Biochemical Evidence for the Oligomeric Arrangement of Bovine Rotavirus Nucleocapsid Protein and Its Possible Significance in the Immunogenicity of This Protein

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SUMMARY

The nucleocapsid protein of bovine rotavirus was shown to exist in trimeric units in both the virus particle and in infected cells, with the subunits linked by non-covalent interactions. These trimeric units complex further by disulphide bridges into larger units which may represent the hexameric structures observed by electron microscopy. Visualization of various nucleocapsid protein complexes was also achieved on polyacrylamide gels by treating virus preparations with urea at 37 °C or boiling in the presence and absence of 2-mercaptoethanol. Since virus particles devoid of nucleic acid were also broken down into trimeric subunits by such treatments, assembly of virus particles appears not to require an RNA–protein interaction. Four nucleocapsid-specific monoclonal antibodies with low neutralizing ability reacted with the monomeric (45 000 mol. wt., 45K), dimeric (90K), trimeric (135K) and trimeric pair (270K) subunits, indicating that a site responsible for neutralization is probably exposed after assembly of these subunits. Analysis of radiolabelled virus revealed that a high proportion (80%) of infectious particles could be immunoprecipitated by these monoclonal antibodies, suggesting that the virus particles are either partially double-shelled or have the nucleocapsid exposed on the surface. The monoclonal antibodies also cross-reacted with the nucleocapsid proteins of simian (SA11), pig (OSU), bovine (NCDV and UK) and human (Wa and ST4) rotaviruses in an immunoblot ELISA reaction. Since these six viruses belong to two different subgroups, it is likely that the antibodies did not recognize the subgroup-specific site, but a shared exposed antigenic determinant. Due to the hexameric configuration of the nucleocapsid in virus particles the neutralizing epitope may be repeatedly presented and, therefore, may contribute to the immunogenicity of this protein.

INTRODUCTION

The most abundant structural protein in the rotavirus particle is the 45000 mol. wt. (45K) nucleocapsid protein. It is also described as the common rotavirus group antigen since some 45K-specific monoclonal antibodies react with all mammalian rotaviruses and polyclonal serum raised against a single rotavirus type can detect most other mammalian rotavirus strains (Greenberg et al., 1983a). In addition, it has been identified as the subgroup antigen by several techniques including complement fixation (Sabara et al., 1985), ELISA (Kalica et al., 1981), immuno-adherence agglutination assay (Kapikian et al., 1981) and specific monoclonal antibodies (Greenberg et al., 1983a). Two distinct subgroup specificities have now been described and are designated subgroup 1 and 2.

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In addition to its antigenic properties, the nucleocapsid protein is very immunogenic and several investigators have found that polyclonal serum raised against this protein has neutralizing ability (Bastardo et al., 1981). The reason for this observation has not yet been identified. However, the availability of nucleotide and amino acid sequence data on nucleocapsid proteins from different rotavirus species (Both et al., 1984; Cohen et al., 1984; Estes et al., 1984) as well as a more detailed investigation of the protein should aid in elucidating this phenomenon.

Since the nucleocapsid protein constitutes approximately 80% of the protein in single-shelled particles, the arrangement of this protein has been the focus of many studies (Chasey & Labram, 1983; Martin et al., 1975; Palmer et al., 1977; Palmer & Martin, 1982; Petrie et al., 1982; Sabara et al., 1985). Electron microscopical examination of virus particles suggested the arrangement of inner capsid subunits into an open mesh exhibiting a regular pattern of holes (Martin et al., 1975). Closer examination of the electron micrographs as well as the data presented in this manuscript and by Gorziglia et al. (1985) provide biochemical evidence for a trimeric arrangement of the bovine and porcine rotavirus nucleocapsid protein. During progressive degradation of rotavirus particles, Palmer & Martin (1982) observed ring-shaped morphological units composed of six smaller wedge-shaped subunits, which they identified as the monomeric form of the nucleocapsid.

