

THE local production of tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) was evaluated in the cerebrospinal fluid (CSF) from ten patients with tuberculous meningitis (TBM). The degree of intrathecal immune activation was also studied by assessing the CSF levels of  $\beta_2$ -microglobulin ( $\beta_2$ -M) and adenosine deaminase activity (ADA). Results indicate that elevated CSF concentrations of TNF $\alpha$ ,  $\beta_2$ -M and ADA were found in all TBM patients. Moreover, TNF $\alpha$  is produced and selectively concentrated for a long period of time, while  $\beta_2$ -M and ADA values progressively decline during the course of TBM. Our findings suggest that in TBM patients, after an early activation of immune cells, there is an enhanced and continuous production of TNF $\alpha$  at the site of infection.

**Key words:** Adenosine deaminase,  $\beta_2$ -microglobulin, Cerebrospinal fluid, Immune activation, Tuberculous meningitis, Tumour necrosis factor- $\alpha$

## Tumour necrosis factor- $\alpha$ production and immune cell activation in tuberculous meningitis

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## Introduction

Tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) is a cytokine mainly released by cells of the monocyte-macrophage lineage in response to lipopolysaccharide (LPS) and other immune and inflammatory stimuli. It has been recognized as a primary mediator in the pathogenesis of infection, injury and inflammation and in the beneficial processes of host response.<sup>1</sup> In particular, TNF $\alpha$  is an important macrophage-activating factor for antibacterial resistance against infections caused by facultative intracellular organisms, such as mycobacteria.<sup>2</sup>

In experimental models it has been reported that TNF $\alpha$  enhances macrophage phagocytic capacity and mycobacterial killing by human macrophages.<sup>3</sup> In addition, recent lines of evidence indicated that TNF $\alpha$  is synthesized in large amounts in pulmonary tuberculosis and it is locally concentrated at the site of disease activity.<sup>4,5</sup> To date, little is known about the intrathecal production of TNF $\alpha$  during the course of tuberculous meningitis (TBM).<sup>6</sup> Therefore, it appears to be of interest to evaluate the local production and release of TNF $\alpha$  in the cerebrospinal fluid (CSF) of patients with TBM.

In the present study, TNF $\alpha$  concentrations were measured in CSF specimens obtained from hospital patients on admission and during the course of TBM. In order to determine the extent to which cell-mediated immunity is involved in the disease, the CSF levels of two markers of immune activation, such as  $\beta_2$ -microglobulin ( $\beta_2$ -M) and adenosine deaminase activity (ADA), were also measured.

## Materials and Methods

**Patients:** Ten patients with TBM (five males, five females) admitted to the Institute of Infectious Diseases of the University of Rome 'La Sapienza', were enrolled in this study. The mean age was 34.4 years (range, 12–55 years). TBM was diagnosed on the basis of findings in the CSF. In six cases CSF smears were positive by acid-fast staining and fluid cultures grew *Mycobacterium tuberculosis*; in the remaining patients, diagnosis was made by compatible cytological and biochemical findings, supported by clinical features and symptomatic improvement with antituberculous therapy. All patients were sero-negative for human immunodeficiency virus (HIV) and did not have other disturbances of immunity.

A total of 66 CSF samples was collected from TBM patients before starting treatment and subsequently at various intervals of time during the course of infection.

CSF specimens were also obtained from 15 patients with non-infectious neurological disorders, including cerebrovascular disease, hydrocephalus, encephalomyelitis and epilepsy.

CSF specimens, upon collection, were placed in a refrigerated centrifuge (4°C) and spun at 3000 rpm for 10 min. Cell supernatants were stored at –80°C until use.

**Assay for TNF $\alpha$ :** TNF $\alpha$  concentrations in CSF were measured by a quantitative immunoenzymatic sandwich assay (Quantikine, R&D Systems, Minneapolis, MN, USA). Samples and standards were

incubated in anti-TNF $\alpha$  monoclonal antibody-coated polystyrene microtiter wells. A horseradish peroxidase-conjugated anti-TNF $\alpha$  polyclonal antibody was then added and allowed to bind the TNF $\alpha$  captured by the first antibody, completing the sandwich. After washing, substrate solution was added to the wells. The reaction was stopped by addition of 2N H $_2$ SO $_4$  and optical density (OD) at 492 nm was measured using an ELISA microreader. To estimate the amount of TNF $\alpha$  in the CSF samples we constructed a standard curve by using concentrations of TNF $\alpha$  ranging from 0 to 1000 pg/ml. Unknown values of TNF $\alpha$  in the samples were determined by referring to the standard curve and expressed as pg/ml. The detection limit of the assay was 4.8 pg/ml.

