MAJOR ARTICLE

Incomplete Immune Reconstitution after Initiation of Highly Active Antiretroviral Therapy in Human Immunodeficiency Virus–Infected Patients with Severe $CD4^+$ Cell Depletion

Howard M. Lederman,[1] Paige L. Williams,[3] Julia W. Wu,[3] Thomas G. Evans,[4,a] Susan E. Cohn,[4] J. Allen McCutchan,[6] Susan L. Koletar,[8] Richard Hafner,[2] Elizabeth Connick,[9] Fred T. Valentine,[5] M. Juliana McElrath,[10] Norbert J. Roberts, Jr.,[11] and Judith S. Currier,[7] for the AIDS Clinical Trials Group 889 Study Team[b]

[1]Eudowood Division of Pediatric Allergy and Immunology, Johns Hopkins University School of Medicine, Baltimore, and [2]Treatment Research Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; [3]Center for Biostatistics in AIDS Research, Harvard School of Public Health, Boston, Massachusetts; [4]Infectious Diseases Unit, University of Rochester Medical Center, Rochester, and [5]New York University School of Medicine, New York; [6]Division of Infectious Diseases, University of California, San Diego School of Medicine, San Diego, and [7]David Geffen School of Medicine, University of California Los Angeles, Los Angeles; [8]Division of Infectious Diseases, Ohio State University, Columbus; [9]Division of Infectious Diseases, University of Colorado Health Sciences Center, Denver; [10]University of Washington School of Medicine, Seattle; [11]University of Texas Medical Branch, Galveston

(See the editorial by Hengel and Kovacs, on pages 1791–3.)

Immune function was observed for 144 weeks in 643 human immunodeficiency virus (HIV)–infected subjects who (1) had nadir $CD4^+$ cell counts of <50 cells/mm^3, followed by a sustained increase to ⩾100 cells/mm^3 after the initiation of HAART, and (2) were enrolled in a randomized trial of continued azithromycin prophylaxis versus withdrawal for prevention of *Mycobacterium avium* complex disease. The median $CD4^+$ cell count was 226 cells/mm^3 at entry and 358 cells/mm^3 at week 144. Anergy (80.2% of patients) and lack of lymphoproliferative response to tetanus toxoid (TT; 73%) after immunization and impaired antibody responses after receipt of hepatitis A (54%) and TT (86%) vaccines were considered to be evidence of impaired immune reconstitution. Receipt of azithromycin did not have an effect on $CD4^+$ cell count but was associated with higher rates of delayed-type hypersensitivity responses to TT (25% of subjects who received azithromycin vs. 15% of those who did not; $P = .009$) and mumps skin test antigen (29% vs. 17%; $P = .001$). Although the subjects had only partial responses to immune function testing, the rate of opportunistic infections was very low, and none of the tests was predictive of risk.

Highly active antiretroviral therapy (HAART) dramatically decreases the rate of AIDS-related opportunistic infections (OIs) and deaths [1, 2]. This protection suggests that increases in $CD4^+$ cell counts in patients receiving HAART reflect restoration of pathogen-specific immunity, even among those with a history of severe immunosuppression [3, 4]. Restoration of in vitro immune responses to microbial antigens after initiation

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[a] Present affiliation: Vical Corporation, San Diego, California.

[b] Study group members are listed at the end of the text.

Reprints or correspondence: Dr. Howard M. Lederman, Div. of Pediatric Allergy and Immunology, Johns Hopkins Hospital, CMSC 1102, Baltimore, MD 21287-3923 (hlederm1@jhem.jhmi.edu).

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of HAART has been documented in the short term [5], although it is often incomplete [6–8]. Longer-term changes in measures of immunologic function associated with HAART in patients with a history of severe immunosuppression have not been well described.

We studied in vitro and in vivo measures of immune function in patients followed up prospectively after initiation of HAART. A subset of patients were studied more intensively, with additional in vitro immunologic tests, including lymphocyte proliferation assays (LPAs) and 3-color flow cytometry. The objectives were to determine whether the results of those assays correlated with risk of *Mycobacterium avium* complex (MAC) disease or other OIs and whether the results of flow cytometry or measurement of HIV-1 RNA levels correlated with LPA or antibody responses to vaccine antigens. The relatively large number of subjects enrolled provided an important opportunity to study immune reconstitution after initiation of HAART during a period of almost 3 years and to determine which tests of immune function are most helpful in assessing immunocompetence.

SUBJECTS AND METHODS

Study population. We studied measures of immunologic function associated with HAART in a sample of 643 human immunodeficiency virus (HIV)–infected patients enrolled between October 1997 and April 1999 at 29 sites in a randomized, double-blind, placebo-controlled trial (AIDS Clinical Trials Group [ACTG] 362) to evaluate the efficacy of continued azithromycin prophylaxis versus withdrawal for the primary prevention of MAC infection and other OIs. The design and results of the study have been described in detail elsewhere [3]. In brief, all subjects had a history of having a $CD4^+$ cell count of <50 cells/mm^3, followed by a subsequent sustained increase in the $CD4^+$ cell count to $\geqslant$100 cells/mm^3, after initiation of potent antiretroviral therapy. One objective of the study was to measure immunologic function in patients with advanced AIDS who had responded to HAART with a significant increase in $CD4^+$ cell counts and to determine whether the results of any of the tests we performed would correlate with risk of developing MAC disease or other OIs.

