

Demographic and immune correlates of human herpesvirus 8 seropositivity in Malawi, Africa

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Background: In the USA, human herpesvirus 8 (HHV-8) is associated with Kaposi's sarcoma (KS) and HIV infection. We examined HHV-8 seroprevalence in a Malawian cohort, and assessed its relationship with HIV, KS, demographic characteristics, and immune findings.

Methods: In 1997 and 1998, blood samples were obtained from 272 hospitalized Malawian patients, for whom demographic information was obtained, and 24 healthy volunteers without demographic data. We used enzyme immunoassays to assess seroprevalence and antibody titers to peptide antigens derived from HHV-8 K8.1 and ORF65-encoded proteins. Intracellular cytokines and cell surface antigens were assessed with four-color flow cytometry. Data were analyzed using non-parametric univariate and regression analytic techniques.

Results: The rates of HHV-8 seroprevalence to either or both HHV-8 peptides were 67% for the patients and 54% for the healthy volunteers. Seroprevalence increased with patients' age ($P < 0.001$) but was not associated with HIV status, percentage of lymphocytes expressing CD4, or KS ($n = 10$). Seropositive females had lower antibody titers to both peptides than did males (medians: 455 versus 1361 for K8.1, $P < 0.001$; and 268 versus 405 for ORF65, $P = 0.044$). For the healthy volunteers, the percentage of CD8⁺ cells producing IFN- γ after stimulation was significantly lower in ORF65-specific antibody-positive persons (medians: 24% versus 57%, $P = 0.008$).

Conclusions: In Malawi, HHV-8 is endemic and is not associated with HIV infection or HIV severity. Seroprevalence rates increase in childhood, and, most steeply in adolescence. Titers are higher in seropositive males than in seropositive females. The immune effects of HHV-8 in healthy adults are consistent with chronic inhibition of type 1 cytotoxic T-cell responsiveness, independent of HIV status.

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INTRODUCTION

Human herpesvirus 8 (HHV-8) is a recently discovered virus. HHV-8 DNA sequences have been found in lesions from persons with Kaposi's sarcoma (KS), suggesting a causative association.^{1–3} In the USA, studies of homosexual men have found associations between HHV-8 and HIV seropositivity.⁴ Although HHV-8 is endemic in many parts of Africa, previous studies suggest that its seroepidemiology varies geographically.^{5–9}

Despite intense research on HHV-8, many questions remain. The data support the sexual transmission of

HHV-8; however, it is unclear whether other modes of transmission also exist.^{4,7–12} More information is needed concerning HHV-8 seroprevalence in non-homosexual populations, variations in HHV-8 seroprevalence by age, gender, and country, and the effect of HHV-8, a chronic herpesvirus, on the immune system.

We evaluated the seroprevalence and titers of antibodies to two HHV-8 antigens in hospitalized patients, 10 of whom exhibited some form of KS, and 24 employed, healthy people in Malawi, Africa. We present results concerning: (1) HHV-8 seroprevalence in a non-US population where HIV is endemic; (2) age- and gender-specific HHV-8 seroprevalence rates; (3) HHV-8 seroprevalence in persons with HIV and/or KS; and (4) for the healthy volunteers, relationships between HHV-8 seropositivity and various immune parameters.

METHODS

Participants

Serum or plasma samples were obtained in 1997 and 1998 from 272 febrile patients (ages 1 month to 64 years, median 25 years) hospitalized at Lilongwe Central Hospital, Malawi, Africa, as part of a study of bloodstream infections.¹³ Demographic and clinical data were

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obtained for these patients, but not information concerning sexual activities or behaviors. Of the 269 patients for whom gender was recorded, 53.5% were male. To assess associations between HHV-8 serostatus and immune findings, HHV-8 serology, HIV serology and immune studies were performed on blood drawn from 24 asymptomatic, apparently healthy, non-hospitalized local volunteers, since these persons would not be expected to have an acute co-infection. Demographic information on these healthy participants was not obtained. The study protocol was approved by the institutional review boards of the Centers for Disease Control and Prevention (CDC) and Lilongwe Central Hospital; informed consent was obtained from all participants or their guardians.

