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LIST OF PROCEDURES DONE

PROJECT REPORT

TITLE OF THE PROJECT: IMMUNOHISTOCHEMICAL DEMONSTRATION OF MYCOBACTERIAL ANTIGENS IN INTRACRANIAL TUBERCULOMAS

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PROGRAMME :.....MCh. NEURO SURGERY

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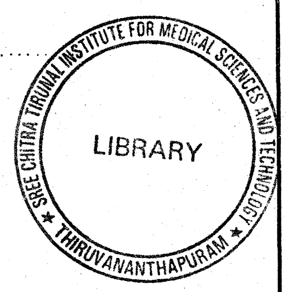

CERTIFICATE

I, Dr...SHANTI..SHANKAR..PRAHARAJ..............hereby declare that I have actually performed all the procedures listed / carried out the project under report.

Signature....................
4.12.91

Place : SCTIMST TRIVANDRUM

Name in ..SHANTI..SHANKAR..PRAHARAJ........
capital letters

Date : 4.12.91.

Forwarded. He has carried out the minimum requirement of procedures / etc.

4/12/91
Signature
Head of the department

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INTRODUCTION

Human tuberculosis is still a major health problem in developing countries like India. In developed countries, the incidence of tuberculosis was low during the past decade, but is now on the increase partly because of Acquired Immune Deficiency Syndrome.

Tuberculosis of the CNS is always secondary to tuberculosis elsewhere in the body and is often difficult to diagnose in the early stages because of its diverse clinical manifestations and infrequent demonstration of M.tuberculosis in clinical specimens.

Intracranial tuberculomas constitute less than 1% of all ICSOL in any large series reported from the United Kingdom, United States or Scandinavia (Dott and Levin, 1939; Grant, 1956; Hoersly and Olivecrona, 1956). The highest incidence has been reported from India. However there is a distinct regional variation in the country. Dastur and Desai, 1965; Dastur, Lalitha and Prabhakar; and Sinh, Pandya and Dastur (1968); all from Bombay, reported an incidence ranging from 18 to 30.5%. The incidence in Madras and Andhra Pradesh (South India) is nearly as high, 19.4% (Ramamurthy, 1956) and 14.5% (Reddy, 1951).

Intracranial tuberculomas manifest certain clinical features which help in making a diagnosis like pyrexia of a few weeks duration; high frequency of convulsions; evidence, past or present, of tuberculosis elsewhere in the body and proximity of tuberculous contacts. However, it

is not possible to distinguish intracranial tuberculomas on clinical grounds alone. Fever, contrary to general expectations is only rarely a feature of tuberculoma of the brain, and in a country where tuberculosis is still a common disease, the discovery of a tuberculous focus elsewhere in the body does not necessarily imply that the intracranial lesion is of the same aetiology. Dastur and Lalitha (1965) had pointed out the high incidence of false localising signs and symptoms in tuberculomas of the brain as compared to gliomas, due to multiplicity of the former lesions.

Histopathologically, classically, a tuberculoma of the brain shows a central zone of caseous necrosis surrounded by tuberculous granulation tissue, consisting of follicles of epitheloid cells, Langhan's giant cells and some lymphocytes, polymorphs and plasma cells. Acid fast bacilli may be seen in both these layers. However, the ultrastructure of brain tuberculoma may have protean manifestations (Dastur and Desai, 1971). Also, brain tuberculomas with unusual pathological features also manifest themselves at times (Sinh, Pandya and Dastur, 1968). The specific diagnosis of a tuberculous granuloma depends upon the demonstration of M.tuberculosis either by culture or by Zeihl-Neelsen stain. But bacteriological methods have a low sensitivity which may be because of the fact that majority of the patients would have already received antituberculous treatment, and moreover, the bacteria are engulfed and phagocytosed by macrophages in the granuloma, which prevents their demonstration by bacteriological methods. Routine laboratory investigations are also not very helpful in the diagnosis of intracranial tuberculomas.

In view of the above, an alternative method of diagnosis possessing greater sensitivity and specificity than the bacteriological methods has been tried.

