Original Article

MYCOBACTERIAL ANTIGEN IN TISSUES IN DIAGNOSIS OF CUTANEOUS TUBERCULOSIS

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Summary:
***Background*:** Cutaneous tuberculosis presents a diagnostic challenge, as it is difficult to demonstrate the causative organism by histopathology and also culture of organisms from skin lesions is a less rewarding and time consuming process**.**
Aim: Present study was undertaken to evaluate the utility of immuno-histochemical staining to demonstrate *Mycobacterium tuberculosis* antigen in tissue sections. This is based on the finding that the mycobacterial antigen is the last to disappear from the tissues and thus can be used as a marker of mycobacterial infections.
Material & Methods: Fifty randomly selected skin biopsy specimens were subjected to routine histopathological examination to corroborate the clinical diagnosis. Immuno-histochemical study was undertaken to demonstrate mycobacterial antigen.
Observations: All the tissue sections were negative for AFB, both by Z-N stain and by culture. Mycobacterial antigen was demonstrable in 68% of cases of cutaneous tuberculosis. The highest positivity was recorded in scrofuloderma (89%), followed by Lupus Vulgaris (69%) and Tuberculosis Verrucosa Cutis (47%).
Conclusions: Mycobacterial antigen was demonstrable in majority of cases of cutaneous tuberculosis using polyclonal antiserum. However, since cross reactivity was reported in cases of leprosy and also in some fungal infections, this test by itself cannot be considered as diagnostic. The results should be considered along with other findings. ***[Indian J Tuberc 2005; 52:31-35]***

***Key Words*:** Cutaneous tuberculosis, Mycobacterial antigens, Immuno-histochemical staining.

INTRODUCTION

Cutaneous tuberculosis is not an uncommon condition in India. The confirmatory diagnosis of this condition depends on the demonstration of AFB in tissues and by culture. However, the minimum number of AFB required for a positive smear is high, being about 5,000 – 10, 000 bacilli per cubic mm of sputum specimen, while exact figure is not available for extra-pulmonary specimens. Demonstration of *M. tuberculosis* antigen in tissues may be useful as an adjunct in the diagnosis and management, more so, if the lesion appears clinically inactive. Immuno-histochemistry cannot be a substitute for the routine laboratory investigations, but may be employed in an academic and research set up, to establish tuberculous etiology in any granuloma.

It was observed that stainable bacilli are the first to disappear, followed by *M.tuberculosis* antigen and finally the granuloma. Thus, the presence of granuloma, in the absence of antigen, in a specimen from a residual lesion, probably indicates that the granuloma is in the resolving stage and the patient may not require further ATT[1].

MATERIAL AND METHODS

Patients from South Arcot Vallalar (SAV) and Villupuram districts of Tamil Nadu with various clinical types of cutaneous tuberculosis, attending the dermatology department of Rajah Muthiah Medical College and Hospital, Annamalai University, between 1991 and 1998, were included in the present study.

There were a total of 117 cases of cutaneous tuberculosis; lupus vulgaris (39), scrofuloderma (38), tuberculosis verrucosa cutis (32) and others (9).

Skin biopsy specimens were subjected to routine HPE to corroborate the clinical diagnosis. Immuno-histochemical study was undertaken to demonstrate *mycobacterial* antigen in 50 randomly

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selected tissue sections.

Principle of Immuno-histochemistry

The principle is similar to that of ELISA. The tissue containing the antigen is treated with an appropriate antibody, to form an antigen- antibody complex. The section is treated with peroxidase conjugated secondary antibody and the antigen – antibody complex binds with the conjugate. When the substrate is added to this, the whole reaction is seen as brown coloured areas. These brown coloured areas are the positive sites of location of antigen-antibody complexes. Anti H_{37} RV serum raised in rabbits was the antiserum employed in the procedure[2].

Preparation of Mycobacterial antigens

M. tuberculosis, which was cultured on Lowenstein-Jensen slopes, was inoculated into Sauton's medium. This medium was kept in the incubator, maintained at 37^0 C for four weeks, after which the growth was terminated. The culture was heat killed at 60^0 C, centrifuged and then filtered using a Seitz filter. The culture sediment and the media containing the mycobacterial antigens were separated. The sediment was resuspended to give a concentration of 2.5 x 10 organisms per microliter[7]. A portion of this preparation was sonicated using a sonicator. The two preparations (whole organisms and sonicated organisms) were stored in aliquots at -20^0 C. The antigen used is a known high virulent strain of *M.tuberculosis* (H_{37}RV), either whole organisms or sonicated organisms.