Enzyme immunoassay

Two enzyme immunoassays (EIAs) using synthetic peptides derived from the antigens HHV-8 ORF65¹⁴ and K8.1¹⁵ were used to determine HHV-8 seroprevalence, and the protocol was followed as previously described.¹⁴

All specimens initially testing HHV-8 positive were tested at least once again with each peptide EIA to confirm seropositivity. The cutoff for positivity was set at five standard deviations above the mean optical density (OD) of sera from 18 KS-negative, HIV-negative US persons. These sera had previously tested negative for HHV-8 with other methods.¹⁴ One positive control, an HIV-positive US homosexual KS patient who had previously tested positive for HHV-8 with other methods, was also included on each EIA plate.

A sample was considered to be HHV-8 positive if either or both antibody assays were positive. Using this criterion, the assay sensitivity was 93% and the specificity was 100% in KS patients and healthy controls from the US population.¹⁵ Fourfold serial dilutions were made of all positive samples. The endpoint titer was determined for the positive samples, from plots of OD versus dilution, and was taken as the dilution value where the titration curve intersected the cutoff value for positivity.

Immune techniques

Serum samples were analyzed for interleukin (IL)-2, IL-4, IL-6, IL-8, IL-10, interferon-gamma (IFN- γ), and tumor necrosis factor alpha (TNF- α) by enzyme-linked immunosorbent assays (ELISAs) (Becton Dickinson Immunocytometry Systems/PharMingen, San Jose, CA, USA). Whole blood was stimulated for intracellular cytokine production using phorbol 12-myristate 13-acetate (PMA)-ionomycin for 5 h at 37°C.¹⁶ Four-color cytometry was done using a FACSort or FACS Calibur flow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems/PharMingen).¹⁶ Fluorescein isothiocyanate (FITC)-conjugated, phycoerythrin (PE)-conjugated, peridinin chlorophyll protein

(PerCP)-conjugated or allophycocyanin (APC)-conjugated murine monoclonal antibodies were obtained from the following sources: (1) Becton Dickinson Immunocytometry Systems/PharMingen (CD8-FITC and CD8-PE (clone SK1), CD3-PerCP and CD3-APC (clone SK7), CD4-APC (clone SK3), CD45-FITC (clone 2D1), CD19-APC (clone SJ25C1), CD14-PE (clone MNP9), CD69 (clone L78), CD16-PE (clone B73.1), CD56 (clone MY31), IL-4-PE (clone 8D4-8), IL-8-PE (clone G265-8), IL-10-PE (clone JES3-9D7), and IL-12-FITC (clone C11.5)); (2) Research and Diagnostics, Minneapolis, MN, USA (IL-6-PE (clone 1927.311)); (3) Immune Source, Reno, NV, USA (CD8-APC (clone KL.12), IL-2-APC (R-56.2), IL-12-FITC (clone 1.A1), TNF- α -FITC (clone DTX.34), and IFN- γ -APC (clone 13.TR)); (4) Sigma (St Louis, MO, USA) microtubulin (MT) (clone DM1A) custom conjugated to FITC by CalTag, South San Francisco, CA, USA; and (5) Endogen, Woburn, MA, USA (T-cell antigen $\gamma\delta$ receptor chains-FITC (clone 5A6.E9)). Isotype controls were obtained from Becton Dickinson Immunocytometry Systems/PharMingen. All antibodies used had been pretested for stability to the permeabilization-fixation protocol.

