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In Vivo Delayed-Type Hypersensitivity Skin Test Anergy in Human Immunodeficiency Virus Type 1 Infection Is Associated with T Cell Nonresponsiveness In Vitro

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In a cross-sectional study, the prevalence of delayed-type hypersensitivity skin test anergy (DTHA) was examined in 136 asymptomatic human immunodeficiency virus–infected participants in relation to immunologic and virologic parameters. DTHA was assessed with a multitest cell-mediated immunity skin test. Of the 136 participants, with a mean CD4 T cell count of 335 $\times$ 10^6/L, 25 were anergic (18.4%). In the stepwise forward multivariate logistic regression models, after adjustment for CD4 T cell counts, depending on whether it was analyzed continuously or after dichotomization (20th percentile), both T cell reactivity to CD2 plus CD28 antibodies or to CD3 antibodies were the most predictive markers of DTHA (odds ratio, 0.80; 95% confidence interval, 0.67–0.94; and odds ratio, 2.97; 95% confidence interval, 1.1–8.3, respectively). This study shows a strong correlation between the decreased T cell responses in vitro and DTHA. Therefore, next to DTHA testing, T cell function assays may be useful to test immune reconstitution observed during antiretroviral treatment.

Infection with human immunodeficiency virus (HIV) induces profound defects of cell-mediated immunity, which will eventually result in AIDS. The hallmark of progression of HIV-1 infection is depletion of CD4 T cells, but several other surrogate markers, such as HIV-1 RNA load [1] and T cell reactivity in vitro [2], have also been shown to provide important additional information regarding the prognosis of HIV-1–infected persons. Although not frequently used, delayed-type hypersensitivity (DTH) skin testing was recognized early on as a marker to assess the immune status of HIV-1–infected persons [3–5].

The DTH reaction is mediated by Th1 cells, which produce interleukin-2 and interferon-γ [6]. It has been shown in asymptomatic HIV-1–infected persons that peripheral blood T cells [7, 8] and lymph node T cells [9] have a reduced capacity to produce interleukin-2 and interferon-γ, which is probably the cause of the diminished capacity to induce DTH reactions. The Th1 dysfunction in HIV-1–infected persons could, at least in part, be caused by diminished release of interleukin-12, a cytokine that directs Th1 responses [10, 11] and that has been reported as able to restore HIV-specific T cell responses in patients [12, 13].

DTH anergy (DTHA) has been observed in 20%–30% [14, 15] of asymptomatic HIV-1–infected persons with normal or slightly decreased CD4 T cell counts. The prognostic significance of DTHA has been recognized in the Walter Reed classification, in which it was an independent indicator for progression to disease [16–18].

We therefore investigated the possible relation between DTHA and T cell function in vitro by using different stimulation protocols, as well as the wider association with the other virologic and immunologic markers used for monitoring progression of HIV-1 infection.

Materials and Methods

Study population. The study population consisted of HIV-1–infected white homosexual men who were participating in the Amsterdam Cohort study on HIV infection and AIDS [19]. Participants were seen at 3-month intervals. At each 3-monthly visit, as well as a physical examination and a medical history, immunologic and virologic laboratory evaluations were done.

A multitest cellular-mediated immunity (CMI) skin test was done on all 136 HIV-1–infected participants who visited the Municipal Health Service in the period from September 1995 through February 1996, at one of the routine visits or shortly afterwards.

DTH skin testing. For evaluation of DTHA, we used the commercially available Multitest CMI applicator (Institut Mérieux, Lyon, France) [20–22]. The recall antigens applied with the multitest were tetanus, diphtheria, *Streptococcus* group C, tubercu-

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Informed consent was obtained from all participants.

