Antigenic Relationships Among Some Bovine Rotaviruses: Serum Neutralization and Cross-Protection in Gnotobiotic Calves

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A method was further developed to screen non-tissue-culture-adapted bovine rotaviruses for serotype, using a neutralization test with infectious fecal rotavirus. One of those rotaviruses (B223) which was not blocked by antiserum to the neonatal calf diarrhea virus (NCDV) serotype was then adapted to cell culture in the presence of the antiserum for two or more passages and hyperimmune antiserum to this isolate had a 60-fold-higher homologous neutralization titer than with the NCDV serotype rotavirus. Seventy-three isolates were serotyped and eight (11%) were not of the NCDV serotype (bovine rotavirus serotype I). Of these eight, five belonged to the new bovine rotavirus serotype II and three were not typed, indicating the existence of one or more further serotypes. Cross-protection studies in gnotobiotic calves showed that cross-protection only occurred between rotaviruses of the same serotype, and even a minor serotype difference was sufficient for the calves to show a lack of cross-protection. The serotypes (I and II and the three untyped isolates) also showed differences in the rate of migration in polyacrylamide gel electrophoresis of some of their RNA segments (no. 4, 6, 7, 8, 9, 10), indicating that they were of different electrophoretotypes.

Bovine rotavirus was first isolated in the United States by Mebus et al. in 1968 (11) and was called reovirus-like (Nebraska or neonatal calf diarrhea virus [NCDV]) (15). Subsequently, the properties of this virus were used for classification of this group of viruses into the genus Rotavirus of the family Reoviridae (2). All other rotaviruses subsequently have been compared antigenically and morphologically with this bovine isolate (NCDV), regardless of species of origin of the virus (5). In 1974 (17) a bovine rotavirus was isolated in England and shown to be closely related to the U.S. isolate, although some differences were reported, e.g., virulence to pigs (7) and the lack of an age-related resistance to the UK isolate in gnotobiotic calves (16). Cross-protection studies also suggested that the two viruses were closely related, although the animals took a longer period of time to develop immune protection when vaccinated with the U.S. vaccine of NCDV and challenged with the UK virulent virus (16). An alternative interpretation of the data in this paper is that the viruses were antigenically different and only showed 62.5% cross-protection, as five of five were protected when challenged 7 to 21 days postvac-
United States were (i) virulent and of the NCDV serotype, (ii) avirulent due to colonization of the country by the NCDV vaccine virus, or (iii) antigenically different from the NCDV serotype. A hyperimmune antiserum to a new isolate of bovine rotavirus together with a hyperimmune antiserum to NCDV were used to serotype rotaviruses isolated from diarrhea of neonatal calves in Pennsylvania, Florida, and midwestern states by the method of Thouless et al. (14). Selected viruses were inoculated orally into gnotobiotic calves, and studies were made on their ability to induce disease and on their cross-protective properties.

**MATERIALS AND METHODS**

**Animals.** Gnotobiotic calves were produced and reared by the method of Matthews et al. (10).

**Cell culture.** Cultures of MA104 cells or BSC-1 cells were prepared as described previously (6). Fetal bovine serum was used for growth of cell cultures, but this was replaced for virus culture with 0.1% pancreatin (4 X N.F., 2.5% [10X; GIBCO Laboratories, Grand Island, N.Y.]); (serum-free [SF] minimum essential medium [MEM]-pancreatin).

**Rotavirus isolates.** The tissue culture-adapted bovine rotavirus (NCDV, B:USA:72:1 Lincoln strain), kindly supplied by R. Wyatt, was originally isolated by C. A. Mebus. R. Wyatt also supplied hyperimmune guinea pig antiserum to NCDV. Other isolates of rotavirus were obtained from diarrheic calf feces samples supplied by R. Whitlock (Pennsylvania) and Braun (Florida); those from Iowa and other midwestern states isolates were obtained by this laboratory or kindly supplied by H. Hill of the Iowa State University Diagnostic Laboratory. Canadian isolate 2352 was identified by L. A. Babiuk and studied in both laboratories (13). The animal-passaged and the tissue culture-adapted porcine rotaviruses (OSU strain P:USA:77:1) were kindly supplied by E. Bohl. The origins of simian and B14 rotaviruses have been described previously (6).