In tuberculosis, it is well recognised that the organism causing tuberculosis (M.tuberculosis) has various antigenic components residing in the cell wall and cytosol fractions. Several investigators have applied the mycobacterial antigens with which they are working in the immunodiagnosis of tuberculosis (Kailash S.B. et al, 1983; Prabhaker and Oomen, 1987). Janicki et al (1971 & 1973), described an analytical immunoelectrophoretical reference system for the culture filtrate antigen for $H_{37}RV$ stain of M.tuberculosis, based on demonstrable precipitin arcs on immunoelectrophoresis. Several investigators have applied this reference system to identify the mycobacterial antigens characterised in their laboratories. Daniel and Affronti (1973) have used this reference system to identify the components of culture filtrate fractions prepared by the procedure earlier described by Siebert (1949).

Immunohistochemical method as a diagnostic tool started by the demonstration in the early 1930's that antibody could be labelled chemically without destroying its specific reactivity with antigen (J.Marrack and others). Albert Coons in 1942 used fluorescien labelled antibody to trace Streptococcal antigens in rheumatic fever patients. Horseradish peroxidase as an immunohistochemical label, giving an insoluble dark brown reaction product with diaminobenzidine, was claimed

to have several advantages over flourescein - including the avoidance of the trouble and expense of darkfield ultraviolet illumination of the viewing microscope and the tendency of the floorescein tissues to fade (Nakane and Pierce, 1967). The PAP (Peroxidase - antiperoxidase) method introduced by Sternberger et al in 1970, is a 3-step procedure : to the mycobacterial antigen in the tissue sample is added the primary antibody (raised in rabbits against commercially available mycobacterial antigen), followed by the secondary antibody (antirabbit IgG raised in sheep). To this is then added peroxidase anti-peroxidase (tertiary antibody) and diaminobenzidine (substrate) which gives brownish pink precipitate, the presence of which thus indicates the presence of mycobacterial antigen in the tissue sample.

Humphrey et al (1987) evaluated an alternative method of diagnosis of tuberculosis other than by detection of AFB and culture, by detecting mycobacterial antigen using immunohistochemistry using the indirect peroxidase anti-peroxidase method and demonstrated excellent results, especially in caseating pulmonary granulomas where there were few or no AFB. Subsequently Barbolini et al (1989) reported immunohistologic analysis of mycobacterial antigens using monoclonal antibodies directed against different proteins of M.tuberculosis using the PAP method.

This present study was an endeavour to establish an alternative method in the laboratory diagnosis of CNS tuberculomas using the immunohistochemical method and to emphasise the potential application of these immunohistochemical methods.

OBJECTIVES

The objectives of this study are:-

1. To establish the mycobacterial aetiology of an intracranial granuloma by standard bacteriological methods and correlate the data with immunohistochemical demonstration of mycobacterial antigens in the lesions.

2. By immunohistochemical methods to differentiate tuberculous versus nontuberculous granulomatous lesions of CNS.

MATERIAL AND METHODS

Specimen collection

In this study, 20 specimens of intracranial tuberculomas were selected and 20 specimens of granulomatous lesions due to nontuberculous aetiology were selected as control. The diagnosis of tuberculoma was made on the basis of clinical manifestations, relevant neuroradiological investigations and operative findings at surgery, supported by a compatible frozen section diagnosis. Representative tissues were subjected to bacteriological and histopathological studies simultaneously. Culture was done in Lowenstein-Jensen media. For histopathological studies 2 representative blocks of tissues were sampled from every specimen and routinely processed : tissued obtained from surgery were fixed in 10% buffered formaldehyde for 24 hours. These tissues were dehydrated in ascending grades (50-100%) of isopropyl alcohol for 4 hours. Then the tissues were cleaned in chloroform for another 4 hours and impregnated with paraffin wax for 3 hourss. Finally 5 micron thick serial sections were made which were stained with Haematoxylin and Eosin for histopathological examination and Zeihl-Neelsen stain for the demonstration of AFB.

Paraffin sections from Aspergillus granulomas (n = 5), parasitic granulomas (n = 5), foreign body granuloma in craniopharyngiomas (n=6) and giant cell variant of glioblastoma multiforme (n = 4) were selected as controls.

