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Protection between Different Serotypes of Bovine Rotavirus in Gnotobiotic Calves: Specificity of Serum Antibody and Coproantibody Responses

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In a previous study, different U.S. isolates of bovine rotavirus were studied for their serotypes and cross-protective properties (G. N. Woode, N. E. Kelso, T. F. Simpson, S. K. Gaul, L. E. Evans, and L. Babiuk, J. Clin. Microbiol. 18:358–364, 1983). Three viruses belonging to two different serotype groups were used as vaccines in gnotobiotic calves, which were subsequently challenged with B641 or B223, representing the two bovine serotypes. In the present work, the experiments were repeated with more calves and the specificity of their antibody responses was measured and compared with the results of the protection studies. Protection between different serotypes occurred under both homologous and heterologous conditions but was not directly serotype dependent. B223 virus showed both homologous and heterologous protection against B223 and B641 challenge viruses. This was a one-way reaction, as B641 did not induce protection against B223. Neonatal calf diarrhea virus vaccine produced neither homologous (against B641) nor heterologous (against B223) protection. The plaque reduction neutralization titers of serum antibody and coproantibody did not predict a state of protection against the challenge virus. Calves vaccinated with neonatal calf diarrhea virus or B641 developed neutralizing antibodies to their respective heterologous challenge viruses but were not protected. After challenge, the boosted coproantibody plaque reduction neutralization response to the original vaccine virus was greater than that to the challenge virus.

There have been several reports of the isolation of different serotypes of bovine rotavirus (15, 22, 29). In one of these studies of bovine rotaviruses isolated in the United States, three viruses representing two major serotypes were compared (29). The neonatal calf diarrhea virus (NCDV) Lincoln strain was shown to be closely related by neutralization to a new isolate (B641) and to be serotypically distinct from another isolate (B223). In studies on heterologous protection, NCDV vaccine failed to induce protection against B223 challenge in experimentally infected gnotobiotic calves, and surprisingly, also failed to protect two calves against B641, despite inducing neutralizing antibodies to B641 prior to challenge.

The current information on heterologous protection induced by different serotypes of rotavirus in different mammalian species is confusing. Most studies show that in infections of antigenically unprimed animals, similar serotypes induce active immune protection to each other, whereas dissimilar serotypes induce relatively poor or no heterologous protection (2, 10, 16, 25, 28, 29). However, in trials with children and in experimental studies with calves and piglets, protection has been reported between NCDV vaccine and experimental or natural infections with human rotavirus, although NCDV does not belong to any of the four main serotype groups of human rotavirus (9, 26, 32, 33). Possible explanations for the differences in the results include the criteria accepted for evidence of protection in the various studies, as well as the comparative lack of virulence of human rotavirus in animals and the possibility that it does not, therefore, provide a sufficiently severe challenge. It is possible that children who were protected had been primed at an earlier age with a strain related to the challenge human rotavirus. However, the degree of protection in the children may also reflect the less-than-optimal protection between different serotypes. More recent reports have shown a lack of protection against rotavirus infection in NCDV-vaccinated children (8), and immunity to repeat infections appears to be serotype dependent (4). Some NCDV vaccine studies have shown that under farm conditions, the vaccine gives little protection against bovine rotavirus infections in calves (1, 7, 27) and against swine strains in pigs (14). The failures are thought to be due to blocking of the vaccine by colostral antibody, poor response to vaccination by the animals, or the presence of different serotypes of rotavirus.

The situation regarding humoral antibody responses may be similarly unclear. Although protection provided by lactogenic immunity in mice is serotype dependent (18, 19), the serum response is usually broader, at least in cattle (22, 29). In one study, cows vaccinated with a particular serotype developed neutralizing responses to the vaccine strain and to strains to which they had been previously exposed, but not to other serotypes (22).

There have been a number of studies on the presence of rotaviral antibodies in feces of human, calves, and pigs after infection, but these have been confined largely to studies, usually by enzyme-linked immunosorbent assay (ELISA), on the immunoglobulin classes (5, 6, 12, 23).

This paper describes further studies on heterologous protection among NCDV, B641, and B223, on the specificity of the serum and intestinal antibody responses as measured by

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neutralization, and on the way these responses correlate with a state of protection.

MATERIALS AND METHODS

Animals. Gnotobiotic calves were obtained and reared as described previously (29).

