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Identification of a Nucleocapsid Protein as a Specific Serological Marker of Human Herpesvirus 6 Infection

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Enveloped whole virions and nucleocapsids of human herpesvirus 6 (HHV-6) strain Z29 were purified from supernatant fluids of infected human cord blood lymphocytes by filtration through polyvinylpyrrolidone-treated filters, banding on a Nycondenz step gradient, and centrifugation through two successive continuous sucrose gradients. More than 20 proteins ranging in molecular weight from less than 30,000 to more than 200,000 were identified in preparations of purified whole virions labeled with [^{35}S]methionine and [^{35}S]cysteine. Immunogenic virion proteins of HHV-6 were identified in immunoblot assays with human immune sera, immune sera generated from mice immunized with purified whole virions or purified nucleocapsids, and a monoclonal antibody generated from a mouse immunized with purified nucleocapsids. The sera and the monoclonal antibody reacted strongly with a 101-kilodalton protein in the immunoblots, suggesting that the protein is a component of the nucleocapsid. Human sera lacking HHV-6-specific antibodies and seropositive for one or more of the other human herpesviruses failed to react with this protein, indicating that it is a specific serologic marker for HHV-6 infection.

Human herpesvirus 6 (HHV-6) is a recently discovered virus (9, 21, 26, 30) of which little is known about its pathogenic, immunologic, or molecular biologic characteristics. Seroepidemiologic studies have found a high prevalence of antibody directed against HHV-6 and suggest that HHV-6 infection occurs early in life (4, 5, 17, 19, 23, 27). The virus has been etiologically associated with the usually mild childhood disease exanthem subitum (31), but its role in other diseases is not clear.

Studies of the properties of the HHV-6 virion are important in understanding the biologic characteristics of HHV-6 as well as for development of diagnostic tools. HHV-6-infected-cell proteins have been studied by analysis of profiles of radiolabeled HHV-6-infected cells (2), immune precipitation experiments with labeled infected-cell extracts (1, 2, 15, 28, 32), and immunoblot analysis of infected-cell extracts (1, 2, 15, 27); however, such experiments are not suitable for identifying virion proteins because of the presence of nonstructural infected-cell proteins in crude infected-cell extracts and because of protein-to-protein variations in antigenicity and immune reactivity.

In this report, we present a preliminary characterization of the proteins found in highly purified HHV-6 strain Z29 virions and the identification of a nucleocapsid protein as a specific serologic marker for HHV-6 infection.

MATERIALS AND METHODS

Purification of whole virions and nucleocapsids. HHV-6 (Z29) was propagated by adding infected cells to phytohemagglutinin-stimulated cord blood lymphocytes (CBL) cultured in the presence of interleukin-2 and hydrocortisone, as previously described (3, 21). Whole virions and nucleocapsids were purified from supernatant media collected from 2.6 liters of culture fluids by low-speed centrifugation and filtered through a 0.2% polyvinylpyrrolidone-treated 0.45-μm-pore-size filter (Fig. 1). The serine protease inhibitor phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM. Filtered supernatants were pelleted at 22,000 × *g* overnight and suspended in a total volume of 24 ml of TNE buffer (0.01 M Tris [pH 7.4], 0.1 M NaCl, 0.001 M EDTA). This suspension was layered onto a Nycodenz (Nyegaard & Co. AS, Oslo, Norway) step gradient (20 and 40%, wt/vol) made up in TNE buffer and centrifuged at 54,000 × *g* for 2 h in a swinging-bucket rotor. The band visible at the 20 and 40% interface was collected and diluted in TNE buffer. The suspension was layered onto a continuous sucrose gradient (20 to 60%, wt/wt) made up in TNE buffer and centrifuged at 56,000 × *g* for 2 h in a swinging-bucket rotor. A single band was visible at approximately 40% sucrose (ρ = 1.176). This band was collected in a volume of approximately 1 ml, diluted to 6 ml with TNE buffer, layered onto a second sucrose gradient, and centrifuged as before. The single resulting band was harvested and diluted 10-fold in TNE buffer, and virions were pelleted by centrifugation at 155,000 × *g* for 1 h in a swinging-bucket rotor. The pelleted material was suspended by being incubated in 0.1 ml of TNE buffer overnight at 4°C. Nucleocapsids were prepared by solubilizing membranes of virions obtained from the Nycodenz step gradient with a mixture of 1% Nonidet P-40 and 1% sodium deoxycholate in TNE buffer for 10 min on ice before purification on two successive sucrose gradients.