Immunohistochemical method to demonstrate mycobacterial antigens

A. Preparation of Mycobacterial antigen:

$H_{37}Ra$ M.tuberculosis (supplied by the kind courtesy of Tuberculosis Research Centre, Chetpet, Madras) was subjected to culture under sterile conditions on Proskauer and Beck medium (Hi-Media, Bombay) for 6 - 8 weeks. Following this the culture was decanted by Whatman filter paper IV and the cell free culture filtrate was subsequently passed through 0.45 micron pore-sized millipore membranes (Millipore, Bedford MA) and dialysed for 48 hours at 4°C against double distilled water. The dialysed material was lyophilised into dried form and then reconstituted in normal saline, and the protein content was adjusted to 2mg/ml. These were stored in aliquots at - 20°C. The bacillary sediment in each culture bottle was resuspended in distilled water and contents of all bottles pooled and autoclaved, and retained for immunization.

B. Immunisation Schedule for Raising Antisera to Mycobacterial antigens:

Adult albiono rabbits were injected with 2mg of antigen and 2.5mg. of autoclaved dried bacillary sediment of M.tuberculosis. A course of 5 such immunisations were administered on the 1st, 14th, 21st, 28th and 35th days. A persistent high antibody titer could be demonstrated following the fifth immunisation schedule. 20ml blood was drawn from each rabbit by intracardiac puncture, serum was separated and stored in aliquots at -20°C. Sodium merthiolate (1:10,000) was added as preservative. This

was passed through protein A-Sepharose column. IgG fraction to M.tuberculosis in the serum was recovered, and reconstituted in normal saline. In Ouchterlony immunodiffusion plate, rabbit immunoglobulin to M.tuberculosis demonstrated multiple precipitin arcs to culture filtrate antigen. This rabbit IgG to M.tuberculosis was dispensed in vials (100ug/ml) and stored at -70°C.

C. Technical Procedure

Immunohistochemical studies for mycobacterial antigens were performed by the Peroxidase anti-peroxidase method. In brief, 5 micron thick histological sections for immunoperoxidase studies were cut from formalin fixed, paraffin embedded tissue specimens. Sections were mounted on glass slides and heated at 60°C for upto 1 hour. After deparaffinisation, sections were carried through a series of three preliminary incubations: (1) 3% hydrogen peroxide and methanol (1:5) for 30 mts.; (2) concentrated hydrochloric acid and methanol (1:500) for 10 mts, and (3) 10% normal rabbit serum diluted in tris-buffererd saline (pH 7.4) for 10 mts.

Next, sections were incubated for 5 hours at 4-6°C with rabbit IgG to M.tuberculosis (5-10 micro gram/ml). Subsequent incubations were for 20 mts at room temperature with swine antirabbit peroxidase - antiperoxidase diluted 1/100 or 1/200 (Dako). The sections were washed thoroughly

with tris-buffered saline containing 1% normal rabbit serum after each incubation.

Next, 2mg of diaminobenzidine tetrahydrochloride (Sigma) was dissolved in 4 ml tris-buffer (0.05M, pH 7.4). 50ul 3% hydrogen peroxide was added just prior to use (substrate) and sections were incubated for 5-10 mts at room temperature. Finally sections were counter stained with haematoxylin, dehydrated and mounted.

The sections were examined uner microscope for the presence of colour reaction within the cytoplasm of macrophages and giant cells in the granuloma.

Specificity of mycobacterial antigen staining

In order to evaluate the specificity of the above reaction, normal rabbit IgG was substituted instead of rabbit IgG to M.tuberculosis. This substitution gave consistently negative results in all the specimens of tuberculomas. This would suggest that the localisation of mycobacterial antigen in the granulomatous lesion was brought about only by addition of rabbit IgG to M.tuberculosis.