Cell culture. Cultures of MA104 cells or BSC-1 cells were prepared as described previously, in medium containing fetal bovine serum for cell growth and in medium with the fetal bovine serum replaced by 0.1% pancreatin (4× NF, 10× concentrated; GIBCO Laboratories, Grand Island, N.Y.) in serum-free (SF) minimum essential medium (MEM) for virus culture (29).

Rotavirus isolates. The tissue-culture-adapted bovine rotavirus (NCDV B:USA:72:1, Lincoln strain) was kindly supplied by R. Wyatt, and the other two bovine rotavirus strains used in this study have been described previously (29). The B641 culture-adapted isolate was cloned once by limiting dilutions, and the B223 culture-adapted isolate was cloned twice by plaque selection. We then examined them for clonal purity by picking 10 plaques, amplifying their titers, and determining electropherotype by high-resolution polyacrylamide gel electrophoresis (11). B641 and B223 viruses belong to rotavirus serotypes groups 6 and 7, respectively (9). For immunofluorescence (IF) and ELISA studies, canine rotavirus (C:USA:81:2) was used as antigen (29). The sources of the virulent viruses (B641 and B223) for challenge of vaccinated calves have been described (29). The viruses were passaged in gnotobiotic calves, and soon after the onset of diarrhea, the calves were autopsied. Bacterium-free filtrates were obtained as 25% suspensions of intestinal contents in phosphate-buffered saline (pH 7.2). The electropherotypes of the uncloned virulent B641 and B223 viruses were similar to the electropherotypes of their culture-adapted viruses respectively. Aliquots of the challenge viruses had been stored at −70°C. In an earlier study, these were shown to be virulent to calves at ages 1 to 30 days (29). They consistently caused diarrhea in unprotected calves within 2 to 3 days after inoculation and had infectivity titers of 10^3 or greater. Rotavirus isolate CD12 was used as a 25% solution of the fecal virus. CD12 was isolated from the diarrhea of a colostrum-deprived calf, and since the virus was not neutralized by antiserum to NCDV or to B223, it was considered to be a new serotype.

Protection studies. The protection studies were performed as described previously (29). Results for calves in the previous study have been included in this paper for determination of the serum and fecal antibody titers. Vaccine (5 ml) at a titer of approximately 10^7 tissue culture infectious doses of the relevant strain of virus per ml was fed to most of the calves at 1 day of age and to two calves at 7 or 14 days of age. Thereafter, the calves were monitored for the onset of diarrhea, virus excretion, inappetence, etc. The diarrhea was graded to indicate severity, including anorexia and dehydration (see Table 1). Virulent challenge virus was fed at 14 or 21 days postvaccination, and the calves were monitored as before, except that a D-xylose absorption test was performed immediately prior to challenge and again at the onset of diarrhea (29). A small sample of each virus inoculated was removed from the calf isolator and checked for infectious virus. Protection was recorded when the calves remained clinically normal and did not shed rotavirus in the feces.

Fecal dry-matter determination. Feces were weighed in aluminum dishes (ca. 3 g per dish) and then placed for 7 to 10 days in a dry incubator at 37°C. The dry weight was calculated as a percentage of the wet weight.

Virus culture and assay. Viruses were cultured and titrated in flasks and microtiter plates as described previously (29). For assay of fecally excreted virus, a microtiter plate of MA104 cells was inoculated with 25% dilutions of fecal supernatants and fixed at 24 h for IF.

Antibody assays. The viruses were serotyped by using hyperimmune antisera prepared in guinea pigs, as described previously (29). In addition, antiserum to each strain of virus, obtained from calves convalescent for 3 weeks, was used for controlling the specificity of the neutralization titer (NT) and plaque reduction neutralization (PRN) tests and for serotyping virus excreted by one of the calves (GC27).

(i) NT. The NT method of Woode et al. (29) was followed. For determination of NT of coproantibody, 25% solutions were prepared in phosphate-buffered saline and centrifuged at 6,000 × *g* for 30 min, and the supernatants were stored at −80°C until assayed as for serum. As a control for this test, a standard calf antiserum (from calf GC5) was titrated in SF-MEM or in a 25% solution of antibody-negative gnotobiotic calf feces in SF-MEM. The NT of this standard serum was not affected by the presence of the fecal supernatant. To confirm that the NT activities of fecal supernatants were due to antibody, all positive samples were also tested by ELISA and IF. Later, it was shown that gnotobiotic calf coproantibody was stable in feces or as a 25% suspension, when stored at 4°C.

