Original article

Anti-mycobacterial recall responses differentiate female patients with genital tuberculosis from patients with other gynecological problems

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Abstract

Background: Female Genital Tuberculosis (FGTB) is one form of extra pulmonary tuberculosis affecting the female reproductive organs, most commonly the fallopian tubes and the endometrium. It affects young women aged between 20 and 40 years of age and is an important cause of infertility. It often occurs as a secondary complication following pulmonary tuberculosis. Diagnosis depends mainly on clinical suspicion in countries where facilities for mycobacterial culture and histopathology are unavailable. Even in places where these facilities exist, diagnosis still remains difficult because of the lower sensitivity and specificity of the methods as well as the invasive procedure of acquiring biopsy specimens.

Objective: To explore the immunological profiles of female genital tuberculosis (FGTB) patients in response to mycobacterial antigens.

Methods: Twenty-five clinically suspected cases of FGTB and 12 control subjects who came to the Black Lion hospital for unrelated gynecological problems were included in the study. Peripheral blood samples were collected from each subject. Plasma was separated by centrifugation and PBMC were isolated over ficoll-hypaque and stimulated in vitro with mycobacterial antigens to examine their proliferative response as incorporation of tritiated thymidine using a β-counter. HIV status and total IgG-, IgA- and IgM- antibody levels were determined by ELISA tests.

Results: In vitro recall responses to M. tuberculosis antigens (PPD and BCG sonicate) as well as plasma levels of IgG-IgA- and IgM-antibodies to MPT59 showed statistically significant differences between the patients and the controls ($p < 0.05$).

Conclusion: The results show that PBMC of FGTB patients recognize M. tuberculosis antigens more strongly than PBMC of patients with other gynecological problems. [Ethiop.J.Health Dev. 2005;19(3):219-224]

Introduction

Female genital tuberculosis (FGTB) is one type of extra pulmonary tuberculosis affecting the female genital organs. In 90% of cases the fallopian tubes are the primary foci of infection. From here the bacilli often disseminate to infect the ovaries (10-30%), endometrium (50%), cervix (<5%) as well as the vagina and vulva (<1%) (1,2). The most affected groups (80-90%) are women between 20 and 40 years of age (1,3).

Prior to the onset of the HIV epidemic, extra pulmonary TB used to occur in relatively a few (about 15 %) FGTB cases (4). With the onset of the HIV epidemic, however, the occurrence has increased both in absolute and relative numbers with up to 62% extra pulmonary involvement in patients with advanced HIV status according to one study (5). Thus, HIV has emerged as a most important risk factor in the progression of new or dormant TB infection to clinical stages of the disease (6).

The most frequent clinical symptoms of FGTB are: infertility, lower abdominal distention and pain, menstrual irregularity, fallopian tube abscess, ectopic pregnancy, weight loss, pelvic mass and signs of TB elsewhere in the body (3).

Diagnosis of FGTB is usually made based on clinical suspicions (7), and in cases where the appropriate facilities are available, microbiological (AFB staining and culture), histopathological (8) and radiological methods (HSG) are deemed valuable (9,10). Also in cases where the required highly skilled personnel and specialized equipment are available, molecular methods such as the Polymerase Chain Reaction (PCR) might be employed to detect mycobacterial DNA using specific primers. The technique is rapid and sensitive (11). In lung tissue PCR for M. tuberculosis DNA is found to be frequently positive during latency (12), and thus it might be difficult to differentiate

between latent infection and active disease by PCR. All the above methods target the organism attempting to directly demonstrate its presence in tissue or reveal morphologically characteristic lesions caused by the bacilli.

An alternative approach is to exploit the specific nature of immune responses to invading pathogens by eliciting the recall response upon secondary exposure in vitro. The use of humoral or cellular responses to detect the presence of tuberculous infection or disease is an area under investigation (13). By using PBMC obtained from FGTB suspected patients and controls, this study attempted to investigate immune responses to mycobacterial antigens (lymphocyte proliferation assay to BCG sonicate and Purified Protein Derivative, PPD, and antibody responses to MPT59, a secreted protein of M. tuberculosis) to assess whether these immunological methods are capable of assisting the diagnosis of FGTB.

