Instability of Tuberculin and Candida Skin Test Reactivity in HIV-infected Ugandans

JOHN L. JOHNSON, SAM NYOLE, ALPHONSE OKWERA, CHRISTOPHER C. WHALEN, PETER NSUBUGA, VUKOSAVA PEKOVIC, ROBIN HUEBNER, ROBERT S. WALLIS, PETER N. MUGYENYI, ROY D. MUGERWA, and JERROLD J. ELLNER for the Uganda-Case Western Reserve University Research Collaboration

Department of Medicine, Division of Infectious Diseases, and Department of Epidemiology and Biostatistics, Case Western Reserve University School of Medicine and University Hospitals of Cleveland, Cleveland, Ohio; National TB and Leprosy Control Programme, Joint Clinical Research Center, and Department of Medicine, Mulago Hospital and Makerere University, Kampala, Uganda; Centers for Disease Control and Prevention, Atlanta, Georgia.

Anergy testing has been used as an adjunct to tuberculin testing for assessing *M. tuberculosis* (MTB) infection and indications for isoniazid preventive therapy in HIV-infected persons. We examined factors associated with the stability of skin test responses to purified protein derivative (PPD) and candida antigens in a cohort of HIV-infected adults followed prospectively in a tuberculosis preventive therapy trial in Uganda. PPD-positive and anergic subjects in the placebo arms of the preventive therapy study underwent repeat skin testing and immunologic testing including measurement of MTB culture filtrate (CF)–stimulated interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) levels in whole-blood culture supernatants. Anergy was present in 27% of 4,058 HIV-infected subjects screened for the tuberculosis preventive therapy trial compared with 10% of 682 HIV-non-infected persons. On follow-up testing of enrolled subjects, 42% of 139 initially anergic subjects were no longer anergic; two thirds of these had PPD reactions $\geqslant$ 5 mm. Stability of anergy was associated with intercurrent opportunistic infections and AIDS-associated dermatitis at baseline. Thirty-five percent of 313 subjects with an initial positive PPD had a negative PPD test at follow-up, 26% of whom had a positive candida skin test at the same time as the negative PPD test. Baseline MTBCF-stimulated IFN-γ levels were significantly higher among PPD-positive subjects who remained PPD-positive than in those who were falsely negative. We conclude first that anergy is unstable and second that anergy testing is unreliable in identifying HIV-infected adults who are not infected with MTB and should not be used routinely for this purpose in assessing indications for isoniazid preventive therapy. Johnson JL, Nyole S, Okwera A, Whalen CC, Nsubuga P, Pekovic V, Huebner R, Wallis RS, Mugyenyi PN, Mugerwa RD, Ellner JJ for the Uganda-Case Western Reserve University Research Collaboration. Instability of tuberculin and candida skin test reactivity in HIV-infected Ugandans.

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Tuberculosis is the most frequent cause of death due to an identifiable infectious pathogen in HIV-infected persons worldwide (1, 2). In 1997, the World Health Organization (WHO) estimated that there were 15.3 million people coinfected with HIV and *Mycobacterium tuberculosis* (MTB) globally; three-fourths of whom live in sub-Saharan Africa (WHO, unpublished data). Isoniazid (INH) preventive therapy is effective for the prevention of tuberculosis in purified protein derivative (PPD)-positive HIV-infected persons (3–5), and has also been recommended for anergic persons in areas where the prevalence of tuberculosis infection is greater than 10% (6). Identification of persons who might benefit from preventive therapy is an important public health issue.

Tuberculin skin testing with PPD is the only currently available test to detect prior infection with MTB. Five or more millimeters of induration is regarded as a positive test among HIV-infected persons (7). Immunosuppressed individuals, including HIV-infected persons, are often unable to mount appropriate delayed-type hypersensitivity (DTH) responses to PPD despite earlier MTB infection. Simultaneous testing with recall antigens such as candida or mumps to which most individuals are presumably sensitized, frequently is used to assess whether an immunosuppressed patient is capable of manifesting DTH responses to other stimuli. Those who respond to

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Correspondence and requests for reprints should be addressed to John L. Johnson, M.D., Division of Infectious Diseases, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106-4984. jlj@po.cwru.edu

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one or more recall antigens but who do not react to PPD are assumed to be uninfected with MTB. Those who do not respond to any skin test antigens are assumed to be anergic and immunosuppressed to such a degree that it cannot be reliably determined whether or not they have been infected with MTB.

