SERO-EPIDEMIOLOGICAL STUDIES ON HUMAN HERPES VIRUS-8

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ABSTRACT

Human herpes virus–8 (HHV-8) is a known carcinogenic agent. This report investigates five populations to determine if HHV-8 was associated with disease onset. Detection and levels of antibodies were measured using an enhanced immunofluorescent assay and were analyzed with the statistical program, SPSS.

In the first study, we tested the hypothesis that HHV-8 was the causative agent in Langerhan's cell histiocytosis (LCH). The seroprevalence of HHV-8 among 159 LCH patients was similar to the control group, indicating that HHV-8 is not the etiological agent of LCH.

In the second study, we tested the hypothesis that HHV-8 reactivation occurs in solid-organ transplant (SOT) patients, following immunosuppression. We found a significant increase in HHV-8 seropositivity when comparing pre-transplant to post-transplant samples ($p<.01$). There was also an overall increase in viral antibody titers following transplantation ($p<.001$), indicating viral reactivation. In the third study, we compare the SOT results to bone-marrow transplant patients (BMT). Longitudinal serum samples from 34 BMT patients did not demonstrate a significant association with HHV-8 as compared to the control ($p=.716$) or the SOT populations ($p=.180$). In addition, HHV-8 reactivation did not occur post-transplantation.

In the fourth study, we tested the hypothesis that HHV-8 is associated with increased risk of prostate cancer (PrCa). There was greater than a 2-fold association between HHV-8 seroprevalence and PrCa among African-Caribbean men from Tobago (p=.003). A similar trend was present in a PrCa cohort from the United States, p>.05.

In a fifth study, we tested the hypothesis that HHV-8 increased the risk of PrCa among men who carried genetic polymorphisms in the androgen (AR) and estrogen receptor (ESR1) genes. This study analyzed an expanded Tobago cohort, which demonstrated an association between HHV-8 and PrCa (OR 1.74, p=.032). An increased association was found among seropositive men carrying the high-risk AR allele (OR=2.46, p=.023) and ESR1 allele (OR=3.10, p=.004). The strongest association was found in seropositive men with both high-risk alleles (OR=5.20, p=.017).

This study demonstrates the use of HHV-8 serology as a marker for an increased public health cancer detectable risk, due to viral prevalence or reactivation.

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1. INTRODUCTION

1.1. Herpes Viruses

Herpesviridae is one of the largest virus families with members isolated from almost all mammalian species. Assignment of a virus into this family is based on morphology of the virus particle and genomic similarity. Viewed by an electron microscope, the virions of different members of the *Herpesviridae* family are indistinguishable. Each consists of four well-defined components including the core, capsid, tegument, and envelope.[1] The core contains a double-stranded DNA genome, arranged in an unusual torus shape, which is located inside an icosadeltahedral capsid that is approximately 100 nm in size and contains 162 capsomeres.[2] Located between the capsid and the viral envelope is a layer of proteins, collectively known as the tegument. This structure is interspersed with numerous embedded proteins. The tegument arrangement is typically asymmetrical with some herpetic members showing less ambiguous tegument structures than others.[3,4] Structurally the tegument connects the capsid to the envelope, while functionally acting as a reservoir for viral proteins which are required during the initial stages of viral infection.[5,6] The outermost framework of the herpes virion is the envelope, which is derived from cell nuclear membranes and is impregnated with various viral glycoproteins. The size of mature herpes viruses ranges from 120 to 300 nm, this variation is mostly due to differences in the size of the individual viral teguments.

All herpes virus infections have a limited cellular tropism within their specific host, which consists of cycling stages between a replicating infection termed lytic and a dormant-like phase known as latency. During a lytic infection, the virus is actively replicated and newly synthesized particles are released into the surrounding medium. In the latency phase, viral replication is suppressed, resulting in the formation of a quiescent state of dormancy. The specific sites, gene expression, and the timing between episodes of lytic and latent infections are type specific among the various members of the human herpes virus family and are the basis for non-serological clinical diagnostics.

1.2. Herpes virus Subfamilies

The Herpes virus Study Group of the International Committee on the Taxonomy of Viruses[7] has divided the herpes viruses into three subfamilies, termed *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae*. Membership into a particular subfamily is based on biologic and genetic properties. These properties have been useful in predicting the inherent and distinct traits of newly discovered isolates (such as HHV-6, HHV-7, and HHV-8).

The *alphaherpesvirinae* are characterized by a variable host range, a short replicative cycle in the host, rapid growth and spread in cell culture, and the establishment of latent infections in sensory ganglia. Members of this subfamily are often referred to as neurotropic herpes viruses. Among the human herpes viruses, herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2), and varicella-zoster virus (VZV) belong to the *alphaherpesvirinae*.

The *betaherpesvirinae* are characterized by a fairly restricted host range, with a long reproductive cycle, both in cell culture and within the infected host, which often results in the development of a carrier state. Latency is established in lymphocytes, secretory glands, and cells

of the kidney as well as other cell types.[8] The human herpes virus cytomegalovirus (CMV), human herpes virus-6 (HHV-6), and human herpes virus-7 (HHV-7) are members of this subfamily.

The *gammaherpesvirinae* are characterized by a restricted host range with replication and latency occurring primarily in lymphoid tissues, although some members have also demonstrated *in vivo* lytic growth and potential latency in epithelial, endothelial, and fibroblastic cells.[7,9] Among the lymphoblastic cells, viral replication is generally restricted to either T or B-cells. The *gammaherpesvirinae* subfamily is further divided into two genera, *Lymphocryptovirus* and *Rhadinovirus*.[10,11] There are currently two human herpes viruses which belong to the *gammaherpesvirinae* sub-family including Epstein-Barr virus (EBV) and human herpes virus-8. EBV is the prototype for the *Lymphocryptovirus* genus while HHV-8 belongs to the genus *Rhadinovirus*. Members of both genera are characterized by their ability to induce cell proliferation *in vivo* resulting in lymphoproliferative disorders.

Latency of the *gammaherpesvirinae* is unique when compared to members of the *alphaherpesvirinae* or *betaherpesvirinae*. For example, HHV-8 and EBV infection of B-cells triggers the expression of several latent-specific genes. The genes are translated into proteins that function primarily to maintain the episomal viral genome in the latently infected cells and to transform the cells to insure long-term survival in a short-lived cell. Contrary to this pattern, HSV latency is established in non-dividing, long-lived sensory neurons of the trigeminal ganglia and therefore the virus does not encode and express specific cellular transforming genes.[12]

2. KAPOSI'S SARCOMA-ASSOCIATED HERPES VIRUS

2.1. Kaposi's Sarcoma

Kaposi's sarcoma (KS) is an atypical form of cancer that develops in supportive connective tissue such as cartilage, bone, fat, muscle, blood vessels, and fibrous tissue. Histopathologically, the lesions are classified as endothelial neoplasms containing not only the primary neoplastic proliferating spindle-shaped cells of endothelial origin, but also an abundance of extravasated erythrocytes and infiltrating inflammatory cells.[13] KS was first described in 1872 by the Hungarian dermatologist, Moritz Kohn Kaposi, as a relatively uncommon tumor of very limited prevalence found almost exclusively in elderly men of Italian or Eastern European Jewish ancestry.[14] Years later, another epidemiological form was reported in young adult and prepubescent equatorial black Africans. In 1969, a third form of KS was documented as associated with immunosuppressant therapy.[15] However, it was not until 1982 when a sudden increase in the prevalence of KS cases, affecting a large variety of individuals who were not previously associated with any known epidemiological cluster, that KS began to illicit amplified concern in the medical community. This novel form of KS was much more aggressive with a clinical course often characterized as disseminating and fulminating. Eventually it was linked to a disease model later denoted as acquired immunodeficiency syndrome (AIDS).[16]

These separate manifestations of KS tumors have been shown to be essentially identical from a histopathological view, however clinical symptoms and the course of the disease are distinguishable in each of the groups.[17,18,19] The distinctive parameters consist of (i) the degree of the anatomic involvement, (ii) the aggressiveness of lesion formation and progression, (iii) the association with patient morbidity and mortality and, (iv) patient risk factors including age of onset, sex, and ethno-geographic origin.[20,21]

2.1.1. Classic Kaposi's sarcoma

The terms classic, Mediterranean, and sporadic KS all refer to the same disease. This is a rare cancer occurring in North America, Eastern Europe, and Mediterranean populations.[20,22] Classic KS historically has an ethno-geographical predominance in the Mediterranean region, with up to a 10-fold higher incidence than in the rest of Europe and the United States (U.S.).[23,24,25] In general, the average onset of the disease is between fifty to seventy years of age with a 15:1 male to female ratio.

Classic KS tumors are described as one or more asymptomatic lesions that occur in the dermal layer of the skin and appear as either brown, blue, purple, or red blotches or nodules. The lesions are usually localized to one or both lower extremities and frequently involve the ankles and soles. Although the skin lesions are normally disfiguring, they often are not disabling or life threatening.[26] Instead, classic Kaposi's sarcoma most commonly runs a moderately benign course of ten to fifteen years or more, with indolent growth of the primary tumors and the gradual development of additional lesions.

Venous congestion and dependant pedal edema are complications that are often seen as a manifestation of slowing of circulation secondary to the physical pressure caused by local tumor

infiltration of tissues surrounding the veins and lymphatics of the affected limb. In chronic cases, systemic lesions can develop in lymph nodes, along the gastrointestinal tract, and in other organs. These visceral lesions are generally asymptomatic and are most often discovered only at autopsy, although clinically, gastrointestinal bleeding can occur. Up to one-third of the patients with classic KS develop a second primary malignancy, most often non-Hodgkin's lymphoma.[27,28,29]

2.1.2. African Endemic Kaposi's sarcoma

In 1953, African endemic KS was documented as a relatively common neoplasm endemic in native populations in equatorial Africa.[30] From 1968 to 1970, KS was associated with approximately 6.6% of all cancers seen in Ugandan males.[31] In addition, it is more frequently seen in equatorial African children and females than anywhere else in the world. In most cases, clinical African endemic KS is identical to classic KS, although it usually strikes at a much younger age. A second more aggressive form of the disease also arises, but at a less frequent rate. This form is characterized by granulating chancroid sores and a fungating tumor that can penetrate from the skin to the underlying bone.[32] Both the indolent and local aggressive forms of African endemic KS occur with a male to female ratio comparable to that observed with the classic KS tumor.

A less frequent lymphadenopathic form of KS is also seen in Africa. This form of the disease develops primarily in prepubescent children with a mean age of three years and a male-to-female ratio of 5:1.[33,34] In these cases, the generalized lymphatic involvement is usually associated with the visceral organs. In all cases, there is a 100% fatality rate within three years.[35,36]

2.1.3. Transplant-Immunosuppressive Therapy-Related Kaposi's sarcoma

In 1969, the first case of an immunosuppression-associated KS was described in a renal transplant patient. Since that time, a number of renal and other organ allograft recipients have developed KS concurrent with the commencement of the drug therapy used to prevent graft rejection by the immune system.[37,38] This iatrogenic form of KS has been reported to occur 500 to 1,000-fold more often in solid-organ transplant recipients then the general population, with 46% of all cases developing in the first year.[39,40,41]

The risk factors for developing immunosuppression-associated KS are wide-ranging, with a specificity of an ethno-geographic focus. For example, only about 0.4% of transplant patients in the U.S. and Western Europe are prone to develop iatrogenic KS, where as up to 5.3% of renal transplant patients in Saudi Arabia are at the same risk.[42,43,44] To date, the increase risk for HHV-8 infection has been associated with the rate of seroprevalence, within the immediate community, and has not been associated with genetic differences. The disease has also appeared to exhibit a relationship with some grafted organs opposed to others. Reports suggest that transplant-associated KS is overrepresented in recipients of an allograft kidney compared to other allograft solid-organs or bone-marrow. This difference, however, has been arguably considered a result of the type and intensity of immunosuppressive therapy rather than the graft tissue specificity.[45]

The course of iatrogenic KS can be either chronic or rapidly progressive. The tumor in these immunosuppressed patients often remains localized to the skin but widespread dissemination with mucocutaneous or visceral organ involvement is also common.[46] Studies have clearly demonstrated not only an increased risk of KS development with the initiation of

immunosuppressive therapy but well defined subsequent regression of the tumor with its elimination.[41,47] In one study, eight of eleven iatrogenic KS patients had complete regression of visceral and cutaneous tumor progression within six months of cessation of cyclosporine. As a result, KS tumor growth is clinically monitored and controlled through changes in immunosuppressive therapy.[48] These studies provided evidence to some of the earliest clinical recognition of the reversibility of KS.

2.1.4. AIDS-Related Epidemic Kaposi's sarcoma

A fulminant and disseminated form of KS first observed in U.S. immuno-compromised homosexual males was reported in 1981.[49,50,51] This marked the beginning of the AIDS epidemic. At that time, greater than 30% of all U.S. homosexual men diagnosed with the acquired immunodeficiency syndrome developed KS.[52] It was this unusual and sudden appearance, of a more aggressive form of KS, which led scientists to realize that a new disease had emerged. The etiology of AIDS was uncovered in 1985 and was denoted as the T-cell leukaemia type III virus, today referred to as the human immunodeficiency virus (HIV).[53,54] HIV infects and eventually destroys CD-4 T-cells, resulting in an immunologic deficiency and immune dysregulation. The immuno-compromised state predisposes the host to a variety of opportunistic infections and unusual neoplasms, including KS. Two recent independent U.S. studies have shown that HIV-infected homosexual men have greater than 10,000 times the risk of developing KS than the general population.[55,56] Compare this to a four-fold increased risk for KS development in HIV-infected women, and it should not be surprising that most of the U.S. cases of AIDS-related epidemic KS have been diagnosed in homosexual or bisexual men.[57]

In highly endemic regions of Africa, KS prevalence had also risen sharply during the last two decades as a result of AIDS. For example, KS incidence rate increased from 6.6 to 48.6% in the Uganda male population, becoming the most frequently reported cancer among men of that region. In comparison, in Ugandan females, KS climbed by more than 17.9% and in the children KS increased by an additional 40%.[31,58] This trend has also been noted within the South African community, with increasing incidence among the female population.[59]

By August 1998, the heightened proportion of U.S. AIDS patients developing KS had diminished.[60,52,61] This new trend followed the introduction of a highly active (triple-agent) antiretroviral drug therapy known as HAART, which has a synergistic multifocal attack mechanism targeting the virus lifecycle. The triple drug therapy delayed or prevented the emergence of drug-resistant HIV strains and consequently decreased viral load. This resulted in a reduced patient mortality as a consequence of an increased immunocyte survival, indirectly decreasing opportunistic infections.[62,63,64] Data collected in the San Francisco area confirmed this observation by demonstrating a significant KS decrease following the introduction of HAART. In 1973, 0.5 KS cases/100,000 persons/yr were reported. From 1987 to 1997, during the AIDS peak, this increased to 33.3 KS cases/100,000 persons/yr that then sharply declined to 2.8 in 1998. The observation was also documented by the Centers for Disease Control and Prevention (CDC) showing an 8.8% annual decline in KS incidence in the U.S. between 1990 and 1998.[65]

Triple-drug therapy was the key element in the re-establishment of the immune system. In one study, nine of ten early-stage AIDS-associated KS patients had complete remission of KS within six months of the commencement of HAART.[66] In a more recent study, an 81% reduction in AIDS-KS patient mortality was observed with HAART. These results were significant

compared to individuals receiving (i) no therapy, (ii) single or, (iii) double therapy.[67] The heightened synergistic effect of the combined triple antiretroviral therapy was dramatically superior to the single- or double-agent therapy.

2.1.5. HIV-negative "contemporary" epidemic homosexual-related Kaposi's sarcoma

Friedman-Kien *et al*. first reported KS in homosexual men who had no evidence of HIV infection.[17] The lesions were most commonly cutaneous and typically found on the extremities and genitalia, but could occur anywhere on the skin. Within this group, disease progression is gradual and chronic with additional lesions appearing every few years. These types of cases may indicate the presence of other behavior or risk factors, aside from HIV, that are common yet specific to homosexual men.