Antiserum used was anti H_{37} *RV and was raised in rabbits as follows*

Rabbits were first immunized with heat killed *M.tuberculosis* (H_{37}RV), in Freund's incomplete adjuvant as an emulsion. Three boosters were given at two weekly intervals and after the last booster blood was collected from the ear vein of the rabbits. This clotted blood was then kept in the water bath at 37^0C for one hour. The clot was then ringed slightly and kept at 40^0C for two hours for full clot retraction. Finally, it was centrifuged and the serum was separated. After testing this antiserum for anti *M.tuberculosis* antibody titre using ELISA, it was stored in aliquots at -20^0C. The quantitation of the antibodies present in the antiserum was done using ELISA.

In the present study, indirect Immunoperoxidase staining method of Heyderman (1979) was employed as follows[3]:

1. The slides were deparaffinised with xylene
2. To block the endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide in water for 5 minutes.
3. Rinsed well in phosphate buffer saline.
4. Non - immune serum from the same species, as that producing the second layer antibody (e.g. normal swine serum), was applied to the section, to block protein binding sites by non-specific adsorption or by binding of specific but unwanted serum antibodies. This was maintained at room temperature for 10 minutes.
5. The primary antibody with its optimum working dilution were added to the sections and kept in the incubator at 37^0 for one hour.
6. Washed three times with phosphate buffer saline.
7. Incubated at 37^0 in appropriate dilution of peroxidase-labelled secondary antiserum.
8. Washed three times with phosphate buffer saline.
9. Peroxidase staining was developed by adding the chromogen diaminobenzidine and the substrate H_2O_2.
10. Counterstained with hematoxyline, washed and mounted.

Controls

1. Treatment of test sections with negative control serum.
2. Parallel staining of negative control sections with test serum .

3. Adsorption of primary antibody with excess antigen should abolish staining.
4. Adsorption of the specific antibody to affinity column should abolish staining reaction.
5. Treatment of primary antibody with inappropriate antigen should produce no impairment of staining
6. Parallel staining of positive control sections.

RESULTS

All the tissue sections were negative for AFB, both by Z-N stain and by culture. In 50 cases, immuno-histochemical staining was done to locate the mycobacterial antigen in the tissues. Cells of the mononuclear phagocyte series containing the mycobacterial antigen stained brown and in some sections the brown stain could be documented within the multinucleated giant cells.

Mycobacterial antigen was demonstrable in 68% of cases of cutaneous tuberculosis. The highest positivity was recorded in scrofuloderma (89%), followed by lupus vulgaris (LV) (69%) and tuberculosis verrucosa cutis (TBVC) (47%). The antigen was demonstrable within the giant cells and also extracellularly, interspersed within the mononuclear cell infiltrate. (Figure).

DISCUSSION

It is reasonable to expect that histopathology should correlate with the clinical and gross pathological aspects of tuberculosis. That, it may not, particularly in the case of tuberculosis, reflect our imperfect interpretation of cellular events in tissues[4]. The advent of immuno-cytochemistry for evaluating the cell types in lesions and some of their functions has helped in the interpretation of these changes.

The demonstration of mycobacterial organisms in sections either by Z-N stain or by fluorescent stain is an important criterion for the diagnosis. Isolation of the organisms by culture is absolutely diagnostic of this disease. Nevertheless, in about 40% of these cases, bacilli are not usually seen by Z-N stain and 25% of these are culture

