Further Biochemical Characterization, Including the Detection of Surface Glycoproteins, of Human, Calf, and Simian Rotaviruses

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Polyacrylamide gel electrophoretic analysis of purified preparations of the simian rotavirus SA-11 indicated eight polypeptide components that migrated in a manner remarkably similar to those of the previously characterized human and calf rotaviruses. Analyses of preparations of single-shelled and double-shelled particles of human, calf, and simian rotaviruses have also permitted assignment of the polypeptides to the inner or outer shells. The major components of the outer shells of each virus have been identified as glycoproteins, and the importance of this in terms of host cell specificity is discussed. Sensitivities of the various rotaviruses to acid, proteases, and glycosidases were also investigated.

The polypeptides of human and calf rotaviruses (infantile gastroenteritis virus and neonatal calf diarrhea virus) have been fractionated in polyacrylamide gels by two groups of workers (26, 28). The two viruses have very similar polypeptide components, although they can be distinguished from each other (28). It is also known that rotaviruses have a double-shelled capsid structure (2, 4, 12), and, in a previous communication, we suggested that particular polypeptides were associated with the inner shell; the remainder most probably comprises the outer shell (28). This communication confirms these observations and also includes comparative investigations on the polypeptides of the simian rotavirus SA-11. In particular, we report that major outer shell polypeptides of calf, human, and simian rotaviruses are glycosylated. Recently, Krystal et al. (20) have shown that the major outer shell polypeptide of reovirus type 3 (μ2) is glycosylated, thus confirming earlier reports by Lerner and co-workers (22, 23) that carbohydrates may be components of reoviruses. This finding illustrates another similarity between rotaviruses and reoviruses, which together with adenoviruses (16) represent examples of nonenveloped viruses shown to contain glycoproteins. The detection of surface glycoproteins in rotaviruses provided a basis for the hypothesis concerning their host cell specificity and mechanism of infection proposed by Holmes et al. (14).

MATERIALS AND METHODS

Viruses. Human rotavirus was extracted from stools collected from infants with acute enteritis. Calf rotavirus was extracted from stools collected from natural outbreaks of "white scours" among young calves. All analyses were conducted on virus from single stools of infants or calves, i.e., not mixed pools.

Simian rotavirus, SA-11, was kindly supplied by H. Malherbe and was propagated in primary cynomolgus monkey (Macaca fascicularis) kidney epithelial cells (Commonwealth Serum Laboratories [CSL], Australia). The cells were grown in medium 199 (CSL, Australia) with lactose plus 5% fetal calf serum. During virus propagation, bovine serum albumin fraction V (CSL, Australia) at a concentration of 0.05% was substituted for the calf serum. SA-11 virus remained largely cell associated during replication, so cells were harvested 5 to 6 days postinfection, depending on the degree of cytopathic effects (11), and frozen and thawed to release virus particles.

Purification of viruses. Human and calf rotaviruses were purified as described previously (28). SA-11 virus was extracted from infected cells and purified in the same way. Single- or double-shelled virus preparations were obtained by centrifuging to equilibrium in cesium chloride and then harvesting the appropriate band (28). Purity of the preparations was judged on criteria previously reported (28).

Electrophoresis of polypeptides and molecular weight determinations. Disassociated polypeptides were fractionated and stained in polyacrylamide gels by the procedure described previously (28). Briefly, the procedure involved electrophoresis of the polypeptides in 8.75% polyacrylamide gels in the discontinuous buffer system described by Laemmli (21). Polypeptides were stained with Coomassie brilliant blue. Molecular weights of the polypeptides...
were estimated by comparing their electrophoretic mobilities with those of known standards. The values listed in Table 1 represent an average of several determinations. The amounts of virus in the samples to be electrophoresed were judged to be approximately equivalent, on the basis of electron microscopic observations, but were not quantitated biochemically.

