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Enhancement in the Histological Diagnosis of Leprosy in Patients with Only Sensory Loss by Demonstration of Mycobacterial Antigens Using Anti-BCG Polyclonal Antibodies[1]

Antonio Pedro M. Schettini, Luiz Carlos de L. Ferreira, Ruth Milagros, Maria da Conceição A. Schettini, Silmara N. Pennini, and Paula B. Rebello[2]

Patients with complaints of only localized skin sensory loss, without any other sign or symptom, may be early leprosy cases in its purely neural form ([4, 22]). The confirmation of the diagnosis or the exclusion of active leprosy infection in these cases is very difficult because there is no palpable nerve trunk, the skin-smear is negative and the other neurological investigations are nonspecific ([6, 12, 15, 16, 22, 29]). The skin biopsy of the sensory loss area is a procedure that has been used with success confirming some of these cases as leprosy and sparing the patient the necessity of undergoing biopsy of a peripheral nerve, but it is difficult to find the bacilli in a sample of skin tissue using conventional histopathology stains (Fite-Faraco, Ziehl Neelsen, Wade) ([8, 11, 23, 26]). However, many authors have shown that immunohistochemical techniques increase the sensitivity of the histopathological diagnosis of early forms of leprosy by detecting mycobacterial antigens ([1, 11, 17, 18, 19, 23, 25, 26, 27, 30]).

We proposed to undertake this study to assess if the immunoperoxidase technique using the avidin-biotin complex and anti-BCG serum is able to confirm the diagnosis of leprosy among patients with only sensory loss in a higher proportion than the routine mycobacterial staining methods (hematoxylin and eosin and Wade staining).

MATERIALS AND METHODS

Fifty-one paraffin blocks containing skin biopsies of fifty-one patients, who in 1994 presented only localized sensory loss and had skin biopsies done for diagnostic purposes, were retrieved from the archives of the "Fundação Alfredo da Matta" in the state of Amazonas, north of Brazil. All the patients were skin smear negative and the biopsies were performed with punches and processed for paraffin embedding in the routine way which did not envisage the immunohistochemical study.

Paraffin blocks of 50 patients who had undergone biopsies during the same period for the diagnosis of other skin conditions were also retrieved from the same archives. All these patients had the histopathological diagnosis of chronic dermatitis which could be considered in the differential histopathological diagnosis of early leprosy.

New cuts of 4 micra were carried out on the paraffin blocks in order to have them submitted to the hematoxylin and eosin (H&E) and Wade stains and also to the immunohistochemical study, as follows. The slides were first treated with polylysine (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) and the sections were initially deparaffinized and rehydrated in alcohol of 70% to 100%. The blockage of endogenous peroxidase was performed using a solution

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[2] Antonio Pedro M. Schettini, M.D., MsC., Maria Da Conceição A. Schettini, M.D. Silmara N. Pennini, M.D., MsC., and Paula B. Rebello, M.D., Fundação Alfredo da Matta, Av. Codajás no. 25, CEP 69065-130. Cachoeirinha, Manaus-Amazonas-Brasil; Luiz Carlos de L. Ferreira, M.D., Ph.D., Universidade Federal do Amazonas, Faculdade de Ciencias da Saúda, Rua Afonso Pena, 1053, Praça 14, Manaus-Amazonas-Brasil; and Ruth Milagros, Fundação de Medicine Tropical do Amazonas Av. Pedro Teixeira s/n, CEP 69040-000 Dom Pedro I, Manaus-Amazonas-Brasil.