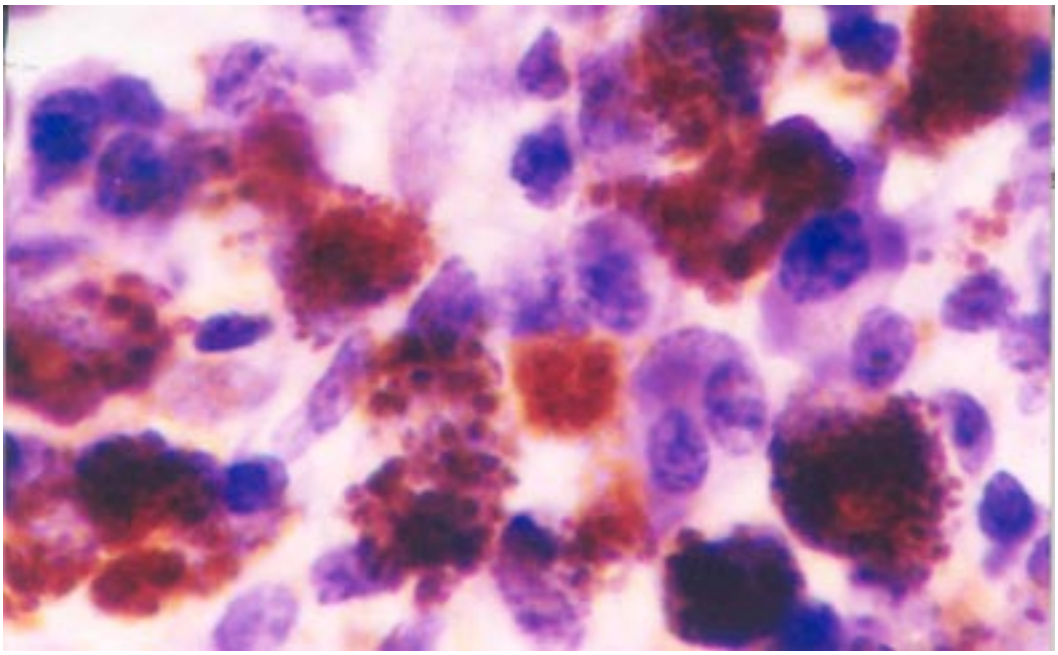

Figure : Skin biopsy from Lupus Vulgaris - showing brown stained *M. tuberculosis* antigen in the Histiocytes and between the cells (Immunoperoxidase stain using anti *M.tuberculosis* antibody) x 1000

negative for *M. tuberculosis*[5]. It is likely that demonstration of the bacillary components using specific probes may help in arriving at an accurate diagnosis of tuberculosis in such atypical situations[6]. An alternative method to identify the presence of the causative microbe may be to demonstrate the bacillary products, using their antigenicity. Varying degrees of positivity are reported in cases of tuberculosis by different workers[5, 7, 8].

Presence of *M. tuberculosis* antigen could be demonstrated in 68% of the 50 cases examined in the present series by immuno-histochemical staining procedures using anti *M. tuberculosis* antiserum. The antigen was demonstrable within the giant cells and also in an extracellular location, interspersed between the mononuclear cell infiltrate.

The bacillary load is relatively high in scrofuloderma as compared to LV and TBVC. It could be presumed that this fact accounts for the higher positivity of 89% in scrofuloderma, and the gradual lower levels of positivity in LV and TBVC at 69% and 47% respectively. According to Lewendowsky's law, the higher the bacterial load, the more the non-specific inflammatory response. This was corroborated in our HPE sections, where scrofuloderma showed a predominantly neutrophilic infiltrate.

In a study employing the anti-BCG immunostain, 100% positive result was reported in 27 cases of mycobacterial infections like tuberculosis, lepromatous leprosy, and atypical mycobacterial infections. Regular skin structures, cellular debris, and necrotic material were not immunostained by anti BCG antibody. This stain cross reacts with many bacteria and fungi and produces minimal background staining. It was concluded that the anti BCG immunostain may be particularly effective in the detection of organisms when obscured by a dense round cell infiltrate and macrophages[9].

Various fungi like sporotrichosis, etc. could be detected by this method, as different groups of pathogens share antigenicity. The cross reactivity could be due to the result of multiple species conservation of specific cell components, that assist the organisms in parasitizing their host[10].

Minute collections of bacteria and fungi in the skin were detected using a polyclonal anti *M.bovis* antibody. It was considered that it could be a clue for an infectious process even in the absence of intact micro organisms in the tissue sections[11]. Antigens in leprosy granulomas were also demonstrated using anti BCG antiserum[12].

Although, mycobacterial antigens can be demonstrated by using polyclonal anti *M. tuberculosis* serum, it cannot be claimed that these are specific to *M. tuberculosis* as weak staining of skin granulomata was observed in lepromatous leprosy patients when stained with anti *M. tuberculosis* antiserum[5].

Since cross reaction is a feature of polyclonal antisera, the test cannot be considered to be specific for diagnosis of tuberculosis and the results should be considered in tandem with other features.

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RADIOLOGY FORUM

As readers would have noticed, we have started a new section "Radiology Forum" from the January 2004, issue of the *Indian Journal of Tuberculosis*. Readers as well as other workers in the field of Tuberculosis & Respiratory Diseases are invited to submit brief report of patients with interesting clinical and radiological features for publication. These will be published, provided that:

(a) the condition is of clinical and radiological interest;

(b) photographs (10 cm x 8 cm) are of excellent quality for printing ;

(c) the diagnosis in each case has been confirmed;

(d) the chest radiograph is accompanied by brief clinical account, not exceeding one page typescript.

All the material received for publication in the Radiology Forum will be peer reviewed as per the Journal policy.