



# Anti-CD95 (APO-1/Fas) autoantibodies and T cell depletion in human immunodeficiency virus Type 1 (HIV-1)-infected children

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Received 23.6.97; revised 28.8.97; accepted 24.9.97

Edited by M.L. Gougeon

## Abstract

Advanced stages of HIV-1-infection are characterized by progressive CD4<sup>+</sup> T cell depletion. Peripheral T cells from HIV-1<sup>+</sup> donors show accelerated apoptosis *in vitro*. The CD95 (APO-1/Fas) receptor/ligand system is involved in this process. To further study deregulation of the CD95 system in peripheral T cells during HIV-1-infection, we measured CD95-expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells together with serum levels of soluble CD95 (sCD95) and anti-CD95 autoantibodies in HIV-1<sup>+</sup> children and healthy controls. Anti-CD95 levels in HIV-1<sup>+</sup> children were significantly elevated when compared to uninfected controls, whereas serum levels of sCD95 were not different. In HIV-1<sup>+</sup> children, CD95-expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells increased with age. A strong correlation between depletion of CD4<sup>+</sup> cells *in vivo* and increase in CD95-expression on CD4<sup>+</sup> T cells was observed. In contrast, such a correlation was not found for CD8<sup>+</sup> T cells. A negative correlation between anti-CD95 autoantibody levels and CD4<sup>+</sup> T cell counts, that was predicted by multiple linear regression analysis of pooled data, was found in individual patients observed longitudinally by repeated measurements. Since anti-CD95 autoantibodies isolated from HIV-infected adults have previously been shown to induce apoptosis of sensitive target cells *in vitro*, we speculate that the interaction of these antibodies with CD95-positive and CD95-sensitive T cells *in vivo* might be involved in progressive T cell loss during HIV-1-infection.

**Keywords:** CD95 (APO-1/Fas); soluble CD95; autoantibodies; HIV; children; CD4<sup>+</sup> T cell depletion

**Abbreviations:** AICD, activation-induced cell death; AZT, Zidovudine; BSA, bovine serum albumin; CD95, CD95

receptor; CD95-L, CD95-ligand; sCD95, soluble CD95; HIV-1, human immunodeficiency virus type 1; HIV-1<sup>+</sup>, HIV-1-infected; HRPO, horseradish peroxidase; Ig, immunoglobulin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; TCR, T cell receptor

## Introduction

HIV-1 infection is characterized by progressive depletion of CD4<sup>+</sup> T lymphocytes. The molecular mechanism of T cell depletion *in vivo* is poorly defined. Recent experimental evidence suggests that depletion of CD4<sup>+</sup> T cells is mediated via apoptosis (for review see Gougeon 1995; Oyaizu and Pahwa, 1995). T cells from HIV<sup>+</sup> donors have been shown to undergo increased spontaneous and activation-induced apoptosis *in vitro* (Meyaard *et al*, 1992; Groux *et al*, 1992) and *in vivo* (Finkel *et al*, 1995). Increased susceptibility to activation-induced cell death (AICD) of peripheral blood T cells correlates with disease progression in HIV-infected adults (Gougeon *et al*, 1996).

It has recently been shown that TCR-induced AICD involves autocrine CD95 (APO-1/Fas) receptor/ligand (L)-mediated suicide (Dhein *et al*, 1995; Ju *et al*, 1995; Alderson *et al*, 1995). The CD95 (APO-1/Fas) receptor (CD95) is a member of the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor superfamily and triggers apoptosis in peripheral T cells (Oehm *et al*, 1992; Itoh *et al*, 1991). CD95 is not expressed on the majority of naive resting T cells in cord blood and shows only low expression on resting adult T cells (Klas *et al*, 1993; Miyawaki *et al*, 1992). Expression is increased after activation *in vitro* (Klas *et al*, 1993). Agonistic anti-CD95 antibodies induce apoptosis in activated but not resting peripheral human T cells (Trauth *et al*, 1989; Yonehara *et al*, 1989).

We and others have provided ample experimental evidence for an involvement of the CD95 receptor/ligand system in T cell apoptosis in HIV-1-infected individuals (Debatin *et al*, 1994; Katsikis *et al*, 1995; Estaquier *et al*, 1995; Bäumlner *et al*, 1996). In particular, we observed an increased expression of CD95 and CD95-L together with an increased sensitivity towards CD95-mediated apoptosis in freshly isolated T cells from HIV-1<sup>+</sup> children (Debatin *et al*, 1994; Bäumlner *et al*, 1996). Since CD95-mediated apoptosis can be blocked by a soluble form of CD95 (Cheng *et al*, 1994) and elevated levels of soluble CD95 (sCD95) may block CD95-L function and apoptosis in patients with leukemias (Knipping *et al*, 1995), accelerated apoptosis of T cells in HIV-1 infection could either result from an increase in pro-apoptotic factors such as expression of CD95 and CD95-L (Bäumlner *et al*, 1996) or from a decrease of apoptosis blockers such as sCD95.

