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Increased expression and occupancy of receptors for tumour necrosis factor on blood monocytes from tuberculosis patients

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SUMMARY

Blood monocytes from tuberculosis patients release high amounts of tumour necrosis factor-alpha (TNF-α). Because the biological efficiency of TNF-α would depend on the expression of TNF-α receptors on target cells, we thought to analyse the capacity of blood monocytes from a group of patients with pulmonary tuberculosis to bind ^{125}I-TNF-α. We report a slight but not significant enhancement in specific binding of ^{125}I-TNF-α on monocytes of 15 consecutively studied patients compared with 10 controls. Per cent cell surface bound and internalized ^{125}I-TNF-α was identical in the two groups. To evaluate the receptor occupancy by endogenously generated TNF-α, similar experiments were performed after cell exposure to low-pH glycine buffer. Under these conditions, specific binding of ^{125}I-TNF-α was significantly higher on tuberculosis monocytes compared with control monocytes. Moreover, the occupancy of TNF-α receptors by endogenously generated TNF-α that was found to be significantly higher on tuberculosis monocytes than on control monocytes, was directly related to the enhanced capacity of mononuclear cells to generate TNF-α *in vitro*. It normalized after 3 months of antituberculous therapy. Scatchard analysis of the binding data revealed that tuberculosis infection caused a significant increase in high affinity ^{125}I-TNF-α binding to monocytes without any significant change in the dissociation constant. Collectively, these results indicate an up-regulation of TNF-α generation and binding to blood monocytes in patients with pulmonary tuberculosis. They provide support to the hypothesis that TNF-α is of critical importance in the pathogenesis of this infection.

Keywords tuberculosis tumour necrosis factor receptor monocytes

INTRODUCTION

Tuberculosis is becoming again a major health problem, especially because of the HIV epidemic [1]. The main clinical features of this infection include fever, weight loss, and raised acute phase reactants. Because of its effects on thermoregulation, protein or lipid metabolism, and acute phase protein synthesis, tumour necrosis factor-alpha (TNF-α) has been suggested as playing potentially important roles in these manifestations [2]. The other reasons why TNF-α has been implicated in the pathogenesis of tuberculosis are that (i) peripheral blood mononuclear cells (PBMC) from tuberculosis patients generate constitutively more TNF-α than do PBMC from control subjects [3,4]; and (ii) *in vitro*, monocytes produce TNF-α in response to cell wall lipoarabinomannan and other components of *Mycobacterium tuberculosis* [5,6]. In turn, by interacting with T cells and monocytes, TNF-α could participate in both killing of *Myco. tuberculosis* [7] and development and maintenance of granulomas [8]. It is believed that these actions are initiated by TNF-α binding to specific receptors. Monocytes, like other myeloid cells, express the type A or type II 75-kD TNF-α receptor which leads to a cross-linked complex of 98–100 kD [9–11]. Because maintenance of the level of these cell-surface receptors is one likely process through which cell responsiveness to TNF-α could be sustained, we studied the expression of TNF-α receptors on human monocytes obtained from patients with pulmonary tuberculosis. We report that these monocytes express a marked increase of TNF-α receptor number compared with monocytes from a control group of healthy blood donors. Moreover, receptor occupancy by endogenous TNF-α appears to be enhanced. The enhancement, directly related to the capacity of tuberculosis monocytes to release TNF-α *in vitro*, is reversible upon anti-tuberculous therapy.

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PATIENTS AND METHODS

Patient selection

Patients admitted to the Department of Respiratory Diseases of the Hôpital Tenon were screened for signs and symptoms of pulmonary tuberculosis, and then selected for entry into the

study on the basis of cultures of sputum or gastric aspirates positive for *Myco. tuberculosis*. Patients with known immunodeficiencies (primary or secondary) were excluded from the study. Once selected, informed consent was obtained and 15 patients (aged 36±13 years) were referred for drawing of a blood sample. Tuberculin skin test was strongly positive in all patients. Seven patients had a high grade fever (> 38·5°C) and a weight loss; eight patients had a low grade fever (< 38°C). Chest x-ray showed parenchymal infiltrates with cavitation(s) in all cases. All the patients were studied before receiving any antituberculous therapy. Five among these patients were also studied after a 3-month therapy consisting in isoniazid, rifampin, pyrazinamide, ethambutol. Ten healthy volunteer donors (aged 32±12 years) served as the control population.