**Virus culture and assay.** Viruses were grown in 3- to 5-day-old MA104 or BSC-1 cells. To adapt fecal rotavirus to cell culture, a 1:3 dilution of the feces in phosphate-buffered saline was incubated at 37°C with an equal volume of EDTA-free trypsin (1,000 μg/ml) and then diluted 1:10 in SF medium and inoculated into cell cultures. Virus was passaged at 24-h intervals by freeze-thawing or trypsinization in MA104 cells with SF MEM-pancreatin medium until a cytopathic effect was evident. For virus assay, 3- to 5-day-old MA104 cell cultures in microtiter plates were washed twice with SF MEM-pancreatin medium. Eight microtiter wells were inoculated with 100 μl each of 10-fold dilutions of virus in SF MEM-pancreatin. The plates were fixed 5 days postinfection with 10% Formalin and stained with 1.0% crystal violet.

**Assay of intestinal or fecal rotavirus.** Unadapted virulent OSU porcine rotavirus and bovine fecal rotavirus preparations were assayed in cell cultures in microtiter plates in the manner described above, except the cultures were fixed at 24 h postinfection in 80% acetone (−20°C) and dried. These plates were examined by immunofluorescence for the presence of rotavirus antigen with gnotobiotic calf antiserum to B14 bovine rotavirus at a 1:40 dilution in phosphate-buffered saline and rabbit anti-bovine gamma globulin conjugated with fluorescein (Cappel Laboratories, Downington, Pa.). The number of immunofluorescent cells was counted in a single field of the 10X objective lens in each of four wells, and the mean number was calculated. The counts were recorded as the mean number of immunofluorescent cells per field and expressed as immunofluorescent cell-forming units (ICFU).

**Serology and serotyping.** Hyperimmune antiserum were prepared in guinea pigs (obtained from the National Animal Disease Center, Ames, Iowa), inoculated twice in the footpads at 3-week intervals with 0.1 ml of cell culture-adapted rotavirus (NCDV or B641) combined with Freund incomplete adjuvant. The method of production of viral antigen has been described before (6). Fecal rotavirus B223 was extracted from feces as a 25% aqueous solution in phosphate-buffered saline and pelleted through 40% sucrose at 80,000 to 100,000 × g. Convalescent antiserum to B2352 virus was obtained from an experimentally infected pig.

(i) NT. NT titers of sera were determined by reacting twofold serum dilutions in SF MEM with 100 to 1,000 tissue culture doses of the respective rotavirus in SF MEM with 0.2% pancreatin. An equal volume of each serum dilution was added to the virus and incubated at 37°C for 1.5 h. Each of eight wells of a microtiter plate containing a 3- to 5-day-old monolayer of MA104 cells was inoculated with 100 μl of virus-serum dilution mixture. After 5 days of incubation at 37°C, the plates were fixed with 10% Formalin, stained with 1.0% crystal violet, and examined for cytopathic effect. The neutralization titer of each serum was expressed as the reciprocal of the highest dilution giving a 50% or greater reduction in the number of cells showing viral cytopathic effect. The reproducibility of this assay was determined by repeatedly testing a standard antiserum. The endpoint varied by a ± twofold dilution. The titers of the hyperimmune antiserum were determined against the homologous virus used for induction of NT antibodies. The homologous titers ranged from 6,400 upwards. Heterologous strains of rotavirus, e.g., the OSU strain of porcine rotavirus, were shown not to be neutralized by these antisera at dilutions of 1:100 (6). For (ii) below, the antisera were diluted at 1:100 and 1:800 or 1:1,000, and the ability of unadapted fecal virus to replicate in the presence of these antisera was determined.

