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Immunologic Function in a Cohort of Human Immunodeficiency Virus Type 1-Seropositive and -Negative Healthy Homosexual Men

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The study objectives were to determine the early effects of human immunodeficiency virus type 1 (HIV-1) infection on both phenotypic and functional immunologic markers in healthy homosexual men, to ascertain the relationships of these markers to each other, and to discover which markers were affected by enrollment in an AIDS study in which HIV-1 serostatus would be determined. The major findings were as follows. (i) The CD4/CD8 ratio and lymphocyte proliferative response to pokeweed mitogen were the characteristics most affected by early HIV-1 infection. (ii) The loss in CD4 cells observed in the HIV-1-positive homosexual men was entirely due to diminished numbers of the memory subset, CD4$^+$ CD29$^+$. The reciprocal subset of CD4, CD4$^+$ CD45RA$^+$, did not differ in the two groups of homosexual men at either time point or in the controls. (iii) Prior to learning their HIV-1 serostatus, HIV-1 antibody-negative risk-group males had lower phytohemagglutinin (PHA) responses than the controls did. In the assays following notification of their seronegativity, however, these men had PHA values which were not different from those of the controls. In the HIV-1-positive group, the responses to both PHA and pokeweed mitogen were below those of both HIV-1-negative groups and did not change after serostatus notification. (iv) The activity of natural killer cells was lower in the risk-group men than in the controls at both pre- and postdiagnosis but was not related to HIV-1 serostatus. (v) In this cohort of homosexual men, the CD4/CD8 ratio correlated significantly with the functional measures of immunologic status in the HIV-1-positive men, but not in the HIV-1-negative men.

There are well-documented progressive quantitative and qualitative decrements in immunologic functioning in individuals who have AIDS or AIDS-related complex or who are clinically asymptomatic but human immunodeficiency virus type 1 (HIV-1) seropositive (4, 9–12, 14, 16, 27, 41). HIV-1-seropositive individuals have been studied at unknown and undoubtedly different lengths of time after seroconversion; with few exceptions, such as recipients of HIV-1-positive blood products (10), precise parallel comparisons of immune dysfunction and disease progression with clear origin points have been impossible to establish.

Studies which have assessed immunocompetence in asymptomatic HIV-1-positive homosexual males relative to seronegative homosexual males have tended to focus on phenotypic markers and were unable to make statements about markers of lymphocyte function (9, 16, 41). In no study to date have both enumerative and functional indices been assessed in healthy HIV-1-seropositive and -negative homosexual males both before and after determination of and notification of HIV antibody status. We have recently reported that a marker of psychological stress, serum cortisol, was elevated in men who entered an AIDS study prior to diagnosis and that in seronegatives, cortisol declined to normal values within a week of serostatus notification. A synchronous decline in the Profile of Mood States Composite scores and in the Impact of Events Scale was observed in these subjects (2). For the present study, a cohort of healthy homosexual men who had not yet been tested for antibodies against HIV-1 was recruited. A panel of immunologic tests was selected to measure the functional potential as well as phenotype of lymphocytes from patients in the study. Immunologic evaluations were done 5 weeks prenotification and 1 week postnotification for these men, as well as for 25 matched non-risk-group controls. Such a design allowed for the removal of the emotional stress caused by knowledge of seropositivity and any immunomodulatory effect that such an affective state might contribute (23).

MATERIALS AND METHODS

Subjects. Healthy homosexual men between the ages of 18 and 40 who had not yet been tested for antibodies against HIV-1 were given a complete physical examination, medical history examination, and fitness test by a physician and were admitted to the study provided they had no clinical signs or symptoms suggestive of HIV-1 infection. Thus, they had none of the following: diagnosis of AIDS or AIDS-related complex, unexplained weight loss of >10% or >15 lb (ca. 6.8 kg) within the past 3 months, generalized lymphadenopathy, oral candidiasis, herpes zoster within the past year, fever of unexplained origin, or unexplained diarrhea. Also excluded were men with a repeated history of drug or alcohol abuse, use of anabolic steroids, or regular use of antihistamines.

The immunologic measurements of the homosexual males enrolled in the study were compared with 25 healthy, age-matched HIV-1 antibody-negative male laboratory controls who were not asked about their sexual habits.

Blood samples. In order to control for diurnal variations, all blood samples were collected from fasting subjects between 7:30 and 10:30 a.m. Peripheral venous blood samples were obtained from each subject or control and placed in appropriate tubes. Heparin tubes were used for in vitro functional assays and flow cytometry (Vacutainer-Sodium

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Heparin; Becton-Dickinson, Rutherford, N.J.). Another blood sample in EDTA was collected from each subject for an automated complete blood count. Whole-blood samples were held for no longer than 4 h at room temperature before complete blood count determinations or functional assay procedures and for no longer than 24 h before preparation and fixation for flow cytometry assay (12, 33). For serum samples, peripheral venous blood was collected in red-top tubes (Vacutainer; Becton-Dickinson) and allowed to clot at 23°C for 30 min. The serum was separated from the clot and stored at −20°C until use. The samples were obtained and analyzed for the risk-group subjects. One sample was obtained upon entry into the study. A serum sample was obtained 4.5 weeks later and was used to determine the presence or absence of antibodies against HIV-1. The results of this testing were communicated to the participant 72 h later. A third sample for immunologic assessment was obtained at 6 weeks postentry. Only one assessment was done for each control.