RESULT

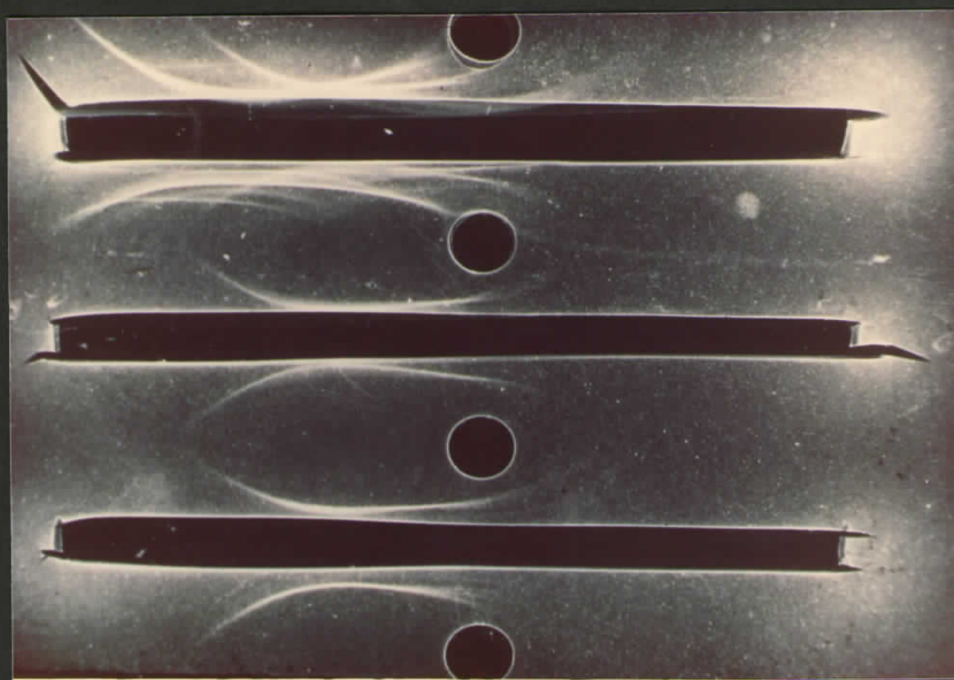
Table I shows the result of the immunohistochemical method for the demonstration of mycobacterial antigen in 20 cases of intracranial tuberculous granulomas. This data was compared with Zeihl-Neelsen method of staining for acid fast bacilli in another section of the same paraffin block.

It was seen that while AFB could not be demonstrated in any specimen of tuberculoma and bacteriological culture was positive in only 2, mycobacterial antigens could be demonstrated in all the 20 specimens by immunohistochemical methods. Furthermore, on comparing this data with the histopathological appearence, it was seen that out of these 20 cases, 4 did not have the classical features of tubercular granuloma, in whom at most only a probable diagnosis of tuberculoma was possible by histopathology. Two of these were on antitubercular treatment preoperatively. Immunohistochemistry could, however, establish their tubercular aetiology irrevocably.

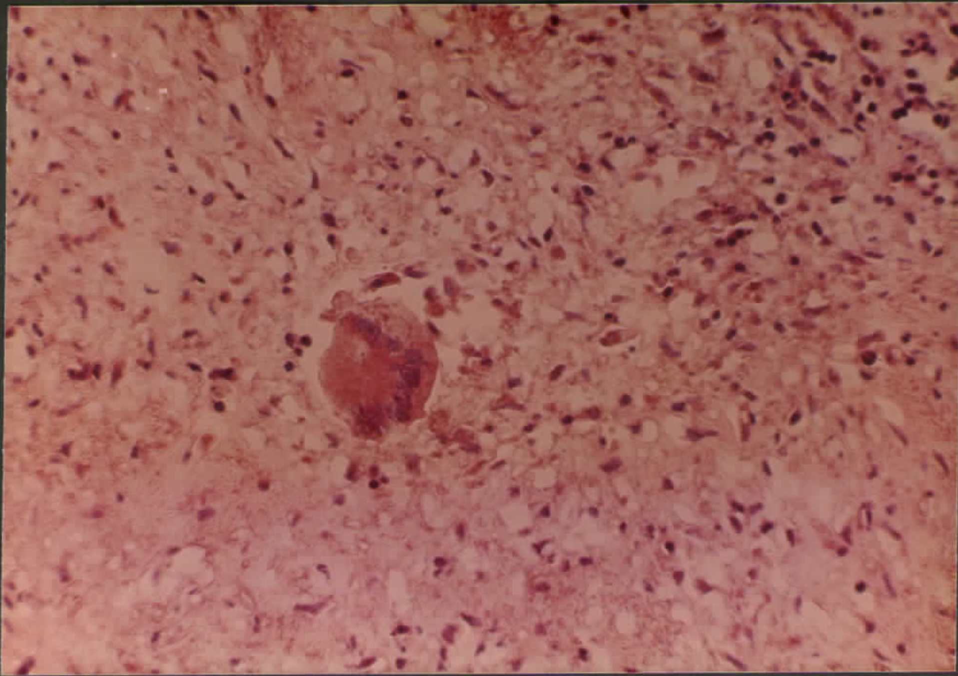
There were two types of distribution of mycobacterial antigen. In the noncaseating zone, the mycobacterial antigens were characteristically located within the cytoplasm of macrophages and giant cells; and in the caseous zone, in which clusters of mycobacterial antigen staining masses contrasted sharply with the remainder of necrotic amorphous debris. In none of the nontuberculous granulomatous lesions was the characteristic intracytoplasmic localisation of mycobacterial antigen present.