(ii) NT of unadapted fecal or intestinal rotavirus. Fecal preparations were diluted with SF MEM with 0.2% pancreatin to give 100 or more ICFU. This was diluted with an equal volume of antiserum, and the mixture was incubated at 37°C for 1.5 h. Four microtiter plate wells were inoculated with each virus-serum mixture or with a virus-medium mixture and incubated at 37°C for 24 h. Cultures then were fixed and examined for the number of immunofluorescent cells. The serum titer was recorded as the dilution which neutralized 80% or more of the ICFU.

**Adaptation to tissue culture of a new serotype of bovine rotavirus.** Fecal rotavirus (B223) which was not neutralized by antiserum to the laboratory strain B641 or antiserum to NCDV was treated with trypsin as described for rotavirus culture and subcultured in...
MA104 cells with SF MEM-pancreatin. Antiserum to B641 and NCDV were incorporated into the media at a final concentration of 0.5% of each serum to prevent any B641/NCDV variants from replicating. After 3 passages in the presence of these antisera, the antiseras were excluded from further passages. The rotavirus was grown in cell culture to passage 23 and used for serotyping and for oral vaccination of calves.

Rotavirus RNA extraction and polyacrylamide gel electrophoresis. The method used for RNA extraction and polyacrylamide gel electrophoresis has been described recently (6).

Cross-protection studies. Rotavirus vaccines were prepared from cell-cultured bovine rotaviruses diluted to contain approximately 10^7 50% tissue culture infective doses of virus per ml. As the B223 isolate was not adapted to culture sufficiently to yield that titer of virus, the vaccine dose was approximately 10^6. Some calves were inoculated first with bacteria-free filtrate prepared from fecal rotavirus diluted threefold in phosphate-buffered saline. Gnotobiotic calves were inoculated intranasally with a vaccine rotavirus or 5 ml of fecal rotavirus, and records were made of food consumption, change of color of feces, and development of diarrhea. This latter characteristic took the form of a large production of feces of a yellow color, semisolid to liquid in consistency. Anorexia was recorded if 50% or less of normal milk consumption occurred per day. Calves were bled at 14 or 21 days postinfection and then challenged with virulent rotavirus (5 ml of a 33.3% [vol/vol] bacteria-free filtrate of fecal rotavirus). This material was obtained from fecal or intestinal samples of naturally infected and conventionally reared calves or from gnotobiotic calves inoculated intranasally with a bacteria-free filtrate (0.45 mm) of rotavirus, lacking other known viruses as far as could be demonstrated by electron microscopic examination and with the absence of fecal hemagglutinin for bovine coronavirus and “Breda” viruses (18). General health, anorexia, and incidence of diarrhea again were recorded. The calves were observed for a minimum period of 5 days after challenge. When cross-protection was not observed, the calves became diarrheic within 48 h. When cross-protection occurred between rotaviruses in animals 3 to 4 weeks of age, the challenge virus was inoculated also into unvaccinated control gnotobiotic calves at that age to ensure its ability to induce diarrhea. Fecal samples were taken twice daily after both vaccination and challenge. Successful immunization was considered to have occurred either in calves which excreted the vaccine rotavirus in the feces and seroconverted or in those calves in which tissue culture-adapted vaccine virus was not detectable in the feces and the calves seroconverted. Cross-protection was recorded when the challenged calf remained clinically normal and did not excrete rotavirus in the feces. Seroconversion was recorded initially by the development of immunofluorescent antibodies, at dilutions of 1:40 to 1:160 to tissue culture-adapted rotavirus, and at a later date by determining the NT titer against vaccine virus and challenge virus.