Cell preparation

Forty millilitres of venous blood were collected into 10-ml vacutainer tubes containing sodium heparin (Vacutainer, Becton Dickinson, Mountain View, CA) and diluted 1:1 (v/v) in calcium-free minimum essential medium (MEM) with Earle's Salts (Boehringer, Mannheim, Germany) supplemented with 0·8% EDTA. PBMC were isolated by density centrifugation of lymphoprep (Nycomed Pharma AS, Oslo, Norway), resuspended in the same medium diluted 1:9 (v/v) in calcium-free MEM, and centrifuged at 900 *g* for 10 min. Thereafter, PBMC were resuspended in culture medium consisting of RPMI 1640 (Flow Labs, Irvine, UK) buffered with 20 mM HEPES to pH 7·4, and supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (culture medium), counted after staining with acridine orange, and adjusted to a concentration of 5×10^6 cells/ml. For binding studies, the cells were plated into 24-well tissue culture plates (Costar, Cambridge, MA) and allowed to adhere for 4 h at 37°C. Thereafter, the wells were extensively rinsed with culture medium, leaving only adherent monocytes. However, numerous platelets were occasionally found in association with monocytes. Thus, in subsequent experiments, the blood suspension was subjected to centrifugation at 100 *g* for 15 min to remove the overlying platelet-rich plasma before isolating PBMC as indicated above. This procedure prevented platelet contamination, but did not modify binding data.

Binding studies

Monocytes in 24-well plates were overlaid with 0·2 ml/well of binding buffer (RPMI 1640 containing 10% FCS), and incubated with 0·5 nM human recombinant (r) ^{125}I-TNF-α (400–600 Ci/mmol; Radiochemical Centre, Amersham, UK) and varying concentrations of unlabelled human rTNF-α (Boehringer). After 2 h at 4°C, the binding buffer was removed and monocytes were washed with ice-cold medium, incubated with 0·05 M glycine-HCl buffer (pH 3·0) containing 0·15 M NaCl for 10 min at 4°C, and finally solubilized in 1 M NaOH. The acid-dissociable radioactivity and the radioactivity present in solubilized cells represented cell surface-bound ^{125}I-TNF-α and internalized ^{125}I-TNF-α, respectively. Non-specific binding determined in the presence of a 100-fold excess of unlabelled TNF-α was 10–20% of total binding, and was subtracted to calculate specific binding. In all the cases, monocytes were pre-exposed for 10 min to low-pH glycine buffer, to reveal possible occupation of TNF-α receptors by endogenously generated TNF-α. The protein content of each well was determined by the method of Lowry *et al.* [12], and specific binding of ^{125}I-TNF-α on monocytes was expressed as fmol/mg of protein.

