ORIGINAL PAPER

S. Govender · K. Annamalai · K.P.S. Kumar
U.G. Govender

Spinal tuberculosis in HIV positive and negative patients: immunological response and clinical outcome

Accepted: 21 February 2000

Abstract We measured cytokine secretion patterns in peripheral blood and granulation tissue by flow cytometry in 16 human immunodeficiency virus (HIV) positive and 26 HIV negative patients with spinal tuberculosis. Anti-retroviral therapy was not prescribed. There were no significant differences in the postoperative morbidity and neurological recovery between the two groups.

Résumé Nous avons mesuré, par cytométrie en flux, la sécrétion de cytokine dans le sang périphérique et le tissu de granulation chez des patients atteints de tuberculose rachidienne, 16 HIV positifs et 26 HIV négatifs. Il n'y avait pas de différence significative dans la morbidité post-opératoire et la récupération neurologique entre les deux groupes.

Introduction

Tuberculous spondylitis, which accounts for up to 60% of all bone and joint tuberculosis (TB) typically affects contiguous vertebral bodies and the intervening disc spaces [7]. Human immunodeficiency virus (HIV) dramatically increases the risk of reactivation of a latent focus of infection and progression of the primary infection to a more atypical and disseminated extrapulmonary manifestation [2,5,12]. The development of TB in this group is associated with accelerated rates of decline in the numbers of CD4 lymphocytes [11]. A recent study on spinal TB concluded that good clinical outcomes could be expected irrespective of the HIV status [12].

The immune response in pulmonary TB has been extensively reported [10,14,16,17]. There has been no study to our knowledge on the immune responses in granulation tissue of spinal TB. We compared the clinical outcome and the cytokine secretion patterns of monocytes and lymphocytes in peripheral blood and granulation tissue in the HIV positive and negative patients who underwent surgery for spinal TB.

Patients and methods

The ethics committee of the medical faculty approved a study to evaluate the immunological response and clinical outcome in patients with spinal TB. The study was conducted between January and December 1998 (Table 1). Twenty-five patients presented with neurological deficit and 17 had progressive pain and deformity. All patients received preoperative nutritional support and anti-TB treatment. Anterior spinal decompression [trans-thoracic (33) and retroperitoneal (9)] was performed on all patients. The diseased bodies were excised and the spinal cord decompressed by removing the extradural granulation tissue. The anterior column was supported with an appropriate length of fresh, frozen allograft. Therapeutic antibiotics (first generation cephalosporins) were administered postoperatively for 7 days in all patients. Anti-retroviral therapy was not instituted in the 16 HIV positive patients.

Sample collection and processing

Prior to induction for surgery two tubes with EDTA anticoagulated blood and one tube heparin anticoagulated blood were obtained from each patient. The extradural granulation tissue was harvested and transported immediately to the laboratory on ice. Full blood counts were done in a Coulter STKS (Beckman Coulter, USA) and differential counts were verified manually.

Immunophenotyping of peripheral blood was performed using commercially available monoclonal antibodies conjugated to fluorescein-isothiocyanate (FITC), or phyco-erythrin (PE) fluorescent molecules on a Facs Vantage Flow Cytometer (Becton-Dickinson, USA). The following monoclonal antibodies (Beckman Coulter) were used: anti-CD3, anti-CD4, anti-CD8, anti-CD56, anti-CD19, anti-CD29, anti-CD45RA. Dual marker staining was undertaken to analyze subsets of CD4-positive memory and naive cells and appropriate isotypic control antibodies were used to define negative threshold values. Lymphocytes and monocytes were gated on their forward and side scatter properties and the fidelity of each gate was assessed using CD45/CD14 staining.

S. Govender (✉) · K.P.S. Kumar
Department of Orthopaedic Surgery, Faculty of Medicine,
University of Natal, P.O.Box 7, Congella, Durban 4013,
South Africa
e-mail: thompson@.med.und.ac.za
Tel.: +27-031-2604293, Fax: +27-031-2604518

K. Annamalai · U.G. Govender
Department of Haematology, Faculty of Medicine,
University of Natal, Durban, South Africa

Isolation and in vitro stimulation of mononuclear cells from peripheral blood and granulation tissue

