Distribution of a Macaque Immunosuppressive Type D Retrovirus in Neural, Lymphoid, and Salivary Tissues

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Simian acquired immune deficiency syndrome (SAIDS) in rhesus macaques (Macaca mulatta) at the California Primate Research Center is caused by a type D retrovirus designated SAIDS retrovirus serotype 1 (SRV-1). This syndrome is characterized by profound immunosuppression and death associated with opportunistic infections. Neurologic signs and lesions have not been described as part of this syndrome. The distribution of SRV-1 in the salivary glands, lymph nodes, spleens, thymuses, and brains of eight virus-infected rhesus macaques was examined by immunohistochemistry. Electron microscopy, in situ RNA hybridization, and Southern blot hybridization were also performed on selected tissues to detect viral particles, RNA, and DNA, respectively. In seven of eight SRV-1-infected animals, the transmembrane envelope glycoprotein (gp20) of SRV-1 was present in three or more tissues, but never in the brain. In the remaining animal, no viral antigen was detected in any tissue. In this same group of animals, viral nucleic acid was detected in the lymph nodes of six of six animals by Southern blot hybridization, in the salivary glands of two of five animals by both Southern blot and in situ hybridizations, and, surprisingly, in the brains of three of three animals by Southern blot and of three or five animals by in situ hybridization, including the one animal in which viral gp20 was undetectable. None of these animals had neurologic signs or lesions. The detection of viral nucleic acid in the absence of viral antigen in the brain suggests latent SRV-1 infection of the central nervous system.

Southern blot analysis for SRV-1 RNA and DNA, respectively. These techniques detect SRV-1 replication from provirus through the whole viral particle, allowing insights into the biological behavior of the virus in addition to topographic distribution.

We found that SRV-1 was widespread in the tissues examined, with an affinity for germinal centers of lymphoid organs and salivary gland acinar cells. We also provide evidence for latent central nervous system infection in four animals and latent systemic infection in one animal.

MATERIALS AND METHODS

Animals, tissues, and virus isolation. Axillary lymph nodes, spleens, thymuses, brains (parietal cortex), and parotid salivary glands were collected at necropsy from eight rhesus monkeys (Macaca mulatta). All animals were euthanatized, four when moribund with terminal SAIDS and four showing only PGL and splenomegaly. All the animals had at least two positive viral cultures prior to necropsy. Infectious SRV-1 was isolated from rhesus monkeys by cocultivation of Raji cells with Ficoll-Hypaque-purified peripheral blood mononuclear cells as previously described (4, 30). All cultures were subcultured and scored as positive when characteristic syncyta were seen. The presence of SRV-1 was confirmed by indirect immunofluorescence with an anti-SRV-1 gp20 monoclonal antibody (18). Viral cultures were repeated at necropsy, at which time one animal (21296) was no longer viremic.

Seven of these animals were intravenously inoculated with SRV-1, and one (20263) was a spontaneously occurring SAIDS case from an outdoor corral where SAIDS is endemic (19, 20). Of the seven experimentally inoculated animals, five (20343, 21292, 21296, 21304, and 21318) received SRV-1-containing plasma and two (21877 and 21824)
received tissue culture-grown SRV-1. The clinical course of SAIDS, virus status, and antibody status of four of these animals (20343, 21318, 21824, and 21877) have been described in detail in previous reports (18, 21, 32). Two additional clinically normal rhesus monkeys that were SRV-1 antibody- and virus-negative were chosen from our SAIDS-free colony as infected controls.

The selection of the salivary gland, brain, and major lymphoid organs was based on the previous detection of retroviruses in analogous tissues from infected mice (9), cats (7, 10, 40), goats (3), sheep (39), gibbons (8, 17), macaques (6, 11, 21), and humans (12, 16, 24, 41, 45).

Tissues were collected immediately after euthanasia, snap frozen in liquid nitrogen-cooled Freon, and stored at −70°C. Adjacent 6-μm cryostat sections were examined by immunohistochemistry and in situ RNA hybridization.