Interpretations of the type of interactions responsible for the formation of the inner capsid subunits have been somewhat conflicting. Bastardo et al. (1981) suggested that intramolecular disulphide bonds could be responsible for maintaining the oligomeric aggregation of 45K, whereas Gorziglia et al. (1985) demonstrated that the 45K protein is not associated in oligomeric structures by disulphide bonding. In the present study, we have used an alternative approach to resolve this conflict by observing the interaction of bovine rotavirus nucleocapsid monomers under various conditions of protein dissociation. Our results demonstrate the occurrence of both disulphide bond-dependent and -independent interactions in the formation of viral subunits. Verification of the nucleocapsid protein composition of these subunits and their accessibility in infectious virus particles was achieved by using specific monoclonal antibodies.

**METHODS**

**Virus and cells.** Bovine rotavirus isolate C486 was cultured from the faeces of an infected calf by a method described previously (Babiuk et al., 1977). The other isolates were kindly provided by H. B. Greenberg (Veterans Administration, Palo Alto, Ca., U.S.A.). Virus was propagated in confluent African monkey kidney cells (MA-104) in the absence of foetal bovine serum (FBS; Gibco) and in the presence of 10 μg trypsin (1:500; Difco) per ml (Babiuk et al., 1977).

The cells were normally cultured in Eagle’s MEM supplemented with 10% FBS.

**Radiolabelling of virus.** Virus was adsorbed to confluent MA-104 monolayers for 1 h, after which time the virus inoculum was replaced with methionine-free MEM. After 3 h of incubation at 37°C, 25 to 50 μCi L-[^35]S]methionine (Amersham) per ml was added to the overlay (Sabara et al., 1985).

**Virus purification and preparation for polyacrylamide gel analysis.** In order to purify virus, the following steps were taken. First, the cellular debris was removed from cell culture supernatant by low-speed centrifugation. The virus was then concentrated by pelleting for 3 h at 100000 g through a 40% sucrose cushion. The resulting pellet was then layered onto 11.5 ml CsCl solution (analytical grade, density 1.3688 g/ml; Sigma) and centrifuged at 38000 r.p.m. in an SW41 rotor (Beckman Model L5-65) for 17 h at 15°C. Complete double-shelled particles containing RNA banded at a density of 1.3692 g/ml and particles devoid of RNA banded at 1.3 g/ml.

**Virus proteins.** Virus proteins were examined by polyacrylamide gel electrophoresis according to the procedure originally described by Laemmli (1970). The effect of three different sample buffer preparations on the virus protein profile was examined: (i) 0.0625 M-Tris–HCl pH 6.8, 4% SDS, 5% 2-mercaptoethanol (2-ME), 8% glycerol, 0.05% bromophenol blue; (ii) 0.0625 M-Tris–HCl pH 6.8, 4% SDS, 8% glycerol, 0.05% bromophenol blue; (iii) 0.0625 M-Tris–HCl pH 6.8, 4% SDS, 6% urea, 8% glycerol. Molecular weight standards included lysozyme (14K) β-lactoglobulin (18.4K), soybean trypsin inhibitor (21K) β-chymotrypsinogen (25.7K), ovalbumin (43K to 45K), bovine serum albumin (68K), phosphorylase B (92K to 97.4K), 13-galactosidase (116K) and myosin (200K).

**Isolation of nucleocapsid protein.** Nucleocapsid protein was isolated by degradation of purified virus with EDTA (Cohen et al., 1979) and CaCl2 (Bican et al., 1982). Briefly, the outer capsid was removed by incubation in 50 mM-EDTA, 0.01 M-Tris–HCl pH 7.4 at 4°C, for 30 min. Subviral particles were recovered by ultracentrifugation (100000 g, 2 h) and resuspended. Removal of EDTA by dialysis against 0.01 M-Tris–HCl pH 7.4 was followed by
treatment with 1.5 m-CaCl$_2$ at 20 °C for 20 to 30 min. Any remaining virus and subviral particles were removed by ultracentrifugation. The solubilized nucleocapsid protein was dialysed against 0.2 m-EDTA-0.01 m-phosphate buffer pH 6.0, and then against 0.01 m-Tris-HCl pH 7.4 at 4 °C. Samples were examined by SDS-PAGE.

For electron microscopy, samples were diluted as required and pipetted onto copper grids covered with a support film of carbon-coated Formvar. After staining with 1% uranyl acetate pH 5.0, they were examined in a Philips EM410LS electron microscope.

