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Homotypic and Heterotypic Serological Responses to Rotavirus Neutralization Epitopes in Immunologically Naive and Experienced Animals

D. R. SNODGRASS,* T. A. FITZGERALD, I. CAMPBELL, G. F. BROWNING, F. M. M. SCOTT, Y. HOSHINO,† AND R. C. DAVIES

Moredun Research Institute, 408 Gilmerton Road, Edinburgh, EH17 7JH Scotland

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Gnotobiotic or specific-pathogen-free animals with no previous exposure to rotavirus were vaccinated with strain UK, serotype G6. The highest serological response was to homologous virus; significant but lower responses occurred to viruses with either VP4 or VP7 related to that of vaccine virus; responses to other viruses were of low titer or infrequent. Adult cows vaccinated with UK virus produced increased titers of antibody to all rotavirus serotypes. The increases in titer to homologous virus and to other natural and reassortant viruses sharing VP7 with the vaccine virus were significantly higher than those to all other viruses. These results suggest the presence of common epitopes which are not well recognized in primary infections.

Rotaviruses are major causes of gastroenteritis in humans and several species of veterinary importance. Control by vaccination in children is a high research priority, and much emphasis is currently placed on animal and animal-human reassortant rotaviruses as oral vaccine candidates (16). In veterinary medicine, successful commercial rotavirus vaccination has been achieved so far only in cattle, through immunization of the pregnant cow with inactivated adjuvanted vaccines and thus passive immunization of the calf through colostral and milk antibodies (1, 19, 26).

Protection against rotavirus diarrhea has been shown to be closely linked to possession of antibodies against either or both of the outer capsid proteins, VP4 (coded for by gene segment 4) and the major outer capsid constituent VP7 (coded for by gene segment 8 or 9) (7). VP4 determines P serotypes (currently largely unclassified), and VP7 determines the dominant G serotype (currently numbered from 1 to 14) (1a).

Protection against reinfection in piglets has been shown to occur only when either VP4 or VP7 of the challenge virus is shared with the initial infecting virus (14). Vaccine trials in children have also suggested serotype specificity (16). Passive immunity due to ingested antibody has also been shown to depend on the VP4 and VP7 specificities of the antibodies. This has been demonstrated both in models of disease in lambs (29) and in experiments using genetic reassortant rotaviruses and monoclonal antibodies (MAbs) in mice (20, 21).

The responses to VP4 and VP7, although crucial to vaccine design, have been complicated by the occurrence of heterotypic as well as homotypic antibody responses in vaccinated individuals. Adult cows vaccinated parenterally with either bovine or nonbovine rotaviruses experienced increases in antibody titer to a wide range of rotavirus serotypes not present in the vaccines (2, 29). Similarly, adult human volunteers vaccinated orally with live rotavirus produced antibodies to homotypic and a wide range of heterotypic rotaviruses (11). However, infants tended to have an antibody response homotypic to the infecting virus strain (10, 11, 32).

The relative contribution of VP4 and VP7 antibodies to the immune response has also been studied. It has been suggested that the VP7 response predominates after parenteral vaccination and the VP4 response predominates after oral infection (9, 30). However, the opposite response with a dominant VP7 response after oral infection has also been reported (31).

In the study reported here, we have attempted to address these issues of immune response to rotavirus vaccines. We have investigated the relationship of the response to the VP4 and VP7 composition of the vaccine, to the route of administration, to the species of animal, and to defined previous rotavirus experience. We have used two serological assays, each with a variety of antigens: the neutralization test (VNT), which detects the total reaction against neutralization epitopes on both VP4 and VP7, and the epitope-blocking assay (EBA), which allows a specific examination of antibodies in a polyclonal serum able to block serotype-specific epitopes.

Vaccines and assays. Gnotobiotic lambs and calves were derived by hysterectomy and hysterotomy, respectively, and reared in positive-pressure plastic film isolators. Rabbits were from a specific-pathogen-free colony from which all of many hundreds of animals have consistently tested negative for rotavirus antibodies, and all individuals were confirmed free of rotavirus antibody before use. Cows were adults of beef type kept under normal commercial conditions, from herds which had no history of usage of rotavirus vaccines.

All vaccines were prepared from UK bovine rotavirus. The dose of the virus used for oral inoculation was $10^{7.3}$ to $10^{8.0}$ 50% tissue culture infective doses per animal. Virus in feces was detected by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (13).

Parenteral vaccines were prepared by inactivation in 0.5% formaldehyde overnight, followed by emulsification in an equal volume of Freund's incomplete adjuvant. The vaccine titer before inactivation was $10^{6.6}$ to $10^{7.0}$ 50% tissue culture infective doses per ml, which constituted a single vaccine dose given intramuscularly.

Serum samples were collected before and 1 month after

* Corresponding author.
† Present address: Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

vaccination from all animals and stored at −20°C. They were assayed by VNTs and EBAs.