Flow cytometry. A single-laser flow cytometer (EPICS C; Coulter Epics Division, Hialeah, Fla.), which discriminates between forward and right-angle light scatter, as well as two colors, was used with a QuadStat (Coulter) software package. Calibration was accomplished with Fullbright grade 1 beads (Coulter) as well as Bench Mark beads (no. 3; Flow Cytometry Standards, Triangle Park, N.C.).

Monoclonal antibody panel. Mononuclear cell populations were determined by two-color direct immunofluorescence, using a whole-blood staining technique with the appropriate monoclonal antibody and flow cytometry (12, 30, 37). The pairs of monoclonal antibodies used were fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (Coulter Immunology, Hialeah, Fla.). Isotypic controls were mouse immunoglobulin G1 (IgG1), IgG2, and IgM (Coulter Immunology).

(i) T11-FITC and Ta1-PE. T11 (CD2) or sheep erythrocyte receptor-bearing cells (26), was combined with TA1 (CD26) (15) to measure the activated $CD2^+$ $CD26^+$ T cells. CD26, a marker for activated T memory cells may be found on $CD4^+$ cells and $CD8^+$ cells (19, 25).

(ii) T4-FITC and 4B4-PE. T4 (34) detects CD4 lymphocytes, the helper/inducer cell and a major target of HIV-1. It was combined with 4B4 to identify a subset of CD4 cells which express CD29, thought to represent the fraction of CD4 cells with the ability to help B cells respond to antigen with specific antibody synthesis (29).

(iii) T4-FITC and 2H4-PE. T4 is also combined with 2H4 to measure a subset of CD4 cells which express CD45RA, thought to be involved in the suppression of specific B-cell responses through the induction of $CD8^+$ suppressor cells (30).

(iv) T8-FITC and I2-PE. T8 which measures CD8 or suppressor/cytotoxic T cells (35) was paired with I2 to measure HLA/DR activation antigen (36) expression on CD8 lymphocytes. The $T8^+$ $I2^+$ cells may indicate activated T-suppressor cells or perhaps an immature subset of $T8^+$ cells which express class II antigen.

(v) NKH.1-PE. CD56 (20) was used in order to assess the numbers of large granular lymphocytes, the natural killer (NK) cells.

(vi) B1-FITC. CD20 (31, 40) was included in the panel to assess the number of mature B cells.

(vii) MO2-FITC and I2-PE. Monocytes were enumerated with MO2, CD14 (42), in combination with I2 to determine the degree of expression of the HLA/DR class II activation antigen on monocytes.

(viii) KC56-FITC and MO2-PE. KC56 (CD45) which is a marker for leukocytes (8) was combined with CD14 to facilitate the setting of the lymphocyte gates.

Flow cytometric analysis. For the lymphocyte markers, gates were set on the lymphocyte population of the forward-angle light scatter versus 90° light scatter histogram, and monocyte and granulocyte areas were excluded. Percent positively stained cells and percent doubly stained cells for each marker pair were determined by using QuadStat software (Coulter Epics). Percentages were corrected for number of cells within a bit map which were positive for the leukocyte marker, CD45, unless the percentage of leukocytes was under 90%. If a preparation could not be regated to yield at least 90% of cells positive for the leukocyte marker, the results were not analyzed and another sample was obtained. Peripheral lymphocyte counts were calculated by multiplying the total leukocyte count and percentage of lymphocytes as determined from a Cell Dyne 1500 (Sequoia-Turner). Estimates of absolute numbers of the lymphocyte or mononuclear cell populations positive for the respective surface markers were determined by multiplying peripheral lymphocyte or mononuclear cell counts by the percentage of cells positive for each surface marker (12).

Lymphocyte proliferation assay. Lymphocyte proliferation to mitogen stimulation was measured by using a whole-blood procedure (12). Mitogens were tested in triplicate at the following level or the final dilution as follows: phytohemagglutinin (PHA) (Wellcome Diagnostics, Dartford, England) at 10 μg/ml and pokeweed mitogen (PWM) (GIBCO, Chagrin Falls, Ohio) diluted 1:40. Three wells for each sample received only culture medium. Plates were incubated for 72 h in a humidified atmosphere of 5% CO_2 at 37°C. During the last 6 h of the appropriate incubation time, cultures were pulsed with 25 μl of tritiated thymidine (1 μCi per well; New England Nuclear, Boston, Mass.). At the end of the pulse incubation, cultures were cooled to 4°C and harvested onto glass filter paper disks (Titertek Cell Harvester; Flow Laboratories, McLean, Va.), immersed in scintillation fluid, and counted on a beta scintillation counter (LKB Instruments, Rockville, Md.). Results were expressed as mean net counts per minute incorporated. The mean net counts per minute was transformed to mean counts per minute per 100,000 lymphocytes. Calculations were done with an ATT program written in-house.

NK cell cytotoxicity assays. NK cell function was evaluated by determining cytotoxicity, using the whole-blood chromium release assay as outlined in detail by Baron et al. (4). This assay requires a small sample amount (<3 ml) per patient and has the advantage that NK cell activity is measured in an environment closer to that in which the cell must function in vivo than is possible using separated mononuclear cells. The target cell line utilized was the NK sensitive erythroleukemic K562 cell line.

