Journal of Infectious Diseases and Immunity Vol. 3(4), pp.64-67, April, 2011
Available online at http://www.academicjournals.org/JIDI
ISSN 2141-2375 © 2011 Academic Journals

Full Length Research Paper

An immunoassay using cord factor antigen of *Mycobacterium tuberculosis* for the confirmatory diagnosis of intracranial tuberculoma

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Accepted March 21, 2011

Demonstration of *Mycobacterium tuberculosis* bacillus in an intracranial tuberculoma by bacteriological culture and staining methods are difficult. Hence the objective of this study is to describe an immunoassay to detect the presence of cord factor antigen of *M. tuberculosis* in intracranial tuberculoma. Cord factor antigen of *M. tuberculosis* in the surgical specimens of intracranial tuberculoma was isolated by a chloroform – methanol precipitation method and its concentration was quantitated by a direct enzyme-linked-immunosorbent assay (ELISA). Cord factor antigen of *M. tuberculosis* was present in the intracranial tuberculous lesions and absent in intracranial fungal granulomas. Demonstration of cord factor antigen of *M. tuberculosis* as described in this study is unique and may be able to distinguish tuberculous from fungal etiology in patients with intra- cranial granulomatous lesions. Thus it has a potential application in the laboratory diagnosis of intracranial tuberculoma.

Key words: Intracranial tuberculoma, *Mycobacterium tuberculosis*, cord factor antigen, specificity, enzyme linked immunosorbent assay.

INTRODUCTION

Central nervous system (CNS) tuberculoma constitutes 1% of extra -pulmonary tuberculosis (Rock et al., 2008). After tuberculous meningitis, intracranial tuberculoma constitutes the second common clinical as well as neuropathological manifestation of CNS tuberculosis. Intracranial tuberculoma clinically may manifest either as single or multiple intracranial space occupying lesions, with the advent of neuroimaging techniques, such as magnetic resonance imaging (MRI) the pre- operative diagnosis of intracranial tuberculoma has been significantly enhanced. However there are still few clinical instances where a confirmative diagnosis of intracranial tuberculoma remained uncertain and the neuro- imaging features in MRI scans could not distinguish tuberculoma from other solitary enhancing lesions such as abscess, metastasis or fungal granuloma (Gupta et al., 2001). Confirmatory laboratory diagnosis of tuberculous etiology that is, the 'gold standard` in the surgical specimens of intracranial tuberculoma depends upon the demonstration of *M. tuberculosis* bacilli either by bacteriological culture or demonstration of acid-fast bacilli (AFB) by the Ziehl-Neelsen (ZN) stain. However, conventional bacteriological methods are not only cumbersome but also less sensitive (Radhakrishnan et al., 1991). Thus the bacteriological confirmation of intracranial tuberculoma is often negative in a vast majority of specimens of intracranial tuberculoma (Sumi et al., 2001).

Histopathological features such as caseous granulomatous lesion can be caused by other microbial agents such as fungus (aspergillus). At times, pathologists are faced with a problem to distinguish tuberculous from fungal etiology in a surgical specimen of

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intracranial granuloma. This distinction become extremely relevant and also assumes greater clinical significance because treatment modalities vastly differ in tuberculous and fungal infections of the CNS. Thus institution of appropriate chemotherapy in patients with intracranial tuberculoma depends upon an accurate laboratory diagnosis. Cord factor (CF) antigen is one of the major lipid antigens of *M. tuberculosis*. It plays a significant role in the immunopathogenesis of human tuberculosis. CF antigen potentiates the macrophage proliferation as well as promotes granuloma formation in tuberculous lesion (Baba et al., 1997; Yamagami et al., 2001). Besides these, several other published studies utilized CF antigen for the immunodiagnosis of pulmonary and ocular tuberculosis (Maekura et al., 1993; Julian et al., 2001; Sakai et al., 2001). However, the potential utility of CF antigen for the immunodiagnosis of intracranial tuberculoma has not been reported. In the present study, we describe a novel diagnostic approach in which CF antigen was isolated in the surgical specimens of intracranial tuberculoma and its concentration was quantitated by a direct enzyme linked immunosorbent assay (ELISA). This approach was found to be very useful and clearly distinguished tuberculous from fungal granulomas of the CNS.

