Sialic Acids on the Surface of Caprine Arthritis-Encephalitis Virus Define the Biological Properties of the Virus

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Received 2 November 1987/Accepted 17 February 1988

The lentivirus caprine arthritis-encephalitis virus (CAEV) is a pathogen of goats. It is transmitted in milk and causes a persistent infection in goats, which often fail to produce neutralizing antibodies to the virus. Native CAEV particles are remarkably resistant to digestion with proteinase K and are neutralized extremely slowly by immune sera. Our studies showed that the virus particles are heavily sialylated. Studies with highly specific sialyltransferase enzymes identified penultimate carbohydrate linkages typical of O- and N-linked oligosaccharides on the virus and suggested that the virus may be more heavily sialylated on O-linked than on N-linked oligosaccharides. Removal of sialic acids from the virus by neuraminidase treatment did not reduce infectivity of the particles. However, desialylation rendered the virus more susceptible to proteolysis by proteinase K. Desialylation also enhanced the kinetics of neutralization of the virus by goat antibodies. These results suggest that the carbohydrates on the viral surface are important both in protecting viral proteins from digestion by proteases and in protecting the virus from rapid neutralization by antibodies.

Caprine arthritis-encephalitis virus (CAEV) is a lentivirus of goats and is closely related to the etiologic agent causing visna-maedi infection in sheep (14, 20). These viruses are replication-competent, nononcogenic retroviruses whose physical, chemical, and biological properties closely resemble those of the human immunodeficiency viruses (5). These viruses have large (approximately 10-kilobase) genomes whose *env* gene encodes a single, large, highly glycosylated protein that forms the envelope of the virion (21, 25, 29). This structure contains the epitopes that bind the virus to cellular receptors and neutralizing antibodies (7, 12, 24, 27). The viruses are transmitted horizontally in body fluids and cause persistent infection in natural hosts by infecting cells of the immune system (3, 9, 16). Viral persistence is associated with minimal or low titers of neutralizing antibodies (15, 23, 31). In addition, these antibodies have low binding affinities for the viruses (7). Natural infections are indolent and give rise to chronic disease weeks to months after the initial infection.

In keeping with the general properties of lentiviruses, CAEV has a tropism for cells of the macrophage lineage (4, 16). The virus is spread artificially or naturally by feeding newborn kids virus-contaminated colostrum (8). Virus is present in colostrum macrophages, and infection of the neonatal animal occurs either by direct absorption of infected macrophages across intestinal villi or by infection of the intestinal lining cells with virus liberated from macrophages previously digested by proteolytic enzymes in the gastrointestinal tract. During the persistent infection that follows, infected goats produce binding antibodies to all viral polypeptides, but most animals do not produce virus-neutralizing antibodies (10). Neutralizing epitopes are present in the virus envelope, but they are not recognized by the immune system of an infected or immunized animal even though the animal remains immunocompetent (15).

The ability of CAEV to escape proteolytic digestion in the gastrointestinal tract and to sequester its neutralization

epitopes during infection suggests that surface components of the virion provide an effective barrier to adverse environmental factors. Carbohydrates have been previously shown to protect viral proteins from protease degradation and also to be responsible for the reduced affinity of influenza virus hemagglutinin for a monoclonal antibody (26, 28). Experiments reported here show that the external surface of the CAEV virion is highly sialylated. These sialic acid molecules appear to be attached to both N- and O-linked oligosaccharides and provide the basis for some of the highly unusual biological properties of the virus.