Detection of glycosylated polypeptides. Virus was dissociated and electrophoresed as described above except that a fivefold increase in the quantity of virus was required for detection of glycoproteins. Alternatively, after extraction of the RNA (28), viral polypeptides were recovered from the phenol phase by a slight modification of a procedure described by Obijeski et al. (27). Briefly, the polypeptides were precipitated by adding 7 volumes of cold ethanol and 5 M NaCl to a final molarity of 0.5 and then leaving the solution to stand overnight at −20°C. The flocculated polypeptides were pelleted at 9,500 × g for 30 min at 4°C, washed twice with cold acetone, and suspended in Laemmli sample dissociation buffer ready for electrophoresis as described above. The fractionated polypeptides were then stained using Grossman and Neville’s modification of the periodic acid-Schiff stain reaction for carbohydrates (13). This modification ensures removal of all sodium dodecyl sulfate from the gel column prior to staining and maintains an acidic environment to avoid simple acid-base staining. Omission of these precautions can lead to false-positive reactions, where all polypeptides would appear to be glycosylated.

Negative-contrast electron microscopy. Virus preparations were negatively stained with 0.1% solution of ammonium molybdate. Specimens were examined in an Hitachi HU11A electron microscope operating at 50 kV with an instrument magnification of ×30,000.

Acid stability of calf and simian rotaviruses. Stability of calf and simian rotaviruses in an acidic environment was examined in the following manner. A loop drop (0.5 μl) of virus was added to 25 μl of the following buffers: (i) Walpole’s acetate buffer at pH 4 and 5 (8); (ii) McIlvaine’s citric acid-phosphate buffer at pH 3, 4, 5, 6, 7, and 8 (8); and (iii) Gomori’s succinate at pH 3, 4, and 5 (8).

As controls, virus was also added to phosphate-buffered saline, pH 7.2 (9), and to doubly distilled water.

One test series was incubated at 37°C for 1 h and a second at room temperature for an hour (30), prior to examination by electron microscopy.

Degradation of human, calf and simian rotaviruses with proteases. The susceptibility of rotaviruses to proteolytic degradation was examined in the following way. Purified (from sucrose and CsCl gradients) and semipurified (from sucrose gradient only) preparations of the viruses were diluted in doubly distilled water and added to an equal volume of each of the following solutions: (i) trypsin, crystallized once, B grade (Calbiochem, La Jolla, Calif.), at a concentration of 400 μg/ml in phosphate-buffered saline, pH 7.4. The effect of trypsin on calf rotavirus was also examined by addilution at a concentration of 1 mg/ml. (ii) Papain, crystallized twice (Sigma Chemical Co.), at a concentration of 200 μg/ml in 0.2 M phosphate buffer, pH 6.0, which contained MgSO4 and cysteine to ensure enzyme activity. (iii) Pronase, B grade (Calbiochem), at concentrations up to 1 mg/ml in PBS, pH 7.4. (iv) α-Chymotrypsin, type II (Sigma), at a concentration of 200 μg/ml in 0.2 M Tris-hydrochloride, pH 7.5 or 8.0, which contained either Na+, K+, Cs+, NH4+, or no added monovalent cations. The monovalent cations were supplied as chloride salts of reagent grade at a concentration of 0.1 M. Calf rotavirus was also examined with other concentrations of α-chymotrypsin varying between 25 and 1,000 μg/ml in the presence of Cs+. The Cs+ cation was chosen in view of its importance in the uncoating of reoviruses (3). (v) Bromelain, grade II (Sigma), at a concentration of 13 mg/ml in 0.1 M Tris-hydrochloride, pH 7.2, which contained 0.001 M EDTA and 0.05 M dithiothreitol (6). The effect of bromelain was only examined on calf rotavirus. For controls, diluted virus was added to an equal volume of the appropriate buffer without enzyme. All solutions were tested for proteolytic activity with a general substrate, Azoceoll (Calbiochem). The tests were conducted according to the manufacturers’ specifications.