From samples of heparinized peripheral blood, triplicate aliquots (150 μl each) were dispensed into the wells on a 96-well flat-bottom tissue culture tray (Costar, Cambridge, Mass.). One row each of assay medium (AM) (RPMI 1640, 10% fetal bovine calf serum, 1 mM L-glutamine, 100 U of penicillin, and 50 μg of streptomycin, 1 ml of nonessential amino acids, 1 mM sodium pyruvate) and 1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) were dispensed to serve as controls for spontaneous release and total release, respectively. Fifty microliters of ^{51}Cr (sodium chromate in 0.85% NaCl New England Nuclear)-labeled log-phase K562 target cells at several dilutions—2×10^6, 1×10^6, 0.5×10^6, and 0.25×10^6 cells per ml—were dispensed to wells of the

specimen and control rows. Plasma controls were performed for each blood sample. Triplicate wells received plasma plus AM and ^{51}Cr-labeled target cells at a dilution of 10^6/ml. The tray was covered, centrifuged at 400 × g for 10 min at room temperature to mix the targets and whole blood, and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 4 h. At the end of incubation, 100 μl of chilled (4°C) AM was added to stop the assay. The tray was then covered and centrifuged, and 100 μl of cell-free supernatant was removed to be counted for 5 min in a gamma counter (LKB Instruments). Natural cell-mediated cytotoxicity expressed as percentage cytotoxicity was calculated as follows:

$$\text{Percent cytotoxicity} = \frac{(\text{ER} - b) \times \left[\dfrac{V_t - (V_b \times H)}{V_t}\right] - (\text{SR} - b)}{(\text{TR} - b) - (\text{SR} - b)} \times 100$$

where ER is the counts per minute mean of experimental release of the specimen, SR is the mean counts per minute of spontaneous release, TR is the mean counts per minute of total release, V_t is the total volume in the well, V_b is the volume of blood in the well, H is specimen hematocrit, and b is instrument background. The percent cytotoxicity at the four target/effector ratios and the number of CD56-positive cells per unit of blood were used to express the results as percent cytotoxicity at a target-to-effector (CD56-positive) cell ratio of 1:1. Calculations were done with an ATT computer program written in-house.

Callewaert et al. (5, 6) have demonstrated that estimates of V_{max} for NK cell activity are equal to the concentration of NK cells tested times the mean lytic activity per NK cell, whether a single- or multistep model of effector cell/target cell interaction and lysis is used. Percent cytotoxicity, determined by ^{51}Cr release assay for the dilutions of target cells to be used, was transformed to number of target cells lysed and T (substrate) is the number of target cells in the assay. The data were then fitted to the following equation, which is analogous to the Michaelis-Menten kinetic equation as described by Cleland (7): $V = (V_{max} \times T)/(K_m + T)$ where V is the number of targets killed, T is the number of targets in the assay, V_{max} is the number of targets killed during the 4-h assay when T is infinite, and K_m is the number of targets required for half V_{max}.

Kinetic lytic units/NK cell is expressed as the maximum number of targets lysed by each NK (CD56-positive) cell during the 4-h assay. Functional efficiency per effector cell was then compared among the study groups.

Viral serology. HIV-1 antibodies were measured by using the enzyme-linked immunosorbent assay (ELISA) and Abbott reagents, reactive samples were retested and doubly positive samples were confirmed by protein immunoblot, using the methodology developed by the Centers for Disease Control.

Data analysis. Descriptive statistics were expressed as medians and range values (25th to 75th percentiles) with nonparametric Mann-Whitney tests used for between-group comparisons and Wilcoxon tests used for within-group comparisons over time. Subsequent regression analyses were performed to determine which immunologic measures were best able to predict antibody status. First, a parametric regression procedure was used with the minimum inclusion criteria of a 0.05 significance level required to enter as a predictor variable. Maintenance of a unique contribution (squared semi-partial correlation) significant at the 0.05 level was required for the variable to remain in the equation. All immunologic measures significantly related ($P < 0.05$) to antibody status by univariate analysis were considered as possible predictors. In addition, a logistic regression analysis was conducted by using the standard logistic transformation as a criteria variable (HIV-1 antibody status) and maximum likelihood estimates. The relationships between phenotypic and functional markers were determined by calculation of the Pearson Product Moment correlations.

RESULTS

Demographic information. Asymptomatic subjects meeting the exclusionary criteria made up the present sample of seropositive and seronegative homosexual males. The healthy laboratory controls included various staff and faculty members of a large university medical center and were HIV-1 antibody negative. The 25 laboratory controls were matched by age with the declared homosexual study subjects (median age = 31 years; range = 18 to 40 years) and included 22 Caucasians and 3 blacks. The men shown ultimately to be seropositive (n = 25) had a median age of 31 years (range = 21 to 40 years) and included 17 Caucasians, 7 Hispanics, and 1 black. Seronegative homosexuals (n = 46) had a median age of 32 years (range = 22 to 40 years) and included 39 Caucasians, 6 Hispanics, and 1 black. Among the two

TABLE 1. T and B lymphocytes and ratio

Group and sampling time	Median[a] (range, 25th to 75th percentile)			
	CD2	$CD2^+$ $CD26^+$	CD20	CD4/CD8 ratio
Controls	1,517 (1,170–1,984)	323 (252–465)	250 (170–346)	1.8 (1.4–2.2)
Week 0				
HIV-1 antibody-negative homosexual males	1,895 (1,550–2,255)	395 (321–524)	218 (159–263)	1.3*+ (1.1–1.7)
HIV-1 antibody-positive homosexual males	1,752 (1,415–2,502)	293 (151–415)	190 (140–253)	0.7* (0.5–0.8)
Week 6				
HIV-1 antibody-negative homosexual males	1,912 (1,434–2,525)	439*+ (302–628)	229 (150–292)	1.4*x+ (1.1–1.9)
HIV-1 antibody-positive homosexual males	1,897 (1,370–2,675)	226* (145–358)	177* (121–222)	0.8* (0.5–1.0)

[a] Symbols: *, significantly different from value obtained for controls ($P < 0.01$); +, significantly different from value obtained for HIV-1 antibody-positive homosexual males at same week ($P < 0.01$); x, significantly different from value obtained for the same group at week 0 ($P < 0.01$).

