Four-Antigen Mixture Containing V-Cyclin for Serological Screening of Human Herpesvirus 8 Infection

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Kaposi sarcoma (KS) is an opportunistic disease in human immunodeficiency virus (HIV) patients and the most common cancer associated with AIDS worldwide (12). Identified a decade ago as the causative agent of KS, human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma-associated herpesvirus, has an approximately 165-kb genome encoding about 90 gene products (21). Many of these gene products allow the virus to evade the human immune system (8). KS primarily affects AIDS patients, but it can also occur in non-HIV-infected individuals and presents as classical, endemic, and posttransplant forms. HHV-8 is also associated with two other rare B-cell lymphoproliferative disorders, primary effusion lymphoma and multicentric Castleman disease, which are primarily found in HIV-infected or other immunosuppressed patients.

Currently, there is a need for sensitive and specific testing to identify HHV-8-infected individuals, especially among potential blood donors (14). Low viral loads in blood limit the sensitivity and thus the usefulness of PCR-based approaches (20). Alternatively, a variety of serological tests, including immunofluorescence assays (23), Western blotting (26), and enzyme-linked immunosorbent assays (ELISAs) (11, 15, 18), have been employed to detect antibodies to HHV-8 proteins and to diagnose infection. Considerable progress has been made in employing defined recombinant HHV-8 antigens, including LANA, K8.1, ORF65, and a LANA fragment (94% sensitivity and 100% specificity) using baculovirus-produced LANA and bacterially produced K8.1. Heat map analysis of KS patient antibody titers revealed marked heterogeneity in humoral responses to this four-antigen panel. Overall, the LIPS assay showed 97% sensitivity, and positive anti-v-cyclin antibodies were detected in approximately 75% of the KS sera. These results suggest that LIPS screening using an antigen mixture is a sensitive and high-throughput method for serological screening of HHV-8 infection in individuals with KS.
ples and protein A/G beads. Following washing, light production is measured, yielding highly quantitative antibody titers. In this study, we used LIPS to evaluate known antigens and potential HHV-8 ORFs for the serological diagnosis of KS. Following the evaluation of pilot and training-serum sets, a four-antigen panel (K8.1, v-cyclin, ORF65, and LANA fragment) was selected. This four-antigen panel, evaluated separately or as a mixture with a validation serum set, showed 100% sensitivity and 100% specificity. These results suggest that a LIPS antigen mixture is an efficient high-throughput method for serological screening of HHV-8 infection in individuals with KS.

MATERIALS AND METHODS

Patient sera. Sera were obtained from patients or volunteers under institutional review board-approved protocols at the Clinical Center, NIH, and at Georgetown University. In the initial pilot set, 6 colon cancer, 15 KS, and 1 HIV-positive sample were analyzed. Subsequent analysis of the single HIV-positive sample revealed that it was strongly positive by both ELISA and LIPS for anti-HHV-8 antibodies, and for simplicity, it was designated a KS sample, making “16” KS samples. The training (HIV-positive sample revealed that it was strongly positive by both ELISA and LIPS for anti-HHV-8 antibodies, and for simplicity, it was designated a KS sample, making “16” KS samples. The training (n = 83 blinded sera) and validation (n = 71) serum sample sets were provided as coded samples for both LIPS and ELISAs, and the code was broken only after titers were established and categorization of the HHV-8 infection status had been done. The training set consisted of 39 KS samples, while the validation set contained 34 KS samples. In the training and validation cohorts, the HHV-8-negative samples represented healthy, HIV-negative volunteers obtained from the NIH Blood Bank and were expected to be at low risk for HHV8 positivity. One volunteer blood donor sample in each of the training and validation sets was also found to be HHV-8 positive by both ELISA and LIPS, and these samples were included as KS- HHV8-positive in the figures.

HHV-8 ELISA. Anti-HHV-8 antibodies were determined using two different ELISAs employing baculovirus-produced LANA (ORF73) and bacterially produced K8.1 antigen as described previously (15).

