JOURNAL OF VIROLOGY, June 1985, p. 791–797
0022-538X/85/060791-07$02.00/0

Vol. 54, No. 3

Rotavirus Neutralizing Protein VP7: Antigenic Determinants Investigated by Sequence Analysis and Peptide Synthesis

PETER R. GUNN,[1] FUMIYASU SATO,[1] KEVIN F. H. POWELL,[1] A. RICHARD BELLAMY,[1]* JAMES R. NAPIER,[2] DAVID R. K. HARDING,[2] WILLIAM S. HANCOCK,[2] LINDA J. SIEGMAN,[3] AND GERALD W. BOTH[3]

Department of Cell Biology, University of Auckland, Auckland,[1] and Department of Biochemistry and Biophysics, Massey University, Palmerston North,[2] New Zealand; and Division of Molecular Biology, Commonwealth Scientific and Industrial Research Organisation, North Ryde, New South Wales 2113, Australia[3]

Received 5 December 1984/Accepted 25 February 1985

The rotavirus neutralizing antigen, VP7, is a 37,000-molecular-weight glycoprotein which is a major component of the outer shell of the virion. The amino acid sequence of VP7 for strain S2 (human serotype 2) and Nebraska calf diarrhea virus (bovine serotype) has been inferred from the nucleic acid sequence of cloned copies of genomic segment nine. Comparison of the amino acid sequences of these two VP7 proteins with those already determined for other rotavirus strains reveals extensive sequence conservation between serotypes with clusters of amino acid differences sited predominantly in hydrophilic domains of the protein. Six peptides have been synthesized that span the hydrophilic regions of the molecule. Antisera to these peptides both recognize the respective homologous peptides in a solid-phase radioimmunoassay and bind to denatured VP7 in a Western blot. However, none of the antisera either recognize virus or exhibit significant neutralizing activity, indicating that these peptide sequences are not available on the surface of the virus.

Acute diarrheal disease in the young of many species, including humans, is frequently caused by rotavirus infections (9, 13, 18). The association between the virus and the disease was first revealed when fecal samples were examined under an electron microscope, although the presence of virus does not always correlate with clinical symptoms (9). Morphological and biochemical analysis of rotavirus isolates has shown them to be members of the family *Reoviridae*: they have an inner core-like structure surrounded by an outer capsid layer and a genome of double-stranded RNA, which for rotaviruses consists of 11 segments. The major antigenic proteins of rotaviruses are VP3, an outer capsid protein which is the viral hemagglutinin, VP6, an inner core antigen which specifies one of two rotavirus subgroups, and VP7, an outer capsid glycoprotein antigen. VP3 and VP6 are coded for by gene segments 4 and 6, respectively (20, 21); VP7 is encoded by gene segment 8 or 9 depending on the virus strain (4, 21). Although monoclonal antibodies against VP3 will neutralize the virus (16), neutralization and serotype specificity is attributed to VP7 (15). Numerous rotavirus strains have been isolated from a wide variety of species (13, 18). To date, four human and three bovine serotypes have been defined by virus neutralization (19, 38, 39). However, the molecular relationship between serotype proteins has not been examined in detail.

In this work we have continued the molecular characterization of VP7 antigens from different serotypes of rotavirus. Our SA11 VP7 sequence (5) is compared here with that for strain Wa (29) and with two other VP7 sequences predicted from nucleotide sequences determined for a human strain, S2, and a bovine isolate, Nebraska calf diarrhea virus (NCDV). These VP7 sequences represent three human serotypes and one bovine serotype defined by neutralization tests (38, 39). We have also attempted to characterize important antigenic regions of SA11 VP7 by synthesizing peptides of defined sequence that correspond to hydrophilic regions of the protein, many of which also show significant amino acid differences from other serotypes. The ability of antisera prepared against these peptides to recognize and neutralize SA11 rotavirus has been investigated.

* Corresponding author.