In addition to T cell deficiency, HIV-1 infection is characterized by a profound dysregulation of the B cell

system (Fauci, 1988). Especially in HIV-infected children a constant polyclonal B cell activation may lead to B cell expansion and elevated Lg levels (Lane *et al*, 1985; Via and Shearer, 1989). The antibody response is not only directed against viral proteins, such as gp120, gp41 and p24, but also against self antigens such as CD4 and MHC (Golding *et al*, 1988; Via and Shearer, 1989; Muller *et al*, 1992). Antibodies against gp41 have been shown to crossreact with MHC class II antigens and crossreactivity has been explained by a sequence identity of seven aminoacids between gp41 and MHC class II molecules (Blackburn *et al*, 1991). Furthermore, gp120 has homologies to CD4 and MHC class I molecules and autoantibodies against both self proteins have been detected in sera from HIV-1 infected individuals (Zagury *et al*, 1993). Likewise, a sequence identity of eight aminoacids between the V3-loop of gp120 and the extracellular part of the CD95 receptor has been described (Szawlowski *et al*, 1993). Therefore, we reasoned that crossreactive antibodies directed against CD95 are generated during the immune response to HIV-1 and might contribute to disease progression by inducing apoptosis in CD95-positive and -sensitive T cells.

In order to evaluate the role of CD95 and sCD95, as well as anti-CD95 autoantibodies in disease progression in HIV-1-infected children, we correlated CD95-expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells with age and *in vivo* CD4<sup>+</sup> T cell depletion by repeated measurements in a cohort of 44 children infected perinatally with HIV-1 and 44 age-matched healthy controls. In addition, we measured anti-CD95 autoantibodies and sCD95-levels in serum samples from these children. We found that CD95-expression in CD4<sup>+</sup> T cells from HIV-1<sup>+</sup> children increased with age and was strongly enhanced during disease progression (as indicated by decreasing CD4<sup>+</sup> T cell counts). Mean serum levels of sCD95 were similar in HIV-1<sup>+</sup> and HIV-1<sup>-</sup> children. Anti-CD95 autoantibody levels, however, were significantly elevated in sera from HIV-1-infected children and increased with disease progression (i.e., decreasing CD4<sup>+</sup> T cell counts).

## Results

### Validity of the anti-CD95 ELISA

To obtain a rapid and reliable system for detection of anti-CD95 autoantibodies in human sera we established a sensitive ELISA. A chimeric antibody containing the CD95 binding sites of the original murine anti-CD95 antibody (Trauth *et al*, 1989) was fused to a human Fc part and used as standard. Using this humanized anti-CD95 antibody the sensitivity of the ELISA was found to be 50 pg (500 pg/ml=OD<sub>492</sub>=0.25=25 U). The detection limit was not affected by addition of human control sera (data not shown). Due to the dilution used (1:100) the detection limit for anti-CD95 in human sera was 5 ng/well (50 ng/ml). Immunoglobulin (Ig) concentrations up to 50 mg/ml human Ig did not change the ELISA results (data not shown). Control sera of age-matched healthy donors had an average level of 25 U/ml. Intraassay variation was less than 7% and interassay variation was less than 16% (data not shown).

### Serum levels of anti-CD95 autoantibodies are increased in HIV-1-infected children

Using the ELISA for detection of anti-CD95 autoantibodies we analyzed 218 serum samples from 44 HIV-1-infected and 37 serum samples from 16 uninfected controls. Anti-CD95 autoantibody levels in sera of HIV-1-infected children (Median: 662 pg/ml, interquartile range: 474 to 954 pg/ml) were significantly higher than in the control group (Median: 460 pg/ml, interquartile range: 342 to 740 pg/ml,  $P=0.029$ ; Figure 1a). Sera from HIV-1<sup>+</sup> children contained anti-CD95 autoantibodies at concentrations up to 3500 pg/ml. This difference between HIV-1<sup>+</sup> and HIV-1<sup>-</sup> children seen in pooled data remained statistically significant even after controlling for repeated measurements and patient age ( $P=0.047$ ). Longitudinal data-analysis from single patients with different patterns of disease progression showed an increase in anti-CD95 autoantibody levels with decreasing CD4<sup>+</sup> T cell count in the rapid progressor (Figure 1c) and low anti-CD95 autoantibody levels with a stably high CD4<sup>+</sup> T cell count in the long-term-non progressor (Figure 1d).