Statistical analyses

Fisher's exact tests or chi-square analyses were performed for comparison of categorical variables, including age group (defined as <13 years old versus \geq 13 years old), gender, blood culture status, HIV status, HHV-8 status, and presence/absence of acute or chronic symptomatology (cough, fever, diarrhea, etc.). Of these categorical variables, only HIV and HHV-8 status were available for the healthy, working volunteers, none of whom were acutely or chronically ill by self-report. Continuous variables of seropositive and seronegative participants were compared with Wilcoxon signed-rank tests. Logistic regression analyses were done to determine which demographic or, for the healthy volunteers, which immunologic parameters were independently associated with HHV-8 seropositivity. Linear regression analyses were done to determine which continuous variables were associated with HHV-8 antibody titers. The significance level for all tests was set at $P=0.05$; however, in logistic regression analyses, levels of retained significance were much higher than this level, ruling against them being an artifact of multiple comparisons.

RESULTS

HHV-8 seroprevalence

The seroprevalence rate of patients for ORF65 was 54%, and that for K8.1 was 61%, resulting in an overall HHV-8 seroprevalence rate of 67% for hospitalized patients. The HHV-8 seroprevalence rate for healthy volunteers was 54%. Seroprevalence rates did not differ

significantly between patients and healthy volunteers (67% versus 54%) or between male and female patients (65% versus 70%). Seroprevalence rates did vary significantly by patients' age group (children, defined as <13 years old=28% versus adults, defined as ≥ 13 years old=75%) ($P < 0.001$) and by actual age in years (Figure 1). The patterns were similar for both antigens (Figure 1). The ages of the seven seropositive children were 0.1, 1.2, 2, 4, 9, 10 and 12 years. Four were male and two were HIV seropositive.

HHV-8 seroprevalence was not associated with the severity of HIV infection, as represented by the percentage of lymphocytes expressing CD4, or with the presence or absence of malaria parasitemia (Table 1). HHV-8 positivity was also not associated with a chronic cough, chronic vomiting, or diarrhea, or the number of people living in a household.

In univariate analyses, HHV-8 seropositivity was significantly associated with HIV seropositivity in the patients. However, in a logistic regression analysis including HIV status, age, and the percentage of lymphocytes expressing CD4 as independent variables, HHV-8 seropositivity was not significantly associated with HIV or the percentage of lymphocytes expressing CD4 when age was taken into account. HHV-8 seropositivity remained highly associated with age ($P < 0.001$, $\beta = 0.066$, 95% CI=0.045, 0.089).

HHV-8 antibody titers

The titers for both peptides increased among patients through adolescence into adulthood (Figure 2). Females had significantly lower antibody titers than did males for both peptides (medians 455 versus 1361 for K8.1, $P < 0.001$; and medians 268 versus 405 for ORF65, $P = 0.044$).

HHV-8 and KS

Ten patients had KS lesions on hospital admission physical examination (four with skin lesions, three with oral lesions, and three with both). Eight of 10 patients with KS were HHV-8 positive, and 65% of those without KS were HHV-8 negative (not significant).

Relationship between HHV-8 seropositivity and immune parameters

These analyses were done for only the healthy volunteers, to avoid confounding due to acute illness. Several immune parameters (see Methods) were examined in univariate analyses of data from the 24 healthy, non-hospitalized participants. Those that appeared to be related to HHV-8 seropositivity were: the percentage of CD3⁺, CD3⁺/CD8⁻ and CD3⁺/CD8⁺ lymphocytes pro-

Table 1. Characteristics of HHV-8 antibody-positive and -negative patients and healthy volunteers

	HHV-8 ⁺	HHV-8 ⁻	P-value
Patients			
% Male	52	57	NS
% HIV ⁺	62	40	0.011
% Malaria smear positive	13	12	NS
Age (median)	30	20	<0.001
% of lymphocytes expressing CD4			
HIV ⁻ participants	29	32	NS
HIV ⁺ participants	8	9	NS
Healthy volunteers			
% HIV ⁺	62	55	NS
% of lymphocytes expressing CD4			
HIV ⁻ participants	43	42	NS
HIV ⁺ participants	34	39	NS

NS, not significant.