All patients enrolled in the study met the criteria at study entry for receipt of MAC prophylaxis. Study participants were assessed at weeks 4 and 8 of the study and at 8-week intervals thereafter. After November 1999, sites were instructed to discontinue blinded treatment with azithromycin or placebo [3], and the study was amended to increase the interval between visits to 16 weeks. Evaluations made at each visit included clinical examination, immunologic assessments, and performance of blood cultures for MAC. For the purpose of this analysis, data from October 1997 through October 2001 were considered. In addition, only the first 144 weeks of follow-up data for each individual were included, because sample sizes were too small during the longer follow-up period.

Immunologic and virologic assessments. At entry, serum antibodies to tetanus toxoid (TT) and hepatitis A virus were measured, and delayed-type hypersensitivity (DTH) responses to *Candida albicans* antigen (Candin; ALK Laboratories), mumps skin test antigen (MSTA; Connaught), and TT US Pharmacopeia fluid (Aventis Pasteur) were assessed through intradermal skin testing. Skin tests were read after 48–72 h, using the ballpoint pen technique [9]. The results of DTH tests were considered to be positive if the area of induration (transverse $\times$ longitudinal) was $\geqslant$25 mm^2. "Anergy" was defined as negative results of DTH tests with *Candida* antigen, MSTA, and TT.

All subjects were immunized with TT vaccine (Connaught; 5 level of flocculation units/0.5 mL) at week 4, unless they had received this immunization within the previous 12 months. Hepatitis A virus–seronegative subjects were also immunized with hepatitis A vaccine (Havrix; SmithKline Beecham; 1440 ELISA units/0.5 mL) at weeks 16 and 40. Levels of antibodies to TT and hepatitis A virus were reassessed at weeks 24 and 48; DTH responses were reassessed at week 24.

Antibodies to hepatitis A virus were measured by a modification of the Abbott HAVAB EIA. Serum samples were screened in duplicate, and positive samples were run again at 3 dilutions based on the optical density of the initial positive value; antibody concentrations were derived from a standard, using dilutions of the World Health Organization reference standard (100 IU). The lower limit of sensitivity of the assay was 0.05 IU/mL, and the upper limit was 150 IU/mL. A positive test result was defined by the standards included with the kit, as specified by the package instructions.

Anti-TT IgG antibodies were measured by EIA, using TT, unadsorbed (Lederle Laboratories), as antigen [11]. A standard curve was established using the International Standard for Tetanus Immunoglobulin (World Health Organization International Laboratory for Biological Standards, Statens Serum Institute, Copenhagen). Antibody concentrations were calculated using linear regression, and the results were expressed as international units per milliliter. The lower limit of detection was 0.01 IU/mL, and a level of 0.1 IU/mL was considered to be protective.

Plasma HIV-1 RNA levels were measured using the Roche Amplicor 1.0 standard assay (lower limit of detection, 500 copies/mL) at study entry, at week 8 of the study, and at 16-week intervals thereafter.

Adherence to HAART was assessed by study personnel at each scheduled visit [12]. We considered a subject to be "nonadherent" at a particular scheduled visit if (1) neither protease inhibitors nor other antiretroviral therapy had been prescribed for that subject or (2) antiretroviral medications had been prescribed but the subject had missed $\geqslant$1 dose in the preceding 48 h.

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A subset of subjects were coenrolled in a prospective, observational in-depth study of immunologic function (ACTG 889). Patients from 18 of the sites were eligible to enroll simultaneously with entry into the larger study, as were any subjects who developed disseminated MAC disease during study follow-up. After November 1999, the study was amended to allow all patients who developed an OI during follow-up to enter this intensive immunologic study. Subjects enrolled in the in-depth immunologic study underwent additional in vitro immunologic tests at entry and at 24-week intervals or at the onset of OI, if applicable.

Lymphocyte subsets were enumerated in whole blood by 3-color flow cytometry, using directly labeled murine monoclonal antibodies (PharMingen). Two-color and 3-color flow cytometry were performed according to ACTG consensus immunology protocols [10].

LPAs were performed according to ACTG consensus immunology protocols [10]; responses to phytohemagglutinin A (PHA; Sigma Chemical Company; 5 μg/mL), pokeweed mitogen (PWM; Sigma Chemical Company; 5 μg/mL), *C. albicans* (Greer Laboratories; 10 μg/mL), TT (Wyeth-Lederle; 1 μg/mL), and *M. avium* sensitin (MAS; Staten Seruminstitut; 0.5 and 1.0 μg/mL) were measured. Results are expressed as stimulation index (SI). SI was the ratio of counts per minute with mitogen or antigen to counts per minute without antigen, where the SI was never assigned a value <1. A positive test result was defined as an SI of >10 for the mitogens PHA and PWM and of >5 for the *Candida* antigen, TT, and MAS.

