Clin. exp. Immunol. (1992) **87**, 287–292

Soluble IL-2 receptor and tumour necrosis factor-α in plasma of haemophilia patients infected with HIV

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(Accepted for publication 25 July 1991)

SUMMARY

We measured plasma concentrations of soluble receptors for IL-2 (sIL-2R) and tumour necrosis factor-α (TNF-α) in 149 haemophilia patients. Soluble IL-2R levels were elevated in 37% of 62 HIV-seronegative patients (mean 570 ± 27 U/ml *versus* 361 ± 17 U/ml in the control group, $P < 0.0001$), in 78% of 68 HIV-seropositive patients (928 ± 49 U/ml, $P < 0.0001$), and in 95% of 19 AIDS/ARC patients (1578 ± 199 U/ml, $P < 0.0001$ compared with controls and with HIV-seronegative patients; $P < 0.005$ compared with HIV-seropositive asymptomatic patients). A negative correlation was observed between sIL-2R, relative and absolute numbers of $CD4^+$ cells ($P < 0.0001$), and CD4/CD8 ratios ($P < 0.0001$). There was also a negative correlation between sIL-2R in plasma and the cellular expression of IL-2R ($P < 0.001$). We found a significant association of sIL-2R and plasma neopterin ($P < 0.0001$). With progression of the disease from HIV-seronegative to seropositive without symptoms and to full manifestation of AIDS/ARC, sIL-2R plasma levels increased. The highest levels were found at the time of diagnosis of AIDS/ARC, but the levels decreased again during the following 18 months. Eight per cent of HIV-seronegative patients, 32% of HIV-seropositive patients, and 24% of patients with AIDS/ARC had increased plasma TNF-α. We conclude that sIL-2R and TNF-α plasma levels are elevated in HIV-infected haemophilia patients and that sIL-2R is a marker for disease progression from asymptomatic HIV-seropositive to AIDS/ARC.

Keywords soluble IL-2 receptors tumour necrosis factor-α haemophilia HIV infection

INTRODUCTION

Cytokines and cytokine receptors play an important role in the regulation of the immune response. IL-2 exerts proliferation and differentiation functions after binding to IL-2 receptors (IL-2R) on the cell surface. Rubin and coworkers [1] demonstrated that IL-2R remain not only on the cell surface, but that a 10 kD smaller protein is released. The soluble IL-2R (sIL-2R) can be measured in blood by means of an ELISA.

Tumour necrosis factor-α (TNF-α) plasma levels were studied to analyse the activation status of monocyte-macrophages. TNF-α levels in haemophilia patients with or without AIDS/ARC are of interest also because TNF-α (also known as cachectin) can inhibit lipoprotein lipase activity and thus may be involved in the pathogenesis of cachexia [2–4].

PATIENTS AND METHODS

Patients and control groups

Serial plasma samples of 149 patients with haemophilia A or B cared for by the Heidelberg Haemophilia Center were analysed.

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They had been treated with lyophilized commercial factor VIII concentrates of intermediate or high purity and were converted to heat-inactivated products in 1984 and 1985. It is impossible to know the exact date when the HIV infection occurred. However, all infections occurred before conversion to heat-inactivated products, *i.e.* before 1985. After 1985 not a single patient turned HIV-seropositive. Blood samples were collected every 2–6 months, plasma was separated, snap-frozen, and stored at −20°C until use. The presence of HIV antibodies was investigated by routine ELISA tests and confirmed by Western blot analysis. At the time of investigation 62 patients were HIV-seronegative (age 27·51 ± 2·09 years, mean ± s.e.m., range 4–62 years) and 87 were HIV-seropositive. Applying the classification of the Center for Disease Control (CDC) [5], 68 HIV-seropositive patients did not have symptoms of AIDS or ARC (age 29·93 ± 1.67, range 3–66 years) whereas 19 patients had AIDS/ARC (age 35·37 ± 3·10 years, range 13–66 years). In the latter group, seven patients were treated with azidothymidine (AZT).

