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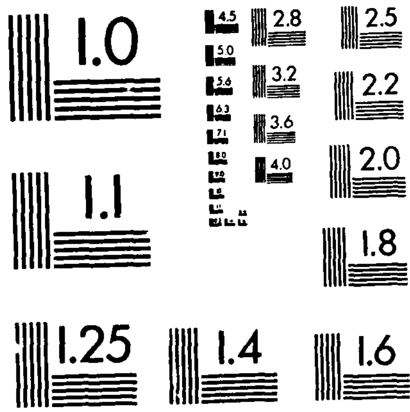
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The Role of Cytomegalovirus As A Cofactor in the Development of ARC-AIDS

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SUMMARY

This report displays the results of one year's longitudinal study of cytomegalovirus (CMV) as a candidate triggering cofactor in the development of acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus (HIV)-infected Air Force personnel. The design of the study is intended to differentiate triggering from opportunistic roles of CMV in HIV-infected people. Saliva and urine specimens are being cultured in MRC-5 human fetal lung cells, white blood cells are being stored in DMSO for later revival and study, and plasma or serum specimens are being studied for CMV-specific IgG and IgM. Western blot (W.B.) analysis is being done on each subject to determine the patterns of antibody response to over 25 CMV antigens. Higher concentrations of CMV-specific IgM were found in WR-6 patients than were found in WR-1 subjects ($p = .005$). The prevalence of culturable CMV in saliva was 18%, compared with 9% in urine specimens. Recovery of CMV from specimens was best if specimens were inoculated into cultures directly (shortly after collection) rather than after storage at -70°C (8/12 stored vs 12/12 fresh). Over 25 different CMV antigens were detectable by W.B., using pools of CMV-positive sera. The most prominent (highest titered, most prevalent) Anti-CMV IgG is directed toward an antigen having a molecular weight in the range of 135-141 Kd. CMV-specific IgM detection by W.B. was not significantly inhibited by CMV-specific IgG. W.B. strips (with CMV antigens adsorbed) were stable on storage for months and, therefore, could be standardized for quality control in these studies. Wild CMV strains (new isolates) were remarkably similar to prototype strains when co-electrophoresed, but there may be minor antigenic differences between some strains, particularly in the 80-90 Kd range. We are particularly interested in comparing the clinical course of the following 3 major groups of HIV-positive Air Force Personnel:

1) CMV-infected subjects: 91% of all personnel tested;

2) CMV-naive subjects: 9% of all personnel tested;

3) CMV-naive subjects who develop their primary infections during the course of these studies (2 such people have been found).

Such studies are truly longitudinal and will require several years to complete.

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A. Statement of the problem.

The main purpose of this study is to test the hypothesis that coinfection with human immunodeficiency virus (HIV) and cytomegalovirus (CMV) results in more rapid progression of AIDS than infection with HIV alone.

Reliable serologic tests for antibodies or antigens (ELISA and Western blots) will be used to identify seropositivity or naiveté to CMV. One goal will be to identify HIV-seropositive individuals who are seronegative (naive) to CMV, the cofactor candidate, and to follow a sufficiently large number of these individuals over several years to be able to determine whether immunosuppression and onset of AIDS is significantly less rapid in CMV-naive individuals than in matched CMV-seropositive individuals. Specific aims include:

1) Initially screen all subjects in the total HIV population for evidence of infection with CMV.

2) Screen all CMV-naive subjects for CMV antibody at least annually to identify seroconversions, indicating recent primary infections; separate CMV-naive and newly seroconverted individuals into subgroups for tracking of their clinical course (Walter Reed staging).

3) Calculate the probability of differences between each group in the rate of AIDS progression (staging) over several years.

4) Determine whether individuals are shedding infectious CMV in their salivas and urines at the time they are tested for antibody.

5) Separate and store white blood cells for recovery and possible culture for CMV at a later time.

6) Determine whether there is a relationship between serum and salivary anti-CMV IgG or IgM and AIDS disease progression (staging).

7) Analyze differences and similarities between new CMV isolates and prototype CMV strains (Ad-169 and Towne strains) by electrophoretic techniques (comigration of proteins and Western blots).

B. Background

Several viruses have been suspected as cofactors with HIV in playing important roles in AIDS. Candidate cofactor viruses include CMV (1), EBV (2-3), HBV (4-5), HSV (6-7), and other DNA viruses (6). So far, however, it has been difficult to distinguish clearly between triggering cofactors, i.e., cofactors which act with HIV to initiate events leading eventually to profound immunosuppression, and opportunistic cofactors, i.e., cofactors which emerge to produce health or life threatening infections after substantial immunosuppression has occurred. CMV, HSV and EBV are each considered significant opportunistics in AIDS patients. All four cofactor candidates are transmitted venereally or by other means of exchanging body fluids. Therefore, seroepidemiologic association of these agents with AIDS patients is not surprising. This, plus the fact that each of them can establish life-long latent-recurrent infections makes it difficult to distinguish possible triggering (initiating) roles from opportunistic roles in HIV-coinfected

persons. The presently proposed study is designed to distinguish a putative triggering role from an opportunistic role of the cofactor candidate CMV.