2.2. Human herpes virus-8

In 1994, using representational difference analysis, Chang and colleagues described the detection of two DNA fragments uniquely associated with diseased dermal tissue from a KS lesion of an AIDS patient.[68] The predicted amino acids encoded by these DNA sequences were found to share 39 and 51% identity to the capsid and tegument proteins of two transforming primate gammaherpesviruses, Epstein-Barr virus and herpes virus saimiri (HVS) respectively. The descriptive name of Kaposi's sarcoma-associated herpes virus (KSHV) emerged. However, the Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses officially re-denoted the virus as human herpes virus-8 and categorized it as part of the *gammaherpesvirinae* subfamily. To date, HHV-8 and EBV are the only known human gammaherpesviruses and both are characterized as causative agents to various, but specific,

tumor development. For example, in addition to KS, HHV-8 has been linked to a rare type of lymphoma known as primary effusion lymphoma (PEL) and with the angiolymphoproliferative disorder termed plasmablastic multicentric Castleman's disease (MCD).[69,70,71]

2.2.1. Patho-physiology and morbidity

HHV-8 primary infection in the immuno-competent population has not been associated with significant concomitant morbidity. However, mild non-specific signs and symptoms have been observed. In a cohort study of HIV-negative homosexual men, 108 men were followed over a fifteen-year period.[72] During this time, five men where shown to seroconvert, as detected by the HHV-8 lytic immunofluorescent assay (IFA). Of these men, four were reported to have experienced fever, diarrhea, fatigue, localized rash, and lymphadenopathy near the time of seroconversion. In a separate case report, an HIV-1 positive man was determined to have seroconverted to HHV-8 five weeks prior to the onset of various symptoms including fever, arthralgia, cervical lymphadenopathy, and splenomegaly.[73] In this patient, polymerase chain reaction (PCR) revealed HHV-8 DNA, vascular hyperplasia, and intense activation and proliferation of B-cells with no detection of EBV gene expression. In addition, prospective HHV-8 studies in children have also shown to link primary infection with a mild febrile illness and accompanying respiratory symptoms.[74,75,76]

2.2.2. Determination of viral infection

2.2.2.1. DNA detection

PCR-based methodology of HHV-8 DNA detection gives the greatest specificity for diagnosis, compared to all other tests currently used for measuring HHV-8 exposure or infection.[77,78] Viral

genomic copy number varies according to cell type and disease status. For example, PEL cells carry 40-150 copies of HHV-8 DNA per cell genome, compared to 1-2 copies per cell in KS tissue. In terms of other body locations, viral DNA is most often found in saliva followed by peripheral blood mononuclear cells (PBMC) and occasionally in semen and the male urogenital tract.[78,79,80,81,82,83,84,85,86,87]

In PEL cells, also referred to as body cavity-based lymphoma cells (BCBL), HHV-8 DNA is detected at 100% efficiency, which corresponds to 100% assay sensitivity. In comparison, PCR of KS lesions from known HHV-8 positive patients is detected in approximately 95% of all cases.[68,88,89,90,91,92] This technological discrepancy, between BCBLs and KS tissues, is believed to be reflective of the viral copy number as well as cellular heterogeneity of the tissue opposed to the relative homogeneity of the single B-cell typed lymphoma. In circulating PBMCs and in the plasma, detection of HHV-8 DNA in seropositive individuals is very rare. As a result of this poor sensitivity, DNA detection is not typically employed to evaluate patient exposure or infection to HHV-8.

Viral DNA detection has been used to analyze HHV-8 disease-associated progression. Reactivation of latent virus and proliferative infection leads to an increase in viral load in the peripheral blood stream. This increased viral load contributes to increased viral dissemination, spreading the infection.[78,93,94] In KS patients, an increase in plasma viral DNA, isolated from other blood products including unlysed cells, has been used as an indicator of a more advanced viral infection. Studies have also demonstrated a strong correlation between viral load within the peripheral blood, including cells, and an increased risk of KS progression.[95,96,97,98] Hence, peripheral blood viral load is used to predict the pathogenic outcome of the infection by measuring active HHV-8 replication through PCR detection of viral DNA.[99]

2.2.2.2. Serological detection

Serologic testing is a more sensitive application for detecting HHV-8 exposure or infection than PCR, especially during virally established latency. Therefore, serological methodology has become the standard tool for establishing HHV-8 patient status.

The seroprevalence of HHV-8 antibodies in the high titer KS population ranges between 90 to 100%, depending on the serological assay employed. This variation in seroprevalence is amplified when measuring the moderate to low titer population, reflecting a lack of assay sensitivity. Although there is variation between assays, the calculations for each population remain consistent when the results from similar assays are compared.[100]

Current HHV-8 serological assays are divided into two groups. The first, the lytic assay, measures antibodies directed against viral proteins expressed in the lytic phase. The second, the latent assay, measures antibodies directed against latent phase viral proteins. The assays utilize either immunofluorescent techniques, using infected cells as targets, or an HHV-8 peptide specific enzyme linked immunosorbent assays (ELISA). In a recent study, the CDC and the National Heart, Lung, and Blood Institute, in a collaborative effort with six separate laboratories, evaluated the current major HHV-8 serological assays.[101] In this study, replicated panels of plasma specimens were prepared from 1000 U.S. blood donors. In addition, each panel included forty positive controls from single aliquots originating from twenty-one different KS patients, with an additional nineteen samples diluted from one specific KS specimen. All six laboratories detected 100% of the positive controls received. There was complete agreement on 894 (89.4%) negative specimens, leaving 106 specimens that were questionably positive. The outcome of the

combined effort provided an HHV-8 seroprevalence ranging from 0.5 to 5.1% among the blood donor group.

The CDC study results were analyzed using three models 1) the gold standard model, specimens positive in two or more laboratories, 2) by a conditional dependence latent-class model, which allowed for the possibility of inter-assay correlations, and 3) a conditional independent latent-class model, which is based on the assumption that all testing laboratories are independent. The collaborative study concluded that the conditional dependence model best fit the data providing a consensus and projection of 3.5% HHV-8 seroprevalence in the general blood donor population. The specificities within the conditional dependence model ranged from 96.6 to 100 percent, with sensitivities ranging widely, but overlapping 95% confidence intervals.

In conclusion, although the laboratories were not in complete agreement of which of the 106 HHV-8 samples were true positives, the net performance of all of the assays was substantially similar. Therefore, no one assay was declared superior. However, the assays designed to detect antibodies to lytic phase proteins had a discernable increase in sensitivity.[102]

2.2.2.3. Serology and DNA detection

Comparison of results from various assays with different detection levels has set the ground work for establishing the relationships between the stage of infection (primary, latent, and lytic) and HHV-8 pathogenesis. For example, a patient who is seropositive without detectable DNA in the peripheral blood is believed to be carrying a latent HHV-8 infection, whereas seronegative patients who are DNA positive are considered to be hosting an early primary infection.[72,76,103,104] Furthermore, those who are positive by both tests are reasoned to have a reactivated infection or a late primary infection. In addition, the dissimilarity between the timely detection of HHV-8

specific antibodies verses viral DNA was compared with the duration to KS development. Using the Kaplan-Meier survival curves, it was revealed that HIV-1 positive men who were sero-reactive to the HHV-8 latent protein, but HHV-8 DNA negative within the peripheral blood, had a 49.6% probability of developing KS with a mean time of ten years.[105] However, in a separate study, quantitation of HHV-8 DNA in peripheral blood correlated to the estimated median time to KS development at 3.5 years. In conclusion, detection of viral DNA requires a higher viral burden and therefore indicates a more advanced infection than detection of HHV-8 seroreactivity. This difference has been employed to follow disease progression in the KS susceptible population.

2.2.3. Epidemiology of transmission

Transmission of HHV-8 is primarily through exchange of bodily fluids, and more readily through sexual then casual contact. Therefore, HHV-8 seroprevalence frequently increases with sexual promiscuity and therefore age. In the non-endemic populations, HHV-8 infection in children under the age of fifteen is rare.[103,106,107,108,109,110] Within the general adult population, seroprevalence of HHV-8 ranges from 3-10%, depending on the assay.[103,109,107,110] In U.S. homosexual men with numerous sexual partners, HHV-8 seroprevalence ranges between 20 to 60%.[105,107,111,112]

2.2.3.1. Horizontal transmission in the male homosexual community

Recent studies have suggested that receptive oro-anal rather than receptive anogenital sex is the most significant behavior risk factor for HHV-8 infection in the male homosexual community.[113] This hypothesis was based on data from 1985 to 1996, in which the HIV-1 seroconversion rate

declined while the rate of seroconversion for HHV-8 remained relatively steady. The trend was correlated to an increasing number of men participating in protected anogenital sex while neglecting to provide the same self-protection during oro-anal interactions. In a longitudinal study of 474 U.S. HIV-negative homosexual men, 3.8/100 person/years seroconverted to HHV-8, which was similar to HSV-1 and considerably higher than HIV-1 seroconversion. In addition, an Amsterdam Cohort Study also showed that participation in oro-anal sex is a strong predictor for HHV-8 seroconversion compared to receptive anal intercourse among homosexual men.[114] These conclusions are further supported by the ease of detection of HHV-8 DNA in saliva opposed to the low viral load in semen, urogenital tract, and within prostate tissue.

2.2.3.2. Horizontal transmission in prepubescent

Although intimate, sexual interactions appear to be the most efficient means of HHV-8 transmission, it is not the only mode.[115,116,117,118] Increased risk of HHV-8 infection in Egyptian children is proportional to an increased rate of casual contact with other HHV-8 positive children or siblings. In the French Guiana of northern South America, HHV-8 seroprevalence among children reached 15% by the age of fifteen years. This study documents an intrafamilial correlation of HHV-8 seropositivity by calculating a high association between children and their mothers, but not their fathers (OR 2.8, 95% CI 1.6-5.0) and between siblings (OR 3.8, 95% CI 1.6-9.5).[119]

A pattern of prepubescent acquisition of HHV-8 infection has been seen to be similar to EBV and hepatitis-B virus in many African countries. In the pre-AIDS population of Uganda HHV-8 seroprevalence reached 57% by ages eleven to thirteen and 89% between ages fourteen to seventeen years old.[120] In Cameroon, seroprevalence increases gradually during childhood

years to 48% by the age of fifteen years. Similarly, the seroprevalence of anti-lytic antibodies to HHV-8 in Egyptian children increased from 16.6% at younger than one year, to 58% at older than twelve years.

These studies, and others, strongly support oral transmission of HHV-8 through casual contact in endemic populations. In addition, these data explain why a higher incidence of HHV-8 transmission is observed in population with higher HHV-8 seroprevalence.[121,122]

2.2.3.3. Vertical transmission - mother to fetus

Several studies argue that a majority, but not all, seropositive infants acquire only passive exposure to maternal HHV-8-specific immunoglobulins across the placenta. This is supported by data demonstrating that there is a significant loss of HHV-8 seropositivity in children after infancy. For example, of the 83% HHV-8 seropositive Zambian infants born to mothers who were also seropositive, only 3% of the infants had detectable HHV-8 DNA in their PBMCs.[123] In addition, 46% of all Cameroon infants are seropositive prior to six months of age, but only 13% remained positive between seven and twelve months of age. In two separate studies, 25 and 58% of Ugandan infants were determined to be HHV-8 seropositive, but all of the infants lost their detectable antibody after one year.[120,124] Finally, fourteen infants born to HIV-1/HHV-8 positive mothers were also seropositive before one month of age, but all had become sero-negative by six months old.[125] Similar results have been reported in Ghanaian and Italian descendents.[126] Taken together these results suggest that vertical transmission of HHV-8, if it occurs, is very rare.

2.2.3.4. Iatrogenic transmission

HHV-8 can also be transmitted during organ transplantation. In one study, HHV-8 seroprevalence had risen sharply from 5.3 to 15.8% after transplantation.[127] The source of the virus was not identified. However, during the transplantation the patient receives an organ and blood products from more then one individual. In addition, the patient is under the care of numerous individuals while their immune system is compromised, due to a stringent immunosuppressant regime to suppress graft rejection. Thus, numerous sources must be considered.

Although HHV-8 DNA has been found in saliva, PBMCs, and semen of infected individuals, the exact nature of an HHV-8 infection including the location of the infectious virus, its dissemination into defined tissue, timing of viral shedding, and location of infectious virus still remains undetermined.[81,128,129,130,131,132] Indirect analysis has demonstrated that HHV-8 is not readily spread through HHV-8 infected blood, within the immunocompetent population.[133,134,135] Therefore, to date it is not understood why HHV-8 transmission in association with solid-organ transplantation is more efficient then through blood or blood products in the general immunocompetent population.

2.2.4. Viral replication

Infection of susceptible cells by HHV-8 involves the specific binding of the HHV-8 virion to the surface of the cell. The laboratories of Charles Rinaldo and Frank Jenkins (University of Pittsburgh) has demonstrated that the dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN) serves as a primary receptor for HHV-8.[136] Following the binding of the virus to DC-SIGN there also appears to be an interaction between glycoprotein-B

of HHV-8 and one or more cellular integrins.[137] Once the nucleocapsid has entered the cell, it migrates to the nuclear membrane where the viral DNA genome enters the nucleus and viral gene transcription begins.

Like all herpes viruses, HHV-8 establishes a latent infection that can be reactivated to a productive lytic cycle. During latency, a few latent-specific genes are expressed. In contrast, most of the genes encoded by the viral genome are transcribed during the active lytic phase. Normally more then 95% of HHV-8 infected cells exhibiting a latent viral infection. However, treatment of HHV-8 infected cell lines with 12-O-tetradecanoyl-13-phorbol-acetate (TPA) causes 10 to 20% of the cells to reactivate to the lytic replicative cycle. Therefore, viral genes that are expressed constitutively, regardless of TPA, are categorized within class-I and termed the latent genes. Viral genes that are expressed at low levels during latency and whose expression is induced by TPA are termed class-II genes. Viral genes that are expressed only after TPA treatment are class-III genes and consist of the late structural and replication genes.

2.2.5. Viral carcinogenesis

KS is a tumor of the endothelial cell origin while PELs are of the B-cell origin. Thus, HHV-8 has the ability to transform at least two different cell types. While the exact mechanism of HHV-8 transformation is not known, both autocrine and paracrine pathways have been postulated. Autocrine pathways require that virions infect the tumor cells and the viral proteins responsible for tumorgenesis are expressed within these cells. On the other hand, paracrine pathways require that the virus infects neighboring cells, not necessarily the tumor cells directly, and viral proteins responsible for tumorgenesis are excreted into the environment where they can interact with nearby tumor cells. The primary viral genes implicated in an autocrine pathway include LANA-

1, viral cyclin D, and vFlip. Viral genes implicated in a paracrine pathway include ORF74, which expresses a G-protein coupled receptor (vGPCR) homologue and a B-cell activating chemokine, viral interlukin-6 (vIL-6).

LANA-1, v-cyc and vFlip are all expressed from the same locus in polycistronic, differentially spliced mRNAs. Therefore, all three transcripts are regulated by a common promoter.[138,139,140,141,142] This promoter is bidirectional, controlling the constitutive expression of the latent genes to the left and lytic, K14 and vGPCR genes, to the right.[141,143,142,139]

2.2.5.1. Latency-associated nuclear antigen-1 (LANA-1)

LANA-1 is encoded by ORF73. The protein functions as a transcript regulator, modifying viral and cellular gene expression during the latency phase. It also binds to its own promoter acting as both and activator and repressor for transcription.[143,144,145,146,147,148,149] In addition, this protein interacts with cellular promoters and directly or indirectly regulates transcription of specific cellular genes, controlling cell cycling.[144,143] In mammalian cell lines, LANA-1 can disrupt normal tumor suppressor protein function and transduction pathways by binding directly to cellular proteins p53 or RB1.[150,151] Through these interactions, LANA-1 can block apoptosis and repress cellular control by stimulating the G1-to-S transition of the cell cycle, resulting in transformation.

2.2.5.2. Viral Cyclin D (v-cyc)

ORF72 encodes viral cyclin D protein, which is 32% identical and 54% similar to the cellular cyclin D2 protein and is a functional homolog.[152] Like its cellular mammalian counterpart, v-cyc

forms a complex with cellular cyclin dependent kinase (cdk)-6, which then phosphorylates RB1. Phosphorylated RB1 then releases E2F, which initiates cellular synthesis. However, unlike its cellular counterpart, this complex is resistant or less sensitive to the cyclin dependent kinase inhibitors.[153] Furthermore, efficient cellular RB1 phosphorylation requires the cycH/cdk7 stimulation of cdk6, where v-cyc is not dependent on cycH/cdk stimulation.[154,155] Therefore, v-cyc expression can bypass normal growth regulatory mechanisms and induce S-phase within the cell cycling cascade of events.[153,152,156,157,158]

2.2.5.3. Viral FLICE-inhibitory protein (vFlip)

vFlip is encode by ORF71 and is homologous to cellular FLICE (caspase-8)-inhibitory proteins (FLips). Like its cellular homologs, vFlip has been shown to protect the cell from programmed cell death by blocking Fas-mediated apoptosis.[159,160] vFlip also constitutively activates the nuclear factor κB (NF-κB) pathway through a direct interaction with the family of inhibitor proteins called LκB.[161,162] The LκB proteins retain NF-κB in the cytosol by masking its nuclear localization signal. vFlip interferes with this interaction, resulting in the phosphorylation of the LκB proteins. The phosphorylation of these proteins initiates their rapid ubiquination and proteasome-mediated degradation, releasing NF-κB.