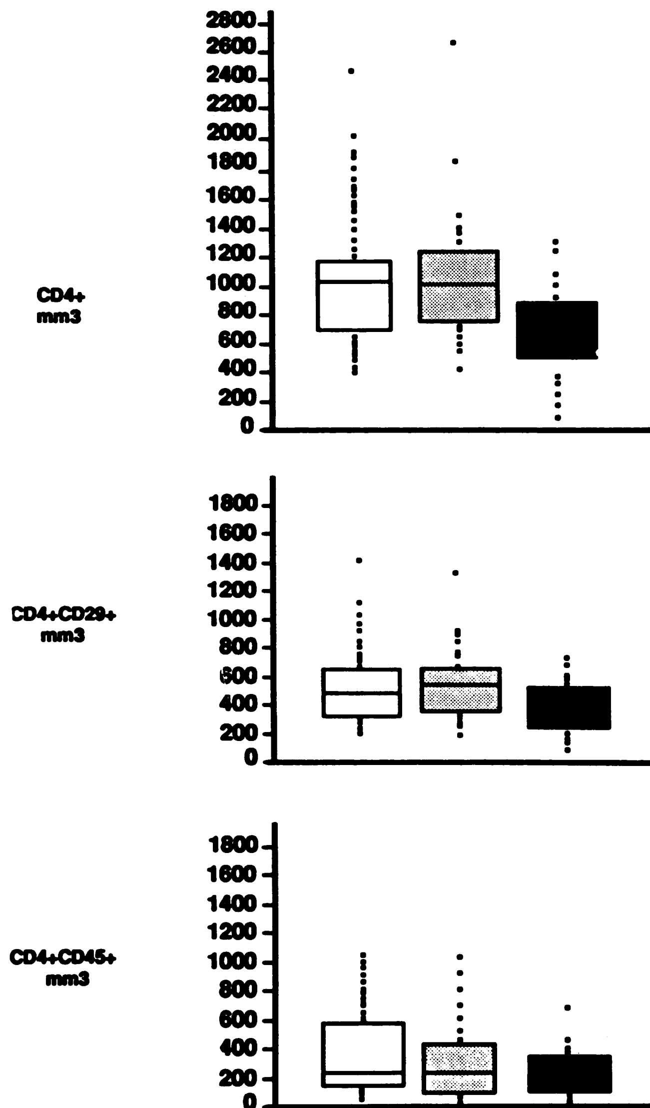

FIG. 1. Box plots of the distribution (numbers of cells per millimeter³) of CD4 lymphocyte markers in the seropositive and seronegative homosexual men (n = 71) and the controls (n = 25) at week 0. Plots show medians and 25th and 75th percentiles and outliers. Symbols: □, control group; ▨, HIV-1 antibody-negative group; ▩, HIV-1 antibody-positive group.

homosexual groups studied, there were no significant differences in age (t [67] = 1.36, $P > 0.05$) or ethnic group distribution (X^2 = 12.2, df = 16, $P > 0.05$). Nearly 95% of the entire sample was college educated, and there were no significant differences in the distributions of education or annual income (modal range = $20,000 to 30,000/year) among seropositives, seronegative homosexuals, and controls.

Serology. Of the 71 risk-group men enrolled in the study, 35%, or 25, were shown to be HIV-1 antibody positive (repeatedly ELISA positive and confirmed by protein immunoblot).

Lymphocyte phenotypes. The total lymphocyte counts of the asymptomatic HIV-1 antibody-positive men in this study did not differ from that of the HIV-1 antibody-negative homosexual men or the non-risk-group controls at either week 0 or week 6. No difference was observed in total monocyte counts (CD14-positive cells) or in total T cells (CD2-positive) in the three groups studied.

Both groups of homosexual men had significantly higher percentages of CD2 cells, but not absolute counts, than did the controls at both week 0 and week 6. Seropositives had significantly less $CD2^+$ $CD26^+$ cells at week 6 than the controls, while seronegatives had significantly more $CD2^+$ $CD26^+$ cells than both seropositive and controls at week 6. Seropositive men showed a significantly reduced number of CD20 cells at week 6, while seronegative risk-group men had a lower percentage of CD20 cells at week 0 (Table 1).

The numbers of CD4 and $CD4^+$ $CD29^+$ cells were significantly depressed in the HIV-1 antibody-positive men compared with the matched seronegative men. There were no differences in the number of $CD4^+$ $CD45RA^+$ cells between groups (Fig. 1).

There was a significant elevation in the percentages and numbers of CD8 and the subsets, $CD8^+$ $I2^+$ and $CD8^+$ $I2^-$, cells for both seronegative and seropositive homosexual men compared with controls at both week 0 and week 6 (Fig. 2).

