

Increased serum neopterin in patients with HIV-1 infection is correlated with reduced *in vitro* interleukin-2 production

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SUMMARY

Recently we have observed that the CD4⁺ T cell response of peripheral blood mononuclear cells (PBMC) to soluble antigens is the first to be lost in the course of HIV-1 infection followed by the loss of response to HLA alloantigens. In this study we compared serum neopterin concentrations of individuals with early stages of HIV-1 infection (stages WR1 and WR2, Walter Reed staging system) with *in vitro* interleukin-2 (IL-2) production of PBMC in response to stimulation with soluble antigens (influenza A virus and tetanus toxoid) and alloantigens. Neopterin concentrations were significantly higher in HIV-1-seropositive individuals who showed deficient IL-2 production in response to recall antigens only or to all of the stimuli tested *in vitro*, compared with HIV-1-seropositive individuals who exhibited no CD4⁺ T cell defects. No difference in serum neopterin concentrations was observed between the group that was functionally deficient to soluble antigens only *versus* those who were unresponsive to both types of stimuli. It appears that the selective loss of the MHC self-restricted CD4⁺ T cell function is associated with an increase in serum neopterin levels. Neopterin concentrations are an estimate of the activation status of macrophages. We conclude that defective *in vitro* production of lymphokines by T lymphocytes is associated with activated macrophages *in vivo*.

Keywords neopterin HIV infection interleukin-2 production *in vitro* macrophage activation *in vivo* cellular immunity

INTRODUCTION

Individuals infected with HIV-1 exhibit several abnormalities of immune function, and advanced stages of HIV-1 infection are associated with profound immune system defects. In particular, cell-mediated immunity is severely reduced, and may be one reason for increased susceptibility of patients to acquire opportunistic infections leading to symptomatic AIDS (Fauci, 1988). Abnormalities of interleukin-2 (IL-2) receptor expression (Prince & John, 1987) and defects in the ability of T lymphocytes to generate cytokines such as interferon-gamma (IFN- γ) and IL-2 upon stimulation *in vitro*, are considered to be of crucial relevance (Margolick & Fauci, 1987). It has been postulated that

defective production of cytokines may predispose individuals to secondary infections (Murray *et al.*, 1985).

In contrast, signs of activated immune cells are apparent in patients with HIV-1 infection, such as increased concentrations of neopterin (Wachter *et al.*, 1983; Fuchs *et al.*, 1988) and soluble IL-2 receptors (Kloster *et al.*, 1987). Recently it was reported that HIV-1-infected individuals also show increased circulating tumour necrosis factor-alpha (TNF- α) (Lähdevirta *et al.*, 1988) and IFN- γ in serum (Fuchs *et al.*, 1989a). These data are consistent with increased spontaneous cytotoxicity and TNF- α production by peripheral blood monocytes of patients (Wright *et al.*, 1988). However, these findings are in contrast to reduced capacity of T lymphocytes to respond to challenge *in vitro*, and raise the possibility that *in vivo*-activated T cells may not respond to subsequent challenge with specific antigens *in vitro* or *in vivo* (Fuchs *et al.*, 1987a).

To investigate this possibility we have compared the *in vitro* response of peripheral blood mononuclear cells (PBMC) to antigenic stimulation and serum neopterin concentrations in patients with established HIV-1 infection.

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MATERIALS AND METHODS

Patients and clinical evaluation

Patients were from the Wilford Hall United States Air Force (USAF) Medical Center, Lackland Air Force Base, Texas, the referral center for all active duty USAF personnel. Individuals were diagnosed as being HIV-1-infected if they had anti-HIV-1 antibodies demonstrated on two samples tested by the HIV-1 enzyme immunoassay and confirmed by Western blot analysis. Patients were classified according to the Walter Reed staging system (Redfield, Wright & Tramont, 1986): 54 patients were Walter Reed (WR) stage 1 (WR1, HIV-1-seropositive, more than 400 T helper cells/mm³), and 35 patients were classified as WR2 (HIV-1-seropositive, chronic lymphadenopathy more than 400 T helper cells/mm³). The patients represent a subgroup of a cohort, whose immunologic profiles were described in more detail elsewhere (Clerici *et al.*, 1989a, 1989b).

Analysis of CD4⁺ T cell subsets

To determine the number of CD4⁺ T cells, PBMC were analysed for surface expression of OKT4 antigen (Ortho reagents, kindly provided by Dr G. Goldstein, Ortho Pharmaceuticals, Raritan, NJ) by indirect immunofluorescence using flow microfluorometry as described (Biddison, Sharrow & Shearer, 1981).

