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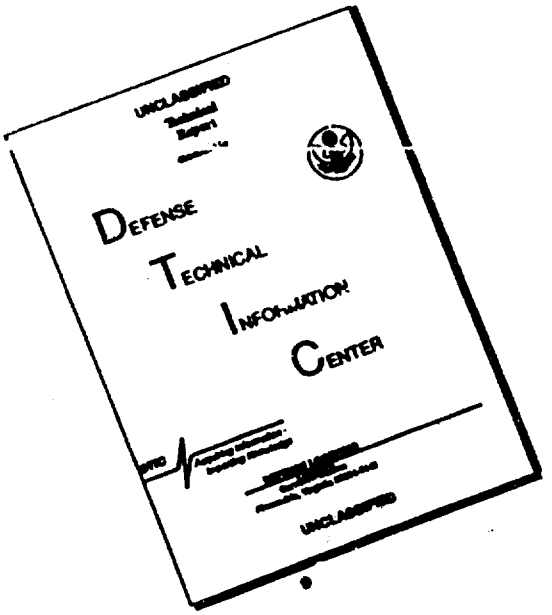

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This was a study to determine whether cytomegalovirus (CMV) might be a cofactor with the human immunodeficiency virus type 1 (HIV-1) to accelerate immunosuppression. Over seven hundred healthy HIV-1-seropositive U.S. Air Force personnel consented to be a part of this study, of whom 6.3% were initially CMV-seronegative. The CMV-seroconversion rate among seronegatives was 19% yr, indicating primary infection through close personal contacts. Three groups of subjects were studied to compare their rates of CD4+T-cell decline: CMV-seronegatives, CMV-seropositives, and CMV-seroconverters. CD4+T-cell depletion in CMV-seropositive subjects whose cell numbers were decreasing has been significantly faster than in CMV-seronegatives ($P = 0.003$). Using a similar means of analysis, herpes simplex virus type 2 (HSV-2) infection was not found to be associated with decline in CD4+T-cell depletion in HIV-1-infected people. The variations in the rates of CD4+T-cell decline among CMV-seropositive subjects were significantly greater than among CMV-seronegative subjects ($P \leq 0.0001$).

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n/a In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

PI Signature Kendall O. Smith Date 9/12/91

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INTRODUCTION

Numerous studies have shown that nearly all AIDS patients are CMV-infected (1-3), that CMV latency often shifts to active infection (4-5), with consequent severe disease in HIV-immunosuppressed people (6-9), and that active CMV-infection itself is immunosuppressive (10-13). These phenomena make it difficult to determine whether there is a "triggering" cofactor role in dual CMV-HIV infection, i.e., whether the synergistic phenomena well documented in doubly infected cells *in vitro* (12,14,15) have any relevance to the clinical course of doubly infected people.

Individual brain cells in AIDS patients are coinfected with CMV and HIV (7,8), which suggests that CMV could modify the pathogenesis of HIV infection, at least in the brain. CMV genomes can be found in both polymorphonuclear and mononuclear human leukocytes, including OKT4 cells. Therefore, dually infected OKT-4 cells are probably present in many HIV-infected subjects and the two viruses might interact *in vivo* in a manner similar to that observed *in vitro*.

The published evidence for the possibility that CMV infection serves as an early, triggering cofactor in AIDS is, not surprisingly, small. At present there seems to be little direct evidence that the *in vitro* interactions between CMV and HIV have any relevance in patients. Perhaps the best published evidence that CMV-infection is associated with a more rapid progression to HIV disease is the report of Webster et al. (35), who studied the effect of CMV and herpes simplex virus (HSV) infection in 108 HIV-infected hemophiliacs. They found that the risk to development of AIDS in CMV-seropositive subjects was 2.5-fold greater than in CMV-seronegative subjects. Seropositivity to HSV was not associated with AIDS progression, making the interpretation of their analysis and their conclusions seem more valid.

Others have encountered difficulty in determining whether CMV infection may accelerate immunosuppression in HIV-infected subjects because most HIV-infected subjects are also CMV-infected, therefore very few CMV-negative, HIV-positive subjects exist for study. Nearly all of the HIV-seropositive Air Force personnel in our

study were physically and immunologically normal, suggesting early stage HIV infections. Many were serologically negative on first screening and seroconverted during this study. This presented interesting opportunities for us to study the possible triggering cofactor (early) role of CMV in subjects who were likely to have been recently infected with HIV, not already clinically immunosuppressed. Of great importance to this study was the fact than about 6% of the HIV-seropositive subjects were CMV-seronegative, which gave us the opportunity to follow these subjects and compare their clinical course with 94% who were dually infected on entry into the study. Some of the CMV-negative subjects seroconverted during the course of the study, presenting an interesting third group to study. The fact that a relatively large number of subjects was involved (over 400 who visited at least twice) possibly makes our study more valid than smaller studies from the statistical point of view.

EXPERIMENTAL METHODS AND RESULTS

Cell culture and viral strains

MRC5 cells were obtained from Whittaker Bioproducts (Walkersville, MD) and were grown in Basal Medium Eagle (Flow Labs, Dublin, VA) with 10% calf bovine serum. The Towne strain of CMV (American Type Culture Collection, Rockville, MD) was used in all the CMV Western blot (WB) studies, HSV-1 (KOS strain) and G strain of HSV-2 (American Type Culture Collection) were used for all HSV Western blot studies.

Antigen production

CMV was grown in a 50-60% monolayer of MRC5 cells and harvested when 100% cytopathic effect was seen. To harvest the antigen, the cells were gently scraped from the T150 (150 cm^2) plastic flask and centrifuged at approximately 400 x *g* for 10 min. The cells were washed with 50 ml of phosphate buffered saline (PBS), centrifuged as above and the pellet was resuspended in 1 ml of PBS (to give a 50x concentration). This was performed on each T150 flask. The antigen aliquots were frozen at -70°C, thawed when needed and sonic oscillated for 3 min before being separated by gel electrophoresis.