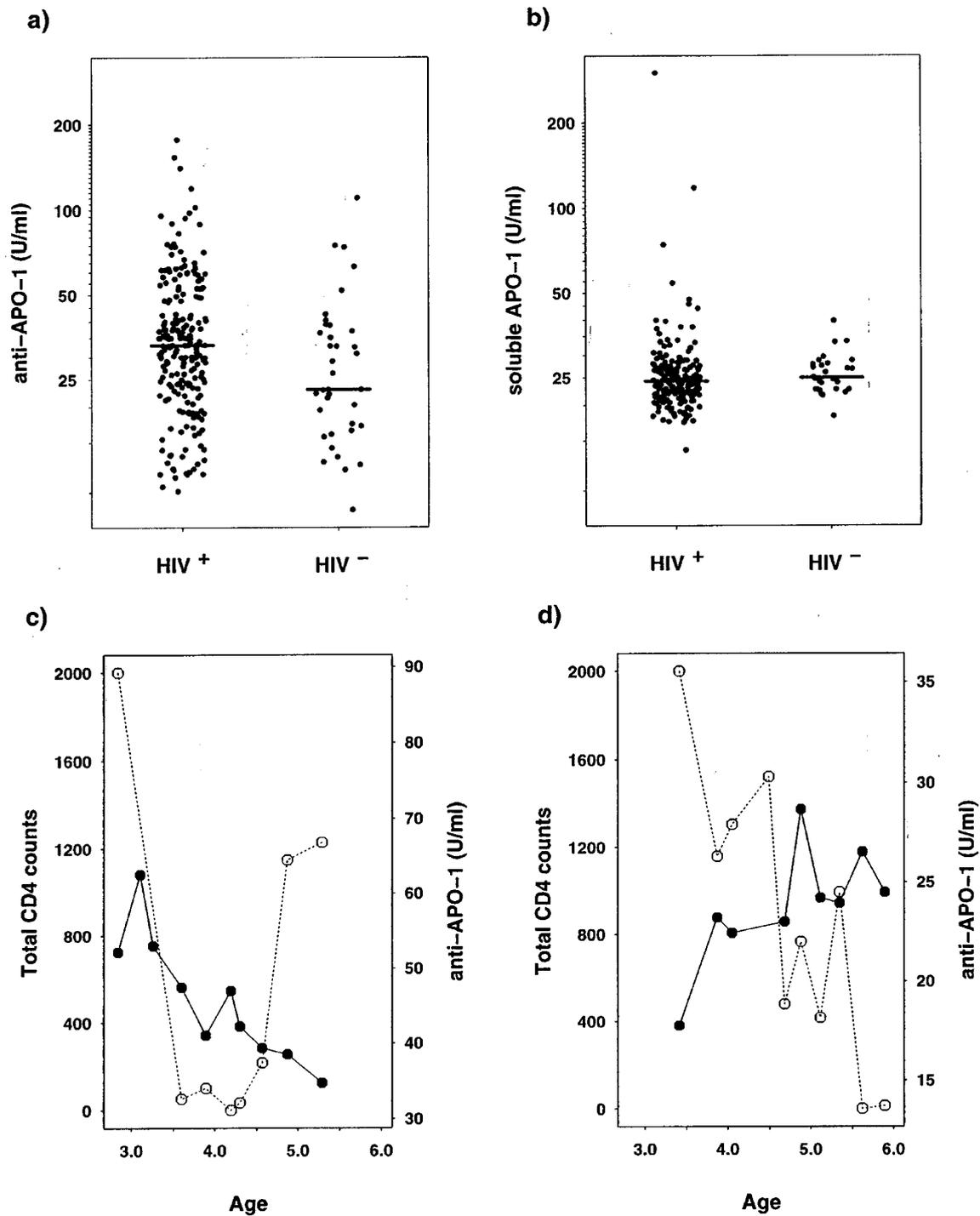
To confirm the specificity of the results obtained by ELISA, purified recombinant CD95 (Knipping *et al*, 1995) was blotted to nitrocellulose membranes and detected by the chimeric humanized anti-CD95 antibody or by anti-CD95 autoantibodies from ELISA-positive serum samples. One representative result is shown in Figure 2. Purified recombinant CD95 was used in all lanes. CD95 incubated with either medium or control serum gave no bands in the immunoblot (lanes 1 and 2). Serum of an HIV-1<sup>+</sup> donor, previously shown to contain anti-CD95 autoantibodies by ELISA, was able to detect also purified recombinant CD95 (lane 3). Humanized anti-CD95 (10 μg/ml) was used as a positive control (lane 4). Samples containing anti-CD95 antibodies (lanes 3 and 4) detected CD95 bands of 48–52 kD in monomeric form and bands of higher molecular weight (multimeric form), reflecting the known tendency of recombinant CD95 to form SDS-stable dimers and multimers (Oehm *et al*, 1992; Knipping *et al*, 1995). Thus, autoantibodies against CD95 recognize different native forms of the molecule.

### Levels of soluble CD95 in HIV-1-infected children are not increased

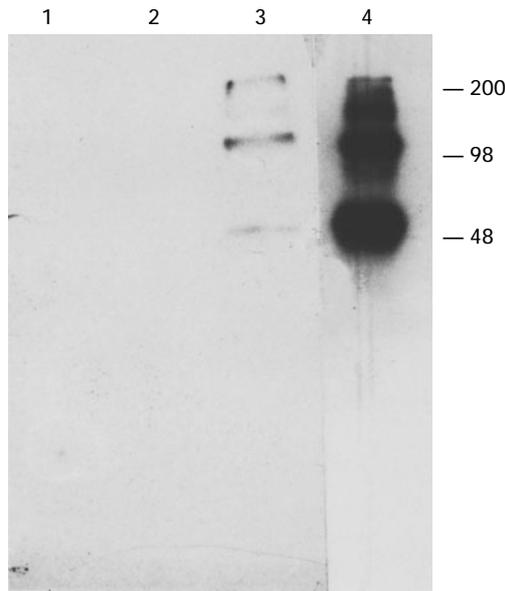
All sera assayed for anti-CD95 autoantibodies were also tested for sCD95. As shown in Figure 1b, serum levels of sCD95 were slightly (but not statistically significantly) lower in the HIV-1<sup>+</sup> group than in the control group.