Peptide mapping (Cleveland digestion). Partial proteolytic digestion of the nucleocapsid protein was performed according to the procedure of Cleveland et al. (1977). A 5 cm 5% acrylamide stacking gel and an 11 cm 17.5% acrylamide resolving gel were employed for the digestion and resolution of the digest.

The enzymes used were (per lane) 50 μg papain (Sigma), 1-0 μg Staphylococcus aureus V8 protease (Miles Laboratories) and 5-0 μg chymotrypsin (Sigma). The resulting Cleveland digests were then electroblotted to nitrocellulose paper as described below and treated with antibodies.

Western blotting of rotavirus polypeptides and reaction with antibodies. Proteins were transferred from polyacrylamide gels to 0-45 μm nitrocellulose paper by electroblotting for 12 h at 4 °C in 25 mM-Tris-HCl pH 8.3, 190 mM-glycine, 20% methanol at 875 V/cm. After transfer, either the nitrocellulose strips were stained in 7% acetic acid containing 1-0% amido black dye (if non-radiolabelled samples were used) or exposed to 3M film (Picker International, Saskatoon, Canada) if radiolabelled samples were used.

The procedure for reacting monoclonal antibodies or rabbit antisera with polypeptides transferred to nitrocellulose was essentially the same as that described by Braun et al. (1983). Each 11 x 10 cm nitrocellulose strip was first incubated with 3 ml phosphate-buffered saline containing 3% bovine serum albumin (fraction V, Sigma) and the appropriate primary antibody for 1 h at 37 °C. After extensive washing, strips were incubated with either 5 μl horseradish peroxidase-coupled goat and anti-rabbit IgG or horseradish peroxidase-coupled rabbit anti-mouse IgG. Subsequent development of the strips with 0.02% o-dianisidine dihydrochloride substrate proceeded for 18 h at room temperature.

Production of monoclonal antibodies. BALB/c mice were immunized with a purified rotavirus isolate C486. After three immunizations each approximately 2 weeks apart, the mice were sacrificed and the spleens were removed for fusions. The procedure followed for fusion of spleen cells with NS-1 cells was essentially that of Greenberg et al. (1983b). After the fusion, wells with visible colonies were tested by ELISA against single-shelled virus. Selected hybrids were subcloned by limiting dilution with a macrophage feeder layer and grown to yield 2 to 5 ml suspensions. To amplify some monoclonal antibodies hybridoma cells were injected intraperitoneally into pristane (Aldrich) primed BALB/c mice at a concentration of 5 x 10$^5$ cells/mouse. Ascites fluids were collected 1 to 2 weeks later, clarified by low-speed centrifugation, and stored at -20 °C.

Plaque reduction assay. Neutralization of bovine rotavirus isolate C486 by rabbit antisera and ascites fluids was determined by a standard 50% plaque reduction assay (Sabara et al., 1985).

Immunoprecipitation. Virus-infected cells were radiolabelled in vitro for 18 h with 50 μCi/ml L-[35S]methionine in 100 mm tissue culture plates. The cells were harvested, washed once in MEM, pelleted and resuspended in 0-5 ml RIPA buffer (0.05 m-Tris-HCl, 0-15 m-MgCl$_2$, 1% deoxycholate, 1% Triton X-100) per plate and then sonicated for 2 s at 100 W. The cell lysates were clarified by centrifugation at 25000 r.p.m. (Beckman SW41 rotor) for 1 h at 4 °C and the lysate was used immediately for immunoprecipitation. Fifty μl of this lysate or of radiolabelled purified virus was added to 50 μl of ascites fluid (1:10 dilution). The mixture was incubated for 1 h at room temperature, then 50 μl of a 50% Protein A-Sepharose CL-4B suspension (Pharmacia) was added for 1 h at 4 °C. The precipitate was pelleted (10000 r.p.m. in an Eppendorf microfuge) and washed with either RIPA buffer or MEM. The Protein A-Sepharose immunoprecipitates were then treated with one of the three sample buffers described above and analysed by PAGE.

