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A Herpesvirus of Rhesus Monkeys Related to the Human Kaposi's Sarcoma-Associated Herpesvirus

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A herpesvirus that is related to but distinct from the Kaposi's sarcoma-associated herpesvirus (KSHV, or human herpesvirus 8) was isolated from rhesus monkeys. The sequence of 10.6 kbp from virion DNA revealed the presence of an interleukin-6 homolog similar to what is present in KSHV and a closer relatedness of the DNA polymerase and glycoprotein B reading frames to those of KSHV than to those of any other herpesvirus. This rhesus monkey herpesvirus replicated lytically and to high titers in cultured rhesus monkey fibroblasts. Antibody testing revealed a high prevalence for at least 10 years in our rhesus monkey colony and a high prevalence in two other colonies that were tested. Thus, rhesus monkeys naturally harbor a virus related to KSHV, which we have called RRV, for rhesus monkey rhadinovirus.

The herpesviruses are a large, complex group that exhibit extensive diversity in their biological properties. Their large DNA genomes allow considerable flexibility in the complement of genes that they carry, and this presumably is a major factor responsible for the diversity in biological properties. Eight distinct human herpesviruses have now been identified. These include herpes simplex virus (HSV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV). Some genes, such as those for DNA polymerase, major capsid protein, and surface glycoproteins, have a related member in each herpesvirus. However, 20% or more of the genes may be unrelated and nonequivalent when distinct herpesviruses are compared (31).

Taxonomic subclassification of herpesviruses into three subfamilies (alpha, beta, and gamma) is based on biological properties, genomic organization, and sequence relatedness (31). The gamma, or lymphotropic, subgroup of herpesviruses includes EBV and related B-cell-tropic viruses from great apes and Old World primates; these have been called the lymphocryptoviruses (31). The gammaherpesviruses also include the T-cell-tropic herpesvirus saimiri of New World primates and related members from horses, cattle, and other species; these have been called rhadinoviruses (31). A newly identified human herpesvirus, called Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 (HHV-8), has herpesvirus saimiri as its closest known relative (7, 33). Epidemiological and molecular studies have associated KSHV with Kaposi's sarcoma (17, 22, 25, 41) and some types of B-cell lymphoma (4, 5, 34, 35). KSHV has been identified in B cells as well as in other cell types in naturally infected humans (2, 3, 36). In this report we describe the isolation and early characterization of a related virus from rhesus monkeys.

MATERIALS AND METHODS

Cell culture. Primary rhesus monkey fibroblast cell lines were established from skin punch biopsies under sterile conditions as previously described (21). Dissected 1- to 2-mm skin pieces were placed under a glass slide on the scored surface of a tissue culture dish. Dulbecco's modified Eagle's medium (Cellgro) supplemented with 20% fetal bovine serum (Sigma), 10 mM HEPES (Cellgro), 2 mM L-glutamine, penicillin-streptomycin (50 IU and 50 μg/ml, respectively), vancomycin (30 μg/ml), and gentamicin (10 μg/ml) was replenished twice per week. Fibroblasts were trypsinized when confluent and were passaged to tissue culture flasks. Early passages were frozen in liquid nitrogen for later use. Subsequent passages of cells used the same medium without the vancomycin and gentamicin. For electron microscopy, cells were fixed, processed, and stained with uranyl acetate and Reynold's lead citrate by routine techniques (30).

Virus stocks. Virion DNA, prepared as described below, was treated with 25 μg of pancreatic RNase (Boehringer Mannheim, Indianapolis, Ind.)/ml for 15 min at 20°C and was transfected into rhesus monkey fibroblasts by a DEAE-dextran procedure (27). Virus produced from transfection was expanded on rhesus monkey fibroblast cultures, and hundreds of vials of clarified, filtered virus stock were frozen at −160°C in the vapor phase of liquid nitrogen.

