Clin Exp Immunol 1996; **105**:409–415

Low T cell reactivity to combined CD3 plus CD28 stimulation is predictive for progression to AIDS: correlation with decreased CD28 expression

M. TH. L. ROOS, F. MIEDEMA, A. A. P. MEINESZ, N. A. S. M. DE LEEUW, N. G. PAKKER, J. M. A. LANGE*, R. A. COUTINHO† & P. TH. A. SCHELLEKENS *Department of Clinical Viro-Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, *Department of Internal Medicine, Academic Medical Centre, University of Amsterdam, and †Municipal Health Service, Amsterdam, The Netherlands*

(Accepted for publication 28 May 1996)

SUMMARY

In 219 HIV-1-infected men of the Amsterdam cohort we measured $CD4^+$ T cell numbers and *in vitro* T cell responses to CD3 MoAbs with or without CD28 costimulation and phytohaemagglutinin (PHA). The value of these markers was estimated for disease progression within 4 years. CD28 expression on T cells has been related to T cell responses. CD28 costimulation considerably enhanced T cell reactivity ($\approx$ 8–10-fold) with lower coefficients of variation compared with reactivity to CD3 MoAb alone (median 5 *versus* 20). T cell reactivity to CD3 plus CD28 MoAb was decreased during HIV-1 infection and was besides $CD4^+$ T cell numbers the only independent predictor for progression to AIDS. Compared with the group with high $CD4^+$ T cell numbers the relative risk (RR) for the group with intermediate levels was 2·28, with low levels 5·20. In the groups with intermediate and low CD3 plus CD28 responses the RR was 2·04 and 4·16, respectively. The combined RR for both was 4·65 and 21·63. The independence of this marker was confirmed when the group with low $CD4^+$ T cell numbers was subdivided into groups with high, intermediate and low T cell responses. The expansion of $CD8^+CD28^-$ T cells was already apparent in HIV^- homosexual men, but $CD8^+CD28^+$ T cells specifically decreased in patients with AIDS. CD28 expression on T cells correlated moderately with T cell responses to CD3 plus CD28 MoAb. T cell reactivity to CD3 MoAb in the presence of CD28 MoAb is a stronger prognostic marker than T cell reactivity to CD3 MoAb alone.

Keywords HIV-1 T cell function combined CD3 plus CD28 MoAb prognostic marker CD28 expression

INTRODUCTION

HIV-1 produces profound phenotypic and functional disturbances within the cells of the immune system. Apart from the decline in $CD4^+$ T cells [1,2] and expansion of $CD8^+$ T cells [3–5] it has already long been recognized that early in the asymptomatic stage, when $CD4^+$ T cell numbers are still in the normal range, functional defects of both $CD4^+$ and $CD8^+$ T cells are present in HIV-1-infected individuals [6–9]. Several groups reported that early in HIV-1 infection, proliferation to soluble CD3 MoAbs or pokeweed mitogen (PWM) is impaired, while responses to phytohaemagglutinin (PHA) are unaffected [10–13]. Shearer *et al.* [7] demonstrated a progressive loss of T cell reactivity first in response to recall antigens presented by autologous MHC (self-MHC) molecules followed by decreased responses to allo-antigens and finally to PHA.

Correspondence: Dr P. Th. A. Schellekens, Department of Clinical Viro-Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands.

The interaction of the membrane antigen CD28, present on T cells with the counterstructure B7, on antigen-presenting cells (APC) supplies a costimulatory activation signal for T cell proliferation [14]. CD28 is expressed on almost all normal $CD4^+$ T cells and on most circulating $CD8^+$ T cells [15]. *In vitro* the MoAb CD28, not being mitogenic of its own, strongly facilitates proliferation of T cells stimulated by CD3 MoAb or CD2 MoAb [16,17]. Gruters *et al.* [18] showed that in HIV-1-infected individuals from seroconversion to appearance of clinical symptoms an increase mainly in the number of $CD8^+CD28^-$ T cells accounted for the increase of $CD8^+$ T cells and paralleled the appearance of cells expressing the CD38 marker. The resulting progressive decline in the proportion of $CD8^+CD28^+$ cells during HIV-1 infection has been confirmed in other studies [19–22]. Also the proportion of $CD4^+/CD28^+$ drops but the decline is much less pronounced than the decrease of $CD8^+/CD28^+$ proportion [19–22]. We have reported as well that in the

Amsterdam cohort of HIV-1-infected men low T cell reactivity correlated with progression to AIDS [12,23].

In this study, besides T cell proliferation induced by CD3 MoAb alone, costimulation by CD28 MoAb was also investigated. We demonstrate that costimulation by CD28 enhanced reactivity and resulted in much less variability. Patients with AIDS fail to respond to CD3 plus CD28 MoAb. We questioned whether the diminished reactivity of T cells from HIV-1-infected individuals is due to a lack of T cells expressing CD28. Thus, we analysed the association between CD28 expression on T cells and T cell function to CD3 MoAb in the presence of the costimulatory signal CD28. Furthermore, we investigated whether T cell responses after costimulation with CD28 and expression of CD28 on $CD4^+$ or $CD8^+$ T cells were predictive for progression to disease.