Disassociated NF-κB migrates into the nucleus and activates transcription of its target genes. Some of the genes activated by NF-κB include those for cytokines and growth factors as well as chemokines, cell adhesion molecules, acute phase proteins, anti-apoptotic proteins, and transcription factors p53 and c-Myc.[163] NF-κB therefore is believed to have both a direct and indirect role in tumor formation.[164]

2.2.5.4. Viral G-protein coupled receptor (vGPCR)

ORF74 encodes a viral G-coupled protein receptor. This protein is homologous to the human IL-8 receptor. vGPCR constitutively engages pathways downstream of multiple G-protein subunits in a phospholipase-C and phosphatidylinositol 3-kinase-dependent manner. These pathways include protein kinase-C, protein kinase-B, Akt, NF-κB, and mitogen-activated protein kinases, leading to increased transcriptional activity of their nuclear targets, stimulation of cellular proliferation, promotion of cell survival, and transformation.[165,166,167,168,169,170,171,172]

Microarray experiments have shown that the large-scale gene expression response to vGPCR expression is very divergent between B-lymphocytes and endothelial cell lines. In B-cells, two CC-chemokines are induced, while (human interlukin-6) IL-6 and growth related oncogene-α (gro-α) are activated in endothelial cells.[173] In transient transfections, vGPCR activates the promoters of multiple latent and lytic HHV-8 genes.[174] Ultimately, cells expressing vGPCR secrete increased levels of autocrine and paracrine cytokines and growth factors (IL-1ß, TNF-, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, VEGF, bFGF, and MCP-1), which produces conditioned medium that is chemotactic.[170,175,176,177,166] In transgenic mice, vGPCR induces multifocal, angioproliferative, KS-like lesions.[172,169,178179,180]

2.2.5.5. Viral Interleukin-6 (vIL-6)

Encoded by K2, vIL-6 retains sequence and functional homology to cellular IL-6. However, unlike the cellular homologue, it functions constitutively, interacting with gp130 independent of the IL-6 receptor. vIL-6 stimulates multiple cellular pathways to induce cell proliferation and

extrahepatic acute-phase responses through the engagement of gp130.[14,181,182,183,184,185,186,187,188] The viral IL-6, but not human IL-6, protects PELs and heterologous cells from the antiviral, cytostatic effects of interferon, which down-regulates the surface expression of gp80 but not gp130.[189] vIL-6 induces human IL-6 secretion, supports the growth of IL-6 dependent cell lines, and is an autocrine growth factor for PEL cells.[190,191,192] Cells stably expressing vIL-6 secrete have a indirect paracrine effect by increasing VEGF, inducing hematopoiesis, tumorigenesis, and angiogenesis when injected into nude mice.[193,194]

3. SPECIFIC AIMS

The seroprevalence of HHV-8 in the general population is between 5 and 11%, depending on the serological assay used for viral detection. This seroprevalence is considerably higher then the prevalence of the virus-associated tumors, implying HHV-8 in a co-factor role in tumor development. Hence, viral infection alone is believed not to be sufficient in promoting a carcinogenic state.[195] One known additional fundamental factor in the development of HHV-8 associated carcinogenesis is immunosuppression. This can be acquired though many different etiological models including but not limited to drug therapy, viral induction, or through the aging process. In addition, while the exact mechanism of HHV-8 induced transformation is not currently known, analysis of the viral genome has revealed several proteins that share significant homology with human cell cycling and anti-apoptotic proteins. These genes include homologs for interleukin-6 (IL-6), cyclin D, Bcl-2, macrophage inhibitory factors, interferon regulatory factor, FLICE inhibitory protein, and a G-protein coupled receptor.[196] Therefore, due to the explicit patterns seen in HHV-8 gene expression and cancer association, it is reasonable to predict that other cancers may also be affected by the presence of HHV-8 as a causative agent or in a co-factor role.

HHV-8 has demonstrated cellular transforming potential via both the autocrine and paracrine pathways. With its recent discovery and tumor-association, we had hypothesized that HHV-8 is a likely candidate, as a direct causative agent or co-factor, in other such carcinomas. Furthermore, little is known about where HHV-8 is harbored and within which specific tissue. We know that the virus infects B-cells, monocytes, endothelium, epithelial, and fibroblastic cells.

Studies have also demonstrated that the virus is capable of infecting dendritic cells and prostate tissue.[197,198] Therefore we investigated HHV-8 association with dendritic and prostate cancer populations to determine if we can correlate viral infection with tumor development. In addition, we studied HHV-8 primary and secondary infections in solid-organ transplant and bone-marrow transplant recipients to better understand disease progression.

Specific Aim 1. Test the hypothesis that HHV-8 is the causative agent of Langerhan's Cell Histiocytosis.

Specific Aim 2. Test the hypothesis that HHV-8 is reactivated following solid-organ and bone-marrow transplantation.

Specific Aim 3. Test the hypothesis that HHV-8 is associated with prostate cancer in the U.S. (low HHV-8 seroprevalence and prostate cancer incidence area) and Tobago (high HHV-8 seroprevalence and high prostate cancer prevalence area).

Aim 3A. Determine if there is a sero-epidemiological association between HHV-8 and the presence of prostate cancer.

Aim 3B. Determine if HHV-8 shows an interaction with genetic polymorphisms in the presence of prostate cancer.

4. HHV-8 SERO-EPIDEMIOLOGICAL STUDIES 1998-2002

STUDY 4.1: Seroprevalence of human herpes virus-8 among Langerhan's cell histiocytosis patients

STUDY 4.2: Reactivation and primary infection of human herpes virus-8 among solid-organ transplant patients

STUDY 4.3: Sero-Prevalence of human herpes virus-8 among solid-organ compared to bone-marrow transplant recipients

STUDY 4.4: Elevated seroprevalence of human herpes virus-8 among men with prostate cancer

STUDY 4.5: Prostate cancer risk is elevated in genetically susceptible men who are seropositive for human herpes virus-8: The Tobago prostate survey

4.1. Seroprevalence of human herpes virus-8 among patients with Langerhan's cell histiocytosis

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Unpublished

4.1.1. Abstract

Langerhan's cell histiocytosis (LCH) is a dendritic cell proliferative disorder, with an unknown etiology. A number of human viruses were screened as potential causative agents. However, there has not been a significant association demonstrated between LCH and any virus tested. Human herpes virus-8 (HHV-8), a recently discovered human virus, can infect some dendritic cells. Therefore, we screened 159 LCH patients for HHV-8 specific antibodies, to determine if the virus is the causative agent to LCH. In addition, we tested nine LCH patient PBMCs for HHV-8 DNA. There was no significant association found between HHV-8 seropositivity and LCH. Furthermore, none of the tissues contained viral DNA. Thus, HHV-8 is not the causative agent of LCH.

4.1.2. Introduction

Langerhan's Cell Histiocytosis has had many names including Hand-Schüller-Christian disease, Letterer-Siwe disease, and Histiocytosis-X.[199] While LCH can occur in individuals at all ages, its peak incidence occurs between 1-3 years of age. LCH is a rare disease, with an incidence rate among children at 3-4 cases/million and less frequent among adults. The disease is characterized by a Langerhan's cell infiltration of skin and bone and is typically diagnosed as a neoplastic process, a reactive disorder, or an aberrant immune response. It can range in severity from a single localized lesion to multi-organ involvement, and even to a fatal leukemia-like disorder (which occurs primarily in infants). Localized lesions have a much better prognosis and response to treatment, than multi-system involvement. Among adults, a pulmonary form is also seen which is associated with smoking. LCH can affect many organs including bone, skin, lymph nodes, spleen, lung, liver, thymus, bone-marrow, central nervous system, and the gastrointestinal tract. The mortality of LCH is approximately 10-20%, but up to half of all LCH survivors will suffer significant sequelae.[200]

Diagnosis of LCH is based histologically on the presence of a large infiltrate of Langerhan's' cells (LC) within the lesion or mass. These cells are in the arrested an early stage of activation and do not function well for antigen-presentation.[201] Instead, the abnormal LCs produce an increased number of cytokines compared to the typical Langerhan's cells.

Viral encoded or induced cytokines have been shown to be capable of stimulating the growth of normal Langerhan's cells, *in vitro*.[202] Therefore, in search of the etiological agent, investigators have looked for association between LCH and several human viruses including HHV-6, adenovirus, cytomegalovirus, EBV, HTLV-I, HTLV-II, and parvovirus. Of all the

human viruses investigated to date, none has been shown to be present in the majority of LCH tissues.

In 1997, Rettig and coworkers reported on the identification of human herpes virus-8 (HHV-8), a known carcinogenic agent, in bone-marrow dendritic cells of multiple myeloma patients. They were the first to suggest that the HHV-8 virus was capable of infecting dendritic cells. In addition, several laboratories have reported the occasional detection of HHV-8 DNA in biopsies of normal skin from KS patients. These results led us to question if HHV-8 could infect other types of dendritic cells such as the Langerhan's cells and as a result be involved in the development of LCH. Therefore, we decided to investigate LCH patient plasma for the presence of HHV-8 antibodies to determine if HHV-8 infection is associated with the disease.

4.1.3. Materials and Methods

Study Subjects. Subject recruitment involved University of Pittsburgh Medical Center (UPMC) and Children's Hospital clinics, as well as nationwide members in the Histiocytosis Association of America (HAA).

Physician provided the relevant information to the patients and/or legal guardians, explaining the purpose of the study. If the patient or guardian was interested, a signed consent form and a medical questionnaire were obtained.

Recruitment from the HAA was initiated by the organization, in letter form. The letter described the study and requested interested individuals to contact Dr. Jenkins' laboratory. Once contacted, the study was explained to them and all questions answered. If the individual was

willing to participate, a package was sent to them containing a consent form, a medical questionnaire, instructions to a physician's office for the blood sample (the blood was drawn in a tube containing sodium heparin as anticoagulant and was not to exceed 20 mls), and a pre-paid federal compliant overnight mailer for shipment of the blood sample to the Jenkins laboratory. Of the 227 HAA members who expressed an interest in the study, 169 registered participants returned their mailers. Any individual between the age of birth and seventy years who had received a diagnosis of LCH, at sometime during their life, and correctly completed the questionnaire were included in the study (n=159). The mean age of the LCH cohort, at the time in which their blood was drawn for the study, was 20.3 (range 0.66-72).

Control Population. The control group consisted of 101 University of Pittsburgh students. The mean age of the control group was 22.8 (range 17-47). The study control group was chosen based on the hypothesis that HHV-8 was the causative agent of LCH. Hence, the control group was not aged-matched due to the lack of necessity and availability of a population of blood samples drawn from health children.

HHV-8 Serology. Plasma and peripheral blood mononuclear cells were isolated from blood samples, aliquotted, and frozen at -70^{o}C. Each serum sample was tested for the presence of HHV-8 antibodies using an indirect immunofluorescence assay that detects antibodies directed against replicative (lytic) viral proteins. This assay employs the B-cell line termed BCBL-1.[203] The BCBL-1 cell line is predominantly infected with the latent HHV-8 virus. Treatment of the cells with a TPA results in the induction of lytic viral replication in 10-20% of the cells. For each batch of serum samples tested, known HHV-8 positive and negative sera were analyzed. All serum samples were tested twice in a blinded fashion and are read by the same person to reduce variation, resulting from multiple interpretations of subjective observations.

HHV-8 DNA Analysis. PBMC. Patient samples testing positive for HHV-8 antibodies were analyzed for viral DNA by PCR analysis as described below. DNA was extracted from patient PBMCs. **TISSUE**. We received paraffin sections from seventeen separate LCH tumor tissues from Ronald Jaffe at Children's Hospital of Pittsburgh. Total DNA was isolated from three paraffin sections of each LCH sample by dissolving the paraffin in xylene and extracting the DNA in a cell lysis buffer (100 mM NaCl, 25 mM EDTA pH 8.6, 10 mM Tris pH 8.3, 0.5% NP.sub.40). The samples were centrifuged at 12,000 rpm for two minutes and the supernatant was siphoned off. The DNA within the supernatant was then extracted with phenol and chloroform, precipitated with 100% ethanol and resuspended in sterile H_2O.

PCR Analysis. Isolated DNA from the tissues and PBMCs were analyzed for the presence of HHV-8 genome, by PCR using DNA primers specific for ORF26 (a minor capsid gene) and/or K1 gene of HHV-8. The K1 gene has been shown to have a hypervariable region. Therefore, individual strains and isolates of HHV-8 can be uniquely identified with this primer. The PCR products were separated on 2% agarose gels, transferred to nytran membrane sheets, and hybridized to P^{32}-labeled oligonucleotide probes specific for each PCR product. Following an extensive washing, the PCR products were visualized using a PhophorImager (Molecular Dynamics).

The following PCR primer sets and probes were used in this study:

ORF26 (KS330)

1. Sense: 5' AGCCGAAAGGATTCCACCAT 3'

2. Antisense:: 5' TCCGTGTTGTCTACGTCCAG 3'

3. Probe: 5' TGCAGCAGCTGTTGGTGTACCACAT 3'

K1

1. Sense: 5' CTGATGAGCCAAACTGAG 3'

2. Antisense: 5' CTGGTTGCGTATAGTCTTC 3'

3. Probe: 5' ATGCAACCACACGTG 3'

The cycling conditions for both primer sets was 35 cycles of 94 deg. C. for one minute, 58 deg. C. for one minute, and 72 deg. C. for one minute.

Data Analysis. Differences between the two HHV-8 seropositivity rate groups were examined using Yates-corrected chi-square analysis.

4.1.4. Results

The total number of patients participating in the study was 169. However, only 159 patients were included in the analysis due to the lack of pertinent data provided in the questionnaires. Of the 159 LCH patients sixteen (10.1%) were HHV-8 seropositive (table 1).

The seroprevalence of HHV-8 among the LCH patients was compared to a control population, which consisted of 101 University of Pittsburgh students. The mean age of the control group was 22.8 (range 17-47). The mean age of the LCH cohort, at the time in which their blood was drawn for the study, was 20.3 (range 0.66-72). There was no difference between the HHV-8 seroprevalence among the LCH population (10.1%) compared to the control group (9.9%) (p=.100). The cohort was divided by sex to determine if the gender of the LCH patient was associated with HHV-8 sero-status, since LCH is observed in more frequently in males than females. The data revealed that 8.3% of the male LCH population compared to 12.0% of the female population was HHV-8 seropositive (table 1). There was not a significant difference between the sero-status of the two populations (p=.615).

The data were divided into groups based on the age at LCH diagnosis to determine if HHV-8 was associated with early verses late LCH onset. We compared HHV-8 sero-status to age at LCH diagnosis (table 2). One seropositive patient was not included in the analysis because they had failed to provide age at LCH diagnosis. Therefore, only 158 patients were compared by in this analysis. The patients were divided by 0-17 years (n=117) with a mean age of 8.07 $\pm$ 4.50 and eighteen or older (n=41), mean age 40.7 $\pm$ 15.14. There was a significant

difference in HHV-8 seroprevalence between age groups (p=.001, odds ratio (OR) 7.226, 95% confidence interval (CI) 2.39-21.74).

We performed a PCR analysis for HHV-8 DNA on isolated PBMCs from nine HHV-8 seropositive LCH patients. Of the nine samples, only one was found to contain HHV-8 DNA (data not shown). The single DNA positive patient was a 69-year-old female with active disease. We also tested 17 LCH tumor tissues from paraffin sections provided by Dr. Ronald Jaffe at Children's Hospital of Pittsburgh. PCR analysis of DNA isolated from three sections of each tumor failed to detect the presence of HHV-8 DNA in any of the seventeen samples (data not shown).

4.1.5. Conclusion

HHV-8 is not the causative agent of LCH, as evidence by the low seroprevalence of HHV-8 among these patients. If HHV-8 played a significant role in the development of LCH, we would have expected to see a seroprevalence similar to KS patients (i.e. 90% or greater).

There was a seven-fold increase in HHV-8 seroprevalence among the eighteen-and-older LCH population, compared to the under eighteen group. However, since HHV-8 has been reported to be spread by sexual routes in the U.S., thus increasing with sexual promiscuity and therefore age, elevated HHV-8 seroprevalvence among the older LCH group is not unexpected.

4.1.6. Tables

Table 1. HHV-8 sero-status among LCH populations.

Gender (n)	% HHV-8 Sero-Status (n)	
	Negative	Positive
Male (84) (52.8%)	91.7 (77)	8.3 (7)
Female (75) (47.2%)	88.0 (66)	12.0 (9)
Total (159)	89.9 (143)	**10.1** (16)

Table 2. HHV-8 sero-status among age defined populations.

