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Human herpesvirus 8 (HHV-8; also designated Kaposi's sarcoma-associated herpesvirus) is the likely etiological agent of Kaposi’s sarcoma (KS). HHV-8 encodes a latent nuclear antigen (LNA) which is the product of the viral gene orf73. LNA is recognized by most infected patient sera and is the basis of current immunofluorescence assays used in epidemiological studies of HHV-8 infection. Here we describe the characterization of four monoclonal antibodies raised to the C-terminal third of LNA-glutathione S-transferase fusion proteins. These monoclonal antibodies recognized discrete linear epitopes within the C terminus and repetitive region of LNA, detected antigen in primary effusion lymphoma (PEL) cells, and precipitated a 220- to 230-kDa protein doublet corresponding to LNA from HHV-8-infected PEL cell lines. In situ immunocytochemistry of KS lesions with these antibodies show that LNA is extensively expressed in KS spindle cells.

Human herpesvirus 8 (HHV-8; also designated Kaposi’s sarcoma-associated herpesvirus) is the first known human member of the genus Rhadivirus within the Herpesviridae (9). HHV-8 is related to the New World primate rhadinovirus herpesvirus saimiri (1), a rhesus macaque rhadinovirus (10), and macaque retroperitoneal fibromatosis virus isolates Mn and Mn (25). More recently, equine herpesvirus 2, bovine herpesvirus 4, alcelaphine herpesvirus 1, and murine herpesvirus 68 have been tentatively classified as rhadinoviruses. HHV-8 is associated with all epidemiological forms of Kaposi’s sarcoma (KS) (4, 6, 28). HHV-8 is also associated with primary effusion lymphoma (PEL) (7) and a subset of multicentric Castleman’s disease (12, 30). Epidemiological studies using PCR detection of specific HHV-8 genomic sequences, and immunological assays for antibodies to the major latent nuclear antigen (LNA) and other antigens, have shown that HHV-8 largely fulfills epidemiological criteria for causation in KS (5, 13, 14, 16, 19, 20, 22, 29, 32).

Sera from HHV-8-infected individuals react with a specific latent nuclear antigen in latently infected PEL cell lines which is characterized by a punctate nuclear immunofluorescence pattern (19). Screening of cDNA libraries with an HHV-8-positive patient serum identified this nuclear antigen as the product of the viral gene open reading frame 73 (orf73), and the encoded protein was designated LNA or LANA (17, 18, 24). LNA is recognized by most infected patient sera and is the basis of current immunological assays for antibodies to the major latent nuclear antigen of HHV-8, and detect LNA expression in PEL cell lines and in KS nodules.

Generation of MAbs against LNA. Glutathione S-transferase (GST) proteins of the C terminus of orf73 (GST-C14 and GST-C17) were described previously (18). Briefly, GST-C14 contains amino acids 803 to 1113 of orf73 encompassing 126 amino acids of the central repeat domain as well as 184 amino acids of the C-terminal basic domain (Fig. 1). GST-C17 contains amino acids 803 to 942 comprised almost entirely of repetitive coding sequence. GST-C14 served as the backbone for making further defined deletions of the C terminus of orf73 by using three unique restriction enzyme sites. GST-C7 was constructed by digesting GST-C14 with NruI (in orf73) and NotI (in pGEX-4T3 [Pharmacia], followed by end repair and ligation. GST-C11 was constructed by digesting GST-C14 with NruI and NotI and cloning the restriction fragment into the SmaI and NotI sites of the vector pGEX-4T1 (Pharmacia). GST-C11 served as the backbone for two further deletion