MATERIALS AND METHODS

Study population

The study population consisted of two subpopulations. For the cross-sectional studies HIV^- heterosexual control individuals, HIV^- healthy homosexual control individuals, HIV-1-infected asymptomatic individuals and patients were selected. Fifty-four heterosexual controls were bloodbank donors apparently being in good health. The HIV^- homosexual control population was originally included in the Amsterdam cohort because of being at high risk of HIV infection ($n = 24$) [24]. The HIV-1-infected asymptomatic population ($n = 219$) was derived from the Amsterdam cohort studies as described below. The selected patients with AIDS classified according to the Centres for Disease Control classification 1987 [25] CDC IV-C-1 ($n = 24$) were recruited from the clinic of the Academic Medical Centre (AMC) in Amsterdam.

For the prognostic study of T cell numbers and T cell function as markers for progression to AIDS, HIV-1-infected asymptomatic participants (CDC II and III) ($n = 219$) from the Amsterdam cohort study were included. In this study blood is sampled and cells are stored every 3 months. The starting date of the present study was 1 May 1989. The study group consisted of 132 HIV-1-infected men who entered the cohort study between October 1984 and April 1985, were seropositive at their first visit [24] and were still in active follow up in May 1989, and 87 men who seroconverted after enrolment between October 1984 and May 1989. Within 4 years of follow up, 67 of these 219 persons developed AIDS. The markers were measured on samples stored between May and August 1989.

T cell reactivity

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen. After thawing, 40×10^3 lymphocytes were cultured in round-bottomed plates, in Iscove's modified Dulbecco's medium (IMDM) supplemented with antibiotics and 20% human pool serum (HPS). Cultures were done in triplicate and stimulated with MoAbs to CD3 (CLB T3/4.E, subclass IgE; final dilution ascites $1:10^4$) [26] in the absence or presence of CD28 MoAb (CLB-CD28/1, IgG1; final dilution ascites $1:10^3$) [27] and PHA (Wellcome, Dartford, UK; final concentration 1 μg/ml). Proliferative responses were measured after 4 days of culture by means of incorporation of ^{3}H-thymidine, added 24 h before harvest.

Immunophenotyping

Lymphocyte immunophenotyping on fresh cells was carried out by flow cytometry. To investigate the expression of CD28 on $CD4^+$ and $CD8^+$ T cells, triple staining was used. CD28 MoAb was coupled to PE (Becton Dickinson, San Jose, CA), CD8 and CD4 were coupled to biotin, together with streptavidin-tricolor (Caltag, San Francisco, CA), and CD3 in conjunction with goat anti-mouse (GAM) immunoglobulin to FITC (Becton Dickinson).

Statistical analysis

The coefficient of variation of the triplicates of each measurement was calculated in the various cultures. Box and whisker plots were used for graphical comparison of the distributions of several data groups. The box and whisker plot is defined in terms of percentiles: the whiskers (or tails) mark the 10th and 90th percentile, the box the 25th and 75th percentile, the dots in the boxes represent the median.

To avoid spurious associations between T cell function and outcome, men were subdivided for each marker into three groups with high, intermediate or low values. The threshold between high and intermediate values was defined using a receiver operating characteristics (ROC) curve [28]. By this method