**Assay for  $\beta_2$ -M:**  $\beta_2$ -M concentrations were assessed by the IMx system (Abbott Laboratories, North Chicago, IL, USA), according to the manufacturer's instructions. The IMx system is based on a rapid microparticle enzyme immunoassay (MEIA), in which submicron particles are coated with a mouse monoclonal antibody specific for  $\beta_2$ -M. The use of microparticles increases the assay kinetics and decreases the assay incubation. The sensitivity of the IMx  $\beta_2$ -M was calculated to be better than 5  $\mu$ g/l. Normal  $\beta_2$ -M values were considered equal or less than 3 mg/l.

**ADA activity measurement:** ADA activity was measured in fresh CSF samples after centrifugation at 300 g for 10 min. ADA assay was performed at 37°C by using the colorimetric method of Giusti,<sup>7</sup> based on the determination of the amount of NH $_3$  released in the reaction mixture. The enzymatic activity was calculated by referring to a standard curve of ammonium sulphate in buffer and was

expressed as IU/37°C/l. Normal ADA values were considered equal or less than 4 IU/l.

**Statistical analysis:** The Mann-Whitney *U*-test was used for statistical analysis.

## Results

CSF levels of TNF $\alpha$ ,  $\beta_2$ -M and ADA were measured in ten patients with TBM and in 15 control subjects with non-infectious CNS disorders. Data are shown in Figure 1 and are expressed as mean  $\pm$  SEM. TNF $\alpha$  concentrations in TBM patients (155  $\pm$  21.62 pg/ml) were significantly higher than those found in controls (5.69  $\pm$  0.77 pg/ml) ( $p$  < 0.0001). Similarly, TBM patients had elevated CSF values of  $\beta_2$ -M (8.69  $\pm$  1.07 mg/l) and ADA (10.83  $\pm$  1.61 IU/37°C/l) in comparison with those observed in the non-TBM cases (1.34  $\pm$  0.12 mg/l and 1.02  $\pm$  0.16 IU/37°C/l, respectively) ( $p$  < 0.0001 for both).

The kinetics of TNF $\alpha$  and both  $\beta_2$ -M and ADA values during the course of TBM were illustrated respectively in Figures 2 and 3.

In all patients CSF levels of TNF $\alpha$  were low on admission, then progressively increased and remained detectable for a long period of time. Antibiotic treatment did not appear to influence the TNF $\alpha$  levels. Similarly, there was no correlation between the cytokine concentrations and the clinical course of the disease.

On the contrary,  $\beta_2$ -M and ADA values resulted to be elevated on admission. Later, a progressive decline was observed. Only two patients with culture-proven TBM showed a further increase in CSF  $\beta_2$ -M and ADA values, despite the administration of antituberculous treatment. The peak of these