The virus-enzyme mixtures and controls were incubated at 37°C, except in the case of the papain-containing mixture, which was allowed to stand at room temperature. Samples were removed after incubation for 1, 2, and 19 h and examined by electron microscopy for any morphological alterations.

Degradation of human, calf, and simian rotaviruses with β-galactosidase or lactase. The susceptibility of double-shelled rotavirus particles to β-galactosidase or lactase was determined as follows. Purified preparations of virus were diluted with doubly distilled water and added to an equal volume of each of the following solutions: (i) β-galactosidase (Escherichia coli), grade IV (Sigma), in 0.05 M phosphate buffer, pH 7.2. Sinus rotavirus β-galactosidase was supplied as a crystalline suspension in 2.2 M ammonium sulfate, the effect of ammonium sulfate on the viruses, at concentrations corresponding to those resulting from the addition of the enzyme slurry to test mixtures, was also examined. (ii) Lactase (Saccharomyces fragilis), grade II (Sigma), at 50 U/ml in 0.05 M phosphate buffer, pH 7.2.

RESULTS

Morphology and buoyant density of simian rotavirus (SA-11). The morphology of SA-11 virus has been described (11) and noted as typically rotaviral in nature (15). When centrifuged to equilibrium in cesium chloride, virus-containing bands were obtained at buoyant densities of 1.32 g/ml, 1.36 g/ml, and 1.38 g/ml. The band at density 1.32 contained single- and/or double-shelled particles penetrated to varying degrees by negative stain, thus corresponding to the empty particles reported by Els and LeCatsas (11). Double-shelled particles (see Fig.

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Polypeptide components of double-shelled simian rotavirus particles and determination of their molecular weights. Instead of retaining the system used previously where the polypeptides of human and calf rotaviruses were numbered 1 through 9 from highest to lowest molecular weight (28), the system suggested by August et al. (1) was adopted. Thus, the prefixes p or gp followed by the molecular weight in thousands represent particular protein or glycoprotein components of the capsid.

Electrophoresis of dissociated purified double-shelled preparations of SA-11 virus yielded eight or nine polypeptides (Fig. 1f), the molecular weights of which are listed in Table 1. For comparison, the fractioned polypeptides of human and calf rotaviruses are shown in Fig. 1a and c. The polypeptides of SA-11 virus show a distribution basically similar to the other two rotaviruses, with the main differences occurring in the low-molecular-weight polypeptide region, i.e., p18 and 14 of SA-11 virus compared with p21 of human rotavirus and p16.5 and 14.5 of calf rotavirus. Whether or not these low-molecular components represent entire polypeptides or breakdown products, they do occur consistently and provide a basis upon which the viruses can be distinguished. The molecular weights of the remaining components of SA-11 virus are virtually identical to the corresponding components of human and calf rotaviruses, with allowance for experimental variation.

Figure 1c shows the fractionation pattern of double-shelled calf rotavirus polypeptides. It should be noted that p97, previously reported by us (28) as absent in some calf rotavirus preparations, was present in this preparation. Subsequent experiments have shown that it is usually present. It is interesting to note that the corresponding polypeptide of SA-11 virus

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**Table 1. Estimated molecular weights of human, calf, and simian rotavirus polypeptides, and their location in a double-shelled virion**

<table>
<thead>
<tr>
<th>Human Inner shell</th>
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<th>Calf Inner shell</th>
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* This polypeptide was not always resolved.
(p99) is also not always resolved on polyacrylamide gels.

Polypeptides of single-shelled human, calf, and simian rotaviruses and identification of inner and outer shell components. Electrophoretic fractionation patterns of dissociated preparations of single-shelled human, calf, and simian rotaviruses are shown in Fig. 1b, e, and g. In each case, five polypeptides were obtained; both the polypeptide patterns and the molecular weight of the corresponding components of the three viruses were virtually identical (28).