D-Xylose absorption test. Before challenge virus was inoculated and again on the first day of diarrhea, calves were fed 26 g of D-xylose prepared as a 10% solution in water at pH 5.5 and autoclaved at 120°C for 60 min. Plasma samples were obtained with EDTA at 1 and 2 h postfeeding. The method of Eberts et al. (4) was followed for assay of D-xylose. Phloroglucinol (1,3,5-trihydroxybenzene) was obtained from Sigma Chemical Co. (St. Louis, Mo.), and D-xylose was from Fisher Scientific Co. (Pittsburgh, Pa.). The color reagent consisted of 0.5 g of phloroglucinol, 100 ml of glacial acetic acid, and 10 ml of concentrated HCl. Xylose standard preparations were prepared by dissolving D-xylose in saturated benzoic acid to make the following concentrations: 10, 12.5, 25, 50, 75, and 100 mg/100 ml. Xylose assay was performed as follows: 50 µl of plasma or xylose solution was placed in a disposable test tube (16 by 100 mm) and 5 ml of phloroglucinol color reagent was added. Tubes were placed in a water bath at 99°C for 4 min and then cooled to room temperature in water. After mixing, the absorbances were read at 520 nm in a spectrophotometer (Coleman Junior). For each sample and for the xylose solutions an unheated preparation was used to obtain a zero blank for each sample. A heated xylose-negative serum plus phloroglucinol was also used as a zero blank. This heated control sample and the unheated test samples had approximately the same zero or near-zero readings. The D-xylose fed to the calf was checked for D-xylose concentration by the above assay. For interpretation of results, the standard xylose solution readings were plotted on a graph, and the plasma-xylose levels were read from this graph and recorded as milligrams per 100 ml of blood.

RESULTS

A study was made on the effect of filtration (0.45 µm) of fecal rotavirus (6,000 x g supernatant fluid) on the ICFU titer of B641 virus and the titer of specific neutralizing antisera (GP48). The titers of unfiltered and filtered preparations were 65 ± 4 x 10^2 and 16 ± 3 x 10^2, respectively. The titer of antisemum (80% NT of ICFU) was 1,600 for both unfiltered and filtered viral preparations. As filtration significantly reduced the infectivity of the virus and many processed fecal samples only produced 10^2 ICFU or less per field in inoculated monolayers, unfiltered virus was used for the remainder of the studies.

The specificity of the NT with fecal rotavirus as antigen was determined by a comparative NT test with B641 fecal virus and OSU fecal virus against their respective specifically prepared antisera (GP48 and GP44). With tissue culture-adapted viruses these antisera had showed 128- and 64-fold higher NT titers, respectively, with the homologous virus, demonstrating a significant antigenic difference between the two viruses (6). In this study antisemur to B641 had a 16-fold-higher NT titer with fecal B641 than with fecal OSU virus. For OSU virus, the homologous titer was 128-fold higher than the heterologous titer. These results confirmed that serotype-specific antisera could discriminate by NT between serotypes of rotavirus when these were used as fecal extracts.
Hyperimmune guinea pig culture-adapted was inoculated 2,400 approximately antisera B641 isolates neutralized both than (GP48) approximately 10-fold-higher dilution, by os or B641, B641, and B641 isolates, and thus it could not be deter-

Serotyping of fecal bovine rotavirus isolates. Hyperimmune guinea pig antisera to NCDV and B641 isolates were used. Four guinea pigs were inoculated with one of the viruses, and the NT titer was determined against each virus. The NCDV antisera neutralized NCDV and B641 tissue culture-adapted viruses to the same titer, approximately 2,400 to 4,800. The B641 antisera neutralized both viruses but showed an approximately 10-fold-higher NT titer, e.g., to B641 (GP48) than to NCDV (2,400 and 150, respectively). From these data we concluded that the two bovine isolates were closely related, although NCDV antisera appeared to possess broader NT activities (Table 1).

For NT of bovine fecal rotaviruses the specific antisera to NCDV (GP56) and B641 (GP48) were diluted 1:100 and 1:1,000. At the 1:100 dilution both fecal virus preparations, NCDV and B641, were neutralized 100% by both antisera and at the 1:1,000 dilution they were neutralized 80% or better. NCDV antiserum at 1:1,000 neutralized all of the NCDV ICFU but only 80% of the ICFU of B641.