MATERIALS AND METHODS

Viruses. Visna virus 1514 and CAEV CO were used in these studies. The history of these viruses has been described previously (2, 13, 15, 18). The viruses were cultivated in cultures of either goat synovial membrane cells or primary goat macrophages (described below). Infectious supernatant fluids were concentrated by Pelicon membrane filtration followed by centrifugation through sucrose gradients, as described in previous reports (6, 15). Purified virus concentrates were dialyzed against 0.1 M sodium chloride–1 mM EDTA–20 mM Tris buffer (pH 7.4) (NET), aliquoted, and stored at -70°C until used. Some batches of virus were radiolabeled with [^{35}S]methionine (1,128 Ci/mM, Du Pont NEN Products, Boston, Mass.) added to the cultures 1 day after inoculation. Supernatant fluids were collected at 8-h intervals for 2 days. Viruses from these preparations were purified as described previously (7). Influenza virus PR8 and the paramyxovirus Sendai virus were kindly provided by R. G. Webster, St. Jude's Children's Research Hospital, Memphis, Tenn. Virus stocks were prepared by inoculation into the allantoic sacs of 10-day-old chicken embryos. Allantoic fluid was harvested 2 days later, aliquoted, and stored at -70°C until used. Vesicular stomatitis virus (VSV) New Jersey was kindly provided by Paula Pitha, Oncology Center, Johns Hopkins University, Baltimore, Md. Virus was propagated in cultures of goat synovial membrane cells. Mouse hepatitis virus (MHV A29) was kindly provided by

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K. Holmes of the Uniformed Services University, Bethesda, Md. This virus was propagated and purified from inoculated NIH 3T3 cell cultures.

Cells. Infectivity assays of visna virus, CAEV, and VSV were performed in monolayers of a cell line of goat synovial membrane cells prepared from the synovium of a colostrum-deprived newborn goat kid, as previously described (13). Primary macrophage cultures were prepared from peripheral blood leukocytes of mature, uninfected goats (16). Assays of MHV were performed on cultures of NIH 3T3 cells. Chicken erythrocytes used for hemagglutination assays were obtained from 7- to 14-day-old chicks and stored at 4°C in 50% Alsever solution for up to 1 week. Prior to being used for these assays, the cells were washed three times in phosphate-buffered saline (PBS) (pH 7.4).

Treatment of virus with neuraminidase. Neuraminidase derived from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, Mo.) was added to purified virus preparations at a concentration of 500 mU/ml in minimal essential medium at 37°C for 45 min. Samples were then either treated with proteinase K (PrK) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) or mock treated and incubated for an additional 30 min at 37°C. Neuraminidase from *Vibrio cholerae* (Calbiochem-Behring, La Jolla, Calif.) was used in the sialyltransferase-labeling experiments described below. The enzyme was used at a concentration of 200 mU/ml in 50 mM sodium acetate–4 mM CaCl₂–155 mM NaCl at a pH of either 5.5 or 7.4 (depending on the specific experimental conditions described below), and the solution was incubated at 4°C for 1 h.

Treatment of virus with PrK. The stock solution of PrK was made at a concentration of 1 mg/ml in 50 mM Tris hydrochloride–1 mM CaCl₂ (pH 8), aliquoted, and stored at –20°C. PrK was mixed with virus at various concentrations and incubated at 37°C for 30 min in a shaker water bath. Each sample was then diluted in cold minimal essential medium supplemented with 1% fetal bovine serum, and serial dilutions of 1:3.2 (0.5 log₁₀) were prepared. These were then inoculated into cultures of indicator cells (GSM cells) in microdilution plates.

Sialyltransferase labeling of virus. A modification of the procedure described by Whiteheart and Hart (32) was used. Purified CAEV (1 ml) was brought up to 5 ml in 5-ml polyallomer tubes with NET buffer and then pelleted in an SW50.1 swinging bucket rotor (Beckman Instruments, Fullerton, Calif.) at 100,000 × g for 1 h in a Sorvall ultracentrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). For some experiments 100-μl samples were pelleted in 250-μl polyallomer tubes at 90,000 × g for 40 min in a Beckman Airfuge centrifuge. The pellets were then either treated with neuraminidase (200 mU of *V. cholerae* neuraminidase per ml at 4°C for 1 h) or mock treated and then repelleted and washed three times in 100 mM NaCl–20 mM Tris (pH 7.4). The pellets were solubilized in 80 μl of 100 mM HEPES (N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 6.5)–0.5% Nonidet P-40–10% aprotinin and vortexed at 4°C. Then either α2,3Galβ1,3GalNAc sialyltransferase (purified from porcine submaxillary salivary gland) (22) or α2,6Galβ1,4GlcNAc sialyltransferase (purified from rat liver) (28) was added at the appropriate concentration. Cytidine 5'-monophospho-*N*-acetyl-[³H]neuraminic acid (CMP-[³H]NANA; 13.6 Ci/mmol; Du Pont, NEN Research Products) was stored in ethanol. Before being used, it was dried and suspended to its original volume (10 μl per sample) in deionized water. CMP-[³H]NANA was then added to each sample to initiate radiolabeling. Samples were incubated at

37°C for 1 h. The reaction was stopped by adding 100 μl of 100 mM ammonium formate–0.2% sodium dodecyl sulfate (SDS)–100 mM EDTA and then boiling the samples for 2 min. Samples were then frozen and stored at –70°C until analyzed.