Electron microscopy. Samples of lymph node, spleen, salivary gland, and brain (parietal cortex) collected at necropsy were also fixed in Karnovsky fixative (M. Karnovsky, J. Cell Biol. 27:137A, 1973) for examination by electron microscopy. Tissues were postfixed in 1% osmium tetroxide, serially dehydrated for infiltration, embedded in epoxy, and further dehydrated with 1% osmium tetroxide, serially dehydrated for infiltration, embedded in epoxy, and sectioned at 40 to 90 nm. Sections were stained with lead citrate and uranyl acetate and examined in a Philips EM-400 electron microscope.

Immunohistochemistry. A monoclonal antibody specific for the transmembrane envelope glycoprotein (gp20) of SRV-1 was produced and characterized as previously described (18). This IgG2a monoclonal antibody was used in an avidin-biotin-peroxidase complex technique (Vector Laboratories, Burlingame, Calif.) with 3-amino-9-ethylcarbazole as the chromogen. Sections of salivary glands and brains from two monkeys (20263 and 21296) were also examined with serum from a rabbit hyperimmunized with whole SRV-1. This serum was reactive with all major viral proteins on Western blot (immunoblot). All sections were lightly counterstained with Mayer’s hematoxylin and mounted in Aquamount (Lerner Laboratories, New Haven, Conn.).

Control procedures included omission of the primary antibody, substitution of a heavy-chain-matched monoclonal antibody of irrelevant specificity, and use of normal rabbit serum for rabbit anti-SRV-1. Additional controls included SRV-1-infected and uninfected Raji cells prepared as previously described (30) and matched tissues from the two normal uninfected rhesus monkeys. To ensure reproducibility, each tissue was tested a minimum of three separate times.

Preparation of [35S]RNA. Radioactive RNA probes for in situ hybridization were prepared by using the Riboprobe SP65 vector (with the Salmonella typhiurium bacteriophage SP6 promoter) containing subcloned full-length DNA from SRV-1. The SP65 plasmids plus linearized were linearized and processed to yield a very pure template for the runoff transcription of radiolabeled RNA. The templates were linearized by using the restriction endonuclease XbaI according to manufacturer guidelines. After restriction endonuclease digestion was complete, the reaction was terminated and the DNA template was extracted with phenol, phenol-chloroform (1:1, vol/vol), and chloroform. The DNA was precipitated twice with 2.5 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 6.0). The DNA template was suspended in RNase-free water (29) and stored at 4°C. The linearized templates were used to generate [35S]RNA by using the Promega Biotec Riboprobe reaction conditions to produce RNA probes of high specific activity (34), with the following modifications: 50 μCi of [35S]UTP (specific activity, 1,200 Ci/mmol) was used as the labeled nucleotide, with the concurrent reduction to 12 μM of the unlabeled UTP, and all other nucleotides were at a final concentration of 500 μM in the reaction; the reaction was allowed to progress for 4 h at 37°C prior to termination with RNase. The resulting RNA was precipitated three times with a mixture of 2.5 volumes of 100% ethanol, 0.1 volume of 3 M sodium acetate, and 20 μg of tRNA. The RNA was suspended in 20 μl of RNase-free water and subjected to mild alkaline hydrolysis to reduce the RNA to lengths of 50 to 400 base pairs (27). The processed RNA was stored at −20°C in 2.5 volumes of 100% ethanol-0.1 volume of 3 M sodium acetate.

In situ RNA hybridization. Glass slides were pretreated to reduce nonspecific binding of probes by methods previously described (13). Tissue sections of salivary gland and brain were collected onto pretreated slides and fixed in ethanol-acetic acid (3:1, vol/vol) at room temperature for 15 min, rinsed in 100% ethanol for 5 min, air dried, and stored desiccated at room temperature.