Age Group	% HHV-8 Sero-Status (n)		
	Negative	Positive	Total
0-17	95.7 (112)	4.3 (5)	117
18+	31 (75.6)	24.4 (10)	41
Total	90.5 (143)	**9.5** (15)	158

4.2. Reactivation and primary infection of human herpes virus-8 among solid-organ transplant patients

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4.2.1. Abstract

Human herpes virus-8 (HHV-8) is the causal agent of all forms of Kaposi's sarcoma, including the iatrogenic form that presents in solid-organ transplant recipients. A longitudinal study of HHV-8 seropositivity was conducted among a cohort consisting of children and adult solid-organ transplant recipients. Antibodies to HHV-8 lytic proteins were detected by an indirect immunofluorescence assay in serum samples of 100 transplant recipients. HHV-8 seropositivity increased significantly from 5.3% pre-transplant to 15.8% post-transplant ($p<.01$). Seropositivity was not related to the age of the patient or type of organ transplanted. HHV-8 seroconversion occurred in both children and adult recipients. None of the seroconversion events was related to the source of the donor organ. These findings suggest that HHV-8 infection is not uncommon among both adult and children transplant recipients and that viral infection may be acquired from an outside source other than the transplanted organ.

4.2.2. Introduction

Viral infections are a major cause of morbidity and mortality following transplantation. Infection or reactivation of several human herpes viruses is responsible for causing serious clinical diseases among transplant recipients. These viruses include herpes simplex virus, cytomegalovirus, Epstein-Barr virus, and human herpes virus-6.[204] Human herpes virus-8, also termed Kaposi's sarcoma-associated herpes virus, is the most recently discovered human herpes virus. HHV-8 has been identified as the causative agent of Kaposi's sarcoma as well as a rare B-cell pleural effusion and some forms of multicentric Castleman's disease.[205,206,207]

Kaposi's sarcoma is a tumor originating from either endothelial or spindle cells that occurs in four forms.[208] The classical form presents in older men of Mediterranean, east European, or Jewish descent while the endemic form is seen in sub-Saharan Africa and affects primarily young adults and young children. An epidemic or AIDS-associated form is the most common cancer seen in HIV-infected homosexual men and an iatrogenic form is seen in solid-organ transplant (SOT) recipients.

The iatrogenic form of KS is a relatively common malignancy of solid-organ transplant recipients occurring 1000 fold more often than in aged matched controls, and with an incidence rate ranging from 0.5 to 5%. KS is more common among transplant recipients from those areas associated with classical and endemic forms of KS (reaching 5% in Saudi Arabia). In the United States, KS occurs in approximately 0.5% of all transplant recipients.

The occurrence of KS among transplant recipients is associated with the immunosuppressive therapy (particularly calcineurin inhibitors) as evidenced by several reports describing the remission of KS lesions following the reduction or withdrawal of the

immunosuppressive therapy.[209, 210, 211, 46, 212] Immunosuppression is known to result in the reactivation of many human herpes viruses including HSV-1 and 2, CMV, EBV, varicella-zoster virus, and HHV-6.

The majority of studies on HHV-8 among transplant recipients have focused on individuals who developed KS with an emphasis on adult renal transplants. Very little is known about the natural history of HHV-8 among U.S. transplant recipients. In addition, none of the published reports has included studies on children or comparisons between different organ transplants. In this report, we performed a longitudinal study of HHV-8 serology in serum samples collected before and after transplant in a cohort of 100 transplant recipients which consisted of both children and adults with different transplanted organs. Our results demonstrate that HHV-8 is reactivated following transplantation and suggest that the virus can be acquired from sources other than the donor organ.

4.2.3. Materials and Methods

Study subjects. A retrospective study was conducted on 100 stratified serum samples belonging to a serum repository of transplant recipients at the University of Pittsburgh that were collected from 1983 through 1994. Aspects of some of the samples from this repository have been reported previously.[213] Samples were selected to fulfill the following criteria: 1) an approximately equal number of men and women, 2) a broad range of ages at the time of transplant, and 3) a broad range of organ types. At the time of this study (1999 to 2001), limited clinical data was available on all of the transplant recipients. These data included the age of the transplant recipient at the time of transplant, gender, and type of organ transplanted. Other data such as the race or ethnicity of the recipient, age and gender of donor, type of

immunosuppressive therapy, clinical outcome of transplant recipient, and number of blood transfusions each recipient received were not available on a sufficient number of the subjects to permit analyses.

Healthy controls consisted of 101 University of Pittsburgh students. The average age of this cohort was 22.8±0.71 with a range of seventeen to forty-seven.

Serologic Assays. Antibodies against HHV-8 lytic antigens were determined by a modification of an indirect immunofluorescence assay (IFA) using the BCBL-1 cell line that contains the HHV-8 genome.[109,203] Briefly, BCBL-1 cells were induced by 20 ng/ml TPA for 3-5 days, collected by centrifugation, rinsed with phosphate buffered saline (PBS) and resuspended in a small volume of PBS. Aliquots of cells were placed in individual wells on a 12-well Teflon-coated glass slide, air-dried, and fixed with ice-cold acetone for twenty minutes. Following fixation, the slides were again allowed to air dry and stored at -20^{o}C. For each assay, the fixed cells were first incubated in PBS containing 10% goat serum for one hr at 37^{o}C to block non-specific binding. The blocking buffer was removed and primary antibody added, which was diluted in PBS containing 10% goat serum. The slides were incubated for one hour at 37^{o}C. Two dilutions (1:50 and 1:100) of primary antibody (participant's sera) were tested for each serum sample. The cells were next washed extensively in PBS, treated with a 1:200 dilution of a mouse monoclonal antibody directed against human IgG (Clone GG-7, Sigma Chemicals), and incubated at 37^{o}C for 0.5-1 hour. The cells were again washed in PBS, treated with a 1:100 dilution of a goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) and incubated at 37oC for 0.5-1 hour. The cells were washed overnight in PBS and glass cover slips added. For each assay, the specificity of the fluorescence was confirmed using cells incubated with primary but no secondary antibody and cells incubated with secondary, but no primary antibody.

For each batch of serum samples tested, known HHV-8 positive and negative sera were included. All serum samples were tested at least twice in a blinded fashion, and assessed microscopically by the same reader. Assays using the induced BCBL-1 cells were examined for the presence of whole cell immunofluorescence. The cutoff for a positive seropositivity was 1:50. HHV-8 antibody titers were determined by performing the IFA on serially diluted serum samples (1:25 to 1:51,200). End-point titers were reported as the reciprocal of the last positive dilution.

Data Analysis. Differences between the two HHV-8 seropositivity rate groups were examined using chi-square or Fisher's exact test, as appropriate. The Wilcoxon signed ranks test was used to examine differences between matched samples. Differences in mean titer levels post-transplant (a continuous variable) by categorical risk factor variables were examined using the Mann-Whitney U test.

4.2.4. Results

Determination of HHV-8 seropositivity. Tables 3 and 4 list several characteristics of each population analyzed in the study, the healthy adult volunteers and solid-organ transplant recipients. Serum samples from 101 healthy adult volunteers and samples from 100 solid-organ transplant recipients taken 6-7 months post-transplant were analyzed for antibodies directed against lytic HHV-8 antigens. Among the transplant recipients, individuals who were negative for HHV-8 antibodies in the sample taken post-transplant (6-7 months) were identified as seronegative while individuals whose post-transplant sample were positive were identified as seropositive. As shown in tables 3 and 4, HHV-8 seropositivity was higher in transplant recipients (20%) than in the healthy controls (9.9%; $p<.05$). Among the twenty seropositive transplant recipients, serum samples taken before or on the day of transplant were available for

fifteen subjects. Analysis of the pre-transplant samples showed that five of the fifteen subjects were seropositive for HHV-8 before and after transplant and were therefore, HHV-8 seroprevalent. The remaining ten subjects that were seronegative at pre-transplant and seropositive at post-transplant were identified as seroconverters. The identification of the five-seroprevalent samples puts the pre-transplant HHV-8 seropositivity at 5.3% (5/95). Thus, the HHV-8 seropositivity among the transplant patients increased significantly from 5.3 to 15.8% ($p<.01$).

Determination of HHV-8 seropositivity by age, gender, type of organ transplanted, and immunosuppressive therapy. In the United States, HHV-8 appears to be primarily sexually transmitted and therefore seropositive individuals have generally been found to be sexually active or adults.[214,215] To determine if the presence of HHV-8 antibodies in the samples taken post-transplant was related to the age of the transplant recipient at the time of transplant, the cohort was divided into those above the age of sixteen and those sixteen years of age and younger. As seen in table 2, there was no significant difference in HHV-8 seropositivity between these groups (20.6% vs. 18.9%; $p>.05$). The group of less than or equal to sixteen years old was then further divided into three subgroups; less than six years old, between six and twelve years of age, and between thirteen and sixteen years old. Surprisingly, there was no significant difference in HHV-8 seropositivity among these age subgroups (20%, 17.6%, 20%; $p>.05$).

The transplant recipients were grouped according to their HHV-8 sero-status and then separated by age, gender, and organ transplanted (table 5). If the post-transplant sample was negative for HHV-8 antibodies, the individual was classified as seronegative. Individuals whose pre-transplant sample was negative and post-transplant sample was positive were classified as seroconverters. Individuals whose pre-transplant and post-transplant samples were positive were

classified as seroprevalent. Individuals whose post-transplant sample was positive and there was no pre-transplant sample available were classified as simply seropositive post-transplant. As shown in table 5, all of the known seroprevalent cases occurred in individuals who were older than sixteen years of age at transplant. At least three of the individuals younger than sixteen seroconverted to HHV-8.

HHV-8 seropositivity (post-transplant) was not associated with the gender of the transplant recipient (table 4; $p>.05$). There was also no association between gender and HHV-8 serology among the healthy adult volunteers (table 3; $p>.05$).

HHV-8 seropositivity (post-transplant) was also not associated with the type of organ transplanted. As shown in table 4, the percentage of HHV-8 seropositive individuals within each organ subgroup was not significantly different among the groups ($p>.05$).

There was limited information on the immunosuppressive therapy given to each transplant recipient. The different regimens used for immunosuppression were 1) FK506 plus steroids, 2) FK506 plus azathioprine and steroids, 3) cyclosporin A plus steroids and 4) cyclosporin A plus azathioprine and steroids. This information was available on only four of the twenty HHV-8 seropositive and eighteen of the eighty HHV-8 seronegative individuals preventing any analyses on the effect(s) of immunosuppressive therapy on HHV-8 reactivation or primary infection.

Determination of HHV-8 antibody titers. The HHV-8 antibody titers of the twenty transplant and ten healthy control HHV-8 seropositive subjects were determined by an end-point IFA. As shown in table 6, the average titer of the transplant recipients was much higher than the healthy controls ($p<.001$). When the seropositive transplant recipients were divided into seroprevalent and seroconverters, the average HHV-8 antibody titer in the seroconverter group was twice as

high as the average titer in the seroprevalent group (table 6). There were no associations between the HHV-8 post-transplant antibody titer and type of organ transplanted ($p>.05$) or age group ($p>.05$). Serologic evidence for viral reactivation was obtained by comparing the viral titers of the five seroprevalent cases in the pre- and post-transplant samples. As shown in table 7, four of the five seroprevalent subjects demonstrated a four fold or greater increase in antibody titer between the pre- and post-transplant samples indicating that viral reactivation had occurred ($p<.05$).

Serum specimens from donors were available from 49% of the transplant cases, including 90% (9/10) of the seroconverters. None of the donor samples from the seroconverter cases tested positive for HHV-8 antibodies (data not shown) suggesting that these individuals did not obtain HHV-8 from the donor organ.

4.2.5. Discussion

This study reports on the seropositivity of HHV-8 among a cohort of 100 solid-organ transplant recipients. Unique to this cohort of North American patients is that it consists of children and adults as well as several different organ types. The HHV-8 seropositivity increased significantly from pre-transplant to post-transplant. This increase in seropositivity suggests that a number of the transplant recipients obtained a primary infection following the transplant procedure. While the transmission of HHV-8 from donor organ to recipient has been documented, the majority of these reports have involved patients from countries where endemic KS is found and where the seropositivity of HHV-8 among the healthy population is higher than that seen in the U.S.[216,217,218,219,220] In our study, none of donors for which serum was available (49% overall and 90% of the seroconverters) were seropositive for HHV-8 indicating that infection from the donor

organ was unlikely. This was further supported by evidence that the increase in seropositivity was not associated with the type of organ transplanted.

HHV-8 seropositivity post-operation was not related to the age of the recipient, at the time of transplant. It is noteworthy that all of the documented seroprevalent cases occurred in individuals above the age of twenty-one. This suggests that the increased seropositivity, particularly among children (<16 years of age), was due to the acquisition of HHV-8 during or following transplantation. HHV-8 infection in children within non-endemic populations and under the age of fifteen is rear, and therefore the mode of non-sexual transmission is not completely understood.[104,117,221,215,214] However, our data suggest that the infection was not acquired from the donor organ and therefore must have come from an outside source such as blood products, or perhaps, a caregiver or family member.

In a separate study using our serological assay, we found 5% of 1000 U.S. blood donor specimens to be HHV-8 seropositive. In addition, HHV-8 has been detected in cell-free plasma suggesting that viral infection could be spread by blood or blood-products.[222] Blood has been shown to not be an efficient means of transmitting HHV-8. However, few cases to the contrary exist. Cannon and coworkers found an association between injection drug use and HHV-8 seropositivity in women with or at risk for HIV infection, suggesting possible blood-borne transmission.[223] In addition, Rosenzwajg *et al.* have also suggested a blood-borne transmission of HHV-8 in their study of allogeneic bone-marrow transplant patients who seroconverted following transplantation.[224] In the latter study, the post-transplant samples were taken one year after transplant and therefore the acquisition of HHV-8 from another person following transplant cannot be ruled out. Countering these arguments are studies such as Cattani *et al*, who studied

100 kidney transplant patients in Italy and found no association between HHV-8 seropositivity and blood transfusions.[225]

Another possible route of HHV-8 transmission is the caregiver or family member. Previous reports have shown that HHV-8 is present in saliva and in several studies analyzing HHV-8 seropositivity in countries where KS is endemic, HHV-8 infection occurs primarily during childhood.[119,124,116,130,226,227,228,229] In addition, a study involving homosexual men in the U.S. suggested that HHV-8 can be transmitted by oral-oral contact. The low seropositivity in the general U.S. population (in comparison to countries where KS is endemic) suggests that oral or saliva-based transmission of HHV-8 may depend on either the viral load (which is presumed to be much higher in the endemic countries) or on other cofactors such as immunosuppression which is present in the transplant population. In support of this, Sitas *et al*., reported that the transmission of HHV-8 from mother to child among African patients increased relative to the mother's HHV-8 antibody titer suggesting that viral load was important for transmission to non-immuno-compromised hosts.

The most commonly reported clinical manifestation of HHV-8 infection among transplant recipients is the development of KS. The rate of KS among solid-organ transplant recipients varies by geographical location such that countries with endemic KS and higher seropositivity rates to HHV-8 have higher rates of KS among the transplant population. In the United States, KS occurs in approximately 0.5% of solid-organ transplant patients. This rate is much lower than the seropositivity rate reported in this study and in a study by Hudnall and coworkers who reported an increase in HHV-8 seropositivity following adult kidney transplants.[230] The discrepancy between development of KS and HHV-8 seropositivity among transplant recipients would seem to suggest that the majority of HHV-8 reactivation or primary

infections in this population do not result in clinical disease. However, Luppi and coworkers recently reported the appearance of an acute virus-like syndrome (fever, splenomegaly, and cytopenia) in a HHV-8 seronegative kidney transplant recipient who received a kidney from an HHV-8 seropositive donor.[231] The patient suffered bone marrow failure and ultimately died of renal and cardiac failure. They also reported an HHV-8 reactivation event in an autologous stem-cell transplant patient that coincided with the appearance of fever and bone marrow aplasia with plasmacytosis.[232] There have not been any reports of longitudinal studies on transplant patients investigating associations of HHV-8 infection or reactivation and non-malignant disease or other clinical syndromes.

This study measured the antibody levels to lytic HHV-8 antigens using an IFA on induced BCBL-1 cells. Other laboratories have reported that this assay is the most sensitive assay available for detecting HHV-8 antibody responses.[233,234] There has been some debate however, regarding the specificity of this assay with some suggestions that the increased sensitivity is confounded by a loss of specificity due to cross-reacting antibodies, especially anti-EBV antibodies. At least two studies however, have demonstrated that there is no detectable EBV cross-reactivity with the HHV-8 lytic IFA.[108,109] In the present study, the same serological assay was used for comparisons between transplant recipients and controls. Any potential overestimation of seropositivity should be uniform throughout the study and therefore, the differences noted between different sample groups remains significant. In addition, the seropositivity rates among the pre-transplant and healthy control samples reported in this study (5.3 and 9.9%) are in line with previous reports from other laboratories using the same assay.[109,108,230,235]

The increased seropositivity of HHV-8 among transplant recipients reported in this study and others and the evidence for both viral reactivation as well as primary infections (as measured by HHV-8 seroconversion) suggests that future studies should be performed to evaluate the role of HHV-8 in graft survival and non-malignant disease. In addition, the potential role of blood transfusions and saliva-borne transmission of HHV-8 in transplant patients should be investigated in more detail.