The median ratio of CD4/CD8 cells was approximately 1.3 in the HIV-1-negative homosexual males, significantly higher than in the HIV-1 antibody-positive group (median = 0.7), but also significantly lower than the controls (median = 1.8). These patterns were also the same at week 6 (Table 1).

Functional assays. The response to PHA in HIV-1 antibody-positive men was significantly lower than in both seronegative groups at both week 0 and week 6. Similarly, the HIV-1 antibody-negative men had significantly lower PHA response values than controls at week 0, but not at week 6 (Fig. 3). In fact, seronegative subjects showed a significant increase in response to PHA from week 0 to week 6. In addition, the response to PWM among the HIV-1 antibody-positive men was approximately one-third that of the seronegative homosexual men and less than one-eighth of the control group at both week 0 and week 6. Response to PWM for seronegatives increased but did not return to control values at week 6 (Fig. 4).

Baseline NK cell markers are shown in Fig. 5. Both the seronegative and seropositive homosexual men had significantly less NK cell cytotoxicity than controls at both week 0 and week 6. Only seronegative subjects had significantly lower in kinetic lytic units per NK cell than controls at both time points. There were no differences among the groups in numbers of CD56-positive cells.

Correlations among phenotypic and functional assays. To assess the degree of association among CD4 and CD8 counts and ratio as well as subsets of these phenotypic markers to functional immunologic markers, we calculated Pearson's correlation coefficients. We found positive correlations between the CD4/DC8 ratio to mitogen response and to NK cytotoxicity in the seropositive men, but not the seronegative men (Table 2).

Regression analyses. All immunologic measures compared between the two groups of homosexual men which were significantly different in univariate analyses were examined by parametric stepwise multiple regression procedures for their ability to predict HIV-1 antibody status. The first variable to enter the regression equation was the $CD4^+$/$CD8^+$ ratio, which explained 49% of the variance (F [1, 36] = 35.1; P = 0.001). The only other immunologic measure to add significantly was response to PWM (t = 2.57; P = 0.01), increasing the proportion of explained serostatus variance to 57%. A nonparametric (logistic) regression analysis yielded

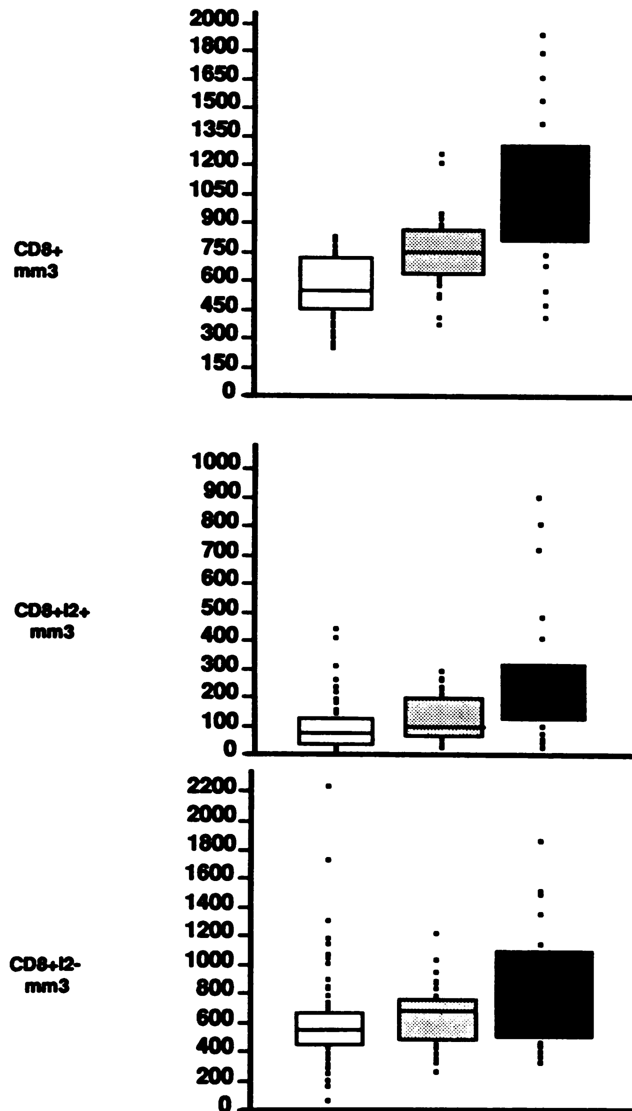

FIG. 2. Box plots of the distribution (numbers of cells per millimeter^3) of CD8 lymphocyte markers in the seropositive and seronegative homosexual men (n = 71) and the controls (n = 25) at week 0. Plots show medians and 25th and 75th percentiles and outliers. Symbols: □, control group; ▨, HIV-1 antibody-negative group; ▩, HIV-1 antibody-positive group.