Table 1: Data on immunohistochemical technique v/s zeihl-Neelsen method, Bacteriological culture and Histopathology.

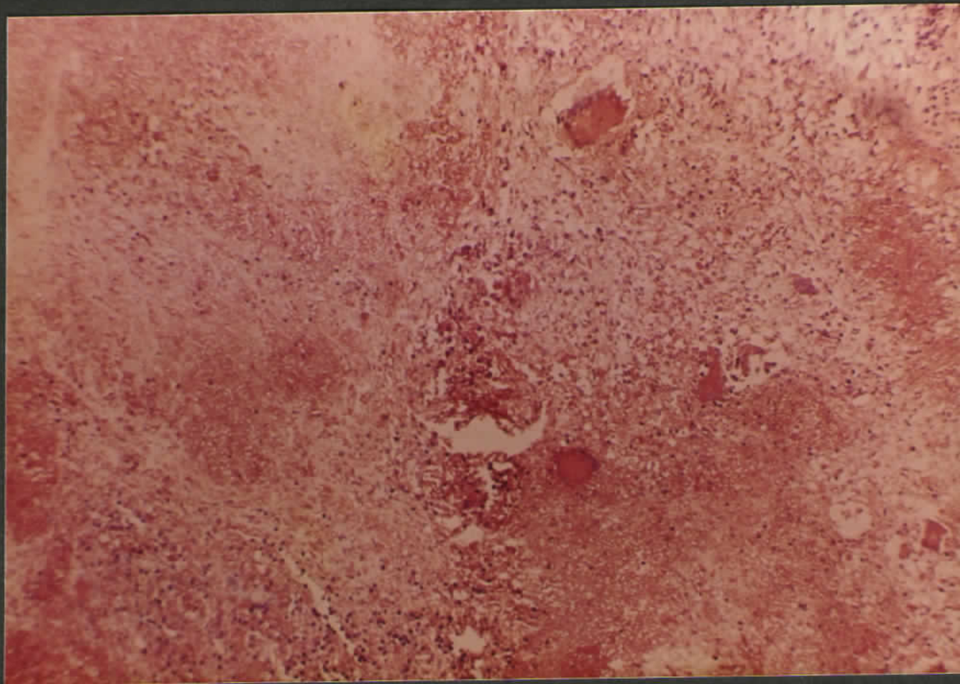
Case No.	Anatomical location of tuberculoma	Mycobact. antigen	Z-N stain for AFB	Bacteriol. culture	H/o ATT	Histopathol. appearence	
						Classi-cal	Probable
1.	Posterior fossa	++	-	+	-		+
2.	Lt. temporal	+	-	-	-	+	
3.	Rt. occipital	++	-	-	-	+	
4.	Rt. frontal	++	-	-	-	+	
5.	Rt.post.frontal	++	-	-	-	+	
6.	Posterior fossa	++	-	-	8 days	+	
7.	Posterior fossa	++	-	-	10 days		+
8.	Rt. parasellar	+	-	-	-	+	
9.	Rt. tentorial	+	-	-	-	+	
10.	Posterior fossa	++	-	-	-	+	
11.	Rt. frontal	++	-	+	-	+	
12.	Rt. parietal	++	-	-	-	+	
13.	Posterior fossa	++	-	-	-		+
14.	Rt. occipital	++	-	-	3 months		+
15.	LT. frontoparietal	+	-	-	-	+	
16.	Rt. frontal	++	-	-	-	+	
17.	Posterior fossa	++	-	-	-	+	
18.	Lt. occipital	++	-	-	-	+	
19.	Posterior fossa	++	-	-	-	+	
20.	Rt. temporal	++	-	-	-	+	



