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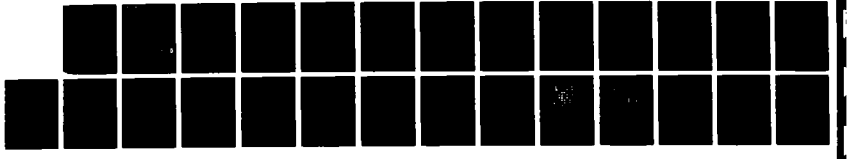

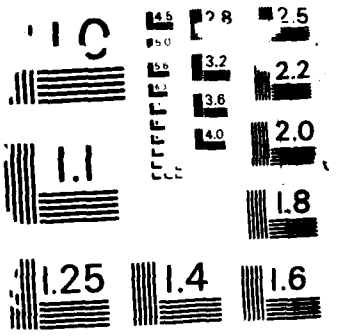

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THE DEFINITION OF EPSTEIN BARR VIRUS (EBV'S) ROLE IN HIV INFECTED USAF PERSONNEL AS RELATED TO DISEASE PROGRESSION

ANNUAL REPORT

Ciro V. Sumaya, M.D.

October 27, 1987

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HIV-III; Epstein Barr Virus; Human Immunodeficiency Virus; Virology; Infectious Disease; AIDS Acquired Immune Deficiency

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
The main objective of this study was to define the role of EBV in the progressive evolution of AIDS by means of a prospective evaluation of persons with HIV infections. Detailed EBV antibody responses and virologic studies to determine the EBV load in body fluids and EBV strains were performed serially. The possible concurrent presence of HIV in EBV cell cultures established from body fluids from HIV infected persons also was examined. The HIV infected participants had an enhanced antibody response to a broad spectrum of EBV antigens. In addition, an increased amount of EBV was detected in oropharyngeal secretions as well as an increased proliferative ability of peripheral blood mononuclear cells compared to normal, healthy adults. The consequences of this highly increased burden of EBV in HIV patients are not well understood but could support the possibility that EBV may be a stimulus to produce tumors or other lymphoproliferative manifestations. No correlation was noted between specific EBV genotypes and progression of the HIV infection, but the numbers tested (and the changes in disease progression) were small at this point in the study. The continuation of
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19. Abstract

this prospective study should allow for a better interpretation and extension of the present findings.

Foreward

For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46.

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administration Practices Supplements.

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Statement of Problem

Infection with human immunodeficiency virus (HIV), the cause of acquired immunodeficiency (AIDS), may remain quiescent for long periods, including years, without producing any clinical signs of disease. The events that stimulate the progression of this indolent infection into clinical disease are not defined. The present study hypothesizes that immunosuppression produced by repeated reactivation of latent infection by strains of Epstein-Barr virus, a member of the herpesvirus group, and other forms of *in vivo* interaction between HIV-1 and EBV provokes clinical progression of the HIV infection.

Background

Although HIV is essential to the development of AIDS, there is evidence suggesting that EBV and possibly other viruses, may have a contributory role in the pathogenesis of AIDS. [1] Preliminary results have shown that patients with AIDS-related complex (ARC) and AIDS have an exaggerated antibody response to EBV antigens and an increased load of EBV in oropharyngeal secretions and peripheral mononuclear cells. [2] These findings by the principal investigator's laboratory suggest that there is recurrent reactivation of the lifelong latent EBV infection in patients with ARC and AIDS. Undifferentiated lymphomas containing EBV-DNA also have been found in increased frequency in homosexuals and AIDS patients. [3,4] Moreover, EBV by itself can produce significant immunosuppression in vivo; different, possibly more virulent, strains of EBV have been detected in AIDS patients; and there is strong evidence of interaction at the cellular level between EBV and HIV.[1]

Approach to Problem (AIMS)

The aims of the current protocol were to determine if changes in EBV serologic findings and the quantity or type of EBV strains in oropharyngeal secretions and peripheral blood mononuclear cells correlated with progression from an asymptomatic HIV antibody state to ARC or AIDS. As the HIV infection evolved into an increasingly severer disease state, it was to be determined if EBV strains were present in pathologic lesions and also if abnormalities in T cell immunoregulation of EBV coincided with this progression.

