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AUTOIMMUNITY IN AIDS

PRINCIPAL INVESTIGATOR: Albert D. Donnenberg, Ph.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University
600 N. Wolfe Street
Baltimore, Maryland 21205

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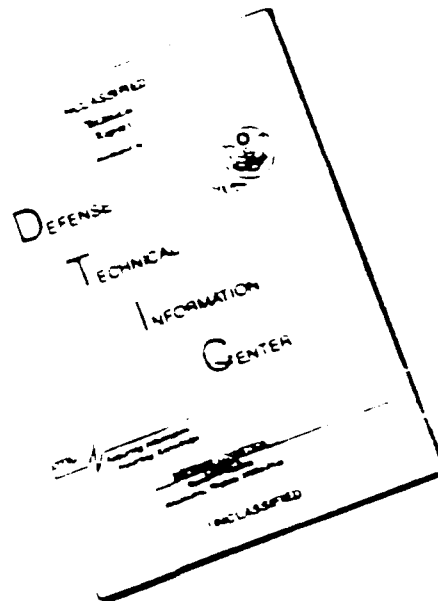
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13. ABSTRACT (Maximum 200 words) Infection with Human Immunodeficiency Virus type 1 (HIV-1), the etiologic agent of the acquired immune deficiency syndrome (AIDS), is increasing at an epidemic rate. The severity of immunologic impairment of infected individuals spans a broad range. It is not currently known whether this heterogeneity reflects distinct outcomes of infection or whether infections progress to a common endpoint at different rates. Developing closer knowledge of immunological events that precede the onset of frank AIDS, as well as the interplay between HIV, immunity and the hematopoietic elements that give rise to the immune system will assist in our understanding of the pathophysiology of AIDS. Such knowledge is also guiding our attempts to develop better therapeutic approaches to AIDS. This proposal focuses on two primary areas reflecting the respective strengths of the two participating laboratories. From their inception these efforts have been highly collaborative. The first section, Interaction of HIV-1 and Bone Marrow, focuses on hematopoietic progenitor cells as possible reservoirs of virus. It is designed to explore the relationship between acquisition of lineage specific				
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differentiated function and viral permissiveness, and examine the interaction of infected myeloid and lymphoid cells as they affect immune function and regulation of bone marrow growth. These studies also bear directly on the use of bone marrow transplantation as a new therapeutic approach to the treatment of HIV associated malignancies and, potentially, AIDS itself. Our project has monitored immune reconstitution in six HIV patients receiving allogeneic bone marrow transplants plus antiviral therapy following full myeloablation. Our second research goal has been the evaluation of immunologic changes related to progression of HIV infection. This aspect of our research draws on the resources of two large prospective cohort studies of the natural history of HIV infection, one in gay/bisexual men (SHARE) and one in intravenous drug users (ALIVE). These studies focus on evaluating changes in phenotypically and functionally defined T cell subpopulations which may represent host responses to HIV infection or may predict decline in immune competence as a result of HIV infection. Specifically, phenotypic studies have examined changes in numbers T cell subsets, and non-T lymphocyte populations such as double negative (CD4⁺, CD8⁻) T cells, natural killer cells, and in vivo activated IL-2 responsive T cells as a function of disease stage. They have also explored expression of HLA-DR on antigen presenting cells, and the immunophenotype of lymphocytes isolated from cerebrospinal fluid. Functional studies have included prospective analysis of antigen and mitogen induced T cell responses and characterization of IL-2 receptor expression.

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Albert D. [Signature] MD 9/15/92
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III. INTRODUCTION

A. Interaction of HIV-1 and Bone Marrow. The severity of immunohematologic impairment of HIV-infected individuals spans a broad range, culminating in a clinical syndrome characterized by hematologic abnormalities and the profound impairment of immune function. Although immunosuppression can be explained in part by the selective depletion of permissive CD4 positive helper/inducer T lymphocytes, the mechanisms by which the virus interferes with the generation of other marrow derived lineages are not known. In addition to lymphopenia, AIDS and AIDS-related complex (ARC) patients often suffer from a variety of peripheral blood abnormalities including granulocytopenia, thrombocytopenia (Spivak et al., 1984, Treacy et al., 1986), and anemia (Castella et al., 1985). Despite this finding, bone marrow from these subjects ranges from normocellular to hypercellular, with distinct abnormalities in the maturation of all lineages similar to those seen in myelodysplastic syndrome (Schneider and Picker, 1985). Similarly, there is no apparent deficit in multipotential hematopoietic progenitor cells as reflected in normal numbers of bone marrow cells giving rise to erythroid and granulocyte-macrophage colonies in vitro (Donahue et al., 1987). Thus it appears probable that HIV-mediated myelosuppression occurs by interruption of maturation, either by direct noncytolytic infection hematopoietic cells, or by interfering with ancillary cell populations that regulate their growth. Studies of in vitro hematopoietic colony growth implicate T lymphocytes in the regulation of neutrophil, macrophage, eosinophil (Chickkappa and Phillips, 1984), and erythroid (Sharkis et al., 1978) lineages. Thus it is conceivable that marrow dysplasia occurs, at least in part, secondary to the loss or alteration of lymphocyte mediated regulation. However, HIV may be capable of infecting myeloid precursors in vivo (Bush et al., 1986) as well as in vitro (Donahue et al., 1987), leaving open the possibility that while not directly cytotoxic to marrow progenitors, HIV may alter the maturational pathway of infected progenitors or affect their ability to receive and/or respond to regulatory signals.

Of equal importance in terms of viral pathogenesis, immature myeloid cells may serve as a reservoir for replicating or latent virus. Myeloid derived cell lines such as U937, normally highly permissive for virus growth, may be rendered nonpermissive by culture in the presence of granulocyte-monocyte colony stimulating factor (GM-CSF) (Hammer et al., 1986).

Understanding the interactions between HIV and hematopoietic cells is also of potential importance to the rational development of therapeutic strategies for the treatment of AIDS and AIDS related malignancies. Bone marrow transplantation (BMT) has been proposed as a possible treatment for AIDS because it presents the opportunity to reconstitute the immune system with cells derived from a healthy donor. The infectious nature of AIDS, however, has justifiably given rise to the concern that the newly developing immune cells would become infected and suffer the same fate as the recipients own cells. This in fact appears to be the case in a limited number of marrow transfusions performed without the benefit of a marrow ablative preparative regimen (Lane and Fauci, 1985). This problem has prevented the initiation of full scale clinical trials of BMT for the treatment of AIDS. However, the development of antiviral agents such as the analog 3'-Azido-3'-deoxythymidine (AZT, BW A509U, Burroughs Wellcome), raises new possibilities for BMT. It is therefore important to determine how anti-retroviral agents, which themselves may be myelosuppressive, affect the balance of lymphocyte-marrow-HIV interactions.

That the failure of previous transplant attempts may be related to the quantitative extent of virus "burden" at the time of transplant is suggested by our study of BMT recipients who were inadvertently infected with transfusion borne HIV shortly after receiving allogeneic marrow transplants (Duff et al., 1985). In two well documented cases, HIV infection at a time when the patients were severely immunocompromised resulted in no detectable alteration of the pace or extent of reconstitution. Both

patients are alive almost 9 years later. One is well and active, with persistent antibody titers and lymphadenopathy. The second developed clinical AIDS more than 7 years after transplant. Although the eventual outcome of infection may be no different in these patients than in individuals infected in health, these results give us reason to hope that under the coverage of appropriate antiviral agents, and with an appropriate marrow ablative regimen, a durable improvement of immunity could be effected in AIDS patients. We have now transplanted 6 HIV seropositive patients, 3 of whom had hematologic malignancy in the absence of opportunistic infection (see Holland et al., 1989, Appendix). Our results indicate not only significant immune reconstitution, but of reduction of virus load to as well.

B. Evaluation of immunologic changes related to progression of HIV infection. Many immune functions decline as HIV infection progresses (Margolick and Fauci, 1987), and the culmination of this general decline is the onset of opportunistic infections and neoplasms. Key questions that remain unanswered are at what point does this decline become important for the health of the infected person, and which functions are critical to this adverse health effect? Answers to these questions are necessary for identifying individuals at high risk for progression of HIV infection and for developing effective therapies for HIV infection.

We have approached these issues in several ways. We hypothesize that a more detailed understanding of the pathogenesis of immune compromise will help us determine which HIV-infected individuals have stable infections and which will progress. We have employed limiting dilution analysis (LDA) because, as detailed below, LDA can be used to measure the frequency of cells belonging to functionally defined lymphocyte subsets, and yields quantitative information at the level of the single precursor.

Of particular interest is the ability of T lymphocytes to respond to IL-2, a lymphokine which is critical for proliferation of T cells; we and others have hypothesized that defects in the ability of T lymphocytes to proliferate in response to IL-2 might contribute to the loss of cell mediated immunity that is the hallmark of AIDS. Therefore, we have conducted studies of this basic T cell function in patients at different stages of HIV infection. The results of these studies formed the basis for our biochemical studies to elucidate the molecular mechanisms responsible for decreased IL-2 responsiveness of cells from patients with advanced HIV infection.

Another approach which we have used to distinguish individuals with different responses to HIV infection is to analyze the immune response to the infection itself. Changes in T lymphocyte subsets in the peripheral circulation after infection with HIV are well documented and include a decrease in CD4⁺ T cells and an increase in CD8⁺ T cells. We have focused attention on other, less well studied lymphocyte populations. We demonstrated that when CD3⁺, CD4⁺, and CD8⁺ lymphocytes are determined independently (one-parameter analyses), an increase in the difference between the number of CD3⁺ lymphocytes and the sum of CD4⁺ and CD8⁺ lymphocytes [i.e., $CD3 - (CD4 + CD8)$] occurs at the time of seroconversion and continues afterward (Margolick et al, 1989). This increase is present in both absolute and relative terms and cannot be accounted for by changes in the CD4⁺ and CD8⁺ populations of T cells. It is not known whether this response to HIV infection, or its underlying cause, has prognostic significance, but our findings do warrant further prospective study.

Finally, in view of the critical nature of loss of antigen-induced immune responses in the pathogenesis of AIDS (Lane et al, 1985), we studied the expression of Class II histocompatibility molecules expressed on antigen-presenting cells (monocytes) from HIV-infected individuals. These molecules (HLA-DR) are essential for normal presentation of antigen to T helper lymphocytes in the initiation of antigen-specific immune responses. Early reports indicated that expression of these molecules by antigen-presenting cells might be diminished in patients with AIDS, suggesting a possible

mechanism for impaired cellular immunity in AIDS (Belisto et al, 1984; Heagy et al, 1984). However, our current findings, as well as those of others (Sei et al, 1986) have not confirmed the early reports.

IV. BODY

A. Methods

1. Patient populations

a. **SHARE patients.** SHARE participants are gay/bisexual men from the Baltimore-Washington area who had not been diagnosed as having AIDS prior to entry into the study (April - November, 1984). At each semi-annual visit participants undergo interview, physical examination, and laboratory testing. Tests include HIV-1 serology (ELISA and immunoblot for confirmation), complete blood count, screening for gonorrhea and syphilis, and T-cell subset analysis (CD3, CD4 and CD8). Several aliquots of plasma, serum, lymphocytes, semen, urine, and mouthwash are obtained for frozen storage at each semi-annual visit. We also have data on serum immunoglobulins, cytomegalovirus serology, hepatitis-B serology, and routine serum chemistries from the first two visits. Volunteers have been sought from the SHARE cohort for the proposed additional prospective studies.

As of February, 1989, SHARE has completed 10 full cycles of follow-up visits. Follow-up rates have been notably high. More than eighty-five percent of the initial cohort are still being followed. There have been 113 cases of AIDS diagnosed thus far in SHARE; 80 participants who were seronegative at baseline have seroconverted.

b. **Moore Clinic Outpatients.** Patients, staged according to the CDC criteria, were seen at the Moore Clinic of the Johns Hopkins Hospital. Peripheral blood was drawn during routine office visits. Control subjects were Johns Hopkins students and employees who denied belonging to HIV risk groups. Informed consent was obtained according to a protocol approved by the Johns Hopkins Joint Committee for Clinical Investigation.

c. **ALIVE study patients.** AIDS Link to IntraVenous Experiences (ALIVE) is a prospective cohort study of 800 intravenous drug users (IVDUs) which was initiated at Johns Hopkins University in February, 1988. The goal of the study is to determine the natural history of HIV infection in this high risk population. The study was designed to enroll 640 HIV - seropositive IVDUs as well as 160 seronegative IVDUs to serve as controls and to prevent stigmatization of study participants. Participants are seen in clinic at 6 month intervals for physical and laboratory examination, including measurement of T cell subsets, as described above for SHARE.

d. **Bone Marrow Transplant Recipients.** Six HIV positive patients undergoing bone marrow transplantation (3 syngeneic, 3 allogeneic) were treated at the Johns Hopkins Oncology Center. All patients met study inclusion criteria approved by the Johns Hopkins Joint Committee on Clinical Investigation. Recipients of syngeneic marrow had HIV seronegative identical twin donors. Recipients of allogeneic marrow had HIV associated nonHodgkin's lymphoma and received grafts from HIV negative MHC identical sibling donors.

2. Virus strains. Low passage monocyctotropic strains SF162 and KW were obtained from, J. Levy and H. Farzadegan, respectively. Strains CD and TD were clinical isolates obtained from H. Farzadegan. HTLV-IIIb was originally obtained from the laboratory of R. Gallo.

3. Antigens and Mitogens. Tetanus toxoid, preservative free refined concentrate, the generous gift of Wyeth Laboratories (Marietta, PA.), was used at a concentration of 1 to 5 ug/ml. CMV antigen (MA Bioproducts, Walkersville, MD) was used at a 1/1000 dilution of stock. PHA, CONA and PWM (Sigma Chemicals) were used at 2, 5, and 5 ug/ml, respectively.

4. Lymphoproliferative Responses. Peripheral blood mononuclear cells (PBML) from HIV-1 positive subjects or healthy volunteers were resuspended to 7.5×10^5 /ml in complete medium (CM) consisting of RPMI 1640, 10% heat inactivated human AB serum, 2 mM L-glutamine, 10 mM HEPES buffer, 50 ug/ml gentamicin, 100 U/ml penicillin, and 5×10^{-6} M 2-mercaptoethanol. The cells were aliquoted (200 ul/well) in U-bottom microtiter plates (Flow Laboratories, Cat. No. 76-013-05). Antigens were added at 10x final concentration (see Antigens, above) in triplicate and the plates incubated at 37°C, 5% CO₂. Cells were cultured with and without the addition of exogenous Interleukin-2 (IL-2, 4 units/ml) on day 3. Serum-free supernatant, obtained from MLA-144 cell culture was used as a source of IL-2. On day 6, the cultures were pulsed with ³HTdR (0.5 uCi/well) and incubated an additional 4 h before being harvested onto glass fiber filters (Whatman, GF/A) with a multiple automatic sample harvester. ³HTdR uptake was measured by liquid scintillation spectrometry. Results were expressed as net counts per minute above background (NET CPM), where background counts were determined by culturing in the presence of diluent alone. Analysis was performed using the logarithm of the NET CPM because CPM data are log-normally distributed.

5. Teflon culture of human bone marrow. Cells were suspended to 1×10^6 /ml in growth medium consisting of alpha-MEM, 30% FCS, 1% BSA plus antibiotics and cultured in 50 ml Teflon Erlenmeyer flasks (Nalgene), 5 ml/flask. Cultures were supplemented with either lymphocyte conditioned medium (10%) or recombinant human growth factors (rhM-CSF, 1/4,500 dilution of stock plus rhGM-CSF, 5 U/ml, Genetics Institute, Cambridge, MA).

6. Methylcellulose culture of human bone marrow. Bone marrow was grown in methylcellulose medium according to a modification (Rowley et al., 1987) by the method of Ogawa et al. (1976). Cells were plated in alpha-MEM medium containing 0.8% methylcellulose, 30% fetal calf serum, 1% BSA, 0.1 mM 2-mercaptoethanol and either 10% lymphocyte conditioned medium or recombinant human growth factors.

7. Limiting dilution analysis of bone marrow progenitor growth. Progenitor enriched bone marrow cells (described above) from healthy subjects (24 h after infection with HIV-1 or Mock infection) or from HIV-1 positive subjects were serially diluted in growth medium (see Teflon cultures) and plated in round bottom wells (200 uL/well). Dilutions were made in half-log₁₀ steps ranging from 300 to 1 cell/well, with one full plate (96 replicates) for each cell concentration. Cultures were evaluated microscopically for colony growth on a weekly basis beginning on day 14. Precursor frequency was determined by the maximum likelihood method as described above. Medium was removed (100 uL) and cells fed every 2 weeks. Medium from colony positive wells was stored at -135°C for p24 assay.

8. Preparation of a progenitor enriched bone marrow fraction by elutriation (positive selection). Mononuclear cells (prepared by Ficoll-Hypaque gradient centrifugation) from heparinized aspirated bone marrow from HIV⁺ or normal donors were elutriated (3000 rpm, standard chamber, JE6 rotor). 100 ml fractions were collected at flow rates of 17 ml/min (load), 24 ml/min (predominately lymphocytes), and 28 ml/min (monocyte enriched). Cells retained in the chamber at 28 ml/min were

flushed by turning off the rotor while maintaining medium flow (R/O fraction, progenitor enriched). Cells from the R/O fraction were resuspended ($80-100 \times 10^6$ cells) in 2 ml Percoll ($\text{sg} = 1.080$) and layered beneath 5 ml of 1.074 Percoll which in turn was layered beneath 1 ml RPMI-1640 medium. Gradients were centrifuged at 1475 rpm for 20 min. The blast cell enriched, lymphocyte and monocyte depleted second band was collected and washed 2x with PBS.

9. Preparation of a progenitor enriched bone marrow fraction (positive selection).

Positive selection of CD34⁺ cells was accomplished by immune adherence. A 60 x 15 mm plastic petri dish was flooded with 3 ml goat anti-mouse IgG (10 $\mu\text{g}/\text{ml}$) in 0.05 M Tris buffer (pH 9.5). After 2 h at room temperature the dish was washed 4 x with PBS-A, 0.2% BSA. Bone marrow mononuclear cells (12×10^6 prepared by Ficoll/Hypaque gradient separation) were incubated for 30 min on ice with anti-MY-10 antibody (20 $\mu\text{L}/1 \times 10^6$ cells), and washed twice with cold PBS-A, 0.2% BSA were centrifuged onto the prepared petri dish (200 RPM for 2 min). The petri dish was then incubated for 30 min at 4°C and subjected to 2 cycles of gentle rinsing (PBS-A, 0.2% BSA) to obtain nonadherent cells, followed by 2 cycles of vigorous rinsing to obtain adherent (positively selected) cells. Phenotype was confirmed by flow cytometry after overnight incubation in growth medium.

1. p24 antigen capture assay. HIV-1 antigen (p24) was detected in culture supernatants using an enzyme-linked immunosorbent (ELISA) antigen capture assay. The capture reagent, a high titer human serum, diluted 1/100,000 (in carbonate buffer pH 9.6) was adsorbed to microtiter plates (75 $\mu\text{L}/\text{well}$) (FLOW Laboratories, McLean, VA, Cat. no. 76-381-04) overnight at 4°C. The plates were washed 2 times in PBS-TWEEN (0.05%) using a Skatron Micro II plate washer. PBS-A containing 3% bovine serum albumin (BSA) was added to each well (100 $\mu\text{L}/\text{well}$) and incubated 1 h at 37°C. The plates were washed as before. Test supernatants and a positive control standard (human recombinant p24, Du Pont Laboratories) were serially diluted in the plates (75 $\mu\text{L}/\text{well}$, 8 two-fold steps) in PBS-A, 1% BSA, 0.5% NP-40. The starting antigen concentration was 5000 pg for the p24 standard and 1/2 for the test supernatants. The plates were incubated overnight at room temperature and washed as before. Rabbit anti-HIV-1 p24 was added (1/3000, 100 $\mu\text{L}/\text{well}$, MicroGeneSys) and the plates were incubated 4 h at 37°C. After 2 washes, goat anti-rabbit IgG alkaline phosphatase conjugate (SIGMA), diluted 1/300 in PBS-NP40 (0.5%) (1% BSA), was added (100 $\mu\text{L}/\text{well}$) and incubated 2 h at 37°C. The plates were washed and p-nitrophenylphosphate substrate (SIGMA) at 1 mg/ml in diethanolamine buffer (pH 9.8) was added (100 $\mu\text{L}/\text{well}$) and incubated 30 minutes in the dark at room temperature. 50 μL of 3M sodium hydroxide was added to each well and the plates read at 405 nm on a Titertek Multiskan Plate Reader. Antigen concentration was determined relative to the recombinant HIV-1 p24 standard. The assay was directly compared to the licensed test provided by Abbott Laboratories and had similar sensitivity (approximately 50 pg/ml).

2. Assessment of antigen presentation in BM derived monocytes. Development of immunocompetent antigen presenting cells from monocyte depleted bone marrow was evaluated after 7 and 14 days culture in the presence of recombinant human colony stimulating factors as described above (5. Teflon culture). Cultured cells (20,000 cells/well) were allowed to adhere to flat bottom microtiter plates for 2 h at 37°C after which nonadherent cells were washed away with PBS-A. The remaining adherent cells were pulsed with antigen (2 $\mu\text{g}/\text{ml}$ tetanus toxoid) and incubated for 2 h at 37°C. The cells were then washed to remove excess antigen, irradiated at 1500 rad and cocultured with 1×10^6 monocyte depleted autologous lymphocytes. The ability of these antigen-pulsed adherent cells to trigger proliferation of T cells was then determined by ^3H thymidine uptake as described above.

3. **Two color flow cytometry.** Cell surface phenotype of bone marrow mononuclear cells was determined by 2 color immunofluorescence modified from the protocol of Loken et al. (1987) using a Becton-Dickinson FACSCAN analyzer. Briefly, indirect staining was first performed using fluorescein isothiocyanate labeled anti-mouse Ig (affinity purified, F(ab')₂, raised in sheep, Sigma Chemicals). Staining with a second, phycoerythrin conjugated, monoclonal antibody was performed after washing the cells in filter sterilized medium containing PBS-A, 1 % normal mouse serum, 4 % newborn calf serum, 2 % normal goat serum and 2 % human AB serum. Monoclonal antibodies were obtained from Ortho Pharmaceuticals (Raritan NJ), Becton-Dickinson Corporation (Mountainview, CA,) and Coulter Immunology (Hialeah, FL). Specimens from SHARE or ALIVE were stained with directly conjugated antibodies as listed under Results using previously described methods (Margolick et al, 1989). Briefly, antibodies were added to 100 ul whole blood for 30 min, the red cells were lysed with a hypotonic ammonium chloride solution, and the cells were washed, fixed in 1 % paraformaldehyde, and analyzed on a Coulter EPICS C flow cytometer.

4. **Flow cytometric analysis of surface expression of IL2R α and β chains:** Peripheral blood lymphocytes expressing CD3, CD4, and CD8 were measured in whole blood using an ammonium-sulfate based lysing technique to remove erythrocytes and a staining technique that has been described (Giorgi et al, 1990; Hoffman et al, 1980) and also in previous reports. Two-color flow cytometry was used, with two combinations of antibodies directly labelled with FITC or phycoerythrin (PE) as follows: CD3-FITC/IgG₁-PE and CD8-FITC/CD4-PE.

For analysis of IL2R α and β subunits on cultured lymphocytes, 10⁶ cells were washed three times in PBS containing 0.1 % BSA and 0.01 % azide (wash buffer), resuspended in 0.1 ml cold wash buffer, and stained with either FITC-labelled anti-IL2R α or a 1:1000 dilution of anti-IL2R β antibody (TU27, graciously provided by Dr. S. Taki, Ajinomoto Co. Inc., Central Research Laboratories, Kawasaki, Japan, (Ohasi et al, 1989) followed by FITC labelled goat anti mouse IgG antibody, with normal mouse IgG as a control. All other antibodies were purchased from Becton-Dickinson (Mountain View, CA), and all incubations with antibodies were performed on ice for 30 min and followed by three washes in wash buffer. Stained cells were fixed in wash buffer containing 0.5 % formaldehyde (Polysciences, Warrington, PA) and stored in the dark at 4° C until analysis on an EPICS C or Profile II flow cytometer (Coulter Electronics, Hialeah, FL) with selection of lymphocytes by gating using forward and side light scatter.

5. **Cell culture:** PBMC were isolated from heparinized blood of HIV⁻ and HIV⁺ homosexual men participating in the SHARE, the Baltimore center of the MACS by centrifugation at 400 g for 30 min over Ficoll-Hypaque (Boyum, 1968). PBMC were washed and suspended at 5 x 10⁶ cells per ml in complete culture medium consisting of RPMI 1640 (Whittaker Bioproducts, Walkersville, MD), 10 % FBS (Hyclone, Logan, Utah) and 100 μ g per ml gentamicin. The cells were stimulated with 0.5 μ g per ml PHA (Wellcome Research Laboratory, Janesville, NC) and, unless otherwise stated, cultivated in 75 cm² tissue culture flasks at 37°C in 5 % CO₂ and 95 % humidity.

6. **Cell proliferation:** 5 x 10⁴ PBMC were cultured in triplicate wells of flat bottom 96 well microtiter plates in 0.2 ml with PHA or complete medium alone for 72 h, with a pulse of 1 μ Ci per well of ³H-TdR (Sp. Act 6.7 Ci/mmol, NEN) for the last 18 h. To study the stimulatory effects of IL2, cells were stimulated with PHA for 55 h, washed, and recultured overnight in the absence of lymphokines. Cells was cultured at 5x10⁴ cells per well with or without 1 nM rIL2 for 48 hrs and pulsed with 1 μ Ci per well of ³H-TdR for the last 4 h. To analyze the role of TGF- β , 1.2 x 10⁶ PBMC in 1.2 ml complete

medium were stimulated with PHA in the presence of predetermined optimal concentration (12.5 μg per ml) chicken anti-TGF- β , neutralizing antibody or control (Chicken IgG) antibody (both from R & D Systems, Minneapolis, MN) and cultured in 24 well flat bottom tissue culture plates. After 55 h in culture, cells were washed and recultured overnight in complete medium. 5×10^4 cells per well were cultured in 0.2 ml complete medium for 48 h in the presence or absence of rIL2, TGF- β , antibody or control antibody with the last 4 h pulse with ^3H -TdR. The amount of ^3H -TdR incorporated into cellular DNA was quantified by standard liquid scintillation counting.

7. Limiting dilution analysis of lymphoproliferative response to Interleukin-2. LDA of lymphocyte proliferation was used to estimate the proportion of activated (i.e. IL-2 receptor bearing) circulating T lymphocytes. Assays were performed in U-bottom 96 well plates (Linbro). PBML obtained from Ficoll-Hypaque separated peripheral blood were serially diluted in CM. For experimental wells the medium was supplemented with 25% MLA-144 supernatant (approximately 20 units/ml of IL 2 final concentration). Twelve replicates were plated per dilution. In subsequent experiments, we used 8 four-fold dilutions ranging from 80,000 to 4.9 cells/well. Control cultures grown in the absence of IL-2 were plated in sextuplicate at each cell dilution. Cultures were incubated at 37°C, 5% CO₂ humid atmosphere. On day 6 each well received 0.5 uCi of methyl-tritiated thymidine and was harvested 4 h later. Thymidine uptake was measured by liquid scintillation spectrometry as described above. Experimental wells were scored as positive or negative by comparison to CPM incorporated in control (i.e. without IL-2) cultures assayed at the same cell concentration. A well was considered positive if it exceeded the greater of the geometric mean control CPM plus three standard deviations or 500 CPM.

The frequency of IL-2 responsive cells was estimated by a two-step procedure. Briefly, a preliminary estimate was made by determining the slope of the least squares line of best fit of the log fraction negative wells versus the responder cell concentration. The frequency was then determined by the maximum likelihood approach using the least squares estimate as an initial value. Net counts per minute per precursor (Net CPMP) was calculated at each cell concentration by dividing the net counts per minute (Net CPM) in each positive well by the average number of precursors per positive well. Net CPM was obtained by subtracting background CPM from experimental CPM, where background CPM represents an N weighted mean of control CPM (N = 6) and CPM obtained in negative experimental wells. Precursors per positive well was calculated from the LDA frequency estimate and approaches unity at limiting dilution.

8. ^{125}I -IL2 binding: After stimulation with PHA for 55 h, PBMC were washed twice and reincubated in complete medium at 37°C overnight (at least 16 h). The cells were isolated, washed, and used for ^{125}I -IL2 binding assay as described previously (Robb et al, 1985). Briefly, 100 μl of serial dilutions of 1.5 - 200 pM of ice cold ^{125}I -IL2 (Sp. Act 33-46 $\mu\text{Ci}/\mu\text{g}$, New England Nuclear, Boston, MA) were mixed with 100 μl of 5×10^5 cells in 1.5 ml microcentrifuge tubes. After 30 min incubation on ice, 1 ml of ice-cold media was added to each tube, and the tubes were then centrifuged at 9000 g for 2 min. The supernatants were saved and the cell pellets suspended in 100 μl of RPMI-1640 and centrifuged through the oil barrier. The tips of the tubes containing the cell pellets were clipped, and radioactivity was counted in a γ counter. The nonspecific binding of ^{125}I -IL2 to the cells was determined in some experiments by adding $\approx$ 100 fold excess cold rIL2 (provided by Cetus Inc, Emeryville, CA). The B max of IL2 binding and the equilibrium dissociation constant (kd) were determined using the ENZYFIT program (Enzfitter Manual).

9. Preparation and analysis of IL2R α , β and TGF- β 1 mRNA: Total cellular RNA was isolated from 20×10^6 unstimulated or 24 h PHA stimulated cells by the RNAzol method (Cinna/Biotec x

Laboratories, Friendswood, TX). For unstimulated cells 12 μ g and for stimulated cells 15 μ g of RNA per lane was electrophoresed, blotted, and hybridized as described previously (Raj and Pitha, 1983) with CDNA probes specific for human IL2R α , IL2R β (plasmids graciously provided by Dr. W. J. Leonard, NICHD, Bethesda, MD), TGF- β 1 (plasmid graciously provided by Dr. R. Derynck, Department of Developmental Biology, Genentech, South San Francisco, CA) and human actin (plasmid graciously provided by Dr. L. H. Kedes, Stanford University School of Medicine, Palo Alto, CA) labelled to high specific activity with ^{32} P CTP (NEN) using the random primer method (Feinberg and Vogelstein, 1983). Air dried filters were exposed at -70° C to Kodak XAR film with intensifying screens, and autoradiograms were scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, CA). The concentrations of mRNA bands were normalized to the concentration of actin mRNA bands (taken as 2000 arbitrary densitometric units).

10. **^{125} I-IL2 cross linking:** After stimulation with PHA for 55 h, and recultivation for overnight in complete medium as described above, $4-8 \times 10^6$ cells were incubated on ice with 1 nM ^{125} I-IL2 in 0.5 ml RPMI 1640 containing 3% FBS and 0.1% Na $_2$ S $_2$ O $_3$ for one hr. After three washes with PBS containing 1 mM MgCl $_2$, the receptor-ligand complexes on cells were covalently cross linked with freshly prepared disuccinimidyl suberate (DSS) (Pierce Chemicals, Rockford, IL) as previously described (Sharon et al, 1986). The cells were lysed with Tris buffer, pH 6.8, containing 0.5% NP-40 and electrophoresed under reducing condition on gels containing 10% polyacrylamide and 0.1% SDS (Saragovi and Malek, 1987). After electrophoresis, the gels were fixed, dried under vacuum, and autoradiographed as described (Chopra et al, 1989). Quantitative analysis of autoradiograms was performed using a scanning densitometer (Pharmacia LKB, Biotechnology, Piscataway, N.J.). The results are presented as arbitrary units corresponding to the relative areas under the peak.

11. **Statistical analysis:** Data analysis was performed using a commercially available statistical package (SYSTAT, Evanston, IL). Student's t-test (two-tailed) was used for comparison of radioactive ^{3} H-TdR and densitometry data. ^{3} H-TdR uptake data was approximately log normally distributed and were log transformed prior to use of Student's "t" test. IL2 receptor data were bimodally distributed; the Kruskal-Wallis rank order test was used to compare IL2R expression on cells from HIV- and HIV+ donors.

C. Results

1. Interaction of HIV-1 and Bone Marrow.

a. **Culture of Human Bone Marrow Cells.** Many of our planned experiments involve assessment of marrow growth and virus growth as a function of time after infection. This is difficult using currently available culture methods that use a semi-solid culture medium such as agar or methylcellulose. We have recently adapted a culture method described for the long term culture of monocytes. By using Teflon culture vessels and highly specific recombinant growth factors we are able to grow sufficient numbers of marrow cells and maintain them in culture for up to three months. Culture vessel and culture density aside, all other culture parameters are identical to that used in a conventional methylcellulose assay.

b. **Lymphocyte and monocyte depleted bone marrow and purified marrow progenitor cells can be infected with HIV-1.** We have used the system described above to grow human bone marrow cells that have been purified using two different methods. The object of these purifications

is to remove cell types that are already known to be permissive to HIV infection. After lymphocytes and monocytes have been removed we can culture their progenitors and determine when during development these cells become infectable. In this way we can determine whether HIV-1 growth is limited to mature peripheral blood cells or alternatively whether virus can be harbored in more primitive progenitor cells.

We have used Counterflow Centrifugal Elutriation and depletion with monoclonal antibodies plus complement (anti-CD4, anti LeuM1, anti-CD3) as a means of lymphocyte and monocyte depletion of bone marrow progenitors. We have infected lymphocyte/monocyte depleted human bone marrow with several strains of HIV and have used in situ hybridization, in collaboration with Dr. Opendra Narayan and his colleagues, to probe for expression of viral GAG sequences. We have detected viral GAG mRNA in the monocytoid progeny of monocyte depleted bone marrow after 14 days culture in the presence of recombinant human M-CSF and GM-CSF, suggesting that cells earlier than those found in the periphery may serve as viral hosts (figure 1).

Although elutriated marrow is depleted of mature lymphocytes and monocytes it contains progenitor cells of all lineages at various stages of differentiation. We have taken advantage of a monoclonal antibody that identifies very early marrow progenitor cells (anti-CD34) to positively select a relatively homogeneous cell population for culture and infection. Studies involving progenitor growth at limiting dilution are now in progress (see below) and will determine whether these primitive multipotential marrow progenitors can be infected and transmit the virus to their progeny.

c. **Effect of HIV-1 infection on growth of purified progenitor cells: comparison of conventional methylcellulose culture and Teflon liquid culture systems.** We compared these two systems using visual colony count to assess progenitor growth in the methylcellulose system and tritiated thymidine uptake to assess proliferation in the Teflon culture system. Purified marrow progenitor cells (CD34⁺ [MY-10⁺] cells isolated by immune adherence) were used in both assays. Replicate cultures were established after 24 h adsorption or mock adsorption with HIV-1 strain KW (a monocyto-tropic strain, the gift of Dr. H. Farzadegan). There was close correlation between the 2 assays (figure 2, A). Although a modest decrease in progenitor growth was detected by both colony count (figure 2, B) and thymidine uptake (figure 2, C), robust growth of both monocytoid and granulocytoid lineages were observed in the cultures derived from infected progenitors. The use of purified progenitor cells and recombinant exogenous growth factors suggest that reports describing severe inhibition of bone marrow colony growth by HIV-1 may reflect secondary effects of the virus on other cell populations present in unfractionated marrow (e.g. lymphocytes, monocytes).

d. **Limiting dilution analysis (LDA) of human bone marrow progenitor growth.** The results described above (section b.), as well as those reported recently by Folks et al (1989), in which monocyte/lymphocyte depleted bone marrow progenitors were shown to be infectable with HIV-1 (which was detectable in their monocytoid progeny 2 weeks later) are subject to a major criticism: that even low levels of contaminating infected monocytes (e.g. <0.1%) could harbor virus and transmit it to newly developing monocytes as they mature. We are now addressing this problem by using LDA, a quantitative method to determine the frequency with which cells having a specific functional property are present in a heterogeneous population. We have used this method extensively to lymphoproliferative responses (see below). Recently we have also adapted this methodology to the growth of human bone marrow progenitor cells; its advantage over bulk culture methods is that at limiting dilution one can determine with a given statistical precision that all cells present in a culture were derived from a single precursor. One can also determine the probability that a cell of a given phenotype (e.g. a lymphocyte or a monocyte) was also present in that culture. We are now using LDA to grow monocyte/lymphocyte

depleted bone marrow (from HIV-1 infected subjects or marrow infected in vitro prior to culture). These studies should help resolve important question concerning the range of viral permissiveness in developing human bone marrow progenitor cells.

e. Development of antigen presenting cells from monocyte depleted bone marrow: a system to study the effects of HIV-1 infection on monocyte ontogeny. In order to study the effects of HIV-1 on the phenotypic and functional ontogeny of antigen presenting cells (APC), we have developed a liquid culture system in which monocyte depleted bone marrow is driven to proliferate and differentiate with recombinant human GM-CSF and M-CSF in Teflon flasks. Ability of plastic adherent cells from fresh and cultured monocyte depleted bone marrow to present the protein antigen tetanus toxoid to autologous monocyte depleted lymphocytes was assessed by measuring $^3\text{HTdR}$ uptake after 5 days co-culture in microtiter wells. Generation of APC function and maximal CD4 expression (as measured in the monocyte scatter gate) required the presence of CSFs and a 14 day culture interval (table 1). In contrast, LeuM3⁺ phenotype developed after 7 days in culture with CSFs. This system provides a simple and rapid assessment of antigen presenting cell ontogeny, demonstrating the appearance of LeuM3 prior to detectable APC function.

In two independent experiments (table 2), monocyte depleted bone marrow was inoculated with HIV-1 (lymphotropic HTLV-III_B or monocyto-tropic SF162 strains), washed after 24 h, cultured with CSFs for 14 days, and transferred to microtiter wells. Cells adhering to the plastic wells were then irradiated and tested for the ability to present tetanus toxoid antigen by co-culture with autologous monocyte depleted lymphocytes. Antigen specific lymphoproliferation was increased 2 to 3-fold over that observed when mock infected adherent cells were used as APC. Intimate contact between APC and lymphocytes during immune interactions may be a mechanism by which the virus facilitates its own transmission and propagation. It is not presently known whether the observed increase in thymidine uptake is due directly to interaction of lymphocytes with APCs that are actively expressing HIV gene products (approximately 10%).

f. Bone Marrow Transplantation in AIDS. One of the stated AIMS of the this contract is to model the use of BMT as a possible treatment for AIDS. This area was advanced with the first transplant of an HIV-1 seropositive patient using full myeloablation and azidothymidine. This laboratory has collaborated in this project by monitoring immune reconstitution after transplantation. The first patient, who in addition to his HIV disease presented with an aggressive lymphoma, died of relapse of his tumor 50 days after transplant. Before his death, several important parameters of antigen specific immunity had been restored. At autopsy we were unable to detect virus using polymerase chain reaction gene amplification, the most sensitive technique available. We initiated a protocol to transplant more HIV-1 positive patients who have hematologic malignancies and explored the possibility of initiating a protocol to transplant a carefully selected population of HIV-1 seropositive patients without cancer. These results were published in abstract form (V International AIDS conference, Montreal) and as a full manuscript (Annals of Internal Med, appended).

The second patient enrolled into our phase I trial of allogeneic BMT plus azidothymidine for HIV-1 positive patients with malignancies was a 36 y/o man with nonHodgkin's lymphoma and a prior history of pneumocystis pneumonia. Because of prior radiation therapy he could not receive our first choice marrow ablative regimen (200 mg/kg cytoxan [CY] plus 1200 rads total body irradiation [TBI]). He instead received busulfan (16 mg/kg) and cytoxan (200 mg/kg). Unlike the first patient transplanted under the CY-TBI protocol, HIV-1 was never cleared. Blood and bone marrow specimens remained virus positive after therapy. Marked restoration of immune responses, due to donor and recipient

immunization (tetanus and diphtheria toxoids) and adoptive transfer of donor immunity, was observed. The patient died 13 weeks after BMT of progressive multifocal leukoencephalopathy. JC virus was isolated from his brain at autopsy.

The third patient recruited into our phase I trial, a 29 y/o HIV-1 seropositive man with nonHodgkins lymphoma, received cytoxan (200 mg/kg) plus total body irradiation (1200 rads) as marrow ablative therapy. Prior to transplant his blood and bone marrow were culture positive for HIV-1. He was placed on AZT one week before initiation of marrow ablation and remained on AZT for the duration of treatment. He and his HLA matched sibling donor were immunized (tetanus and diphtheria toxoids) according to our protocol. His immune reconstitution was evaluated in his 5th month post-transplantation. He remained virus culture negative since one week after transplant (blood and bone marrow, 16 cultures), but virus was first detected in peripheral blood at 12 weeks after transplant by polymerase chain reaction gene amplification (40 days after BMT).

After determining, in the first 3 patients, that bone marrow engraftment was not compromised by continuous treatment with azidothymidine, we initiated a new protocol evaluating the use of syngeneic BMT plus AZT for treatment of AIDS without malignancy. Despite the relatively low frequency of AIDS patients with HIV negative identical twin donors, we were able to recruit 3 patients into this study since. Preparative regimen and supportive care was identical to that used for the study described above (including the Cyclosporine A regimen). As in the study described above, Dr. Donnenberg's lab monitored the reconstitution of immune responses to specific antigens, and Dr. Margolick's performed detailed flow cytometric evaluations.

g. Immune Reconstitution in HIV infected BMT Recipients. In order to facilitate adoptive transfer of immunity to tetanus toxoid, donors were immunized 7-10 days before marrow harvest, recipients were immunized immediately after marrow infusion. Figures 3 and 4 show tetanus specific antibody titers and lymphoproliferative responses, respectively, for 2 patients. The phase I trials of BMT plus azidothymidine (AZT) for HIV-1 positive patients were closed after enrolling 3 allogeneic and 3 syngeneic patients. These studies are ongoing at Emory University, where Drs. Rein Saral and Kent Holland, the principal clinical investigators, relocated as of July 1, 1991. The results of the Hopkins trial are summarized below in tabular form (table 3).

h. SCID/Hu Model for adoptive transfer of HIV-1 specific responses. One of our major goals is to develop bone marrow transplantation as a therapeutic modality for HIV-1 and HTLV infection. As discussed above, our findings (Annals of Internal Med. 111(12):973-981, 1989) suggest that it may be possible to reduce HIV-1 burden by many orders of magnitude through total marrow ablation and reconstitution under the coverage of azidothymidine. These results also indicated that antigen specific responses (to tetanus and diphtheria toxoids) could be restored through adoptive transfer of donor memory T and B cells. These data suggest that a similar strategy of transfer of HIV-1 specific immunity could be perused as an adjunct to the antiviral effects mediated by marrow ablation (cytoxan plus total body irradiation) and antiviral agents. Since we have a collaborative relationship with Dr. R. Markham, who heads the Hopkins SCID (severe combined immune deficiency) mouse facility, we were able to determine whether SCID mice reconstituted with human lymphocytes could accurately reproduce the results obtained in human marrow transplant patients, and therefore serve as a valid model for future experiments exploring transfer of HIV-1 specific responses.

The results of the first 5 experiments are detailed here. We have recently published the definitive findings (Markham and Donnenberg, 1992). CB.17 (scid/scid) SCID mice (2 mice/group) were given 1.5×10^7 peripheral blood mononuclear cells from a single normal human volunteer. Three days after

transfer the recipient mice were bled and some of the mice were then immunized intraperitoneally with 0.1 ml tetanus toxoid (10 Lf units/ml). Seven days after immunization the mice were bled to determine the levels of human anti-tetanus IgG. Mice that received human PBMC, but no tetanus and mice that received tetanus toxoid, but no human PBMC were also evaluated. Using an ELISA assay specific for human anti-tetanus IgG, the optical density of the pre- and post-immune sera was determined at serial two-fold dilutions. These data demonstrate the ability of the reconstituted SCID mice to generate secondary antibody responses shortly after adoptive transfer of cells.

In the preliminary studies described above, we demonstrated that low titer (1/8) tetanus specific antibody could be detected by ELISA in Hu-PBL/SCID chimeric mice only if the mice were boosted with tetanus toxoid following transfer of human lymphocytes. Since our studies in human bone marrow transplant recipients indicate that recent booster immunization of the donor prior to marrow harvest favors the adoptive transfer of immune memory to the marrow recipient, we examined the effect of donor and recipient booster immunization on transfer of tetanus specific human antibody responses in SCID mice reconstituted with human peripheral blood cells. Groups of 3 six to eight week old SCID mice were transplanted intraperitoneally with 1.25×10^7 peripheral blood mononuclear cells obtained from a single human donor either before or after booster immunization of that donor with tetanus toxoid. The recipient SCID mice were immunized 3 or 35 days after cell transfer with 0.1 ml tetanus toxoid (10 Lf/ml) or with 0.5 ml sheep red blood cells (SRBC, 10% vol/vol). The tetanus toxoid protocol was repeated with cells obtained from the same donor 10 days after he received a TT booster immunization (0.5 ml, 10 Lf/ml). Sera, pooled by experimental group, were obtained from recipient mice (0, 14, 35 and 45 days after cell transfer) and assayed by ELISA for human IgG to tetanus toxoid, and for SRBC specific hemagglutinating antibody.

Primary immunization of Hu-PBL/SCID chimeric mice with SRBC did not elicit a detectable antibody response. In contrast, adoptive transfer of secondary responses to T were consistently observed. The magnitude of this response was highly dependent on the immunization protocol (table 4).

These data indicate that: 1) Hu PBL/SCID chimeric mice could not generate a primary antibody response to a novel antigen (SRBC); 2) Adoptive transfer of secondary antibody responses depend on both the donor immune status and require early recipient immunization (< day 35) for optimal responses; 3) Titers achieved in optimally immunized chimeric SCID mice equaled those observed in high the boosted human donor; and 4) This model system may be of use in characterizing adoptive transfer of HIV-1 specific responses, an area of importance to the development of bone marrow transplantation as anti-retroviral therapy. These data were presented at the 6th International AIDS conference.

2. Evaluation of immunologic changes related to progression of HIV infection.

a. Flow Cytometric Studies.

(1) **Changes in T cell subsets.** Analyzing data acquired over the first three years of the multicenter AIDS cohort study (MACS), we demonstrated that when CD3⁺, CD4⁺, and CD8⁺ lymphocytes are determined independently (one-parameter analyses), an increase in CD3⁺ (CD4⁺ + CD8⁺) occurs at the time of seroconversion and continues afterward. This increase is present in both absolute and relative terms and cannot be accounted for by changes in the CD4⁺ and CD8⁺ populations of T cells. The most likely explanations for these findings were that either 1) there is an increase in "double negative" T cells, i.e., lymphocytes expressing the CD3⁺CD4⁻CD8⁻ phenotype, or 2)

there is a decrease in natural killer (NK) cells, some of which express the CD3⁺CD4⁺CD8⁺ phenotype. A paper describing these findings and discussing these possible explanations for the findings was published (Margolick et. al., Clin. Immunol. Immunopathol. 57:348-361, 1989) and a reprint is appended.

To test these hypothetical explanations for the increase in calculated double negative cells present in seropositive individuals compared to seronegatives, we studied seropositive and seronegative subjects participating in SHARE (homosexual men) and ALIVE (a cohort of intravenous drug users also being followed prospectively at Johns Hopkins beginning in February, 1988). Preliminary results derived from the 65 patients (34 seronegative for HIV-1 and 31 seropositive, including 4 with AIDS) are shown in table 5. It is important to note in this table that the expected increase in absolute number of calculated double negative cells was indeed observed and was statistically significant. This result permits a more detailed analysis of the relative contributions of the two cell populations which we expect to account for this increase.

To evaluate the possibility of an increase in true (as opposed to calculated) double negative T cells, we have stained lymphocytes with antibodies to CD3 (fluorescein-conjugated) and a mixture of CD4 and CD8 (phycoerythrin-conjugated) in a single specimen, so that lymphocytes with the CD3⁺CD4⁺CD8⁺ phenotype can be measured directly and unambiguously (figure 5). In this figure, the double negative lymphocytes are those in quadrant 4 (lower right quadrant) which fluoresce positively with anti-CD3 (green) but not anti-CD4 or anti-CD8 (red). We hypothesize that these cells may represent a T cell precursor population.

As shown in Table 5, the absolute number (cells/mm³) of lymphocytes with the double negative (CD3⁺CD4⁺CD8⁺) phenotype were significantly increased in seropositive individuals as compared to seronegatives. A similar increase was also present in relative (percent) terms (not shown). Most of this change appeared to be due to an increase in T cells (CD3⁺) expressing the gamma/delta-T cell receptor rather than the more common alpha/beta-receptor, as indicated by significant increases in the absolute number and proportion (not shown) of lymphocytes staining with an antibody (TCR δ -1, T Cell Sciences, Cambridge, MA) to the delta chain of the T cell antigen receptor.

To evaluate the possibility of a fall in NK cells expressing the CD3⁺CD4⁺CD8⁺ phenotype, we have taken advantage of the fact that some NK cells express CD8 at low levels. Therefore, we have measured the proportion and number of dim CD8⁺ lymphocytes. Although the proportion of such cells was not decreased in seropositive individuals (not shown), the absolute number was significantly decreased, consistent with this possibility. In further support of this interpretation, there was a large (67%) decrease in the absolute number of lymphocytes expressing the Leu19⁺CD3⁺ phenotype characteristic of NK cells in seropositive as compared to seronegative subjects. This decrease was statistically significant despite the smaller number of subjects studied.

Taken together, these results suggest that the increase in calculated CD3 minus (CD4 plus CD8) is due to both an increase in double negative T cells and a decrease in natural killer cells. On the basis of these preliminary data, the contribution of the decline in NK cells appears to be about twice that of the increase in true double negative T cells. A role for NK cells in host defense against HIV has been postulated, but most studies have not found a decrease in NK cell numbers until after the onset of AIDS. Our results suggest that this decline occurs much earlier than has been thought, and it will therefore be important to determine if this decrease is of prognostic significance. Studies to investigate this possibility using the MACS data are underway. In addition, our data are consistent with the hypothesis that double negative T cells, as well as T cells expressing the $\gamma\delta$ -TCR, may play a role in defense against HIV. These results were presented and published in abstract form (annual meeting of the Clinical Immunology Society, Alexandria, VA, November 1989 see appended abstract).

(2) Natural killer (NK) cells and $\gamma\delta$ T cells in participants in SHARE. We have continued to study proportions and numbers of natural killer (NK) cells and $\gamma\delta$ T cells in participants in SHARE, the Baltimore chapter of the Multicenter AIDS Cohort Study (MACS). The cells being quantified include $\gamma\delta$ T cells, both CD8+ and CD8-, natural killer cells as defined by the CD56+ CD3- phenotype, and the standard CD3, CD4, and CD8 lymphocytes. In addition, we have also specifically measured the number of T cells expressing CD8 but at dim levels rather than the bright levels of immunofluorescence characteristic of mature cytotoxic T cells. Findings on the first 64 AIDS-free participants are summarized in table 6. Sample size has subsequently been increased to 232 (table 5), with identical conclusions. The results were accepted for publication by Clinical Immunology and Immunopathology (Margolick, J.B., Scott, E.R., Odaka, N., and Saah, A.J. Flow cytometric analysis of $\gamma\delta$ T cells and natural killer cells in HIV-1 infection. Clin. Immunol. Immunopathol., 58:126-138, 1991). The findings are notable for showing a marked decrease in NK cells in seropositive individuals compared to seronegative, which is relatively independent of stage of HIV infection since it is seen at all levels of CD4 cell numbers (figure 6). In addition, the findings demonstrate that changes in dim CD8 cells and $\gamma\delta$ cells are of smaller magnitude, although there is a proportionately large increase in $\gamma\delta$ cells that co-express CD8 (at dim levels). The function of these cells is not known, although they have been reported to recognize "stressed" lymphoid cells. Finally, it appears that although dim CD8 cells have traditionally been regarded as NK cells, in HIV-1 sero-positive individuals a high proportion of them may actually be $\gamma\delta$ T cells.

We have also performed a preliminary analysis of the relationship between changes in CD4 and CD8 subsets in HIV-1 seroconverters which indicate a relative excess of CD8 cells in the first 6 months after seroconversion but not thereafter. Specifically, the rise in CD8 cells that follows seroconversion appears to exceed in magnitude the fall in CD4 cells that occurs during the same time period. The meaning of this change is not obvious, but it is another example of the fact that larger changes in T cell subsets appear to occur early after seroconversion than later on, and this finding points to the importance of understanding this early time period of HIV infection. An abstract describing these findings was submitted to the VI international conference on AIDS in San Francisco, June 1990 (appended).

We are continuing to extend these studies. We have begun looking at the subset of $\gamma\delta$ T cells recognized by the monoclonal antibody δ TCS-1, i.e., the V δ 1 subset. These cells were reported by others to be increased in HIV-1 seropositives (Autran et al, Clin. Exp. Immunol. 75:206,1989). Our data on 30 seropositive individuals also show this difference, and in the seropositives the data suggest that higher numbers of these cells, with or without expression of the CD8 molecule, may be associated with higher numbers of CD4 lymphocytes (figure 7). The differences observed are obviously very preliminary, but it should be noted that no similar differences were seen in total lymphocyte count or total T cell count.

Studies of T cell regulation are continuing using the MACS seroconverters as a source of data on T cell subset measurements on the same individuals before and after seroconversion to HIV-1. The analysis is not concluded but our preliminary observations on the Baltimore (SHARE) cohort of gay men are supported so far: that T cells change primarily in the first year or two after seroconversion and are quite stable thereafter, and that non-T cells show a pattern of decline which resembles that of CD4 cells: rapid at first, then slower but still declining. We are continuing to monitor NK cells (CD3⁺CD56⁺) in members of the SHARE Baltimore cohort of gay men so that we can determine to what degree changes in non-T cells are accounted for changes in NK cells.

We have begun to analyze the relationship between numbers and percents of natural killer (NK) cells and non-T lymphocytes (i.e., lymphocytes which are CD3⁺) in the Baltimore cohort of gay men (the

SHARE study). Preliminary observations on SHARE participants indicate that there is a high correlation ($r = 0.50$ approximately) between measurements of non-T cells at a given visit and natural killer (NK) cells, as identified by the $CD3^+CD56^+$ phenotype, at the same visit (figure 8). This relationship holds for both HIV-1-seropositive and HIV-1-seronegative men. The next phase of the analysis will be to determine the relationship between changes in NK cells and changes in non-T lymphocytes. This will require continued analyses of SHARE participants over at least two visits.

(3) Expression of HLA-DR by monocytes of HIV-seropositive individuals.

To test the possibility that decreases in HLA-DR expression which lie within the normal range may contribute to immune suppression in HIV infection, we prospectively evaluated expression of HLA-DR by peripheral blood monocytes of homosexual men who are seropositive for HIV. This study is being conducted prospectively in participants in SHARE, the Baltimore chapter of MACS. We are evaluating subjects who have developed antibodies during the study, so that the time of infection with HIV is known to within four months. Seronegative and long-term seropositive subjects were also studied for purposes of quality control and establishing normal levels of expression of HLA-DR by monocytes. For these studies, the monocyte population is defined by light scatter characteristics and by positive staining with the monoclonal antibody anti-LeuM3 which stains most monocytes but not lymphocytes.

We have studied monocytes from 72 seroconverters. All but one of these have had normal percentages of monocytes expressing HLA-DR, i.e., more than 85% of monocytes positive for HLA-DR. These levels have not changed when these subjects have returned for follow-up evaluations. We also measured intensity of HLA-DR expression on a per cell basis, so that not only number of positive monocytes but degree of positivity could be factored into this analysis in the future. This particular aspect of the study was initially delayed because it required re-calibration of the flow cytometer.

(4) Analysis of cerebrospinal fluid from HIV-seropositive individuals. In keeping with one of the major goals of this project, the prospective evaluation of risk factors for development and progression, we developed a method for phenotypic analysis of lymphocytes in cerebrospinal fluid (CSF) by 2-color flow cytometry. In collaboration with Dr. Justin McArthur of the Department of Neurology, Johns Hopkins Hospital, we found that in all stages of HIV-associated neuropathology, as well as in control subjects without neurologic disease, the proportions of $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes in the CSF are virtually the same as those in the peripheral blood (Margolick et al, 1988). This result suggests the absence of preferential recruitment of lymphocyte subtypes to the CSF, and the lack of preferential T cell proliferation in the CSF. After our initial report, we extended the study to include activation markers on paired peripheral blood and cerebrospinal fluid specimens from 32 HIV⁺ and HIV⁻ individuals. The finding that there is a consistent lack of preferential expression of putative activation markers HLA-DR and CD38 on either the $CD4$ or $CD8$ subset of T cells in the CSF as compared to the peripheral blood, held true (figure 10) in single and subsequent 2-color analyses. It is surprising in view of the general belief that the lymphocytes in the CSF are more likely than those in the peripheral blood to be activated. Either this belief is false, or the markers being tested are not useful as activation markers, or, more remotely, HIV-1⁺ individuals do not generate activated T cells well in CSF. To test the latter, individuals with other neurologic diseases are needed and these will be sought for analysis.

(5) Development of improved software for flow cytometric data acquisition and analysis. We have long recognized the need for an inexpensive and versatile system for acquiring and storing data files of raw flow cytometric data (listmode data) that can be re-analyzed after

sample data have been obtained. In collaboration with Dr. Robert Vogt of the Centers for Disease Control, we tested a new system of hardware and software that provides such capability for the EPICS C flow cytometer in our laboratory. The system interfaces the flow cytometer with an analogue-to-digital converter and a personal computer equipped with a new software package called Acmeocyte, written with our input by Dorn Hetzel. With this system, we can analyze the same flow cytometric data on the EPICS C itself and on our computer simultaneously. Preliminary study indicated that virtually identical results were obtained for several phenotypes of interest for the natural history of HIV-1 infection (table 7). Subsequent analysis revealed very small biases, none of biological importance, in the following phenotypes: CD3⁺, CD4⁺, CD8⁺, CD3⁺CD56⁺ (natural killer cells), CD8⁺TCR δ -1⁺ ($\gamma\delta$ T cells not expressing CD8), and CD8⁺TCR δ -1⁺ ($\gamma\delta$ T cells expressing CD8). The results were presented at the Conference on Clinical Applications of Cytometry, Charleston SC and were submitted to Cytometry as the first simultaneous comparison by two analytic methods of data acquired by one flow cytometer. A copy of the abstract and manuscript are attached. In addition to data acquisition software, new analytical approaches were evaluated, including the use of spline statistics, with the collaboration of Dr. S. Jay Smith of the U.S. Centers for Disease Control (Atlanta, GA).

b. Functional Studies.

(1) **Magnitude of antigen- and mitogen-induced lymphoproliferation.** To determine whether loss of antigen-induced T cell proliferative responses are predictive of, or merely coincident with disease progression, we followed these responses among SHARE seroconverters. This population was chosen because they are still in the early stages of decline and therefore can provide baseline data against which subsequent values may be compared. Such a foundation is needed in view of the notorious variability of lymphoproliferative responses among individuals and between individuals, especially with respect to antigens. We also evaluated responses to T cell dependent mitogens (PHA, PWM, Con A) both as positive controls for the soluble protein antigens and to evaluate the prognostic value of these parameters as well. Another strength of the experimental design was that a seronegative participant was studied concurrently with each seroconverter, in order to provide a positive control for reagents and cell preparation. Most seroconverters were tested as they came to clinic for their routine visits. Whenever a seroconverter was tested, a seronegative study participant was analyzed concurrently, as a control for assay-to-assay variation, if needed. We analyzed 36 seropositive specimens, mostly from seroconverters, and 30 seronegatives. There was, as would be expected, a large amount of variation across individuals, but certain trends could be recognized. Mitogen responses declined with time from seroconversion, and this is clearest with Pokeweed mitogen (figure 9), which is consistent with other studies which have emphasized the utility of this test for prognostic purposes. In addition, responses to tetanus toxoid also declined with time, although this measure, which in theory should be the best predictor of future progression clinically, was not as good as PWM because of its greater variability in immunologically normal individuals.

(2) **Limiting Dilution Analysis of In Vivo Activated (IL-2 Responsive) Peripheral Blood Lymphocytes in HIV-1 Infected Subjects.** Using conventional bulk culture of peripheral blood mononuclear cells (PBMC), we confirmed the results of others that HIV infected patients manifest a loss of T cells that can respond to IL-2. Thus PBMC from AIDS patients respond more poorly than those from patients with AIDS related complex (ARC), which respond more poorly than cells from asymptomatic HIV-infected subjects; all HIV-infected groups have weaker responses than uninfected people. Using a novel highly quantitative assay to estimate the proportion of IL-2 responsive cells and examine responsiveness on a "per cell" basis, we found in a cross-sectional study of a

spectrum of HIV-infected homosexual men that there was a progressive decrease in the number of IL-2 responsive cells decreases as the disease progressed. However, a potentially very important finding was that individual IL-2 responsive cells from asymptomatic infected persons or ARC patients responded as well as individual IL-2 responsive cells from healthy noninfected persons. In contrast, AIDS patients not only had fewer IL-2 responsive cells, but those that did respond gave rise to fewer progeny cells. These results suggest that an impairment in responsiveness to IL-2 may limit the ability of AIDS patients to amplify responding immune cells. A manuscript describing our findings has been published in *Clinical Immunology and Immunopathology*, and a reprint of the paper is appended.

To confirm and extend these results, and to evaluate rigorously the value of measurement of IL-2 responsive lymphocytes for staging and prognostication of HIV infection, we initiated such a study using PBMC from seroconverters in the Baltimore chapter of the Multicenter AIDS Cohort study. This was supervised by Dr. Rajesh Chopra who joined our laboratory as a Research Associate with the support of this contract. The analysis of IL-2 responsive T cells in SHARE participants continued to demonstrate that there were reductions in both the number and responsiveness of cells with progressive HIV infection as reflected in declining numbers of CD4 T cells. These deficits were not reversed by the addition of large mounts of IL-2 to the cultures, nor by the addition of PHA as well as IL-2. These results show that there is an intrinsic inability of T cells in HIV infection to be activated by PHA at the level of the single T cell in a monocyte-dependent system. An abstract describing these findings was submitted to the annual meeting of the American Association of Immunologists, New Orleans in June, 1990.