Gel electrophoretic analysis of radiolabeled virions. CBL were infected with HHV-6 in growth medium (3) containing [^{35}S]methionine and [^{35}S]cysteine at 3.8 μCi/ml. Whole virions were purified from culture supernatants collected 10

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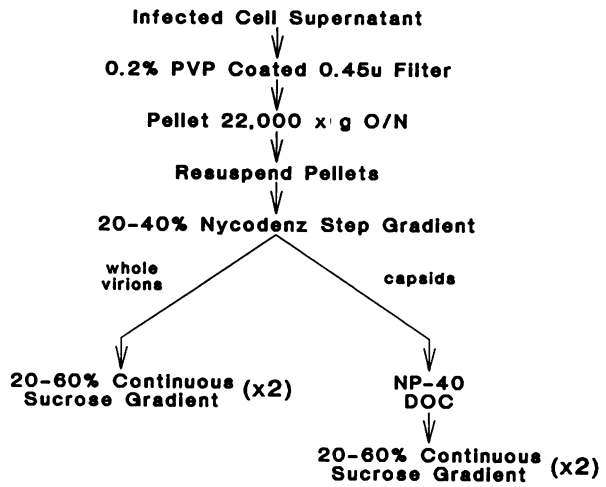

FIG. 1. Flow diagram of the procedures used in the purification of HHV-6 whole virions and nucleocapsids from the extracellular supernatant of HHV-6-infected cord blood cultures. PVP, Polyvinylpyrrolidone; NP-40, Nonidet P-40; DOC, deoxycholate.

to 12 days after infection, as described above. This material was solubilized in sodium dodecyl sulfate (SDS) sample buffer (0.05 M Tris [pH 6.8], 1% SDS, 1% deoxycholate, 1% 2-mercaptoethanol, 10% glycerol) and heated at 100°C for 2 min, and virion proteins were separated by electrophoresis in an SDS–9% polyacrylamide gel cross-linked with 0.24% bisacrylamide. After electrophoresis, the gels were fixed in 40% methanol-10% acetic acid in distilled water, treated with a fluorographic enhancer (Amplify; Amersham Corp., Arlington Heights, Ill.), and dried, and the labeled proteins were visualized by autoradiography. Molecular weight markers included [^{35}S]methionine-labeled proteins from cells infected with herpes simplex virus type 1 (HSV-1) in the presence or absence of cycloheximide, as previously described (22, 24).

Production of HHV-6-specific MAbs and mouse immune serum. BALB/c mice were immunized intraperitoneally a total of five times with 50 μg of purified nucleocapsids emulsified with adjuvant containing trehalose dimycolate and monophosphoryl lipid A (Ribi Immunochem Research, Inc., Hamilton, Mont.) per immunization. Spleen cells from immunized mice were fused with SP 2/0 myeloma cells, as previously described (25). Hybridoma cell culture supernatants were tested for the presence of HHV-6 antibody reactivity against HHV-6-infected or uninfected CBL by immunofluorescence assay with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (Caltag Laboratories, San Francisco, Calif.). Sera were collected from these mice and used as a monoclonal antibody (MAb) source.