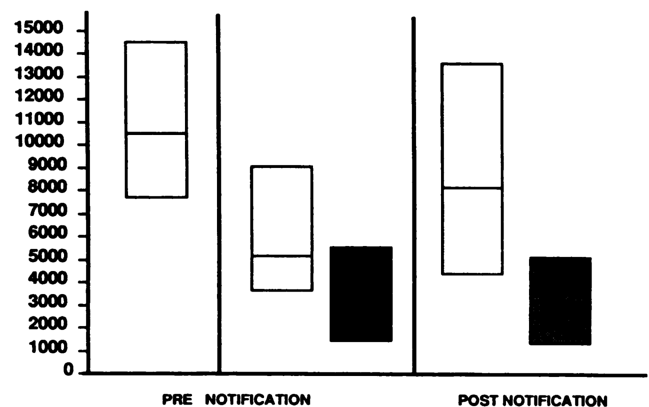

FIG. 3. Box plots of the distribution of PHA proliferative responses, expressed as counts per minute × 10, in the controls and the risk-group males pre- and postnotification. Plots show medians and 25th and 75th percentiles. Symbols: □, control group; ▨, HIV-1 antibody-negative group; ▩, HIV-1 antibody-positive group.

results quite consistent with the parametric analysis. The logistic model with the two predictors fit the observed data well (likelihood ratio, X^2 = 29.74, df = 47, probability = 0.98). Associated chi-square tests of 48.5 (P = 0.027) for PWM counts per minute and 8.44 (P = 0.004) for the $CD4^+/CD8^+$ ratio were obtained.

DISCUSSION

It is clear from many studies that HIV-1 infection has a profound effect on many aspects of immunologic function, beyond a simple depletion of CD4 cells (12, 13, 27). The present study aimed to delineate the immunologic state of an AIDS risk group, homosexual men, prior to the appearance of any physical signs related to HIV-1 status. Phenotypic and functional indices were compared among asymptomatic seropositives, seronegatives, and age- and gender-matched laboratory controls.

Phenotypic findings. In terms of overall CD4 cells, the HIV-1 antibody-positive homosexual men had a relatively high median count of 721/mm^3 at week 0 (baseline) and 813/mm^3 at 1 week postdiagnosis, which is consistent with the data from the Multicenter AIDS Cohort Study (MACS) (16) that reported a similar value of 715 T4 cells/mm^3 in 33 homosexual men within 6 months after seroconversion. MACS found a further drop after 1 year to 626/mm^3; whereas longer-term-seropositive, asymptomatic men in the MACS study had a CD4 count of 530/mm^3 (16). Klimas et al. (27) reported a CD4 value of 463 cells/mm^3 in 56 homosexual men with lymphadenopathy syndrome. Thus, on the basis of previous findings, it would appear that the seropositive homosexual males in the present study are representative of individuals with early HIV-1 infection.

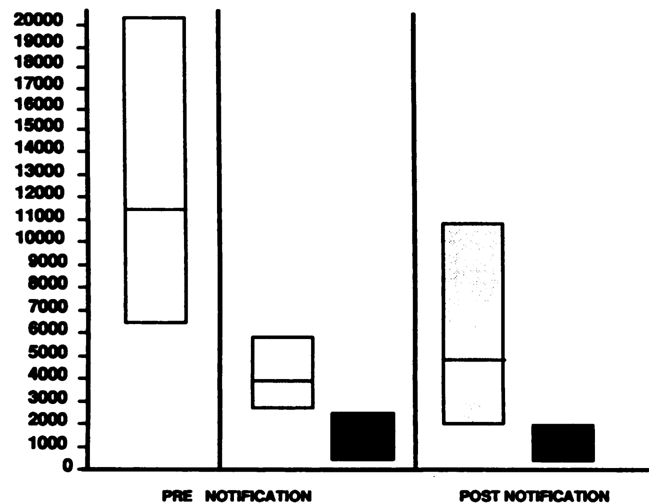

FIG. 4. Box plots of the distribution of PWM proliferative responses, expressed as counts per minute, in the controls and the risk-group males pre- and postnotification. Plots show medians and 25th and 75th percentiles. Symbols: □, control group; ▨, HIV-1 antibody-negative group; ▩, HIV-1 antibody-positive group.

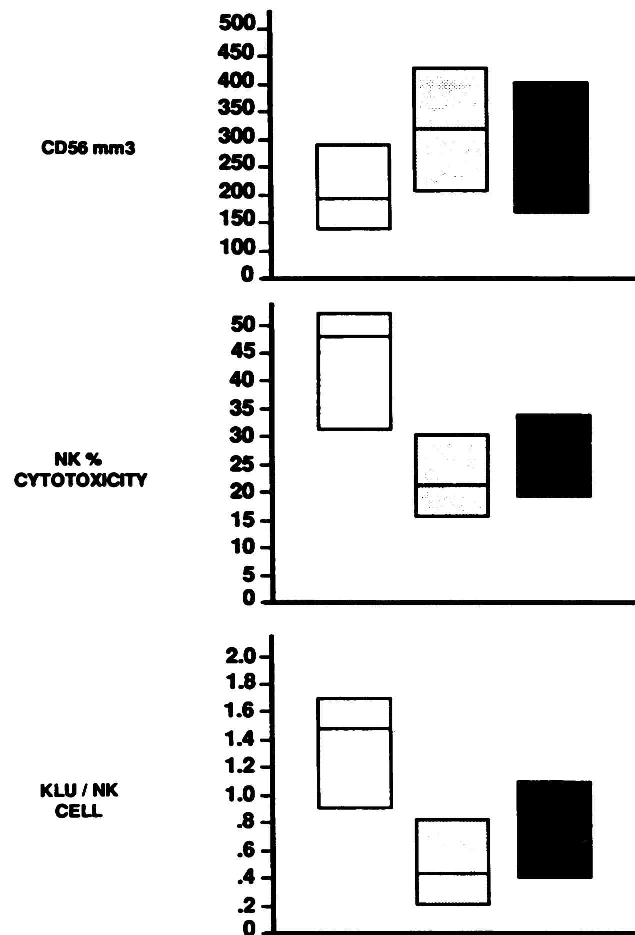

FIG. 5. Box plots of the distribution of NK cell markers in the controls and the risk-group males at week 0. Plots show medians and 25th and 75th percentiles of number of CD56 cells per millimeter^3, cytotoxicity of NK cells, and kinetic lytic units per NK cell (KLU/NK cell). Symbols: □, control group; ▩, HIV-1 antibody-negative group; ▩, HIV-1 antibody-positive group.