Virus purification. Procedures for the purification of virus have been published previously (9). Briefly, cells and debris were removed by low-speed centrifugation following complete cell lysis. The supernatant was then filtered through a 0.45-μm-pore-size filter to remove any additional cells and debris. The filtered supernatant was then centrifuged for 3 h at 17,000 rpm in a Sorvall type 19 rotor in order to pellet the virus. Resuspended virus was then fractionated by Sepharose 4B column chromatography, and virus contained in the void volume was used as a source of virion DNA for cloning and as a source of viral antigens for antibody testing.

Cloning and sequencing. Purified virions were lysed in Sarkosyl and digested with proteinase K; DNA was extracted with phenol and chloroform and was precipitated with ethanol. Virion DNA was digested with restriction endonucleases according to the recommendations of the manufacturers. *Pst*I fragments were cloned into vector pSP72. *Pst*I inserts in two of the clones were randomly sheared by sonication, cloned into pUC18, and sequenced by using an ABI Prism automated sequencer. Sequences were aligned with Sequencher 3.0 software, and open reading frames were identified with MacVector. Related genes were identified by BLAST search of GenBank sequences. The degrees of amino acid identity and similarity were calculated with Genetics Computer Group software.

Antibody testing. Procedures for coating enzyme-linked immunosorbent assay (ELISA) plates and performing ELISAs have been described previously (9, 23). Eight micrograms of protein from detergent-lysed virions was used to coat each plate. Procedures for indirect immunofluorescence tests have also been described previously (20).

Nucleotide sequence accession number. The RRV sequence was deposited in GenBank under accession no. AF029302.

RESULTS

A large number of rhesus monkeys in our colony were found to have antibodies reactive with herpesvirus saimiri by ELISA. Several lines of evidence indicated to us that this antibody reactivity was real and not artifactual, so we set out to try to recover a virus. Peripheral blood mononuclear cells from rhe-

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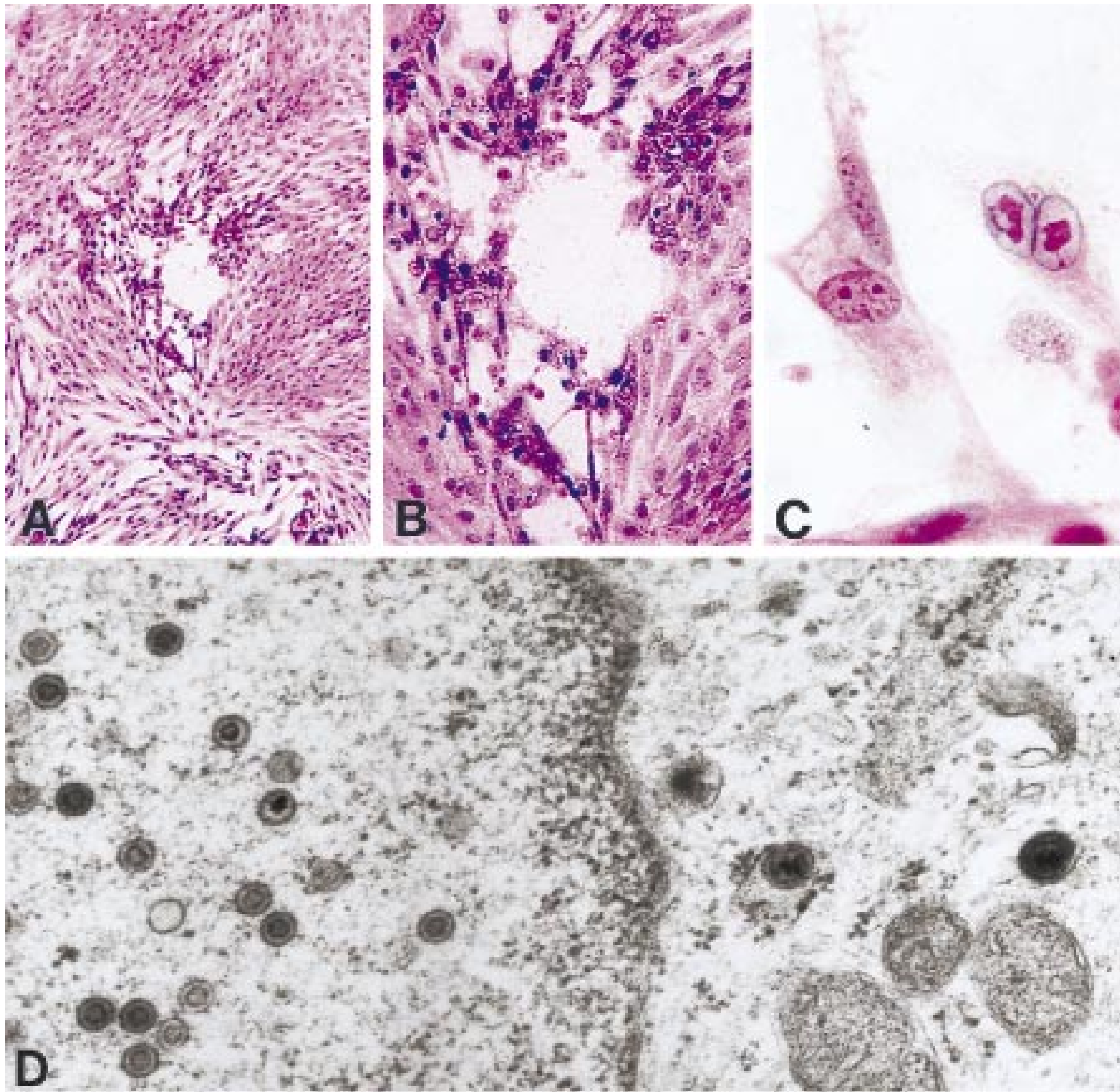