Reprint requests to Dr. Antonio Pedro M. Schettini, Rua Benjamim Lima nº 16, Q36, Manaus/AM—Brazil 69.044-040, or FAX 55-92-2342115, e-mail: apms@argo.com.br

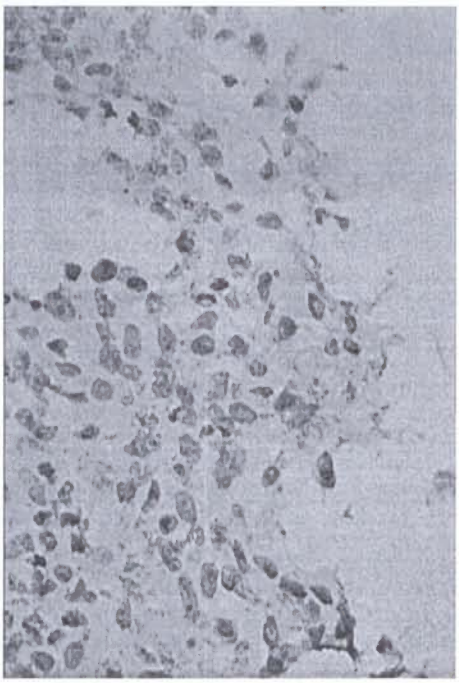

FIG. 1. Appearance of a positive control (LL) immunostained for BCG antigen (×200).

TABLE 1. *Baseline characteristics of the patients with sensory loss areas and patients with chronic dermatitis.*

	Patients with	
	Sensory loss (N = 51)	Chronic dermatitis (N = 50)
Age[a]		
Mean (standard deviation)	34.4 (14.7)	25.7 (17.7)
Range	12–72	01–66
Sex[b]		
Males	32 (64%)	20 (40%)
Females	18 (36%)	30 (60%)
Duration of sensory loss[c]		
<1 year	8 (28.6%)	
1–5 years	17 (60.7%)	—
>5 years	3 (10.7%)	
Site of sensory loss		
Upper limbs	10 (19.6%)	—
Lower limbs	41 (80.4%)	

[a] Data missing for 1 patient of the study group and for 2 patients of the control group.
[b] Data missing for 1 patient of the study group.
[c] Data missing for 23 patients.

of 3% hydrogen peroxide in methanol (1:1). After being washed in phosphate-buffered saline (PBS), pH 7.2, the slides were incubated with normal swine serum (Dako Corp., Carpinteria, California, U.S.A., X901) for 20 minutes. Following this, the slides were placed in a rack containing citric acid, pH 6.0, for the stage of antigenic recuperation, carried out in a pressure cooker for 5 minutes. The polyclonal rabbit antibody against *Mycobacterium bovis* (BCG) (Dako B124, Lot 115, U.S.A.), in a dilution of 1:5000, was applied for 16 hours in a humid layer. After being washed with PBS, the slides were incubated with the secondary antibody (biotinylated goat anti-rabbit IgG 1:5000) (Dako, X 936, Lot 033, U.S.A.). Next the complex of avidin-biotin-peroxidase (Dako K0355, Lot 1060, U.S.A.) was applied. The antigens were visualized after incubation with the diaminobenzidine (Sigma) and the slides counterstained with H&E ([10]).

The reading of the slides carried out in a "blind" manner, that is without the examiner knowing whether the slides were part of the group from patients with suspected early leprosy or the group with chronic dermatitis. The identification of the acid-fast bacilli by Wade stain, and/or the demonstration of inflammation in the neurovascular bundles and around skin appendages with the H&E stain, were taken as parameters for the confirmation of the diagnosis of leprosy. In the immunohistochemical study, the diagnostic criterion was the demonstration of intracellular yellow-brown pigmentation (Fig. 1).

In the statistical analysis, the McNemar paired χ-squared test was applied for one degree of freedom in order to compare the results obtained by routine stains (using H&E and Wade) and by the immunohistochemical stain.

Sixty-four percent of the patients of the study group were males and 36% were females. The ages of the subjects varied between 12 and 72, with a mean age of 34.4 years. The localization of the anaesthetic area was most frequently in the lower limbs in 80% of the cases. Sixty percent of the cases presented symptoms within 1 to 5 years of the medical consultation. In the control group, 60% of the patients were female, 40% were males, and the mean age was 25.7 years (Table 1).