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lin, *Proteus, Candida albicans,* and *Trichophyton* along with a glycerine control. The result of the skin test was assessed after 48 h. The test was administered and interpreted by trained nurses [23] of the tuberculosis prevention department of the Municipal Health Service of Amsterdam. Furthermore, for a reference, the multitest was also applied to 20 staff members of the Municipal Health Service of Amsterdam and to 4 HIV-negative homosexual men. No blood was drawn from these controls. DTHA is frequently defined as none of the skin test indurations >2 mm [24]. However, this definition was not feasible because the number of anergic persons was too small to perform an analysis. Therefore, the following DTHA definition was used: A skin induration ⩾2 mm to an individual recall antigen was considered a response. The mean DTH score was defined as the sum of all of the indurations ⩾2 mm (nominator), divided by the number of responding antigens (denominator). DTHA was defined as a mean DTH score ⩽2 mm.

Laboratory methods. Sera were tested for the presence of HIV-1 antibodies with two commercially available ELISAs (Abbott Laboratories, Abbott Park, IL; Vironostika Teknika, Organon, Oss, The Netherlands) and confirmed with Western blot. HIV-1 p24 antigen was detected with a solid-phase, sandwich-type EIA (Abbott Laboratories). The HIV-1 RNA virus load was assessed with the nucleic acid sequence–based amplification [25] assay in cryopreserved sera. All serologic and immunologic tests were carried out in fresh specimens. T cell subsets were enumerated by a direct immunofluorescent technique using monoclonal antibodies (MAbs) and a flow cytometry system. Cocultivation of peripheral blood mononuclear cells with MT-2 cells was used to detect the presence of syncytium-inducing HIV-1 variants [26]. To measure T cell reactivity, fresh cells were stimulated in a whole blood culture system with CD3 MAb, CD3 plus CD28 MAb [27], CD2 plus CD28 MAb, and phytohemagglutinin [28]. Reactivity was expressed as the percentage of median responses detected in concurrently running cultures of 5 healthy controls.

Statistical analysis. In a cross-sectional design, continuous variables with an approximately normal distribution were tested with Student's t test. Variables with a skewed distribution were tested with the Mann-Whitney test or, after the most appropriate transformation (log or square root), tested with the Student's t test. In addition, categorical variables were examined with the χ^2 test. The predictive value for developing DTHA was assessed in two ways, continuously and after dichotomization. In the latter analysis, the continuous variables were dichotomized at the 20th percentile. This level was chosen because it approximates the lower reference range that is used by our laboratory. In this analysis, the odds ratio (OR) of DTHA was calculated in the group with marker levels below the 20th percentile compared with others in the study group (⩽20th vs. >20th percentile). Plasma HIV-1 RNA virus load was log-transformed, and the dichotomization threshold was set at 10^4 copies/mL, the level at which antiretroviral treatment is recommended [1].

ORs with 95% confidence intervals (95% CIs) were calculated with logistic regression. The associations between the presence of DTHA and the various immunologic and virologic variables were analyzed univariately and after adjustment for CD4 T cell count. Finally, the variables that predicted DTHA significantly were analyzed in a stepwise forward model. The following levels of significance were used: $P \geqslant .10$ for removal and $P < .10$ for addition.

Results

The mean age of the 136 HIV-1–positive homosexual men was 41.5 years (range, 25–59). Ninety-four were HIV-1–positive at entry and 42 seroconverted during follow-up. The mean HIV-positive follow-up period was 6.4 years, with a range of 3 months to 11 years. The mean age of the 24 controls (15 men and 9 women) was 41 years (range, 28–56). In 60% of subjects, time between blood drawing at one of the routine visits and applying the multitest was <2 weeks, with a maximum range of 3 months.

Of the 136 HIV-1–positive men included in this study, 25 were found to be anergic (18.4%), compared with 1 in the control group (4.2%) ($P = .13$). However, the sum of skin test indurations, the number of responding antigens per subject, and the mean DTH score were all significantly lower in the HIV-1–positive subjects than in controls ($P < .001$, $P < .001$ [figure 1], and $P = .002$, respectively). Eleven subjects showed no response whatsoever against any of the antigens (8%), compared with none of the controls ($P = .16$).

Table 1 shows that the mean induration and the percentage of responding subjects per recall antigen was in general lower—except for *Trichophyton* antigen—in the HIV-1–infected group than in the control group, although statistical significance was not always reached.