Radiolabeled macromolecules were separated from unincorporated label on a Sephadex G-50 column and collected in 1-ml aliquots. Fractions containing radiolabeled macromolecules from the void volume were pooled for each sample. These samples were then lyophilized, suspended in a 1-ml volume of water, and precipitated with 8 volumes of cold acetone for 15 h at 4°C. The precipitate was pelleted at 2,800 × g for 10 min and suspended in SDS-polyacrylamide gel electrophoresis sample buffer.

Hemagglutination tests. Serial twofold dilutions of PR8 or Sendai virus suspensions were prepared in 0.5 volumes of PBS. Then 100 μl containing 0.1 mg of protein from purified CAEV or visna virus was added to each well. PBS (100 μl) was added to the control dilutions, and all samples were incubated for 2 h at 4°C. Then 100 μl of 0.5% erythrocytes (RBCs) in PBS was added to each well. The suspensions were mixed, and the plates were incubated at 4°C for approximately 1 h.

Kinetic neutralization tests. These tests were performed by methods described previously (15). Briefly, a 10⁶ 50% tissue culture infective dose of CAEV was mixed with a 1:10 dilution of neutralizing serum, and at appropriate time points aliquots were removed and immediately diluted in cold minimal essential medium with 1% fetal bovine serum. Serial 0.5 log₁₀ dilutions were then prepared in maintenance medium and inoculated into indicator GSM cell cultures in microdilution plates. Cytopathic end points were determined 2 days later for VSV assays and 14 days later for CAEV assays.

Thiobarbituric acid assay. A high-pressure liquid chromatography-adapted assay was performed as previously described by Powell and Hart (19). Briefly, standards were prepared from NANA (Sigma Chemical Co.). Sodium periodate solution (20 μl; stock solution consisted of 128 mg of sodium metaperiodate, 1.7 ml of phosphoric acid, 1.3 μl of water) was added to 40-μl samples or standards and incubated for 20 min at room temperature. Sodium arsenite (100 μl of a 10% solution in 0.1 N H₂SO₄) was added slowly while the tube was vortexed gently. After 5 min of incubation at room temperature, 600 μl of 0.6% thiobarbituric acid solution was added. The tubes were then boiled for 15 min, cooled to room temperature, and centrifuged for 2 min at 10,000 × g immediately before injection of a sample (250 μl) for high-pressure liquid chromatography analysis on a C18 reverse-phase column (Alltech Associates, Inc., Deerfield, Ill.). The sialic acid-chromagen peak was read with the wavelength detector set at 549 nm.

Sensitivity of viral proteins to PrK. ³⁵S-labeled CAEV purified as described above was lysed in a buffer containing 0.5% SDS, 1 mM CaCl₂, and 50 mM Tris (pH 7.6). Portions of this lysate were treated with different concentrations of PrK, and all samples were analyzed by electrophoresis in a 5 to 20% acrylamide gel containing 0.1% SDS (11). The gels were fluorographed and exposed to X-ray film at –70°C.

RESULTS

Resistance of infectious CAEV particles to inactivation with PrK. Preliminary studies on the physical properties of CAEV showed that this virus resisted inactivation by trypsin at concentrations used to dissociate cells (data not shown).

FIG. 1. Comparative sensitivities of CAEV (■), visna virus (▨), and MHV (▤) to PrK after incubation at 37°C for 30 min.

This property was investigated more thoroughly by studying the effects of PrK on purified virus particles. CAEV was purified on sucrose gradients and diluted in minimal essential medium to contain approximately 10^6 50% tissue culture infective doses per ml. Samples of these virus preparations were mixed with increasing concentrations of PrK, incubated at 37°C for 30 min, and then assayed for residual infectivity. The virus resisted inactivation with PrK at concentrations between 10 and 500 μ g/ml (Fig. 1). Visna virus, which is genomically and antigenically closely related to CAEV, was purified under conditions similar to those used for CAEV and was used as a control in these experiments. Purified MHV A29, a coronavirus, was also used as an unrelated enveloped virus. Both of the latter two agents were inactivated by 10 μ g of PrK per ml.