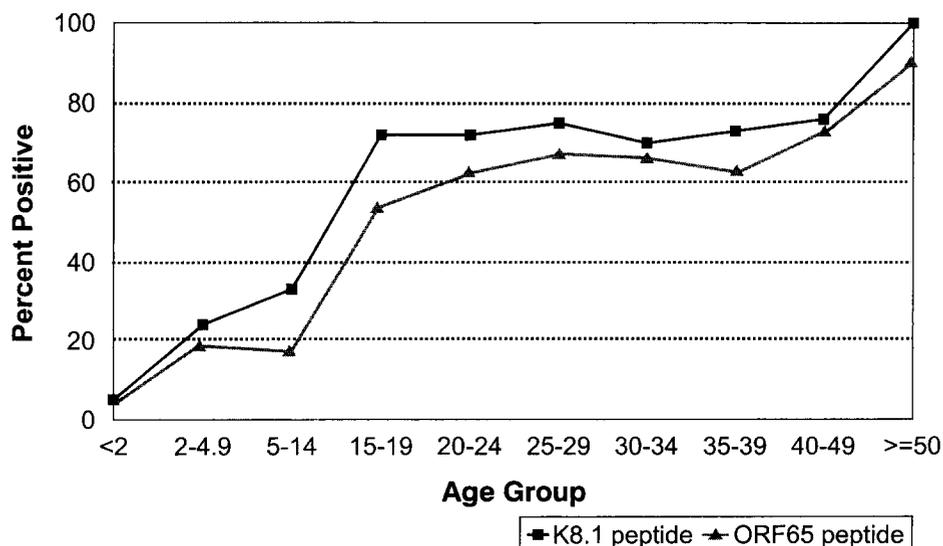


Figure 1. HHV-8 seroprevalence, by age group, among hospitalized patients in Malawi, Africa. Symbols represent median values for all participants within that age group. Numbers of patients in each age group by year were as follows: <2, n=20; 2-4.9, n=21; 5-14, n=12; 15-19, n=18; 20-24, n=47; 25-29, n=32; 30-34, n=43; 35-39, n=30; 40-49, n=37; >50, n=8.

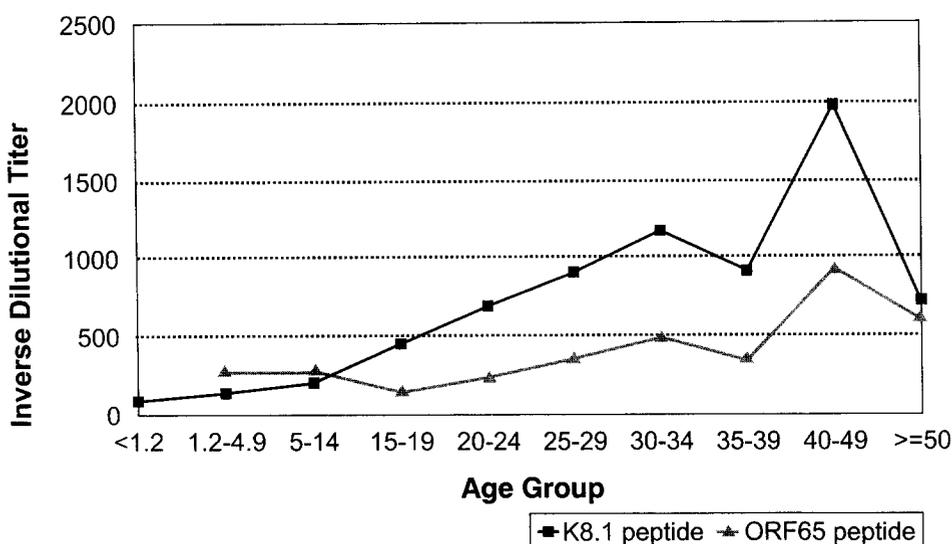


Figure 2. HHV-8 antibody titer, by age group, among hospitalized patients in Malawi, Africa. Symbols represent median values for all participants within that age group. Numbers of patients in each age group who were seropositive for K8.1 peptide and ORF65 peptide respectively were: <1.2, n = 1, 0; 1.2-4.9, n=5, 5; 5-14, n=4, 2; 15-19, n=13, 9; 20-24, n= 31, 29; 25-29, n=24, 21; 30-34, n=29, 28; 35-39, n=22, 20; 40-49, n=28, 27; >50, n=8, 7.