The HIV-1–infected subjects were divided into 2 groups, anergic ($n = 25$) and responders ($n = 111$), and compared regarding virologic and immunologic markers (table 2). The mean CD4 T cell count for the 136 HIV-1–positive participants was 335×10^6/L, with a mean for responders and anergic men of 355×10^6/L and 246×10^6/L, respectively ($P = .01$). Moreover, T cell responses induced by CD3 MAb, CD3 plus CD28 MAb, and CD2 plus CD28 MAb were significantly higher in the responders than in the anergic men. Of the virologic markers, only HIV-1 p24 antigen differed significantly between the groups. Plasma HIV-1 RNA virus load did not. There was no association of DTHA with age or length of follow-up ($P = .37$ and .67, respectively).

In the univariate logistic regression analysis, the following continuous markers were shown to be significantly associated with DTHA (table 3): CD4 T cell count and low T cell reactivity after stimulation with CD3 plus CD28 MAb and CD2 plus CD28 MAb. The presence of HIV-1 p24 antigen was also significantly associated with DTHA. In the stepwise forward multivariate model (including CD4 T cell count, T cell reactivity after stimulation with CD3 plus CD28 MAb and CD2 plus CD28 MAb, and HIV-1 p24 antigen), low T cell reactivity after stimulation with CD2 plus CD28 MAb appeared to be the most predictive marker, with an OR of 0.80 (95% CI, 0.67–0.95) ($P = .01$).

In the univariate logistic regression analysis, the following dichotomized markers were shown to be significantly associated with DTHA (table 3): low T cell reactivity after stimulation with CD3 MAb and CD2 plus CD28 MAb. In a bivariate

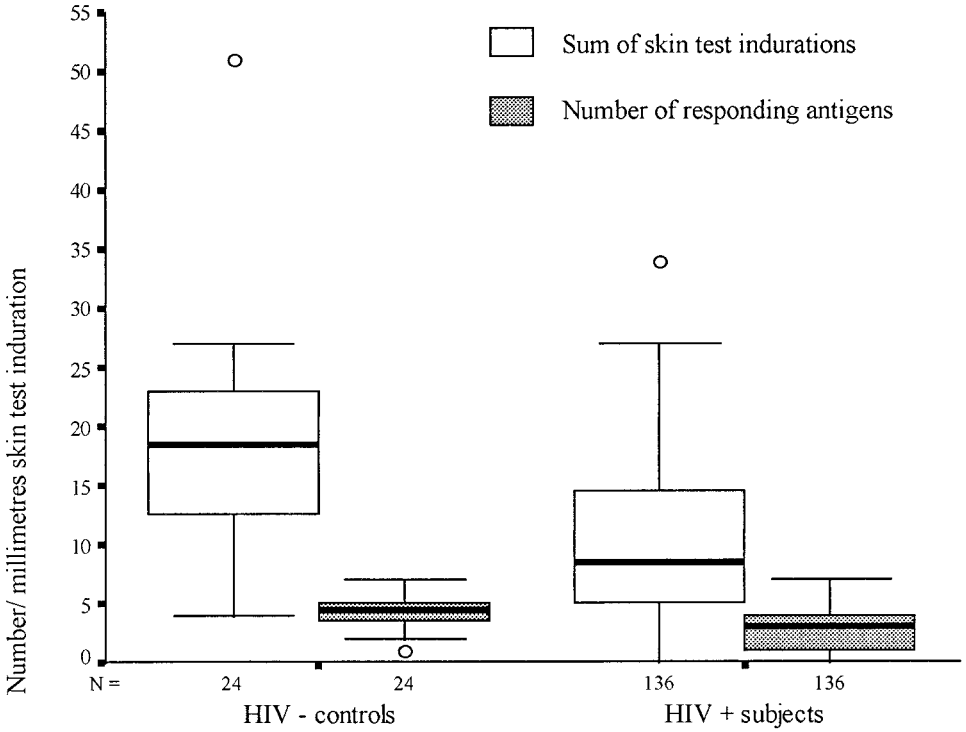

Figure 1. Median, quartiles, and extreme values (○) for sum of skin test indurations and number of responding recall antigens observed in HIV-seronegative controls and HIV-seropositive participants.

analysis with CD4 T cell count forced into the logistic model, the ORs for the other markers remained similar. In the final stepwise forward multivariate model, including CD3 MAb, CD2 plus CD28 MAb, and HIV-1 p24 antigen, low T cell reactivity to CD3 MAb appeared to be the best discriminating marker, with an OR of 2.97 (95% CI, 1.1–8.3) ($P = .04$).

