CONCISE COMMUNICATION

Evaluation of the Latency-Associated Nuclear Antigen (ORF73) of Kaposi's Sarcoma–Associated Herpesvirus by Peptide Mapping and Bacterially Expressed Recombinant Western Blot Assay

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Kaposi's sarcoma (KS)–associated herpesvirus open-reading frame (ORF) 73 encodes a latency-associated nuclear antigen (LANA) that is the basis for several serologic assays. Immunoreactive epitopes were searched for by peptide mapping, and 171 cleavable, biotinylated 17-mer peptides offset by 5 residues were synthesized and screened with human serum samples by ELISA. The initial screen, which used highly reactive serum diluted 1:500, identified 38 immunoreactive peptides. These were subsequently tested on additional serum samples diluted 1:40. Thirteen peptides were more reactive with serum samples from patients with KS than with control serum samples. No single epitope was recognized by most KS patient serum samples. Combined use of these peptides did not increase test sensitivity to that of current indirect immunofluorescence assays for LANA (80%–90%). For comparison, full-length ORF73 was expressed in bacteria and analyzed by Western blot. The overall sensitivity was 67% (range, 100% among US patients with classic KS to 52% among Italian patients with classic KS). These studies suggest that LANA immunoreactivity may be due to variations in patient response or conformational epitopes.

Kaposi's sarcoma (KS)–associated herpesvirus (KSHV) is a γ herpesvirus [1] that is etiologically related to KS [2]. Although KSHV DNA is found by polymerase chain reaction (PCR) in >95% of all types of KS, current serologic assays used to detect antibodies to KSHV are only 80%–90% sensitive [3–8]. Two of the first serologic assays, an indirect immunofluorescence assay (IFA) and a Western blot, detect antibodies to a latent nuclear antigen [3, 4, 8]. When reacted against serum from patients with KS, the IFA produces a specific speckled, nuclear staining pattern in cell lines latently infected with KSHV, whereas the Western blot produces a doublet of high molecular mass (range, 204–234 kDa) in the nuclear component of similar latently infected cells. The antigen identified by Western blotting, also called the latency-associated nuclear antigen (LANA, LNA, LNA-1), is encoded by open-reading frame (ORF) 73 [9, 10].

LANA is located on the right end of the genome and contains an acidic repeat domain [11]. The protein migrates aberrantly on a denaturing gel (204–234 kDa vs. predicted 135 kDa), which, in part, may be due to the highly negative charge of the repeat region [8]. LANA also may be posttranslationally modified through covalent interaction with the ubiquitin-like molecule SUMO-1 (small ubiquitin-related modifier; also termed sentrin), as well as through phosphorylation (J. Wiezorek, R. Sarid, P. S. Moore, and Y. Chang, unpublished data).

For the detection of most viral infections, serologic assay is a more powerful tool than PCR since it can detect both past and current infections. Recombinant technology can be used to overcome the inherent difficulties of whole-cell assays [5, 12]. Both full-length and truncated (carboxyl terminus) recombinant ORF73 are reactive against KS serum samples in Western blot assays [9]. The truncated protein was evaluated for use as an antigen and was only 66% sensitive on endemic (human immunodeficiency virus [HIV]–negative) KS serum samples. In an attempt to design a new assay with higher sensitivity that could be used to efficiently screen patients for KSHV infection, we epitope mapped ORF73 to identify the immunogenic regions. We also evaluated the use of full-length recombinant ORF73 in a Western blot assay.

Materials and Methods

Peptide mapping of ORF73. In another study, the start and stop nucleotides for KSHV were identified at positions 127,296 and 123,808, respectively, of the BC-1 sequence [11]. The amino acid (aa) sequence was generated using MacVector software (version 4.5.2; MacVector, New Haven, CT). In total, 171 cleavable, biotinylated (N-terminus SGSK) 17-mer peptides offset by 5 were