The following parameters were examined in all patients: plasma sIL-2R, plasma TNF-α, cellular expression of IL-2R, peripheral blood T cell subsets (CD4, CD8 and CD4/CD8 ratio) and neopterin plasma concentration. The most recent plasma

samples of HIV-seronegative and asymptomatic seropositive haemophilia patients were chosen for statistical analysis. In the group of HIV-seropositive patients with AIDS/ARC the analysis was based on samples obtained at the time of AIDS/ARC diagnosis. The control group comprised 60 healthy blood donors (age 26·58 ± 1·90 years, range 19–50 years) for the determination of sIL-2R plasma levels. TNF-α levels were analysed in 30 of these healthy donors.

Soluble IL-2 Receptor Assay

Soluble IL-2R in plasma was determined using a sandwich ELISA (T Cell Sciences, Cambridge, MA). The first monoclonal antibody (MoAb) (Tac equivalent) was coated on polystyrene microtitre plate wells. Standards or plasma samples (50 μl) were added to 100 μl of diluent and incubated for 2 h. The wells were washed and incubated again with an anti-IL-2R MoAb (directed against a second epitope) conjugated with horseradish peroxidase. After 2 h, the wells were washed and a substrate solution composed of o-phenylenediamine was applied. The reaction was stopped with 2 N sulfuric acid and absorbance was measured at 490 nm using an ELISA reader (Dynatech Laboratories, Alexandria, VA). A standard curve was prepared from five IL-2R standards and the sample values were determined by means of a computer program (EIA-Microsoft, Dynatech Laboratories, Chantilly, VA), and expressed as units/ml.

TNF-α assay

TNF-α was determined by an immunoradiometric assay (IRE-Medgenix SA, Fleurus, Belgium). According to the manufacturer's instructions, 200 μl of plasma sample of standard were dispensed into tubes coated with moAbs directed against epitopes of TNF-α. [^{125}I]-labelled anti-TNF-α antibody (50 μl) were added and the reaction was incubated for 18 h at room temperature. The content of each tube was aspirated and the tubes were washed with Tween 2%. The radioactivity bound to the tubes was determined in a gamma counter (LKB, Münich, Germany). Two uncoated tubes were incubated only with the tracer in order to estimate the total counts. The standard curve was obtained from seven standards and the concentration values of the samples were determined using a semi-log graph function and expressed in pg/ml.

Other immunologic parameters

CD4, CD8 and CD25 lymphocytes subsets were analysed in the same samples by indirect immunofluorescence and flow cytometry using monoclonal CD4 and CD8 antibodies (Ortho Pharmaceuticals, Raritan, NJ), and CD25 (IL-2R; IL-2 receptor expressing activated T lymphocytes; Becton Dickinson, Sunnyvale, CA). Whole blood (100 μl) was incubated with 10 μl of antibody for 30 min at 4°C. Erythrocytes were lysed with ammonium chloride. After washing with phosphate-buffered saline (PBS) the cells were incubated with 50 μl of goat anti-mouse IgG plus IgM FITC-conjugated antibody (Medac, Hamburg, Germany), diluted 1/20, for another 30 min at 4°C. After two washes, the cells were resuspended in 0·5 ml PBS and analysed using a flow cytometer (Spectrum III, Ortho).

Plasma neopterin levels were determined using a radioimmunoassay (Neopterin-RIA, Henning, Berlin, Germany). Values were expressed in nmol/*l* (control measurements in 60 individuals: 6 ± 5·6 nmol/*l*, mean ± s.d.). The upper limit of normal was considered as mean + 2 s.d. with 95% confidence: 18 nmol/*l*.

Statistical analysis

Data were expressed as mean and standard error of mean (mean ± s.e.m.). The standard deviation (s.d.) was calculated for the determination of the 95% confidence interval in the control group. Patients whose levels were > 2 s.d. above the mean were considered abnormal.

Statistical comparisons were made by the Wilcoxon rank-sum test and the χ^2-test (two-tailed). Correlation coefficients were determined using the Spearman rank test.