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4.2.6. Tables

Table 3. Characteristics and HHV-8 seropositivity of healthy adult volunteers.

		HHV-8 Seropositivity		
Characteristic		**% Positive**	**95% CI**	**P**
Full Cohort (n=101)		9.9 (10/101)	6 - 17	.045[A]
Gender				.725
Male	26.7%	11.1% (3/27)	4 - 28	
Female	73.3%	9.5% (7/74)	4 - 18	

Note. CI, confidence interval; HHV-8, human herpes virus-8

A.Chi-square comparing HHV-8 seropositivity among healthy controls to post-transplant samples of transplant recipients.

Table 4. Characteristics and HHV-8 seropositivity of solid-organ transplant recipients.

Characteristic	HHV-8 Seropositivity Post-Transplant			
	% of Total (n=100)	HHV-8+ within group	95% CI	P
Full Cohort	100%	20% (20/100)	13 - 29	.045[A]
Age at Transplant Mean 26.25 ± 1.52 (Range 1 - 50)				
>16	63%	20.6% (13/63)	13 - 33	.84[B]
≤16	37%	18.9% (7/37)	10 - 34	
<6	10%	20% (2/10)	6 - 51	.984[C]
6 – 12	17%	17.6% (3/17)	6 - 41	
13 – 16	10%	20% (2/10)	6 - 51	
Gender				.367
Male	46%	23.9% (11/46)	14 - 38	
Female	54%	16.7% (9/54)	9 - 29	
Organ Transplanted				.98
Liver	46%	21.7% (10/46)	12 - 36	
Kidney	19%	15.8% (3/19)	6 - 38	
Heart	13%	23% (3/13)	8 - 50	
Multi-Organ	12%	24% (2/13)	8 - 52	
Lung	10%	20% (2/10)	6 - 17	

Note. HHV-8, human herpes virus-8; CI, confidence interval.

[A] Chi-square comparing HHV-8 seropositivity among transplant patients post-transplant to healthy controls.

[B] Chi-square analysis comparing HHV-8 seropositivity among individuals>16 years old to those ≤16 years old at transplant.

[C] Chi-square analysis comparing HHV-8 seropositivity between age subgroups ≤16 years old at transplant.

Table 5. HHV-8 sero-status among solid-organ transplant recipients.

Characteristic	HHV-8 Sero-status				
	Sero-Negative	Sero-Prevalent	Sero-Converter	Seropositive Post-Tx (No Pre-Tx Sample)	Overall
Age					
>16	50 (79.4%)	5 (7.9%)	7 (11.1%)	1 (1.6%)	63
≤16	30 (81.1%)	0	3 (8.1%)	4 (10.8%)	37
<6	8 (80%)	0	1 (10%)	1 (10%)	10
6 - 12	14 (82.3%)	0	1 (5.9%)	2 (11.8%)	17
13 -16	8 (80%)	0	1 (10%)	1 (10%)	10
Gender					
Male	35 (76.1%)	2 (4.3%)	5 (10.9%)	4 (8.7%)	46
Female	45 (83.3%)	3 (5.6%)	5 (9.3%)	1 (1.9%)	54
Organ Transplanted					
Liver	36 (78.3%)	1 (2.2%)	5 (10.9%)	4 (8.7%)	46
Kidney	16 (84.2%)	1 (5.3%)	1 (5.3%)	1 (5.3%)	19
Heart	10 (76.9%)	1 (7.7%)	2 (15.4%)	0	13
Multi-Organ	10 (83.3%)	1 (8.3%)	1 (8.3%)	0	12
Lung	8 (80%)	1 (10%)	1 (10%)	0	10

Note. HHV-8, human herpes virus-8; Tx, transplant

Table 6. HHV-8 antibody titers.

Group	n	Average Titer*
Healthy Adults	10	125 ± 17
Solid-organ Transplant Recipients	20	4828 ± 2589
Seroprevalent	5	3600 ± 2312
Seroconverter	10	7710 ± 4995

Note. HHV-8, human herpes virus-8
* Mean ± Mean S.E.

Table 7. HHV-8 antibody titers of pre and post-transplant samples.

Patient No.	Antibody Titer		Time after Tx
	Pre-Tx	Post-Tx	
17	50	400	6 months
60	200	3200	6 months
75	50	1600	6 months
78	800	1600	7 months
89	800	12,800	6 months

Note. HHV-8, Human herpes virus-8; Tx, transplant.

4.3. Sero-prevalence of human herpes virus-8 among bone marrow transplant recipients compared to solid-organ

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4.3.1. Abstract

Human herpes virus-8 (HHV-8) has been associated with the development of Kaposi's sarcoma. Serological and epidemiological studies have indicated that infection with HHV-8, along with immunosuppression, increases the risk of KS development. The iatrogenic form of KS is seen among solid-organ transplant (SOT) recipients in the U.S., but rarely in bone-marrow transplant (BMT) recipients. In this study, we compared the seroprevalence of HHV-8 among bone-marrow (n=34) and solid-organ (n=61) transplant recipients. Seroprevalence rates for HHV-8 were approximately 2-4-fold lower among the BMT patients (5.9%) when compared to the control population (11%), or the SOT patients (20%), respectively. Our results reveal that BMT population has a decreased seroprevalence to HHV-8, following transplantation, compared to either the SOT or the healthy adult control populations.

4.3.2. Introduction

Human herpes virus-8 is a recently discovered member of the human herpes virus family. At the DNA level, HHV-8 is closely related to the simian virus, herpes virus saimiri (HVS) and to a lesser extent the human virus, Epstein Barr (EBV). Based on its ability to infect B-lymphocytes and endothelial cells, along with its genetic relatedness to EBV and HVS, HHV-8 has also been classified in the *gammaherpesvirinae* sub-family of herpes viruses. These viruses are capable of immortalizing cells and persisting in lymphocytes.

HHV-8 has been strongly associated with the development of Kaposi's sarcoma, the plasmablastic variant of the multicentric Castleman's Disease, and a rare primary effusion lymphoma.[70,69,71] The iatrogenic form of KS occurs following transplantation. KS develops in approximately 0.5-5% of all solid-organ transplants, but rarely among bone-marrow transplants. The seroprevalence of HHV-8 in BMT and SOT recipient populations correlate to the risk of KS. The purpose of this study was to determine 1) if the differences in incidence of KS among BMT patients compared to SOT patients is reflected in the HHV-8 seroprevalence and 2) if HHV-8 reactivates in BMT patients following transplantation.

4.3.3. Materials and Methods

Subjects. Plasma samples from twenty-six bone-marrow transplant patients collected from 1992-1993 were acquired from Dr. Madhavi Kadakia, University of Pittsburgh. An additional eight samples were collected in year 2000, through collaboration with Dr. Albert Donnenberg (University of Pittsburgh Cancer Institute). Serum or plasma samples from 61 patients receiving

solid-organ transplants from 1983-1994 were obtained from Dr. Monto Ho at the University of Pittsburgh, School of Public Health. At the time of this study, limited clinical and demographic data were available on all of the transplant recipients. The control population consisted of 101 University of Pittsburgh students.

The average age of the BMT patients was 40±11 with a range of 21-69. The average age of the SOT patients, for this study, was 37±8 with a range of 21-50 and the control population's average age was 23±1 with a range of 17–47. Within the BMT cohort, 41% was male and 59% female, compared to 48% male and 52% female in the SOT cohort. In the control group, 27% were male and 74% female. The BMT and SOT sample populations were from retrospective studies and therefore not all pre-transplant, post-transplant, and donor serum samples were available for analysis. The BMT study provided all 34 pre and post transplant serum aliquots, with thirteen out of thirty-four donor sera. However, within the SOT study, we obtained fourteen pre out of 61 post-transplant serum aliquots and thirty-one out of 61 donor sera.

Serologic Assays. Each serum sample was tested for the presence of HHV-8 antibodies using an indirect immunofluorescence assay that detects antibodies against replicative (lytic) viral proteins. This assay uses a B-cell line termed BCBL-1. The BCBL-1 cell line contains the HHV-8 genome in a latent infection. Treatment of the cells with a phorbol ester results in the induction of lytic viral replication in approximately 20% of the cells. For each batch of serum samples tested, known HHV-8 positive and negative sera were included. All serum samples were tested twice in a blinded fashion with the same person reading all of the slides.

Data Analysis. Differences between the two HHV-8 seropositivity rate groups were examined using chi-square or Fisher's exact test, as appropriate. Differences in mean titer levels post-

transplant (a continuous variable) by categorical risk factor variables were examined using the Mann-Whitney U test.

4.3.4. Results

HHV-8 antibodies were detected 5.9% (2/34) among BMT patients compared to 18% (11/61) of the SOT subjects and 9.9% (10/101) among healthy adults. These results were based on the analysis of serum or plasma samples taken before and several months after transplantation. HHV-8 seropositivity among BMT patients post-transplant was lower than the healthy adult controls ($p=.716$) or the SOT group ($p=.180$). Although the difference in HHV-8 seropositivity compared to both the SOT patients and the controls was insignificance, there was a trend with a 3-fold risk of HHV-8 exposure for the SOT compared to the BMT recipients. The presence of HHV-8 antibodies did not appear to be related to gender ($p>.05$) (table 8).

The serum samples taken both before and after transplant were analyzed for HHV-8 antibodies and compared. Subjects determined to have at least one seropositive sample were classified according to the following scheme (results shown in table 9).

1) Seronegative; all samples were HHV-8 seronegative.
2) Seroprevalent; samples before and after transplant were HHV-8 seropositive.
3) Seroconverter; sample prior to transplant was HHV-8 seronegative, samples after transplant were HHV-8 seropositive.
4) Seropositive; no pre-transplant sample was available; samples after transplant were HHV-8 seropositive.

Table 10 shows the endpoint titers of the pre-transplant and post-transplant samples from the seroprevalent subjects. The seroprevalent patients in the SOT group demonstrate an increase

in antibody titer following transplantation, while those in the BMT group did not. The average HHV-8 antibody titer in the first sample taken following transplantation (3-6 months) was greatly increased in the SOT patients (both among seroprevalent and seroconverters) as compared to the BMT patients or healthy adults (table 10). These results suggest that HHV-8 is reactivating either from the donor organ or within the recipient among SOT, but not BMT patients.

4.3.5. Conclusion

The data obtained in this study have demonstrated that HHV-8 seroprevalence post transplant was higher among solid-organ transplant patients compared to either bone-marrow transplant patients or our control population. However, this study suffers from a small sample size, especially in regard to the BMT population, and therefore we can only address the observed trend. Although there was not a significant difference between HHV-8 sero-status and age groups, another inherent flaw within this retrospective study design was that the populations were not age matched, This discrepancy could become significant in a larger study. By inference, this study represents a potential difference between the tested populations and this difference is the groundwork for a future prospective study. Therefore, our laboratory investigated the possibility of setting up a prospective study; however, we failed to acquire the necessary sample size in regard to the BMTs, with appropriated aged matched populations.

Although this study suffered from the lack of aged matched controls and a small sample size in the BMT population, these results are consistent with the increased incidence of KS among SOT patients as compared to BMT patients. HHV-8 antibody titers increased in the SOT population following transplant while those in the BMT group remained unchanged or

decreased. We believe that these results may be reflective of the immunosuppression regime in the patients receiving a SOT, which resulted in a reactivation of latent virus (from either donor or recipient). However when comparing this to the lack of reactivation in two immunosuppressed BMT patients we believe that this comparison suggests a type of ablation occurring in the BMT population, possibly causing the destruction of the cell(s) harboring latent HHV-8.

4.3.6. Tables

Table 8. Post-transplant HHV-8 serology separated by subgroups.

	BMT (n=34)			SOT (n=61)		
	n (%)	**Male (%)**	**Female (%)**	**n (%)**	**Male (%)**	**Female (%)**
Gender	**34 (100)**	**14 (41.2)**	**20 (58.8)**	**61 (100)**	**11 (47.5)**	**32 (52.5)**
HHV-8* Seropositive	**2 (5.9)**	**1 (50)**	**1 (50)**	**11 (18)**	**5 (45.5)**	**6 (54.5)**

* Chi-square or Fisher's Exact Test p>.05 for both the SOT and BMT populations

Table 9. Sero-diagnosis among the SOT and BMT populations.

Descriptives	**SOT**		**BMT**	
	HHV-8 serology (%)	**n**	**HHV-8 serology (%)**	**n**
Sero-negative	50 (82.0)	61	32 (9.7)	34
Sero-prevalent	5 (8.2)	61	2 (5.9)	34
Sero-converter	5 (9.1)	55	0 (0)	32
Sero-positive	1 (100)	1	0 (100)	0

Table 10. HHV-8 antibody titers from select SOT and BMT patients.

Patient	Pre-Tx	Post-Tx	Months (post-tx)
SOT			
17	50	400	6
60	200	1600	6
75	50	1600	6
78	800	1600	7
89	800	12,800	6
BMT			
11	800	400	4
15	100	50	4

Table 11. Average antibody titer in both BMT and SOT populations.

	Population Size	Titer
Controls	9	127
SOT	20	5060 +/- 2717
Sero-prevalent	4	4400 +/- 2800
Sero-converter	9	8522 +/- 5510
BMT	2	225

4.4. Elevated seroprevalence of human herpes virus-8 among men with prostate cancer

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4.4.1. Abstract

Background. Prostate cancer (PrCa) is the most common cancer among men in the United States. Additional studies have suggested an elevated risk among men with sexually transmitted diseases (STD). Human herpes virus-8 (HHV-8) is the causative agent of Kaposi's sarcoma and has been demonstrated to have oncogenic potential. HHV-8 prevalence increases with sexual promiscuity and is therefore classified as an STD. Viral DNA has been detected in prostate tissue in some but not all reports; therefore, it is readily accepted that HHV-8 is not the causative agent of PrCa. However, HHV-8 may have a co-factor role because of its presence within the prostate. To investigate an epidemiologic association between HHV-8 and prostate cancer, we

determined the prevalence of HHV-8 seropositivity among prostate cancer cases and controls in the U.S. and in Trinidad & Tobago, two countries with differing rates of prostate cancer.

Methods. Antibodies to HHV-8 were detected in two independent laboratories using either an indirect immunofluorescence assay or a combination of ELISA and IFA procedures. Serum samples were analyzed from men with and without prostate cancer in the United States and the Caribbean nation of Trinidad & Tobago.

Results. Among 138 Tobago men with prostate cancer, HHV-8 seroprevalence was 39.9% which was significantly higher than the seroprevalence among 140 age-matched controls (22.9%; p=.003, odds ratio (OR) 2.24, 95% confidence interval (CI) 1.29-3.90). Among 100 U.S. men with advanced prostate cancer, HHV-8 seroprevalence was 20%, which was significantly higher than 177 blood donors (5.1%; p=.001, OR 4.67, 95% CI 1.91-11.65) and higher than 99 men with a diagnosed cancer not related to HHV-8 (13%; p=.253, 95% CI .77-3.54).

Conclusions. HHV-8 seropositivity is elevated among men with prostate cancer compared to controls suggesting a possible role in the development of prostate cancer.

4.4.2. Introduction

Prostate cancer is the most common cancer among men in the United States and is second to lung cancer as a cause of cancer death.[236,237] The incidence of and mortality from prostate cancer varies across population groups within the United States. For example, African-Americans have both a higher incidence and mortality compared to Caucasian-Americans.[237,,238] The increased risk for prostate cancer among men of African descent is not limited to the United States. In a recent study from the Caribbean nation of Trinidad & Tobago, the prevalence of screening-detected prostate cancer was 3-fold higher among men of African descent compared to men of

Asian-Indian descent.[239] While the reason for the population differences in prostate cancer incidence is not known, several studies have suggested a genetic factor.[240,241,242]

In addition, Hayes and colleagues reported an elevated risk of prostate cancer among men with sexually transmitted diseases.[243] Their study suggested that an infectious agent might be acting as a co-factor in the development of this cancer. However, the results from studies investigating several human viruses, notably herpes simplex virus, cytomegalovirus, and papillomaviruses have not supported this hypothesis.[244]

Human herpes virus-8 is the causative agent of Kaposi's sarcoma as well as a rare lymphoma (primary effusion lymphoma).[245] Transmission of HHV-8 can occur through sexual contact as well as non-sexual routes.[76,105,228,246,247] While HHV-8 DNA has been found by some laboratories in semen, and in both normal and cancerous prostate tissues, others have failed to detect viral DNA in any prostate related fluids or tissues.[85,129,128,198,248,249,250,251]

HHV-8 has oncogenic potential, can be spread sexually, and has been detected in some prostate tissue. Therefore, HHV-8 is a likely infectious co-factor candidate in the development of PrCa. In this study, we have tested this hypothesis by determining the seroprevalence of HHV-8 among prostate cancer cases and controls in populations differing in ethnicity and environment in Trinidad & Tobago and the United States. If HHV-8 serves as a co-factor for the development of prostate cancer, then HHV-8 seroprevalence should increase among men with the cancer as compared to age-matched controls.