Materials and Methods

Study group. Individuals eligible to enroll in this study were required to have a documented HIV infection as determined by the screening HIV antibody program instituted by the Armed Services. Those individuals that were Air Force personnel were then evaluated for their HIV infection at Wilford Hall Medical Center, San Antonio, Texas, and asked to sign the consent form for entry into the collaborative prospective study directed by Dr. (Col.) R. Neal Boswell and which includes this protocol. With the on site evaluation, the HIV infection of the participants was then staged according to the Armed Forces (Walter Reed or WR) Disease Classification (Table 1).[5] Detailed EBV serologic and virologic studies for the present protocol were to be performed serially, every 6-12 months routinely, and at time of clinical changes.

EBV Serologic testing. Specific EBV serologic testing included antibody determinations to EBV-capsid antigen (to include IgM, IgG, and IgA antibodies), diffuse and restricted components of EBV-early antigen, and to EBV-nuclear antigen. This testing involves standard indirect immunofluorescent techniques that have been described by the principal investigator and other workers.[2,6]

EBV virologic testing. This testing included the following procedures: prevalence and titer of infectious EBV in oropharyngeal secretions according to a transforming assay[7], quantity of EBV-DNA by spot hybridization[8], and prevalence of EBV antigens utilizing reference sera or monoclonal EBV antibodies and a spontaneous proliferation assay[9] in peripheral blood lymphocytes and pathologic tissues.

Isolates of EBV were examined for strain differences by performing blot hybridization with cloned probes on agarose gel-separated products of extracted DNA that has undergone restriction enzyme digestions[10].

In addition, the presence of in vitro HIV antigen and infectious virus was searched for in established EBV cell cultures that were derived from specimens obtained from individuals infected with HIV.

Results

Serologic findings. Although low levels (<20) of serum IgM antibodies to EBV capsid antigen were found in a few specimens from patients with HIV infections, actually 3 persons in WR 1 or 2 class, the prevalence of this antibody response was not greater than that noted in healthy controls (Table 2). In 2 patients the serum IgM antibody response to EBV coincided with the onset of clinical manifestations of the underlying HIV infection. The prevalence of serum IgG antibody to EBV capsid antigen was similar among specimens from patients in the individual WR classes and controls; the geometric mean titers (GMT) were also similar among specimens from patients in all the WR classes but in all cases were greater than that found in controls (minimal $p < .01$).

The prevalence of serum IgG antibody to EBV early antigen components was similar among the WR classes but uniformly greater compared to controls

(minimal $p < .0005$). There was a trend toward a greater GMT of this serum antibody response in WR classes 4-6 compared to 1-3 but this was not statistically significant. The GMT of the serum antibody response in all WR classes group were greater than controls (minimum $p < .025$). No intra-class differences in GMT were statistically significant of the HIV infected persons with an IgG antibody response to EBV early antigen components, the response appeared to change from predominantly anti-R to a response directed equally to either D or R component.

The prevalence of antibodies to EBV nuclear antigen were similar among the WR classes and controls. There was a decline in the GMT of this antibody response with an increase in the numerical WR class. The GMT of WR 1 and 2 as a combined group were significantly greater than controls ($p < .05$. Of 19 individuals that changed (increased) in their WR classification, only 1 had a significant change in their EBV serology (antibody to EBV early antigen declined from 20 [anti-R] to nondetectable). Otherwise the antibody responses in these individuals and in those without any change in their WR classification (n=23) remained relatively stable.

Virologic findings. The positive rates of EBV in oropharyngeal secretions from persons in all 6 WR classes were similar and in all cases greater than that in controls (minimum $p < .05$ (Table 3). The titrations of the several positive specimens tested were at levels considerably greater than seen in controls (minimum $p < .005$). No significant differences were noted in EBV titrations performed on oropharyngeal specimens obtained from patients in different WR classes.

The positive rate of spontaneous proliferation of peripheral blood mononuclear cells in vitro was similar among the WR classes and uniformly greater than that of controls (minimum $p < .01$) (Table 3). The estimated mean

number of EBV-infected lymphocytes from peripheral blood (determined by the endpoint dilution procedure) specimens were similar among the WR classes and in all cases except for WR 5 were greater than that in controls (minimum $p < .05$). There were no significant changes in the prevalence and titer of EBV in oropharyngeal specimens, positive rate of spontaneous proliferation of peripheral blood mononuclear cells, or estimated mean number of EBV-infected lymphocytes from peripheral blood specimens from patients that progressed (majority only 1 or 2 classes) or did not progress in WR class.