RESULTS

sIL-2R in haemophilia patients

The data for sIL-2R in plasma from normal individuals and in haemophilia patients are shown in Fig. 1. HIV-seronegative haemophilia patients showed elevated mean sIL-2R levels (570 ± 27 U/ml; $P < 0{\cdot}0001$ compared with normal individuals 361 ± 17 U/ml). Higher levels of sIL-2R were observed in HIV-seropositive haemophilia patients without AIDS/ARC (928 ± 49 U/ml; $P < 0{\cdot}0001$ compared with normal individuals and with HIV-seronegative haemophilia patients). The highest levels were observed in HIV-seronegative haemophilia patients with AIDS/ARC (1578 ± 199 U/ml; $P < 0{\cdot}005$ compared with HIV-seropositive asymptomatic patients). The range of variation is illustrated in Fig. 1. In order to evaluate the percentage of

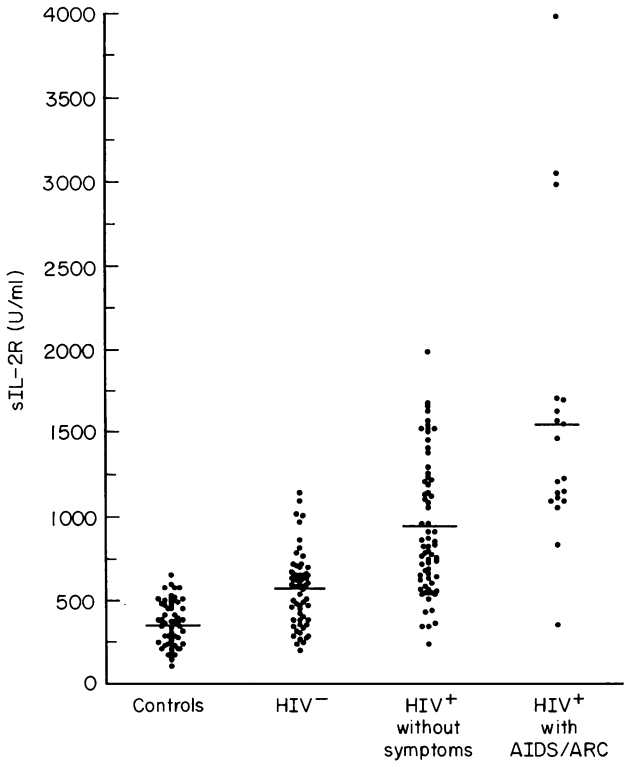

Fig. 1. sIL-2R plasma values in haemophilia patients and healthy controls. Wilcoxon rank-sum-test: Haemophilia patients *versus* healthy controls: $P < 0{\cdot}0001$; HIV-seronegative *versus* HIV-seropositive without symptoms: $P < 0{\cdot}0001$; HIV-seronegative *versus* AIDS/ARC: $P < 0{\cdot}0001$; HIV-seropositive without symptoms *versus* AIDS/ARC: $P < 0{\cdot}005$.

Table 1. T cell subsets, IL-2R cell expression and plasma neopterin in haemophilia patients and in controls

	Controls ($n=60$)	HIV$^-$ ($n=55$)	HIV$^+$ asymptomatic ($n=56$)	HIV$^+$ AIDS/ARC ($n=19$)
CD 4$^+$%	43±1·1	42±1·8	25±1·6*†	11±1·5‡§¶
CD 8$^+$%	27±0·9	31±1·1	46±2·0*†	52±3·2**††
CD4/CD8 ratio	1·69±0·08	1·52±0·09	0·62±0·05*†	0·28±0·03‡§‡‡
Neopterin (nmol/*l*)	6±0·7	8±0·6	18±1·5*†	45±9·4‡§‡‡
CD 25$^+$%	5·1±0·4 ($n=40$)	5·9±0·7 ($n=17$)	2·7±0·4§§¶¶ ($n=24$)	1·6±0·2***††† ($n=10$)

Data expressed as mean±s.e.m.

