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Production of tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) in patients with AIDS. Enhanced level of TNF-α is related to a higher cytotoxic activity

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SUMMARY

We measured simultaneously circulating and cell-generated TNF-α and IL-1 after lipopolysaccharide (LPS) stimulation of peripheral blood mononuclear cells (PBMC) by radioimmunoassay (RIA) in HIV-infected individuals at different stages of infection, classified according to CDC classification. TNF-α production, both *in vitro* and endogenous in sera, remained at the normal level in group II patients but was significantly increased in most patients in group IV ($P < 0.05$). Most patients of group II and IV displayed normal level of IL-1 in their sera, whereas the level of this monokine generated *in vitro* was significantly reduced in both groups ($P < 0.05$). The cytotoxic effect of factor(s) secreted by PBMC from HIV-infected individuals was evaluated towards a fibroblast cell line L929. The higher titre of cytotoxicity was directly related to a higher production of TNF-α by the cells from group IV patients and the effect could be removed by pre-absorption with anti-TNF-α monoclonal antibody.

Keywords cytokines AIDS radioimmunoassay cytotoxicity treatment

INTRODUCTION

AIDS, caused by the human immunodeficiency virus (HIV), is associated with both quantitative and qualitative defects in the T4 lymphocyte subpopulation (for review see Spickett & Dalgleish, 1988; Fauci, 1988). However, monocytes/macrophages can be infected with HIV (Ho, Rota & Hirsch, 1986; Gartner *et al.*, 1986; Koenig *et al.*, 1986) and a number of monocyte functional abnormalities are reported in patients with AIDS, including defective chemotaxis and killing of certain organisms (Smith *et al.*, 1984; Poli *et al.*, 1985; Nielson, Kharazmi & Faber, 1986).

TNF-α and IL-1 are two important macrophage-derived endogenous mediators of numerous host responses to infection, inflammation, and neoplasia. Development of Kaposi's sarcoma in AIDS patients may occur through TNF-α-induced angiogenic activity (Ruszczak, Mayer de Silva & Orfanos, 1987; Leibovich *et al.*, 1987). Entry of virus into brain through infected monocytes may result in local release of TNF-α which can in turn cause lesions of neurons (Lee, Ho & Gurney, 1987; Gallo, 1987).

These observations, along with the studies indicating the importance of TNF-α in tumour killing and destruction of target cells infected with certain viruses (Lloyd, 1985; Baruy, 1985; Wong *et al.*, 1988) have led us to investigate the production of TNF-α and IL-1 in individuals infected with HIV at different stages of infection. We describe elevated levels of TNF-α in advanced stages of AIDS as detected in the sera and in *in vitro* secretion of peripheral blood mononuclear cells (PBMC) after lipopolysaccharide (LPS) stimulation; and depressed levels of IL-1 in the patients of group II and IV in the face of normal circulating levels. To our knowledge, the simultaneous measurement of circulating and cell-generated cytokines in HIV-infected individuals has not been reported. In addition, we describe an enhanced ability of TNF-α by PBMC from AIDS patients to lyse tumour cells.

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MATERIALS AND METHODS

Subjects

HIV-infected individuals were divided into four groups according to the classification proposed by the CDC (Centers for Disease Control, 1986). Patients of group II are seropositive carriers with asymptomatic infection. Group III includes patients who have palpable lymphnodes of more than 3 months in the absence of a concurrent illness or condition other than HIV infection to explain the findings. Patients of group IV are subdivided into four subgroups: subgroup A, with constitu-

tional disease; subgroup B, with neurologic disease; subgroup C, with one of the 12 infectious diseases in category C.1 and/or with other specified secondary infectious diseases in category C.2; and subgroup D, with one of the cancers (Kaposi's sarcoma, non-Hodgkin's lymphoma) known to be associated with HIV infection.

Blood samples from individuals infected with HIV were obtained from the Department of Infectious Diseases, Tourcoing, France. Sera were also collected from control individuals employed at our Institute, who were negative for HIV. Sera were stored at −20°C.