In vitro tests for CD4⁺ T cell function

Whole blood from HIV-1-seropositive individuals was drawn in Vacutainer tubes containing preservative-free heparin (Becton Dickinson, Rutherford, NJ). PBMC were separated on Lymphocyte Separating Medium (Organon Teknika, Durham, NC). The separated PBMC were washed twice in phosphate-buffered saline (PBS), and the number of viable cells was determined by trypan exclusion and haemocytometer. Cells were then resuspended at 3×10^6 /ml in RPMI 1640 (Gibco, Grand Island, NY) containing 0.5% penicillin and 1% glutamine. One millilitre of PBMC was added per well to 24-well flat-bottomed Linbro tissue culture plates (Flow Laboratories, McLean, VA). The PBMC were cultured without stimulation, or were stimulated with (i) influenza A virus (FLU) (A/Bangkok RX73; final dilution was 1/1000 of virus in egg allantoic fluid); (ii) tetanus toxoid (TET) at a final dilution of 40 lf/ml; or (iii) HLA alloantigens (ALLO) by stimulation with a pool of 5000-rad irradiated PBMC from at least two unrelated HIV-1 seronegative donors (2×10^6 /well). Pooled AB⁺ plasma was added to each well (final dilution 1/20). Supernatants of stimulated and unstimulated cultures were harvested 7 days later and frozen at -20°C . The anti-IL-2-receptor antibody, monoclonal anti-TAC (gift from Dr T. A. Waldmann, NCI, Bethesda, MD), was added at the initiation of the cultures (final concentration 10 µg/ml) to adequately prevent IL-2 consumption (Clerici *et al.*, 1989b). The supernatant IL-2 activity was assessed as the ability to stimulate the proliferation of the IL-2-dependent CTL cell line. Results were determined for three replicate wells for five different two-fold supernatant dilutions.

Determination of responsive and unresponsive patients

Proliferation of the IL-2-dependent CTL-cell line was quantified by measurement of ³H-thymidine uptake. Patients were defined as responsive to a given antigen if the mean ct/min of their stimulated cultures was > 3 s.d. above the mean unstimu-

Table 1. Serum neopterin concentrations and CD4⁺ T cell counts in 89 HIV-1-seropositive individuals*

	WR1 (n=54)	WR2 (n=35)	Kruskal-Wallis test
Serum neopterin (nmol/l)			
median	16.0	14.9	<i>H</i> = 0.03
interquartile range	9.1-19.6	10.0-30.1	NS
CD4 ⁺ (ct/mm ³):			
median	678	696	<i>H</i> = 1.47
interquartile range	556-877	525-848	NS

* Classified according to the Walter Reed (WR) staging system (Redfield *et al.*, 1986); see Materials and methods.

NS, not significant.

lated ct/min of HIV-1-seronegative controls. The cut-off value was 7300 ct/min and was determined using 70 HIV-1-seronegative donors (Clerici *et al.*, 1989a).

Neopterin measurements

Serum samples of HIV-1 seropositive patients were kept frozen until measurement of neopterin by radioimmunoassay (Neopterin RIAcid, Henning, Berlin, FRG). Neopterin concentrations were compared with normal levels obtained from 359 healthy HIV-1-seronegative heterosexual blood donors (Werner *et al.*, 1987) and to HIV-1-seronegative homosexual donors (Fuchs *et al.*, 1987b).

Statistical analysis

For comparison of grouped data, the Kruskal-Wallis one-way analysis of variance was applied. For possible correlations we tested by Spearman's rank correlation. Frequencies were compared using the χ^2 test. *P* values < 0.05 were considered significant.

RESULTS

Serum neopterin concentrations were increased in 75 of the 89 HIV-1 seropositives (84.3%) compared with the 95th percentile (8.7 nmol neopterin/l) of adult HIV-1-seronegative blood donors (Werner *et al.*, 1987). Serum neopterin concentrations were also found to be significantly higher (*H* = 47.29, *P* < 0.001) than that found earlier in 51 sera of HIV-1-seronegative homosexual men (Fuchs *et al.*, 1987a).

Patients with WR1 exhibited slightly higher median serum neopterin than those with WR2; however, the differences were not significant (Table 1). By definition, all WR1 and WR2 patients have CD4⁺ T cells above 400/mm³ (Redfield *et al.*, 1986). CD4⁺ T cell counts were not different between the two groups. CD4⁺ T cell counts correlated inversely with neopterin concentrations ($r_s = -0.277$, *P* = 0.0095).