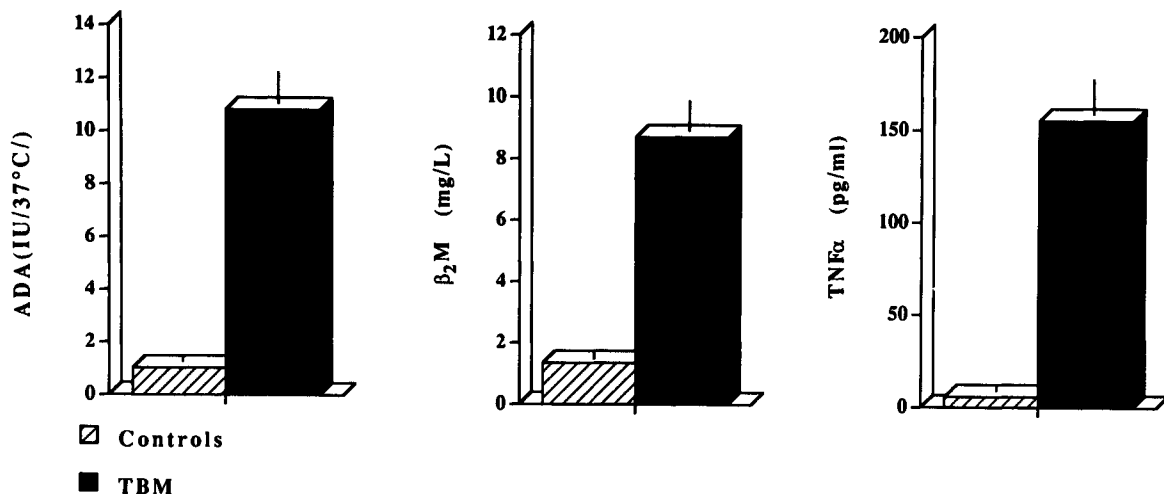


FIG. 1. Levels of  $\beta_2$ -M, ADA and TNF $\alpha$  in the CSF samples ( $n = 66$ ) from TBM patients compared with those ( $n = 15$ ) obtained in controls with non-infectious neurological diseases. Data are expressed as the mean  $\pm$  SEM. Comparisons between groups are made by non-parametric test (Mann-Whitney *U*-test) and differences in CSF levels of  $\beta_2$ -M, ADA and TNF $\alpha$  were statistically significant ( $p$  < 0.0001 for all).

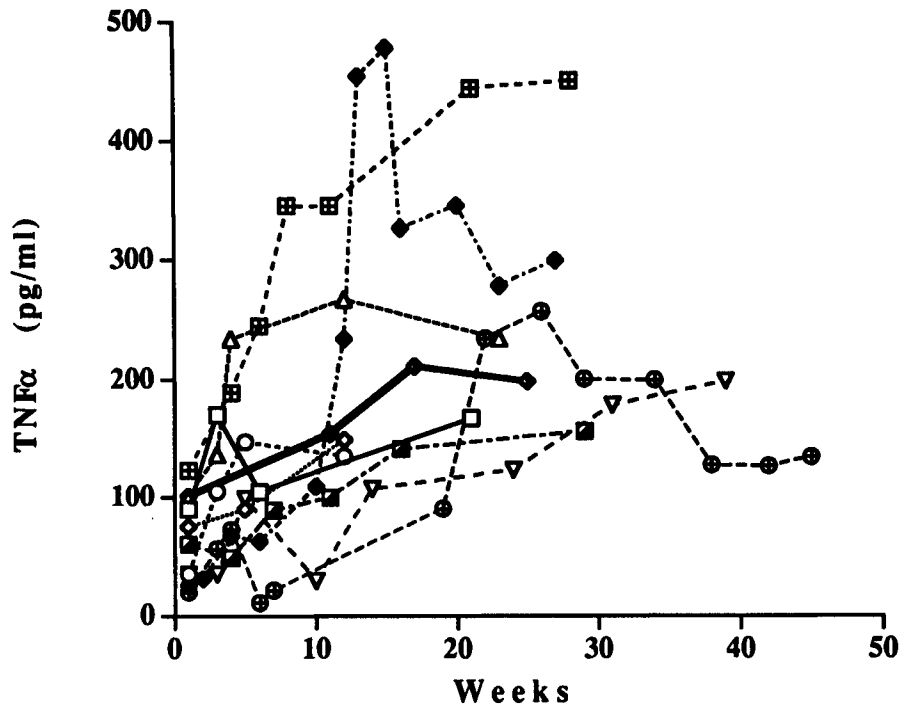


FIG. 2. Time course of TNF $\alpha$  levels in sequential CSF samples from the ten TBM patients.