ducing TNF- α with stimulation, the percentage of monocytes spontaneously producing IL-8, and the percentage of CD3⁺/CD8⁺ lymphocytes producing IFN- γ with stimulation. However, in a series of logistic regression analyses, only the percentage of CD8⁺ lymphocytes producing IFN- γ with stimulation remained significantly—and strongly—related to K8.1-specific antibody positivity

($P=0.008$, $\beta = -0.107$, 95% CI= -0.206 , -0.041) (Figure 3). HIV status and the percentage of lymphocytes expressing CD4 were not significant in these analyses. A similar trend was found for anti-ORF65 peptide positivity, although this did not reach statistical significance.

DISCUSSION

HHV-8 infection occurs relatively infrequently in US, European, and Japanese pediatric and heterosexual populations.¹⁷⁻²² In Cameroon, HHV-8 DNA sequences were reported to be present in 62% of healthy adults (aged 30-40 years), 52% of adolescents (aged 15-20 years), and 40% of healthy children (aged 5-10 years).⁷ This suggests that HHV-8 is endemic to Cameroon, with many infections occurring in preadolescence. However, this age distribution is very different from that found in US and European studies. A low rate of HHV-8 seropositivity was found in HIV-infected US children (aged 0.5-18 years) enrolled in HIV clinical trials, supporting the suggestion that, in the USA, primary HHV-8 infection occurs in adulthood.²¹ Similarly, HHV-8 seropositivity was unusual in the children in our cohort. In our study, HHV-8 seropositivity rates increased most precipitously among patients between 15 and 19 years of age, indicating that primary infection in Malawi usually occurs during adolescence.

Non-sexual modes of viral transmission comprise intrauterine, perinatal, aerosol, fecal-oral, casual contact and vectors. We did not study specific modes of transmission; however, our results concerning HHV-8 age distribution and the lack of association of HHV-8 seropositivity with malaria, respiratory symptoms, gastrointestinal symptoms and the number of household members suggest that the above-mentioned possibilities are uncommon modes of HHV-8 transmission in Malawi.

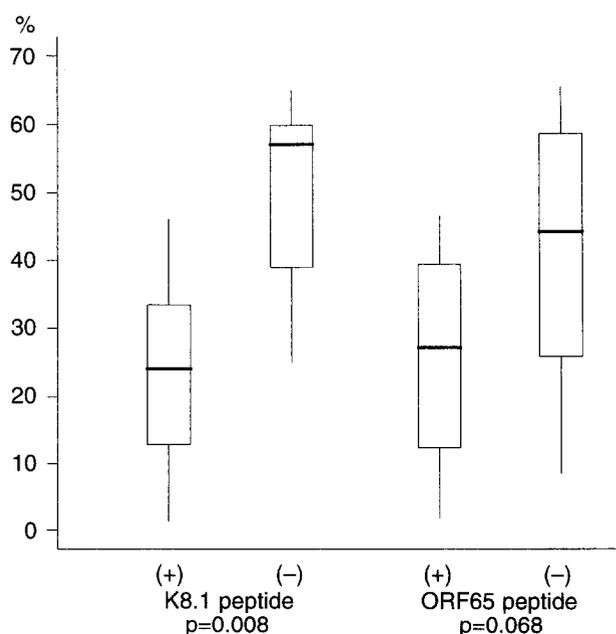


Figure 3. Percentage of CD8⁺ cells producing IFN- γ with stimulation, by HHV-8 status and peptide serology. Boxes include medians (lines) and values between the 25th and 75th percentiles for the following participant groups: anti-K8.1 antibody positive (n=13); anti-K8.1 antibody -negative (n=11); anti-ORF65 antibody positive (n=8); and anti-ORF65 antibody negative (n=16). Lines extend to the farthest value within ± 1.5 times the interquartile range from the 25th and 75th percentiles.