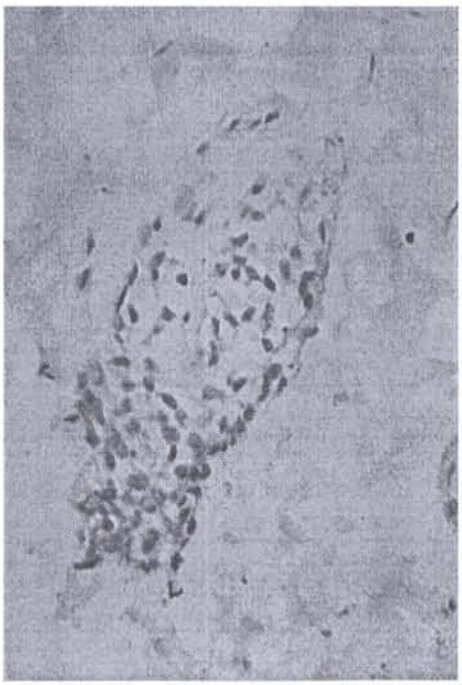

FIG. 2. Appearance of a positive control (LL) immunostained BCG antigen (×200).

RESULTS

From the group of patients with sensory loss, the diagnosis of leprosy using routine stains (H&E and Wade) was confirmed in 9/51 cases (17.6%). Seven cases exhibited features of tuberculoid (TT) or borderline tuberculoid (BT) leprosy. The inflammatory infiltrate consisted of well-circumscribed granulomas made up of epithelioid cells, macrophages, giant cells and lymphocytes in the periphery and were classified as TT. The BT classification was considered when there were similar granulomas but with lymphocytes within it ([6, 23]). All the readings presented granulomatous infiltrates around dermal nerves and there were no acid-fast bacilli.

Indeterminate (I) leprosy was observed in two cases which showed mild lymphohistiocytic infiltration around vessels, appendages and nerves. In only one case acid-fast bacilli were present in the nerves. In 42/51 patients (82%) the skin biopsies showed non-specific inflammatory changes, observed only in small lymphohistiocytic collections around upper dermal blood vessels.

Using the immunoperoxidase, by means of anti-BCG polyclonal antibodies, it was possible to confirm the diagnosis of leprosy in 24 cases (47.1%) of localized sensory loss. The mycobacterial antigens were seen at various sites in the dermis and in several cases at multiple locations: a) around blood vessels—22 cases, b) hair follicles and in either the sweat glands or the sebaceous glands—14 cases, and c) dermal nerves—7 cases (Fig. 2).

TABLE 2. *Results of immunohistochemistry with anti-BCG and routine stains on the patients with sensory loss.*

Immunohistochemistry (Anti-BCG)	Routine stains (H&E/Wade)		
	Positive	Negative	TOTAL
Positive	8	16	24
Negative	1	26	27
Total	9	42	51

All cases of sensory loss except one showed alterations compatible with leprosy by the routine stains, and also showed mycobacterial antigens recognized by immunostaining. On the other hand, 16 cases that showed only a nonspecific infiltration by conventional histopathology had antigenic positivity recognized by the immunohistochemical study (Table 2).

Presumed antigenic positive test was seen in 8 patients (16%) among the patients with the histopathological diagnosis of non-mycobacterial chronic dermatitis (Table 3).

The observed difference between the techniques (immunoperoxidase and routine histopathology) was statistically significant by McNemar's test ($p = <0.05$).

DISCUSSION

Leprosy is diagnosed histologically on finding acid-fast bacilli in the sites of predilection, such as in the nerves, arrector pili muscles, just under the epidermis or in macrophages around vessels. In the early

TABLE 3. *Results obtained from the use of anti-BCG polyclonal antibodies in the study and control groups.*

Anti-BCG	Patients with		Total
	Sensory loss	Chronic dermatitis	
Positive	24	08	32
Negative	27	42	69
Total	51	50	101

stages of the disease, the diagnosis is difficult because of the low bacillary load and because the bacilli may present morphological alterations and may have lost their acid-fast staining quality ([3, 8, 9, 21]). The utilization of the immunohistochemical method, principally by using polyclonal anti-BCG antibody, has increased the sensitivity of the diagnosis of leprosy in paucibacillary (PB) forms ([11, 17, 18, 19, 23, 24, 25]). In the cases where bacilli or bacillary antigens were not shown, but where there was inflammatory infiltrate around skin appendages and nerve involvement was prevalent, it was considered to be compatible with the diagnosis of leprosy.