Other studies pursued by Dr. Chopra focused on the structure of the IL-2 receptor in human lymphocytes, specifically expression of the α and β subunits, to see if these are normally expressed on resting and activated (stimulated) T cells from participants in our NIAID-funded prospective studies who are at different stages of HIV infection. We hypothesized that there may be correlations between the expression of these receptor subunits and the stage of immunodeficiency and the number of CD4 lymphocytes present in these individuals. With the recent report by Taniguchi et al of the development of antibodies to the β , we asked for and received a donation of the antibodies from this group. The antibodies stain weakly compared to the familiar tac antibody to the α chain, which complicated quantification of the number of positive cells and the intensity of positivity of these cells. The expression of the β chain and α chain of the T cells from SHARE participants was also analyzed in both resting and stimulated T cells, in combination with antibodies to CD4 and CD8 so that defects in specific T cell subsets could be detected.

In addition to flow cytometry these studies were continued through the use of several techniques: 1) flow cytometric analysis of expression of the α and β subunits of the high affinity IL-2 receptor (IL2R) by the use of monoclonal antibodies specific for these subunits; 2) flow cytometric detection of fluorescent-labeled IL-2 (fluorescein conjugated to IL-2); and 3) analysis of binding of radiolabeled IL-2 to lymphocytes. A fourth method was also initiated, namely analysis of α and β expression in IL2R bound by IL-2 cross-linked to the high affinity receptors. Results can be summarized as follows: as previously reported, there is reduced expression of the α subunit by stimulated PBMC from HIV-1+ individuals; 2) expression of the β unit is difficult to quantify using monoclonal antibodies. The intensity of fluorescence obtained by indirect methods is too low to permit accurate quantification of the percent of positive cells, both in seropositives and seronegatives, as shown in figure 10. However, there was no gross difference in expression of the β subunit between seropositives and seronegatives, either by resting or stimulated PBMC. Similarly, use of fluorescence labeled IL-2 did not permit accurate quantification of cells expressing high affinity IL2R.

We have also analyzed the subunit composition of high affinity IL-2 receptors crosslinked to radiolabeled IL-2 and analyzed by SDS-PAGE. Findings in 12 seropositive-seronegative pairs indicate

that there is no difference in the α/β ratio by HIV-1-serostatus. Thus, other explanations besides IL2R dysfunction still are needed for the reduced lymphoproliferative responses seen in HIV-1⁺ individuals. Based on a recent report (Kekow et al, Proc Natl. Acad. Sci. 87:8321, 1990), we undertook to determine whether these differences could be accounted for by excessive production of transforming growth factor-beta (TGF- β). We have found no evidence of increased TGF- β production in the HIV-1⁺ individuals studied to date, compared with seronegative controls. This has been studied at the functional level, using the CCL64 assay, based on proliferation of a cell line which is inhibited by TGF- β , and at the RNA level using a cDNA probe specific for TGF- β , in collaboration with Dr. Babu Raj (figure 11) of the Johns Hopkins Oncology Center.

Analyses of IL2R subunit mRNA, conducted with the collaboration of Dr. Raj, has shown in small numbers of individuals 1) that the α subunit is greatly reduced in stimulated cells from HIV-1⁺ donors as compared to HIV-1⁻, as previously reported; and 2) the β subunit is also reduced, but by a proportionately smaller amount (figure 11), which has not been reported. Of note, no individuals were found with absent IL2R- β subunit mRNA expression, as was reported by Sahraoui et al at the San Francisco AIDS Conference. There does appear to be a fraction of T cells that responds poorly to PHA, even after 3 days of culture, but the basis for this remains to be determined.

D. Discussion related to the goals of this contract.

a. **Original Goals.** This contract proposal was submitted under the Broad Agency Announcement and underwent two cycles of review prior to funding (original submission date February, 1986; funded December, 1987). In response to the requests of Drs. Bancroft and Noyes two major revisions were made to the goals as originally submitted: 1) studies specifically dealing with potential autoimmune mechanisms of HIV-1 pathogenesis were deemed not to be of sufficient interest and were therefore omitted, 2) studies on the in vitro effects of the drug Cyclosporine A on HIV-1 growth were deemphasized on the basis of newly available data indicating that Cyclosporine A had no apparent clinical role in the management of HIV-1 infection.

b. **Amended Goals.** We have amended our research goals in keeping with progress in the field and the unique resources available to us through our involvement in the Johns Hopkins Marrow Transplantation Program and Hopkins-based cohort studies of the natural history of HIV infection. These revised goals are faithful to the original intent of the proposal in that they are directed toward an understanding of interactions of HIV-1 and the hematopoietic system and toward the establishment of immunologic markers of disease progression. These goals were discussed in detail with our project officer, Dr. Peter Gomas, at our site visit of April, 1989.

V. CONCLUSIONS

A. Summary of implications

1. **Newly adapted marrow culture techniques provide improved experimental capabilities.** The adaptation of the Teflon culture system to bone marrow growth has provided a means to obtain quantitative recovery of cells of the monocyte macrophage lineage that have differentiated in bone marrow cultures. These were difficult to recover using other methods owing to their adherent properties. The adaptation of a quantitative assay of precursor growth by limiting dilution analysis has

provided a powerful method to assess cloning efficiency and to isolate cells that are the progeny of a single progenitor cell.

2. Development of an inexpensive p24 assay. We have determined optimal conditions for a p24 antigen capture assay using widely available inexpensive reagents. Although sensitive and specific assay kits are commercially available, these cost up to \$20 per individual test. Our assay compares favorably in sensitivity with the Abbott test and costs approximately \$7 per 96 replicates.

3. Bone marrow progenitor cells can be infected with HIV-1. Our early results indicate that monocytes arising from cultured monocyte/lymphocyte depleted bone marrow express HIV-1 mRNA. These data suggest that a cell developmentally earlier than an identifiable monocyte was infected at the initiation of culture and that viral expression occurred concomitantly to differentiation. These results must be interpreted with caution, however, since contamination of the initial cell inoculum with very small numbers of monocytes (and subsequent spread to newly differentiated cells) could potentially explain these results.

4. HIV-1 exerts minimal effect on progenitor cell growth and function of progeny. Colony forming efficiency and proliferative capacity of purified (CD34⁺) progenitor cells were only mildly reduced when progenitor cells were incubated with virus prior to culture. Mature monocytes derived from infected progenitor cells were able to present antigen to autologous monocyte depleted lymphocytes.

5. Adoptive transfer of donor immunity and transient disappearance of virus in HIV + bone marrow transplant recipients. A total of 6 patients were transplanted. Recipients of allogeneic grafts (n = 3) had HIV related lymphomas; recipients of syngeneic grafts (n = 3) were without malignancy and had seronegative identical twin donors. Adoptive transfer of donor immunity was assessed by boosting marrow donors with tetanus and diphtheria toxoids one week prior to marrow harvest, and immunizing recipients with tetanus toxoid on the day of transplant. Adoptive transfer and reconstitution of humoral and cell mediated (lymphoproliferative) responsiveness to tetanus toxoid were observed in all subjects. The use of AZT before during and after transplant did not interfere with the tempo of engraftment. Our virological results were encouraging, but clearly the goal of HIV-1 eradication was not achieved. It must be noted that, prior to therapy, virus was easily recovered from the peripheral blood of all patients, and all had greatly reduced CD4 counts (50-200/mm³) at the initiation of therapy. Allogeneic BMT resulted in reduction of virus burden to the extent that virus could not be detected (by PCR or cocultivation) for 6 to 12 weeks. As far as we are aware, no other therapeutic modality has been able to totally eclipse the virus for any length of time. In syngeneic BMT treatment failed to ablate virus. This could be due to several factors including the use of a busulfan/cytosine regimen, which is demonstrably less immunoablative than cytosine/total body irradiation; 2) the absence of a graft-versus-virus effect analogous to the well documented graft-versus-leukemia effect; 3) greater virus burden in these patients, who by our study entry criteria had more advanced HIV-1 disease than the patients with HIV-1 related lymphomas.

Future studies will concentrate on allogeneic BMT for patients with HIV related malignancies. Areas for refinement of the procedure include the inclusion of HIV specific immunization in the regimen, the use of combined antiviral regimens (AZT plus DDI).

6. SCID/Hu model for adoptive transfer of HIV-1 specific responses. In collaborative studies with Dr. Richard Markham, we were able to determine that SCID mice reconstituted with human lymphocytes could accurately reproduce the results obtained in human marrow transplant patients, and therefore serve as a valid model for future experiments exploring transfer of HIV-1 specific responses. Our results indicated that: 1) Hu PBL/SCID chimeric mice could not generate a primary antibody response to a novel antigen (SRBC); 2) Adoptive transfer of secondary antibody responses depend on both the donor immune status and require early recipient immunization (< day 35) for optimal responses; 3) Titers achieved in optimally immunized chimeric SCID mice equaled those observed in high the boosted human donor; and 4) This model system may be of use in characterizing adoptive transfer of HIV-1 specific responses, an area of importance to the development of bone marrow transplantation as anti-retroviral therapy. These data were presented at the 6th International AIDS conference.

7. Regulation of lymphocyte populations in HIV-1 infection. In collaboration with the other MACS centers, we have analyzed the relation among changes in several lymphocyte populations after seroconversion to HIV-1. In our initial studies, we found that in the first year after seroconversion, the positive change in CD8 lymphocytes was proportionately greater than the negative change in CD4 lymphocytes (Margolick et al, 1990). On further analysis, it was apparent that these changes were quite dependent on the magnitudes of changes in several different lymphocyte populations, including not only the predominant CD4 and CD8 T lymphocyte subsets, but also non-T lymphocytes. Therefore, we analyzed changes in all of these lymphocyte populations, as well as changes in non-lymphocyte (granulocyte and monocyte) populations.

a. T cells. With respect to T lymphocytes, the data show that despite the familiar dramatic changes in CD4 and CD8 T lymphocyte subsets, total (CD3) T lymphocytes decrease only slightly (figure 12). Specifically, the changes in total T cells in the first 18 months after HIV-1 seroconversions is much smaller than the changes in CD4 or CD8 lymphocytes, and after 18 months total T cells remain constant despite continuing changes in CD4 and CD8 cells. These data suggest a regulatory mechanism that maintains constant numbers of CD3 lymphocytes in the face of loss of CD4 cells. They also suggest that the rise in CD8 lymphocytes is part of a compensatory response to loss of CD4 cells, rather than a specific anti-HIV response. Thus, regulatory mechanisms are likely to be at work when seroconversion occurs, and we will soon be initiating studies to identify the newly produced T lymphocytes that would be expected if this is true. These data have been accepted for publication (J. Aids, in press, 1992).

b. Non-T lymphocytes. The above analysis also demonstrated that a decrease occurs in non-T (CD3⁻) lymphocytes, defined as [total lymphocytes] - [CD3 lymphocytes]. Of note, this decrease in non-T cells parallels that in CD4 lymphocytes, in that it is relatively rapid in the first 18 months after seroconversion and continues more slowly thereafter. Since non-T lymphocytes are not infected with HIV to any significant extent in HIV⁺ individuals, the most probable explanation for this finding is the existence of another regulatory mechanism that is perturbed by HIV-1 infection. These data are also quite consistent with the lower levels of NK cells in HIV-1⁺ individuals that we described above.

c. Potential importance of lymphocyte subset regulatory mechanisms in HIV-1 infection. Recognition of the possible regulatory basis for the lymphocyte subset changes described above is important because it may help to clarify whether these changes are helpful or harmful with

respect to pathogenesis of immune deficiency, and whether alternative approaches to therapy might be appropriate. For example, it could be hypothesized that if a constant number of circulating CD3 cells is maintained physiologically, removal of CD8 cells might stimulate the production of new CD4 cells. The existence and specificity of such regulatory phenomena is supported by the occurrence of certain nonspecific phenomena, such as the nonspecificity of elevations in all circulating leukocyte populations due to cigarette smoking, which we have demonstrated in a separate collaboration with MACS.

8. Analysis of T- and non-T lymphocyte populations in couples discordant for HIV-1.

Among the SHARE (Baltimore MACS) cohort there are a number of couples involved in sexual relationships, in which one member of the couple is HIV⁺ and the other is HIV⁻. The seropositivity and seronegativity of the various individuals have been repeatedly confirmed by ELISA, Western blot, PCR, and viral culture, in studies performed within SHARE and not shown here. It was also shown by questionnaire that the couples were engaged in activities that could spread the virus, such as unprotected anal insertive or receptive intercourse.

Analyses were performed to determine whether there were differences between the seropositive members of the discordant couples, and other seropositives in SHARE, and also whether there were differences between the seronegative members of the discordant couples and other seronegatives. 36 discordant and 24 concordant couples were studied. We found that there were no differences between the two groups of seropositives, and the seronegatives did not differ with respect to absolute numbers of circulating CD4 lymphocytes. Discordant and concordant seronegatives also did not differ with respect to $\gamma\delta$ T cells (69 ± 40 (SD) vs 81 ± 37 cells/mm³, respectively) or natural killer cells (246 ± 176 vs 231 ± 125 cells/mm³, respectively). Interestingly, there was a slightly higher absolute number of circulating CD8 lymphocytes in the seronegative discordants than in the seronegative concordants at the initiation of the study (734 vs 592 cells/mm³, respectively), although this was not statistically significant ($p = 0.09$) and did not persist throughout the one-year followup period of the study. The data suggest that CD8 lymphocyte numbers may be useful as an indicator of exposure to HIV-1, and/or in host defense against the virus. The results to date have been submitted for publication (appendix D), and further studies to characterize the functions of the CD8 lymphocytes in these individuals have been initiated on the basis of these results.

9. Software and hardware for acquisition and analysis of flow cytometric data. Due to limitations in the hardware and software on the EPICS C flow cytometer in the SHARE laboratory, we were unable to store flow cytometric data for listmode analysis or perform analyses while data were being acquired. Hardware and software to perform some of these functions to a limited extent were available from Coulter Corporation, but were prohibitively expensive. In an attempt to provide these functions for maximum flexibility in data acquisition and analysis at a reasonable and affordable cost, we collaborated with Robert Vogt and Dorn Hetzel at Centers for Disease Control and others in the development and implementation of 1) a hardware interface to the EPICS C and 2) a version of operating software (called "Acmeocyte") that could be used for downloading raw data from the C to a more powerful and cost-effective personal computer. This project was also part of the development of the Center for AIDS Research (CFAR) at Johns Hopkins University.

With USAMRUD support, we were able to test the hardware-software interface we implemented on a wide panel of monoclonal antibodies. The goal of these experiments was to validate the system we had installed on the EPICS C so that we could use it exclusively. To do this, we split the electronic impulses generated by each cell as it passed through the flow cell and the laser beam of the flow cytometer, so that the impulses generated could be processed, stored, and analyzed by both the EPICS

software and the Acmeocyte software. Thus, we performed what we believe is the first side-to-side comparison of two systems for acquiring and analyzing the same flow cytometric raw data. The results showed excellent agreement between the two systems (figure 13), though the Acmeocyte system was much more powerful for all aspects of data acquisition, storage, and analysis. This study is now in press in Cytometry. A copy of the preprint is attached (appendix E). Based on these results, we now use the Acmeocyte system for all our flow cytometric studies done in one or two colors.

10. HIV-1 infection is associated with changes in minor T cell subsets. In initial studies, we showed that SHARE participants (homosexual or bisexual men in the Baltimore-Washington area, whose HIV-1 serostatus is tested every six months as part of the Multicenter AIDS Cohort Study (MACS)) who were HIV-1⁺ had higher numbers of CD3⁺CD4⁺CD8⁻ (double negative, or DN) T cells, as calculated according to the formula $CD3 - [CD4 + CD8]$. These studies were carried out using the measurements previously obtained of CD3, CD4, and CD8 positive cells in SHARE participants. Next, with the support of the present contract, we studied serial results from SHARE seroconverters and found that seroconversion with respect to HIV-1 was associated with an increase in the calculated DN cells. Finally, these studies were subsequently confirmed and extended in the entire MACS seroprevalent, seronegative, and seroconverter cohorts; data from this MACS-wide study were published (Margolick et al, 1989). These studies showed that seroconversion to HIV-1 was associated with a rise of approximately 60 cells/mm³ in the number of calculated DN lymphocytes, and this difference continued to increase over the first three years of HIV-1 infection (as defined by seropositivity).

Next, we asked whether the absolute rise in calculated $CD3 - [CD4 + CD8]$ cells was due to a decrease in CD3⁺CD8⁺ natural killer cells, or an increase in a newly recognized class of T cells expressing the CD3⁺CD4⁺CD8⁻ phenotype (i.e., true "double negative", or DN T cells). For these studies, seropositive and seronegative members of the SHARE cohort were analyzed to determine the effects of seropositivity with respect to HIV-1. The studies were carried out by flow cytometry using monoclonal antibodies defining the natural killer (NK) lymphocyte subset (CD3⁺CD56⁺) and the $\gamma\delta$ T cell subset (TCR δ -1⁺). True DN (CD3⁺CD4⁺CD8⁻) T cells were measured using two-color flow cytometry by adding to a single specimen anti-CD3 antibody conjugated to fluorescein isothiocyanate (FITC) and anti-CD4 and anti-CD8 antibodies both conjugated to phycoerythrin (PE).

Our results (table 5) indicated that the rise in calculated DN was attributable to both a rise in true DN T cells and a fall in NK cells, with the latter effect predominating. The rise in DN T cells correlated closely with a rise in T cells expressing the $\gamma\delta$ -T cell antigen receptor (TCR), especially those $\gamma\delta$ -T cells which also express low density CD8. These results, some of which have been described in previous progress reports, have also been published (Margolick et al, 1991; manuscript appended).

The preceding results indicated that T cells expressing the $\gamma\delta$ T cell receptor are increased in seropositives as compared to seronegatives. This conclusion was supported by preliminary data showing an increase in T cells staining with the monoclonal antibodies TCS-1 and TCR δ -1, which recognize some or all δ receptor chains, respectively. These studies have been continued and expanded. Results demonstrating a 30% increase in the geometric mean number of T cells expressing the δ chain have now been published, as noted above. Most of this increase was attributable to an increase in cells co-expressing low levels of CD8, statistically significant at the level of $p = 0.009$. The meaning of this increase is still under investigation, but it could represent an increase in precursor T cells, cells exerting anti-HIV activity, or an epiphenomenon related to HIV-1 infection but not representing a specific host response to the infection. We are currently investigating the specific subsets of $\gamma\delta$ cells that are increased in HIV⁺ SHARE participants, and the relation, if any, between numbers of these cells and numbers of CD4 lymphocytes. Very preliminary data suggest that a high number of $\gamma\delta$ cells may be

associated with a high CD4 count (figure 7), but more data are needed to confirm this. If confirmed, this finding would be consistent with either the production of new CD4 cells in direct relation to the number of $\gamma\delta$ cells, or with a role for $\gamma\delta$ cells in anti-HIV host defense.

11. HLA-DR expression appears to be within normal limits on monocytes from HIV-1 infected individuals. Although the possibility of changes within individuals over time has not been ruled out, this important component of the immune system appears to be relatively unaffected by HIV infection.

12. The proportion of activated (CD38⁺ or HLA-DR⁺) T lymphocytes in the blood and cerebrospinal fluid of HIV-infected homosexual men are indistinguishable. Results of our initial studies were corroborated in a series of 32 CSF/peripheral blood paired samples. Our previous collaborations with Dr. Justin McArthur and the other investigators and staff of the Neuropsychologic study of MACS have developed and applied a flow cytometric method for the analysis of paired specimens of peripheral blood and cerebrospinal fluid (CSF). Specifically, the method we have used for flow cytometric analysis of peripheral blood lymphocytes was adapted so that CSF could be analyzed using the flow cytometer, leading to greater precision in such analyses. Most of the individuals studied have been members of SHARE. We reported an extremely close correlation between the proportions of CD3⁺, CD4⁺, and CD8⁺ lymphocytes in peripheral blood and CSF (Margolick et al, 1988), and are now in the final stages of a followup study addressing the question of whether there is an increased proportion of activated T cells among the lymphocytes in the CSF in HIV-seropositive homosexual men.

By double-staining lymphocytes with T cell markers and the lymphocyte activation markers CD38 and HLA-DR, we analyzed CSF and peripheral blood from 33 individuals, including 17 from SHARE. Surprisingly, we again found that the proportions of activated CD4⁺ and CD8⁺ T lymphocytes were virtually identical in the blood and CSF for both putative activation markers, within both CD4 and CD8 T cell subsets, as shown in figure 14 for HLA-DR in combination with CD8. This result was true for HIV⁺ individuals with no neurologic findings, those with nonspecific neurologic abnormalities, and also for individuals with no HIV infection. We are currently testing individuals with HIV-related manifestations of the central nervous system, such as dementia or opportunistic infections of the central nervous system. At present, the available data strongly suggest that monitoring of T cell markers in the CSF is not prognostically useful in terms of adding information that could not be obtained from analysis of peripheral blood lymphocytes. However, it should be kept in mind that this could change when HIV⁺ individuals with advanced disease are studied. When enough such subjects have been studied, these results will also be prepared for publication.

In view of the lack of prognostic usefulness of T cell markers in the CSF so far, we plan to initiate studies aimed at characterizing the B cells in the CSF. These cells are of interest in view of the increased incidence of B cell lymphomas in the central nervous system (CNS) of individuals with HIV-1 infection. The markers of interest will be markers of B cell activation, based on the hypothesis that these B cell lymphomas develop from activated B cells. It will therefore be important to know whether B cells in the CSF are activated compared to those in the peripheral blood.

13. IL-2 responsive cells decline in number and individual proliferative capacity with progression of HIV infection. Our previous studies of T lymphocyte proliferative responses at the clonal (single-cell) level showed that the number and function of T cells declined through the course of HIV-1 infection, as indicated in a cross-sectional study (Donnenberg et al, 1989). Specifically, we found that in the early stages of HIV infection, the number of interleukin 2 (IL-2) responsive cells, as reflected in

the number of proliferating cells, was reduced in HIV⁺ individuals as compared to HIV⁻ individuals, but that the response on a per cell basis was not different. In contrast, in individuals with AIDS both the number and quantitative response per cell were reduced compared to HIV⁻ individuals. Therefore, further studies were carried out to address the mechanism of these differences, and whether they had prognostic value for the progression of HIV-1 infection.

14. Exogenous rIL2 does not fully reconstitute proliferative response of cells from HIV⁺ donors, possibly due to reduction of high affinity IL2R expression. To determine whether receptors for IL-2 are abnormal in PBMC from HIV-1 infected individuals, we have used several approaches. We first verified that the cells' responsiveness was not changed by the addition of IL-2, PHA, or both to the limiting dilution cultures (Chopra et al, 1990). Next, we analyzed the subunit expression of the IL-2 receptor on lymphocytes from HIV⁺ donors. Using a variety of methods for quantifying expression of both the α and β subunits of this receptor, we have found that both subunits are expressed in stimulated T cells from HIV-1 seropositive individuals, and that the receptors expressed, although decreased in number, appear to be normal in structure.

HIV⁺ homosexual men in the present study showed significantly decreased ³H-TdR incorporation ($p < 0.006$) in response to PHA stimulation in 72 h cultures. The geometric mean ³H-TdR incorporation ($\pm$ SEM) in 15 donors in each group was 11,940 (10186, 13996) in HIV⁻ and 6442 (5623, 7379) in HIV⁺. As shown in figure 15, the proliferative response of cells from 22 HIV⁻ and HIV⁺ donors was augmented by the addition of exogenous IL2 (HIV⁻, mean difference = 49,556 cpm, $p < 0.001$; HIV⁺, 28,008 cpm, $p < 0.001$, paired t-test). However, even in the presence of IL2, cells from HIV⁺ donors showed a proliferative response that was significantly lower than that of similarly treated cells from HIV⁻ donors ($p < 0.001$). To evaluate the possibility of an insufficient expression of high affinity IL2R, the binding of ¹²⁵I-IL2 to IL2R was studied. The high affinity IL2R were significantly reduced on cells from HIV⁺ donors as compared to the HIV⁻ donors (figure 16, median IL2R per cell = 2885 in HIV⁻ and 924 in HIV⁺; $P < 0.031$, Kruskal-Wallis rank order test). Although there appeared to be a relationship between IL2 mediated augmentation of proliferation and IL2R expression (figure 16C), in two cases of HIV⁺ donors there was reduction of the proliferative response despite elevated levels of high affinity IL2R expression.

15. IL2R α , β and TGF- β , mRNA expression in unstimulated or PHA activated PBMC. Attempts to quantify expression of IL2R β on PHA stimulated lymphocytes by flow cytometry showed no consistent differences between cells from 12 HIV⁻ and 14 HIV⁺ donors in 5 separate experiments (figure 10). Only weak staining with anti-IL2R β antibody was observed, which probably reflects a low level of IL2R β expression per cell in both groups (Ohasi et al, 1989; Takeshita et al, 1989; Yagita et al, 1989; Phillips et al, 1989), thus limiting the accurate assessment of IL2R β . Therefore, we examined relative expression of the IL2R α and β subunits. For these experiments, ¹²⁵I-IL2 was covalently cross-linked to its receptors and the relative expression of IL2R α and β subunits was determined by SDS-PAGE/autoradiography. Both IL2R α and β chains were present in cells from all HIV⁻ and HIV⁺ individuals studied (figure 17), and the degree to which these subunits were expressed appeared to be strongly interrelated as demonstrated by the ratio of α/β expression in both groups of donors (figure 17B).

One possible mechanism for decreased responsiveness to IL-2 could be overproduction of the cytokine transforming growth factor- β (TGF- β), which has been reported to reduce lymphocyte responsiveness to IL-2, possibly through a negative effect on the expression of IL-2 receptors. Indeed, overproduction of TGF- β was recently reported by Kekow et al who studied individuals with HIV-1 infection (1990). However, we have obtained data that do not agree with this report. We studied TGF- β , and IL2R mRNA expression in PHA stimulated and unstimulated PBMC. Figure 11 shows a typical Northern blot pattern for 24 h PHA stimulated cells from 7 HIV⁻ and 7 HIV⁺ individuals. As shown by the densitometric analysis, TGF- β , mRNA was not expressed at higher amounts in the HIV⁺ compared to the HIV⁻ donors ($p < 0.23$, figure 18A). However, there was a significant reduction in the expression of IL2R α ($p < 0.006$, figure 18B) and β ($p < 0.02$, figure 18C) mRNA in HIV⁺. However as shown by densitometric analysis in figure 18A and B, TGF- β , and IL2R β mRNA expression were not significantly different for unstimulated cells from HIV⁻ and HIV⁺ donors. Furthermore, IL2R α mRNA was not detected in unstimulated cells. We considered the possibility that cells from HIV⁺ individuals may express more TGF- β receptors or could produce higher concentrations of active TGF- β . To examine this

possibility, cells were stimulated with PHA in the presence of TGF- β_1 antibody or control antibody. As shown in table 8, TGF- β_1 antibody had no effect on the proliferative response of cells cultured in the presence or absence of rIL2 when used at a concentration that neutralized up to 10 ng per ml TGF- β_1 .

Thus, production of TGF- β by either stimulated or unstimulated PBMC from HIV⁺ individuals was not elevated compared to PBMC from healthy controls (HIV-1-seronegative gay men from SHARE). These data suggest that the mechanism of decreased lymphocyte proliferation in HIV⁺ individuals may not be due to overproduction of TGF- β and thus remains unexplained. All of these studies have been submitted for publication (see appendix).

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VII. APPENDICES

Appendix A. Figure Legends.

Figure 1. In situ hybridization for detection of HIV-1 GAG mRNA. Box plots show the distribution of grains in positive cells. Monocyte depleted BM was infected with 3 different clinical isolates of HIV-1 and processed after 14 days in culture. The majority of positive cells were morphologically identifiable as macrophages. A few cells of blast morphology also evidenced grains. Data are summarized in notched box plots which indicate median values (waist), simultaneous 95% confidence intervals about the medians (span of notch), interquartile range (bottom to top of box) and extreme values (bars) exclusive of outliers. Outliers are defined as data points more than 1.5 times the interquartile range beyond the top or bottom of the box and are plotted individually as asterisks.

Figure 2. Effect of HIV-1 infection on the growth of purified CD34+ progenitor cells.

Figure 3. Tetanus specific antibody was measured by ELISA before and at weekly intervals after BMT. Marrow donors (data not shown) were boosted 7-10 days prior to marrow harvest; recipients were immunized on the day of transplant. Both patients evidenced a greater than 4-fold increase in titer. From previous studies in HIV seronegative BMT recipients we would expect anti-tetanus titer to decline 4-16 fold, in the absence of immunization.

Figure 4. Tetanus specific lymphoproliferative responses as measured by thymidine uptake after 5 days in culture with antigen. In the absence of adoptive transfer of immunity, we expect from our previous studies that tetanus would not elicit a significant in vitro response (> 1000 CPM).

Figure 5. Use of 2 color immunofluorescent staining to detect CD3 positive CD4-, CD8- T cells (lower right quadrant). These cells represent 3% of the total lymphocytes.

Figure 6. Scattergram of relationship between absolute numbers of NK cells and CD4+ lymphocytes in HIV-1 seronegative subjects (open circles) and HIV-1 seropositive subjects (closed circles). Line represents distance weighted least squares curvefit.

Figure 7. Plot of absolute number of CD4 lymphocytes per mm³ in 15 seropositive SHARE participants, divided into two groups according to whether the individual had more or fewer than 60 δ TCS-1 + $\gamma\delta$ T cells per mm³ of peripheral blood.

Figure 8. Scattergram of absolute number of NK cells (CD3-CD56+ lymphocytes) vs. number of non-T lymphocytes (CD3- lymphocytes) per mm³ of peripheral blood. Data shown are from 252 seropositive SHARE participants. The line is the distance-weighted least squares regression line, showing that the relationship is linear even when it is not forced to fit a linear model. The correlation coefficient was 0.49.

Figure 9. Pokeweed mitogen induced lymphoproliferative responses as a function of time after seroconversion. Thymidine uptake was measured after 5 days in culture. Solid line indicates distance weighted least squares curvefit.

Figure 10. Representative histograms of the fluorescence distribution of IL2R β on PHA stimulated PBL from 2 HIV- (left) and 2 HIV+ (right) individuals. Shown are histograms from two donors with strong IL2R β fluorescence (top) and two with weak IL2R β fluorescence (bottom). For each case stained and control histograms are superimposed, with the left peak representing lymphocytes stained with an isotype control antibody and the right peak lymphocytes stained with anti-IL2R β antibody (TU27) as the first antibody. In most cases, a large population of positively stained lymphocytes was seen but could not be accurately quantified due to overlap with the unstained lymphocytes. Similar patterns of fluorescence were seen in a total of 15 HIV- and 15 HIV+ individuals.

Figure 11. Northern blot analysis of TGF- β 1, IL2R α and β mRNA from 24 hrs PHA stimulated cells from HIV- and HIV+. Total cellular RNA was extracted by lysing the cells in RNAzol. Following electrophoresis on 1% formaldehyde denaturing gel and capillary transfer to nitrocellulose membrane, the blots were hybridized with 32P-labeled cDNA probe prepared by the random primer method. The blots were first hybridized with cDNA probe specific for human TGF- β 1. After developing the autoradiograms, the TGF- β 1 cDNA probe was stripped off and the IL2R α mRNA was probed with 32P-labelled IL2R α cDNA. Finally, after the IL2R α probe was stripped off, the IL2R β and actin mRNA were probed by using 32P-labeled IL2R β and actin cDNA.

Figure 12. Changes in geometric mean numbers of lymphocytes per mm³ in MACS seroconverters as a function of time intervals (in months) since seroconversion. Points represent geometric means of observations within the indicated time intervals. Error bars represent 95% confidence intervals about the mean. Note that changes in CD3 numbers are much smaller than those in CD4 and CD8 numbers.

Figure 13. Distributions of the differences between Acmeocyte and EPICS C results as a function of the EPICS C result. Each graph shows the percent positive result for a particular phenotype from the EPICS C on the X-axis, which has a varying scale for each phenotype. The arithmetic difference between results (Acmeocyte result - EPICS C result) is shown on the Y-axis, which has a constant scale for all phenotypes. The "mean bias" for each marker is the average of all differences (positive and negative), and the p value indicates the probability that the mean bias is zero. The best-fit spline curve, shown in thick grey, represents the relation between bias and EPICS C result.

Fig. 14. Correlation between percent of lymphocytes co-expressing CD8 and HLA-DR in paired specimens of blood (horizontal axis) and cerebrospinal fluid (vertical axis) obtained simultaneously from homosexual men with HIV-1 infection. There is little difference in the proportions, except for slight differences in two specimens that were run on the same day.

Figure 15. Scattergram of ³H-TdR incorporation in PHA stimulated lymphoblasts from HIV- and HIV+. Individual data points (○) and geometric group means (*) are shown. In the absence of rIL2, the geometric mean ³H-TdR incorporation (minus and plus the SEM) was 22,803 (19634, 26485) and 16,181 (14125, 18535) for HIV- and HIV+, respectively. Addition of rIL2 yielded ³H-TdR incorporation of 70795 (65013, 77090) and 40,179 (35318, 45709) for HIV- and HIV+, respectively.

Figure 16. Relationship between ³H-TdR incorporation and IL-2R expression in HIV- (open circles) and HIV+ (closed circles) donors. High affinity IL2R was measured under high affinity IL2 binding conditions. IL-2 augmentation was calculated as the difference between CPM obtained in the presence and absence of exogenous IL2. Lines represent distance weighted least squares curve smooths of the data from both groups. The Kd of ¹²⁵I-IL2 binding to receptors in HIV- was 26.7 ± 9.83 (mean $\pm$ SEM, n = 10) and 30.301 ± 17.8 (n = 9) in HIV+.

Figure 17. Left panel (a): SDS-PAGE analysis of ¹²⁵I-IL2 bound and cross linked to IL2R on PHA stimulated PBL from three HIV- and three HIV+ individuals. Stimulated cells were bound, crosslinked with ¹²⁵I-IL2, and then lysed. The cell lysates were resolved on 10% PAGE. The gels were fixed, dried and autoradiogrammed.

Right panel (b): The ratio of IL2R α and β bound to ¹²⁵I-IL2. IL2R α and β bands on autoradiograms were scanned by densitometer. The mean ($\pm$ SEM) ratio of IL2R α/β was 1.46 ± 0.06 in HIV- 1.68 ± 0.10 in HIV+ donors (n = 25 in each group).

Figure 18. Scattergram representation of densitometric analysis of TGF- β 1 (A) IL2R α (B) and IL2R β mRNA (C). There were 14 HIV- and 13 HIV+ individuals for each mRNA detected. After developing autoradiograms, the mRNAs bands were quantified by densitometry. The mean TGF- β 1 mRNA in HIV- was 2892 ± 341 (mean $\pm$ SEM) and 2375 ± 272 in HIV+. The mean IL2R α mRNA in HIV- was 7168 ± 778 and 4286 ± 480 in HIV+. The mean IL2R β mRNA in HIV- was 1634 ± 159 and 997 ± 179 in HIV+.

Appendix B. Figures. Eighteen figures are appended (pp 31-43).

Figure 1

Figure 1. In situ hybridization for detection of HIV-1 GAG mRNA. Box plots show the distribution of grains in positive cells. Monocyte depleted BM was infected with 3 different clinical isolates of HIV-1 and processed after 14 days in culture. The majority of positive cells were morphologically identifiable as macrophages. A few cells of blast morphology also evidenced grains. Data are summarized in notched box plots which indicate median values (waist), simultaneous 95% confidence intervals about the medians (span of notch), interquartile range (bottom to top of box) and extreme values (bars) exclusive of outliers. Outliers are defined as data points more than 1.5 times the interquartile range beyond the top or bottom of the box and are plotted individually as asterisks.

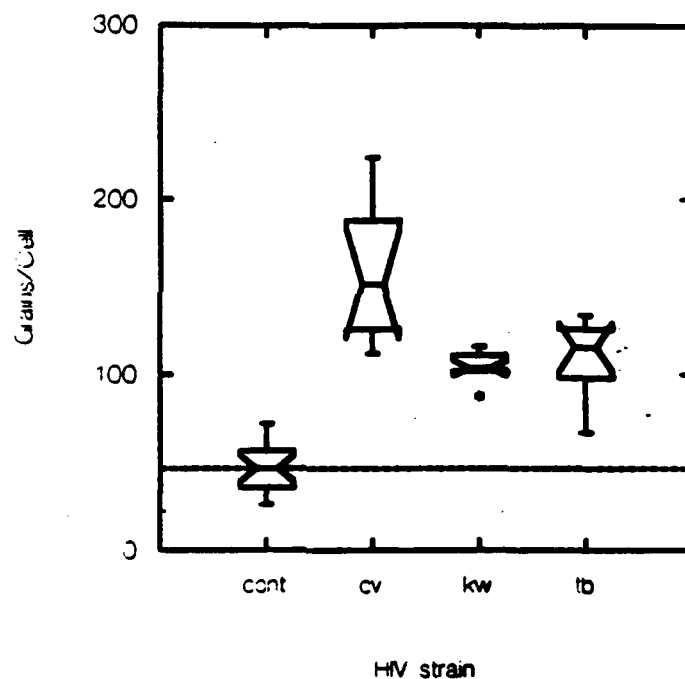


Figure 2

Figure 2. Effect of HIV-1 infection on the growth of purified CD34⁺ progenitor cells.

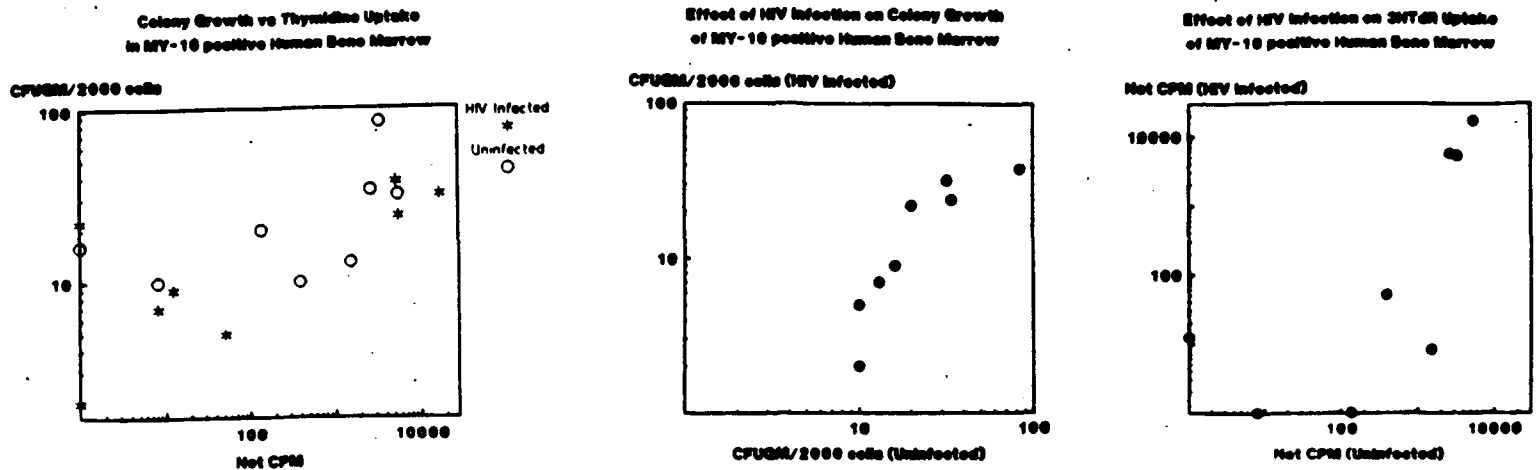
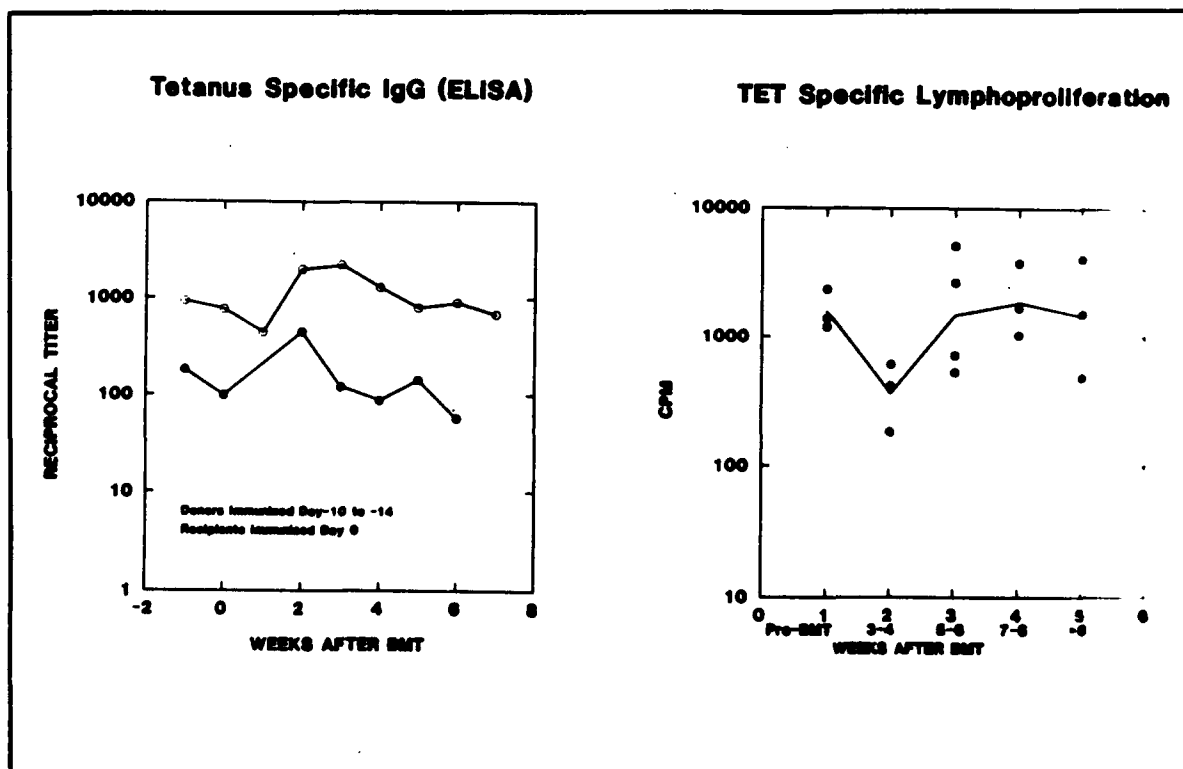


Figure 3 and 4



Reconstitution of antigen specific humoral and cell mediated immunity in 2 HIV-1 seropositive subjects undergoing BMT for lymphoma.

Figure 5

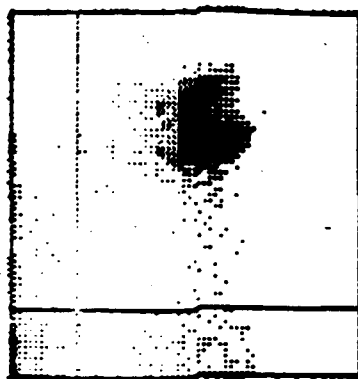


Figure 6

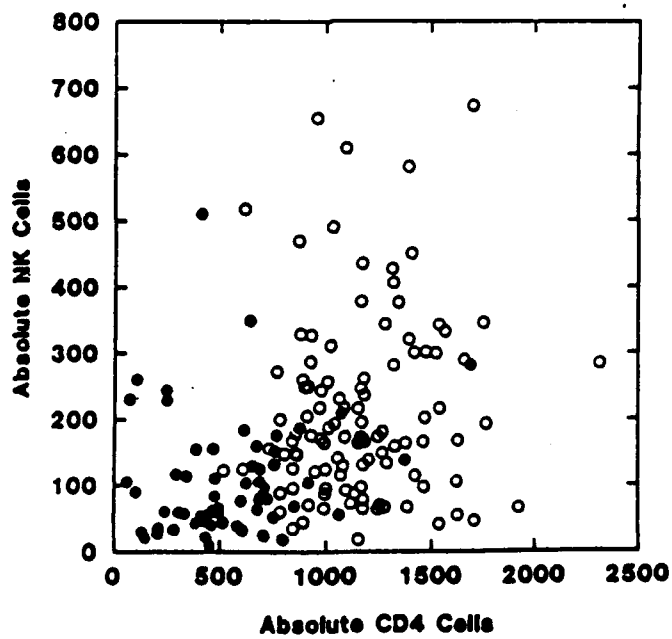


Figure 7

CD4 Cells by δ TCS-1+ Cells

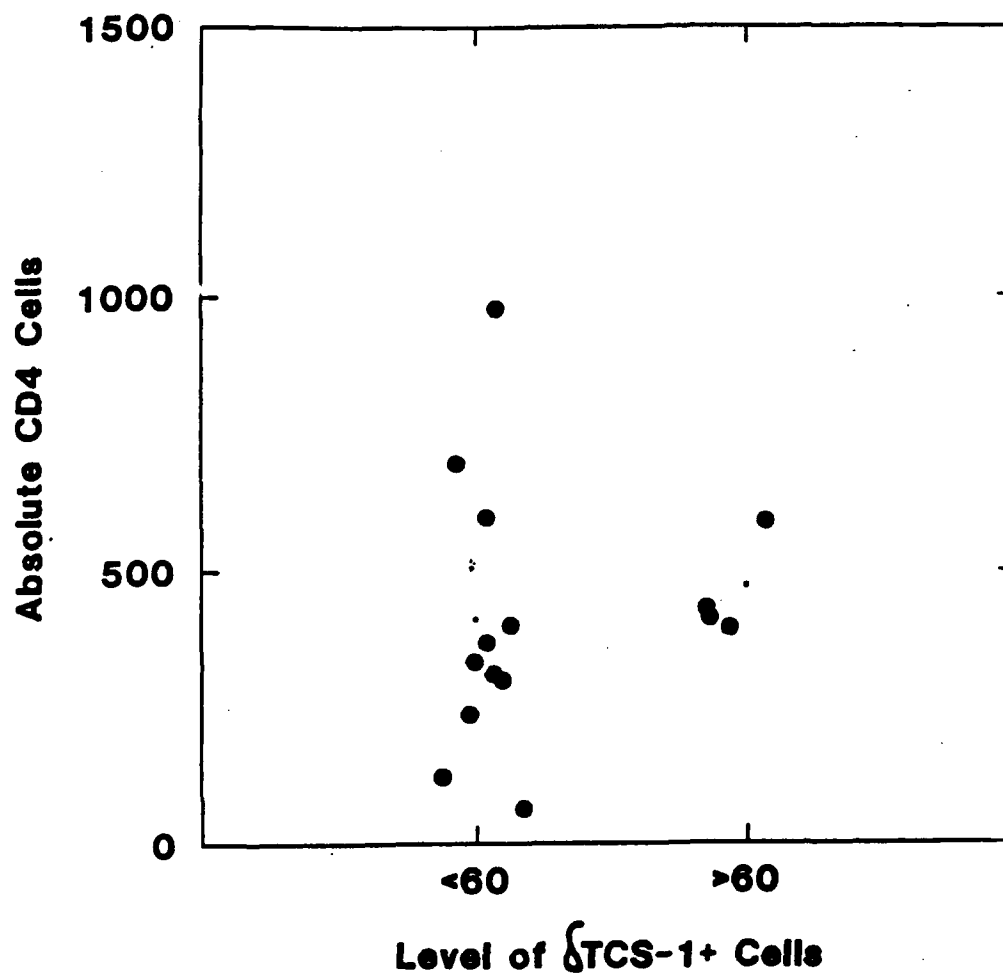


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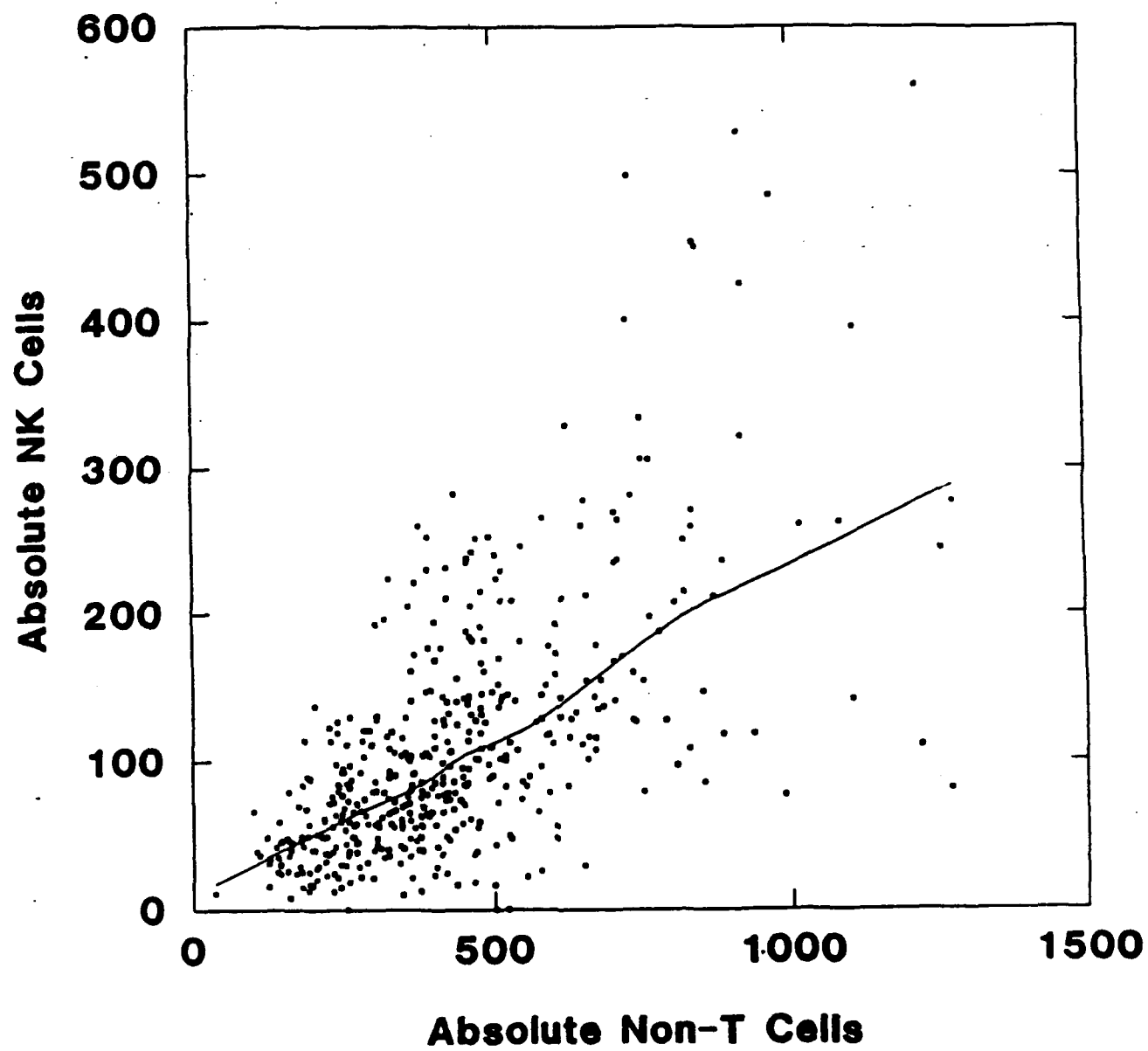
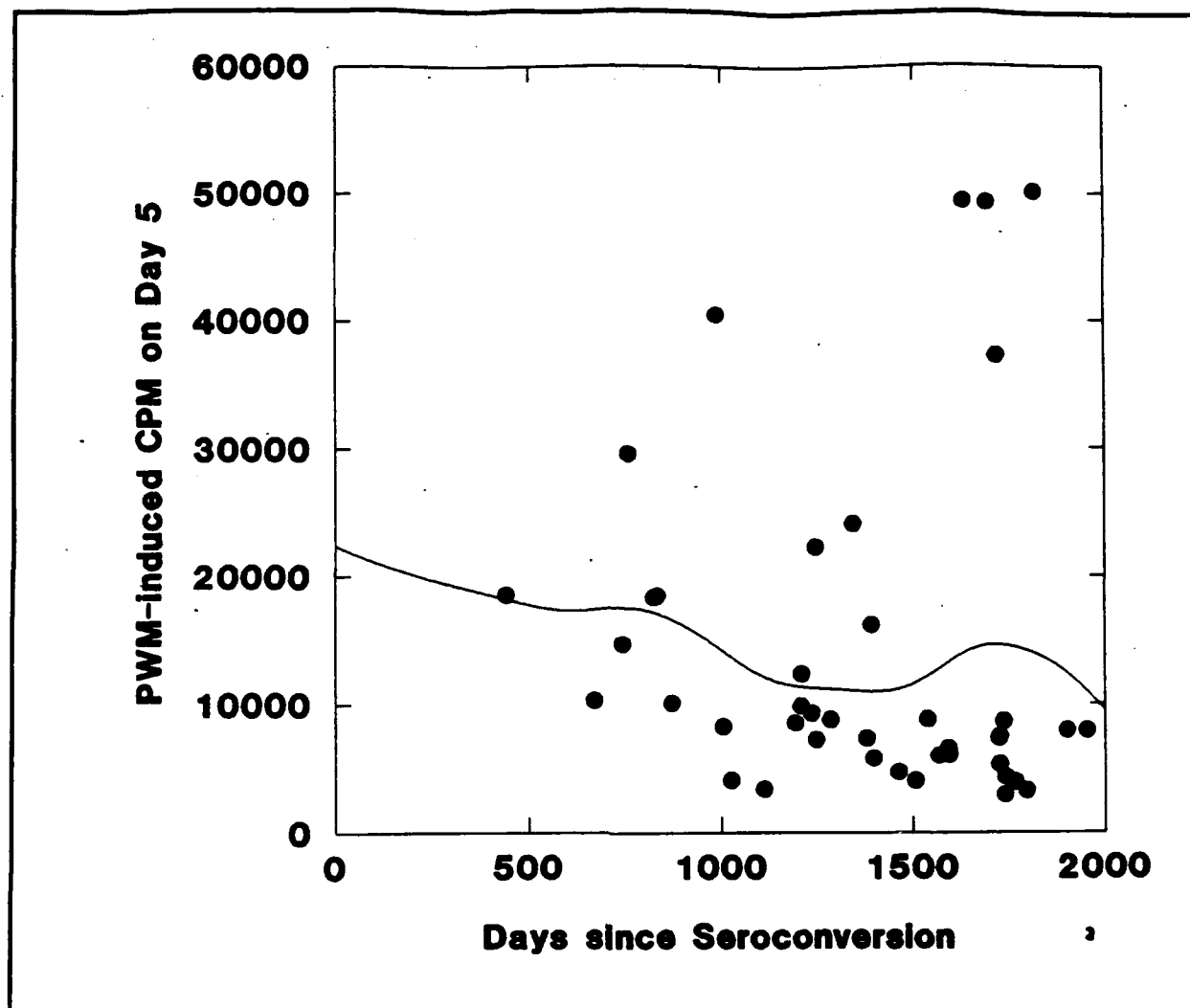


Figure 9

- 36 -



PWM induced lymphoproliferation as a function of time since seroconversion.

