

IMMUNOLOGICAL INVESTIGATIONS IN TUBERCULOUS ASCITES

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Summary: Cell mediated immunity was assessed in seven patients with bacteriologically and/or histologically confirmed tuberculous ascites. Eight non-tuberculous ascites patients were included as controls. Anti-PPD antibody levels were also estimated by ELISA. Macrophage from tuberculous ascitic fluid showed increased production of H_2O_2 when compared with ascitic fluid macrophages from controls. Proliferative response of lymphocytes to PPD antigen was greater in ascitic fluid than in peripheral blood in tuberculous patients, while the responses were reversed in control patients. Tuberculous ascitic fluid had higher levels of anti-PPD antibodies than ascitic fluid from controls, though their levels in peripheral blood were similar in the two groups. It is concluded that the results provide support to the concept of immunologic localization.

Introduction

There have been several reports which provide evidence for the concept of immunologic localization at the site of lesion in tuberculous pleural effusion and in tuberculous meningitis (Ellner, 1978; Fujiwara *et al.*, 1982; Higashi *et al.*, 1981; Kinnman *et al.*, 1981; Pettersson *et al.*, 1978; Stead, 1977). Ascites, the accumulation of abnormal volumes of fluid in the peritoneal cavity, is a frequent manifestation of tuberculosis of abdomen and provides ready access to large volumes of exudate containing immuno-competent cells. To examine the concept of immunologic localization in tuberculous ascites, an investigation was carried out to study the functions of lymphocytes and macrophages drawn from ascitic fluid (AF) and peripheral blood (PB) in patients with tuberculous (TB) and non-tuberculous (NTB) ascites. The findings of this investigation are reported in this paper.

Materials and Methods

The diagnosis of tuberculous ascites was provisionally made on the basis of history, clinical and radiological examinations. The final diagnosis was made when the results of smear and culture for *Mycobacterium tuberculosis* in AF and histopathology of liver or peritoneum became available. The NTB patients were negative for *M. Tuberculosis* by smear and culture and had no histopathological evidence of tuberculosis by liver biopsy. From patients attending the Tuberculosis Research Centre Clinic, Madras, 7 patients with TB ascites and 8 patients with NTB ascites (7 transudative and 1 exudative) were investigated.

The sexes were equally distributed in both the groups and the age range was 18-57 years.

Isolation of mononuclear cells (MNC) : Five hundred ml of AF and 20 ml of PB were collected from each patient at the same time under sterile condition in bottles containing citric acid and dextrose (Sodium citrate 2.63 g, anhydrous dextrose 2.9 g, citric acid 327 mg and monobasic sodium phosphate 251 mg in 100 ml distilled water). AF was centrifuged at 1200 rpm for 10 minutes. Cell-free AF was stored at $-20^{\circ}C$. The cells were suspended in 10 ml RPMI-1640 and MNC were separated by density gradient centrifugation on Ficoll Hypaque (Boyum, 1968) PBMNC were also separated by the same method. The MNC from PB and AF were suspended at a concentration of 5×10^6 cells/ml in RPMI-1640 containing 10% heat inactivated pooled human AB serum.

Non-specific esterase staining : Smear for non-specific esterase was prepared with the cell suspension for determining the percentage of monocyte population in each specimen, according to the method of Horwitz *et al.*, (1977). A number of fields to account for a total of 100 cells were examined in the smear, and the number of esterase positive cells was determined. The mean of three such determinations was taken as the percentage of macrophages/monocytes for the specimen.

In a 96 well flat bottom plate (Falcon), 100 ul of MNC from AF or 200 ul of MNC from PB was added per well. This corresponded to a concentration of 0.5×10^6 MNC per well for AF and 1×10^6 MNC for PB. After

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incubating the plate for 1 h at 37°C, the non-adherent cells were removed by aspiration and the wells were washed three times with fresh medium. The adherent cells were used for hydrogen peroxide (H₂O₂) production assay and non-adherent cells for lymphocyte proliferation assay.