Combined PPD and anergy testing with recall antigens was at one time recommended for screening HIV-infected persons for INH preventive therapy (6). The reliability of anergy testing in assessing prior MTB infection is, therefore, a critical issue in the implementation of preventive therapy in HIV-infected patients. Chin and colleagues (8) recently reported that 30% of anergic patients followed in the U.S. Pulmonary Complications of HIV Study cohort subsequently reacted to one or more antigens on follow-up testing. Additionally, 39% of subjects in this study with a negative PPD test after an initial positive test had a reactive candida skin test at the time of repeat DTH testing (8). Centers for Disease Control recommendations for anergy skin testing in HIV-infected individuals in the United States were revised in September 1997 to reflect accumulating data concerning the variability and lack of reproducibility of anergy testing with available methods and reagents. Anergy testing is no longer routinely recommended in screening programs to identify MTB infection in HIV-infected persons (9).

Tuberculosis is highly endemic in Uganda, with reported rates of active tuberculosis of 120 cases per 100,000 population in 1995 (10). Fifty-eight percent of 3,674 adults older than 15 yr of age were tuberculin-positive in the 1970 Ugandan national tuberculin survey (11).

The validity of anergy testing is an even more important concern in areas with a high prevalence of tuberculosis where misclassification of MTB infection may result in underutilization of preventive therapy. To address this question, we measured the prevalence of PPD positivity and anergy to PPD and candida antigens among HIV-infected and HIV-non-infected adult volunteers screened for participation in a large tuberculosis preventive therapy study in Uganda. The stability of PPD and candida skin test reactivity among the HIV-infected adults followed in the placebo arms of the trial, as well as clinical and immunologic correlates of anergy, also were studied. Interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) are critical cytokines regulating macrophage activation and killing of intracellular mycobacteria (12–14) and granuloma formation (15). The capacity to express IFN-γ and TNF-α in response to MTB antigens may reflect host susceptibility to tuberculosis and the degree of HIV-related immune dysfunction. IFN-γ and TNF-α levels in MTB culture filtrate-stimulated, whole-blood cultures were measured in a subset of subjects in the parent tuberculosis preventive therapy study to assess the association of these *in vitro* correlates of host immunity with PPD reactivity and anergy.

METHODS

Study Population

The study was conducted in Uganda, a nation with a high prevalence of HIV and MTB infections. Volunteers 18 to 50 yr of age were screened for participation in a prospective randomized, placebo-controlled tuberculosis preventive therapy trial between March 1993 and April 1995 at five clinics and voluntary HIV counseling and testing centers in Kampala, Uganda. Initially, only those with a PPD skin test reaction $\geq$ 5 mm were recruited; however, in October 1993 enrollment was expanded to include anergic subjects (both PPD and candida skin test reactions = 0 mm). Other inclusion criteria included positive HIV serology and Karnofsky performance scale score > 50% (16). Subjects with active TB or a prior history of the disease, TB treatment, or preventive therapy (except BCG vaccination, which is routinely given shortly after birth in Uganda as part of the United Nations Expanded Programme on Immunizations), total WBC < 3.0 × 10^9 cells/L, hemoglobin < 8.0 gm/dl, serum aspartate aminotransferase > 90 IU/L, serum creatinine > 160 μM/L, and WHO clinical Stage IV AIDS (17) were excluded. Ten percent of HIV-non-infected subjects also were enrolled in the study cohort to avoid stigmatization of study subjects. The study protocol was approved by the institutional review boards at University Hospitals of Cleveland and Case Western Reserve University and the Ugandan National AIDS Research Subcommittee. All subjects gave oral informed consent before screening and enrollment in the study.

Skin testing was the initial screening procedure. PPD and candida skin testing was performed by the Mantoux method by the injection of 5 tuberculin units of PPD (Tubersol; Connaught, Swiftwater, PA) and 0.1 ml of a 1:500 wt/vol dilution of *Candida albicans* allergic extract (Berkeley Biologicals, Berkeley, CA) into the skin of the volar aspect of the left and right forearms. The number of millimeters of induration at each skin test site was measured after 48 to 72 h. Testing and reading was performed by five highly experienced technicians using standardized procedures.

The interobserver reliability of skin test reading by the team of skin testing technicians was studied in an earlier community survey. In this survey, PPD reactions in 19 subjects were read by two independent readers on the same day. The correlation coefficient of the two readings was 0.98, with a regression slope of +1.03 (C. Whalen, unpublished observation). Intraobserver reliability for the lead PPD technician for the study (SN) also has been studied earlier by measuring skin test responses to PPD and candida twice in 10 subjects on the same day in a blinded fashion (18). The mean difference between the two readings was 0.7 mm, with a standard deviation of 1.1 mm.