4.4.3. Materials and Methods

Subject Populations. A case control study was conducted with participants from the Caribbean nation of Trinidad & Tobago and from the United States. Informed, written consent was

obtained using forms and procedures approved by the Institutional Review Boards of the University of Pittsburgh, the Tobago Ministry of Health and Social Services, and Caroni (1975) Ltd, Trinidad.

The Trinidad & Tobago participants consisted of three groups: 138 men from Tobago with biopsy-confirmed prostate cancer (Tobago prostate cancer), 140 age-matched controls consisting of men from Tobago with no evidence of prostate cancer (normal digital rectal exams (DRE) and prostate specific antigen (PSA) values < 4.0 ng/ml) (Tobago controls), and 174 men from Trinidad with no evidence of prostate cancer (Trinidad controls). Among the Tobagonians, 97% were of African descent, while among the Trinidadians, 90% were of Asian-Indian descent with the remaining 10% being of African descent. Asian-Indian ancestry in this population reflects the immigration of agricultural workers from India in the late 1800's to early 1900's. Aspects of these groups have been reported previously.[239,252]

The U.S. participants consisted of three groups: 100 men with advanced prostate cancer who were seen in a prostate cancer clinic at the University of Pittsburgh Medical Center (Pittsburgh prostate cancer), 99 men with a diagnosed cancer not related to (or suspected to be related to) HHV-8 (Pittsburgh cancer controls), and 177 male blood donors (U.S. blood donors).

The Pittsburgh cancer control group was selected from a serum repository of cancer patients maintained by the University of Pittsburgh Cancer Institute. Selected individuals were aged 40 years and older, male, and at the time of the blood draw they were diagnosed with a cancer that was not associated with HHV-8. We excluded men with a diagnosis of prostate cancer, KS, multi-centric Castleman's disease, primary effusion lymphoma, and multiple myeloma. Detailed medical information such as previous history of cancer, PSA values, and DRE were not available for the Pittsburgh cancer control group. The U.S. blood donors were

selected from a subset of a larger repository collected from five major U.S. centers by the National Heart, Lung, and Blood Institute Retrovirus Epidemiology Donor Study and represent all of the men whose age was within the age range of the Pittsburgh prostate cancer group (46–99 yrs).[101,253]

HHV-8 Serology. Detection of HHV-8-specific antibodies was performed in two separate laboratories, the University of Pittsburgh and the Centers for Disease Control and Prevention. Sera from twenty-one patients with histologically confirmed Kaposi's sarcoma were included as an HHV-8 positive laboratory control.

The University of Pittsburgh used an HHV-8 monoclonal antibody-enhanced immunofluorescent assay with a cutoff value for seropositivity of 1:100. HHV-8 antibody titers were determined using the same IFA on serially diluted serum samples. End-point titers were reported as the reciprocal of the last positive dilution. All samples were analyzed blinded, in duplicate, a minimum of three times by the same reader.

HHV-8 serology performed at CDC involved three separate assays. The first two employed a peptide-based enzyme-immunosorbent assay (EIA). The peptides used for detection by EIA are based on proteins encoded by HHV-8 open reading frames 65 and K8.1 (serum dilution of 1:100).[235,254] Samples that were negative by both EIAs were then screened by the monoclonal antibody-enhanced IFA at a serum dilution of 1:40.[109,235] A specimen was defined as seropositive if it was positive in any of the three assays.

Data Analysis. Differences in HHV-8 seroprevalence were examined by Chi-Square analysis or Fisher's exact test, as appropriate. Comparisons of HHV-8 titer levels by categoric risk factor variables and mean PSA values by HHV-8 sero-status were performed with the Mann-Whitney U test. Student's T test was employed to examine differences in age, height, weight, and body

mass index (BMI) by HHV-8 sero-status. Logistic regression analyses were used to analyze interactions of HHV-8 sero-status and various categoric variables on the presence of prostate cancer.

4.4.4. Results

HHV-8 Seropositivity. HHV-8 seroprevalence was higher among both of the prostate cancer groups compared to their corresponding controls (table 14). In Tobago, men with prostate cancer were more likely to be HHV-8 seropositive than their age-matched cancer-free controls (OR 2.24, 95% CI 1.29-3.90) or healthy men from the neighboring island of Trinidad (OR 2.63, 95% CI 1.54-4.50). HHV-8 seropositivity was also significantly higher among Pittsburgh men with prostate cancer than U.S. blood donors (OR 4.67, 95% CI 1.9-11.65), and was also higher compared to the Pittsburgh cancer controls, although this difference was not significant (OR 1.65, .77-3.54).

To confirm these results, HHV-8 serology was performed on all of the Trinidad, Tobago, Pittsburgh, and KS specimens at CDC using the serology screening algorithm described in Materials and Methods. There was strong agreement between the results from the University of Pittsburgh and the CDC laboratory (Kappa=.742). As shown in table 15, the HHV-8 serological assay from the University of Pittsburgh detected an average of 6.5 percentage points more HHV-8 seropositives among both cases and controls than that of the CDC, but these differences were not significant. The CDC laboratory independently confirmed the significant differences between the Tobago prostate cancer cases and Tobago controls (p=.020, OR=1.94, 95% CI 1.12-3.34). In addition, the elevated HHV-8 seroprevalence in the Pittsburgh prostate cancer group compared to the Pittsburgh cancer control group was also confirmed by the second laboratory.

Thus, the association of HHV-8 seropositivity with prostate cancer in both Trinidad & Tobago and the U.S. was confirmed by two independent laboratories.

HHV-8 Antibody Titers. To compare the levels of HHV-8 antibodies among the different groups, antibody titers were measured using an end-point IFA. While the mean HHV-8 antibody titer was higher in each of the prostate cancer groups compared to their corresponding control groups (table 16), with one exception, the differences observed were not statistically significant. The average antibody titer of the Tobago prostate cancer group was significantly higher than any of the U.S. cancer or control groups ($p<.02$). This difference was not seen when comparing the mean titers from either the Tobago or Trinidad control groups with any of the U.S. groups.

HHV-8 seropositivity by age. We determined the average age of the HHV-8 seropositive and HHV-8 seronegative men in each group. As shown in table 17, with the exception of the U.S. blood donors, there was no significant difference in the average age of the HHV-8 seropositive men compared to the HHV-8 seronegative men.

HHV-8 seroprevalence by population. The background rate of seropositivity, i.e. seropositivity in controls, was lower in the U.S. blood donors (5%) and Pittsburgh cancer controls (13%) compared with the Tobago (25%) and Trinidad (20%) controls. Within the Trinidad group, the HHV-8 seroprevalence among men of African descent was 33% (6/18) compared to 19% (29/156) among the Asian-Indians. In the Pittsburgh prostate cancer group, ethnicity was available for eighty-seven men. Within this subgroup, 42.9% (3/7) of the African-Americans were HHV-8 positive compared to 18.8% (15/80) of the Caucasian-Americans. This elevated HHV-8 seroprevalence among African-American men was not present among the U.S. blood donors (0/7). The Pittsburgh cancer controls did not include any African-Americans.

Future studies of larger populations will be required to determine the significance of these interesting results.

Association between HHV-8 seropositivity and PSA values and Gleason Scores. Gleason scores from prostate tissues were available for 55% of the Pittsburgh prostate cancer cases and 100% of the Tobago prostate cancers. The Gleason score is a histological grading system used in the U.S. that assigns histological patterns on a scale of 1-to-5 based on the degree of differentiation. The distribution of the Gleason scores for the HHV-8 seropositive individuals in Pittsburgh and Tobago was not different from that of the corresponding seronegative subjects (data not shown).

PSA levels were available from every Tobago prostate cancer case and from 84% of the Pittsburgh prostate cancer cases. The mean PSA values (excluding two values that were above or below two standard deviations from the mean) for the HHV-8 seropositive men in Pittsburgh (124.2+/-183.0) and Tobago (39.4+/-107.31) were not significantly different from the corresponding seronegative men in either Pittsburgh (70.20+/-127.02) or Tobago (65.24+/-243.58), based on the Mann-Whitney U test. There was also no correlation between PSA values and HHV-8 antibody titer levels in either population (data not shown). The higher PSA values seen in the Pittsburgh men likely reflect these men having advanced prostate cancer.

Associations between HHV-8 Seropositivity and Medical History Parameters in the Tobago Cohorts. Among the Tobago prostate cancer cases and controls, self-reported information for several medical history variables were available including history of benign prostatic hyperplasia (BPH), prostatitis, syphilis, gonorrhea, smoking, and family history of cancer, as well as their BMI (kg/m^2). This information was available on a majority of the Tobago subjects ranging from 81 to 99%, depending on the particular variable. There was no association between HHV-8

seropositivity and any of the medical history variables (data not shown). In addition, using logistic regression analyses, we determined that there were no interactions between HHV-8 seropositivity and these different health variables on the presence of prostate cancer (data not shown).

4.4.5. Discussion

Our study is the first to document elevated HHV-8 seropositivity among men with prostate cancer, compared to controls. This relationship was observed in two independent laboratories, using assays and algorithms that have been demonstrated to be reliable in identifying meaningful risks associated with HHV-8 infection.[72,255,256,223,233] In this study, we analyzed populations from Trinidad & Tobago and the U.S. that have dramatically different rates of prostate cancer. In both populations, there was an elevation in HHV-8 seroprevalence between the cancer and their associated control groups corresponding to a 2-4 fold higher prostate cancer risk in men with either a past or a current HHV-8 infection.

Among the Tobago prostate cancer cases there was a two-fold higher HHV-8 seropositivity compared to a well-defined, age-matched control group. An increase in HHV-8 seropositivity between prostate cancer cases and controls was also seen in the U.S., even though the control groups were not as well defined as the Tobago controls. Among the Pittsburgh prostate cancer cases there was a four-fold higher HHV-8 seropositivity compared to a control group selected from U.S. blood donors. However, the U.S. blood donor population is a selected group which excludes individuals with specific infections and behaviors.[257] Thus, this group does not fully represent the general population, in contrast to the recruitment for the Trinidad & Tobago controls. The Pittsburgh cancer control group represents unhealthy men, who presented

to the University of Pittsburgh hospital system with a non-HHV-8-related cancer. Detailed medical information is not available on these subjects and while we know that at the time of the blood draw they were not diagnosed with an HHV-8 related cancer, we cannot determine whether they had a prior history of prostate or any other HHV-8-related cancer. Nonetheless, our serology results show a trend in the association of HHV-8 to prostate cancer in the U.S. using this control group. While the HHV-8 seropositivity among the U.S. cancer controls (13%) is higher than the seroprevalence of the U.S. blood donors (5.1%), it is not significantly higher than the HHV-8 seroprevalence of 100 University of Pittsburgh students (10%). The lower seroprevalence among the blood donor group is probably a result of the screening process used in selecting blood donors.

Thus, it appears that the prostate cancer association with HHV-8 is similar across populations with different HHV-8 seroprevalence rates. In addition, HHV-8 seropositivity is higher among men of African descent in both the U.S. and Trinidad. These results suggest that HHV-8 infection may be endemic in these populations and therefore may help explain their higher prevalence (Tobago) or incidence (U.S.) of prostate cancer in men of African descent.

The fact that not all men with prostate cancer are HHV-8 seropositive probably indicates that the virus is not associated with all cases of prostate cancer. In addition, these results may explain discrepancies reported in the literature regarding the presence of HHV-8 in semen and prostate tissue.[258] None of these studies examined the seroprevalence of HHV-8 among the individuals tested. The discrepancies of viral detection in these reports may reflect the fact that the seroprevalence in the general population is low (5-10%) and that HHV-8 may not be associated with every case of prostate cancer. This lack of serological data prompted the current study.

One interpretation of our data is that HHV-8 directly elevates risk for prostate cancer. Under this scenario, we would speculate that the virus serves to increase either the risk of developing prostate cancer or the tumorigenicity of an existing prostate cancer through autocrine or paracrine paths. There was no significant difference in HHV-8 antibody titers between seropositives in the prostate cancer groups and their corresponding controls. This suggests that the virus is not actively replicating (beyond the basal rate needed to maintain a latent repository) in men with prostate cancer. If vigorous viral replication was occurring, we would have expected to see much higher levels of HHV-8 antibodies as seen with KS patients.[110,259,260]

An alternative interpretation of our results is that HHV-8 antibodies are a marker for some other infectious agent or a genetic, behavioral, or environmental factor that is responsible for cancer development. This scenario is similar to that seen with HSV-2 and cervical cancer, where HSV-2 seroprevalence was higher among women with cervical cancer compared to age-matched controls.[261,262] The elevated HSV-2 seroprevalence was not due to a direct role of HSV-2 in development of the cancer, but instead reflected similar risk factors in the acquisition of HSV-2 and the actual viral etiological agent of cervical cancer, human papillomavirus. Distinguishing between a causative or casual role for HHV-8 will require prostate tissue screening for viral DNA, to discern whether HHV-8 is present in the tumor, surrounding normal cells, or infiltrating B-cells.

In conclusion, while the data presented in this study do not explain what role(s) HHV-8 may play in prostate cancer, they do suggest an association with this cancer. Thus, the detection of HHV-8 seropositivity may prove to be a useful marker for an increased risk of prostate cancer.

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4.4.6. Tables

Table 12. HHV-8 serological comparisons.

Group	HHV-8 Serology (%)	p*	OR (95% CI)
Tobago Prostate Cancers	554002/138 (39.9)	.003	2.24 (1.29-3.90)
Tobago Controls	32/140 (22.9)		
Trinidad Controls**	35/174 (20.1)	<.001**	2.63 (1.54-4.50)
Pittsburgh Prostate Cancers	20/100 (20)	<.001	4.67 (1.91-11.65)
U.S. Blood Donors	9/177 (5.1)		
Pittsburgh Cancer Controls	13/99 (13)	.253***	1.65 (.77-3.54)

* Fisher's Exact Test
** Comparison with Tobago Prostate Cancers
*** Comparison with Pittsburgh Prostate Cancers

Table 13. Comparison of HHV-8 serological assays.

	HHV-8 Serology (%)		
Group	**University of Pittsburgh**	**CDC**	**p***
Tobago Prostate Cancers	55/138 (39.9)	45/138 (32.6)	.214
Tobago Controls	32/140 (22.9)	28/140 (20)	.565
Pittsburgh Prostate Cancers	20/100 (20)	12/100 (12)	.129
U.S. Blood Donors	9/177 (5.1)	ND	ND
Pittsburgh Cancer Controls	13/99 (13)	8/99 (8.1)	.261
KS Patients	21/21 (100)	19/21 (90.5)	.244

* Fisher's Exact Test
ND=Not Determined

Table 14. HHV-8 antibody endpoint titers.

Group	N	Mean +/- S.E.M.	P*
Tobago Prostate Cancers	56	5416.96 +/- 1461.51	
Tobago Controls	32	4634.38 +/- 1796.89	<.106
Trinidad Controls	49	1006.12 +/- 291.79	<.001# <.466##
Pittsburgh Prostate Cancers	20	870.00 +/- 214.73	
U.S. Blood Donors	9	466.67 +/- 158.99	<.285
Pittsburgh Cancer Controls	13	553.85 +/- 111.30	<.842** <.334***

Comparison with Tobago Prostate Cancers
Comparison with Tobago Controls
* Mann Whitney U Test
** Comparison with Pittsburgh Prostate Cancers
*** Comparison with U.S. Blood Donors

Table 15. Age and HHV-8 sero-status.

	N	Mean Ages (years +/- S.D.)			p*
		Total Group	HHV-8 Seropositive	HHV-8 Seronegative	
Tobago Prostate Cancers	138	66.64 +/- 7.98	67.25 +/- 6.86	66.22 +/- 8.68	.436
Tobago Controls	140	66.53 +/- 8.08	67.74 +/- 7.74	66.07 +/- 8.16	.278
Trinidad Controls	174	56.9 +/- 4.2	56.07 +/- 3.31	57.04 +/- 4.41	.225
Pittsburgh Prostate Cancers	100	68.7 +/- 8.8	67.85 +/- 7.89	68.96 +/- 9.04	.619
U.S. Blood Donors	177	53.9 +/- 6.73	58.89 +/- 6.68	53.65 +/- 6.65	.022
Pittsburgh Cancer Controls	98**	54.8 +/- 6.7	56.62 +/- 3.97	54.58 +/- 7.06	.313

* Student's T-Test comparing means of HHV-8 seropositive to seronegative individuals
** Age data missing on one Pittsburgh Cancer Control Subject

4.5. Prostate cancer risk is elevated in genetically susceptible men who are seropositive for human herpes virus 8: The Tobago prostate survey

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Paper to be Submitted for Publication

4.5.1. Abstract

We have previously demonstrated that the human herpes virus-8 (HHV-8) seroprevalence is elevated among African-Caribbean men with prostate cancer, on the island of Tobago. In the present study, we tested the hypothesis that prostatic cancer associated polymorphisms in the androgen receptor (AR) and estrogen receptor-α (ESR1) genes, could modify the prostate cancer risk in association with HHV-8 seropositivity.