Wilcoxon rank-sum test: * HIV-seropositive asymptomatic *versus* controls: $P<0·0001$. † HIV-seropositive asymptomatic HIV-seronegative: $P<0·0001$. ‡ HIV+AIDS/ARC *versus* controls: $P<0·0001$. § HIV+AIDS/ARC *versus* HIV-seronegative: $P<0·0001$. ¶HIV+AIDS/ARC *versus* HIV-seropositive asymptomatic: $P<0·0001$. ** HIV+AIDS/ARC *versus* controls: $P<0·001$. †† HIV+AIDS/ARC *versus* HIV-seronegative: $P<0·001$. ‡‡ HIV+AIDS/ARC *versus* HIV-seropositive asymptomatic: $P<0·01$. §§ HIV-seropositive asymptomatic *versus* controls: $P<0·01$. ¶¶ HIV-seropositive asymptomatic *versus* HIV-seronegative: $P<0·01$. *** HIV+AIDS/ARC *versus* controls: $P<0·01$. ††† HIV+AIDS/ARC *versus* HIV-seronegative: $P<0·01$. HIV-seronegative *versus* controls: differences not significant.

patients in each group with increased levels of sIL-2R, 95% confidence intervals were calculated for the control group. The mean sIL-2R value of the controls was 361 U/ml with a standard deviation of 134. Therefore, the mean+2 s.d. was 629 U/ml. By this definition (>mean+2 s.d.), 37% of HIV-seronegative patients (23/62), 74% of HIV-seropositive patients (50/68), and 95% (18/19) of HIV-seropositive patients with AIDS/ARC had abnormally elevated levels of sIL-2R.

Relationship between sIL-2R levels and other immunologic parameters

In accordance with literature reports, HIV-infected patients demonstrated decreased numbers of CD4$^+$ cells and CD4/CD8 ratios, most strikingly in the group of HIV-seropositive patients with AIDS/ARC, and increased percentages of CD8$^+$ cells (Table 1). Significantly increased neopterin levels were found in HIV-seropositive patients with AIDS/ARC (45±9·4 nmol/l; $P<0·001$ compared with controls and HIV-seronegative haemophilia patients; $P<0·01$ compared with HIV-seropositive asymptomatic). Fifty-six asymptomatic HIV-seropositive haemophilia patients exhibited higher neopterin levels (18±1·5 nmol/*l*;) than the 55 HIV-seronegative patients or controls (8±0·6 and 6±0·7 nmol/*l*, respectively; $P<0·0001$).

Comparison of sIL-2R levels with relative CD4$^+$ cells showed a significant negative correlation (coefficient $r=-0·54$; $P<0·0001$; Fig. 2), although significance was not reached within the subgroups ($r=0·015$ in controls, $r=-0·09$ in HIV-seronegative, $r=-0·242$ in HIV-seropositive asymptomatic, and $r=-0·139$ in AIDS/ARC haemophilia patients). When this analysis was performed for sIL-2R and relative CD8$^+$ cells, a significant correlation was found between sIL-2R and CD8$^+$% ($r=0·15$; $P<0·001$). A significant correlation was also observed between plasma sIL-2R levels and the CD4/CD8 ratios ($r=-0·54$; $P<0·0001$), and between sIL-2R and plasma neopterin levels ($r=0·68$; $P<0·0001$).

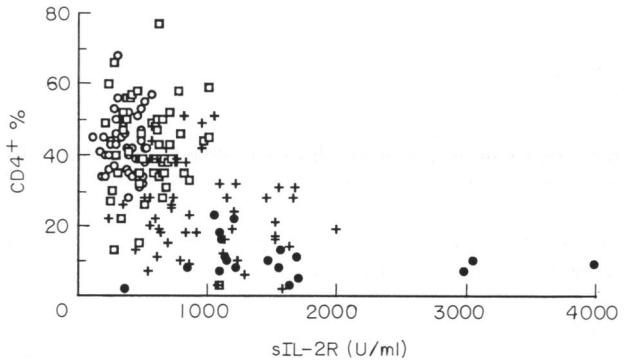

Fig. 2. Correlation between sIL-2R plasma values and relative CD4$^+$ cells. ○, CD4$^+$% controls; □, CD4$^+$% HIV$^-$; +, CD4$^+$% HIV$^+$; ●, CD4$^+$% AIDS/ARC. $r=-0·54$; $P<0·0001$.