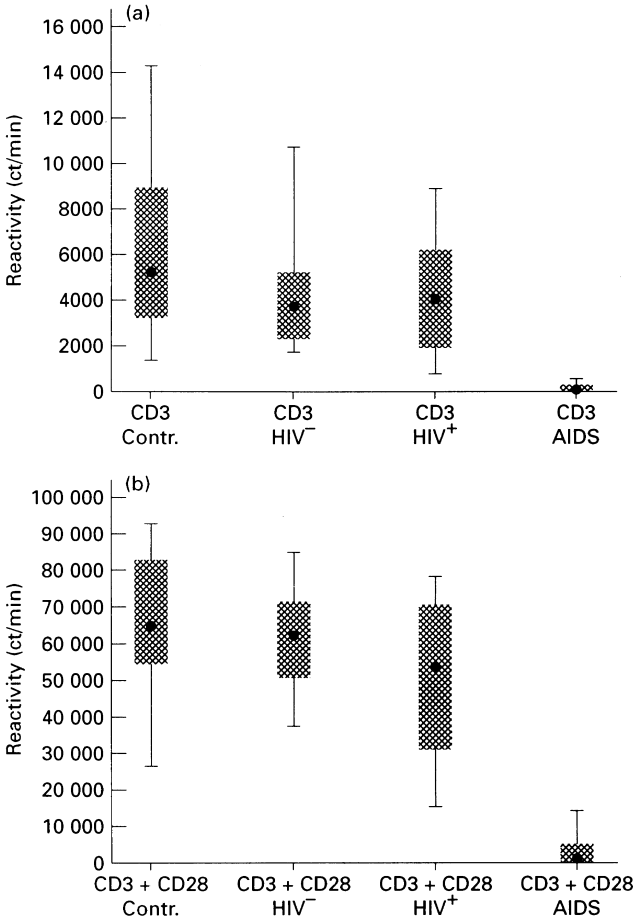

Fig. 1. Lymphocyte reactivity was measured as described in Materials and Methods. Peripheral blood mononuclear cells (PBMC) from HIV^- healthy heterosexual individuals (Contr.), HIV^- healthy homosexual individuals (HIV^-), HIV-1-infected asymptomatic homosexual individuals (HIV^+) and AIDS patients (AIDS) were stimulated with CD3 MoAb alone (a), and with CD3 plus CD28 MoAb (b).

Table 1. Relative risk for AIDS in a Cox proportional hazards analysis. Men subdivided into three groups according to levels of parameters measured at entry; 4 years follow up

	Total AIDS		Univariate		Multivariate	
Parameters	*n**	*n*	RR†	CI‡	RR	CI
$CD4^+$ T cells $10^9/l$						
> 0·53	112	20	1·00		1·00	
0·25–0·53	85	32	2·57	2·17–3·06	2·28	1·90–2·73
< 0.25	22	15	6·62	4·69–9·35	5·20	3·62–7·46
Responses to CD3 plus CD28 MoAb, ct/min						
> 52 800	109	18	1·00		1·00	
15 100–52 800	88	35	2·41	2·02–2·87	2·04	1·71–2·43
< 15 100	22	14	5·81	4·10–8·24	4·16	2·92–5·92
Responses to CD3 MoAb, ct/min						
> 3300	132	33	1·00		1·00	
800–3300	65	21	1·65	1·40–1·94	NS§	
< 800	22	13	2·71	1·95–3·78	NS	
Responses to PHA, ct/min						
> 5400	134	30	1·00		1·00	
1800–5400	65	24	1·99	1·69–2·35	NS	
< 1800	20	13	3·97	2·85–5·54	NS	

* Numbers.
† Relative risk.
‡ 95% confidence interval.
§ Not significant.
For all values $P < 0·01$.

optimal cutoff points for T cell reactivity and for $CD4^+$ T cell numbers were based on the sensitivity and specificity in predicting the development of AIDS. The analysis of the curve permits the choice of a value that maximizes sensitivity conditionally to specificity or *vice versa*. $CD4^+$ T cell count of $< 0·53 \times 10^9/l$, T cell responses to CD3 MoAb of < 3300 ct/min, to CD3 plus CD28 MoAb of < 52 800 ct/min and to PHA of < 5400 ct/min discriminated optimally. The threshold between intermediate and low values was defined by determination of the positive predictive value of 70%, i.e. the proportion of individuals with a positive test which progressed to disease [29]. This strategy implied the following threshold for the different parameters: $< 0·25 \times 10^9/l$ for $CD4^+$ T cell count, < 800 ct/min for T cell responses to CD3 MoAb, < 15 100 ct/min for T cell responses to CD3 plus CD28 MoAb, and < 1800 ct/min for responses to PHA.

The proportional hazards model (Cox model) was used to calculate the significant contribution ($P < 0·05$) of the above mentioned parameters for development of AIDS in uni- and multivariate stepwise analyses. Disease-free survival curves were composed by using the Kaplan–Meier method. Significant differences ($P < 0·05$) were tested using the statistics of the generalization of Wilcoxon's two-sample rank sum test by Peto & Peto [30].

RESULTS

T cell reactivity in HIV-1 infection

Studies were performed to compare T cell responses to CD3 MoAb in the absence or presence of CD28 MoAb in HIV^- healthy heterosexual and healthy homosexual controls, HIV-1-infected asymptomatic individuals and patients with AIDS. Figure 1 illustrates that the responses to CD3 plus CD28 MoAb were considerably enhanced compared with CD3 MoAb alone ($\approx$ 8–10-fold) in other than patients with AIDS, while the responses in patients with AIDS remained very low. Responses to CD28 MoAb alone demonstrated levels just above background (median for all groups 176 and 138 ct/min, respectively). Figure 2 shows that the decrease in reactivity towards CD3 plus CD28 MoAb was only weakly correlated with the decline of $CD4^+$ T cells.

Furthermore, the coefficients of variation of the responses to CD3 MoAb (median 18, 10th and 90th percentile: 7 and 39) were much smaller after CD3 plus CD28 stimulation (median 5, 10th and 90th percentile: 2 and 15).