The percentage of blood donors found to have antibodies to CMV in one study was 62%, in contrast to 90% in male homosexuals (5). This indicates that promiscuous male homosexuals are repeatedly exposed to CMV infections through sexual contacts. The probable reason for this is that CMV is found frequently and in high concentrations in semen (9) and infects the G.I. tract of homosexual men (10). A high titer of cytomegalovirus antibody has been found to be a predictor of AIDS development in HIV sero-positive homosexual men (11). These observations are consistent with our own findings in HIV-infected U.S. Air Force personnel (majority probably being homosexual men) and matched HIV seronegative U.S. Air Force personnel controls (see results section below). African AIDS patients show about the same prevalence of CMV antibodies as U.S. AIDS patients and U.S. homosexual men (12); however, the prevalence of CMV antibodies in the African heterosexual population is high (13).

CMV infections are immunosuppressive and immunodysregulatory, affecting the immune system in a manner similar to HIV infections, i.e., through suppressing the number and/or altering the functions of T-4 helper lymphocytes (27). It has been reported that 94-100% of AIDS patients are CMV-seropositive (1,14), which led to the conclusion by some that AIDS and possibly Kaposi's sarcoma (KS, common in AIDS patients) could be etiologically associated with CMV (1). It is clear now, of course, that HIV is the primary etiological agent responsible for destruction of T-4 helper lymphocytes and irreversible immunosuppression in AIDS patients, but it is not clear why some individuals progress much more rapidly in disease development than others after primary HIV infection.

We postulate that primary CMV infection, chronic exacerbation of latent CMV infections, or sequential CMV infections with different strains from different sexual partners (15-17), can be triggering cofactors with HIV to immunosuppress profoundly and consequently leave coinfected individuals more susceptible to opportunistic infections. Co-infections with different CMV strains (15-17) have been well demonstrated. It is also well known that CMV itself can produce serious or lethal opportunistic infections after onset of immunosuppression caused by HIV infection. We hope to determine in the present study whether CMV and HIV act synergistically to produce initial profound immunosuppression, in addition to playing an opportunistic role.

It has been reported that different "wild" (newly isolated) strains of CMV are sufficiently different in antigenic composition to require antigen pools from various strains to insure adequacy for detecting serum antibody in all persons to all strains (19). Therefore, CMV antigens have been prepared from prototype laboratory strains (Ad-169, Towne) and from CMV strains isolated recently from U.S. Air Force personnel; 25). These different antigens will be tested with serially diluted sera from Air Force personnel to determine which single CMV strain, or combination of strains, would be best to use routinely as antigen(s). Studies to optimize the selection of CMV strains for antigen(s) will be done with the use of both ELISA and by Western blot so as to identify possible antigenic differences between CMV isolates. Such a study has not been done and reported, to our knowledge. Latex agglutination screening tests will be studied for feasibility (20-21), but all sera will be tested by Western blot as described by Landini *et al* (22-23).

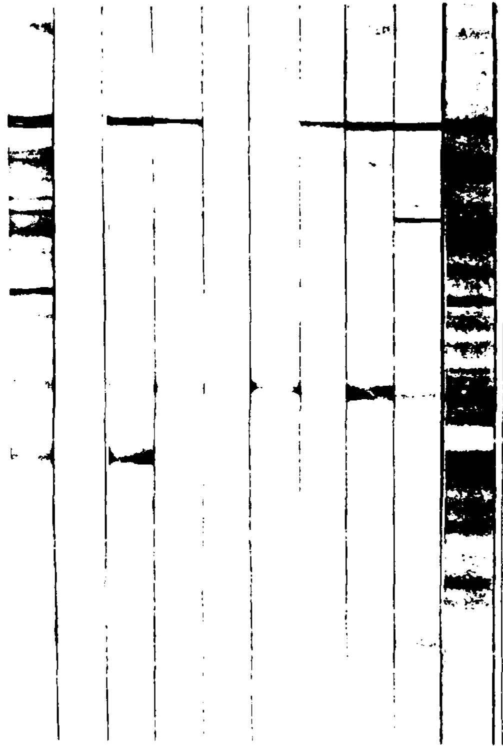
Western blot testing is being done as follows: CMV is inoculated into MRC-5 cells, incubated until 3-4 + cytopathic effects are seen (25); the infected cells are washed free of medium by centrifugation-resuspension, the cells lysed by sonic oscillation and kept at -70°C until needed. Thawed antigens are treated with SDS, mercaptoethanol and boiled, electrophoresed on verticle slab acrylamide gels, and transferred to nitrocellulose paper (22-23). The banded CMV antigens are stabilized by treating the paper in 5% skim milk-PBS, rinsing the blots free of excess milk, drying and storage in a dry place. Such CMV-antigen nitrocellulose blots have been shown (see results section) to be stable for several months at room temperature (25°C). Strips are cut (3mm width), soaked in 5% skim milk-PBS, incubated in individual troughs with 1:100 dilutions of sera in 5% skim milk-PBS, washed 4 times in water, incubated at room temperature in horseradish peroxidase (HRP) tagged anti-human IgG, washed again 4 times in water, incubated at room temperature in substrate (4-chloro-1-naphthol) for 15 minutes, washed in water, dried, stored and read for tagged (blue green) bands (see appendix for display of Western blots). In some cases serial serum dilutions are made and used as above so as to determine end-point titers to each of the major protein antigens (see results section).