MATERIALS AND METHODS

Growth and purification of viruses. Rotavirus strains SA11, S2, and NCDV were kindly provided by I. Holmes (University of Melbourne, Victoria, Australia), S. Urasawa (Department of Hygiene and Epidemiology, Sapporo Medical College, Sapporo, Japan), and C. Mebus (University of Nebraska, Lincoln, Neb.) respectively. Viruses were propagated in MA104 cells and purified on CsCl gradients as previously described (32). The upper virus band was diluted 10-fold with a solution containing 20 mM Tris (pH 8.0), 150 mM NaCl (TBS), and 1 mM $CaCl_2$, and 5 ml of the solution was underlaid with 0.5 ml of 50% sucrose in the same buffer. The samples were centrifuged at 45,000 rpm for 2 h in an SW50.1 rotor, and the viruses in the sucrose cushion and pellet were recovered. This procedure yielded a preparation which retained most of the VP7, whereas other procedures often generated viral cores that had lost the outer proteins.

Cloning of double-stranded RNA segments. Clones corresponding to genomic segment 9 of rotavirus strains S2 and NCDV were obtained essentially as described previously (4) but with the following modifications. For S2 rotavirus, single-stranded cDNAs were transcribed, digested with ribonuclease, and separated by polyacrylamide gel electrophoresis in the presence of urea (4). cDNAs corresponding to genes 7 through 9 were eluted, extracted with phenol-chloroform, and precipitated with ethanol. Double-stranded DNAs were formed by annealing at 65°C for 5 h, followed by slow cooling. For NCDV genomic segment 9, cDNA-RNA hybrids were cloned (7). Briefly, after cDNA transcription, the reaction was stopped with 25 mM EDTA, and the DNA-RNA hybrids were recovered by Sephadex G-50 chromatography. The hybrids were further purified by phenol-chloroform extraction and ethanol precipitation. Hybrids and double-stranded cDNAs were tailed with dCMP by

using terminal transferase and cloned into the *Pst*I site of dGMP-tailed pBR322 (4).

Nucleic acid sequence determination. Initially, a partial clone of the NCDV VP7 gene was obtained by previously published procedures (4). These 616 nucleotides were sequenced by the method of Sanger et al. (30) after *Sau*3A1 fragments from the clone were subcloned into *Bam*HI-cut M13 vector mp7.1 (28). The sequence of the remaining bases of a full-length NCDV clone, obtained by the modified procedure outlined above, was determined by the method of Maxam and Gilbert (27). The S2 gene was completely sequenced by this method (27). Each clone was sequenced for the most part on both strands, particularly if the data were not clear and unambiguous.

Western transfer analysis. Viral proteins (about 200 μg on a 10-cm-wide polyacrylamide slab gel) were separated by electrophoresis (25) and transferred to nitrocellulose paper (BA83, pore size, 0.2 μm; Schleicher & Schuell, Inc., Keene, N.H.) essentially by the method of Towbin et al. (34). After blocking nonspecific binding sites on the filter with either 3% (wt/vol) ovalbumin or 3% (wt/vol) bovine serum albumin plus 1% (wt/vol) gelatin in TBS containing 0.1% NaN_3, strips were incubated overnight at room temperature with serum diluted 100-fold in TBS-azide-blocking protein. Filters were then washed six times with 100 ml of TBS (10 min each) and probed with 0.1 μCi of ^{125}I-labeled protein A per ml in TBS containing 3% ovalbumin. Unbound protein A was removed by washing the filters six times with 100 ml of TBS, and the filter was dried and autoradiographed.

Production of antisera. Peptides were synthesized by the Merrifield solid-phase method on a Schwartz/Mann Automatic Peptide Synthesizer, cleaved from the resin with hydrogen fluoride, and partially purified by chromatography on Sephadex G-10 resin (17). Peptide composition was checked by amino acid analysis. Partially purified peptides were coupled to keyhole limpet hemocyanin as follows. Peptide (10 mg) and keyhole limpet hemocyanin (50 mg) were mixed with 1-ethyl-(3,3-dimethylaminopropyl) carbodiimide (2.3 mg) in a total volume of 2 ml of water, left at room temperature overnight, and stored at 4°C without separation of free peptide. The efficiency of coupling, measured by gel electrophoresis in a duplicate reaction with ^{125}I-labeled peptides, varied between 10 and 70% for different peptides.

