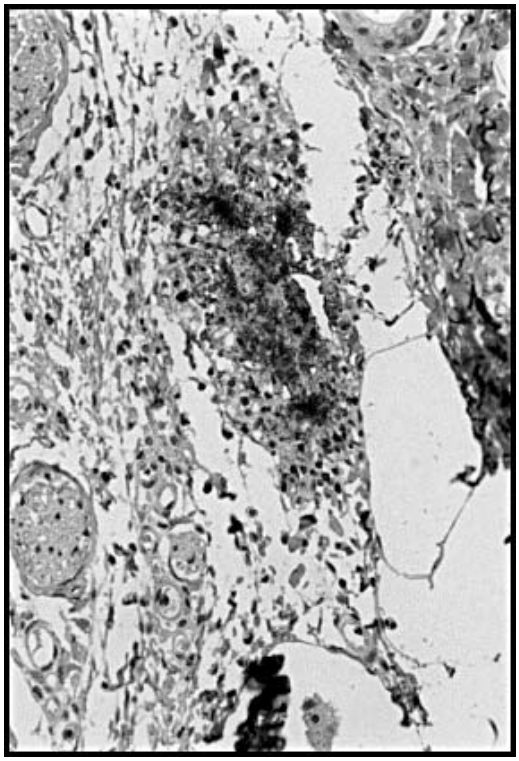

FIG. 2. Immunohistochemistry findings in ear fragment. Inflammatory infiltration of the lymphohistiocytes and neutrophils, and Gram positive bacteria as coccus presenting immunoexpression to the anti-BCG marker (Objective 20×).

Guérin) antibodies. Using the same technique, Barbosa Jr., *et al.* ([1]) found acid-fast bacilli (AFB) in 50% of the skin biopsies where they did not find AFB before by Fite-Faraco stainning. The antigens were detected in several sites of the dermis, mainly in the perivascular region. Kutzner, *et al.* ([4]) described the anti-BCG immunostaining technique as a promising screening tool for the detection of common infectious microorganisms in humans. The anti-BCG antibodies do not react with the normal structures from human skin nor with inflammatory cells, and produce little background, although Schettini, *et al.* ([6]) observed that melanophages and mast cells could be stained. Some authors have demonstrated cross reaction between polyclonal antibodies and a large spectrum of fungi and bacteria ([2, 3, 4, 9]). Bonenberger, *et al.* ([2]) established that some microorganisms such as *Mycobacteria* and *Nocardia* were strongly stained by immunostaining with anti-BCG; *Criptococcus*, *Pythium* and *Prototheca* were partially stained; spirochetes and protozoa *(Leishmania)* were not stained.

We analyzed 66 wild nine-banded armadillos, *Dasypus novemcinctus* species, from the State of Espírito Santo, localized in Southern region of Brazil. The purpose of this research was to evaluate if im-