(ii) PRN. Confluent monolayers of MA104 cells prepared in eight-well plastic plates (Costar, Cambridge, Mass.) were washed twice with SF-MEM. Each virus was pretreated with SF-MEM containing trypsin (10 μg/ml; Difco Laboratories, Detroit, Mich.) at 37°C for 1 h. For virus assay, virus dilutions were adsorbed onto MA104 cells for 1 h at 37°C, and the cells were washed once with SF-MEM and overlaid with 2 ml of SF-MEM–1.0% agar (special Noble agar; Difco), containing 2.5 μg of trypsin and 75 μg of DEAE-dextran per ml. The plates were incubated for 2 to 3 days, and 2 ml of a second overlay was added (SF-MEM with 1.0% agar and 0.01% neutral red). Plates were incubated at 37°C overnight and read or fixed with 10% Formalin and stained with 1% crystal violet. For PRN, virus was diluted to approximately 60 PFU/ml. This was mixed with equal volumes of serum or fecal dilutions in SF-MEM, incubated at 37°C for 1 h, and adsorbed to the plates, and the procedure was completed as for the virus assay. Antiserum titers were expressed as the highest dilution which reduced the plaque count by 50% or greater. Each PRN test was controlled with positive and negative standard antisera or standard fecal preparations, specific for the viral serotype being studied.

(iii) IF. An indirect IF test was performed (28) with antiserum or fecal 1:20 dilutions, followed by rabbit anti-bovine immunoglobulin G conjugated with fluorescein (Cooper Biomedical, Inc., West Chester, Pa.) at a dilution of 1:200. As controls, the standard positive antiserum, GC5, and a negative antiserum, GC76, were diluted 1:20 in 5% antibody-negative fecal preparation. Canine rotavirus-infected MA104 cells served as antigen.

(iv) ELISA. The ELISA method has been described previously (31). Canine rotavirus antigen, purified by pelleting at 100,000 × *g* through 30% sucrose, was adsorbed overnight to flat-bottomed microtiter ELISA plates (Immulon 11 TM; Dynatech Laboratories, Inc., Alexandria, Va.). For certain tests, B641, NCDV, or B223 rotavirus antigen was used. After 0.1% ovalbumin adsorption to the plate for 0.5 h, 50 μl

TABLE 1. Studies on virulence and homologous and heterologous protection

Calf	Vaccine virus (age [days])[a]	Diarrhea[b]	Virus excretion	Challenge virus (age [days])	Diarrhea[b]	D-Xylose malabsorption	Virus excretion	Protection[c]	
								Hom	Het
GC28	B223 (1)	+++	+	B223 (22)	−	NA[d]	−	+	
GC30	B223 (1)	+++	+	B223 (22)	−	NA	−	+	
GC19	NCDV (1)	−	+	B223 (22)	++	+	+		−
GC22	NCDV (1)	−	−	B223 (22)	++	+	+		−
GC24	NCDV (1)	−	−	B223 (22)	++	+	+		−
GC25	NCDV (1)	−	−	B223 (22)	++	+	+		−
GC27	NCDV (1)	−	+	B641 (22)	+++	+	+	−	
GC29	NCDV (1)	−	+	B641 (15)	+++	+	+	−	
GC52	NCDV (1)	−	−	B641 (22)	+++	+	+	−	
GC26	B223 (7)[e]	+++	+	B641 (29)	−	NA	−		+
GC32	B223 (1)	+++	+	B641 (15)	−	NA	−		+
GC47	B223 (1)	+++	+	B641 (22)	−	NA	−		+
GC55	B223 (1)	+++	+	B641 (22)	−	NA	−		+
GC15	B641 (14)[e]	+++	+	B223 (36)	++	ND[f]	+		−
GC17	B641 (1)[e]	+++	+	B223 (22)	++	ND	+		−

[a] Tissue-culture-grown rotavirus unless otherwise specified.
[b] + to +++, Increasing severity of diarrhea; −, no diarrhea.
[c] Hom, Homologous; Het, heterologous.
[d] NA, Not applicable.
[e] Fecal rotavirus (virulent).
[f] ND, Not done.

of each dilution of antiserum or fecal preparation in phosphate-buffered saline was added, and the mixture was incubated for 1 h at room temperature with shaking. The plates were then washed, and peroxidase-labeled goat anti-bovine immunoglobulin G (heavy and light chains; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) and then substrate were added. The four different rotavirus antigens were titrated with the standard antiserum (GC5) and were all diluted to give the same optical density (OD) reading when GC5 was diluted to 1:4,096. As a control for the ELISA on fecal samples, GC5 antiserum diluted in the antibody-negative fecal preparation used as a control for the NT was compared with GC5 diluted in phosphate-buffered saline. The titers in the two diluents were similar (titer of 4,096 at an OD of 0.500). A negative antiserum (GC76) was incorporated into the assay in like manner. These, together with a selected positive fecal antibody preparation (calf GC32), were included for each ELISA.