EBV-DNA findings. EBV DNA content in oropharyngeal secretions from HIV infected patients was determined by a DNA hybridization method using a virus specific probe. In this case, the patients were placed in three groups: asymptomatic, ARC (mild to moderately symptomatic), and frank AIDS (severe opportunistic infection or AIDS-related cancer). Controls included healthy individuals and patients with acute EBV infectious mononucleosis. Of 124 mouthwash specimens from 111 patients, 33% were positive for EBV DNA with concentrations from $10^5 > 10^8$ genome equivalents/ml (Figure 1). Ten (40%) of 25 specimens from patients with AIDS and 24 (43%) of 56 specimens from patients with ARC were positive for EBV DNA. In contrast, only 7 (23%) of 30 specimens from HIV antibody positive asymptomatic patients were positive. A positive correlation existed between the concentration of EBV DVA detected and the severity of the diagnosis (Table 4). The sensitivity and specificity of the hybridization assay compared to a qualitative bioassay (lymphocyte transformation) was 48% and 95% respectively. EBV DNA was not detected in healthy controls and persons with acute EBV infectious mononucleosis, including those with oropharyngeal EBV detected by the bioassay. By the quantitative modification of the lymphocyte transformation assay, it was noted that the patients with AIDS tested had higher titers of transforming EBV in

oropharyngeal secretions compared to patients with acute infectious mononucleosis (Table 5).

EBV genotyping findings. Southern analysis using the Epstein-Barr virus EBV Eco RI "A" probe was performed to determine the EBV genotype in orpharyngeal specimens and/or peripheral blood lymphocytes from HIV infected patients. The genotypes were classified as standard, if the pattern was identical to the non-permissive EBV infected, Burkitt tumor derived cell line denoted Raji, or otherwise as unique.

Results are summarized according to EBV genotype and patient classification in Table 5. A unique variant EBV isolate was present in the positive oropharyngeal washing of 8 of 21 patients. The numbers were too few to detect differences by WR class. In patients with matched oropharyngeal and blood lymphocyte specimens, the EBV genotype was identical in both specimens. A representative Southern analysis of oropharyngeal washings and peripheral blood lymphocytes from HIV infected patients is depicted in Figure 2. Genotypic examination was also made of patients with acute or "chronic" infectious mononucleosis, healthy family contacts of acute infectious mononucleosis patients, and healthy individuals with an old quiescent EBV infection. A similar degree of genomic variability was detected in the latter controls compared to HIV infected patients. In 70% of cases with unique genotypes, there was at least an alteration in the Bam HI H region of the EBV genome.

Surveillance for HIV infection in EBV cell cultures in vitro. All 24 EBV cell cultures established from oropharyngeal washings and 54 peripheral blood mononuclear (lymphocytic) cell cultures that developed spontaneous proliferation, i.e., were EBV infected, collected from HIV infected individuals were negative for HIV DNA as determined by the p32

labeled DNA probe (Dupont de Nemours & Company, Boston, Massachusetts). None of 22 supernates from EBV cell cultures established from oropharyngeal washings or proliferating transformed peripheral blood lymphocytes collected from HIV infected individuals have yielded infectious HIV isolates as determined by cytopathogenic effect of inoculated Alec cells; results from the examination of reverse transcriptase activity are pending.

Discussion

HIV-infected patients in general have an enhanced antibody response to a broad spectrum of EBV antigens, an increased amount of EBV in oropharyngeal secretions and increased lymphoproliferative ability of the peripheral blood compared to normal, healthy adults. Interestingly, these findings are usually already present early in the HIV infection (WR class 1) and remain so in higher classes, at least from a non-cohort analysis. There are, however, some changes in EBV status that are apparent with higher classes in this predominantly non-cohort analysis. An inverse correlation between the intensity of the antibody response to EBV nuclear antigen and increasing WR class was noted, a finding previously seen in other forms of immunodeficiency/immunosuppression or in aberrant immunoregulation of EBV, as possibly in chronic infectious mononucleosis. There also was a trend in the increased development of an antibody response to EBV early antigen complex directed to diffuse (D) component instead of restricted (R) component with higher WR classes. The reason for these serologic findings is not clear yet, but it is well known that there exists a proclivity for the antibody response to early antigen complex to be directed to one or the other component in selected oncogenic processes, ie anti-D is characteristically seen in nasopharyngeal carcinoma while anti-R is seen in Burkitt's lymphoma. The EBV

IgM antibody responses detected in a few individuals coincidental with the initial manifestations related to clinical progression of their HIV infection, suggesting a possible association with this progresstion. However, too few individuals changed in their WR classification, and usually only from 1 to 2 classes, during this first year of the study to determine if there existed any correlation with significant changes in EBV serologic or virologic findings.