Subjects meeting the skin test inclusion criteria (PPD $\geq$ 5 mm or nonreactive to both PPD and candida antigens) were then asked to undergo medical history and physical examination, chest radiography, HIV testing, sputum examination, and hematologic and biochemical testing. Complete blood counts were performed using a Coulter T-540 automated system (Coulter Electronics, Hialeah, FL). CD4 lymphocyte counts were done by flow cytometry using a Becton-Dickinson FACScan (Becton-Dickinson, Santa Rosa, CA). Serum neopterin and β_2-microglobulin levels were measured by enzyme immunoassay (EIA) (Neopterin-MW EIA; ICN Pharmaceuticals, Costa Mesa, CA and β_2-microglobulin EIA; Coulter, Miami, FL, respectively). HIV infection status was assessed by HIV-1 EIA (Recombigen HIV-1 env + gag EIA; Cambridge Bioscience, Worcester, MA). One out of 10 positive and one out of 25 negative HIV EIA tests were routinely confirmed by HIV-1 Western immunoblotting (Novapath HIV-1 Western Blot Kit; BioRad Novapath, Hercules, CA).

After excluding active tuberculosis by clinical, chest radiographic, and sputum examination, eligible subjects were randomly assigned to preventive therapy or placebo and followed prospectively for the development of tuberculosis. Subjects were evaluated in the study clinic monthly during preventive therapy and every 3 mo thereafter. Follow-up examinations included chest radiography, symptom survey, and yearly DTH skin testing. MTB culture filtrate-stimulated *in vitro* whole-blood culture for generation of supernatants for cytokine assay and serial CD4 lymphocyte counts was performed in subjects enrolled in the placebo arms of the preventive therapy trial. Detailed information about study enrollment and follow-up procedures has been published elsewhere (5).

In Vitro Induction of MTB Culture Filtrate–stimulated IFN-γ and TNF-α

The whole-blood culture method (19, 20) was used to measure *in vitro* induction of IFN-γ and TNF-α. Peripheral blood was collected in sterile Vacutainer tubes anticoagulated with lithium heparin (Becton-Dickinson). The blood was diluted with RPMI-1640 medium with 1 mM HEPES buffer, penicillin, and streptomycin in 24-well Corning tissue culture plates (Corning, Corning, NY). MTB culture filtrate (MTBCF), prepared in the laboratory of Dr. Robert Wallis (21), was added to selected wells at a final concentration of 5 μg/ml. Selected wells were left unstimulated as a negative quality control to detect accidental contamination of the cell cultures or spontaneous production of IFN-γ

and TNF-α. The plates were then cultured at 37° C in 5% CO_2 in air. Culture supernatants were harvested after 24 h for TNF-α assay and after 72 h of incubation for IFN-γ assay. Samples were stored at −80° C until assay. IFN-γ and TNF-α were measured by enzyme-linked immunosorbent assay using commercially available kits (IFN-γ; Endogen, Worcester, MA, and TNF-α; R&D Systems, Minneapolis, MN) following the manufacturer's instructions. The sensitivity of the enzyme immunoassays for IFN-γ and TNF-α were 15 and 4.4 pg/ml, respectively. IFN-γ and TNF-α levels in the unstimulated negative control wells were 2 ± 11 pg/ml (range, 0 to 25 pg/ml) and 20 ± 4 pg/ml (range, 15 to 27 pg/ml), respectively.

Statistical Analysis

First, we determined the prevalence of DTH anergy (both PPD and candida skin test reactions = 0 mm) among all subjects screened for the tuberculosis preventive therapy trial, based on their initial skin test results. Second, all HIV-infected anergic and PPD-positive (PPD ⩾ 5 mm) subjects (as determined at screening) enrolled in the placebo arms of the parent preventive therapy study were compared by baseline demographic, clinical, and laboratory characteristics. Third, we determined the stability of DTH testing among initially PPD-positive and anergic HIV-infected subjects in the placebo arms of the preventive therapy trial. We also examined the association of reversion of anergy (PPD and/or candida > 0 mm) and initial PPD-positivity with factors measured at baseline and during follow-up. Finally, for a subset of subjects for whom *in vitro* MTBCF-stimulated, whole-blood cultures for cytokine expression were performed, we compared levels of MTBCF-stimulated IFN-γ and TNF-α in culture supernatants at baseline and at the time of subsequent DTH testing.

Statistical significance among groups was assessed using χ^2, χ^2 for trend, Student's *t* test, and analysis of variance. The paired *t* test was used for comparison of parameters among reverters and subjects with false negative PPD skin tests before and after changing their DTH status. All statistical tests were two-tailed.

RESULTS

Prevalence of Anergy

Between March 1993 and April 1995, 9,095 adults were screened for the tuberculosis preventive therapy trial at five clinics and voluntary HIV testing and counseling centers by skin testing with PPD and candida antigens. Seven thousand six-hundred ten (84%) subjects returned for skin test reading and further evaluation. HIV testing was performed after skin test reading for PPD-positive and anergic subjects. Ten percent of all subjects skin tested had PPD reactions of < 5 mm induration and were not anergic (candida > 0 mm). These subjects were not studied further. Among all subjects skin tested, 4,086 were HIV-infected and 682 were HIV-non-infected, and in 4,237, the HIV serostatus was unknown. Skin test reactivity to PPD and candida by HIV status is presented in Figure 1. Of the 4,058 HIV-positive subjects with complete PPD and candida skin test results, 27% were anergic. Among 682 HIV-non-infected subjects, the prevalence of anergy was 10%, and in 2,877 subjects of unknown HIV status, 19%.