Coupled peptide was diluted fivefold with phosphate buffer (10 mM, pH 7.0) containing 150 mM NaCl and emulsified with an equal volume of Freund complete adjuvant. For each peptide, three New Zealand White rabbits were inoculated intradermally at 6 to 10 sites along their backs with the equivalent of 200 μg of peptide, given boosters in the same way at monthly intervals, and bled from the ear 7 days after each boost.

Virus neutralization assay. MA104 cells were harvested from roller bottles, washed three times with serum-free medium 199 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% tryptose phosphate (D-medium) and resuspended in the same medium containing 5 μg of trypsin per ml at a density of 2×10^5 cells per ml. Portions (0.1 ml) were dispensed into each well of a 96-well culture plate and incubated at 37°C in a CO_2 incubator to allow a monolayer to form.

An infectious stock of SA11 was diluted into D-medium plus 0.1% gelatin, and portions were first incubated for 1 h with equal volumes of appropriately diluted anti-rabbit antisera prepared in the same medium. A 50-μl sample of each virus-antiserum mix was then added to the cell monolayers. After 4 h of adsorption, the medium was removed, each well was washed three times with D-medium–gelatin, and the cells were covered with medium containing 8 μg of trypsin per ml. After incubation for 5 days at 37°C to allow the virus to replicate, the culture medium was removed, the cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet, and virus neutralization endpoints were determined.

Radioimmunoassays (RIAs). Antigen (5 μg of peptide or 1 μg of whole virus) suspended in 50 μl of 0.1 M sodium phosphate (pH 8.0) was applied to each well of a 96-well microtiter dish that had been pretreated for 3 h at room temperature with 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 5.0). Wells were then blocked with 1% bovine serum albumin in TBS, the solution was reacted for 3 h at 37°C with serum diluted in TBS, and bound antibody was detected by reaction for 3 h at 37°C with 0.5 μCi of ^{125}I-labeled staphylococcal protein A per ml (approximately 100 ng/ml in TBS–1% bovine serum albumin). All steps were separated by three washes with TBS containing 0.1% Tween 20. Individual wells were then cut out, and the bound radioactivity was measured.

RESULTS

Comparison of VP7 gene sequences. The complete nucleotide sequence for a number of rotavirus VP7 proteins has been determined previously (5, 10, 11, 29). Therefore, the detailed nucleic acid sequences of the S2 and NCDV VP7 genes are not presented here. They have been sent to the major data bases (Fig. 1), and copies are also available on request (from G.W.B. or A.R.B.).

The S2, Wa, and NCDV gene sequences are 74.2, 76.3, and 80.4% homologous with the SA11 sequence, having 272, 252, and 208 nucleotide differences, respectively. However, the majority of these nucleotide changes are silent and do not result in changes in the inferred amino acid sequences. For each gene, the first AUG at bases 49 through 51 is conserved, and it seems likely that it is used for initiation (5), although this has not been proved. The termination codon at bases 1027 through 1029 is also conserved, yielding a single open reading frame of 326 codons: this predicts a maximum size of the unmodified VP7 of 37.1 kilodaltons, in close agreement with the apparent size of 37 kilodaltons estimated by gel electrophoresis.

Comparison of VP7 protein sequences. The S2, Wa, and NCDV VP7 protein sequences are 75.4 (80 changes), 82.2 (59 changes), and 84.7% (50 changes) homologous with SA11 VP7, indicating extensive conservation of VP7 proteins between the four serotypes (Fig. 1). Notable features include five conserved stretches of amino acids at residues 101 through 121, 151 through 177, 247 through 260, 283 through 302, and 310 through 316, all of which contain only a few, conservative amino acid changes. Similarly, all cysteine residues in the proteins are unchanged, with the exception of one which is unique to SA11 (residue 32). This cysteine is unlikely to be of great structural significance, as it lies within the second of two runs of amino acids whose hydrophobic nature is conserved between serotypes, despite many amino acid changes. These hydrophobic domains are probably involved in the transport and processing of VP7 and therefore may not be present in the mature protein.