Furthermore, we performed a third logistic analysis in which we defined DTHA as none of the indurations >2 mm (11 anergic men vs. 125 responders). In this analysis, low T cell reactivity to CD2 plus CD28 MAb appeared to be the strongest predictor of DTHA, with an OR of 3.0 (95% CI, 0.8–11.9) ($P = .1$).

Discussion

Increasingly, sets of progression markers are being used to stage HIV-1–infected patients. Most of these surrogate markers, for example, plasma HIV-1 RNA load and CD4 T cell count, have proven to be useful as predictors of progression to symptomatic HIV-1 infection and death. DTH skin induration

Table 1. Results of multitest CMI: mean DTH induration and % of responding subjects per recall antigen.

	Skin test induration, mm (SE)			No. of responders (%)		
DTH antigen	HIV-infected ($n = 136$)	Control ($n = 24$)	P	HIV-infected ($n = 136$)	Control ($n = 24$)	P
Candida	2.5 (0.20)	3.3 (0.39)	.11	82 (60)	20 (83)	.03
Proteus	2.0 (0.14)	2.8 (0.29)	.02	86 (64)	21 (88)	.02
Tetanus	2.3 (0.22)	4.2 (0.84)	.03	74 (54)	16 (67)	.26
Trichophyton	1.1 (0.16)	1.0 (0.44)	.15	38 (28)	5 (21)	.46
Streptococcus	1.0 (0.12)	2.0 (0.34)	.007	43 (32)	14 (58)	.02
Diphtheria	1.1 (0.14)	2.7 (0.59)	.008	48 (54)	15 (63)	.01
Tuberculin	1.0 (0.15)	3.3 (0.75)	<.001	21 (15)	13 (54)	<.001
Glycerol control	0 (0)	0 (0)	—	0	0	—

NOTE. Responder is defined as skin test induration for specific recall antigen ≥2 mm. P values were determined with Mann-Whitney or χ^2 test.

Table 2. Immunologic and virologic characteristics of anergic and non-anergic HIV-1–positive participants.

Marker of progression	Anergic (n = 25)	Non-anergic (n = 111)	P
T cell subset			
CD4	246 (31)	355 (33)	.01
CD8	1073 (511)	1174 (577)	.43
CD3	1409 (707)	1618 (683)	.18
T cell reactivity to			
phytohemagglutinin	39.7 (4.6)	53.1 (4.9)	.06
CD3 MAb	15.4 (4.6)	33.7 (4.0)	.03
CD3 plus CD28 MAb	27.3 (5.9)	41.3 (6.2)	.04
CD2 plus CD28 MAb	45.9 (4.0)	64.5 (3.9)	.008
Virologic marker			
HIV-1 RNA virus load	4.4 (0.95)	4.2 (0.78)	.26
HIV-1 p24 antigen	11/25 (44)	25/111 (23)	.02
HIV-1 syncytium-inducing variant	5/25 (20)	13/111 (12)	.26

NOTE. Data are mean (SD) or no. positive/total (%). T cell subsets are cells $\times 10^6$/L. T cell reactivity is % in relation to median of responses of 5 healthy controls cultured simultaneously. HIV load is $\log_{10}$-transformed copies/mL. Categorical values were analyzed with χ^2 test; continuous variables were analyzed with Student's t test.

following a standardized amount of intracutaneously applied recall antigens is a direct in vivo measure of cellular immunity. In the present study, we examined the association between several immunologic and virologic laboratory markers of progression and the occurrence of DTHA.