The heparin anticoagulated blood was diluted 1:1 with 0.1% fetal calf serum (FCS)/RPMI, layered onto Ficoll Hypaque (Histopaque, Sigma Chemicals, St. Louis, MO, USA; specific gravity 1.007 g/ml) and centrifuged at 400xg for 40 min at room temperature. The mononuclear cell layer was removed, washed twice with 0.1% FCS/RPMI 1640 (GIBCO-BRL, USA), counted and tested for viability using propidium iodide staining (Sigma) by flow cytometry. 10^6 viable cells per ml complete RPMI (10% FCS, (GIBCO), 10 mmol/l L-glutamine, 10 mmol/l hepes, (GIBCO penicillin/streptomycin 50 U) (Sigma) were cultured for various times (1–12 h) in the presence or absence of 10 ng/ml phorbol-myristate-acetate (PMA) + 1 micromol/l ionomycin (Sigma Chemicals) Brefeldin A 1 mg/ml (Boehringer Mannheim, Germany) was used in cultures to inhibit the secretion of proteins into the culture supernatants, enabling the detection of intracellular cytokines by flow cytometry [13,15]. The granulation tissue was mechanically teased with a scalpel in RPMI-1640 tissue culture medium. The homogenate was aspirated a few times with an 18-gauge needle, layered on a lymphocyte gradient (Histopaque Sigma) and processed as for peripheral blood.

Intracellular cytokine determination by flow cytometry

Cells from the tissue culture (peripheral blood and granulation tissue) were harvested, fixed in 4% paraformaldehyde, permeabilised in 0.1% Triton X100 in phosphate buffered saline (PBS pH 7.2), (Sigma) and washed in 0.1% FCS/PBS. After an initial blocking step with Fc-Block (purified anti-mouse CD16/CD32), (PharMingen San Diego, Calif., USA) the cells were stained with anti-IL2-FITC, γ -interferon FITC, IL4-PE, IL6-PE (Beckman-Coulter), IL12-PE, IL10-PE, TNF α -FITC (PharMingen). Isotypic controls were used to determine negative responses and double positive cells were stained with combinations of the above antibodies. Anti-vimentin antibodies/FITC labelled (Dako, Copenhagen, Denmark), which stain intra-cellular microtubules were used to determine the permeability action efficiency. The cells were incubated in the dark at room temperature and washed in 0.1% FCS/PBS and analyzed by flow cytometry on a FACS Vantage flow cytometer (Becton Dickinson). Ten thousand events were acquired and analyzed in Cell Quest software (Becton Dickinson)

Statistical analysis

The means and standard deviation were calculated and statistically analyzed using the Mann-Whitney U-Test for unpaired non-parametric data. A *P*-value of <0.05 was considered to be statistically significant.

Results

Clinical outcome measures in the HIV positive and HIV negative patients

The patients were followed-up for a minimum of 11.5 months (range 8.5–19 months). There were no significant differences in the postoperative complications in both groups (Table 1). A patient developed recurrent pleural effusion, which resolved with tapping and anti-TB medication and another had superficial wound sepsis, which healed without any sequelae. Death due to keto-acidosis occurred in a non-insulin-dependent diabetic patient at 4 months post-surgery. The neurological recovery improved by two Frankel grades [8] at 6-months' follow-up in the majority of patients.

Comparison of cytokine secretion patterns between HIV positive and negative patients

The HIV positive group had a significantly lower CD4 count ($P<0.05$, Table 2). There were no differences in the cytokine secretion patterns in peripheral blood, except for an increase in IL12 positive monocytes in the HIV negative group ($P<0.05$, Table 3). The mononuclear cells isolated from the granulation tissue did not show a preferential expression pattern for most cytokines measured in the HIV positive and negative patients. However, the expression of IL4 positive lymphocytes was higher in the negative patients ($P<0.05$, Table 4). The cyto-

Table 1 Preoperative evaluations, postoperative complications and neurological recovery

	HIV positive <i>n</i> =16	HIV negative <i>n</i> =26
1. Patient characteristics		
Age (average)	28.5 years	46.3 years
Male:Female	1:7	1:2.6
2. Hematological investigations (average)		
Hemoglobin (g/dl)	10.25	12.1
Platelet count (/ μ l)	314	347
White cell count (/ μ l)	4.9	7.3
Albumin (g/dl)	31.2	32.3
Erythrocyte sedimentation rate (mm/h, Westergren)	71	45
3. Pulmonary TB	4	4
4. Neurological recovery (>2 Frankel grades)	10	23
5. Postoperative complications		
Pulmonary infection	0	1
Wound sepsis	0	1
Death	0	1

Table 2 Peripheral blood lymphocyte subsets in HIV positive and HIV negative patients

Lymphocyte subsets	Markers	HIV positive n=16	HIV negative n=26
T-helpers	CD4% CD4 count**	27.1±12.6 496±253	41.1±9.6* 933±210*
T-suppressors/cytotoxic	CD8% CD8 count	51.5±13.0 813±501	32.0±12.0* 650±330
Ratio	CD4/CD8	0.53	1.3
Total T-cells	CD3% CD3 count	77.4±5.3 1470±463	71.9±9.0 1465±841
Natural killer cells	CD56% CD56 count	12.0±4.7 238±153	14.9±7.6 310±167
B-cells	CD19% CD19 count	10.4±3.7 171±101	13.9±6.0 354±305