### Anti-CD95 autoantibody levels do not reflect global B cell activation

Increased levels of anti-CD95 autoantibodies may be a consequence of global polyclonal B cell stimulation. Therefore, we analyzed autoantibody levels in correlation to the total number of B cells and to total serum Ig (IgG+IgA+IgM) in individual patients. No clear correlation between the number of B cells and anti-CD95 autoantibodies was observed (Figure



**Figure 1** Anti-CD95 (APO-1/Fas) autoantibodies and sCD95 (sAPO-1/Fas) in sera from HIV-1-infected children and healthy controls. (a) Anti-CD95 autoantibody levels in patient sera (HIV-1<sup>+</sup>, n=218) compared to healthy controls (HIV-1<sup>-</sup>, n=37). Horizontal bars indicate the median of anti-CD95 concentrations for the respective group. (b) Soluble CD95 levels in patient sera (HIV-1<sup>+</sup>, n=178) compared to healthy controls (HIV-1<sup>-</sup>, n=28). Horizontal bars indicate the median of sCD95 concentrations for the respective group. (c) Repeated measurements of anti-CD95 autoantibodies (○) and absolute CD4<sup>+</sup> T cell counts (●) in a patient with rapid disease progression despite therapy with zidovudine (AZT). A steady increase in anti-CD95 autoantibody levels was seen at CD4<sup>+</sup> T cell counts below 500 cells/ $\mu$ l. (d) Longitudinal data of a long-term non-progressor with slight increase in absolute CD4<sup>+</sup> T cell counts (●) and slightly decreasing serum levels of anti-CD95 autoantibodies (○) following start of antiretroviral treatment with AZT

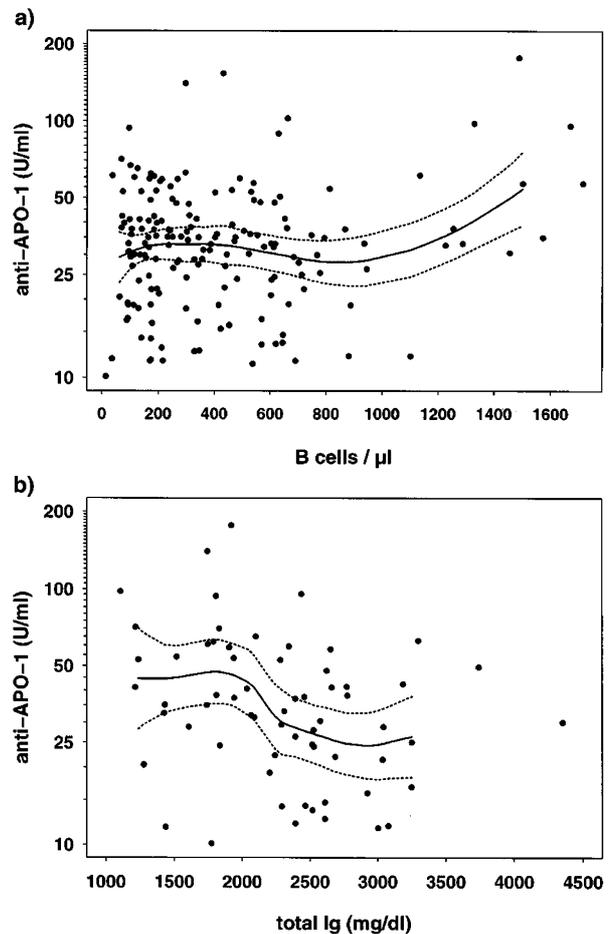


**Figure 2** Western blot analysis of anti-CD95 in sera from HIV-1-infected children. Lanes 1–4: Purified recombinant CD95 (2 µg/lane) was run on a 12% SDS-PAGE under nonreducing conditions. CD95 was transferred onto nitrocellulose membranes by electroblotting. Lane 1 was incubated with culture medium. Lane 2 was incubated with serum from a HIV<sup>-</sup> donor (ELISA negative). In lane 3 recombinant CD95 was detected by anti-CD95 autoantibodies present in a representative serum sample from an HIV-1-infected individual (ELISA positive, 100 U/ml). In lane 4 recombinant CD95 was detected by the humanized anti-CD95 antibody (10 µg/ml). Subsequently, antibodies specific for CD95 were detected with a goat anti-human HRPO-conjugated IgG F(ab')<sub>2</sub>-fragment. The enzyme reaction was performed using the ECL chemoluminescence method

3a), indicating no direct influence of B cell expansion on the level of anti-CD95 autoantibodies. Furthermore, a negative correlation between anti-CD95 autoantibody level and Ig levels was found (Figure 3b). These data indicate that high levels of anti-CD95 autoantibodies are not a direct consequence of global B cell stimulation during HIV-1 infection.

### Correlation of increased CD95-expression with CD4<sup>+</sup> T cell depletion

In children perinatally infected with HIV-1 a progressive depletion of CD4<sup>+</sup> T cells was found with increasing age ( $r = -0.58$ ,  $P < 0.001$ ). Figure 4a shows this linear age-dependent decrease of CD4<sup>+</sup> T cell counts in comparison to the lowest numbers of CD4<sup>+</sup> T cells associated with normal immune function in these patients (dashed line). In contrast, numbers of CD8<sup>+</sup> T cells remained within the normal range (dashed line, Figure 4b). We recently described an upregulation of CD95-expression in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells of HIV-1<sup>+</sup> children (Debatin *et al*, 1994). Detailed analysis of these data revealed an age-dependent increase in CD95-expression in CD4<sup>+</sup> ( $r = 0.47$ ,  $P = 0.003$ ; Figure 4c) and CD8<sup>+</sup> T cells ( $r = 0.31$ ,  $P = 0.049$ ; Figure 4d). When CD95<sup>+</sup> T cells were analyzed with respect to total T cell numbers, a strong correlation was found between depletion of CD4<sup>+</sup> T cells *in vivo* and the increase in CD95-expression ( $r = -0.81$ ,  $P < 0.001$ ; Figure 4e). In contrast, such a