Our result, a DTHA percentage of 18.4% with a relatively high CD4 T cell count of 335 $\times$ 10^6/L, is in accordance with previously published results [14, 15]. Furthermore, studies by MacDonell et al. [16], Blatt et al. [17], and Birx et al. [29] demonstrated that DTHA is an independent marker for progression to AIDS. DTHA can therefore be useful in distinguishing progressors from nonprogressors in the group of HIV-1–infected asymptomatic persons.

Depending on whether analyses were done with continuous variables or after dichotomization, we found a strong association between DTHA and depressed T cell responsiveness to activation signals induced by CD2 plus CD28 MAb or CD3 MAb. However, T cell reactivity after stimulation with CD2 plus CD28 MAb appeared to be a more universal predictor of DTHA than was T cell reactivity after stimulation with CD3 MAb. This can be explained by the fact that, in addition to activation via the CD3 T cell receptor complex or the CD2 T cell receptor, CD28 MAb delivers a second signal to T cells [27]. In vitro, the CD28 MAb, not being mitogenic on its own, strongly enhances proliferation of T cells stimulated by CD3 MAb or CD2 MAb. Therefore, in patients with more advanced HIV infection, T cell reactivity to CD2 plus CD28 MAb is longer preserved and more robust than T cell reactivity to CD3 MAb.

Although a significant association between DTHA and HIV p24 antigen was found, surprisingly, no association with plasma HIV-1 RNA at the level of 10,000 copies/mL could be found. However, if a higher cutoff value was used, the plasma HIV RNA level became more predictive (data not shown). From this we have concluded that HIV RNA is only capable of predicting DTHA at higher RNA levels.

In previous studies, we have demonstrated that early in infection, CD3 MAb–induced T cell responses are depressed in

Table 3. Results of univariate logistic regression analyzing predictive value of various immunologic and virologic markers in predicting DTH skin test anergy.

	Results, univariate logistic regression						
	Continuous			Dichotomized (20%)			
Marker of progression	OR	95% CI	P	Cutoff*	OR	95% CI	P
T cell subset							
CD4	0.75	0.57–0.97	.03	192 $\times$ 10^6/L	2.5	0.9–6.7	.07
CD8	0.97	0.88–1.05	.43	662 $\times$ 10^6/L	0.8	0.2–2.5	.68
CD3	0.95	0.88–1.02	.18	996 $\times$ 10^6/L	1.1	0.4–3.3	.87
T cell reactivity to							
phytohemagglutinin	0.85	0.72–1.01	.07	30.0%	1.1	0.4–3.4	.84
CD3 MAb	0.91	0.82–1.02	.10	9.0%	3.0	1.1–8.3	.04
CD3 plus CD28 MAb	0.83	0.69–0.99	.04	16.0%	1.9	0.7–5.3	.21
CD2 plus CD28 MAb	0.80	0.67–0.95	.01	37.2%	3.0	1.1–8.2	.03
Virologic marker							
HIV-1 RNA virus load	1.41	0.78–2.55	.25	10^4 copies/mL	1.7	0.6–4.8	.29
HIV-1 p24 antigen	—	—	—	Yes/no	2.9	1.2–7.2	.02
HIV-1 syncytium-inducing variant	—	—	—	Yes/no	1.9	0.6–6.0	.26

NOTE. Continuous markers were dichotomized at 20th percentile. T cell subsets are changes in odds ratio (OR) per 100 $\times$ 10^6/L; T cell reactivity is changes in OR per 10%; HIV-1 load is changes in OR per 1 log HIV-1 RNA level. CI, 95% confidence interval.

* Marker value found at 20% cutoff.

asymptomatic persons who are at risk of progression toward clinical symptoms or AIDS [2, 30]. Years before the critical reduction in CD4 T cell numbers, subtle defects in T helper cell function can be detected [2, 27, 31]. On the basis of these and other observations, it can be suggested than an early decline in T cell function may contribute to a poor control of virus infection, which will result in a more rapid depletion of CD4 T cells and, hence, faster progression to AIDS.