Figure 10

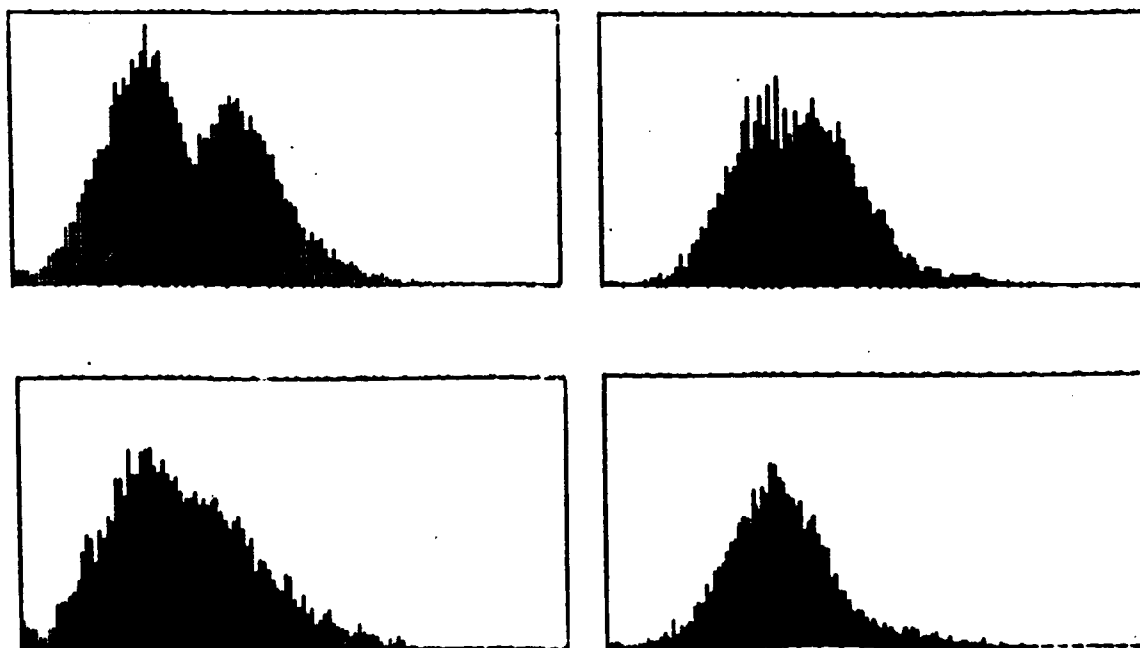


Figure 12

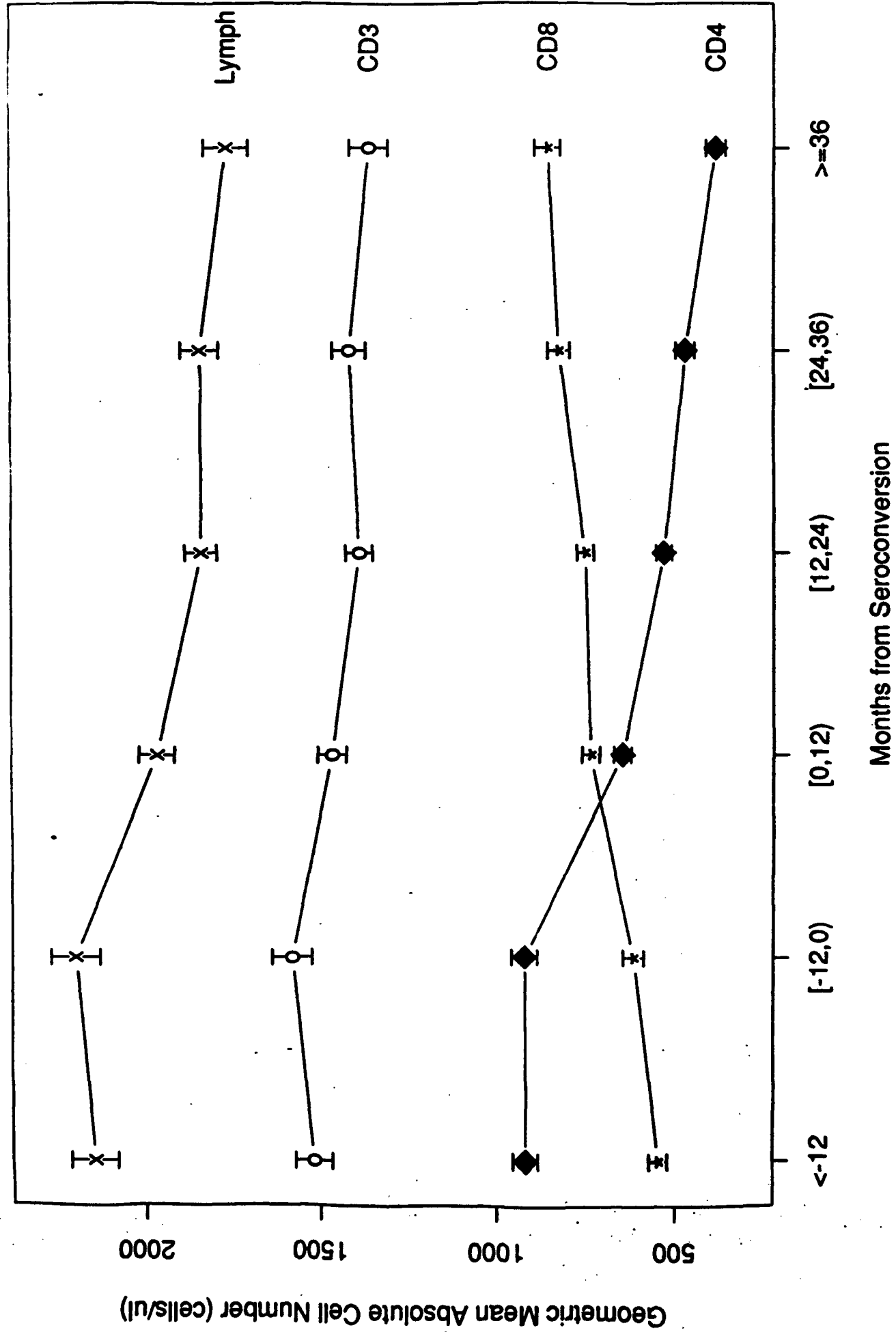
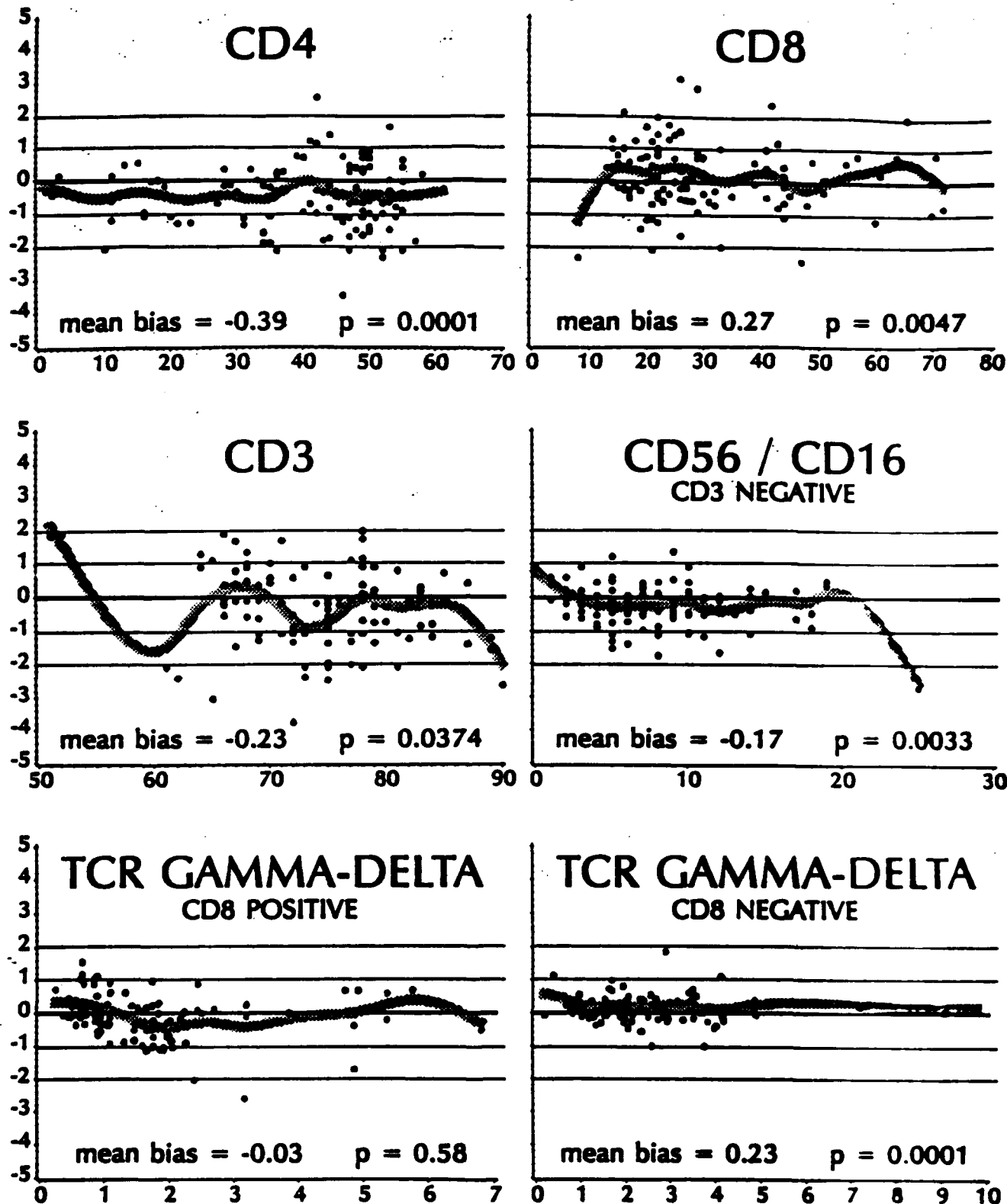


Figure 13

DIFFERENCE IN PERCENT POSITIVE CELLS
(ACMECYTE - EPICS C)



PERCENT POSITIVE CELLS
(EPICS C)

Figure 14

-40-

Percent DR-Positive CD8 Cells in Blood and CSF

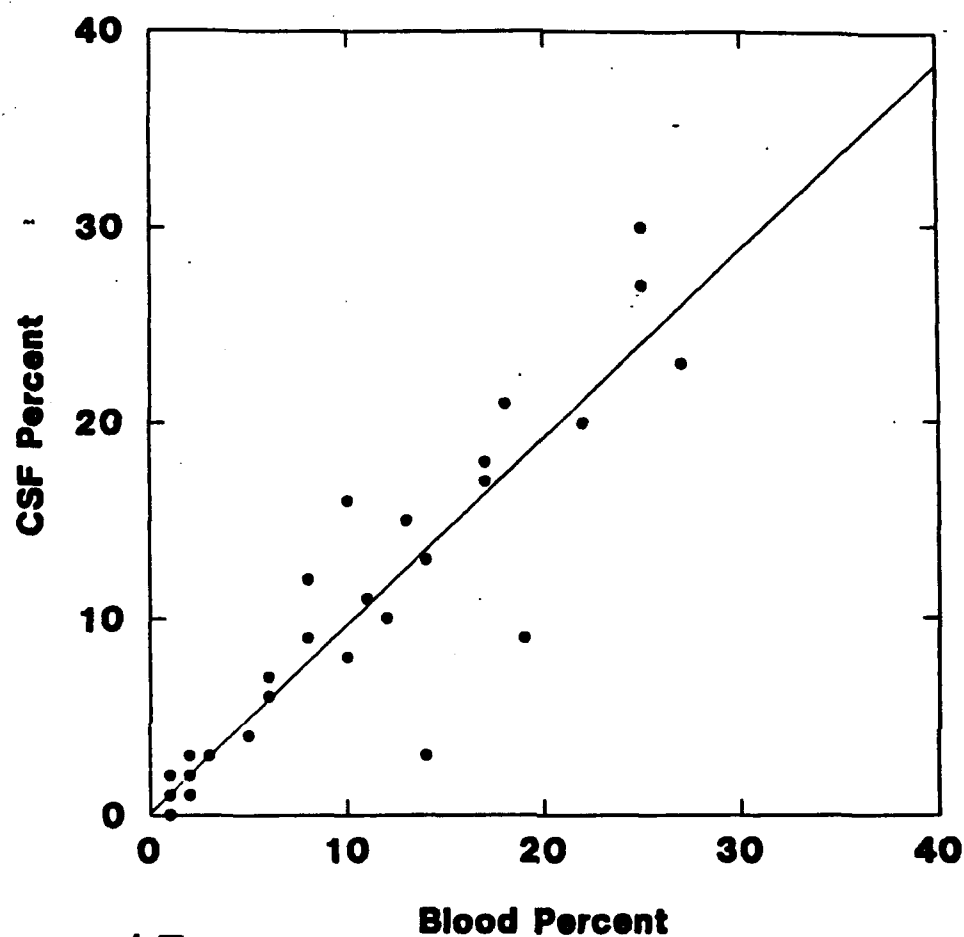


Figure 15

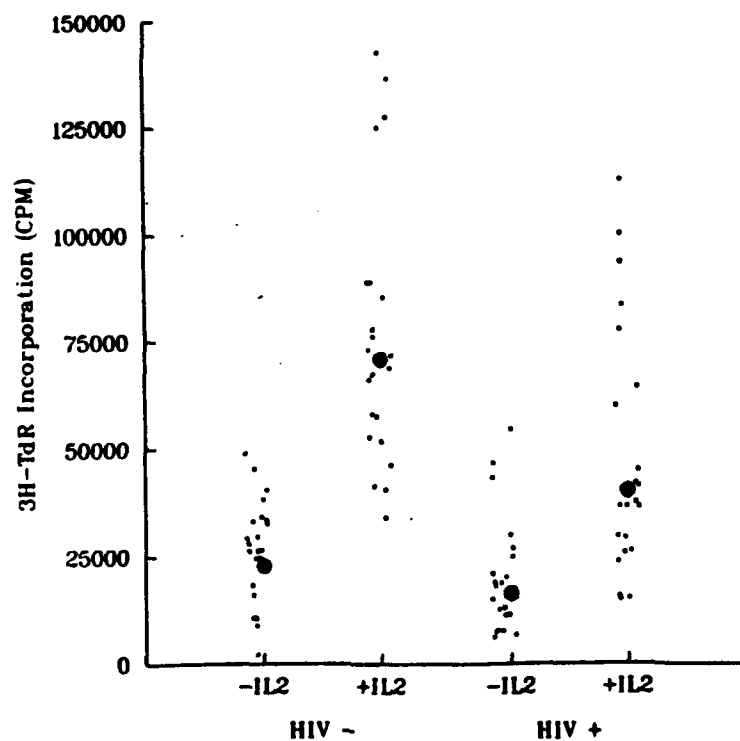


Figure 16

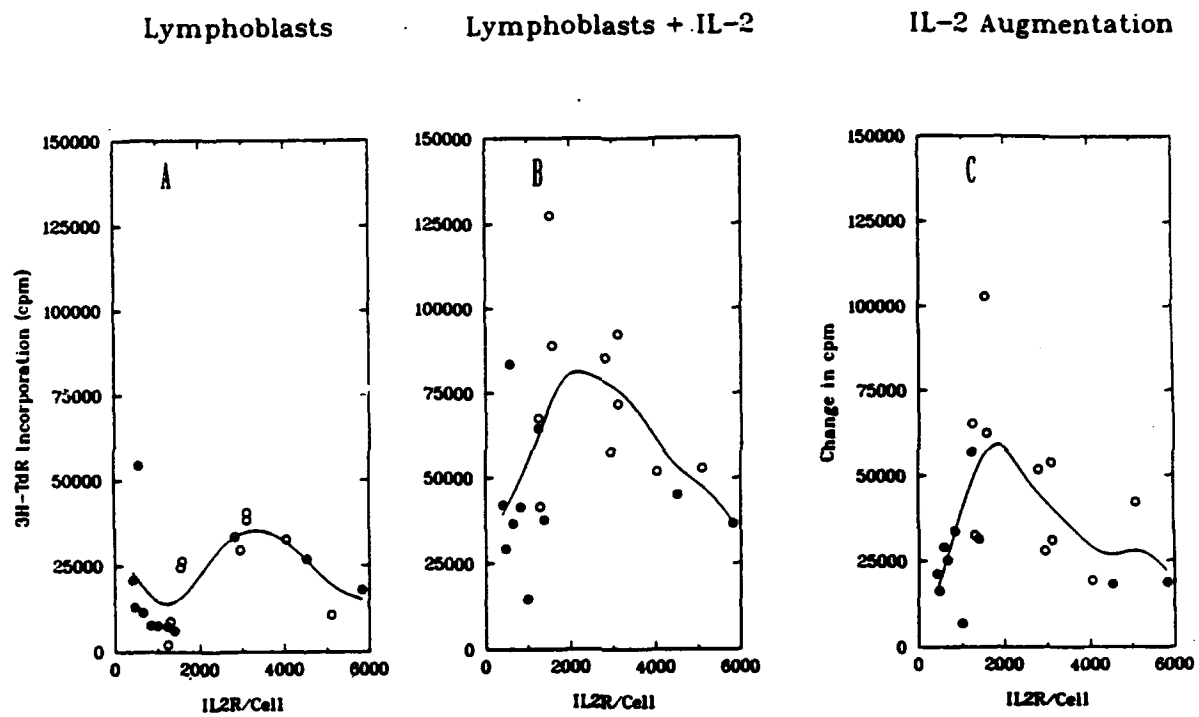


Figure 17

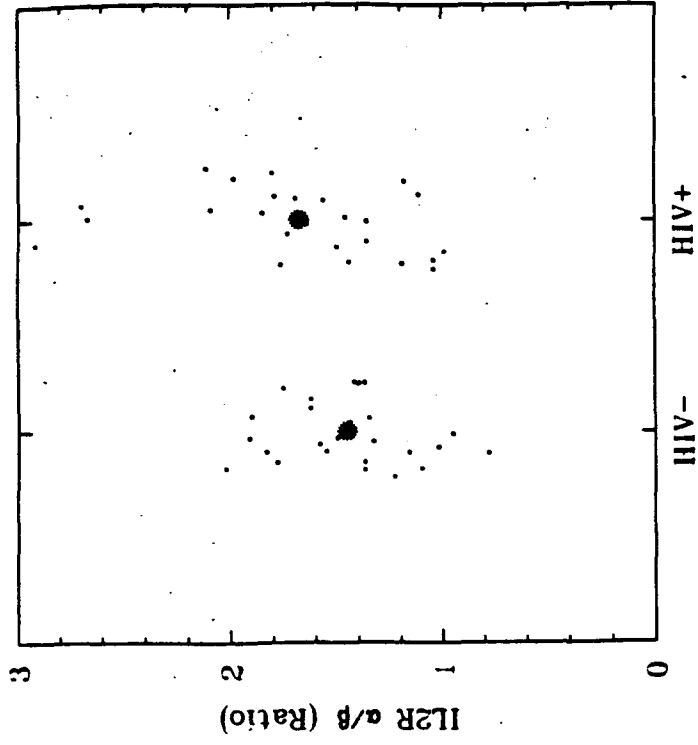
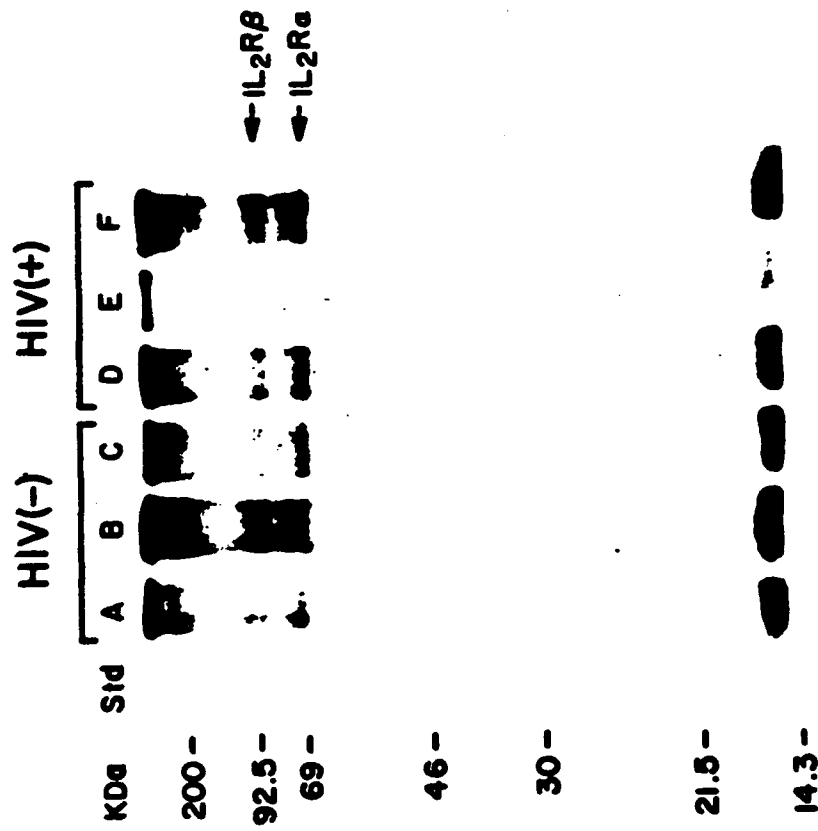
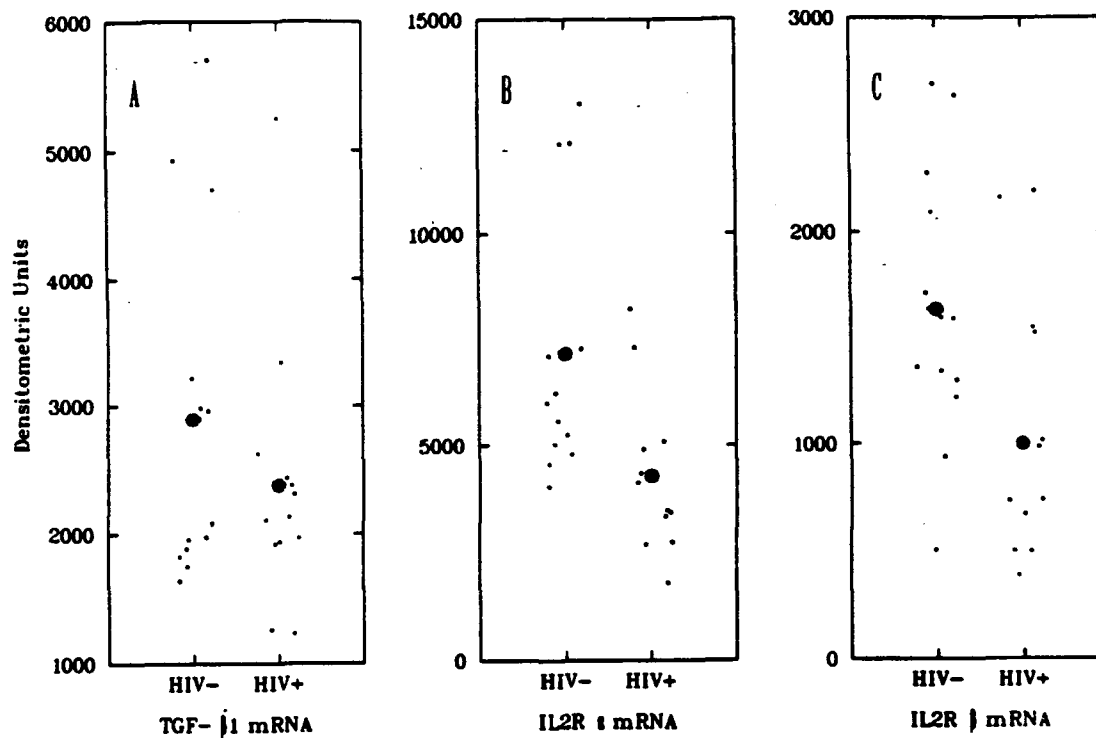


Figure 18



Appendix C. Tables. Eight tables are appended (45-49).

Table 1. Development of Immunocompetent Monocyte/Macrophages from Cultured Monocyte Depleted Marrow: Antigen presenting capacity and Immunophenotype.

Day	CSFs	³ HTdR Uptake*	Leu M3 % Pos	CD4 %Mono Gate
0	No	314 (274, 424)	0.8	0.0
7	No	109 (52, 228)	5.4	28.0
7	Yes	150 (45, 498)	24.4	27.7
14	No	1953 (1734,2192)	15.9	39.9
14	Yes	5336 (4428, 6430)	45.9	61.2

*. Geometric mean CPM, parentheses: lower, upper 95% confidence intervals.

Table 2. Ability of HIV-1 Infected and Uninfected Adherent Cells Derived from Adherence Depleted Cultured Marrow to Present Tetanus Antigen to Autologous Monocyte Depleted Lymphocytes

BM Treatment		NET CPM Mean (SEM)
M + GMCSF	HIV IIIB	
No	No	1,358 (309)
No	Yes	3,444 (634)
Yes	No	2,946 (836)
Yes	Yes	10,507 (617)
No APC		1,360 (32)
No APC, No TET		196 (49)

Table 3. Allogeneic BMT for Lymphoma in HIV+ patients, Syngeneic BMT for AIDS.

Dx/BMT	Prep Tx ¹	DTE ²	Neuro ³	Status ⁴	HIV ⁵
Lymph/ALLO	CY TBI	17	ok	D tumor d47	neg 6 wks
Lymph/ALLO	BU CY	25	PML	D CNS bleed d97	+ 7 wks
Lymph/ALLO	CY TBI	25	ok	D d279 GVHD	+ 12 wks
AIDS/SYN	BU CY	28	ok	A d310 +	+
AIDS/SYN	BU CY	14	Dementia	D d106 CNS	+
AIDS/SYN	BU CY	18	Dementia	D d80 CMV IP	+

- 1 Preparative therapy. CY TBI: cyclophosphamide, 200mg/kg and total body irradiation, 1200 rads. BU CY: Busulfan, 16mg/kg and cyclophosphamide, 200mg/kg.
- 2 Days to engraftment (absolute neutrophil count to 500/mm³).
- 3 Neurological status. PML: progressive multifocal leukoencephalopathy, JC virus was isolated from the brain at autopsy.
- 4 Status as of February 3, 1991. D: deceased; GVHD: graft-v-host disease. CMV IP: cytomegalovirus interstitial pneumonitis.
- 5 HIV: time of first positive sample by culture or PCR. Peripheral blood was assayed weekly by quantitative cocultivation. Weekly specimens were frozen and processed in batch for PCR. Patient 3 was negative by co-culture through week 24, but proved PCR positive on week 12. Patients 4-6 were never virus negative.

Table 4. Effect of booster regimen of on adoptive transfer of human antibody responses in Hu-PBL/SCID chimeric mice.

Donor Boost*	Mouse Day 3 TT	Mouse Day 35 TT	Reciprocal Titer			
			Day 0	Day 14	Day 35	Day 45
none	none	none	0	8	nd	0
none	YES	none	0	173	nd	873
none	none	YES	0	8	0	0
YES	none	none	0	1,691	3,324	930
YES	YES	none	0	30,064	24,079	22,901
YES	none	YES	0	1,691	1,118	171

* Prior to boost the donor had a TT specific IgG titer of 1/2,600. Ten days after booster immunization this titer rose 19 fold to 49,700.

Table 5. Absolute Numbers of Lymphocytes in Peripheral Blood of HIV-1 Seronegative and Seropositive Individuals Studied

	Seronegative			Seropositive			P
	Mean ^a	(95% C.I.) ^b	n	Mean	(95% C.I.)	n	
CD3	1750	(1673, 1831)	132	1589	(1471, 1715)	78	0.022
CD4	1125	(1075, 1177)	132	428	(365, 501)	78	<0.001
CD8	564	(524, 606)	132	970	(879, 1071)	78	<0.001
CD3 - (CD4 + CD8) ^c (calculated DN)	28	(8, 48)	34	87	(59, 115)	30	<0.001
CD3 ⁺ CD4 ⁻ CD8 ⁻ ^c (calculated DN)	56	(46, 69)	34	67	(53, 87)	30	0.257
NK (CD56 ⁺ CD3 ⁻)	170	(150, 193)	110	81	(66, 99)	60	<0.001
δ-TCR	62	(56, 70)	132	81	(68, 96)	77	0.010
δ-TCR/CD8 ⁺	15	(12, 20)	50	27	(19, 37)	41	0.009

^a Geometric mean numbers of cells/mm³.

^b 95% confidence interval of the mean.

^c Data for these two groups were derived from the same individuals.

Table 6. Comparison of Lymphocyte Subsets in 64 Seronegative and Seropositive SHARE participants.

	SN	SP	P
Lymphocytes	2387 ± 503 (33)	1928 ± 755 (30)	0.006
Calculated	26.1 ± 57.0 (33)	95.8 ± 74.1 (29)	<0.001
Double Neg.			
Measured	68.2 ± 41.2 (33)	90.1 ± 50.1 (29)	0.064
Double Neg.			
γδ-TCR	59.5 ± 39.9 (33)	86.5 ± 63.0 (29)	0.046
Dim CD8	153.7 ± 68.7 (20)	112.7 ± 63.8 (22)	0.052
NK	227.1 ± 174.4 (10)	79.1 ± 55.0 (10)	0.048

Table 7. Summary of results of comparison study

Phenotype	CD4	CD8	CD3	CD56/CD16 CD3 neg	TCR- $\gamma\delta$ CD8 pos	TCR- $\gamma\delta$ CD8 neg
Mean Epics C Result ^a	40.13	30.42	74.10	7.47	2.44	1.71
Mean Bias ^b	-0.39	0.27	-0.23	-0.17	-0.03	0.23
Mean % Bias ^c	-0.90	0.84	-0.29	-2.18	-2.22	8.66
Mean Discrepancy ^d	0.83	0.79	0.89	0.47	0.32	0.49
Mean % Discrepancy ^e	2.73	3.26	1.24	8.07	21.98	41.41

^aResult for percent cells positive for each phenotype as analyzed on the Epics C.

^bMean bias is the average of all differences (Acmeocyte-Epics C) for each phenotype.

^cMean % bias is the average of all differences expressed as a percentage of their respective Epics C results.

^dMean discrepancy is the average of all absolute values for the difference (Acmeocyte-Epics C).

^eMean % discrepancy is the average of all absolute value differences expressed as a percentage of their respective Epics C results.

Table 8. Anti TGF- β 1 Antibody Does Not Increase the IL2 Induced Proliferative Response of Cells from HIV⁺

Expt #	HIV Serostatus	Control antibody	Control antibody + IL2	TGF-β1 antibody	TGF-β1 antibody + IL2
3H-TdR incorporation (cpm)					
1	-	ND	78,309	41,836	89,316
1	-	ND	84,105	60,247	75,711
1	+	ND	84,652	42,859	87,877
1	+	ND	19,840	7,830	17,670
2	-	4,777	28,905	5,357	25,837
2	-	11,021	82,408	11,701	77,682
2	+	10,471	61,885	8,389	84,566
2	+	10,073	81,714	5,395	65,879
3	-	17,156	70,698	18,356	76,031
3	-	13,729	87,900	13,860	106,113
3	+	24,394	71,339	23,909	82,870
3	+	13,670	60,383	21,077	79,943
4	-	54,147	227,465	32,120	252,233
4	+	35972	93,539	24,760	89,604

PBMC from HIV⁻ and HIV⁺ donors (1.2×10^6 cells per 1.2 ml complete medium) were cultured in 24 well microculture plates in the presence of PHA and control chicken IgG or polyclonal anti TGF- β 1 antibody (12.5 μ g per ml). After 55 h in culture, cells were washed, and recultured overnight in complete medium. The cells were then washed and incubated for 48 h in the presence or absence of 1 nM rIL2 (5×10^4 cells per well in triplicate cultures in 96 well microculture plates). In these triplicate cultures, control antibody was added to the cells initially cultured in presence of control antibody while anti TGF- β 1 antibody was added to cells initially cultured in presence of anti TGF- β 1 antibody. ³H-TdR incorporation was assessed during the last 4 h in culture. ND, not determined. Responses were not significantly improved by the addition of anti TGF- β 1 antibody along with rIL2 compared to rIL2 alone (mean augmentation = 5,585 CPM, $p = 0.122$, paired t-test) in cultures from HIV⁻ or HIV⁺ donors.

Appendix D. Staff with percent effort by quarter**Staff as of 8/31/89 with percent effort of each on project:**

Principal Investigator	
Albert D. Donnenberg, Ph.D.	30%
Co-Investigator	
Joseph B. Margolick, M.D., Ph.D. ...	20%
Technical Staff	
Suzanne Eacker	100%
Elvia Scott	25%
Lisa McCall	25%
Veena Chatterjee	20%
Jack Towsley	25%

Staff as of 12/15/89 with percent effort of each on project:

Principal Investigator	
Albert D. Donnenberg, Ph.D.	30%
Co-Investigator	
Joseph B. Margolick, M.D., Ph.D.	5%
Research Associate	
Rajesh Chopra, Ph.D.	90%
Technical Staff	
William R. Cappuccio	20%
James P. Barber	100%
B. Taylor	10%

Staff as of 3/15/90 with percent effort of each on project:

Principal Investigator	
Albert D. Donnenberg, Ph.D.	30%
Co-Investigator	
Joseph B. Margolick, M.D., Ph.D. ...	5%
Research Associate	
Rajesh Chopra, Ph.D.	90%
Technical Staff	
William R. Cappuccio	20%
James P. Barber	100%
B. Taylor	10%

Staff as of 6/15/90 with percent effort of each on project:

Principal Investigator	
Albert D. Donnenberg, Ph.D.	30%
Co-Investigator	
Joseph B. Margolick, M.D., Ph.D. ...	5%
Research Associate	
Rajesh Chopra, Ph.D.	90%
Technical Staff	
William R. Cappuccio	20%
James P. Barber	100%
B. Taylor	10%

Staff as of 9/15/90 with percent effort of each on project:

Principal Investigator		
Albert D. Donnenberg, Ph.D.	20%	
Co-Investigator		
Joseph B. Margolick, M.D., Ph.D. ...	5%	
Research Associate		
Rajesh Chopra, Ph.D.	100%	
Technical Staff		
William R. Cappuccio	20%	(34% as of 8/1/90)
James P. Barber	100%	
B. Taylor	10%	(20% for month of August only)

Staff as of 12/15/90 with percent effort of each on project:

Principal Investigator	
Albert D. Donnenberg, Ph.D.	20%
Co-Investigator	
Joseph B. Margolick, M.D., Ph.D. ...	5%
Research Associate	
Rajesh Chopra, Ph.D.	100%
Technical Staff	
William R. Cappuccio	34%
James P. Barber	100%
B. Taylor	10%

Staff as of 3/15/91 with percent effort of each on project:

Principal Investigator	
Albert D. Donnenberg, Ph.D.	20%
Co-Investigator	
Joseph B. Margolick, M.D., Ph.D. ...	5%
Research Associate	
Rajesh Chopra, Ph.D.	100%
Technical Staff	
William R. Cappuccio	34%
James P. Barber	100%
B. Taylor	10%

Graduate degrees granted: None.

Appendix E. Abstracts, manuscripts and publications resulting from this project. Copies have been appended to this report.

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1. Margolick, J.B., McArthur, J.C., Scott, E.R., McArthur, J.H., Cohn, S., Farzadegan, H., and Polk, B.F. Flow Cytometric Quantitation of T Cell Phenotypes in Cerebrospinal Fluid of Homosexual Men With and Without Antibodies to Human Immunodeficiency Virus. J. Neuroimmunol. 20:73-81, 1988.
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9. Chopra, R.K., Raj, N.B.K., Scally, J.P., Donnenberg, A.D., Adler, W.H., Saah, A.J., and Margolick, J.B. IL2 Receptor α and β Chain mRNA Expression is Decreased in PHA Stimulated PBMC from HIV Type 1 (HIV-1) Infected Homosexual Men. Clin. Exp. Immunol. (in press).
10. Palenicek, J., Fox, R., Margolick, J., Farzadegan, H., Odaka, N., Taylor, E., Ward, L., Harris, J., Armenian, H., and Saah, A.J. Longitudinal Study of Homosexual Couples Discordant for HIV-1 Antibodies in the Baltimore MACS Study. J. AIDS (in press).

Abstracts

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10. **Margolick, J.B., Donnenberg, A.D., Muñoz, A., Odaka, N., and Saah, A.J. Altered Replacement of CD4 Lymphocytes by CD8 Lymphocytes Early After Seroconversion to HIV-1. 6th Intl. Conf. on AIDS, San Francisco, June 1990.**
11. **Markham, R.B., Barber, J.P., and Donnenberg, A.D. Adoptive Transfer of Human Immune Memory Responses to Hu/PBL/SCID Mice: Effect of Donor and Recipient Immunization. 6th Intl. Conf. on AIDS, San Francisco, June 1990.**
12. **Scott, E., Chadwick, K., Margolick, J., Shapiro, H., Hetzel, A., Vogt, R. Comparison of Lymphocyte Immunophenotypes Obtained from Two Different Data Acquisition and Analysis Systems Simultaneously on the Same Flow Cytometer. 5th Annual Meeting, Clinical Applications of Flow Cytometry, Charleston, SC, September 1990.**
13. **Chopra, R.K., Adler, W.H., Saah, A., and Margolick, J.B. Co-ordinate Expression of IL2R α and β Chains on Stimulated T Cells from HIV⁺ Homosexual Men. FASEB, 1991.**

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Flow cytometric quantitation of T cell phenotypes in cerebrospinal fluid and peripheral blood of homosexual men with and without antibodies to human immunodeficiency virus, type I

Joseph B. Margolick, Justin C. McArthur, Elvia R. Scott, Julie H. McArthur, Sylvia Cohn, Homayoon Farzadegan and B. Frank Polk

Departments of Environmental Health Sciences, Neurology, and Epidemiology, Johns Hopkins Medical Institutions, Baltimore, MD 21205, U.S.A.

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Summary

Two-color flow cytometry was used to analyze T cell subsets (total (CD3), helper-inducer (CD4), and suppressor-cytotoxic (CD8)) in paired specimens of cerebrospinal fluid (CSF) and peripheral blood of 66 homosexual men, including 62 with antibodies to human immunodeficiency virus, type 1 (HIV-1). With the exception of one traumatic specimen, all of the CSF specimens, 52 of which had ≤ 5 lymphocytes/mm³, were evaluated fully, with the number of lymphocytes counted for each antibody ranging from 200 to 2933 (mean = 1129). Proportions of CD3, CD4, and CD8 lymphocytes in CSF were very highly correlated with the proportions of these cells in the peripheral blood ($r = 0.87, 0.96$, and 0.94 , respectively), as was the CD4/CD8 ratio ($r = 0.96$). These strong correlations were present in each of seven subgroups of study subjects defined on the basis of detailed neurologic examination, neuropsychological testing, and the presence or absence of antibodies to HIV-1. In the population studied, T cell phenotypes in CSF as analyzed by two-color flow cytometry were largely determined by the corresponding proportions in the peripheral blood.

Introduction

The etiologic agent for acquired immunodeficiency syndrome (AIDS), now known as hu-

man immunodeficiency virus, type 1 (HIV-1) (Coffin et al., 1986), infects the central nervous system (CNS) as well as cells of the immune system (Shaw et al., 1985; Johnson and McArthur, 1986). Although our understanding of the pathogenesis of CNS damage due to HIV-1 is incomplete (Barnes, 1987; Price et al., 1988), there appears to be a local immune response against HIV-1 in the CNS, as evidenced by the intrathecal synthesis of immunoglobulins directed against

Address for correspondence: Joseph B. Margolick, M.D., Ph.D., Department of Environmental Health Sciences, Johns Hopkins School of Public Health, 615 North Wolfe St., Room 7032, Baltimore, MD 21205-2179, U.S.A.

HIV-1 (Resnick et al., 1985; Ackermann et al., 1986; Goudsmit et al., 1986) and by cerebrospinal fluid (CSF) pleocytosis in some cases (Ho et al., 1985; Hollander and Levy, 1987; McArthur et al., 1988a). It is well established that this cellular response is predominantly lymphocytic (Hollander and Levy, 1987; McArthur et al., 1988a), but the specific types of lymphocytes involved have been analyzed in only a few cases (Steck and de Flaingergues, 1987; McArthur et al., 1988b). One reason for the paucity of such studies in HIV-1-related disorders, as well as in neurological disease in general, may be the relatively small numbers of cells present in the CSF and available for analysis.

Flow cytometry has provided greatly improved accuracy and sensitivity, compared to manual methods, for the analysis of T cell subsets in peripheral blood (Parks and Herzenberg, 1984), other body fluids such as bronchoalveolar lavage specimens (Davidson et al., 1985), and, in one study, CSF (Vandenberg et al., 1985). We examined whether the low concentrations of lymphocytes present and the small volumes of fluid available for analysis would permit the use of this technique to quantitate lymphocyte subsets in CSF from individuals infected with HIV-1. This report presents a comparison of T cell phenotypes in the CSF, as determined by flow cytometry, with those in simultaneously obtained specimens of peripheral blood from homosexual and bisexual men participating in a prospective study of the neuropsychologic manifestations of HIV-1 infection.

Materials and methods

Study subjects

The study population consisted of 66 homosexual/bisexual men: 15 patients who underwent lumbar puncture for clinical (diagnostic) indications (McArthur, 1987), and 51 volunteers from SHARE, the Baltimore participants in the Multicenter AIDS Cohort Study (MACS) (Kaslow et al., 1987). All subjects were screened for antibodies to HIV-1 by ELISA (Sarnagadharan et al., 1984), and all positive specimens were confirmed by Western blot (Ustrup et al., 1986). The SHARE subjects, 47 of whom were seropositive and four

seronegative for HIV-1, underwent neurologic and psychologic testing as well as lumbar puncture (McArthur et al., 1988a) after giving informed consent. Neurological assessment included standardized neurological examination by a board-certified neurologist, administration of a neurological questionnaire, and a battery of neuropsychological tests including the Shipley Hartford Scale, WAIS-R Block Design, Warrington Recognition Memory Test, Rey Complex Figure, and the Trailmaking Tests (Lezak, 1983). Electroencephalography and magnetic resonance imaging were also performed on a majority of the individuals. Results of these studies are reported in detail elsewhere (McArthur et al.: Low prevalence of neurological and neuropsychological abnormalities in healthy HIV-1-infected individuals: results from the Multicenter AIDS Cohort Study, submitted).

The study subjects were classified as described below, according to findings on neurological examination, neuropsychological testing, and serology for HIV-1. All subjects except for the four in group 7 were seropositive for HIV-1.

(1) Dementia - signs of cognitive, behavioral or motor dysfunction consistent with HIV-1 encephalopathy (Navia et al., 1986) ($n = 9$).

(2) Myelopathy - spastic paraparesis (Petito et al., 1985) ($n = 3$).

(3) Cryptococcal meningitis (Kovacs et al., 1987) ($n = 2$).

(4) HIV-1-related meningitis (Hollander and Levy, 1987) ($n = 2$).

(5) Neuropsychologic findings - mild abnormalities on neurologic examination or neuropsychological testing, but insufficient to categorize definitively as HIV-1 encephalopathy ($n = 33$).

(6) Asymptomatic - no neurologic symptoms or abnormalities on neuropsychological testing, and no other HIV-1-related symptoms ($n = 12$).

(7) Seronegative - seronegative for HIV-1 and normal neurologic examination and neuropsychological testing ($n = 4$).

Specimen collection

Peripheral blood was obtained by venipuncture from the antecubital vein using a heparinized syringe. CSF was obtained by lumbar puncture using aseptic techniques and a standardized proto-

col. The CSF cell count was performed by hemocytometer on the 3rd 1 ml aliquot of CSF collected. The last 3–5 ml of CSF collected were reserved for flow cytometry as described below. The CSF was considered to be normocellular if the WBC count was ≤ 5 cells/mm³ and the neutrophil count was ≤ 1 cell/mm³.

Flow cytometric analysis of T lymphocytes

Within 3 h, the specimens were stained with monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) for two-color fluorescence analysis. For blood specimens, the whole blood-lysis method was employed as described (Hoffman et al., 1980), using three monoclonal antibodies and an isotype control antibody in two test tubes, as follows: anti-Leu4 (CD3)-FITC and control antibody (IgG₁)-PE in tube 1, and anti-Leu 2a (CD8)-FITC and anti-Leu3a (CD4)-PE in tube 2. Antibodies were obtained from Becton-Dickinson, Mountain View, CA. The stained lymphocytes were fixed in paraformaldehyde (Polysciences, Warrington, PA) and analyzed within 48 h. Analysis was performed using an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a sealed flow tip and specimen collection system (Coulter) to eliminate aerosolization of the specimens. The flow cytometer was gated on peripheral blood lymphocytes by forward- and 90°-light scatter characteristics, along with software for two-color analysis (Coulter). 5000 cells were counted for each tube of each peripheral blood specimen.

For CSF, the fluid was centrifuged at 1200 rpm for 5 min, and the cells were washed once with phosphate-buffered saline containing 1% bovine serum albumin and 0.1% azide and resuspended in 300 μ l of the same buffer. 100 μ l were added to test tubes containing the above antibody combinations and incubated for 30 min on ice. The stained cells were then analyzed as above, except that it was never possible to count 5000 cells. Instead, cells were counted until the specimen was exhausted, and the number of cells counted was recorded.

Statistical analysis

Percentages and ratios of lymphocyte subsets among the study groups were compared by analysis of variance.

Results

Study subjects and clinical laboratory results

Paired specimens of peripheral blood and CSF were obtained on 66 subjects from February, 1987, to April, 1988. All cases were included in the study except one case in which the lumbar puncture was traumatic and the specimen was grossly bloody. The clinical/neurologic classification of the remaining 65 subjects is shown in Table 1. Also shown are the cell counts in the 65 CSF specimens, 52 of which were within the normocellular range. It should be noted that none of the study subjects had Kaposi's sarcoma, and only two had opportunistic infections (cryptococcal meningitis).

Flow cytometric analysis of lymphocytes

The numbers of lymphocytes counted in each of the two tubes of CSF from each subject were equivalent, ranging from 200 to 2933 with an overall mean of 1129 (Table 1). In all cases it was possible to analyze sufficient cells to determine proportions of CD3, CD4, and CD8 lymphocytes, and their ratios, in the CSF, even though the number of lymphocytes per mm³ was generally not elevated and in 24 cases was 0 or 1 (Table 1).

The correlations between the proportions of a given type of lymphocyte in the peripheral blood and in the CSF were extremely high, as shown in Fig. 1 (A–C for CD3, CD4, and CD8 lymphocytes, respectively). The correlation coefficient for CD4 cells was 0.96 and that for CD8 cells was 0.94. The slightly lower value of 0.87 for CD3 cells

probably reflects the relatively narrow range of values for the percent of CD3 cells present in the specimens studied. In addition, the regression lines for CD4 and CD8 cells had slopes very close to 1.0 and intercepts very close to 0 (legend to Fig. 1). Part D of Fig. 1 shows the regression line for the helper-suppressor ratio measurements in blood and CSF, which had very similar characteristics ($r = 0.98$).

Table 2 shows the T cell proportions and CD4/CD8 ratios in peripheral blood and CSF of study subjects classified according to the results of neurological examination and neuropsychological testing. As expected, all seropositive groups had lower proportions of CD4 cells and higher propor-

tions of CD8 cells than did the seronegative group. In addition, all neurologically symptomatic seropositive groups, with the exception of the three

patients with HIV-1 myelopathy, had lower proportions of CD4 cells and higher proportions of CD8 cells than did the asymptomatic seropositive

TABLE 1

CLINICAL AND LABORATORY CHARACTERISTICS OF STUDY SUBJECTS AND SPECIMENS

Subject number	Clinical features	CSF cell counts (cells/mm ³)			No. of cells counted	
		WBC ^a	Lymphocytes	RBC ^b	Tube No. 1 ^c	Tube No. 2 ^d
34	Seronegative control	1	1	0	519	602
35	Seronegative control	1	1	0	1441	1205
36	Seronegative control	1	1	0	1540	1526
56	Seronegative control	0	0	0	305	313
29	Asymptomatic	5	5	0	2024	2043
30	Asymptomatic	4	4	2	2025	2134
31	Asymptomatic	4	4	0	1908	1612
32	Asymptomatic	3	1	0	724	837
33	Asymptomatic	15	15	1	2030	2076
39	Asymptomatic	0	0	0	613	625
44	Asymptomatic	3	3	0	432	515
45	Asymptomatic	5	5	2	1235	1274
47	Asymptomatic	9	9	0	2534	2770
51	Asymptomatic	6	6	0	833	855
64	Asymptomatic	2	2	1	498	522
65	Asymptomatic	6	5	0	1997	2023
11	NP findings ^e	0	0	0	1021	1133
12	NP findings	6	6	6	2012	2020
13	NP findings	1	1	0	966	844
14	NP findings	0	0	0	642	1014
15	NP findings	5	5	90	1834	1573
16	NP findings	6	5	0	1419	1508
17	NP findings	0	0	0	1996	1817
18	NP findings	3	3	0	2502	2704
19	NP findings	5	3	0	1703	1627
20	NP findings	3	3	1	1179	1351
21	NP findings	3	3	2	1028	1217
22	NP findings	2	2	0	268	283
23	NP findings	8	8	9	2018	1800
24	NP findings	3	3	0	666	597
25	NP findings	12	12	3	1010	1028
26	NP findings	4	4	2	200	212
27	NP findings	1	1	6	328	356
28	NP findings	12	12	1	1096	1022
37	NP findings	5	5	0	1594	1642
38	NP findings	1	1	0	770	796
40	NP findings	0	0	1	352	341
41	NP findings	2	2	3	501	523
42	NP findings	nd ^f	nd	nd	693	722
43	NP findings	11	11	0	544	624
46	NP findings	3	3	0	698	708
48	NP findings	1	1	7	1405	1493
49	NP findings	4	4	0	902	915
50	NP findings	3	3	0	918	921
52	NP findings	1	1	1	1011	1032
53	NP findings	0	0	0	733	741
54	NP findings	2	2	1	937	983

(continued)

TABLE 1 (continued)

Subject number	Clinical features	CSF cell counts (cells/mm ³)			No. of cells counted	
		WBC ^a	Lymphocytes	RBC ^b	Tube No. 1 ^c	Tube No. 2 ^d
62	NP findings	13	13	0	1986	1973
63	NP findings	3	2	473	1126	1153
10	HIV-meningitis	11	11	0	1314	1297
59	HIV-meningitis	0	0	0	948	1051
8	Cryptomeningitis	2	2	0	1425	1573
9	Cryptomeningitis	5	5	0	2802	2933
6	Myelopathy	1	1	0	1242	1478
7	Myelopathy	62	62	2	415	398
57	Myelopathy	0	0	12	405	515
1	Dementia	0	0	0	966	1276
2	Dementia	3	3	0	1904	1670
3	Dementia	6	6	13	426	354
4	Dementia	0	0	0	306	277
5	Dementia	1	1	0	320	510
55	Dementia	1	1	0	943	971
58	Dementia	21	21	0	604	709
60	Dementia	1	1	10	1264	1273
61	Dementia	2	2	1	514	528

^a WBC = white blood cells; ^b RBC = red blood cells; ^c Tube No. 1 = Leu4 and IgG₁ control antibody; ^d Tube No. 2 = Leu3a and Leu2a; ^e NP findings = neuropsychologic findings, as defined in text; ^f nd = not done.

group. Among the three largest groups studied, there was a progressive decrease in percentage of CD4 cells when subjects with neuropsychologic findings and subjects with dementia were compared with the asymptomatic seropositive group; the difference between the latter two groups was statistically significant ($P = 0.021$ by analysis of variance). There were no significant differences between the groups in percentage of CD3 or CD8 cells, or in CD4/CD8 ratios. As indicated by the very close correlation between T cell proportions in peripheral blood and CSF, no differences between T cell proportions in peripheral blood and CSF were observed in any of the subject groups.

Discussion

This study represents the first flow cytometric analysis of T cells present in the CSF of individuals infected with HIV-1. Our results confirm the applicability of flow cytometry to cells in the CSF, as shown in one previous study (Vandenbark et al., 1985) which used a one-color method to analyze 25 patients with a variety of neurologic conditions, both immunologic and nonimmunologic, which

were not related to HIV-1. Together, that study and the present study demonstrate the advantages of flow cytometry over manual methods for immunotyping intrathecal lymphocytes: (1) rapid processing of specimens, permitting the analysis of many specimens; (2) efficient counting of cells, permitting normocellular specimens to be analyzed; and (3) quantitation of larger numbers of cells than the 50–200 cells per antibody typically counted by manual methods. Specifically, Vandenbark et al. (1985) counted an average of 750 cells per antibody with at least 150 cells counted for each of the four antibodies studied, using only 0.8–8.0 ml of CSF per patient. In the present study, we counted at least 200 cells per antibody in all specimens, more than 300 in 62 of 65, and a mean of 1129 (Table 1) using 3–5 ml of CSF. Based on the numbers of lymphocytes in the specimens containing 0 or 1 white blood cell/mm³, it can be established that we counted approximately 10–50% of the cells in the specimen. This percentage could have been increased by counting monocytes as well as lymphocytes. In addition to increasing the efficiency of cell counting, two-color flow cytometry has the potential to permit a more detailed phenotypic characterization of the

lymphocytes present in the CSF than one-color analysis can accomplish.

In the present study, the most prominent finding was the very high correlation between T cell proportions in CSF and peripheral blood. This high correlation was seen over a wide range of values for the T cell proportions and lymphocyte counts/mm³, and did not appear to be influenced by the clinical status of the study subjects as indicated by detailed neurological and neuropsychological assessment. Moreover, the regression lines closely resembled the line of equivalence, with slopes nearly equal to 1 and intercepts nearly equal to 0, particularly for CD4 and CD8 cells and CD4/CD8 ratio. This suggests that the proportions of T cells in the CSF were determined

almost entirely by the proportions present in the peripheral blood. These results are consistent with recent manual immunocytochemical studies which found a general correlation between CD4/CD8 ratios in CSF and peripheral blood in four cases of AIDS (Steck and de Flaugergues, 1987) and in 31 HIV-1-infected individuals with a variety of neurologic disorders (McArthur et al., 1987, 1988b). These data suggest that selective recruitment of specific T cell populations to the CSF, and/or proliferation of these populations in the CSF, do not play a primary role in the pathogenesis of HIV-1-related neurological disorders. Our results also have the support of a recent study in which peripheral blood lymphocytes from patients with multiple sclerosis were labelled *in vivo* with

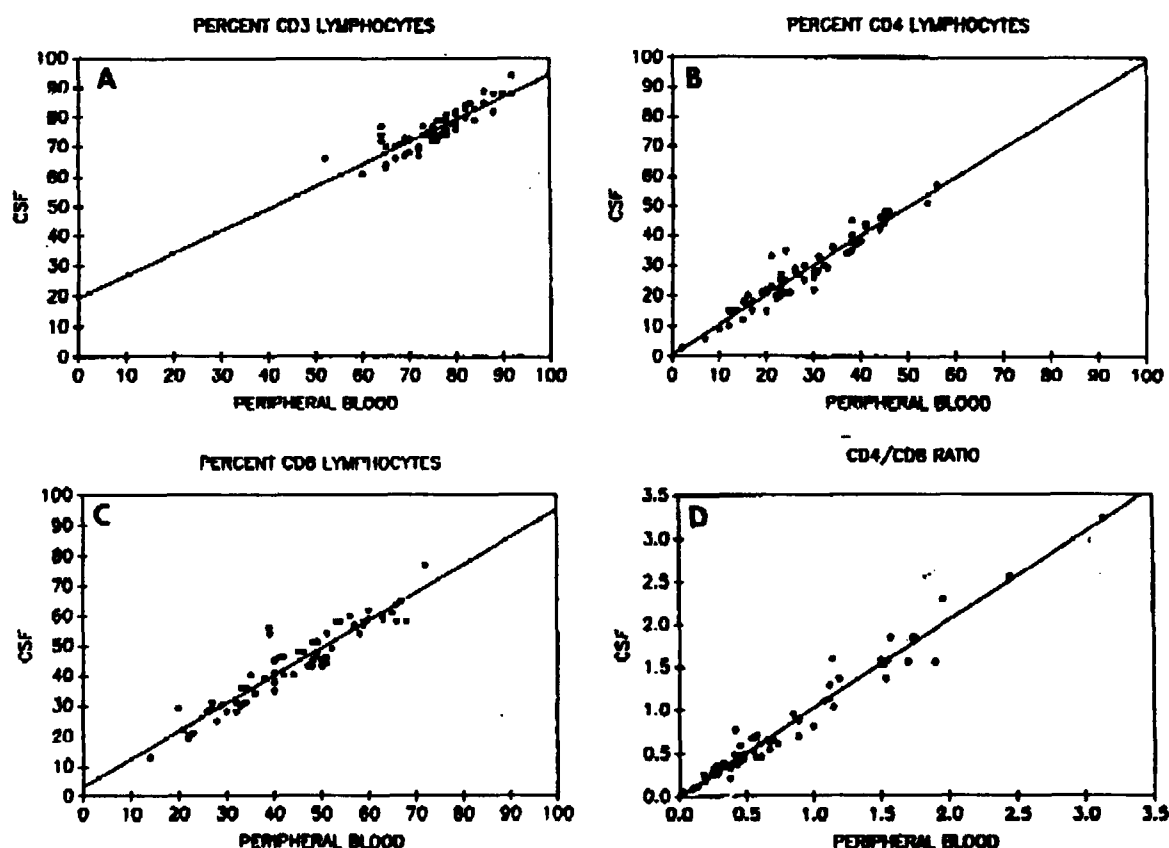


Fig. 1. Linear regressions of T cell subset proportions (A-C) and CD4/CD8 (D) ratio in peripheral blood (horizontal axes) against the corresponding measurements in cerebrospinal fluid (vertical axes). Least-squares regression lines are shown. The regression and correlation coefficients are as follows: A: $y = 19.12 + 0.75x$; $r = 0.87$; B: $y = 0.68 + 0.98x$; $r = 0.96$; C: $y = 3.23 + 0.92x$; $r = 0.94$; D: $y = -0.003 + 1.02x$; $r = 0.98$.

TABLE 1

PERCENTAGES OF LYMPHOCYTES EXPRESSING T CELL MARKERS IN PERIPHERAL BLOOD (PB) AND CEREBROSPINAL FLUID (CSF) OF STUDY SUBJECTS

Group	CD3		CD4		CD8		CD4/CD8 ratio	
	PB	CSF	PB	CSF	PB	CSF	PB	CSF
Seronegative (n = 4)	68.3 ^a (5.7)	69.5 (5.0)	42.5 (3.2)	42.5 (5.4)	24.5 (7.0)	22.3 (6.2)	1.95 (0.75)	2.12 (0.76)
Asymptomatic (n = 12)	77.5 (5.1)	78.4 (5.5)	31.5 ^a (7.6)	32.3 (7.1)	40.6 (10.5)	41.4 (11.3)	0.88 (0.41)	0.88 (0.39)
NP findings (n = 33)	76.0 (6.3)	75.9 (6.3)	27.2 (10.7)	27.2 (10.9)	44.7 (13.0)	44.1 (12.7)	0.76 (0.56)	0.76 (0.55)
HIV-1 meningitis (n = 2) ^b	85.0	81.0	18.0	17.5	62.5	57.0	0.29	0.31
Cryptococcal meningitis (n = 2) ^b	76.5	76.5	10.5	12.0	62.0	59.0	0.18	0.22
HIV-1 myelopathy (n = 3)	73.7 (11.6)	75.3 (9.3)	31.7 (17.6)	30.0 (19.4)	37.7 (4.2)	44.3 (10.3)	0.90 (0.61)	0.84 (0.72)
Dementia (n = 9)	76.3 (11.9)	77.7 (7.4)	21.1 ^a (7.7)	22.0 ^a (8.9)	50.1 (13.8)	49.9 (12.6)	0.49 (0.30)	0.54 (0.43)

^a Data given as mean (standard deviation).^b HIV omitted because of small numbers.^a $P = 0.021$ compared to asymptomatic seropositive group.

OKT-11 monoclonal antibody, and T cell percentages in CSF and peripheral blood did not differ (Hafler and Weiner, 1987).

These results contrast with many previous reports dealing with disorders unrelated to HIV-1 infection, which indicated that the proportion of T cells is higher in CSF than in peripheral blood. In addition, a wide variety of neurological conditions have been reported to be characterized by relative increases in specific T cell subsets in the CSF as compared to the peripheral blood: either the CD4⁺ subset (Sandberg-Wollheim, 1975; Fryden, 1977; Brinkman et al., 1983; Hauser et al., 1983; Griffin et al., 1985; Johnson et al., 1986; Jozsevicus and Rynes, 1986; Stern et al., 1987) or the CD8 subset (Marrow et al., 1983; Czlonkowska et al., 1986; Johnson et al., 1986; Richert et al., 1987). In particular, Vandenberg et al. (1985) reported that elevated proportions of T cells in CSF compared to peripheral blood reflected an elevation of CD4-positive cells in specimens containing > 3 cells/mm³. The discrepancy between these results and the present findings may be due to methodologic differences, or to differences in the patient populations studied. In the latter respect, it is possible that HIV-1 infection of the central

nervous system may elicit different patterns of T cell trafficking into the CSF than other infections, or that the ability of T cells from HIV-1-infected individuals to proliferate in the CSF may be reduced compared to cells from HIV-1-negative individuals with other infections of the central nervous system. Further studies involving functional as well as phenotypic characterization of CSF lymphocytes, and analysis of individual subjects over time, will be necessary to resolve these questions.

In the present study there was a progressive decrease in proportion of CD4 lymphocytes in the peripheral blood, and hence the CSF, when the seropositive groups with no symptoms, non-specific neuropsychological abnormalities, and dementia were compared. This trend was statistically significant, suggesting that the proportion of CD4 cells in these compartments may help determine the progression of HIV-1 infection of the central nervous system. In this study, examination of the CSF did not appear to add significant information to analysis of peripheral blood. However, the possibility remains that changes in CSF that are independent of changes in peripheral blood may occur in some individuals and may help identify

HIV-1-infected individuals with progressive neuropsychological disease. In addition, it is possible that other markers not examined in this study may differ between peripheral blood and CSF, as was observed in a study comparing peripheral blood and bronchoalveolar lavage fluid (Davidson et al., 1985). Further analyses of our study population will help to clarify these points and to determine the extent to which nonspecific neuropsychological abnormalities in HIV-1-positive homosexual men are due to HIV-1 rather than other, potentially nonprogressive factors (e.g., alcohol or drug use).

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Limiting Dilution Analysis of *in Vivo*-Activated (IL-2 Responsive) Peripheral Blood Lymphocytes in HIV-1-Infected Subjects¹

ALBERT D. DONNENBERG,*† JOSEPH B. MARGOLICK,‡ AND B. FRANK POLK§²

**The Johns Hopkins University School of Medicine, Oncology Center, Bone Marrow Transplantation Program and †The Johns Hopkins University School of Public Health, Departments of Immunology and Infectious Diseases, ‡Environmental Health Sciences, and §Epidemiology, Baltimore, Maryland 21205*

The progression of infection with human immunodeficiency virus, type 1 (HIV-1), is associated with a loss of helper T cell function, but the mechanism for this loss (e.g., decreased absolute number of helper cells, altered function of helper cells, or both) has not been delineated. Many studies have suggested that T-cell production of and/or responsiveness to the T cell growth factor interleukin-2 (IL-2) declines over the course of HIV-1 infection. Using a highly quantitative 6-day limiting dilution assay (LDA), we investigated whether the number and the proliferative capacity of circulating IL-2 responsive cells in patients with AIDS differ from those in patients in earlier stages of HIV-1 infection (asymptomatic or AIDS-related complex) and healthy seronegative individuals. The frequency of IL-2 responsive cells declined progressively in asymptomatic seropositive subjects, those with ARC, and those with AIDS. In contrast, the proliferative capacity of individual IL-2 responsive cells, as reflected by the magnitude of thymidine uptake per precursor, was reduced only in patients with frank AIDS and was normal in asymptomatic subjects and in those with ARC. These results suggest that the development of AIDS in the setting of HIV-1 infection may reflect a combination of qualitative as well as quantitative changes in lymphocyte function. They also suggest that analysis of lymphocyte responsiveness to IL-2 may provide a useful approach to prediction of the development of AIDS in individuals infected with HIV-1. © 1989 Academic Press, Inc.

INTRODUCTION

Several lines of evidence indicate that decreased responsiveness to antigenic stimulation is critical to the development of AIDS, as demarcated by the onset of opportunistic illnesses. The lack of antigen-induced T cell functions such as proliferation (1, 2) and lymphokine production (1, 3) appears to distinguish most reliably between HIV-1-infected individuals with AIDS and those who do not have AIDS by clinical criteria. In addition, Murray *et al.* (4) reported that antigen-induced production of interferon- γ was more strongly associated with progression of AIDS-related complex to frank AIDS within 1 year than was CD4 cell number.

The mechanisms responsible for this loss of antigen-induced immunity have not been fully defined. However, a defect in the function of CD4+ helper T cells, which play a pivotal role in the induction of antigen-specific immune responses,

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² Dr. B. Frank Polk died on October 11, 1988. This article is dedicated to his memory.

has been implicated. Such a defect could be due to either decreased number or altered function of the CD4 cell population. Although a decrease in the number of CD4 lymphocytes is statistically associated with the development of AIDS (5), the range of CD4 numbers present at the time of development of AIDS is great, suggesting that functional as well as numerical changes in CD4 cells contribute to the onset of AIDS. Thus, Lane *et al.* (2) found that defective antigen-induced proliferation of CD4 lymphocytes from patients with AIDS persisted even after isolation of CD4 cells and adjustment *in vitro* to the same proportions of CD4 cells and autologous antigen-presenting cells as control subjects. In contrast, CD4 lymphocytes isolated from AIDS patients exhibited normal production of and responsiveness to interleukin-2 (IL-2) after mitogen stimulation. Giorgi *et al.* (6) reported that antigen-induced lymphocyte proliferation, when adjusted for the number of circulating CD4 cells, was normal in short-term seropositive subjects and most long-term seropositive subjects, and absent in the vast majority of patients with AIDS. Of note, three of four long-term seropositive subjects who had absent antigen-induced proliferation developed AIDS within 4 months of the study, strongly suggesting that an event critical to disease progression was associated with the loss of antigen-induced lymphoproliferative responses. Using flow cytometry, Prince *et al.* (7) demonstrated decreased expression of interleukin-2 receptors (IL-2R) by individual T cells after antigenic stimulation of peripheral blood mononuclear cells *in vitro*. Maggi *et al.* (8) reported decreased production of IL-2 by cloned T cells from patients with AIDS as opposed to those from healthy donors.

Taken together, these data support the hypothesis that defective production of and/or responsiveness to IL-2 by individual CD4⁺ cells after antigenic stimulation play an important role in AIDS pathogenesis. The present study was therefore undertaken to determine whether the number and/or proliferative capacity of circulating IL-2 responsive cells differ in patients with AIDS as compared to those in earlier stages of HIV-1 infection.

MATERIALS AND METHODS

Patients. Patients were seen at the Moore Clinic of The Johns Hopkins Hospital from February to April, 1986 and were classified as having no symptoms, AIDS-related complex, or AIDS according to the CDC criteria (9). Peripheral blood was drawn during routine clinic visits. Control subjects were Johns Hopkins students and employees who denied belonging to HIV risk groups. Control and HIV-1 seropositive subjects were matched for age and sex. Informed consent was obtained from all study participants according to a protocol approved by the Johns Hopkins Joint Committee for Clinical Investigation.