H₂O₂ Assay : This was done by the method of Pick and Mizel (1981). H₂O₂ production in nmol/10⁵ macrophages/monocytes was calculated for each specimen using a standard curve.

Lymphocyte proliferative assay : The non-adherent cells at a concentration of 0.5x10⁶ cells per ml RPMI were stimulated with phytohaemagglutinin (PHA, 5 ug/ml) or PPD (50 ug/ml) or none (control) in a 96 well microtitre plate (Falcon) (Rajiswamy, 1987), and the results expressed as stimulation index (S.I.) :

$$\text{S.I.} = \frac{\text{Mean log count of stimulated cells}}{\text{Mean log count of control cells}}$$

ELISA for anti PPD antibodies : This was done on 1 : 80 dilution of AF and serum. 25 ul of 3% glutaraldehyde in 0.15 M phosphate buffer pH 7.4 was placed in each well of a 96 well microtitre plate (Falcon) and 50 ul of PPD at a concentration of 10 ug/ml in distilled water was added to each well. The plates were incubated at 4°C for 24 h. Anti-PPD antibody (IgG) was estimated by using anti human IgG peroxidase as described by Narayanan. *et al.*, (1981).

Results

The results of the different tests performed

are presented in Table. 1 Although the mean levels presented were based on all the available specimens, the tests of significance were done by paired t-test for within group comparison and unpaired t-test for between group comparisons. Some of the comparisons involving PB are of limited value in view of the small numbers tested; nevertheless, they have been presented to facilitate comparisons.

The mean percentage of lymphocytes in AF appears to be higher in TB patients than in NTB patients, the mean levels in PB being similar in the two groups of patients. The statistically significant difference between AF and PB in the mean percentage of lymphocytes within the NTB patients and no such difference in TB patients may reflect a true situation, suggesting selective recruitment of lymphocytes in TB AF.

The mean H₂O₂ production by AF macrophages was 8.4 nmol/10⁵ cells in TB patients as against the corresponding mean of 2.1 nmol/10⁵ in NTB patients, the difference being statistically significant (P<0.01). Also, AF macrophages appear to produce more H₂O₂ than PB monocytes in TB patients as against similar levels in NTB patients.

The response in lymphocyte proliferation test with the mitogen PHA was normal in lymphocytes from AF as well as PB in each group. On the contrary, differences were observed in lymphocyte proliferation response to the antigen PPD in the two groups of patients. The following inferences can be made from the mean S.I. values and statistical significance of their differences :

1. The low mean S.I. values of 1.06 for PB in TB patients and 1.05 for AF in

Table 1.

Comparison of immunological parameters in ascitic fluid and peripheral blood from tuberculous and non-tuberculous ascites patients :

Parameter	TB Ascites Patients			Non-TB Ascites Patient			TB Vs. Non-TB	
	Fluid	Blood	P - Value*	Fluid	Blood	P Value*	P-value**	
	Mean ± S.D. (n)	Mean ± S.D. (n)		Mean ± S.D. (n)	Mean ± S.D. n		Fluid	Blood
1. Percentage of Lymphocytes	60 ± 21 (7)	74 ± 14 (3)	NS	42 ± 23 (8)	88 ± 7 (4)	<.05	NS	NS
2. H ₂ O ₂ Production (nmol/10 ⁵ macrophages or monocytes)	8.4 ± 3.7 (7)	4.6 ± 0.1 (2)	NS	2.1 ± 1.5 (6)	2.0 ± 2.3 (2)	NS	<.01	NS
3. Stimulation Index (PHA)	1.25 ± 0.18 (7)	1.25 ± 0.21 (5)	NS	1.19 ± 0.14 (6)	1.42 ± 0.09 (2)	NS	NS	NS
4. Stimulation Index (PPD)	1.19 ± 0.07 (7)	1.06 ± 0.07 (5)	<.05	1.05 ± 0.07 (7)	1.11 ± 0.10 (4)	<.01	<.01	NS
5. ELISA Optical Density	0.111 ± 0.19 (7)	0.180 ± 0.025 (7)	<.001	0.057 ± 0.049 (8)	0.174 ± 0.019 (7)	<.001	<.05	NS

NS - Not significant (p > .05) * and ** correspond to paired and unpaired t-tests, respectively.