The different sensitivities of CAEV and visna viruses to PrK were obtained with viruses grown in GSM cell cultures. Since macrophages are the natural host cells for virus replication *in vivo*, it seemed relevant to ask whether the same effect would be obtained with virus cultivated in primary cultures of macrophages. Macrophage-derived CAEV was therefore examined under the experimental conditions described above. CAEV resisted inactivation by more than 600 μ g of PrK per ml. Thus, infectious CAEV particles were innately resistant to extremely high concentrations of PrK.

We next investigated whether the denatured polypeptides of disrupted CAEV particles have a similar resistance to proteolytic degradation as the intact virions and also whether purified CAEV contained any protease inhibitors which could adversely affect the enzymatic properties of PrK. Various concentrations of PrK were incubated with purified CAEV for 30 min at 37°C. The virus particles were then pelleted at $90,000 \times g$. The supernatant, which contained 0, 10, and 100 μ g of PrK per ml, was mixed with purified ^{35} S-labeled virus particles which had been solubilized with 0.5% SDS before PrK treatment. After incubation at 37°C for 30 min, proteolysis was monitored by SDS-polyacrylamide gel electrophoresis. The gel was subsequently fluorographed and autoradiographed to identify polypeptide species. The major polypeptide bands of the virus were labeled distinctly and visibly after treatment with 0.5% SDS alone (Fig. 2, lane 1). However, the lanes con-

taining similar numbers of counts of electrophoresed PrK-treated material were blank, suggesting that the denatured viral proteins of the lysed virus were completely degraded by treatment with as little as 10 μ g of PrK per ml. These data suggested that our CAEV preparations did not inhibit PrK activity, since preincubation with unlabeled CAEV did not interfere with PrK activity on the radiolabeled, lysed virus. In addition, these data suggested that the infectious particle might be covered with surface structures that prevent access of the protease to labile protein structures within the particle.

Identification of sialic acids on the surface of CAEV. Carbohydrate moieties on the surfaces of the virus particles are logical candidates for preserving infectivity of the agent in the presence of proteolytic enzymes. We tested initially for the presence of these molecules on CAEV by determining whether preincubation of the purified particles with a myxovirus, PR8, and a paramyxovirus, Sendai virus, would cause reduction in the ability of these two viruses to cause hemagglutination. Both of these viruses have surface hemagglutinin proteins which have a high affinity for sialic acids on the surfaces of erythrocytes. This binding results in hemagglutination. Purified CAEV was added to each of the two virus suspensions to determine whether putative sialic acid molecules on CAEV would bind the hemagglutinin proteins of each of the two viruses and thus prevent the latter from binding to erythrocytes. CAEV caused an 8- to 16-fold reduction in hemagglutination by Sendai and PR8 viruses, respectively (Table 1). This suggested that considerable amounts of sialic acids are present on the surfaces of CAEV particles. Sialic acids were also demonstrated on visna virus, confirming an earlier report (1). However, the visna virus preparation was less efficient in reducing hemagglutination in the two hemagglutinating viruses than an equivalent (based on protein content) concentration of CAEV. The suggested that visna virus contains fewer sialic acids than CAEV.

FIG. 2. Proteolysis of ^{35} S-labeled CAEV which had been lysed and denatured by SDS. Various concentrations of PrK were preincubated with unlabeled virus. The samples were centrifuged to pellet the virus particles. Supernatant containing PrK was used to digest ^{35} S-labeled CAEV which had been lysed in 0.5% SDS. The products were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The numbers at the left represent the positions of prestained molecular weight markers. Lane 0, SDS-disrupted ^{35} S-labeled CAEV with no PrK treatment. Lanes 10 and 100, SDS-disrupted ^{35} S-labeled CAEV that had been treated with 10 and 100 μ g of PrK per ml, respectively.