FIG. 1. Productive infection of rhesus monkey fibroblasts with herpesvirus. Infected cells were stained with hematoxylin and eosin. Early CPE is shown at low magnification (×50) (A) and at a higher magnification (×125) (B). Several syncytial and rounded cells can be seen at the margin of the lytic area. Higher magnification (×750) shows margination of nuclear chromatin and eosinophilic intranuclear viral inclusions (C). Electron microscopy revealed numerous nonenveloped herpesvirus particles in the nucleus and enveloped particles in the cytoplasm (magnification, ×50,000) (D).

sus monkeys were cocultured with passage 8 to passage 15 rhesus monkey fibroblasts derived from skin punch biopsy. Six independent cultures from six different animals developed cytopathic effect (CPE). CPE first became detectable 8 to 11 days after the initiation of coculture and completely destroyed the culture by 13 to 16 days. The nature of the CPE was reminiscent of that produced by herpesvirus saimiri infection of monolayer cells. At a low multiplicity of infection, early destruction of cells was focal and produced holes in the monolayer around which rounded and syncytial cells could be observed in the spreading infection (Fig. 1A and B). Intranuclear inclusions were observed in the regions of localized CPE (Fig. 1C). Herpesvirus particles were observed by electron microscopy in all six cultures (Fig. 1D). Large numbers of herpesvirus particles were observed in the nucleus (nonenveloped), cytoplasm (enveloped), and extracellular space.

To help ensure the absence of any potential contaminating agents, working stocks of virus were produced by transfection of cells with RNase-treated virion DNA. Virus was pelleted from the clarified supernatant of three of the new herpesvirus isolates and was used to prepare virion DNA. The virion DNA was treated with 25 μg of pancreatic RNase/ml for 15 min at 20°C and was transfected into rhesus monkey fibroblasts by a DEAE-dextran procedure. Virus produced from transfection was expanded on rhesus monkey fibroblast cultures, and hundreds of vials of clarified, filtered virus were frozen at −160°C in the vapor phase of liquid nitrogen. Virus produced from transfection induced CPE that first became evident 4 to 7 days after infection, and total CPE occurred 8 to 11 days after infection. Virus contained in the clarified supernatant of one culture with complete CPE was found to contain 5×10^5 50% tissue culture infective doses per ml when titered on rhesus