T cell reactivity as prognostic marker for development of disease

Uni- and multivariate proportional hazards analyses (Cox) were used to determine the predictive value for progression to AIDS of reactivity induced by CD3 MoAb, CD3 plus CD28 MoAb and PHA compared with $CD4^+$ T cell numbers. All markers were predictors of progression to AIDS in a univariate analysis (Table 1). The number of $CD4^+$ T cells and responses to CD3 plus CD28 MoAb were stronger predictors than responses to CD3 MoAb and to PHA. After subdivision into three groups a trend of increased risk was observed when groups with intermediate and low values were compared with groups with high values. A multivariate stepwise analysis indicated that besides intermediate and low $CD4^+$ T cell numbers, only intermediate and low T cell reactivity to CD3 plus CD28 MoAb were grossly independent of each other, resulting in the group with the highest risk, i.e. very low $CD4^+$ T cell numbers and very low reactivity, of a relative risk of 21·63

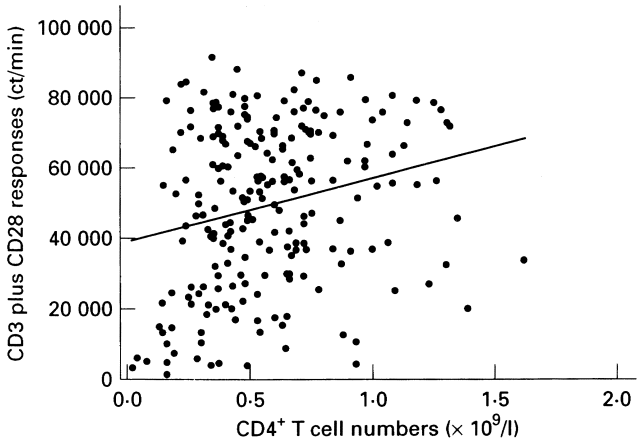

Fig. 2. Regression analysis of $CD4^+$ T cell numbers compared with CD3 plus CD28 responses. P ($\beta <> 0$) $< 0{\cdot}001$, $r^2 = 0{\cdot}05$.

(95% CI 10·57–44·16). In this analysis the responses to CD3 MoAb and PHA did not contribute any more to progression to clinical disease.

To underscore the independence of the predictive value of these markers the attack rate of AIDS was determined by the product-limit method of Kaplan–Meier. Individuals with low $CD4^+$ T cell numbers were subdivided into groups with either high, intermediate or low responses to CD3 plus CD28 MoAb. Also individuals with low responses to CD3 plus CD28 MoAb were subdivided into groups with high, intermediate or low $CD4^+$ T cell numbers. Figure 3a shows that although subgroups were rather small, individuals with low $CD4^+$ T cell numbers and high responses induced by CD3 plus CD28 MoAb were relatively protected from progression to AIDS. Indeed only $\pm 30\%$ developed AIDS ($P < 0{\cdot}05$). In contrast, in persons with low $CD4^+$ T cell numbers 80% and 90% of individuals with intermediate and low responses to CD3 plus CD28 MoAb, respectively, progressed to disease within 4 years. The same holds when in the group with low responses to CD3 plus CD28 MoAb, individuals were subdivided into groups with high, intermediate and low $CD4^+$ T cell numbers. As shown in Fig. 3b the progression rate for these subgroups was 40%, 43% and 90%, respectively.