TABLE 1. Hemagglutination inhibition of PR8 and Sendai viruses by CAEV and visna virus^a

Virus	Hemagglutination titer
Sendai virus	1:2,560
Sendai and CAEV	1:320
Sendai and visna virus	1:1,280
PR8	1:320
PR8 and CAEV	<1:20
PR8 and visna virus	1:40

^a CAEV and visna viruses were purified on sucrose density gradients and used at equivalent concentrations on the basis of protein content. PR8 and Sendai viruses were used as infectious allantoic fluids from inoculated chicken embryos.

We next performed experiments to confirm the presence of sialic acids on the virus particles and to identify the oligosaccharides to which these molecules are attached. Increasing concentrations of the exoglycosidase *V. cholerae* neuraminidase were added to purified virus particles, and the mixtures were examined for release of sialic acids by using a high-pressure liquid chromatography-thiobarbituric acid assay. Increasing amounts of sialic acids were removed by corresponding increases in the concentration of neuraminidase enzyme (Fig. 3). In independent experiments, it was shown that saturation of removal of sialic acid by *V. cholerae* neuraminidase occurs at concentrations of >20 mU of the neuraminidase per sample. To quantitate the total sialic acid present in a sample (100 μ l of gradient-purified virus; 1 mg of protein per ml; 10^7 50% tissue culture infective doses per ml), virus was incubated in 0.1 N hydrochloric acid at 80°C for 1 h. Approximately 4,300 pmol of sialic acid was released. This suggested that nearly all of the sialic acid on the virus is sensitive to hydrolysis by *V. cholerae* neuraminidase (Fig. 3).

In order to identify the penultimate oligosaccharide chains to which the sialic acids had been attached, we used linkage-specific sialyltransferase enzymes. The sialyltransferases are highly specific for the transfer of radiolabeled sialic acids from a nucleotide-sugar donor to specific oligosaccharide acceptor sites on the particles. We chose one sialyltransferase which recognizes acceptors found almost exclusively on N-linked oligosaccharides (rat liver $\alpha 2,6$ Gal $\beta 1,4$ GlcNAc

FIG. 3. Release of native sialic acid from 100 μ l of gradient-purified CAEV by neuraminidase as monitored by a high-pressure liquid chromatography-adapted thiobarbituric acid assay.

FIG. 4. Saturability of sialyltransferase-mediated incorporation of ^{3} H-sialic acids into CAEV. For each data point, 100 μ l of gradient-purified CAEV was pelleted, treated with *V. cholerae* neuraminidase, and spin washed three times. The pellets were solubilized and incubated with CMP-[^{3} H]NANA. Increasing concentrations of either $\alpha 2,3$ Gal $\beta 1,3$ GalNAc sialyltransferase ($\square$) or $\alpha 2,6$ Gal $\beta 1,4$ GlcNAc sialyltransferase ($\blacklozenge$) were added and the products were fractionated on a Sephadex G-50 gel filtration column. The number of counts incorporated was plotted against the amount of enzyme used.

sialyltransferase) (30) and another sialyltransferase whose acceptor linkage is found almost exclusively on O-linked oligosaccharides (porcine submaxillary $\alpha 2,3$ Gal $\beta 1,3$ GalNAc sialyltransferase) (22). Purified CAEV was treated with neuraminidase at twice the levels (40 mU) described above to remove the native sialic acid from the surfaces of the particles. The sialyltransferase and CMP-[^{3} H]NANA were then used to radiolabel either the N- or the O-linked oligosaccharides. Macromolecules were separated from unincorporated radioactivity by gel filtration chromatography. The results of these resialylation experiments are shown (Fig. 4). The products of gel filtration were electrophoresed by SDS-polyacrylamide gel electrophoresis and subjected to fluorography and autoradiography.

Figure 5 shows that [^{3} H]NANA was incorporated into penultimate structures typically specific for O- or N-linked oligosaccharides on the virus particles. Almost no counts were incorporated into the enzyme-alone control, showing that little autosialylation of the enzyme had occurred. In the native state, these carbohydrates were apparently fully sialylated, since only a small amount of [^{3} H]NANA was incorporated when transferase and CMP-[^{3} H]NANA were added to virus particles that had not been pretreated with *V. cholerae* neuraminidase. Removal of the native sialic acids with neuraminidase made available the proper acceptor linkages. These results thus suggested that CAEV is highly glycosylated on both N-linked and O-linked oligosaccharides. They also indicated that CAEV contains many more penultimate Gal $\beta 1,3$ GalNAc moieties than Gal $\beta 1,4$ GlcNAc moieties, suggesting that the envelope protein may contain more O-linked oligosaccharide side chains than N-linked side chains.