Informed consent. The study protocol was approved by the institutional review board at each participating ACTG site, and each patient provided written informed consent before enrollment. Clinical research was conducted in accordance with the guidelines for human experimentation specified by the US Department of Health and Human Services and by the institutions that participated in this study.

Statistical analysis. The immunologic substudy ACTG 889 was designed to enroll 200 subjects to provide at least 80% power for detecting a relative risk of $\geqslant 3.0$ in the development of MAC disease or another AIDS-defining illness, based on estimated yearly rates of 22% and 8% for those without and with a particular positive immunologic response at entry. In these power calculations, it was assumed that subjects would be accrued over the course of 1 year and followed up for an additional 1.5 years and that the percentage of subjects lost to follow-up or for whom immunologic data were missing would be ~20%. In addition, it was assumed that the proportion of subjects with a positive immunologic response at entry would be $\geqslant 30\%$. In practice, despite the longer period of accrual and follow-up, the actual power for detecting a relative risk of $\geqslant 3$ was lower because of the overall rarity of AIDS-defining illnesses.

The primary statistical analysis of the immunologic objectives was based on comparison of the rate of AIDS-defining illnesses among subjects with and without a positive immunologic response to each test at entry; significance was calculated using a standard log-rank test. The distributions of baseline $CD4^+$ cell counts were compared, using a Wilcoxon rank sum test, among subgroups of subjects defined by baseline DTH response or response to vaccines. Immunologic response rates to DTH tests were compared between treatment regimens using Fisher's exact test. For immunologic outcomes for which there were repeated measurements over time (hepatitis antibody responses, LPA responses, and flow cytometric measures), a generalized estimating equation (GEE) model was used to evaluate the impact of treatment and other baseline covariates ($CD4^+$ cell count, HIV-1 RNA detectability, Karnofsky score, and the duration of previous HAART at study entry), while taking into account the correlation among repeated measurements for the same subject over time [13]. The GEE model also allowed evaluation of trends over time in immunologic responses. In evaluating the effects of azithromycin treatment on LPA responses and flow cytometric results, only data from weeks 0, 24, and 48 were used, because the majority of subjects were still receiving blinded treatment at that point and the number of subjects evaluated thereafter was small. For binary outcomes, a logistic link was used, under the assumption that equal correlation existed between any pair of measurements for the same subject. For outcomes measured at a single time point (anti-TT antibody response and DTH responses), standard logistic regression models were fit. Because so many statistical comparisons were conducted, the P values presented should be considered to be exploratory and interpreted with caution. $P < .05$ was considered to be statistically significant.

RESULTS

Baseline characteristics of patients. Six hundred forty-four patients were enrolled between October 1997 and April 1999. One ineligible subject was inadvertently enrolled as a result of a site processing error, leaving 643 eligible subjects. During the same time period, 200 subjects, including the ineligible subject, were enrolled in the in-depth study of immunologic function, leaving 199 subjects. Among all subjects at entry, 87% were male, 85% had no history of injection drug use, the median age was 40 years, the median Karnofsky score was 90, and the median duration of previous HAART was 40 weeks (table 1). At entry, the median baseline $CD4^+$ cell count was 226 cells/mm^3; the lowest preentry $CD4^+$ cell count was 20 cells/mm^3. Among subjects for whom virus load data were available, 64% had undetectable loads ($\leqslant 500$ copies/mL). Fifty-eight percent of subjects were anergic in response to a panel of DTH skin test antigens at entry. These factors did not differ significantly

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Table 1. Demographic and clinical characteristics at baseline of subjects included in a study of immune reconstitution after initiation of highly active antiretroviral therapy (HAART).

Characteristic	Parent study (*n* = 643)	Subjects in immunologic substudy: Total (*n* = 199)	Azithromycin (*n* = 94)	Placebo (*n* = 105)	Subjects with AIDS-defining illness (*n* = 28)
Male sex, % of subjects	87	87	91	83	79
No history of injection drug use, % of subjects	85	83	85	81	89
Age, median years	40	40	41	39	40
$CD4^+$ cell count, median cells/mm^3	226	228	236	222	234
Lowest preentry $CD4^+$ cell count, median cells/mm^3	20	19	18	23	23
HIV-1 RNA level,[a] median copies/mL	⩽500	⩽500	⩽500	⩽500	1113
Undetectable HIV-1 RNA level,[a] % of subjects	64	65	63	66	37
Karnofsky score, median	90	90	90	90	90
Receipt of previous MAC prophylaxis, % of subjects	64	66	68	66	71
Duration of previous HAART, median weeks	40	40	40	40	35
Anergy,[b] % of subjects *(n/N)*	58 (353/611)	55 (105/192)	49 (44/90)	60 (61/102)	64 (16/25)

NOTE. HIV-1, human immunodeficiency virus type 1; MAC, *Mycobacterium avium* complex.

[a] The lower level of detection of the assay was 500 copies/mL.