HSV was inoculated onto an 80% monolayer of MRC5 cells and harvested at approximately 48 hr (100% cytopathic effect was seen). The procedure for harvesting and storing HSV-1 and HSV-2 was the same as described for CMV antigen.

WB procedure

CMV or HSV antigens were separated by gel electrophoresis by the method described by Laemmli [18]. The separated proteins were then electrically transferred to nitrocellulose as described by Burnette [19]. The nitrocellulose blots were cut into

2.5 mm width strips. These strips were "blocked" (excess non-specific proteins added) for 10 min with 3.75% milk (Carnation dry non-fat milk) in isotonic PBS (which is called blotto, pH = 7.4) [20]. Dilutions (1:100 for CMV antigen-treated strips and 1:50 for normal cell antigen-treated strips) of the subjects' plasma samples were made in blotto and the strips were left in the dilutions for 1 hour with shaking. The strips were rinsed X 3, transferred to clean trays and left in deionized (di)H_2O for 15 min. A 1:100 solution of labeled anti-human IgG, γ-chain specific, affinity purified antibodies conjugated with horseradish peroxidase (obtained from Calbiochem, San Diego, CA), diluted in blotto was placed over the strips and incubated for 1 h. Another X3 diH_2O rinse followed and the strips were transferred to clean trays and left with diH_2O on them for 15 min. Finally the water was removed and the strips were developed with the substrate 4-chloro-1-naphtol (Sigma, St. Louis, MO).

Interpretation of WB's

In order to interpret the results of WB's used to test samples for CMV antibodies, we developed guidelines based upon published reports [21,22] and after having examined several hundred strips. It was noted that there were 3 bands which were common in almost all of the obviously positive strips. We considered the presence of any of these "major" bands (140, 48 and 34 Kd) to indicate a CMV-seropositive sample. However, in most cases 2 or all 3 of the major bands were easily recognized in WB's of CMV-seropositive samples. If any other bands were present while major bands were absent, and these "minor" bands matched the bands on a cellular antigen strip (on which electrophoresis was done simultaneously), the sample was considered CMV-seronegative. If any minor bands were present on the CMV antigen strip and there were no corresponding bands on the cellular antigen strip, the sample was labeled "indeterminant." Only 8 of over 1,200 samples had to be classified indeterminant. All the other subjects were classified by WB into 3

groups: group 1 - CMV-seronegatives; group 2 - CMV-seropositives; and group 3 - CMV-seroconverters (seroconverted during the course of the study).

Regarding HSV-1 and HSV-2 antibody status, we noted the characteristic banding patterns with known control sera and devised guidelines similar to those above for making determinations. Samples tested for HSV-1 were considered positive if either or both 140 and 45 Kd bands were present on nitrocellulose strips with HSV-1 antigens. Samples tested for HSV-2 were considered positive if 2 or more of these bands were present on nitrocellulose strips with HSV-2 antigens: 55, 50 or 45 Kd. This approach to identifying banding patterns for differentiating HSV-1 and 2 antibody reactions was similar to the procedures described by Bernstein et al. [23], although our molecular weight determinations of the bands were slightly different from those reported by Bernstein et al.

Plasma samples

Over 700 different subjects' blood samples containing EDTA anticoagulant were taken at Wilford Hall Medical Center (WHMC, Lackland Air Force Base, San Antonio, Texas) and the plasma was separated at the University of Texas Health Science Center (UTHSC). Each plasma was aliquotted and frozen at -70°C until used. In view of our use of calcium - containing diluent in all assays (blotto), the plasma was converted to serum upon dilution and is therefore referred to hereafter as serum. No troublesome clotting was encountered at the plasma dilutions used.

Procedure for determining changes in CD4 + T-cell concentration (number/mm^3)

The CD4 + T-cell number was determined on blood collected at about 7:30 AM, which may have helped avoid diurnal variations [24]. Flow cytometry was done at WHMC, as previously described (Clerici et al. [25]). CMV WB antibody evaluations were done at the UTHSC on serum samples which had been collected from the subject

at the same time. A blood sample was received from each subject for CD4+T-cell count and CMV WB on an average of every 13 months. The only subjects selected for this study were those classified as Walter Reed (WR) 1 or 2 at the time of entry into the program. The WR staging evaluation-classification of each subject was done at WHMC and followed the protocol described by Redfield et al. [26]. Each of the subjects included in the study reported here returned to WHMC for reassessment-medical evaluation at least once. CD4+T-cell numbers obtained on a subject within 6 months of each other were not considered; multiple CD4+T-cell number determinations within one month were averaged.

We asked the question: "among subjects whose CD4+T-cell numbers were declining, were there significant differences in the rates of CD4+T-cell decline between the 3 CMV-serologic groups?".

All of the subjects in the cohort classified as WR 1 or 2 whose CD4+T-cell numbers were declining at the last visit (compared with the highest number found at any previous visit) were included for analysis. These subjects were then placed in CMV serologic groups 1, 2 or 3, as indicated above, on the basis of WB analysis. The CD4+T-cell number at the last visit was subtracted from the highest number obtained at any prior visit and the rate of decline was calculated (giving the cell number decline/time interval between these visits). This rate of decline was then normalized to decline/12 months. Then the mean ($\bar{x}$) rate of decline and standard deviation(s) for each group were calculated and Student's t-test was used to determine whether the rates of decline were significantly different between the groups. T-tests were computed taking unequal variances into account when appropriate (due to differences between the groups in standard deviations; see Results section) and a two-tail chart was used to determine the level of significance. Stat-sak, "The Statisticians Swiss Army Knife", was the computer software used to determine all t-test values. Since only CD4+T-cell declining subjects were included

for decline rate calculations, a one-tail chart might appropriate. However, we used a two-tail chart in order to interpret the results conservatively.

Chi-square and Fisher's exact caluclations were also made using Stat-sak. Results were two-tailed.