Baseline Characteristics

Of the 9,095 screened subjects, 2,736 HIV-positive subjects were enrolled in the parent Phase III tuberculosis preventive therapy trial. Of the 2,736 enrolled, 786 were randomized to the placebo arms of the trial. Baseline demographic and clinical characteristics of these anergic (n = 322) and PPD-positive (n = 464) subjects are presented in Table 1. Absolute lymphocyte counts, CD4 lymphocyte counts, serum β_2 microglobulin, and serum neopterin levels at baseline were available for 780, 731, 676, and 200 subjects, respectively. PPD-positive subjects had higher mean absolute CD4 lymphocyte counts and body mass index than did anergic subjects ($p < 0.05$). The proportion of subjects with CD4 lymphocyte counts less than 200 μL^{-1} was greater among anergic subjects (39%) than among PPD-positive subjects (20%) ($p = 0.001$, χ^2 for trend). The odds of anergy in HIV-infected subjects increased with declining baseline CD4 lymphocyte count. The odds of anergy in subjects with baseline CD4 counts of 200 to 499 μl^{-1} were one and one-half times greater compared with those in the reference group of HIV-infected subjects with CD4 counts ⩾ 500

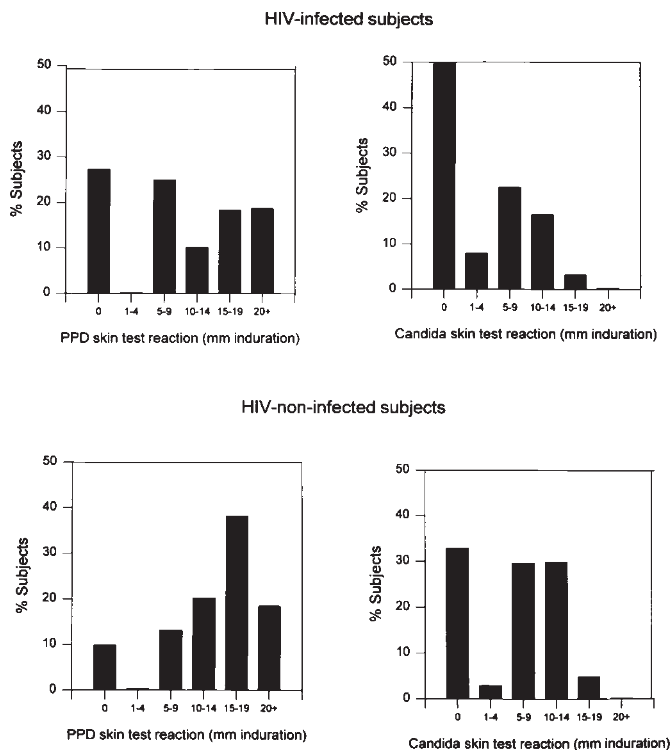

Figure 1. Initial PPD and candida skin testing results for all subjects screened with known HIV infection status. Four thousand fifty-eight HIV-infected subjects had PPD test results available and 3,242 had candida skin test results available. Among 682 HIV-non-infected subjects, 682 and 507 had PPD and candida skin test results available, respectively.

TABLE 1

BASELINE CHARACTERISTICS OF ANERGIC AND PPD-POSITIVE HIV-INFECTED STUDY SUBJECTS

Characteristic	Anergic (*n = 322*)	PPD-positive (*n = 464*)	p Value
Age, yr*	30 ± 7	30 ± 7	0.81†
Male sex, %‡	102 (32)	146 (32)	0.95†
PPD skin test, mm*	0	13 ± 6	—
Candida skin test, mm*	0	6 ± 5	—
Body mass index, kg/m^{-2}*	22 ± 3	23 ± 4	0.001†
Hemoglobin, g/dl*	12.5 ± 1.8	12.6 ± 1.9	0.29†
Absolute lymphocyte count, mm^{-3}*	2,006 ± 872	2,252 ± 837	0.0001†
CD4 lymphocyte count, μl^{-1}*	410 ± 480	563 ± 453	0.0001†
< 200 μl^{-1}, %‡	117 (39)	88 (20)	0.001§
200–499 μl^{-1}, %‡	93 (31)	140 (33)	
⩾ 500 μl^{-1}, %‡	91 (30)	202 (47)	
Serum β_2 microglobulin, mg/dl*	5.5 ± 3.1	4.8 ± 2.6	0.002†
Serum neopterin, mg/dl*	7.1 ± 7.9	5.9 ± 4.8	0.18†

* Values are means ± SD.
† By *t* test.
‡ Percentages are shown in parentheses.
§ χ^2 for trend.