Methods

Patients

Thirty-seven women, 25 of whom are suspected to have genital TB on clinical grounds (patients), and 12 controls were included in the study. Most patients came to the hospital and TB was suspected on clinical basis due to infertility problem, and laboratory findings. The controls were gynecological patients who underwent surgery due to other gynecological problems (mainly with cancer) having no clinical signs or symptoms suggestive of tuberculosis. Surgically excised tissue was taken from both groups for laboratory examination, including AFB using light microscopy following concentration and staining by ZN as well as culture and M. tuberculosisspecific PCR (14).

The study was approved by the Ethical Review Committees of AHRI/ALERT, the Department of Obstetrics and Gynecology (Medical Faculty of Addis Ababa University), and the National Ethical Review Committee (Ethiopian Science and Technology Commission). Informed consent was obtained from all the women included in the study.

Antigens: MPT59 (Antigen 85B, Rv1886c, is a major secreted protein in M. tuberculosis culture filtrates containing reactive B cell epitopes) (15, 16) and BCG sonicate were obtained as previously described (15). PPD was obtained from the Statens Serum Institute, Copenhagen, Denmark, and PHA was purchased from Sigma. The antigens were aliquoted and frozen at -20^0C before use.

Isolation of PBMC

Venous blood (10ml) was drawn in heparinized tubes, centrifuged, and the plasma separated. The pellet was diluted with RPMI-1640 in a one to one ratio and layered over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). It was then centrifuged for 30 minutes at 1800 rpm and at room temperature. Peripheral Blood Mononuclear Cells (PBMC) were collected and washed three times with RPMI-1640, each time centrifuged at 1500 rpm for five minutes at 4^0C. PBMC were resuspended in two ml of 5% Normal Human

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Serum (NHS) and stored in liquid nitrogen. Plasma was stored frozen at -20^0C until it was used.

HIV antibody testing by ELISA

The study subjects were tested for HIV antibodies using a microelisa system kit (Vironostika, HIV Uni-Form 11 Plus, Organon Teknika GmbH, Eppelheim, The Netherlands in accordance with the manufacturer's instructions.

Lymphocyte Stimulation Test (LST) PBMC were cultured in 96 well tissue culture plates (Flow laboratory, Irvine KA1 28NB, Scotland) at a concentration of 10^5 cells/well in the presence of PHA (1µg/well) or PPD (10 µg/ well (Statens Serum Institute, Copenhagen, Denmark) or BCG sonicate in 5% NHS supplemented with 5% glutamine and 5% PenicillinStreptomycin in RPMI as culture medium and incubated at 37^0C in 5% CO_2. Then, the cells were pulsed with tritiated thymidine (Amersham, Little Chalfont, UK) (1µCi/well) at day three (PHA) and day six (BCG and PPD). After 18 hrs, the cells were harvested on to a filter mat (cat. No. 11731). Finally, proliferation was measured on a β-liquid scintillation counter (LKB Wallac 1216 Rackbeta II, Uppsala, Sweden) and the stimulation index (SI) was calculated by dividing the CPM (counts per minute) of antigen stimulated cells with the CPM of non stimulated controls.

ELISA (Enzyme Linked Immuno Sorbent Assay) Flat-bottomed 96 well plates (Immulon-2 Dynatech Laboratories, Chantilly, VA, USA) were coated with 0.5 µg/ well of purified antigen, MPT59 in phosphate buffered saline (PBS) (pH 7.2) and incubated overnight at 4^0C. The plates were then washed four times with 0.1% Tween in PBS (PBS-T) and blocked with 100 µl of blocking buffer (5mg bovine serum albumin/ml in PBS) and incubated for 2 hrs at room temperature (RT). Serum samples were diluted 1:50 for IgG- and IgM- and 1:10 for IgA antibody assay and added at 100 µl/well and incubated for 1 hr at RT. After washing four times in 0.1% PBS-T, 100 µl of peroxidase conjugated secondary antibody (anti-human IgG/IgA/IgM) (Sigma) (1 mg/ml) diluted 1:10,000 was added and incubated for 1 hr at RT. Finally, 100 µl/well of the substrate o-Phenylenediamine Dihydrochloride (OPD) was added and incubated for 30 minutes. The reaction was stopped by adding 50 µl stop solution (1M H_2SO_4) to each well. The optical density (OD) was read at 492 nm using an ELISA reader (Titertek Multiskan Plus, Helsinki, Finland).

Statistical analysis

Statistical analysis was carried out using the Mann-Whitney rank sum test. Fisher's exact test was also used to compare proportions. Differences among groups were considered to be significant when the p-value was found to be less than or equal to 0.05.