Limiting dilution analysis (LDA) of IL-2 responsive cells. LDA of lymphocyte proliferation was used to estimate the proportion of IL-2R-bearing circulating T lymphocytes. Assays were performed in U-bottom 96-well plates (Linbro). Peripheral blood mononuclear leukocytes (PBML), obtained from Ficoll-Hypaque separated peripheral blood, were serially diluted in complete medium (CM) consisting of RPMI 1640, 10% heat-inactivated human AB serum, 2 mM L-glutamine,

10 mM Hepes buffer, 50 μ g/ml gentamicin, 100 U/ml penicillin, and 5×10^{-5} M 2-mercaptoethanol. CM for experimental wells was supplemented with 25% MLA-144 supernatant (12.5 units/ml of IL-2 final concentration). MLA-144, a gibbon lymphoma line which constitutively secretes IL-2 (10) but not IL-1 or interferons (H. Rabin, personal communication), was also grown in CM. Under the assay conditions used for these studies, MLA-144 supernatant-induced proliferation of human PBML could be completely abrogated by the addition of monoclonal antibody to the IL-2 receptor (anti-CD25 antibody, 50 ng/ml). Our preparation of MLA-144 supernatant also failed to sustain hematopoietic colony growth of human bone marrow.

Approximate log-linearity of the assay in the absence of a constant concentration of feeder cells was confirmed in the first two experiments, in which we assessed 16 serial twofold dilutions ranging from 80,000 to 2.45 cells/well, with 12 replicates/dilution. In subsequent experiments, we used 8 fourfold dilutions ranging from 80,000 to 4.9 cells/well. Control cultures without IL-2 were plated in sextuplicate at each cell dilution. Cultures were incubated at 37°C, 5% CO₂ humid atmosphere. On Day 6 each well received 0.5 μ Ci of methyl-tritiated thymidine and was harvested 4 hr later onto glass fiber filters (Whatman GF/A) with a multiple automatic sample harvester (Mini-Mash II, Whittaker M.A. Bioproducts, Walkersville, MA). The thymidine uptake was measured by liquid scintillation spectrometry. Experimental wells were scored as positive or negative by comparison to CPM incorporated in control cultures (without IL-2) assayed at the same cell concentration. A well was considered positive if it exceeded the greater of the geometric mean control counts per minute (CPM) plus three standard deviations or 500 cpm.

The frequency of IL-2 responsive cells was estimated by a two-step procedure (11) which was based on the method of Taswell (12). Briefly, a preliminary estimate was made by determining the slope of the least squares line of best fit of the log fraction negative wells versus the responder cell concentration. The frequency was then determined by the maximum likelihood approach using the least squares estimate as an initial value. Net cpm per well was obtained by subtracting background cpm from experimental cpm, where background cpm represents an *N* weighted mean of control cpm (*N* = 6) and cpm obtained in negative experimental wells. Precursors per positive well were calculated from the LDA frequency estimate and approaches unity at limiting dilution. Net counts per minute per precursor (net cpmp) were calculated at each cell concentration by dividing the net cpm in each positive well by the average number of precursors per positive well.

Statistics. Statistical analysis was performed on a personal computer (PCs Limited 286, Austin, TX) using SYSTAT Data, MGLH, Stats, and Graph modules (SYSTAT Inc., Evanston, IL). The parameters net cpm, net cpmp, and precursor frequency were approximately log normal in distribution as determined by probability plots, and were log transformed prior to analysis. A limiting dilution analysis was performed using software developed in-house with the assistance of Dr. Ronald Brookmeyer, Johns Hopkins University School of Public Health, Department of Biostatistics.

RESULTS

The frequency of IL-2 responsive PBML was assayed concurrently in 10 seronegative nonrisk group members and a total of 20 HIV-1 seropositive subjects (7 asymptomatic, 5 with ARC, and 8 with AIDS). All subjects were tested on a single occasion. All demonstrated IL-2 responsive cells as indicated by significant tritiated thymidine uptake compared to control cultures incubated without IL-2. However, there was a progressive decline in both the frequency of IL-2 responsive cells at time 0 (precursors) and the magnitude of thymidine uptake by proliferating lymphocytes on Day 6 (net cpm/well) in relation to the clinical stage of HIV-1 infection (Fig. 1). Precursor frequency and net thymidine uptake were well correlated over the entire study population ($r = 0.893$, $P < 0.001$) and within each of the HIV-1 seropositive clinical groups ($r = 0.945$, $P = 0.001$ for asymptomatic; $r = 0.936$, $P = 0.019$ for ARC; and $r = 0.883$, $P = 0.004$ for AIDS), but not in the seronegative control group ($r = 0.133$, $P = 0.741$).

Thymidine uptake at 8×10^4 cells/well and LDA estimates of precursor frequencies of IL-2 responsive PBML were compared between clinical groups by ANOVA. Although both assays detected the same overall trend (Fig. 1), LDA was more sensitive for detecting differences among the clinical groups (Table 1). Specifically, precursor frequencies of control subjects differed significantly from those of all HIV-1 seropositive subject groups. In contrast, the measurement of thymidine uptake alone revealed no significant differences between seronegative subjects and HIV-1 seropositive subjects who either were asymptomatic or had ARC. Highly significant differences between control subjects and AIDS patients were detected by both methods ($P < 0.001$).

The above analyses suggested a quantitative change in circulating IL-2 responsive cells as a function of disease progression. In order to determine whether these cells also undergo a qualitative change in IL-2 responsiveness (i.e., a decrease in proliferative capacity), we estimated the magnitude of thymidine uptake on a per

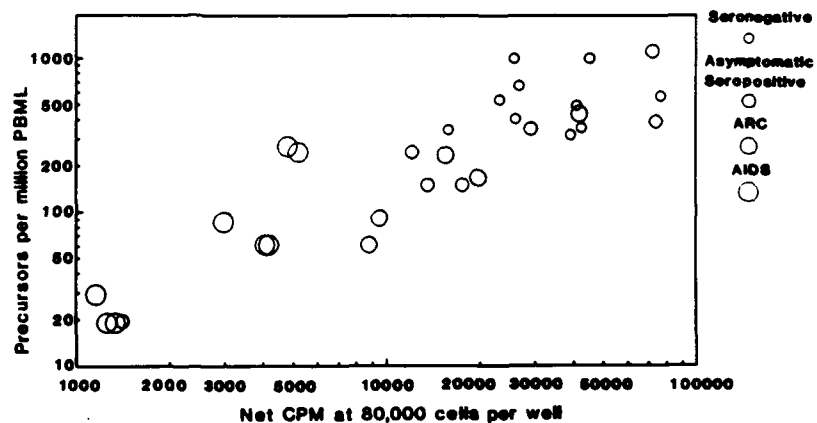


FIG. 1. Correlation between LDA frequency of IL-2 responsive PBML and thymidine uptake per well (8×10^4 cells/well). Symbol size reflects the stage of HIV-1 disease with the smallest circles representing responses of seronegative controls and the largest indicating those of AIDS patients.

TABLE 1
THYMIDINE UPTAKE PER WELL AND PRECURSOR FREQUENCY OF IL-2 RESPONSIVE PERIPHERAL
BLOOD MONONUCLEAR CELLS BY CLINICAL GROUP

Group	N	Net cpm ₈₀ ^a	Precursor frequency ^b
Seronegative	10	33.3 (22.9, 48.5)	524 (347, 790)
Seropositive	7	18.2 (11.6, 28.5)	206 (126, 336)*
Asymptomatic			
ARC	5	16.2 (9.5, 27.4)	157 (88, 280)**
AIDS	8	2.7 (1.8, 4.0)***	62 (39, 98)***

Note. Asterisks indicate significance of differences between HIV-1 positive groups and the seronegative subjects: * $P < 0.05$; ** $P < 0.025$; *** $P < 0.001$.

^a Geometric mean net cpm in thousands, measured at the highest cell concentration tested (80,000 cells/well). Parentheses indicate lower and upper 95% confidence intervals about the mean as determined by ANOVA.

^b Proportion of PBML responsive to IL-2 in the absence of antigen expressed as number of responsive cells per million PBML as measured by limiting dilution analysis. Parentheses indicate lower and upper 95% confidence intervals.

precursor basis. For each subject, the average number of IL-2 responsive cells plated at each dilution was calculated from the LDA frequency estimate as described above. The net cpm/precursor (net cpmp) were then computed for each cell dilution by dividing the net cpm in positive wells by the estimated number of precursors plated per positive well. The results of this analysis are shown in Fig. 2. Net cpmp were dependent on culture density, but at any given density was indistinguishable among controls, seropositive asymptomatics, and individuals with ARC (Fig. 2; $P = 0.280$ by ANOVA). In contrast, PBML from AIDS patients had significantly lower net cpmp than members of the other groups ($P < 0.001$).

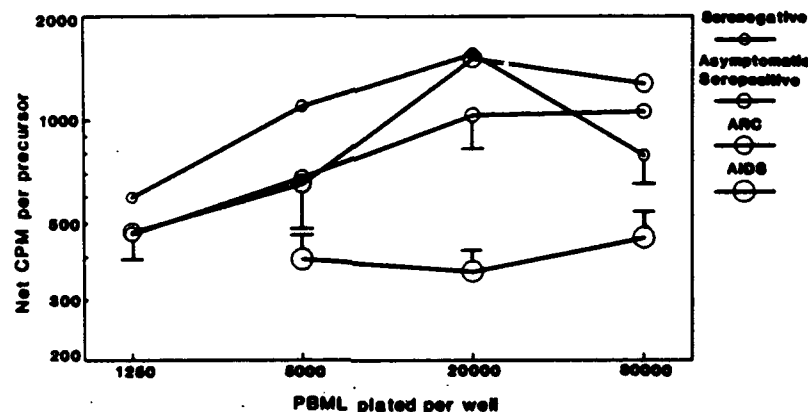


FIG. 2. Thymidine uptake (net cpm) per IL-2 responsive precursor expressed as a function of the number of PBML plated per well. Net cpm per precursor were calculated as described under Materials and Methods. Symbol size indicates disease stage as indicated in Fig. 1.

In addition, net cpm did not increase significantly as a function of cell density in the AIDS group as it did in all of the other groups.

The failure to detect a relationship between cell concentration and net cpm in AIDS PBML does not reflect a dose-response curve shift, since the difference in mean precursor frequencies between ARC and AIDS patients is only 6-fold (Table 1). A minimum of two 4-fold higher dilution steps (16-fold) would be required to bring the results observed using AIDS PBML into register with those obtained in the other clinical groups (Fig. 2).

DISCUSSION

Proliferation of activated T cells requires the growth factor IL-2 and the expression of specific IL-2R. Since IL-2R are not expressed on resting T cells, the proportion of cells which proliferate *in vitro* in the presence of IL-2 without exogenous activation signals (i.e., antigen, alloantigen, or mitogen) reflects the number of T cells bearing functional IL-2R *in vivo*. With this in mind, we quantified IL-2 responsive cells by LDA to determine the number of *in vivo*-activated T cells in the peripheral circulation of subjects in various stages of HIV-1 infections as well as in seronegative controls.

Two types of differences in T cell responsiveness to IL-2 were present in the HIV-1-infected individuals as compared to the seronegative controls. First, apparent frequencies of IL-2 responsive cells declined progressively according to the stage of HIV-1 infection. Second, the IL-2 responsive T cells from subjects with AIDS also exhibited a decrease in their capacity to proliferate in response to IL-2. This decrease was not observed in the other seropositive subjects. These observations suggest that while decreases in the number of activated (IL-2 responsive) cells may occur *in vivo* in the "early" stages of HIV-1 infection, a further decrease in the proliferative capacity of the surviving IL-2 responsive cells is associated with the onset of frank AIDS. These results extend our previous findings and those of other investigators who have shown decreased T cell colony formation (1, 13) and cloning efficiency (8, 14) in association with AIDS.

Several mechanisms could explain the decreased responsiveness observed in the present study, reflecting either the history of the activated cell *in vivo* or subsequent events occurring during the 6-day period of *in vitro* cultivation. Possible inhibitory events occurring *in vivo* include decreased accessory cell function (15, 16), exposure to inhibitory serum factors (17, 18), HIV-1 (19-21), or peptides with homology to HIV-1 (22). Alternatively, the development of intrinsic defects in T cell activation and/or differentiation could result in the decreased expression of IL-2R (7). Finally, the observed results could be explained by the selective depletion in AIDS of subpopulations that are capable of proliferating vigorously in response to IL-2, resulting in a higher proportion of cells which respond with slow kinetics.

Possible factors that could act *in vitro* to contribute to the observed decrease in proliferative capacity include abnormal regulation of IL-2R number and function (7, 23, 24), elaboration of suppressor or cytotoxic factors (25) which could inhibit IL-2 production and receptor function (18, 26), or induction of latent HIV-1 gene products concomitant to IL-2-induced proliferation (27). A role for accessory cells

in vitro is unlikely in view of the one-hit limiting dilution curves obtained and the fact that IL-2-induced proliferation of IL-2R-bearing lymphocytes is independent of accessory cells.

In addition to identifying an AIDS-related functional defect in *in vivo*-activated T cells, this study quantified these cells *in vitro* by functional criteria. The decline in the number of *in vivo*-activated cells as a function of disease stage may indicate a progressive reduction in the baseline activity of the immune system. When this reduction reaches a critical level, other qualitative changes related to the onset of AIDS may occur as well. Thus the incapacitation of the immune system that occurs when AIDS supervenes may reflect a combination of qualitative as well as quantitative changes in lymphocyte function. In addition, the observed decrease in circulating activated T cells with an increasing stage of HIV-1 infection is consistent with the interpretation that activated T cells are not the primary reservoir of HIV-1 infection *in vivo*.

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Allogeneic Bone Marrow Transplantation, Zidovudine, and Human Immunodeficiency Virus Type 1 (HIV-1) Infection

Studies in a Patient with Non-Hodgkin Lymphoma

H. Kent Holland, MD; Rein Sarai, MD; John J. Rossi, PhD; Albert D. Donnenberg, PhD; William H. Burns, MD; William E. Beschoner, MD; Homayoon Farzadegan, PhD; Richard J. Jones, MD; Gerry V. Quinnan, MD; Georgia B. Vogelsang, MD; Huibert M. Vriessendorp, MD; John R. Wingard, MD; John A. Zaia, MD; and George W. Santos, MD

Human immunodeficiency virus type 1 (HIV-1)-infected patients with non-Hodgkin lymphoma are classified as having the acquired immunodeficiency syndrome (AIDS). Allogeneic bone marrow transplantation is a successful therapy for patients with lymphoma who have a poor prognosis. Combined therapy with allogeneic bone marrow transplantation and the antiviral drug zidovudine has the potential advantage of protecting the new donor hematopoietic-lymphoid and monocyte-macrophage cells from HIV-1 infection. A 41-year-old man infected with HIV-1 who had lymphoma was treated with high-dose cyclophosphamide and total body irradiation followed by allogeneic bone marrow transplantation. Before transplantation he received high-dose zidovudine for 2 weeks (5 mg/kg body weight intravenously every 4 hours) and after transplantation he received a lower maintenance dose (1.33 mg/kg body weight intravenously every 4 hours). No untoward toxicities attributable to zidovudine were observed. Bone marrow engraftment occurred on day 17. Chromosome and restriction fragment length polymorphism analyses demonstrated complete chimerism. Peripheral blood mononuclear cells and bone marrow samples were negative for HIV-1 by culture and polymerase chain reaction gene amplification 32 days after transplantation. The patient died 47 days after transplantation because of tumor relapse. Analysis of autopsy tissue showed no evidence of HIV-1 by either culture (brain, bone marrow, lymph node, and tumor specimens) or by polymerase chain reaction gene amplification for HIV-1 RNA and DNA sequences (brain, bone marrow, heart, kidney, liver, lung, rectosigmoid, spleen, and tumor specimens). Immunologic monitoring showed loss of HIV-1 antibody. Adoptive immunologic transfer was shown to be present to both tetanus and diphtheria antigens. Our case suggests that the HIV-1-infected recipient cells may have been eradicated secondary to the bone marrow ablative chemo-radiotherapy and that zidovudine may be able to prevent the establishment of HIV-1 infection in donor hematopoietic-lymphoid cells.

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From The Johns Hopkins School of Medicine, Baltimore, Maryland; the City of Hope National Medical Center, Duarte, California; and the Food and Drug Administration, Bethesda, Maryland. For current author addresses, see end of text.

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that infects CD4+ T-cell lymphocytes (1-6) and other bone marrow-derived cells including monocyte-macrophages (7), Langerhan-dendritic cells (8), microglial cells (9), and B-lymphocytes transformed by Epstein-Barr virus (10, 11). Infection with HIV-1 can ultimately result in the acquired immunodeficiency syndrome (AIDS).

Restoration of the cellular immune system in the setting of HIV-1 infection has been attempted. Bone marrow and lymphocyte infusions in patients infected with HIV-1 have resulted in transient improvement of some in-vitro immune parameters, but no significant clinical benefit or effects in patients with HIV-1 infection have been shown (12-14). Allogeneic and syngeneic bone marrow transplantation have also been evaluated but fail to prevent HIV-1 from infecting the donor marrow graft (15-18). Zidovudine, an inhibitor of the retroviral reverse transcriptase process, is able to inhibit HIV-1 replication and prevent the establishment of retroviral infection of uninfected CD4+ lymphocytes (19).

We report the results of a combined modality approach using allogeneic bone marrow transplantation and zidovudine in a patient infected with HIV-1 who had non-Hodgkin lymphoma (20-22). We used a bone marrow ablative regimen (cyclophosphamide and total body irradiation) to eliminate both lymphoma cells and HIV-1-containing hematopoietic-lymphoid cells and replaced these cells with bone marrow derived from the patient's histocompatibility locus antigen (HLA)-identical donor. The patient was maintained on zidovudine in an attempt to prevent donor-derived hematopoietic-lymphoid cells from becoming infected by HIV-1.

Case History

A 41-year-old man was diagnosed as having HIV-1 infection and malignant, small non-cleaved undifferentiated cell lymphoma (23). He presented with a left axillary mass measuring 20 × 25 cm and a mass in the liver measuring 7 × 7 cm (biopsy positive for lymphoma). Cytologic analysis of the cerebrospinal fluid was positive for tumor cells; bone marrow aspirate was nondiagnostic for lymphoma. A computed tomographic scan of the head was normal. He had a neurologic examination that was normal and a Karnofsky

Table 1. Detection of Human Immunodeficiency Virus-Type 1 (HIV-1) in Peripheral Blood Mononuclear Cells and Bone Marrow before and after Bone Marrow Transplantation Using HIV-1 Culture and HIV-1 RNA Polymerase Chain Reaction Gene Amplification*

Method of HIV-1 Detection	Day 24 before Transplantation	After Transplantation						Autopsy Tissue
		Day 20	Day 25	Day 32	Day 41	Day 45	Day 46	
HIV-1 culture								
Peripheral blood mononuclear cells or bone marrow	++	-	++		-		-	-
HIV-1 RNA PCR								
Peripheral blood mononuclear cells	++			-				-
Bone marrow			++	+		-		-

* PCR = polymerase chain reaction. ++, positive sample; +, trace positive; -, negative sample. CD4 RNA expression was detected in all polymerase chain reaction gene amplification samples.

performance score of 100. He had no history of opportunistic infection. Phenotypic lymphocyte studies showed an absolute T4-cell count of $0.116 \times 10^9/L$. After chemotherapy with methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, and decadron; and intrathecal chemotherapy with cytosine arabinoside and methotrexate, no tumor was detected except for a 1-cm left axillary node (24). After being advised of the potential risks and benefits of marrow transplantation and administration of zidovudine, he gave informed consent in accordance with the guidelines of the Joint Committee of Clinical Investigation of The Johns Hopkins University School of Medicine.

Zidovudine was begun before bone marrow transplantation (day -14 through day -1) at a dose of 5 mg/kg body weight intravenously every 4 hours (given over 1 hour), which was reduced at the time of marrow infusion (day 0) to 1.33 mg/kg body weight intravenously every 4 hours. Zidovudine was given as scheduled except on day 12 when the dosage was reduced for 24 hours by 50% because of transient azotemia secondary to nephrotoxic drugs (cyclosporin, aminoglycosides, and amphotericin) and on day 44 when two doses were not given. The preparative regimen for transplantation included cyclophosphamide, 50 mg/kg daily given intravenously on day -9 through day -5, and total body irradiation, 300 Gy per day on day -4 through day -1 with lung shielding on day -2 (25). On day 0 the patient received 3.7×10^6 nucleated cells/kg of unmanipulated donor marrow harvested from his HLA-identical ABO-compatible sister. Cyclosporin was administered as prophylaxis for graft-versus-host disease, as previously reported (26, 27). Prophylactic acyclovir was administered at a dose of 500 mg/m² body surface area intravenously every 8 hours beginning on day -4 and was continued throughout the hospitalization.

Although the patient developed profound neutropenia (neutrophil count, less than $0.5 \times 10^9/L$) and required empiric antibiotic therapy for fever (temperature greater than 38.3 °C), no documented bacterial, fungal, protozoal, or viral infection developed. Mild, stage I graft-versus-host disease was noted after transplantation. On day 17 the absolute neutrophil count rose above $0.5 \times 10^9/L$. Engraftment studies of peripheral blood cells (day 17) using restriction fragment length polymorphism analysis demonstrated complete chimerism of donor cell origin. (detection

sensitivity, 99.9%) (28, 29). A chromosome analysis of bone marrow done on day 31 (two metaphases observed; $n = 46, XX$) was also consistent with donor origin. Platelet counts from days 20 to 40 ranged from $17 \times 10^9/L$ to $47 \times 10^9/L$. A reticulocyte count greater than 2% was not achieved.

The patient continued to improve, but a biopsy sample from a palpable left axillary node obtained on day 39 showed recurrent lymphoma of recipient origin, as determined by restriction fragment length polymorphism analysis. The patient refused further therapy, and all treatment support was discontinued except for the intravenous zidovudine. He died on day 47 from respiratory failure. An autopsy was done. The gross examination was remarkable for a tumor mass in the left axilla measuring $5 \times 4 \times 4$ cm. There were no other areas of gross tumor involvement or lymphadenopathy, and the brain appeared normal. Bacterial, fungal, and routine viral cultures of the liver, spleen, kidney, and lung were all negative. There was no histologic evidence for fungal, viral, mycobacterial, or *Pneumocystis carinii* infection. Immunoperoxidase staining of fresh frozen tumor tissue showed strong staining for Leu 12, B1, IgM, and lambda light chains consistent with B-cell lymphoma (30). Microscopic foci of tumor were also observed in spleen, liver, and kidney tissue, but tumor was not observed in the brain. Graft-versus-host disease was not observed (27).

Methods

HIV-1 Cultures

Peripheral blood mononuclear cells and autopsy tissues were cocultivated for 4 to 6 weeks with phytohemagglutinin-stimulated lymphocytes from a person who was not infected with HIV-1. Presence of HIV-1 was determined by a p24 antigen-capture assay and by reverse transcriptase assays of culture supernatants (1, 2, 31).

HIV-1 p24 Antigen-Capture Assay

Abbott (North Chicago, Illinois) and Du Pont (Wilmington, Delaware) HIV-1 p24 antigen-detection kits were used, and assays were done as described by the manufacturer.

HIV-1 Western Blot

Using the Biotech Western immunoblot kit (Rockville, Maryland) and the manufacturer's procedure, we did the

Western blots batch-wise (32, 33). Antibody to specific HIV proteins (p17, p31, gp41) were quantified by image analysis of immunoblot banding patterns and were expressed as relative area.

Drug Levels of Zidovudine

Peak drug levels of zidovudine were assessed using serum and Omayya reservoir cerebrospinal fluid samples (drawn 10 minutes after the 1 hour of continuous infusion of zidovudine was completed); trough levels (15 minutes before the next zidovudine dose) were also determined. Zidovudine was assayed as described previously (34).

Polymerase Chain Reaction Gene Amplification

The polymerase chain reaction (Perkin-Elmer-Cetus, Norwalk, Connecticut) analyses for detecting HIV-1 DNA and RNA LTR, 3'ORF, *gag*, and *env* sequences were done as previously described (35), but modified for divalent cation concentration optimal for *Taq* I polymerase (36). More specific details are provided in the Appendix.

Immunologic Assessment

Serum IgG was measured by nephelometry. Tetanus and diphtheria toxoid-specific IgG was measured by enzyme-linked immunosorbent assay (ELISA) (37). Antigen-specific in-vitro lymphoproliferative responses were assessed by thymidine uptake (37). Lymphocyte CD3, CD4, and CD8 determinations were done using flow cytometry and commercially available reagents (Becton Dickinson, Mountain View, California).

Autopsy

A complete examination after death was done in a sterile isolation room following recommended procedures (38). Cultures for HIV-1 were done on fresh brain, lymph node, bone marrow, and tumor. Tissue specimens for polymerase chain reaction gene amplification analysis were immediately frozen to -70°C and included coronal brain sections (including basal ganglia) and samples of bone marrow, heart, liver, lung, lymph nodes (all nodes were atrophic and without evidence of gross tumor), rectosigmoid, spleen, and tumor.

Results

Zidovudine Pharmacokinetics and HIV-1 Detection

The patient received a zidovudine dose of 5 mg/kg body weight every 4 hours intravenously before transplantation and a dose of 1.33 mg/kg body weight every 4 hours intravenously after transplantation. Peak and trough serum levels of zidovudine before transplantation were 5.44 and 0.988 $\mu\text{mol/L}$, respectively. Peak and trough cerebrospinal fluid levels of zidovudine before transplantation were 2.84 and 0.921 $\mu\text{mol/L}$, respectively. Peak and trough serum levels of zidovudine after transplantation were 2.64 and 0.588 $\mu\text{mol/L}$, respectively. Peak and trough cerebrospinal fluid levels of zidovudine after transplantation were 0.958 and 0.715 $\mu\text{mol/L}$, respectively.

The HIV-1 p24 antigen assays of the patient's serum were done before transplantation and weekly thereafter. All samples were negative for p24 antigen. Cere-

brospinal fluid samples tested on day -5 and day $+24$ were negative.

A culture of peripheral blood mononuclear cells was HIV-1 positive 24 days before bone marrow transplantation (Table 1). After transplantation, a culture of peripheral blood mononuclear cells was positive on day 25; however, cultures were negative 20, 41, and 46 days after transplantation. Autopsy tissue cultures of bone marrow, brain, lymph nodes, and tumor were all negative for HIV-1. Cultures of cerebrospinal fluid samples obtained 5 days before and 12 days after transplantation were negative for HIV-1.

Polymerase chain reaction gene amplification analysis for HIV-1 RNA was done using 5'LTR primers on peripheral blood mononuclear cells, tumor and bone marrow specimens, and autopsy tissue. The HIV-1 RNA sequences were detected using polymerase chain reaction gene amplification in peripheral blood mono-



Figure 1. The HIV-1 RNA polymerase chain reaction gene amplification probes on peripheral blood and bone marrow before and after allogeneic bone marrow transplantation. A sample of peripheral blood mononuclear cells isolated 24 days before transplantation was positive (Lane A). Samples of bone marrow isolated 25 days (Lane B) and 32 days (Lane C) after transplantation were positive. A sample of bone marrow isolated 45 days after transplantation was negative (Lane D). Control samples of HIV-1-infected H9 cells (Lane E) and uninfected H9 cells (Lane F) were positive and negative, respectively.

nuclear cells isolated 24 days before transplantation but were not detected in a sample isolated 32 days after transplantation (Table 1). The HIV-1 RNA sequences were similarly detected in bone marrow specimens obtained on days 25 and 32 after transplantation but could not be detected in a marrow sample from day 45. (Figure 1; Table 1). The CD4 RNA receptor sequences were detected in all samples of peripheral blood mononuclear cells and bone marrow. The HIV-1 RNA sequences were not detected in any autopsy

tissue specimens (brain, heart, kidney, liver, lung, lymph node, rectosigmoid, spleen, and tumor) (Figure 2). The CD4, beta-actin, and T-cell receptor RNA sequences, however, were detected in all autopsy specimens except the lymph node samples, which were markedly atrophic (a frequent finding after bone marrow transplantation). Peripheral blood mononuclear cells from the donor were negative for HIV-1 RNA.

Polymerase chain reaction gene amplification analyses for HIV-1 DNA *env*, *gag*, and 5'LTR primers were done for autopsy specimens from brain, heart, kidney, liver, lung, lymph node, spleen, rectum, and tumor. Under the highest stringency conditions for filter washing, only the positive control (HIV+ H9 cells) signal remained (Figure 3), and all tissue samples were negative. Under lowered stringency conditions for filter washing, a positive *gag*-specific signal was present in the sequences amplified from the rectosigmoid DNA (data not shown). The rectosigmoid tissue was negative using primers for 5'LTR, 3'ORF, and *env* sequences. As a control for the integrity of the DNA samples, CD4 primers were used and all samples were positive (Figure 3).

Immune Reconstitution

Before bone marrow transplantation, total plasma IgG was elevated, and antibody to HIV-1 *env*, *gag*, and *pol* gene products was detected by Western blot. Although the patient had an immunization history, antibody titers to tetanus and diphtheria (1/130 and 1/360, respectively) were approximately tenfold lower than control reference values. In-vitro lymphoproliferative responses to tetanus and diphtheria were absent, but the patient retained the ability to mount a proliferative response to alloantigen in mixed lymphocyte culture (Figure 4).

The marrow donor's tetanus- and diphtheria-specific lymphoproliferative responses and antibody titers were within normal limits (not shown). To facilitate adoptive transfer of donor immunity, the donor was boosted with tetanus and diphtheria 8 days before marrow harvest (37). The recipient was immunized with tetanus toxoid immediately after marrow infusion. Lymphocytes were first detected in the periphery on week 3 and were present in numbers sufficient for analysis by week 4. The relative number of CD4+ (helper-inducer) T cells remained low at this time. Total serum IgG declined by approximately half but remained within normal limits; HIV-specific IgG declined in a manner consistent with loss of recipient cell-derived antibody. In contrast, the tetanus- and diphtheria-specific antibody levels rose transiently to more than four times the trough values and were maintained above (tetanus) or at (diphtheria) pretransplant levels. More important, significant lymphoproliferative responses to both antigens were detected 5 weeks after transplantation.

Discussion

Allogeneic bone marrow transplantation is an effective therapy for patients with lymphoma who have a poor

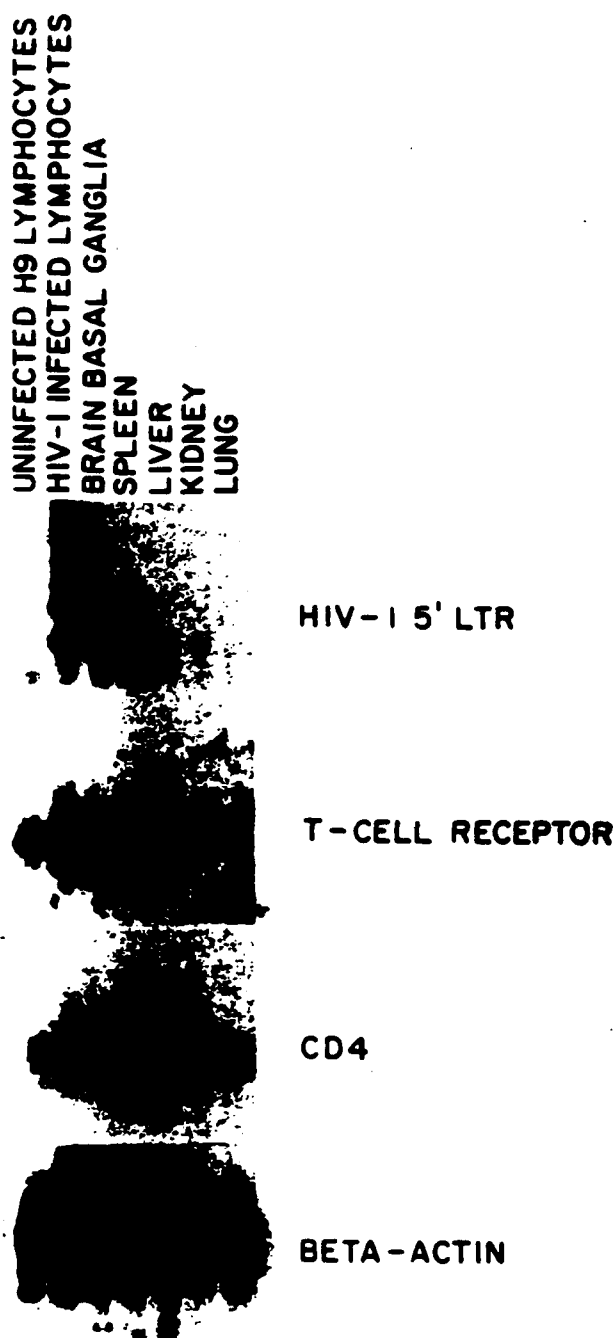


Figure 2. The HIV-1 RNA polymerase chain reaction gene amplification done on autopsy tissue. The results of four probes are shown: an HIV-1 RNA probe for 5'LTR sequences, a T-cell receptor RNA probe, a CD4 RNA probe, and beta-actin RNA probe. All tissues are negative for HIV-1 RNA, whereas all control standards for endogenous sequences are positive.

prognosis (25% to 50% long-term survival rate) (39, 40). Persons with HIV-1 infection are at risk for developing lymphoma and have a poor prognosis despite responses to chemotherapy (20, 22, 41). Therefore, many of these patients are eligible for allogeneic bone marrow transplantation, although their tolerance to the intensive preparative therapy administered before transplantation and their recovery from infection and the other complications that often follow transplantation are of concern. Furthermore, despite intensive chemotherapy and irradiation therapy, a reservoir of cells containing HIV-1 might persist after transplantation, leading to the infection of donor cells. Previous experience with allogeneic bone marrow transplantation has shown that immunocompromised patients who are free of active infection can tolerate the intensive therapy (25, 42). The preparative regimens used for allogeneic bone marrow transplantation do eradicate recipient lympho-hematopoietic cells and cells of monocyte-macrophage lineage; follow-up studies have shown complete chimerism (donor cells) in these cells from long-term survivors of bone marrow transplantation (25, 43-46). Although there may be a transient appearance of recipient cells early after transplantation, long-term, mixed chimerism (donor and recipient cells) has rarely occurred in our population of patients who had bone marrow transplantation.

Although HIV-1-infected cells derived from recipient bone marrow can be eradicated by ablative bone marrow transplantation therapy, donor-derived bone marrow cells remain susceptible to HIV-1 infection after transplantation. In-vitro evidence indicates that zidovudine is able to prevent CD4⁺ cells from being permissively infected by HIV-1, and feline and murine retroviral models have shown that zidovudine inhibits retroviral infection of the host's hematopoietic cells if the drug is administered at the time of initial viral exposure (47-49). These data suggest that donor marrow cells may be protected from HIV-1 infection in vivo by zidovudine. The known marrow-suppressive effects of this compound, however, might adversely affect engraftment of donor marrow. In-vitro studies have shown a colony forming unit-granulocyte macrophage (CFU-GM) fraction survival of $45\% \pm 10\%$ (mean $\pm$ standard deviation) when normal bone marrow was grown in the presence of $10 \mu\text{mol/L}$ zidovudine (Jones RJ. Unpublished data.), which suggests that bone marrow engraftment should not be prevented. In addition, administration of zidovudine in an animal allogeneic bone marrow transplantation model did not prevent bone marrow engraftment (Holland HK. Unpublished data.) We postulated that the combination of bone marrow ablative chemo-radiotherapy, allogeneic bone marrow transplantation, and the coadministration of zidovudine would eradicate recipient cells containing HIV-1 and protect donor cells from infection with this virus.

As the clinical and laboratory results from this case show, the patient did tolerate the intensive therapy associated with bone marrow transplantation. His death resulted from tumor relapse and not from untoward toxicity associated with the transplant process.

In addition, the administration of intravenous zidovudine at a dose schedule that maintained serum levels significantly above the known in-vitro HIV-1 inhibitory dose concentration of zidovudine did not inhibit bone marrow engraftment (19). The kinetics of marrow engraftment in this patient was similar to that observed in patients who had allogeneic bone marrow transplantation without zidovudine therapy (50).

Our studies suggest that the therapeutic approach we used may have eradicated the HIV-1 reservoir of infected recipient cells by 6 weeks after transplantation. We surmise that the infected cells detected within the first 5 weeks after transplantation were residual, nonproliferating recipient cells that were lethally damaged but had not yet been cleared from the body (although analysis by restriction fragment length polymorphism was consistent with only donor cells, polymerase chain reaction gene amplification is several magnitudes more sensitive in detection) (35, 36, 51). The inability to detect HIV-1 after day 32 by culture or polymerase chain reaction gene amplification strongly suggests the loss of recipient cells harboring HIV-1, as the likelihood that HIV-1 was spontaneously cleared from the viable donor cells is nil (52). The only traces of HIV-1 detected after day 32 were *gag* sequences present in autopsy rectum tissue and only under low stringency conditions. It has been suggested that HIV-1 infection cannot be confirmed by polymerase chain reaction unless assays with two or more primer pairs from different regions of the virus are

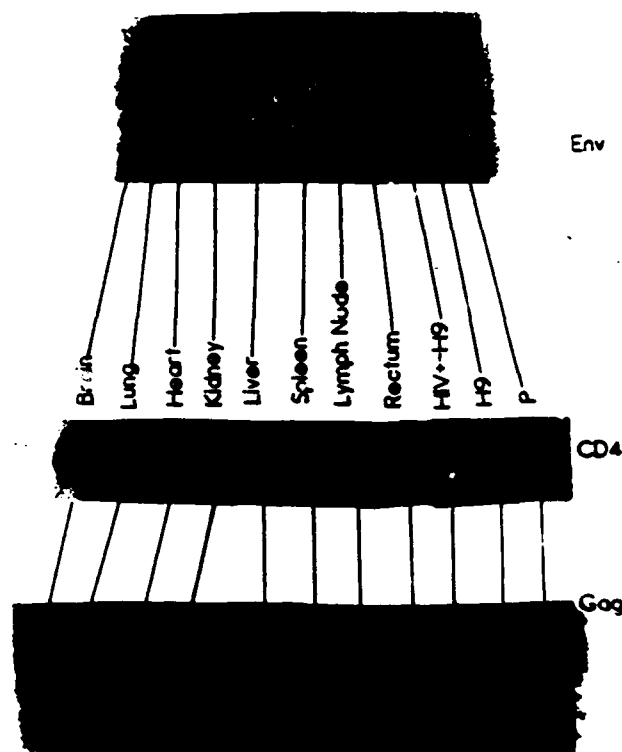


Figure 3. The HIV-1 DNA polymerase chain reaction gene amplification for *gag* and *env* probes (5'LTR data not shown) on autopsy tissue. The conditions for amplification are described in Methods. The autoradiographs are aligned to indicate the positions of each of the experimental samples. The DNA amplified from HIV-1-infected (HIV+ - H9) and uninfected (H9) cells and from primers alone (P) are shown as controls.

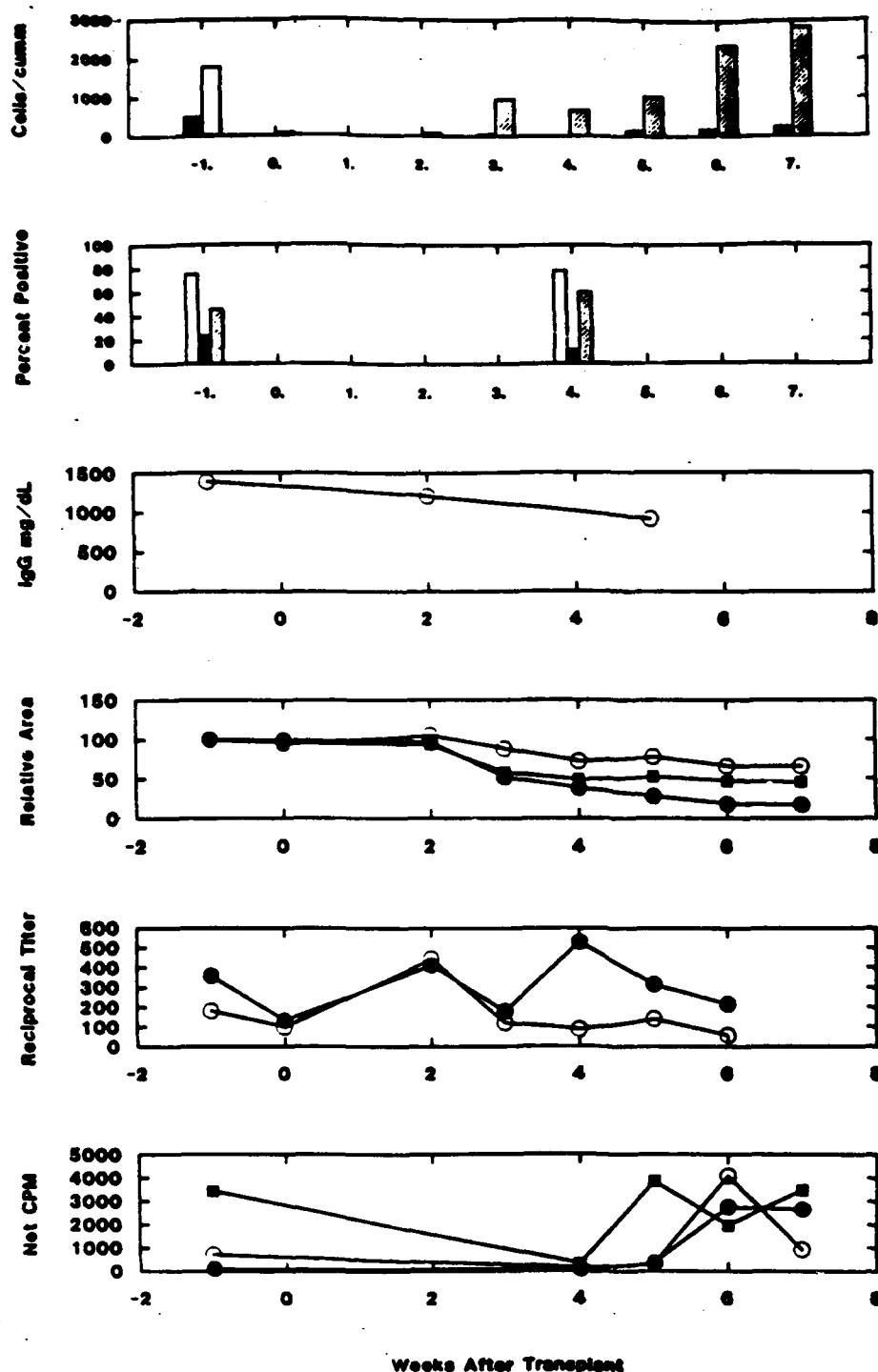


Figure 4. Immune reconstitution as a function of time after bone marrow transplantation. Top panel. Recovery of peripheral leukocytes (shaded bar) and lymphocytes (closed bar). Second panel. Immunophenotype of peripheral blood mononuclear cells. Open bars denote the CD3 phenotype; closed bars, the CD4 phenotype; and shaded bars, the CD8 phenotype. Third panel. Total serum IgG. Fourth panel. Change in HIV-1-specific serum antibodies relative to pretransplant levels. Antibodies to gp41 (closed squares), p31 (closed circles), and p17 (open circles) were quantified by densitometric evaluation of immunoblot banding patterns. Fifth panel. Serum reciprocal titers for antibody specific to tetanus (open circles) and diphtheria (closed circles). Bottom panel. In-vitro lymphoproliferation in response to irradiated allogeneic peripheral blood mononuclear cells (closed squares) and to tetanus (open circles), and diphtheria (closed circles) antigens. Responses were measured by tritiated thymidine uptake and are expressed as counts per minute above background (Net CPM).

positive (36). That only the *gag* sequence occurred in a single sample could be explained by cross-reactivity to a non-HIV-1 endogenous retrovirus sequence. If this is true, it is not clear why similar sequences were not detected in other tissues. It is more likely that this *gag* sequence was a remnant of a defective HIV-1 proviral sequence. It has been suggested that defective provirus might be present in specimens from patients with AIDS (53). The polymerase chain reaction assay is a highly sensitive method for detection of HIV-1 sequences (35, 36). It has been noted that the sensitivity of this assay increases the risk for false-positive

results, and this has been a major concern with the clinical application of the assay (54). The negative results with the great amount of clinical matter tested in this study suggests that false-positive results were not a problem. The question of false-negative results was addressed by the use of primers to endogenous cellular sequences. In addition, the determination of sensitivity by in-vitro methods in our laboratory and the detection of HIV-1 in specimens obtained before and early after transplantation suggest that sensitivity was not a problem. It has been suggested that RNA detection by polymerase chain reaction is potentially

more sensitive because of the likelihood for multiple RNA copies per cell and lower overall sequence complexity (36). For this reason, we included the RNA assay in our study. We do not have direct evidence, however, that latently infected cells would express HIV-1 RNA, although there is theoretical reason to believe that this might be possible (55). The use of RNA from frozen material may have diminished the sensitivity, but the ready ability of the assay to detect endogenous sequences suggests that this was not a problem.

The hypothesis that zidovudine would protect donor cells from HIV-1 infection is supported by the absence of detectable latent HIV-1 by culture or polymerase chain reaction assay after day 32 in either peripheral blood or bone marrow and by the absence of the virus in multiple organs at autopsy. The absence of HIV-1 detection in the autopsy specimens offers further insight into the cellular reservoir for HIV-1 and the effectiveness of zidovudine. Investigators have shown that central nervous system macrophages and microglial cells express CD4 receptor and can be infected by HIV-1 (56-58), whereas neurons have rarely been shown to be a reservoir for HIV-1 except in patients with AIDS who have terminal disease (59, 60). Hicky and Kimura (43) showed that perivascular microglial cells of the central nervous system are bone marrow-derived and replete the central nervous system within 2 months. Lymphocytes also migrate into the central nervous system. Alveolar macrophages (61), Langerhan-dendritic cells (62), and leukocytes from cervical secretions have been shown to harbor HIV-1, but all these cells would be replaced by donor-derived cells (63). Grataña and colleagues (64) have reported that latent Epstein-Barr virus can be eradicated in seropositive recipients of bone marrow transplantation who receive seronegative donor marrow. By using bone marrow ablative therapy and zidovudine treatment, the autopsy data suggest that these reservoirs were destroyed and reconstituted by donor-derived bone marrow cells that did not contain HIV-1 because of treatment with zidovudine.

Immunologic adoptive transfer for lymphoproliferative response to specific recall antigen and antibody production was observed in our patient. Before bone marrow transplantation, the patient had a blunted response to antigenic challenge, which would be expected in a subject with lymphoma or AIDS. After transplantation, the recipient developed an appropriate in-vitro lymphoproliferative and serum antibody response to these antigens. Similar responses have been observed in our other patients who had allogeneic bone marrow transplantation. In addition, Western blots in our patient showed a persistent decline of antibodies to all HIV-1 antigens, which implies an in-vivo loss of HIV-1 antigen expression.

The results observed in our patient have at least two potential implications. First, the therapeutic approach outlined in this paper deserves further study to determine whether this therapy has a role in treatment of HIV-1-infected patients with lymphoma. It is possible that the number of HIV-1 proviral DNA-harboring

cells was so low that it precluded detection by polymerase chain reaction. We will not know if this is true until patients can be followed for a longer period after bone marrow transplantation. Second, if additional studies evaluating a larger number of patients confirm our results, this approach may be applicable to a larger group of patients who are infected with HIV-1. Additional studies are necessary to determine the ultimate role of bone marrow transplantation and antiviral chemotherapy as a therapeutic modality in patients exposed to or infected with HIV-1 or other retroviruses.

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Requests for Reprints: Rein Saral, MD, Room 173, The Johns Hopkins Oncology Center, 600 North Wolfe Street, Baltimore, MD 21205.

Current Author Addresses: Drs. Holland, Saral, Donnemberg, Burns, Beschoner, Parzadegan, Jones, Vogelstein, Vriesendorp, Wingard, and Santos: The Johns Hopkins School of Medicine, 600 North Wolfe Street, Baltimore, MD 21205.

Dr. Rossi: Department of Molecular Genetics, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010.

Dr. Quinman: Center for Biologics Evaluation and Research, Food and Drug Administration, U.S. Public Health Service, Department of Health and Human Services, Bethesda, MD 20892.

Dr. Zain: Division of Pediatrics, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010.

Appendix

Polymerase Chain Reaction Gene Amplification

The analysis by polymerase chain reaction was done for HIV-1 LTR, 3'ORF, *gag*, and *env* as described previously (35), but modified for divalent cation concentration optimal for *Taq* I polymerase (36). Primers from *gag* (SK38 and SK39) and *env* (SK68 and SK69) were selected for detection of HIV-1 sequences as described previously (36).

RNA was purified according to the method of Chirgwin and colleagues (65) as modified for use with cesium chloride by Maniatis and colleagues (66). Total RNA, 0.3 to 1.0 µg, was mixed with 50 pmol of each polymerase chain reaction primer in 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.5 mM MgCl₂, 0.01% gelatin, 200 µM of each deoxynucleoside triphosphate in 48-µL final volume. This mixture was overlaid with 50 µL mineral oil and subjected to repeated rounds of amplification in a thermal cycler (Perkin-Elmer-Cetus, Norwalk, Connecticut). The mixture was heated to 92 °C and held for 5 min, cooled to 42 °C over a 3-min period, at which time 1 µL containing 2.0 U avian myeloblastosis virus reverse transcriptase was added; incubation then continued for an additional 3 min. One µL of *Taq* polymerase (2.5 U) (Perkin-Elmer-Cetus) was added and the cycling program linked to the following pattern: 94 °C, 2 min; 3-min cooling to 42 °C; an additional 3 min at 42 °C; rapid heating

to 72 °C and 3 min at 72 °C. Each successive polymerization round at 72 °C had an additional 10 s added to a total of 34 cycles. The DNA was extracted from tumor specimens and autopsy tissue using the phenol-chloroform-proteinase K method as described previously (66). Except for the addition of the reverse transcriptase, all of the cycling conditions for purification of DNA were identical to those described for RNA, and 0.5 to 1.0 µg ethanol-washed DNA was used in each assay. All specimens were prepared in a building separate from the polymerase chain reaction laboratory in a room free of cloned HIV-1 sequences. Reconstruction experiments using plasmid or in-vitro prepared RNA mixed with total cellular DNA or RNA have shown that the sensitivity of these assays in our laboratory is approximately 100 molecules (6.023×10^{-21} mol) RNA (35) and 10 to 100 molecules (approximately 10^{-21} mol) DNA.

Filter Hybridization and Washing Conditions

With the exception of *gag* polymerase chain reaction assays, all filters were hybridized and washed at 55 to 65 °C according to published procedures (35). For the *gag* probe, which is much longer than the others used in these studies, hybridizations were done at 65 °C, but washing stringencies had to be increased to 70 °C in $2 \times$ SSC (standard saline citrate = 0.15 M NaCl and 0.015 M sodium citrate).

HIV-1 LTR

Primer A: 5' -TGA GTG CTT CAA GTA GTG TGT GCC C-3'
Primer B: 5' -GTC GCC GCC CCT CGC CTC TTG CCG T-3'
Probe: 5' -CGA AAG GGA AAC CAG AGC TCT CTC G-3'

HIV-1 *gag* (SK38, SK39, and S19)

Primer A: 5' -ATA ATC CAC CTA TCC CAG TAG GAG AAA T-3'
Primer B: 5' -TTT GGT CCT TGT CTT ATG TCC AGA ATG C-3'
Probe: 5' -ATC CTG GGA TTA AAT AAA ATA GTA AGA ATG TAT AGC CCT AC-3'

HIV-1 *env* (SK68, SK69, and SK70)

Primer A: 5' -AGC AGC AGG AAG CAC TAT GG-3'
Primer B: 5' -CCA GAC TGT GAG TTG CAA CAG-3'
Probe: 5' -ACG GTA CAG GCC AGA CAA TTA TTG TCT GGT ATA GT-3'

CD4:

Primer A: 5' -CTG AAT GAT CGC GCT GAC TCA AG-3'
Primer B: 5' -TTG GCA GTC AAT CCG AAC ACT AG-3'
Probe: 5' -GTA TCT GAG TCT TCT ATC TTA AG-3'

T-Cell receptor

Primer A: 5' -TAA TAC GAC TCA TAT AGG GAC TCC AGA TAC TGC CTG AGC-3'
Primer B: 5' -GTC CAC TCG TCA TTC TCC GAG-3'
Probe: 5' -CAG AAG GTG GCC GAG ACC CTC CGG C-3'

Beta-actin:

Primer A: 5' -CTC ATT GCC AAT GGT GAT GAC CTG-3'
Primer B: 5' -GCT ATC CCT GTA CGC CTC ACC G-3'
Probe: 5' -CGG TGA GGA TCT TCA TGA GCT AGT C-3'

3'ORF

Primer A: 5' -ATG CTG ATT GTG CCT GGC TA-3'
Primer B: 5' -TGA ATT AGC CCT TCC AGT CC-3'
Probe: 5' -AAG TGG CTA AGA TCT ACA GCT GCC T-3'

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Development of Antibodies to HIV-1 Is Associated with an Increase in Circulating CD3⁺CD4⁻CD8⁻ Lymphocytes¹

JOSEPH B. MARGOLICK,* VINCENT CAREY,† ALVARO MUÑOZ,†
B. FRANK POLK,†² JANIS V. GIORGI,‡ KENNETH D. BAUER,§
RICHARD KASLOW,¹ AND CHARLES RINALDO¶

**Department of Environmental Health Sciences and †Department of Epidemiology, Johns Hopkins School of Public Health, Baltimore, Maryland 21205; ‡Department of Medicine, University of California School of Medicine, Los Angeles, California 90024; §Department of Medicine, Northwestern University School of Medicine, Chicago, Illinois 60611; ¶National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892; and †Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261*

This study investigated whether seroconversion with respect to human immunodeficiency virus, type 1 (HIV-1) was associated with an increase in lymphocytes expressing the CD3⁺CD4⁻CD8⁻ phenotype. Proportions and absolute numbers of CD3⁺, CD4⁺, and CD8⁺ lymphocytes were determined prospectively over a 2.5-year period on 4954 homosexual and/or bisexual men participating in the Multicenter AIDS Cohort Study. Of the 4806 men whose serostatus at entry could be verified, 1745 were seropositive (SP) for antibodies to HIV-1 at entry into study, 2795 were uniformly seronegative (SN) for HIV-1 for 30 months, and 268 were seroconverters (SC) with respect to HIV-1 during this period. For each of six semiannual evaluations, proportions and numbers of CD3⁺CD4⁻CD8⁻ lymphocytes (calculated as CD3 - (CD4 + CD8)) were both significantly greater in the SP group than in the SN group ($P < 0.001$). Mean CD3⁺CD4⁻CD8⁻ levels in the SC group were indistinguishable from those in the SN group before seroconversion, but by 3-9 months after seroconversion the SC group demonstrated absolute numbers of CD3⁺CD4⁻CD8⁻ lymphocytes which were significantly increased ($P < 0.001$) compared to the SN group using linear regression methods with adjustment for correlation of measurements within an individual over time. An additional significant increase occurred by 21-27 months after seroconversion ($P = 0.006$). These results are consistent with an association of HIV-1 seroconversion with an increase in circulating T lymphocytes expressing the CD3⁺CD4⁻CD8⁻ phenotype (double negative T cells), a decrease in CD3⁺CD4⁻CD8⁺ natural killer cells, or both. An increase in double negative T cells could reflect a host defense mechanism against HIV-1 or effects of HIV-1 on T cell development. © 1989 Academic Press, Inc.

¹ Investigators: Baltimore: Johns Hopkins School of Public Health: B. F. Polk, principal investigator; J. Margolick, H. Farzadegan, R. Fox, J. McArthur, and A. J. Saah. Chicago: Howard Brown Memorial Clinic-Northwestern University Medical School: J. P. Phair, principal investigator; J. S. Chmiel and S. Wolinsky. Pittsburgh: University of Pittsburgh School of Public Health: C. R. Rinaldo, Jr., principal investigator; L. A. Kingsley, D. W. Lyter, P. Gupta, M. Ho, R. O. Valdeserri, and A. Winkelstein. Los Angeles: University of California (Los Angeles) Schools of Public Health and Medicine: R. Detels, principal investigator; B. R. Visccher, coprincipal investigator; J. L. Fahey, D. Imegawa, J. Taylor, J. V. Giorgi, and J. Dudley. Data coordinating center: Johns Hopkins School of Public Health: A. Muñoz, principal investigator; V. Carey, S. Su, L. P. Jacobson, L. Park, and S. Zeger. National Institutes of Health: National Institute of Allergy and Infectious Diseases: J. Kilen, R. A. Kaslow, W. C. Blackwelder, and M. J. van Raden. National Cancer Institute: I. O'Brien.

² Dr. B. Frank Polk died on October 11, 1988. This article is dedicated to his memory.

INTRODUCTION

Seroconversion with respect to human immunodeficiency virus, type 1 (HIV-1), the etiologic agent of acquired immune deficiency syndrome (AIDS) (1, 2), has been associated with changes in several lymphocyte populations. Some of these changes, such as a decrease in helper-inducer CD4 (T4) T lymphocytes (3), are believed to contribute to the development of immune deficiency. Other changes, such as an increase in suppressor-cytotoxic CD8 (T8) T lymphocytes (4, 5), which can kill HIV-1-infected target cells (6, 7) or suppress HIV-1 replication (8) *in vitro*, may represent host resistance to HIV-1. Changes in subsets of CD4 and CD8 lymphocytes have also been reported in patients with AIDS as well as earlier stages of HIV-1 infection (3, 9-14).

Since 1984, the Multicenter AIDS Cohort Study (MACS) has prospectively monitored numbers and proportions of CD3⁺, CD4⁺, and CD8⁺ lymphocytes in almost 5000 homosexual and bisexual men. In the course of these studies, we suspected that the difference between the proportion of CD3⁺ lymphocytes and the sum of the proportions of CD4⁺ and CD8⁺ lymphocytes was greater in individuals who were seropositive (SP) for HIV-1 than in those who were seronegative (SN). This observation was of interest because of the recent recognition that a small proportion of circulating T cells normally express the CD3⁺CD4⁻CD8⁻ phenotype (15-18), which has been referred to as "double negative" (DN), and that a subset of these DN T cells expresses a unique T cell antigen receptor (TCR) composed of γ and δ chains rather than the $\alpha\beta$ receptors found on most CD4⁺ and CD8⁺ T cells (15, 18, 19). The physiologic function of DN T cells is not yet known, but DN T cell clones have been reported to exert cytolytic activity *in vitro* against a variety of tumor cell lines in a non-MHC restricted manner and thus resemble natural killer (NK) cells (15, 20).

With this in mind, we investigated the hypothesis that the proportion and number of CD3⁺CD4⁻CD8⁻ lymphocytes are higher in individuals who are infected for HIV-1 than in those who are SN. Using the serial measurements of numbers and proportions of CD3⁺, CD4⁺, and CD8⁺ lymphocytes which had been obtained on the MACS cohort, we calculated serial differences between numbers and proportions of CD3⁺ lymphocytes and numbers and proportions of CD4⁺ and CD8⁺ lymphocytes combined. This report compares the calculated numbers and proportions of CD3⁺CD4⁻CD8⁻ lymphocytes in subjects who were seropositive for HIV-1 throughout the study to corresponding data from subjects who were seronegative throughout the study. In addition, the temporal relationship between seroconversion with respect to HIV-1 and the number and proportion of circulating CD3⁺CD4⁻CD8⁻ lymphocytes was analyzed using data from subjects who were seronegative at baseline but seropositive at later evaluations (seroconverters, SC).

MATERIALS AND METHODS

Study subjects. The MACS was initiated in Baltimore, Chicago, Los Angeles, and Pittsburgh with the goal of defining the natural history of AIDS and related illnesses. Four thousand nine hundred fifty-four homosexual or bisexual men were

enrolled in 1984-1985 and since that time have undergone clinical and immunologic evaluations twice yearly or, in a high proportion of seroconverters (defined below), four times yearly. The study design has been described in detail (21). This report is based on data from the first 2.5 years of the study.

Measurement of serologic status and definition of seroconversion. Antibodies to HIV-1 were assayed by ELISA; positives were confirmed by repeat ELISA and Western blot tests (22). Subjects who consistently lacked antibodies to HIV-1 during the study period were defined as prevalent SN ($n = 2795$), subjects who had such antibodies at entry into the study were defined as prevalent SP ($n = 1745$), and subjects who lacked such antibodies at the first study visit but subsequently developed them were defined as SC ($n = 268$). For SC, the time of seroconversion was defined as the time halfway between the last serum specimen negative for antibodies to HIV-1 and the first specimen positive for such antibodies. The time of seroconversion was known to within ± 4 months for 228 (85%) of the SC.

Measurement of T cell proportions and numbers. Proportions of circulating $CD3^+$, $CD4^+$ and $CD8^+$ T cells were determined by two-color flow cytometry, using either anticoagulated (heparin or EDTA) whole blood (23) or purified peripheral blood mononuclear cells (PBMC) obtained from heparinized whole blood by density gradient centrifugation (24). Specimens were stained with monoclonal antibodies (Becton-Dickinson, Mountain View, CA) conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) as follows: anti-Leu 4 FITC ($CD3$; total T cells) and control IgG, PE in one tube and anti-Leu 3a PE ($CD4$; helper-inducer T cells) and anti-Leu 2a FITC ($CD8$; suppressor-cytotoxic T cells) in another tube. Proportions of stained cells were quantitated using the EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) with software (Quadstat) for two-color analyses. The proportion of $CD3^+CD4^-CD8^-$ cells among lymphocytes (% D) in each specimen was then calculated according to the formula: $\%D = \%CD3^+ - (\%CD4^+ + \%CD8^+)$. The symbol D was used for this difference to distinguish the calculation performed from a direct measurement of lymphocytes expressing the DN or $CD3^+CD4^-CD8^-$ phenotype (see Discussion).

Each study participant also had a complete blood count and differential performed at each clinic visit by a commercial laboratory. Absolute numbers of circulating $CD3^+CD4^-CD8^-$ lymphocytes were calculated by multiplying the $\%D$ by the total lymphocyte count (white blood count $\times$ % lymphocytes).

Data analysis and display. Graphical methods of exploratory data analysis (25) were used to demonstrate differences between SN and SP subject populations with regard to calculated proportions and absolute numbers of $CD3^+CD4^-CD8^-$ lymphocytes over time (Figs. 1 and 2); robust locally weighted scatterplot smoothing (26) was used to exhibit graphically the change in $CD3^+CD4^-CD8^-$ lymphocytes over time among SC (Fig. 3). Briefly, the line in this figure is achieved by slicing the data vertically, calculating robust summaries of the data in each slice, and joining the summaries.