Generation of Ruc-antigen fusion constructs. pREN2, a mammalian Ruc expression vector, was used to generate all plasmids (2). HHV-8 clones were amplified by PCR-specific linker-primer adapters using HHV-8 genomic DNA and a LANA cDNA template. Using gene-specific primers in PCR amplifications, the full-length coding sequences, as well as subfragments, were generated for fusions at the C terminus of Ruc. For each construct, including deletion mutants, a stop codon was included at the end of the coding sequence. The primer adapter sequences used to clone each protein or protein fragment are presented (see Fig. 4) are log10-transformed values and are coded using a color palette ranging from red to green, indicating high and low titers, respectively. For the antigen mixture tests, the assay was modified slightly. In these tests, the Ruc-antigen extracts were harvested in lysis buffer without glycerol and used immediately upon collection. Antigen mixtures were added to each well and processed as described above using similar inputs of LU for each antigen.

Data analysis. The GraphPad Prism software (San Diego, CA) was used for statistical analysis. Results for qualitative antibody titers between the controls and KS individuals are reported as the mean ± standard deviation (SD). Mann-Whitney U tests were used to compare the antibody titers among the groups. For the calculation of sensitivity and specificity, a cutoff limit of approximately 9,000 LU was used, which was derived from the combined value of the mean of the six control samples plus 5 SD for each antigen from the pilot set.

RESULTS

Patient antibody responses to known HHV-8 antigens. Evaluation of the utility of LIPS in HHV-8 diagnosis began by screening serum samples from 16 KS and 6 control patients for antibodies against known HHV-8 antigens, including LANA, ORF65, and K8.1. Because of the small pilot set, the discriminatory potential of each of these HHV-8 antibody tests was evaluated by a variety of statistical and diagnostic methodologies, including receiver operator characteristics (ROC), area under the curve, Mann-Whitney U tests, and using sensitivity and specificity calculations derived from the mean plus 5 SD of the control samples (Table 1).

In the case of LANA, we generated three different protein fragments spanning amino acids 6 to 286, 274 to 1089, and 863 to 1068, designated LANA-Δ1, LANA-Δ2, and LANA-Δ3, respectively. Using the N-terminal LANA-Δ1 fragment, only one of the 16 KS sera showed positive results by the cutoff compared to the controls (Table 1). In contrast, LANA-Δ2, representing the central region of LANA, detected positive antibody responses in 10 of 16 KS sera and none of the 6 controls (Table 1). The LANA-Δ3 fragment, representing the C terminus of LANA, performed slightly better than LANA-Δ2 and reacted with 13 of the 16 KS samples (Table 1). Several other larger LANA constructs did not increase the diagnostic sensitivity of the LIPS test over the values obtained with the LANA-Δ2 and LANA-Δ3 fragments (data not shown).

Analysis of full-length ORF65 by LIPS correctly identified

<table>
<thead>
<tr>
<th>Antigen</th>
<th>AUCa</th>
<th>P valueb</th>
<th>Sensitivityc</th>
<th>Specificityd</th>
</tr>
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<tbody>
<tr>
<td>K8.1-Δ4</td>
<td>0.96</td>
<td>0.0011</td>
<td>13/16</td>
<td>0/6</td>
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<tr>
<td>K8.1-Δ1</td>
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<td>0.0028</td>
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<td>0.0052</td>
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<td>0.89</td>
<td>0.0068</td>
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<td>0/6</td>
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<tr>
<td>ORF65</td>
<td>0.84</td>
<td>0.0105</td>
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<tr>
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<td>0.52</td>
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<td>1/16</td>
<td>0/6</td>
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</table>

a ROC as determined by the area under the curve (AUC).
b The P value was calculated using the Mann-Whitney U test.
c For LIPS, the cutoff limit for calculating the sensitivity and specificity for each antigen was derived from the value of the mean plus 5 SD of the six control samples.