Of the 20 children less than 2 years of age in our study, two were HHV-8 seropositive (aged 0.1 and 2 years); this is consistent with either mother-child transmission, or placental transfer of maternal antibody. Reports of intrauterine or perinatal HHV-8 transmission are largely anecdotal.¹¹ In one cohort study, no infants of 17 HHV-8-seropositive mothers were infected with the virus.¹² In our study, seropositivity in childhood was less common than in studies done in Cameroon⁷ and Uganda;⁸ only 5 of 29 Malawian children 2–12 years of age were HHV-8 seropositive. Varying cultural practices, (e.g. sharing or non-sharing of eating utensils), as well as varying rates of viral endemicity in adults, may play a role in the different seroprevalence rates among these three African pediatric populations.

Both HIV and HHV-8 appear to be endemic in Malawi; however, contrary to findings for US homosexual cohorts, we found no independent association between HHV-8 and HIV seropositivity when we controlled for age. For healthy volunteers, all of whom were employed adults, HIV status did not differ significantly between the HHV-8-positive and HHV-8-seronegative participants. This result is consistent with findings in South Africa.⁹

Moore et al observed that HHV-8 antibody titers are significantly higher among HIV-positive/KS-positive patients than among HIV-negative/KS-negative US control patients.²³ High titers of HHV-8 antibodies among patients in South Africa have been associated with KS.⁹ In our study, 8 of 10 KS patients tested positive for lytic HHV-8 antibodies, compared to 65% of patients without KS. Although HHV-8 seroprevalence and antibody titers did not differ significantly between KS and non-KS patients in our cohort, the number of KS patients in our study was small, and thus the power of our analysis was low.

Two of our findings appear not to have been previously reported. First, we found that HHV-8 antibody titers were related to gender, with females having lower titers of antibodies to both antigens. To our knowledge, our study is the first to report gender-specific differences in HHV-8 antibody titers. Second, we found that the percentage of CD8 cells producing IFN- γ after stimulation with PMA and ionomycin was lower in healthy persons with HHV-8 infection, irrespective of their HIV status or proportion of lymphocytes expressing CD4. This immune finding suggests that HHV-8 infection may be associated with inhibition of type 1 cytokine production by cytotoxic T cells, irrespective of any effects of potential coexistent HIV infection. This would be consistent with the proposed importance of CD8 cells and IFN- γ in maintaining the chronicity of HHV-8 infection.²⁴

In summary, we found HHV-8 to be endemic in Malawi, with primary transmission usually occurring in adolescence. Antibody titers were lower in seropositive females. Seropositivity was unrelated to HIV, and its relationship with KS could not be confirmed, perhaps

because of the small number of KS patients. The immune effects of HHV-8 are consistent with chronic infection and suppressed type 1 cytokine production by CD8⁺ T cells.