The mean titers of EBV in oropharyngeal washings in the few individuals tested (from all WR classes) were considerably higher than that noted in patients with acute EBV infectious mononucleosis. On the other hand, and for reasons unknown, the mean estimated number of EBV infected cells in the peripheral blood was greater than that noted in the general population but still not at the high level seen during acute infectious mononucleosis. The intense amount of lytic virus produced in HIV infected patients, presumally from epithelial cells in the oropharynx may account for the increased EBV DNA genomic content in this body fluid in patients with that disorder. However, increased titers of infectious virus as detected by the lymphocyte transformation bioassay does not explain the very highly increased genomic content seen in AIDS patients that would fall in the counterpart WR 6 classification scheme. Also the titer determined by the lymphocyte transformation bioassay oftentimes appeared to be disproportionately low to account for the amount of EBV DNA estimated by the hybridization assay. These latter findings, together with the rare event of transformation-negative, hybridization positive results, raise the intriguing possibility that the salival secretions of patients with HIV infections contain EBV that is defective or nontransforming, in addition to transforming virus. Alternatively, it could be speculated that the results were due to the presence of neutralizing antibody in oropharygeal secretions of HIV positive

patients which prevented or decreased the efficiency of transformation. The latter possibilities will be examined in the next year of the study.

No detectable correlation was noted between a specific EBV genotype and progression of the HIV infection taking into account the quite small number of patients tested to date. The preliminary results of these HIV infected individuals and controls, patients with acute infectious mononucleosis and healthy individuals, suggest that the genomic variability noted is due to a population based molecular heterogeneity of the EBV genome. The interesting observation of consistent alterations of the Bam IH H region of the EBV genome may correlate with the potential for molecular recombination and/or deletion among the tandemly repeated sequences known to be present in this region. These findings need to be greatly extended to determine if there are any unique EBV genotypes that may be correlated with HIV the progression of the HIV infection or the development pathologic lesions related to EBV.

The consequences of a highly increased burden of EBV in HIV patients, even early in their infection (class 1) as documented in this study, are not well understood, but they could support the possibility that EBV may be a stimulus to produce tumors or other lymphoproliferative manifestations. The continuation of this prospective study should allow for a better interpretation and extension of the present findings.

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ARMED FORCES (WALTER REED OR WR)
Disease Classification of Infection
by Human Immunodeficiency Virus (HIV)

Class	HIV ANTIBODY *and/or* VIRUS ISOLATION	CHRONIC LYMPH-ADENOPATHY	T HELPER CELLS/mm3	DELAYED HYPER SENSITIVITY	THRUSH	OPPORTUNISTIC INFECTION
1	+	-	>400	Normal	-	-
2	+	+	>400	Normal	-	-
3	+	+/-	<400	Normal	-	-
4	+	+/-	<400	Partial anergy	-	-
5	+	+/-	<400	Partial/ complete anergy	+	-
6	+	+/-	<400	Partial/ complete anergy	+/-	+

2

The prevalence and geometric-mean titer (GMT) of serum Antibodies to Epstein-Barr Virus (EBV) according to the Walter Reed (WR) classification.

	Class					
	1	2	3	4	5	6
	(n=231)	(n=60)	(n=27)	(n=13)	(n=25)	(n=21)
IgM-CA						
Prevalence*	0.9	2.6	0	0	0	0
GMT	2.5	1.7	-	-	-	-
IgG-CA						
Prevalence	99.6	100	100	92.31	100	100
GMT	333.0	323.5	382.9	319.9	367.5	411.6
IgG-EA						
Prevalence	75.8	63.3	59.3	92.3	60.0	76.2
Anti-D*	14	21.6	31.3	50	33.3	50
Anti-R	81.5	64.9	56.3	33.3	53.3	50
Anti-DR	4.5	13.5	6.3	16.7	13.3	0
GMT	22.1	17.5	16.2	40.0	20.0	20.7
Anti-EBNA						
Prevalence	99.1	96.7	96.3	92.3	96.0	100
GMT	27.8	27.4	16.5	13.1	16.3	11.8

* Percent (%) of positive specimens

+ The positive reactions were divided into these directed to the diffuse (D) component or restricted (R) component of EBV early antigen complex or an undifferentiated (DR) response.