TABLE 1. LIPS performances of top 10 HHV-8 antigens for KS diagnosis

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10 of 16 KS samples and showed no immunoreactivity in the controls (Table 1). Initial tests of K8.1 utilized two different K8.1 exon fragments. Both of these protein fragments, K8.1-H90041 (containing 123 amino acid residues) and K8.1-H90042 (containing 89 amino acids), identified the same 12 KS samples as positive (Table 1 and data not shown). Subsequent testing of K8.1-H90044, containing the complete coding sequence of the K8.1 protein, demonstrated diagnostic superiority in comparison to the two smaller K8.1 fragments and detected 13 of 16 KS samples (Table 1).

Identification of v-cyclin as a new informative HHV-8 antigen. To identify potentially new HHV-8 antigens, the pilot serum set was screened with a panel of 14 different HHV-8 fusion proteins, i.e., v-cyclin, vFLIP/ORF71/K13, v-BCL2/ORF16, vIL-6/K2, v-GPCR/Orf74, ORF48, ORF52, Kaposin/K12, ORF57, vIRF-1/ORF K9, vIRF-3/LANA2, ORF45, ORF47, and MCP-1. Most of these HHV-8 proteins showed weak or nonexistent antibody signals with the KS sera (Table 1 and data not shown). For example, ORF52 showed weak positive signals with only 3 of the 16 KS sera (Table 1), while ORF74 showed no signal with any of the sera tested (data not shown). However, the latent HHV-8 protein, v-cyclin, displayed positive results with 12 of 16 KS samples (Table 1). The geometric mean titer of the anti-v-cyclin antibody in the 16 KS samples was 14,281 LU (95% confidence interval [CI], 1,318 to 154,759 LU), 1,000-fold higher than the geometric mean titer of 15 LU (95% CI, 0.65 to 56) in the controls (Mann-Whitney U test, \( P < 0.0068 \)). The anti-v-cyclin antibody-positive KS patients did not simply reflect high anti-HHV8 antibody responding patients, as 2 of 16 anti-v-cyclin-positive samples were from KS sera that were negative for anti-LANA, anti-K8.1, and anti-ORF65 antibodies. These results suggest that anti-v-cyclin antibodies might represent a highly sensitive, independent marker of HHV-8 infection in KS patients.

Selection of an HHV-8 antigen panel. We focused our efforts on defining a diagnostic antigen panel and utilized the six most informative antigens: LANA-Δ2, LANA-Δ3, v-cyclin, K8.1-Δ1, K8.1-Δ2, and ORF65, with sensitivity values of 63%, 81%, 75%, 75%, 75%, and 63%, respectively. We derived a cutoff value of 9,000 LU from the approximate sum of the values of the means plus 5 SD. Using this parameter and the combined results of each singly tested antigen, LIPS correctly identified 15 of the 16 KS patient sera (94% sensitivity and 100% specificity). The panel missed only one KS patient sample, later identified as an individual with a low CD4 count of 40. In comparison, the HHV-8 ELISA, based on two separate LANA and K8.1 tests (25), detected 13 of the 16 KS samples as positive (81% sensitivity and 100% specificity), with 3 KS sam-
samples designated indeterminate. Interestingly, one of the ELISA-indeterminate sera was the same serum sample missed by LIPS.

**Initial testing of the training set with an HHV-8 panel.** Based on the results of the pilot set, an independent training set of 83 blinded sera was tested with the six-antigen panel identified above. We screened the six antigens separately and also simultaneously as a single antigen mixture. The sum of the six separate tests and the value of the six-antigen mixture were closely correlated (Pearson $R = 0.97; P < 0.00001$), and using the 9,000-LU cutoff, both formats identified the same 44 potential positives. Following unblinding, LIPS performance showed 95% sensitivity (38/40) and 88.4% specificity (38/43). In addition, all six separate tests showed robust antibody titers in many of the KS samples compared to the volunteers (Fig. 1). Compared to LIPS, the optimized ELISA showed slightly higher performance, with 97.5% (39/40) sensitivity and 88.4% (38/43) specificity.