The technical protocol used for the isolation of CF antigen in tuberculous lesion is also highlighted.

MATERIALS AND METHODS

In this prospective study, twelve surgical specimens of intracranial tuberculoma were collected over a period of two years (2009 to 2010). The pre-operative diagnosis of the intracranial tuberculoma in each patient was made by the relevant clinical features and was supported by neuro-imaging features in MRI scan. Representative specimens obtained during surgery were subjected to histopathological and bacteriological studies simultaneously. Under aseptic conditions the tissue homogenate of the surgical specimens were inoculated onto Lowenstein-Jensen (LJ) medium for culturing *M. tuberculosis* and the smears from the homogenates were simultaneously stained with ZN stain for the demonstration of AFB. For histopathological studies, two representative blocks of tissue sampled from every surgical specimen and they were routinely processed for histopathology. Five micron - thick paraffin sections under hematoxylin and eosin (H&E) stain showed the presence of characteristic caseating epithelioid granulomatous lesion and they were suggestive of tuberculous etiology. In four surgical specimens, diminished inflammatory reaction was seen along with extensive caseous necrosis. ZN staining for demonstration of AFB was also performed on paraffin sections.

For the 'disease' controls twelve surgical specimens of non-tuberculous intracranial granulomas were selected (aspergillus granuloma - 6, mucor granuloma -2, cryptococcal granuloma-2, cholesterol granuloma -1, foreign body granuloma (suture induced) -1).

Isolation of cord factor antigen from M. tuberculosis

M. tuberculosis $H_{37}Rv$ strain in this study was obtained from a clinical isolate and The Tuberculosis Research Institute, Chetpet, Chennai confirmed this strain. Sauton culture medium (250 ml) in 1000 ml Roux culture bottle with an off set neck was inoculated with one ml of tubercle bacilli from a 10 to 14 day old 'seed' culture and incubated at 37°C for 6 to 8 weeks. The optimum growth of the cultures at the end of 8 weeks was ascertained by CFU. Following this the cultures were autoclaved at 120°C for 30 min and centrifuged at 10,000 g x 30 min. The bacillary sediments were suspended in chloroform- methanol (4:1 by volume) and subjected to ultrasonic disruption. The chloroform layer containing mycobacterial lipids was separated and allowed to dry at room temperature. The mycobacterial lipids were precipitated in acetone, subsequently suspended in chloroform- methanol (2:1 by volume). The crude mixture of mycobacterial lipids were then loaded on to silica gel thin-layer chromatography plates (TLC; Uniplate, Analtech, Inc.; Newark, Del). CF fraction in the TLC plate was visualized with iodine vapour and then recovered from TLC plates and dissolved in chloroform. Subsequently the chloroform containing CF was run by passage through a column of silica gel (Wakogel C-200; Wako Pure Chemical, Osaka, Japan).

The purification procedure was repeated until a single spot was seen in TLC. CF thus obtained by this procedure was preserved in aliquots in n-hexane (500 µg/in n- hexane) at +4°C.

Antibody to cord factor

Two adult male albino rabbits were immunized with 300 µg of CF antigen mixed thoroughly in 1 ml of 0.1 M phosphate buffered saline (PBS) containing 0.2% Tween-80 and 1 ml of Incomplete Freunds Adjuvant- (IFA) obtained from Sigma Chemicals, St. Louis, USA. The immunization was repeated on 14th, 21st and 27th days following primary immunization (Anie et al., 2007). The rabbits were bled on 35th day and an indirect ELISA was performed to measure antibody titers in immune rabbit sera. An end- point antibody titer at 1:2400 dilution was obtained following the 4th immunisation. 10 ml of venous blood collected from each rabbit through ear vein. The sera were separated, dispensed in 1 ml sterile aliquots and stored at - 70°C until used in the ELISA.