(v) Blocking ELISA. Fecal samples which reacted with rotavirus antigen by ELISA were confirmed by a blocking test. Before antiserum or fecal dilution was added, 50 μl of preimmune or hyperimmune goat rotavirus antiserum (G75), at a 1:100 dilution, was reacted with antigen. Plates were incubated with shaking for 30 min at room temperature and then washed eight times, and the procedure was continued as for ELISA. Blocking was recorded if the percentage ([OD reading with hyperimmune goat antiserum/OD reading with preimmune goat antiserum] × 100) gave a result of 50% or less. To investigate whether the diluting effect of diarrheic fluids would produce variation among calves, the dry-matter content of the calf feces were determined. As this varied only between 12 and 15% for the 3-week postvaccination period, no correction was made.

Rotavirus RNA extraction and polyacrylamide gel electrophoresis. The methods used for rotavirus RNA extraction and polyacrylamide gel electrophoresis have been described recently (10). For analysis of the subclones of B641, B223, and GC27, the method of Gombold and Ramig (11) was followed. Two virus isolates were considered to have the same electropherotype if no significant difference could be observed between the rate of migration of any of the 11 RNA segments.

RESULTS

Clonal purity of culture-adapted rotaviruses. Ten subclones of B223 were all homogeneous for electropherotype (data not shown). Seven distinguishable electropherotypes were obtained from 10 subclones of B641 (Fig. 1A). Uncloned B641 constituted one major electropherotype, with segments 2, 3, 5, 6, 7, 8, and 10 having electrophoretically variant minor species also detectable. The seven electropherotypes subcloned from the uncloned B641 population simply represented segregation of the heterogeneous genome segments, as none contained segments with mobilities different from mobilities identified in the uncloned B641.

Animal studies. The results of the vaccination and challenge studies in calves are summarized in Table 1. With the exception of results for four calves, the results have been reported previously (29). The additional calves were vaccinated with NCDV and challenged with B641 (GC52) or vaccinated with B223 and challenged with B641 (GC32, GC47, and GC55). The effects of the various combinations on the calves were the same as previously reported for calves GC27, GC29, and GC26, respectively. The use of the additional calves confirmed the earlier results that protection did not correlate directly with the serotype of the vaccine and challenge viruses, except in the truly homologous situation for B223 with calves GC28 and GC30. Viruses of the same serotype (B641 and NCDV) did not induce protection, at least as a one-way reaction, in calves GC27, GC29, and GC52, whereas a heterologous serotype (B223) induced protection against B641 in calves GC26, GC32, GC47 and GC55. This latter result was a one-way reaction, as B641 did not protect calves GC15 and GC17 against B223. The failure of NCDV to induce protection in calves GC19, GC22, GC24, and GC25 against B223, a different serotype, was predictable.

Although one might expect virulent virus as vaccine to induce heterologous protection more readily as a conse-