In univariate analyses, neither the AR nor ESR1 polymorphisms were significantly associated with prostate cancer risk. However, an increased association was found in seropositive men carrying the high-risk AR allele (OR=2.46, p=.023) and in seropositive men with the high-risk ESR1 allele (OR=310, p=0.004). The strongest association was found in seropositive men with both high-risk alleles (OR=5.20, p=0.017). The data suggest that HHV-8 infection increases prostate cancer risk in genetically susceptible men.

4.5.2. Introduction

Prostate cancer risk is higher in populations of African descent in the U.S., and on the Caribbean islands of Jamaica and Tobago, compared to Caucasian-Americans and Asia-Indians of Trinidad & Tobago.[263,264,252] Clinical prostate cancer risk ranges from most prevalent among African descendants to least prevalent within the far East-Asian communities[265], with Caucasian-Americans measured between these two populations. These studies suggest that population differences in prostate cancer incidence maybe partially due to genetic factors.[266] In addition, the individual risk for developing prostate carcinoma reflects the incidence of this cancer within their family history. Finally, risk for PrCa also increases with age. Prostate cancer rate averages from 16% during a man's average life span to 80%, in the 80-year-old population seen at autopsy (80%).[267] Currently, these three factors, ethnicity, family history of PrCa, and age are used as the primary indicators for increased monitoring for PrCa.

Two specific genetic polymorphisms have been linked to PrCa. The first is the androgen receptor (AR) located on the X-chromosome at Xq11-12. This gene contains within exon-1, a polymorphic CAG repeat which codes for a polyglutamine tract. The activity of the AR can range from high ($\geq$20 repeats) to low (<20 repeats). Studies demonstrate that polymorphic variation in the length of a glutamine (CAG) repeat (range 9–38 repeats) in exon-1 is associated with mild modulation of receptor activity and the growth of prostate epithelium cells.[266,268,269,270] The shorter CAG repeat length in exon-1 of the androgen receptor gene correlated with an earlier age of onset of prostate cancer and a higher tumor grade and aggressiveness, in some studies.[271,272,273,274] However, in other epidemiological studies of prostate cancer patients, these

associations are not consistent.[275,276] Therefore, due to the lack of consistent data, it is believe that a second co-factor may exist.

The second genetic link to prostate development is the estrogen receptor (ER).[277] This gene ESR1 maps on chromosome 6, at 6q25.1. There are two ER alleles present, one for each chromosome. Estrogen in men increases with age and is related to PrCa development in animal models.[278,279] The ER has two forms, alpha (ESR1) and beta (ESR2) that are expressed within the prostate.[280,281] ESR1 is expressed mainly in stroma while ESR2 is predominately expressed in epithelium. ESR1 has been the target of prostate cancer research. The polymorphisms within the gene that encodes the ESR1 are distinguished by the presence (+/+), the presence within only one allele (-/+), or the complete absence (-/-) of the Xba-1 restriction sequence (TCTAGA). Recent studies from Japan have suggested that the absence or partial absence of the Xba-1 site in the ESR1 may contribute to prostate cancer risk.[282,283]

Human herpes virus-8 is a known carcinogenic agent. It was first discovered 1994 from Kaposi's sarcoma lesions. Since then, it has also been linked to two other cancers as the causative agent of a lymphoma pleural effusion lymphoma (PEL) and Multicentric Castleman's disease. Human herpes virus-8 has been associated as a potential co-factor with prostate cancer.[284] In a recent study, HHV-8 seropositive status was more frequently observed among the prostate cancer cases compared to the cancer-free controls (p=.003, OR 2.24, 95% CI 1.29–3.90).

4.5.3. Materials and Methods

Study Population. Prostate cancer cases and non-cases (controls) were drawn from the population-base prostate cancer screening study, the Tobago Prostate Survey. This survey was

conducted among African-Caribbean men aged 40-79 on the island of Tobago, as described previously.[285]

HHV-8 Serology. HHV-8 seropositivity was determined by an HHV-8 monoclonal antibody-enhanced immunofluorescent assay, a cutoff value at a 1:100 dilution was used to designate HHV-8 seropositivity. All samples were analyzed blinded, in duplicate, a minimum of three times by the same reader.

Genotyping. High molecular weight genomic DNA was isolated from the residual clot from the PSA sample. The AR and ESR1 polymorphisms were genotyped as previously described.[286] AR polyglutamine repeats were dichotomized at the median (< 20 (short), ≥20 repeats); the ESR1 genotypes for the single nucleotide polymorphism located at IVS1-354 were designated +/+, -/+, or -/-, correlating to the presence or absence of the Xba-1 restriction site. All genotyping was performed in the laboratory of Dr. Robert Ferrell, University of Pittsburgh, School of Public Health.

Data Analysis. Chi-square tests were used to compare the distributions of seropositivity and genotypes between cases and controls. Logistic regression models were employed to estimate the odds ratio (OR) for the higher risk categories compared to the lowest risk category in each univariate analysis. Each of the genotype categories were then combined with HHV-8 seropositivity status to result in four HHV-8/AR categories, four HHV-8/ESR1 categories, and three HHV-8/AR/ESR1 categories. SPSS 11.0 was used for data analysis. Data analysis was performed by Dr. Clareann H. Bunker, University of Pittsburgh, School of Public Health.

4.5.4. Results

Characteristics of the cases and non-cases did not differ significantly, these included age, height, weight, and body mass index (kg/m^2) (data not shown). However, HHV-8 seropositivity was significantly associated with PrCa. Among the men with PrCa, 34% were HHV-8 positive compared to 23% of the non-cases, p=.031. The OR for the seropositivity was 1.74 with a 95% confidence interval (CI) of 1.05-2.90, demonstrating a significantly increased risk of PrCa among Toboggonian men who are also HHV-8 seropositive (figure 1).

There was an equal distribution of the number of AR CAG repeat lengths within both of the PrCa cases and controls (p=.78) (figure 2). The mean CAG repeat among cases was 19.6 and among non-cases it was 19.9 (Mann-Whitney U test, p=.54). In addition, although the complete absence of the Xba-1 sequence was higher in the cases compared to the controls, there was no significant difference between the complete or partial absence or presence of the Xba-1 restriction site within the ESR1 alleles among the populations study (p=.2) (figure 3). Both genetic polymorphisms were compared to the presence of prostate cancer in univariate analyses. Neither the CAG repeat length in the AR nor the polymorphism in the ESR1 gene were independently associated with PrCa (data not shown).

However, there was a greater than two-fold increased risk of PrCa among the HHV-8 seropositive men carrying the AR short CAG repeats as compared to the seronegative men with the longer CAG repeat (p=.023, OR=2.46, 95% CI 1.13–5.35) (figure 4). Among seropositive men with ESR1 -/- or -/+ genotypes, PrCa risk was elevated (p=.004, OR=3.10, 95% CI 1.42–6.77) compared with seronegative men with the -/- ESR1 genotypes (OR=1.12, 95% CI 0.66-1.94) (figure 5). Analysis of HHV-8 seropositive men with both the short AR CAG repeats and

the ESR1 -/- or -/+ genotype demonstrated the highest risk for PrCa (p=.017, OR=5.20, 95% CI 1.34–20.17) compared to seronegative men with the long AR CAG repeat and the ESR1 +/+ polymorphisms (figure 6).

4.5.5. Discussion

Despite the absence of a univariate association of the AR CAG repeat length with PrCa risk in the Tobaggonian population, the association of the HHV-8 with the short variation of this genetic polymorphism resulted in an increased prostate cancer risk. Thus, our data suggest that a high frequency of the shorter AR CAG repeat may contribute to an eleveated PrCa susceptibility, through the additional interaction with HHV-8, in men of African descent.

Our study was in agreement with other recent investigations of the ESR1 IVS1-354 polymorphism, which did not find a univariate association with PrCa. However our data demonstrated an approximant 3-fold risk for PrCa in HHV-8 seropositivemen with the ESR1 -/- or -/+ polymorphisms and HHV-8 seropositive status. Thus, HHV-8 seropositive men who carry at least one of the Xba-1 negative ESR1 alleles are at an increased risk for PrCa.

Analysis combining the high-risk genetic polymorphisms (short AR CAG repeats and ESR1 -/- or -/+) with HHV-8 seropositivity demonstrated a greater than five-fold increase risk for PrCa. This significant cancer risk exceeds the risk associated with HHV-8 alone or in combination with either polymorphism, and represents the highest risk association with PrCa reported to date.

These data suggest that HHV-8 infection increases prostate cancer risk in genetically susceptible men. The high seropositive rate, with short AR CAG repeats and the absence or

partial absence of the ESR1 Xba-1 restriction sequence, may be significant contributors to the high prostate cancer risk in this African-Caribbean population.

The public health significance of the correlations presented in this study arises from the need to monitor highly susceptible populations for an elevated PrCa risk. Screening for HHV-8 sero-status is relatively non-invasive, since blood is already being drawn to measure prostate specific antigen levels within at risk populations. Therefore, PrCa monitoring should include HHV-8 screening.

4.5.6. Figures

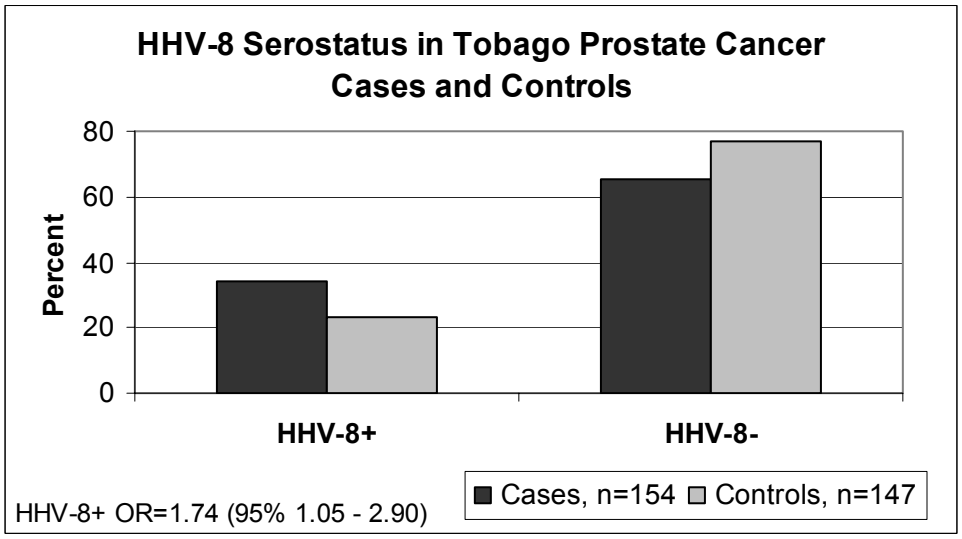

Figure 1. Increased risk of prostate cancer among men who are HHV-8 seropositive

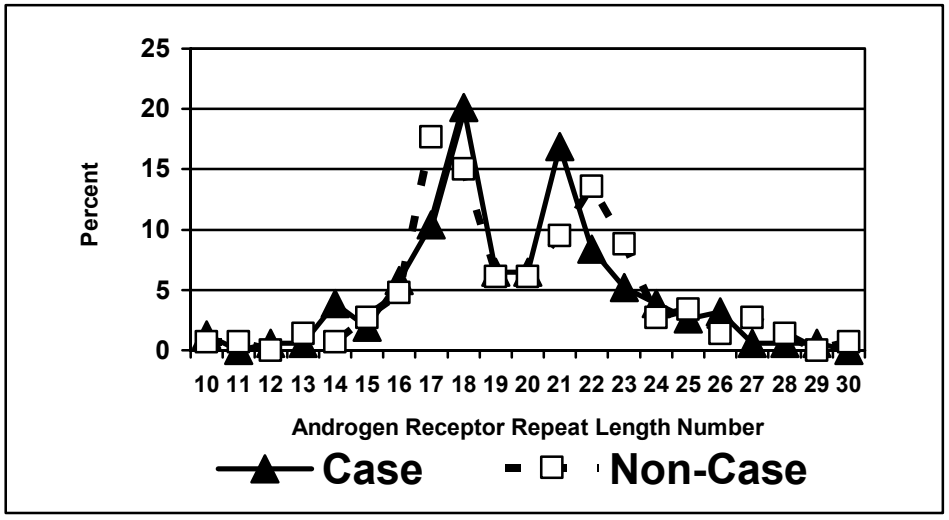

Figure 2. Distribution of the number of AR CAG repeats between PrCa cases and non-cases.

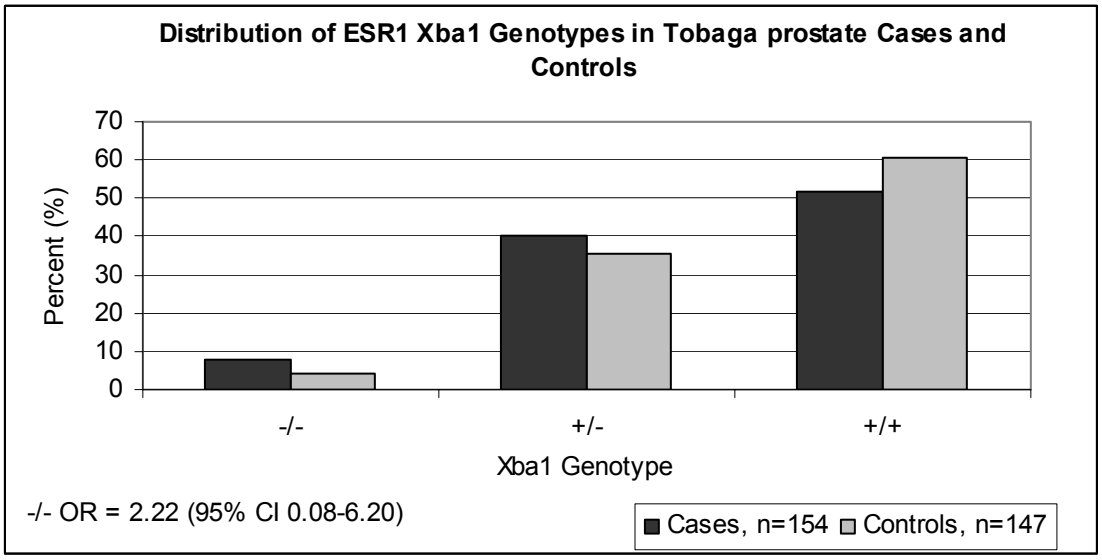

Figure 3. ESR1 polymorphism is not associated with prostate cancer risk.

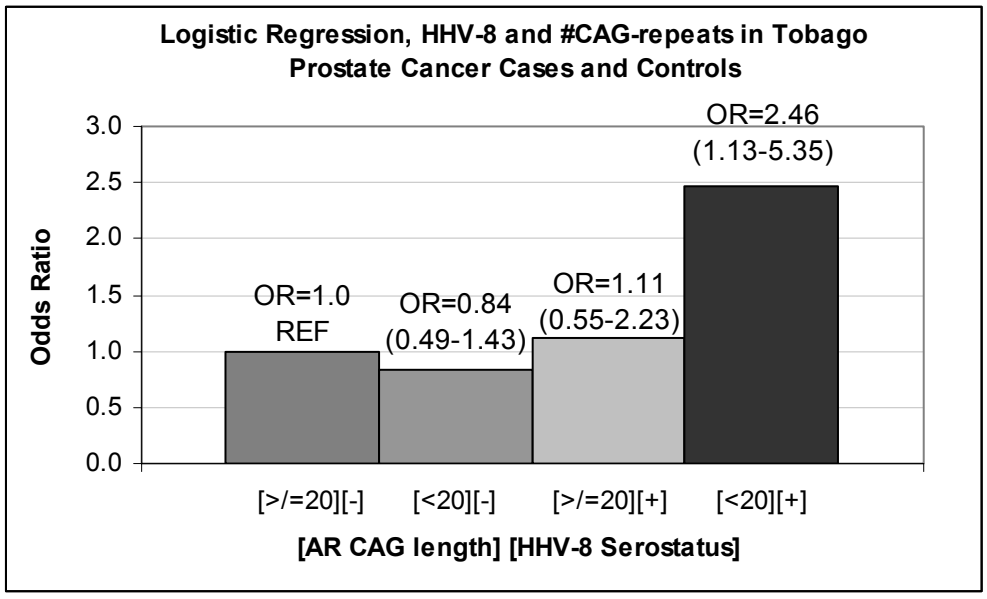

Figure 4. Prostate cancer risk is elevated among HHV-8 seropositive men with shorter AR CAG repeats.