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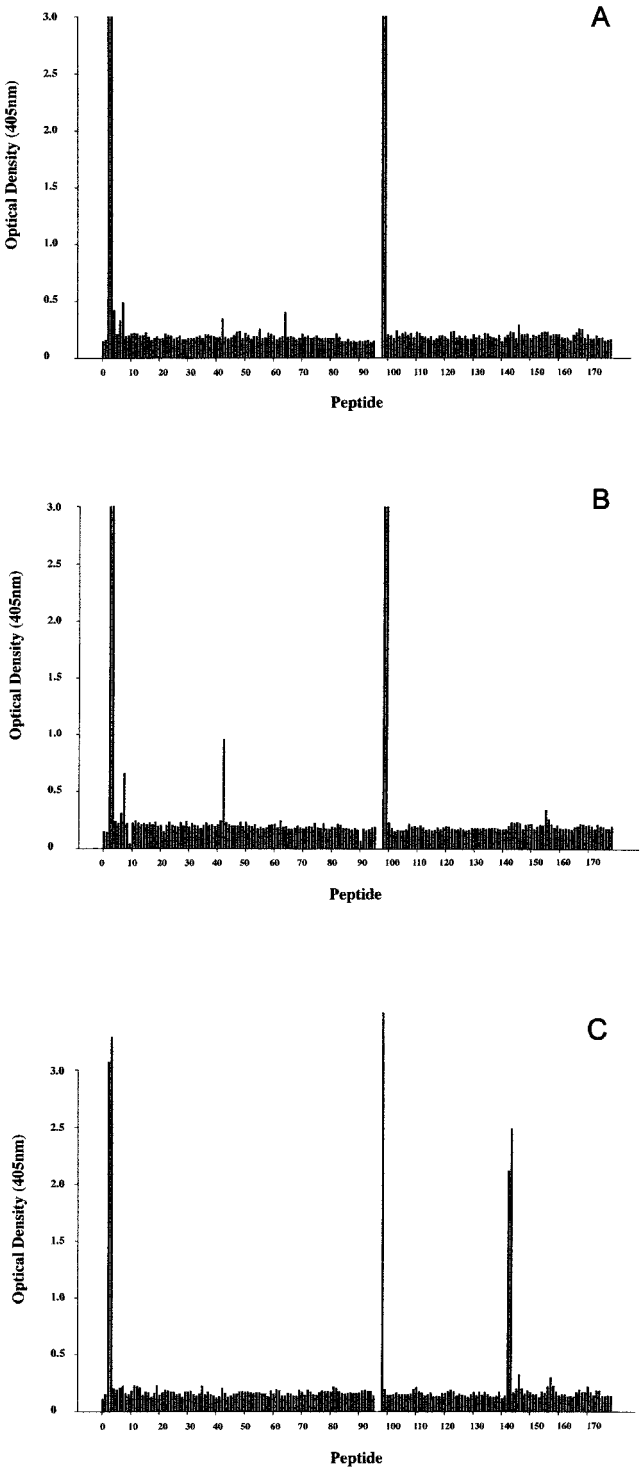

Figure 1. Examples of peptide screen *(A–C).* Initial peptide screen against serum samples diluted 1:500 from blood donor *(A),* patients with AIDS and Kaposi's sarcoma (KS) *(B),* and patient with classic KS *(C).* Peptides 3, 4, 99, and 100 are internal human immunodeficiency virus (HIV) control peptides reacted against HIV-positive serum samples, except for P100 in *(C),* which is reacted against HIV-negative serum samples.

synthesized by Chiron Technologies (Clayton, Australia). Beginning at the amino terminus, these peptides were sequentially numbered P5 to P96 and P101 to P179. Identical peptides from the repeat region were eliminated. Two biotinylated (N-terminus SGSK and SGSG) 10-mer peptides from the HIV gp41 protein (IWGCSGKLIC) were synthesized (P3, P4, P99, and P100) and used as internal controls [13]. An ELISA was performed according to the manufacturer's protocol by using a panel of human serum. To identify highly reactive peptide, the initial screen used serum samples diluted 1:500. Because the objective was to develop a sensitive screening assay, peptides that did not react to serum samples from patients with KS at 1:500 were deemed unlikely to react against serum samples from KSHV-infected, KS-negative persons and were, therefore, not studied further. Peptides that produced a significant peak when compared with the lowest 25% were then screened at a 1:40 serum dilution. All peptide screening at 1:40 used a ratio cutoff to determine positivity. Serum samples from US blood donors previously shown to be KSHV seronegative by LANA immunofluorescence and ORF65 Western blot [5] were used as negative controls. The optical density for each test sample was divided by the average of the optical density of 2 blood donors for that peptide. If this ratio was ≥ 2, the sample was scored as positive.

Expression of ORF73. pTrcHis plasmid (Invitrogen, San Diego), described elsewhere [9], that contained an ORF73 insert (except for the first 4 aa) was used for bacterial expression of LANA. Recombinant LANA expression in TOP10 cells (Invitrogen) was induced with 1 m*M* isopropyl-β-D-thiogalactopyranoside, and protein was purified by using nickel resin (ProBond; Invitrogen) under denaturing conditions in accordance with the manufacturer's instructions.