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clones. GST-C9 and GST-C10 were constructed by restriction enzyme digestion with StuI plus NotI and AgeI plus NotI, respectively, followed by end repair and ligation. Recombinant protein-expressing clones were confirmed by DNA sequence analysis. In addition, two polyhistidine fusion proteins were constructed. The entire BamHI-to-NotI C-terminal restriction fragment from GST-C14 was cloned into the polyhistidine fusion protein vector pTrcHis2C (Invitrogen) via BamHI and HindIII following conversion of the NotI and HindIII sites to blunt ends to produce clone TH-C14. The NruI-to-NotI fragment corresponding to GST-C11 was PCR amplified and cloned to produce TH-NN. Large-scale production and single-step affinity purification of fusion proteins (GST [Pharmacia] and polyhistidine [Invitrogen]) were performed according to the manufacturer’s instructions. All fusion proteins were recognized by antibodies to GST (B14; Santa Cruz) or to the Myc epitope present in the polyhistidine fusion proteins (data not shown). Pools of affinity-purified GST fusion proteins were used for intraperitoneal immunization of rats at 3-week intervals. Three days following the final boost, rats were sacrificed to obtain splenocytes for hybridoma production by standard procedures. Hybridomas were screened by enzyme-linked immunosorbent assay (ELISA) and Western blotted against the polyhistidine fusion protein TH-C14, and positive hybridomas were subcloned. Following a second ELISA screening, reactive MAbs were bulk cultured and antibodies were purified from culture supernatants by antibody affinity chromatography. This procedure produced MAbs ranging in concentration from 1 to 2 mg/ml. The isotype of each MAb was determined by standard methods. Four MAbs, LN20, LN53, LN69, and LN72, with isotypes immunoglobulin M (IgM), IgG2c, IgG1 and IgG2b, respectively, were raised to these GST fusion proteins.

Mapping of the epitopes recognized by the MAbs. The antigenic epitopes recognized by these MAbs were mapped by using the panel of GST and polyhistidine fusion proteins (Fig. 1) in an ELISA format. Briefly, fusion proteins were immobilized onto 96-well plates (Immulon 2; Dynex Technologies) in 10 mM sodium phosphate buffer (pH 7.0) by overnight incubation at 4°C. A standard ELISA protocol was followed, using appropriate dilutions of anti-LNA MAbs and 1:2,000 dilution of goat anti-rat alkaline phosphatase-conjugated secondary antibody (Sera-Lab) as described elsewhere (15). Reactions were visualized by using p-nitrophenyl phosphate (Sigma Fast) according to the manufacturer’s instructions. Antibody LN20 recognized all recombinant proteins to various degrees, as shown by reactivity to clones of the repetitive and nonrepetitive C-terminal regions (Fig. 1). The recombinant proteins represent a nonoverlapping set and contain no apparent common epitopes, which suggests that LN20 is a mixed antibody population, requiring further rounds of monocloning. Antibody LN53 recognized recombinant proteins GST-C14, GST-C17, GST-C7, and TH-C14 (Fig. 1), demonstrating that the
The minimum epitope recognized by this antibody is the repetitive region of orf73 encompassing GCT-C17 or the 13 amino acids C terminal to the repetitive region. Antibody LN69 recognized only GST-C9, GST-C10, TH-C14, and TH-NN. This mapped the LN69 minimal epitope to the 37 amino acids (amino acids 981 to 1018 of LNA) present in GST-C10 (Fig. 1). It is unclear why LN69 did not recognize GST-C14 or GST-C11, as both contained the minimal epitope. Antibody LN72 recognized GST-C14 and -C7 but not GST-C17 and thus recognizes a 39-amino-acid region (from amino acids 942 to 981) of LNA.

To confirm and further refine the minimal epitopes for each MAb, multiple overlapping peptides corresponding to the regions of LNA identified by ELISA were synthesized by using the SPOTS system (Genosys). Peptide mapping revealed that LN69 recognized the epitope THPPKPHPDAYQQ, LN72 recognized the epitope EVDPYPV, and LN53 recognized the epitope EQEQE. These epitopes are contained within the minimal LNA recombinant proteins identified by ELISA, thus confirming the binding sites of the three MAbs. The LN53 epitope is contained within the repetitive region of LNA, resulting in 23 copies of the LN53 epitope in full-length LNA. Database searching revealed that this epitope is present in human proteins, including the alpha-type calcitonin gene-related peptide precursor (P06881), drebrin (Q16643), translation initiation factor IF-2 (P46199), and aldehyde dehydrogenase (P30838), but only in single copy. The LN69 and LN72 epitopes have only partial homologies to known cellular proteins.