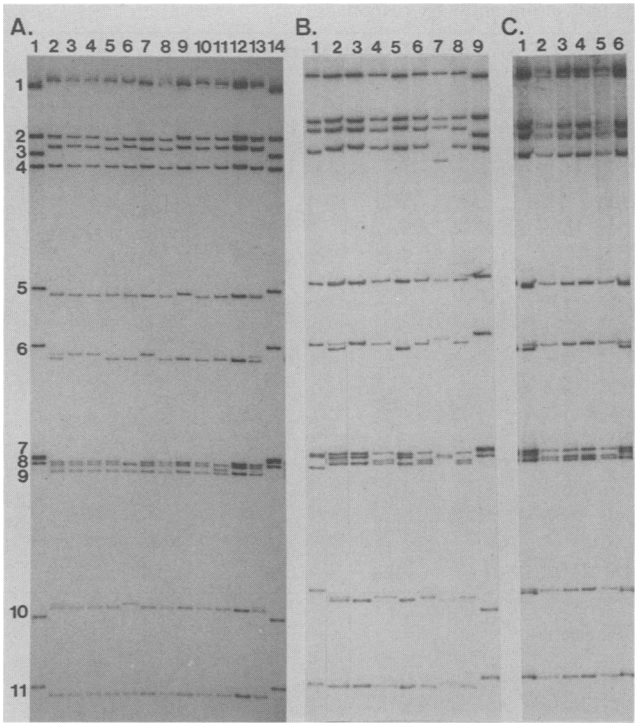

FIG. 1. Electrophoresis of ^{32}P-labeled double-stranded RNAs from selected rotaviruses used in this study. RNAs were labeled with ^{32}P and resolved in 8% Tris-glycine gels 0.75 mm thick and 45 cm long. The distance between segment 1 and segment 11 of SA11 marker RNAs was 20.0 to 20.5 cm, depending on the gel. The genome segments are labeled in order of increasing electrophoretic mobility on the left of the figure. (A) Uncloned tissue culture-adapted B641 and subclones derived from tissue culture-adapted B641. Lanes 2 and 13, uncloned B641. Lanes 3 to 12, subclones 1 to 10, respectively, derived from uncloned B641. Note the heterogeneity that is clearly visible among the subclones for segments 2, 3, 5, 6, 7, 8, and 10. Note also the presence of faint companion bands for the same segments in the uncloned B641. Lanes 1 and 14, SA11 marker RNA. (B) Electropherotypes of selected viruses used in this study. Lane 1, B223; lane 2, tissue culture-adapted uncloned B641; lane 3, tissue culture-adapted B641 subclone 5; lane 5, tissue culture-adapted B641 subclone 7; lane 7, NCDV; lanes 4, 6, and 8, uncloned culture-adapted virus isolated from calf GC27. Note the distinct electropherotypes of B223, B641, and NCDV. Also note the apparent selection of minor species present in the uncloned B641 in the virus isolated from calf GC27. Lane 9 contains SA11 marker RNA. (C) Comparison of subclones derived from culture-adapted virus from calf GC27 to the uncloned B641 virus. Lanes 1 and 6, uncloned culture-adapted B641; lanes 2 to 5, subclones 1 to 4, respectively, from virus shed by calf GC27. Note the identity of electropherotype among the subclones and the presence in the subclones of minor variants present in bands heterogeneous in the uncloned B641. All other subclones derived from GC27 had electropherotypes identical to those shown here.

quence of a greater antigenic stimulation when compared with the avirulent vaccine (NCDV), this was not the case. Virulent B641 did not induce protection against B223 in calves GC15 and GC17.

Coproantibody responses. The coproantibody responses as determined by ELISA were similar to those reported by Hess et al. (12). There was considerable variation among calves, both in the day of first detectable antibody and in the titer. A selection of samples representing strong, moderate, and weak titers were titrated against the standard virus (canine rotavirus) or against the homologous antigen. There was no significant difference between the heterologous and homologous titers. As the viral antigens had been standardized with one serum (GC5), this result can be interpreted to mean that the ELISA was measuring the common antigen(s) of bovine rotaviruses. Comparative titers were determined from the dilution giving a reaction at an OD of 0.200 or greater. Some samples had an OD of <0.200 at 1:4 dilutions but were positive when compared with OD values of known negative preparations and by the blocking test.

The titer of the coproantibody response did not correlate with the severity of the diarrhea induced by the respective virus. As an example, of B223-vaccinated calves, calf GC28 had a peak titer response of 4, whereas that of calf GC30 was 32. A similar variation in response was seen with calves vaccinated with avirulent NCDV (Table 2).

Selected ELISA-positive fecal samples were tested by IF at 1:20 or 1:80 dilutions, and as most were positive at 1:20, these were then tested against culture-adapted NCDV, B641, and B223 for their PRN titers (Table 2). There was poor correlation between the serotype used for vaccination and the specificity of the fecal antibody immune response as measured by PRN. The samples tested were selected because of their ELISA titers. It is possible that responses on days when the calves were not tested might have shown a better correlation, as shown by results for calves vaccinated with B223 (GC28, GC30, and GC55), for GC29 vaccinated with NCDV, and for GC17 vaccinated with B641. The serum antibody responses showed a better correlation with vaccine serotype. There was little or no PRN response to B223 in calves vaccinated with B641 or NCDV. In contrast, calves vaccinated with B223 showed moderate to good PRN titers to B641 and to NCDV, although in all calves there was a 2- to 32-fold higher titer to B223.