Of the inner shell components, i.e., p127, 103, 97, 88, and 32 for human rotavirus, p131, 103, 97, 92, and 32 for calf rotavirus, and p133, 102, 99, 92, and 31 for SA-11 virus, p102 to 103 and 31 to 32 are the major components. Of the outer shell components, p26 of human rotavirus, p22 of calf rotavirus, and p23 of SA-11 are major. These results confirm our earlier suggestions concerning the locations of the various polypeptides within the double-shelled structure (28).

It should also be noted that the band corresponding to p58 (Fig. 1a), an outer shell component of human rotavirus, is faint by comparison with p58 of calf and simian viruses (Fig. 1c and f), respectively. Similarly, p26 of human rotavirus is faint compared with p22 and 23 of calf and simian viruses, respectively (Fig. 1a, c, and f). This result does not imply any real difference in composition of the outer shells of the three rotaviruses, but simply reflects the fact that the band of human rotavirus at a density of 1.36 in CsCl gradients did not contain such a high percentage of double-shelled particles as were obtained from calf and simian virus preparations (see above and (28)).

Identification of glycoproteins in the outer shells of human, calf, and simian rotaviruses. Periodic acid-Schiff staining of the fractionated polypeptides prepared by either of the two procedures described in this text indicated the presence of carbohydrate in one (calf and simian viruses) or two polypeptides (human virus). To identify the glycoprotein with which the positive reaction was associated, the molecular weight of the positively staining band was determined, or, alternatively, the gel was compared with one electrophoresed simultaneously but stained with Coomassie brilliant blue for protein (Fig. 1c and d). The intensity of the periodic acid-Schiff staining reaction was not high, although large quantities of virus were used; nevertheless, there was no background staining, and definite identification of the glycoproteins was possible. It was concluded that p26 and 21 of human rotavirus, p22 of calf, and p23 of simian rotaviruses were glycosylated and henceforth will be referred to as gp26, etc. Thus, the major outer shell components of all three rotaviruses are glycoproteins.

The necessary experiments for determination of the exact molecular weights of these glycoproteins have not yet been conducted, i.e., electrophoresis and molecular weight determination in gels of varying concentration. Such experiments would require greater quantities of virus than are available at present, and it appears preferable to wait for a supply of radioactively labeled virus.

Acid stability of rotaviruses. No morphological changes were noted in preparations of calf rotavirus examined as described above. However, a large percentage of simian rotavirus particles were clumped or degraded to amorphous protein at pH 3 and 4 in the buffers described (Fig. 2a and b). The effect was more dramatic at pH 3 than at pH 4, and it was also more obvious in preparations incubated at 37°C than at room temperature.

Degradation of rotavirus by proteases. No alteration in morphology of the double-shelled calf or simian rotavirus particles was observed when they were treated with bromelain, pepsin, Pronase, trypsin, or a-chymotrypsin under any of the conditions listed. The double-shelled human rotavirus particle also resisted degradation by papain, Pronase, and trypsin as above, but was degraded or uncoated to a stage indistinguishable from the single-shelled particle form by a-chymotrypsin in the presence of Cs+ (Fig. 3). This effect was observed on three of four occasions. On each occasion a different virus preparation was used. The uncoated particles were analyzed by polyacrylamide gel electrophoresis and found identical in polypeptide composition to the naturally occurring single-shelled particles described above.

Although the virus preparations used in these experiments contained a vast majority of double-shelled particles, some single-shelled particles were present, so the effect of the various proteases on these particles could also be observed. In all cases, the single-shelled particles resisted degradation under the conditions described.