NTB patients suggest deficiency of sensitized lymphocytes.

2. The mean S.I. values of 1.19 for AF in TB patients and 1.11 for PB in NTB patients indicate the presence of sensitized lymphocytes.
3. Whereas the mean S.I. was higher in PB than in AF ($P < 0.01$) in NTB patients, the reverse was true in TB patients, the difference being significant ($P < 0.05$). The individual values are also shown in Figure-1.

The level of antibodies to PPD was measured by optical density (O.D.). The mean O.D. Values of 0.180 and 0.174 in PB in the two groups of patients were similar. Although the mean O.D. of 0.111 in AF is significantly ($P < 0.01$) less than that in PB in TB patients, it is significantly higher ($P < 0.05$) than the mean O.D. of 0.057 in AF from NTB patients.

Discussion

This investigation highlights the status of cell mediated immunity in bacteriologically and/or histopathologically diagnosed TB ascites patients; NTB ascites patients served as controls. Some functions of lymphocytes and macrophages obtained from the site of lesion as well as from peripheral blood were assayed as indicators of cell mediated immunity. Anti-PPD antibody levels were estimated by ELISA method.

Enhanced production of H_2O_2 is known to be one of the characteristics of macrophage activation. Klebanoff and Sheppard (1984) have shown that *M. leprae* is susceptible to peroxidase mediated H_2O_2 killing in vitro, and similar finding was reported for *M. tuberculosis* by Jacket et al. (1978). Hence, H_2O_2 production by macrophages was chosen for studying its function. It has been found in this study that AF macrophages produced higher