The effect of the serotype of the challenge virus on the specificity of the coproantibody-boosted response was then studied. As before, fecal samples taken after challenge of the calves and shown to have the highest positive results by ELISA for the particular calf were tested by PRN (Table 3). Generally, the calves showed a higher PRN response to the vaccine virus than to the challenge virus.

Typing of fecally shed rotavirus. The rotavirus shed in the feces after vaccination or challenge matched the inoculated virus for electropherotype. When protection did not occur between viruses of the same serotype (NCDV and B641), it was considered possible that the challenge fecal virus which was uncloned contained a contaminating strain of virus of the same electropherotype but with different serotype properties. One calf, GC27, vaccinated with NCDV and challenged with B641, was selected for further study. A postchallenge fecal sample, containing rotavirus, was cultured in MA104 cells. Although the infectivity titer was low, approximately 10/ml, the virus was successively adapted to cell culture. Unlike for most bovine rotaviruses, adaptation was slow, and it was not until passage 25 that the virus reached a titer sufficient for serotyping. At 100 tissue culture infective doses, the virus was neutralized by antisera to B641 (GC51) and NCDV (GC54) to similar titers for their respective viruses (200 and 800, respectively). These sera at a 1:50 dilution did not neutralize B223, and B223 antiserum (GC47), with an homologous titer of >2,560, had a titer of 50 with GC27 virus. The electropherotype patterns of 15 plaque clones of GC27 virus were compared with the patterns of uncloned B641 and three B641 subclones, with B223 and with NCDV (Fig. 1C). All 15 selected plaques of GC27 virus had apparently identical electropherotype patterns, implying that a single clone was selected by passage in calf GC27 from

TABLE 2. Specificity of coproantibody and serum antibody responses

Calf	Vaccine[a]	ELISA titer (days postvaccination)[b]	IF[c] at 1:20	PRN titer to given virus					
				Coproantibody			Serum antibody[d]		
				B641	NCDV	B223	B641	NCDV	B223
GC28	B223	4 (21)	+	8	8	32	160	320	1,280
GC30	B223	32 (18)	+ + +	64	<4	1,024	80	80	640
GC19	NCDV	<4 (12)	+ +	32	32	16	320	640	<20
GC22	NCDV	4 (20)	−	32	<16	16	320	1,280	20
GC24	NCDV	4 (3)	−	16	64	32	640	640	<20
		<4 (20)	−	32	8	8			
GC25	NCDV	4 (3)	−	8	64	32	320	1,280	<20
		<4 (19)	−	32	64	64			
GC27	NCDV	<4 (20)	+	32	<32	32	160	320	<20
GC29	NCDV	8 (6)	−	128	256	8	640	640	<20
		8 (15)	+	32	32	32			
GC52	NCDV	12 (12)	+	64	128	64	320	640	20
GC26	B223[e]	8 (21)	+ +	32	32	128	20	80	320
GC32	B223	4 (8)	+ + +	64	32	64	40	160	320
GC47	B223	16 (6)	+	8	64	64	160	320	10,240
GC55	B223	8 (19)	+ + +	<8	<8	128	80	320	640
GC15	B641[e]	<4 (12)	+ +	16	<8	<8	640	1,280	40
GC17	B641[e]	24 (9)	+	1,024	1,024	128	640	640	20

[a] Tissue-culture-grown rotavirus unless otherwise specified.
[b] Titer (coproantibody) determined from the dilution producing an OD of 0.200 or greater.
[c] Coproantibody response.
[d] Serum antibody at 21 days postvaccination.
[e] Fecal rotavirus (virulent).

the heterogeneous B641 challenge virus. The GC27 virus clone was interesting because it was different from any of the B641 subclones generated in vitro. Specifically, all subclones isolated from calf GC27 contained the minor component for segments heterogeneous in the uncloned B641 virus (Fig. 1B). These data confirm that the excreted virus was present in the challenge preparation, was not a reassortant with NCDV, and had the serotype properties of the NCDV-B641 group.