Immunoblot assay. Proteins from purified whole virions were separated by SDS-polyacrylamide gel electrophoresis as described above. In some experiments, filtered supernatants of HHV-6-infected CBL cultures were pelleted at 22,000 × *g* overnight and used as antigens in place of purified whole virions. Proteins were transferred to nitrocellulose sheets by transverse electrophoresis in 25 mM Tris–192 mM glycine–20% (vol/vol) methanol (pH 8.3) buffer. Strips cut from the sheets were blocked in 5% skim milk in 1% Tween-phosphate-buffered saline for 1 h and incubated with human serum specimens obtained from patients of an Ohio community hospital who were between the ages of 1 and 99 years, laboratory control serum specimens for anticomplement immunofluorescence assay (ACIF), or serum specimens obtained from mice immunized as described above. The strips were then reacted with alkaline phosphatase-conjugated anti-human immunoglobulin G (Bio-Rad Laboratories, Richmond, Calif.) or similarly labeled anti-mouse immunoglobulin G (Cappel, Organon Teknika Corp., Westchester, Pa.) and visualized with a commercial kit according to the instructions of the manufacturer (alkaline phosphatase Immune-Blot assay kit; Bio-Rad).

RESULTS

Purification of whole virions and nucleocapsids. By using the purification scheme described in Materials and Methods and the legend to Fig. 1, we found that whole virions and nucleocapsids sedimented as single, sharp bands in the second sucrose gradient (Fig. 2A). The bands were collected, and a portion was negatively stained and then examined with an electron microscope (Fig. 2B through F). Each band consisted of intact virions (ρ = 1.176) or nucleocapsids (ρ = 1.186) and contained little visible debris. The second sucrose gradient was critical, because material obtained after a single sucrose gradient contained a large amount of debris and clumps of aggregated particles, as visualized by electron microscopy. A third sucrose gradient resulted in wide, diffuse bands containing virions with damaged envelopes, as observed by electron microscopy (not shown). Material purified through two successive sucrose gradients was used in subsequent studies. Supernatant media from uninfected cell cultures treated in an identical manner did not yield a visible band in the second sucrose gradient (not shown).

HHV-6 virion and nucleocapsid proteins. The molecular weights of the proteins found in the [^{35}S]cysteine- and [^{35}S]methionine-labeled preparations of purified HHV-6 virions were determined by SDS-polyacrylamide gel electrophoresis using both [^{35}S]methionine-labeled HSV-1-infected cell polypeptides and unlabeled molecular weight standards. The plot of the logarithm of molecular weight versus relative migration distance of these proteins was nearly linear (not shown).

More than 20 bands were visible in the autoradiogram of purified whole virions of HHV-6 (Fig. 3A). The apparent molecular weights of proteins in these bands range from less than 30,000 to more than 200,000. The 191-, 143-, 108-, 101-, 80-, 57-, 47-, and 41-kilodalton (kDa) proteins were the most prominant (Fig. 3A). One band with an electrophoretic mobility too low to allow an accurate estimation of its apparent molecular weight was visible near the top of the gel. No bands were seen in an autoradiogram of material obtained from ^{35}S-labeled mock-infected cell cultures processed through the same purification scheme in parallel with material from infected cell cultures.

Small amounts of highly purified nucleocapsids could be obtained (Fig. 2F). Three proteins, of 143, 101, and 41 kDa, were faintly visible in autoradiograms of a gel containing ^{35}S-labeled purified nucleocapsids after a 30-day exposure (data not shown).

Identification of HHV-6 virion and nucleocapsid proteins reactive in immunoblot assays. Serum from a mouse immunized with purified virions reacted in immunoblots (Fig. 3B, lane 1) with several bands of electrophoretic mobilities similar to those of bands seen in gels containing ^{35}S-labeled virions. Among them were bands migrating with apparent molecular masses of 143 and 101 kDa and broad bands centered at 85 and 70 kDa. When identical nitrocellulose