3

The prevalence and titers of EBV in oropharyngeal washings and peripheral blood mononuclear cells from HIV infected patients according to WR class.

	Class					
	1	2	3	4	5	6
Oropharyngeal Washings						
Prevalence*	109/149	32/42	14/16	6/10	14/18	13/17
%	73.2	76.2	87.5	60	77.8	76.5
Mean Titer $\log_{10}$	1.73 (n=3)	3.23 (n=1)	2.03 (n=2)	2.33 (n=1)	2.3 (n=5)	2.23 (n=5)
Peripheral mononuclear cells						
Prevalence*	122/314	30/59	11/33	6/14	6/26	5/22
%	38.9	50.8	33.3	42.9	23.1	22.7
Mean estimate	+ 24.8 (±104.8)	44.8 (±141.6)	48.9 (±195.8)	186.9 (±511.8)	10.4 (±41.5)	56.8 (±124.2)

* no. of positive specimens/total no. of specimens. All the oropharyngeal washings have not yet been processed.

+ no. of infected cells/10^7 mononuclear cells (±1 SD)

Hybridization of EBV DNA Probe to Oropharyngeal Specimens of Patients with AIDS, ARC or Asymptomatic HIV antibody Positivity

Signal Intensity:	Strong	Moderate	Weak	Negative
EBV Genome Equiv/ml:	10^7 - $\geq 10^8$	10^6 - 10^7	10^5 - 10^6	$<10^5$
Clinical Diagnosis:		n = # specimens		
AIDS	5	3	2	17
ARC	1	9	14	43
Asymptomatic HIV Antibody Positive	0	2	5	23

Note: The strength of each hybridization signal was assigned relative to all specimens in the assay. EBV genome equivalents/ml were estimated by visual comparison to the serial Raji cell dilutions.

5

Variation in DNA patterns of EBV isolates in oropharyngeal washings or peripheral blood mononuclear cells according to WR class

	Class						
	1	2	3	4	5	6	Total
Oropharyngeal Washings							
No. with							
Prototype (Raji-like isolate)	7	5	...	1	0	0	13
Variant isolate	5	0	...	0	2	1	8
Peripheral blood mononuclear cells							
No. with cells							
Prototype	8	4	...	1	1	0	14
Variant isolate	4	0	...	0	1	1	6

