PATTERN OF MYCOBACTERIAL ANTIGEN DETECTION IN LEPROSY

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SUMMARY

Immunohistochemistry reaction (Peroxidase anti-peroxidase - PAP) was carried out on fifty-two skin biopsies from leprosy patients with the purpose to identify the antigenic pattern in mycobacteria and to study the sensitivity of this method. Five different patterns were found: bacillar, granular, vesicular, cytoplasmatic and deposits, classified according to the antigenic material characteristics. Deposits (thinely particulate material) appeared more frequently, confirming the immunohistochemistry sensitivity to detect small amounts of antigens even when this material is not detected by histochemical stainings.

KEYWORDS: Immunohistochemistry; Leprosy; Mycobacterial antigen.

INTRODUCTION

Mycobacteria are associated to many human infections such as leprosy which is one of the most important infectious diseases in our country.

The diagnosis is usually made by identification of mycobacteria in tissue and the most common staining technique used are Kinyon, Faraco-Fite and Ziehl-Neelsen that is a good and fast method for identification of the microorganisms. However these methods can only identify either whole or fragments of bacilli [8] and when mycobacterial load is low ($<10^4$ ml $^{-1}$), bacilli are rarely seen in Ziehl-Neelsen staining [5]. It has been demonstrated that in lesions where bacilli are rare or absent, mycobacterial antigens can be found; it means that debris of mycobacteria mantain the antigenic properties even when the acid-fast staining properties are lost.

Based on this, the detection of mycobacterial antigenic material with mono and polyclonal antibodies has been used in order to enhance the sensitivity for the diagnosis of this infection [1].

ORRELL et al., using a rabbit polyclonal anti-BCG antiserum (DAKO) with avidin-biotin immunoperoxidase, demonstrated a granular and diffuse staining in lung of mice infected with *Mycobacterium tuberculosis* [9].

HIGUSHI et al. demonstrated fragments and wall components of BCG in the inoculation site where bacilli could not be detected by histochemical techniques [3]. HUMPHREY and WEINER, using antibody against *Mycobacterium tuberculosis* with Peroxidase anti-peroxidase method (PAP) demonstrated antigenic material in fragments of bacilli of tuberculosis [4]. MSHANA et al. used Peroxidase antiperoxidase method to demonstrate mycobacterial antigens in skin biopsies of leprosy patients [7]. They found a

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positive reaction in biopsies material where no mor-phologically preserved bacilli were identified. In an-other paper, using the same method with the anti-*Mycobacterium bovis* (DAKO), mycobacterial antigens in peripheral nerve biopsies from patients with leprosy were detected [8]. WILLEY et al. using the Peroxidase anti-peroxidase method with three different antibodies, among them the anti-*M. bovis* (DAKO) found higher positivity compared with histochemical stains [10].

Since immunohistochemistry is a sensitive tech-nique to detect mycobacterial antigens in tissue and that these antigens can show many patterns, we propose to identify a profile of antigenic patterns in myco-bacteria in cases of leprosy.

MATERIAL AND METHODS

Fifty-two skin biopsies from leprosy patients were selected from the Dermatology Department of the Clinical Hospital of the University of São Paulo. Twenty-nine were paucibacillar, twenty multibacillar and three Mitsuda reaction biopsies.

Fourteen of the biopsies came from follow up pa-tients after treatment and six were initial biopsies. Posi-tive controls used were selected from cases in which mycobacteria had been identified with the use of routine acid-fast stains. The histochemical study of the biopsies was performed using the Ziehl-Neelsen staining.

Four-micra sections were cut from paraffin blocks, deparaffinized twice in xylene for 60 minutes at 56°C and 20 minutes at room temperature, respectively. After that they were hydrated in alcohol (from 100% to 70%) and washed in tap water and distilated water. Endogenous peroxidase was blocked incubating twice in hydrogen peroxidase 0.3% with Methanol (1:1) for 10 minutes each.

Slides were washed in tap water, distilated water and PBS and incubated with Swine Normal Serum (DAKO X901-USA), 1/20 for 20 minutes at 37°C and then incubated with specific antibody. Polyclonal rab-bit antibody against *Mycobacterium bovis* (BCG) is known to cross-react with *Mycobacterium leprae* [2] and it was used as primary antibody (DAKO B124-USA), at the dilution of 1/50000. After over-night incubation in refrigerator the slides were washed in PBS twice during 15 minutes and incubated with Swine anti-rabbit anti-body (DAKO Z196-USA) 1/160 for 30 minutes at 37°C. Slides were washed in PBS and incubated with Rabbit peroxidase anti-peroxidase (DAKO Z113-USA) 1/160 for 30 minutes at 37°C.