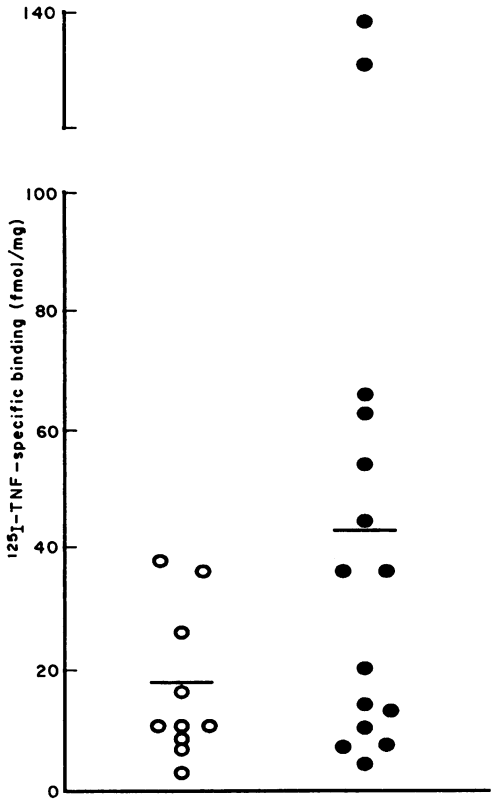

Fig. 1. ^{125}I-tumour necrosis factor-alpha (TNF-α) binding to monocytes from control group ($n=10$; ○) and tuberculosis group ($n=15$; ●). Monocytes were incubated for 2 h at 4°C with 0·5 nM ^{125}I-TNF-α, in absence or presence of unlabelled TNF-α, before specific ^{125}I-TNF-α binding was determined. The mean of ^{125}I-TNF-α specific binding was slightly higher on monocytes from tuberculosis group ($P=0·06$).

Cytokine assays

PBMC were adjusted to a concentration of $0·5 \times 10^6$ cells/ml in culture medium. Two-hundred-microlitre volumes of this suspension were added to 96-well microplates (Nunc, Roskilde, Denmark). After incubation of the cells at 37°C for 18 h, cell-free supernatants were frozen at −70°C until analysed for TNF-α and interferon-gamma (IFN-γ). Concentrations of TNF-α were measured by immunoradiometric assay (TNFα-IRMA; Ire-Medgenix, Fleurus, Belgium), and concentrations of IFN-γ were measured by ELISA (Intertest-γ; Genzyme, Cambridge, MA). For IFN-γ assay, samples should be concentrated 10 times (Centricon-10; Amicon, Beverly, MA) to be within the linear range of the standard curve. Samples were assayed in duplicate.

Statistical analysis

Data were expressed as the mean±s.d. They were analysed using Student's *t*-test for unpaired values. Regression analysis was performed to estimate the relationship between two parameters. $P<0·05$ was considered significant.

RESULTS

The proportion of monocytes in the PBMC population was 32±15% in the tuberculosis group and 39±6% in the control

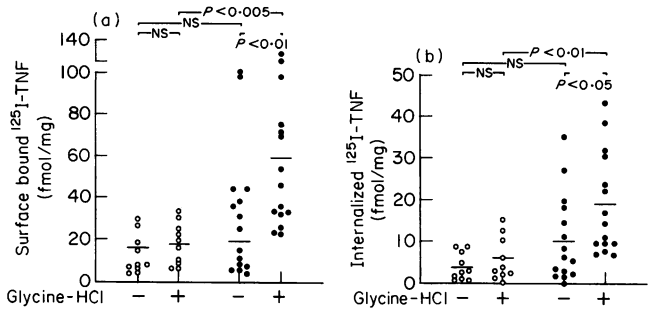

Fig. 2. Effect of low-pH treatment on ^{125}I-tumour necrosis factor-alpha (TNF-α) binding to monocytes from control group (○) and tuberculosis group (●). Monocytes were incubated in the presence or absence of low-pH glycine buffer before cell surface-bound ^{125}I-TNF-α (a) and internalized ^{125}I-TNF-α (b) were determined. Under these conditions total amount of bound ^{125}I-TNF-α was significantly higher in monocytes from tuberculosis group ($P<0·005$).