**Improving the performance of the HHV-8 antigen panel.** During the course of our studies, a new K8.1 antigen, K8.1-$\Delta 4$, was found to have the highest discriminatory potential in comparison to other HHV-8 antigens (Table 1). In an effort to improve the performance of the HHV-8 LIPS panel, we replaced the K8.1-$\Delta 1$ and K8.1-$\Delta 2$ protein fragments with the full-length K8.1-$\Delta 4$. After unblinding the 83 sera from the training set, we reevaluated the sera by LIPS using the K8.1-$\Delta 4$ antigen alone and with the new five-antigen mixture (K8.1-$\Delta 4$, v-cyclin, LANA-$\Delta 2$, LANA-$\Delta 3$, and ORF65). Following unblinding, the anti-K8.1-$\Delta 4$ antibody test showed promising results, detecting all but 4 of the 40 KS samples (90% sensitivity) with 100% specificity (Fig. 2A). Furthermore, the new five-antigen panel showed improved diagnostic performance, demonstrating 95% (38/40) sensitivity and 93% (40/43) specificity (Fig. 2B). Despite the improved performance of the new five-antigen mixture, three false-positive sera were still found above the cutoff of 9,000 LU. Inspection of the separate results from these five antigen tests revealed that the LANA-$\Delta 3$ test was responsible for all the false samples above the 9,000 LU cutoff. Since the anti-LANA-$\Delta 3$ antibody-positive signals were also positive for the anti-LANA-$\Delta 2$ antibody test, we omitted the LANA-$\Delta 3$ result and recalculated the sum of the four antigen tests (LANA-$\Delta 2$, K8.1-$\Delta 4$, v-cyclin, and ORF65). This new four-antigen panel showed 95% sensitivity and 100% specificity for discriminating KS-positive from KS-negative samples (Fig. 2C). These results suggest that this four-antigen panel assayed separately or potentially as a mixture might be a simple and useful test for diagnosis.

**A four-HHV-8-antigen panel shows 100% sensitivity and 100% specificity.** To test the effectiveness of the new four-antigen panel, a new validation cohort of 71 blinded sera were evaluated with the four antigens individually and as a mixture. The four HHV-8 antigens assayed separately or as a mixture perfectly distinguished (100% sensitivity and 100% specificity) the 35 KS-positive samples from the 36 controls (Fig. 3). The sum of the four separate tests and the four-antigen mixture were also closely correlated (Pearson $R = 0.95; P < 0.00001$). LIPS also detected two more positive samples than the ELISA (94% sensitivity and 100% specificity), but ROC analysis of LIPS and ELISA showed no significant difference in diagnostic performance.

Further analysis of anti-K8.1, anti-v-cyclin, anti-LANA-$\Delta 2$, and anti-ORF65 antibody titers measured by LIPS in the assays described above revealed that the patient antibody responses to these different antigens varied markedly. For example, using the Spearman rank test, the correlation, $R$, between the anti-K8.1 and anti-v-cyclin antibodies was $-0.13 (95\%$ CI, $-0.4511$ to $0.2237)$, while the correlation of anti-cyclin and anti-LANA antibodies was 0.06 (95% CI, $-0.2861$ to $0.3964$). To easily visualize these differing patient antibody responses to the panel of four HHV-8 antigens, a heat map was employed (Fig. 4). In this graphical format, the marked differences in patient antibody responses to this panel become obvious. For example, several samples, including 513, 327, and 35, showed high level of antibodies to all four antigens, while several sam-
DISCUSSION