The antigen was visualized after incubation for 3 minutes with 0.03% 3,3'- Diaminobenzidine (SIGMA) in PBS with 300µl hydrogen peroxidase (20V). The slides were washed in tap water, counterstained with hematoxylin and mounted with Permount.

RESULTS

We found five different antigenic patterns: bacil-lar, granular, vesicular, cytoplasmatic and deposits.

Bacillar pattern was represented by large bacilli fragments found out in nerves or macrophages cyto-plasm (Fig. 1). Particulate material forming cytoplas-matic granules was named granular pattern (Fig. 2).
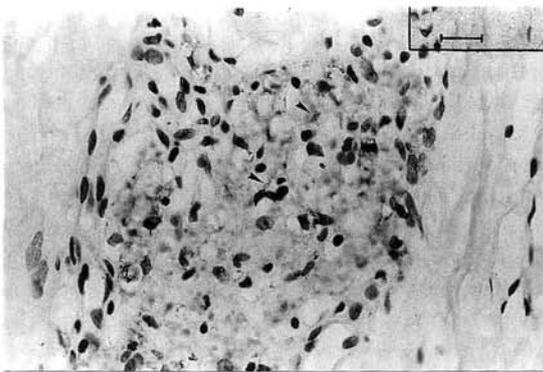

Fig. 1 - Leprosy skin biopsy. Little organized epithelioid granuloma. Bacillar pattern in the macrophages cytoplasm (→). PAP, x63.
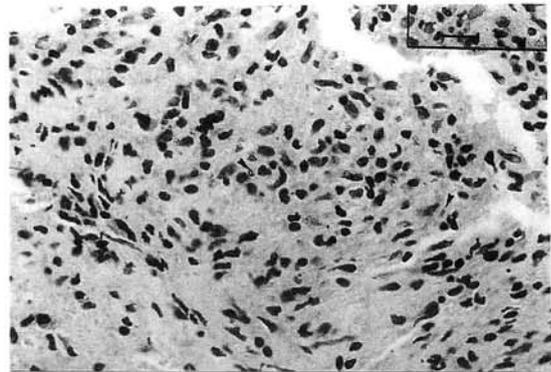

Fig. 2 - Leprosy skin biopsy. Tuberculoid granuloma. Granular pattern in the macrophage cytoplasm (→). PAP, x63.

The vesicular pattern was represented by stained circles in nerves or macrophages cytoplasm (Fig. 3). Cytoplasm stained homogeneously was classified as cytoplasmatic pattern (Fig. 4) and deposits were constituted by thinely particulate material (TPM) in nerves and macrophages cytoplasm. (Fig. 5).

Bacillar pattern appeared eigth times; granular pattern 17 and the vesicular pattern (circles) 13. Deposits (TPM) appeared 23 times and the cytoplasmatic pattern eight.

Association of two or more patterns was observed in the same slide or the patterns appeared individually. We found fifteen samples showing only deposits (TPM), nine samples showing only granular pattern, seven samples with cytoplasmatic pattern, four samples showing granular pattern associated to vesicular pattern in the same slide, four samples with vesicular
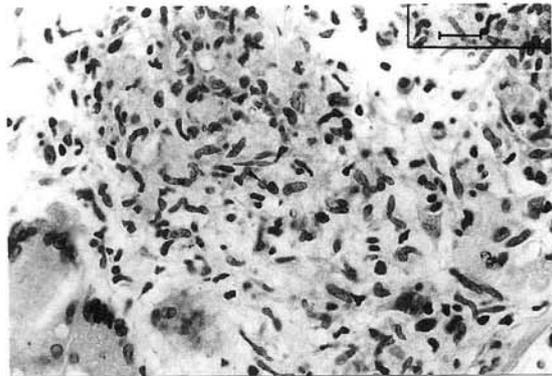

Fig. 5 - Leprosy skin biopsy. Deposits (thinely particulate material) in macrophages cytoplasm (→). PAP, x63.

Fig. 3 - Leprosy skin biopsy. Tuberculoid granuloma with macrophages showing vesicular pattern in the cytoplasm (→). PAP, x63.