[b] "Anergy" was defined as negative delayed-type hypersensitivity skin test responses to *Candida* antigen, tetanus toxoid, and mumps skin test antigen. Data were not available for all subjects. *n/N,* no. of subjects with anergy/no. for whom data were available.

between subjects enrolled in the parent study and those enrolled in the intensive immunologic substudy, between those receiving azithromycin and those receiving placebo, or between those who developed AIDS-defining illnesses during the trial and those who did not.

Changes in $CD4^+$ lymphocyte subpopulations over time. The mean $CD4^+$ cell count increased over the course of follow-up (figure 1). It was estimated, by fitting a repeated-measures GEE model, that the mean $CD4^+$ cell count increased by 19 cells for every 24 weeks ($P < .0001$). The median number of naive $CD4^+$ cells was 54 cells/mm^3 at entry; this number increased to 93 cells/mm^3 at week 144. During the same time period, the median number of memory $CD4^+$ cells increased from 157 to 234 cells/mm^3. The percentage of naive $CD4^+$ cells increased slightly, from 26% at entry to 33% at week 144. The median number of resting $CD4^+$ T cells increased from 194 to 315 cells/mm^3, and the median number of activated $CD4^+$ cells increased from 23 to 28 cells/mm^3. The percentage of activated $CD4^+$ cells remained constant (10% at entry and 9% at week 144).

Responses to DTH skin tests. Fifty-eight percent of subjects were anergic at entry in response to DTH skin tests, and 46.7% were anergic at week 24. The decrease in the percentage of anergic subjects is largely explained by an increase in positive responses to TT, from 5.1% to 19.8% of tested subjects, although increases also were seen in the percentage of subjects with positive responses to *Candida* antigen (from 31.9% to 37.6%) and MSTA (from 16.6% to 22.8%). Among subjects who were immunized with TT vaccine, the percentage with positive DTH responses to TT increased from 5% to 19% ($P < .0001$), whereas the changes in the percentages of subjects with positive responses to *Candida* antigen (from 30.7% to 37.4%) and MSTA (from 18.4% to 20.5%) were small.

Responses to LPAs. LPA responses were measured at 24-week intervals throughout the study; the number of subjects for whom LPA data were available ranged from 188 at week 0 to 61 at week 144. The percentage of subjects with positive responses varied for the stimulants tested but did not change significantly over the course of the study (for PHA, 84%–92%; for PWM, 81%–100%; for *Candida* antigen, 69%–71%; for MAS, 46%–47%; and for TT, 7%–14%). However, among those subjects who were immunized with TT vaccine, the percentage who had LPA responses to TT increased significantly between baseline and week 24 (from 8% to 27%; $P < .0001$) and between baseline and week 144 (from 8% to 29%; $P < .0001$).

Responses to vaccines. A total of 210 subjects were seronegative for hepatitis A virus antibodies at baseline, were immunized with hepatitis A vaccine, and then were retested at week 24. Of these subjects, only 59 (28%) seroconverted. By week 48, 76 subjects had received 2 immunizations with hepatitis A vaccine, and 35 (46%) had seroconverted. Three hundred forty-five subjects were immunized with TT vaccine and had measurements of anti-TT antibody for weeks 0 and 24. At week 0, 92% of subjects had protective levels of antibody (>0.1 IU/mL), and at week 24, 94% of subjects had protective levels of antibody. The median antibody level was 1.02 IU/mL at week 0 and 1.75 IU/mL at week 24. When vaccine response was defined as an increase in antibody levels of at least 4-fold over baseline, only 14% of subjects (47/345) responded to the TT booster vaccine. When response to tetanus vaccine was defined

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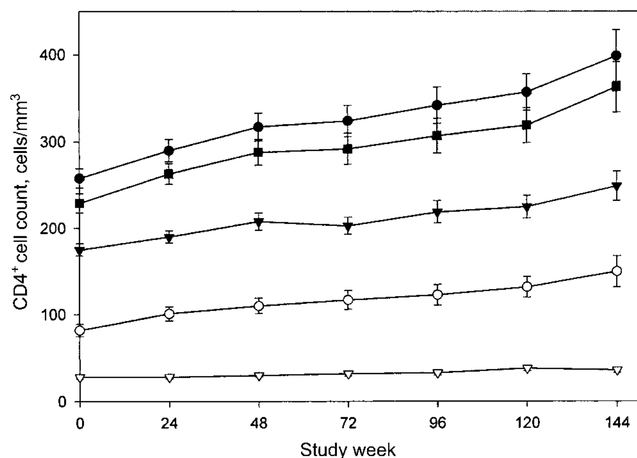

Figure 1. Changes in $CD4^+$ cell subpopulations over time among human immunodeficiency virus–infected patients. Results are mean cell count ± SE for each time point. *Black circles,* $CD4^+$ cells; *white circles,* naive ($CD45RA^+CD62L^+$) $CD4^+$ cells; *black triangles,* memory ($CD45RA^-CD45RO^+$) $CD4^+$ cells; *white triangles,* activated ($CD38^+$HLA-DR^+) $CD4^+$ cells; *black squares,* resting ($CD38^-$HLA-DR^-) $CD4^+$ cells. One hundred thirty-nine patients were tested at week 0, 173 at week 24, 138 at week 48, 107 at week 72, 90 at week 96, 74 at week 120, and 62 at week 144.

as an antibody level of ⩾1.0 IU/mL, 68% of subjects (236/346) responded after booster immunization.