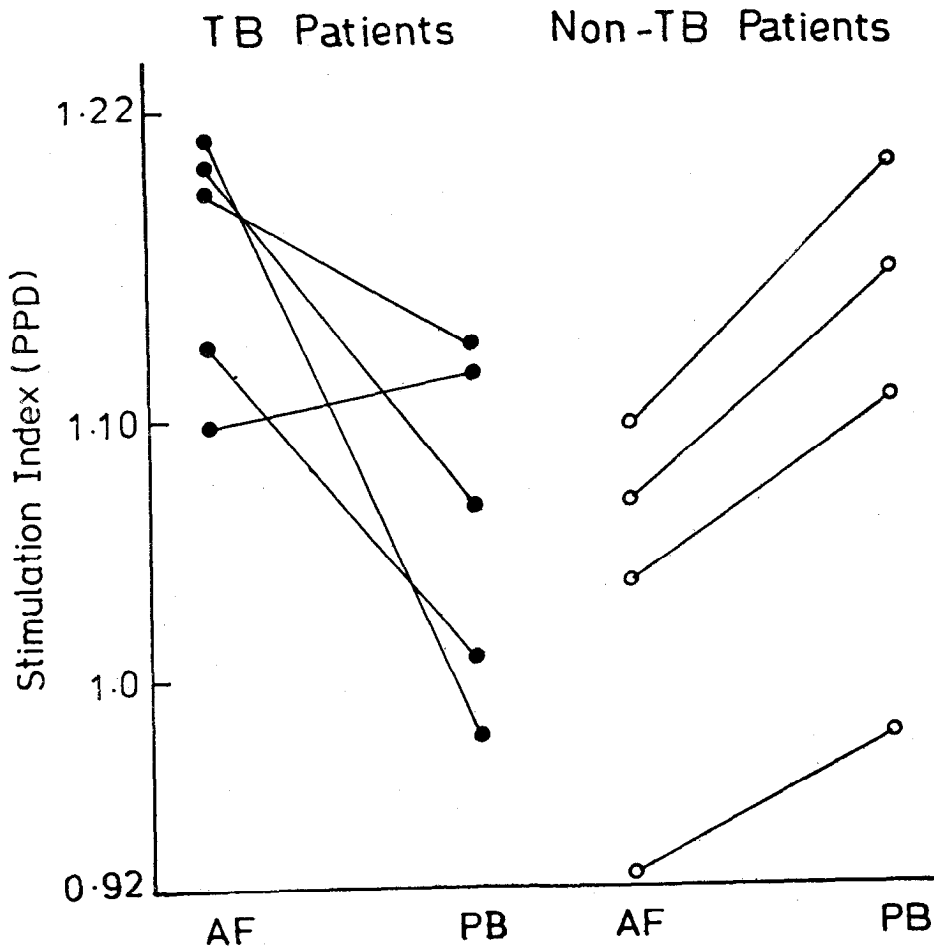


Fig 1 Proliferative response to PPD of non-adherent cells from AF and PB in tuberculous ascites. Each line represents the response from the same individual. Non-adherent cells from both PB as well as AF MNC were cultured with 50 ug/ml PPD for 6 days. 3 H-Thymidine was added 18 h. before harvesting.

amount of H₂O₂ in comparison with the monocytes from PB in TB patients and also from AF or PB in NTB ascites patients. The increase in H₂O₂ production by TB AF macrophages may be due to the activation of the macrophages by phagocytosis of *M. tuberculosis* or as suggested by Gately *et al.*, (1983) due to a lymphokine following T-cell stimulation by *M. tuberculosis* antigens.

Kinman *et al.* (1981) reported higher proliferative response of lymphocytes from the cerebral spinal fluid to PPD antigen than the peripheral blood lymphocytes in 2 patients with TB Meningitis. Fujiwara *et al.* (1982) and also Ellner (1978) have reported similar findings for lymphocytes from tuberculous pleural fluid. In this study, lymphocyte proliferative response was assessed not only to antigen PPD but also to the mitogen PHA. The assays with PHA served as controls. While the proliferative response to PHA was similar in both the groups of patients in AF as well as PB, AF lymphocytes from TB patients showed a much higher response to PPD than their corresponding PB lymphocytes and either category of lymphocytes from NTB patients. This could be attributed to either a selective sequestration of sensitized T-cells from the blood to the site of inflammation or due to clonal expansion of PPD responding T-lymphocytes (Rohrbach and William, 1986). In the control group, there is neither evidence of selective sequestration of PPD sensitized cells to the peritoneal cavity, nor does the peritoneal lesion provide antigenic challenge.

This study has also indicated a higher proportion of lymphocytes in AF in TB patients. This finding is expected in view of the fact that lymphocytosis in tuberculous exudates is well-documented in literature.

Using radioimmunoassay, Samuel *et al.* (1984) found elevated levels of tuberculous antigens and antibodies in TB AF and TB pleural fluid. With ELISA method, we estimated anti-PPD antibody levels both in AF and PB and found that, though the antibody levels were similar in PB in both the groups of patients, their levels in AF in TB patients was significantly higher than in NTB patients. As mycobacterial infections are known to be endemic in South India, the similarity of antibody levels in PB is expected; but what is significant is the higher antibody levels in AF in TB patients and this finding is in conformity with that of Samuel *et al.*, (1984).

The results of none of these tests is suggestive of a defect in cell mediated immunity in TB. On the other hand, our study, has shown an enhancement of both

macrophage and lymphocytes function in TB at the site of lesion. Bactericidal properties of macrophages stimulated with appropriate T-cell factors could be the possible approach to study the defect. Our observations provide further support to the concept of immunologic localization at the site of lesion in tuberculous ascites.

Acknowledgement

We thank all the Clinic Staff, especially S.P. Tripathy, P. Joseph, R. Rajeswari & R. Sundararaman for collection of ascitic fluid and blood. We also thank R. Paranjape and P.R. Somasundaram for their critical assessment of the manuscript; S. Ramanujam for the excellent technical assistance throughout the script and P. Karthigayan, for typing the script.

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