* $P<0.05$; **count per microliter blood

Table 3 Intracellular cytokine analysis of peripheral blood, showing percentage positive lymphocyte and monocyte secreting cells

Cells	Cytokines	HIV positive	HIV negative
Lymphocytes	Interleukin 2	1.6±0.9	2.9±2.0
	IL4	2.7±1.2	2.3±1.7
	IL6	5.2±3.4	7.8±4.5
	Interferon- γ	4.4±2.4	4.4±3.8
	IL12	2.0±0.69	3.3±2.4
	Tumor necrosis factor- α	2.9±0.35	2.2±0.8
	IL10	9.6±6.7	14.4±12.2
Monocytes	IL6	14.1±7.6	26.0±21
	Interferon- γ	20.3±9.6	31.6±30.5
	IL12	4.6±1.4	10.8±4.4*
	IL10	23.1±14.8	28.4±24.6

* $P<0.05$

Table 4 Intracellular cytokine analysis of granulomatous tissue, showing percentage positive lymphocyte and monocyte secreting cells

Cells	Cytokines	HIV positive	HIV negative
Lymphocytes	Interleukin 2	0.73±1.1	0.9±0.7
	IL4	2.3±1.2	6.6±2.5*
	IL6	7.8±6.8	7.2±4.9
	Interferon- γ	2.2±1.9	4.1±3.7
	IL12	7.0±4.4	6.5±3.2
	Tumor necrosis factor- α	19.8±16	9.0±8.4
	IL10	13.2±11.2	12.5±9.0
Monocytes	IL4	13.7±6.4	18.2±8.1
	IL6	24.1±14.0	35.8±20.0
	Interferon- γ	46.9±20.4	50.7±13.5
	IL12	10.7±5.8	13.4±4.1
	IL10	37.8±26.7	37.7±26.0

* $P<0.05$

kine secretion patterns showed that both cell-mediated (IL6, IL12) and humoral immune responses (IL4, IL10) were greater in the granulation tissue when compared to peripheral blood in both HIV-positive and HIV-negative patients with spinal TB.

Discussion

The recent resurgence of TB worldwide has several underlying causes, but the HIV infection has undoubtedly been a key co-factor in the current TB epidemic [12]. A survey in 1998 revealed 22.8% of the women attending antenatal clinics of the public health facilities in South Africa were infected with HIV [1,4]. Since TB is endemic, the influence of HIV is of concern, particularly with the emergence of multi-drug-resistant strains. The remarkable susceptibility of patients with AIDS to develop TB has shown the critical role of CD4 lymphocytes in protective immunity [2]. The dually infected individual in most cases is infected with TB before HIV and has 170-fold increased risk of reactivation of TB [5]. In the absence of immunological surveillance by CD4 cells 5–10% of persons with latent foci of TB have the potential to break down and reactivate each year.

In a study on tuberculous osteomyelitis (12 patients) of whom 58% were HIV positive the immunological response was assessed in whole blood by using indirect cytokine proliferation assays [6]. Plasma IL6 levels were significantly elevated in TB as compared to controls. There were no differences in the cytokine levels between bacterial and TB osteomyelitis. The authors used peripheral blood leukocytes in their study and were unable to demonstrate the cell types responsible for cytokine secretion. They recommended measuring cytokines at local areas of infection as opposed to measuring plasma levels since systemic inflammatory markers correlate poorly with clinical and laboratory parameters. The disadvantages of measuring cytokines in plasma either directly or indirectly are that the potential sources of cytokine are unknown and in addition cytokines in plasma are labile with short half-lives.

This is the first study to our knowledge describing the cytokine expression patterns of lymphocytes and monocytes in the granulation tissue of patients with spinal tuberculosis who were HIV positive and negative. The rationale for culturing lymphocytes and monocytes from granulation tissue was to increase the potential of these cells to secrete the cytokines marked

for their initial commitment response (TB) [15]. Significant increases in IL6 and IL12 in lymphocytes and similar increases in IL4, IL6, IL10, IL12 secreting monocytes in granulation tissue were suggestive of a mixed immune response. The secretion of IL6 is important for the pro-inflammatory process, while IL12 is central for true cell-mediated immunity while enhancing the secretion of cytokines (IL4) involved in antibody formation, which is necessary for the clearance of non-proteinaceous TB antigens [14]. Cytokines, which play an important role in granuloma formation, are increased at the site of pathology, due to increased activity of pro-inflammatory and anti-inflammatory cells, which were responsive to the initial insult [3]. However, the increase in pro-inflammatory cytokines (IL6, IL12) may contribute to extensive tissue destruction mediated through tumor necrosis factor- α (TNF- α), as seen in our HIV positive patients. The increase in both TNF- α and IL6 may potentiate HIV infection by increasing viral replication and load, as demonstrated by other studies [9]. Although TNF- α was elevated in some patients, the increase in IL10 may have exerted negative regulation on its secretion especially in the HIV negative patients. It should be noted that all our patients were on pre-operative anti-TB treatment, and the influence of this on the cytokine expression is unknown.