Cell cultures

Mononuclear cells were separated from heparinized peripheral blood by sedimentation on Lymphoprep™ (Nycomed A, Oslo, Norway). The cells were then cultured in RPMI-1640 medium supplemented with glutamine, antibiotics and 10% heat-inactivated fetal calf serum (FCS). Cultures were set up in duplicates at a concentration of 10^6 cells/ml and stimulated with LPS. The dose of LPS and the duration of incubation for the optimum production of the cytokines (TNF-α and IL-1) were predetermined in preliminary experiments. The maximum production of TNF was obtained after incubation of PBMC with LPS (10 μg/ml) for 18 h. Cell-free supernatants were filtered through a 0·45-μm millipore filter and either used immediately or stored at −20°C. Cell viability at 18 h of culture ranged from 80 to 95% by trypan blue dye.

Target cell cultures

The target used was a murine fibroblast cell line L929 (kindly provided by Dr P. Gosset), which was sensitive to the effects of TNF, but non-permissive for HIV-1 replication and cytopathic effects (Maddon *et al.*, 1986). Monolayer cultures were maintained in the medium supplemented with HEPES 20 mM, 1 mM pyruvate, 2 mM glutamine and 10% FCS. L929 cells 3×10^4 were added in 100 μl of culture medium to each well of a 96-well, flat-bottomed microtest II plate (Falcon, Becton Dickinson, Lincoln Park, NJ), and incubated at 37°C.

Assay for cytotoxicity

Actinomycine D (1 μg/ml, Sigma, St Louis, MO) was added to target cells and incubated for 2 h. Then, 50 μl of supernatant sample was added and a serial three-fold dilution was made. Following 18 h of culture, the dilution of sample that caused 50% destruction of target cells was determined. The anti-TNF-α monoclonal antibody (MoAb), (provided by Médgenix, Brussels, Belgium) was diluted at 1:100. The dilution of supernatant that resulted in 50% destruction was absorbed with antu TNF-α MoAb (vol:vol) by incubation at 37°C for 1 h.

Radioimmunoassay (RIA)

The level of TNF in supernatant samples or in sera of subjects, was determined by using the RIA kit for TNF-α (Médgenix). An aliquot of 100 μl of sample was pre-incubated with 100 μl of anti-TNF-α monospecific polyclonal antiserum in a tube at room temperature for 24 h. Then, 100 μl of ^{125}I-TNF-α (tracer) were dispensed into each tube and incubated for 4 h at room temperature. The complex formed was precipitated by adding anti-rabbit gammaglobulin antiserum plus polyethylene glycol (PEG) to each tube. The tubes were incubated for 20 min at room temperature and were centrifuged. The globulin–PEG bound radioactivity was determined. A standard curve was constructed and the TNF-α concentrations of the samples were determined by close interpolation from this curve.

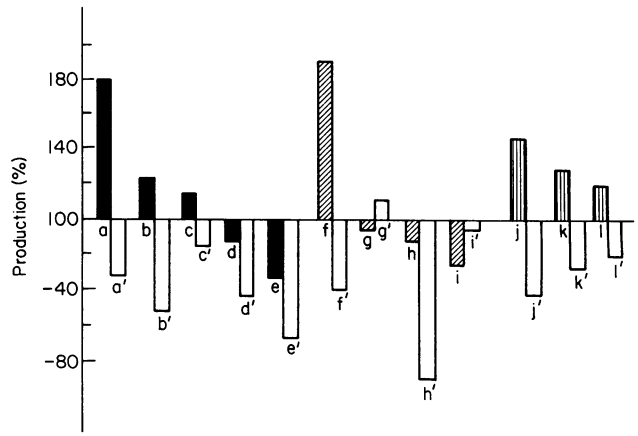

Fig. 1. Inverse relation between levels of tumour necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) generated *in vitro* by peripheral blood mononuclear cells (PMBC) from group IV patients: 10^6 cells were stimulated with lipopolysaccharide (LPS) (10 μg/ml) for 18 h (see Materials and Methods). a–l represent TNF-α levels for each individual and a′–l′ represent IL-1 levels for the same individuals. ■, TNF-α levels in subgroup IVD patients; ■, TNF-α levels in subgroup IVC patients; ■, TNF-α levels in subgroup IVC+IVD patients; and □, IL-1 levels in group IV patients. Results are shown as percentage production (mean control values are equivalent to 100).

The presence of IL-1 in supernatant samples and in sera of patients was assessed by using RIA kit for IL-1 (MÉDGENIX). The assay was developed in the same way as for TNF-α-RIA.

Percentage production of TNF-α or IL-1 was determined by the following formula:

$$\% \text{ production} = \frac{\text{ct/min (RIA) in samples from HIV infected individuals}}{\text{ct/min (RIA) in samples from control individuals}} \times 100$$

Statistical analysis

Experimental results were analysed for their statistical significance by the Student's *t*-test.