Relationship between immunologic test results at baseline and subsequent risk for AIDS-defining illnesses. On the basis of immunologic test results at baseline, subjects were considered to be "responders" or "nonresponders" to each test and then were monitored prospectively for the development of AIDS-defining illnesses. Among all 643 subjects, 28 (18 subjects randomized to placebo and 10 randomized to azithromycin) developed ⩾1 new AIDS-defining illness (36 AIDS-defining illnesses total). The most common diagnoses were *Pneumocystis carinii* pneumonia (9 subjects), cytomegalovirus infection (7 subjects with retinitis, gastrointestinal disease, pneumonitis, and/or esophagitis), candidiasis (5 subjects), and MAC disease (3 subjects). At the time of development of an AIDS-defining illness, the mean $CD4^+$ cell count for the 28 subjects was 147 cells/mm^3 (range, 2–845 cells/mm^3). Eleven of the subjects (39%) developed the illness when they had $CD4^+$ cell counts of >200 cells/mm^3. Among the 199 subjects included in the in-depth immunologic study, 13 subjects (7 randomized to azithromycin and 6 randomized to placebo; $P = .709$, by log-rank test) developed ⩾1 AIDS-defining illness (20 AIDS-defining illnesses total).

DTH response at baseline, antibody response to the neoantigen, hepatitis A vaccine, antibody response to the recall antigen TT vaccine, and LPA responses at baseline were not helpful in predicting risk for subsequent AIDS-defining illness (table 2). Median $CD4^+$ cell counts at baseline for subjects who developed AIDS-defining illnesses were similar to those for subjects who did not develop such illnesses (234 vs. 226 cells/mm^3; $P =$ .805); so were counts of naive $CD4^+$ cells (50 vs. 55 cells/mm^3; $P = .986$), memory $CD4^+$ cells (181 vs. 157 cells/mm^3; $P =$ .986), and activated $CD4^+$ cells (19 vs. 23 cells/mm^3; $P = .499$). Subjects who developed AIDS-defining illnesses had higher baseline median HIV-1 RNA levels than did those who did not develop such illnesses (1113 vs. <500 copies/mL; $P < .01$).

Relationship between baseline $CD4^+$ cell count or baseline virus load and immune function. Higher $CD4^+$ cell counts were found in subjects with positive baseline DTH responses than in those with negative responses to TT (median, 275 vs. 222 cells/mm^3; $P = .009$), MSTA (median, 259 vs. 222 cells/mm^3; $P = .050$), and *Candida* antigen (231 vs. 217 cells/mm^3; $P = .216$), but for *Candida* antigen, the difference did not achieve statistical significance (table 3). Responders to the recall antigen TT vaccine at week 24 had higher baseline $CD4^+$ cell counts than did nonresponders (271 vs. 221 cells/mm^3; $P =$ .018). Similar trends were observed for responders and nonresponders to hepatitis A vaccine at week 24, although the results did not achieve statistical significance (254 vs. 215 cells/mm^3; $P = .066$).

The proportion of subjects with undetectable HIV-1 RNA levels was slightly higher among those with a positive DTH response than among those with negative responses to TT (76% vs. 67%) or MSTA (74% vs. 66%). However, proportions were not significantly different for any of the 3 antigens tested.

Comparison of DTH and LPA responses to TT. T cell

Table 2. Rates of AIDS-defining illnesses by immunologic status at baseline.

	No. of subjects with AIDS-defining illness/total no. with indicated test result (%)		
Immunologic test, antigen	Negative	Positive	P^a
Baseline DTH skin test			
Tetanus toxoid	25/581 (4)	0/31 (0)	.231
Mumps skin test antigen	19/521 (4)	7/104 (7)	.170
Candida antigen	19/420 (5)	7/197 (4)	.533
⩾1 of the above	16/353 (5)	9/258 (4)	.494
Week 24 antibody response			
Hepatitis A virus	8/194 (4)	3/69 (4)	.954
Tetanus toxoid	15/532 (3)	4/53 (8)	.222
Baseline LPA			
Tetanus toxoid	12/175 (7)	1/13 (8)	.943
Candida antigen	3/58 (5)	10/131 (8)	.478
Phytohemagglutinin A	4/31 (13)	9/157 (6)	.232
Pokeweed mitogen	3/30 (10)	9/132 (7)	.650
Mycobacterium avium sensitin	5/90 (6)	5/78 (6)	.675

NOTE. DTH, delayed-type hypersensitivity; LPA, lymphocyte proliferation assay.

[a] Calculated using the log-rank test.

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Table 3. Relationships between baseline $CD4^+$ cell count and the results of tests of immune function.