μL^{-1} (OR = 1.5; 95% CI: 1.0 to 2.2). The odds of anergy were 3-fold higher in HIV-infected subjects with CD4 counts < 200 μL^{-1} compared with those in subjects with CD4 counts ⩾ 500 μL^{-1} (OR = 3.0; 95% CI: 2.0 to 4.3). The mean serum β_2-microglobulin level, a marker of increased risk of progression to AIDS (22), also was significantly higher among anergic subjects ($p < 0.05$). A greater proportion of anergic subjects presented with another concomitant HIV-associated opportunistic infection (oral thrush, herpes zoster, or genital sores) or a history of AIDS-associated papular dermatitis than did PPD-reactive subjects ($p < 0.05$).

Stability of Anergy

DTH testing was repeated in 139 subjects who were anergic at baseline. The median duration of time between baseline and subsequent DTH testing was 568 d (interquartile range, 420 to 644 d). Fifty-nine (42%) of the 139 initially anergic subjects were no longer anergic (PPD > 0, n = 29; candida > 0 mm, n = 14; both PPD and candida > 0, n = 16) when skin testing was repeated. Thirty-nine (66%) of 59 reverters had a PPD ⩾ 5 mm on repeat testing.

The baseline CD4+ lymphocyte count was not associated with stability of anergy (p = 0.4, χ^2) (Table 2). Subjects with AIDS-associated papular dermatitis at baseline and subjects who developed one or more opportunistic infections (oral thrush, herpes zoster, or genital sores) occurring between initial and repeat skin testing were more likely to remain anergic ($p < 0.05$, χ^2) (Table 2). Baseline sex, age, body mass index, hemoglobin, absolute lymphocyte count, and serum β_2-microglobulin and neopterin levels were not associated with stability of anergy.

Stability of a Positive PPD Skin Test

Of the 313 initially PPD-positive subjects who had at least one subsequent DTH test performed, 203 (65%) remained PPD-positive, 94 (30%) became PPD-negative and remained negative, and in 16 (5%) the PPD test changed back and forth between positive and negative. The median time between baseline and follow-up DTH skin testing was 591 d (interquartile range, 367 to 757 d).

Thus, a total of 110 initially PPD-positive subjects developed a negative PPD skin test (PPD < 5 mm) during follow-up ("false negative PPD test"). Of these, 81 (74%) had a negative candida test (0 mm) and 29 (26%) had a positive candida test at the same time.

Baseline CD4+ lymphocyte counts were available for 292 initially PPD-positive subjects. PPD-positive subjects with higher baseline CD4+ counts were less likely to have a negative PPD test on one of their subsequent evaluations (p = 0.001, χ^2 for trend) (Figure 2). Age, sex, body mass index, absolute white blood cell count, hemoglobin, serum β_2-microglobulin and neopterin, and the presence of a BCG scar were not associated with stability of an initially positive PPD skin test result. At follow-up, the level of serum β_2-microglobulin was higher in subjects who tested PPD-negative at the time of their first PPD-negative result (3.7 ± 2.5 mg/dl) compared with subjects who remained PPD-positive at the time of their last PPD test (3.0 ± 1.5 mg/dl, p = 0.01, *t* test). Hemoglobin levels were not different between the two groups at follow-up. Uganda is a region holoendemic for falciparum malaria, and all subjects reported at least one episode of malaria during follow-up. The frequency of reported episodes of malaria did not differ between reverters and nonreverters.

CD4 lymphocyte counts were available for 85 of the 110 initially PPD-positive subjects at the time of their first negative tuberculin test result and were not associated with candida skin test reactivity. Nineteen, 35, and 20% of subjects with CD4 counts of < 200, 200 to 499, and ⩾ 500 μL^{-1} and a negative repeat PPD, had candida skin test reactions ⩾ 1 mm (p = 0.3; χ^2 for trend).

In Vitro MTB Culture Filtrate–stimulated IFN-γ and TNF-α in Culture Supernatants of Initially Anergic and Initially PPD-positive Subjects

Baseline levels of IFN-γ and TNF-α were measured in supernatants from whole-blood cultures stimulated with MTBCF from 67 initially anergic and 48 initially PPD-positive subjects. At the time of repeat skin testing, MTBCF-stimulated IFN-γ and TNF-α measurements were available for 52 initially anergic subjects. MTBCF-stimulated IFN-γ and TNF-α levels were available for 56 and 50 initially PPD-positive subjects, respectively.