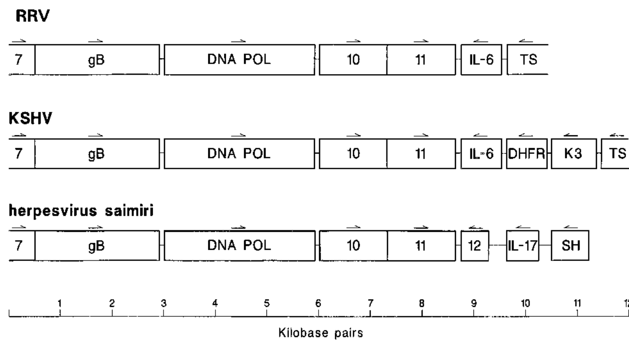

FIG. 2. Schematic representation of open reading frames in 10.6 kbp of RRV. The genomic organization of the RRV region that was sequenced is compared to those of the corresponding regions of KSHV (33) and herpesvirus saimiri (1). The numbers 7, 10, and 11 refer to the sequentially numbered open reading frames in herpesvirus saimiri and the corresponding genes in KSHV and RRV. gB, glycoprotein B; DNA POL, DNA polymerase; IL-6 and IL-17, IL-6 and IL-17 homologs, respectively; SH, superantigen homolog; K3, unique open reading frame 3 of KSHV.

monkey fibroblasts. These three herpesvirus isolates appear to be free of other infectious agents on the basis of the manner in which they were prepared, by the lack of other agents observed by electron microscopy, by the consistent nature of the CPE, and by the lack of evidence for other viral DNAs in the analyses described below. One isolate (H26-95) was selected for more detailed analysis.

Column-purified virus was used for the preparation of virion DNA from H26-95. Agarose gel electrophoresis of virion DNA without any restriction endonuclease digestion revealed only large DNA of >25 kbp. Digestion of virion DNA with *Pst*I, *Hin*dIII, and other restriction endonucleases produced a broad range of different-sized fragments with a level of genetic complexity expected for a herpesvirus (data not shown). Fragments were cloned as plasmids in *Escherichia coli*, and clones with larger inserts of 2 to 10 kbp were selected for sequencing. We have completed the sequencing of two *Pst*I clones that represent 10,595 bp of contiguous genetic information containing the DNA polymerase and glycoprotein B genes (Fig. 2).

BLAST searches revealed the highest levels of similarity of the new sequence to sequences of KSHV, herpesvirus saimiri, and other rhadinoviruses. The predicted amino acid sequence over the full length of each open reading frame was compared in detail to amino acid sequences of other herpesviruses contained in the GenBank database (Table 1). Overall, the predicted amino acid sequences over this region of the H26-95 isolate were slightly closer to those for KSHV than to those for herpesvirus saimiri or the other rhadinoviruses (Table 1). The DNA polymerase open reading frame of H26-95 showed amino acid identities of 67.1% with that of KSHV, 62.5% with that of herpesvirus saimiri, 59.5% with that of equine herpesvirus 2, and 54.8% with that of EBV, and considerably lower levels of amino acid identity with those of HSV and CMV. Similarly, the glycoprotein B open reading frame of H26-95 showed amino acid identities of 65.5% with the glycoprotein B open reading frame of KSHV, 54.4% with that of herpesvirus saimiri, 54.3% with that of bovine herpesvirus 4, 50.6% with that of equine herpesvirus 2, and 43.0% with that of EBV, and considerably lower levels of amino acid identity with those of HSV and CMV (Table 1). The G+C content of the 10.6-kbp fragment is 59.9%, which compares with 53.5% for the long unique region of KSHV and 34.5% for that of herpesvirus saimiri (1, 33).