DISCUSSION

The separation of bovine rotaviruses into different serotypes is based on the arbitrary choice of a 20-fold or greater difference between the homologous and heterologous NT of hyperimmune guinea pig antiserum (29). With many rotavirus isolates, there is some neutralization with heterologous convalescent-phase calf antisera at low dilutions, and it is usually not possible to observe more than 4- to 16-fold differences between homologous and heterologous titers. The results of this study also showed much heterologous neutralization by the convalescent-phase gnotobiotic calf sera, particularly when B223 was used as the vaccine strain. This heterologous PRN activity may be directed toward the minor neutralizing antigen of rotavirus, a product of gene segment 4 (19). As discussed previously (29), the presence of neutralizing antibody to the challenge virus in the serum immediately prior to challenge did not correlate directly with protection. The coprantibody titers also showed heterologous reactivity in most of the samples, although calves GC26, GC28, GC30, GC55 for B223 vaccine, GC29 for NCDV, and GC15 and GC17 for B641 showed a fourfold or greater homologous response (Table 2). The coproantibody titers showed even less correlation with protection, since calves GC19, GC22, GC24, GC25, GC27, GC29, and GC52

TABLE 3. Coproantibody response after challenge

Calf	Vaccine[a]	Challenge virus (days postvaccination)	DPV[b]	Prechallenge PRN titer to:			DPC[c]	Postchallenge PRN titer to:		
				B641	NCDV	B223		B641	NCDV	B223
GC28	B223	B223 (21)	21	8	8	32	5	8	8	64
GC30	B223	B223 (21)	20	8	16	512	2	8	8	256
GC24	NCDV	B223 (21)	20	32	8	8	2	16	512	32
GC25	NCDV	B223 (21)	19	32	64	64	2	<8	128	8
GC27	NCDV	B641 (21)	20	32	<32	32	4	8	16	16
GC29	NCDV	B641 (14)	13	32	32	32	4	128	128	<8
GC52	NCDV	B641 (21)	19	32	16	16	2	64	32	8
GC32	B223	B641 (21)	12	<4	8	16	9	16	4	128
GC47	B223	B641 (21)	19	32	32	128	2	64	16	512
GC55	B223	B641 (21)	19	<8	<8	128	4	32	16	128
GC15	B641[d]	B223 (21)	21	16	<8	<8	6	32	16	16
GC36	B223	CD12 (21)	19	8	8	16	4	8	32	256
GC54	NCDV	CD12 (21)	19	32	64	32	4	128	256	8

[a] Tissue-culture-grown rotavirus unless otherwise specified.
[b] DPV, Days postvaccination.
[c] DPC, Days postchallenge.
[d] Fecal rotavirus (virulent).

were not protected, although they developed neutralizing intestinal antibodies to the challenge strains, and calf GC55 was protected against B641 despite lacking neutralizing antibodies. The coproantibody titers after challenge were particularly interesting, in that there appeared to be an anamnestic response with a fourfold or greater response to the vaccine virus serotype(s) in 13 of 15 samples tested (Table 3) than to the challenge virus. In previous study (29), B641 was shown to be a minor serotype variant of the NCDV group, as one hyperimmune guinea pig serum had a 16-fold higher titer to B641 than to NCDV, but this difference was not observed with a guinea pig antiserum to NCDV. Results for a few of the coproantibody samples tend to support the evidence of a minor difference between B641 and NCDV (GC30 prechallenge and GC24 and GC25 postchallenge).

The electropherotyping experiments provided useful information about the tissue-culture-adapted viruses (B223 and B641) used for vaccination and the virus shed by a calf challenged with B641 (GC27). Using high-resolution electrophoresis (11), we found that B223 was electropherotypically homogeneous. This result was not surprising, as this virus had been cloned by plaque purification. B641 was found to be heterogeneous in the migration of seven genome segments (segments 2, 3, 5, 6, 7, 8, and 10), and seven electropherotypes were identified among 10 B641 subclones examined. Although heterogeneous, the B641 culture-adapted virus contained a major electropherotype. The heterogeneity of B641 was less surprising, since it had been subjected to limiting dilution passage but not single-plaque cloning. The electropherotype of virus shed by calf GC27 was determined, since homologous protection had not been shown by this calf. This virus was serotypically related both to the vaccine (NCDV) and to the challenge (B641) viruses and was electropherotypically distinguishable from but related to the culture-adapted B641. The electropherotype of GC27 virus represented a subset of the segments observed in uncloned B641. Thus it appeared that a single electropherotype had been selected by growth in calf GC27. It is interesting that this electropherotype included the minor variant of all heterogeneous bands present in B641. As the heterogeneities of the challenge and culture-adapted B641 viruses were similar, it is reasonable to conclude that the virus shed by calf GC27 originated from B641 and not from a persistent infection with the vaccine virus (NCDV).