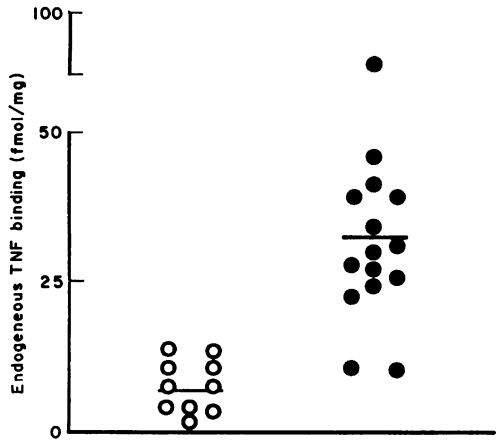

Fig. 3. Receptor occupancy by endogenously generated tumour necrosis factor-alpha (TNF-α). Monocytes from control group (○) and tuberculosis group (●) were incubated in the presence or absence of low-pH glycine buffer before total bound ^{125}I-TNF-α was determined. Receptor occupancy by endogenously generated TNF-α was determined by measuring binding recovery following low-pH treatment of the cells. Its mean value was significantly higher in monocytes from tuberculosis group ($P<0·001$).

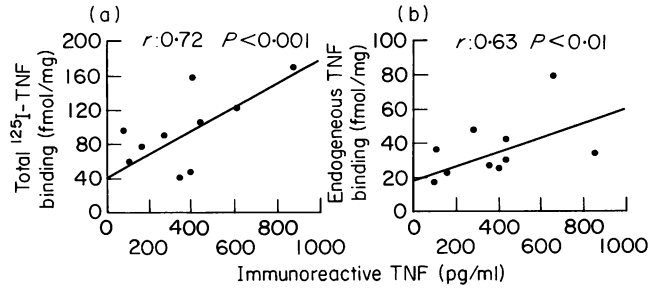

Fig. 4. Correlations between tumour necrosis factor-alpha (TNF-α) levels in culture supernatants of peripheral blood mononuclear cells (PBMC) and both specific ^{125}I-TNF-α binding (a) and receptor occupancy by endogenously generated TNF-α (b) on monocytes obtained from 10 tuberculosis patients.

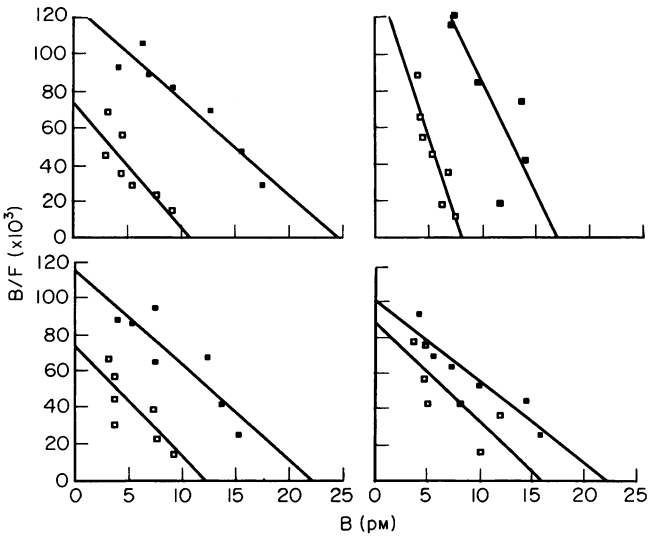

Fig. 5. Competitive displacement of ^{125}I-tumour necrosis factor-alpha (TNF-α) bound on monocytes from control group (□) and tuberculosis group (■). Monocytes were incubated for 2 h at 4°C with 0·5 nM ^{125}I-TNF-α and varying amounts of unlabelled TNF-α. Scatchard analysis of the specific binding data obtained on monocytes from four tuberculosis patients and corresponding controls is shown.

group. Monocytes from both groups were compared for their ability to bind ^{125}I-TNF-α. The results shown in Fig. 1 demonstrate that monocytes from the tuberculosis group expressed slightly higher specific binding than monocytes from the control group ($P=0·06$). In both groups, the non-specific binding was 10–20% of the total binding. The specific binding was found to include both cell surface-associated ^{125}I-TNF-α and internalized ^{125}I-TNF-α (Fig. 2). The extent of internalization was low and similar in monocytes from both groups (22·7 ± 13·3 and 21·9 ± 7·6, in monocytes from the control and tuberculosis group, respectively).