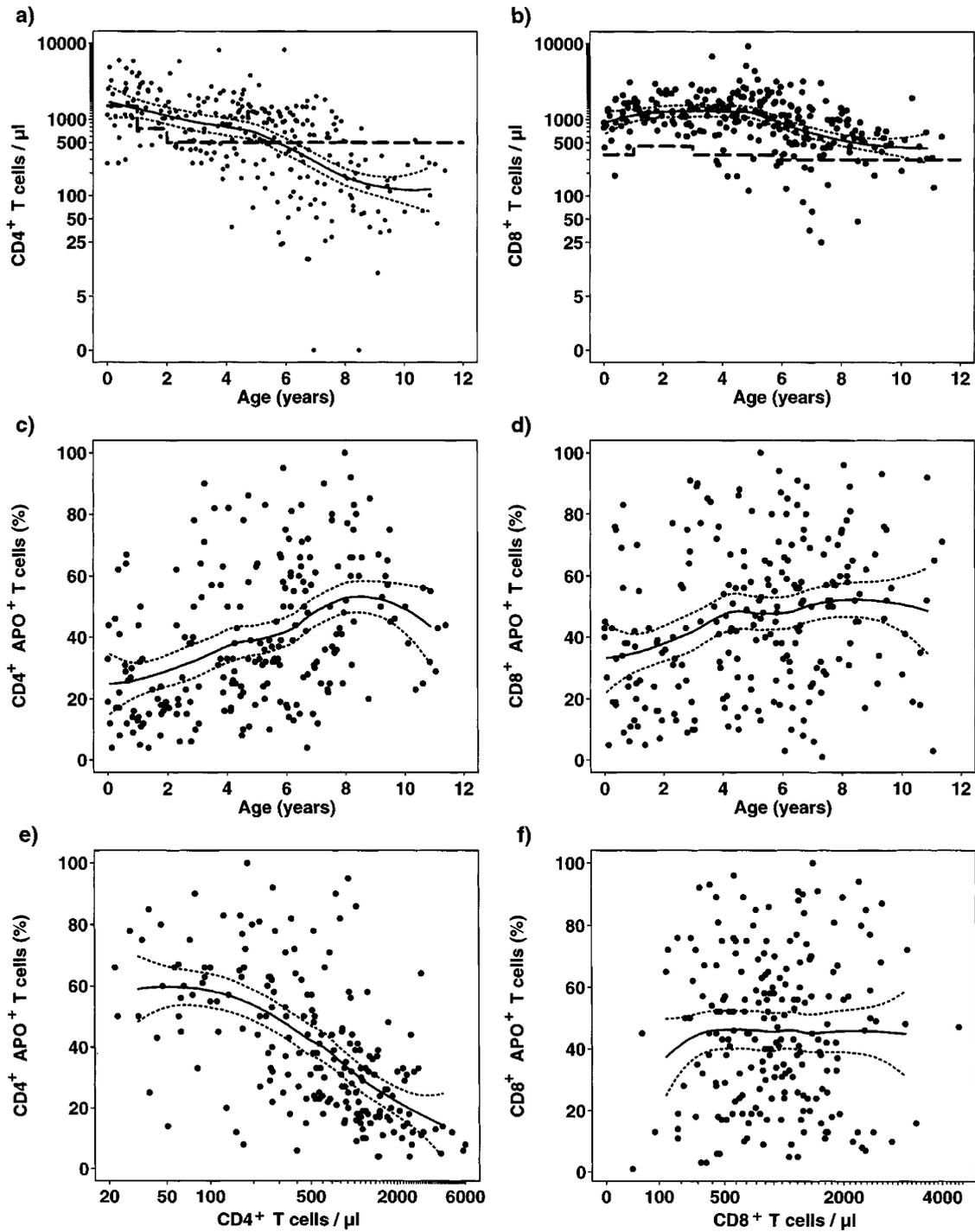


**Figure 3** Univariate analysis of anti-CD95 concentration and total number of B cells or total serum immunoglobulin concentration (IgM, IgG and IgA). Scatterplots of the observed data are superimposed by a smooth curve representing the *loess* fit (solid line) and pointwise 95% confidence intervals (dotted lines). Spearman's rank correlation was additionally computed to measure the strength of the association of anti-CD95 level and either B cell numbers (a)  $r = 0.031$ ;  $P = 0.68$ ) or total serum Ig concentrations (b)  $r = -0.31$ ;  $P = 0.014$ )

correlation was not observed for CD8<sup>+</sup> T cells ( $r = -0.17$ ,  $P = 0.28$ ; Figure 4f).

### Correlation of anti-CD95 autoantibody levels with CD4<sup>+</sup> T cell depletion

Multiple regression analysis was performed to evaluate parameters that may determine the level of anti-CD95 autoantibodies in sera of HIV-1<sup>+</sup> children. We included the age of the patient, total number of lymphocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T cell count, and total number of CD95<sup>+</sup> CD4<sup>+</sup> and CD95<sup>+</sup> CD8<sup>+</sup> T cells. Multiple dependencies between anti-CD95-autoantibody levels and these covariates were analyzed in HIV-1<sup>+</sup> children using generalised linear models for dependent data (Liang and Zeger, 1986) applying a backward elimination procedure. The final model obtained by this approach includes the total number of CD4<sup>+</sup> T cells, the