Relationship between sIL-2R and cellular expression of IL-2R

Peripheral blood mononuclear cells (PBMC) were assayed by indirect immunofluorescence with the MoAb anti-CD25. As shown in Table 1, percentages of IL-2R$^+$ cells were similar in HIV-seronegative haemophilia patients and healthy controls. The mean expression of IL-2R was 90±15 cells/μl (5·9±0·7% of PBMC) in 17 HIV-seronegative and 131±0·4 cells/μl (5·1±0·4% of PBMC) in 40 healthy individuals. In 24 HIV-seropositive asymptomatic haemophilia patients, however, the expression of IL-2R was significantly lower (53±12 cells/μl, 2·7±0·4%, $n=24$; $P<0·01$ compared with HIV-seronegative patients and controls), and there was a striking decrease in patients with AIDS/ARC symptoms (22±9 cells/μl, 1·6±0·2%, $n=10$; $P<0·01$ compared with HIV-seronegative patients and controls). Interestingly, a negative relationship was observed between cellular expression and the soluble plasma levels of IL-2R as demonstrated in Fig. 3 ($r=-0·38$; $P<0·001$). The differences did not reach statistical significance within the different subgroups ($r=0·309$ in controls, $r=0·092$ in HIV-

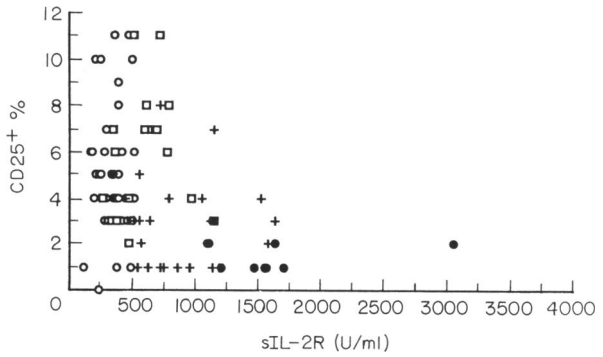

Fig. 3. Correlation between sIL-2R plasma values and cellular expression of IL-2R. ○, CD25$^+$ % controls; □, CD25$^+$% HIV$^-$; +, CD25$^+$% HIV$^+$; ●, CD25$^+$% AIDS. $r = -0{\cdot}38$; $P < 0{\cdot}001$.

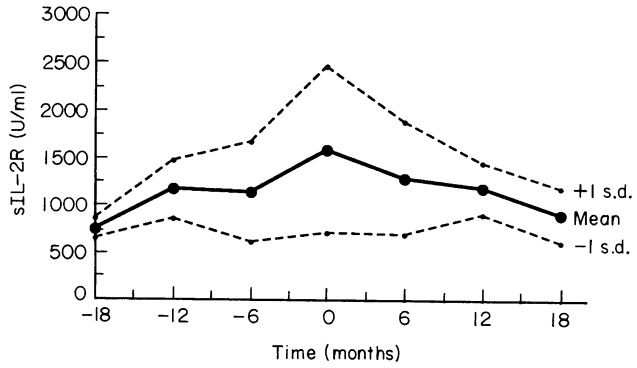

Fig. 4. Sequential analysis of sIL-2R plasma levels in HIV-seropositive haemophilia patients before and after the diagnosis of AIDS/ARC.

seronegative, $r = -0{\cdot}12$ in HIV-seropositive, and $r = -0{\cdot}041$ in AIDS/ARC haemophilia patients).

Sequential analysis of sIL-2R in HIV-infected patients with AIDS/ARC before and after the diagnosis

To investigate whether there is a relationship of sIL-2R plasma levels with progression from the asymptomatic HIV-seropositive state to clinical AIDS/ARC, sIL-2R levels were analysed in the same individuals 6–18 months before, and 6–18 months after the appearance of clinical symptoms. The results are shown in Fig. 4. Eighteen months before the appearance of AIDS/ARC symptoms the sIL-2R levels were similar to those in HIV-seropositive patients who did not develop AIDS/ARC 18 months later. With disease progression the sIL-2R levels increased. The highest levels were found at the time of AIDS/ARC diagnosis. Thereafter, within the next 18 months, the sIL-2R levels progressively decreased.