To facilitate probe diffusion, postfixed cryostat sections were pretreated with HCI, SSC (0.15 M NaCl plus 0.015 M sodium citrate; pH 7.4), and proteinase K by previously established methods (13). Each tissue section was covered with 20 μl of hybridization solution (0.3 M NaCl, 0.1 M Tris [pH 7.5], 5 mM EDTA, 2× Denhardt solution, 10% dextran sulfate, 50% formamide) containing radiolabeled RNA probe (1000 dpm per slide), overlaid with a siliconized cover slip, and incubated in a moist chamber for 72 h at room temperature in the dark. After hybridization, cover slips were removed in 2× SSC, and sections were incubated with RNases A (20 μg/ml) and T1 (2 μg/ml) for 30 min in 10 mM Tris (pH 7.5) with 5 mM EDTA and 300 mM NaCl at 37°C. Sections were then washed in hybridization wash medium (0.6 M NaCl, 10 mM phosphate buffer [pH 7.4], 1 mM EDTA, 50% formamide) for 10 min, transferred to 2× SSC at 55°C and incubated for 1 h. After an additional hour of washing in hybridization wash medium with gentle stirring, sections were dehydrated in graded alcohols containing 300 mM ammonium acetate (70%, twice; 95%, once) and air dried. The finished sections were coated with nuclear track emulsion (NTB2; Eastman Kodak Co., Rochester, N.Y.) diluted 1:1 (vol/vol) with 0.03 M ammonium acetate, air dried, and stored desiccated in the dark at 4°C for 48 h. The emulsion was developed with D-19 developer (Eastman Kodak), and development was stopped with water. The emulsion was fixed in Kodak fixer and washed in water. Slides were counterstained with hematoxylin and eosin. dehydrated through graded ethanol, air dried, and mounted in Aquamount (Lerner Laboratories).

Several levels of controls were implemented. These include hybridization to anti-complementary (5′-to-3′) RNA of SRV-1, run in parallel with hybridization to the complementary (3′-to-5′) RNA strand, for all samples. An additional section was pretreated with RNase prior to hybridization to demonstrate that hybridization was due to RNA-RNA hybrids. Sections were also incubated with labeled RNA of no demonstrable homology to eliminate the possibility of nonspecific binding. Tissue controls included matched tissues from the two uninfected control rhesus monkeys, as well as SRV-1-infected and uninfected Raji cells. The Raji cells were infected and prepared as previously reported (30), had known levels of SRV-1 infection, and served as a batch control. Reproducibility was demonstrated by three repeat examinations of each tissue. A cell or tissue was judged infected according to methods previously described (13).
(parietal cortex), and axillary lymph node cryopreserved at necropsy were homogenized under liquid nitrogen. The homogenate was incubated overnight at room temperature in DNA extraction buffer (20 mM Tris hydrochloride [pH 7.6], 10 mM EDTA, 100 mM NaCl, 0.5% sodium dodecyl sulfate, 200 μg of proteinase K per ml). Further purification was done by multiple phenol-chloroform extractions and treatment with proteinase K and RNase A as previously described (26). DNA from these tissues (10 μg) was analyzed for the presence of SRV-1 by Southern blot hybridization after digestion with SphI as previously described (2, 42). Cloned DNA from the envelope region of SRV-1 was used as the hybridization probe. Posthybridization washes were performed under high stringency conditions consisting of three changes of 2× SSC for 10 min at room temperature and two 30-min washes in 2× SSC at 68°C, followed by one 30-min wash in 0.1× SSC at 68°C. Blots were then air dried and autoradiographed at −70°C for 1 to 2 days with X-Omat XAR5 film (Eastman Kodak) with Cronex Lightning-Plus (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) intensifying screens.

RESULTS

Immunohistochemistry. For seven of eight SRV-1-infected rhesus monkeys, the transmembrane envelope glycoprotein (gp20) of SRV-1 was detected by immunohistochemistry in three or more tissues, but never in the brain (Table 1). One animal with PGL and splenomegaly (21296) and the two uninfected controls had no detectable gp20 in any tissue. Seven animals had detectable SRV-1 gp20 in the lymph node and spleen. In these tissues, antigen was detected within germinal centers in a reticular or dendritic staining pattern (Fig. 1a). In animals with terminal SAIDS, most germinal centers were positive, whereas in animals with PGL and splenomegaly, only scattered germinal centers stained. SRV-1 gp20 was also detected in aberrant germinal centers in the thymic medulla of one of four animals with PGL and splenomegaly. Detection of SRV-1 in the thymus of the four monkeys with terminal SAIDS was limited to clusters of lymphocytes and rare unidentified stellate cells (Fig. 1b). For these four animals with terminal SAIDS, it was not possible to determine whether the gp20-positive cells were in the cortex or in the medulla of the thymus because of marked thymic atrophy characterized by lobular collapse and loss of distinction between cortex and medulla. In the spleen of the four animals with terminal SAIDS, perifollicular sinusoidal lining cells were intensely stained (Fig. 1c), as were germinal centers. Staining of sinusoidal lining cells of the spleen was not seen in the four animals with PGL and splenomegaly.