**Figure 4** Correlation analysis of CD95-expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells with age and total numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The correlation structure between the different variables was explored by depicting the pairwise scatterplots with corresponding *loess* curves (solid lines) and pointwise 95% confidence intervals (dotted lines). In **a** and **b** the data points were compared with the lower limits of absolute CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts associated with normal immune function (dashed lines). Total numbers of CD4<sup>+</sup> decrease with age in HIV-1<sup>+</sup> children (**a**). No such relationship is seen with total numbers of CD8<sup>+</sup> T cells and age (**b**). CD95-expression on CD4<sup>+</sup> (**c**) and CD8<sup>+</sup> T cells (**d**) increases with age in HIV-1<sup>+</sup> children. The percentage of CD95<sup>+</sup> CD4<sup>+</sup> T cells increases with disease progression as indicated by decreasing numbers of total CD4<sup>+</sup> T cells (**e**). No such relationship is observed between the percentage of CD95<sup>+</sup> CD8<sup>+</sup> T cells and total numbers of CD8<sup>+</sup> T cells (**f**)

total number of CD95<sup>+</sup> CD4<sup>+</sup> T cells, chronological age and interaction terms of these covariates and revealed a strong negative correlation between serum levels of anti-CD95 and absolute CD4<sup>+</sup> T cell counts (Figure 5). Thus, the results of multivariate analysis of pooled data correspond to longitudinal observations in individual patients and suggest a role of anti-CD95 autoantibodies in CD4<sup>+</sup> cell depletion.

## Discussion

Apoptosis in CD4<sup>+</sup> T cells is considered to be an important mechanism for CD4<sup>+</sup> T cell depletion during HIV-1-infection (Gougeon, 1995). In normal T cells a tight balance between survival, expansion and apoptosis is necessary to maintain homeostasis and control of immune responses. Regulation of apoptosis in peripheral T cells crucially involves the CD95 cell surface receptor and its ligand (Krammer *et al*, 1994). In previous studies we had observed an increased expression of CD95 and CD95-L together with an increased sensitivity towards anti-CD95- and anti-CD3-induced apoptosis in freshly isolated T cells from HIV-1-infected children (Debatin *et al*, 1994, Bäumlner *et al*, 1996).

These findings were extended in the present study by measuring CD95-expression on T cells together with serum levels of anti-CD95 autoantibodies (as potential inducers of

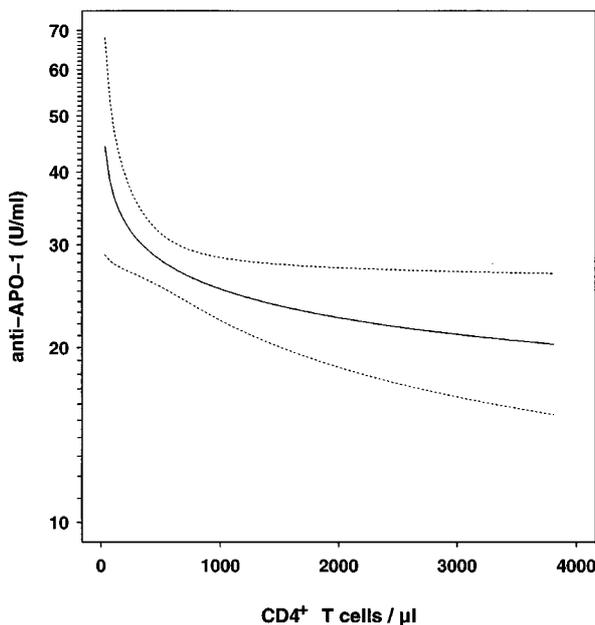
apoptosis) and sCD95 (that may counteract CD95-L function) in relation to disease progression (as indicated by decreasing CD4<sup>+</sup> T cell counts) in these patients. In addition to increased expression of CD95 on the surface of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells we found increased serum levels of anti-CD95 autoantibodies, while serum levels of sCD95 were not elevated in HIV-1-infected children. Recently, increased levels of sCD95 have been found in a cohort of HIV-1-infected adults (Fadeel *et al*, 1997). It remains unclear whether these different findings result from differences in the patient cohorts (adults vs children) or in the methods used (different anti-CD95 antibodies for sCD95-ELISA). However, no correlation of sCD95 levels with clinical disease progression was observed in the adult patients.

In HIV-1<sup>+</sup> children, the increase of CD95-expression strongly correlated with the decline of total CD4<sup>+</sup> T cell counts. Since all of these children had acquired HIV-1 perinatally from their HIV-1<sup>+</sup> mothers, the age of the patients corresponds directly to the duration of infection. Thus, expression of CD95 on the surface of CD4<sup>+</sup> and CD8<sup>+</sup> T cells of these patients increased also with age. In advanced stages of HIV-infection only CD95-positive CD4<sup>+</sup> T cells were found.

In HIV-1 infection expression of CD95 is upregulated in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The mechanisms that lead to an increase in CD95-expression (as well as to susceptibility towards CD95-mediated apoptosis) in peripheral blood T cells from HIV-1<sup>+</sup> patients may involve accelerated activation and 'physiologic aging' of T cells (Plaeger-Marshall *et al*, 1993; Gougeon *et al*, 1996). Additionally, dysregulation of the CD95 receptor/ligand system may be directed towards CD4<sup>+</sup> T cells by immunomodulating HIV-gene products such as Tat and gp120 (Banda *et al*, 1992; Finkel and Banda, 1994; Oyaizu *et al*, 1993; Westendorp *et al*, 1995). Interestingly, we could show in a small group of asymptomatic and oligosymptomatic HIV-1-infected children, that increased CD95-expression in peripheral T cells was due to an increased percentage of CD95-highly positive, CD45RO<sup>+</sup> CD45RA<sup>-</sup> primed/memory CD4<sup>+</sup> T cells, which are also sensitive towards anti-CD95-induced apoptosis *in vitro* (Böhler *et al*, 1997a).