A total of 129 U.S. isolates of fecal bovine rotavirus and one Canadian tissue culture-adapted rotavirus isolate (B2352) were screened against B641 antisera. Of the 129 fecal viruses, 72 were shown to have a sufficiently high ICFU count to produce reliable data. All viruses showing an 80% or greater reduction of ICFU at a 1:100 antisera dilution were recorded as neutralized. Of these 72, 15 were not neutralized by B641 antisera at a 1:100 dilution, but of these 15, 8 were neutralized by NCDV antisera at 1:100. In addition, B2352 virus was not neutralized by B641 and NCDV antisera. Thus, 8 of 73 (11%) were antigenically distinct from both NCDV and B641, and again a difference was noted in the antigenic relationships of NCDV and B641. Rotavirus isolates B681 and B720 used in later studies belonged to the NCDV serogroup.

Serotyping of B223 bovine rotavirus serotype II. B223, one of the non-NCDV/B641 rotaviruses, was selected for further studies. Hyperimmune antiserum was prepared in a guinea pig (GP67) with semi-purified fecal B223 rotavirus, and this antiserum was used in comparative NT with the following tissue culture-adapted rotaviruses: B641, NCDV, and B223. B223 tissue culture-adapted virus was tested by NT with antisera to simian, OSU, B641, NCDV, and B223 and their respective antisera. B223 differed from all other rotaviruses by 48- to 96-fold with their respective antiserum and from B641 and NCDV by 32- and 60-fold, respectively, with B223 antiserum. The antigenic relationship of B223 to other animal rotaviruses is summarized in Table 1.

Serotyping of non-NCDV/B641 fecal rotavirus isolates. Seven of eight fecal rotaviruses (excluding B223) were tested by NT with hyperimmune antiserum (GP67) to B223 at 1:100 and 1:1,000 dilutions. Four of seven showed 80% or greater NT at a 1:100 antiserum dilution and were considered to be B223 serotypes. One of the remaining three, rotavirus isolate B2352 (tissue culture-adapted Canadian rotavirus isolate), was tested by NT with the antiserum to B641, NCDV, and B223 and differed from the other three by 96- to 240-fold. This virus was designated tentatively as bovine serotype III. However, B2352 convalescent antiserum had a low titer of homologous NT (240) and did not differentiate B2352 from NCDV, B641, and B223 by NT. The two remaining viruses, which were not neutralized by antiserum to NCDV, B641, and B223 were considered either as additional serotypes or as possibly belonging to serotype III; further studies with these viruses and B2352 are in progress.

Comparative RNA segment migration patterns of B223, NCDV, and B641. B223 differed from B641 in segments 7 or 8 or 9 (probably 9) 10, and possibly 4. B223 differed from NCDV in segments 4, 6, 7 or 8 or 9 (probably 9), and 10. In both studies, segments 7, 8, and 9 migrated closely together, and thus it could not be deter-

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Animal no.</th>
<th>Simian</th>
<th>OSU</th>
<th>B641</th>
<th>NCDV</th>
<th>B223</th>
<th>2352</th>
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<td>36</td>
<td>12,800</td>
<td>&lt;100</td>
<td>ND*</td>
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<td>&lt;100</td>
<td>ND</td>
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<tr>
<td>OSU</td>
<td>44</td>
<td>&lt;100</td>
<td>6,400</td>
<td>ND</td>
<td>ND</td>
<td>&lt;10</td>
<td>ND</td>
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<td>B641</td>
<td>48</td>
<td>ND</td>
<td>ND</td>
<td>2,400</td>
<td>150</td>
<td>50</td>
<td>&lt;10</td>
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<tr>
<td>NCDV</td>
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<td>ND</td>
<td>ND</td>
<td>4,800</td>
<td>4,800</td>
<td>&lt;100</td>
<td>50</td>
</tr>
<tr>
<td>B223</td>
<td>67</td>
<td>ND</td>
<td>ND</td>
<td>150</td>
<td>80</td>
<td>4,800</td>
<td>50</td>
</tr>
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</table>