The region selected for sequencing is informative not only because it contains a conserved gene block (including genes for DNA polymerase and glycoprotein B) for comparison to other herpesviruses but also because it contains a nonconserved stretch with genes that are variably present or variably located even among rhadinoviruses. The sequence of the rhesus monkey herpesvirus contained a homolog of the interleukin-6 (IL-6) gene immediately following the open reading frame corresponding to open reading frame 11 of herpesvirus saimiri (Fig. 2). KSHV has recently been shown to contain a functional IL-6 homolog at the equivalent genomic location (24, 28, 33), but no such homolog is present in herpesvirus saimiri or any other herpesvirus that has been studied. The IL-6 homolog in the rhesus herpesvirus exhibited 18.5% amino acid identity and 44.4% similarity with the corresponding reading frame in KSHV (Table 1) and 19.6% identity and 41.2% similarity with human IL-6. All four of the cysteines present in human IL-6 were uniformly conserved in the rhesus herpesvirus. By way of comparison, the KSHV and human cell IL-6 reading frames were found to exhibit 24.7% amino acid identity and 49.7% similarity (24, 28, 33). The IL-6 gene in KSHV is followed by genes for dihydrofolate reductase (DHFR), K3 (a relative of the immediate-early gene of bovine herpesvirus 4), and thymidylate synthase (TS) (33). TS genes have been found in some rhadinoviruses and in varicella-zoster virus (HHV-3) but not in other herpesviruses (19, 37). DHFR genes have been found in KSHV and herpesvirus saimiri but not in other herpesviruses (33, 38). The rhesus herpesvirus also contained a TS gene in this general vicinity (Fig. 2). In herpesvirus saimiri, the TS gene is located far (57 open reading frames) downstream. The rhesus herpesvirus did not contain a DHFR gene at a position equivalent to where it is found in KSHV (Fig. 2). In herpesvirus saimiri, the DHFR gene is the second open reading frame, considerably upstream of the genomic region shown in Fig. 2 (1, 26, 38).

The prevalence of this herpesvirus in rhesus monkeys was assessed by screening for the presence of antibodies. Column-purified H26-95 was used to coat ELISA plates, and serum samples from rhesus monkeys were tested for reactive antibodies by the same procedures that we have used successfully with

TABLE 1. Amino acid identity/similarity of RRV with other herpesviruses[a]

Reading frame	Identity/similarity with:						
	KSHV	Herpesvirus saimiri	EBV	HSV-1	CMV	BHV4	EHV2
gB	65.5/77.4	54.4/72.1	43.0/61.0	30.4/50.3	34.0/54.3	54.3/73.1	50.6/67.4
DNAPol	67.1/80.0	62.5/76.7	54.8/71.9	40.4/60.8	40.5/60.4	NA	59.5/76.0
ORF 10	34.6/55.9	23.8/47.0					
ORF 11	33.3/54.5	32.4/54.5	29.2/48.2				
IL-6	18.5/44.4						

[a] Abbreviations: HSV-1, HSV type 1; BHV4, bovine herpesvirus 4; EHV2, equine herpesvirus 2; gB, glycoprotein B; DNA Pol, DNA polymerase; NA, not available; ORF, open reading frame.