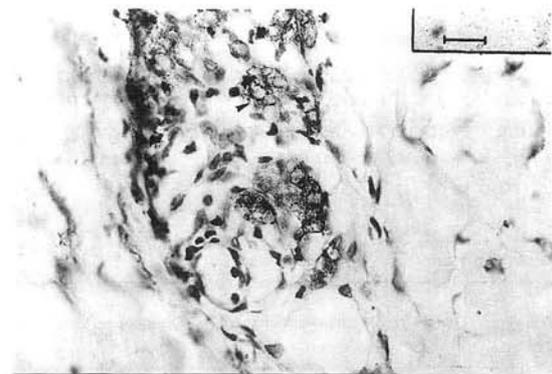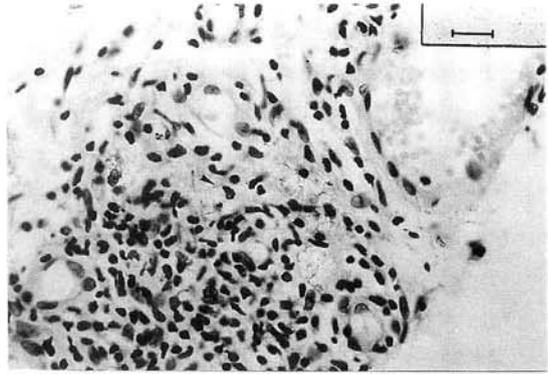Fig. 4 - Leprosy skin biopsy. Perivascular macrophagic accumulation with cytoplasmatic pattern (→). PAP, x63.

pattern associated to deposits (TPM) in the same slide, three with granular pattern associated to bacilli, three samples showing bacilli associated to deposits, three samples showing only the vesicular pattern, two showing only bacilli, one showing cytoplasmatic pattern associated to vesicular pattern and one sample where appeared the patterns vesicular, granular and deposits.

In percents, the results are represented in graphic 1.

Among the 52 biopsies, 29 showed the Paucibacillar form, 20 showed the Multibacillar form and three were from Mitsuda reaction.

The distribution of the different patterns among the biopsies with Paucibacillar and Multibacillar form is represented in Table 1.

Among the three biopsies with Mitsuda reaction, we found 1 cytoplasmatic pattern, 1 bacillar pattern associated to deposits and 1 biopsy showing only deposits (TPM).

Twenty-three biopsies were initial and deposits (TPM) were more frequent appearing in eight of them. The granular pattern was predominant among the biopsies from patients in treatment, appearing in seven of them. The distribution of the patterns among the initial biopsies and those from patients in treatment is represented in Table 2.

We found three different kinds of positive results in histochemistry, classified as "whole bacilli", "whole bacilli + granules" and "granules".
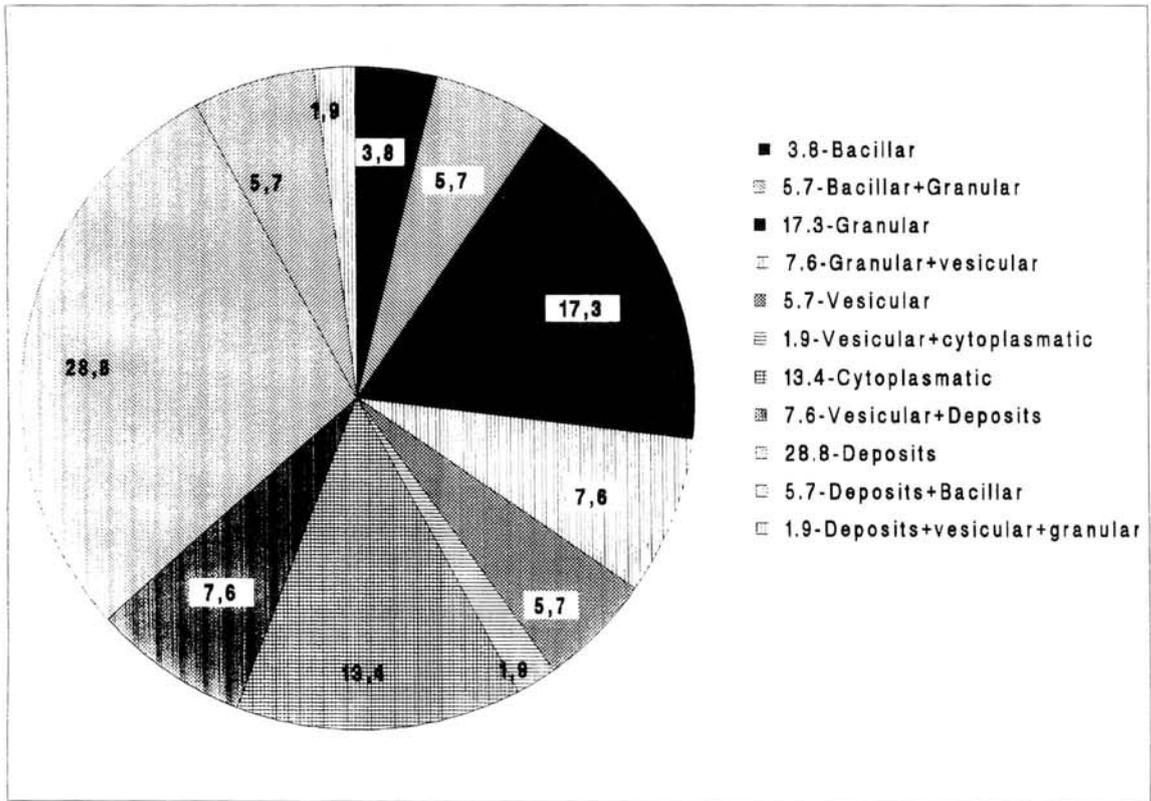
Graphic 1 - Association of different antigenic patterns. Representation in percents.