RESULTS

Characterization of the nucleocapsid protein using neutralizing monoclonal antibodies

Four monoclonal antibodies specific for the bovine rotavirus (isolate C486) 45K nucleocapsid protein were identified by immunoprecipitation of infected cell lysates (Fig. 1). The four monoclonal antibodies demonstrated neutralizing activity similar to that observed for monospecific antiserum to the nucleocapsid protein, although lower than that exhibited by antisera to the two outer capsid proteins (Table 1). In addition, each of these four monoclonal antibodies recognized the nucleocapsid protein of a porcine rotavirus (OSU, belonging to subgroup 1), simian rotavirus (SA11, belonging to subgroup 1), bovine rotaviruses (UK and NCDV, belonging to subgroup 1) and human rotaviruses (Wa and ST4, belonging to subgroup 2) in an immunoblot ELISA reaction. The reaction of nucleocapsids with only one monoclonal antibody (1B4) is illustrated in Fig. 2 since all the monoclonal antibodies reacted in a similar manner.
Table 1. Neutralizing antibody titres of polyclonal and monoclonal antibodies

<table>
<thead>
<tr>
<th>Antibody type</th>
<th>Neutralizing titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal</td>
<td></td>
</tr>
<tr>
<td>Anti-45K</td>
<td>500</td>
</tr>
<tr>
<td>Anti-38.2K/41.9K</td>
<td>200000</td>
</tr>
<tr>
<td>Anti-82K/84K</td>
<td>5000</td>
</tr>
<tr>
<td>Monoclonal</td>
<td></td>
</tr>
<tr>
<td>1D7</td>
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<td>1B4</td>
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</tr>
<tr>
<td>1B9</td>
<td>800</td>
</tr>
<tr>
<td>1D10</td>
<td>500</td>
</tr>
</tbody>
</table>

* Neutralizing titre determined by the reciprocal of antibody dilution necessary to produce 50% plaque reduction. Normal ascites fluid has a neutralizing titre of 1:10.

In order to localize the antigenic determinants recognized by these monoclonal antibodies, immunoblot ELISA reactions were carried out on nitrocellulose-blotted 45K partial protein digests using monoclonal antibodies and monospecific antiserum (Fig. 3). Examination of the digests of each different enzyme revealed that all four monoclonal antibodies recognized essentially the same peptides of the 45K protein in that digest. In the case of chymotrypsin and
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Fig. 2. Immunoblot ELISA reaction of bovine, simian, pig and human rotavirus nucleocapsid proteins with monoclonal antibody IB4. Lane 2, bovine isolate C486; lane 3, porcine isolate OSU; lane 4, bovine isolate NCDV; lane 5, human isolate Wa; lane 6, bovine isolate UK; lane 7, human isolate ST4; lane 8, simian isolate SAI1. Human isolates Wa and ST4 belong to subgroup 2, and the other isolates belong to subgroup 1. Mol. wt. values are indicated on the right-hand side and ascites control sample is in lane 1.

Fig. 3. Reaction of bovine rotavirus nucleocapsid protein digests with anti-rotavirus serum (lanes 1), monoclonal antibodies ID7 (lanes 2), 1B4 (lanes 3), 1B9 (lanes 4) and 1D10 (lanes 5). The enzymes used were (a) none, (b) chymotrypsin, (c) papain and (d) V8 protease. Digestion of the nucleocapsid protein occurred during electrophoresis through the 5% stacking gel. The resulting digest was then fractionated on the 17.5% resolving gel. After electrophoresis, digest patterns were transferred to nitrocellulose according to the procedure described in Methods. Immune reactions of the digests with monoclonal antibodies and monospecific antiserum were carried out according to the procedure also described there.
Fig. 4. Effect of various sample buffers on the arrangement of the bovine rotavirus nucleocapsid protein. (a) \[^{35}S\]Methionine-labelled protein profile on a 10% resolving gel. (b) \[^{35}S\]Methionine-labelled protein profile on a 7.5% resolving gel and 3% stacking gel. Lanes 1, virus preparation treated with 2-ME-containing buffer and boiled; lanes 2, virus preparation treated with buffer without 2-ME and boiled; lanes 3, virus preparation treated with urea buffer at 37 °C. The components of each buffer are outlined in Methods. The positions of mol. wt. standards are shown on the left side, and arrows indicate positions of major viral proteins, other than those consisting of the 45K protein. (c) Electron micrograph illustrating the hexamers and hexameric aggregates produced upon disassembly of virus particles with EDTA and CaCl\(_2\) as described in Methods. Arrows indicate obvious hexameric structures.