RESULTS

Existence of an inverse relation between the in vitro *production of TNF-α and IL-1 in group IV patients*

Supernatants from the PBMC cultures that were stimulated with LPS, were tested for the production of both TNF-α and IL-1. The TNF-α production in nine out of 12 patients of group IV was either increased ($n=6$; $P<0·05$) or remained ($n=3$) at the same level observed in the healthy individuals. In the rest ($n=3$), the TNF-α production was significantly reduced compared with controls ($P<0·05$). Among different subgroups of stage IV, all the patients of subgroup IVC+IVD ($n=3$) displayed an elevated level of TNF-α. In the subgroup of IVD, two patients (designated as b and c), who showed RIA values of 7·13 and 3·53 ng/ml, respectively, were not treated. The mean TNF-α RIA value for normal individuals was 6·04+0·43 ng/ml.

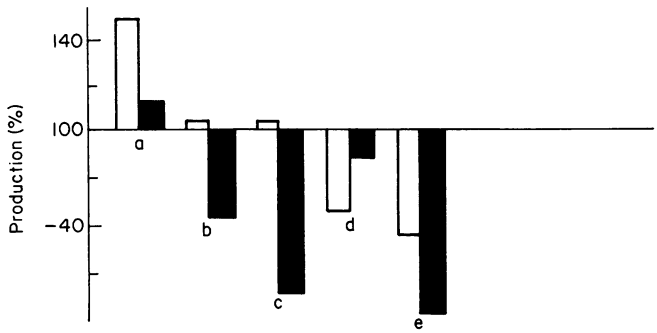

Fig. 2. Production of tumour necrosis factor alpha (TNF-α) and its comparison with that of interleukin-1 (IL-1) by peripheral blood mononuclear cells (PMBC) from each individual of group II. The assay was performed under the same conditions. Blank bars represent TNF-α and solid bars represent IL-1 level for each individual (a–e).

Compared with the level of TNF-α, a lower level of IL-1 was observed in majority ($n=12$) of group IV patients ($P<0.05$). The RIA values for IL-1 in group IV patients and in normal subjects were 4·8+0·81 and 9·07+0·99 ng/ml, respectively. These results are summarized in Fig. 1.

Comparison between levels of in vitro *produced TNF-α and IL-1 in patients of group II*

The level of TNF-α generated *in vitro* in four patients of group II was neither increased nor decreased significantly when compared with that in healthy controls (means ± s.e.m. 5·2 ± 1·2 ng/ml; $P>0.05$). Only one patient of this group displayed a high level of TNF-α (10·5 ng/ml). In contrast, four out of five patients from group II showed significantly reduced *in vitro* production of IL-1 ($P<0.05$). Again, one patient showed a normal production of IL-1. These results are presented in Fig. 2.

Serum levels of TNF-α and IL-1 in subgroups of group IV

The levels of TNF-α in the sera of subgroup IVD patients ranged from 130 to 59 pg/ml and were significantly higher than in controls ($P<0.05$) (Table 1, patients 1–5). Circulating TNF-α level was significantly increased in subgroup IVC compared with healthy controls ($P<0.05$) (Table 1, patients 6–9). Serum TNF-α level ranged from 133 to 59 pg/ml in subgroup IVC+IVD and was significantly higher than in controls ($P<0.05$) (Table 1, patients 10–12). The serum level of TNF-α in six healthy individuals varied from 37 to 65 pg/ml is (mean ± s.e.m. 49 ± 4) (for convenience, the data for healthy subjects are not included in the table). There was no difference in the levels of circulating TNF-α between patients of group IV and healthy controls ($P>0.05$). These results along with information regarding treatment of the patients are shown in Table I.