The richest sources of SRV-1 gp20 were the serous acinar cells of salivary glands, in which up to 80% of the cells in animals with terminal SAIDS were positive for gp20 (Fig. 1d). Only two animals with PGL and splenomegaly had salivary gland staining, and far fewer acinar cells were positive. For all animals, cells lining striated and intercalated ducts did not stain for SRV-1 gp20. Interestingly, an increase in gp20 antigen-positive acinar cells in salivary glands appeared to correlate with the advanced stage of the disease and the length of time the animal had been viremic (Table 2). Thus, while each animal with terminal SAIDS was positive regardless of the length of viremia, the percentage of positive

| TABLE 1. Tissue localization of SRV-1 transmembrane envelope glycoprotein (gp20) and nucleic acidsa |
|---|---|---|---|---|---|---|---|---|
| Rhesus monkey | LN | Thymus | Spleen | SG | Brain | LN | Thymus | Brain |
| With terminal SAIDS | | | | | | | | |
| 20263 | + | + | + | + | + | + | + | + |
| 20343 | + | + | + | + | + | + | + | + |
| 21877 | + | + | + | + | + | + | + | + |
| 21824 | + | + | + | + | + | + | + | + |
| With PGL and splenomegaly | | | | | | | | |
| 21292 | + | + | + | + | + | + | + | + |
| 21296 | - | - | - | - | - | - | - | - |
| 21304 | + | - | + | + | + | + | + | + |
| 21318 | - | - | + | + | + | + | + | + |
| Uninfected controls | | | | | | | | |
| 20245 | - | - | - | - | - | - | - | - |
| 20448 | - | - | - | - | - | - | - | - |

a SG, Parotid salivary gland; LN, axillary lymph node; ND, not determined

| TABLE 2. Correlation of stage of disease and length of viremia with the percentage of SRV-1 transmembrane envelope glycoprotein (gp20) antigen-positive cells in the salivary gland |
|---|---|---|
| Rhesus monkey | Length of viremia (mo)a | Result of salivary gland stainingab |
| With terminal SAIDS | 20263 | 24' | ++++ |
| 20343 | 12 | ++++ |
| 21824 | 8 | +++ |
| 21877 | 3 | + |
| With PGL and splenomegaly | 21318 | 20 | + |
| 21304 | 18 | + |
| 21292 | 6 | + |
| 21296 | 5d | + |

a Viremia was determined by Raji cell assay and confirmed by immunofluorescence as described previously (4, 14, 30). The length of viremia was determined from the date of inoculation to euthanasia.

b Percentage of cells staining for SRV-1 gp20: -, negative; +, 1 to 25%; + +, 25 to 50%; + + +, 50 to 75%; + + + +, >75%.

c A spontaneously occurring SAIDS case. The length of viremia was the time from the first positive viral culture to euthanasia.

d Virus negative at necropsy. The length of viremia was the time from inoculation to the last positive viral culture.
acinar cells increased with the length of viremia to approximately 80% in animal 20263 which had been viremic for at least 24 months. This observation was also made with two of four animals with PGL and splenomegaly that had positive salivary gland staining. These two animals had been viremic for 18 and 20 months (Table 2).

Rabbit anti-whole SRV-1 gave the same staining pattern as did the monoclonal antibody on sections of salivary gland and brain (data not shown) in that viral antigens were detected in salivary gland acinar cells but not in the brain. This polyclonal antiserum was used to confirm the results of the monoclonal antibody which recognizes only one epitope on one protein (gp20) of SRV-1. If there were alterations in the epitope of gp20 recognized by the monoclonal antibody or if gp20 was absent, this polyclonal antiserum still would have detected the virus.