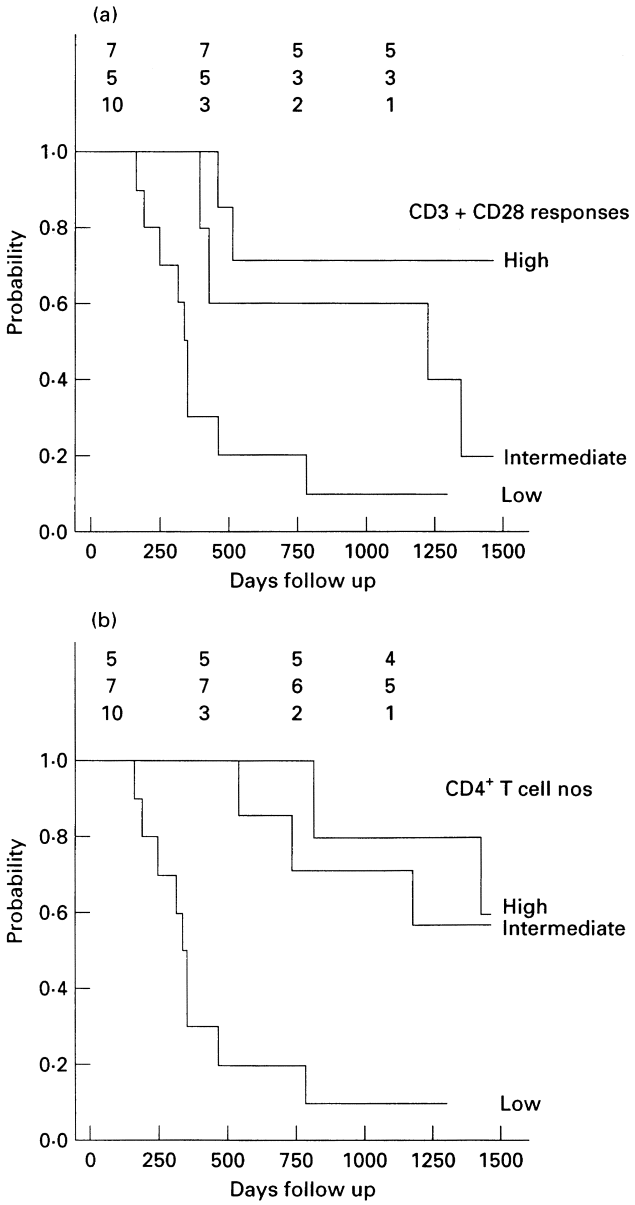

Fig. 3. Kaplan–Meier plot of cumulative progression to AIDS. Subjects were stratified according to the parameters: low $CD4^+$ T cell numbers subdivided into groups with high, intermediate and low CD3 plus CD28 responses (a) and low CD3 plus CD28 responses subdivided into groups with high, intermediate and low $CD4^+$ T cell numbers (b). Number of men at risk at each time point is indicated.

Expansion of $CD8^+/CD28^-$ and decrease of $CD8^+CD28^+$ T cells

Qualitative and quantitative expression of CD28 has been studied on both $CD4^+$ and $CD8^+$ T cells in HIV^- healthy heterosexual and homosexual controls, HIV-1-infected asymptomatic individuals and patients with AIDS.

In order to assess qualitative differences in the different study groups we measured the mean fluorescence intensity (MFI) and we also used quantitative FITC and PE microbeads as a control. The CD28 MoAb neither showed bimodal reactivity with a dim and bright positive peak nor showed an even more complex staining. We did not find significant differences in the MFI in the different study groups, which is in agreement with results obtained in the Leucocyte Typing V Workshop 1993 [19]. In healthy controls, HIV^- homosexual controls, HIV-1-infected asymptomatic individuals and patients with AIDS the median of the MFI was 114, 120, 114 and 111 in the $CD4^+CD28^+$ population and 127, 133, 127 and 126 in the $CD8^+CD28^+$ population. The distribution of $CD4^+CD28^+$ T cells within the study groups is shown in Fig. 4. Compared with healthy heterosexual controls the proportion of CD28, expressed as percentage of $CD4^+$ T cells, was slightly decreased in HIV^- homosexual men (mean 98% and 91%, respectively) and in HIV-1-infected asymptomatic individuals (mean 90%) (Fig. 4a). In patients with AIDS the range was very broad, but this finding has to be considered with caution, due to the low percentages of $CD4^+$ T cells present in the sample. As was expected, the decline in $CD4^+CD28^+$ T cell numbers during HIV-1 infection is proportionally related to the decline in $CD4^+$ T cell numbers, because nearly all $CD4^+$ T cells express CD28 (Fig. 4a,b).

In agreement with previous studies [18–22], the proportion of $CD8^+CD28^-$ T cells was increased in both HIV-1-infected

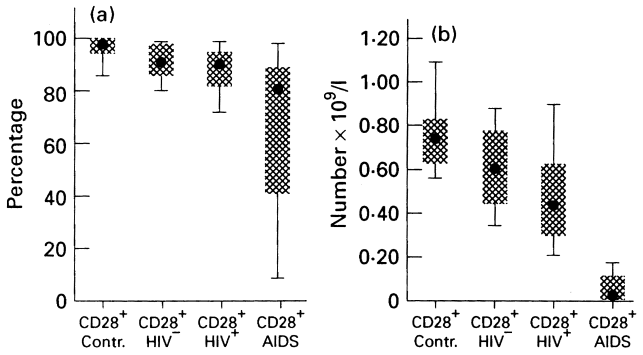

Fig. 4. CD28 expression was measured on $CD4^+$ T cells in the groups as described in the caption to Fig. 1. Results of $CD4^+CD28^+$ T cells are expressed in percentages (a) and numbers (b).

asymptomatic individuals and patients with AIDS (mean in HIV-1-infected asymptomatic individuals 61%, in patients 80%, in healthy controls 24%) (Fig. 5a). This increase in proportion of $CD8^+CD28^-$ T cells is already apparent in HIV^- homosexual controls (mean 52%). However, when expressed in numbers, there is virtually no difference between $CD8^+CD28^-$ T cell numbers among HIV^- homosexual con-trols, HIV-1-infected asymptomatic individuals and patients with AIDS (mean in heterosexual controls $0{\cdot}11 \times 10^9/l$, in HIV^- controls $0{\cdot}37 \times 10^9/l$, in HIV-1-infected asymptomatic men $0{\cdot}40 \times 10^9/l$ and in patients with AIDS $0{\cdot}44 \times 10^9/l$) (Fig. 5b). In contrast, $CD8^+CD28^+$ T cell numbers appeared to decrease in patients with AIDS (mean in heterosexual controls $0{\cdot}33 \times 10^9/l$, in HIV^- homosexual men $0{\cdot}37 \times 10^9/l$, in HIV-1-infected asymptomatic men $0{\cdot}28 \times 10^9/l$ and in patients with AIDS $0{\cdot}13 \times 10^9/l$) (Fig. 5c).