Results

Among the 37 study subjects, 25 were suspected clinically to have FGTB and out of these 25 suspected cases, 16 were confirmed to be positive for M. tuberculosis based on laboratory findings (14). The main clinical symptoms detected in the patient group were infertility. Out of the 17 patients whose infertility status was known, six (35%) had primary and 11 (65%) had secondary infertility. Other symptoms included lower abdominal pain, irregular menstrual bleeding and pelvic mass, (Table 1). The age range of the patients was between 18-39 years and the median age was 28.

Immunological assays

HIV status

To determine the HIV status of the study subjects, HIVELISA test was performed for 34 patients (22 patients and 12 controls). The result showed that 15 out of the 34 (43%) patients were HIV positive. The proportion of seropositivity among patients was found to be 44% compared to 25% among the controls ($p<0.05$).

Antibody Assay

An antibody assay was performed using MPT59 as an antigen on the solid phase in the ELISA test. Fig. 1 shows the plasma level of the three antibody isotypes irrespective of their HIV status.

Table 1: Clinical presentation and laboratory findings in 25 women who visited Tikur Anbessa Hospital for infertility problem and who are suspected to have genital tuberculosis

Patient	Signs and Symptoms	AFB	Culture	Histology	PCR
M-003	2^0 infertility, bilateral tubal blockage			+	
M-006	2^0 infertility, right tubal blockage, irregular menses, vaginal				

	bleeding, adhesion			+	
M-007	2^0 infertility, bilateral tubal blockage, irregular menses, pelvic pain, vaginal discharge, adhesion				
M=012	1^0 infertility	+	+	+	+
M-017	1^0 infertility, bilateral tubal blockage				+
M-020	1^0 infertility, vaginal discharge, adhesion, lower abdominal mass, pain		+		+
M-024	2^0 infertility, bilateral tubal blockage, amenorrhoea		+	+	+
M-028	2^0 infertility, bilateral tubal blockage				+
M-032	2^0 infertility. bilateral tubal blockage, lower abdominal pain				+
M-037	Adhesion				+
M-041	Pelvic pain				
M-045	1^0 infertility, bilateral hydrosalpinx, ammenorrhoea				
M-049	2^0 infertility, bilateral tubal blockage, vaginal discharge				+
M-053	2^0 infertility, bilateral tubal blockage			+	+
M-057	1^0 infertility				+
M-060	1^0 infertility				
M-063	2^0 infertility, infertility, abdominal swelling				
M-066	Right tubal blockage, adhesion				+
M-069	Irregular menses, vaginal bleeding, lower abdominal pain			+	
M-072	2^0 infertility				
M-077	1^0 infertility				
M-081	1^0 infertility, bilateral tubal blockage				
M-086	1^0 infertility, bilateral tubal blockage, vaginal discharge, adhesion			+	
M-089	1^0 infertility, bilateral hydrosalpinx, dysmenorrhoea				+
M-093	2^0 infertility, tubo-peritionial factor				
	Total positive	1	3	7	12

Table 1 continued.....

Patient	Signs and Symptoms	TB status	HIV	CD4/CD8 (ratio)
M-003	2^0 infertility, bilateral tubal blockage	+		ND
M-006	2^0 infertility, right tubal blockage, irregular menses, vaginal bleeding, adhesion	+		ND
M-007	2^0 infertility, bilateral tubal blockage, irregular menses, pelvic pain, vaginal discharge, adhesion			ND
M=012	1^0 infertility	+	ND	ND
M-017	1^0 infertility, bilateral tubal blockage	+	+	ND
M-020	1^0 infertility, vaginal discharge, adhesion, lower abdominal mass, pain	+	ND	ND
M-024	2^0 infertility, bilateral tubal blockage, amenorrhoea	+	+	ND
M-028	2^0 infertility, bilateral tubal blockage	+		27/33 (0.8)
M-032	2^0 infertility. bilateral tubal blockage, lower abdominal pain	+	+	37/14 (2.6)
M-037	Adhesion	+	+	23/53 (0.4)
M-041	Pelvic pain		+	51/18 (2.8)
M-045	1^0 infertility, bilateral hydrosalpinx, ammenorrhoea			36/38 (0.9)
M-049	2^0 infertility, bilateral tubal blockage, vaginal discharge	+		ND