To address questions regarding receptor occupation by *in vivo* generated TNF-α, a possible membrane-associated ligand was first removed by cell exposure to low-pH glycine buffer (Fig. 2). Under these conditions, the ^{125}I-TNF-α binding capacity of monocytes from the control group was not modified, whereas that of monocytes from the tuberculosis group was significantly enhanced ($P<0·01$ and $P<0·05$, for cell-surface associated ^{125}I-TNF-α and internalized ^{125}I-TNF-α, respectively). The binding recovery, representing receptor occupancy by endogenously generated TNF-α, is depicted in Fig. 3. There was a significant correlation between ^{125}I-TNF-α-specific binding or receptor occupancy by endogenously generated TNF-α on monocytes obtained from 10 tuberculosis patients and the capacity of PBMC from the same patients to generate TNF-α *in vitro* (Fig. 4).

In order further to compare the characteristics of ^{125}I-TNF-α binding on monocytes in both groups, rTNF-α competition curves were obtained after cell exposure to low pH glycine buffer. The Scatchard analysis of the data indicated an approximately two-fold increase of high affinity ^{125}I-TNF-α binding in monocytes from the tuberculosis group (11·75 ± 3·21 pM and 21·47 ± 3·18 pM for monocytes from the control group and the tuberculosis group, respectively; $P=0·01$), without any significant difference in the affinity constant (0·68 ± 0·27 nM and

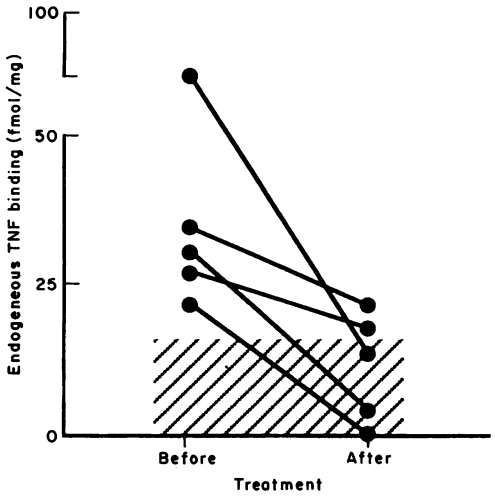

Fig. 6. Effect of anti-tuberculous therapy on endogenous tumour necrosis factor-alpha (TNF-α) binding to monocytes from five tuberculosis patients. The shaded area represents values obtained from control group.

0·86±0·31 nM for monocytes from the control group and the tuberculosis group, respectively) (Fig. 5). T cell-derived cytokines including IFN-γ have been shown to increase TNF-α receptor on cells [13]. Therefore, it was of interest to determine the capacity of PBMC from tuberculosis patients to generate IFN-γ *in vitro*. IFN-γ levels were detectable in most samples (15–30 pg/ml), but did not correlate with the increased number of ^{125}I-TNF-α-binding sites on monocytes.

Finally, to assess the role of anti-tuberculous therapy on the increased capacity of monocytes to bind endogenous TNF-α, ^{125}I-TNF-α binding to monocytes from five tuberculosis patients was measured before and after cell exposure to low-pH glycine buffer both at the admission and after administration of isoniazid, rifampin, pyrazinamide, ethambutol for 3 months. As demonstrated in Fig. 6, endogenous TNF-α binding to monocytes almost normalized in treated patients.