* ND, Not done.
TABLE 2. Studies on virulence, convalescent antibody response, and cross-protection

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<th>Calf no.</th>
<th>Virus (age at vaccination [days])</th>
<th>D*</th>
<th>D-X*</th>
<th>VE*</th>
<th>Virus (age at challenge [days])</th>
<th>D</th>
<th>D-X</th>
<th>VE</th>
<th>Cross-protection</th>
<th>NT titer 3 weeks postvaccination to given virus</th>
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<tr>
<td>GC6</td>
<td>B720d (5)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>B641d (26)</td>
<td>-</td>
<td>NA</td>
<td>-</td>
<td>+</td>
<td>320 160 160</td>
</tr>
<tr>
<td>GC11</td>
<td>B681d (4)</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>B641d (25)</td>
<td>-</td>
<td>NA</td>
<td>-</td>
<td>+</td>
<td>320 320 20</td>
</tr>
<tr>
<td>GC8</td>
<td>B641d (28)</td>
<td>++</td>
<td>ND*</td>
<td>+</td>
<td>B223d (36)</td>
<td>++</td>
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<td>+</td>
<td>+</td>
<td>480 480 10</td>
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<td>B641d (30)</td>
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<td>+</td>
<td>B223d (22)</td>
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<td>+</td>
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<td>80 240 10</td>
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<td>B641d (15)</td>
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<td>+</td>
<td>200 200 10</td>
</tr>
<tr>
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<td>+</td>
<td>B641d (15)</td>
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<td>ND*</td>
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<td>-</td>
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<td>GC30</td>
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<td>+++</td>
<td>ND*</td>
<td>+</td>
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<td>-</td>
<td>NA</td>
<td>-</td>
<td>+</td>
<td>160 160 640</td>
</tr>
</tbody>
</table>

* D, Diarrhea developed.
* D-X, D-Xylose malabsorption demonstrated.
* VE, Virus excreted in feces.
* Fecal rotavirus (virulent).
* NA, Not applicable.
* ND, Not done.
* Tissue culture-grown rotavirus.

mined with absolute certainty which of the three segments migrated differently.

The comparative RNA migration patterns have not been performed between the Canadian isolate and NCDV, B641, and B223.