C. Results

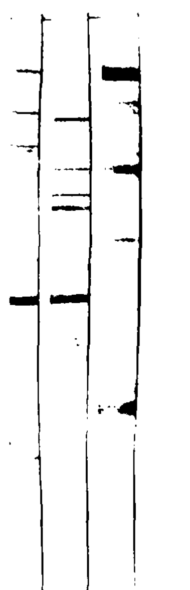
(1) Western blots have been done on the sera of nearly all patients whose specimens have been received, and 29/339 (8.6%) have been found to be sero-negative. Most of these results have been confirmed by ELISA. These results compare well with published results of studies in homosexual men (10%; reference 5).

(2) HIV-seronegative control subjects (a sample of 36 Air Force personnel matched with HIV-seropositives for age and sex) were tested by Western blot for CMV antibody, and 14/36 (39%) were seronegative, or about 4 times the frequency among HIV-seropositive personnel. Again, these results compare closely with published results on sera from blood donors (38%; reference 5).

(3) A typical random series of Western blots to detect CMV-specific IgG is shown below:



(4) Typical Western blots to detect CMV-specific IgM are shown below:



(5) Prevalance of "strong" (several dark bands showing) Western blots for CMV-specific IgM in Walter Reed stage 6 patients

IgM Western Blot	Walter Reed Stage 1	Walter Reed Stage 6	Raw Totals
High	24*	5	29
Low	191	4	195
Column totals	215	9	224

*No. of patients
Chi Square: $p = .005$ that there is no difference between WR-1 and WR-6 groups

It is concluded that high concentrations of CMV-specific IgM are more prevalent in high WR stage AIDS patients than in relatively healthy patients (WR-1) who are HIV seropositive.

(6) Initially, it was believed that saliva and urine specimens containing CMV could be effectively stabilized by mixing with 10% calf serum and 10% DMSO, then freezing and storing at -70°C. To test this, specimens were inoculated freshly, aliquots stored as above, known positive specimens then thawed and inoculated after storage for a period of 2-5 weeks. The results are as follows:

CMV isolation from specimens inoculated freshly (not frozen-stored)

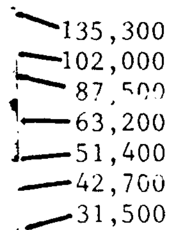
Specimen	No. CMV-Positive / Total number cultured
Saliva	9/51 (18%)
Urine	5/57 (9%)

Success of reculture from known CMV-positive frozen-stored specimens

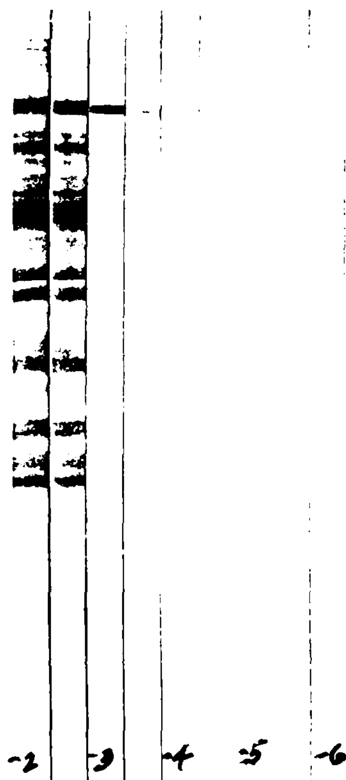
Specimen	Number successfully recultured / Total number positive
Saliva	4/7
Urine	4/5

It can be concluded that maximum recovery of virus is accomplished when fresh specimens are inoculated and that storage at -70°C results in about 1/3 loss of capability to isolate CMV from specimens. Operationally, therefore, specimens need to be processed for isolation of virus the same day as they arrive. Technical difficulties in maintaining availability of tissue cultures in appropriate vessels in the right numbers may sometimes require short-term storage of specimens. This, however, is not preferable.