In a retrospective review of 26 patients with spinal TB (7 HIV positive), Liebert et al. found no differences in the clinical outcome in both groups of patients [12]. They reported significant neurological recovery in 1 HIV positive patient who underwent anterior spinal decompression. The use of anti-retroviral therapy is not mentioned in their study and compared to our group the HIV positive group of patients were older (average age 43 versus 28.5 years). We treat approximately 200 new cases of spinal TB per annum and the protocol includes nutritional support, chemotherapy and surgery. No anti-retroviral therapy is available for the HIV positive patients in state hospitals due to economic constraints. Although there were differences in the CD4 counts and cytokine secretion patterns between the two groups, the clinical outcome and complications following surgery were similar despite the lack of anti-retroviral therapy. This may be attributed to adequate pre-operative nutritional support, anti-TB therapy and adequate local cellular immune response.

Acknowledgements This study was supported by grants from the Faculty of Medicine, University of Natal and the Medical Research Council of South Africa.

References

1. AIDS Scan (1999) 11(2):5–17
2. Barnes PF, Bloch AB, Davidson PT, Snider DE Jr (1991) Tuberculosis in patients with human immunodeficiency virus infection. *N Engl J Med* 324:1644–1649
3. Bergeron A, Bonay M, Kambouchner M, Kambouchner M, Lecossier D, Riquet M, Soler P, Hance A, Tazi A (1977) Cytokine patterns in tuberculosis and sarcoid granulomas: Correlations with histopathologic features of the granulomatous response. *J Immunol* 159:3034–3043
4. Department of Health and Population Development (1996) 6th National HIV survey of women attending antenatal clinics of the Public Health Services in the Republic of South Africa. *Epidemiol Comm* 23:3–14
5. Ellner JJ (1997) Review: The immune response in human tuberculosis – implications for tuberculosis control. *J Infect Dis* 176:1351–1359
6. Evans CA, Jellis J, Hughes SP, Remick DG, Friedland JS (1998) Tumor necrosis factor- α , interleukin-6, and interleukin-8 secretion and the acute-phase response in patients with bacterial and tuberculosis osteomyelitis. *J Infect Dis* 177:1582–1587
7. Fam AG, Rubenstein J (1993) Another look at spinal tuberculosis. *J Rheumatol* 20:1731–1740
8. Frankel HL, Hancock DO, Hyslop G, Melzak J, Michaelis LS, Ungar GH, Vernon JD, Walsh JJ (1969) The value of postural reduction in initial management of closed injuries of the spine with paraplegia and tetraplegia. *Paraplegia* 7:179–192
9. Garrait V, Cadranet J, Esvant H, Herry I, Morinet P, Mayaud C, Israel-Biet D (1997) Tuberculosis generates a micro environment enhancing the productive infection of local lymphocytes by HIV. *J Immunol* 159:2824–2830
10. Gerosa F, Nisii C, Righetti S, Micciolo R, Marchesini M, Cazzadori A, Trinchieri G (1999) CD4 (+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients. *Clin Immunol* 92:224–234
11. Laurence J (1993) T-cell subjects in health, infectious disease, and idiopathic CD4+ T lymphocytopenia. *Ann Intern Med* 119:55–62
12. Liebert E, Schluger NW, Bonk S, Rom WN (1996) Spinal tuberculosis in patients with human immunodeficiency virus infection: clinical presentation, therapy and outcome. *Tuber Lung Dis* 77:329–334
13. Openshaw P, Murphy EE, Hosken NA, Maino V, Davis K, Murphy K, O'Garra A (1995) Heterogeneity of intracellular cytokine synthesis at the single-cell level in polarised T helper 1 and T helper 2 populations. *J Exp Med* 182:1357–1367
14. Orme IM, Cooper AM (1999) Cytokine/chemokine cascades in immunity to tuberculosis. *Immunol Today* 20:307–312
15. Prussin C (1996) Cytokine flow cytometry: Assessing cytokine production at the single cell level. *Clin Immun* 16:85–91
16. Somoskovi A, Zissel G, Zipfel PF, Ziegenhagen MW, Klauche J, Haas H, Schlaak M, Müller-Quernheim J (1999) Different cytokine patterns correlate with the extension of disease in pulmonary tuberculosis. *Eur Cytokine Netw* 10:135–142
17. Swaminathan S, Gong J, Zhang M, Samten B, Hanna LE, Narayanan PR, Barnes PF (1999) Cytokine production in children with tuberculosis infection and disease. *Clin Infect Dis* 28:1290–1293