TNF-α levels in haemophilia patients

In the majority of the measurements TNF-α was undetectable. Figure 5 shows the results of TNF-α levels in the different groups. HIV-seronegative haemophilia patients showed a mean TNF-α level of $2 \pm 1{\cdot}0$ pg/ml, similar to the value in the control group ($2 \pm 0{\cdot}7$ pg/ml). HIV-seropositive patients had higher TNF-α levels (11 ± 2 pg/ml, $n = 66$; $P < 0{\cdot}0001$ compared with HIV-seronegative patients and controls). The highest levels were found in the group of HIV-seropositive haemophilia patients with AIDS/ARC (12 ± 5 pg/ml, $n = 17$; $P < 0{\cdot}001$ compared with HIV-seronegative; not significant compared to

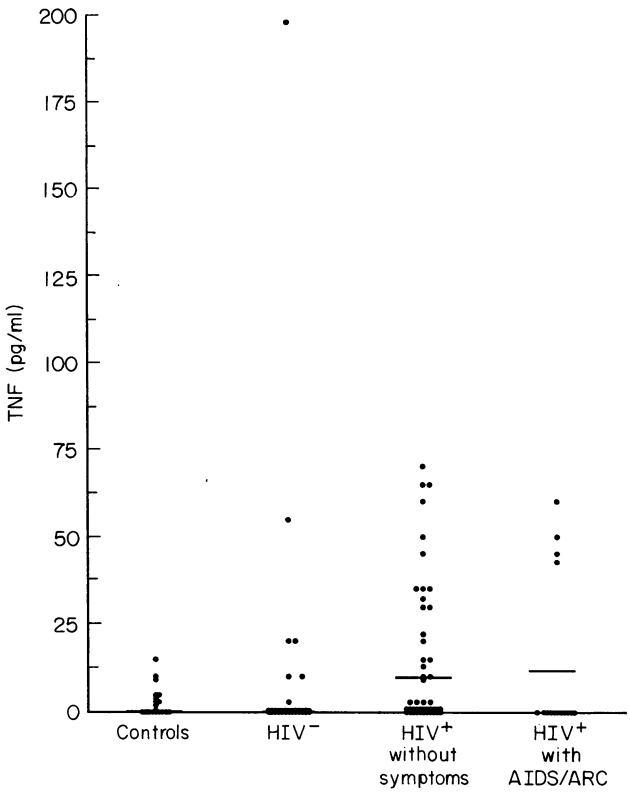

Fig. 5. TNF-α plasma levels in haemophilia patients and in healthy controls. Wilcoxon rank-sum-test: HIV-seronegative *versus* healthy controls: NS; HIV-seronegative *versus* HIV-seropositive asymptomatic: $P < 0{\cdot}0001$; HIV-seronegative *versus* AIDS/ARC: $P < 0{\cdot}001$; HIV-seropositive asymptomatic *versus* healthy controls: $P < 0{\cdot}0001$; HIV-seropositive asymptomatic *versus* AIDS/ARC; NS; AIDS/ARC *versus* healthy controls: $P < 0{\cdot}0001$.

HIV-seronegative asymptomatic). The data are illustrated in scatter plot form in Fig. 5.

The 95% confidence interval for TNF-α was calculated for the control group composed of 30 healthy individuals. The mean TNF-α value was 1·9 pg/ml $\pm$ 3·7 s.d. The mean + 2·045 s.d. was 9·5 pg/ml. By this definition (> mean + 2 s.d.) 8% (5/59) of the HIV-seronegative, 32% (21/66) of the HIV-seropositive, and 24% (4/17) of the AIDS/ARC patients had abnormally elevated TNF-α levels.