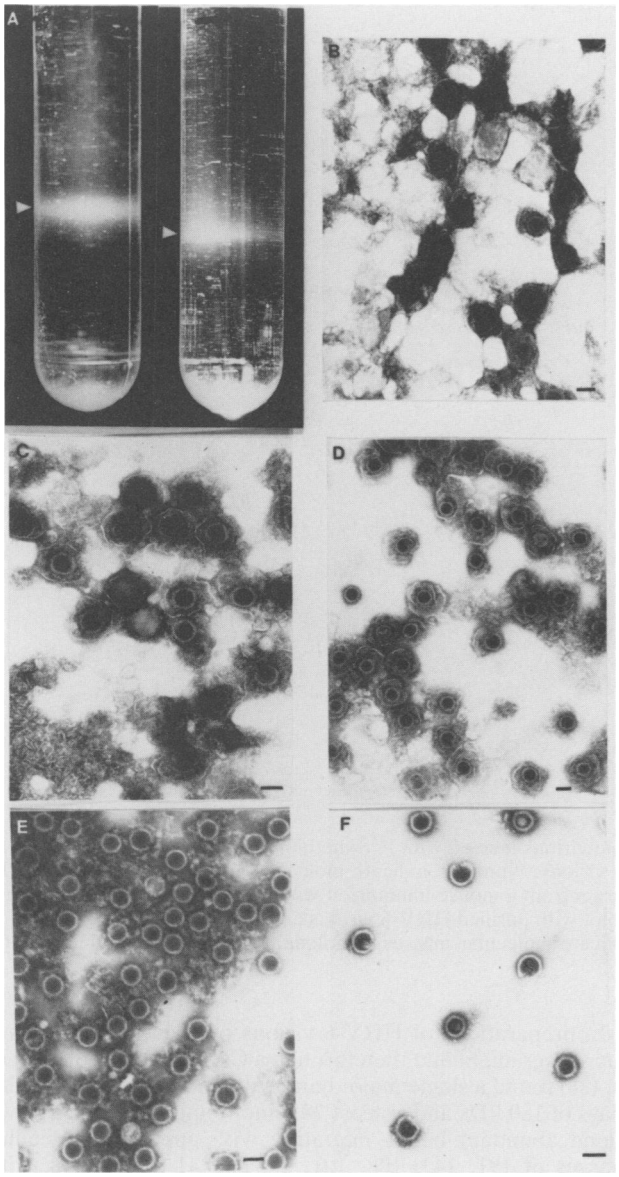

FIG. 2. Purification of HHV-6 whole virions and nucleocapsids. (A) Photograph of the second sucrose gradient. The upper band (left, arrow) contains whole virions and the lower band (right, arrow) contains nucleocapsids. (B through F) Electron micrographs of negatively stained material collected from various steps of the purification procedure. Purified whole virions collected from the Nycodenz step gradient (B) and the single band found in the first (C) and the second (D) sucrose gradients are shown. Purified nucleocapsids collected from the single band found in the first (E) and the second (F) sucrose gradients are also shown. Bars, 100 nm.

strips were reacted with serum from a mouse immunized with purified nucleocapsids (Fig. 3B, lane 2), a subset of the bands seen in the reaction with the antivirion serum was observed. Little reactivity was observed with the broad bands centered at 85 and 70 kDa, as seen in the strip reacted with antibodies against whole virions, suggesting that the proteins making up these bands may be virion envelope glycoproteins. The most prominent band in the reaction with nucleocapsid antiserum was the 101-kDa band.

A MAb (C3108-103) obtained after immunization of a mouse with purified nucleocapsids reacted with a band that comigrated with the 101-kDa protein recognized by the antivirion and antinucleocapsid sera (Fig. 3B, lane 3). Preincubation of blot strips with the MAb markedly reduced the level of reaction with human sera, and preincubation with human sera reduced the level of reaction with the MAb, indicating that the 101-kDa protein recognized by both sources of antibody is the same protein (data not shown). This MAb showed intense nuclear staining of HHV-6-infected cells (Fig. 4), diffuse cytoplasmic staining of cytomegalovirus (CMV)-infected cells, and no staining of Epstein-Barr virus-, varicella-zoster virus-, or HSV-infected cells by immunofluorescence assay (data not shown).