Other serologic test

FIAX tests were performed at WHMC for detecting CMV, HSV-1, and HSV-2 antibodies. FIAX is a commercially produced fluorescence system (Whittaker M.A. Bioproducts, Walkersville, MD).

Method for calculating the rate (incidence) of CMV-seroconversion

The number of CMV-seroconverters was divided by the total number of CMV-seronegatives per unit time. Six subjects who seroconverted, and the time interval between blood sample testing was 18 months or more, were not included in these calculations. Seroconversion rate was calculated (normalized) to percent seroconversion per 12 months.

Description of subject population

The U.S. Air Force mandatory HIV-1-screening between 1985 and 1990 yielded approximately 1000 HIV-1-seropositive persons. Most of this cohort were asymptomatic. Fifty-five percent (55%) of the subjects were Caucasian, 39% Black and 7% Hispanic, Asian or of other racial groups; 4.5% have been female. From this cohort, 773 had volunteered to be a part of this study by July, 1990 and this is the larger pool from which subjects were taken.

The subjects were an average of 29 years old ($\sigma = 5.7$) upon entry into the study and the average length of time each subject had spent in the study was 32 months; the shortest amount of time was 9 months and the longest was 52 months. None of

the subjects admitted into this study were taking azidothymidine (AZT) at any time before or during the study.

There was a total of 509 subjects who visited WHMC at least twice for medical evaluation and volunteered blood samples at the time of their visits. At the time of this writing, 26 of these were CMV-seronegative, 465 were seropositive and 11 were seroconverters (see Table 1). The remaining 7 were labelled indeterminant (see Results section). Of the 439 subjects who had visited Wilford Hall at least twice and were classified WR1 or 2 at time of entry into the program, 20 were CMV-seronegative, 409 were seropositive and 10 were seroconverters. There were 317 subjects who had visited WHMC at least twice, were WR1 or 2 at time of entry and whose CD4+T-cell numbers were declining. Twelve of these 317 were CMV-seronegative, 298 were seropositive and 7 were seroconverters.

Basis for selecting the WB technique for evaluating serologic status.

We assumed that WB analysis would be the most reliable technique for analysis of the CMV serologic status in HIV-infected subjects because CMV antibodies react in highly characteristic patterns in immunoblots [21, 22]. Thirty-nine subjects were selected who initially gave negative results by WB and 200 were selected (first 200 subjects in the study) who were CMV-seropositive by WB. For several months a commercially available fluorescence system (FIAX), was used at the WHMC for routinely detecting CMV antibodies. The results obtained by FIAX were compared with those obtained by WB, in a double-blind mode (see Table 2). Those samples, which were WB positive for CMV antibody, showed only 7 of 200 were FIAX negative-WB positive, indicating 97% agreement between the 2 tests. This suggests that the 2 tests, as used in this study, are approximately equal in sensitivity for detecting CMV antibody and that FIAX is reliable for identifying CMV-seropositive subjects. However, only 29 of 39 WB CMV antibody negatives were negative by

FIAX, showing a 74% agreement (26% discrepancy), which suggests a specificity problem with FIAX. We suspected that the false positives obtained with the FIAX technique were due to reactions of some subjects' sera with normal cell antigens contaminating the CMV antigen used in the FIAX.

To test the hypothesis that antibodies to cellular antigens contaminating commercial CMV antigen preparations might give false positive results, we tested 189 sera, taken in chronological order of receipt into the laboratory, from HIV-1-seropositive subjects, for the presence of antibodies to cellular antigens (one hundred and seventy-six of the 189 HIV-1-seropositive subjects were CMV-seropositive by WB, i.e., they demonstrated highly characteristic banding patterns with CMV antigens). Table 3 shows WB analysis to determine the presence of antibodies to uninfected cellular antigens. Approximately one-third (65/189) of these sera demonstrated 1 or more bands on uninfected cellular antigen blots. Fourteen percent (26/189) showed strong banding with cellular antigens. There was no apparent correlation between the presence of CMV antibodies and cellular antibodies in this study, i.e., CMV-seropositive subjects were no more likely than CMV-seronegative subjects to possess antibodies to normal cell antigens ($P = >.30$).

A normal population of people (HIV-1-seronegative subjects visiting a local blood bank) was studied similarly by WB to determine the presence or absence of antibodies to cellular antigens. Eleven of 46 (24%) had strong WB reactions, 6 of 46 (13%) had weak reactions and 29 of 46 (63%) were negative for cellular antibodies. One of the strongly reacting serum samples displayed 6 distinct bands. Since this data was similar to that in Table 3, it was concluded that HIV-1-seropositive and HIV-seronegative subjects have antibodies to normal cells in similar prevalence and types. The 6 most strongly reactive samples (by WB), however, came from HIV-1-seropositive subjects.

The facts that (a) about one third of human sera contain antibodies to normal cells, (b) some sera are strongly reactive to normal cellular antigens, and (c) serologic cross reactions reportedly occur between members of the Herpesvirus family [23], suggested to us that caution be exercised in interpreting serologic data to determine CMV antibody status. It appeared that the WB technique was more reliable than other methods because the highly characteristic WB banding patterns with CMV are valuable for differentiation from other antigen-antibody reactions.

Typical WB banding patterns for CMV, HSV-1, HSV-2 and normal cellular antigens are shown in Figures 1 and 2. The criteria we used for identifying CMV, HSV-1 and HSV-2 antibodies are given in Materials and Methods. Figure 3 shows serial dilution titrations by WB of 4 different sera which were strongly reactive to normal cellular antigens. Two sera were chosen from each of the 2 most obvious banding patterns seen throughout this part of the study. Serum samples were diluted 1:50, 1:150, 1:450, 1:1350, 1:4050, 1:12,150, and 1:36,450. The endpoint dilution (last dilution showing a banding pattern) for 3 of the 4 subjects' sera was 1:4,050. The two sera on the left (Fig. 3) showed a narrower banding pattern, i.e., mainly in the 29-45 Kd molecular weight range; the two on the right showed a broader pattern extending over a range of low molecular weights (bottom of the blots) to high (about 100 Kd). Some of the bands occurred in areas very close to bands seen with CMV, HSV-1, and HSV-2, suggesting the value of evaluating the entire WB banding pattern for each subject's sample and establishing criteria for specific antibody identification.