Predictive value of T cell CD28 expression

We examined whether a correlation existed between the presence of CD28 on T cells in the samples of HIV-1-infected individuals and T cell responses to CD3 MoAb in presence of CD28 MoAb. In a regression analysis there was moderate correlation between CD28 expression on T cells and responses to CD3 plus CD28 MoAb. (Regression equation: response $= 949{\cdot}7 \times \%CD28^+$ T cells $+ 10\,247$; $P < 0{\cdot}001$; $r^2 = 0{\cdot}30$.)

Given the predictive value of T cell responses for progression and the moderate correlation between T cell function and CD28 expression, we finally investigated whether CD28 expression on both $CD4^+$ and $CD8^+$ T cells had predictive value for progression to disease. In a univariate Cox analysis CD28 expression was weakly predictive (data not shown), but this was lost in a multivariate analysis when this marker was analysed in combination with T cell function.

DISCUSSION

For optimal mitogenic activation of T cells secondary signals are needed. Besides signalling through the T cell receptor/CD3 complex costimulatory molecules play an essential role. Both *in vivo* and *in vitro* it has been demonstrated that interaction of CD28 on T cells with its ligand B7 on APC fulfils this role [14]. In the present study, performed in cultures on Ficoll–Hypaque-isolated PBMC, in $HIV\text{-}1^+$ asymptomatic and in HIV^- individuals, T cell responses to CD3 plus CD28 MoAb were considerably enhanced ($\approx$ 10-fold) compared with T cell responses to CD3 MoAb alone. This was accompanied by notably reduced coefficients of variation. However, in patients with AIDS the responses remained greatly impaired. The larger range of responses to the combination of CD3 and CD28 MoAb with less intra-individual variability, together with the nearly absent reactivity in patients with AIDS, warranted an investigation to compare the predictive value in HIV-1-infected individuals for progression to AIDS of stimulation of lymphocytes *in vitro* by CD3 MoAb or CD3 plus CD28 MoAb. In a 4-year follow-up study $CD4^+$ T cell numbers, responses to CD3 MoAb in the absence or presence of CD28 MoAb and to PHA were all predictive in a univariate analysis. In a multivariate analysis $CD4^+$ T cell numbers was the strongest predictor, followed by T cell reactivity to CD3 plus CD28 MoAb. In this setting T cell reactivity to CD3 MoAb alone and PHA lost their significance. The regression analysis demonstrated that the correlation between $CD4^+$ T cell numbers and reactivity to CD3 plus CD28 MoAb was very weak. Apparently, in some individuals when the number of $CD4^+$ T cells declines the proliferative capacity of $CD8^+$ T cells remains relatively preserved. Furthermore, the independence of $CD4^+$ T cell numbers and T cell responses to CD3 plus CD28 MoAb has been confirmed in a Kaplan–Meier analysis when individuals with low $CD4^+$ T cell numbers or low responses to CD3 plus CD28 MoAb were subdivided into groups with high, intermediate and low values for CD3 plus CD28 responses and $CD4^+$ T cell numbers, respectively.

Decreased expression of CD28 on $CD8^+$ T cells, concomitantly resulting in increased percentages of $CD28^-$ T cells,

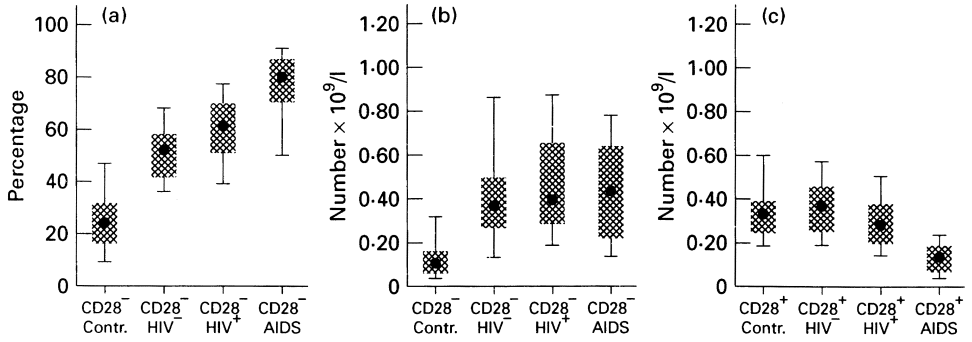

Fig. 5. CD28 expression was measured on $CD8^+$ T cells in the groups as described in the caption to Fig. 1. Results of $CD8^+CD28^-$ T cells are expressed in percentages (a) and numbers (b), results of $CD8^+CD28^+$ T cells are expressed in numbers (c).

has been repeatedly described in HIV-1-infected individuals [18–22]. We show that the expansion of $CD8^+CD28^-$ T cells existed already in HIV^- homosexual control individuals and remained at comparable levels in HIV-1-infected asymptomatic individuals and patients with AIDS. A likely explanation for this observation is that the immune system of the HIV^- population, in the past selected because of being at high risk of HIV-1 infection [24], is continuously stimulated as a consequence of frequent viral infections. It may be important to investigate homosexual men who are not at high risk of HIV-1 infection. Several investigators have demonstrated that $CD3^+CD28^-$ T cells accumulate in HIV-1 infection but are unresponsive to CD3 MoAb, mitogens, CD28 MoAb and staphylococcal superantigens [20–22,31]. It has been suggested that $CD8^+CD28^-$ T cells are generated as a result of an immunological event in the periphery [31]. Subsequently $CD28^-$ T cells were supposed to be a population of activated terminally differentiated effector cells that are cytotoxic only in short-term cultures [20,31].