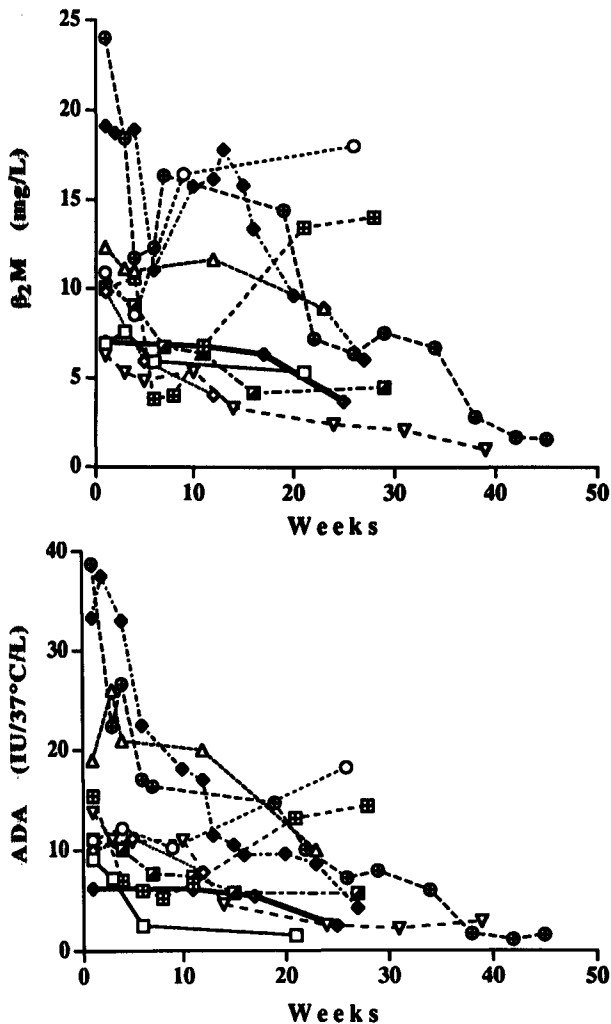


FIG. 3. Time course of  $\beta_2$ -M and ADA levels in sequential CSF samples from the ten TBM patients.

immune activation markers was associated with the development of complications (hydrocephalus, hemiplegia) and an unfavourable clinical course of meningitis.

### Discussion

Cell-mediated immunity plays an important role in the control of infections by *M. tuberculosis*. It is widely assumed that T-lymphocytes, through lymphokine-mediated macrophage activation, are the major immune cells involved both in the pathogenesis and protective mechanisms in human tuberculosis.<sup>8-10</sup>

In the case of TBM, it is conceivable that the involvement of cellular immune system mainly occurs in the central nervous system (CNS). In this respect, the dosage in the CSF of two markers of immune activation, such as  $\beta_2$ -M and ADA, represents a useful tool to investigate the degree to which cell-mediated immunity is stimulated in the CNS.  $\beta_2$ -M is a portion of the major histocompatibility complex class I antigen (MHC-1) and is expressed on the surface of lymphocytes and macrophages. With regard to the adenosine deaminase, the activity of this enzyme is increased in lymphoid cells, especially during T-cell proliferation and differentiation.

Previous investigations have already demonstrated a close correlation between elevated CSF ADA values and TBM,<sup>11,12</sup> while an increase in the CSF concentrations of  $\beta_2$ -M has been reported only in viral and pyogenic meningitis.<sup>13</sup> In the present

study, both  $\beta_2$ -M and ADA levels were found to be elevated in the CSF of all TBM patients, thus indicating a marked stimulation of immune system within the CNS. The increased CSF levels of  $\beta_2$ -M were possibly a consequence of cell-mediated cytotoxicity directed against macrophages infected by *M. tuberculosis*.<sup>14</sup> The elevation of CSF ADA values was most likely related to the local immune response as the result of proliferating lymphocytes in response to mycobacterial antigen.

Intrathecal immune activation appears to be also associated with increased cytokine expression in the CNS. Indeed, our results showed that TBM patients had significantly higher CSF TNF $\alpha$  concentrations than those found in control subjects with non-infectious neurological diseases. The local production of TNF $\alpha$  is low in the initial phase of TBM, while the peak in the CSF concentration was obtained later. Moreover, the CSF levels of this cytokine did not decrease during the course of the disease, but they were elevated for a long time in all patients, irrespective of the antibiotic therapy and the clinical course of TBM. This pattern is quite different from that observed in the case of  $\beta_2$ -M and ADA, whose levels declined rapidly during the course of the disease. Only two patients who developed neurological complications showed a further increase in the CSF  $\beta_2$ -M and ADA values.

Taken together, our findings suggest that a great stimulation of immune T-cells primarily occurs in the acute phase of TBM and may account for the early increase in the CSF  $\beta_2$ -M and ADA values. On the contrary, the enhancement of cytokine expression related to TNF $\alpha$  production is a later but more prolonged phenomenon. It is likely that brain macrophages activated by T-cell-mediated pathway are the source of TNF $\alpha$  in TBM patients. This hypothesis is also supported by previous investigations that have demonstrated that *M. tuberculosis* cell wall components can trigger the release of TNF $\alpha$  from human and murine macrophages, as well as from pleural fluid mononuclear cells of patients with pulmonary tuberculosis.<sup>5,15,16</sup>

In conclusion, our data provide direct evidence that TNF $\alpha$  is produced and selectively concentrated

for a long period of time in the CSF from patients with TBM. The chronic release of TNF $\alpha$  at the site of the infection suggests that this cytokine may be involved in the complex immunoregulatory mechanism that contributes to mycobacterial containment and elimination.

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