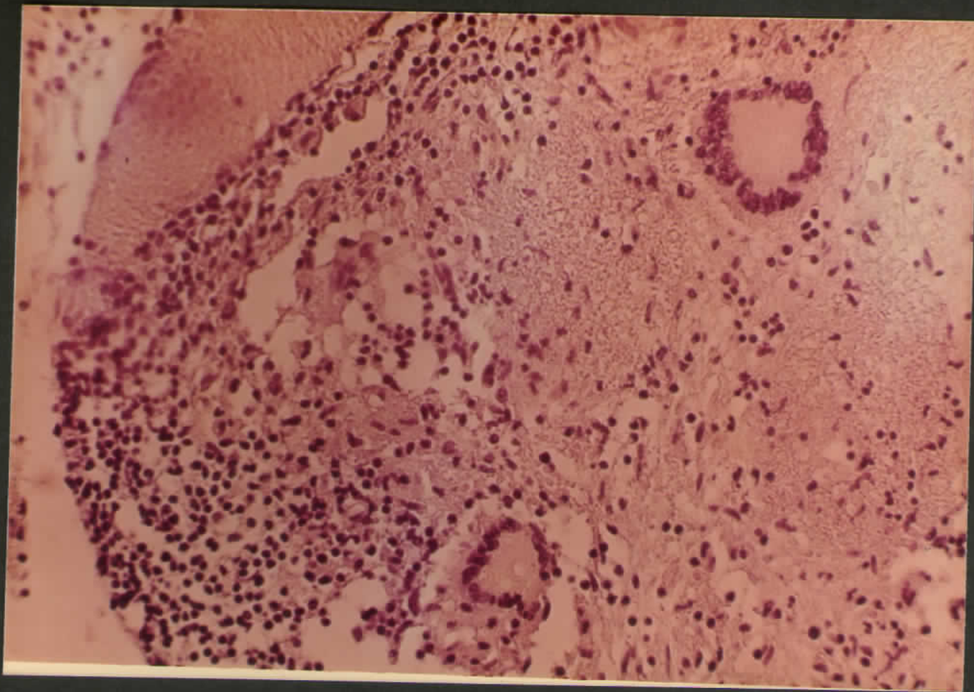
Immunoelectrophoretic pattern showing multiple precipitin arcs with polyvalent antibody and single precipitin arc with monovalent antibody to mycobacterial antigen.