Convalescent antibody response, virulence, and cross-protection studies in gnotobiotic calves. Five recently isolated bovine rotaviruses (B641, B720, B681, CD15, and B223) were tested in gnotobiotic calves for their virulence and for the specificity of the immune response of the animals. All five and the tissue culture-adapted B223 caused diarrhea of varying severity, and some calves developed anorexia and marked dehydration. The diarrhea was graded + to ++++, indicating severity including anorexia and dehydration. Diarrhea persisted for 3 to 5 days. All of those calves in rotavirus diarrhea which were tested showed more than 70% D-xyllose malabsorption when the absorption rate was compared with preinoculation rates. This was not tested in those calves which were inoculated at 0 to 1 day of age. In addition to those animals recorded in Table 2, calves GC7 and GC12 were inoculated with B641 and calf GC21 was inoculated with CD15. The severity of diarrhea was: GC7, ++++; GC12, ++; and GC21, ++++. All three calves developed D-xyllose malabsorption of 70% or greater. The wild-type rotaviruses B681 and B720 showed cross-protection against challenge with B641 in calves GC6 and GC11, and their sera showed cross-neutralization with NCDV and B641 with both convalescent calf antisera and the respective hyperimmune guinea pig antisera. Thus, B681 and B720 are of the same serotype as NCDV/B641. B641 was confirmed in two calves (GC8 and GC9) as being capable of causing diarrhea at 28 and 30 days of age, respectively. For B223 virus this was not confirmed experimentally, as calves challenged with the virus at 22 to 36 days of age, despite vaccination, developed diarrhea (calves GC15, GC17, GC19, GC22, GC24, GC25). Calves vaccinated with NCDV (GC19, GC22, GC24, GC25) and with B641 (GC15, GC17) developed diarrhea and excreted virus after challenge with fecal B223. The tissue culture-adapted B223 virus caused severe diarrhea (but without anorexia) for 5 days in calves GC28 and GC30. These calves were protected against B223 fecal challenge. The observation that calves GC27 and GC29, vaccinated with NCDV and challenged with B641, developed diarrhea and excreted virus was surprising with regard to their close NT relationship. To test whether the challenge viruses may have been of mixed serotypes, the RNA electropherotype patterns of the viruses excreted by those calves not showing cross-protection (GC15, GC17, GC19, GC22, GC24, GC25, GC27, GC29) were tested by coelectrophoresis with the challenge virus RNA. In all cases, the patterns were identical, implying that the challenge viruses used were not mixtures of different types and that the calves excreted the
challenge virus when in diarrhea. Thus, from the data obtained of both disease induction and viral excretion in challenged calves, we conclude that cross-protection did not occur in these animals. Thus, at least three distinct types of rotavirus exist which show cross-protective differences, and these are represented by NCDV, B641, and B23. Calf GC26, which was vaccinated with virulent B223 at 7 days of age (and developed severe diarrhea), was protected against challenge with B641 virus (a different serotype). Convalescent serum responses of some of the calves were recorded in Table 2. In addition, two calves (GC5 and GC13) were inoculated with another tissue culture-adapted bovine rotavirus, B14, which is of the same serotype as NCDV. Of 17 calves (including GC7, GC12, and GC21), 13 immunized with rotaviruses other than B223 produced low NT responses to B223 (10 to 20), with three (GC6, GC7, and GC21) responding as for NCDV/B641 (NT of 80 to 320). In contrast, calves immunized with B223 developed high-titer antibodies to B641 and NCDV (GC26, GC28, GC30). The presence or absence of neutralizing antibody to the challenge virus in vaccinated calves in general correlated with cross-protection. However, calves GC27 and GC29 vaccinated with NCDV succumbed to B641 challenge despite possessing serum-neutralizing antibody to the challenge virus.

**DISCUSSION**

For the recognition of different serotypes we have used a 20-fold or greater difference of NT titers in two-way cross-reactions (6). In general, convalescent antisera discriminate poorly between strains, and hyperimmune antisera are required (6).

The NT test performed with fecal rotavirus as antigen proved to be a useful screening method. It permitted the recognition of eight rotavirus isolates which appeared to be of different serotypes from NCDV/B641 (bovine serotype I group). One of these (B223), after subculture in the presence of antisera to NCDV/B641, proved to be a distinct rotavirus serotype showing at least a 48-fold NT difference from all other rotavirus serotypes used, and a further four isolates appeared to be of the same serotype as B223. These we called bovine serotype II group. The Canadian isolate B2352 may represent bovine serotype III. Two further isolates were distinctly different from the NCDV/B641 (bovine serotype I) and B223 (bovine serotype II) isolates, but we were unable to confirm whether or not they belonged to the serotype III group. Antisera are being prepared to these three viruses and their serotype classifications will be determined. Thus, from this study 89% of isolates were similar to bovine serotype II, and 4% remained untyped.

This screening test for serotypes should be confirmed by adapting the variant rotaviruses to cell culture and then performing NT on the adapted virus with hyperimmune homologous and heterologous rotavirus strain antisera.

All five wild-type rotaviruses tested for virulence in gnotobiotic calves caused diarrhea and D-xylose malabsorption. As these viruses were isolated from calves in Florida, Pennsylvania, and the Midwest, these limited data appear to deny the hypothesis that the predominant natural rotavirus infection of diarrheal calves is the vaccine strain. However, some vaccinated calves will excrete detectable quantities of vaccine rotavirus in the feces, and diagnostic laboratories should consider the vaccination history of the calf in question before concluding that the isolate is a virulent strain.