Formal hypothesis testing was carried out using the following model for counts of $CD3^+CD4^-CD8^-$ lymphocytes in SC over time. $DN_{v,i}$, the calculated number of $CD3^+CD4^-CD8^-$ lymphocytes at visit v for the i th subject ($v = 1, \dots, 11; i =$

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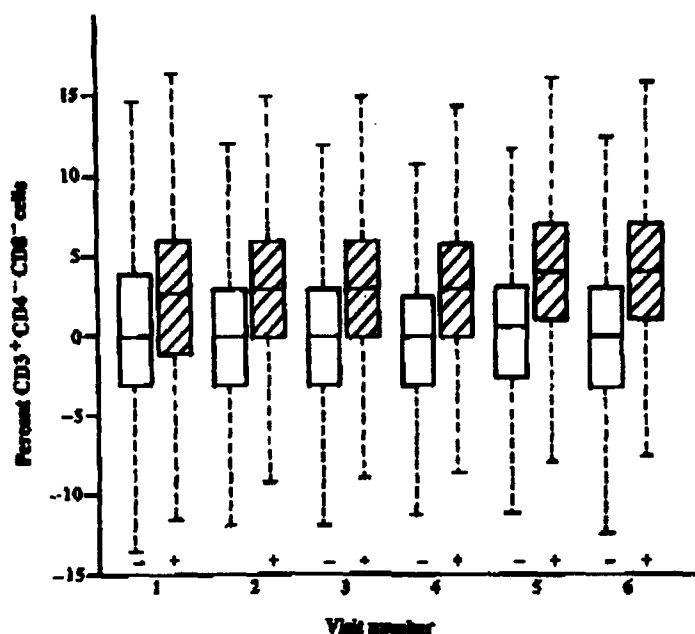


FIG. 1. Box plot showing calculated proportions of CD3⁺CD4⁺CD8⁻ lymphocytes as the percentage of total lymphocyte population for seronegative (-; open boxes) and seropositive (+; shaded boxes) study participants at each of six semianual evaluations. Proportions were calculated from individually determined proportions of CD3⁺, CD4⁺, and CD8⁺ lymphocytes according to the formula $\%CD3^{+}CD4^{+}CD8^{-} = \%CD3^{+} - (\%CD4^{+} + \%CD8^{+})$ and were adjusted by subtracting the median value for $\%CD3^{+}CD4^{+}CD8^{-}$ for the seronegative participants at the corresponding center and visit as described under Results. Horizontal lines in each box indicate first quartile (Q1), median, and third quartile (Q3). The dotted lines extend an additional 1.5 times (Q3-Q1), corresponding approximately to numbers in percentile 1-5 (lower line) or 95-99 (upper line) of the values for each group.

1, . . . 268), was taken to be a normal variate with mean depending on the time since seroconversion. The time of seroconversion was taken as $t = 0$, and time elapsed since seroconversion was broken into 6-month periods as: more than 9 months prior to seroconversion ($t < -9$), from 9 to 3 months before seroconversion ($-9 < t < -3$), and so on, up to more than 27 months after seroconversion ($t > 27$). Repeated numbers of CD3⁺CD4⁺CD8⁻ lymphocytes from a single individual were assumed to follow an intraclass correlation structure (27). In this approach, maximum likelihood methods are used to estimate mean levels of CD3⁺CD4⁺CD8⁻ lymphocytes according to time since seroconversion, adjusting for correlation among those CD3⁺CD4⁺CD8⁻ values contributed by the same individual. This methodology allows for imbalance in the number of observations contributed by each individual. In addition, an intraclass correlation structure does not require complete or equidistant data. Linear contrasts among the estimated means are then used to test the hypothesis that the mean number of CD3⁺CD4⁺CD8⁻ cells increases with time elapsed from seroconversion (i.e., that the mean CD3⁺CD4⁺CD8⁻ number in one of (or a collection of) the afore-

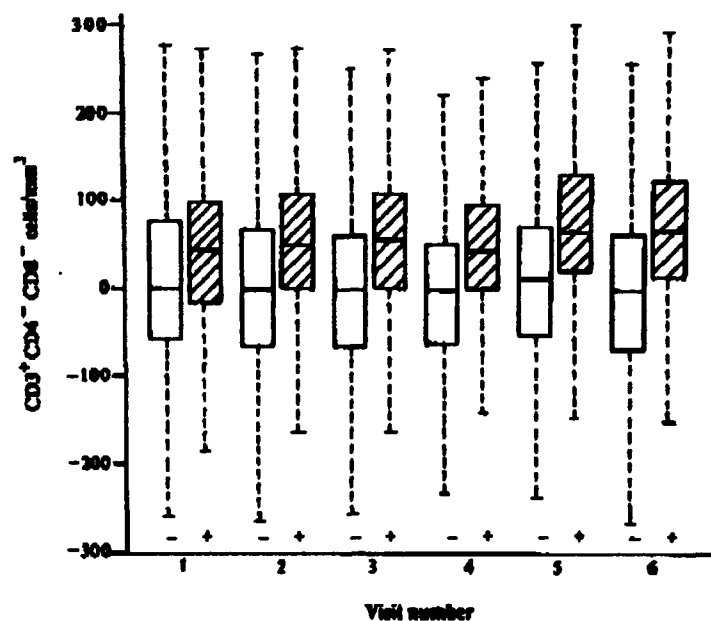


FIG. 2. Box plot showing calculated absolute numbers (cells/mm³) of CD3⁺CD4⁻CD8⁻ lymphocytes for seronegative (-; open boxes) and seropositive (+; shaded boxes) study participants at each of six semianual evaluations. Calculations were performed and adjusted, and boxes were derived, as described in legend to Fig. 1.

mentioned interval(s) is greater than the mean CD3⁺CD4⁻CD8⁻ number in one of (or a collection of) earlier interval(s)).

RESULTS

Cross-sectional relationship between CD3⁺CD4⁻CD8⁻ lymphocytes and serostatus. A total of 11,337 observations was available on the 2795 SN subjects and 8311 observations on the 1745 SP subjects. In order to compare proportions and numbers of CD3⁺CD4⁻CD8⁻ lymphocytes in these groups, we first evaluated median percentages and numbers of these cells in the SN group at each center at each of the six clinic visits of these individuals. As shown in Table 1, there was a range of 4.7% (-2.2% to 2.5%) in the medians of percentages and a range of 104.1 cells/mm³ (-46.02 to 58.08 cells/mm³) in the medians of numbers of calculated CD3⁺CD4⁻CD8⁻ lymphocytes in the SN group. The overall means were 0.1% for the percent and 3.7 cells/mm³ for the number of CD3⁺CD4⁻CD8⁻ lymphocytes. To compensate for this intercenter and intervisit variation, the median values shown in Table 1 were subtracted from all data points for the corresponding center and visit in subsequent analyses, thus yielding adjusted values for the calculated proportions and numbers of CD3⁺CD4⁻CD8⁻ lymphocytes which reflected only the difference between SP and SN individuals.

The distributions of percentages and numbers of CD3⁺CD4⁻CD8⁻ lympho-

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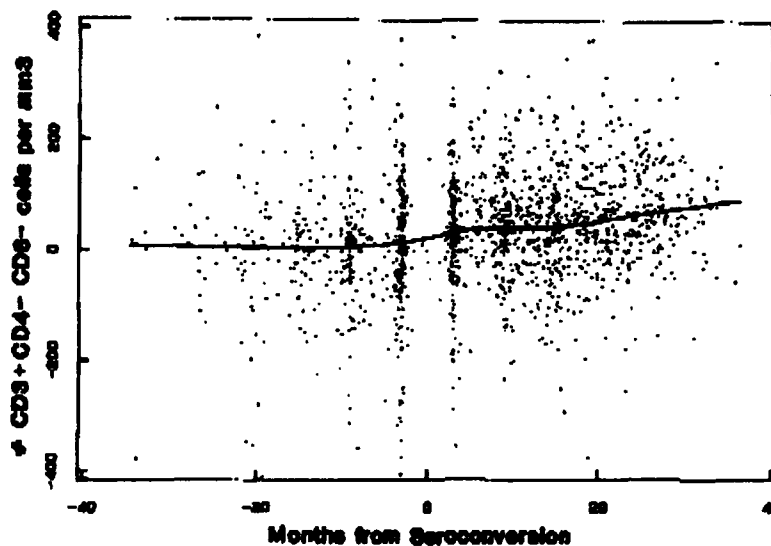


FIG. 3. Scattergram showing individual data points ($n = 1571$) for calculated absolute number of CD3⁺CD4⁺CD8⁻ lymphocytes in relation to estimated time of seroconversion ($t = 0$) for seroconverter population ($n = 268$). Data points were calculated and adjusted as described in legend to Fig. 1 and under Results. Robust locally weighted scatterplot line was derived as described under Materials and Methods. Nineteen data points lie outside the range displayed (i.e., < -400 or > 400) and are not shown.

cytes at each clinic visit for the serologically stable SN or SP subjects after this adjustment are shown in Figs. 1 and 2, respectively. The adjusted proportion of CD3⁺CD4⁺CD8⁻ lymphocytes was higher for the SP than for the SN at all visits, with overall means of 0.01% for SN and 3.0% for SP. This difference was highly statistically significant ($P < 0.001$). Similarly, the adjusted mean absolute number

TABLE I
MEDIAN CALCULATED LEVELS OF CD3⁺CD4⁺CD8⁻ CELLS IN SERONEGATIVE SUBJECTS

Center	Visit number					
	1	2	3	4	5	6
A. Percent						
Baltimore	1.0	1.0	1.0	0.0	0.0	2.5
Chicago	-1.5	-2.2	-1.7	-1.0	0.9	0.4
Pittsburgh	1.9	1.3	0.2	0.4	1.0	1.3
Los Angeles	0.0	0.0	0.0	-0.1	0.0	-1.0
B. Cells/mm ³						
Baltimore	20.24	15.00	23.20	0.38	-0.16	58.08
Chicago	-30.60	46.02	-31.31	-15.28	12.38	9.78
Pittsburgh	39.08	28.99	2.92	9.29	20.15	27.15
Los Angeles	0.43	0.29	-0.12	20.48	0.20	-29.48

of $CD3^+CD4^-CD8^-$ lymphocytes was consistently higher in the seropositive population, with an overall mean of 0.72 cells/mm³ for SN and 55.3 cells/mm³ for SP. This difference was also highly statistically significant ($P < 0.001$).

Longitudinal relationship between $CD3^+CD4^-CD8^-$ and HIV-1 seroconversion. The above results strongly suggested a direct relationship between the presence of antibodies to HIV-1 and the absolute number of $CD3^+CD4^-CD8^-$ lymphocytes. To test this hypothesis, we analyzed the numbers of $CD3^+CD4^-CD8^-$ lymphocytes present in study subjects for whom data were available before and after seroconversion with respect to HIV-1, i.e., the SC group. For each of the 268 SC identified, the time (t) of seroconversion, as defined above, was taken as $t = 0$. The results of the lymphocyte evaluations for each subject before or after this time ($t < 0$ or $t > 0$, respectively) were analyzed; a total of 1571 observations was available on these 268 subjects. To eliminate any effect of intervisit and inter-center variation in numbers of $CD3^+CD4^-CD8^-$ lymphocytes, the median number of $CD3^+CD4^-CD8^-$ lymphocytes in the SN group at each center at each visit was subtracted from the value calculated for each seroconverter at the corresponding center and visit, as described above. This adjusted number of $CD3^+CD4^-CD8^-$ lymphocytes, corresponding to the difference between subjects who seroconverted and subjects who remained seronegative during the study period, was used in the subsequent analysis.

Figure 3 shows both the primary adjusted data and the robust smoothed line for this analysis. It can be seen that although the range of the values is large, an increase occurs following the calculated time of seroconversion. Figure 4 shows

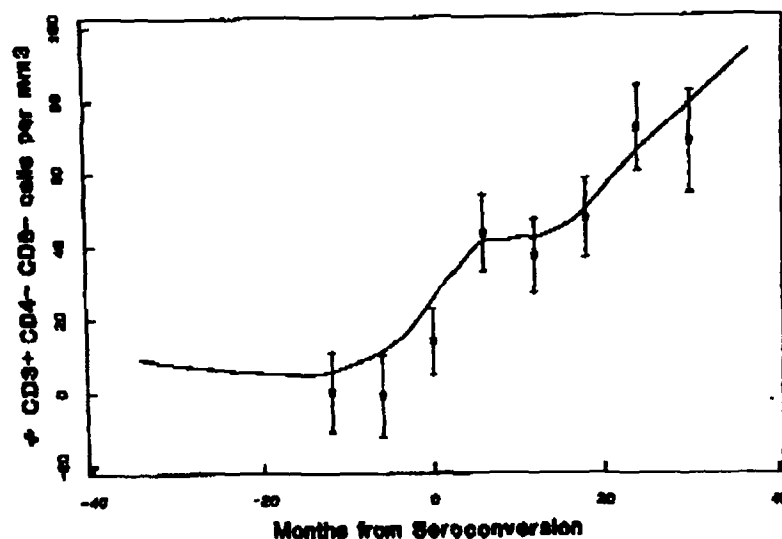


FIG. 4. Plot showing relation between robust locally weighted scatterplot of observed data (Fig. 3) and model for number of $CD3^+CD4^-CD8^-$ lymphocytes as a function of time since seroconversion. The asterisks indicate the estimated value for $CD3^+CD4^-CD8^-$ for the 6-month intervals, and the bars around the asterisks indicate ± 1 SE of this estimate.

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the same robust smoothed line along with the estimated means ($\pm$ one standard error), derived from the parametric regression methods, for each time interval in relation to seroconversion. The *P* values for significance testing associated with these data are shown in Table 2. Before seroconversion (i.e., when $t < 0$), the adjusted number of CD3⁺CD4⁺CD8⁺ lymphocytes was very close to 0, indicating that SC exhibited numbers of CD3⁺CD4⁺CD8⁺ lymphocytes which were indistinguishable from the SN. By 3–9 months after seroconversion, however, SC had adjusted numbers of CD3⁺CD4⁺CD8⁺ lymphocytes which were similar to those seen in the SP population (compare Fig. 4 with Fig. 2). The change in number of CD3⁺CD4⁺CD8⁺ from before seroconversion (–9 to –3 months) to 3–9 months after seroconversion was highly statistically significant ($P < 0.001$). In addition, there was a further increase by the end of the study period (>21 months) which was also highly statistically significant compared to the value at 3–9 months after seroconversion ($P = 0.006$). Thus, the increase in CD3⁺CD4⁺CD8⁺ after seroconversion progressed with time. It should be noted that the values of the robust smoothed line derived from the adjusted primary data all lie within or very close to one standard error of the mean estimates using the parametric regression model (Fig. 4). This result supports the validity of the parametric approach.

Lack of correlation between numbers of CD3⁺CD4⁺CD8⁺ lymphocytes and numbers of CD4⁺ and CD8⁺ lymphocytes. Since the number of CD3⁺CD4⁺CD8⁺ lymphocytes was calculated using a formula that included the numbers of CD4⁺ and CD8⁺ lymphocytes, we were interested to determine if the number of CD3⁺CD4⁺CD8⁺ lymphocytes was significantly correlated with the numbers of CD4⁺ and CD8⁺ lymphocytes. To evaluate this, correlation coefficients for the numbers of lymphocytes at each clinic visit were calculated for the group of 268 seroconverters ($n = 1571$ observations). Little association was observed between the number of CD3⁺CD4⁺CD8⁺ lymphocytes and the number of T cells belonging to either subset ($r = -0.047$ for CD4⁺

TABLE 2
RESULTS OF REGRESSION ANALYSIS OF CALCULATED NUMBERS OF CD3⁺CD4⁺CD8⁺
LYMPHOCYTES IN SEROCONVERTERS IN RELATION TO TIME OF SEROCONVERSION

Months from seroconversion ^a	Estimated no. of CD3 ⁺ CD4 ⁺ CD8 ⁺ cells/mm ³	Standard error of estimate	<i>P</i> value ^b
< -9	-0.03	8.25	NS ^c
-9 to -3	-1.01	8.23	NS
-3 to +3	13.71	9.13	0.13
3 to 9	43.60	7.35	<0.001
9 to 15	37.17	9.31	<0.001
15 to 21	47.39	7.99	<0.001
21 to 27	71.94 ^d	9.00	<0.001
>27	68.06 ^d	12.04	<0.001

^a Time of seroconversion (defined as in text) taken as 0 months.

^b Relative to estimated number of CD3⁺CD4⁺CD8⁺ lymphocytes/mm³ before seroconversion.

^c NS = $P > 0.20$.

^d $P = 0.006$ for CD3⁺CD4⁺CD8⁺ at >21 months compared to CD3⁺CD4⁺CD8⁺ at 3 to 9 months.

lymphocytes and 0.147 for CD8⁺ lymphocytes), suggesting that the numbers of CD3⁺CD4⁻CD8⁻ lymphocytes were not related to the numbers of CD4⁺ lymphocytes, and only weakly related to CD8⁺ lymphocytes.

DISCUSSION

This study shows that HIV-1 seroconversion in a large cohort of gay/bisexual men was associated with statistically significant relative and absolute increases in circulating CD3⁺CD4⁻CD8⁻ lymphocytes. These increases were seen in prevalent seropositive subjects as compared to prevalent seronegative subjects and in seroconverters after seroconversion as compared to before seroconversion. In the seroconverters, the increase in CD3⁺CD4⁻CD8⁻ lymphocytes was approximately 43 cells/mm³ within 9 months of estimated seroconversion and was further increased to approximately 70 cells/mm³ beyond 21 months from seroconversion. These observations confirm and extend a recent analysis of lymphocyte subset data in 42 published studies, in which increased percentages of CD3⁺CD4⁻CD8⁻ lymphocytes were calculated among individuals infected with HIV-1 (28). In the present study, increased percentages and numbers of these cells were found in a large cohort of individuals followed over time.

Technical explanations for the observed increase in CD3⁺CD4⁻CD8⁻ lymphocytes should be considered but seem unlikely. If expression of CD4 is down-regulated in HIV-1-infected T cells (29, 30), infection of approximately 40–80 CD4⁺ T cells/mm³ with HIV-1 might account for the observed increase in CD3⁺CD4⁻CD8⁻ lymphocytes. However, using the most sensitive methods available, the number of peripheral blood lymphocytes actively infected with HIV-1 appears to be on the order of 1/1000 or less (31, 32), a number far too small to support this explanation. Another theoretical possibility is that HIV-1 envelope proteins (gp120) bound to CD4 *in vivo* could block the anti-CD4 antibody used in this study (anti-Leu3a), yielding a spuriously low %CD4. While this possibility cannot be excluded, the correlation between the numbers of CD3⁺CD4⁻CD8⁻ and CD4⁺ cells was extremely low (-0.047), and the intensity of CD4 positivity was not decreased in SP as compared to SN subjects (data not shown). For these reasons, we favor the interpretation that these results reflect a true increase in the population of CD3⁺CD4⁻CD8⁻ lymphocytes rather than a technical inability to measure CD4⁺ lymphocytes.

According to the methods used in this study, a true increase in CD3⁺CD4⁻CD8⁻ lymphocytes could be explained by three mechanisms: (i) an increase in cells expressing the CD3⁺CD4⁻CD8⁻ phenotype; (ii) a decrease in cells expressing the CD3⁺CD4⁻CD8⁺ phenotype; or (iii) a decrease in cells expressing the CD3⁺CD4⁺CD8⁻ phenotype. In support of the first possibility, Baseler *et al.* (33) reported that HIV-1 seropositive individuals demonstrated an increase in circulating T cells expressing the δ T cell receptor chain; the δ chain is associated with the DN phenotype (15, 18, 19), and many of the δ ⁺ cells expressed this phenotype (M. W. Baseler, personal communication). In healthy HIV-1 seronegative individuals, DN T cells represent approximately 3–5% of circulating lymphocytes (16). Assuming 2000 lymphocytes/mm³ in the peripheral

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blood, an increase of 40% or more in DN T cells would be required to account for the increase of 43-68 cells/mm³ we observed in the HIV-1 SC group.

The second possibility, that a decrease in CD3⁺CD8⁺ cells contributes to the observed rise in calculated CD3⁺CD4⁺CD8⁻ cells, is also tenable. Because some NK cells express low levels of CD8 without expressing CD3 (34, 35) and because CD8⁺ cells were determined separately from CD3⁺ cells in this study, CD8⁺CD3⁻ NK cells reduced the calculated percent or number of CD3⁺CD4⁺CD8⁻ lymphocytes. Thus, NK cells probably account for the discrepancy between the 0.1% mean proportion of CD3⁺CD4⁺CD8⁻ lymphocytes in SN in the present study and the reported 3-5% proportion of DN T cells in healthy HIV-1 seronegative individuals (16), and a decrease in NK cells could account for a rise in calculated CD3⁺CD4⁺CD8⁻ lymphocytes. However, arguing against this possibility are several studies indicating that proportions and numbers of circulating NK cells are normal or elevated in HIV-1-infected individuals, at least until the development of AIDS (11, 36-41). In this connection, Vuiller *et al.* (42) recently reported that HIV-1-infected individuals exhibit a decrease in numbers of lymphocytes coexpressing CD16 or Leu19 and CD8 at low density, a phenotype characteristic of NK cells. However, these cells were also decreased, though to a lesser extent, in seronegative individuals at high risk for HIV-1 infection, and the data from the HIV-1-infected subjects were not subclassified according to the stage of HIV-1 infection. Thus, the question of whether HIV-1 infection is associated with a reduction of NK cells is unresolved at present. The third possibility, that a decrease in CD3⁺ monocytes expressing low levels of CD4 (43, 44) contributes to our findings, seems remote because the proportion of contaminating monocytes within the lymphocyte gate, as measured with the anti-Leu M3 antibody, is generally <1% (data not shown) and therefore could not decrease enough to account for our findings.

Thus, the present findings appear to be due to some combination of an increase in CD3⁺CD4⁺CD8⁻ T lymphocytes and a decrease in NK cells. These mechanisms can be distinguished by the use of more accurate formulas for calculating DN T cells, such as $DN = Leu4 - (Leu3 + Leu2[T-NK])$, or $DN = Leu4 - (Leu3 + Leu2[strong fluorescence])$, and by direct measurement of CD3⁺CD4⁺CD8⁻ lymphocytes in one tube, using anti-CD3 conjugated to one fluorochrome and anti-CD4 and -CD8 conjugated to another. These calculations were not possible in the present study because the previously obtained measurements included only CD3, CD4, and CD8 positive cells.

Despite the small size of the normal pool of circulating DN T lymphocytes, an increase in these cells in association with HIV-1 seroconversion could be important for several reasons. First, DN T lymphocytes have been reported to exert non-MHC restricted, NK-like cytotoxicity against a variety of tumor cell lines (15, 20). Preliminary data suggest that T lymphocytes not expressing WT31 antigen under standard flow cytometry conditions, many of which are $\gamma\delta$ -TCR positive and express the CD3⁺CD4⁺CD8⁻ phenotype, can kill target cells coated with inactivated HIV-1 (45). Thus, CD3⁺CD4⁺CD8⁻ lymphocytes may play a role in host defense against HIV-1, raising the possibility that individuals who generate a large increase in CD3⁺CD4⁺CD8⁻ lymphocytes in response to HIV-1 serocon-

version may have a delayed progression of HIV-1 infection compared to those who generate a small increase. Second, human thymocytes expressing the CD3⁺CD4⁺CD8⁻ phenotype have been demonstrated to develop *in vitro* into mature T cells expressing CD3, the $\alpha\beta$ -TCR, and either CD4 or CD8 (46, 47). Thus, a rise in CD3⁺CD4⁺CD8⁻ lymphocytes could reflect increased production of T cell precursors in response to the fall in CD4⁺ T cells and/or the rise in CD8⁺ T cells that follow HIV-1 seroconversion (4, 5). In this connection, an increase in the number and proportion of CD3⁺CD4⁺CD8⁻ lymphocytes in lymph nodes of mice which had been depleted of circulating CD4⁺ lymphocytes by injection of anti-CD4 monoclonal antibody has been reported (48). These data suggest that the increase in circulating CD3⁺CD4⁺CD8⁻ lymphocytes in HIV-1-infected individuals might reflect an increased rate of T cell production, and that measurement of this number could have a utility in assessing T cell turnover analogous to that of the reticulocyte count in assessing the severity of hemolytic states. A third possibility is that the increase in CD3⁺CD4⁺CD8⁻ lymphocytes is a nonspecific result of generalized immune stimulation following HIV-1 seroconversion. For example, DN T cells can proliferate in the presence of IL-2 (19, 20). Thus, a further study of this population may yield insight into the normal mechanisms for regulation of lymphocyte populations.

The clinical significance of the increase in CD3⁺CD4⁺CD8⁻ lymphocytes observed in the present study remains to be determined. Because of the long incubation period for HIV-1-induced illnesses, it is still too soon to determine if the magnitude of this increase in individual seroconverters correlates with the clinical courses of those individuals. Further studies are needed to answer this important question. To date, HIV-1 infection is the only human disorder, aside from congenital immune deficiencies (18), that has been associated with an increase in CD3⁺CD4⁺CD8⁻ lymphocytes. It remains to be determined whether infection with viruses other than HIV-1 can lead to an increase in these cells.

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Flow Cytometric Analysis of $\gamma\delta$ T Cells and Natural Killer Cells in HIV-1 Infection

JOSEPH P. MARGOLICK, ELVIA R. SCOTT, NANCY ODAKA, AND
ALFRED J. SAAH

Departments Mental Health Sciences and Epidemiology, Johns Hopkins School of
Hygiene and Public Health, Baltimore, Maryland 21205

We have previously shown that HIV-1 seropositivity is associated with an increase in the difference between the number of $CD3^+$ lymphocytes and the total number of $CD4^+$ and $CD8^+$ lymphocytes [$CD3 - (CD4 + CD8)$] among peripheral blood lymphocytes (PBL). To investigate the cellular basis of this increase, PBL from seronegative (SN) and AIDS-free seropositive (SP) homosexual men and intravenous drug users were analyzed by two-color flow cytometry. Results showed that SP compared to SN manifested the expected elevation in calculated [$CD3 - (CD4 + CD8)$] cells (87 vs 28 cells/mm³; $P < 0.001$). Only small differences in lymphocyte populations that could contribute to this increase were observed, namely lymphocytes expressing the $CD3^+CD4^-CD8^-$ phenotype (67 vs 56 cells/mm³; $P > 0.10$) or the $CD8^{dim}$ phenotype (135 vs 142 cells/mm³; $P > 0.10$). However, SP had significantly lower numbers of cells expressing the $CD56^+CD3^-$ phenotype characteristic of natural killer cells (81 vs 170 cells/mm³; $P < 0.001$) and significantly higher numbers of T cells expressing the $\gamma\delta$ T cell receptor (TCR) (81 vs 62 cells/mm³; $P = 0.010$). The latter difference was primarily due to higher numbers of cells coexpressing $\gamma\delta$ -TCR and low levels of CD8 (27 vs 15 cells/mm³; $P = 0.009$). These data suggest that HIV-1 seropositivity is associated with low numbers of natural killer cells and high numbers of $CD8^+ \gamma\delta$ -TCR lymphocytes. Changes in these populations may reflect altered host defense against HIV-1 or altered T cell kinetics in the presence of HIV-1 infection. © 1991 Academic Press, Inc.

INTRODUCTION

Infection with human immunodeficiency virus (HIV), type 1, the etiologic agent of acquired immune deficiency syndrome (AIDS), is associated with changes in the proportions and absolute numbers of T lymphocytes circulating in the peripheral blood. In absolute terms, the largest changes are a decrease in $CD4$ lymphocytes and an increase in $CD8$ lymphocytes (1), but changes in numerically smaller T cell subsets have also been documented (2-7). Depletion of $CD4$ lymphocytes has prognostic value for the progression of HIV-1 infection to AIDS (8, 9), but whether changes in other T cell subsets have prognostic or mechanistic importance in this regard remains unknown.

In a recent study of 268 seronegative (SN) homosexual men who developed antibodies to HIV-1 (seroconverted), we found that the difference between the total number of T lymphocytes, as measured by the number of $CD3^+$ lymphocytes, and the number of lymphocytes expressing either $CD4$ or $CD8$ increased significantly at the time of seroconversion (10). This difference was denoted as calculated $CD3^+ - (CD4^+ + CD8^+)$. Although the specific cell population(s) responsible for this increase could not be defined from the data available, we

hypothesized that the increase in calculated $CD3^+ - (CD4^+ + CD8^+)$ could have been due to either (i) an increase in T cells expressing the $CD3^+CD4^-CD8^-$ (double negative, or DN) phenotype or (ii) a decrease in non-T lymphocytes expressing the $CD3^-CD4^-CD8^{dim}$ phenotype seen on some natural killer (NK) cells (11, 12).

The goal of the present study was to distinguish between these two alternatives. Therefore, we analyzed numbers of DN and NK lymphocytes in HIV-1 seropositive and seronegative individuals. In addition, we also examined $\gamma\delta$ -T cell receptor (TCR) T cells, because the DN phenotype is associated with expression of the $\gamma\delta$ -TCR (13–15) and some $\gamma\delta$ -TCR⁺ T cells express $CD8^{dim}$ (16).

MATERIALS AND METHODS

Study subjects. The study population consisted of 210 participants in two prospective studies of the natural history of HIV-1 infection. Of these, 204 were homosexual/bisexual men participating in the Baltimore–Washington, DC, chapter of the Multicenter AIDS Cohort Study (MACS) (17), known as the Study to Help the AIDS Research Effort (SHARE); the remaining 6 were intravenous drug users (IVDUs) participating in ALIVE (AIDS Link to IntraVenous Experiences), a longitudinal study of HIV-1 infection in IVDUs (18). Results included in the present analysis were derived from participants who did not have AIDS at the time of testing and whose serological status for antibodies to HIV-1 (established by commercial ELISA and Western blot kits (19)) was known; 132 were SN and 78 were SP for HIV-1. Except for seroconverters, seropositive subjects from SHARE have been seropositive at least since 1984, and seropositive subjects from ALIVE have been seropositive at least since 1988–1989.

Subjects in both SHARE and ALIVE visit an outpatient clinic twice yearly and undergo a physical examination and laboratory testing. The physical examination includes determination of (i) symptoms related to stage of HIV-1 infection (i.e., sweats, weight loss, fever, diarrhea, or thrush); (ii) other symptoms; and (iii) medications taken. Of the 78 seropositive individuals studied, only 7 had two or more HIV-related symptoms and 13 were known to have taken AZT within the last 6 months. The laboratory examination includes determination of HIV-1 serostatus, as above, and measurement of T cell subsets as described below.

Measurement of T cell subsets. Specimens for the present study were selected randomly from those sent to the laboratory for measurement of T cell subsets. On any given day, specimens from both seronegative and seropositive individuals were run. Specimens of heparinized whole blood were stained with monoclonal antibodies using the whole blood method of Hoffman *et al.* (20) as described (21). Briefly, antibodies conjugated to either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were added and, after staining for 20 min on ice and 10 min at room temperature, the erythrocytes were lysed using an ammonium chloride-based lysing solution (NIH natural history kit, Becton-Dickinson, Mountain View, CA). The stained lymphocytes were fixed in 1% paraformaldehyde (prepared at the time of use from 10% ultrapure EM grade formaldehyde, Polysciences, Warrington, PA) and proportions of stained cells were quantitated

within 48 hr using the EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) with software (Quadstat) for two-color analyses.

For the flow cytometric analyses, lymphocytes were gated based on forward and 90° light scatter on linear scales; a wide gate was intentionally set to maximize inclusion of large granular lymphocytes. Five thousand cells were counted for each antibody combination. The percentage of positive cells for each marker or combination of markers was determined by dividing the number of positive events by 5000, after subtracting the number of background events obtained with an appropriately conjugated isotype control antibody, as described below. Absolute numbers (cells/mm³) of lymphocyte subsets were calculated by multiplying the percentage of positive cells for a given subset by the number of lymphocytes per mm³ in the peripheral blood, as determined from a complete blood count with an automated 10,000 cell differential.

Several types of controls were used for assessing background staining and setting fluorescence cutoffs. First, nonspecific staining was detected with a reagent combination of IgG₁-FITC and IgG₁-PE. In addition, to assure that fluorescence measurements in the two-color preparations were accurate, fluorescence cutoffs were also checked using each FITC- and PE-conjugated antibody by itself to set the PE and FITC cutoffs, respectively, and to set color compensation. At the same time, background levels of green fluorescence were also determined in antibody combinations with a distinct negative population, such as anti-CD4/anti-CD8 and anti-CD3/anti-TCR δ -1 (see below). This latter method was used exclusively after early experiments demonstrated clearly that the cutoffs for both green and red background fluorescence obtained from the single color controls were equivalent to those obtained from the negative populations in the two-color specimens. Finally, after the cutoffs were set any events counted as positive in unstained specimens were subtracted from the counts of positive events in the stained specimens. Contamination of the lymphocyte population with unlysed erythrocytes and with neutrophils and monocytes was assessed using the combination of anti-CD45-FITC and anti-CD14-PE. All of the specimens contained at least 95% lymphocytes (brightly positive for CD45 and negative for CD14) and less than 2% monocytes (brightly positive for both CD45 and CD14).

The antibody combinations used for each specimen, in addition to the above controls, were (i) anti-Leu 4-FITC (CD3; total T cells) and control IgG₁-PE; (ii) anti-Leu 3a-PE (CD4; helper-inducer T cells) and anti-Leu 2a-FITC (CD8; suppressor-cytotoxic T cells); (iii) anti-TCR δ -1-FITC (TCR δ chain (22), T Cell Sciences, Cambridge, MA) and anti-Leu 4-PE; (iv) TCR δ -1-FITC and anti-Leu 2a-PE; and (v) anti-CD3-FITC and anti-Leu 19-PE (CD56) or, in later experiments, a combination of anti-CD3-FITC with both anti-CD56-PE and anti-Leu 11c (CD16)-PE. In a sixth tube, anti-CD3-FITC, anti-CD4-PE, and anti-CD8-PE were added for the direct identification of CD3⁺CD4⁻CD8⁻ cells; each antibody was added at the manufacturer's recommended concentrations, which gave equivalent results when used separately or in combination. NK cells were identified as CD3⁻CD56⁺ or CD3⁻CD16⁺CD56⁺ cells (23); specimens stained with both antibody combinations generally agreed to within 1%. All antibodies except TCR δ -1 were obtained from Becton-Dickinson. Aside from the CD4-PE/CD8-FITC and

anti-CD3-FITC/anti-CD56-PE/anti-CD16-PE combinations, which were purchased premixed, all antibody combinations were prepared at the time of staining by adding the antibodies to the staining tubes immediately prior to the addition of the whole blood.

For the determination of numbers of CD8^{dim} cells, one-color histograms derived from cells stained with anti-CD8-PE alone were used. As shown in Fig. 1, the left cursor was set from background staining, and the right cursor was set by mutual agreement of two observers based on the apparent lower boundary of the bright population. In the few cases where there was not a clear demarcation between the bright and dim populations of CD8⁺ cells, this cursor was set conservatively, i.e., to include only cells clearly not part of the bright population.

During this study, our laboratory participated in a quality control program conducted by the four flow cytometry laboratories in the MACS. Every week since July, 1988, lymphocyte subsets have been measured in aliquots of two samples of peripheral blood shipped to each laboratory within 24 hr of venipuncture. Our measurements of CD3⁺, CD4⁺, CD8⁺, CD20⁺, and CD3⁻CD56⁺ lymphocytes have been similar to those of the other centers in the program (data not shown), supporting the validity of the measurements of the dim CD56 reported in this study. In addition, as part of this quality control program the flow cytometer used in these studies underwent a thorough calibration and adjustment for the detection of dim markers such as CD56 and CD8^{dim}.

Statistical analysis. Analyses and graphics were performed on a personal computer using a commercially available software package (24, 25). Subjects were divided into two groups: seronegative ($n = 132$) and seropositive without AIDS ($n = 78$). Data from the two groups were compared using descriptive data analysis techniques, as described by Tukey (26). Significance of differences between the groups was determined by analysis of variance (ANOVA) as described by Snedecor and Cochran (27). Absolute numbers of cells were log normally distributed and were log transformed for testing of significance of differences and estimation of 95% confidence intervals for geometric means.



FIG. 1. Representative histogram showing measurement of CD8^{dim} lymphocytes. The boundary between unstained and stained cells (left cursor) was set using cells incubated with an isotype control antibody. The boundary between dimly and brightly stained cells (right cursor) was set empirically assuming a normal distribution of brightly stained cells.

RESULTS

Table 1 shows the geometric means for the absolute numbers of lymphocyte subsets for the HIV-1 SN and SP groups. As expected, SP had lower CD4 levels and higher CD8 levels than SN, and these differences were statistically significant. SP also demonstrated a markedly higher geometric mean calculated $CD3^+ - (CD4^+ + CD8^+)$, consistent with our previous report (10). Thus, it was possible to assess the extent to which differences in $CD8^{dim}$ cells and directly measured $CD3^+CD4^-CD8^-$ (true DN) cells contributed to the difference in calculated $CD3^+ - (CD4^+ + CD8^+)$.

Figure 2 and Table 1 summarize the numbers of $CD8^{dim}$ cells and true DN cells observed in the two groups. Compared to the SN group, the geometric mean number of $CD8^{dim}$ cells in the SP group (125 ± 66 cells/mm³) was 7 cells/mm³ lower than in the SN group (132 ± 64 cells/mm³), and the mean number of DN cells was 11 cells/mm³ greater (Table 1); neither difference was statistically significant, and these analyses were discontinued after the 34 SN and 30 SP shown in Table 1 had been studied. However, initial analyses of NK and $\gamma\delta$ cells showed larger differences and these analyses were therefore conducted on additional subjects. Using the $CD56^+CD3^-$ phenotype to define NK cells, SP individuals manifested a mean number of NK cells that was 52% lower than in SN (Table 1), a difference that was highly statistically significant ($P < 0.001$). Numbers of NK cells were low even for SP subjects who had relatively high numbers of CD4 lymphocytes (>500 cells/mm³; Fig. 3). Interestingly, the two SP individuals who had the highest numbers of NK cells (Fig. 3) had both recently begun taking AZT, suggesting an effect of AZT on NK cell number.

$\gamma\delta$ -TCR T cells were detected in all individuals using the TCR δ -1 antibody. All TCR δ^+ cells were also $CD3^+$ (data not shown), so that the $CD8/TCR\delta$ -1 antibody combination could be interpreted to delineate those $\gamma\delta$ -TCR $^+$ cells that did or did

TABLE 1
ABSOLUTE NUMBERS OF LYMPHOCYTES IN PERIPHERAL BLOOD OF HIV-1 SERONEGATIVE AND SEROPOSITIVE INDIVIDUALS STUDIED

	Seronegative			Seropositive			P
	Mean ^a	(95% C.I.) ^b	n	Mean	(95% C.I.)	n	
CD3	1750	(1673, 1831)	132	1589	(1471, 1715)	78	0.022
CD4	1125	(1075, 1177)	132	428	(365, 501)	78	<0.001
CD8	564	(524, 606)	132	970	(879, 1071)	78	<0.001
$CD3 - (CD4 + CD8)^c$							
(calculated DN)	28	(8, 48)	34	87	(59, 115)	30	<0.001
$CD3^+CD4^-CD8^-^c$							
(calculated DN)	56	(46, 69)	34	67	(53, 87)	30	0.257
NK ($CD56^+CD3^-$)	170	(150, 193)	110	81	(66, 99)	60	<0.001
δ -TCR	62	(56, 70)	132	81	(68, 96)	77	0.010
δ -TCR/ $CD8^+$	15	(12, 20)	50	27	(19, 37)	41	0.009

^a Geometric mean numbers of cells/mm³.

^b 95% confidence interval of the mean.

^c Data for these two groups were derived from the same individuals.

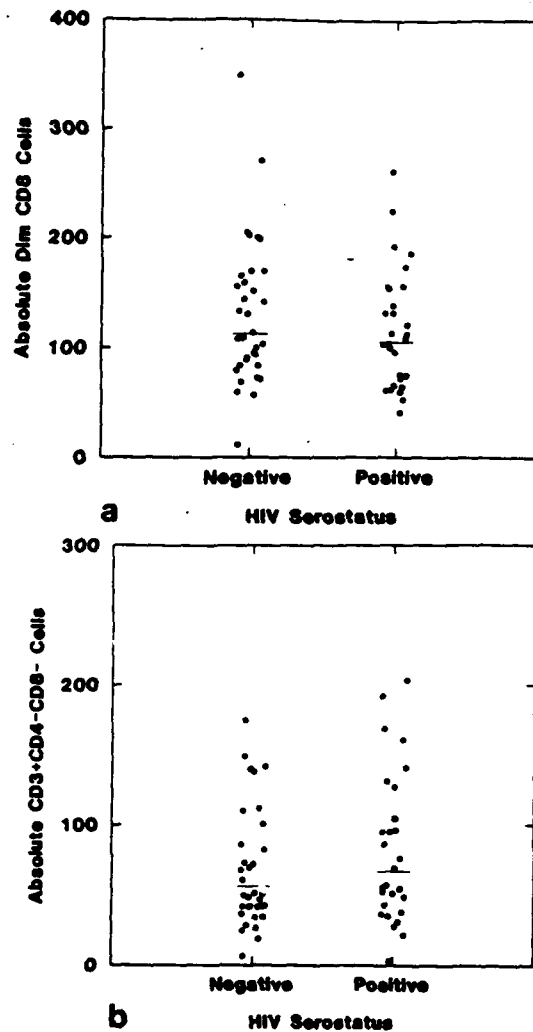


FIG. 2. Plot of absolute numbers (per mm³) of lymphocytes in peripheral blood of HIV-1 seronegative and seropositive individuals studied: (a) CD8^{dim} lymphocytes and (b) CD3⁺CD4⁻CD8⁻ (double negative) lymphocytes. Solid lines indicate the geometric means for each group. Points have been displaced slightly laterally and vertically for clarity.

not express CD8. All CD8⁺ $\gamma\delta$ -TCR⁺ cells expressed CD8^{dim} (data not shown). As shown in Table 1 and Fig. 4a, SP had slightly higher numbers of $\gamma\delta$ cells than SN, and this difference was statistically significant. Of note, the subset of $\gamma\delta$ T cells expressing CD8 was statistically higher in the SP than in the SN (Table 1 and Fig. 4b) and accounted for most (12 out of 19 cells/mm³) of the difference in $\gamma\delta$ -TCR⁺ cells. Because of these differences, CD8⁺ cells represented a geometric mean of 30% of $\gamma\delta$ cells in the SN but more than 41% of $\gamma\delta$ cells in the SP.

Because of their lower levels of NK cells and higher levels of CD8^{dim} $\gamma\delta$ T cells, SP had a higher percentage of $\gamma\delta$ cells expressing CD3 among the CD8^{dim} popu-

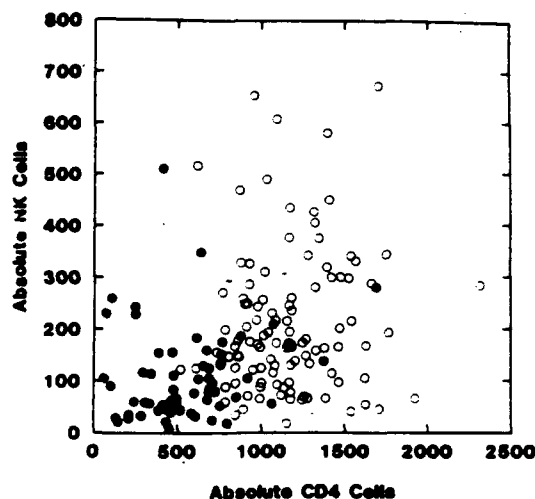


FIG. 3. Scattergram of relationship between absolute numbers (per mm^3) of NK cells and CD4^+ lymphocytes in HIV-1 seronegative subjects (open circles) and HIV-1 seropositive subjects (closed circles).

lation than the SN group (31% vs 14%, $P = 0.002$; Fig. 5). In 7/30 SP cases, the proportion of $\gamma\delta$ T cells among the CD8^{dim} population exceeded 50%, and in 15/30 cases the proportion was more than 25%. In contrast, only 3 of 34 SN exceeded 25%. Thus, the CD8^{dim} cells were composed predominantly of NK cells in the SN group, but frequently contained a large proportion of $\gamma\delta$ T cells in the SP group.

The relationship between the number of calculated [$\text{CD3}^+ - (\text{CD4}^+ + \text{CD8}^+)$] cells and the number of directly measured $\text{CD3}^+\text{CD4}^-\text{CD8}^-$ (true DN) cells is shown in Fig. 6. These parameters were significantly correlated ($r = 0.48$; $P < 0.001$), and this correlation was similar for both SN and SP groups, although the range of values observed was greater in the latter group. In contrast, there was no significant correlation between the number of calculated $\text{CD3}^+ - (\text{CD4}^+ + \text{CD8}^+)$ and the number of CD8^{dim} cells when all study participants were analyzed ($r = 0.016$; $P > 0.10$). However, significant correlations for these parameters were observed when the serostatus of the individual was taken into account ($r = -0.47$, $P = 0.029$ for SN; $r = 0.42$, $P = 0.037$ for SP). That these parameters were oppositely correlated in the two serostatus groups probably reflects the differing types of CD8^{dim} cells present in each group, as noted above.

DISCUSSION

In this study we analyzed differences in numbers of NK cells and $\gamma\delta$ T cells in groups of HIV-1 seronegative and AIDS-free HIV-1 seropositive individuals, primarily homosexual or bisexual men. The most striking finding was a much lower number of NK cells in the SP group, as measured by the $\text{CD56}^+\text{CD3}^-$ or $\text{CD56}^+\text{CD16}^+\text{CD3}^-$ phenotypes. SP also had higher numbers of $\gamma\delta$ T cells, most of this difference being accounted for by a 80% rise in the subset of these cells which expresses dim levels of CD8. These differences are most likely due to

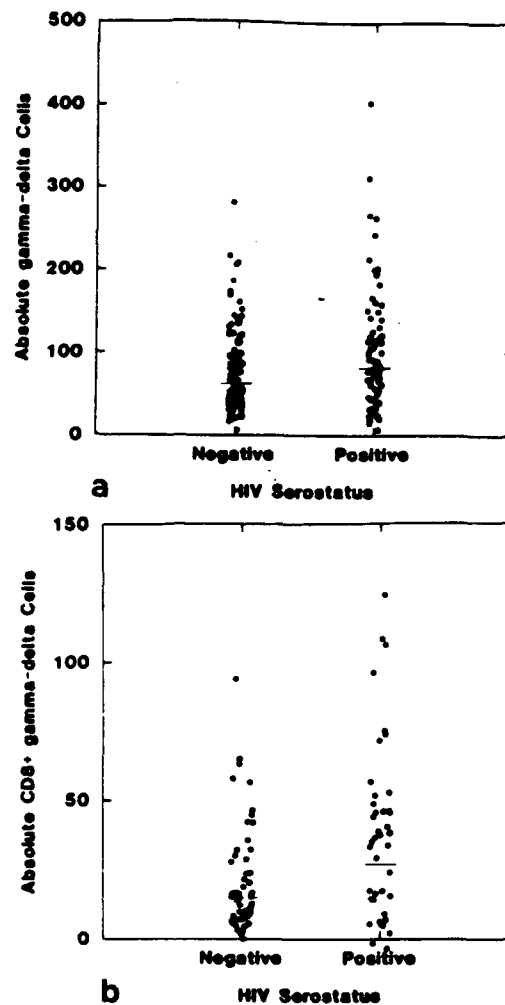


FIG. 4. Plot of absolute numbers (per mm³) of circulating (a) $\gamma\delta$ -TCR T cells and (b) $\gamma\delta$ -TCR T cells expressing CD8 in peripheral blood of HIV-1 seronegative and seropositive individuals studied. Solid lines indicate the geometric means for each group. Points have been displaced slightly laterally and vertically for clarity.

infection with HIV-1, because the SP studied had few clinical symptoms or infections other than HIV; however, this conclusion remains to be directly demonstrated in prospective studies.

Although it is sometimes assumed that CD8^{dim} lymphocytes represent NK cells, our data indicate that in SN a mean of 14% of these cells were actually $\gamma\delta$ T cells expressing CD3, and that this figure was substantially higher in many SP. However, these data should be considered approximate since they are based on a somewhat arbitrary method for discriminating between CD8^{dim} and CD8^{bright} cells. Imprecision in this discrimination may account for our inability to find a

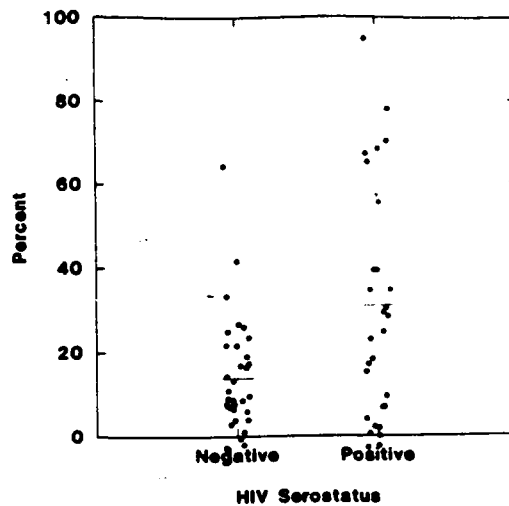


FIG. 5. Plot of proportion of circulating $CD8^{dim}$ lymphocytes in peripheral blood of HIV-1 seronegative and seropositive individuals studied which express the $CD3^{+} \gamma\delta-TCR^{+}$ phenotype. Data are calculated as $(\%TCR\delta-1^{+} \div \%CD8^{dim}) \times 100$. Solid lines indicate the geometric means for each group. Points have been displaced slightly laterally and vertically for clarity.

difference in $CD8^{dim}$ cells between the SN and SP subject groups: alternatively, it is possible that some NK cells in some individuals express levels of CD8 that are bright enough to overlap with those found on T cells. Although technical factors can dramatically affect the measurement of dimly positive markers such as $CD8^{dim}$ or CD56 on lymphocytes, these factors were minimized in the present

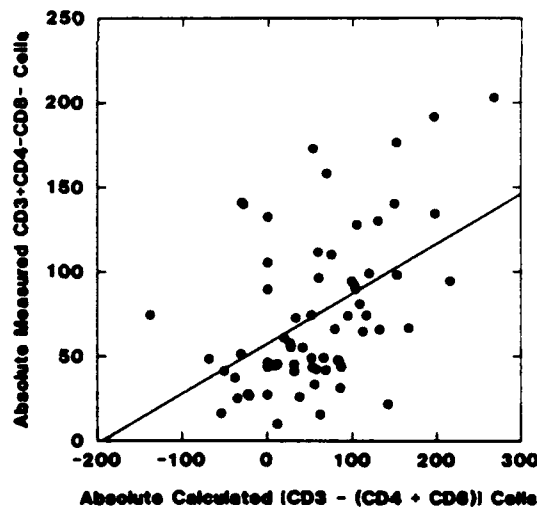


FIG. 6. Scattergram showing relationship between calculated $CD3^{+} - (CD4^{+} + CD8^{+})$ and directly measured DN (i.e., $CD3^{+}CD4^{-}CD8^{-}$) lymphocytes in subjects studied. Calculated regression line is $y = 57.4 + 0.295x$; correlation coefficient is 0.48.

study by participation in the flow cytometry quality program of the MACS, as described under Materials and Methods.

The data obtained in this study support the interpretation that the large rise in calculated $CD3^+ - (CD4^+ + CD8^+)$ observed in the first year after seroconversion in our earlier study (10) is due primarily to a fall in $CD8^{dim}$ NK cells. The magnitude of the increase observed in that study (approximately 40 cells/mm³) is too large to be explained by changes in $CD3^+CD4^-CD8^-$ or $\gamma\delta$ lymphocytes, but would be entirely consistent with the magnitude of the difference in the number of NK cells observed in the present study and reported data that approximately 30–50% of NK cells express CD8 (11, 12). There are no reported prospective studies of changes in NK cell numbers in relation to HIV-1 seroconversion, and most cross-sectional studies have found little or no change in $CD16^+Leu7^+$ cells in the early stages of HIV-1 infection (reviewed in (28)). However, Vuillier *et al.* (29) found a decrease in NK cell number in HIV-1 infection using anti-CD56 antibodies, which most accurately identify cells with NK activity. Our results extend these by demonstrating that the decrease is in $CD56^+$ cells that are $CD3^-$. On the other hand, several studies have shown a decrease in NK cell function in HIV-1 SP compared to SN (reviewed in (28)), and anti-HIV-1 NK activity has been reported (30, 31). Taken together, the available data suggest that NK cells decrease very soon after HIV-1 seroconversion, along with NK cell function, and that this decrease in NK cell number may predispose to progression of HIV-1 infection. The more gradual rise in calculated $CD3^+ - (CD4^+ + CD8^+)$ seen beyond the first year after seroconversion could be due to a further decline in NK cells, to the increase in true DN T cells observed in the present study, or a combination of both.

The present findings of higher levels of $\gamma\delta$ T cells, and particularly the $CD8^-$ subset of these cells, confirm and extend the recent study of Autran *et al.* (32), who reported that the total number of $\gamma\delta$ cells was slightly, although not significantly, higher in HIV-1 seropositive individuals compared to seronegatives. These authors also found significantly higher levels of the subset of $\gamma\delta$ cells recognized by the monoclonal antibody δ TCS-1. Thus, it appears that infection with HIV-1 may preferentially alter the expression of distinct populations of $\gamma\delta$ cells out of proportion to relatively small effects on the total numbers of these cells. While the importance of these changes in subsets of $\gamma\delta$ T cells remains to be determined, anti-viral activity and anti-mycobacterial activity by $\gamma\delta$ T cells have been reported (13, 33, 34) and could represent important mechanisms of host defense against HIV-1 and/or opportunistic infections. Moretta *et al.* (35) have reported that $CD8^+ \gamma\delta$ cells express a 55-kDa product of the $C_{\gamma 2a}$ gene instead of the 42-kDa protein more commonly found on $\gamma\delta$ cells, and that expression of CD8 was associated with expression of the BB-3 antigen found on thymic $\gamma\delta$ cells. These observations suggest that $CD8^+ \gamma\delta$ cells may represent immature T cells and possibly precursors of mature T cells in the peripheral blood.

Whether any of the differences in lymphocyte populations observed between SP and SN have prognostic importance for HIV-1 infection remains to be determined from prospective studies. Such studies will be facilitated by a better un-

derstanding of the changes in lymphocyte populations occurring in relation to HIV-1 infection.

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Effect of Donor and Recipient Immunization Protocols on Primary and Secondary Human Antibody Responses in SCID Mice Reconstituted with Human Peripheral Blood Mononuclear Cells

RICHARD B. MARKHAM^{1,2*} AND ALBERT D. DONNENBERG^{1,3}

*Departments of Immunology and Infectious Diseases,¹ Medicine,² and Oncology,³
Johns Hopkins Health Institutions, Baltimore, Maryland 21205*

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We have examined the ability of mice with severe combined immunodeficiency (SCID mice) reconstituted with human peripheral blood mononuclear cells (PBMC) to generate human antibody responses after specific immunization. SCID mice reconstituted with cells from a keyhole limpet hemocyanin (KLH)-naive donor are unable to generate specific human antibody responses after immunization with that antigen. After KLH immunization, SCID mouse recipients of human PBMC from a KLH-immune subject develop specific human antibody levels exceeding those of the donor. Human antitetanus antibody titers in reconstituted, immunized mice are also equivalent to those of the donor, provided that the mice are immunized within days of human cell transplantation. The ability of reconstituted mice to generate high titers of specific human antibody is lost within 35 days of human cell reconstitution, even though titers of total human immunoglobulin (Ig) are preserved. SCID mice reconstituted with tetanus-immune donor cells fail to generate IgA responses after booster immunization, and IgM responses are low or nonexistent. These data indicate that early exposure of the adoptive recipients of human cells to antigen is required to transfer specific human humoral responses. These findings are also consistent with a requirement for persistence of antigen for the maintenance of B-cell memory. The ability to achieve specific human antibody levels equivalent to those obtained with humans indicates that reconstituted mice may be useful for the evaluation of human antibody-mediated mechanisms of resistance to infection. The data indicate, however, that cells from immunized donors will have to be used for such studies.

CB.17 *scid/scid* mice (SCID mice) lack functional B and T lymphocytes and therefore are unable to reject allogeneic and xenogeneic tissue grafts (1). Two groups of investigators have demonstrated that human lymphocytes can be transferred into these mice, with resultant spontaneous production of human immunoglobulin (Ig). In one of these models (the SCID/Hu mouse model), the mice are reconstituted with human fetal liver, thymus, and lymph nodes (5). In the other, the Hu-PBL/SCID mouse model, the mice are reconstituted by intraperitoneal (i.p.) transplantation of human peripheral blood mononuclear cells (PBMC) (6). In both the Hu-PBL/SCID and SCID/Hu mouse models, circulating human Ig can be detected within a few weeks of transplantation of human cells or tissues. In the case of the Hu-PBL/SCID mice, specific antibody responses to tetanus toxoid (TT) immunization have also been demonstrated (6).

Much of the interest in reconstituted SCID mice derives from their potential use for studying immunoprophylactic and therapeutic treatments of cells infected with human immunodeficiency virus. Since human lymphocytes survive and function in the mice for periods of several months (6), the mice potentially provide a practical alternative to studies with primates, which are more expensive and much less accessible.

Use of this mouse model to analyze immunoprophylactic regimens will depend on the generation in the mice of human immune responses that are equivalent in magnitude to those obtained with immunized humans. Previous studies of specific immune responses to TT in Hu-PBL/SCID mice dem-

onstrated antibody levels which were 5 to 10% of those observed with immunized humans (6). The current studies were undertaken to determine the optimal conditions under which SCID mice reconstituted with human PBMC generate specific human antibody in response to *in vivo* antigenic challenge.

Our results indicate that donor immune status and the timing of recipient immunization are critical to the successful transfer of human humoral immunity to murine graft recipients. Hu-PBL/SCID mice are unable to generate primary immune responses to exogenous antigens to which the donor has never been exposed. However, with appropriate immunization regimens for human donor and recipient mice, Hu-PBL/SCID mice can generate human antibody responses which are equivalent in magnitude to those observed with immunized humans.

MATERIALS AND METHODS

Mice. SCID mice were raised in a colony maintained at the Johns Hopkins Hospital Oncology Center. The colony was derived from mice kindly provided by Leonard Shultz of the Jackson Laboratory, Bar Harbor, Maine. The mice were maintained in microisolator cages (Lab Products, Maywood, N.J.) were fed autoclaved mouse chow, and received trimethoprim-sulfamethoxazole (Roche Laboratories, Nutley, N.J.) (5 ml of a suspension of 80 mg of trimethoprim and 400 mg of sulfamethoxazole in 500 ml of acidified drinking water) 3 consecutive days per week. Donors of human cells were normal subjects who had been immunized with TT or keyhole limpet hemocyanin (KLH). In the case of TT, the exact time of immunization is known only for the recently

* Corresponding author.

immunized subjects (indicated in Results). For subjects not recently immunized, the times since their last immunizations exceed at least 3 years. The KLH-immune donor had last been immunized 18 months previously as part of a regimen involving three KLH immunizations over a 1-year period. Heparinized blood was obtained from these donors, and PBMC were separated from erythrocytes and granulocytes by Ficoll-Hypaque density gradient centrifugation. Eight-week-old mice were reconstituted by administration of 1.25×10^7 of these PBMC by the i.p. route.

Immunization of mice. Mice were inoculated i.p. with 1.0 Lf units of TT (Wyeth Laboratories, Marietta, Pa.) or 25 μ g of KLH (Sigma Chemicals, St. Louis, Mo.) adsorbed to alum at the indicated time intervals after transplantation of human PBMC.

Antigens for in vitro assays. Preservative-free TT was the generous gift of Frank McCarthy, Wyeth Laboratories.

ELISA antibody detection. Total human anti-TT and anti-KLH antibodies, as well as specific IgA and IgM antibodies, were detected by enzyme-linked immunosorbent assay (ELISA). Purified TT (0.09 μ g per well) and KLH (0.2 μ g per well) antigens were diluted in carbonate buffer (pH 9.6) and adsorbed to 96-well microtiter plates (Immulon 2; Dynatech, Chantilly, Va.). After being washed three times in phosphate-buffered saline (PBS)-Tween, the plates were blocked with 5% bovine serum albumin (BSA) (1 to 2 h, 37°C). The plates were then washed and serial serum dilutions were added (eight fourfold dilutions starting at 1:8). Sera were incubated overnight at 4°C and washed again. Goat anti-human IgG (heavy- and light-chain specific) alkaline phosphatase conjugate (Kirkegaard & Perry, Gaithersburg, Md.), diluted 1:50,000 in PBS-Tween-BSA, was added to the plates and incubated for 2 h at 37°C. For measurement of IgA and IgM antibodies, alkaline-phosphatase-conjugated heavy-chain-specific goat anti-human IgA (diluted 1:4,000) and IgM (diluted 1:10,000) antibodies were used (Jackson Immuno-Research Company, Inc., West Grove, Pa.). The plates were washed, and *p*-nitrophenylphosphate substrate in diethanolamine buffer (pH 9.8) was added. After incubation for 30 min in the dark at room temperature, 3 M sodium hydroxide was added to each well and the plates were read at 405 nm. The titers of antibody in the donors and the mice are expressed as the reciprocal of the highest dilution at which optical density values above background were observed. Because no individuals who had not been immunized against TT could be identified, control wells for the human sera obtained directly from the human subjects were simply microwells to which no experimental sera were added. Control wells for measurement of human antibody in sera from Hu-PBL/SCID mice contained normal mouse serum. Human sera for titers of preexisting antibody in the donors were obtained at the time that the PBMC were obtained for reconstitution of the mice. For some studies, quantities of specific antibody were determined by comparing optical density readings of the experimental sample with those generated by standard curves of human Ig run in the same assay.