The following rotavirus strains were used: Wa (serotype G1), DS-1 (G2), rhesus rotavirus (RRV) (G3), ST-3 (G4), OSU (G5), UK and NCDV (G6) (these viruses share the same VP7s but have distinct VP4s [15]), ch2 (G7), 69M and 678 (G8), WI-61 (G9), and B223 (G10). In addition, the following rotavirus reassortants were used: DS-1 × UK containing 10 genes from DS-1 and a UK VP4 gene (G serotype DS-1, P type UK, kindly donated by R. Wyatt) and B223 × UK containing B223 gene segments 1, 3, 4, and 11 and the other segments from UK (G serotype UK, P type B223, determined by coelectrophoresis and reaction with specific MAbs; data not shown).

All viruses were propagated in MA104 cells as previously described (22). All virus stocks were checked for identity by comparing their double-stranded-RNA electrophoretypes with those of the parent virus in silver-stained polyacrylamide gels (13).

Neutralization assays were fluorescent focus reduction tests in MA104 cells in microplates, with end-point titers determined as a 60% reduction in foci. VP7 MAbs 4F8 to RRV serotype 3 (24), UK/7 to UK bovine rotavirus serotype 6, and B223/3 to B223 bovine rotavirus serotype 10 (28) were used. These MAbs belonged to immunoglobulin G2a (IgG2a), IgM, and IgG2b isotypes, respectively. It has been suggested that B223/3 reacts with an epitope on VP4 (28), but we have now definitively shown by the use of reassortants that B223/3 reacts with neutralizing epitopes on VP7. MAbs were raised in mouse ascitic fluid. UK/7 was purified by gel filtration, and the IgG MAbs were purified by affinity chromatography on protein A-Sepharose. No MAbs to UK VP4 were available.

The EBA was based on that described by Shaw et al. (23), to test the ability of polyclonal sera to react with specific neutralization epitopes. Briefly, a polyclonal antirotavirus capture antibody at optimal dilution was used to coat immunoassay plates, which were then incubated with diluent. Neat cell culture fluid containing the appropriate virus (RRV, UK, or B223) was added to each well. In the next stage, the test serum sample was added in duplicate to wells in doubling dilutions from 1/10. Finally, the appropriate MAb was added at the optimal dilution. A test was developed either by using a biotinylated preparation of the purified MAb and reacting the antigen-antibody complex with streptavidin-peroxidase (4F8 and UK/7) or by adding the underivatized MAb and then anti-mouse IgG conjugated with peroxidase (B223/3). In each case, color was developed by adding substrate H_2O_2 and *o*-phenylenediamine dihydrochloride to the bound peroxidase. Controls for each test incorporated specific blocking by the homologous MAb and optimization of the test by incorporating phosphate-buffered saline (PBS) instead of serum as the blocking stage. All incubation steps were for 1 h at 37°C, except that virus was incubated for 3 h. The diluent used throughout was PBS–0.05% Tween 20–2% fetal bovine serum, and plates were washed three times between stages. The titer of each serum was the highest dilution that reduced the optical density by at least 50%.

Serological responses were compared by Mann-Whitney tests and two-sample *t* tests.

Vaccination of naive animals. Calf rotavirus UK replicated efficiently in the gnotobiotic lambs and calves, as demonstrated by virus excretion in feces over several days (data not shown), but no diarrhea was produced.

Preexposure antibody was not detected in any animal. The VNT responses to different rotaviruses after vaccination were broadly consistent between species and vaccine regimens and could be categorized as follows.

(i) Homologous virus. The mean VNT titer to UK virus was 443, and all 17 animals responded (Table 1).

(ii) Viruses sharing VP4 or VP7. At least 15 animals responded to each of NCDV, B223 × UK, and DS-1 × UK, which share either VP7 or VP4 with UK virus. The mean titers were 87, 115, and 65, respectively, which were not significantly different from each other ($P > 0.05$). However, titers were significantly lower than that to UK virus ($P < 0.01$).

(iii) Unrelated viruses. There was a more variable but generally lower response to the unrelated viruses. To five strains (Wa, DS-1, OSU, ch2, and B223) there was no response in any animal. It is therefore likely that no significant neutralization epitopes on either VP4 or VP7 are shared between UK and these viruses. Only 3 animals responded with a low titer to RRV and ST-3 viruses, but 12 to 14 animals responded with a low titer to 69M, 678, and WI-61 viruses. A consistent but minor neutralization epitope is therefore shared between UK virus and these serotype G8 and G9 viruses.

The EBA results generally mirrored those obtained by VNT with the same antigen (Table 1); i.e., for UK/7 MAb and UK virus, 16 of 17 animals had a fourfold increase in titer to a mean titer of 231, while two animals responded to RRV and none responded to B223.