\textit{S. aureus} V8 protease digests, the reactivity of monospecific serum was almost identical to that demonstrated by the monoclonal antibodies.

\textit{Characterization of the structural arrangement of the nucleocapsid}

In order to elucidate the structural arrangement of the 45K protein in the virus particle and the infected cell, whole virus preparations and immunoprecipitated cell lysates were examined by PAGE using various sample buffer systems. Fig. 4 illustrates the protein profile of purified
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Fig. 5. Immunoprecipitation of [³⁵S]methionine-labelled bovine rotavirus-infected cell lysate by monoclonal antibodies and examination of resulting polypeptide profiles under three different sample buffer conditions. Lanes 1 to 4 represent samples treated with urea sample buffer at 37 °C; lanes 5 to 8 represent samples treated with sample buffer not containing 2-ME but boiled; lanes 9 to 11 represent samples treated with sample buffer containing 2-ME and boiled. Immunoprecipitations were performed using the following monoclonal antibodies: lanes 1, 5 and 10, 1D7; lanes 2, 6 and 10, 1B4; lanes 3, 7 and 11, 1B9; lanes 4, 8 and 11, 1D10; lane 9, rabbit hyperimmune anti-bovine rotavirus serum. Electrophoresis was carried out on 10% resolving and 3% stacking polyacrylamide gels. Positions of mol. wt. standards are shown by arrowheads on the right-hand side. Arrows next to lane 9 denote position of major rotavirus proteins other than those associated with 45K.

Virus treated with or without 2-ME and boiled, or treated with urea. Under conditions of urea denaturation, the 45K protein was found in a trimeric arrangement (mol. wt. 135K), whereas boiling disrupted this unit into its 45K monomeric components. A faint higher mol. wt. protein (approximately 270K) was also visualized in the boiled, 2-ME-untreated preparations and appeared to be sensitive to the effects of 2-ME since it was not present in boiled 2-ME-treated samples. In addition, the 3% stacking gel contained a protein band at approximately 270K and a very high mol. wt. protein (HMW), both of which disappeared with 2-ME treatment. In this gel system, the 45K monomer was also observed to shift slightly in its mobility. Further proof of the ordered arrangement of the 45K protein is illustrated by the electron micrograph in Fig. 4(c), where hexameric structures and aggregates thereof can be visualized upon disassembly of the virus particle with EDTA and CaCl₂.

Immunoprecipitation of cell lysates with monoclonal antibody specific for the nucleocapsid protein enabled detection of trimers (135K) and dimers (90K) in the cell prior to assembly of the virus (Fig. 5). However, in contrast to the virus particle profile (Fig. 4a, b; lanes 3), where only one 135K protein species was observed in urea-treated samples, it appeared that there were several species with slightly different mol. wt. in infected cells (Fig. 5, lanes 1 to 4). A parallel observation was made in boiled, 2-ME-untreated immunoprecipitated samples which illustrated heterogeneity in the 45K region. An approximately 270K mol. wt. species was also efficiently immunoprecipitated by monoclonal antibodies to the 45K protein in preparations which were not treated with 2-ME but boiled (Fig. 5, lanes 5 to 8) and to a lesser extent in preparations treated with urea (Fig. 5, lanes 1 to 4). However, after boiling in the presence of 2-ME this
Table 2. Effect of sample treatments on the nucleocapsid protein in infected cells and the virus particle

<table>
<thead>
<tr>
<th>Sample treatment*</th>
<th>Urea/37 °C</th>
<th>2-ME/100 °C</th>
<th>100 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected cell</td>
<td>Virus particle</td>
<td>Infected cell</td>
</tr>
<tr>
<td>HMW aggregate (hexamer)</td>
<td>+‡</td>
<td>+</td>
<td>−‡</td>
</tr>
<tr>
<td>270K hexamer (trimeric pair)</td>
<td>+</td>
<td>+§</td>
<td>−</td>
</tr>
<tr>
<td>135K (trimer)</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>90K (dimer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45K (monomer)¶</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