Isolation of cord factor from surgical specimens

Fresh surgical specimens were collected during surgery from patients with tuberculoma and from disease control groups. Using a cryostat (Leica CM 3000, Lowa city, Lowa, USA) a ribbon containing five 20 µm- thick cryostat sections was cut from each specimen and they were directly transferred onto a solution containing chloroform methanol (3:1). This was followed by ultrasonic disruption and followed by centrifugation at 5000 rpm for 30 min. The precipitate was discarded. The supernatant containing the lipids were allowed to dry at room temperature and to which 200 µl of n- hexane was added and stored at + 4°C. A direct ELISA was performed for the quantitation of CF antigen in individual specimens.

Direct ELISA for the estimation CF antigen in tuberculoma

Prior to assay in tuberculous lesions, a direct ELISA was standardized with decreasing concentration of CF antigen (2000-1 pg) with rabbit antibody to CF. Briefly, each well in the microtitre ELISA plates (Nune, Roskilde, Denmark) was individually coated with CF antigen (at different concentrations) in 100 µl n-hexane and incubated at 37ºC for 2 h. Following that, the plates were washed thoroughly with 0.15 M PBS with 0.05% Tween 20 (PBS-T) and quenched with 1% bovine serum albumin (BSA) in 0.15 M PBS for 1 h. 100 µl of rabbit antibody to CF (1:20,000 dilution) was added to each well and incubated for 2 h. The wells were washed thoroughly and 100 µl of (1:50,000 dilution) anti-rabbit IgG biotin conjugate

Table 1. Absorbance obtained in the standardization of ELISA.

CF antigen pg/ml	Absorbance of CF antigen at 450nm mean ± SD	Absorbance of blank at 450nm	Difference in absorbance between CF antigen and blank (mean ± SD)
1	0.02 ±0.006	0.02	00
10	0.04 ± 0.009	0.02	0.02 ± 0.0027
50	0.21 ± 0.014	0.02	0.19 ± 0.0031
100	0.39 ± 0.012	0.02	0.37 ± 0.0038
1000	0.61 ± 0.011	0.02	0.59 ± 0.0039
2000	0.90 ± 0.019	0.02	0.88 ± 0.0045

(Sigma) was added and incubated for 2 h. Subsequently the plates were washed and 100 µl (1: 1000 dilution) of avidin- peroxidase (Sigma) was added and incubated for 1 h. The plates were then washed for five times with PBS-T and 100 µl of substrate containing o-phenylenediamine dihydrochloride added and incubated for 30 min. A colour reaction was developed and it was stopped at the end of 30 min by adding 100 µl of 2N HCl. The blank well in the ELISA plate contained all the aforementioned reagents minus CF antigen. The absorbances in all the wells in the microtitre plates were measured at 450 nm with an ELISA reader (Bio-Rad, model number- 550, Bio-Rad Laboratories, Inc, Hercules, California, USA). The absorbances at each CF antigen concentration (2000- 1 pg/ml) in the ELISA were measured and the differences in absorbance between blank and CF antigen wells were calculated (Table 1) and plotted in a semi-log paper. A linear graph was obtained.

The ELISA detected the presence of CF antigen as low as 10 pg/ml. Direct ELISA for the quantitation of CF antigen in all the surgical specimens from tuberculous and non-tuberculous granulomas was identically performed as described in the standardization assay and the concentration of CF antigen in each specimen was directly quantitated from the standard graph. The assay was performed in all the specimens on two different occasions to assess the reproducibility of the assay.

RESULTS

Standardization ELISA detected the presence of CF antigen and ranged between 10-2000 pg/ml. In other words, the assay is sensitive to detect the presence of CF antigen as low as 10 pg/ml and above. The absorbances obtained in all the twelve patients with intracranial tuberculoma ranged between 0.51 to 0.71(mean- 0.56±0.016). The CF antigen concentration in these specimens measured from the standard graph and it ranged between 550 to 1450 pg/ml (mean 760 pg/ml). The absorbances in ELISA in specimens from twelve patients in 'disease control' group were less than the absorbances in the blank wells and hence they were regarded negative for tuberculous etiology. Thus the results of this study suggest that a positive result in ELISA in a specimen from intracranial granuloma study can be regarded as highly specific for tuberculous etiology. The assay in specimens from tuberculoma as well as in 'disease control' was repeated on two different occasions and no variation in the CF concentration were encountered in any one of the specimens. Thus the assay is also reproducible.