Because our results showed a direct correlation between T cell reactivity to CD3 MAb and CD2 plus CD28 MAb in vitro and the observed skin test anergy in vivo, this relatively simple DTH test could be useful in determining the extent of immune reconstitution. The extent of skin test induration following a standardized amount of intracutaneously applied recall antigens may be considered as a close reflection of the quality of cellular immunity. However, there are several drawbacks to the application of DTH tests for clinical practice as a tool for intensive monitoring. For instance, the application of the antigens and the skin induration can cause discomfort, and patients have to present again after 48 h. On the other hand, in vitro T cell reactivity can be determined along with CD4 T cells in blood samples and is therefore more suitable for frequently monitoring the course of the cellular immunity.

We and others previously showed improved T cell function, on a per-cell basis, in zidovudine-treated patients [32] and in patients treated with combination therapy [33, 34]. From this, we conclude that T cell reactivity in vitro can be useful for determining effectiveness of antiretroviral treatment and the extent of in vivo immune reconstitution.

In summary, to our knowledge, this is the first study to examine the direct relationship between DTH skin tests and the various virologic and immunologic in vitro markers of progression of HIV. We found, independent of CD4 T cells, associations between DTHA and T cell reactivity to CD3 MAb and CD2 plus CD28 MAb. For a more intensive monitoring of cellular immunity, determination of T cell reactivity in vitro, next to CD4 T cells and HIV-1 RNA load, may be included.

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References

1. Mellors JW, Kingsley LA, Rinaldo CR Jr, et al. Quantitation of HIV-1 RNA in plasma predicts outcome after seroconversion. Ann Intern Med **1995**;122:573–9.
2. Roos MTL, Miedema F, Koot M, et al. T cell function in vitro is an independent progression marker for AIDS in human immunodeficiency virus–infected asymptomatic subjects. J Infect Dis **1995**;171:531–6.
3. Kornbluth RS, McCutchan JA. Skin test responses as predictors of tuberculous infection and of progression in HIV-infected persons. Ann Intern Med **1993**;119:241–3.
4. Razzaque A, Douglas A. Delayed-type hypersensitivity skin testing. Arch Dermatol **1983**;119:934–45.
5. Dvorak HF, Galli SJ, Dvorak AM. Expression of cell-mediated hypersensitivity in vivo—recent advances. Int Rev Exp Pathol **1980**;21:119–94.
6. Cher DJ, Mosmann TR. Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. J Immunol **1987**;138:3688–94.
7. Clerici M, Hakim FT, Venzon DJ. Changes in interleukin-2 and interleukin-4 production in asymptomatic human immunodeficiency virus seropositive individuals. J Clin Invest **1993**;91:759–65.
8. Meyaard L, Hovenkamp E, Keet IPM, et al. Single-cell analysis of IL-4 and IFN-gamma production by T cells from HIV-infected individuals. J Immunol **1996**;157:2712–8.
9. Graziosi C, Pantaleo G, Gantt KR, et al. Lack of evidence for the dichotomy of TH1 and TH2 predominance in HIV-infected individuals. Science **1994**;265:248–52.
10. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. Science **1993**;250:547–9.
11. Uherova P, Connick E, MaWhinney S, Schlichtemeier R, Schooley RT, Kuritzkes DR. In vitro effect of interleukin-12 on antigen-specific lymphocyte proliferative responses from persons infected with human immunodeficiency virus type 1. J Infect Dis **1996**;174:483–9.
12. Clerici M, Lucey M, Berzofsk JA. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. Science **1993**;262:1720–4.
13. Chehimi J, Starr SE, Frank I, et al. Impaired interleukin 12 production in human immunodeficiency virus–infected patients. J Exp Med **1994**;179:1361–6.
14. Sears SD, Fox R, Brookmeyer R, Leavitt R, Polk BF. Delayed hypersensitivity skin testing and anergy in a population of gay men. Clin Immunol Immunopathol **1987**;45:177–83.
15. Selwyn PA, Hartel D, Lewis VA, et al. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. N Engl J Med **1989**;320:545–50.
16. MacDonell KB, Chmiel JS, Goldsmith J, et al. Prognostic usefulness of the Walter Reed staging classification for HIV infection. J Acquir Immune Defic Syndr **1988**;1:367–74.
17. Blatt SP, Hendrix CW, Butzin CA. Delayed-type hypersensitivity skin test predicts progression to AIDS in HIV infected patients. Ann Intern Med **1993**;119:177–84.
18. Trauger RJ, Giermakowska WK, Ferre F, et al. Cell-mediated immunity to HIV-1 in Walter Reed stages 1–6 individuals: correlation with virus burden. Immunology **1993**;78:611–5.
19. de Wolf F, Lange JMA, Houweling JTM, et al. Numbers of $CD4^+$ T 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.
20. Frazier TC, Wagner JM, Wray BB, Stafford CT. Multitest CMI skin test compared with standard intradermal skin testing in evaluation of cell-mediated immunity [abstract]. J Allergy Clin Immunol **1993**;91:213.
21. Pesanti EL. The negative tuberculin test. Tuberculin, HIV, and anergy panels. Am J Respir Crit Care Med **1994**;149:1699–709.
22. Sokal JE. Measurement of delayed skin-test responses. N Engl J Med **1975**;293:501–2.
23. Bates SE, Suen JY, Tranum BL. Immunological skin testing and interpretation. A plea for uniformity. Cancer **1979**;43:2306–14.
24. Kniker WT, Anderson CT, Roumiantzeff M. The multitest system: a standardized approach to evaluation of delayed-type hypersensitivity and cell-mediated immunity. Ann Allergy **1979**;43:73–9.
25. Gemen B, Kievits T, Huisman HG, Jurriaans S, Goudsmit J, Lens P. Qualitative and quantitative detection of HIV-1 RNA by nucleic acid sequence–based amplification. AIDS **1993**;7(suppl):S107–10.