Time, test, antigen	Negative test result: Baseline $CD4^+$ cell count, median cells/mm^3	Negative test result: No. of subjects	Positive test result: Baseline $CD4^+$ cell count, median cells/mm^3	Positive test result: No. of subjects	P[a]
Baseline, DTH					
Tetanus toxoid	222	581	275	31	.009
Mumps skin test antigen	222	521	259	104	.050
Candida antigen	217	420	231	197	.216
Week 24					
DTH, tetanus toxoid[b]	221	227	240	47	.762
Antibody assay					
Hepatitis A virus[c]	212	162	254	61	.039
Tetanus[d]	220	298	270	47	.041

NOTE. DTH, delayed-type hypersensitivity.

[a] Calculated using the Wilcoxon rank sum test.

[b] Among subjects vaccinated against tetanus by week 24 who had negative baseline DTH responses to tetanus toxoid.

[c] Among subjects vaccinated against hepatitis A by week 24.

[d] Among subjects vaccinated against tetanus by week 24.

responses to antigen can be assessed in a number of ways, including by DTH skin tests and LPAs. A GEE model based on longitudinal data (weeks 0 and 24) showed a significant association between DTH and LPA responses to TT ($P =$ .001), but the significance was based almost entirely on the correlation between negative DTH and negative LPA responses (table 4). A positive result of either test was not predictive of a positive result of the other, and neither seemed to be consistently more likely to detect positive responses. The small number of AIDS-defining illnesses prevented us from assessing whether a positive DTH or LPA response was predictive of lower risk for such illnesses.

Effect of adherence to prescribed HAART regimen. We assessed adherence to potent antiretroviral therapy at week 48, because that was the last point at which a majority of subjects were still receiving blinded study treatment, and at week 144, because that was the point through which we had adequate follow-up data. Through week 48, 5.9% of subjects were reported to be nonadherent at $\geqslant 2$ visits, and 13.4% of subjects were reported to be nonadherent at $\geqslant 2$ visits through week 144. Subjects who were adherent through week 48, compared with those who were not adherent, had more memory $CD4^+$ cells (median, 194 vs. 63 cells/mm^3; $P = .009$) and resting $CD4^+$ cells (median, 247 vs. 88 cells/mm^3; $P = .015$). No differences were detected between adherent and nonadherent subjects at week 144 with respect to total, naive, memory, activated, or resting $CD4^+$ cell counts. Subjects who were adherent through week 48 had a higher LPA response rate to *Candida* than did subjects who were not adherent (67% vs. 18%; $P = .002$). This difference persisted through week 144 (68% vs. 25%; $P =$ .044). No differences were detected in responses to other antigens at either week 48 or week 144.

Effect of azithromycin treatment on immunologic function. The percentage of subjects with a DTH response to TT, MSTA, or *Candida* antigen at baseline was similar in subjects randomized to azithromycin and those randomized to placebo (table 5). However, at week 24, the percentage of subjects who responded to TT was higher in the azithromycin group than in the placebo group (25% vs. 15%; $P = .009$), and the same was true for MSTA (29% vs. 17%; $P = .001$). A similar trend was seen for the *Candida* antigen (41% vs. 34%; $P = .090$). Because of a difference in baseline $CD4^+$ cell counts between treatment arms noted in the parent study [3], the effect of treatment was also evaluated, using logistic regression models that were adjusted for both baseline $CD4^+$ cell count and baseline DTH response for the antigen of interest. In these models, the significant effect of treatment persisted for TT and MSTA ($P = .009$ and $P = .010$, respectively), and the effect of treatment became significant for *Candida* antigen ($P = .034$). The effect of treatment on each of the 3 DTH responses at week 24 remained significant after controlling for other potentially influential baseline covariates, including baseline virus load detectability, Karnofsky score, and duration of HAART before entry.

Among subjects who were immunized, the percentage of responders (those who had a $\geqslant 4$-fold increase in antibody level) in the treatment arms (azithromycin vs. placebo) was found by Fisher's exact test to be similar for TT vaccine (14% vs. 12%; $P = .56$) and hepatitis A vaccine (25% vs. 28%; $P = .68$). LPA responses to each of the 5 tested stimulants at weeks 0, 24, and 48 did not differ significantly between treatment arms.

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Table 4. Comparison of delayed-type hypersensitivity (DTH) skin test and lymphocyte proliferation assay (LPA) responses to tetanus toxoid before and after immunization.

Time, LPA result	Baseline DTH skin test result, no. (%) of subjects	
	Positive (n = 9)	Negative (n = 175)
Baseline[a]		
Positive (n = 13)	4 (2.2)	9 (4.9)
Negative (n = 171)	5 (2.7)	166 (90.2)
	Week 24 DTH skin test result, no. (%) of subjects	
	Positive (n = 20)	Negative (n = 124)
Week 24[b]		
Positive (n = 33)	14 (9.7)	19 (13.2)
Negative (n = 111)	6 (4.2)	105 (72.9)

[a] Includes data only from subjects who had both LPA and DTH measurements at baseline.
[b] Includes data only from subjects who had both LPA and DTH measurements at week 24.