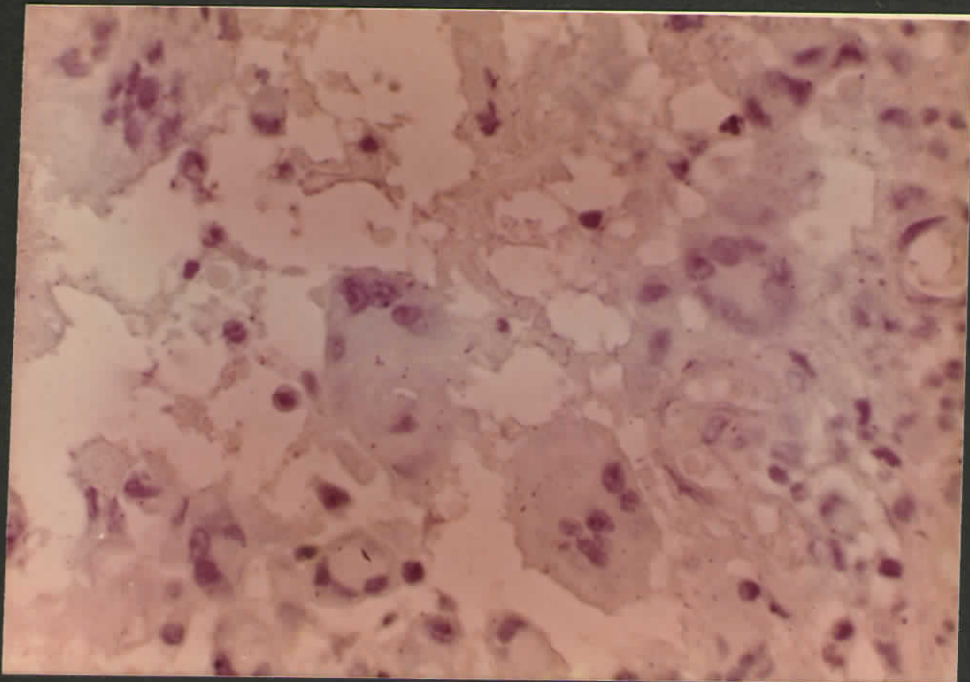
Photomicrograph showing the presence of mycobacterial antigen within the giant cells in tuberculous granuloma by immunohistochemistry.



Photomicrograph showing the presence of mycobacterial antigens in the caseous zone characterised by amorphous homogenous brownish pink precipitates. Mycobacterial antigens are also seen in the giant cells.



Photomicrograph of a tubercular granuloma showing the absence of staining reaction when primary antibody was excluded in the technique.



Photomicrograph showing nontuberculous (fungal) granuloma. Note the absence of immunostaining for mycobacterial antigen within the cytoplasm of giant cells.

DISCUSSION

It is essential to establish the aetiological nature in all intracranial tubercular granulomatous lesions because specific chemotherapy are available for the management of these lesions and complete cure is possible. Intracranial granuloma in our country is most commonly caused by M.tuberculosis. Because of the previous chemotherapy and the long duration of these lesions, the histopathological nature of these tuberculomas often gets altered, and sometimes do pose diagnostic difficulties. However, the absolute criterion to establish the diagnosis of tuberculoma depends upon the specific demonstration of M.tuberculosis by either bacteriological culture or by Zeihl-Neelsen stained smears.

In our country, several studies of tuberculoma have documented the incidence, clinical manifestation and bacteriological data. Dastur and Desai (1965) had pointed out the high incidence of false localising signs and symptoms in tuberculomas of the brain as compared to gliomas. It is not always possible to distinguish tuberculoma on clinical grounds alone. Routine laboratory investigations are also not always helpful in the diagnosis of intracranial tuberculomas. Bacteriological methods have a low sensitivity and false negative results can be frequently obtained. Also the demonstration of AFB in paraffin section is often negative.

There could be two important reasons for the low positivity of AFB in paraffin sections. Firstly, the majority of these patients have already

received antituberculous chemotherapy for varying intervals prior to surgery as a result of which the number of viable tubercular bacilli in these lesions is small. Secondly, AFB are often engulfed and phagocytosed by the macrophages in the granuloma as a result of which the morphological characteristics of AFB are often distorted. Hence the Zeihl-Neelsen preparation often shows a negative result.

In the absence of a definite criteria to establish the mycobacterial aetiology in granulomas an alternative method is necessary. Such a method should not only possess a sensitivity which is greater than the bacteriological method but should also cary more specificity. Besides these, the newly developed test should be rapid as well as feasible to be carried out in most of the routine laboratories where the laboratory resources are rather limited. In an attempt to achieve the above objectives we have standardised an immunohistochemical method to demonstrate mycobacterial antigens in the paraffin sections.