Immunoprecipitation and immunofluorescence assay (IFA) of HHV-8 LNA. Previous studies have shown that the authentic LNA protein encoded in PEL cell lines is a doublet with an apparent molecular mass of 220 to 230 kDa (13, 18, 24), in contrast to the predicted molecular mass of 135 kDa. The reasons for the size difference are unclear. MAbs LN53, LN69, and LN72 produced in this study were used to immunoprecipitate LNA from the PEL cell line BC-3 (2). Briefly, cells were starved for 1 h in methionine- and cysteine-free RPMI (Sigma), washed, and then labeled for 4 h in fresh medium.
supplemented with a mixture of [35S]methionine and [35S]cysteine (70 μCi/ml in medium; Amersham Pro-mix) plus 10% dialyzed FCS. After being washed, the cell pellets were lysed for 30 min on ice in 1 ml of lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris-HCl [pH 8]) supplemented with the protease inhibitor phenylmethyisulfonyl fluoride (100 μg/ml), pepstatin A (0.7 μg/ml), and leupeptin (5 μg/ml), and lysates were cleared by centrifugation at 14,000 × g for 10 min at 4°C. Extracts from the equivalent of 10^7 cells were precleared for 1 h with protein G-Sepharose beads (Sigma) equilibrated in PBST (phosphate-buffered saline [PBS] plus 1% Triton X-100) and then immunoprecipitated overnight at 4°C with protein G-Sepharose beads precocated with saturating amounts of the indicated MAbs. Immunoprecipitates were washed and loaded onto an SDS–8% polyacrylamide gel. Only MAb LN53 was able to precipitate a doublet protein of 220 to 230 kDa. LN69 and LN72 precipitated proteins present in both BC-3 and control Ramos cells but did not precipitate LNA (Fig. 2a). The identity of the major 105- to 110-kDa protein precipitated by LN69 from both cell types is unclear, and a database search using the LN69 epitope failed to identify any candidate proteins. Antibodies LN20, LN53, and LN72 reacted with a protein doublet of 220 to 230 kDa by Western blotting (Fig. 2b). In addition, a protein of approximately 180 kDa was recognized by the antibodies by Western blotting (Fig. 2b). Reactivity to proteins of this size had previously been seen in Western blots with HHV-8-positive patient sera but had not been assigned as LNA specific (13, 18, 24).

IFAs are used extensively in the seroepidemiology of HHV-8 (6). Under standard IFA conditions, MAbs LN20, LN53, and LN72 were able to produce the punctate nuclear fluorescence pattern associated with HHV-8 LNA in PEL cell lines (Fig. 3a to c). The antibodies were specific for HHV-8 LNA, with no apparent cross-reaction to other proteins present in control B-cell lines Daudi and Ramos (Fig. 3d and e). The best antibody for IFA was LN53, which could be diluted to 1:10,000 without significantly affecting the characteristic LNA IFA pattern in BCP-1 cells. All antibodies were also able to recognize LNA expressed in BCP-1 cells by fluorescence-activated cell sorting analysis (FACS) analysis (Fig. 4a). No cross-reactivity was observed for each antibody with the control B-cell lines Ramos (EBV negative) and Daudi (EBV positive) (representative controls [Fig. 4b]). FACS analysis was also performed with LN53 on one other PEL, BC-3, and on the HHV-8/EBV-coinfected B-cell lymphoma line HBL-6 (Fig. 4b). The BC-3 cell line exhibited the characteristic FACS pattern showing that the majority of cells express LNA. Analysis of HBL-6 showed that most cells expressed LNA, but a subpopulation appeared to be nonexpressing. Whether this is a consequence of HHV-8/EBV coinfection is currently being studied.

HHV-8 LNA is abundantly expressed in KS lesions. To date few protein-specific antisera or MAbs have been raised to HHV-8 proteins. Antibodies to virus-encoded interleukin-6 (vIL6) (21) and to orf26 (23) and orf59 (8) protein products have been used to study protein expression in a variety of HHV-8-infected cell types. However, no antibodies to latent proteins have been used in such studies. LN53 and LN72 but not LN20 and LN69 reacted with antigen in paraffin-embedded KS tissue. As MAb LN53 was assessed to be the best antibody raised, it was used further in the examination of the expression of HHV-8 LNA in KS lesions. LNA was extensively expressed in late-stage nodular KS, predominantly in cells of spindleshaped morphology (Fig. 5b to d). LNA-positive cells account for more than 90% of all cells present in nodular KS lesions. The staining was exclusively nuclear, and in most cases a punctate nuclear staining, reminiscent of the nuclear IFA staining seen in PEL cell lines (Fig. 3), was seen (Fig. 5d). No expres-
sion of LNA was detected in surrounding normal dermis (Fig. 5b). No staining was observed in the control tumors angiosarcoma and hemangioma (not shown).