* Sample treatments refer to buffers used to dissociate virus proteins prior to electrophoresis. Buffer formulations are listed in Methods.
† Lysate components refer to the different nucleocapsid protein structures visualized in Fig. 4 and 5.
‡ + Indicates the presence of the component and − indicates its absence after treatment with the indicated sample buffer.
§ The 270K protein was visible in gels but did not reproduce well in Fig. 4 due to the small amounts present.
¶ The 45K monomer was not present in infected cell lysates since they were prepared from a late infection of cells. Therefore, the presence of monomers in various preparations is due solely to the treatments.

complex disappeared (Fig. 5, lanes 9 and 11) as did the 90K dimeric complex. As demonstrated for the virus particle profiles (Fig. 5), the HMW protein band was also immunoprecipitated and localized in the upper stacking gel (Fig. 5, lanes 5 to 8). This HMW protein was less evident in urea-treated samples (Fig. 5, lanes 1 to 4) and disappeared in the presence of 2-ME (Fig. 5, lanes 9 to 11). Table 2 summarizes the related proteins present in the virus particle and infected cell lysates under various denaturing conditions.

**Possible mechanism of virus neutralization by anti-nucleocapsid antibodies**

Since it appeared that a neutralizing epitope on the 45K protein was exposed on larger nucleocapsid complexes, we investigated whether it was also exposed on the virus particle. To test this possibility, radiolabelled preparations composed of complete particles migrating at a density of 1.3692 g/ml (i.e. double-shelled) were reacted with monoclonal antibodies and then Protein A was added in order to immunoprecipitate the immune complexes. Approximately 80 to 85% of the infectious virus particles in various preparations were able to bind the monoclonal antibodies and were immunoprecipitated, indicating that a high proportion of the infectious virus had the 45K epitope sufficiently exposed such that antibodies could recognize and bind to it.

**DISCUSSION**

The demonstration of a rotavirus nucleocapsid protein trimeric complex in virus particles and in infected cells supports previous reports suggesting this type of nucleocapsid arrangement (Chasey & Labram, 1983; Gorziglia et al., 1985; Martin et al., 1975; Novo & Esparza, 1981; Palmer et al., 1977; Petrie et al., 1982). Novo & Esparza (1981) first suggested that this was the case, on the basis of velocity sedimentation and gel filtration studies of infected cell extracts. Electron microscopic studies done by Palmer & Martin (1982) identified large ring-shaped morphological units on the surface of single-shelled particles which were formed by sharing small wedge-shaped trimeric units. They also observed that the capsomeres were held together by some type of intrasubunit linkage, further supporting this concept.

Based on all the data available, a probable assembly process for the nucleocapsid is proposed in Fig. 6. Since the trimeric complex (mol. wt. 135K) could only be disrupted by boiling in the presence of SDS and not at 37 °C in the presence of 6 M-urea our studies suggest that the
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Putative assembly process

45K monomer

135K trimer

270K trimer pair

HMW protein (i.e. hexamers and hexameric aggregates)

Artefacts of sample processing

100 °C

90K dimer

270K perihexagon

Fig. 6. The probable process by which the nucleocapsid assembles into bovine rotavirus particles (left-hand column). The monomer (45K) forms trimeric units (135K) via non-covalent interactions, since boiling of samples in the absence of 2-ME disperses the trimer into monomeric units. The trimers then assemble into trimer pairs and hexameric units by virtue of disulphide bridges (S-S), since these complexes can be dispersed upon boiling in 2-ME. The dimeric unit (90K) and the perihexagonal unit (270K) are likely to be artefacts produced under conditions where samples containing trimers and hexamers are boiled.