PBMC of patients were tested for IL-2 production in response to FLU, TET, and ALLO. Of the 89 HIV-1 seropositives, 26 responded in the same range as HIV-1-seronegative donors to all three stimuli (group ++, Table 2); 40 failed to respond to FLU and TET but responded to ALLO (group -+), and 23 were unresponsive to all three antigens (group --). From group ++, 15 (57.7%) belonged to WR1. From

Table 2. Incidence of neopterin concentrations and CD4⁺ T cell counts above and below median correlated to *in vitro* response of cells

	<i>In vitro</i> response			Significance
	++	-+	--	
Response to FLU/TET*	12621 ± 1629	2704 ± 262	1463 ± 326	
Response to ALLO	36195 ± 5207	27109 ± 2299	4148 ± 533	
Above median neopterin (16.0 nmol/l)	3/26 (11.5%)	26/40 (65.0%)	15/23 (56.2%)	$\chi^2 = 21.11$ $P < 0.0001$
Below median CD4 ⁺ (680 ct/mm ³)	9/26 (34.6%)	23/40 (57.5%)	13/23 (56.5%)	$\chi^2 = 3.74$ NS

* *In vitro* IL-2 production by PBMC (mean ± s.e.m. ct/min).

† Incidences of levels above (below) median in different groups was compared.

NS, not significant. For groups, see Fig. 1.

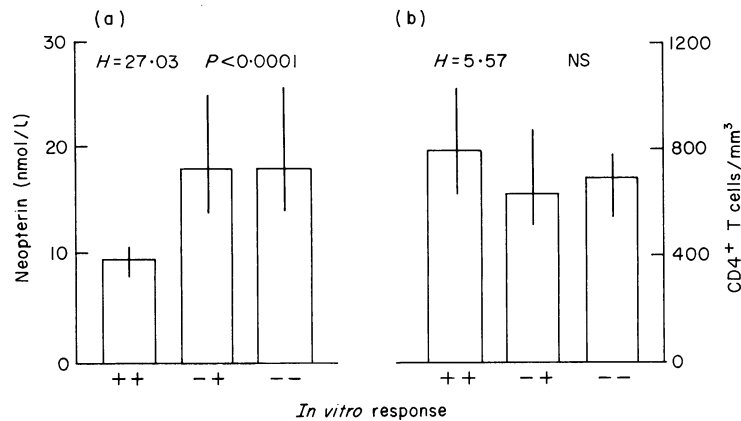


Fig. 1. Serum neopterin concentrations (a) and CD4⁺ T cell counts (b) compared with the *in vitro* response of patients with HIV-1 infection (median and interquartile ranges are shown). ++, positive response to FLU/TET, and ALLO ($n=26$); -+ positive response to ALLO but negative response to FLU/TET ($n=40$); and -- negative response to FLU/TET, and ALLO ($n=23$). NS, not significant (Kruskal-Wallis test).

group -+, 24 (60.0%) were WR1; from group --, 15 (65.2%) were WR1.

Neopterin concentrations were significantly higher in patients who showed deficient response to stimuli used compared with those who responded well (Fig. 1). The increase in neopterin levels was observed in both the -+ and -- group compared with the ++ patients, and the increased neopterin levels were not different between the two groups that were functionally deficient by IL-2 production. CD4⁺ T cell counts were higher in responders, compared with those with deficient response to one or both antigens, but these differences were not significant (Fig. 1). Combining neopterin with CD4⁺ data (ratio neopterin per CD4⁺ T cell numbers; see Fuchs *et al.*, 1989c) did not improve the statistics compared with neopterin alone ($H=25.91$, $P<0.0001$). When comparing neopterin results and CD4⁺ T cell counts at their median levels, significantly lower incidences of levels above medium were observed for neopterin in the group which responded well to both types of stimuli (Table 2). CD4 counts below median were more often observed in those with defective response, but the differences were not significant.

DISCUSSION

In this study, serum neopterin concentrations, CD4⁺ T cell counts, and *in vitro* response of PBMC to antigenic stimulation did not differ between WR1 and WR2 HIV-1-infected individuals. Also, no difference of CD4⁺ T cell counts was observed between patients with intact *in vitro* responses to antigenic and allogeneic stimuli compared with patient who expressed defective *in vitro* response to one or all stimuli. In contrast, neopterin concentrations were found to be significantly higher in patients who exhibited deficient *in vitro* T cell responses.