TABLE 2

FACTORS ASSOCIATED WITH STABILITY OF THE ANERGIC STATE IN HIV-INFECTED SUBJECTS ON SUBSEQUENT DTH TESTING

Variable	Remained Anergic (*n* = 80) (*n*)	(%)	Reverters* (*n* = 59) (*n*)	(%)	p Value†
CD4 count ⩽ 200 μl^{-1}	19	24	11	17	0.4
Pruritic papular rash at baseline	38	48	10	17	0.01
Interim opportunistic infection‡	31	39	12	20	0.02

* Reversion from PPD = 0 mm, candida = 0 mm to PPD and/or candida > 0 mm.
† χ^2 test.
‡ Occurrence of one or more of the following conditions at one or more interim visits: thrush, genital sores, herpes zoster.

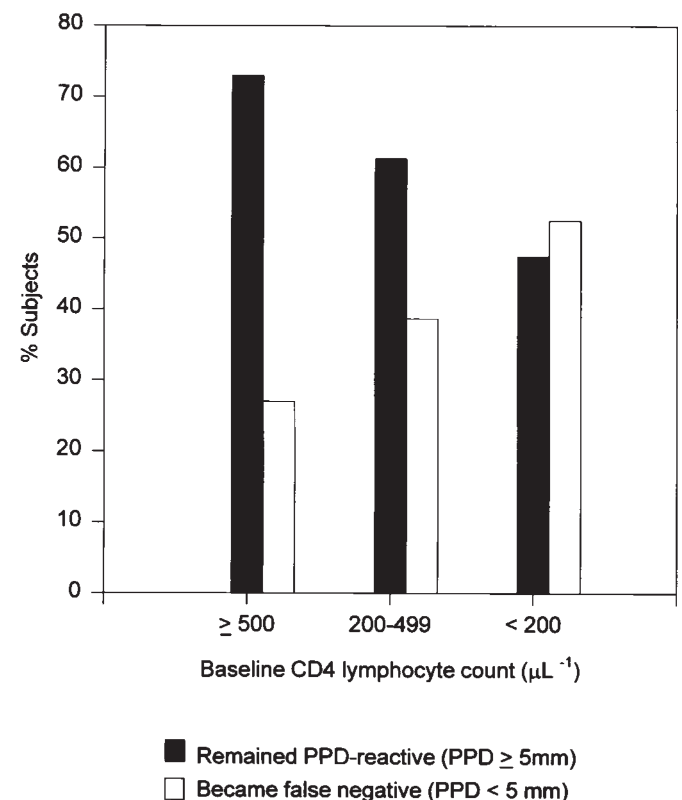

Figure 2. Stability of PPD skin testing among initially PPD-positive HIV-infected subjects, by CD4 lymphocyte count.

The mean baseline level of MTBCF-stimulated IFN-γ was significantly higher among the 27 initially PPD-positive subjects who remained PPD-positive during follow-up (2,047 pg/ml) than among the 21 subjects who became false negative (454 pg/ml), 14 subjects who reverted from anergy (295 pg/ml), and 53 subjects who remained anergic (99 pg/ml, p = 0.001, ANOVA) (Table 3). The differences between the latter three groups were not significant. Similarly, the mean level of MTBCF-stimulated IFN-γ at the time of repeat DTH testing was highest in subjects who remained PPD-positive (p = 0.08, ANOVA) (Table 3). There were no significant differences in levels of MTBCF-stimulated TNF-α between the four groups at baseline or at the time of subsequent skin testing (Table 3). Mean MTBCF-stimulated TNF-α levels were twofold higher at the time of a subsequent positive PPD test (1,746 ± 927 pg/ml) than at baseline (854 ± 793 pg/ml) among the 13 initially anergic reverters who had both baseline and follow-up TNF-α levels measured (p = 0.02, paired *t* test). The levels of MTBCF-stimulated IFN-γ did not change significantly before and after skin test reversion.

MTBCF-stimulated cytokine levels were measured at baseline and at follow-up in 14 subjects who changed their status from PPD-positive to PPD-negative. In these subjects, baseline levels of MTBCF-stimulated TNF-α (656 ± 661 pg/ml) were significantly lower than at the time when they first tested PPD-negative (1,448 ± 1,218 pg/ml, p = 0.05, paired *t* test). In these subjects, the change in levels of MTBCF-stimulated IFN-γ from baseline (729 ± 2,120 pg/ml) to the time of first negative PPD result (111 ± 215 pg/ml) was not statistically significant.