Western blot analysis. Western blot analysis was done as described elsewhere [8] except by using recombinant LANA instead of nuclear cell lysates. In brief, recombinant LANA (~100 ng of protein per lane [12]) was electrophoresed on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was saturated with Blotto (Pierce, Rockford, IL), cut into strips, and incubated overnight with patient serum diluted 1:100. The strips were washed and then were incubated with anti–human polyvalent immunoglobulins conjugated to alkaline phosphatase (Sigma, St. Louis) diluted 1:2500. After a repeat wash, the strips were developed with 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium chloride (Gibco BRL, Grand Island, NY). Serum reactive to both p226 and p234 was scored as positive [8]. The blots were scored by 2 independent readers, 1 of whom was blind to the code. All negative results were repeated twice for confirmation; 1 blood donor serum sample with discordant results after 2 blots was repeated a total of 4 times and was judged to be indeterminate.

Human serum samples. Peptide screening of LANA was done on a panel of human serum samples. The initial peptide screening was performed on human serum samples from 6 persons with AIDS and KS (AIDS-KS), 2 US patients with classic KS, 1 HIV-positive, KS-negative patient, and 3 blood donors. The subsequent screen of 38 peptides used serum samples from 9 US patients with AIDS-KS and 4 US blood donors. Western blot analysis was performed for 58 KS patients (12 African patients, 11 patients with AIDS-KS, 25 Italian patients with classic KS, 3 US patients with classic KS, and 7 posttransplantation patients with KS) and 61 controls

Table 1. Immunoreactive peptides from open-reading frame 73 latency-associated nuclear antigen of Kaposi's sarcoma (KS)–associated herpesvirus and their reactivity to serum from 9 US patients with AIDS-KS.

Peptide[a]	Position	Amino acid sequence	Pt 1	Pt 2	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9
P8	16–32	APLTRGSCRKRNRSPER	−	−	−	−	−	−	+	+	−
P10	26–42	RNRSPERCDLGDDLHLQ	−	−	−	−	−	−	−	−	+
P43	191–207	LAPSTLRSLRKRRLSSP	−	−	+	−	−	+	+	−	−
P108	751–767	DEQEQQEEQEQQEEQEQ	+	−	−	−	−	−	−	−	−
P109	756–772	QEEQEQQEEQEQELEEQ	+	−	−	−	−	−	−	−	−
P121	851–867	VEEQEQEQEEQELEEVE	+	−	−	−	−	−	−	−	−
P122	856–872	QEQEEQELEEVEEQEQE	+	−	−	−	−	−	−	−	−
P124	866–882	VEEQEQEQEEQEEQELE	+	−	−	−	−	−	−	−	−
P143	971–987	PDDDPQPGPSREYRYVL	+	−	−	−	−	−	−	−	−
P144	976–992	QPGPSREYRYVLRTSPP	+	−	−	−	+	−	+	−	+
P146	986–1002	VLRTSPPHRPGVRMRRV	−	−	−	−	−	−	+	+	−
P147	991–1007	PPHRPGVRMRRVPVTHP	−	−	−	−	−	−	−	+	−
P156	1035–1051	HIFYRRFLGKDGRRDPK	−	−	−	−	−	−	+	+	+

NOTE. Pt, patient; −, negative; +, positive.
[a] Amino terminus begins with P5.

without KS (10 Africans, 39 US blood donors, 3 patients with high Epstein-Barr virus [EBV] viral capsid antigen titers, 2 Italians, and 7 posttransplantation patients).

Statistical analysis. Computation of mean, SD, and significant peaks for each ELISA plate was calculated by using software provided by Chiron Technologies (Read and Plot Software).

Results

The primary analysis of all 171 peptides studied using serum diluted 1:500 identified 38 potential immunoreactive peptides. An example of the initial peptide screen by ELISA is shown in figure 1. Subsequent analysis of these 38 peptides on serum diluted 1:40 identified 13 candidate peptides (table 1). These 13 peptides are distributed equally over the entire protein, including 3 peptides from the amino terminus, 5 from the repeat region, and 5 from the carboxyl terminus. When screened against serum from 9 US patients with AIDS-KS, no specific linear epitope was identified (table 1). The sensitivity of the individual peptides was 11%–44%; 7 (78%) of 9 serum samples were positive on $\geq$1 peptide.