Figure 2 shows parallel WB banding patterns produced by 7 sera from HIV-1-seropositive subjects using CMV antigens (V) and cellular antigens (C). It is seen that some of the antibodies to cellular antigens gave banding patterns similar to those seen with viral antigens, as might be expected, because the crude viral antigen separated by gel electrophoresis also contained cellular antigen. As a result of these studies, we concluded that critical judgments on CMV serologic status required that

WB's be run in parallel for antibodies to CMV and to normal cellular antigens. The result of this parallel assay approach was that a few subjects believed to be CMV-seropositive by FIAX were actually seronegative (they possessed antibodies to normal cells in sufficient quantities to give false positive results).

The WB technique was, therefore, used to assess the CMV antibody status of all HIV-1-seropositive subjects who volunteered to participate in this study. In most subjects' serum samples tested for CMV antibodies, the characteristic CMV antibody banding patterns were sufficiently obvious for easy identification of subjects as either seropositive or seronegative. However, 38 HIV-1-seropositive subjects' sera of 773 needed further evaluation to discriminate between overlapping banding patterns with CMV and cellular antigens, i.e., patterns obtained on initial testing were not sufficiently typical and the reactions were therefore temporarily designated "weakly positive", "indeterminate" or "equivocal." WB electrophoresis was done simultaneously with CMV and normal cellular antigens. WB staining was then done simultaneously. Two of the 38 retested serum samples were then reclassified as negative for CMV antibodies because each of the WB bands on the CMV antigen strips matched bands on the normal cell antigen strips. An example of this is shown in Figure 4, where serum antibodies to normal cellular antigens initially confused interpretation of the WB. The presence of 2 distinct bands in the CMV antigen strip was due to reactions to cellular antigens, which was demonstrated in the cellular antigen strip. Therefore, the serologic status of this subject was CMV-seronegative. Thirty of the remaining 38 sera were reclassified as CMV-seropositive, and only 6 of the 38 remained indeterminant (patterns of banding were so indistinct as to make classification decisions by WB analysis too difficult). Accurate serologic assessment to allow classification of subjects into groups 1, 2 and 3 was of great importance for our study because so few subjects were initially CMV-seronegative (6.3%).

Comparisons between subjects in groups 1, 2 and 3 in their rates of CD4+T-cell decline.

The primary goal of this study was to identify HIV-1-seropositive individuals who were CMV-seronegative (group 1), to follow a sufficiently large number of these individuals over time, identify any subjects whose CD4+T-cell numbers were declining and to compare their rate of CD4+T-cell decline and the prevalence of decliners with HIV-1-seropositive people who were CMV-seropositive and declining (group 2). The third group of subjects studied were people initially HIV-1-seropositive and CMV-seronegative who developed primary infection with CMV (seroconverted) during the course of the study. Forty-nine (6.3%) of the 773 HIV-1-seropositive subjects were CMV-seronegative at the time of their first visit. Five hundred and nine (509) of 773 subjects returned at least once for serologic re-assessment. Of the 26 initially CMV-seronegatives who returned and volunteered blood samples at least once, 12 of these (19% per year) seroconverted to CMV-seropositive, indicating intimate physical contacts with CMV shedding people.

Table 4 presents data and calculations on the average rate of decline in CD4+T-cell numbers observed in all subjects whose numbers were declining (see Materials and Methods). Calculations showed an average rate of decline in group 1 and 2 of 108 and 188 CD4+T-cells/mm^3 per year, respectively. The significance of the difference between the changes in CD4+T-cell numbers between groups 1 and 2 was .003. This indicates that co-infection with CMV and HIV-1 results in accelerated decline in CD4+T-cell numbers among subjects whose numbers are declining. When all subjects in the cohort, declining or not, were averaged, the average rates of decline from first to last visits for groups 1, 2 and 3 were 31, 28, and 30, respectively. There was no significant difference between these numbers ($P \geq .9$). The difference between rates of decline in groups 1 and 3 were -108 and -162, respectively, not significant probably because of low numbers in both groups.

Table 5 shows the frequency of CD4+T-cell declining subjects in groups 1 and 2 using all WR1 and 2 subjects in the cohort. There was no significant difference between groups 1 and 2 in the fraction (prevalence) of subjects whose CD4+T-cell numbers were declining to any extent (60% and 73%, respectively, see footnote). However, the fraction of subjects declining rapidly, i.e., more than 244 cells/year (see Table 5), was significantly different in groups 1 and 2 (P = .019).

It seemed unlikely that age differences of subjects influenced this outcome, since the average ages of groups 1-3 were 27.6, 28.7 and 29.5 years, respectively, on entry into the study. However, the following data were collected and calculations made to verify this assumption. The average rate of decline of CD4+T-cells/mm^3 in the 30 youngest members of group 2 ($\bar{x}$ age = 22.8), subjects selected as above, was 155 (σ = 102), compared with the 30 oldest members ($\bar{x}$ age = 39.2), which was 186 (σ = 252). The P value comparing the oldest and youngest members of group 2 was .53, showing no significant difference in the rates of decline of their CD4+T-cell numbers.

The calculated standard deviation (σ) from the mean rate of CD4+T-cell decline in group 1 was 71, compared with 166 for group 2. This rather large difference in CD4+T-cell decline fluctuation between the two groups suggested that we calculate the standard deviation of individual subject's deviation from the mean CD4+T-cell decline in the two groups and thus determine whether the fluctuations in CD4+T-cell decline rates were significantly different between the 2 groups. The average deviation from the mean CD4+T-cell decline in group 1 (N = 12) was 57, with a standard deviation (σ) of 38. Similar calculations in group 2 (N = 298) were $\bar{x}$ = 117, with σ = 119, indicating much greater fluctuations in CD4+T-cell decline than in group 1. The Student's t-test calculation using a program accounting for unequal variance in the two groups, two-tailed, gave a P value of <.0001, confirming that

group 2 subjects showed greater fluctuations in the degree of CD4+T-cell decline than those in group 1.