Specific antimycobacterial antibodies were raised in rabbits and this rabbit serum served as the primary antibody. The Ludwig Sternberger's method was basically applied in our study. Antirabbit IgG raised in sheep served as secondary antibody, and antirabbit peroxidase anti-peroxidase served as the labelling antibody. By this 3-layered antibody technique we have been able to demonstrate mycobacterial antigen within the cytoplasm of the macrophages and Langhan's giant cells in all the 20 intracranial tuberculomas. Thus the method yielded 100%

sensitivity. To evaluate the specificity of the immunohistochemical reaction we had applied the same antibody in nontuberculous granulomatous lesions due to fungi, parasites, giant cell glioblastomas and craniopharyngiomas. We observed that the characteristic intracytoplasmic antigen was absent in these nontuberculous lesions. Thus the test was also considered to be specific. This observation is very relevant because at times the fungal granuloma may mimic closely the histopathological features of a tubercular granuloma and a clear distinction may not be possible particularly when the organism cannot be demonstrated in the lesions. Also, in 4 of our patients, histopathology did not reveal classical tuberculoma features. In these patients the diagnosis of tuberculoma could be established by the demonstration of mycobacterial antigen by immunohistochemical methods.

There have been only a few studies regarding application of immunohistochemical methods in the demonstration of mycobacterial antigen in CNS tuberculosis. Shanker et al (1989) have used several monoclonal antibodies in their study (ML-30, ML-34, ML-03, TB-23) and have observed the vascular changes in tuberculous involvement. However, in this study, the specificity of these immunohistochemical methods were not evaluated in nontuberculous granulomatous lesions. Secondly, since this study has been done in postmortem material, its relevance to invivo situations is yet to established. Apart from this study there is no other published report regarding the application of immunohistochemical studies in CNS tuberculosis.

However, there are two important studies on the demonstration of mycobacterial antigen in pulmonary and lymph node tuberculosis by immunohistochemical methods. Humphrey et al (1987) used a rabbit polyclonal antibody to $H_{37}Ra$ M.tuberculosis in their immunohistochemical analysis of 59 cases of pulmonary tuberculosis and have emphasised that this technique was useful for establishing specific mycobacterial aetiology in caseating pulmonary granuloma.

It is relevant to mention that crossreacting antigens from atypical mycobacteria may react with polyclonal antibody to M.tuberculosis. Using 4 different monoclonal antibodies Barbolini et al (1989) have evaluated monoclonal antibodies by dot-immunobinding technique. These monoclonal antibodies reacted with culture filtrate antigens from 7 different mycobacterial strains and none of these monoclonal antibodies were found to be specific for N.tuberculosis. So the atypical mycobacterial CNS involvement, though rare, cannot be ruled out by this technique. Thus Barbolini et al have emphasised the usefulness of this immunohistochemical technique in establishing the mycobacterial aetiology of a caseating granuloma and also in avoiding false negative results obtained in Zeihl-Neelsen staining method.

The results obtained in immunohistochemical methods are reproducible. However the technical error at times may result in inconsistent staining reactions, which include: (a) heating the paraffin sections to more than 60°C; (b) improper removal of background staining;

(c) an optimal working dilution of antibody to M.tuberculosis; (d) last but not the least is the diaminobenzidine substrate which may at times not give the final colour reaction due to improper preservation. If these technical errors can be avoided and if the technique is done under ideal laboratory conditions the reaction often give consistent results. Moreover the immunological reagents can be stored at 4°C in the refrigerator for several months. As this technique carries higher sensitivity and specificity than the conventional bacteriological methods it is recommended that the immunohistochemical technique be used as a routine method in establishing the laboratory diagnosis of a caseating granulomatous lesion.

CONCLUSIONS

1. An alternative method for the laboratory diagnosis of CNS tuberculomas was developed by immunohistochemical demonstration of mycobacterial antigen in the lesions.
2. This method carries greater sensitivity than the routine bacterilogical methods namely Zeihl-Neelsen stain for AFB and bacteriological culture of M.tuberculosis.
3. Nontuberculous granulomatous lesions due to fungi and parasites, giant cell glioplastomas and craniopharyngiomas give a consistently negative result with this technique. Thus the test is highly specific.
4. In cases with a doubtful histopathological picture this method could be used to confirm the tubercular aetiology as was done in 4 cases in the present study.
5. The results obtained by the immunohistochemical methods are reproducible.
6. It can be used as a routine method in establishing the laboratory diagnosis of caseating granulomatous lesions.

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