No increase in circulating level of TNF-α and IL-1 in group II patients

In four out of five patients, the serum TNF-α value ranged from 42 to 76 pg/ml and was not significantly different from that in healthy controls ($P>0.05$). Only one patient in this group showed an increased level of circulating TNF-α (126 pg/ml). Serum TNF-α value for five healthy subjects was 50·8 ± 4·17 pg/ml (mean ± s.e.m.). No significant difference in the level of

Table 1. Serum TNF-α and IL-1 levels in sera of HIV-infected patients in group IV, determined by radioimmunoassay

Patients	Treatment	TNF-α level (pg/ml)	IL-1 level (pg/ml)
Subgroup D			
Patient 1	IFN-α	130	210
Patient 2	—	67	349
Patient 3	AZT, IFN-α	59	246
Patient 4	AZT	82	216
Patient 5	—	75	190
Mean ± s.e.m.		82 ± 12	242 ± 63
Subgroup C			
Patient 6	AZT, pentamidine	60	184
Patient 7	AZT, pentamidine	103	258
Patient 8	AZT, TMX, AP	200	304
Patient 9	TMX	62	314
Mean ± s.e.m.		106 ± 32	265 ± 59
Subgroup C+D			
Patient 10	IFN-α, TMX	59	161
Patient 11	AZT, IFN-α, AP	122	214
Patient 12	IFN-α, TMX, AP	133	222
Mean ± s.e.m.		104 ± 23	199 ± 33

Patients were grouped according to the Centers for Disease Control (CDC) classification, as detailed in Materials and Methods (*Subjects*).

Mean level of TNF-α in controls was 49 ± 4 and of IL-1, 270 ± 3 pg/ml.

IFN, interferon; AZT, azidothymidine; TMX, trimethoprime sulphamethoxazole; AP, acid phosphonoformic.

circulating IL-1 was observed between group II patients and healthy controls ($P>0.05$). These results are presented in Table 2.

Cytotoxic effect of TNF-α secreted by PBMC from patients with AIDS (group IV)

When a correlation was made between the RIA values and the cytotoxicity titres, we observed a clustering of samples obtained from patients of group IV, group II or from healthy, control individuals at the titre of 81. The TNF-α values in the supernatant samples that gave 81 cytotoxic titre ranged from 3 to 8 ng/ml. When the TNF-α content in the supernatant was increased to 10 ng/ml, the titre of cytotoxicity was elevated to 243. This higher titre was obtained with the samples collected mainly from group IV patients. Only one sample from group II patients demonstrated this high titre of cytotoxicity. The supernatant samples were absorbed with anti-TNF-α MoAb (vol:vol). This treatment resulted complete removal of cytotoxic effect (shown in the box, Fig. 3). These results are shown in Fig. 3.

DISCUSSION

The results of our study show that *in vitro* TNF-α production was augmented in a majority of group IV patients, compared with seropositive asymptomatic carriers (group II) or healthy individuals. However, there existed a heterogeneity in the population of group IV, because 25% of the patients studied in this group produced a feeble quantity of TNF-α. There was also

Table 2. *In vitro* production of TNF-α by peripheral blood mononuclear cells from, and serum levels of endogenous TNF-α and IL-1 in HIV-infected patients of group II and their healthy controls

	TNF-α *in vitro* (ng/ml)	TNF-α level in sera (pg/ml)	IL-1 level in sera (pg/ml)
Patient 1	10·59	76	204
Control	7·35	53	300
Patient 2	5·54	126	142
Control	5·04	51	256
Patient 3	5·48	42	200
Control	5·04	41	260
Patient 4	3·66	53	216
Control	5·20	44	313
Patient 5	3·15	58	278
Control	5·30	65	226

HIV-infected patients were grouped according to the Centers for Disease Control (CDC) classification, as detailed in Materials and Methods (*Subjects*).

No significant difference in TNF-α and IL-1 levels between patients and controls ($P > 0{\cdot}05$).

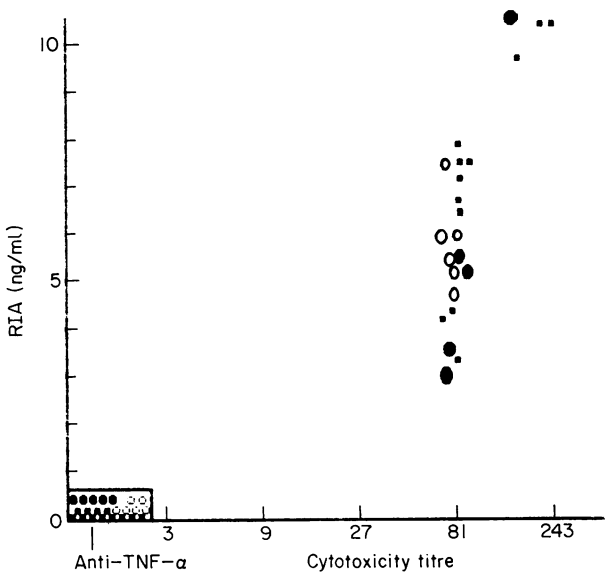

Fig. 3. Correlation between the values from tumour necrosis factor alpha (TNF-α) produced *in vitro* assayed by radioimmunoassay (RIA), and cytotoxicity titres of the supernatant samples. TNF-α values were expressed in ng/ml and the cytotoxicity titre was determined by the dilution of sample that caused 50% destruction of target cells. ■, Group IV (HIV$^+$); ●, Group II (HIV$^+$); and ○, healthy individuals (HIV$^-$). The box indicates contents of TNF-α and cytotoxicity titres after absorption with anti-TNF-α monoclonal antibody.