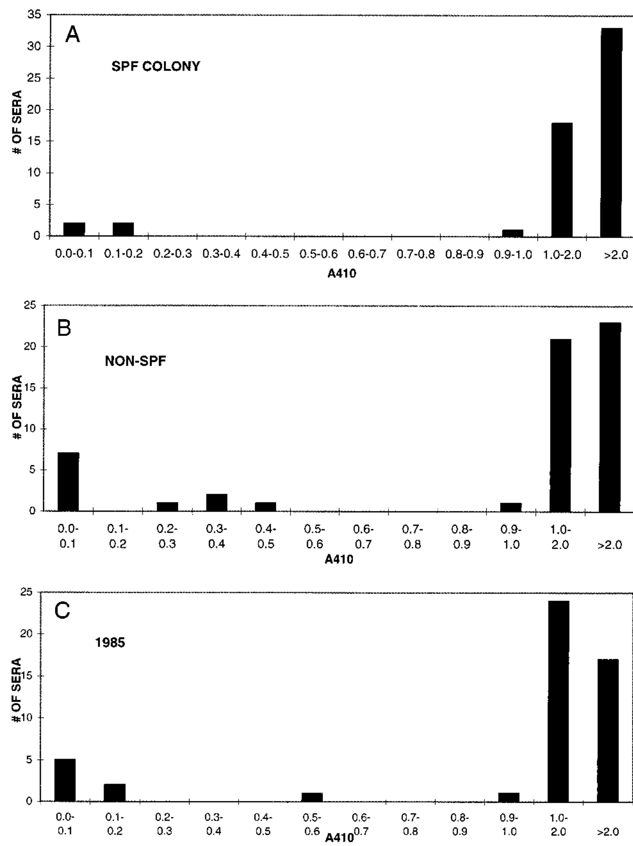

FIG. 3. Reactivity of rhesus monkey serum samples to RRV by ELISA. Shown are results for samples taken from the SPF (A) and conventional (B) rhesus monkey colonies at NERPRC and results for frozen samples taken from rhesus monkeys at NERPRC in 1985 (C).

other viruses (9, 11, 23). Fifty-six randomly selected rhesus monkeys from our specific-pathogen-free (SPF) rhesus monkey colony at the New England Regional Primate Research Center (NERPRC) were analyzed first. These monkeys are housed in breeding groups of 10 to 14 animals and are maintained free of herpes B virus, simian T-cell lymphotropic virus type 1, type D retrovirus, and simian immunodeficiency virus (SIV) (11). Fifty-two of these 56 SPF monkeys (92.8%) were strongly positive for the presence of antibodies to the H26-95 herpesvirus (Fig. 3A). Similarly, a high percentage of rhesus monkeys from our conventional colony at NERPRC were also strongly antibody positive (Fig. 3B).

Several lines of evidence indicate that the high frequency of antibody positivity in these tests reflects a high prevalence of infection in our colony with the herpesvirus. (i) Serum samples from rhesus monkeys that tested negative at <0.1 absorbance unit in the experiment for which results are given in Fig. 3 remained negative on repeated testing. (ii) The six monkeys from whom the new herpesvirus was recovered scored strongly positive (1.1 to >2) for the presence of antibodies. (iii) Ten monkeys that were hand reared from birth and kept in isolation all scored negative (<0.1) for the presence of antibodies. (iv) We have documented four cases of seroconversion among young rhesus monkeys in our colony. Serum samples taken from these four monkeys 4 to 7 months after birth were negative for the presence of antibodies (<0.1), but subsequent serum samples taken when they were 1 year old or older were strongly positive (>1.3). (v) Pre- and postseroconversion serum samples from one of these monkeys (255-95) were used for confirmatory testing by indirect immunofluorescence. The ELISA-positive serum samples from this animal showed strong reactivity to cells infected with H26-95 (Fig. 4). There was no reactivity of the ELISA-negative serum to infected cells, and there was no reactivity of either serum to uninfected cells (data not shown). (vi) Our previous experience with these same procedures with other viruses has provided extensive evidence for their reliability (9, 11, 23).

Additional testing demonstrated that the high prevalence of infection with this herpesvirus is not unique to our colony or to recent times. Serum samples taken from rhesus monkeys at NERPRC in 1985 (9) and stored frozen since that time also showed a high frequency of strong antibody positivity (Fig. 3C). We also tested serum samples obtained in 1996 from rhesus monkeys at two other institutions. Forty of 40 serum samples from one primate facility and 25 of 28 from a commercial supplier gave strong reactivity, with ELISA values of >1.0. Additional serological testing indicated that other species of macaques are also infected with the same or a similar agent (data not shown).