M-053	2^0 infertility, bilateral tubal blockage	+		42/42 (1)
M-057	1^0 infertility	+		41/33 (1.2)
M-060	1^0 infertility		+	20/51 (0.4)
M-063	2^0 infertility, infertility, abdominal swelling			21/30 (0.7)
M-066	Right tubal blockage, adhesion	+		44/30 (1.5)
M-069	Irregular menses, vaginal bleeding, lower abdominal pain	+	+	33/28 (1.2)
M-072	2^0 infertility		+	ND
M-077	1^0 infertility		ND	10/20 (1)
M-081	1^0 infertility, bilateral tubal blockage		+	34/35 (1)
M-086	1^0 infertility, bilateral tubal blockage, vaginal discharge, adhesion	+		30/39 (0.8)
M-089	1^0 infertility, bilateral hydrosalpinx, dysmenorrhoea	+		ND
M-093	2^0 infertility, tubo-peritionial factor		+	ND
	Total positive	16	10	

ND=not done; AFB=Acid Fast Bacilli

All the clinically suspected FGTB cases were found to be reactive (IgG, IgA, IgM) to this antigen with variably high levels of ODs. The difference in median antibody levels between the two groups was significantly different for all the three immunoglobulins: IgG, IgA and IgM ($p<0.05$). The data were analyzed based on HIV status, and showed that there was no statistically significant difference between HIV positive and HIV negative individuals regarding the antibodies of the three isotypes ($p= 0.6$).

Lymphocyte Stimulation Test (LST)

PBMC were subjected to LST test using the mitogen phytohaemaglutinin (PHA) as a control for proper cell viability and the mycobacterial antigens BCG sonicate and PPD. Fig. 2 shows the proliferative response of patients and controls to these antigens irrespective of their HIV status.

Although there was a variation in the degree of response to stimulation, all subjects responded well to PHA, mean SI = 39 in patients and SI =31 in controls. This difference was not significant ($p > 0.05$). There was however, a statistically significant difference between the patient and the control groups in response to both BCG ($p = 0.03$) and PPD ($p = 0.025$).

Discussion

Although the magnitude of the prevalence of FGTB in Ethiopia is not known, it will remain an important gynecological problem as long as pulmonary TB is rampant. PBMC from FGTB patients react to mycobacterial antigens more strongly than PBMC from the controls (patients with other gynecological problems, mainly cervical cancer). This shows that although FGTB is a localized disease, there are

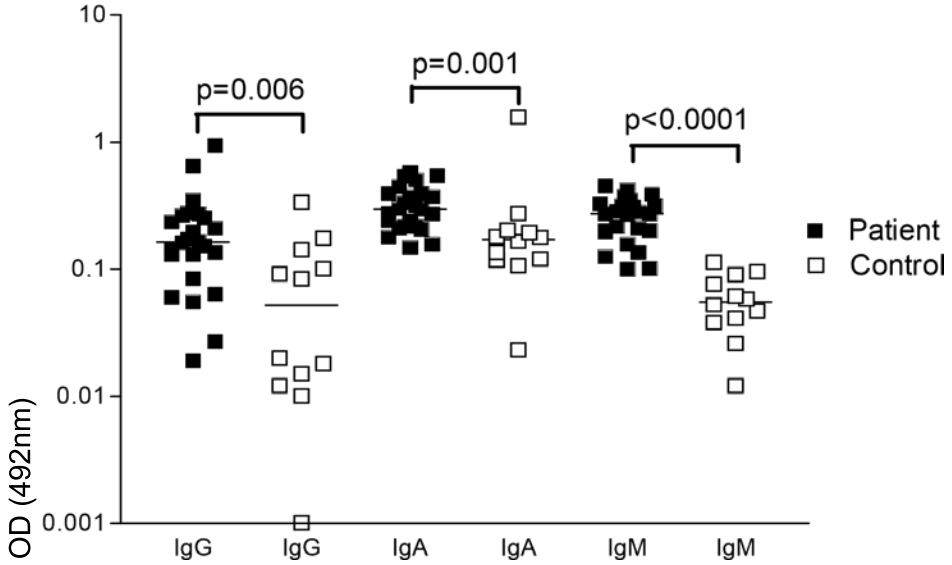

Figure 1: Levels of antibodies to MPT 59 antigen in the plasma of patients suspected to have female genital tuberculosis and the control gynecological patients, Addis Ababa, 2002 OD=Optical Density