Human subjects. Fifty to 200 ml of human peripheral blood was obtained from subjects before and after immunization with TT (10 Lf units in 0.5 ml) or KLH (100 μ g) adsorbed to aluminum phosphate, administered intramuscularly. Immunizations and phlebotomy for reconstitution of SCID mice were approved by institutional human studies review committees.

TABLE 1. Effect of donor immune status and recipient booster immunization on generation of human antibody to KLH in Hu-PBL/SCID chimeric mice

Day after cell transfer	Booster immunization 3 days after transfer	Reciprocal titer ^a in mice immunized with cells from:	
		KLH-immune donor ^b	KLH-nonimmune donor
3	No	<4	<4
10	No	64	<4
10	Yes	640	<4
17	No	2,560	<4
17	Yes	>10,240	<4

^a Geometric mean titer of three microwells containing pooled serum samples from three mice.

^b Immunized 18 months previously; anti-KLH titer, 1:6,000.

RESULTS

Response of Hu-PBL/SCID mice to primary immunization.

Twenty SCID mice were each reconstituted i.p. with 2×10^7 human PBMC from either a KLH-immune donor (with an anti-KLH titer of 1/6,000; donor was immunized with KLH for the third time over a 1-year period ending 18 months prior to study) or a nonimmune donor. Three days after cell transfer, mice were randomized to receive KLH (i.p.; 25 μ g adsorbed to aluminum phosphate) or no treatment. Two mice from each group were bled and sacrificed on days 3, 10, and 17 after cell transfer. Anti-KLH antibody in serum was measured by ELISA. The antibody levels achieved with different immunization protocols are shown in Table 1. By 17 days after transfer of cells, mice reconstituted with cells from the KLH-immune donor achieved a titer of circulating KLH-specific human Ig that was approximately one-third that observed with the donor. However, if the mice received a booster immunization 3 days after the human cells were transferred, the human antibody response in the mice exceeded that which existed in the donor. Mice that received PBMC from a human donor who lacked antibody to KLH failed to generate any detectable antibody response, even if the recipient mice received booster immunizations *in vivo* with KLH. Mean levels of total circulating human Ig in recipients of KLH-immune and KLH-nonimmune PBMC were both $>5 \mu$ g/ml, indicating that both groups of mice were engrafted with human cells.

Adoptive transfer of human immune memory responses to SCID mice: effect of donor and recipient immunization. The previous experiments indicated that donor immune status was a critical determinant of whether Hu-PBL/SCID mice could generate any specific human antibody response. We next evaluated the influence of timing of donor and recipient immunization on the magnitude and maintenance of immune responsiveness subsequent to xenografting human PBMC into SCID mice. Groups of three 6- to 8-week-old SCID mice were transplanted i.p. with 1.25×10^7 PBMC obtained from a single human donor either before or after booster immunization of that donor with TT. Prior to receiving a booster immunization for these studies, the human donor had not received TT immunization in at least 10 years. The recipient SCID mice were immunized 3 or 35 days after cell transfer with 0.1 ml of TT (10 Lf/ml). The TT protocol was repeated with cells obtained from the same donor 10 days after he received a TT booster immunization (0.5 ml [10 Lf/ml]). Sera, pooled by experimental group, were obtained from recipient mice 0, 14, 35, and 45 days after cell transfer and assayed by ELISA for human IgG to TT.

TABLE 2. Effect of time after reconstitution with human cells of booster immunization on the magnitude of human antibody response observed

Donor boost ^a	Booster immunization 3 days after reconstitution	Booster immunization 35 days after reconstitution	Reciprocal titer ^b on:			
			Day 0	Day 14	Day 35	Day 45
No	No	No	0	8	ND	0
No	Yes	No	0	173	ND	873
No	No	Yes	0	8	0	0
Yes	No	No	0	1,691	3,324	930
Yes	Yes	No	0	30,064	24,079	22,901
Yes	No	Yes	0	1,691	1,118	171

^a Prior to booster immunization, the donor had a TT-specific IgG titer of 12,600. Ten days after booster immunization, this titer rose 19-fold to 49,700. At this time point, cells were harvested from the donor for reconstitution of the mice.

^b Geometric mean titer of sera in three microwells. Serum samples from five mice were pooled. ND, not determined.

Adoptive transfer of secondary responses to TT was consistently observed. The magnitude of this response was highly dependent on the immunization protocol (Table 2). Mice that were reconstituted with cells from the donor prior to booster immunization of the donor and that received no immunization had barely detectable specific human anti-TT responses. Similarly, reconstituted mice that were immunized 3 days after engraftment with human cells had a response that was 20-fold higher than that observed with unimmunized, reconstituted mice. However, the specific human antibody levels achieved with the mice were still more than 1 log unit less than those circulating in the human donor. Waiting until 35 days after human cell reconstitution to give the TT booster immunization to the reconstituted mice resulted in a loss of effect of the booster immunization, suggesting a loss of the memory B cells producing anti-TT antibody.

Use of cells from the same human donor which were obtained 10 days after donor immunization resulted in a marked enhancement of the specific antibody responses that could be obtained with reconstituted SCID mice. At the time that cells were obtained from the donor, the donor had a reciprocal antibody titer of 49,000. By giving the mice booster immunizations of TT 3 days after cell transfer a reciprocal antibody titer of 30,064 was obtained with mice, a level essentially equivalent to that observed with the human donor. This level of antibody was effectively maintained for 45 days, the last time point examined. Again, waiting until 35 days after transplantation of human cells into the mice to provide the booster immunization of the mice resulted in a significant reduction in the magnitude of the human antibody response observed compared with the response of mice given booster immunizations 3 days posttransplantation.

Induction of human IgA and IgM antibodies by secondary immunization of Hu-PBL/SCID mice receiving PBMC from TT-primed donors. To further characterize the secondary antibody response in Hu-PBL/SCID mice, mice were reconstituted with cells from TT-immune donors other than those described above, and quantitative levels of total TT-specific Ig and specific IgA and IgM antibody levels were determined. The time of TT immunization of these donors was not known beyond the fact that they were not recently immunized, and their immune status was therefore reflected in their preexisting TT-specific antibody levels. None of the three subjects used for these studies had preexisting anti-

TABLE 3. Magnitude of specific total Ig, IgM, and IgA responses observed with Hu-PBL/SCID mice given booster immunizations of TT

Donor	TT-specific antibody in donor (µg/ml)			TT-specific antibody in TT-boostered ^a mice (µg/ml)		
	Total Ig	IgM	IgA	Total Ig	IgM	IgA
A	5	4	<1	28	<1	<1
A	5	4	<1	73	<1	<1
A	5	4	<1	56	1	<1
B	23	<1	<1	18	<1	<1
C	11	<1	<1	32	<1	<1
C	11	<1	<1	6	<1	<1

^a TT-boostered mice, mice given booster immunizations of TT.

body to KLH. Mice reconstituted with cells from these subjects and given booster immunizations of KLH (three mice with cells from subject A, one mouse with cells from subject B, and two mice with cells from subject C) failed to develop any detectable anti-KLH antibody. The data in Table 3 confirm that the level of antibody generated by giving the reconstituted mice booster immunizations of TT, to which the donors were immune, was related to the preexisting antibody level of the donor. Although some of the human cell donors possessed low levels of TT-specific IgA or IgM, only one of the mice responded to secondary immunization with either an IgA or IgM TT-specific antibody response, and that IgM response was minimal (1 µg/ml). It thus appears that Hu-PBL/SCID mice are capable of generating only IgG secondary responses. The data in this table also demonstrate the variability in the immune responses observed with different mice reconstituted with cells from the same donor. The basis for this variation is unclear.

DISCUSSION

These studies were designed to characterize the ability of Hu-PBL/SCID mice to generate human antibody responses. We initially examined the response to an antigen to which most humans are not immune, KLH. SCID mice reconstituted with cells from nonimmune individuals were unable to produce any detectable specific antibody response, even when recipient mice were given booster immunizations within 3 days of cell transfer. Mice reconstituted with cells from an individual who had been immunized with KLH 18 months earlier attained, after *in vivo* booster immunization of the mice, levels of circulating specific human IgG that exceeded those of the donor of the human cells (Table 1). However, mice that received cells from a KLH-nonimmune donor failed to generate any KLH-specific antibody upon immunization.

Similarly, mice reconstituted with PBMC from an individual whose last immunization against TT had occurred more than 10 years previously had a minimal anti-TT response (Table 2). After the donor, whose TT immunization status had declined over 10 years, was given a booster immunization, mice reconstituted with his cells and immunized *in vivo* were able to generate an anti-TT response that yielded levels of circulating specific antibody approaching those observed with the human donor (Table 2). It thus appears that reconstituted mice are unable to generate primary immune responses to exogenous proteins and that the magnitude of the secondary response observed with boosted mice can be directly correlated with the immune status of the cell donor.

Furthermore, the TT immunization studies (Table 2) indi-

cate that the timing of the booster immunization of the mice is critical to the generation of an adoptive response. While SCID mouse recipients of immune cells generated a significant antibody response if given booster immunizations 3 days after receipt of immune cells, similar mice given booster immunizations 35 days after cell transfer failed to demonstrate any significant response.

At least two possible explanations for the failure of reconstituted SCID mice to develop primary human antibody responses could be proposed, and both are related to a deficiency in critical cells required for the generation of an antigen-specific response. It should be emphasized that the cells being placed in the mice are derived from peripheral blood. Therefore, the ability to generate a specific response is going to depend on the number of antigen-specific circulating lymphocytes. This number is likely to be higher in recently immunized individuals than in individuals who were immunized in the past (2).

The ability of mice to generate an antibody response might also be related to the presence in the peripheral blood of a number of antigen-presenting cells sufficient to activate the helper T cells involved in the response to either KLH or TT, both of which are T-cell-dependent antigens (3, 7). The available data indicate that macrophages survive less well in PBMC-reconstituted SCID mice than do lymphocytes (6). If sufficient antigen-specific B cells were present in the transplanted cells, these cells might function as antigen-presenting cells, obviating the need for macrophages. Nonimmune donors would lack such a pool of expanded antigen-specific B cells and would therefore be more dependent for the activation of helper T cells on the presence of other antigen-presenting cells. Thus, the generation by primary or secondary immunization of circulating specific B cells in the peripheral blood of the human cell donor might be essential for both generation of T-cell help and for actual antibody production.

The requirement for in vivo boosting within a short time period after cell transfer suggests that the failure to stimulate the specifically reactive clones results in the loss of the ability to generate secondary responses as well. This rapid disappearance of immune response capability is unlikely to represent a general loss of human cells from reconstituted mice, as immunized mice continue to produce human antibody for a period of at least several months (6 and unpublished observations). However, this observation may reflect preferential loss of memory cells in these mice. The maintenance of clones of a given specificity appears to require stimulation of that clone at a time when specific responsive cells are most abundant. This finding is consistent with the observations of others, indicating that the presence of continuous antigenic stimulus is required for persistence of memory B cells (8), although a loss of specific helper T cells in this setting cannot be excluded.

Mazingue et al. (4) have recently reported the obtainment of primary immune responses to schistosome antigens in SCID mice reconstituted with PBMC from a healthy donor. While this donor was free of schistosomiasis, the authors provided no evidence that the healthy donor had no preexisting immunity to the schistosome antigens, which could have been elicited by exposure to a cross-reacting antigen. Thus, it is unclear that the response obtained with the mice was truly primary. The investigators also employed complete Freund's adjuvant in their immunization protocol, which might be an alternative explanation for their ability to

elicit primary responses. Since the mice in the study by Mazingue et al. were reconstituted with only 5×10^6 to 10×10^6 human PBMC, however, it seems unlikely that the unprimed donor would contain enough circulating precursors to reconstitute a detectable specific response in the mice.

Because it is difficult to recover human cells from reconstituted mice sufficient to perform in vitro assays of effector T-cell responses, we have not evaluated functions such as cytotoxic T-cell activity, mediated by CD8⁺ T cells, in reconstituted SCID mice. However, as indicated, antibody responses to the antigens being studied are known to require the activity of CD4⁺ helper T cells, and the presence of such responses is indicative of helper T-cell as well as B-cell functions.

These studies indicate that reconstituted SCID mice could prove to be useful for the evaluation of candidate vaccines designed to elicit protective humoral immune responses, but only under defined conditions. The cells used to reconstitute the mice must be obtained from individuals who have already been vaccinated. In addition, optimization of the human antibody response requires early booster immunization of the reconstituted mice. The ability to challenge such mice will be particularly useful in evaluating the protective efficacy of vaccines against pathogens, such as human immunodeficiency virus, which could not be used in human clinical trials.

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Revised

Margolick-1

Comparison of lymphocyte immunophenotypes obtained simultaneously from two different data acquisition and analysis systems on the same flow cytometer¹²

Joseph B Margolick³, Elvia R Scott³, Karen R Chadwick³, Howard M Shapiro⁴, A Dorn Hetzel⁵, S Jay Smith⁶, Robert F Vogt⁶

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³ Johns Hopkins School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, MD 21205 Telephone: 301-955-4712 Fax: 301-955-0116

⁴ Howard M. Shapiro, M.D., P.C., 283 Highland Ave., West Newton, MA 02165

⁵ Fabscale Systems, Inc., Box 77030, Atlanta GA 30357

⁶ Div. of Environmental Health Laboratory Sciences, Centers for Disease Control, Atlanta GA 30333

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Running Title: Data acquisition and analysis comparisons

Abstract

Immunophenotyping of different lymphocyte populations was carried out in parallel on 113 consecutively received specimens of human peripheral blood using two different data acquisition and analysis systems (EPICS C and 4Cyte-Acmecyte) on the same flow cytometer (EPICS C). The phenotypes analyzed were CD3⁺, CD4⁺, CD8⁺, CD56⁺CD16⁺CD3⁻, TCR- $\gamma\delta$ ⁺CD8⁻, and TCR- $\gamma\delta$ ⁺CD8⁺. Both HIV⁻ and HIV⁺ specimens were used for this study, including some with CD4 levels as low as 2% of all lymphocytes. Despite differences in gating procedures and shapes of bitmap (rectilinear vs. "amorphous"), the two methods agreed to within 2% positive cells in 97% of the cases. Although some statistically significant biases in the methods were observed, these were small and not biologically important. We conclude that both methods of data acquisition and analysis, as employed by experienced operators on the EPICS C flow cytometer, gave essentially equivalent results for lymphocyte sub-populations in peripheral blood preparations.

Key terms: Immunocytometry, data acquisition, data analysis, flow cytometry, T cell subsets, NK cells, $\gamma\delta$ T cells, HIV

Introduction

Flow cytometry has become the accepted method for immunophenotyping clinical specimens. The results obtained using this technology depend critically on both the hardware (e.g., laser, lenses, photomultiplier tubes) and software (e.g., for setting of displays, bitmaps, and gates, and tabulating statistics) used for data acquisition and analysis. In theory, variations in data could arise from either of these independent systems. However, the lack of instrument bias noted in previous surveys (3,9) suggests that differences in hardware are not a major source of variation. In contrast, differences in data analysis, particularly the selection of gated populations, can be major contributors to variation in results (4).

To evaluate separately the importance of the data acquisition and analysis aspects of the flow cytometer, it is necessary to compare data obtained simultaneously on the same events by two different data acquisition and analysis systems. However, to our knowledge there are no reports where this has been done. Therefore, the present study was undertaken to determine whether measurements of lymphocyte phenotypes obtained on two parallel systems of acquisition hardware and software were equivalent or biased. Specifically, phenotypes of clinical or research importance were examined, and comparisons were made of both overall means and the variability of the measurements obtained with the two parallel systems.

Methods

Sample preparation. Heparinized peripheral blood was obtained from participants in the Baltimore center of the Multicenter AIDS Cohort Study (MACS). Participants in MACS are homosexual or bisexual men, of whom approximately one-third have antibodies to HIV-1 as determined by ELISA with confirmatory Western blot (7). Specimens were prepared according to MACS protocols (1), based on an ammonium chloride-based whole blood-lyse method described previously (2). Monoclonal antibodies (see below) were added to 100 μ l of whole blood for 30 min. The blood was lysed for 10 min (2 x 5 min) and washed, and the cells were resuspended in 1% formaldehyde (Ultrapure EM grade, Polysciences, Warrington, PA) for at least 10 min at 4°C before 2-color flow cytometric analysis.

The monoclonal antibodies used (obtained from Becton-Dickinson, Sunnyvale, CA, except as noted) were CD3-FITC and CD8-FITC/CD4-PE (NIH Natural History of AIDS Kit), CD3-FITC/CD56-PE/CD16-PE (Natural Killer Cell Kit), and TCR δ -1 (anti δ -chain of T cell receptor; T cell Sciences, Cambridge, MA)/CD8-PE. The phenotypes measured were CD3⁺, CD4⁺, CD8⁺, CD3⁻CD56⁺CD16⁺, TCR δ -1⁺CD8⁻, and TCR δ -1⁺CD8⁺. The CD45-FITC/CD14-PE reagent (Leucogate) was also included to assess the proportions of monocytes and unstained debris within the lymphocyte bitmap.

Acquisition of flow cytometric data. All studies were performed on an EPICS C Flow Cytometer (Coulter Electronics, Hialeah, FL 33015) equipped with the biohazard closed flow tip (250 μ m orifice). The argon laser adjusted for 488 nm emission was used at 700-900 mW power. Four signals (FS, SS, FL1 - FITC, FL2 - PE) were collected. The FITC fluorescence signals came through a

488 nm long pass dichroic filter, a 488 nm long pass blocking filter, a 550 nm short pass dichroic filter, and a 525 nm bandpass filter. PE fluorescence signals came from the second dichroic filter through a 575 nm bandpass filter. PMT voltages were 230-260 for side scatter, 1250-1275 for FITC fluorescence, and 1170-1200 for PE fluorescence. Forward and side scatter signals were directed through linear amplifiers, and fluorescence signals through 3-decade logarithmic amplifiers.

The first software system for data acquisition and analysis was the EPICS C software. Data acquired by this system went through 256-channel analog-to-digital converters (ADCs) and were displayed, saved, and analyzed as histograms (see below). The second software system was the 4Cyte-Acmecyte system, composed of 4Cyte hardware (6) and Acmecyte data acquisition and analysis software we have recently developed for pooling calibrated data from interlaboratory collaborative studies (8). Data acquired by this system were taken simultaneously from test points on the EPICS C amplifier circuit boards for the four parameters. The signals were sent through a front end signal processor (4Cyte FE, HM Shapiro, M.D., P.C., West Newton, MA). Signals were then sent to the 256-channel ADCs mounted on a data acquisition board (4CyteI, H.M. Shapiro, MD., P.C.) in a 486/25 IBM-compatible personal computer. Buffer memory on the acquisition board temporarily accumulated up to 2048 events of digitized data, which were then written to list mode files on a hard disk.

Display and Analysis of acquired data. Data acquired on the EPICS C were displayed on a monochrome monitor as 2-dimensional (2-D) histograms showing 64x64 channels (4-channel resolution of the original 256-channel data). One of the 2-D histograms showed FS by SS at three different intensities that discriminated histogram coordinates containing 1 event, 2-6

events, and 12 or more events (Fig. 1a). The lymphocyte cluster was selected by visual inspection by one of the authors (KC or ES) and surrounded by an "amorphous" bitmap, i.e., a bitmap formed by an n-sided polygon with n ranging from 8 to 15 in most cases. 5000 events within the FS-SS bitmap were acquired and displayed in the FL1-FITC and FL2-PE 2-D histogram, and "quadstat" cursors were set by visual inspection to select the negative, single positive and double positive populations. For most markers, results to the nearest 1% were transcribed manually from the screen display and typed into a computer spreadsheet program. For the TCR- $\gamma\delta$ phenotypes, the total event counts in each quadstat region were transcribed, and results to the nearest 0.1% were computed from the total lymphocyte event count. All manually transcribed results were cross-checked for accuracy.

Data acquired on the 4Cyte-Acmecyte system were displayed on a VGA color graphics screen as 2-D histograms at full resolution (256x256 channels at one pixel per channel), using the Acmecyte software program (Fabscale Systems, Atlanta, GA). The number of events per histogram coordinate (pixel) was depicted by three different colors: one event was displayed as gray, 3 or 5 events as magenta, and more than 6 events as yellow (Fig. 1b). Coordinates with event counts of 2, 4, or 6 were not shown (i.e., displayed as black) in order to sharpen the perceived boundaries of the clusters so that they appeared as similar as possible to clusters on the EPICS C. The lymphocyte cluster was selected on the FS-SS histogram by visual inspection and surrounded by a rectangular gate with the sides parallel to the horizontal and vertical axes (referred to as a "rectilinear" gate). Boundary selection for the gate using the 4Cyte-Acmecyte system was performed by the same operator as for the EPICS C, and was aided by 1-dimensional histogram displays

superimposing FS or SS histograms from gated and ungated events (Fig. 1b). Events within the FS-SS gate were acquired and displayed in the FL1-FITC and FL2-PE 2-D histogram and "quadstat" cursors were set as above. Resulting statistics were automatically accumulated in a delimited ASCII file, then imported directly into the spreadsheet program. Percent positive results for all phenotypes were rounded to the nearest 0.1%.

Statistical analysis. The Epics C result was designated as the reference result, and the Acmeocyte result as the test result. The bias for each pair of results was calculated as the arithmetic difference (Acmeocyte - Epics C); thus, a lower Acmeocyte result was defined as a negative bias. The discrepancy for each pair of results was calculated as the absolute value of the bias, reflecting the difference between results in either direction. The percent bias and percent discrepancy were calculated from the ratio of the bias or discrepancy to the reference (Epics C) result. Mean values for bias, percent bias, discrepancy, and percent discrepancy were calculated for each phenotype from all pairs of results.

The data were analyzed and plotted with the SAS System (5). The univariate procedure with a two-tailed t-test was used to determine whether the mean bias for each phenotype was significantly different from zero. To determine if bias varied with respect to the reference result, a distribution plot of all individual biases versus their respective reference results was constructed for each phenotype. This plot was fitted with a spline curve to inspect the results for any biases localized to particular ranges of data. To test formally for overall biases, t-tests were used to determine if slopes and intercepts for the regression of results against biases were significantly different from 0.

Results

The percentages of cells expressing the 5 phenotypes measured were obtained simultaneously on the EPICS C and the 4Cyte-Acmecyte systems on 113 specimens received consecutively in the laboratory. Samples included in the study met the following criteria on both data analysis systems: (1) at least 90% of the cells in the selected lymphocyte population were stained brightly with CD45, and (2) the light scatter histograms showed adequate separation between the major leukocyte populations and debris.

Results are shown in Figure 2 and summarized in Table 1, Table 2, and Figure 3. It should be noted from Fig. 2 that many of the specimens analyzed had markedly depressed levels of CD4⁺ lymphocytes, and thus a significant proportion of abnormal specimens was included in this study. Nevertheless, as shown in Table 1, mean biases for CD3, CD4, and CD8 were less than 0.5% in magnitude, and mean discrepancies were less than 1%. Of the 678 paired results for percent positive cells, 92% differed by no more than 1% positive, and 97% by no more than 2% positive (Figure 3). Three results differed by more than 3% positive, and the largest discrepancy observed was 3.7% positive on a CD3 phenotype.

Depending on phenotype, the mean biases ranged from -0.39% to +0.27%. For five phenotypes, these small biases were consistent enough to show statistical significance (Fig. 1). No definite trends in bias as a function of the magnitude of Epics C result were apparent. When analyzed by linear regression, both the slopes and intercepts of the plot of bias against EPICS C result were very close to 0 for all phenotypes (Table 2). However, again three of the phenotypes had either a slope or an intercept that was statistically different from 0 at $p < 0.05$, although the 95% confidence

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intervals for these values were still quite close to 0.

Discussion

This study provided a rigorous test for the equivalence of results obtained when identical events were acquired and analyzed by two different hardware and software systems. The panel of phenotypes measured ranged from the frequent (e.g., CD3) to the very infrequent (TCR- $\gamma\delta^+$), and from the bright (CD8) to the dim (CD56 $^+$ CD16 $^+$). It also encompassed comparisons of T cells and NK cells, which have differing light scatter distributions. Moreover, many of these samples were from HIV-infected donors, whose blood sometimes yielded less separation between cell clusters and more debris than did normal blood. Nonetheless, despite the inclusion in this study of many HIV $^+$ specimens with greatly decreased proportions of CD4 $^+$ lymphocytes (as low as 2%), comparison of the results showed only small differences which had no biological importance. However, it would be premature to generalize these results to other types of abnormal specimens, such as those from leukemic or severely leukopenic patients.

Some methodological implications of the observed differences may be drawn. First, the magnitude of the biases and discrepancies was relatively consistent across the different phenotypes, despite the large differences in percent positive results between phenotypes. Thus, the influence of the difference between methods was negligible on all of the CD3, CD4 and CD8 results, greater on some of the CD56/CD16 results, and notable on many of the TCR- $\gamma\delta$ results. Similar results are seen in inter-laboratory comparison studies, where CVs for low-proportion phenotypes are considerably higher than those for phenotypes with higher proportions. Much of this increased variance is due to an enlargement in the stochastic counting error, which contributes 14% to the coefficient of variation (CV) when 1% positive cells are counted in

a total of 5000 events, but only 1.4% to the CV when 50% positive cells are counted in 5000 events.

A second methodologic point concerns the small but statistically significant biases for five of the six phenotypes. These differences are probably related to the varying pattern of phenotype distributions within the lymphocyte light scatter cluster. Variance in these patterns represents information which has not been yet been systematically addressed in searching for indicators of pathology or therapeutic responses.

Finally, the similarity of the results from the two systems bears particular relevance to the issue of gating on light scatter clusters to select the population of cells from which the "percent positive" will be determined. The use of "amorphous" bitmaps to select gated populations has become popular and is often considered superior to rectilinear gates because the contours of the cluster can be followed more closely. However, amorphous bitmaps may be drawn too tightly around the cluster for proper measurement of all lymphocyte subsets (4). In addition, their selection may be quite arbitrary and variable, while rectilinear gates can be selected and reproduced in a more consistent fashion. In this study, when samples were well prepared and boundaries for both methods were determined by the same experienced operator for any given specimen, either method gave essentially equivalent results for lymphocyte sub-populations in peripheral blood preparations. This close agreement provides very strong support to the validity of each of the two systems used. Moreover, the results demonstrate the feasibility of augmenting a commercial flow cytometer with third-party hardware and software without loss of reliability or accuracy.

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Figure Legends

Figure 1. Histograms showing data displays of a) EPICS C and b) 4Cyte-Acmecyte. In a) two separately displayed histograms are needed to view light scatter (x-SS, y-FLS) and fluorescence (x-FITC, y-PE) histograms. In b) both histograms are displayed simultaneous along with superimposition of FS of gated and ungated events (shown in upper right). A similar display for SS can be displayed by toggling a switch. These superimposed displays are useful in setting gates.

Figure 2. Distributions of the differences between Acmecyte and EPICS C results as a function of the EPICS C result. Each graph shows the percent positive result for a particular phenotype from the EPICS C on the X-axis, which has a varying scale for each phenotype. The arithmetic difference between results (Acmecyte result - EPICS C result) is shown on the Y-axis, which has a constant scale for all phenotypes. The "mean bias" for each marker is the average of all differences (positive and negative), and the p value indicates the probability that the mean bias is zero. The best-fit spline curve, shown in thick grey, represents the relation between bias and EPICS C result.

Figure 3. Cumulative probability distributions by phenotype for the absolute difference between Acmecyte and EPICS C results. The absolute difference (absolute value of Acmecyte result - EPICS C result) represents the discrepancy (i.e., magnitude of the difference) between the two results. The cumulative probability is expressed as the percentage of results that are

equal to or less than a given absolute difference. The thick grey line shows the distribution for all results.

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Use of trade names is for identification only and does not constitute endorsement by the Public Health service or the Dept. of Health and Human Services.

TABLE 1 Summary of results of comparison study

Phenotype	CD4	CD8	CD3	CD56/CD16 CD3 neg	TCR- $\gamma\delta$ CD8 pos	TCR- $\gamma\delta$ CD8 neg
Mean Epics C Result ^a	40.13	30.42	74.10	7.47	2.44	1.71
Mean Bias ^b	-0.39	0.27	-0.23	-0.17	-0.03	0.23
Mean % Bias ^c	-0.90	0.84	-0.29	-2.18	-2.22	8.66
Mean Discrepancy ^d	0.83	0.79	0.89	0.47	0.32	0.49
Mean % Discrepancy ^e	2.73	3.26	1.24	8.07	21.98	41.41

^aResult for percent cells positive for each phenotype as analyzed on the Epics C.

^bMean bias is the average of all differences (Acmeocyte-Epics C) for each phenotype.

^cMean % bias is the average of all differences expressed as a percentage of their respective Epics C results.

^dMean discrepancy is the average of all absolute values for the difference (Acmeocyte-Epics C).

^eMean % discrepancy is the average of all absolute value differences expressed as a percentage of their respective Epics C results.

Table 2. Linear regression analysis of EPICS-C results (x) and bias (y).

Marker	Slope	Intercept	R-square	P value	
				Slope	Intercept
CD3	-0.03 ^a	2.27 ^b	.046	.0219	0.037
CD4	0.00	-0.39	.000	.9963	0.168
CD8	0.00	0.39	.003	.5400	0.074
TCR- $\gamma\delta$ CD8 neg	-0.02	0.28 ^c	.009	.3250	<0.001
TCR- $\gamma\delta$ CD8 pos	-0.09	0.12	.031	.0639	0.250
NK (CD56/16 pos)	-0.03 ^d	0.03	.042	.0285	0.760

^a95% Confidence Interval (CI) = (0.006, 0.062)

^b95% CI = (0.1, 4.5)

^c95% CI = (0.14, 0.42)

^d95% CI = (0.002, 0.052)

Changes in T and non-T lymphocyte subsets following seroconversion to HIV-1: stable CD3⁺ and declining CD3⁻ populations suggest regulatory responses linked to loss of CD4 lymphocytes^{1,2}

Joseph B. Margolick*, Albert D. Donnenberg[†], Alvaro Muñoz[‡], Lawrence P. Park[‡], Kenneth D. Bauer[§], Janis V. Giorgi^{||}, John Ferbas[#], Alfred J. Saah[‡], and the Multicenter AIDS Cohort Study**

*Departments of Environmental Health Sciences and [‡]Epidemiology, Johns Hopkins School of Hygiene and Public Health, and [†]Department of Oncology, Johns Hopkins School of Medicine, Baltimore MD; [§]Department of Pathology, Northwestern University School of Medicine, Chicago, IL; ^{||}Department of Medicine, UCLA School of Medicine, Los Angeles, CA; [#]Department of Infectious Diseases and Microbiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA.

Address for correspondence: Joseph B. Margolick, M.D., Ph.D., Dept. of Environmental Health Sciences, Johns Hopkins School of Hygiene and Public Health, 615 N. Wolfe St, Room 7032, Baltimore, MD 21205; telephone: 410-955-4712, fax 410-955-0116

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** Centers and Investigators: Baltimore: The Johns Hopkins University School of

Hygiene and Public Health: Alfred J. Saah; John Palenicek, Haroutune Armenian, Homayoon Farzadegan, Neil Graham, Joseph Margolick, Justin McArthur. *Chicago*: Howard Brown Memorial Clinic-Northwestern University Medical School: John P. Phair; Joan S. Chmiel, Kenneth Bauer, Bruce Cohen, Daina Variakojis, Jerry Wesch, Steven M. Wolinsky. *Los Angeles*: University of California, UCLA Schools of Public Health and Medicine and the Jonsson Comprehensive Cancer Center, Los Angeles: Roger Detels; Barbara R. Visscher, Irvin Chen, Jan Dudley, John L. Fahey, Janis V. Giorgi, David T. Imagawa, Oto Martinez-Maza, Eric N. Miller, Pari Nishanian, Jeremy Taylor, Jerry Zack. *Pittsburgh*: University of Pittsburgh Graduate School of Public Health: Charles R. Rinaldo, Jr.; Lawrence Kingsley. James T. Becker, Phalguni Gupta, Monto Ho. *Data Coordinating Center*: The Johns Hopkins School of Hygiene and Public Health: Alvaro Muñoz; Terri Beaty, Noya Galai, Donald R. Hoover, Lisa P. Jacobson, Curtis Meinert, Kenrad Nelson, Lawrence Park, Steven Piantadosi, Sol Su. *NIH*: National Institute of Allergy and Infectious Diseases: Lewis Schrager, Sten H. Vermund; Richard A. Kaslow, Mark J. VanRaden; National Cancer Institute: Iris O Abrams, Daniela Seminara.

ABSTRACT

We investigated changes in lymphocyte subsets (total, CD4, and CD8 T cells, as well as non-T cells) associated with HIV-1 seroconversion in 321 homosexual or bisexual men in the Multicenter AIDS Cohort Study (MACS). These subjects had serial lymphocyte characterizations for up to 4 years before and 5 years after seroconversion. CD4 lymphocytes declined rapidly in the first 18 months following seroconversion, and less rapidly thereafter, while CD8 lymphocytes increased with similar kinetics. In contrast, total T (CD3) lymphocytes declined only slightly in the first 18 months following seroconversion, and then remained stable. These results support the hypothesis of physiologic regulation of total number of circulating T cells, such that lost CD4 lymphocytes are replaced by newly generated CD4 and CD8 lymphocytes; over time, continued loss of CD4 lymphocytes due to HIV-1 infection would result in net replacement of lost CD4 lymphocytes with CD8 lymphocytes. Non-T (CD3⁻) lymphocytes also declined after seroconversion, and this decline paralleled that of CD4 lymphocytes. Thus, changes in both T- and non-T lymphocytes after HIV-1 seroconversion may reflect the operation of homeostatic or regulatory mechanisms. Whether these mechanisms contribute to the development of immune deficiency requires further study.

Key words: Lymphocyte subsets, lymphocyte regulation, CD4 lymphocytes, non-T lymphocytes, HIV-1 seroconversion

INTRODUCTION

Infection with human immunodeficiency virus, type 1 (HIV-1), the etiologic agent of AIDS, leads to profound changes in numbers of circulating T lymphocytes. Most notable are a large absolute decrease in circulating CD4⁺ T cells, which is believed to be one of the key processes in the development of AIDS, and a large absolute increase in circulating CD8⁺ T cells. The decrease in CD4 lymphocytes is attributed to the tropism of HIV-1 for these cells, although the specific mechanism for the depletion remains unknown. Comparatively little attention has been paid to the increase in CD8 lymphocytes after HIV-1 seroconversion. This has generally been assumed to represent an adaptive antiviral host response to HIV-1, because these cells can kill HIV-1 infected target cells (1-4) and suppress HIV-1 replication in vitro (5). Under this interpretation, the chronicity of the increase in CD8 T cells is due to the persistence of HIV-1 in the host.

However, an alternative hypothesis is that the increase in CD8 lymphocytes represents a compensatory response to the loss of CD4 lymphocytes which effectively maintains a constant number of circulating CD3 lymphocytes. This hypothesis predicts that the absolute number of CD3 lymphocytes should remain constant over time irrespective of changes in CD4 and CD8 T cell subsets in individuals infected with HIV-1. Seroconverters in the Multicenter AIDS Cohort Study (MACS) have had serial T cell subset measurements for up to 6 years, including both pre- and post-seroconversion measurements, and thus afford a unique opportunity to determine the short- and long-term effects of HIV-1 infection on these lymphocyte populations. Therefore, we have used the MACS seroconverters to test the hypothesis by analyzing in detail their longitudinal data on CD3, CD4, and CD8 lymphocytes. To obtain a more complete picture of the

effect of seroconversion on lymphocyte populations, we also analyzed changes in numbers of non-T lymphocytes, i.e., lymphocytes not expressing the CD3 cell surface marker.

MATERIALS AND METHODS

Study population and definition of seroconverters. The study population consisted of homosexual/bisexual men in the MACS who have developed antibodies to HIV-1 (seroconverted) since the initiation of the study in 1984. MACS participants have visited an outpatient clinic twice yearly (four times yearly for some seroconverters) and at each clinic visit have undergone a physical examination and laboratory testing, including determination of T cell subsets (see below) and testing for antibodies to HIV-1. The study design and procedures for MACS have been described in detail (6). Antibodies to HIV-1 were measured using a commercially available ELISA kit (Genetic Systems, Seattle, WA), and positive specimens were confirmed by repeat ELISA and Western Blot (Dupont, Wilmington, DE) (7). Criteria for seropositivity were a positive ELISA along with a Western blot with bands corresponding to two of the following three gene products of HIV-1: gag, pol, and env. Of the 4954 men enrolled, 3145 were seronegative at baseline and 321 of these were observed to seroconvert during the first 5 years of the study. The time of seroconversion was taken as the date halfway between the last seronegative and the first seropositive visit.

Immunofluorescence analysis. Similar procedures were used at the four MACS laboratories, as described (8). Briefly, specimens of heparinized whole blood were stained with monoclonal antibodies using the method of Hoffman et al (9) as modified (8). Antibodies conjugated to either fluorescein

isothiocyanate (FITC) or phycoerythrin (PE) were added to whole blood at appropriate concentrations, and the erythrocytes were lysed using an ammonium-chloride based lysing solution. Proportions of lymphocytes that were stained were quantified using EPICS C flow cytometers (Coulter Electronics, Hialeah, FL) with software (Quadstat) for two-color analyses, gated on lymphocytes by light scatter characteristics. The antibody combinations used were as follows: anti-Leu 4-FITC (CD3; total T cells) and control IgG₁-PE in one tube, and anti-Leu 2a-FITC (CD8; suppressor-cytotoxic T cells) and anti-Leu 3a-PE (CD4; helper-inducer T cells) in another tube. For each antibody combination, 5000 cells were counted; all antibodies were obtained from Becton-Dickinson Immunocytometry, Inc. (San Jose, CA). Absolute numbers (cells/mm³) of lymphocyte subsets were calculated by multiplying the percent positive cells for a given subset by the number of lymphocytes/mm³ in the peripheral blood, as determined from a complete blood count with automated 10,000 cell differential. Non-T lymphocytes were quantified as the difference between the total lymphocyte and CD3⁺ cell counts.

Statistical Methods. Scatterplots of immunologic markers by time from seroconversion were smoothed by robust locally weighted regression (also known as lowess) (10). To investigate the relationship between the non-T-lymphocyte and CD4 lymphocyte counts at different times from seroconversion, we grouped the data into 6 time periods (more than 12 mos. before seroconversion, 0-12 mos. before seroconversion, 0-12 mos. after seroconversion, etc.) and produced scatterplots corresponding to each time period. These scatterplots were then summarized by plotting the convex hulls (11) which enclosed 80%, 67% and 5% of the data points. Convex hulls provide a simple, non-parametric means to summarize the location and density of distributions.

To formally test whether relationships between non-T and CD4 lymphocyte counts were different at various times from seroconversion, we modelled NT_{ij} (the non-T cell measurements of the i th individual at the j th category of time from seroconversion) as follows:

$$NT_{ij} = \alpha + \beta_j + (\gamma + \delta_j) CD4_{ij} + e_{ij}$$

where $CD4_{ij}$ are the CD4 cell measurements of the i th individual at the j th category of time. For simplicity, the categories of time (in months) relative to seroconversion were defined as: <0 , $[0,12)$, $[12,24)$, $[24,36)$ and ≥ 36 . The residuals e_{ij} are assumed to be normally distributed with unknown variance σ^2 and correlation ρ between e_{ij} and $e_{ij'}$ for all j and j' . Under this structure, correlations of repeated measurements within and between categories of time are handled in a unified fashion. This corresponds to the simplest case of regression with intraclass correlation (12) or the two-way ANOVA (13). The parameters of interest are the β_j 's and δ_j 's. Values of β_j different from zero were indicative of different intercepts for different times from seroconversion, and values of δ_j different from zero correspond to different slopes. If all the β_j 's and δ_j 's are zero, the model reduces to a simple regression with intercept α and slope γ . The intraclass correlation ρ measures the within-individual correlation of the measurement. The regression methods incorporate the repeated measurements on a given individual and provide a unified approach for the testing of hypotheses of interest. In particular, the statistical methods used provide valid estimates in the presence of differences in the number and timing of observations for different individuals.

RESULTS

This study is based on analysis of 3354 observations provided by 321

seroconverters. The time of seroconversion was known to within ± 4 months for 272 (85%) of the seroconverters. Of the study participants, 46 (14.3%) had taken AZT at some time during the period of study.

Effect of seroconversion and continued HIV-1 infection on total lymphocytes, T lymphocytes and T lymphocyte subsets. Fig. 1 shows the raw data and locally smoothed regression line for numbers of CD3⁺ lymphocytes (total T lymphocytes) in MACS participants as a function of time relative to seroconversion. It can be seen that in the first 1-2 years following seroconversion there was a slight fall in T cells around the time of seroconversion. For comparison, geometric means of cell numbers for total lymphocytes (LYMPH) as well as CD3, CD4 and CD8 T cells are shown in Fig. 2. It can be seen that the change in total T cells was small in comparison with the reciprocal changes that occurred over the same time period in the CD4⁺ and CD8⁺ subsets, or the somewhat less dramatic decline observed in total lymphocytes.

These changes in lymphocyte populations were analyzed in more detail using regression methods, and the results are shown in Table 1. The models in Table 1 correspond to the model indicated in the Methods section, except that CD4 was not included as an independent variable. The intercepts in the table correspond to the α s of the respective regressions, and the other entries correspond to the β s. The decrease in CD3 lymphocyte number in the first 18 months after seroconversion (i.e., the midpoint of the 12-24 mo interval) was statistically significant. After the level achieved by 18 months after seroconversion, the CD3 count remained stable at approximately 1500 cells/mm³ (i.e., approximately 160 cells below the level before seroconversion). This contrasted with the continuing fall in CD4 lymphocytes (268 cells in the first year, followed by 106 (i.e., 374-268), 49, and 100 cells per year in the next 3

called

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years) and the continuing rise in CD8 lymphocytes. The continued changes in CD4 and CD8 lymphocytes were both highly significant. Essentially identical results were obtained when the analysis was repeated using the interval changes in T cell subset measurements for an individual subject as the dependent variable. In addition, and as depicted in Fig. 2, the total number of circulating lymphocytes, like the number of CD4 lymphocytes, fell significantly both in the first year after seroconversion and in later years.

The finding of a fall in total lymphocytes which exceeded that in T lymphocytes in magnitude and duration suggested a fall in non-T cells. This was verified by direct analysis of changes in the difference [total lymphocytes - T cells], as shown in Fig. 3. From Table 1, non-T cells had a significant decrease of 122 cells/mm³ within the first year after seroconversion, and this continued at a rate of approximately 30 cells/mm³/year (Table 1). Because the fall in total lymphocytes exceeded that in T cells both around the time of seroconversion and thereafter, the percentage of T cells (CD3⁺) among lymphocytes actually rose slightly (3-5%; $p < 0.001$) with seroconversion and remained elevated, reflecting the fact that total lymphocytes are generally taken as the denominator for the flow cytometric measurements of percentages of lymphocyte subsets.

Relationship between changes in non-T lymphocytes and CD4 lymphocytes.

The finding that non-T lymphocytes, like CD4 lymphocytes, declined significantly within the first year after seroconversion as well as during later intervals suggested that changes in these two populations might be correlated. Therefore, these two populations were analyzed further. Fig. 4 shows their relationship as a function of time before or after seroconversion. At all time intervals, both before (Fig. 4, a-b) and after seroconversion (Fig.

4, c-f), numbers of non-T and CD4 lymphocytes were strongly correlated ($r=$ 0.38, 0.42, 0.41, 0.36, 0.43, 0.43, respectively, all $p < 0.001$). The effect of time from seroconversion on the relationship between numbers of non-T cells and numbers of CD4 cells is shown in Fig. 4 by the movement of the centers of the distributions (i.e., the means of the data contained within the 5% convex hulls), which are summarized in Fig. 5A. It can be seen that in the first year after seroconversion (i.e., from a and b to c), both CD4 and non-T numbers showed a large decline; after the first year, these declines (from c to d, d to e, and e to f) continued, though at slower annual rates, in essentially the same relationship, in which approximately 3 non-T cells were lost for every 10 CD4 cells lost. Importantly, similar trends were seen in the relation between non-T percent and CD4 percent (Fig. 5B). Because non-T percent and CD4 percent are independent flow cytometric measurements, this observation rules out the possibility that the correlation between numbers of non-T lymphocytes and numbers of CD4 lymphocytes could have been an artifact due to the use of the absolute lymphocyte count in calculating both of these numbers.

The relationship between non-T lymphocytes and CD4 lymphocytes is summarized by the regression results in Table 2. Models were fit to test whether the values of the β_j 's (intercepts) and δ_j 's (slopes) (see methods) were equal to zero. The β_j 's were significantly different from zero ($p < 0.001$), and the parameter estimates are provided in Model 1. However, the δ_j 's (i.e. different slopes of CD4 for non-T cells for different times from seroconversion) were not significantly different from zero ($p = 0.078$). That is, the changes of non-T cells per unit change of CD4 were the same for all times from seroconversion. Although Model 1 indicates that the intercepts (i.e., β_j 's) were different from zero, when Model 1 was compared with Model 2

there was no indication that the β_j 's after seroconversion were different among themselves ($p = 0.094$ for model 1 vs. model 2). In summary, Model 2 provides an appropriate and parsimonious description of the data. It indicates that the changes of non-T cells per change in CD4 are the same for all the times from seroconversion (i.e., loss of 3 non-T cells for every 10 CD4 cells lost) and that seroconversion induces a drop of 51 non-T cells/mm³. In addition, model 2 summarizes the regression lines depicted in fig. 4 which suggest a common slope and a lower intercept after seroconversion. Inferences were not changed by using a logarithmic transformation of the data.

DISCUSSION

In this study, we have analyzed the effect of HIV-1 infection on numbers of circulating T and non-T lymphocytes in 321 homosexual men who seroconverted to HIV-1 during the study period. These individuals provided data for up to 4 years before and up to 5 years after seroconverting, so that both short- and long-term effects of incident HIV-1 infection could be analyzed. Our results confirm and extend previous studies (14,15) by unequivocally demonstrating the extent and biphasic pattern of the changes in CD4 and CD8 lymphocytes. In addition, we have demonstrated the relative stability of CD3 lymphocyte numbers and characterized the decline in non-T lymphocytes following seroconversion.

Changes in non-T lymphocytes, which consist primarily of B cells and natural killer (NK) cells, have not been reported previously, although numbers of both cell types have been reported to be lower in HIV-1-seropositive individuals than in -seronegatives (16-19). Like CD4 lymphocytes, non-T cells declined relatively rapidly in the first year after seroconversion and more slowly thereafter. Analysis of the changes in these two lymphocyte populations

showed that they were closely linked in terms of both absolute numbers and relative percents of lymphocytes. Although some non-T lymphocytes can be infected with HIV in vitro (20,21), they do not appear to be major targets of this virus in vivo. Therefore, the parallel nature of the declines in CD4 and non-T lymphocytes after HIV-1 seroconversion provides evidence for co-ordinate regulation of CD4 and non-T lymphocyte populations. Whether this depends on CD4 cell function or an independent mechanism remains an open question. Changes in numbers of circulating non-T lymphocytes probably reflect primarily changes in NK cells, which are more abundant than B cells in normal peripheral blood and are proportionately more reduced in HIV-1-seropositive individuals (16-19).

Beyond the first 1½ years after seroconversion, levels of circulating total T lymphocytes changed remarkably little despite continuing dramatic changes in the numbers of circulating CD4 and CD8 lymphocytes. Specifically, total T lymphocytes declined by about 10% (164/1661 cells/mm³) in the first 1½ years after seroconversion, while CD4 lymphocytes declined 38% (374/995 cells/mm³) and CD8 lymphocytes rose by 28% during this time. Furthermore, T lymphocytes did not change significantly in the ensuing two years, while the changes in CD4 and CD8 lymphocytes continued, albeit at a slower rate. The relative long-term stability of CD3 lymphocytes suggests the existence of another regulatory mechanism which maintains CD3⁺ T cell levels, such that 1) loss of CD4 lymphocytes is detected as loss of CD3 lymphocytes, and 2) lost CD3 lymphocytes are replaced without regard to CD4⁺ or CD8⁺ phenotype. Such a mechanism would be reflected in healthy individuals by a long-term stability of numbers of circulating CD3⁺, CD4⁺, and CD8⁺ lymphocytes, consistent with available data implying little or no change in these values as a function of time in cross-sectional (22,23) or longitudinal (8) studies of HIV-1-

added

L seronegative adults. However, in HIV⁺ individuals loss of CD4 lymphocytes would stimulate production of new CD4⁺ and CD8⁺ lymphocytes. Loss of the new CD4 lymphocytes (but not the new CD8 lymphocytes) due to HIV-1 over a period of months to years would result in a gradual replacement of CD4 lymphocytes by new CD8 lymphocytes while preserving circulating T (CD3⁺) lymphocytes.

This long-term regulatory mechanism would be distinct from other regulatory mechanisms that might pertain in acute conditions such as infectious mononucleosis due to infection with Epstein-Barr virus (24) or cytomegalovirus (25), or other viral infections (26). These conditions produce a transient lymphocytosis, primarily of CD8 lymphocytes, which returns to baseline values

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Hed in weeks to a few months. In addition, some HIV⁺ individuals treated with AZT and related drugs experience a short-lived rise in CD4 lymphocytes which appears to be due primarily to a rise in CD3⁺ lymphocytes, because both CD4 and CD8 lymphocytes increase and the CD4/CD8 ratio does not change appreciably (27-29). A partial reversal of the depression in CD3⁺ lymphocytes seen after HIV-seroconversion could account for this phenomenon. In contrast to these situations, HIV-1 infection has an average incubation period of many years, although the more pronounced changes in CD3, CD4, CD8, and non-T lymphocytes in the first year after HIV-1 seroconversion could also reflect acute regulatory phenomena.

The hypothesis of long-term regulation at the CD3 lymphocyte level is also supported by the presence of normal numbers of circulating CD3 lymphocytes, despite markedly reduced or absent CD4 lymphocytes, in some congenital immune deficiency states (30-32). More direct evidence that depletion of CD4 lymphocytes results in production of new T cells is provided by mouse experiments in which animals depleted of CD4 lymphocytes with monoclonal

antibodies generated an increase in CD8 lymphocytes, thus maintaining normal or near-normal levels of CD3 lymphocytes (33,34)¹. An increased rate of T cell lymphopoiesis would lead to an abnormally high frequency of newly generated T lymphocytes of both phenotypes in the circulation. Studies of regenerating lymphocytes after bone marrow transplantation indicate that newly produced T lymphocytes frequently express phenotypic markers associated with activated lymphocytes, such as HLA-DR and CD38 (35-39). Thus, the well-documented increase in T lymphocytes expressing CD38 and/or HLA-DR in HIV-1 infection (40-42) could be due in part to turnover of T cells, measurement of which could provide a useful indication of disease activity.

An alternative explanation for the persistent increase in CD8 lymphocytes after HIV-1 seroconversion, namely an increase in HIV-1-specific cytotoxic T lymphocytes (CTL), seems unlikely for two reasons. First, the number of CD8 lymphocytes approximately doubles after seroconversion; to account for such an increase, antigen-specific CD8 cells would have to represent about half of the CD8 lymphocytes circulating in a HIV-1-seropositive individual. However, the frequency of antigen-specific CTL, even after immunization, represents a very small proportion of total T cells and could not account for such a large increase in circulating CD8 lymphocytes (43). Second, this alternative does not explain the constancy of numbers of T lymphocytes, rather than an increase or decrease, in the face of large changes in numbers of CD4 and CD8 lymphocytes. An inherently more rapid reproduction rate of CD8 lymphocytes compared to CD4 lymphocytes, as suggested by earlier reconstitution by CD8 cells after bone marrow transplantation (reviewed in (44)), may also contribute to the rise in CD8 lymphocytes in HIV-1 infection.

Taken together, the available data suggest that the alterations in

lymphocyte populations that are characteristic of HIV-1 infection are due at least in part to the operation of normal regulatory mechanisms which are triggered in response to HIV-1 mediated destruction of CD4 lymphocytes. Further, these alterations may contribute to the development of immune deficiency because a progressively larger proportion of both CD4 and CD8 lymphocytes will be composed of newly generated, functionally unresponsive cells.

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Legends

Fig. 1. Scatterplot of number of CD3 lymphocytes as a function of time relative to seroconversion in MACS seroconverters. The estimated time of seroconversion is taken as 0, with negative numbers representing time points before seroconversion. Each dot represents one observation on one individual (n=3198). A locally weighted regression line is shown.

Fig. 2. Changes in geometric mean numbers of lymphocytes per mm^3 in MACS seroconverters as a function of time relative to seroconversion. Points represent geometric means of observations within the indicated time intervals. Error bars represent 95% confidence intervals of the mean (i.e., approximately twice the standard error of the mean). The numbers of observations for each time point are as follows: for lymphocyte number n=423, 499, 700, 717, 529, 466; for T cell subsets n=394, 457, 649, 706, 524, 463.

Fig. 3. Scatterplot of numbers of non-T lymphocytes (per mm^3 of blood) for MACS seroconverters as a function of time relative to seroconversion. Each dot represents one observation on one individual. A locally weighted regression line is shown. 151 of 3354 individual observations lie above the vertical limit; these are not shown but are included in the derivation of the regression line.

Fig. 4. Scatterplots of number of non-T lymphocytes vs. number of CD4 lymphocytes (per mm^3 of blood) for MACS seroconverters at different times relative to seroconversion. Figs. a-f display data from the indicated time

periods in relation to the estimated time of seroconversion. The polygons enclose 5%, 67%, and 80% of the data, from the center of the clusters moving outward. The solid straight lines indicate the least squares regression lines for each time period separately. The dashed straight lines indicate the regression lines derived from the overall regression model described in the text. The numbers of observations in each figure are: a) 393, b) 436, c) 648, d) 703, e) 523, and f) 465.

Fig. 5. Movements of the centers of the distributions of numbers (A) and percents (B) of non-T lymphocytes in relation to CD4 lymphocytes over time. The data displayed correspond to the centers of the distributions shown in Fig. 4 for part A, and to the corresponding raw data for percents (not shown). Letters in graph indicate the time intervals corresponding to parts a-f of Fig. 4. Thus, a and b represent pre-seroconversion data, and c to f represent data post seroconversion.

Table 1. Results derived from regression of lymphocyte populations on time from seroconversion (SC). The intercepts are the estimated values prior to SC and correspond to the α s in the regression models. The coefficient for a given time period after SC is the estimated change from the intercept value and corresponds to the β for that time point in the regression model. For example, CD4 measurements made between 24 and 36 months after SC average $572 \text{ cells}/\mu\text{l} - 995 - 423$.

Table 2. Results derived from regression of non-T lymphocytes vs. CD4 number and time from seroconversion (SC). The intercept is the estimated number of

added
*
w-
written

non-T cells prior to SC, corresponding to a CD4 count of zero cells/ μ l and corresponds to the α for the regression model. The coefficient of #CD4 is the estimated number of non-T lymphocytes lost for each CD4 cell lost and corresponds to the γ for the regression model (δ s were all not significantly different from 0). The estimate for each time period after SC is the difference in non-T cell number between the pre-SC value and measurements made during the indicated period, and corresponds to β in the regression model. Model 1 provides estimates for 4 time periods. Model 2 provides a single estimate combining all times after SC, and is not significantly different from Model 1 (see text).