Ultrastructure. Electron microscopy was performed to determine whether the immunohistochemical localization of SRV-1 gp20 correlated with productive infection. Typical type D viral particles (36) were observed in salivary gland, lymph node, and spleen but not in brain. Salivary glands contained large numbers of mature viral particles in acinar lumina and lateral intercellular canaliculi, with lesser numbers of intracytoplasmic A-type particles and budding type D particles in serous acinar cells (Fig. 2a). Neither budding particles nor intracytoplasmic A-type particles were seen in any other cell type in the salivary gland. In spleen and lymph node, rare extracellular mature viral particles were found (Fig. 2b and c). In lymph nodes, the viral particles were seen in germinal centers associated with cellular processes (Fig. 2b), possibly belonging to follicular dendritic cells. Thus, the ultrastructural localization of viral particles correlated with the immunohistochemical localization of SRV-1 gp20 in the salivary gland and lymphoid tissue.

**Distribution of viral nucleic acid and evidence of viral latency.** In situ RNA hybridization and Southern blot analyses were performed to explore the possibility of latent infections. In situ hybridization was done on sections of salivary gland and brain adjacent to those used for immunohistochemistry. This process revealed SRV-1 RNA in brain and salivary gland acinar cells (Fig. 3) in two of two animals with terminal SAIDS and in the brain of one of three animals...
FIG. 2. Electron microscopic demonstration of type D retroviral particles in salivary gland (a), germinal center of a lymph node (b), and spleen (c). (a) In the salivary gland, numerous mature extracellular particles in a small acinar lumen, as well as intracytoplasmic A particles (arrows) and a budding particle (inset), are seen. (b) In the germinal center, a single mature particle adjacent to a lymphoid cell and numerous cellular processes possibly belonging to follicular dendritic cells are seen. (c) In the spleen, a single mature particle is seen in the extracellular space.
with PGL and splenomegaly (Table 1). Most cells in the sections had no more than the background of 1 to 2 grains per cell, but in other areas, significant hybridization was evident. In these foci, discrete collections of grains were observed over and around nuclei of salivary gland acinar cells and rare unidentified, scattered cells in the brain. The numbers of silver grains over positive cells in the brain and salivary gland (Fig. 3) were comparable, indicating the presence of comparable amounts of viral RNA. Brain and salivary glands from the two uninfected control animals were negative.

Southern blots were performed on DNA extracted from adjacent areas in these same tissues (Fig. 4). The salivary glands and brains of those animals positive for SRV-1 RNA by in situ hybridization were also positive for SRV-1 DNA, as was the brain of one animal with terminal SAIDS not tested by in situ hybridization (Table 1). Southern blots of lymph nodes from each infected animal served as positive controls, and lymph nodes from the two uninfected animals served as negative controls. The intensity of the bands indicated that salivary glands and lymph nodes contained more viral DNA than did the brain. Thus, viral nucleic acid was identified by in situ hybridization, Southern blot, or both in the brains of four of six animals. None of these animals had detectable antigen in the brain (Table 1), suggesting that SRV-1 infection of the central nervous system was latent. One of these animals (21296; Table 1) was not viremic at necropsy and lacked detectable viral antigen in any tissue but did have viral nucleic acid in the lymph node and the brain, suggesting latent systemic infection.

**DISCUSSION**

We have surveyed the tissue distribution of SRV-1 in the salivary glands, lymph nodes, spleens, thymuses, and brains of eight rhesus monkeys with terminal SAIDS or PGL and splenomegaly by immunohistochemistry, in situ RNA hybridization, Southern blot analysis, and electron microscopy. Immunohistochemistry revealed that SRV-1 was commonly found in germinal centers of lymphoid organs. This finding, in conjunction with the demonstration of abundant viral DNA in lymph nodes by Southern blot and the ultrastructural identification of type D retroviral particles in...
germinal centers, suggests that germinal centers may be an important target of SRV-1. Infection of germinal centers could facilitate spread of the virus to other lymphocytes which circulate through germinal centers. In addition, infection of follicular dendritic cells, if present, could result in the disruption of the microenvironment necessary for appropriate antigen presentation. Detection of virus particles in this location has also been noted in other animal retrovirus models (8, 39, 40) and in humans infected with human immunodeficiency virus (45).