We also compared MTBCF-stimulated IFN-γ expression between subjects who remained PPD-positive and subjects who became PPD-negative, but remained reactive to candida antigen. MTBCF-stimulated TNF-α expression was measured in 31 subjects at baseline and 40 subjects at the time of repeat testing. MTBCF-stimulated IFN-γ measurements were available for 31 subjects at baseline and 38 subjects at follow-up testing. There were no significant differences between baseline levels of MTBCF-stimulated TNF-α between subjects who remained PPD-positive and those who subsequently became PPD-negative, but remained reactive to candida antigen. The mean level of baseline MTBCF-stimulated IFN-γ was significantly lower in four subjects who became PPD-negative (54 ± 62 pg/ml) compared with 27 subjects who remained PPD-positive (2,047 ± 3,030 pg/ml, p = 0.002, *t* test). At follow-up, the mean level of MTBCF-stimulated IFN-γ in the four subjects at the time of their first PPD-negative result was 168 ± 314 pg/ml compared with a mean level of 853 ± 1,658 pg/ml in 34 subjects who remained PPD-positive at the time of their last DTH test (p ± 0.04, *t* test).

DISCUSSION

The natural history of HIV infection is associated with progressive immunosuppression and diminished delayed-type hypersensitivity responses. Anergy to PPD and candida antigens was present at initial testing in 27% of the HIV-infected and 10% of HIV-non-infected subjects in our study population. Anergy was associated with lower CD4 lymphocyte counts, lower BMI, higher serum β_2-microglobulin levels, and previous AIDS-associated opportunistic infections or HIV-associated papular dermatitis.

Identifying HIV-infected persons who are infected with MTB is difficult because of the decreased sensitivity of tuberculin skin testing in HIV-infected persons (23–28). Anergy testing has been used as an adjunct to PPD skin testing in HIV-infected persons to identify those likely to benefit from INH preventive therapy despite the limited information available regarding the reliability of anergy testing in this population. Testing with recall antigens has been proposed as a means to allow the identification of HIV-infected persons with advanced HIV infection who are unable to express DTH responses to any antigen. Such subjects are termed anergic and have been targeted for preventive therapy based on observational studies suggesting a high risk for the development of active tuberculosis among anergic persons residing in areas with a high prevalence of tuberculosis or belonging to high risk groups such as intravenous drug users (29, 30). The utility of anergy testing would be less if this status was not stable and if patients fluctuated between anergy and nonanergy. The role of anergy testing in identifying and excluding tuberculous infection also must be tempered by recent data from two randomized clinical trials by Gordin and colleagues in the United States (31) and our trial in Uganda (5) that demonstrated no protective efficacy of isoniazid preventive therapy against tuberculosis in anergic HIV-infected adults.

Our data confirm those of Chin and colleagues (8) who noted reversion of anergy on follow-up skin testing in 30% of HIV-infected subjects in the United States. We observed that 42% of anergic Ugandan adults who underwent follow-up skin testing with PPD and candida reverted to a nonanergic status. Two thirds of the reverters had a PPD ⩾ 5 mm on follow-up skin testing. The rate of reversion of anergy in our East African study population is higher than the 15% rate reported in a predominantly inner city intravenous-drug-using population followed in Baltimore (32) and that described by Chin and colleagues (8) and is likely due to a lesser degree of immunosuppression at baseline in our study cohort and/or possibly to greater transmission of tuberculosis in crowded urban settings in sub-Saharan Africa. In our study, subjects who re-

TABLE 3

IFN-γ AND TNF-α LEVELS IN MTBCF-STIMULATED WHOLE BLOOD CULTURE SUPERNATANTS FROM INITIALLY PPD-POSITIVE AND ANERGIC SUBJECTS AT BASELINE AND AT THE TIME OF SUBSEQUENT PPD AND CANDIDA ANTIGEN SKIN TESTING*

Delayed-type Hypersensitivity (DTH) Status		MTBCF-stimulated IFN-γ (*pg/ml*)				MTBCF-stimulated TNF-α (*pg/ml*)			
Baseline	Follow-up	Baseline†	n	Follow-up‡	n	Baseline	n	Follow-up	n
PPD ⩾ 5 mm	PPD ⩾ 5 mm	2,047 ± 3,030	27	852 ± 1,658	34	641 ± 660	27	1,717 ± 1,264	36
PPD ⩾ 5 mm	PPD < 5 mm	454 ± 1,607	21	336 ± 1,037	21	535 ± 592	21	1,511 ± 1,110	22
Anergic	Nonanergic	295 ± 773	14	374 ± 1,042	22	854 ± 793	13	1,594 ± 921	20
Anergic	Anergic	99 ± 266	53	115 ± 381	30	914 ± 985	53	1,130 ± 922	30

* Values are means ± SD.

† p = 0.001, ANOVA, comparing means of MTBCF-stimulated IFN-γ at baseline among the four DTH groups.

‡ p = 0.08, ANOVA, comparing means of MTBCF-stimulated IFN-γ at follow-up among the four DTH groups.

verted were less likely to have baseline CD4 counts $\leqslant 200\ \mu l^{-1}$ and less likely to have had other intercurrent opportunistic infections during the time interval between skin tests. We found no significant difference in MTBCF-stimulated TNF-α or IFN-γ levels in *in vitro* whole-blood culture supernatants at baseline or follow-up testing between persons who remained anergic and those who were PPD-positive on follow-up testing.