26. Koot M, Vos AH, Keet IPM, et al. HIV-1 biological phenotype in long-term infected individuals evaluated with a MT-2 co-cultivation assay. AIDS **1992**;6:49–54.
27. Roos MTL, Miedema F, Meinesz AAP, de Leeuw NASM, Pakker NG, Lange JMA. Low T cell reactivity to combined CD3 plus CD28 stimulation is predictive for progression to AIDS: correlation with decreased CD28 expression. Clin Exp Immunol **1996**;105:409–15.
28. Bloemena E, Roos MTL, Heijst JLAM, Vossen JMJJ, Schellekens PTA. Whole-blood lymphocytes cultures. J Immunol Methods **1989**;122:161–7.
29. Birx DL, Brundage J, Larson K, et al. The prognostic utility of delayed-type hypersensitivity skin testing in the evaluation of HIV-infected patients. J Acquir Immune Defic Syndr **1993**;6:1248–57.
30. Schellekens PTA, Koot M, Roos MTL, Tersmette M, Miedema F. Immunologic and virologic markers determining progression to AIDS. J Acquir Immune Defic Syndr Hum Retrovirol **1995**;10(suppl 2):S62–6.
31. Miedema F, Chantal Petit AJ, Terpstra FG, et al. Immunological abnormalities in human immunodeficiency virus (HIV)–infected asymptomatic homosexual men. J Clin Invest **1988**;82:1908–14.
32. Koot M, Schellekens PT, Mulder JW, et al. Viral phenotype and T cell reactivity in human immunodeficiency virus type 1–infected asymptomatic men treated with zidovudine. J Infect Dis **1993**;168:733–6.
33. Autran B, Carcelain G, Li TS, et al. Positive effects of combined antiretroviral therapy on $CD4^+$ T cell homeostasis and function in advanced HIV disease. Science **1997**;277:112–6.
34. Pakker N, Notermans DW, de Boer RJ, et al. Biphasic kinetics of peripheral blood T cells after triple combination therapy in HIV-1 infection: a composite of redistribution and proliferation. Nature Med **1998**;4:208–14.