Since most of the subjects in this study have so far not declined much in CD4+T-cell numbers and, therefore, most of the subjects were probably HIV-1-infected relatively recently, changes in clinical staging classification would be expected to be small in all groups. This was confirmed by the following calculations: the weighted average progression for all subjects was +0.61 WR Unit/12 months, with an average change of 0.30, 0.62 and 0.85 for groups 1, 2 and 3, respectively (no significant differences; $\underline{P} = 0.21$, groups 1 and 2; $\underline{P} = .13$, groups 1 and 3). The latter calculation ($\underline{P} = .13$) approaches significance.

Comparisons between HSV-seronegative and seropositive subjects

Herpes simplex virus has been shown to be a common agent infecting HIV-seropositive subjects [1]. Consistent with this report, we found that 105/134 (78%) of randomly selected HIV-1-seropositive subjects were also HSV-2-seropositive. Since HSV-2 infection is usually local and superficial and CMV infections are systemic and may expose many organs to the virus, we compared HSV-seronegative subjects with HSV-2-seropositive subjects for rates of CD4+T-cell number decline, i.e., we studied the possible cofactor relationship of HSV-2 and HIV-1 in suppressing CD4+T-cell numbers. The subject selection method and the significance calculation method used in the CMV cofactor study (already described) were used for this study. Table 6 shows that there were no significant differences between HSV-seronegative and HSV-2 seropositive subjects in their rates of CD4+T-cell decline ($\underline{P} = 0.193$).

CONCLUSIONS

Hirsch et al. predicted in 1984 [12] that CMV might interact with HIV to stimulate multiplication of the agent. Skolnik et al. in 1988 [14] showed bidirectional interaction in vitro between CMV and HIV-1, with mutual enhancement of replication. Davis et al. [15] demonstrated the mechanism by which CMV can transactivate promoter genes of HIV and they postulated that "CMV gene functions could enhance the consequences of HIV infection in persons with previous or concurrent CMV infection." Our studies were designed to explore this possibility, i.e., to determine whether the in vitro interactions demonstrated by these investigators [14,15] were demonstrable in HIV-infected subjects, i.e., in vivo. The hope was to study subjects sufficiently soon after infection that their general health was unaffected. Ideally, infected subjects would be initially recognized serologically rather than by the effects of immunosuppression.

The Armed Forces of the United States began, in 1985, a systematic ELISA screening of all their uniformed personnel for antibodies to HIV-1. To date, the U.S. Air Force has found approximately 1000 of its personnel to be HIV-1-seropositive, confirmable by WB. Of these, 773 had volunteered to be part of our investigation and 509 of this group had returned for further visits (on the average, every 13 months).

First, it was necessary to obtain the most reliable evaluation of the CMV serologic status of each indiviuual in this study, i.e., to determine whether each person did or did not have CMV-specific antibodies indicating prior infection with this agent. It was assumed that persons with such antibodies probably have a subclinical CMV infection (they maintain a steady-state relationship between viral replication and their immune defenses). Assays for CMV antibodies might be misinterpreted so as to give false positives for the following reasons: the difficulty in separation of viral antigens from normal cell antigens, and the presence of antibodies

to normal cell antigens in many subjects' sera (Figs. 2, 3, above [24, 27, 28]); serologic cross-reactions seen between some members of the human Herpesvirus family [29]; and the unavailability at the present time of well defined, purified CMV antigens, e.g., fusion proteins produced by genetic engineering [21]. We followed the WB testing strategy which had been used before for confirming presumptively HIV-1-seropositive subjects (found by ELISA screening), i.e., we used the characteristic CMV banding patterns seen with the WB technique. In order to avoid possible loss of valuable CMV-seronegative subjects (valuable because of their small numbers) through false-positive FIAX assays, we did CMV WB assays on all subjects entered into this study. Use of the WB procedure, and the fact that most of the HIV-seropositive subjects in this study were young, probably accounts for the finding that 6.3% of subjects entering our study were CMV-seronegative, compared to somewhat lower figures in some other studies of HIV infected subjects [2, 30]. Our finding of 6.3% CMV-seronegatives among HIV-1-infected young people compares well with the report of Halbert et al. [1], who used the Cordis Laboratories ELISA, and found 6.1% of AIDS patients (4/66) to be CMV-seronegative.

The CMV seroconversion rate of 19% per year (CMV-seronegative to seropositive) in our study is rapidly depleting the number of subjects in our group 1, but is providing additional information concerning the extent of physical contact behavior of healthy HIV-1-infected young people. These data suggest substantial intimate contact between some HIV-1-infected, CMV-naive subjects and CMV-shedding subjects. The observed 19% incidence of CMV-infection in our study is comparable with the 18.4% incidence of HIV infection among HIV-seronegative homosexual men in San Francisco during the period of 1982-84, before the promiscuous male homosexual population became fully aware of the mode of HIV transmission [31]. Continued close physical contacts between identified HIV-seropositive subjects and others raises the question whether these contacts are

selective (i.e., with already identified HIV-infected subjects) or with HIV-uninfected, CMV-shedding subjects. Primary CMV infections are probably undesirable for HIV-1-infected people, since such infections were found in our study (see Table 4) possibly to accelerate the decline in their CD4+T-cell numbers, compared with CMV-seronegative subjects (statistical significance of this difference was limited because of the small number of seroconverters). This accelerated decline in CD4+T-cell numbers among subjects with primary infections is not surprising, in view of the known short-term immunosuppressive effects of primary CMV infections, with associated inverted T-4/T-8 cell ratios [12, 13, 32]. The long-term consequences of acquiring primary CMV infections after having become HIV-1-infected (as in subjects in group 3) will be interesting to follow and to compare with subjects in groups 1 and 2.