Potential carbohydrate attachment sites differ between strains. For SA11, there is a single glycosylation site at Asn 69, which carries carbohydrate of the high mannose type (6), whereas for S2, NCDV, and Wa proteins, there are several potential carbohydrate attachment sites (Fig. 1), although

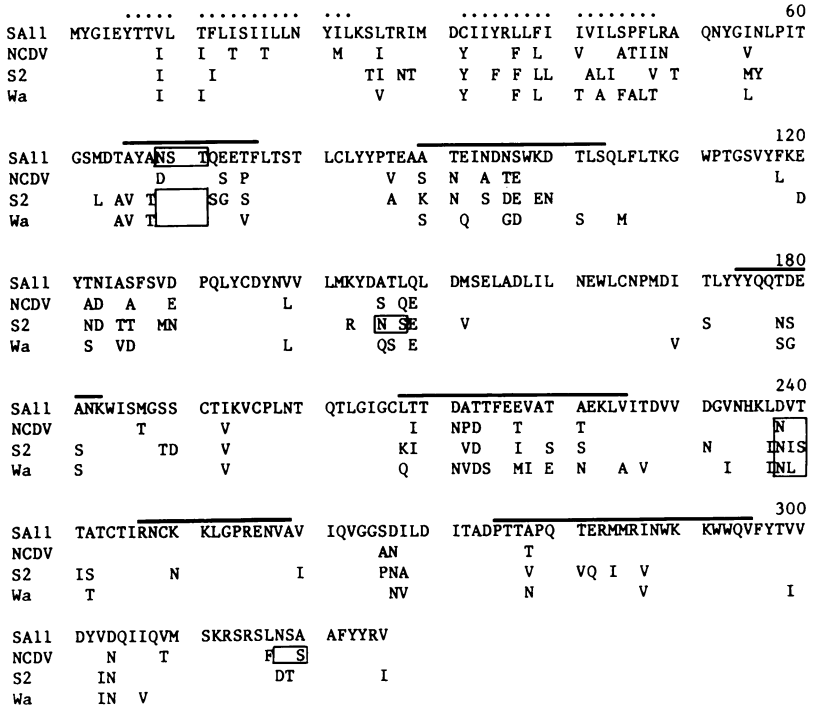

FIG. 1. Comparison of rotavirus VP7 amino acid sequences. The single-letter amino acid code is used. Only amino acid residues which differ from those found for SA11 are indicated for the other strains. Data for rotavirus strain Wa are from reference 29. SA11, NCDV, and S2 are simian 11, Nebraska calf diarrhea, and S2 rotavirus strains, respectively. Symbols: ▭, potential glycosylation sites; ——, SA11 peptides synthesized. Nucleic acid sequence information on which the amino acid sequences in Fig. 1 are based has been lodged with Genbank, c/- Computer Systems Division, Bolt Beranek and Newman, Inc., Cambridge, MA 02238; European Molecular Biology Laboratory, D-6900 Heidelberg, Federal Republic of Germany; National Biomedical Research Foundation (Dayhoff, Data Base), Georgetown University Medical Center, Washington, DC 20007.

which of these is used is not known. The site at residues 69 through 71 is retained in the UK bovine VP7 sequence (11) but is missing in NCDV as a result of an Asn-Asp change at residue 69. Unique sites are present in S2 at Asn 146, and in NCDV at Asn 318. SA11 is the only strain lacking a site at Asn 238. There is therefore considerable potential for variation in the location and amount of carbohydrate attached to VP7 in different rotavirus serotypes.

There is significant amino acid variation between the four serotypically distinct VP7 proteins. Many of the differences are clustered and located in relatively hydrophilic regions of the protein, e.g., residues 66 through 75, 90 through 100, and 146 through 149 (Fig. 2). The hydrophilicity of these regions is consistent with a possible surface location for the variable residues, and, given the otherwise basic similarity of the VP7 proteins, some or all of the variable regions are likely to contribute to antigenic differences between the serotypes.