The characteristic high-molecular-mass doublet of LANA was strongly visible in both whole-cell bacterial lysates and nickel-purified protein when the membrane was reacted against serum from a patient with KS, whereas the doublet was absent when reacted against control serum (figure 2). Overall, 39 (67%) of 58 patients with KS were seropositive by this assay, compared with 50 (86%) of 39 by the LANA IFA. However, the sensitivity varied greatly with the recombinant Western blot. Among persons with KS, the sensitivities for the recombinant Western blot and IFAs were as follows: 83% versus 83% among African patients with KS, 82% versus 100% among US patients with AIDS-KS, 52% versus 84% among Italian patients with classic KS, 100% versus 100% among US patients with classic KS, and 57% versus 71% among posttransplantation patients with KS. Of interest, all Italian KS serum samples examined by recombinant Western blot assay had low sensitivity, compared with other KS serum samples, which suggests possible differences in sample preparation or local strain variation. Of serum samples tested from 39 US blood donors, 1 was indeterminate by recombinant Western blot assay (2 positive and 2 negative readings); the other 38 samples were KSHV seronegative. All 39 were seronegative by IFA. Three patients with high EBV titers also were seronegative on both assays, as were 2 Italians without KS.

Discussion

Several serologic assays, including an IFA and a Western blot assay, detect antibodies to LANA. We peptide mapped ORF73 in an attempt to identify immunoreactive epitopes. Our analysis revealed 13 potential peptides for use in an ELISA. Individually these peptides were insensitive (11%–44%); combined use of these peptides increased the sensitivity among patients with AIDS-KS to only 78%. We were unable to identify a combination of peptides for use in an ELISA that performed better than current methods. Peptide mapping identifies linear epitopes. Therefore, our results suggest that LANA immunoreactivity may be due to conformational or posttranslational epitopes. Although our inability to find a linear epitope by peptide mapping may be due to the loss of the epitope, we cannot rule out a negative result due to peptide instability, despite the strong response of the HIV peptide controls (figure 1).

In addition to peptide mapping of ORF73, we evaluated the use of full-length recombinant ORF73 protein in a Western blot assay. The overall sensitivity of this assay (67%) was also lower than preexisting assays, but there was a substantial difference among the KS populations studied. The sensitivity of the recombinant assay both in Italian patients with classic KS and posttransplantation patients with KS (52% and 57%, respectively) was much lower than the sensitivity of the LANA IFA using the same serum samples [5]. The sensitivity of the recombinant assay in other populations with KS was 82%–100%, which is consistent with the sensitivity estimates of current serologic assays. We speculate that there may be a vari-

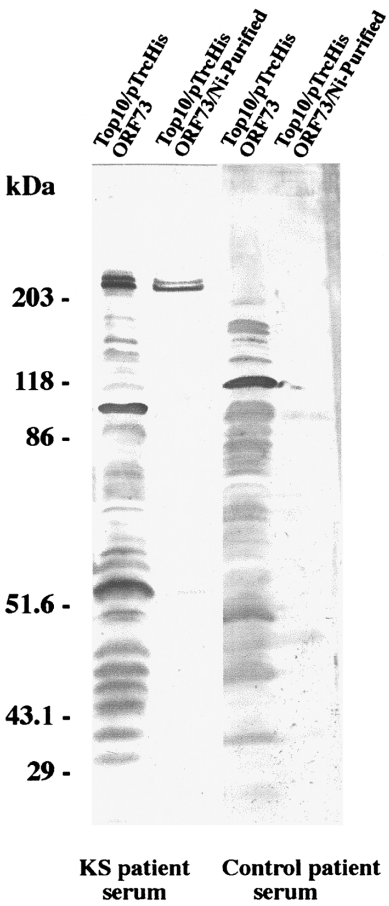

Figure 2. *Escherichia coli* TOP 10 cells transformed with pTrcHis plasmid that contained an open-reading frame 73 insert. Whole-cell lysates *(lanes 1* and *3)* and nickel-purified protein *(lanes 2* and *4)* were immunoblotted against serum samples from patient with Kaposi's sarcoma and from a control patient.

ation in the response of KSHV-infected subjects to different epitopes, which may be dependent on individual sensitivities to different viral antigens. However, the failure to find a unique immunoreactive peptide in our study suggests that the immunodominant LANA epitope may be conformational (even on Western blot assays, because of the highly charged protein) or related to interactions with cellular proteins. Finally, different KSHV strains have variable numbers of internal acidic repeats (MOI) that make up the acidic repeat region of LANA [11]. At least 4 distinct phylogenetic clades of KSHV have been characterized [14]; however, to date there is no evidence for differing seroreactivity among persons infected with different KSHV strains.