In previous studies we demonstrated that in HIV-1 infection T cell reactivity to soluble CD3 MoAb measured in whole-blood lymphocyte cultures is an independent prognostic marker for progression to disease [12,23]. However, T cell responses to CD3 MoAb are relatively low with a tendency to have high coefficients of variation. Our present data demonstrate that T cell reactivity to CD3 plus CD28 MoAb is a more suitable marker for progression to disease than T cell reactivity to CD3 MoAb alone, probably due to the higher level of responses and lower coefficients of variation, and correlates moderately to the disappearance of $CD28^+$ cells. Nevertheless, when compared with stimulation by CD3 plus CD28 MoAb, CD28 expression on $CD4^+$ and $CD8^+$ T cells was not an independent marker in a multivariate Cox analysis. Furthermore, the expansion of $CD8^+CD28^-$ cells was not specific for HIV-1 because this expansion was already seen in HIV^- homosexual men at high risk. As we reported before, it is well feasible to test T cell reactivity to PHA and CD3 MoAb in whole-blood lymphocyte culture assays [12,23,32]. This is a simple, reproducible and suitable method for large routine screening/ monitoring of cohorts and patients enrolled in anti-retroviral drug trials. We are currently evaluating T cell reactivity induced by CD3 and CD28 MoAb in this whole-blood lymphocyte culture system.

ACKNOWLEDGMENTS

We thank Dr René Keet for providing clinical data, Dr Maarten Koot for critical comments, and Linda Dekker, Antoon Hekman, Nel Albrecht-van Lent, and Margreet Bakker for excellent technical assistance. Financial support was provided by the Netherlands Foundation for Preventive Medicine and the Netherlands Ministry of Health (grants 28-1079, -1026).

REFERENCES

1 Fahey JL, Prince HE, Weaver M, Groopman J, Visscher B, Schwartz K, Detels R. Quantitative changes in T helper or suppressor/cytotoxic lymphocyte subsets that distinguish acquired immune deficiency syndrome from other immune subsets disorders. JAMA 1984; **76**:95–100.

2 Melbye M, Biggar RJ, Ebbesen P *et al.* Long-term seropositivity for human T-lymphotropic virus type III in homosexual men without the acquired immunodeficiency syndrome: development of immuno-logic and clinical abnormalities. Ann Intern Med 1986; **104**:496–500.

3 Kornfeld H, Vande Stouwe RA, Lange M, Reddy MM, Griego MH. T-lymphocyte subpopulations in homosexual men. N Engl J Med 1982; **307**:1563–6.

4 Hersh EM, Mansell PWA, Reuben JM. Leukocyte subset analysis and related immunological findings in acquired immunodeficiency disease syndrome (AIDS) and malignancies. Diagn Immunol 1983; **1**:168–73.

5 Walker CM, Moody DJ, Stites DP, Levy JA. $CD8^+$ lymphocytes can control HIV infection *in vitro* by suppressing virus replication. Science 1986; **234**:1563–6.

6 Lane HC, Depper JL, Greene WC, Whalen G, Waldmann TA, Fauci AS. Qualitative analysis of immune function in patients with the acquired immunodeficiency syndrome. N Engl J Med 1985; **313**:79–84.

7 Shearer GM, Bernstein DC, Tung KSK, Via CS, Redfield R, Salahuddin SZ, Gallo RC. A model for the selective loss of major histocompatibility complex self-restricted T cell immune responses during the development of acquired immune deficiency syndrome (AIDS). J Immunol 1986; **137**:2514–21.

8 Giorgi JV, Fahey JL, Smith DC, Hultin LE, Cheng HL, Mitsuyasu RT, Detels R. Early effects of HIV on CD4 lymphocytes *in vivo*. J Immunol 1987; **138**:3725–30.

9 Ballet JJ, Couderc LJ, Rabian-Herzog C, Duval-Roy C, Janier M, Danon F, Clauvel JP, Seligmann M. Impaired T-lymphocyte-dependent immune responses to microbial antigens in patients with HIV-1-associated persistent generalized lymphadenopathy. AIDS 1988; **2**:291–7.

10 Miedema F, Petit AJC, Terpstra FG *et al.* Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. HIV affects the immune system before $CD4^+$ T helper cell depletion occurs. J Clin Invest 1988; **82**:1908–14.