The most highly significant difference in rates of CD4+T-cell decline ($P = 0.003$) was between CMV-seronegatives (group 1) and originally CMV-seropositive (group 2; see Table 4). The probable reason for the high statistical significance is that the number of subjects in each of these 2 groups was relatively larger (12 and 298) than the number of subjects in group 3 ($N = 7$). However, the rates of CD4+T-cell decline of originally CMV-positives (group 2) and CMV-seroconverters (group 3) were similar 188 and 162, respectively). As this study progresses in time, more CMV-seronegatives (in group 1) will likely acquire primary infections and will be placed into group 3, and it is predictable from these data that the significance of the differences between groups 1 and 3 will become greater. Also, the rates of CD4+T-cell decline could then be calculated over longer time periods, possibly making the group differences larger and more significant.

The difference in the frequencies (prevalence) of rapid CD4+T-cell decliners, i.e., subjects declining more than 244 cells/year, was significant ($P = .019$) when groups 1 and 2 were compared (see Table 5). Perhaps the difference in these

frequencies will increase as the study progresses. At this point in our study, however, CMV and HIV-1 coinfection was associated with a higher frequency of rapid CD4+T-cell decliners (group 2). This chi-square calculation to determine possible significance of decliner prevalence differences between groups 1 and 2 was of particular value because it eliminated the quantitative effect of extremely high "outliers" in either group upon the mean rate of decline in CD4+T-cell numbers.

We examined the possibility that the statistically significant differences in CD4+T-cell decline/year between groups 1 and 2 were due to some systematic data collection strategy error, subject selection strategy error or calculation errors. To clarify this issue we chose 2 other groups of subjects to compare: HSV-1 and-2-seronegative and HSV-2-seropositives (some of these were also HSV-1-seropositive). This seemed to be a useful choice because, as indicated earlier, HSV infections are usually superficial, not systemic (as with CMV). Therefore, coinfection of individual cells in vivo with HSV and HIV would seem less likely, even though ulcerative genital lesions caused by this agent have been shown to be an HIV-transmission cofactor [33]. Holmberg et al [33] reported 68% of HIV-seroconverters were seropositive for HSV-2, which agrees reasonably well with our observation that 78% of randomly selected HIV-1-seropositive subjects were HSV-2-seropositive by WB. The differences in CD4+T-cell decline/year in HSV-seronegatives and HSV-2-seropositives was not significant (see Table 6), and the weighted average decline of the two groups, 177, was almost identical to that seen in the CMV-seropositive groups 2 and 3 (Table 4). This indicates no detectable early triggering cofactor role for HSV-2 in HIV-1-infected people (postulated after in vitro studies [29]) and gives additional support to the conclusion that we have drawn, that CMV probably does play this important role.

Our observations confirm the predictions of Hirsch et al. [12], Davis et al. [15] and Skolnik et al. [14], that CMV might play an important cofactor role in HIV-

infected people. The data we have presented suggest that this cofactor role is played early in the natural history of HIV-1 infection, while subjects are still healthy and that this earlier "triggering" role in immunosuppression is different from the well known opportunistic role of CMV after substantial immunosuppression has already occurred. This conclusion is consistent with the observations of Eyster et al. [34], who studied the natural history of HIV infections in hemophiliacs. They found that only 18% of HIV-infected hemophiliacs had progressed to AIDS in 6 years. The prevalence of subclinical CMV infections in HIV infected 20-29 year-old hemophiliacs is only 38%, compared with 94% in our studies in Air Force men and in studies in HIV-infected male homosexuals [1]. Webster et al. [35] recently reported that the age-adjusted risk of progression to AIDS in CMV-seropositive, HIV-infected hemophiliacs was 2.5 times the risk in a comparable CMV-seronegative group.

There may be cofactors other than CMV infection that accelerate the decline of CD4+T-cell numbers in HIV-1 infected subjects, but CMV infections appear to be important. The drug DHPG (gancyclovir) has been used in suppressing CMV replication in ophthalmic and lung infections in AIDS patients, but it is probably too toxic for long-term prophylactic use and must be administered parenterally [36]. This makes use of DHPG in outpatients impossible or very difficult. Delaying onset of immunosuppression in HIV-1 infected subjects by suppressing CMV replication may be an interesting strategy to consider, however, and might stimulate a more vigorous search for less toxic anti-CMV drugs that can be given orally. The data we have presented in this paper reinforce the recommendation of DiNubile [37] that in "cytomegalovirus-seronegative patients with HIV infection, only cytomegalovirus-negative cellular blood products should be used." Need for blood transfusions in HIV-infected subjects given AZT might be increased because of bone marrow suppression. Subjects infected with HIV who are CMV-seronegative, and who are suffering the toxic effects of AZT may be particularly vulnerable to primary CMV infection.

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Table 1. Description of the subject population

Subject status	Numbers of subjects in various CMV study groups			
	Group 1*	Group 2*	Group 3	Total
Classified WR1 → 6†	26	465	11	502
Classified WR1 or 2†	20	409	10	439

* Mean CD4 + T-cell numbers on entry were: group 1 - 624; group 2 - 717; there was no significant difference between these means ($\underline{P}$ = .117, using Student's $\underline{t}$-test).

† Visited WHMC at least 2X, medically evaluated, volunteered blood samples at time of visit.

Table 2. Comparative study of CMV antibody detection by Western blot (WB) and FIAX*

Study Group Selected	WB Negative/ Total Tested	FIAX Negative/ Total Tested	% in Agreement	% Discrepant
All WB Negative	39/39	29/39	74	26
All WB Positive	0/200	7/200	97	3

*FIAX: a commercially available fluorescence system obtainable from Whittaker M.A. Bioproducts, used at WHMC for detecting CMV antibodies.