Particularly in endemic areas, health professionals are confronted with patients with only a localized sensory loss area ([13, 22, 28]). These patients may have a very early stage of purely neural leprosy and the diagnosis can only be confirmed if the acid-fast bacilli or its antigenic components are observed, or if there are the well-known histological changes due to leprosy in a biopsy from the peripheral nerve or from the hypesthetic yet normal-appearing skin ([4, 13, 21, 26]).

The results of this study showed that a skin biopsy from a hypesthetic area could show specific histological changes due to leprosy and corroborates the trends reported in other studies ([13, 26]). This is very important in endemic areas because the skin biopsy is a simple technique with minimal morbidity and it spares the patient the necessity of undergoing a biopsy of a peripheral nerve ([12, 13, 18, 26]).

Studies regarding an enhancement in the histological diagnosis of leprosy by the demonstration of mycobacterial antigens with anti-BCG polyclonal antibodies have been reported, and the present study has shown that it is possible to demonstrate antigenic determinants of *M. leprae* with good quality even with biopsies collected years ago and fixed in formalin (for periods of over 3 days). It has also shown that the immunostaining could demonstrate the presence of mycobacterial antigens in all patients except one, who presented histopathological alterations specific to leprosy by the routine stains and in more than 16 patients whose routine histopathology presented a nonspecific inflammatory infiltration. These results show that mycobacterial antigens could be identified in many cases in which no bacilli were visible and where there was no suspicion of infiltration of leprosy in the routine stains.

The statistical analysis of the comparison of the results obtained from the staining used in routine histopathological leprosy diagnosis and the results obtained using the immunostaining method demonstrated a significant difference in the number of leprosy cases diagnosed. The superiority of the performance of the immunohistochemical method in the histological diagnosis of leprosy had already been demonstrated by other authors in paucibacillary forms of the disease ([1, 7, 11, 17, 19, 23, 27]), however, it had not been demonstrated in cases presenting with only localized sensory loss.

The most commonly observed site of antigenic detection was in macrophages around blood vessels, and is comparable with observations by others in cases of indeterminate (I) leprosy ([1]). In contrast to other authors, we found immunoreactivity in some skin diseases that have a differential diagnosis of leprosy, from the histopathological point of view ([1, 27]). Probably it was a misinterpretation due to the fact that mast cells and melanophages may resemble immunoreactive cells. Moreover, there are some reports of cross-reaction with other mycobacteria and some species of fungi ([2, 14, 31]).

The demonstration of mycobacterial antigens in hypesthetic normal-appearing skin alone is not necessarily diagnostic of purely neural leprosy, but this result must be analyzed together with clinical-epidemiological information, as well as with the routine histopathological findings. Also failure to detect mycobacterial antigens in some biopsies of patients with only sensory loss does not necessarily mean exclusion of leprosy. The patients should be followed up by the health team to see if they will develop cardinal signs of leprosy ([23]).

Finally the present study suggests that immunostaining, by means of anti-BCG polyclonal antibodies, may increase the proportion of the histological diagnosis of leprosy in patients who present with only sensory loss. This is the case even with biopsies obtained in fieldwork conditions, and is very advantageous in hyper-endemic areas of leprosy, such as Brazil ([5, 20]). Furthermore, in the post-elimination period of

leprosy control, the sensory loss may be a sentinel sign of the disease.