Synthetic peptides and antisera. To investigate whether any of the hydrophilic regions of the VP7 protein are capable of inducing neutralizing antibodies, peptides corresponding to amino acids 66 through 76, 90 through 103, 174 through 183, 208 through 225, 247 through 259, and 275 through 295 were synthesized, coupled to keyhole limpet hemocyanin, and used to immunize rabbits. Pre-immune and post-immune sera were collected, and the reactivity of each antiserum to the homologous peptide was determined. The results of a typical solid-phase RIA are presented in Table 1 for the set of animals that best responded to immunization with the peptide. All six sera exhibited similar reactivity to the respective homologous peptide, at dilutions down to at least 1:810. The ability of these sera to recognize VP7 was also investigated: Western blotting confirmed that the antipeptide sera recognized the denatured form of the protein (Fig. 3). However, in a solid-phase RIA, a high-titer polyclonal neutralizing antiserum directed against SA11 did not recognize any of the seven peptides when they were immobilized on microtiter dishes pretreated with glutaraldehyde (data not shown).

The reactivity of the peptide antisera in an endpoint dilution neutralization assay was then investigated. In this assay a polyclonal antiserum prepared against whole virus yielded a neutralization titer of 10^6 U/ml, but little or no neutralizing activity was detected for the peptide antisera at dilutions as low as 1:4 (Fig. 4, rows A through G).

Paradoxically, an antiserum to peptide 247 through 259, prepared in one rabbit (A2; Fig. 4, row H) exhibited a low but reproducible neutralization titer (ca. 1:32), and this was confirmed in a plaque reduction assay (data not shown). However, antisera to the peptide 247 through 259 prepared in other rabbits did not exhibit neutralizing activity, and the significance of the weak neutralization shown in Fig. 4 must therefore remain in doubt, in view of the variation commonly found between the immune responses generated by individual rabbits.

As anticipated from the lack of neutralizing activity found for the peptide antisera, none of the sera recognized whole virus in a solid-phase RIA (titers of less than 1:9), whereas the neutralizing polyclonal antiserum exhibited strong affinity for the virus, yielding an endpoint titer of greater than 10^4 (data not shown). Antiserum A2, which exhibited weak neutralizing activity, also weakly recognized virus (titer of 1:27). The possible significance of the failure of the antipep-