Several immune measures collected in the present study yielded no differences among the three groups at either week 0 (baseline) or at 6 weeks including total lymphocyte counts, numbers of T (CD2) cells, and numbers of total monocytes (CD14) and of monocytes expressing the class II activation marker (I2$^+$). Other reports of early HIV-1 infections parallel some of these findings (9). At the 6-week mark, after disclosure of HIV-1 test results, the number of T cells expressing CD26, a marker associated with activated memory lymphocytes (39), was depressed in the group which received a positive test result. Similarly, the number of B cells (CD20) did not differ between the groups at week 0 (baseline) but was significantly depressed at 6 weeks in the seropositive group compared with non-risk-group control group.

TABLE 2. Correlation of CD4$^+$/CD8$^+$ with functional measures

Functional measure	HIV-1 antibody-negative homosexual males		HIV-1 antibody-positive homosexual males	
	r	*P*	*r*	*P*
PHA	−0.02	0.44	0.46	0.01
PWM	−0.06	0.37	0.36	0.05
NK cell cytotoxicity	−0.10	0.30	0.48	0.02

Examination of the results of the analyses for the subsets of CD4 cells revealed that the decline in CD4 count was entirely accounted for by the decrease in numbers of the memory subset CD4$^+$ CD29$^+$. A decrease was noted following diagnosis in the HIV-1-positive men in CD26-expressing T cells. This surface marker is associated with T-cell activation and memory T cells (19). A decrease in CD2$^+$ CD26$^+$ lymphocytes has been noted in other HIV-1-infected cohorts (11). In contrast, there was no significant difference in number of CD4$^+$ CD45RA$^+$ cells between seropositive and seronegative homosexual men. These results, which indicate an early selectivity in the effect of HIV-1 infection on helper and inducer subsets, are in conflict with those of Giorgi and colleagues (16, 17), who reported no selective decrease in the subsets of CD4 cells. However, they are in agreement with those of DeMartini et al. (9) and Vuillier et al. (43). The Transfusion Study Group has found that these two subpopulations of CD4 are reduced at different rates in HIV-1 antibody-positive patients with a congenital clotting disorder (11, 14). Our present results indicate that monitoring by two-color immunofluorescence with CD4 monoclonal antibody together with the CD29 and CD45RA monoclonal antibodies may provide more useful information for evaluation of disease progression in HIV-1 infection than that provided by CD4 enumerations alone.

A possible consequence of the imbalance of CD4 subsets in early HIV-1 infection may be reflected in the change in CD8 cells which were observed. Although the seronegative homosexual men had elevated numbers of CD8 cells compared with the controls, the CD8 counts of the seropositive homosexual men were significantly elevated over those of both seronegative groups. Both subsets of CD8 cells were elevated including CD8$^+$ I2$^+$, which are possibly activated suppressor cells (22) or perhaps an immature T-cell subset (38), and CD8$^+$ I2$^-$, a subset reported to have a predominant cytotoxic function (21). It has been speculated that among HIV-1-infected men, elevated cytotoxic cell (CD8$^+$ I2$^-$) levels may reflect effective control of HIV-1 (32). If so, our seropositives, showing a large degree of variability in this subset (25th to 75th percentile range = 493 to 1,085), should be expected to show a good deal of variance in the clinical course of the disease. The CD4$^+$/CD8$^+$ ratio was significantly different for all three groups and was the best predictor of HIV-1 serostatus of all of the phenotype indices as determined by logistic stepwise regression analysis. The subset ratio of CD4$^+$ CD29$^+$ number to CD8$^+$ I2$^+$ number was also significantly lower in seropositive subjects compared with both seronegative homosexual males and controls.

Functional findings. Lymphocyte proliferative responses to the plant mitogens PHA and PWM were determined as a measure of functional immunocompetence. The baseline median response of the HIV-1 antibody-positive homosexual men to PHA, principally a T-cell mitogen, was significantly lower than those of both seronegative groups. This disparity was more pronounced for PWM responses with seropositives evincing values which differed from seronegative risk-group men by nearly a factor of 3 and from laboratory controls by a factor of 8. In addition to the CD4$^+$/CD8$^+$ ratio, proliferative response to PWM was the only other immune variable measured which was significantly predic-

tive of HIV-1 antibody status by multivariate stepwise regression analyses.

It would seem likely that the relatively huge decrease in response to PWM (which normally stimulates both T and B cells to proliferate) that is observed so early in HIV-1 infection would be related to the early decrement in the B-cell-regulating T-cell subset, the $CD4^+$ $CD29^+$ lymphocytes. However, of all the phenotypic parameters, only the CD4/CD8 ratio was significantly correlated to the functional markers, mitogen response and NK cytotoxicity. This suggests that the proportion of helper/inducer lymphocytes to suppressor/cytotoxic lymphocytes may be more related to functional activity and by inference to immunologic status than absolute counts of CD4 cells or subsets of CD4.