**Conclusions.** One of the predominant proteins encoded by HHV-8 during the latent phase of the virus life cycle is LNA, the product of orf73 (17, 18, 24). Here we describe the characterization of four MAbs to the C terminus of LNA. The properties of the MAbs are summarized in Table 1. These antibodies recognize LNA in immunofluorescence, immunoprecipitation, in situ immunocytochemistry, and FACS analyses. Three of the MAbs recognized different discrete epitopes in the C terminus of LNA. The most reactive MAb, LN53, recognized the minimal epitope EQEQE present as 23 copies in LNA. This provides an explanation for this antibody’s high reactivity in all assays used, as the repeat units of LNA should provide multiple epitopes for the antibody. The majority of the EQEQE epitopes are located in the extensive leucine zipper motif of LNA. Structure predictions of this region and knowledge of coiled-coil leucine zipper structures suggest that the EQEQE motif is located in a regular pattern on the leucine zipper alpha helices, allowing accessibility of the antibody under native protein folding conditions. This correlates with the ability of LN53 to immunoprecipitate LNA from cell extracts.

Immunoprecipitation studies confirmed that LNA exists as a protein doublet of 220 to 230 kDa in BC-3 cells (Fig. 2a). A protein of similar size was recognized by Western blotting (Fig. 2b). As with HHV-8-positive patient sera, the LNA antibodies produce a punctate immunofluorescence pattern in PEL cell

**TABLE 1. Properties of MAbs to LNA**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>ELISA</th>
<th>Western blotting</th>
<th>Immunoprecipitation</th>
<th>IFA</th>
<th>Immunocytochemistry</th>
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<tbody>
<tr>
<td>LN20</td>
<td>IgM</td>
<td>+</td>
<td>+</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>LN53</td>
<td>IgG2c</td>
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<td>IgG2b</td>
<td>+</td>
<td>+</td>
<td>−</td>
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</tr>
</tbody>
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<sup>a</sup> BC-3 cells.
<sup>b</sup> BCP-1 cells.
<sup>c</sup> Paraffin-embedded KS lesion.
<sup>d</sup> ND, not determined.

FIG. 5. LN53 reactivity to LNA in nodular KS. Immunocytochemistry was performed on a paraffin-embedded tumor from classical KS. Permeabilization was performed by microwave. Negative control biopsies were from angiosarcomas and hemangiomas (not shown). After incubation with normal rabbit serum (DAKO), MAbs were applied for 1 h at 22°C followed by two washes in PBS–0.1% (vol/vol) Tween. The secondary biotin-conjugated antibody (rabbit anti-rat; DAKO) was applied for 30 min followed by washing. Antibody reactions were visualized with streptavidine-alkaline phosphatase (Vector Laboratories) and a substrate red chromogen (Vector). Adjacent sections were stained by standard methods. (a) Hematoxylin and eosin staining showing KS nodule (left) and surrounding dermis (right). (b) Adjacent sections stained with LN53 showing clear demarcation between LNA expression in the KS lesion and not in the surrounding dermis (magnification, ×40). Almost all spindle-shaped cells are positive for LNA (×60) (c), with a stippling pattern reminiscent of the staining of PEL cells (×160) (d).
lines (Fig. 3). Using LN53, we demonstrated extensive LNA expression in nodular KS. There is a clear demarcation between the KS nodule and surrounding dermis (Fig. 5b). The majority of cells showed punctate nuclear staining reminiscent of the nuclear staining in HHV-8-positive PEL cells (Fig. 5d), which suggests that the nuclear structures containing LNA present in PEL cells are also present in HHV-8-infected KS spindle cells. LNA expression has recently been demonstrated in all tissue and cell types associated with HHV-8 infection and disease (12). At present it is unclear which LNA associates with EBNA1 and butyrate-inducible 32-kilodalton protein described for HHV-8, and use of our MAbs to understand the relationship between the KS nodule and surrounding dermis (Fig. 5b). The KS tumors. Antibodies to vIL6 showed expression in approximately 50% of uninduced BCP-1 cells and primary ascitic PELs, suggesting constitutive vIL6 expression in latently infected PEL cells (21). However, vIL6 protein was rarely detected in KS lesions, and when detected, it accounted for less than 2% of HHV-8-infected cells, indicating that vIL6 could be differentially expressed in hematopoietic versus mesenchymal cells. Whether LNA is involved, through interaction with different tissue-specific cellular factors, in the differential expression of HHV-8 genes and whether this contributes to HHV-8 latency remains to be determined. LNA is the first latent protein described for HHV-8, and use of our MAbs to understand its function is likely to be crucial to the understanding of HHV-8 cellular transformation and virus pathogenesis.

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