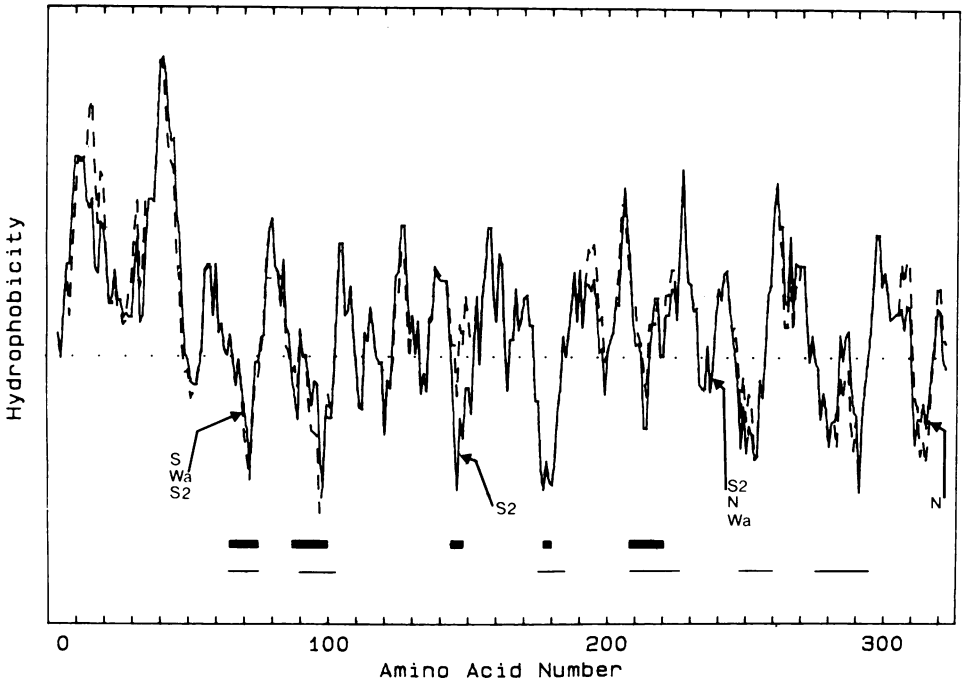

FIG. 2. Hydrophobicity profiles for SA11 and NCDV VP7 proteins computed by the method of Kyte and Doolittle (24). Symbols: ——, NCDV; - - -, SA11. Solid bars indicate hydrophilic regions that exhibit amino acid variability between VP7 sequences of differing serotype (refer Fig. 1). Thin lines indicate SA11 peptides synthesized. Arrows indicate the location of potential glycosylation sites for S2, Wa, SA11 (S), and NCDV (N). Regions above the datum line are hydrophobic: these below the line are hydrophilic.

tide sera to either recognize or neutralize virus is discussed further below, but these results provide evidence that the continuous antigenic determinants presented by the peptides are not available on the surface of the virus.

DISCUSSION

Comparison of VP7 proteins. The nucleotide sequences determined for the rotavirus strains S2 and NCDV, when combined with that for the Wa strain (29), permit the comparison of isolates representing three human and one bovine neutralization serotype. In addition, the NCDV and S2 sequences presented here are closely related to those determined for the UK bovine strain (11) and HU/5 strain (10), respectively, as might be anticipated from antigenic studies (38, 39). There are only 51 base and 11 amino acid changes between UK and NCDV and 56 base and 11 amino acid changes between S2 and HU/5. Taken together, all the VP7 sequences exhibit extensive sequence conservation, with regions of difference clustered in potential surface locations (Fig. 2). The sequence conservation found among different VP7 molecules presumably reflects structural and functional constraints imposed during evolution and argues for a basic similarity in the architecture of VP7 between serotypes, analogous to that observed for the hemagglutin of influenza virus (35).

TABLE 1. Antipeptide activity in serum from immunized animals

Peptide[a] antigen serum	Serum dilution	Radioactivity bound[b] in:	
		Immune serum	Pre-immune serum
247–259	1:10	10,074	55
	1:30	3,963	42
	1:90	1,539	30
	1:270	638	22
	1:810	272	1
	1:2,430	113	27
	1:7,290	48	23
66–76	1:10	3,893	57
	1:810[c]	135	9
90–103	1:10	4,261	45
	1:2,430[c]	164	19
174–183	1:10	4,264	39
	1:2,430[c]	123	34
208–225	1:10	3,300	38
	1:810[c]	121	29
275–295	1:10	9,501	70
	1:7,290[c]	464	141

[a] Peptide (5 μg) was applied to each well of a glutaraldehyde-treated microtiter dish.

[b] Counts per minute I^{125}-labeled protein A (see text). Data for antisera which reacted most strongly with VP7 by Western analysis (see Fig. 3 and text).

[c] The endpoint dilution. Intermediate dilutions not included.

Although most of the sequence variation found between different VP7 molecules is located in hydrophilic regions, significant variation also occurs between serotypes in about the first 50 residues, particularly between residues 40 and 50 (Fig. 1). This variation is probably unrelated to antigenic properties because the region is highly hydrophobic in character (Fig. 2). Furthermore, depending on which AUG is used for initiation of protein synthesis, one (or both) of these putative signal peptides is almost certainly removed during processing and transport of the molecule (12).

Antigenic determinants of VP7. We have also attempted to identify important immunogenic regions of glycoprotein VP7, since it is against this polypeptide that neutralizing antibodies are directed (9, 13, 18, 31). By analogy with the influenza hemagglutinin, for which both the three-dimensional structure and the location of antigenic sites are known (37), potential rotavirus antigenic sites should be located on the surface of VP7. Since the nucleotide sequence for the SA11

VP7 gene had been determined (5), the predicted amino acid sequence was surveyed for hydrophilic domains with potential surface locations on the protein. Six of these regions were synthesized as peptides, coupled to keyhole limpet hemocyanin, and used to produce antisera. As other VP7 sequences became available from our work and from other reports (1, 10, 11, 29), it became apparent that many of these hydrophilic peptides were coincident with regions of the protein which varied extensively in amino acid sequence between serotypes. A peptide spanning the variable region centered on amino acid 148 was not synthesized initially, because for SA11, it did not coincide with a peak of hydrophilicity (Fig. 2).