This study demonstrated that LIPS can robustly detect antibody titers to a panel of HHV-8 antigens with high diagnostic sensitivity and specificity. Our approach of using a four-antigen panel is consistent with ELISA studies showing the diagnostic usefulness of two or more different HHV-8 antigens (18, 22). Using either single-antigen tests or an HHV-8 antigen mixture, the LIPS assay consistently showed high sensitivity and specificity in identifying individuals with KS. The titer values obtained using both strategies closely matched each other, suggesting that in the LIPS format, diagnosis is highly reproducible regardless of assay conditions. The use of an antigen mixture by LIPS is highly convenient because it simplifies data collection and analysis. Furthermore, increasing the number of antigens within a mixture is generally a favorable condition if each member of the antigen has a comparable low background signal. In contrast, ELISAs perform poorly when more than one antigen is immobilized, and the alternative is to employ complicated multiepitope hybrid molecules. The positive results obtained here by LIPS with the HHV-8 antigen mixture are also consistent with our previous finding that a two-antigen mixture is a simple and sensitive method for diagnosis of *Strongyloides stercoralis* infection (24). Thus, this general approach of combining antigens in a mixture is likely to be useful for diagnosing many other infections.

Overall, the LIPS assay showed 97% (88/91) sensitivity in detecting the KS samples. The K8.1-Δ4 protein was the most...
useful antigen in the LIPS format, identifying 94% (86/91) of the KS-positive samples with 100% specificity. While not statistically significant, ROC comparisons of LIPS and ELISA in the detection of anti-K8.1 antibodies in the validation cohort revealed that LIPS performed slightly better than the ELISA (data not shown), which might be due to the detection of more conformational epitopes by LIPS. The anti-ORF65 antibody test did not increase sensitivity, and this antigen could have been omitted without affecting performance. Further modifications of the HHV-8 LIPS tests, such as the addition of new HHV-8 antigens/fragments, leaving out antigens (e.g., ORF65), and adjusting cutoffs, may further simplify testing and improve assay performance. Nevertheless, one limitation of our study is that mainly KS patients were used to assess the diagnostic performance of the LIPS assay. The use of only KS samples may cause performance to be overestimated because of the known higher antibody titers in KS compared to non-KS, HHV-8-infected samples. Future studies directed at testing other cohorts, including asymptomatic HHV-8-infected individuals, should further establish the utility of LIPS screening.

We report here for the first time that many KS patients have high levels of anti-v-cyclin antibodies. The relatively high titer and high prevalence (75%) of anti-v-cyclin antibody responses is consistent with mRNA expression showing that v-cyclin is highly expressed in KS tumors (10). v-Cyclin may be involved in HHV-8 malignancy because it is similar to the human cyclin D oncogene (17). It is worth noting that Katano et al. screened a number of glutathione S-transferase fusions of HHV-8 proteins for their serologic reactivities, including the v-cyclin (ORF72), but did not detect immunoreactivity to v-cyclin by Western blotting (16). The detection of diagnostically useful antibodies to v-cyclin by LIPS, but not with a bacterial recombinant protein, supports the notion that the LIPS antigens may have many more conformational epitopes, because these antigens were produced in mammalian cells. Particularly useful was the finding that some of the positive anti-v-cyclin antibody responses were detected in sera that were negative by ELISA and/or LIPS for anti-LANA, anti-K8.1 and anti-ORF65 antibodies. Nevertheless, 3% (3/91) of the total KS serum samples studied here by LIPS showed undetectable antibodies to v-cyclin, K8.1, LANA, and ORF65, which is likely consistent with the weak or undetectable anti-HHV-8 antibody responses that are associated with low CD4 counts in HIV-infected patients (9).

Although a few of the HHV-8 antigens screened in the initial small pilot set did not show immunoreactivity, it is possible that some of these proteins may show informative humoral targets when larger numbers of HHV-8-infected sera are studied. Along these lines, LIPS detection of patient-specific antibody responses to a large panel of HHV-8 antigens might have additional utility for understanding immune responsiveness, the severity and duration of infection, and response to drug therapy. It will also be of interest to determine whether the antibody profiles against v-cyclin and other HHV-8 proteins described here are different in other cohorts, including in asymptomatic HHV-8-infection, KS, and HHV-8 B-cell lymphoproliferative disorders, such as primary effusion lymphoma and multicentric Castleman disease.

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