Effect of azithromycin treatment on blood lymphocyte subsets. The median CD4+ cell count at baseline for subjects receiving azithromycin was ~20 cells/mm^3 higher than that for subjects receiving placebo, and the CD4+ cell counts remained higher in the azithromycin group, but the difference was not statistically significant ($P = .10$). Azithromycin recipients, compared with placebo recipients, had a higher median number (82 vs. 66 cells/mm^3; $P = .08$) and percentage (34% vs. 26%; $P = .09$) of naive CD4+ cells at week 48, but neither difference reached significance when the Wilcoxon rank sum test was used. We fit a repeated-measures GEE model to the lymphocyte subset data, which accounted for the correlation among repeated measures for the same subject. After the analysis was adjusted for baseline CD4+ cell count and other potentially confounding baseline covariates, treatment did not appear to affect the number or percentage of naive CD4+ cells or the number or percentage of memory CD4+ cells ($P = .20$, $P = .58$, $P = .62$, and $P = .61$, respectively).

DISCUSSION

This study is among the largest to assess laboratory measures of immune reconstitution among HIV-infected patients with severe CD4+ cell depletion (CD4+ cell count of <50 cells/mm^3) before initiation of HAART. Both naive and memory CD4+ cell counts increased consistently over a period of 144 weeks, without an obvious plateau. The percentage of naive CD4+ cells increased slightly, from 26% to 33%, during this interval. Nevertheless, the majority of patients were anergic and unresponsive to immunization with a neoantigen, hepatitis A vaccine, given at weeks 24 and 48. Those who did not initially have DTH or LPA responses to TT were unlikely to develop those responses, despite booster immunization with TT. Similarly, tetanus immunization rarely led to an increase in anti-TT antibody levels. These data are in general agreement with previous reports in which the patient populations were much smaller [7, 8, 14–16]. Higher rates of antibody [7] and LPA [8] responses have been reported among subjects whose CD4+ cell count nadirs were >100 cells/mm^3, whereas the responses were comparably low among subjects with CD4+ cell count nadirs of <100 cells/mm^3 [14, 15]. For example, Kroon et al. [14] reported seroconversion after administration of a trivalent influenza vaccine in 30%–60% of subjects with median CD4+ cell count nadirs of 23 cells/mm^3, compared with seroconversion rates of 64%–74% among subjects with median CD4+ cell count nadirs of 152 cells/mm^3.

Fortunately, only a very few patients in the present study developed OIs. Restoration of immunity to AIDS-related OIs during HAART has been reported elsewhere to be correlated with increased numbers of CD4+ cells [2–4] but not with in vitro and in vivo assays of immunologic function [17, 18]. We have considered several potential explanations for the remarkable discordance between susceptibility to infection and our measures of immune function. It may be that laboratory assays do not measure immune responses to the important antigens and relevant pathogens. Although lymphocyte proliferation in response to antigens may be used to assess general cellular immunocompetence, a specific pattern of cytokine production or other lymphocyte function that is not measured may be required for host protection. LPAs and DTH skin tests may be insensitive to levels of antigen-specific T lymphocyte function that are adequate for protection. For example, measurements

Table 5. Association between use of azithromycin and delayed-type hypersensitivity (DTH) response.

Antigen, time	No. of subjects with positive DTH response/ no. tested (%)		
	Azithromycin recipients (n = 322)	Placebo recipients (n = 321)	P[a]
Tetanus toxoid			
Week 0	18/305 (6)	13/307 (4)	.363
Week 24	60/243 (25)	36/241 (15)	.009
Mumps skin test antigen			
Week 0	59/315 (19)	45/310 (15)	.164
Week 24	71/245 (29)	40/242 (17)	.001
Candida antigen			
Week 0	98/310 (32)	99/307 (32)	.931
Week 24	100/242 (41)	80/237 (34)	.090

[a] Calculated using Fisher's exact test.

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of single-cell responses by ELISPOT or CD69 expression [19] and of induced cytokine secreted into the culture medium [15] have detected antigen-specific responses in HIV-infected patients who do not mount a lymphoproliferative response to the same antigen. Alternatively, control of viral replication, accompanied by small increases in CD4$^+$ cell counts, may lead to restoration of innate immune function mediated by NK lymphocytes and phagocytic cells. In fact, neutrophils and monocytes from HIV-infected patients have impaired chemotaxis and oxidative metabolism [20], which normalize when patients are treated with HAART. In addition, HIV-1 infection of cultured human macrophages inhibits Fcγ receptor–mediated phagocytosis [21]. Finally, HAART regimens may have direct antimicrobial effects on some pathogens; for example, *C. albicans* virulence enzymes are inhibited by anti-HIV protease inhibitors [22].

The small number of incident OIs that occurred in these HAART recipients limited the ability of the present trial to achieve the primary objective of assessing the value of the immunologic tests performed to predict risk for MAC disease and other AIDS-defining illnesses. In addition, for the first year of the protocol, only those subjects who developed MAC disease, and not those who developed other OIs, were assessed in the immunologic substudy, which further limited the number of subjects with AIDS-defining illnesses for whom the full battery of immunologic tests had been performed.