The reactions of human immune sera with purified whole virions was examined (Fig. 3C) in immunoblots. These sera had earlier been identified as positive for HHV-6 antibody in an ACIF (21). All three sera reacted strongly with only one protein, which migrated with an apparent molecular mass of 101 kDa and which comigrated with the protein recognized by MAb C3108-103 (Fig. 3C, lane 4). As described above, this protein appears to be a component of the HHV-6 nucleocapsid. The reaction of these sera with other virion bands was much weaker, and although serum 1 reacted detectably with more than 10 additional bands, sera 2 and 3 each reacted detectably with only 2 other bands.

Specificity of seroreactivity to the 101-kDa protein. The specificity of the seroreactivity of the 101-kDa protein was examined by testing more than 200 human serum samples for antibodies to HHV-6 by ACIF, immunoblot, and enzyme immunoassay and for the presence of antibodies specific for CMV, Epstein-Barr virus, HSV, and varicella-zoster virus (J. Stewart, K. Sanderlin, J. Black, J. Patton, M. Yamamoto, and P. Pellett, unpublished data). Since MAb C3108-103 detected the 101-kDa protein by immunoblot when pelleted supernatants from HHV-6-infected CBL were used as antigens, it was used as an antigen source in this experiment. A subset of the results is presented in Table 1. A serum sample (sample 5) with no detectable antibody to the other human herpesviruses but positive in the HHV-6 ACIF reacted with the 101-kDa protein, but serum samples negative for antibody to HHV-6 by ACIF and positive for antibody to one or more of the other human herpesviruses did not. It is important to note that human serum samples containing CMV-specific antibodies but no HHV-6 antibodies detectable by ACIF (serum samples 1, 6, and 7) did not react with the 101-kDa protein.

DISCUSSION

We obtained preparations of purified HHV-6 virions and nucleocapsids from the supernatant of infected-cell cultures. More than 20 proteins ranging in molecular weight from less than 30,000 to more than 200,000 were identified in autoradiograms of SDS-polyacrylamide gels of ^{35}S-labeled proteins obtained from purified virions. On the basis of these data, together with the lack of visible debris in purified virion preparations examined by electron microscopy, we conclude that most, if not all, of the bands seen in the autoradiogram of purified radiolabeled virions arose from components of HHV-6 virions. It is possible, however, that some of the bands represent cellular proteins or nonvirion viral proteins which copurified with the virions. It is also possible that some of the components of complete HHV-6 virions were lost during the purification procedure, but the virions we obtained did retain infectivity (data not shown). Insufficient