DISCUSSION

Our results indicate that rhesus monkeys are naturally infected with a herpesvirus in the rhadinovirus subgroup. Our sequence analysis of H26-95 clearly places this herpesvirus as a close relative of KSHV (HHV-8) and herpesvirus saimiri, but it is also clearly distinct from them. The genomic organization of the sequenced region, its G+C content, and the extent of amino acid identity and similarity of its conserved genes with those of other herpesviruses suggest that the rhesus monkey virus is somewhat closer to the human KSHV than it is to herpesvirus saimiri. Most telling is the presence of a homolog

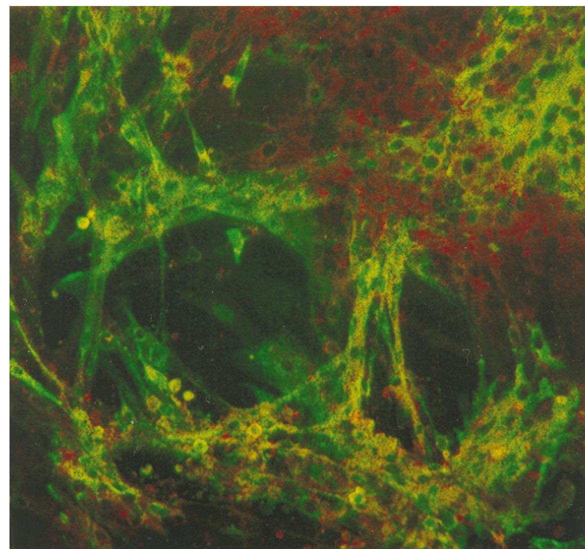

FIG. 4. Indirect immunofluorescence confirmatory testing. Serum samples taken from monkey 255-95 after seroconversion to positivity for antibodies to RRV by ELISA were used for indirect immunofluorescence tests on rhesus monkey fibroblasts infected with RRV according to published procedures (20). The photomicrograph (magnification, ×125) was taken under epifluorescence illumination and shows an area of the culture with typical CPE as demonstrated in Fig. 1. The majority of the cells at the margin of the lytic area (black central area) are positive for viral antigens (green to yellow). Uninfected fibroblasts in relatively normal portions of the monolayer are faintly red (Evans blue counterstain).

of the IL-6 gene, which was found at equivalent positions in KSHV and the KSHV-related rhadinovirus of rhesus monkeys (RRV); this gene is not present in herpesvirus saimiri or any other herpesvirus that has been analyzed. Nonetheless, the genomic organizations of KSHV and RRV are not identical over the 11-kbp region that was sequenced. How differences in the complement of genes may contribute to differences in biological properties remains to be determined.

Diseases that may be associated with RRV infection remain to be elucidated. By analogy to diseases associated with KSHV in humans, there are some intriguing candidates. Macaques at NERPRC have a relatively high frequency of spontaneous lymphomas and lymphoproliferative diseases, and the frequency appears to be considerably higher in the context of SIV- and type D virus-induced immunodeficiency (8, 10, 13). Early testing suggested that many of these may not be EBV related (14). SIV-infected monkeys at NERPRC also often exhibit an unusual arteriopathy (6) that is not seen outside the context of SIV-induced immunodeficiency. A transmissible, subcutaneous fibromatosis with similarities to Kaposi's sarcoma has been observed in macaques at the Washington Regional Primate Research Center (18, 32, 39, 40). A causative role for RRV in these or other diseases would greatly facilitate studies of pathogenesis and vaccine and drug development.

The ability to grow RRV lytically and to high titer in cell culture is a distinct advantage of this system. Analogous conditions for permissive growth of KSHV have not been identified (16, 29). The availability of permissive cell culture conditions greatly facilitates the construction of gene deletion and point mutants, an approach that has been utilized effectively for the study of oncogenic transformation by herpesvirus saimiri (12, 15, 26). Permissive growth in culture also allows for study of the lytic cycle and production of large amounts of purified virus for study of virion composition, as a source of virion DNA, and as a source of structural antigens for antibody testing.

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