(7) Molecular weight estimates of electrophoresed antigens. Below is a Western blot of CMV antigen (Towne strain) showing the location of various molecular weight bands determined by co-electrophoresing markers of known molecular weights.



(8) <u>End-point titers of CMV-specific antibodies by Western blot</u>. Half log unit or log unit seral dilutions of low and high WR-stage subject's sera were reacted by WB and the end-point titers recorded for each band observable. Such a half-log unit serial dilution assay is shown below (10^{-2}-10^{-6}):



Although higher overall IgG and IgM antibody titers in Walter Reed stage 6 patients could be observed, distinctive patterns of reaction with various CMV bands were not apparent in advanced AIDS cases. The above technique seems powerful for revealing anomalies in humoral antibody responses to CMV infection and will be used more extensively for this purpose later. This method allows end point titration of 10-20 different CMV antibodies simultaneously and gives a great deal of information from a relatively modest effort. Some of these Western blot titrations will be compared with neutralizing antibody titrations to determine if CMV neutralizing capability is lost in some patients due to loss of biological specificity resulting from dysregulation. The major antibody produced in most CMV infected people is against the 135-141 Kd band; often the end points reach 1:100,000.

(9) We needed to determine whether anti-CMV <u>IgM</u> in sera would be masked by competition with anti-CMV <u>IgG</u> for antigen sites on Western blot strips. Therefore, protein A from staphylococci was used to absorb out serum IgG to determine whether IgM specific reactions would be enhanced in sera known to contain both classes of CMV antibody. This experiment demonstrated that almost complete removal of CMV-specific IgG antibodies did not signficantly increase the Western blot reactivity of CMV-specific IgM antibodies in a given serum.

(10) Comparison of serum and salivary antibodies to CMV in the same individuals. Twenty HIV antibody negative, CMV serum antibody positive healthy control individuals were tested by Western blot for salivary IgG and IgA antibodies in their saliva. 15/20 of these individuals were Western blot positive for CMV-specific salivary antibodies. Serial dilution to end-point titration suggests the presence of about a 10^3 fold greater concentration of CMV-specific IgG in serum than in saliva. Salivas negative for CMV antibodies in every case were from individuals with weak Western blot serum antibody reactions. These experiments were undertaken primarily to determine whether salivary antibodies may neutralize CMV secreted in the saliva, hence obscure shedding of the virus. Our conclusion is that this may be a significant problem, in view of the fact that some saliva contains anti-CMV IgG at levels over 1:1,000 by Western blot. We are now attempting to deal with this potential interference to the isolation of CMV from saliva by devising a strategy to neutralize the anti-CMV IgG with anti-human IgG soon after specimen collection and before attempting to isolate the virus. Also, these results suggest that efforts to detect CMV antigens in saliva by ELISA would be justified (there may be large amounts of neutralized virus in the saliva).

(11) Technical experiments. It was determined early in this work that nitrocellulose blots of electophoresed CMV antigens were stable for more than three months after soaking one hour in 5% skim milk-phosphate buffered saliva ("Blotto"), rinsing in water, drying, and keeping in a dry place. Strips used with a pooled anti-CMV serum at 1, 2 and 3 months (left to right, respectively) after drying are shown below:

This technique of preparing blots, storage, then testing sample strips before use of the whole blot, has facilitated the standardization of our Western blot studies and is extremely valuable for quality control of this phase of the project.

(12) Comparison of co-migrated CMV strains by Western blot. Prototype CMV strains (Ad-169 and Towne) and numerous fresh CMV isolates from HIV sero-positive people have been compared for antigenic differences by Western blot. The most obvious outcome of this work is the remarkable similarity between different CMV strains regarding their gene products, which is at variance with one published report (19). However, we want to examine at least 20 more new isolates and compare them with prototype strains before drawing conclusions about homogeneity-heterogeneity. We have seen some heterogeneity in antigens in the 80,000-90,000 D range, but definitive conclusions must await further study with more new isolates.

(13) CMV seroconversions. At least 2 originally CMV antibody negative individuals have been identified as seroconverting, hence have experienced their primary CMV infection recently. Seroconverting individuals now have both CMV-specific IgG and IgM antibodies in their second serum. Prospecting for more CMV naive subjects and observing their clinical course and possible seroconversion will be of great interest to us during the course of these longtitudinal studies.

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