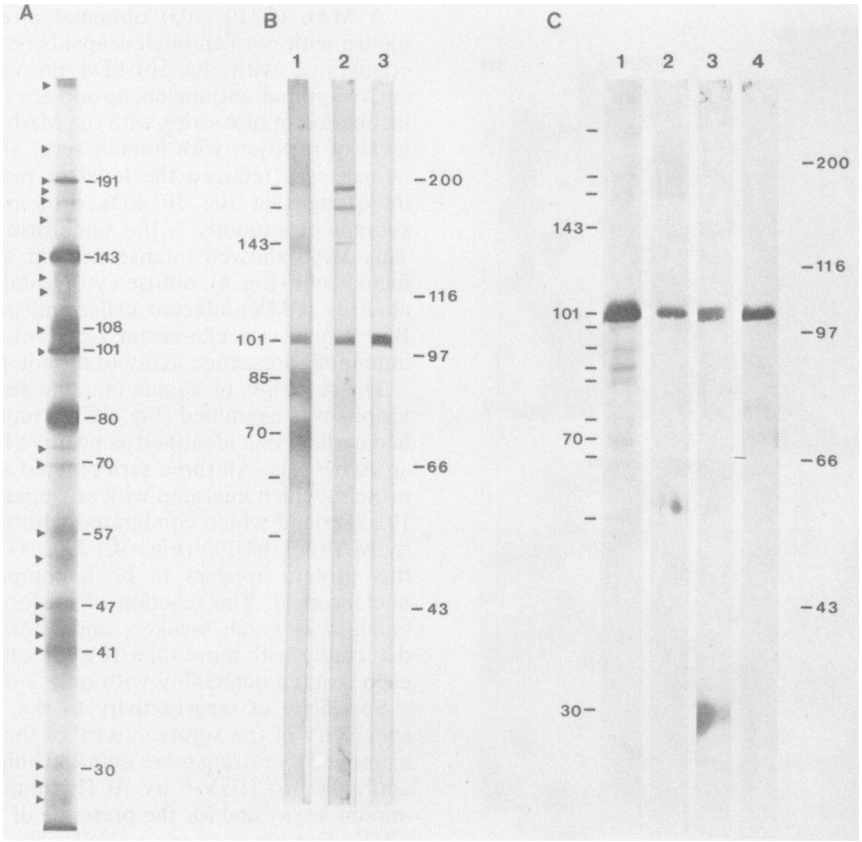

FIG. 3. Virion proteins of HHV-6. (A) Proteins of purified HHV-6 whole virions labeled with [^{35}S]methionine and [^{35}S]cysteine for 10 to 12 days. Arrows indicate proteins identified from purified HHV-6 whole virions. Numbers indicate molecular masses in kilodaltons. (B) Immunoblot with purified HHV-6 virions as an antigen. Lanes 1 and 2, Serum from a mouse immunized with purified whole virions or with purified nucleocapsids, respectively; lane 3, MAb C3108-103. (C) Immunoblot with purified HHV-6 virions as an antigen. Lanes 1 through 3, Human sera; lane 4, MAb C3108-103. (B and C) Numbers on the left indicate molecular masses (in kilodaltons) of proteins identified by immunoblots. Numbers on the right indicate molecular mass standards.

quantities of virions were obtained for quantification of either infectious titers or particle-to-infectious-unit ratios. Further work will be necessary to identify minor proteins, proteins smaller than 30 kDa, glycoproteins, and other posttranslationally modified proteins; to resolve closely comigrating molecules; and to rigorously quantify the relative molar abundance of each protein. A subset of the virion proteins was identified as nucleocapsid components.

The 143-kDa protein seen in both the virion and nucleocapsid preparations may correspond to the major capsid protein of herpesviruses (16), given its size, relative abundance in the purified virions, and presence in the nucleocapsid. In addition, Lawrence et al. (18) determined the nucleotide sequence of the portion of the HHV-6 genome encoding the homolog of the herpesvirus major capsid protein and found that the HHV-6 homolog of this protein is approximately 3 kDa smaller than its HSV-1 counterpart, ICP5. This is consistent with our observation that the apparent molecular weight of the HHV-6 protein is less than that of HSV-1 strain F ICP5, which migrated with an apparent molecular weight of 151,000 in our gel system.

The 143-kDa protein reacted weakly in immunoblot assays, as has been observed by others for an abundant HHV-6 protein of similar mobility (2, 20). This is similar to the weak reactivity of the CMV counterpart of this protein (14) and is in contrast to the strong reactivity seen with the HSV-1 major capsid protein (10).

In preparations of HHV-6 virions purified first through a sucrose gradient and then through a CsCl gradient, Shiraki et al. (28) found a single major band with an apparent molecular mass of 180 kDa and at least 28 minor bands. In contrast, we found abundant bands migrating with apparent molecular masses of 191, 143, 108, 101, 80, and 41 kDa, along with nearly 20 other less abundant bands. Differences in the gel systems used do not account for the differences in the results. The sizes and relative abundances of the proteins in our virion preparations were within the range of results obtained with other well-characterized herpesviruses (8, 13, 16, 29).