DISCUSSION

Increased expression of TNF-α receptors on monocytes from tuberculosis patients

In this report, we show first that monocytes isolated from the blood of patients with pulmonary tuberculosis have elevated numbers of both occupied and freely accessible receptors for TNF-α, compared with normal monocytes (Figs 2 and 5). Enhanced expression of TNF-α receptors, which was noted in most patients, was not related to clinical manifestations of the infection including fever, weight loss, and chest x-ray abnormalities. Two separate TNF-α-binding proteins are expressed in various relative amounts on different cells, but 75-kD TNF-α receptor has been shown to be predominant on human monocytes [11,14]. It is thus conceivable that tuberculosis-induced up-regulation of TNF-α receptors implicates more 75-kD TNF-α receptor than 55-kD TNF-α receptor. Because there is some evidence that the extracellular domains of both receptors are shed from the cell surface in response to various inflammatory stimuli [15], the presence of an inhibitor of TNF-α cytotoxicity in most sera from tuberculosis patients [16] could reflect the higher expression of this molecule at the surface of circulating monocytes. This phenomenon could parallel the amplified capacity of blood monocytes from tuberculosis patients to express functional IL-2 receptor molecules on their surface, and to release soluble IL-2 receptor molecules into their supernatant without *in vitro* induction [17].

While a constitutive expression of TNF-α receptors has been demonstrated in most cells, positive and negative regulatory mechanisms have also been shown to exert a control at the receptor level. The mechanisms involved include changes in affinity, number, internalization, shedding, or recruitment from a latent pool of TNF-α receptor molecules. In normal PBMC, activators of protein kinase A signal transduction pathway enhance TNF-α receptor expression [18], whereas in the same cells activation of protein kinase C leads to opposed effects [19]. Besides these agents, both exogenous (lipopolysaccharides, lectins) and endogenous (cytokines) stimuli contribute to modulate TNF-α binding and activities [20–22]. For example, IFN-γ, a lymphokine produced by T cells, has been shown to increase the total number of TNF-α receptors, especially at the latest stages of cell differentiation in monocyte-macrophage lineage cells [22]. Because mycobacterial components stimulate local generation of IFN-γ [23], this cytokine may participate *in vivo* in an amplification process that results in increased expression of TNF-α monocytes. However, our data indicated that monocyte expression of TNF-α receptors was not directly related to the capacity of PBMC to generate IFN-γ *in vitro*. Thus, further work needs to be done to determine the possible implication of other cytokines (i.e. IL-2) simultaneously generated in the immune response to mycobacterial infection.

Increased occupancy of TNF-α receptors on monocytes from tuberculosis patients

The existence of elevated levels of TNF-α in the culture medium of monocytes from tuberculosis patients [3,4] suggested that TNF-α receptors on these cells could be partly occupied by *in vivo* or *in vitro* generated TNF-α. To test this hypothesis, membrane-associated TNF-α was removed by cell exposure to low-pH glycine buffer, before determining ^{125}I-TNF-α binding (Figs 2, 3). Under these conditions, the increase in total number of ^{125}I-TNF-α binding sites on monocytes, representing receptor occupancy by endogenously generated TNF-α, was directly related to the increase in TNF-α release by corresponding PBMC (Fig. 4). Because pleiotropic activities are induced by TNF-α binding to less than 10% of cell receptors [24], the observed level of occupancy would be sufficient to mediate biologic response to TNF-α. Among the effects of TNF-α on monocytes are its ability to enhance migration [25] and expression of receptors for urokinase [26], and to prevent a sequence of molecular events leading to mononuclear cell death [27]. All together these effects may be responsible for increased invasiveness of the activated monocytes within the lung. Thereafter, TNF-α may locally prime monocytes for enhanced release of platelet-activating factor [28], superoxide anion [29], lysozyme [30] and neutrophil chemotactic factor IL-8 [31]. In turn, IL-8 could be responsible for the neutrophil infiltrates described in severe pulmonary tuberculosis [2]. Because activated alveolar macrophages have been shown to release several-fold more TNF-α than do blood monocytes [32,33] it is conceivable that TNF-α receptor occupancy is similarly increased at the surface of pulmonary macrophages. Neverthe-

less, receptor expression could be regulated differently in circulating monocytes and tissue macrophages. For instance, Brennan *et al.* [34] have demonstrated enhanced expression of TNF-α receptor mRNA and protein in mononuclear cells isolated from rheumatoid arthritis synovial joints, but not in corresponding circulating cells. Thus, further studies will need to measure expression and occupancy of TNF-α receptors in tuberculosis patients, not only on blood monocytes, but also on alveolar macrophages.

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