The studies on protection were concerned with active rather than passive protection. Passive protection, in mice and possibly in calves (18, 19, 21, 22), appears to be restricted to homology of serotype between the vaccine and challenge rotaviruses, presumably owing to the neutralization of the challenge virus by antibody in the milk. Gene segments 4 and 9 code for viral protein products VP3 and VP7, respectively, which induce a serotype-specific neutralizing antibody response in serum and milk and serotype-specific protective passive immune responses in milk of murine dams (17–19). However, in a recent study, monoclonal antibodies to VP3 induced passive protection to three serotypes of rotavirus in mice (20). The apparent discrepancy between these results was not explained, and it is possible that it is an artifact of the monoclonal system. Likewise, active-immunity studies have usually shown serotype-homologous protection with poor or no heterologous protection (2, 4, 10, 16, 28, 29). However, in these studies, vaccine viruses were of the same isolation origin as the challenge viruses and thus were truly homologous. For experimental piglets (34) and experimental calves (32, 33) and in a field trial with children (25), there are reports of heterologous protection between bovine (NCDV) and human rotaviruses. However, the strict criterion for protection (that no demonstrable virus is shed by protected animals after challenge) was not applied, and the human viruses appear to be of relatively low virulence and possibly of low host adaptability, when passaged in animals (3, 25, 28).

The results of this study of protection are surprising and demonstrate not only that vaccine viruses must be tested against viruses of the same as well as of different serotypes, but also that the challenge viruses should have a different isolation history. From the data presented, there was no in vitro test which predicted whether the vaccine virus, or whether the specificity of the response of the animal, would protect against the particular challenge virus. Both heterologous and homologous protection was obtained, but the protection was selective. There was no homologous protection between NCDV and B641, and there was a one-way heterologous protection between B223 and B641. The intestinal response to NCDV may have been poor because the virus is avirulent and apparently replicates less well than the virulent strains, at least in some calves. These experimental results of lack of vaccine protection have been supported to some extent by field trial studies of rotavirus vaccine protection in calves, piglets, and children. Most field studies of protection produced in calves of active immunity by rotavirus vaccine (NCDV serotype) failed to show protection (1, 8, 27), although there is at least one report claiming that protection was induced (24). The majority of isolates of bovine rotavirus, including NCDV, are thought to belong to the rotavirus serotype 6 group (22, 29). In a study with children (8), the vaccine virus was also NCDV (serotype 6) and failed to produce a significant reduction of rotavirus-associated diarrhea. In this report, the human rotavirus isolates were not serotyped, but they were probably among the known human serotypes, none of which are believed to belong to the serotype group of the vaccine. In another study, the incidence of postweaning diarrhea associated with rotavirus was compared for vaccinated and unvaccinated piglets (13). There was no difference between the groups in the geometric mean titer of serum, the incidence of diarrhea, or the incidence of rotavirus excretion in the feces. The challenge viruses, although of different electropherotypes from the vaccine virus, were classified within the same serotype group as the vaccine virus (serotype 5). It is not known whether heterologous active immune protection can be achieved under field conditions with a vaccine with the properties demonstrated by B223.

After these studies were nearly completed, the B641 virulent virus, but not the culture-adapted virus, was found to be contaminated with a low concentration of bovine astrovirus. This virus does not cause clinical disease or D-xylose malabsorption, but it does infect and destroy the M cells of the Peyer's patch dome epithelium and thus may affect intestinal immune responses (30). However, the presence of this virus did not inhibit the protective effect of B223 vaccine in calves subsequently challenged with B641, although it may have influenced the response of the two calves vaccinated with B641 fecal preparation and reduced their ability to control the B223 challenge. The presence of astrovirus apparently did not influence the antibody response to rotavirus in the serum or feces, neither from the vaccine nor from the boosting effect of the challenge virus, since these responses were similar, although there were variations between animals that were unrelated to the presence or absence of astrovirus.

The mechanism of active immunity to rotavirus is not

known and was not elucidated in these studies. However, the antigenic specificity for protection appears to be different from neutralization, and the mechanism may not be dependent on the gut neutralizing antibodies. Studies are in progress to determine the range of serotypes of rotavirus against which B223 can induce heterologous protection and the gene-coding assignments for this protection.

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