Removal of sialic acids from CAEV and effects of PrK inactivation. In this experiment we determined whether sialic acids from the virus particles were responsible for the resistance of the particles to proteolysis. We compared mock-treated virus with neuraminidase-treated virus, with PrK-treated virus, and with virus treated with both neuraminidase and PrK. The results of this experiment are shown

FIG. 5. SDS-polyacrylamide gel electrophoresis of CAEV radio-labeled with CMP-[^3H]NANA and sialyltransferases. Lanes: 1, molecular weight markers (in thousands; 200, 92.5, 69, 46, 30, and 14.3); 2, blank; 3, CAEV pretreated with neuraminidase followed by treatment with the N-linked specific sialyltransferase ($\alpha 2,6$ Gal $\beta 1,4$ GlcNAc sialyltransferase) and CMP-[^3H]sialic acid; 4, the same transferase-labeled CAEV as in lane 3 without neuraminidase pretreatment; 5, CAEV pretreated with neuraminidase followed by treatment with the O-linked specific sialyltransferase ($\alpha 2,3$ Gal $\beta 1,3$ GalNAc) and CMP-[^3H]NANA; 6, the same transferase-labeled CAEV as in lane 5 without neuraminidase pretreatment.

in Fig. 6. Treatment of the virus with neuraminidase did not result in loss of infectivity. Since the virus particles were treated with a higher concentration of enzyme than that which had denuded the particles of sialic acids as described above, we inferred that sialic acids were not essential for infectivity of the virus. The increased inactivation of the virus preparation treated with PrK after treatment with neuraminidase in comparison with viruses treated with PrK alone suggested that the particles deficient in sialic acids

FIG. 6. Effect of neuraminidase on sensitivity of CAEV to inactivation by PrK. Infectivity of mock-treated virus was compared with that of virus treated with neuraminidase for 45 min at 37°C and virus treated with a mixture of both enzymes for 30 min at 37°C. Values are the geometric means of three experiments.

FIG. 7. Kinetics of neutralization between CAEV (■) and VSV (●). Immune serum was used at dilutions of 1:100 for VSV and 1:10 for CAEV. Serum-virus mixtures were incubated at 37°C, and at specified intervals aliquots were removed, diluted 1:100 in cold medium, and assayed for infectivity. Similar dilutions of normal lamb serum mixed with CAEV (□) and VSV (○) were used as controls.

were more susceptible to proteolytic digestion than were naturally sialylated particles. Therefore, sialic acids on the virus particles may be responsible in part for the remarkable resistance of the virus to inactivation with PrK.

Removal of sialic acids from CAEV enhanced neutralization of the virus. Since removal of sialic acids from CAEV did not affect infectivity, we performed further experiments to determine whether removal of these molecules would affect the kinetics of neutralization of the virus. Kinetic neutralization tests showed that the virus was neutralized at extremely slow rates, requiring incubation of virus and antibody for 30 min before any reduction of infectivity of the virus occurred (Fig. 7). The animal which produced these poor anti-CAEV neutralizing antibodies was immunized with VSV, and the kinetics of neutralization of this virus were determined with serum from the animal. The antibodies neutralized VSV extremely rapidly (Fig. 7). We hypothesized, therefore, that the slow kinetics of neutralization of CAEV may be due in part to the high level of sialylation of this virus. We therefore

FIG. 8. Kinetics of neutralization of neuraminidase-treated CAEV. Neuraminidase-treated virus (■) and mock-treated virus (◆) were mixed with CAEV-neutralizing serum. At specified time points aliquots of each were removed, diluted into a 0.5 log₁₀ series, and assayed for infectivity. Neuraminidase-treated virus mixed with normal lamb serum (□) was used as a control.

performed kinetic neutralization tests using stock CAEV and neuraminidase-treated CAEV. Figure 8 shows that the desialylated CAEV was neutralized much more rapidly than the native infectious particles were. These results suggested that the extensive amounts of sialic acid on the virus particles prevented the CAEV from being neutralized at a rapid rate.