When we related Histochemistry to Immunohistochemistry we found the results showed in Table 3.

DISCUSSION

From 52 cases, only eight appeared as bacillar pattern and two composed exclusively by bacilli. The relatively low occurrence of this pattern was expected because the biopsies were mostly from patients in treatment. On the other hand, we have to consider some evidences that mycobacteria are impermeable to antibodies when preserved and so, they do not penetrate to the whole bacilli cell wall [9] and only fragments (even in large size) can be detected.

That is similar with the impermeability of mycobacteria to tinctorial stains. In histochemical stains the whole bacilli can be detected only if the tissue is previously treated with oily substances to evidence the bacilli wall [6].

It was not possible to conclude the sequential pattern of bacilli decomposition after fragmentation during treatment and regression of the leprosy because

Table 1

Distribution of different antigenic patterns among the biopsies taken from Pauci and Multibacillary patients.

	Bacillar	Granular	Vesicular	Cytopl.	Deposits	Bacillar+ Granular	Cytopl+ Vesicular	Bacillar+ Deposits	Vesicular+ Granular+ Deposits	Granular+ Vesicular	Deposits+ Vesicular
Paucibac.	2	5	2	4	11	0	0	1	0	1	3
Multibac.	0	4	1	2	3	3	1	1	1	3	1

Table 2

Distribution of the different antigenic patterns among the initial biopsies and biopsies from patients in treatment.

	Bacillar	Granular	Vesicular	Cytopl.	Deposits	Bacillar+ Granular	Cytopl+ Vesicular	Bacillar+ Deposits	Vesicular+ Granular+ Deposits	Granular+ Vesicular	Deposits+ Vesicular
Initial	1	1	3	1	8	2	0	1	1	2	3
Treatment	1	7	0	4	4	1	0	1	0	2	1

the cases came from patients in different steps of the disease.

When we compared the results from Immuno-histochemistry with those obtained from Histoche-mistry (Ziehl-Neelsen), we could verify that from 28 biopsies with negative result in the Acid-Fast Bacilli stain (53% of the cases), 13 showed deposits (TPM) as antigenic pattern in Immunohistochemistry confirming that it had the sensitivity to detect small amounts of antigens and show that mycobacterial debris can keep their antigenic characteristics even when they are not detected by histochemical stainings.

Among the 29 biopsies taken from Paucibacillary patients, 11 showed deposits as antigenic pattern and so, we conclude that the Immunohistochemistry can be a good method for the diagnosis of those forms, as they do not show many bacilli in histochemical stainings.

Therefore, we could conclude that Immuno-histochemistry is an auxiliary procedure for diagnosis of leprosy, specially important to the control of the disease treatment, where the presence of antigenic material is not detectable by histochemical stainings. All patterns represent the presence of the antigens in

the lesions, showing that it is not necessary to identify the whole forms of the bacilli for the etiological diag-nosis in leprosy.

RESUMO

Detecção de padrões antigênicos de micobactérias na hanseníase

Foi realizada reação imunohistoquímica (Pe-roxidase anti-peroxidase-PAP) em 52 biópsias de pele de pacientes com hanseníase com o objetivo de verificar quais os padrões antigênicos das mico-bactérias e estudar a sensibilidade deste método como auxílio diagnóstico da hanseníase.

Foram encontrados cinco padrões diferentes, clas-sificados como bacilar, granular, vesicular, citoplas-mático e depósitos, de acordo com a característica do material antigênico.

Os depósitos (material finamente particulado) apareceram com maior frequencia confirmando a sensibilidade da imunohistoquímica na detecção de pequenas quantidades de antígeno, cujo material não é mais visto por colorações histoquímicas usuais.

Table 3

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Relationship among the results found in Acid-Fast Bacilli stain (A.F.B.) and Immunohistochemistry reaction.

A. F. B.	Bacillar	Granular	Vesicular	Cytopl.	Deposits	Bacillar+ Granular	Cytopl+ Vesicular	Bacillar+ Deposits	Vesicular+ Granular+ Deposits	Granular+ Vesicular	Deposits+ Vesicular
Whole Bacilli	1	0	2	1	2	1	1	0	0	0	2
Granules	0	2	0	1	0	1	0	1	0	1	1
Whole Bacilli+ Granules	0	0	0	0	0	1	0	0	1	2	1
Negative	1	6	1	4	13	0	0	2	0	1	0

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