a patient in group II who displayed an elevated production of TNF-α. We have not been able to explain this heterogeneity, which appears to be independent of any particular clinical characteristic or of concurrent parasitic and other viral infections. The treatment may not have an essential effect on this heterogeneity, since most of the patients of group IV were treated. In one of the non-treated patients (subgroup IVD), the *in vitro* production of TNF-α was quite high and also demonstrable in the serum of the same patient.

In an earlier study, a similar increase in *in vitro* production of TNT-α by patients with AIDS was described (Wright *et al.*, 1988); however, our studies are different in many respects from those described by Wright *et al.*: firstly, we examined TNF-α production in individuals infected with HIV at all stages of infection, whereas these authors had investigated primarily AIDS patients with Kaposi's sarcoma; secondly, we used LPS for stimulation whereas these workers used IFN-γ for activation; and thirdly, we applied whole PBMC, whereas they used highly enriched adherent monocytes exclusively. The reasons why we used whole PBMC were the recent observations implicating both T (Sung *et al.*, 1988a) and B (Sung *et al.* 1988b) lymphocytes in the production of TNF-α. Moreover, the whole PBMC include non-adherent monocytes that may also be involved in the production of TNF-α.

In contrast to our observation, Amman *et al.* (1987) reported a diminished production of both TNF-α and TNF-β in patients with AIDS-related complex (ARC) and AIDS. These authors measured TNF-α by using a MoAb conjugated with peroxidase in ELISA. In our study, we applied RIA based on the competition between a labelled antigen TNF-α (tracer) and an unlabelled antigen (TNF-α) for monospecific polyclonal antibodies. It is possible that the polyclonal antibodies used in our assay recognized several epitopes of the antigen, allowing a more global determination. These authors used both PMA and PHA for stimulation, whereas we used LPS for activation. The differences in the nature of stimulus and the subsequent factors produced by different cell populations may affect the production of TNF-α.

The present study clearly distinguishes from those of others by investigating the serum levels of endogenous TNF-α and IL-1 in the same patients whose cells were used for *in vitro* production of TNF-α and IL-1. It is important to examine whether the augmented *in vitro* generation of these monokines represents *in vivo* production of the same. The results of our experiments clearly demonstrate that the group IV patients who displayed augmented *in vitro* production of TNF-α by their cells had also elevated level of endogenous TNF-α in their sera. In contrast, most of the group IV and group II patients secreted *in vitro* decreased amounts of IL-1, while the levels of endogenous IL-1 in the sera of these patients were essentially the same as in the sera of normal individuals. An increased or normal level of IL-1 produced *in vitro* may be masked by the presence of IL-1 inhibitor (Berman *et al.*, 1987). Earlier reports described both defective (Roy *et al.*, 1988) and essentially normal generation of IL-1 activity (Sei *et al.*, 1986; Murray, Jacobs & Bovbyerg, 1987) in AIDS patients. In these studies, IL-1 activity in supernatants was determined in murine thymocyte proliferation assay with PHA, whereas we used a sensitive immunodetection assay (RIA) to measure IL-1 itself.

We report that a factor(s) was present in the supernatants of PBMC obtained from HIV-infected individuals, which was able

to kill a murine fibroblast cell line (L929). We have identified this cytotoxic factor as TNF-α since absorption with anti TNF-α MoAb could eliminate this cytotoxic effect. Considering the results obtained by RIA for TNF-α and cytotoxicity assay, we conclude that an elevated level of TNF-α resulted a higher titre for cytotoxicity. This result is particularly interesting, since a higher level of TNF-α was reported in those patients who were showing a significant depletion of T4 cells (data not shown). It has not yet been clearly established whether macrophage-derived TNF can participate in the the destruction of HIV-infected T4 cells. We are currently exploring this possibility.

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