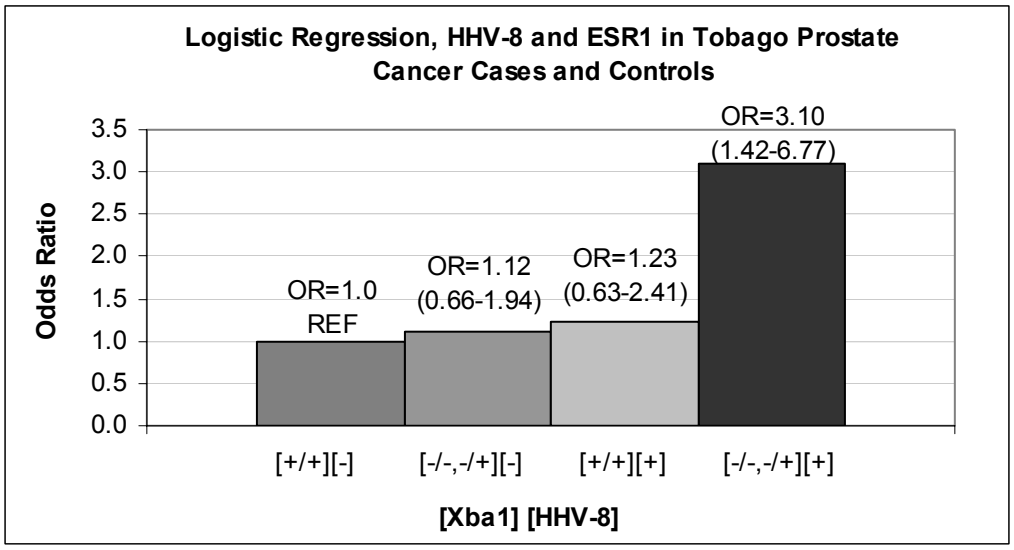

Figure 5. Prostate cancer risk is elevated among HHV-8 seropositive men with ESR1 -/- or -/+ genotypes.

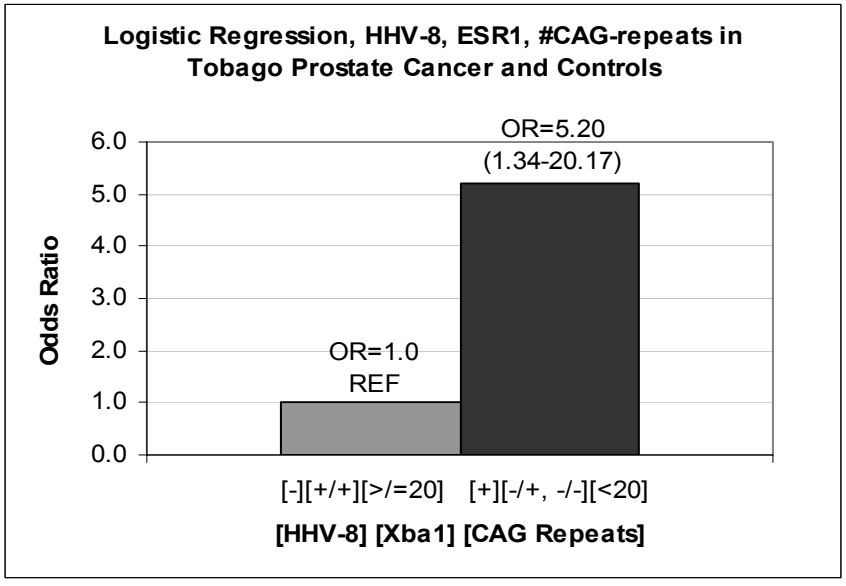

Figure 6. Prostate cancer risk in HHV-8 seropositive men with both the short AR CAG repeats and the ESR1 -/- or -/+ genotype.

5. CONCLUSION

Human herpes virus-8 has been demonstrated to be the causative agent of KS, PEL, and the plasmablastic variant of Multicentric Castleman's Disease (pMCD). While the virus is clearly necessary for development of these tumors, its presence alone is not sufficient. This is most clearly demonstrated by the fact that seroprevalence of HHV-8 is far more copious then the incidence of any given HHV-8 associated cancer. Thus, simply being infected with the virus does not result in cancer formation. Therefore, it can be inferred that other co-factors must play an important role in HHV-8-induced tumorigenesis. One such co-factor is immunosuppression that can be induced through either an iatrogenic chemical means, seen in solid-organ transplantation therapy, viral-induction, as in HIV-1-associated AIDS, or a waning of the immune response due to advancing age. A key assumption associated with the role of immunosuppression in the development of a herpes virus-induced cancer is that the state of immunosuppression results in the reactivation of a latent viral infection. This type of reactivation causes a significant increase in the viral load, which has been shown to correlate with cancer development.

We have clearly demonstrated that immunosuppression, as a result of solid-organ transplantation and subsequent suppressive therapies, results in the reactivation of HHV-8 among individuals who were infected prior to transplant surgery. Thus, like many of the human herpes viruses, HHV-8 is reactivated as a result of immunosuppression. Interestingly, we did not find viral reactivation among patients receiving bone-marrow transplants. While there were only two

seropositive patients detected in our study, neither one demonstrated viral reactivation (as measured by antibody responses) following immunosuppression. The lack of viral reactivation among BMT patients may explain the findings of various epidemiological studies that show the absence of KS among these patients. It is not clear from our results as to why reactivation did not occur. However, we believe that the difference in HHV-8 reactivation, between the solid-organ and the bone-marrow recipients, is directly related to the types of conditioning therapy that each group is subjected to before and after transplantation.

The target goal of immunosuppressive therapy given to SOT patients is to modulate in a non-immunocytotoxic manner the human immune response to the foreign major histocompatibility complexes (MHC) on the transplanted tissue. However in BMT patients, chemotherapy is designed to eradicate the native diseased lymphocytes. As a result, HHV-8 latently infected B-cells are theoretically eliminated, which could explain the lack of virus reactivation in the two HHV-8 seropositive BMT patients examined in our study. An alternative possibility is that the virus does reactivate among BMT patients, but secondary to the destruction of the B-cell population, the patient does not elicit a humoral immune response. In this scenario, the serological assay results would be mis-leading. However, evidence against this theory exists from a prior study, which demonstrated reactivation of HHV-6 in these same samples and within the same time-period tested by the current study.[287] Prospective studies using a larger cohort of BMT patients are necessary to further explore these possibilities.

We also demonstrated that SOT immunosuppressed individuals have a significantly higher susceptibility to a primary viral infection, as seen in the elevated HHV-8 seropositivity following transplant. The seroconversion was not due to a neo-infection of virus from donor organs since the donors (for whom blood samples were available) were found to be seronegative.

Therefore, we have postulated that the source of virus may have come from either the blood donors, through transfusions, or from caregivers through exchange of body fluids such as saliva. Both of these hypotheses need to be tested and examined in greater detail. In addition, the fact that a number of non-sexually active children seroconverted demonstrates that HHV-8 can be spread by non-sexual routes in the U.S., as has been reported for other areas of the world.

The three human cancers types associated with HHV-8 (KS, PEL, and pMCD) share the common causative agent HHV-8 which has been shown to be present in the tumor cells of all cancer cases. While the precise mechanism of HHV-8 tumorigenesis is not completely understood, there are at least two possible pathways. The first involves a direct mutagenic effect in which the tumor cell is infected by the virus that transforms the cell, resulting in carcinogenesis. Using PEL as an example, every B-cell from this lymphoma is infected with HHV-8. However, the vast majority of these cells are latently infected and therefore carcinogenesis is presumably due to the expression of one or more latent genes. In this regard, it has been demonstrated that one of the viral latent proteins, vFlip, causes the activation of NF-κB, a key player in sustaining the transformed B-cell. A second possible pathway involves a more indirect role of HHV-8 in tumorigenesis, one in which the virus causes transformation in a paracrine manner. For this scenario, using KS as an example, the spindle cell is the tumor cell of KS and has also been shown to be infected with HHV-8. In a manner similar to PEL, the vast majority of the spindle cells are latently infected with virus. However, it has been reported that a small percentage of these cells, at any given time, undergo viral reactivation and therefore within the KS lesion, there is always a few spindle cells expressing the lytic phase viral proteins. It has been reported by several laboratories that some of these lytic phase proteins are capable of inducing transformation and have potential for angiogenesis and for hematopoiesis, most notably

a G protein-coupled receptor (vGPCR) and the viral interlukin-6 (vIL6) cytokine. It has been shown that the presentation of the vGPCR on the membrane of HHV-8 infected cells can bind to cytokines interlukin-8 and growth related oncogene-α, stimulating the production of the vascular endothelium growth factor (VEGF). Secretory VEGF has both angiogenetic and hematopoiesis properties which can support tumor growth within the local area. Thus, the vGPCR is an example of HHV-8-induced carcinogenesis through an indirect paracrine pathway. On the other hand, vIL-6 is secreted from the HHV-8 infected cells and is directly measurable *in vitro* within growth media. The secretory vIL-6 has a direct effect on surrounding cells, it has been shown to activate B-cell proliferation as well as stimulate VEGF production. This is an example of a direct paracrine factor in carcinogenesis.

The notion that HHV-8 can act in a paracrine manner to support tumor formation underlies our hypothesis, involving HHV-8 in some cases of prostate cancer, but not all. Studies have demonstrated that the majority of men who live to the age of eighty have undiagnosed prostate cancer. Thus, most men die with prostate cancer rather than from prostate cancer. A key question is why some men in their late forties thru mid sixties develop clinically apparent prostate cancer. Our studies have clearly demonstrated that HHV-8 infection (defined by the presence of viral antibodies) is associated with prostate cancer. In the Caribbean island of Tobago, men with prostate cancer were almost twice as likely to have antibodies to HHV-8 compared to controls. In addition, recent unpublished work in our laboratory has demonstrated that HHV-8 is present in columnar epithelial cells of normal prostates from seropositive men.[288] Thus, the virus establishes a latent infection in prostate glands of seropositive men. It is clear that HHV-8 does not cause all cases of prostate cancer, since the majority of men with prostate cancer are seronegative. We believe that rather than acting as a causative agent, HHV-8 serves

the role of co-factor in men who have been infected. In this regard, we hypothesize that the presence of the virus and production of latent viral proteins, and few lytic proteins, may act in a paracrine manner to increase the tumorigenicity of existing cancers with some but not all cancer cases.

In support of HHV-8 association with prostate cancer, our second study in collaboration with Dr. Clareann Bunker and Dr. Robert Ferrell at the University of Pittsburgh School of Public Health discovered an interaction between HHV-8 and genetic polymorphisms in the androgen and estrogen receptor. These genetic polymorphisms have been associated with increased risk of prostate cancer in some but not all reports. In our study, while neither genetic polymorphism was found to result in an increased risk for prostate cancer, when HHV-8 infection was factored into the analysis, the risk of prostate cancer increased above the risk of HHV-8 alone. These results indicated an interaction between HHV-8 and these genetic polymorphisms with prostate cancer risk. Ongoing research in our laboratories is investigating these interactions to determine how the virus increases risk among men with either or both of these polymorphisms. The results of both prostate cancer studies strongly suggest that HHV-8 is a co-factor for some prostate cancers and opens the door for future research.

Earlier studies implicated HHV-8 as a causative agent for multiple myeloma, suggesting that the virus could infect bone-marrow dendritic cells. While this research is very controversial with several laboratories reporting an inability to confirm these findings, our laboratory was interested in the possibility that HHV-8 could infect dendritic cells. Work from the laboratory of Dr. Charles Rinaldo (University of Pittsburgh School of Public Health) has demonstrated that a primary infection of HHV-8 in immunocompetent men results in a less than robust immune response, both cellular and humoral. We speculated that one explanation for this was the ability

of HHV-8 to infect dendritic cells and disrupt their antigen presenting capabilities (APC). The possibility that HHV-8 could infect dendritic cells (DCs) led us to ask if the virus was the causative agent for Langerhan's cell histiocytosis, an extremely rare cancer of DCs. Our results have clearly demonstrated that HHV-8 is not the causative agent for LCH. In support of this, very recent work from the laboratory of Dr. Charles Rinaldo (University of Pittsburgh School of Public Health) in collaboration with our laboratory, has demonstrated that the cellular receptor for HHV-8 is DC-SIGN, a cell surface receptor present on monocyte derived dendritic cells (MDDC), but absent from Langerhan's cells. Thus while HHV-8 can infect MDDCs, it would be unable to infect Langerhan's cells through this same mechanism and thus would not have a role in LCH.

Our studies are the first to use HHV-8 serology to investigate increased risk through a paracrine mechanism of various cancers. We believe that viral serology should provide the ground work for additional investigations with other cancers that are known to involve cells or organs known to harbor HHV-8. Previous studies that have looked for an association between HHV-8 and other tumors, thus far, have neglected to specifically identify HHV-8 seropositive populations prior to looking for viral DNA. These studies were attempting to identify HHV-8 as the causative agent of the cancer rather than a co-factor. Our studies have demonstrated that an indirect association of HHV-8 with tumorigenesis must also be considered.

5.1. Future implications and public health significance

The results from the transplantation studies, on viral reactivation following immunosuppression in solid-organ and bone-marrow transplant patients, suggests that additional studies are need to

be performed. Among the SOT patients, it will important to determine if HHV-8 reactivation results in any serious sequelae such as episodes of high fever and acute or chronic graft rejections. It will also be important to determine if the reactivation of one latent lymphotrophic herpes virus, such as HHV-8, results in reactivation of other lympotrophic herpes viruses, such as EBV, CMV, or human herpesvirus 6 (HHV-6). Interactions between one or more reactivating lymphotrophic herpes viruses may increase patient morbidity and mortality. Among the BMT patients, our results need to be confirmed with a larger study designed to look at both viral antibody titers and viral DNA load in circulating PBMCs. This would allow us to distinguish between the absence of viral reactivation compared to the absence of a humoral response. This information could be used to determine the appropriate post-transplantation protocol.

The results from the prostate studies, demonstrate an increased risk for prostate cancer among HHV-8 seropositive men and genetically susceptible men. This is a novel finding. Evidence that HHV-8 serves as a co-factor in prostate cancer is very important and is the foundation for future research investigating the mechanism(s) and pathways by which the virus increases prostate cancer risk. These studies would focus initially on the role of IL-6 cytokine receptors, androgen receptors, and estrogen receptors. Determination of how HHV-8 increases prostate cancer risk will lead to new anti-viral therapies for the treatment of this most common cancer.

We demonstrated that measuring HHV-8 antibody with our enhanced immunofluorescent assay was a useful tool for differential diagnosis, distinguishing the disease from the possible cause or attributing factor. From the data gathered, we were able to calculate increased cancer-risk associated with HHV-8 seropositive status and viral reactivation. Therefore, from a public health perspective, we believe that HHV-8 screening should be further investigated as a potential

indicator of increased risk of advanced disease onset. This includes, but not limited to, regular monitoring of high-risk cancer populations, specifically prostate cancer and solid-organ transplant recipients.

6. PUBLICATIONS

6.1. Published manuscripts

Hoffman, L.J., Bunker, C.H., Pellet, P.E., Trump, D.L., Patrick, A.L, Dollard, S.C., Keenan, H.A., Jenkins, F.J. 2003. Elevated Seroprevalence of HHV-8 Among Men with Prostate Cancer. J Inf Dis. 189(1):15-20.

Jenkins, F.J., Hoffman, L.J., and Liegey-Dougall, A. 2002. Reactivation and Primary Infection of Human Herpes virus-8 (HHV-8) Among Solid-Organ Transplant Patients. J. Infect. Dis. 185:1238-43

6.2. Manuscripts in progress

Bunker, C.H., Ferrell, R.E., Jenkins, F.J., Zmunda, J.M., Shea, P.R., Hoffman, L.J., Kuller, L.H., Patrick, A.L. Prostate Cancer Risk is Elevated in Genetically Susceptible Men who are Seropositive for Human Herpes Virus 8: The Tobago Prostate Survey. In Progress.

6.3. Published reviews

Jenkins, F.J. and Hoffman, L.J. 2001. Surgical Oncology. Viral Carcinogenesis. Bland ed. *et al*. McGraw-Hill (NY): Medical Publishing Division.

Jenkins, F.J. and Hoffman, L.J. 2000. Infectious Causes of Cancer: Targets for Intervention. J.Goedert, ed. Totowa (NJ): Humana Press.

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