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REFERENCES

1. Chang Y, Cesarman E, Pessin MS, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 1994; 266:1865–1869.
2. Huang YQ, Li JJ, Kaplan MH, et al. Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. *Lancet* 1995; 345:759–761.
3. Moore PS, Chang Y. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and those without HIV infection. *N Engl J Med* 1995; 332:1181–1185.
4. O'Brien TR, Kedes D, Ganem D, et al. Evidence for concurrent epidemics of human herpesvirus 8 and human immunodeficiency virus type 1 in US homosexual men: rates, risk factors, and relationship to Kaposi's sarcoma. *J Infect Dis* 1999; 180:1010–1017.
5. Chang Y, Ziegler J, Wabinga H, et al. Kaposi's sarcoma-associated herpesvirus and Kaposi's sarcoma in Africa. *Arch Intern Med* 1996; 156:202–204.
6. Kennedy MM, Lucas SB, Jones RR, et al. HHV-8 and Kaposi's sarcoma: a time cohort study. *J Clin Pathol Molec Pathol* 1997; 50:96–100.
7. Rezza G, Tchamgomena OB, Andreoni M, et al. Prevalence and risk factors for human herpesvirus 8 infection in northern Cameroon. *Sex Transm Dis* 2000; 27:159–164.
8. Mayama S, Cuevas LE, Sheldon J, et al. Prevalence and transmission of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in Ugandan children and adolescents. *Int J Cancer* 1998; 77:817–820.
9. Sitas F, Carrara H, Beral V, et al. Antibodies against human herpesvirus 8 in black South African patients with cancer. *N Engl J Med* 1999; 340:1863–1871.
10. Martin JN, Ganem DE, Osmond DH, Page-Shafer KA, Macrae D, Kedes DH. Sexual transmission and the natural history of human herpesvirus 8 infection. *N Engl J Med* 1998; 338:948–954.
11. Sitas F, Newton R, Boshoff C. Increasing probability of mother-to-child transmission of HHV-8 with increasing maternal antibody titer for HHV-8. *N Engl J Med* 1999; 340:1923–1924.
12. Lyall EG, Patton GS, Sheldon J, et al. Evidence for horizontal and not vertical transmission of human herpesvirus 8 in children born to human immunodeficiency virus-infected mothers. *Pediatr Infect Dis J* 1999; 18:795–799.
13. Jason J, Buchanan I, Archibald LK, et al. Natural T, $\gamma\delta$, and natural killer cells in mycobacterial, salmonella, and HIV infections. *J Infect Dis* 2000; 182:474–481.

14. Pau C-P, Lam L, Spira TJ, et al. Mapping and serodiagnostic application of a dominant epitope within the human herpesvirus 8 ORF 65-encoded protein. *J Clin Microbiol* 1998; 36:1574–1577.
15. Spira TJ, Lam L, Dollard SM, et al. Comparison of serological assays and PCR for the diagnosis of human herpesvirus 8 (HHV-8) infection. *J Clin Microbiol* 2000; 38:2174–2180.
16. Jason J, Archibald L, McDonald LC, et al. Immune determinants of organism and outcome in febrile hospitalized Thai patients with bloodstream infections. *Clin Diagn Lab Immunol* 1999; 6:73–78.
17. Lin S-F, Sun R, Heston L, et al. Identification, expression, and immunogenicity of Kaposi's sarcoma-associated herpesvirus-encoded small viral capsid antigen. *J Virol* 1997; 71:3069–3076.
18. André S, Schatz O, Bogner JR, et al. Detection of antibodies against viral capsid proteins of human herpesvirus 8 in AIDS-associated Kaposi's sarcoma. *J Mol Med* 1997; 75:145–152.
19. Regamey N, Cathomas G, Schwager M, Wernli M, Harr T, Erb P. High human herpesvirus 8 seroprevalence in the homosexual population in Switzerland. *J Clin Microbiol* 1998; 36:1784–1786.
20. Gao S-J, Kingsley L, Hoover DR, et al. Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. *N Engl J Med* 1996; 335:233–241.
21. Blauvelt A, Sei S, Cook PM, Schulz TF, Jeang KT. Human herpesvirus 8 infection occurs following adolescence in the United States. *J Infect Dis* 1997; 176:771–774.
22. Katano H, Iwasaki T, Baba N, et al. Identification of antigenic proteins encoded by human herpesvirus 8 and seroprevalence in the general population and among patients with and without Kaposi's sarcoma. *J Virol* 2000; 74:3478–3485.
23. Moore PS, Gao S-J, Dominguez G, et al. Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. *J Virol* 1996; 70:549–558.
24. Virgin HW, Speck SH. Unraveling immunity to γ -herpesviruses: a new model for understanding the role of immunity in chronic virus infection. *Curr Opin Immunol* 1999; 11: 371–379.