Footnote

¹Adelman, L.A. and D. Wofsy, T cell homeostasis: implications in HIV infection
(manuscript submitted)

Table 1 : Regression of Numbers of Lymphocyte Subsets on Time from Seroconversion

		Numbers of Cells			
		CD4	CD8	CD3	Non-T
Time from SC	Intercept	995 $\pm$ 16*	648 $\pm$ 39	1661 $\pm$ 28	637 $\pm$ 11
	[0,12) months	-268 $\pm$ 13	167 $\pm$ 11	-82 $\pm$ 23	-122 $\pm$ 10
	[12,24) months	-374 $\pm$ 13	181 $\pm$ 12	-164 $\pm$ 23	-158 $\pm$ 10
	[24,36) months	-423 $\pm$ 14	276 $\pm$ 17	-112 $\pm$ 26	-192 $\pm$ 12
	($\geq$ 36) months	-523 $\pm$ 16	316 $\pm$ 18	-188 $\pm$ 28	-216 $\pm$ 13
Standard Deviation (σ)**		338	361	584	251
Intraclass Correlation (ρ)		0.53	0.44	0.49	0.43

* Regression coefficient $\pm$ standard error

** Standard deviation of cell number measurements

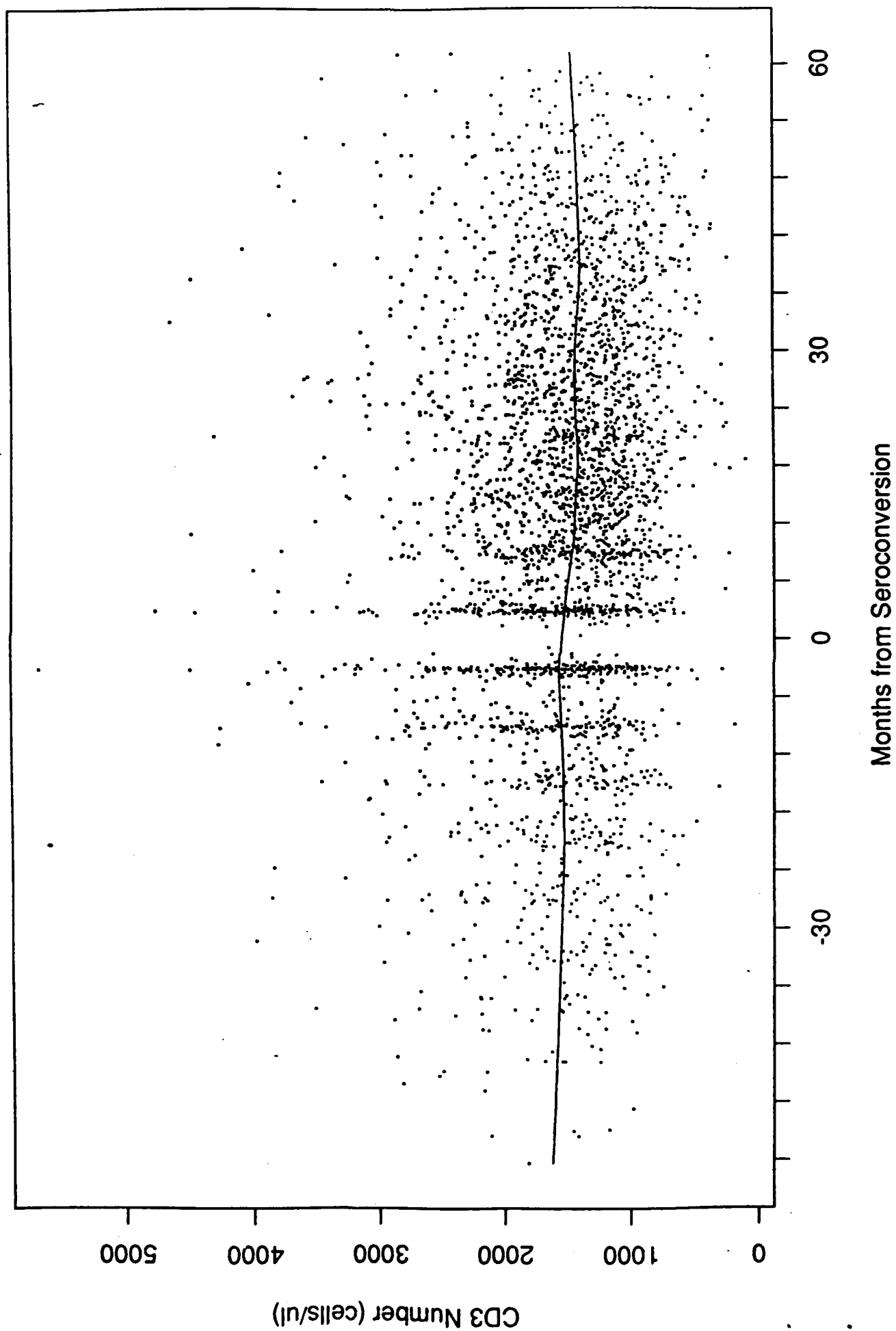
Table 2 : Results of Regressing the Number of Non-T Lymphocytes
on Number of CD4 Lymphocytes and Time from Seroconversion

		Model 1	Model 2
Intercept		$350 \pm 17^*$	342 ± 16
# CD4 Lymphocytes		0.29 ± 0.01	0.30 ± 0.01
Time from SC	[0,12) months	-45 ± 10	
	[12,24) months	-50 ± 11	
	[24,36) months	-70 ± 12	
	(≥ 36) months	-65 ± 14	
	(≥ 0) months		-51 ± 9
Standard Deviation (σ)**		230	230
Intraclass correlation (ρ)		0.41	0.41
-2 log likelihood		42764.5	42770.9
deviance; p-value		6.4; 0.094	

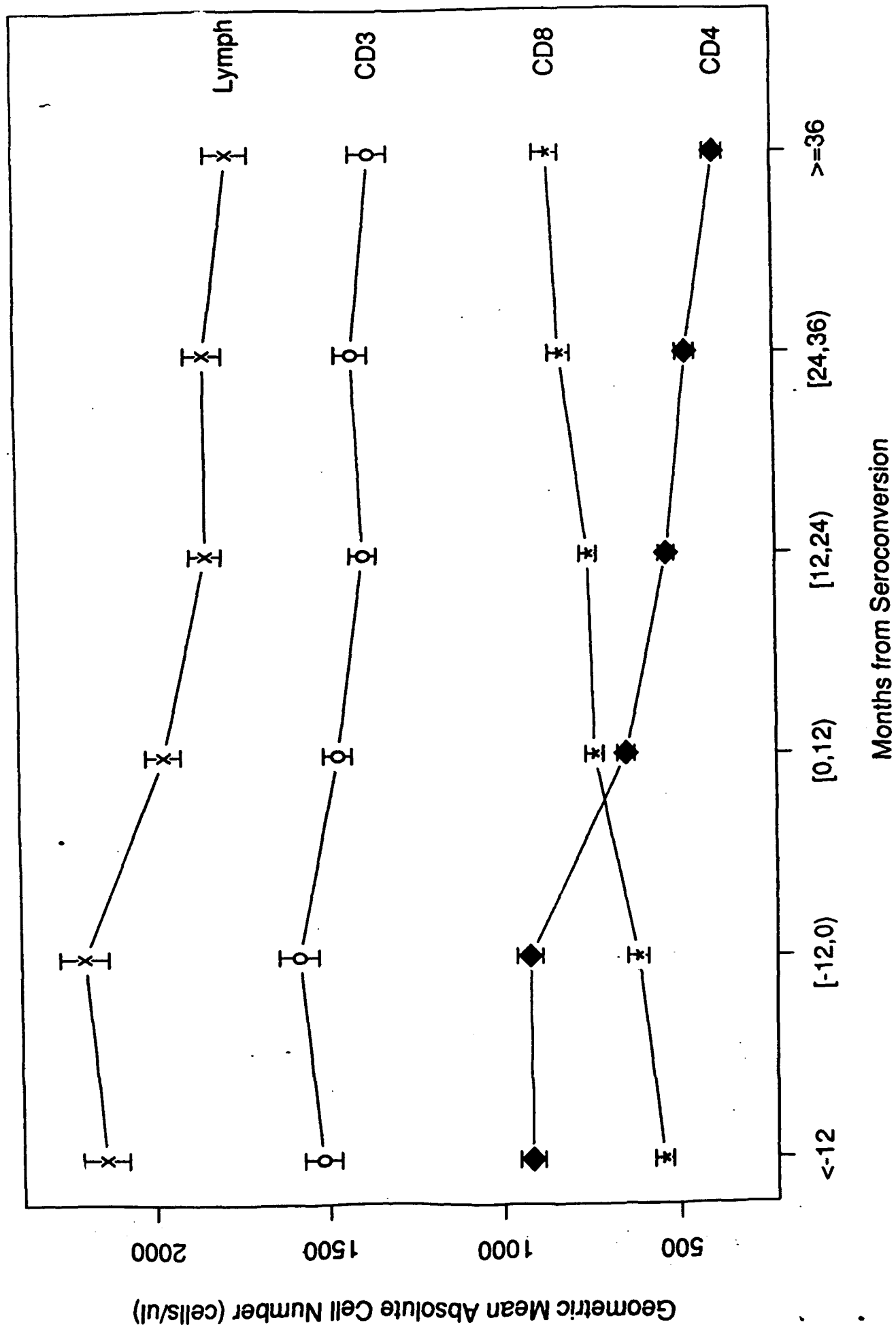
* Regression coefficient $\pm$ standard error

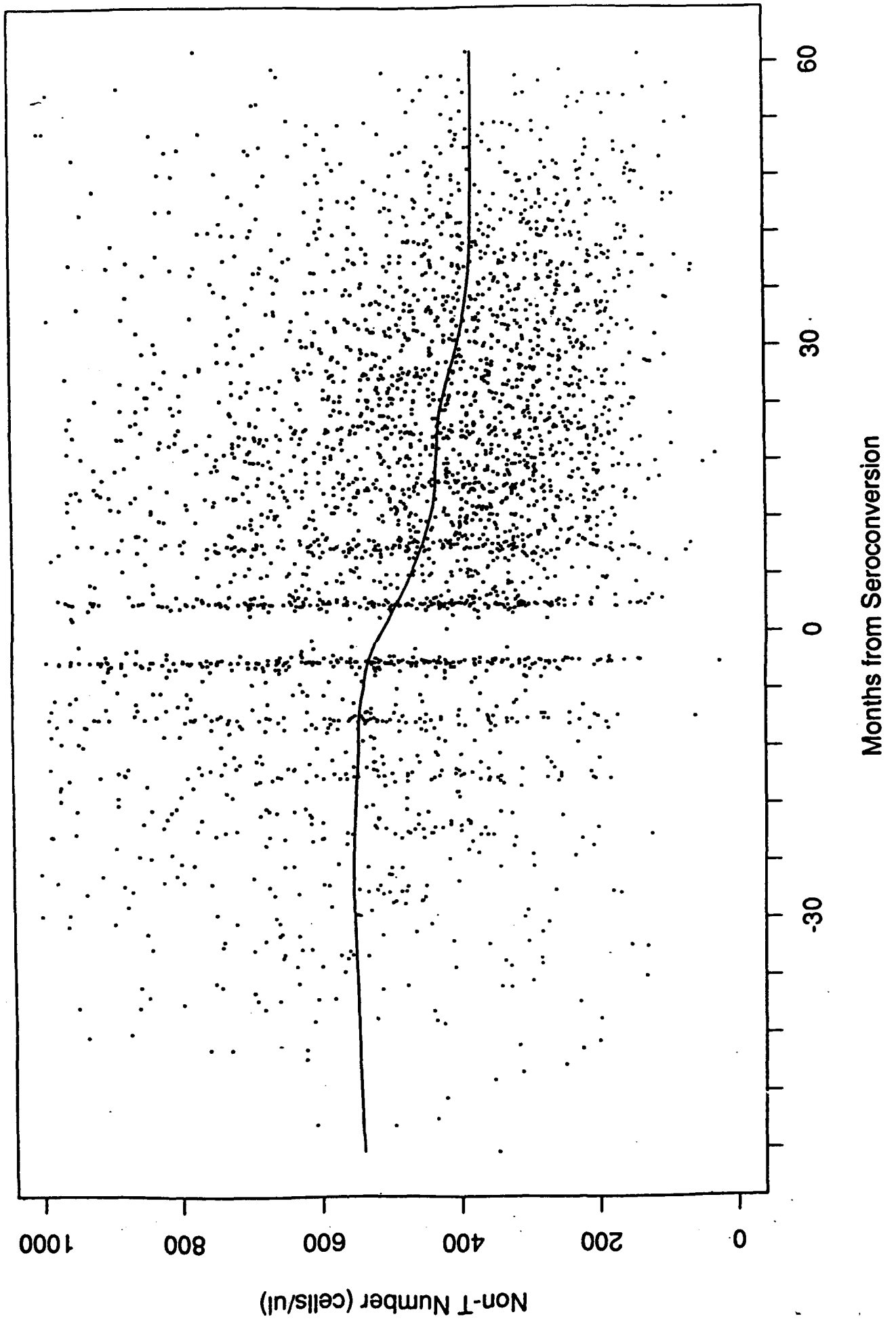
** Standard deviation of cell number measurements

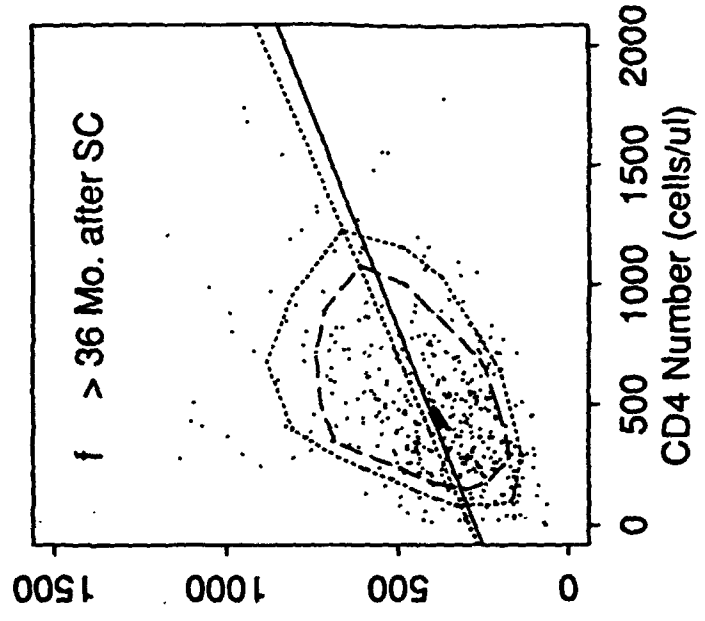
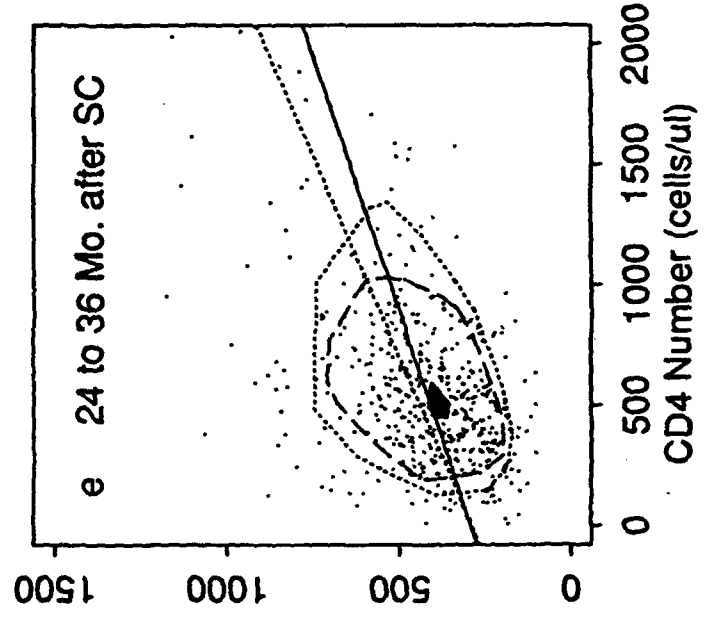
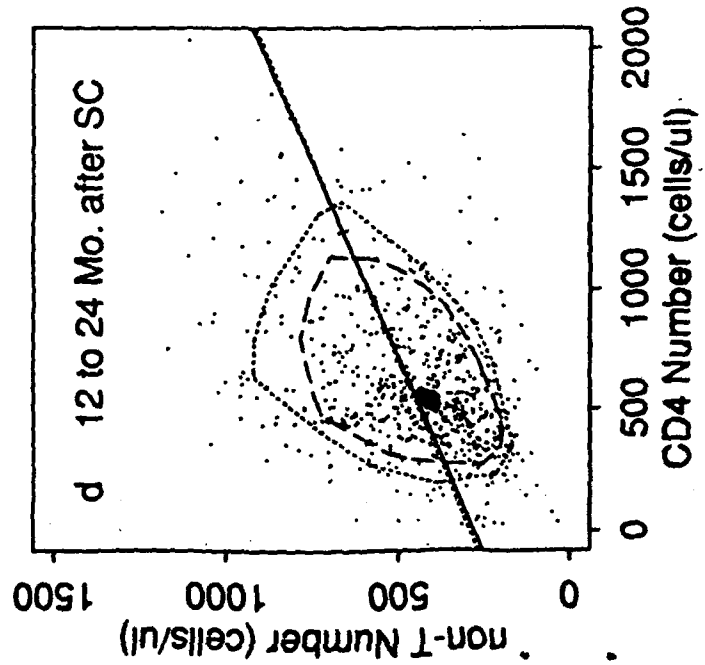
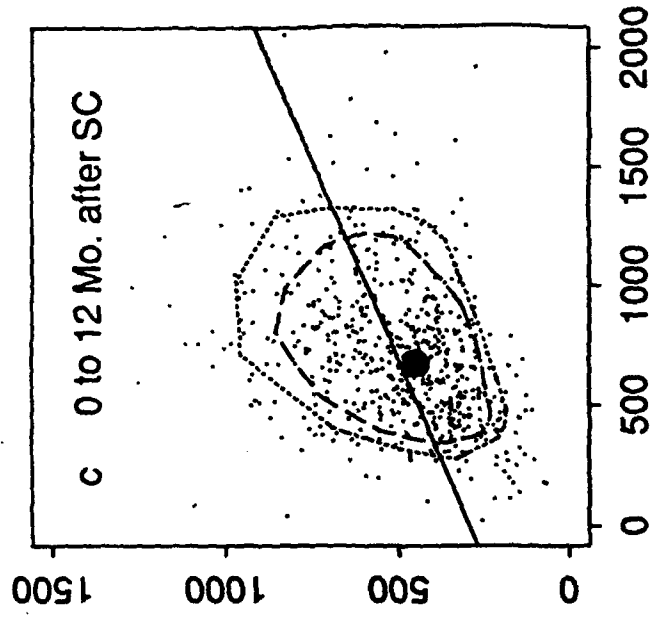
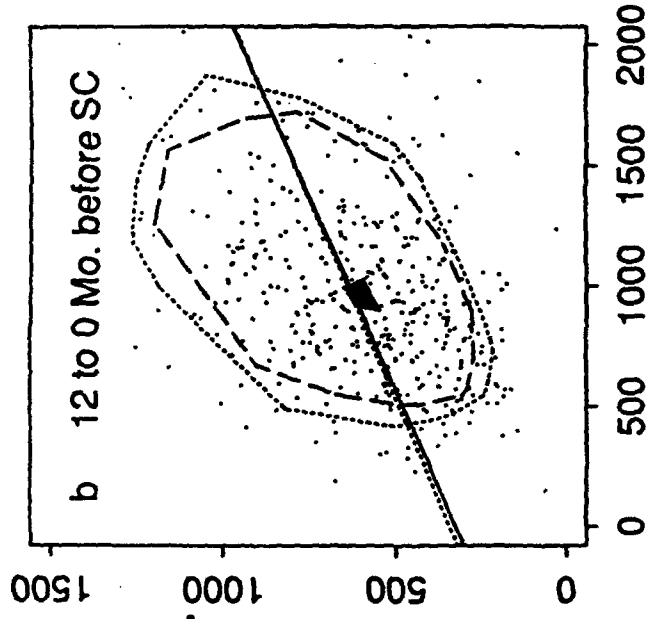
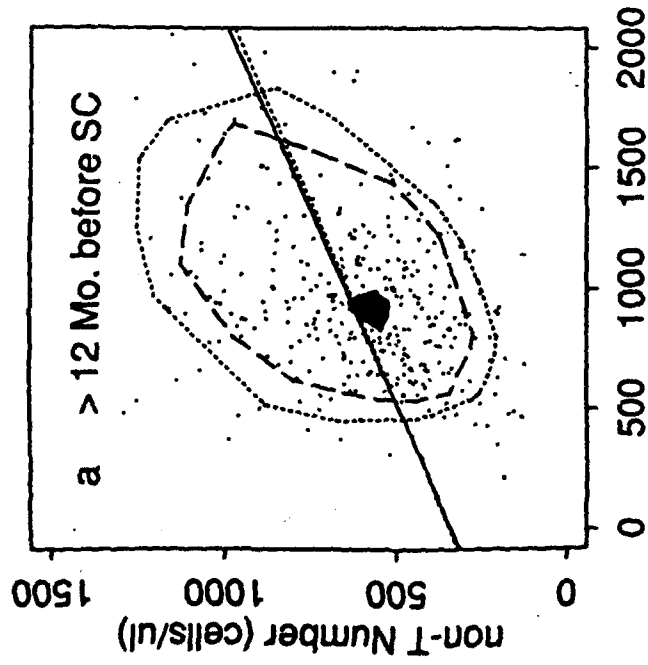
Margolick - 24
Figure 1

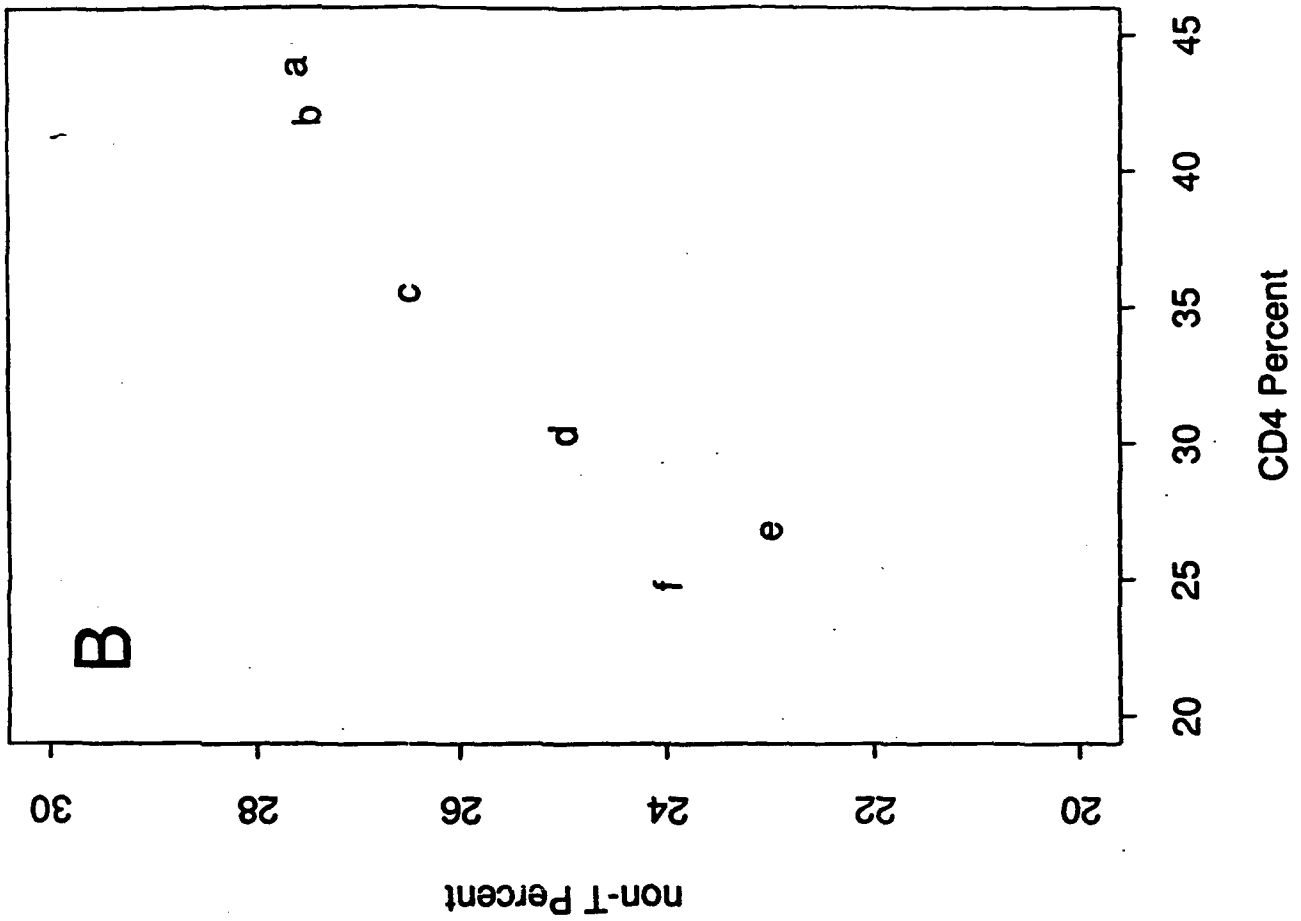
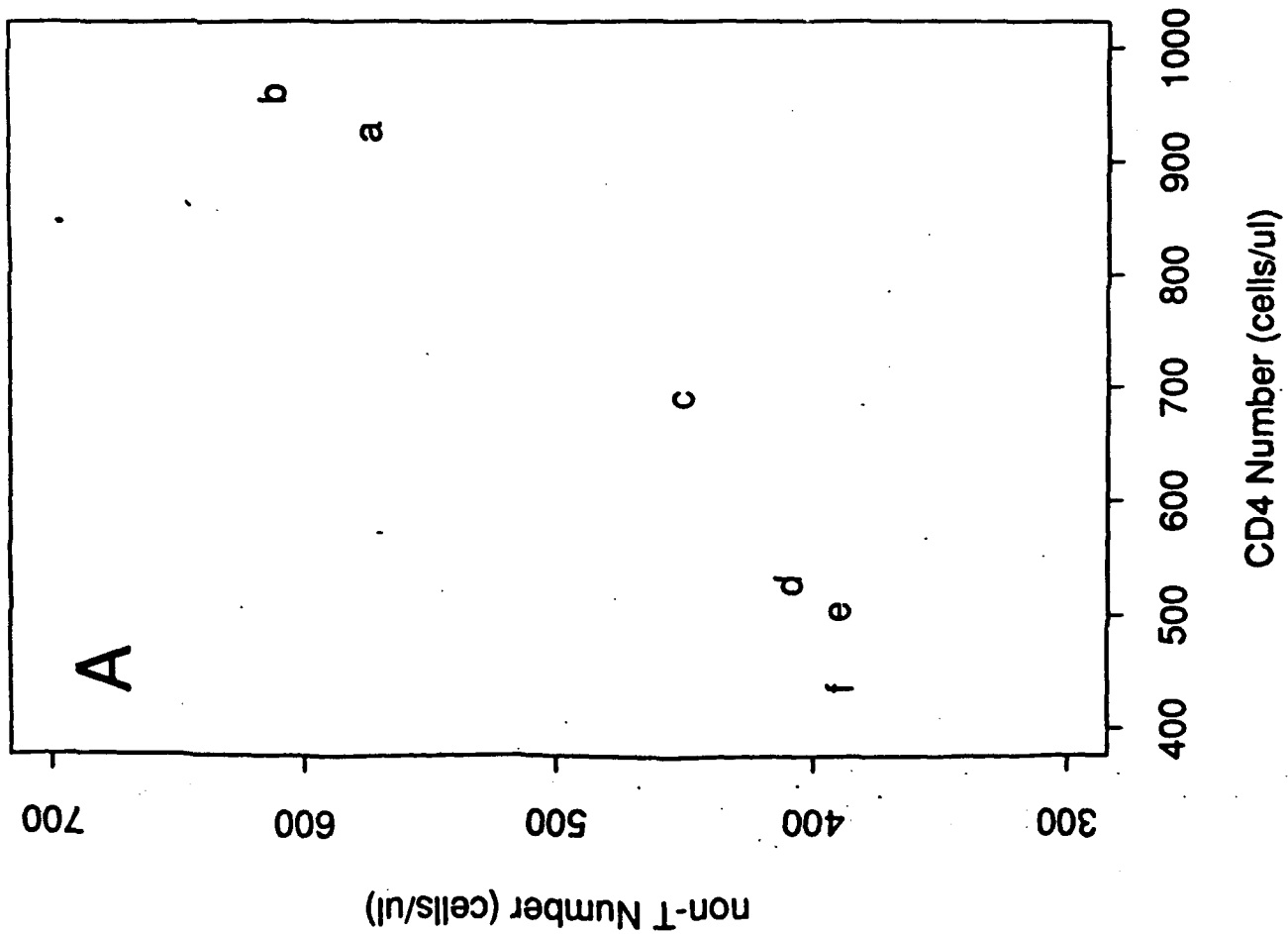


Margolick - 25
Figure 2









Running Title:

IL2R and TGF- β 1 mRNA expression in HIV⁺ homosexuals.

Full title:

**IL2 RECEPTOR α AND β CHAIN mRNA EXPRESSION IS DECREASED IN PHA
STIMULATED PBMC FROM HIV TYPE 1 (HIV-1) INFECTED HOMOSEXUAL MEN¹**

Authors' full names:

Rajesh K. Chopra, N. Babu K. Raj*, John P. Scally, Albert D. Donnenberg||,
William H. Adler‡, Alfred J. Saah‡, and Joseph B. Margolick

Affiliations:

Departments of Environmental Health Sciences and †Epidemiology, School of
Hygiene and Public Health, 615 N. Wolfe Street, and *Department of Oncology,
School of Medicine, 418 N. Bond Street, The Johns Hopkins University and the
Baltimore Multicenter AIDS Cohort Study, Baltimore, MD 21205, || The Johns
Hopkins Oncology Center, Bone Marrow Transplantation Program, 600 N. Wolfe
St., Baltimore MD, 21205, ‡Clinical Immunology Section, Gerontology Research
Center, National Institute on Aging, 4940 Eastern avenue, Baltimore, MD 21224

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Correspondence: ² Correspondence should be addressed to Dr. Rajesh K. Chopra, Room 7032, Department of Environmental Health Science, The Johns Hopkins University School of Hygiene and Public Health, 615 N. Wolfe Street, Baltimore, MD 21205.

A.D.D. present address: ³ Hematology/Bone Marrow Transplantation, University of Pittsburgh, Montefiore Hospital 2N, 3459 5th Ave, Pittsburgh, PA 15213.

Abbreviations used in this paper: ⁴ DSS, disuccinimidyl suberate; kD, equilibrium dissociation constant; HIV-1, HIV type-1; IL2R, IL2 receptor; MACS, multicenter AIDS Cohort Study; PE, phycoerythrin; TGF- β 1, transforming growth factor- β 1;

ABSTRACT

Previous studies have shown that exogenous IL2 can not correct the reduction in PHA-induced proliferation of lymphocytes from HIV type-1 (HIV-1) infected (HIV⁺) individuals. We investigated the mechanism of this reduction to determine if a reduced expression of complete IL2 receptor (IL2R) and/or over production of transforming growth factor- β 1 (TGF- β 1) were responsible. PHA stimulated T cells from 89 HIV⁻ and 93 HIV⁺ homosexual men from the Baltimore Multicenter AIDS Cohort Study (MACS) were studied using Northern blot analysis of IL2R α , IL2R β , and TGF- β 1 mRNA expression, binding of ¹²⁵I-IL2 to high affinity IL2R, and determination of IL2 dependent cell proliferation. Compared to HIV⁻ donors, HIV⁺ donors demonstrated reductions in high affinity IL2R expression and in the IL2 induced proliferative response of PHA stimulated PBMC. Cells from HIV⁺ donors also evidenced a reduction in IL2R α - and β -chain mRNA expression which may be responsible for decreased high affinity IL2R expression. However, there was no reduction in TGF- β 1 mRNA expression in unstimulated or PHA stimulated PBMC from HIV⁺ compared to the HIV⁻ donors. Furthermore, anti TGF- β 1 antibody did not increase the IL2 augmentation of proliferative response of cells from HIV⁺ donors. These results suggest that T cell activation defects associated with decreased IL2R expression contribute to the lower proliferative response of cells from HIV⁺ donors. TGF- β 1 does not appear to contribute to this deficit.

INTRODUCTION

Reduction of T cell proliferative responses to antigens, mitogens, and IL2 occurs in the early stages of HIV-1 infection (1-4). The mechanism responsible for these reduced responses is not well understood. A direct viral effect seems unlikely, since the frequency of HIV infected cells is very low (10^{-3} to 10^{-6}) (5-7). Possible mechanisms, in addition to a variable degree of CD4 lymphocytes depletion include the possibility of T cell inhibitory factors present in serum from HIV⁺ individuals (8,9); inhibitory effects of HIV proteins such as gp120, gp41, and tat (10-12); elevated serum levels of soluble IL2R α (13) which can compete with cellular IL2R for free IL2 (14); reduced production of IL2 and other growth factors (15-19); and overproduction of inhibitory factors such as TGF- β_1 (20).

T cells stimulated by either antigen or mitogen enter the G₁-S phase of the cell cycle after receiving a signal transduced by the binding of IL2 to the high affinity IL2R, a noncovalently associated heterodimer composed of α and β chains which responds to physiologic concentrations of IL2 (K_d = 1-50 pM) (21,22). Activated T cells express at least 20-40 times more IL2R α chain than required to associate with β chains (21,22), and as such the β chain is the limiting component for the formation of the high affinity IL2R. In addition, the β chain is essential for transduction of the IL2 signal (23-25). These observations suggest that a defect in expression of either the α or the β subunit of the high affinity IL2R provides a possible explanation for the decrease in lymphoproliferative responses. In support of this possibility, it has been

demonstrated that reduction of antigen- and mitogen-induced lymphoproliferative responses in short term cultures of PBMC from HIV⁺ donors is not reversed by exogenous IL2 (2,16,18,26-28). Similarly, we found lower precursor frequencies of IL2-responsive cells in PBMC from HIV⁺ donors (4,29), and expression of IL2R α has recently been reported to be reduced on circulating lymphocytes from HIV⁺ donors (30). However, expression of the IL2R α subunit on mitogen activated T cells from HIV⁺ donors, though reduced (1,18,26-28), should still be sufficient to form a normal number of high affinity IL2R, because the α subunit is normally expressed in such great excess. In contrast, the effect of HIV-1 infection on the expression of the β subunit has been less well studied, although a preliminary report suggested that expression of the β subunit by stimulated PBMC from HIV⁺ donors is diminished, or absent in some cases (31). In this study, we have investigated expression of the β subunit by PHA stimulated lymphocytes from HIV⁺ donors.

Recently it has been suggested that an excess of TGF- β 1, a proliferation inhibitory cytokine produced by a wide variety of cells (32-34), could contribute to a reduced proliferative potential in T cells from HIV⁺ donors (20). Therefore, we also investigated the relation between the expression of IL2R α and β mRNA and resultant high affinity IL2R, the expression of TGF- β 1 mRNA, and the effect of neutralization of TGF- β 1 on T cell proliferative ability.

MATERIALS AND METHODS

Study participants: 89 HIV⁻ and 93 HIV⁺ homosexual men participating in the Baltimore center of the MACS, were studied. As described in detail elsewhere (35), participants in this study are followed at 6-month intervals with clinical and laboratory evaluations, including testing for the presence of antibodies to HIV-1 by ELISA with confirmatory analysis using Western blot, and measurement of T cell subsets by flow cytometry as described below.

Cell culture: PBMC were isolated from heparinized blood by centrifugation at 400 g for 30 min over Ficoll-Hypaque (36). PBMC were washed and suspended at 5×10^5 cells per ml in complete culture medium consisting of RPMI 1640 (Whittaker Bioproducts, Walkersville, MD), 10% FBS (Hyclone, Logan, Utah) and 100 μ g per ml gentamicin. The cells were stimulated with 0.5 μ g per ml PHA (Wellcome Research Laboratory, Janesville, NC) and, unless otherwise stated, cultivated in 75 cm² tissue culture flasks at 37°C in 5% CO₂ and 95% humidity.

¹²⁵I-IL2 binding: After stimulation with PHA for 55 h, PBMC were washed twice and reincubated in complete medium at 37°C overnight (at least 16 h). The cells were isolated, washed, and suspended in ice cold complete medium at 5×10^6 viable cells per ml. ¹²⁵I-IL2 binding to the cells was assayed as described previously (37). Briefly, 100 μ l of serial dilutions of 1.5 - 200 pM of ice cold ¹²⁵I-IL2 (Sp. Act 33-46 μ Ci/ μ g, New England Nuclear, Boston, MA) were mixed with 100 μ l of 5×10^5 cells in 1.5 ml conical microcentrifuge tubes. The tubes were gently vortexed and then incubated on ice for 30 min with 2-3 intermediate vortexings.

After incubation, 1 ml of ice-cold media was added to each tube, and the tubes were then centrifuged at 9000 g for 2 min. The supernatants were saved and the cell pellets were suspended in 100 μ l of RPMI-1640 and were then layered over dibutylphthalate/dioctylphthalate (1.1:1) in chilled microcentrifuge tubes. The samples were centrifuged to pellet the cells through the oil barrier. The tips of the tubes containing the cell pellets were clipped, and radioactivity was counted in a γ counter. The nonspecific binding of ^{125} I-IL2 to the cells was determined in some experiments by adding $\approx$ 100 fold excess cold rIL2 (graciously provided by Cetus Inc, Emeryville, CA). The B_{max} of IL2 binding and the equilibrium dissociation constant (kd) were determined using the ENZYFIT program (38)

Preparation and analysis of IL2R α , β and TGF- β 1 mRNA: Total cellular RNA was isolated from 20×10^6 unstimulated or PHA stimulated cells by the RNazol method (Cinna/Biotech Laboratories, Friendswood, TX). Twenty-four hours after PHA stimulation there is maximal IL2R β mRNA expression (24) and at this time period TGF- β 1 mRNA is also maximally expressed (34). Although IL2R α mRNA is maximally expressed by 16 h after PHA stimulation, its expression still remains nearly maximal at 24 h after PHA stimulation (39, Personal observations). Thus cells were stimulated with PHA for 24 h to study the expression of these three genes in the same individuals. For unstimulated cells 12 μ g and for stimulated cells 15 μ g of RNA per lane was electrophoresed, blotted, and hybridized as described previously (40) with cDNA probes specific for human IL2R α , IL2R β (plasmids graciously provided by Dr. W. J. Leonard, NICHD, Bethesda, MD), TGF- β 1 (plasmid graciously provided by Dr. R. Derynck, Department of Developmental Biology, Genentech, South San Francisco, CA) and human actin (plasmid graciously provided

by Dr. L. H. Kedes, Stanford University School of Medicine, Palo Alto, CA) labelled to high specific activity with ^{32}P CTP (NEN) using the random primer method (41). For unstimulated cells, blots were first hybridized with probes for IL2R α . The IL2R α probe was then stripped (40) and the blots were hybridized with TGF- β 1 probe, after which the TGF- β 1 probe was stripped and the blots probed with IL2R β and actin probes together. For PHA stimulated cells, blots were similarly hybridized with probes for TGF- β 1 followed by IL2R α , and finally IL2R β and actin together. Air dried filters were exposed at -70°C to Kodak XAR film with intensifying screens, and autoradiograms were scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, CA). The concentrations of mRNA bands were normalized to the concentration of actin mRNA bands (taken as 2000 arbitrary densitometric units).

Flow cytometric analysis of surface expression of IL2R α and β chains: Peripheral blood lymphocytes expressing CD3, CD4, and CD8 were measured in the whole blood using staining technique as previously described (42,43) using two-color flow cytometry and two combinations of antibodies directly labelled with FITC or phycoerythrin (PE) as follows: CD3-FITC/IgG₁-PE and CD8-FITC/CD4-PE. For analysis of IL2R α and β subunits on cultured lymphocytes, 10^6 cells were washed three times in PBS containing 0.1% BSA and 0.01% azide (wash buffer), resuspended in 0.1 ml cold wash buffer, and stained with either FITC-labelled anti-IL2R α or a 1:1000 dilution of anti-IL2R β antibody (TU27, graciously provided by Dr. S. Taki, Ajinomoto Co. Inc., Central Research Laboratories, Kawasaki, Japan, (44) followed by FITC labelled goat anti mouse IgG antibody, with normal mouse IgG as a control. All other antibodies were purchased from Becton-Dickinson (Mountain View, CA), and all incubations with antibodies were performed on ice for 30 min

and followed by three washes in wash buffer. Stained cells were fixed in wash buffer containing 0.5% formaldehyde (Polysciences, Warrington, PA) and stored in the dark at 4° C until analysis on an EPICS C flow cytometer (Coulter Electronics, Hialeah, FL) with selection of lymphocytes by gating using forward and side light scatter.

¹²⁵I-IL2 cross linking: After stimulation with PHA for 55 h, cells were pelleted, washed twice and recultivated in complete medium as described above. The cells were then pelleted and washed, and 4-8 x 10⁶ cells were incubated on ice with 1 nM ¹²⁵I-IL2 in 0.5 ml RPMI 1640 containing 3% FBS and 0.1% NaN₃ for one hr. After three washes with PBS containing 1 mM MgCl₂, the receptor-ligand complexes on cells were covalently cross linked with freshly prepared disuccinimidyl suberate (DSS) (Pierce Chemicals, Rockford, IL) as previously described (45). The cells were lysed with Tris buffer, pH 6.8, containing 0.5% NP-40 and electrophoresed under reducing condition on gels containing 10% polyacrylamide and 0.1% SDS (46). After electrophoresis, the gels were fixed, dried under vacuum, and autoradiographed as described (47). Quantitative analysis of autoradiograms was performed using a scanning densitometer (Pharmacia LKB, Biotechnology, Piscataway, N.J.). The results are presented as arbitrary units corresponding to the relative areas under the peak.

Cell proliferation: 5 x 10⁴ PBMC were cultured in triplicate wells of flat bottom 96 well microtiter plates in 0.2 ml with PHA or complete medium alone for 72 h, with a pulse of 1 µCi per well of ³H-TdR (Sp. Act 6.7 Ci/mmol, NEN) for the last 16 h. To study the stimulatory effects of IL2, cells were stimulated with PHA for 55 h, washed, and recultured overnight in the absence of

lymphokines. An aliquot of these cells was cultured at 5×10^4 cells per well with or without 1 nM rIL2 for 48 hrs and pulsed with 1 μ Ci per well of ^3H -TdR for the last 4 h. To analyze the effects of TGF- β 1 antibody, 1.2×10^6 PBMC in 1.2 ml complete medium were stimulated with PHA in the presence of 12.5 μ g per ml chicken anti TGF- β 1 neutralizing or control (Chicken IgG) antibody (both from R & D Systems, Minneapolis, MN) and cultured in 24 well flat bottom tissue culture plates. In preliminary experiments, 12.5 μ g per ml anti TGF- β 1 antibody neutralized the inhibitory effects of 10 ng per ml of TGF- β 1 on CCL64 epithelial cell proliferation (data not shown). Also this concentration of anti TGF- β 1 antibody was several fold in excess of the one described by the manufacturer (2-3 μ g for 0.25 ng per ml TGF- β 1) to ensure complete neutralization of TGF- β 1 in the cultures. After 55 h in culture, cells were washed and recultured overnight in complete medium. The cells were then isolated and washed, and 5×10^4 cells per well were cultured in 0.2 ml complete medium for 48 h in the presence or absence of rIL2, TGF- β 1 antibody or control antibody (chicken IgG) with the last 4 h pulse with ^3H -TdR. The amount of ^3H -TdR incorporated into cellular DNA was quantified by standard liquid scintillation counting.

Statistical analysis: Data analysis was performed using a commercially available statistical package (SYSTAT, Evanston, IL). Student's t-test (two-tailed) was used for comparison of radioactive ^3H -TdR and densitometry data. ^3H -TdR uptake data was approximately log normally distributed and were log transformed prior to use of Student's "t" test. IL2 receptor data were bimodally distributed; the Kruskal-Wallis rank order test was used to compare IL2R expression on cells from H I V - a n d H I V + d o n o r s .

RESULTS

In addition to the 89 HIV⁻ individuals who served as control donors, 93 HIV⁺ individuals who did not have AIDS were studied. The mean percentage and number of CD4 lymphocytes per mm³ were 48.22 ± 1.12 , 1323 ± 54 (mean $\pm$ SEM, $n = 74$), respectively, for the seronegatives and 30.59 ± 1.57 , 640 ± 41 ($n = 81$) for the seropositives, both significantly less in HIV⁺ ($p < 0.001$, t-test). Due to limited availability of cells, not all individuals could be studied in all experiments. Consistent with previous reports, the HIV⁺ homosexual men in the present study showed significantly decreased ³H-TdR incorporation ($p < 0.006$) in response to PHA stimulation in 72 h cultures. The geometric mean ³H-TdR incorporation (minus and plus the SEM) in 15 donors in each group was 11,940 (10186, 13996) in HIV⁻ and 6442 (5623, 7379) in HIV⁺.

EXOGENOUS rIL2 DOES NOT FULLY RECONSTITUTE PROLIFERATIVE RESPONSE OF CELLS FROM HIV⁺

We considered the possibility that in vitro spread of HIV-1 might lead to increased cell mortality which could account at least in part for the reduced proliferative response seen with cells from HIV⁺ donors. Therefore to find the additional mechanisms that contribute to the defective proliferative response, activated cells from HIV⁻ and HIV⁺ donors were recultivated at equal viable cell density. In these experiments, cells from 22 donors in each group were stimulated with PHA for 55 h, washed to remove endogenously produced lymphokines, and recultured overnight in complete medium to allow the optimal expression of IL2R on cells. The proliferative response of these cells was examined after 48 h in culture in the presence or absence of 1 nM rIL2. These conditions were

determined to be optimal for IL2 induced cell proliferation. As shown in Fig. 1, the proliferative response of cells from HIV⁻ and HIV⁺ donors was augmented by the addition of exogenous IL2 (HIV⁻, mean difference = 49,556 cpm, $p < 0.001$; HIV⁺, 28,008 cpm, $p < 0.001$, paired t-test). However, even in the presence of saturating concentrations of IL2, cells from HIV⁺ donors showed a proliferative response that was significantly lower than that of similarly treated cells from HIV⁻ donors ($p < 0.001$). Therefore, the decrease in proliferative response in IL2 treated cultures persisted even when numbers of viable cells per culture were standardized. These results suggest that cells from HIV⁺ donors have decreased expression of high affinity IL2R, or alternatively, a subpopulation of these cells fails to express IL2R.

HIGH AFFINITY IL2R EXPRESSION BY CELLS FROM HIV⁺ DONORS IS DECREASED

To evaluate the possibility of an insufficient number of high affinity IL2R expressed on PHA stimulated cells from HIV⁺ donors, the binding of ¹²⁵I-IL2 to IL2R was studied under high affinity IL2 binding conditions. The high affinity IL2R were significantly less on cells from HIV⁺ donors as compared to the HIV⁻ donors (Fig. 2, median IL2R per cell = 2885 in HIV⁻ and 924 in HIV⁺. $P < 0.031$, Kruskal-Wallis rank order test). Although there appeared to be a relationship between IL2 mediated augmentation of proliferation and IL2R expression (Fig. 2C), in two cases of HIV⁺ donors there was reduction of the proliferative response despite elevated levels of high affinity IL2R expression. This suggested that reduction of high affinity IL2R may not be sufficient in itself to explain the lower proliferative response of HIV⁺. Since the number of high affinity IL2R in these experiments represents an average number per cell, it is possible that there is an accumulation of IL2R α or β chain negative cells in the PHA

stimulated cell population of cells from HIV⁺ donors that contributes to the overall reduced average number of high affinity IL2R per cell.

EXPRESSION OF IL2R α AND β CHAINS IN CELLS FROM HIV⁺ INDIVIDUALS

The lower ¹²⁵I-IL2 binding to the cells from HIV⁺ donors suggested that there could be an accumulation of a certain T cell subpopulation in HIV⁺ donors that do not express IL2R β chain, the limiting component of the high affinity IL2R. Attempts to quantify expression of IL2R β on PHA stimulated lymphocytes by flow cytometry showed no consistent differences between cells from 12 HIV⁻ and 14 HIV⁺ donors in 5 separate experiments (Fig. 3). Only weak staining with anti-IL2R β antibody was observed, and the percentages of stained cells expressing IL2R β could not be accurately assessed. These results are consistent with previous studies of healthy donors (44,48-50), and probably reflect a low level of IL2R β expression per cell in both HIV⁻ and HIV⁺ donors.

Therefore, we examined relative expression of the IL2R α and β subunits. For these experiments, ¹²⁵I-IL2 was covalently cross-linked to its receptors and the relative expression of IL2R α and β subunits was determined by SDS-PAGE/autoradiography. In order to ensure that all β chains would bind IL2, we selected an intermediate concentration of ¹²⁵I-IL2 (1 nM), which would saturate both the high affinity ($\alpha\beta$) IL2R as well as the intermediate affinity (β alone) IL2R on the cells. Both IL2R α and β chains were present in cells from all HIV⁻ and HIV⁺ individuals studied, and the degree to which these subunits were expressed appeared to be strongly interrelated (Fig. 4A). To verify this, we analyzed the ratio of α/β expression in 25 HIV⁻ and 25 HIV⁺ individuals. As shown in Fig. 4B, there was no significant difference in the ratio of IL2R α/β

between HIV⁻ and HIV⁺ individuals ($p < 0.07$). No individuals with very low or very high ratios, which would have suggested selective reduction in the expression of the α or β subunit, respectively, were found.

IL2R α , β AND TGF- β 1 mRNA EXPRESSION IN PBMC ACTIVATED WITH PHA

Because TGF- β 1 inhibits IL2R α expression as well as IL2 induced T cell proliferation (34) and has been reported to be overexpressed by unstimulated PBMC from HIV⁺ donors (20), we considered the possibility of excessive production of TGF- β 1 as one of the possible mechanisms leading to the reduction of IL2R expression and T cell proliferative response in HIV⁺ donors. Therefore, we studied TGF- β 1 mRNA expression in PHA stimulated PBMC from HIV⁺ donors. Also, in the same individuals we studied IL2R mRNA to examine the relationship between IL2R and TGF- β 1 mRNA expression. Fig. 5 shows a typical Northern blot pattern of TGF- β 1, IL2R α , β and actin mRNA in cells stimulated with PHA for 24 h from 7 HIV⁻ and 7 HIV⁺ individuals. As shown by the densitometric analysis, TGF- β 1 mRNA was not expressed at higher amounts in the HIV⁺ compared to the HIV⁻ donors ($p < 0.23$, Fig. 6A). However, there was a significant reduction in the expression of IL2R α ($p < 0.006$, Fig. 6B) and β ($p < 0.02$, Fig. 6C) mRNA in HIV⁺ compared to the HIV⁻ donors. Thus it appears that a tandem reduction in the expression of mRNA for IL2R α and β subunits contributes to the deficiency of IL2R chains and high affinity IL2R.

IL2R α , IL2R β AND TGF- β 1 mRNA EXPRESSION IN UNSTIMULATED PBMC FROM HIV⁺ DONORS

In contrast to the present findings in PHA activated PBMC, a previous study has reported that unstimulated PBMC from asymptomatic HIV⁺ donors express significantly more TGF- β 1 mRNA compared to HIV⁻ donors (20). We considered the

possibility that there may be more in vivo activated T cells in HIV+ donors which might lead to over expression of TGF- β 1 mRNA as well as its production. Therefore, RNA was harvested from the unstimulated PBMC of 14 HIV⁻ and 13 HIV⁺ donors. The TGF- β 1 and IL2R β mRNA was detected in RNA preparations from unstimulated cells from all individuals. Fig 7A and B shows the densitometric analysis of the mRNA bands for TGF- β 1 and IL2R β in cells from HIV⁻ and HIV⁺ individuals. The TGF- β 1 and IL2R β mRNA expression were not significantly different for cells from HIV⁻ and HIV⁺ donors. Furthermore, the IL2R α mRNA was not detected in unstimulated PBMC from any HIV⁻ or HIV⁺ individual. Therefore there was no unusual expansion of an in vivo activated T cell population which express IL2R α mRNA. Similarly there was no excessive base line TGF- β 1 mRNA or reduction in the expression of IL2R β mRNA in unstimulated PBMC from HIV⁺ donors.

TGF- β 1 ANTIBODY DOES NOT REVERSE THE IMPAIRED T CELL PROLIFERATION OF HIV+

Although TGF- β 1 mRNA was not expressed at higher concentrations in unstimulated or PHA stimulated PBMC of HIV⁺ individuals, we considered the possibility that cells from HIV⁺ individuals may express more TGF- β 1 receptors or could produce higher concentrations of active TGF- β 1. To examine this possibility, cells were stimulated with PHA in the presence of TGF- β 1 antibody or control antibody for 55 h, washed and recultured overnight in absence of any lymphokines to induce optimal IL2R. These cells were then cultivated for 48 h in the presence of 1 nM rIL2 and either anti TGF- β 1 or control antibody. As shown in Table 1, TGF- β 1 antibody had no effect on the proliferative response of cells cultured in the presence or absence of rIL2 when used at a concentration that neutralized up to 10 ng per ml TGF- β 1.

DISCUSSION

The results of this study show that: 1) high affinity IL2R expression is decreased on PHA stimulated cells from asymptomatic HIV⁺ individuals and it appears to cause reduction of the IL2 induced proliferative response, 2) IL2R α mRNA is not expressed by unstimulated PBMC of HIV⁺ individuals but a steady state IL2R β and TGF- β 1 mRNA is expressed and their expression is not affected by the HIV serostatus, 3) the levels of IL2R α and β mRNA are lower in PHA stimulated PBMC of HIV⁺, but the expression of TGF- β 1 mRNA by these cells is not affected by the HIV serostatus, 4) expression of the IL2R β subunit is reduced but does not appear to be absent in PBMC from HIV⁺ individuals, as has previously been demonstrated for the α subunit. Moreover, flow-cytometry and cross-linking studies did not suggest that there are significant numbers of cells expressing the α or β subunit singly, and 5) TGF- β 1 does not appear to cause reduction of IL2 mediated proliferative response of cells from HIV⁺ because TGF- β 1 antibody failed to reverse the low proliferative response.

Despite the fact that there was a general correlation between reduction of high affinity IL2R expression and IL2 induced proliferation, 2/11 HIV⁺ individuals showed reduction of IL2 induced proliferative response despite no apparent deficiency in the expression of high affinity IL2R. This discrepancy could be due to the fact that the amount of ¹²⁵I-IL2 binding was quantified on 5×10^5 cells. The number of high affinity IL2R which was determined from the B_{max} of ¹²⁵I-IL2 binding represents an average number of receptors per cell with an assumption that high affinity IL2R are uniformly expressed on all cells in the test sample.

However there may be a heterogeneous T cell subpopulation that did not express high affinity IL2R, or a certain T cell subpopulation may have expressed supraoptimal number of high affinity IL2R but ceased to incorporate more ^3H -TdR after incorporating a threshold level of ^3H -TdR. Other possible explanations for reduction of proliferative response of HIV⁺ individuals could be: 1) a defect in the post IL2-IL2R signalling, 2) certain defects in T cell activation independent of IL2-IL2R interaction 3) defects in the dynamics of high affinity IL2R expression during the 48 hrs culture with rIL2 or 4) some combination of these possibilities.

The deficiency in the expression of IL2R α and β mRNA may be related to the reduction of high affinity IL2R on the cells from HIV⁺ individuals. It is also possible that selective reduction or absence of IL2R α or β chain on certain populations of T cells may have contributed to the reduction in the high affinity IL2R expression on PHA stimulated T cells from HIV⁺. By flow cytometry, lymphocytes stained with anti- β chain antibody as shown in previous reports (24,44,48-50), but generally fluoresced dimly and overlapped with the negative population, precluding precise quantification of β^+ cells. However there was no obvious difference in staining pattern of HIV⁻ and HIV⁺ donors. With ^{125}I -IL2 cross linking, we could not find any evidence of selective absence of the IL2R β chain. These results of the cross linking experiments do not agree with those of Sehraoui et al, who reported selective absence of IL2R α and/or β chains on PHA stimulated cells from HIV⁺ individuals in similar cross linking experiments (31). The reasons for these differences are not known. However, due

to limited efficiency of cross linkers (generally less than 10%), the absence of IL2R α or β chain, or both, on some PHA stimulated T cells from HIV⁺ cannot be ruled out.

TGF- β 1 has a wide variety of immunosuppressive effects on the immune response including inhibition of IL2R expression and IL2 induced proliferation of T cells (32,33). However, unstimulated as well as PHA stimulated PBMC of both HIV⁻ and HIV⁺ donors showed TGF- β 1 mRNA expression and its expression was not affected by the HIV serostatus. However there was a deficiency in the expression of IL2R α and β chain mRNA which suggests that these reductions are not related to the over expression of TGF- β 1. Furthermore TGF β 1 antibody failed to restore the IL2 induced proliferative response of HIV⁺ individuals, which also suggests that the impaired proliferation is not caused by the over production of active TGF- β 1 by the stimulated cells from HIV⁺ donors. These results on TGF- β 1 mRNA expression in unstimulated cells are contradictory to the findings of Kekow et al (20), who reported an increased expression of TGF β 1 mRNA by unstimulated PBMC of HIV⁺ donors compared to the HIV⁻ after PCR amplification. It is possible that different stages of HIV-1 infection may be responsible for this discrepancy.

In addition to the loss of CD4⁺ T cells, several phenotypically defined T cell subpopulations are known to be elevated or depressed in HIV⁺ individuals. Recently it has been reported that there is a selective depletion of CD29^{high} T cells in men with early HIV-1 infection (51). This has been interpreted as evidence for a decrease in the number of memory T cells, and has been associated with the loss of proliferative response to mitogenic antibodies in asymptomatic HIV⁺ men. Furthermore Pantaleo et al (52) demonstrated that there is an

expansion of CD3⁺CD8⁺DR⁺CD25⁻ T cell subpopulation in patients with AIDS (but not in asymptomatic HIV⁺ individuals), and that CD8⁺DR⁺ cells are severely deficient in their ability to express IL2R α chain and proliferate in the presence or absence of IL2 after stimulation with various mitogenic stimuli. Although we did not analyze phenotypic markers in the present study, it is possible that an increased representation of CD29^{low} and CD8⁺DR⁺ populations in the HIV⁺ individuals studied may have contributed to the reduction of PHA induced IL2R expression and proliferation.

In summary we have shown that IL2R α - and β -chain mRNA, their product, and IL2 induced cell proliferation are decreased in PHA stimulated cells from HIV⁺ individuals. However reductions in IL2R are not sufficient in some HIV⁺ individuals to explain completely the inhibition of proliferative responses. It is possible that accumulation of antigen nonresponding T cell populations in HIV⁺, as has been demonstrated in elderly donors (53), or additional defects in the signal transduction mechanism contribute to the reductions in the proliferative responses.

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LEGENDS.

Fig. 1. Scattergram of ^3H -TdR incorporation in PHA stimulated lymphoblasts from HIV⁻ and HIV⁺ (n = 22 in each group) individuals. PBMC were stimulated with PHA for 55 h, washed and recultivated for 16 h in complete medium. After washing, the cells were counted and recultivated at 5×10^4 cells per well in triplicate with or without 1 nM rIL2 for 48 h. Individual data points (O) and geometric group means (*) are shown. In the absence of rIL2, the geometric mean ^3H -TdR incorporation (minus and plus the SEM) was 22,803 (19634, 26485) and 16,181 (14125, 18535) for HIV⁻ and HIV⁺, respectively. Addition of rIL2 yielded ^3H -TdR incorporation of 70795 (65013, 77090) and 40,179 (35318, 45709) for HIV⁻ and HIV⁺, respectively.

Fig. 2. Relationship between ^3H -TdR incorporation and IL-2R expression in HIV⁻ (open circles) and HIV⁺ (closed circles) donors. Fifty-five hours after stimulation with PHA, cells were washed and recultivated in complete medium (A) or complete medium plus 1 nM rIL2 (B). ^{125}I -IL2 binding was measured under high affinity IL2 binding conditions and IL2 induced proliferation was measured after 48 h in culture. IL-2 augmentation (C) was calculated as the difference between CPM obtained in the presence and absence of exogenous IL2. Lines represent distance weighted least squares curve smooths of the data from both groups. The Kd of ^{125}I -IL2 binding to receptors in HIV⁻ was 26.7 ± 9.83 (mean $\pm$ SEM, n = 10) and $30.3 \text{ 01} \pm 17.8$ (n = 9) in HIV⁺.

Fig. 3. Representative histograms of the fluorescence distribution of IL2R β on PHA stimulated PBL from 2 HIV⁻ (left) and 2 HIV⁺ (right) individuals. Shown are

histograms from two donors with strong IL2R β fluorescence (top) and two with weak IL2R β fluorescence (bottom). For each case stained and control histograms are superimposed, with the peak representing lymphocytes stained with an isotype control antibody and the right peak lymphocytes stained with anti IL2R β antibody (TU27) as the first antibody. In most cases, a large population of positively stained lymphocytes were seen but could not be accurately quantified due to overlap with the unstained lymphocytes. Similar patterns of fluorescence were seen in a total of 12 HIV⁻ and 14 HIV⁺ individuals.

Fig. 4. A) SDS-PAGE analysis of ^{125}I -IL2 bound and cross linked to IL2R on PHA stimulated PBL from three HIV⁻ and three HIV⁺ individuals. Cells were stimulated with PHA for 55 h, washed and recultured for 16 h without stimulus. Cells were then washed, counted and incubated with 1 nM ^{125}I -IL2 for 1 h on ice. After incubation, cells were extensively washed and lysed with 80 μl lysis buffer for 30 min. The cell lysates were centrifuged at 16000g for 10 min. The supernatant was mixed with 2x sample buffer containing 2% SDS, denatured by incubation in boiling water bath for three min and resolved on 10% PAGE. The gels were fixed, dried and autoradiogrammed.

B) The ratio of IL2R α and β bound to ^{125}I -IL2. IL2R α and β bands on autoradiograms were scanned by densitometer. The mean ($\pm$ SEM) ratio of IL2R α/β was 1.46 ± 0.06 in HIV⁻ donors and 1.68 ± 0.10 in HIV⁺ donors (n=25 in each group).

Fig. 5. Northern blot analysis of TGF- β 1, IL2R α and β mRNA from 24 h PHA stimulated cells from HIV⁻ and HIV⁺. Total cellular RNA was extracted by lysing the cells in RNazol. Following electrophoresis on 1% formaldehyde denaturing gel

and capillary transfer to nitrocellulose membrane, the blots were hybridized with a random primer ^{32}P -labelled cDNA probe specific for human TGF- β 1. For IL2R α mRNA detection, cDNA probe for TGF- β 1 was stripped off and the blots were reprobed with ^{32}P -labelled IL2R α cDNA. For IL2R β and actin mRNA detection, cDNA probe for IL2R α was stripped off and the blots were reprobed simultaneously with ^{32}P -labelled IL2R β and actin cDNA.

Fig. 6. A-C). Scattered representation of densitometric analysis of TGF- β 1 (A) IL2R α (B) and IL2R β mRNA (C) bands. There were 14 HIV $^{-}$ and 13 HIV $^{+}$ individuals for each mRNA detected. After developing autoradiograms, the mRNAs bands were quantified by densitometry. The concentrations of TGF- β 1, IL2R α and IL2R β mRNA were recalculated with respect to 2000 densitometric units for actin in each individuals. The mean TGF- β 1 mRNA in HIV $^{-}$ was 2892 ± 341 (mean $\pm$ SEM) and 2375 ± 272 in HIV $^{+}$. The mean IL2R α mRNA in HIV $^{-}$ was 7168 ± 778 and 4286 ± 480 in HIV $^{+}$. The mean IL2R β mRNA in HIV $^{-}$ was 1634 ± 159 and 997 ± 179 in HIV $^{+}$.

Fig. 7. A and B). Scattered representation of densitometric analysis of TGF- β 1 (A) and IL2R β mRNA (B) bands in unstimulated PBMC from HIV $^{-}$ and HIV $^{+}$ donors. The procedure for isolation of total cellular RNA, electrophoresis and transfer to nitrocellulose papers and ^{32}P -labelling of cDNA probes were similar to the one described in Fig. 5. After developing autoradiograms, the mRNAs bands were quantified by densitometry. The concentrations of TGF- β 1 and IL2R β mRNA were recalculated with respect to 2000 densitometric units for actin in each individuals. The mean TGF- β 1 mRNA in HIV $^{-}$ was 4485 ± 774 (mean $\pm$ SEM, $n = 13$) and 3559 ± 310 ($n = 13$) in HIV $^{+}$. The mean IL2R β mRNA in HIV $^{-}$ was 1884 ± 308 ($n = 14$) and 1290 ± 241 ($n = 13$) in HIV $^{+}$.