Another alternative explanation for reversion from the anergic to the PPD-positive state is boosting whereby the initial PPD skin test stimulates cell-mediated immune memory responses that had waned over time in an individual previously infected with MTB. Boosting from an initial PPD < 5 mm to a PPD $\geqslant 5$ mm occurred in 29% of HIV-infected Ugandan adults who underwent repeat PPD testing 1 wk later in an earlier study and was associated with higher CD4 count and body mass index (18). It is unlikely that the booster effect accounts for reversions to PPD positivity in our study for the following reasons. Boosting is less frequent when longer periods of time occur between initial and repeat testing (33). In our study the mean time between DTH testing was 588 d in reverters and 567 d in nonreverters. Furthermore, we found no relationship between baseline and follow-up CD4 lymphocyte counts and reversion of anergy. The decline in CD4 lymphocyte count over time did not differ between reverters and nonreverters. We cannot, however, exclude possible roles for exogenous reinfection and differences in exposure to environmental mycobacteria and candida species in reversion from anergy based on our data.

We also studied the stability of positive tuberculin reactions on follow-up skin testing results in subjects known to be PPD-reactive at baseline. Thirty-five percent of all PPD-positive subjects who were retested were PPD-negative ("false negative PPD test") on repeat testing. Thirty percent remained consistently PPD-negative; however, the PPD skin test in the other 5% fluctuated between positive and negative over time. Importantly, 26% of subjects with a negative PPD test were still reactive to candida antigen at the time of the negative PPD skin test and would have been misclassified as not being infected with MTB. MTBCF-stimulated IFN-γ levels were higher at baseline in subjects who remained PPD reactive than those who were falsely negative at repeat testing.

The whole-blood culture method used in our study measures cytokine production by both lymphocytes and monocyte cell populations. The main source of TNF-α production is the monocyte-macrophage population and the high levels of TNF-α measured in the culture supernatants after stimulation with MTB culture filtrate reflect the capacity for induction of TNF expression by monocytes and macrophages. Our data are consistent with the hypothesis that during the course of HIV infection there is progressive loss of antigen-specific (i.e., MTBCF) T-cell responses, leading to impaired interferon gamma expression, despite relative preservation of nonspecific inflammatory responses exemplified by the release of TNF-α by activated monocyte-macrophages. Baseline levels of MTBCF-stimulated IFN-γ were highest in subjects who remained PPD-reactive during follow-up ($p = 0.001$, ANOVA) (Table 3). The changes in IFN-γ expression we observed in MTBCF-stimulated cell cultures over time were most marked in subjects who were PPD-positive on initial and follow-up skin testing and may represent clonal deletion of activated T-cells via apoptosis in the setting of immune surveillance of latent tuberculosis infection. The decline in MTBCF-stimulated IFN-γ expression present before decreased DTH skin test responses also may reflect susceptibility to reactivation of tuberculosis at relatively high CD4 counts. These hypotheses warrant further study.

Our study has several important limitations. Because of the screening algorithm of our original tuberculosis preventive therapy study, we did not perform HIV testing until after skin testing. We do not, therefore, know the HIV infection status of nonanergic subjects with small PPD reactions and also did not follow those in this group prospectively. We were, however, able to prospectively follow a large group of PPD-positive and anergic HIV-infected adults residing in an area with a high prevalence of tuberculosis and examine the stability of PPD and candida skin reactions over time. All skin testing was done by five highly experienced skin testers without knowledge of previous results to minimize ascertainment bias and interobserver variation. We also used only one recall antigen during skin testing in contrast to other studies using multiple antigens. Because of logistical constraints, we were able to perform *in vitro* cell cultures on only a subset of subjects. Nonetheless, this is the first time that these *in vitro* correlates have been studied prospectively in relationship to DTH skin testing.

Our data are concordant with those of Chin and colleagues and other investigators and they confirm the unreliability of concomitant anergy testing with candida antigen as a means to identify or exclude prior infection with MTB in HIV-infected persons. Nearly one half of anergic subjects were nonanergic on follow-up testing. Just as importantly, candida skin tests were positive in one quarter of those with false negative PPD skin tests, based on a previously known positive PPD skin test. Our data support recently revised U.S. national guidelines, which no longer recommend the routine use of anergy testing in conjunction with PPD skin testing for screening HIV-infected persons for infection with MTB (9). On the basis of our earlier study of boosting in HIV-infected adults (18), two-step skin testing of HIV-infected adults who are initially PPD-negative by repeat PPD testing 1 wk after the initial test, when logistically feasible, may be a more useful strategy to improve the sensitivity of tuberculin testing in HIV-infected adults.

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