A remarkable finding in our study is the detection of autoantibodies directed against the CD95 cell surface receptor. By multivariate analysis a strong negative correlation between CD4<sup>+</sup> T cell counts and the presence of anti-CD95 autoantibodies was found in particular at CD4<sup>+</sup> T cell counts below 500 cells/ $\mu$ l. This predicted relationship was indeed confirmed by observations in individual patients that were followed longitudinally over a 2–3 year period. In a patient with rapid progressive disease an increase in anti-CD95 autoantibodies was seen together with a decrease in CD4<sup>+</sup> T cell counts.

Development of anti-CD95 autoantibodies may be a consequence of polyclonal B cell activation and global elevation of Ig levels observed predominantly during the early phases of HIV-1 infection (Fauci, 1988). However, we did not find a positive correlation between anti-CD95 autoantibody levels and total Ig levels in serum, total numbers of lymphocytes, B cells or CD8<sup>+</sup> T cells or



**Figure 5** Multiple linear regression analysis for evaluation of parameters that determine serum levels of anti-CD95 autoantibodies in HIV-1-infected children. Statistically significant factors influencing anti-CD95 autoantibody levels were: total CD4<sup>+</sup> T cell counts, total number of CD95<sup>+</sup> CD4<sup>+</sup> T cells, age of the patients and interaction terms between these covariates. Graphical description of the model generated by backward elimination procedures shows the partial effect plot of anti-CD95 autoantibody concentration vs total CD4<sup>+</sup> T cell count (adjusted to the median values of total CD95<sup>+</sup> CD4<sup>+</sup> T cells and age). The solid line represents the predicted values for the anti-CD95 autoantibody concentration, the dotted lines show the pointwise 95% confidence limits of the predicted values. The model describes that anti-CD95 autoantibody levels rise steeply once absolute CD4<sup>+</sup> T cell counts fall below a critical value of 500 cells/ $\mu$ l

CD95<sup>+</sup>CD8<sup>+</sup> T cells, respectively. These findings suggest that the presence of anti-CD95 autoantibodies is not directly related to global activation of the immune system.

Autoantibodies against various cell surface molecules including HLA have been found in sera of most HIV-1 infected individuals. Some of these autoantibodies may reflect crossreactivity between antibodies against viral proteins such as gp120 and cellular proteins. HIV-1 gp120 has been found to bear sequence homology to CD95 and CD4 (Szawlowski *et al*, 1993; Zagury *et al*, 1993). The identical eight amino acid sequence shared by gp120 and CD95 may be responsible for the generation of autoantibodies that react to CD95 on the surface of CD95-expressing T cells. Thus, anti-CD95 autoantibodies present *in vivo* may contribute to CD95-mediated T cell depletion in HIV-1 infection.

Since other autoantibodies also increase during late stages of HIV-1 infection, our observation may simply reflect an epiphenomenon of acquired immunodeficiency due to loss of functional CD4<sup>+</sup> T cells leading to impaired control of specific antibody synthesis. However, autoantibodies directed against CD95 may be of functional importance. In advanced stages of the disease, peripheral T cells express high amounts of CD95 and CD95L and are sensitive towards CD95-mediated apoptosis (Gougeon *et al*, 1996; Bäumlner *et al*, 1996; Böhler *et al*, 1997a, b). Anti-CD95 autoantibodies may interfere with CD95-mediated AICD *in vivo* either by directly inducing apoptosis in sensitive T cells or by blocking CD95/CD95L interactions, thus blocking autocrine or paracrine cell death. Recently, Silvestris *et al* (1996) demonstrated in a cohort of HIV-1-infected adults that anti-CD95 autoantibodies purified from patients' sera were capable of inducing apoptosis in CD95-sensitive CEM lymphoblasts. Thus, anti-CD95 autoantibodies may be functional in HIV-infected individuals and may contribute to increased T cell loss by causing apoptosis of uninfected T cells either via pro-apoptotic signaling through the CD95-receptor or by inducing antibody-dependent cytotoxic T cell responses. Indeed, cytotoxic T cells recognizing the peptide sequence common to CD95 and gp120 have been found in the peripheral blood of HIV-1-infected individuals (Moukrim and Achour, 1995). Thus, an immune response to HIV-1 envelope proteins might directly contribute to T cell depletion involving CD95-mediated apoptosis.

In conclusion, the present study provides further evidence for an involvement of the CD95 system in T cell depletion in HIV-1-infected individuals. First, CD95-expression is strongly enhanced during CD4<sup>+</sup> T cell depletion. Second, anti-CD95 autoantibody levels significantly increase with progressive CD4<sup>+</sup> T cell depletion. In addition, serum levels of sCD95 molecules that may counteract CD95-L function are not elevated. Increased CD95-L production and sensitivity towards CD95-induced apoptosis have previously been demonstrated in freshly isolated peripheral blood T cells from our patients (Bäumlner *et al*, 1996). Thus, in HIV-1 infection the balance between pro-apoptotic and anti-apoptotic mechanisms that critically involve the CD95 receptor/ligand system in T cells are disturbed and shifted towards apoptosis. It remains to be evaluated whether increased anti-CD95 autoantibodies and

other parameters of hyperreactivity of the CD95 system (e.g., expression of CD95/CD95-L in T cells) may serve as a prognostic factor in HIV-1<sup>+</sup> patients.