Of interest was our observation that before learning of their serostatus, seronegative homosexual men had lower $CD4^+/CD8^+$ ratios and significantly depressed responses to both PHA and PWM than the controls. This suggested either a certain degree of chronic immunologic impairment in this risk group prior to infection with HIV-1 or the effects of being enrolled in the study including the anticipation of receiving a diagnosis. Within 1 week of receiving news of a negative test for HIV-1 antibodies, the PHA responses of these men climbed to a value not significantly different from that of the controls. In fact, when we compared $CD4^+/CD8^+$ ratios and PHA and PWM responses between the present sample of seronegative homosexual males and controls after the risk-group men had learned their diagnosis, the differences in PHA values disappeared and the $CD4^+/CD8^+$ ratio and PWM differences were reduced. In contrast to the upward movement of median proliferative response is the seronegative group, with a downward trend in both PHA and PWM responses in the seropositives following notification. These findings suggest that the differences noted at week 0 in the seronegatives may have been a function of psychosocial factors to the homosexual group stemming from entry into an AIDS study in which serostatus would be determined (1, 3, 23). The growing body of literature relating psychosocial stressors and immune function has recently been reviewed (18), and empirical work has suggested that marked changes in immunologic functioning may occur during the period preceding impending stressors, such as medical school examinations (18) and death of spouse (24). The men in the present study were recruited from an area with a very high incidence of HIV-1 infection in the gay community, and subjects were, therefore, likely to have perceived themselves to be at considerable risk for infection. In fact, we have recently reported that serum cortisol levels were significantly higher in seronegative men prior to entering the study than 6 weeks later after being informed of negative results (2).

We have previously reported depressed NK cell cytotoxicity in HIV-1-infected drug users (27) and in patients with AIDS and AIDS-related complex (4). In those studies, NK cell numbers were found not to be depressed, yet cytotoxic activity was greatly reduced relative to normal values. In the present study of early HIV-1 infection, we saw no difference between the seropositive and seronegative risk-group men in numbers of CD56 cells or in NK cytotoxic activity. Compared with non-risk-group controls, the homosexual men had similar numbers of CD56 cells. However, these putative NK cells had a significantly depressed ability to kill tumor targets. Our observation of impaired cytotoxicity in the seronegative homosexual males suggests that some degree of chronic immunologic impairment which is unrelated to infection with HIV-1 may exist in this risk group. This poor NK cell function in the seronegative homosexual males does not appear to be attributable to the stress of entry into an AIDS study because it did not change over the course of 6 weeks, when the men were informed of their negative serostatus. This finding is unrelated to undiagnosed seroconversion because at the 1 year follow-up, no changes in status occurred within this group.

The paucity of significant correlations between the lymphocyte phenotypic markers and the assays which measured in vitro function of lymphocytes suggests that the particular panel of phenotypic markers selected for use in this study, even though extensive, does not adequately predict lymphocyte function—in either HIV-1-infected or uninfected men.

Importance of matched comparison groups. This study provided descriptive information on immunologic subset enumerations and functional competence among early HIV-1-seropositive and -seronegative homosexual males and is not the first to do so. A unique contribution of this work, however, was the collection of these measures in a design that attempted to minimize the sources of error variance which have been prevalent in previous studies of this nature.

The sources of error variance in prior studies include but are not limited to group nonequivalence on demographic variables (gender and socioeconomic status), medical status variables (symptoms related to HIV-1 and symptoms not related to HIV-1 but associated with changes in immunocompetence), and adaptation-to-diagnosis variables (length of time since diagnosis was learned).

All three of these sources of error variance may contribute to the variability in immune measures collected and may therefore distort apparent group differences or mask real differences. Gender and age differences in immunologic measures have been noted (19, 28, 34, 35) as have immunologic differences as a function of HIV-1 symptomatology (11, 14) and period of time since learning of HIV-1 serostatus (3).

In the present study, we compared enumerations and functional measures among HIV-1-seropositive homosexual males, HIV-1-seronegative homosexual males, and HIV-1-seronegative laboratory control males. All comparison groups were equivalent on the variables of age and gender as well as several other demographic variables (ethnic group, education, annual income). All subjects were asymptomatic at the time of data collection and hence were equivalent in terms of medical status. Finally, all of our homosexual subjects were unaware of their serostatus at the time immune markers were collected (they learned their antibody status approximately 5 weeks later). These subjects were therefore matched on adaptation burden in that they were all in a state of anticipation over their soon-to-be-learned HIV-1 serostatus, yet none of them were, as yet, coping with a seropositive diagnosis. It is true that the HIV-1-seronegative laboratory controls in our study were aware of their antibody-negative status and hence were not subject to anticipatory or adaptation burden. Therefore, adaptation-to-diagnosis equivalence can be claimed only for our homosexual groups. Our pre- and postdiagnosis measures for seronegative homosexuals allowed us to demonstrate immunologic differences in the presence of this anticipatory effect (versus controls) and the disappearance of these differences in its absence.

In order to reduce potential sources of noise, we recommend that investigators in this area employ rigorous yet practical control procedures, such as exclusionary criteria (e.g., asymptomatic status, gender matching), sociodemographic interviewing (e.g., age, socioeconomic status), and HIV-1 disease history information (e.g., time since learning

of serostatus). The incorporation of such methodological considerations will facilitate the comparison of group measures within and across such studies.

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