SUMMARY

This study was undertaken to assess whether the immunoperoxidase technique using anti-BCG serum is able to confirm the diagnosis of early leprosy among patients whose unique clinical manifestation is a localized area of sensory loss, in a higher proportion than the routine mycobacterial staining methods, namely hematoxylin-eosin and Wade. The study was held in the north of a hyper-endemic area of leprosy, Manaus, Amazonas (Brazil). Fifty-one paraffin-embedded skin biopsy blocks were retrieved and processed for the immunohistochemical study, by means of anti-BCG polyclonal antibodies for the detection of mycobacterial antigens. The routine stains confirmed the leprosy diagnosis in 17% of the cases, while the immunostaining method confirmed it in 47%. The McNemar test showed that the observed difference between these two techniques was statistically significant ($p = <0.05$). In the same way, 50 blocks of skin conditions considered in the differential histopathological diagnosis of early leprosy were processed for the immunohistochemical test to analyze the possibility of false-positive results which occurred in 8 (16%) patients. The study suggests that immunostaining may increase the proportion of the routine histological diagnosis of leprosy in patients who have sensory loss only, even while using biopsies obtained in fieldwork conditions. This is very advantageous in hyper-endemic areas and in areas that are in the post-elimination period of leprosy control where sensory loss may be a sentinel sign of the disease.

RESUMEN

El presente estudio se realizó para establecer si la técnica de la inmunoperoxidasa, usando un suero anti-BCG, era mejor que los métodos de tinción de rutina, principalmente Hematoxilina-Eosina y Wade, para confirmar el diagnóstico de lepra temprana en los pacientes cuya única manifestación clínica es un área localizada de pérdida sensorial. El estudio se realizó en el norte de Manaus, una zona del Amazonas (Brasil) hiperendémica en lepra. Se prepararon cortes de 51 biopsias incluidas en parafina y se procesaron para su estudio inmunohistoquímico usando anticuerpos policlonales anti-BCG para la detección de antígenos micobacterianos. Las tinciones de rutina confirmaron el diagnóstico de la lepra en el 17% de los casos, mientras que el método inmunohistoquímico permitió el diagnóstico en el 47% de los mismos. La prueba de McNemar mostró que la diferencia observada entre estas dos técnicas fue estadísticamente significativa ($p = <0.05$). Para analizar la posibilidad de reacciones falso-positivas también se incluyeron cortes de 50 biopsias de piel con otras condiciones diferentes a la lepra. En 8 casos (16%) se encontraron resultados falso-positivos. El estudio sugiere que la tinción inmunohistoquímica puede aumentar el grado de detección de la lepra en los pacientes que sólo muestran pérdida sensorial, aun cuando las biopsias se hayan tomado en condiciones de campo. Esto es muy ventajoso en áreas hiperendémicas y en las áreas que están en el periodo de post-eliminación del control de la lepra, donde la pérdida sensorial puede ser un signo esencial de la enfermedad.

RÉSUMÉ

Cette étude a eu pour but d'évaluer si la technique d'immunopéroxydase utilisant un sérum anti-BCG permettait de confirmer le diagnostic de lèpre précoce, parmi des patients dont l'unique manifestation clinique est une perte localisée de sensibilité; et ceci dans une plus grande proportion que les méthodes de coloration de routine des mycobactéries, telles que l'hémalun éosine et la méthode de Wade. Elle fut réalisée dans le nord d'une région hyper-endémique de lèpre à Manaus, en Amazonie (Brésil). Cinquante et un blocs en paraffine de biopsies cutanées furent utilisés pour l'étude immunohistochimique, au moyen d'anticorps polyclonaux pour la détection des antigènes de mycobactéries. Les colorations de routine ont confirmé le diagnostic de lèpre dans 17% des cas, tandis que la méthode immunohistochimique l'a confirmé dans 47%. Le test de McNemar a montré que la différence observée entre les deux techniques était statistiquement significative ($p = <0,05$). De la même manière, 50 blocs provenant de lésions de patients avec maladies cutanées où la lèpre précoce peut être considérée comme diagnostic différentiel furent utilisés pour même analyse immunohistochimique afin d'analyser la possibilité de l'existence de faux positifs, qui apparut de fait chez 8 (16%) de ces patients. Cette étude suggère que l'immunomarquage pourrait augmenter le taux de diagnostic histologique de routine de maladie de Hansen, exclusivement chez les patients qui présentent des pertes de sensibilité, même en utilisant des biopsies obtenues dans des conditions de terrain. Ceci est très avantageux dans les régions hyper-endémiques, ainsi que dans les zones en période de post-éradication des programmes d'élimination de la lèpre, où la perte de sensibilité nerveuse représente souvent un signe d'appel de la maladie.

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