11 Bentin J, Tsoukas C, McCutchan JA, Spector SE, Richman DD, Vaughan JH. Impairment in T-lymphocyte responses during early infection with the human immunodeficiency virus. J Clin Immunol 1989; **9**:159–68.

12 Schellekens PThA, Roos MThL, De Wolf F, Lange JMA, Miedema F. Low T-cell responsiveness to activation via CD3/TCR is a prognostic marker for AIDS in HIV-1 infected men. J Clin Immunol 1990; **10**:121–7.

13 Hofmann B, Orskov Lindhardt B, Gerstoft J *et al.* Lymphocyte transformation response to pokeweed mitogen as a marker for the development of AIDS and AIDS related symptoms in homosexual men with HIV antibodies. Br Med J 1987; **295**:293–6.

14 Linsley PS, Clark EA, Ledbetter JA. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. Proc Natl Acad Sci USA 1990; **87**:5031–5.

15 June CH, Ledbetter JA, Linsley PS, Thompson CB. Role of the CD28 receptor in T-cell activation. Immunol Today 1990; **11**:211–6.

16 Van Lier RAW, Brouwer M, deJong R, Groot M, de Groot E, Aarden LA. Functional properties of the human T-cell differentiatiation antigen CD28. In: Knapp W, ed. Leukocyte typing, Vol. IV. Oxford: Oxford University Press, 1989.

17 De Jong R, Brouwer M, Kuiper HM, Hooibrink B, Miedema F, Van Lier RAW. Maturation- and differentiation-dependent responsiveness of human $CD4^+$ T helper subsets. J Immunol 1992; **149**:2795–802.

18 Gruters RA, Terpstra FG, De Goede REY *et al.* Immunological and virological markers in individuals progressing from seroconversion to AIDS. AIDS 1991; **5**:837–44.

19 Giorgi JV, Boumsell L, Autran B. Reactivity of Workshop T-cell section mAb with circulating $CD4^+$ and $CD8^+$ T cells in HIV disease and following *in vitro* activation. In: Schlossman SF, ed. Leucocyte typing V. Oxford: Oxford University Press, 1995:446–61.

20 Borthwick NJ, Bofill M, Gombert WM *et al.* Lymphocyte activation in HIV-1 infection. II. Functional defects of $CD28^-$ T cells. AIDS 1994; **8**:431–41.

21 Brinchmann JE, Dobloug JH, Heger BH, Haaheim LL, Sannes M, Egeland T. Expression of costimulatory molecule CD28 on T cells in human immunodeficiency virus type 1 infection: functional and clinical correlations. J Infect Dis 1994; **169**:730–8.

22 Vingerhoets JH, Vanham GL, Kestens LL *et al.* Increased cytolytic T lymphocyte activity and decreased B7 responsiveness are associated

with CD28 down-regulation on $CD8^+$ T cells from HIV-infected subjects. Clin Exp Immunol 1995; **100**:425–33.

23 Roos MTL, Miedema F, Koot M, Tersmette M, Schaasberg WP, Coutinho RA, Schellekens PTA. T-cell function *in vitro* is an independent progression marker for AIDS in human immunodeficiency virus (HIV)-infected asymptomatic individuals. J Infect Dis 1995; **171**:531–6.

24 De Wolf F, Lange JMA, Houweling JTM, Coutinho RA, Schellekens PThA, Van der Noordaa J, Goudsmit J. Numbers of $CD4^+$ cells and the levels of core antigens of and antibodies to the human immunodeficiency virus as predictors of AIDS among seropositive homosexual men. J Infect Dis 1988; **158**:615–22.

25 Centers for disease control. Revision of the CDC surveillance case definition of AIDS. MMWR 1987; **36**:3–15.

26 Van Lier RAW, Boot JHA, Verhoeven AJ, De Groot ER, Brouwer MA, Aarden LA. Functional studies with anti-CD3 heavy chain isotype switch variant monoclonal antibodies. J Immunol 1987; **139**:2873–9.

27 Van Lier RAW, Brouwer M, Aarden LA. Signals involved in T-cell activation. T-cell proliferation induced through the synergistic action of anti-CD28 and anti-CD2 monoclonal antibodies. Eur J Immunol 1988; **18**:167–72.

28 Metz CE. 'Basic principles of ROC analysis', seminars in nuclear medicine. Stat Med 1978; **10**:283–98.

29 Viaggi S, Frigo AC. Threshold determination methods with diagnostic tests: a practical approach for medical decision making. Poster, ISCB-13 1992:1–12.

30 Peto R, Peto J. Asymptotically efficient rank invariant test procedures (with discussion). J Roy Stat Soc, Series A 1972; **135**:185–206.

31 Azuma M, Phillips JH, Lanier LL. $CD28^-$ T lymphocytes. Antigenic and functional properties. J Immunol 1993; **4**:1147–59.

32 Bloemena E, Roos MThL, van Heijst JLAM, Vossen JMJJ, Schellekens PThA. Whole-blood lymphocyte cultures. J Immunol Methods 1989; **122**:161–7.