Although the variable hydrophilic regions of VP7 were obvious candidates to account for the antigenic differences between serotypes, peptide antisera which clearly bound homologous peptides and specifically recognized VP7 by Western analysis failed to bind intact virions or to exhibit significant neutralizing activity, with one interesting exception. Antiserum (A2) prepared against peptide 247 through 259 derived from a conserved but hydrophilic area of VP7 provided low but reproducible neutralizing activity. However, antisera to the same peptide derived from two other rabbits did not show any reactivity. The significance of this result is therefore uncertain. Peptides corresponding to other possible antigenic regions on the molecule (residues 138 through 155 and the C terminus) are currently under further investigation.

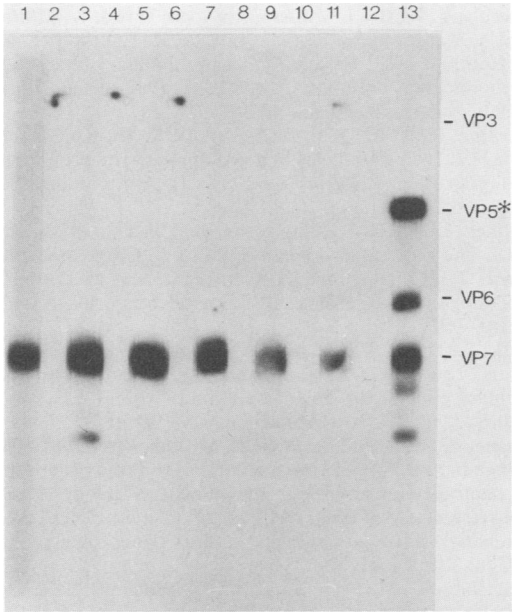

FIG. 3. Reactivity of antipeptide antisera with SA11 viral proteins. SA11 viral proteins, separated on a polyacrylamide-sodium dodecyl sulfate gel, were immobilized by blotting onto nitrocellulose and incubated overnight with 100-fold dilutions of antisera to peptide 66 though 76 (lane 1), peptide 90 through 103 (lane 3), peptide 174 through 183 (lane 5), peptide 208 through 225 (lane 7), peptide 247 through 259 (lane 9), and peptide 275 through 295 (lane 11). Lanes 2, 4, 6, 8, 10, and 12 were reacted with corresponding pre-immune sera, and lane 13 was reacted with 1:200-fold dilution of antiserum to whole virus. Antibody-protein interactions were identified by incubation with 0.1 μCi of iodinated staphylococcal protein A per ml before autoradiography. The position of marker SA11 viral proteins is indicated at the side of the figure. Designation of polypeptides follows the method of Estes et al. (13).