The specificity of the recombinant ORF73 Western blot assay in US blood donors was very similar to that of other assays used in our laboratory and by others [3, 5, 8]. Only 1 of the 39 US blood donors was potentially seropositive by this assay; however, repeat testing was unable to confirm the positive result. Although the true KSHV seroprevalence in other populations without KS is unknown, the recombinant ORF73 assay may be more specific than the IFA. There is a greater likelihood for cross-reactivity in a whole-cell IFA than in a recombinant assay. In this study, fewer African and posttransplantation controls were KSHV seropositive by the recombinant ORF73 assay than by the LANA IFA, which suggests that the recombinant protein may be more specific.

The results of our evaluation indicate that neither the peptide ELISA nor the bacteria-expressed full-length recombinant ORF73 Western blot is useful as a screening assay to detect KSHV infection in patient serum samples. In contrast, Zhu et al. [12] generated a LANA Western blot assay with both high sensitivity and high specificity by using a baculovirus-expressed preparation. These differences may be due to differences in antigen concentration and also by use of a baculovirus expression construct that may allow posttranslational modifications not found in bacterial expression systems. These assays may have utility as confirmatory assays, because of their high sensitivity.

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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: 1865–69.
2. Olsen SJ, Moore PS. Kaposi's sarcoma–associated herpesvirus (KSHV/HHV8) and the etiology of KS. In: Freidman H, Medveczky P, Bendinelli M, eds. Herpesviruses and immunity. New York: Plenum, **1999**:115–47.
3. Kedes DH, Operskalski E, Busch M, Kohn R, Flood J, Ganem D. The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma–associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nat Med **1996**; 2:918–24.
4. Moore PS, Gao SJ, Dominguez G, et al. Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. J Virol **1996**; 70:549–58.
5. Simpson GR, Schulz TF, Whitby D, et al. Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen. Lancet **1996**; 348: 1133–8.
6. Chandran B, Bloomer C, Chan SR, Zhu L, Goldstein E, Horvat R. Human herpesvirus-8 ORF K8.1 gene encodes immunogenic glycoproteins generated by spliced transcripts. Virology **1998**; 249:140–9.
7. Raab MS, Albrecht JC, Birkmann A, et al. The immunogenic glycoprotein gp35-37 of human herpesvirus 8 is encoded by open reading frame K8.1. J Virol **1998**; 72:6725–31.
8. Gao SJ, Kingsley L, Hoover DR, et al. Seroconversion of antibodies to Kaposi's sarcoma–associated herpesvirus-related latent nuclear antigens prior to onset of Kaposi's sarcoma. N Engl J Med **1996**; 335:233–41.
9. Rainbow L, Platt GM, Simpson GR, et al. The 226- to 234-kilodalton latent nuclear protein (LNA) of Kaposi's sarcoma–associated herpesvirus (KSHV/HHV8) is encoded by ORF73 and is a component of the latency-associated nuclear antigen. J Virol **1997**; 71:5915–21.
10. Kedes D, Lagunoff M, Renne R, Ganem D. Identification of the gene encoding the major latency-associated nuclear antigen of the Kaposi's sarcoma–associated herpesvirus. J Clin Invest **1997**; 100:2606–10.

11. Russo JJ, Bohenzky RA, Chien MC, et al. Nucleotide sequence of the Kaposi sarcoma–associated herpesvirus (HHV8). Proc Natl Acad Sci USA **1996**;93:14862–7.
12. Zhu L, Wang R, Sweat A, Goldstein E, Horvat R, Chandran B. Comparison of human sera reactivities in immunoblots with recombinant human herpesvirus (HHV)–8 proteins associated with the latent (ORF73) and lytic (ORFs 65, K8.1A, and K8.1B) replicative cycles and in immunofluorescence assays with HHV-8–infected BCBL-1 cells. Virology **1999**;256: 381–92.
13. Gnann JJ, Schwimmbeck PL, Nelson JA, Truax AB, Oldstone MB. Diagnosis of AIDS by using a 12–amino acid peptide representing an immunodominant epitope of the human immunodeficiency virus. J Infect Dis **1987**; 156:261–7.
14. Zong JC, Ciufo DM, Alcendor DJ, et al. High-level variability in the ORF-K1 membrane protein gene at the left end of the Kaposi's sarcoma–associated herpesvirus genome defines four major virus subtypes and multiple variants or clades in different human populations. J Virol **1999**;73: 4156–70.