Table 3. Western blot analysis of HIV-1-seropositive subjects to determine presence of antibodies to cellular antigens

Experiment #	Number of subjects giving WB reactions			
	Strong*	Weak*	Negative	Total
1	10	12	26	48
2	5	5	37	47
3	6	12	29	47
4	5	10	32	47
Total	26	39	124	189†
%	14%	21%	66%	100%

* 58% of positive reactors had 1 band, 28% had 2 bands, 5% had 3 bands and 9% had 4 or more bands.

† 176 of 189 (93.1%) of these HIV-seropositive subjects were CMV-seropositive by WB.

Table 4. Average rate of decline in CD4 + T-cell numbers and significance calculations for groups 1, 2 and 3 using all declining* WR1 and 2 subjects in cohort

Calculated $\bar{x}$ decline in CD4 + T-cell numbers per mm^3 per 12 months			Significance (two-tailed)	
Group 1	Group 2	Group 3	comparing groups 1 and 2	comparing groups 1 and 3
-108 (N = 12; σ = 71)	-188 (N = 293; σ = 166)	-162 (N = 7; σ = 120)	$\underline{P}$ = 0.003	$\underline{P}$ = 0.231

* When all subjects in cohort, declining or not, were averaged, the average rates of decline from first to last visits for groups 1, 2 and 3 were 31, 28, and 30, respectively. There was no significant difference between these numbers ($\underline{P} \geq .9$).

Table 5. Frequency (prevalence) of CD4+T-cell declining* subjects in groups 1 and 2 using all WR1 and 2 subjects in cohort

Group	Fraction of subjects declining more than indicated rate	
	>188 cells/year†	>244 cells/year
1	2/20 (10%)	0/20 (0%)
2	109/409 (27%)	84/409 (21%)
Significance of difference between groups 1 and 2‡ (two-tailed)	P = .098	P = .019

* When all subjects in the cohort who were declining at any rate were included, the fraction in group 1 was 12/20 (60%) and in group 2, 298/409 (73%). There was no significant difference between these number (P = .21).

† 188 was the mean CD-4 cell decline per year in group 2 (see Table 4)

‡ Chi-square; Fisher's exact calculation was used where 0 subjects occurred

Table 6. Comparative decline of CD4+T-cell numbers in the two groups: HSV-seronegatives and HSV-2-seropositives per year

Group	Calculated $\bar{x}$ decline in CD4+T-cell number per 12 months*	Standard deviation (σ)	Number of subjects
HSV-seronegative†	-153	112	25
HSV-2-seropositive†	-182	175	107
Weighted $\bar{x}$	-177		

* Calculated from data obtained at the time of the visit at which the CD-4+T-cell number was highest and the last visit.

† Significance of difference in rate of decline between HSV-seronegatives and HSV-2-seropositives ($P = 0.193$, two-tailed)

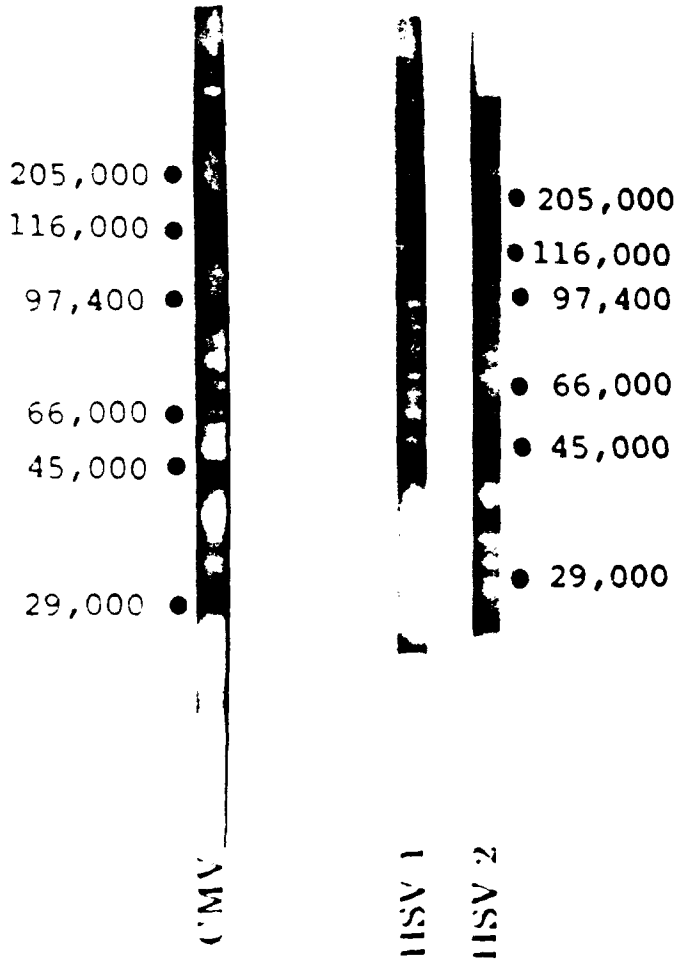

Figure 1.

Figure 1. Western blot banding patterns characteristic for CMV (left) and HSV-1 and HSV-2 (right).