B641 virus appears to be a minor serotype variant of the NCDV serotype group. Antiserum to NCDV neutralized both NCDV and B641 to similar titers but B641 antisera had a 10-fold higher NT titer to B641 than to NCDV. However, this one-way cross-reaction did not correlate with cross-protection, as calves GC27 and GC29 vaccinated with NCDV were not protected against challenge with B641 virus. In general, the cross-protection studies confirmed the serotyping by NT and the results were similar to previously reported work with rotaviruses from calves, pigs, dogs, and monkeys (6). With the exception of NCDV and B641, all viruses that showed NT serotype relationships were cross-protected, whereas B223 only showed cross-protection with its homologous tissue culture-adapted vaccine. It is possible that NCDV vaccine virus produced a poor gut immune response, as it did not cause diarrhea in any calf and three of six NCDV-vaccinated calves did not excrete detectable quantities of NCDV in the feces. However, all six calves seroconverted to the vaccine virus and calves GC27 and GC29 did excrete vaccine virus, although they were not protected from challenge with B641 virus. Cross-protection may be a more sensitive method for studying the antigenic relationships between rotaviruses than NT in vitro. To study this in more depth, copro-antibody responses are being studied, following the method of Crouch (Ph.D. thesis, University of Surrey, Surrey, U.K., 1980), to determine whether the specificity of gut origin antibody shows a stronger relationship with cross-protection than does serum antibody.

The data from the serological responses and cross-protection induced by calves inoculated with fecal or tissue culture-adapted B223 rotavirus (GC26, GC28, GC30) suggest that this
virus may have a broader antigenic makeup than the other viruses used. It is possible that B223 fecal virus (GC26) may have been contaminated with rotaviruses of NCDV/B641 serotypes. However, we feel it is unlikely that these serotypes persisted in the cultivation of B223 in the presence of antisera to these viruses, and this tissue-cultured-adapted virus did induce protection to the fecal virus (GC28, GC30). It is being studied whether tissue-culture-adapted B223 virus is a suitable vaccine against the serotypes NCDV/B641 in addition to B223 serotypes. Studies have shown that convalescent serum (6) or bovine colostrum (1) have a broader antigenic specificity than hyperimmune serum and active immune responses of the gut and will neutralize or block different rotavirus serotypes. We have studied three samples of bovine colostrum for neutralizing antibody to canine rotavirus (6) and have found that these have the same NT titer against both bovine rotavirus and canine rotavirus (titers, 160 to 320; unpublished data). It is possible that the wide colostral antibody activity to rotavirus serotypes is a reflection of repeated infections of cattle with different serotypes of rotavirus, but we feel that it also reflects, at least in part, the broad response to a single infection, as shown by convalescent serum antibody (6). To test this hypothesis, we are planning cow vaccinations with different serotypes to determine whether colostral protection in calves against different serotypes can be induced by one or a limited number of serotypes.

Detailed results of the RNA electropherotype relationship of the bovine rotaviruses studied will be published (T. F. Simpson and G. N. Woode, submitted for publication). That NCDV and B641 viruses differed from B223 in segments 4, 6, 7 or 8 or 9 (probably 9), and 10 is interesting. A previous study by us (6) suggested that, from coinfection data with a number of different rotavirus serotypes, the NT specificitities may be in the coding of segment 11 for major differences and 2 or 3 or 9 segments for minor differences. Studies have been reported that the coding of segments 10 and 11 can be reversed with different rotaviruses (3). Working with reassortants of bovine (UK) and human (WA) rotaviruses, Kalica et al. (8) showed that segment 9 codes for NT specificity of serotypes. From analysis of coinfection data, the data in this paper agree that probably segment 9 codes for the NT-specific antigen of bovine rotavirus. Studies are being done to test the hypothesis that bovine rotaviruses of the same serotype will show coinfection of these various segments, but particularly of segment 9. If this hypothesis is confirmed, the RNA electropherotype could be used to predict the serotype of a particular isolate.

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LITERATURE CITED