Among subjects immunized with hepatitis A vaccine in this study, only 28% seroconverted after the first dose of vaccine, and 46% seroconverted after the second dose, whereas response rates are 85%–97% and 100%, respectively, when the same dose schedule is administered to subjects presumed to be healthy [23, 24]. Ninety-four percent of patients developed protective levels of anti-TT antibody after booster immunization, whereas the response rate of demographically similar but not HIV-infected adults is virtually 100% [25–28]. Furthermore, the fold increase in anti-TT IgG antibodies was only 1.7 in our patient population, whereas it typically exceeds 10 among healthy control subjects. The low rate of antibody response to immunization in this and other studies [7] indicates that many patients in whom CD4$^+$ cell numbers have been restored by HAART may have gaps in protection against vaccine-preventable diseases. Restoration of responses is, at least in part, related to the CD4$^+$ cell count nadir that precedes the initiation of HAART [8, 14–15, 29], the CD4$^+$CD28$^+$ T lymphocyte count at the time of immunization [8], and patient age [30]. The low rate of responses to immunization with recall antigens and neoantigens highlights the need for assessment of immunity to vaccine-preventable pathogens in HIV-infected patients who have experienced significant CD4$^+$ cell depletion (CD4$^+$ cell count of <200 cells/mm^3) before initiation of HAART. In those individuals who require it, immunization does not reliably provide protective levels of antibody equivalent to those seen in healthy, age-matched subjects. Antibody responses should be measured after immunization to verify that protective antibody levels have been achieved.

The slow or incomplete recovery of pathogen-specific antibody responses also may have important implications for the future use of therapeutic HIV vaccines in this and similar populations of HIV-infected patients. Successful immunization may not occur until the generation of naive CD4$^+$ T lymphocytes by the thymus restores the repertoire of HIV-specific T lymphocytes. Based on our data, such a reexpansion may not occur for years after recovery of CD4$^+$ cell counts in association with HAART. Which laboratory tests would best predict recovery of function before immunization is not certain.

We found that receipt of azithromycin improved rates of DTH responses to TT, MSTA, and *Candida* antigen and antibody responses to a booster dose of TT and to a neoantigen, hepatitis A vaccine. These effects of azithromycin were unexpected. Macrolide antibiotics are known to have immunomodulatory properties but are generally immunosuppressive. Macrolides inhibit chemotaxis [31] and the generation of reactive oxygen species by polymorphonuclear neutrophils [32]. They inhibit the release of interleukin (IL)–1 and tumor necrosis factor–α by lipopolysaccharide-stimulated peripheral blood monocytes [33]. They inhibit the production of the Th2 cytokines IL-4 and IL-5, without affecting the production of Th1 cytokines IL-2 and interferon (IFN)–γ [34], and they inhibit the in vitro proliferation of lymphocytes to *Dermatophagoides farinae* (dust mite) antigen [35]. The only reported proinflammatory activity of a macrolide is the augmentation of IL-12 and IFN-γ production in bronchoalveolar lavage fluid from mice infected with influenza virus [36]. It is possible that azithromycin had an indirect effect on immune recovery, perhaps by eradicating an unrecognized pathogen, but this is entirely speculative.

We conclude that immune recovery after initiation of HAART, which provides protection from OIs, is measured by increased numbers of circulating CD4$^+$ lymphocytes but does not correlate with a variety of functional assays for Th1 and Th2 responses. At baseline, only increased HIV-1 RNA levels in plasma, and not baseline CD4$^+$ cell count or response in those assays, correlated with risk for subsequent OIs. Although immune function recovered sufficiently to protect against OIs, responses to immunization with recall and neoantigens remained deficient in a majority of patients.

AIDS CLINICAL TRIALS GROUP 889 STUDY TEAM

Charles Knirsch (Pfizer, New York, NY); Graham Ray and Beverly Putnam (University of Colorado Health Sciences Center, Denver); Anna Paolino and Janet Forcht (New York University, New York); Thomas Hooten, Sheryl Storey, and N. Jeanne Conley (University of Washington, Seattle); Gerianne Casey and

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Jean A. Niles (University of Texas Medical Branch, Galveston); Robert L. Murphy (Northwestern University, Chicago, IL); Alan Landay (Rush–Presbyterian–St. Luke's Medical Center, Chicago); Charles van der Horst and David Ragan (University of North Carolina, Chapel Hill); Ronald Johnson and Gregory Gagliano (Case Western Reserve University, Cleveland, OH); Carol Hamilton and Sherry Swan (Duke University Medical Center, Durham, NC); Michael P. Dube and Connie Olson (University of Southern California, Los Angeles); Mary Sarah Dolan and Eileen Chusid (Mt. Sinai Hospital, New York); Andrea Swift, Ilene Wiggins, and Denise Wright (Johns Hopkins University School of Medicine, Baltimore, MD); Mario Guerrero and Gildon Beall (Harbor–University of California Los Angles Medical Center, Los Angeles); Lori Panther and Carol DeQuattro (Beth Israel Deaconess–West Campus, Boston, MA); and Richard Reichman, Jane Reid, and Tammy O'Hara (University of Rochester Medical Center, Rochester, NY).

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