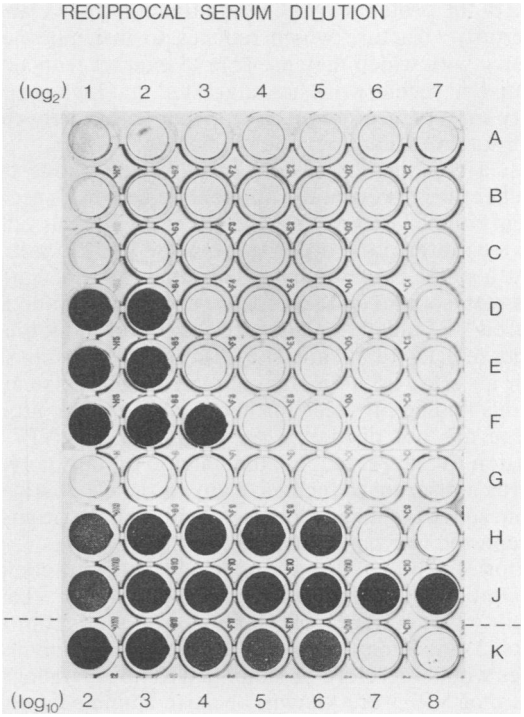

FIG. 4. Neutralizing activity of antipeptide antisera. Standard aliquots of an infective stock of SA11 were preincubated with dilutions of antisera before being applied to monolayers of MA104 cells. Serial twofold dilutions of antisera were applied to rows A through H. Row K received 10-fold dilutions. Row A, pre-immune serum; row B, antiserum to peptide 66 through 76; row C, antiserum to peptide 90 through 103; row D, antiserum to peptide 174 through 183; row E, antiserum to peptide 247 through 259; row F, antiserum to peptide 275 through 295; row G, antiserum to peptide 208 through 225; row H, serum A2 (peptide 247 through 259; see text); row J, no virus or serum; row K, polyclonal antiserum to whole virus. Monolayers were incubated for 5 days and then stained with 0.5% crystal violet to determine the neutralization endpoints.

There are a number of possible reasons for the rather disappointing inability of antipeptide antisera to either recognize or neutralize virus, but all are speculative because of the absence of detailed crystallographic structural information on VP7. First, the regions of VP7 synthesized as peptides may not be located on the surface of the virus but instead could be buried by interaction with other parts of VP7 or with other proteins such as VP3. The possibility of shielding by VP3 gains some support from the fact that neutralizing monoclonal antibodies directed against VP7 almost invariably inhibit hemagglutination (16, 31), a function ascribed to VP3 (15, 20). These two proteins therefore presumably lie in close proximity on the surface, and some regions of VP7 may therefore be unable to interact with the antibody. Second, some parts of VP7 could be covered by carbohydrate that might mask antigenic epitopes in a manner analogous to that described for the influenza hemagglutinin (37). However, glycosylation is unlikely to result in the masking of the whole surface of SA11 VP7, because the molecule possesses but a single glycosylation site: clearly, there is room on the surface for more than one carbohydrate moiety, since other strains have multiple glycosylation sites which are filled (22, 23). Third, the regions of VP7 on the

surface of the protein may be discontinuous and constrained in a tertiary structure which reduces to insignificance the frequency with which they are able to interact with antipeptide antisera: recent work has suggested that high segmental mobility may be a prerequisite for interaction between the native protein and antipeptide antibodies (33, 36).

Thus, a combination of any or all of the above factors could effectively prevent the antipeptide sera from recognizing their homologous peptides in the virus. In this context, we have confirmed the observation made by Bastardo et al. (3) that a polyclonal serum raised after the inoculation of rabbits with sodium dodecyl sulfate and β-mercaptoethanol-denatured VP7 failed to neutralize SA11; nor did it bind in a solid-phase RIA (data not shown). As this serum should contain an array of antibodies directed against linear peptides, this finding provides further evidence that epitopes of this type are not displayed on the surface of VP7. This conclusion is supported by the failure of our polyclonal anti-virus antiserum to recognize any of the six peptides in a solid-phase RIA (data not shown). Similar observations have been reported for the influenza virus system (14).

A priori, the probability of antigenic sites being composed of discontinuous determinants is much higher than the probability that epitopes are composed of continuous determinants (2, 36). Identification of the amino acids involved in epitopes would therefore be simplified if the crystallographic structure of VP7 were known, because amino acid changes found between serotypes might be clustered in patches on the surface. However, in the absence of that information, it may be possible to identify some key residues by selecting variant rotaviruses propagated in the presence of neutralizing monoclonal antibodies specific for that particular serotype (31). Sequence analysis of the viral RNA should then enable identification of amino acid changes which permit the virus to escape neutralization. This approach proved successful for the influenza virus hemagglutinin and neuraminidase antigens (8, 26, 35) and should be applicable to rotaviruses.

ACKNOWLEDGMENTS

This work was supported by grants from the Auckland Medical Research Foundation, the New Zealand Medical Research Council, and the Diarrhoeal Diseases Control Programme of the World Health Organization.

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