The association between neopterin levels and *in vitro* response is interesting because only patients with early stage of HIV-1 infection were investigated. It has been recently demonstrated that the T cell response of PBMC from healthy, HIV-1-seropositive donors to FLU and TET is mediated exclusively by an MHC self-restricted CD4⁺ T helper pathway (Clerici *et al.*, 1989a, 1989b). In contrast, the CD4⁺ T cell response to HLA alloantigens can be mediated by three different pathways which include self-restricted, CD4⁺ T cells; ALLO-restricted, CD4⁺

T cells; and ALLO-restricted, CD8⁺ T cells. We have recently observed that the self-restricted, CD4⁺ pathway is selectively deficient in more than 60% of asymptomatic HIV-seropositive patients, and appears to be the first response to be lost in the progression of asymptomatic, HIV-1 seropositive individuals towards AIDS, followed by the loss of the other pathways (Clerici *et al.*, 1989b). It is noteworthy that there was a two-fold increase in the level of serum neopterin in those patients who exhibited a selective loss in self-restricted, CD4⁺ mediated T-cell function; and no additional increase in the sera of patients who exhibited more severe defects in T cell function that involved the other pathways. Thus, it appears that the selective loss of only the MHC self-restricted T cell function in asymptomatic patients is associated with an increase in serum neopterin levels.

Neopterin is produced by human monocytes/macrophages on stimulation with IFN- γ *in vitro* (Huber *et al.*, 1984). Other cytokines such as TNF- γ are able to amplify the effect of IFN- γ on neopterin release by macrophages (Troppmair *et al.*, 1988; Werner-Felmayer *et al.*, 1989). *In vivo*, stimuli that induce cellular immune cascades leading to production of IFN- γ cause neopterin release (Fuchs *et al.*, 1988). In agreement, clinical conditions that are associated with increased cellular immune activation also lead to increased neopterin concentrations in serum and urine of patients (Wachter *et al.*, 1989). Recently, increased circulating IFN- γ was reported in patients with HIV-1 infection and a strong correlation with neopterin concentrations was observed (Fuchs *et al.*, 1989a). Thus, high neopterin concentrations in our HIV-1-seropositive patients indicate increased endogenous production of IFN- γ *in vivo*.

Higher neopterin concentrations were associated with defective response of T cells to soluble antigens. It is possible that cells exposed to these antigens *in vivo* are refractory to further stimulation *in vitro*, as well as to further antigen-specific T cell activation *in vivo*. The inverse relationship of *in vitro* and *in vivo* data is not restricted to HIV-1 infection (Fuchs *et al.*, 1989b). *In vitro* and *in vivo* IFN production was found to be inversely correlated in patients with graft-versus-host disease (Cleveland, Annable & Klimpel, 1988). Similar results have been reported in patients with systemic lupus erythematosus (SLE) (Preble *et al.*, 1983). Furthermore, defective IL-2 production in response to *in vitro* mitogenic stimulation was shown to be improved when cells from SLE patients were rested for 2 or 3 days prior to *in vitro* stimulation (Huang, Miescher & Zubler, 1986). This finding suggests that activation of cells *in vivo* may cause their refractory status. Lymphocyte autoantibodies and alloantibodies (Daniel, Schimpf & Opelz, 1989) as well as anti-class II MHC antibodies (De la Barrera *et al.*, 1987) were detected in HIV-1 seropositives. Eventually, antibodies could interfere with the interaction of self-antigen-presenting cells with T cells. In addition, infection of dendritic cells by HIV-1 may contribute to inappropriate antigen presentation and cause immunosuppression (Macatonia, Patterson & Knight, 1989).

Defective *in vitro* production of cytokines by T lymphocytes indicates that T cells of the patients do not adequately respond to antigenic challenge *in vitro* and in patients. However, cellular immunity may be already activated as measured by, e.g. increased neopterin and circulating IFN- γ . Activated immune cells may play a crucial role in the pathogenesis of AIDS (Ascher & Sheppard, 1988). It has been shown that production of HIV-1 can be induced via antigenic stimulation in infected T lymphocytes (McDougal *et al.*, 1985) and that entry of HIV-1 in CD4⁺

lymphocytes is more efficient in activated T cells (Gowda *et al.*, 1989). With this respect the finding of an association between activated cellular immunity in patients and deficient *in vitro* T cell responses is particularly interesting.

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