Degradation of rotaviruses with β-galactosidase or lactase. The morphology of the human and simian double-shelled rotavirus particles was unaltered by treatment with β-galactosidase (E. coli). On the other hand, preparations of the double-shelled calf rotavirus particles were degraded to the single-shelled stage (uncoated), or partially so on some occasions by this enzyme (Fig. 4). The degree or amount of uncoating that had occurred was measured in
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FIG. 2. (a) Untreated double-shelled particles of purified SA-11 virus taken from a band at density 1.36 g/ml in a CsCl gradient. (b) Partially degraded and clumped particles of SA-11 virus following exposure to pH 3 or 4 for 1 h at 37°C. ×120,000. The magnification marker represents 100 nm.

FIG. 3. Particles of human rotavirus degraded or uncoated to a stage indistinguishable from the single-shelled particle by α-chymotrypsin in the presence of Cs+. Compare these with the double-shelled particles shown in Fig. 2a. ×120,000. The magnification marker represents 100 nm.

FIG. 4. Particles of calf rotavirus degraded or uncoated to a stage indistinguishable from the single-shelled particle by β-galactosidase (E. coli). Compare these with the double-shelled particles shown in Fig. 2a. ×120,000. The magnification marker represents 100 nm.

terms of an observed increase in the percentage of single-shelled particles in the preparation. Of five different calf rotavirus samples examined, one was uncoated completely by concentrations of the enzyme as low as 2 μg/ml (Fig. 4); three preparations were uncoated to varying degrees by intermediate concentrations of the enzyme; and the last was not uncoated at all, even by concentrations as high as 1 mg/ml. One of the three preparations that was partially uncoated by the E. coli enzyme was also partially uncoated by the lactase from S. fragilis. Ammonium sulfate, at concentrations as high as 1 M, did not induce any morphological changes in calf or human rotaviruses. About 25% of the particles in a preparation of SA-11 virus were uncoated, but only at a concentration of 1 M. Thus, we believe that the observed
uncoating of calf rotavirus was due to the enzyme and not to the ammonium sulfate. On polyacrylamide electrophoretic analysis, the uncoated particles were found to have the same polypeptide composition as naturally occurring single-shelled particles.

**DISCUSSION**

Although SA-11 has never been associated with any simian disease, morphological and serological studies indicate that it can almost certainly be classified within the genus rotavirus (12, 19, 28) along with agents that characteristically cause diarrhea of the young (10). To more formally classify this agent, we have presented the results of biochemical and biophysical tests supporting this classification. In addition, we have fractionated the RNA of SA-11 in polyacrylamide gels and shown it to be typically rotaviral in nature (M. Rodger and I. H. Holmes, manuscript in preparation), although on this basis the agent is distinguishable from both human and calf rotaviruses (Rodger and Holmes, manuscript in preparation; 17, 29). It is also interesting to note that although the pathogenicity of SA-11 virus has never been tested in young monkeys, the agent does produce diarrheal disease in young calves and gnotobiotic piglets (S. Tzipori, personal communication). By comparison then with human and calf rotaviruses, the various particle forms of SA-11 have buoyant densities similar to those of the corresponding particle forms of these two agents, and electrophoresis showed that the polypeptides of the three viruses are strikingly similar. In view of its long history of passage through cell cultures, it was important to establish that SA-11 has retained properties similar to those of the noncultivable rotaviruses since it is an obvious candidate to be used in serology as a substitute antigen, especially for the human rotavirus (18). Also, in our laboratories SA-11 is being used as a model rotavirus since supplies of others are limited and unpredictable.

Comparison of the polypeptide components of single-shelled and double-shelled rotaviruses by polyacrylamide gel electrophoresis has allowed identification of the polypeptides that are components of the inner shell; the remainder are components of the outer shell of the complete double-shelled structure. Of the eight (human and SA-11) (28) or nine (calf) (28) components, five are located internally and three or four comprise the outer shell. When the three rotaviruses were compared on the basis of the components of the inner shell, they were virtually indistinguishable from each other, with the molecular weights of most of the corresponding components identical (allowing for experimental variation). Comparison of the outer shell components revealed that, although p58 was constant, variations occurred among the low-molecular-weight polypeptides. These variations gain importance when considered from a serological point of view. It has been reported that rotaviruses of different species origins cross-react with each other in serological tests (18, 19), although Flewett et al. (12) suggested that although the single-shelled particles of human and calf rotaviruses were serologically indistinguishable, antigenic variations were detected when the cross-reactivity of double-shelled particles were examined. More recently, this group of workers has presented the results of further studies on a wider range of rotaviruses to substantiate this point (32). Their findings presumably reflect the differences between rotaviruses that we have detected by polyacrylamide gel electrophoresis.