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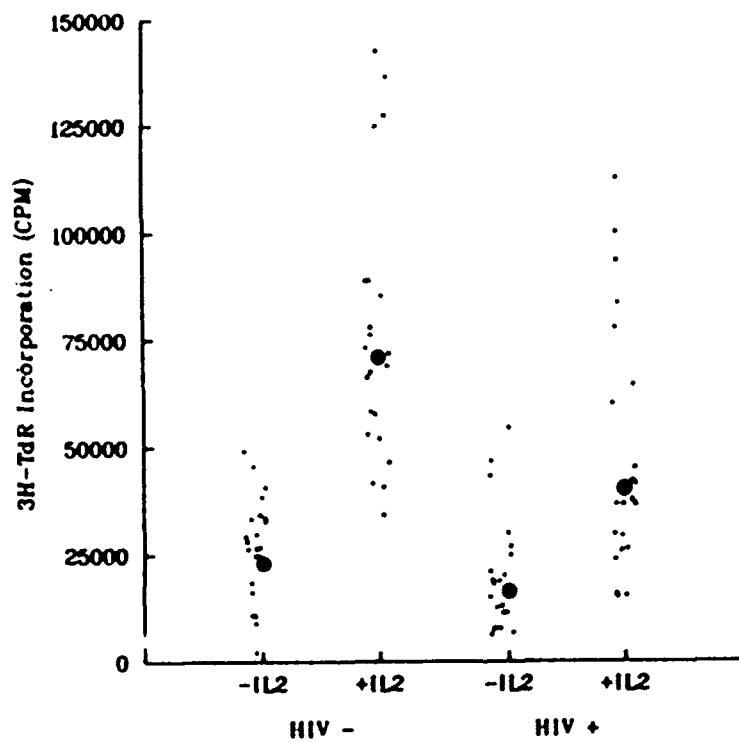
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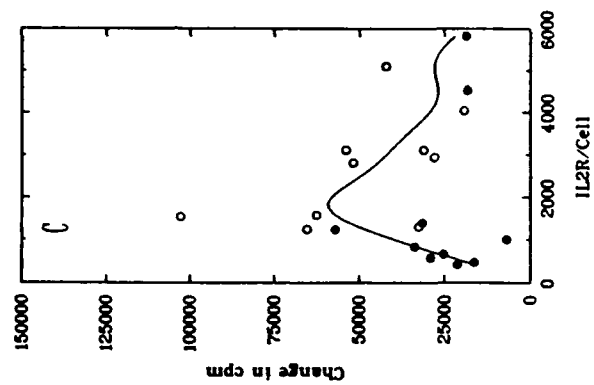
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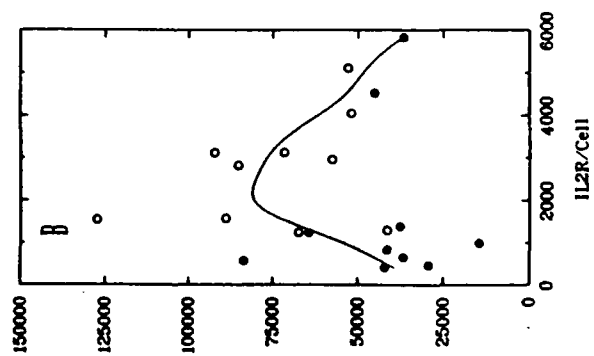
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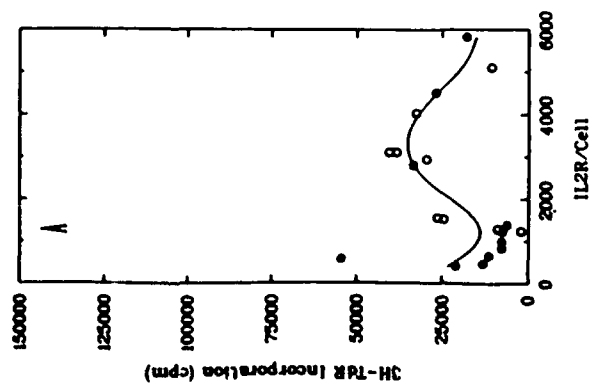
IL-2 Augmentation

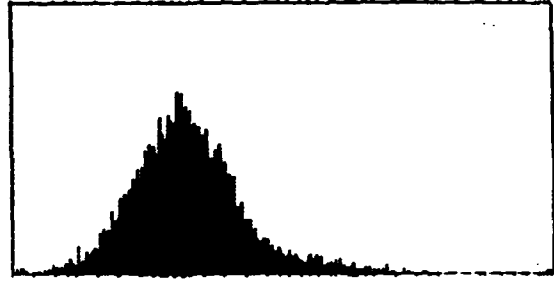
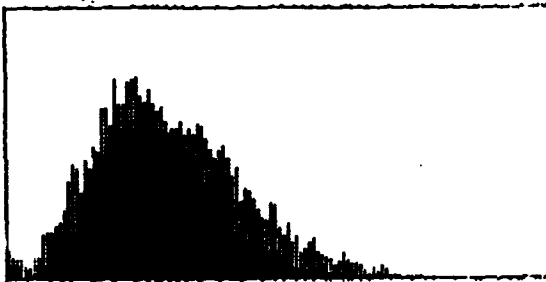
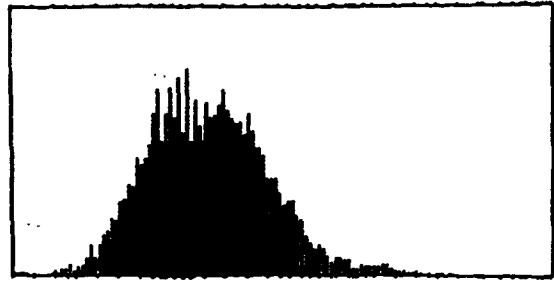
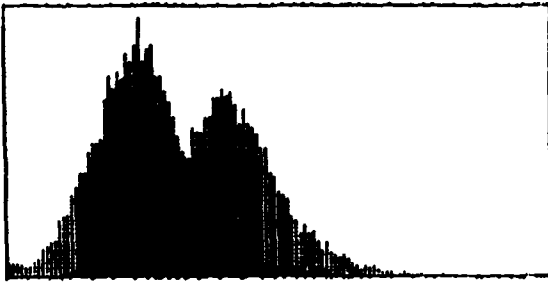


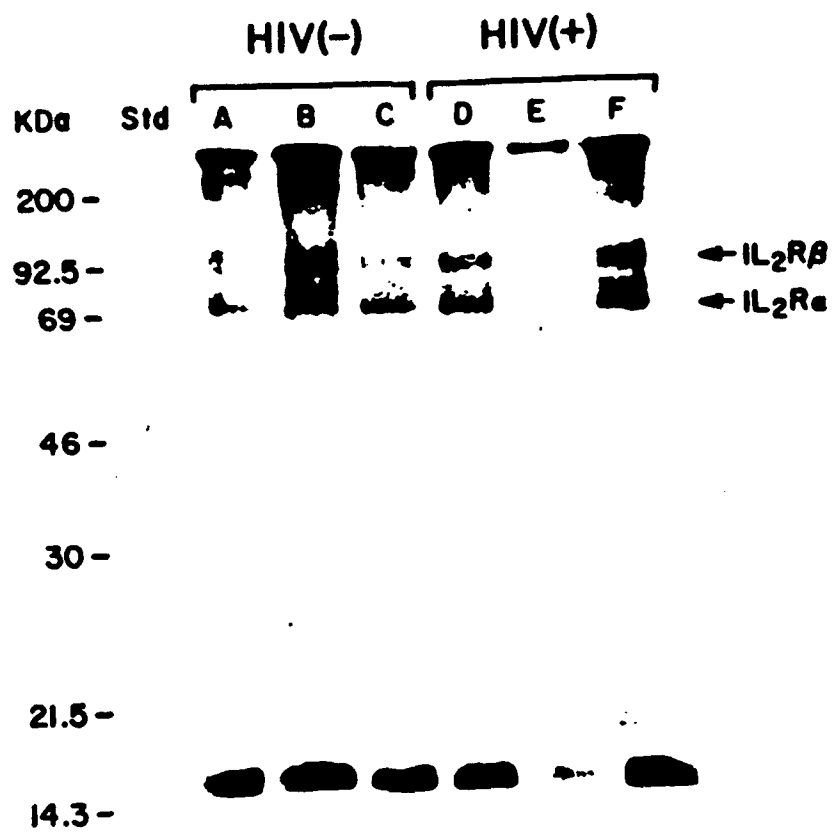
Lymphoblasts + IL-2



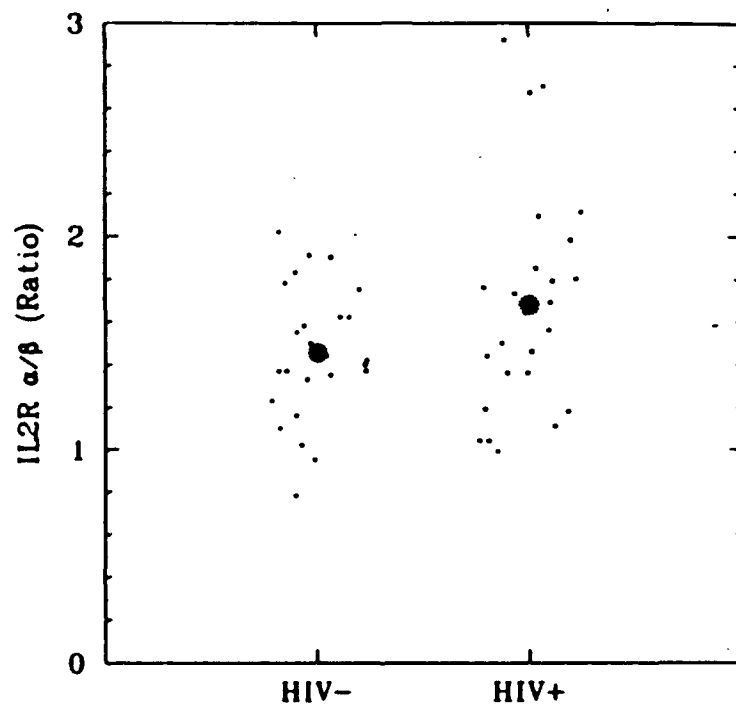
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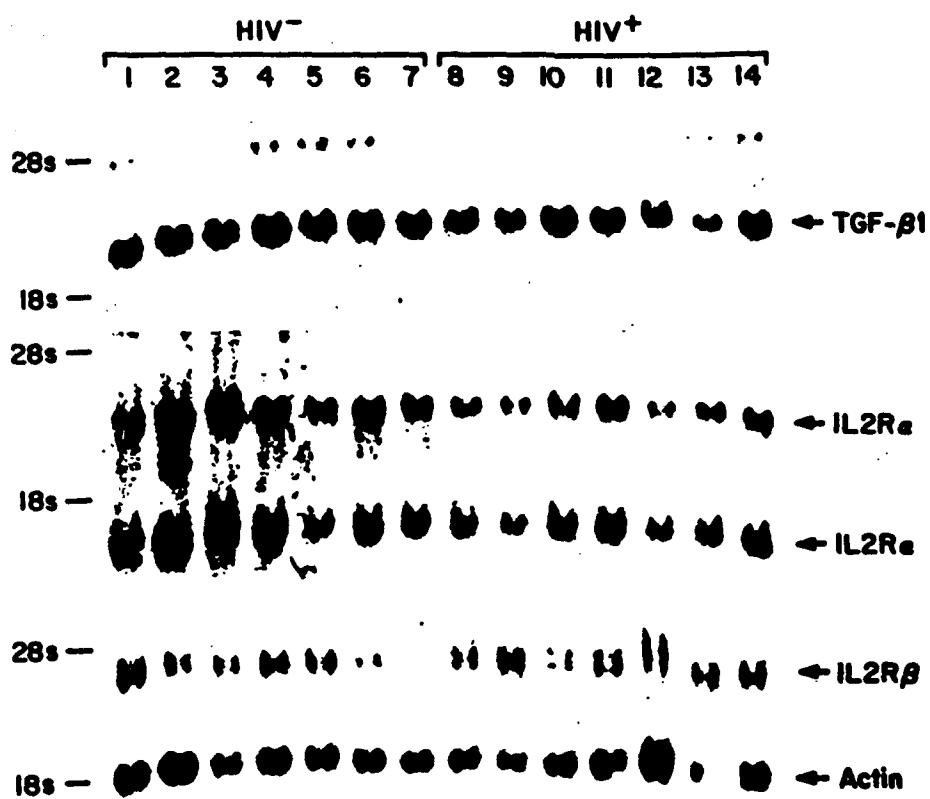




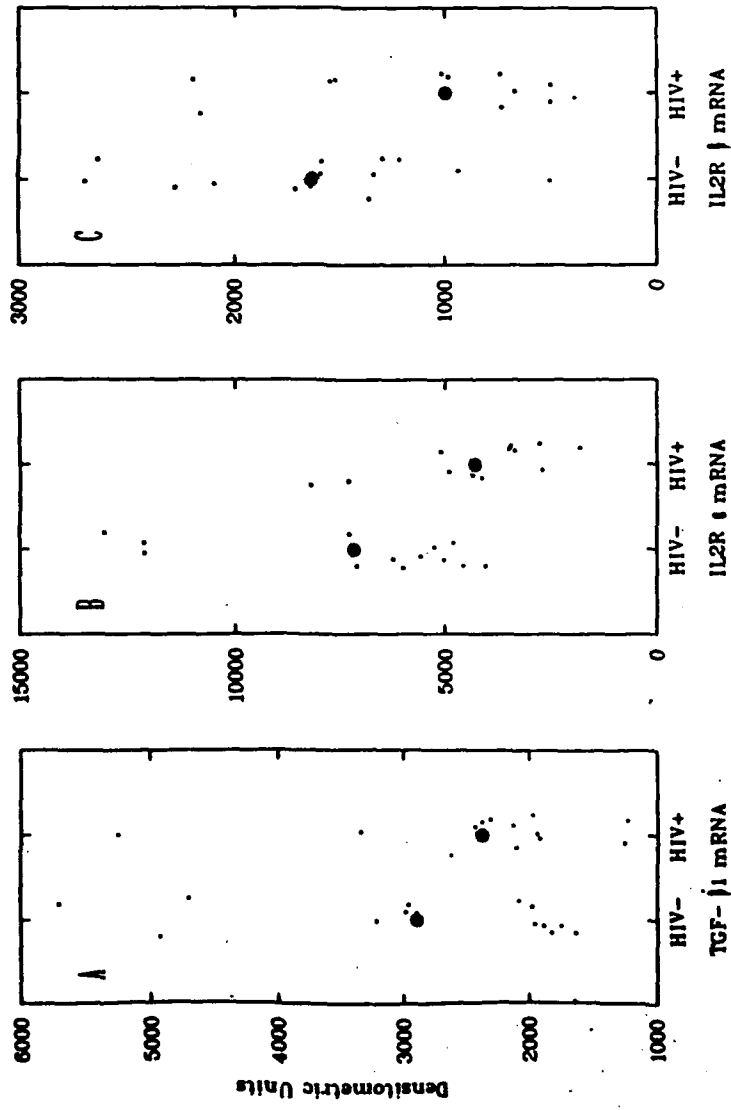
4A

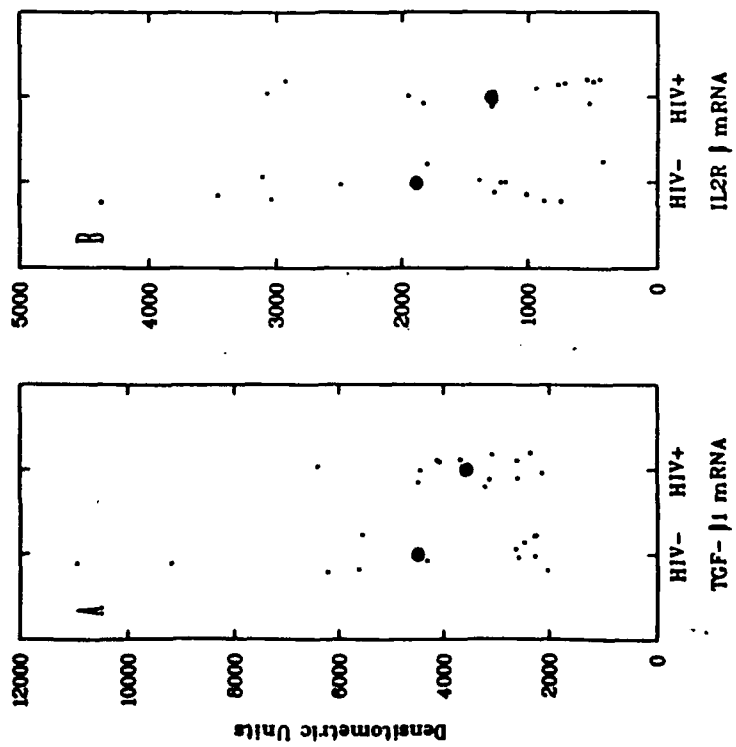


4B



6A-C





7A-B

TABLE 1

ANTI TGF- β 1 ANTIBODY DOES NOT INCREASE THE IL2 INDUCED PROLIFERATIVE RESPONSE OF CELLS FROM HIV+

Expt #	HIV Serostatus	Control antibody	Control antibody + IL2	TGF-β1 antibody	TGF-β1 antibody + IL2
3H-TdR incorporation (cpm)					
1	-	ND	78,309	41,836	89,316
1	-	ND	84,105	60,247	75,711
1	+	ND	84,652	42,859	87,877
1	+	ND	19,840	7,830	17,670
2	-	4,777	28,905	5,357	25,837
2	-	11,021	82,408	11,701	77,682
2	+	10,471	61,885	8,389	84,566
2	+	10,073	81,714	5,395	65,879
3	-	17,156	70,698	18,356	76,031
3	-	13,729	87,900	13,860	106,113
3	+	24,394	71,339	23,909	82,870
3	+	13,670	60,383	21,077	79,943
4	-	54,147	227,465	32,120	252,233
4	+	35972	93,539	24,760	89,604

PBMC from HIV- and HIV+ donors (1.2×10^6 cells per 1.2 ml complete medium) were cultured in 24 well microculture plates in the presence of PHA and control chicken IgG or polyclonal anti TGF- β 1 antibody (12.5 μ g per ml). After 55 h in culture, cells were washed, and recultured overnight in complete medium. The cells were then washed and incubated for 48 h in the presence or absence of 1 nM rIL2 (5×10^4 cells per well in triplicate cultures in 96 well microculture plates). In these triplicate cultures, control antibody was added to the cells initially cultured in presence of control antibody while anti TGF- β 1 antibody was added to cells initially cultured in presence of anti TGF- β 1 antibody. ^{3} H-TdR incorporation was assessed during the last 4 h in culture. ND, not determined. Responses were not significantly improved by the addition of anti TGF- β 1 antibody along with rIL2 compared to rIL2 alone (mean augmentation = 5,585 CPM, $p = 0.122$, paired t-test) in cultures from HIV- or HIV+ donors.

submitted

**LONGITUDINAL STUDY OF HOMOSEXUAL COUPLES DISCORDANT FOR
HIV-1 ANTIBODIES IN THE BALTIMORE MACS STUDY**

**John Palenicek, Robin Fox, Joseph Margolick,
Homayoon Farzadegan, Nancy Odaka, Ellen Taylor, Linda Ward,
Jeanette Harris, Haroutune Armenian, Alfred J. Saah**

**The Johns Hopkins School of Hygiene and Public Health
Baltimore, Maryland**

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**Requests for reprints to: J.G. Palenicek, Department of
Epidemiology, The Johns Hopkins School of Hygiene and Public
Health, 624 N. Broadway, Ste 763, Baltimore, MD 21205.**

ABSTRACT

Thirty-six sexually active couples serologically discordant for HIV-1 within the Baltimore Multicenter AIDS Cohort Study (MACS) were studied to determine (1) if evidence of HIV-1 infection could be detected in the HIV-1 antibody negative partners, and (2) if factors associated with lack of transmission of HIV from the seropositive to the seronegative partner could be identified. Six HIV-1 seropositive couples and 18 seronegative couples were followed concurrently for comparison. None of the seropositive subjects had an AIDS defining illness at entry into the study, and all subjects were followed for one year. Despite having engaged in high-risk sexual activities (unprotected anal receptive and/or insertive intercourse) for a median of 40 months with an HIV-1 seropositive partner, none of the HIV-1 seronegative men in discordant couples had evidence of HIV-1 infection by viral culture, p24 antigen testing, or PCR for HIV-1 DNA. Discordant seronegatives and seropositives did not differ from concordant seronegatives and seropositives in numbers of circulating CD4, CD8, and natural killer lymphocytes, or in prevalence of antibodies to HSV-1, EBV, or CMV, except that discordant seronegative men were less likely than their seropositive partners to have antibody to HSV-2. The reason for the apparent lack of HIV-1 infection in seronegative discordant individuals remains unexplained and may offer an opportunity to identify factors in other seronegative or seropositive persons

that protect against transmission of HIV-1.

Key Words: HIV-1 Transmission, HIV-1 Epidemiology, HIV-1 Immunology, and Sexual Partners

INTRODUCTION

Since the recognition of the epidemic of acquired immune deficiency syndrome (AIDS) in the early 1980's and the discovery of the human immunodeficiency virus, type 1 (HIV-1) as the cause of AIDS, it has become clear that the predominant mode of transmission of HIV-1 is through sexual activity with an infected person (1,2). There have been numerous attempts to determine factors that increase or decrease the risk of HIV-1 transmission. Characteristics of infected individuals which are associated with transmission of HIV-1 include late stage HIV-related disease and duration of infection (3-6), presence of other viruses such as cytomegalovirus (CMV) and herpes simplex virus, type 2 (HSV-2) (11-15), presence of other sexually transmitted diseases (29), and the route by which the seropositive person was infected (2,3,5-9,16-18). Transmission of HIV-1 may also be effected by viral characteristics which include variability in *gag* and *tat* proteins and their functions (10-15).

We identified a group of gay male couples in the Baltimore/Washington, D.C. center of the Multicenter AIDS Cohort Study (MACS) who are serologically discordant for antibody to HIV-1 (i.e., one partner is seropositive and the other seronegative). We conducted a case-control investigation in comparison with serologically HIV-1 concordant (positive-positive or negative-negative) gay couples, to identify factors associated with the lack of transmission of HIV-1. Factors studied included:

safe or protected sexual activities, incidence of other sexually transmitted diseases, numbers of immunologic cells, and clinical status of the infected partner. All couples in this study have been followed for one year.

METHODS AND MATERIALS

Population

All participants in this study were among the 1153 members of SHARE, the Baltimore/Washington, D.C. site of the MACS, a prospective study of the natural history of HIV-1 infection in gay/bisexual men. Participants in SHARE have been seen every six months since the first visit in 1984, at which time 31% of them were HIV-1 seropositive. Further details of the study methods and findings from SHARE/MACS have been published (17). Between August, 1988 and June, 1989, sixty gay male sexual partners of men already in MACS were enrolled by self-referral, forming a cohort of 60 couples who were classified as follows: (1) discordant (one partner seronegative and the other seropositive) (2) concordant seropositive (both seropositive), and (3) concordant negative (both seronegative). All couples were in a primary sexual relationship of at least three months' duration, with or without secondary sexual contacts. 116 of the 120 men knew their HIV-1 serostatus at entry into the study.

Participants were evaluated at the Johns Hopkins Hospital and completed an extensive interview regarding sexual practices during the entire relationship with the primary partner. The interview included questions on the frequency of masturbation, oral-genital activity, protected and unprotected anal insertive and receptive intercourse, and changes in the frequency of these activities over time. The same questions were also asked about

secondary partners. Past and current medical diagnoses, including sexually transmitted diseases and genitourinary infections, were also recorded. Each participant was asked about events that may have exposed him to a partner's body fluids such as shared needles, razor blades, and trauma. At follow-up visits, which occurred approximately every 6 months, interval sexual and medical histories were obtained.

Laboratory Tests

HIV-1 antibody was measured at each visit by Enzyme Immunoassay (Genetic Systems, Seattle, WA) and a Western blot (Bio-Rad, Hercules, CA). Western blots were interpreted as negative if no bands were detected, intermediate if bands from one of three major gene regions were present (gag p17, p24, p55; pol p31, p51, p66; env gp41, gp120/160), and positive if bands from two gene regions were detected.

Other laboratory tests included measurement of antibodies to HSV-1, HSV-2, EBV, and syphilis (Rapid Plasma Reagin-RPR) (Smith Kline Beecham, King of Prussia, Prussia, PA); measurement of total T-lymphocytes (CD3⁺), CD4 lymphocytes (CD4⁺), CD8 lymphocytes (CD8⁺), and NK cells (CD3⁻ and CD56⁺ or CD16⁺) by flow cytometry with use of monoclonal antibodies (Becton Dickinson, Mountain View, CA) as described (19); a complete blood count and a 10,000 cell automated differential (SmithKline Beecham); serum IgG, IgA, and IgM (Whittaker Bioproducts, Inc., Walkersville, MD); and determination of HIV-1-p24 antigen using an

immunocapture method (Abbott Laboratories, Chicago, IL). For HIV-1 detection by culture, peripheral blood mononuclear cells obtained by density gradient centrifugation of heparinized whole blood were co-cultivated with phytohemagglutinin-stimulated lymphocytes for up to 28 days (20) and HIV-1 in culture supernatants was detected by HIV-1-p24 antigen capture (Abbott Laboratories); all cultures were done under code. For HIV-1 detection by polymerase chain reaction (PCR), the presence of amplifiable DNA was determined with the amplification of an HLA site and of a 210 bp target in the gag gene (V. Sanders Sevall and Karen Young, Specialty Laboratories, Inc., Santa Barbara, CA; per written communication). Samples showing variable levels of reactivity were retested using a primer pair complementary to conserved regions of gag (SK 38/39) and pol (SK 101/145) (21).

Data analysis

To test the statistical significance of differences between groups, the Chi-square test for 2 X 2 tables, Fisher's Exact Test, or Student's t-test was used. For comparison of median ages and average durations of relationships, the Wilcoxon test was used. Matched-paired analysis was used to assess the significance of differences within discordant couples for specific sexual activities and demographic characteristics. ANOVA was used to test differences in T-lymphocyte values within and among groups for baseline and followup visits.

RESULTS

Based on the results of HIV-1 antibody testing, of the 60 sexually active homosexual couples enrolled, 18 were HIV-1 concordant negative, 6 were HIV-1 concordant positive, and 36 were HIV-1 discordant. Of note, the 36 members of the HIV-discordant couples who had negative ELISA tests for antibodies to HIV-1 all had negative cultures for HIV-1, negative PCR tests for HIV-1 proviral DNA, and negative antigen capture tests for serum p24 (Table 1). In contrast, most of the latter tests were positive in the known seropositive members of this study (Table 1). Thus, there was no evidence that any of the seronegative discordant couple members had been infected with HIV-1.

These results suggested the possibility that the seronegative discordants had had a low rate of exposure to HIV-1. Therefore, we determined the frequency and duration of high risk sexual activity within the discordant relationships. As shown in Table 2, the frequency of unprotected anal insertive and anal receptive intercourse was quite high for both partners in the discordant relationships. Seronegative discordant partners reported a lower frequency and duration of anogenital receptive intercourse than their seropositive partners. Condom use was inconsistent or absent in most cases, and more than half of the seropositive men had ejaculated into their seronegative partner's rectum while not using a condom. Twenty-seven of 36 (75%) of the discordant couples had entered into their relationship before

November, 1985. Based on these data, and the fact that the seropositive discordant couple members had been seropositive since 1984, it is highly likely that the seronegative partners had been exposed to HIV-1 as a result of sexual contact with their primary seropositive partner.

In order to identify factors that could account for the persistent seronegativity of these discordant partners, we compared the seronegative and seropositive discordant couples to members of the other groups with respect to demographic characteristics, history of sexually transmitted diseases, and antibodies to herpesviruses. As shown in Table 3, there were no outstanding demographic differences among these groups, which were similar in terms of race, median age, median body mass index, and former or current cigarette smoking. The same was true of antibodies to other herpesviruses and RPR for syphilis (Table 3). However, a significant association between HIV-1 seropositivity and the presence of HSV-2 antibody was found in the discordant couples ($p = 0.04$).

We next looked at possible cellular immunological factors that could protect against HIV infection or transmission. Measurement of CD4 lymphocytes, CD8 lymphocytes and NK cells were performed on all partners for baseline and the 2 six month follow-up visits (Table 4). The seropositive discordant partners' mean numbers of CD4 and CD8 lymphocytes were not significantly different from those of the seropositive concordant partners (Table 4 and Figs. 1 and 2). In addition, the

seronegative discordant and concordant individuals had similar absolute CD4 cell counts at all visits. Baseline CD8 cell counts for seronegative discordants (734/cells/mm³) were higher than in seronegative concordants (592/cell/mm³), and this difference was marginally significant ($p = 0.09$). This difference was not seen during the follow-up visits. Between the initial and one-year follow-up visit, both discordant and concordant seropositive individuals experienced similar declines in CD4 counts (Table 4). No decline in CD4 cell counts occurred among seronegative men.

Followup study. During the one-year follow-up period, 2 of 34 (6%) seronegative discordant partners engaged in unprotected anal receptive intercourse; however, no seronegative partner reported ejaculation into his rectum from any partner. Seven of thirty-four (21%) seronegative discordant partners reported unprotected anal insertive intercourse. One seronegative partner engaged in both unprotected anal insertive and receptive intercourse without ejaculation into his rectum from his partner. In total, 10 of 34 (30%) of the seronegative discordant partners engaged in either insertive or receptive anal intercourse without protection during the follow-up period.

Despite these risky sexual practices during the follow-up period, all discordant seronegative partners remained negative for HIV by serology and PCR (Table 4). Three (8%) of the seropositive discordant men developed an AIDS-defining illness, and 14 (40%) experienced AIDS-related symptoms such as oral

candidiasis or night sweats.

DISCUSSION

In this study we have identified a population of HIV-1 negative men who have practiced high risk sexual activity, i.e. unprotected anal receptive or insertive intercourse, over a long period of time with a known HIV-1 positive partner. Detailed sexual histories of these discordant couples indicated that the majority of the seronegative discordant partners were involved in these behaviors for a median of 40 months, and at a high frequency (median of 4 times per month) as reported by the seropositive partner, and a median duration of 34.5 months and frequency of 2.5 times per month reported by the seronegative partner. Even though the frequency of these behaviors decreased in most cases when the couples learned of their discordant serostatus, approximately 30% of the seronegative discordants still engaged in high risk behaviors during the one-year period of this study. Despite this, the seronegative men in discordant relationships remained uninfected by HIV-1 according to all of the tests commonly used to diagnose this infection, including sensitive and well standardized detection assays for anti-HIV antibody, p24 antigen, culturable virus, and proviral DNA of HIV-1. Moreover, no seroconversions were detected among the seronegative participants during the one year follow-up interval, eliminating the possibility that some of the seronegatives were in the "window" period at the first evaluation in this study.

Given the long relationships and sexual practices of the

couples studied, it seems highly unlikely that the lack of spread of HIV-1 to the discordant seronegative person can be explained solely on the basis of lack of exposure. While some individuals have become infected with HIV-1 after a small number of sexual encounters with infected partners (1,3,18,22,23), others have remained uninfected despite large numbers of unprotected contacts with infected sexual partners (2,3,4). The explanation for these diverse outcomes is not known. Possible explanations could be related to characteristics of the transmitter, the transmitter's specific viral isolate, or the uninfected sexual partner. With respect to the transmitter, an increased likelihood of transmission has been associated with clinical stage of disease (5,24), decreased CD4 cell levels (1,5,6,24,26), p24 antigenemia (31), and elevated numbers of CD8 cells (2,5,25,27,28). Within our study population, high risk sexual activity between discordant partners occurred primarily during asymptomatic stages of infection. The low prevalence of p24 antigenemia, along with the relatively high mean CD4 cell count of 561 cells/mm³ at baseline, suggests that relatively low infectiousness due to early stage of disease among the seropositive partners may be part of the reason for lack of transmission of HIV-1 infection in this study. However, it should be noted that during the followup period 3 of the seropositive discordant partners developed AIDS and 14 developed new AIDS-related symptoms. Thus, the stage of infection was not uniformly early in the discordant seropositives. Also, these clinical data show that the specific

HIV isolates present in these individuals were capable of causing progressive immunodeficiency suggesting that the lack of transmission is not due to nonpathogenic HIV-1 isolates. Factors in an HIV-uninfected sexual partner that may predispose toward infection include specific sexual practices (1,2,6,22), concurrent sexually transmitted diseases (7,8,9), anorectal injury (4), and variation in immunologic make-up (25). In our cohort, the seronegative discordant partners engaged in unprotected anal receptive and insertive intercourse over an extended period of time. Most of the discordant couples studied had few or no sexually transmitted diseases during their primary relationships, and the prevalence of antibodies to CMV, EBV, and HSV-1 was similar to that of the other couples studied, with the possible exception of antibodies to HSV-2. Specifically, the prevalence of antibodies to HSV-2 was lower among the discordant seronegatives than the discordant seropositives ($p = 0.04$). This would be consistent with the possibility that genital ulcerations may play a role in susceptibility to infection with HIV after exposure (7,8,9), although the actual self-reported prevalence of active herpes infections in our subjects was low.

Another possible explanation for lack of transmission would be protective immunity in the uninfected seronegative partner. However, we found no consistent differences between the seronegative discordant individuals, who had presumably been exposed to HIV, and the seronegative concordant individuals, who had presumably not been exposed, with respect to numbers of T-

lymphocyte subsets (CD4 and CD8) and natural killer cells. Of interest, CD8 cells in discordant seronegatives were increased at baseline and one year later compared to concordant seronegatives, but this difference was not present at the 6-month evaluation and overall was not statistically significant. More data, including functional tests of CD8 cells in suppression of HIV replication and anti-HIV cytotoxicity, are needed to define the role of CD8 lymphocytes in protection against HIV-1 infection.

In conclusion, the persistent HIV-1-seronegative status of the discordant seronegatives studied remains unexplained but potentially important. Other factors not investigated in this study, such as the amount of virus in semen (30), genetic or phenotypic variations in the CD4 molecule on T cells and monocytes, and mutations in HIV over time (10-15), may provide the needed explanation. Further investigation of the unusual cohort of discordant couples reported here may help identify factors which contribute to nontransmission of HIV by infected individuals. This information might be useful in reducing the spread of HIV by sexual contact.

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Figure Captions

Figure 1. CD4 cell counts from baseline SHARE Couples Study: medians, confidence intervals and ranges.

Figure 2. CD8 cell counts from baseline SHARE Couples Study: medians, confidence intervals and ranges.

Figure 1. Legend: SN-DC - seronegative discordant; SN-CC - seronegative concordant; SP-DC -seropositive discordant; SP-CC - seropositive concordant; * outlier - > 1.5 hinge spreads from upper hinge; 0 far outlier - > 3 hinge spreads from upper hinge.

Figure 2. Legend: (as above, Fig.1 Legend) A single data point is not seen at >2500 CD8 cells.

Table 1. Baseline laboratory results: HIV-1 tests

	<u>Discordant partners</u> Total of 36 pairs		<u>Concordant partners</u> Total of 24 pairs	
	HIV Ab+ partner (n=36)	HIV Ab- partner (n=36)	HIV Ab+ 6 pairs (n=12)	HIV Ab- 18 pairs (n=36)
HIV-1 culture positive	86% (31/36)	0% (0/36)	67% (8/12)	0% (0/36)
Presence of HIV-1 DNA (PCR)	87% (7/8)	0% (0/36)	100% (1/1)	0% (0/8)
Presence of p24 serum antigen	14% (5/36)	0% (0/36)	8% (1/12)	0% (0/36)

Table 2. Activities reported at baseline. Anal receptive and insertive intercourse with primary sexual partner.

	<u>Discordant partners</u>			
	<u>HIV Ab+ partner</u> (n=36)		<u>HIV Ab- partner</u> (n=36)	
	Receptive	Insertive	Receptive	Insertive
Number of persons	31 (86%)	29 (81%)	31 (86%)	31 (86%)
-median length of time doing activity (mos)	39.8	39.8	34.5	47.9
-median frequency of activity (times/mo)	4.0	4.0	2.5	4.0
Number of persons doing activity unprotected all or some of the time	27	24	29	27
-median frequency of activity (times/mo)	4.0	2.0	1.0	4.0
-ejaculation into partner's rectum (%)	-	63	-	82
-receiving partner's ejaculation in rectum (%)	93	-	63	-

* No significant difference at $p \leq .05$

Table 3. Characteristics of Study Participants Reported at Baseline.

	<u>Discordant partners</u> Total of 36 pairs		<u>Concordant partners</u> Total of 24 pairs	
	HIV Ab+ partner (n=36)	HIV Ab- partner (n=36)	HIV Ab+ 6 pairs (n=12)	HIV Ab- 18 pairs (n=36)
Median age at entry	36	37	35.5	37
Race (white)	89%	94%	100%	94%
Cigarette smoking				
- ever	53%	44%	50%	58%
- current	11%	22%	8%	22%
Median Body Mass Index (kg/m ²)	24	24	22	24
History of sexually transmitted disease during relationship				
- hepatitis B	0%	0%	0%	3%
- syphilis	14%	8%	0%	8%
- gonorrhea	14%	14%	17%	11%
- genital/perianal herpes	8%	3%	0%	6%
Presence of antibody to:				
- HSV ₁ (≥ 0.25 OD)	83%	67%	100%	75%
- HSV ₂ (≥ 0.25 OD)	90%	67%	100%	75%
- CMV (FIAX titer >30.0)	100%	97%	100%	97%
- EBV (capsid IgG $\geq 1:320$)	97%	100%	100%	89%
IgA (≥ 340 mg/dl)	28%	11%	42%	3%
RPR - reactive	3%	0%	0%	3%

*p = 0.04 Fisher's exact two-tail when comparing discordant seronegatives to discordant seropositives

Table 4. Laboratory results from baseline, 6 month and one year visits.

	<u>Discordant Partners</u>		<u>Concordant Partners</u>	
	HIV Ab+ partner	HIV Ab- partner	HIV Ab+ pairs	HIV Ab- pairs
<u>Baseline</u>				
Number of persons at visit	36	36	12	36
Mean CD4 lymphocyte (per mm3)	561	1224	630	1167
Range	(49-1425)	(684-2244)	(162-1332)	(610-1796)
Mean CD8 lymphocyte (per mm3)	1054	734*	962	592
Range	(170-3139)	(150-1912)	(473-1594)	(319-1190)
Natural Killer cells (per mm3)				
Number of persons tested	24	24	9	31
Mean ($\pm$ SD)	119 ($\pm$ 104)	246 ($\pm$ 176)	136 ($\pm$ 86)	231 ($\pm$ 125)
<u>6 months</u>				
Number of persons at visit	33	31	10	31
Mean CD4 lymphocytes (per mm3)	538	1146	547	1255
Range	(39-1757)	(544-2703)	(119-1188)	(653-2288)
Mean CD8 lymphocytes (per mm3)	1080	684	984	704
Range	(442-2498)	(237-1290)	(417-1385)	(274-1210)
ELISA or Western blot positive	32	0	10	0
<u>One year</u>				
Number of persons at visit	33	32	12	31
Mean CD4 lymphocytes (per mm3)	479	1225	595	1232
Range	(22-1487)	(478-2136)	(61-1607)	(711-1882)
Mean CD8 lymphocytes (per mm3)	1029	722	936	616
Range	(430-1892)	(193-1347)	(438-1394)	(333-1134)
ELISA or Western blot positive	33	0	12	0
PCR reactive (HIV-1 DNA)		0/32**		

* p = 0.09 when comparing discordant negatives with concordant negatives

** One of four men missing the one year follow-up visit was tested at the first six month visit and found negative on PCR.

DEVELOPMENT OF ANTIGEN PRESENTING CELLS (APC) FROM MONOCYTE DEPLETED BONE MARROW (MDBM): A SYSTEM TO STUDY THE EFFECTS OF HIV-1 INFECTION ON MONOCYTE ONTOGENY.

A. dela Torre,* A. Donnenberg,* and S. Noga.* (Intr. J. Spivak). The Johns Hopkins Oncology Center, Baltimore, MD.

In order to study the effects of HIV-1 on the phenotypic and functional ontogeny of APC, we have developed a liquid culture system in which MDBM is driven to proliferate and differentiate with recombinant human GM-CSF and M-CSF in Teflon flasks. Ability of plastic adherent cells from fresh and cultured MDBM to present tetanus toxoid to autologous monocyte depleted lymphocytes was assessed by measuring ³HTdR uptake after 5 day co-culture in microtiter wells. Generation of APC function and maximal CD4 expression (monocyte gate) required the presence of CSFs and a 14 day culture interval (table). In contrast, LeuM3⁺ phenotype developed after 7 days in culture with CSFs.

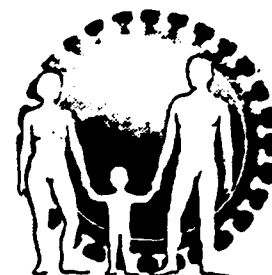
Day	CSFs	³ HTdR Uptake*	Leu M3 % Pos	CD4 %Mono Gate
0	No	314 (274, 424)	0.8	0.0
7	No	109 (52, 228)	5.4	28.0
7	Yes	150 (45, 498)	24.4	27.7
14	No	1953 (1734,2192)	15.9	39.9
14	Yes	5336 (4428, 6430)	45.9	61.2

* Geometric mean CPM, parentheses: lower, upper 95% confidence intervals.

This system provides a simple and rapid assessment of APC ontogeny, demonstrating the appearance of LeuM3 prior to detectable APC function. In a preliminary experiment, MDBM was inoculated with HIV-1 IIIB (24 h), washed, cultured with CSFs for 14 days, and transferred to microtiter wells. Cells adhering to the plastic wells were then irradiated and tested for APC activity by co-culture with autologous monocyte depleted lymphocytes. Antigen specific lymphoproliferation was increased 3-fold over that observed when mock infected MDBM adherent cells were used as APC. Intimate contact between APC and lymphocytes during immune interactions may be a mechanism by which the virus facilitates its own transmission and propagation.

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DO NOT TYPE ANTI-TETANUS ANTIBODY (ATA) IN UMBILICAL CORD BLOOD (UCB) FROM HIV-1 SEROPOSITIVE HAITIAN WOMEN

Neal A Halsey¹, R Boulos², A D Donnenberg¹, A Ruff¹, E Holt³, J Chamandy¹, J Job¹, T Quinn^{1,4}, C Boulos²
Johns Hopkins, Baltimore, MD; ²Centers for Development and Health, Port-au-Prince, Haiti; ³Tulane University, New Orleans, LA; ⁴NIH, Bethesda, MD

Objective: To determine if infants born to HIV-1 positive Haitian women receive protective levels of ATA.

Methods: Tetanus Toxoid (TT) is administered to pregnant women to prevent tetanus neonatorum. ATA were measured in 470 serially diluted UCB specimens by ELISA, standardized with WHO reference serum and capable of detecting 0.008 IU/ML. Protective levels of ATA were defined as ≥ 0.01 IU/ML.

Results. Eight (6.0%) of 134 UCB specimens from HIV-1 positive women and 24 (7.1%) of 336 specimens from HIV-1 negative women contained less than 0.01 IU/ML ATA. 13% and 9.5% of HIV negative specimens had borderline levels (0.01-0.04 IU/ML). The GMT of ATA in HIV-1 positive specimens was not significantly lower than that of HIV-1 negative specimens (0.39 vs. 0.54 respectively). Of the women whose immunization records have been reviewed to date, there are no differences in frequency of ATA < 0.01 IU/ML for HIV-1 seropositive vs. HIV-1 seronegative individuals when stratified by doses of tetanus toxoid received.

Conclusion: Although 6.8 % of infants in this population do not receive protective levels of ATA, infants born to HIV-1 positive mothers were protected as frequently as those born to HIV-1 negative mothers.

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ADOPTIVE TRANSFER OF IMMUNITY TO RECALL ANTIGENS FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION (BMT) IN AN AIDS PATIENT WITH NON-HODGKIN'S LYMPHOMA (NHL)

Donnenberg AD, Holland HK, Burns WH, Santos GW and Saral R
The Johns Hopkins University, Baltimore, MD, USA

Objectives. We have previously shown that transfer of donor immunity can be reproducibly achieved after allogeneic BMT by boosting the BM donor before transplant and/or the recipient on the day of BMT. The present study was conducted on a 41 yo HIV-1 culture⁺ man with NHL who received combined modality therapy of allogeneic BMT plus azidothymidine (see separate abstract, this meeting). The main objective was to determine whether transfer of donor cell mediated and humoral responses to tetanus (TET) and diphtheria (DIP) toxoids could be attained in a patient in whose natural responses to these antigens had been ablated by HIV-1 disease.

Methods. Serum IgG was measured by nephelometry. HIV-1 antibody was measured by immunoblot and quantified by densitometry. TET and DIP antibody was measured weekly by ELISA. Antigen specific proliferating lymphocytes (ASPL) were measured weekly by ³HTdR uptake. The BM donor was boosted i.m. with TET and DIP on day -8; the recipient with TET alone on the day of transplant.

Results. Before BMT IgG was elevated (1440 mg/dL) but TET and DIP antibodies were 10-fold lower than control reference values. TET and DIP ASPL were absent. After BMT, total IgG and HIV-1 antibody declined by half, consistent with clearance of host antibody. TET and DIP antibody rose >4-fold and were maintained above pretransplant levels. TET and DIP ASPL were detected 5 wks after BMT (>3000 CPM) and persisted until death from tumor relapse at wk 8.

Conclusion. These data indicate successful transfer of immunogen specific donor immunity and reconstitution of responses ablated by HIV-1 disease.

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ALLOGENEIC BONE MARROW TRANSPLANTATION (BMT) PLUS AZIDOTHYIMIDINE (AZT) IN AN AIDS PATIENT WITH NON-HODGKIN'S LYMPHOMA (NHL)

Holland HK*, Rossi JJ**, Donnenberg AD*, Zaia JA**, Santos GW*, and
Saral R*

*The Johns Hopkins University, Baltimore, MD, USA

**City of Hope National Medical Center, Duarte, CA, USA

Objective. To evaluate the effect of combined modality therapy (AZT plus
allogeneic BMT) on HIV-1 infection in a lymphoma patient with AIDS.

Methods. The patient, a 41 yo HIV-1 culture⁺ man with NHL, received
cyclophosphamide and total body irradiation to ablate tumor, bone marrow and
marrow derived cells. AZT (5 mg/kg q 4h) was begun 2 wks prior to BMT. Nine
days after initiation of ablative therapy he received 4×10^8 nucleated BM
cells/kg from his HLA identical sister. At this time AZT was reduced to 1.3
mg/kg q 4h and maintained at that level for the duration of treatment. HIV-
1 was monitored by culture and polymerase chain reaction gene amplification
(PCR, LTR and ENV, DNA and reverse transcribed RNA).

Results. Despite continuous AZT, engraftment was prompt (17 days to
neutrophils $> 500/\text{mm}^3$). Peripheral blood mononuclear cells and BM samples
became HIV-1 negative by culture and PCR 32 days after BMT. The patient died
of tumor relapse 47 days after BMT. Complete autopsy showed no evidence of
HIV-1 by PCR (brain, BM, spleen, tumor, heart, kidney, liver, lung, colon)
or culture (brain, BM, lymph node, tumor).

Conclusions. 1) The patient tolerated intensive therapy associated with BMT
2) Prompt engraftment was attained despite AZT therapy 3) PCR and culture
data suggest clearance of host cells harboring virus and prevention of
infection of repopulating donor cells.

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**EQUALLY POOR RESPONSE TO TETANUS VACCINE IN HIV
SEROPOSITIVE AND SERONEGATIVE MOTHERS IN ZAIRE**

Baende, E.*; Ryder, Robert**; Halsey, N.***;
Donnenberg, A.***; Quinn, T.***. *Projet SIDA, Kinshasa,
Zaire; **CDC, Atlanta, GA; ***Johns Hopkins University, Baltimore, MD.

Objective. To determine if current WHO recommendations (one dose in the last trimester if the mother has been vaccinated in the previous 3 years otherwise 2 doses with the last one at least 15 days before delivery) for use of tetanus toxoid vaccine (TTV) in pregnant women are appropriate for HIV(+) women in Zaire.

Methods. Before and after (at the time of delivery) TTV samples were obtained from 111 HIV(+) women and 207 HIV(-) women at a Kinshasa antenatal clinic. Absolute T4 lymphocyte counts (HIV(+) women only) and anti-tetanus antibody titers (ATAT) measured by an ELISA capable of detecting 0.0008 IU/ml, were determined on the paired blood samples. ATAT were classified as non-protective (<0.01 IU/ml), borderline (0.01-0.04 IU/ml) or protective (≥0.04 IU/ml).

Results. Maternal pre- and post-ATAT by serostatus.

	Before TT vaccine		After TT vaccine	
ATAT	HIV(+)	HIV(-)	HIV(+)	HIV(-)
Non-Protective	45%	26	15	13
Borderline	20	21	14	8
Protective	35	53 (p<.05)	29 71	19 (P=NS) 79

(p values compare pre-vaccination ATAT in HIV(+) and HIV(-) mothers and post-vaccination ATAT in the same groups)

In HIV(+) women, HIV-induced immunosuppression (ABS T4 counts) did not correlate with ATAT in pre- and post-vaccination determinations.

Conclusion. A total of 24% of the pregnant women (29% of 111 HIV(+) women and 21% of 207 HIV(-) women (P=NS) with documented appropriate receipt of TTV at a Kinshasa antenatal clinic had less than protective levels of ATAT at the time of delivery. Inability to document a difference in TTV response between HIV(+) and HIV(-) mothers suggests that current TTV practices should be reviewed for both groups of women.

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THE INCREASE IN CALCULATED CD3⁺CD4⁺CD8⁺ LYMPHOCYTES IN HIV-1 INFECTION REFLECTS BOTH A DECREASE IN NATURAL KILLER CELLS AND AN INCREASE IN TRUE CD3⁺CD4⁺CD8⁺ T CELLS EXPRESSING THE $\gamma\delta$ -T CELL RECEPTOR. J Margolick, E Scott, D Vlahov, and A Saah, Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205.

HIV-1 seropositivity is associated with an increase in the difference between the number of CD3⁺ lymphocytes and the total number of CD4⁺ and CD8⁺ lymphocytes [CD3 - (CD4 + CD8)] among peripheral blood lymphocytes (PBL) (Clin. Immunol. Immunopathol. 57:348, 1989). To determine whether this increase reflects an increase in lymphocytes expressing the CD3⁺CD4⁺CD8⁺ phenotype or a decrease in non-T (CD3⁺) lymphocytes expressing CD8, PBL from seronegative (SN; n=36) and AIDS-free seropositive (SP; n=32) homosexual men and intravenous drug users were analyzed by 2-color flow cytometry. True CD3⁺CD4⁺CD8⁺ lymphocytes were detected using anti-CD3-fluorescein and anti-CD4- and anti-CD8-phycoerythrin in one tube. SP showed the expected increase, compared to SN, in calculated [CD3 - (CD4 + CD8)] cells/mm³ (91.5 vs. 28.0; p<0.001). Directly measured CD3⁺CD4⁺CD8⁺ cells/mm³ were increased (85.0 vs. 66.6; p=0.099), and dim CD8⁺ cells were decreased (120.5 vs. 154.3; p=0.066), accounting for 90% of this difference. SP also had increased numbers of cells/mm³ expressing the δ TCR chain (84.9 vs 59.5; p=0.046) or coexpressing δ -TCR and low levels of CD8 (35.0 vs. 16.4; p=0.003), and greatly decreased Leu19⁺CD3⁺ cells (78.1 vs. 204.9; p=0.032). These data suggest that the absolute increase in CD3 - (CD4 + CD8) associated with HIV-1 seropositivity may be due primarily to a decrease in natural killer cells, with a smaller contribution from increased $\gamma\delta$ -TCR lymphocytes. Changes in these populations may reflect altered host defense against HIV or altered T cell kinetics in the presence of HIV infection.

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RECOMBINANT IL-2 (rIL-2) DOES NOT REVERSE THE REDUCED PHA
RESPONSIVENESS OF PBMC FROM HIV-1+ DONORS IN A QUANTITATIVE
LIMITING DILUTION ASSAY.

JB Margolick, JT Sherwood, R Centeno, LD McCall, AJ Saah, AD
Donnenberg*, and RK Chopra, School of Hygiene and Public Health
and *School of Medicine, Johns Hopkins Medical Institutions,
Baltimore, MD 21205

To analyze the effect of rIL2 on defective PHA
responsiveness of peripheral blood mononuclear cells (PBMC)
from HIV-1 infected donors, the PHA responsiveness of PBMC from
HIV-1-seronegative (SN) and -seropositive (SP) homosexual men
was analyzed in limiting dilution experiments done with and
without the addition of 200 pM rIL-2 (25 BRMP units/ml). rIL2
was not mitogenic to PBMC from SP donors after 3 days of
culture, and these donors showed the expected decreases in ³H-
thymidine incorporation, frequency of responsive cells, and
incorporation of ³H-thymidine per precursor compared to SN
donors. Exogenous rIL-2 at 200pM consistently failed to
augment these reduced responses. Moreover, PBMC from SN and SP
donors had similar increases in IL2R expression by flow
cytometry after PHA stimulation, although SP had a lower
percentage of CD4+ cells both before and after stimulation.
These results suggest that the loss of frequency and function
of PHA responsive cells in SP donors cannot be reversed in
vitro by rIL2 even in a very quantitative assay system, and
they provide further evidence that loss of T cell
responsiveness in SP individuals is due at least in part to
factors other than reduced IL-2 secretion or IL2R expression.

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ALTERED REPLACEMENT OF CD4 LYMPHOCYTES BY CD8 LYMPHOCYTES EARLY AFTER SEROCONVERSION TO HIV-1.

Margolick, Joseph B., Donnenberg AD, Muñoz A, Odaka N, Saah AJ.

Johns Hopkins School of Public Health and School of Medicine, Baltimore, MD

Objective. To determine if there is 1:1 replacement of lost CD4 lymphocytes with increased CD8 lymphocytes in HIV-1 infected individuals.

Methods. Data from 83 men who seroconverted (SC) to HIV-1 during a prospective study of gay/bisexual men (part of the Multicenter AIDS Cohort Study) were studied. Percentages (%) of total (CD3), helper (CD4), and suppressor-cytotoxic (CD8) T lymphocytes were determined by flow cytometry. Changes (Δ) in these cell populations were calculated between each clinic visit ($\approx$ 6 month intervals or semesters) and were analyzed by multiple linear regression in relation to the time of SC, with adjustment for repeated measures. 704 phenotypic evaluations over 10 visits yielded 503 valid observation pairs for this analysis.

Results. Estimated $\Delta\%$ CD4 and $\Delta\%$ CD8 per semester, and their sum, were as follows:

Change in Percent Per Semester after Seroconversion (SC)

	Pre-SC	1	2	3-5	6-9
$\Delta\%$ CD3	0.0	2.8*	1.2	0.0	-0.1
$\Delta\%$ CD4	-1.8	-6.4**	-2.8	-1.8	-2.2
$\Delta\%$ CD8	1.1	8.7**	3.4	1.3	2.3
$\Delta\%$ CD4 + $\Delta\%$ CD8	-0.7	2.3*	0.6	-0.5	0.1

Significantly different from Pre-SC value at $p \leq 0.03^*$ or 0.001^{**} .

Conclusions. The data demonstrate that the semester immediately after SC is characterized by large changes in T cell subsets and a significant imbalance (increase in CD8 > decrease in CD4) in the size of these changes. The fact that this imbalance was not seen before SC or at later times after SC indicates marked dysregulation of T cells in the period immediately following SC. The excess CD8 lymphocytes produced immediately after SC could represent either mature suppressor-cytotoxic cells or nascent lymphocytes produced in response to HIV-1 induced loss of CD4 cells.

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ADOPTIVE TRANSFER OF HUMAN IMMUNE MEMORY RESPONSES TO HU-PBL/SCID MICE: EFFECT OF DONOR AND RECIPIENT IMMUNIZATION. Markham, Richard B., Barber JP and Donnenberg AD. Johns Hopkins Medical Institutions, Baltimore, MD.

Objective: To study the effect of donor and recipient booster immunization on transfer of specific human antibody responses to mice with severe combined immunodeficiency (SCID mice) reconstituted with human peripheral blood cells (Hu-PBL/SCID).

Methods: Groups of 3 (6-8 wk old) SCID mice were transplanted i.p. with 1.25×10^7 peripheral blood mononuclear cells (PBMC) obtained from a human donor either before or after booster immunization of that donor with tetanus toxoid (TT). The recipient SCID mice were immunized at 3 or 35 days after cell transfer with 0.1 ml TT (10 Lf/ml) or with 0.5 ml 10% (v/v) sheep red blood cells (SRBC). The TT protocol was repeated with cells obtained from the donor 10 days after he received a TT booster immunization (0.5 ml, 10 Lf/ml). Sera, pooled by experimental group, were obtained from the recipient mice (0, 14, 35 and 45 days after cell transfer) and assayed by ELISA for human IgG antibody to TT and by hemagglutination for antibody to SRBC.

Results: Primary immunization of Hu-PBL/SCID mice with SRBC did not elicit detectable antibody responses. In contrast, adoptive transfer of secondary response was consistently observed, the level of responsiveness being markedly dependent on immunization protocol (Table). With TT immunization the donor antibody titer increased from 2,600 to 49,700 (inverse of greatest serum dilution at which antibody could be detected).

DONOR BOOST	MOUSE		RECIPROCAL TITER			
	DAY 3 TT	DAY 35 TT	DAY 0	DAY 14	DAY 35	DAY 45
-	-	-	0	8	ND	0
-	+	-	0	173	ND	873
-	-	+	0	8	0	0
+	-	-	0	1,691	3,324	930
+	+	-	0	30,064	24,079	22,901
+	-	+	0	1,691	1,118	171

Conclusions: 1) Hu-PBL/SCID could not generate 1° responses to SRBC. 2) Adoptive transfer of 2° responses a) depend on donor immune status b) require early (< Day 35) recipient immunization for optimal responses. 3) Titers achieved in optimally treated Hu-PBL/SCID mice approached those obtained in the recently boosted human donor. 4) This model system may prove useful for evaluating protective efficacy of immune responses generated in human HIV vaccine recipients.

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COMPARISON OF LYMPHOCYTE IMMUNOPHENOTYPES OBTAINED FROM
TWO DIFFERENT DATA ACQUISITION AND ANALYSIS SYSTEMS
SIMULTANEOUSLY ON THE SAME FLOW CYTOMETER. Scott, E.,

Chadwick, K., Margolick, J., Shapiro, H., Hetzel, A.,
Vogt, R. Johns Hopkins University, Baltimore, MD 21205
and Centers for Disease Control, Atlanta, GA 30333

Lymphocytes expressing CD3, CD4, CD8, CD16, CD45,
CD56, and TCR-Delta chain were quantified in parallel by
two different data acquisition and analysis systems
operating on the same flow cytometer. Blood samples
obtained from two AIDS research cohorts were stained
with FITC- or phycoerythrin-conjugated antibodies within
4-24 hrs of collection, lysed with hypotonic ammonium
chloride - sodium bicarbonate, and fixed with 1%
formaldehyde before flowing on an EPICS C cytometer.
Analysis with the EPICS C software was conducted by: 1)
collecting 2500 cells in a provisional lymphocyte bitmap
defined on a 64 x 64 channel FS-SS histogram; 2)
adjusting the bitmap if necessary; and 3) directing the
log fluorescence data from 5000 new gated events to a
64 x 64 channel quadstat display for determining
percent FITC- and PE-labeled cells. At the same time,
data collection and analysis of the four parameters was
performed with AcmeCyte software on a 486-based MS-DOS
computer connected to the EPICS through a 4Cyte hardware
interface and a 256-channel analog-to-digital circuit
board. The listmode data were written directly to disk
and displayed on a 256 x 256 channel histograms using 5
colors to reflect event counts. A rectilinear gate on
the FS-SS two-dimensional histogram determined the
lymphocyte population selected for the fluorescence
quadstat display. If adjustments were made while
recording, listmode data were reanalyzed through the new
settings. All gates and quadstat cursor selections were
determined by one of two people, usually the same person
on both systems for a given sample. Despite the
differences in display formats and gate shapes, the
results for positive cells agreed within 2% in nearly
all the samples analyzed. Thus, in the two systems
tested, with careful attention to display parameters
(levels and colors), very close agreement was obtained
between amorphous and rectilinear gating.

NAME Elvia Scott

ADDRESS Johns Hopkins School of
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CO-ORDINATE EXPRESSION OF IL2R α and β CHAINS ON STIMULATED T CELLS FROM HIV+ HOMOSEXUAL MEN. RK Chopra, WH Adler, A Sash and JB Margolick, for the Baltimore MACS, Johns Hopkins Univ. School of Public Health, Baltimore, MD 21205, and Gerontology Research Center, Baltimore, MD 21224

The interleukin 2 receptor (IL2R) β chain (IL2R β) is an essential and IL2 signalling component of the high affinity IL2R. Previous studies have shown that exogenous IL2 did not correct the reduction in PHA-induced lymphocyte proliferation seen in HIV⁺ individuals, despite abundant expression of the IL2R α chain (IL2R α). These data could be explained by a decreased expression of IL2R β on HIV⁺ donor lymphocytes. Therefore, we studied IL2R α and IL2R β expression by PHA-stimulated cells from 10 HIV⁻ and 10 HIV⁺ homosexual men from the Baltimore MACS, using ¹²⁵I-IL2 binding, cross-linking of IL2 and IL2R, and SDS-PAGE analysis. Although 3-day PHA blasts from HIV⁺ donors bound fewer molecules of IL2, this difference was largely due to lower cell yield in the cultures. Moreover, cells from the HIV⁺ donors showed no decrease in expression of IL2R β compared to IL2R α after three days of culture (i.e., the ratio of IL2R α /IL2R β expression was not different between HIV⁻ and HIV⁺ individuals). Despite this normal balance between IL2R α and β expression, 3-day blasts from HIV⁺ donors showed greatly reduced IL2-induced proliferation when washed and cultured with exogenous IL2 for an additional 24 hr, and this reduction was not corrected by adjustment for cell numbers. These results suggest the presence of either a requirement for other cytokines, a defect in accessory cell function, and/or defects in post IL2/IL2R signalling in cells from HIV⁺ individuals.

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ANALYSIS OF EXPRESSION OF $\gamma\delta$ -T CELL RECEPTOR AND $CD3^+CD4^-CD8^-$ PHENOTYPE BY LYMPHOCYTES FROM HIV-1 SEROPOSITIVE AND SERONEGATIVE HOMOSEXUAL MEN IN THE BALTIMORE MACS.

Margolick, Joseph, Scott, E., and Sash, A., The Johns Hopkins School of Hygiene and Public Health, Baltimore, MD, USA

Objective: To determine the degree to which the increase in calculated $CD3^+CD4^-CD8^-$ (double negative; DN) lymphocytes associated with HIV-1 seropositivity (Margolick et al., *Clin. Immunol. Immunopathol.*, in press) is due to an increase in lymphocytes expressing the $\gamma\delta$ T-cell receptor (TCR).

Methods: Peripheral blood lymphocytes from 17 SN and 19 SP (17 AIDS-free) homosexual men were analyzed by 2-color flow cytometry using the following combinations of fluorescein (FITC)-or phycoerythrin (PE)-conjugated monoclonal antibodies: 1) $CD3$ -FITC; 2) $CD4$ -PE/ $CD8$ -FITC; 3) $CD3$ -FITC/ $CD4$ -PE + $CD8$ -PE; 4) $CD3$ -PE/ α -chain-FITC; and 5) $CD8$ -PE/ α -chain-FITC. Calculated DN (CDN) were determined by the formula $\%CD3 - (\%CD4 + \%CD8)$.

Results: Compared to SN, SP had increased proportions of calculated DN (3.5% vs. 1.5%; $p=0.03$ by 2-sample T test), as expected, and δ^+CD3^+ cells (3.7% vs. 2.3%; $p=0.05$). Directly measured DN (DDN) cells ($CD3^+CD4^-CD8^-$ in antibody combination #3) were also increased in SP, but not significantly (4.6% vs. 3.5%; $p=0.13$); however, $\%DDN$ was significantly correlated with $\%CDN$ ($r=0.484$; $p=0.003$). DDN cells not expressing the δ chain ($\%DDN - (\delta^+CD3^+ - \delta^+CD8^+)$) were present in both SP and SN (2.3% vs 1.6%; $p=0.15$).

Conclusions: These data suggest that 1) the increase in calculated $\%DN$ lymphocytes which is associated with HIV-1 seropositivity is at least partly due to an increase in lymphocytes expressing the DN phenotype and the $\gamma\delta$ -TCR; and 2) changes in other lymphoid populations, such as a decline in natural killer cells, may also contribute.