nucleocapsid monomers (45K) are held as trimers by strong non-covalent interactions. This has also been observed by Gorziglia et al. (1985) for the porcine rotavirus nucleocapsid protein. However, the presence of a 270K protein representing a trimeric pair, and a high mol. wt. protein representing hexameric units, i.e. three trimeric pairs and aggregates thereof, in both urea-treated and boiled samples suggests another type of interaction. A clue to the nature of this interaction is provided by the presence of a dimeric unit (90K) in these sample preparations. This dimer is likely to be an artefact of the sample treatment rather than a step in the assembly of monomers since tetramers, rather than trimers, would be formed as prerequisites for larger complexes. The disappearance of the 270K and HMW proteins after treatment with 2-ME and boiling indicates that intermolecular disulphide bridging is involved in nucleocapsid assembly at some time after trimer assembly, but is not involved in the formation of trimers. These results then explain the fact that Gorziglia et al. (1985), who primarily examined trimers, did not identify disulphide bridging as being involved in the association of monomers into trimers. However, Bastardo et al. (1981) did indicate that disulphide bonds could be involved in intermolecular links, which may represent the bridging involved in the formation of the 270K and the higher mol. wt. proteins identified in our experiments. One mechanism for this assembly could be provided by the interaction of opposing monomers in hexameric complexes via disulphide bridging (Fig. 6). Alternatively, adjacent monomers in hexameric units may be linked by disulphide bridges to form the high mol. wt. aggregates. In either case, disulphide-linked dimers could be produced under appropriate sample preparation conditions.

Fig. 6 also illustrates the possibility of there being two different arrangements producing a 270K protein. As discussed above, the first may represent a trimeric pair whereas the second may be an artefact of sample processing and may represent a perihexagonal unit. This may help explain the decreased intensity of the 270K protein in both infected cell lysates (Fig. 5) and in virus particles (Fig. 4) when subjected to urea sample buffer as compared to boiling sample buffer, since only the latter treatment could probably disrupt hexamers to produce perihexagonal units.
Protein heterogeneity in the 45K region was evident in 2-ME-untreated/boiled preparations of both the virus particle and immunoprecipitated infected cell lysates. This diffuse band merged into a tight, slightly slower migrating band upon 2-ME treatment, suggesting the presence of intramolecular disulphide bridging within the monomer. The identification of similar protein heterogeneity in the 135K mol. wt. range may indicate that intramolecular disulphide bridging of the 45K monomer is not a prerequisite for trimer formation. Since this banding of the trimer was only evident in infected cells and not in the virus particle, it is likely either that only the fully oxidized trimers are incorporated into the virion or that at the time of assembly (i.e. during budding of the particles into the endoplasmic reticulum) all the trimers become fully oxidized (Petrie et al., 1982).

The identity of the reactivity patterns of the four neutralizing nucleocapsid-specific monoclonal antibodies with chymotrypsin, papain and S. aureus V8 protease digests of the nucleocapsid protein suggests that they recognize the same antigenic region. Furthermore, these antibodies identify an epitope which is immunogenic when presented in the virus particle because the reactivity pattern of anti-rotavirus serum with the proteolytic digests was comparable to that observed with the monoclonal antibodies. However, this antigenic determinant does not appear to be subgroup-specific since the monoclonal antibodies reacted in immunoblot ELISA with the nucleocapsid proteins of subgroup 1 and 2 rotaviruses. These results support the identification of the nucleocapsid protein as having a common antigenic determinant for rotaviruses originating from different animal species. The observations that this epitope is exposed in infectious virus particles (as illustrated by the fact that 80% of the infectious virus particles could be immunoprecipitated by nucleocapsid-specific antibodies) and that antibodies directed against this epitope have neutralizing ability, may help explain the observed cross-reactivity between rotaviruses belonging to different serotypes and of serotypes among VP3/VP7 reassortants (Hoshino et al., 1985; Offit & Blavat, 1986). Since the neutralizing epitope of the 45K protein is located on the exposed portion of the nucleocapsid and since the nucleocapsid is arranged in ring-like units, it is possible that a neutralizing epitope is present repeatedly on the virion. This type of presentation may explain the high immunogenicity of this protein.

The finding that 80% of the infectious virus particles were immunoprecipitated could be explained by the fact that the virus particles were only partially double-shelled. Alternatively, it may be that the hexameric unit is partly exposed in double-shelled particles as is the case for reoviruses, where the δ2 protein penetrates the reovirus outer capsid shell (Lee et al., 1981). Electron microscopical studies are currently underway in order to resolve this issue.

Further studies include investigations into the conditions necessary for nucleocapsid assembly to occur in vitro using both native protein and recombinant DNA-produced protein. These studies should allow us to understand better the mechanisms involved in virus assembly.

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