Studies on the fractionation of calf rotavirus polypeptides in polyacrylamide gels have also been conducted by others (4, 26), who reported that it is composed of five polypeptides, four in the inner shell and a fifth comprising the outer shell, compared with our observed total of nine polypeptides. These differing results appear difficult to reconcile, but may be explained by the quite different electrophoretic systems employed. Analyses of calf rotavirus polypeptides by Bridger and Woode (4) and Newman et al. (26) were conducted in gels with a continuous buffer system, whereas all our analyses were conducted using Laemmli's discontinuous buffer system (21). As discontinuous systems are reputed to be more sensitive and give higher resolution than continuous systems (24), the larger number of bands observed by us may thus be explained. This difference in sensitivity between the two electrophoresis systems has been illustrated recently where some previously unresolved reovirus-specified polypeptides were detected when a discontinuous electrophoresis system was used (7). Also, Obijeski et al. (27), using a discontinuous buffer system for electrophoretic analysis of human rotavirus polypeptides, have obtained fractionation patterns remarkably similar to ours.

By periodic acid-Schiff staining, major components of the outer shells of human, calf, and simian rotaviruses have been identified as glycoproteins, and detailed chemical characterization of the carbohydrate moieties of these glycoproteins is currently being carried out. The suggestion that these glycoproteins may play a role in governing virus infectivity and host cell specificity is explained in a previous communication by Holmes et al. (15), who postulate that
rotaviruses contain a lactase or β-galactosidase substrate on their surface, and it is the intestinal brush border enzyme, lactase, that acts as the host cell receptor for the virus. Also, as attempts to degrade rotaviruses with proteolytic enzymes met with little success, we suspect that the carbohydrate moiety may be affording them some protection. This phenomenon has been reported for murine mammary tumor virus (5). In such cases where the double-shelled rotavirus particle was degraded to the single-shelled form (human rotavirus by α-chymotrypsin and calf rotavirus by β-galactosidase), we were able to gain some insight into possible early events involved in rotavirus replication. As the in vitro uncoated particles were indistinguishable from naturally occurring single-shelled particles, we were unable to determine whether such particles obtained from feces or cell culture represent incomplete rotavirions released prematurely from a bursting cell or previously double-shelled particles uncoated in the gut by exogenous proteases or glycosidases.

The single-shelled particles can also be likened to the subviral or intermediate subviral particles obtained by controlled digestion of reovirus by α-chymotrypsin (3). Fully uncoated reovirions exhibit an RNA-dependent RNA polymerase activity (3), but, as yet, the appropriate conditions required for demonstration of the activity of such an enzyme in uncoated rotavirions have not been found. In fact, it was the repeated failure of attempts to uncoat rotaviruses with proteases in search of this enzyme that led us to look for carbohydrate on the surface of rotaviruses.

The observation that calf rotavirus appeared morphologically unaltered at a pH of 3 agrees with Welch and Twiehaus (31), who found that a cell-adapted strain of calf rotavirus did not lose infectivity after similar acid treatment. Our finding that SA-11 particles were clumped and degraded at low pH is consistent with the observation of Malherbe and Stickland-Cholmley (25) that acid treatment caused a loss of infectivity. Although SA-11 virus is thus more acid labile than calf rotavirus, it would not appear reasonable to separate them on this criterion in a conventional classification; at least the difference detected here does not appear to be significant in vivo.

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LITERATURE CITED


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