While germinal centers were the most consistent location for the detection of viral antigen, serous acinar cells of salivary glands were the richest source of viral particles and viral antigens, as detected by electron microscopy and by immunohistochemistry, respectively. This finding correlates with that of previous studies which showed that saliva is a major source of infectious virus and that SAIDS can be transmitted via saliva (11, 21). In addition, the percentage of SRV-1-infected cells in the salivary glands tended to correlate with the stage of the disease and the length of viremia. A similar situation occurs in feline leukemia virus-infected cats, for which epithelial infection and viral shedding characterize the final phase of progressive infection (40).

The result showing a relative abundance of viral particles and antigen in salivary glands compared with those in lymph nodes contrasts with the results of the Southern blot, in which equal amounts of cellular DNA (10 μg) from the lymph node and salivary gland gave signals of comparable intensities. Further in situ hybridization studies are necessary to determine whether this result is due to a higher percentage of infected cells or due to higher copy numbers of viral DNA per cell in the lymph node. Regardless, this finding suggests that whereas large amounts of viral DNA are present in lymph nodes, the salivary gland is more permissive to viral replication.

Differences in the intensity and prevalence of viral antigen were also seen between animals with terminal SAIDS and those with PGL and splenomegaly. In less severely ill animals (those with PGL and splenomegaly), viral antigen was not as prevalent, occurring in fewer tissues and in fewer loci within those tissues. Thus, in animals with PGL and splenomegaly, viral antigen was limited to germinal centers of lymphoid organs and scattered salivary gland acinar cells, whereas in animals with terminal SAIDS, viral antigen was also found in sinusoidal lining cells of the spleen, in stellate cells in the thymus, and in a higher percentage of cells in the salivary gland. This restricted distribution of viral antigen in animals with PGL and splenomegaly may be a reflection of the ability of the animals to control the spread of the virus and, thus, may explain the resulting milder disease.

One animal with PGL and splenomegaly that was particularly interesting was 21296 (Table 1). This animal was not viremic at necropsy and lacked detectable viral antigen in any tissue but did have SRV-1 nucleic acid in the brain and lymph node, suggesting viral latency. Alternatively, this animal may have been in the process of clearing itself of the virus. If the necropsy had occurred a few weeks later, it was possible that this animal would have been clinically normal without detectable SRV-1 nucleic acid in any tissue. Additional experiments involving SRV-1 antibody-positive, virus-negative rhesus macaques will be necessary to answer the question of viral latency.

Neuropathology and neurologic signs are not features of SRV-1-induced SAIDS. Coupled with the presence of SRV-1 RNA and/or DNA but no detectable antigen or viral particles in the brain of these monkeys, these data suggest that SRV-1 may be latent in the central nervous system. The lack of inflammation in the brain suggests that the SRV-1 genome-positive cells detected in the brain are resident cells and not inflammatory cells that have migrated into the central nervous system from the peripheral circulation. The amounts of viral RNA present in individual cells in the brain and in the salivary gland are comparable as assessed by in situ hybridization (Fig. 3). In contrast, no viral antigen or viral particles were detected in the brain, whereas the salivary glands were the richest source of viral particles and antigen, as revealed by electron microscopy and by immunohistochemistry, respectively. This finding supports the idea of viral latency in the central nervous system and makes it unlikely that the inability to detect viral antigen in the brain is due to differences in the sensitivity of detecting viral gp20 by immunohistochemistry and viral RNA by in situ hybridization. It does, however, suggest the possibility of postsynaptic restriction of SRV-1 gene expression, similar to that postulated for visna virus in sheep (43). Additional evidence in support of viral latency was the inability to detect viral antigens when rabbit polyclonal SRV-1 antiserum was used on the brains of two animals with evidence of SRV-1 RNA in the brain by in situ hybridization.

In addition, a partial transcriptional block was suggested by the in situ RNA hybridization results, in that very few cells in the brain were positive, seemingly too few to account for the signal seen by Southern blot. Further studies are needed to confirm the extent of viral latency in the brain, to identify the specific cells infected, and to see whether reactivation of virus from this latent state eventually occurs. It will also be of interest to determine whether a similar pattern of viral latency occurs in the central nervous system of humans infected with human immunodeficiency virus, in which the process of viral replication in different cell types remains largely unknown.

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LITERATURE CITED