We found a 101-kDa nucleocapsid protein to be highly immunoreactive in the immunoblot assay. This protein may be the same as the strongly reactive 120-kDa protein seen in immunoblots by Josephs et al. (15). The differences in apparent size may be due to differences in virus strains or gel systems. We see this apparent size of the major reactive band consistently in experiments with different-percentage gels and different preparations of antigen (data not shown). Ashley et al. (R. Ashley, H. Abbo, J. Militoni, and L. Corey, Abstr. 13th Int. Herpesvirus Workshop, abstr. no. 251, 1988) have noted similar reactivity with a band of apparent molecular weight of 104,000 in proteins prepared by using the same strain of virus as was used here. An epitope of this protein recognized by MAb C3108-103 was found to be cross-reactive between HHV-6 and CMV. Although HHV-6 has

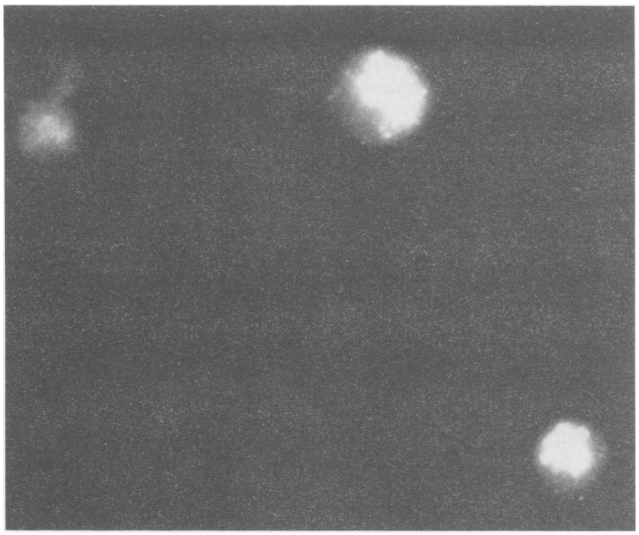

FIG. 4. HHV-6-infected cells stained with MAb C3108-103.

been shown to be clearly antigenically distinct from other herpesviruses, including CMV (6, 9, 18, 19, 21, 26, 27, 29), limited antigenic cross-reactivity of this nature is not surprising, since sequence similarities between HHV-6 and CMV have been observed by others (6, 7, 11).

The specificity of the immunologic reaction of human sera with the 101-kDa protein of HHV-6 was examined by using sera of known reactivity with human herpesviruses. The reaction of human sera with the 101-kDa HHV-6 protein in immunoblot assays coincided with reactivity in the HHV-6 ACIF regardless of the reaction with other herpesviruses (Table 1). This suggests that, despite the cross-reactivity of MAb C3108-103 with CMV-infected cells, the 101-kDa protein may serve as a specific serologic marker for infection by HHV-6. The sensitivity of detecting HHV-6 antibody by using the 101-kDa protein as a marker in immunoblot assays was at least as high as that obtained by using the ACIF (Stewart et al., unpublished data).

Values for HHV-6 seroprevalence vary widely from report to report, in large part because of differences in assay systems and the cutoff values used. It is necessary to develop a standard diagnostic test of both high specificity and high sensitivity. The results obtained from this study suggest that the 101-kDa protein might serve well as a target antigen in the next generation of HHV-6 serodiagnostic assays. This will assist in developing a better understanding of the natural history of HHV-6 infection.

TABLE 1. Specificity of immune reactivity with HHV-6 101-kDa protein

Serum sample	Presence of antibodies against[a]:						Reactivity[b] with 101-kDa protein
	HHV-6[c]	HSV-1[d]	HSV-2[d]	CMV[d]	VZV[d]	EBV[e]	
1	−	ND	ND	+	−	−	−
2	+	ND	ND	−	−	−	+
3	+	ND	ND	−	+	+	+
4	+	+	+	−	−	+	+
5	+	−	−	−	−	−	+
6	−	+	+	+	+	+	−
7	−	+	−	+	−	+	−
8	−	+	−	−	−	−	−

[a] VZV, Varicella-zoster virus; EBV, Epstein-Barr virus. −, No antibodies; +, antibodies present; ND, not done.
[b] −, No reactivity; +, reactivity. Determined by immunoblot.
[c] Determined by ACIF (3, 21).
[d] Determined by enzyme immunoassay (Stewart et al.).
[e] Determined by immunofluorescence assay (12).

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