DISCUSSION

These studies showed that carbohydrate moieties on the surfaces of infectious CAEV particles contribute immensely to the biological properties of the virus. Sialic acids conferred a remarkable degree of resistance to degradation of the virus by proteolytic enzymes and to neutralization of the agent by antibodies in postinfection serum. Both characteristics are probably important for the survival of the agent in nature, because resistance to proteolytic enzymes in the gastrointestinal tract would facilitate infection in intestinal cells, and resistance to neutralizing antibodies would facilitate escape from humoral immune control and thus potentiate persistence of the virus. Although lentiviruses are known to have highly glycosylated envelopes, the function of the carbohydrate elements had not previously been identified. This is the first report showing the biological importance of these molecules.

The envelope of CAEV is poorly characterized. However, the close relationship between this virus and the more thoroughly studied visna virus has provided a basis for predicting some of its properties. The two viruses have extensive homology in the *gag* and *pol* regions of their genomes but more limited homology in the *env* gene which encodes the envelope glycoprotein. The envelope glycoprotein is approximately 135 kilodaltons in size and consists of a hydrophobic transmembrane portion and a larger, hydrophilic external portion. The nucleotide sequence of the *env* gene of visna virus indicates the existence of 28 potential N-linked glycosylation sites (29). Presumably all of these are in the hydrophilic external region of the glycoprotein molecule. The sequence of the *env* gene of CAEV has not yet been published, but it may contain as many if not more glycosylation sites than visna virus. This may be a common factor among lentiviruses, since the human immunodeficiency virus has nucleotide sequences that indicate the existence of 31 potential N-linked glycosylation sites (21, 25).

Biologically, the envelope of visna virus contains the determinants for binding of the virus to cell receptors as well as to neutralizing antibodies. Earlier studies by August et al. (1) had shown that this virus contains surface sialic acids that bind to the hemagglutinin of influenza virus. The envelope of CAEV has similar but more potent biological properties. The fact that CAEV inhibited myxovirus and paramyxovirus hemagglutination to a greater degree than visna virus suggested that the former virus contains either more sialic acids than visna virus or that its sialic acids are arranged in a configuration different from those of visna virus. Such structural differences may explain the vast difference between the two viruses in resistance to inactivation by proteases. The different sialylation of the two viruses must be related to the nucleotide sequences of the *env* gene, because the disparity between the two viruses was maintained whether they were propagated in GSM or macrophage cell cultures. It is possible that the sialylation pattern of the CAEV envelope is responsible for the poor antigenicity of neutralization epitopes because it conceals the protein components of these

molecules. It is of interest that visna virus, which seems to have fewer sialic acids than CAEV, is a better inducer of neutralizing antibodies (15).

The sialyltransferase assay provided a novel and accurate method for identifying specific penultimate structures that are typically found on O- or N-linked oligosaccharides. The biological significance of these two types of complex saccharides is not clearly understood. O-linked sugars have only recently been identified as constituents of viral glycoproteins (17). Since our data suggests that these molecules predominate over N-linked sugars on the surface of CAEV, they may be responsible in part for the unique biological properties of the virus. Further studies on the structural analysis of the viral coat glycoprotein and its oligosaccharides and comparison with studies of the closely related visna virus will shed more light on this subject. Regardless of this, however, data in this report show that carbohydrate molecules on the surfaces of these viruses may temper the biological properties of the virus such that chances for survival of the virus in an immunocompetent host are greatly enhanced. Further, strategies for vaccine production against these viruses and the human immunodeficiency viruses will have to take into account the fact that carbohydrate molecules on the virus surface may impede swift neutralization by antibodies and thus reduce the effectiveness of such vaccines.

ACKNOWLEDGMENTS

These studies were supported by Public Health Service grants AI25744, RR00130, NS21916, NS12127, and CA42486 from the National Institutes of Health.

We thank Antonino Passaniti, Wally Whiteheart, Gordon Holt, and Robert Haltiwanger for helpful discussions; Mark Bamberger for assistance with hemagglutination experiments; and Linda Kelly for assistance in preparation of the manuscript.

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