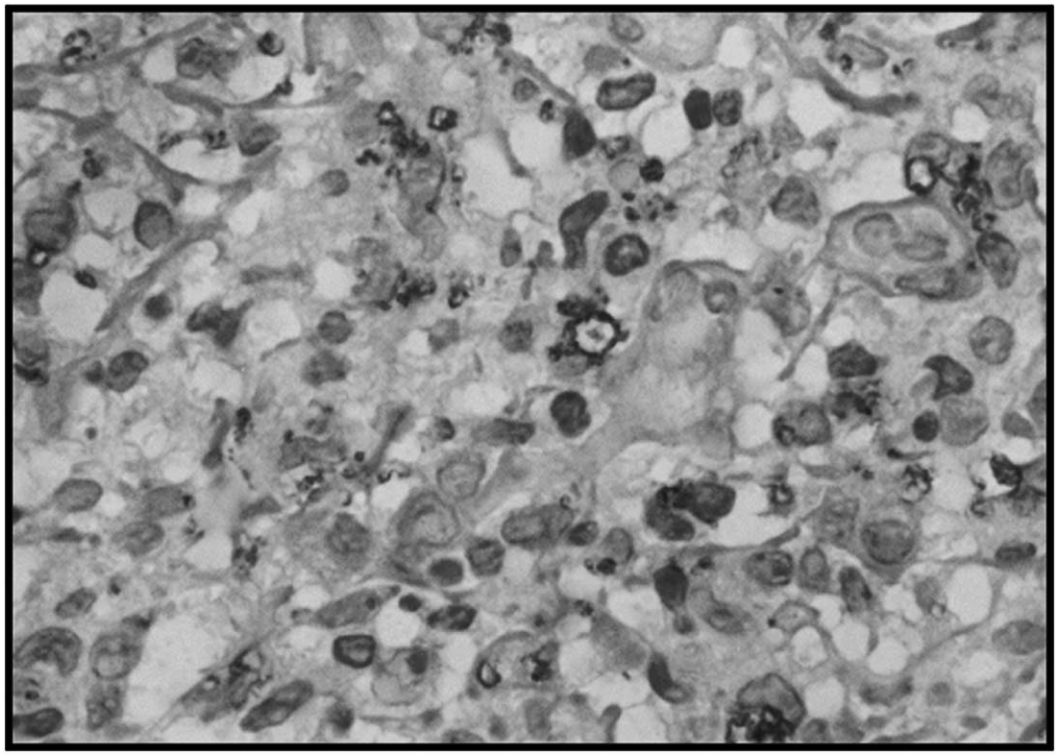

FIG. 3. Immunohistochemistry findings in human skin fragment from leprosy multibacillary patient, the human positive control (Objective 40×).

munostaining with a polyclonal anti-BCG antibody was an appropriate diagnostic tool for *M. leprae* detection in these animals. After the armadillos had been captured, the ears, margins were collected and fixed in 10% neutral buffered formalin.

The paraffin-embedded samples were sectioned and slides were stained with Ziehl-Neelsen or Fite-Faraco stains and immunohistochemistry. We used the immunostaining with the anti-BCG on human positive-control biopsies from multibacillary patients, to compare with the samples from the armadillos. After blocking of endogenous peroxidase, the slides were incubated with the polyclonal rabbit antibody (anti-*Mycobacterium bovis*, DAKO A/S, Denmark), Dilution of 1:100,000 was done in PBS buffered pH 7.4 with bovine serum albumin (BSA). After this, the slides were incubated with the secondary antibody, biotinylated goat anti-mouse/rabbit Ig (StrepABComplex/HRP Duet mouse/rabbit, DAKO A/S, Denmark), diluted 1:200 in PBS and with the strepavidin-Biotin-peroxidase complex 1:200 in PBS. The antigens were visualized after incubation with the diaminobenzidine tetrahydrochloride 60 mg% (SIGMA, EUA), DMSO 1 ml, 6% H_2O_2 20 volume and PBS 100 ml for five minutes at room temperature. Negative controls were prepared by omission of the primary antibody and substitution with BSA. The readings were carried out on coded, "blinded" slides.

The histopathological examination of the ear biopsies showed an inflammatory infiltrate of the lympho-histiocytes and neutrophils, but no AFB was found by Ziehl-Neelsen or Fite Faraco staining, or by immunohistochemical study. However, nuclei of many normal cells, such as condrocytes, condroblasts, fibroblasts, endothelial cells (Fig. 1), and Gram positive bacteria cocci produced immunostaining with the anti-BCG marker (Fig. 2). In all the material, the primary antibody was replaced by BSA to demonstrate that there are no reactions due to the secondary antibody or slide background. The human controls showed macrophages immunostained with the anti-BCG marker (Fig. 3).

The immunohistochemical technique using polyclonal anti-BCG antibody for detection of *M. leprae* antigens in ears from

wild armadillos, or other armadillo tissues, has not been reported before. We found several structures presenting immunoexpression to the anti-BCG marker in the epidermis and the dermis of the ear biopsies, without the morphologic features presented by the *M. leprae* immunostained antigens previously described. Probably in wild nine-banded armadillo, microorganisms other than *Mycobacteria* could be responsible for false positive results in these tissues. These findings agree with previous reports ([2, 4]). Another interesting feature observed is that the polyclonal anti-BCG antibody stains Gram positive bacteria. Some animals showed very similar structures to the morphology of coccus. This similarity can create doubt about the use of the immunohistochemical methods using the polyclonal anti-BCG alone to detect the *M. leprae* infection in wild armadillos. We do not know if this technique would be useful to detect mycobacterial antigens in tissue other than ears, such as skin, lymphnodes, or liver. In conclusion, the immunohistochemical technique, using anti-BCG antibodies, is a very useful tool for the diagnosis of human paucibacillary or indeterminate forms of leprosy, but in wild nine-banded armadillos immunostaining of normal structures of the ear and cross reaction with different infectious agents could difficult its utilization for Mycobacterial antigen detection difficult.

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