## Materials and Methods

### Patients

The cohort of 44 HIV-1-infected children and 44 HIV-1-exposed but -uninfected children was part of the 'German Multicenter Study on HIV Infection in Children', supported by the Federal Ministry of Health and approved by the local Ethical Committee. Children between 1 month and 12 years of age born to HIV-1 infected mothers were eligible for the study. Parents and, when appropriate, patients gave informed consent for participation in the study. Blood samples were obtained by venipuncture in conjunction with routine laboratory investigations. Both HIV-1<sup>+</sup> patients and HIV-1<sup>-</sup> controls were followed up at 3 month intervals until 2 years of age and at least yearly thereafter (total number of investigations: 342 in the HIV-1<sup>+</sup> group and 183 in the HIV-1<sup>-</sup> control group). Children considered to have AIDS-related complex were treated with AZT and intravenous IgG. Controls had been repeatedly shown to be HIV-1<sup>-</sup> as defined by negative results of HIV-1 Western blot, HIV-p24 antigen assay, and viral culture. All patients and controls or their relatives gave informed consent prior to venipuncture.

### ELISA for detection of anti-APO-1 autoantibodies in serum

Purified recombinant CD95 (2 µg/ml) in 100 µl NaHCO<sub>3</sub> pH 9.6 was coupled to ELISA plates (Falcon, Heidelberg, Germany) for 12 h at 4°C. Plates were washed three times with 100 µl PBS containing 0.05% Tween-20 (PBS/Tween) and blocked with 2% BSA in PBS/Tween for 30 min at room temperature. After removing the BSA solution human sera were added at a 1 : 100 dilution in a 2.5% non-fat dry milk powder/PBS solution. Serum samples had been centrifuged for 10 min at 17 300 × g. After 1.5 h incubation plates were washed and horseradish-peroxidase (HRPO)- conjugated goat-anti human IgG<sup>+</sup> IgM F(ab)<sub>2</sub> fragment, adsorbed against bovine serum proteins (Dianova, Hamburg, Germany), was added in PBS/Tween for 1.5 h. After another wash cycle staining was developed with 75 µl substrate solution (0.1% orthophenyldiamine in 0.05 M citric acid, 0.1 M Na<sub>2</sub>HCO<sub>3</sub>) for 10 min and the reaction was stopped by addition of 25 µl 3 N H<sub>2</sub>SO<sub>4</sub>. The results were read with an ELISA reader (EAR 400AT LAB Instruments, Grödig, Austria) at 492 nm. For standardisation all assays were controlled with serum from healthy donors and a chimeric anti-CD95 antibody containing the human instead of the murine Fc part. The detection limit for humanized anti-CD95 was 50 pg/well (500 pg/ml). Human sera were used at a dilution of 1 : 100. Thus, the detection limit for anti-CD95 in human sera was 5 ng. ELISA results were confirmed by detection of recombinant CD95 in a Western blot.

### ELISA for detection of soluble CD95 (sCD95) in serum

The ELISA for sCD95 was used as previously described (Knipping *et al*, 1995) using monoclonal anti-CD95 and a rabbit polyclonal antiserum against recombinant CD95.

### Western blotting

Recombinant CD95 (2 µg/ml) in 8 µl SDS-PAGE sample buffer was denatured for 2 min at 95°C. Samples were run on a 12% SDS-

PAGE under nonreducing conditions and transferred to nitrocellulose membranes by electroblotting (1.5 h, 0.8 mA/cm<sup>2</sup>). Subsequently, membranes were saturated with 5% non fat dry milk powder in PBS. After 30 min blocking solution was removed and samples were added for 1.5 h. Membranes were washed with PBS containing 0.05% Tween-20 and subsequently incubated for 1 h with a goat anti-human HRPO-conjugated IgG F(ab')<sub>2</sub>-fragment (Dianova, Hamburg, Germany). Protein bands were developed using the ECL method (Amersham, Frankfurt, Germany) and light emission was detected on Kodak XAR-4 X-ray films (Kodak, Stuttgart, Germany).

### Immunofluorescence studies of CD95-expression on peripheral blood lymphocytes

CD95-expression on isolated peripheral blood lymphocytes was investigated as described (Debatin *et al*, 1994) by staining with FITC-labelled anti-CD95 and PE-labelled anti-CD4 or anti-CD8 (Dianova, Hamburg, Germany) monoclonal antibodies and flow cytometry using a FACScan equipped with the LYSIS II software (Becton Dickinson, Heidelberg, Germany).

### Determination of total serum immunoglobulin

Serum concentrations of IgM, IgG and IgA were determined using standard procedures (nephelometry on Beckman LCS, München, Germany).

### Statistics

For comparison of measurements between control and experimental groups the two-tailed Wilcoxon rank sum test was used. To describe the association between two quantitative variables a smooth curve using the *loess* fitting method (Cleveland and Grosse, 1991) was added to the respective scatterplot. Spearman's rank correlation coefficients (*r*) were calculated in order to measure the strength of linear association (Lehmann, 1975). The statistical computations also include methods for repeated measurements, especially generalised regression models for dependant data (Liang and Zeger, 1986). *P*-values below the 5% level indicate a statistically significant result.

### Acknowledgements

This work was supported by grants from 'HIV-Verbund' Heidelberg, Tumor Center Heidelberg/Mannheim and Project 'Kinderplanet'. We thank Drs S Enenkel-Stoodt, W Kreuz and A Kynast (University Children's Hospital Frankfurt, Germany) for providing patient samples and Mrs Gabi Hölzl-Wenig for expert technical assistance.

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