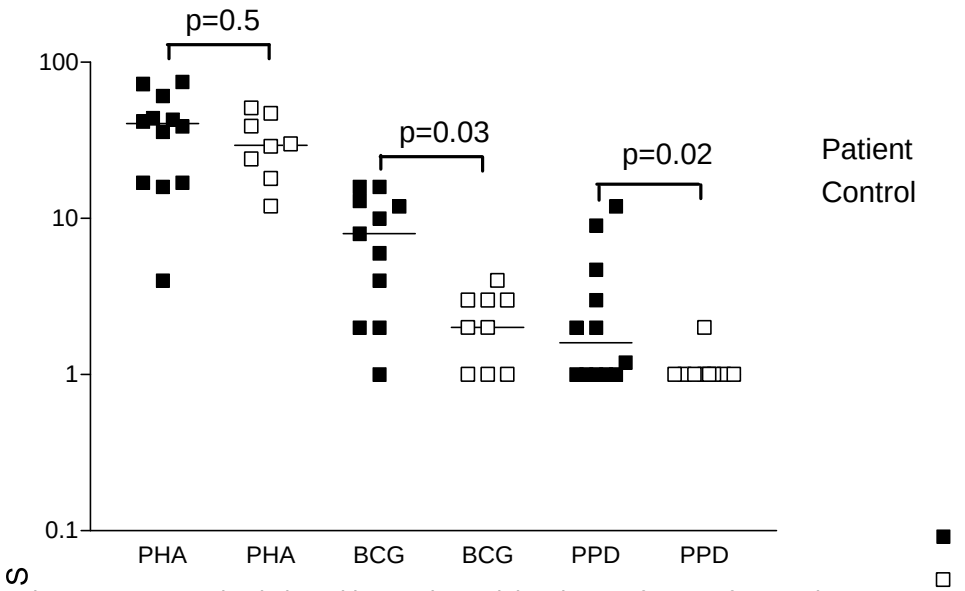

Figure 2: Proliferative response to stimulation with mycobacterial antigens of PBMC from patients suspected to have female genital tuberculosis and control gynecological patients, Addis Ababa, 2002. SI=stimulation Index PBMC=Peripheral Blood Mononuclear cells

circulating memory T and B cells in the peripheral blood, IgA antibodies to a different type of M. tuberculosis which could be targeted for immune based diagnostic antigen denoted as A60. The 100 % reactivity was methods. achieved when the three antibody isotypes were tested in each individual patient since a patient without IgG
Plasma from all the 25 patients clinically suspected of antibodies could have IgM- or IgA antibodies and vice suffering from FGTB contained antibodies to the versa. Antibody positivity does not, however, indicate mycobacterial antigen, MPT59. Another study by Parikh which organ is affected by tuberculosis.
et al. conducted in Indian women (17) has produced similar results. This study confirms that all of their 20 A similar result was obtained concerning the proliferative clinically diagnosed FGTB patients had IgG-, IgM- or response of PBMC from patients to PPD and BCG. The
SI of patients was significantly higher than that of the control group to both PPD ($p = 0.02$) and BCG ($p = 0.03$).

One limitation in the study is the relatively small sample size employed and the fact that most of the controls were cancer patients. This could not be avoided because it is only from such patients that a biopsy material could be obtained for investigation. It is also possible that the weaker immune response seen in the control group could be due to a possibly immunocompromised status in these individuals. Another confounder could be the expected different levels of progression in the HIV- infected individuals in the two groups. This is probably not the case, as the difference in the CD4:CD8 ratios in the patients and the controls were found to be statistically significant.

FGTB patients are often diagnosed lately because of the lack of early detection methods, difficulties with confirmatory tests, and the need for surgical intervention to obtain the appropriate specimens for diagnosis. Early detection is crucial for the proper management of FGTB. Since diagnosis on clinical grounds alone is unreliable, less invasive techniques than surgical interventions are needed. Tests based on the immune response patterns of the subjects using PBMC would be helpful in this regard. The findings of this study also provide the baseline information that PBMC from FGTB patients recognize mycobacterial antigens more strongly than cells from patients with other gynecological problems. Further studies are required to test whether immune response assays could serve as additional diagnostic tests in situations where the confirmation of localized tuberculosis becomes difficult using more thoroughly screened controls and additional selected M. tuberculosis antigens.

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