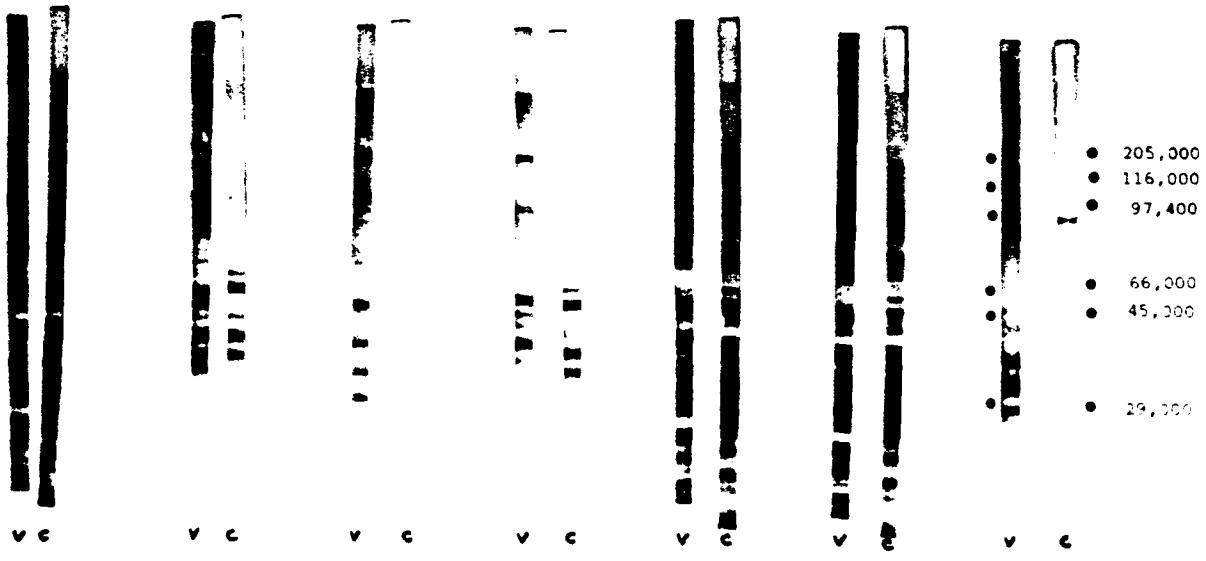

Figure 2.

Figure 2. Titrations of four plasmas which are strongly reactive to normal cellular antigens.

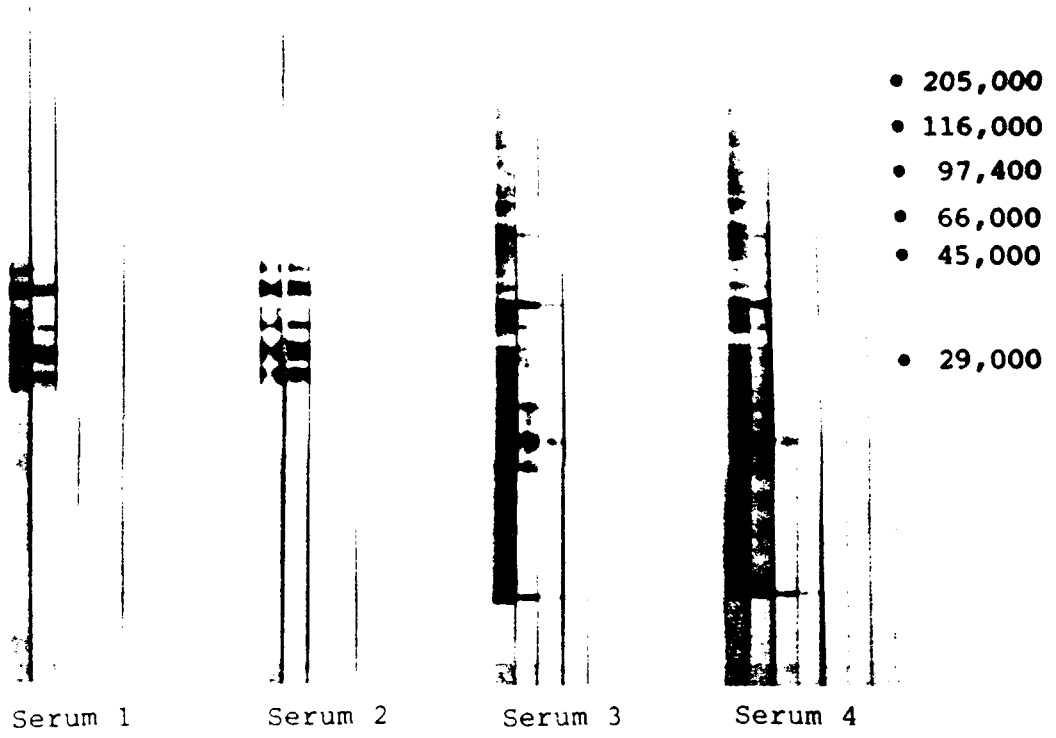

Figure 3.

Figure 3. Comparison of banding patterns in Western blots of 7 sera from HIV-1 seropositive subjects using CMV antigens (V) and cellular antigens (C).

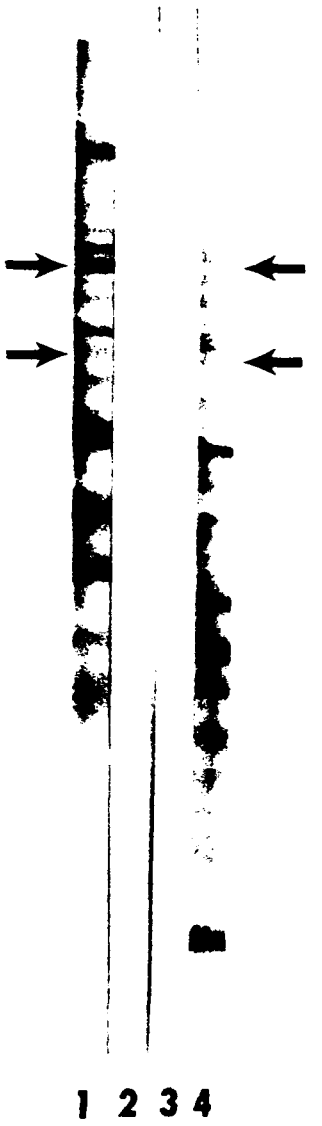

Figure 4. Demonstration of antibodies to normal cellular antigens by WB, seen in a CMV-seronegative serum (center strips).

Strip - 1: CMV antigen plus CMV-seropositive serum control.

Strip - 2: CMV antigen plus CMV-seronegative serum.

Strip - 3: Uninfected cell antigen plus CMV-seronegative serum.

Strip - 4: Uninfected cell antigen plus serum highly reactive against cell antigen.