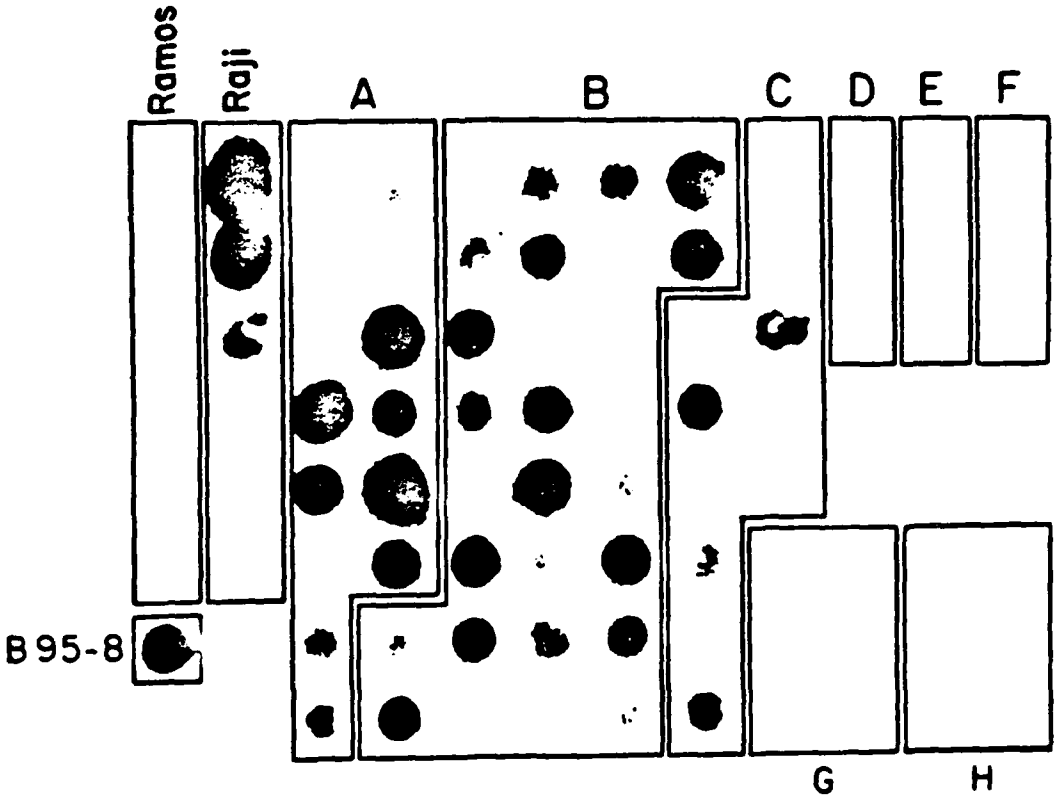

Figure 1. 124 oropharyngeal specimens were assayed for the presence of EBV DNA in an initial screen. All presumptive positive and representative negative specimens from each group were rescreened on the filter shown above. Ramos (negative control) and Raji (positive control) cells were spotten in six, serial 10-fold dilutions (10^6 to 10^1 cells per spot, from top to bottom).

300 µl of each mouthwash and the B95-8 (positive control) cell supernatant were spotted as indicated.

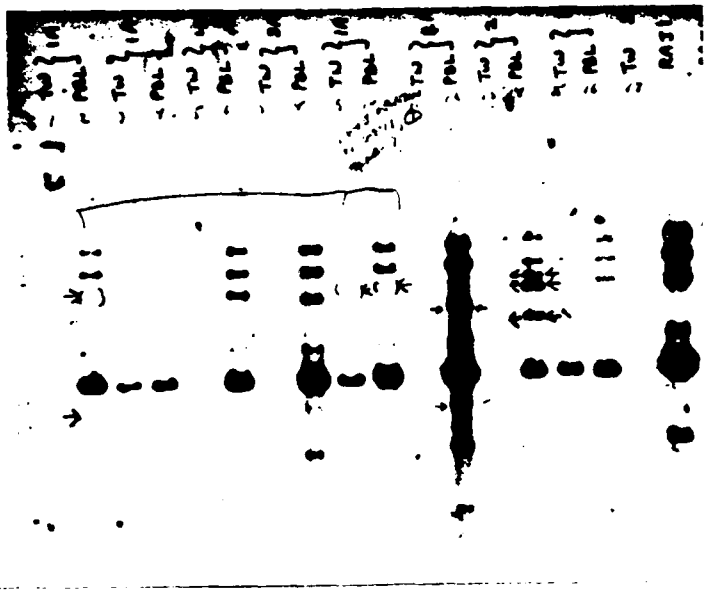

Figure 2. Southern analysis using EBV Eco RI "A" probe to determine the EBV genotype in oropharyngeal (throat) washings (TW) and peripheral blood lymphocytes (PBL) in HIV infected individuals of different WR classes. The genotypes were classified as standard if the pattern was identical to the non-permissively EBV infected Burkitt's tumor derived cell line, Raji (right edge). Arrows (→) point to new DNA bands and with parenthesis [( )] signify loss of bands in comparison to the Raji pattern.

Publications Resulting from Protocol

1. Alsip GR, Ench Y, Sumaya CV, Boswell RN: Increased Epstein-Barr virus DNA in oropharyngeal secretions of patients with AIDS, AIDS-related complex or asymptomatic human immunodeficiency virus infections. J Infect Dis (in press).

2. Sumaya CV, Boswell RN, Ench Y, Reuben JM, Mansell PWA: Epstein-Barr virus IgM antibody responses in acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC). Sumitted to AIDS Research and Human Retroviruses.

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4. Alsip GR, Ench Y, Sumaya CV, Boswell, RN: Increased Epstein-Barr virus DNA in oropharyngeal secretions of patients with AIDS, AIDS-related complex or asymptomatic human immunodeficiency virus infections. In Epstein-Barr Virus and Human Disease, PH Levine, DV Ablashi, M Nonoyama, GR Pearson. R Glaser (eds.). The Humana Press Inc., Clifton, New Jersey, pp. 119-120.

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