DISCUSSION

The data indicated that *M. tuberculosis* was not demonstrated in any one of the twelve specimens of tuberculoma by standard bacteriological methods. There could be a relevant reason for the low sensitivity for the bacteriological method for the isolation of tubercle bacilli in intracranial tuberculoma. AFB are engulfed and phagocytosed by the macrophages present in the tuberculous granulomatous lesions. As a result, the morphology of the AFB becomes distorted and fragments of the bacilli only present in the lesion. In a Z- N stained preparation, fragments of AFB are not identified. The same phenomenon can also explain the infrequent negative culture for *M. tuberculosis* in the surgical specimen of intracranial tuberculoma. In the absence of a confirmatory bacteriological diagnosis, an alternate method to establish in the intracranial tuberculoma has become essential. In an attempt to distinguish tuberculous and fungal etiology in intracranial granuloma, we evaluated two methods, polymerase chain reaction (PCR) and immunohistochemistry (IHC) in our earlier study (Sumi et al., 2001). In that study the result of PCR supported tuberculous etiology only in 60% of cases. The assay showed false negative in 40% of cases and this could be due to sub-optimal quantities of amplifiable mycobacterial DNA present in formaline fixed tissue of tuberculoma. In IHC studies, an attempt was made to demonstrate mycobacterial antigen within the cytoplasm of Langhan's giant cells and macrophages present in tuberculous lesions. IHC method was found to be less sensitive in tuberculous lesions with extensive caseous necrosis. Besides, non-specific background staining also affected the results in immunohistochemical methods for the demonstration of mycobacterial antigens in tuberculous lesions. In the present study, CF antigen of *M. tuberculosis* was quantitated in the surgical specimens of intracranial tuberculoma. CF was found to be positive in all the twelve specimens of intracranial tuberculoma and its concentration ranged between 550 to 1450 pg/ml.

The absorbances in the ELISA in all the twelve specimens from intracranial non-tuberculous granuloma were less than the absorbances obtained in the blank wells and hence CF antigen is absent in specimens of

patient with non-tuberculous granuloma. The technical protocol used in the isolation of CF antigen from the tuberculous lesions by cryostat section as used in this study is simple, user-friendly and less cumbersome than isolation of CF from the tissue homogenate of tuberculous lesions. There was no loss of CF antigen during the technical procedure in our study. The cryostat sections from tuberculous granuloma containing the CF antigen can be stored in *n* hexane at + 4°C. It is stable and CF present in the cryostat sections can be extracted without any significant alteration in its concentration. The assay is highly specific and there is no potential danger of false-positive results in granulomas with non-tuberculous etiology. The results of the assay can be made available within 24 h after the receipt of the specimen in the laboratory. The assay is highly reproducible and there is no inter-observation variation. The clinical recovery in intracranial tuberculoma is highly dependent upon an accurate diagnosis as well as institution of ATT.

The assay as designed in this study for the demonstration of CF antigen from tuberculous lesion is novel and hitherto has not been described in the literature. The technical protocol can be performed in any laboratory and does not require any elaborate instrumentation or technical expertise. Thus we advocate that this diagnostic approach is one of the benchmarks for the laboratory diagnosis of intracranial tuberculoma. This approach can also be extended to tubercular granuloma occurring in extra-cranial locations such as lymph node, lungs etc.

ACKNOWLEDGEMENTS

The authors thank the Director of this Institute for the kind permission to publish this paper. The authors are indebted to Kerala State Council for Science and Technology and Environment (KSCSTE) 005|SRSLS|2009 for the financial support to undertake this project. The authors are indebted to Ms. Devisavitha for secretarial assistance.

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