DISCUSSION

The results presented here demonstrate that sIL-2R plasma levels are significantly elevated in haemophilia patients infected with HIV, and even higher in patients with AIDS/ARC. Elevated sIL-2R levels in HIV infected patients have been shown previously in other studies [6–8], not only in serum but also in the cerebrospinal fluid of patients with AIDS [9]. We found augmented sIL-2R levels not only in AIDS/ARC, but also in asymptomatic HIV-seropositive patients and in 37% of HIV-seronegative patients. It is not clear why some HIV-seronegative haemophilia patients had higher sIL-2R levels than normal individuals. A possible explanation is that the increased sIL-2R levels result from immunostimulation by previous exposure of these polytransfused patients to other

viruses, or blood products, including factor VIII [10]. Other immunologic abnormalities in HIV-seronegative patients have been described by our group [11–13].

In this study we observed a significant difference between the TNF-α plasma values of HIV-seronegative and HIV-seropositive patients. Fifty-six per cent of HIV-seropositive but only 8% of HIV-seronegative patients presented augmented TNF-α values. It is of not that TNF-α was undetectable in many patients, a finding that is not surprising since it is known that TNF-α can be found only transiently due to its short half-life [14,15]. Our results are different from those reported by Lähdevirta and co-workers [16] who reported elevated TNF-α levels in all nine AIDS patients studied.

Many hypotheses have been advanced to explain the high plasma sIL-2R levels found in haemophilia patients infected with HIV. The possibility that HIV infection could *per se* be responsible for the release of sIL-2R has been refused by some authors, who demonstrated that, at least *in vitro*, T cell lines, B cell lines, and macrophages neither released nor expressed IL-2R after infection with HIV. In contrast, some T cell lines previously infected with other viruses such as HTLV I or II, are able to release sIL-2R after infection with HIV [8].

In patients with HIV infection the number and function of $CD4^+$ lymphocytes as well as the *in vitro* responses to mitogens are severely impaired [17,18]. These abnormalities are associated with the development of opportunistic infections, particularly viral and parasitic. The resulting state of chronic immunostimulation may induce an amplification of the HIV infection [19–21], contributing to the progress of the disease [22]. Other cells, such as T and B lymphocytes and monocytes-macrophages, are subsequently activated and can release sIL-2R. Since B cells are massively activated during HIV infection [23,24], the possibility that they release sIL-2R seems reasonable. Indeed, Sugamura and colleages [25] demonstrated that B cells infected with HTLV express IL-2R.

Macrophages can also be infected by HIV [26–29] and, when activated, express IL-2R [30] and produce TNF-α [4]. It has been demonstrated that monocytes from AIDS patients were able to produce and release high levels of TNF-α into the culture supernatant after stimulation *in vitro* [31–33]. Moreover, TNF-α was shown to increase HIV gene transcription [34,35]. Therefore, TNF-α may induce the reproduction of HIV in infected cells [35]. Whether IL-2R gene expression also is a result of TNF-α induction is still under investigation [36].

In contrast to the high levels of sIL-2R in HIV-seropositive patients is the significant decrease of $IL\text{-}2R^+$ cells. Gupta [37] showed that T cells from patients with AIDS, even when stimulated with phytohaemagglutinin (PHA), had a deficient expression of the Tac antigen when compared with asymptomatic homosexual controls. A possible explanation is that the decrease of IL-2R expression might be due to the decreased number of $CD4^+$ lymphocytes in AIDS/ARC patients, although the sIL-2R plasma levels were elevated. To what extent this negative association might be of pathophysiological importance remains to established [38].

The inverse relationship between sIL-2R and the number of $CD4^+$ cells seems to reflect the progression of the disease. Disease progression is accompanied by a decrease in $CD4^+$ cell counts, a decrease in the number of $IL\text{-}2R^+$ cells, and an increase in plasma sIL-2R. Fahey and coworkers [39] reported that $CD4^+$ cell counts, but not sIL-2R levels, are an important marker for progression to AIDS. We agree that $CD4^+$ cell counts are important; however, our results indicate that there is also a relationship between the rise of sIL-2R and progression to AIDS.

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