Vaccination of mature cows. Eleven mature cows vaccinated with UK virus parenterally all had preexisting VNT antibody titers to each of the 14 virus strains tested (Table 2).

The mean prevaccination VNT titer to ch2 serotype G7 was 21, which was significantly lower than that to all the mammalian rotaviruses (347; $P < 0.01$).

The homologous response to UK virus vaccination measured by the VNT showed a mean 9.1-fold increase in titer, with 10 of the 11 cows experiencing at least a 4-fold increase (Table 3). Measured against this response, the magnitudes of increase in titer of those viruses with VP7 related to VP7 of UK (NCDV and B223 × UK) were similar ($P > 0.05$). The response to all other viruses, including DS-1 × UK with related VP4, was significantly less ($P < 0.05$).

From the response of the naive animals, it was apparent that UK virus shared no neutralizing epitopes with Wa, DS-1, OSU, ch2, or B223 virus. As examples of mammalian nonbovine rotaviruses unrelated to UK, the mean response to Wa, DS-1, and OSU was calculated and used as a basis for comparison (Table 3). Only the responses to UK and NCDV were significantly higher ($P < 0.01$), while the greater response to B223 × UK did not reach statistical significance.

The magnitudes of the increases were also compared for other groupings of rotaviruses. The mean increase in titer to nonbovine subgroup I viruses was similar to that for nonbovine subgroup II viruses (5.0- and 3.4-fold, respectively; $P > 0.05$). The mean response to those viruses sharing with the vaccine virus the cross-reacting neutralizing epitope defined by MAb 57-8 (G3, G4, G6, and G10) (17) was not significantly different from the mean response of those without this shared epitope (4.4-fold and 3.8-fold, respectively; $P > 0.05$). The response to ch2 virus was significantly lower than the mean response to all mammalian rotaviruses (2.6- and 4.6-fold, respectively; $P < 0.05$).

When assayed by EBA, all cows had preexisting antibody (Table 4). After vaccination, 9 of 11 cows experienced 4-fold increases in titer to UK virus in the homologous EBA, with a mean increase of 5.1-fold. In the EBAs with MAbs to VP7

TABLE 1. Primary antibody response to vaccination of naive animals with rotavirus strain UK (G6)

Species (route of exposure)	Animal no.	Titer by VNT[a] to:									Titer by EBA[b] to:	
		RRV (G3)	ST-3 (G4)	UK (G6)	NCDV (G6)	69M (G8)	678 (G8)	WI-61 (G9)	DS-1 × UK (G2)	B223 × UK (G10)	4F8 (RRV)	UK/7 (UK)
Lambs (parenteral)	N993	—[c]	—	640	80	—	40	20	160	80	—	80
	N994	—	—	320	80	40	40	10	10	80	40	40
	N995	—	—	320	80	10	80	40	40	160	—	160
	N996	—	—	640	160	10	40	10	80	320	—	640
	L933	—	20	80	80	80	320	—	320	40	—	2,560
	L934	—	40	80	80	160	320	—	320	40	—	2,560
	L935	160	40	160	160	40	320	—	640	80	—	1,280
Lambs (oral)	N997	—	—	160	20	10	10	80	40	80	—	160
	N998	—	—	320	—	—	—	20	40	160	—	160
	N999	—	—	160	10	10	—	160	40	40	—	40
	N1000	—	—	160	—	10	—	20	10	20	—	40
Rabbits (parenteral)	22	—	—	1,280	1,280	—	20	—	40	320	—	160
	48	—	—	1,280	320	20	40	10	40	320	—	320
	49	—	—	640	320	—	20	20	40	160	40	640
	51	—	—	1,280	640	—	40	10	40	80	—	320
Calves (oral)	N1003	40	—	5,120	160	20	80	320	640	640	—	160
	N1006	20	—	2,560	160	160	20	160	20	320	—	80
No. responding		3	3	17	15	12	14	13	17	17	2	16[d]
Mean titer		—	—	443	87	16	35	21	65	115	—	231

[a] All animals showed by VNT a titer of <10 to Wa (G1), DS-1 (G2), OSU (G5), ch2 (G7), and B223 (G10); the number responding was zero.
[b] All animals showed by EBA a titer of <10 to B223/3 (B223); the number responding was zero.
[c] —, <10.
[d] One calf reacted nonspecifically in this assay before inoculation.

of RRV and B223, five and seven animals, respectively, showed 4-fold increases in titer, with overall mean increases of 2.7- and 3.7-fold, respectively.

The rotavirus-free status of the naive animals used allows significant interpretation to be placed on the results.

The response to homologous UK virus was significantly higher than that to all other viruses. The three viruses which shared either VP4 or VP7 with UK (NCDV, DS-1 × UK, and B223 × UK) had increases in titer that were smaller but similar in magnitude to one another and greater than for all other unrelated viruses. It has been suggested that under conditions under which no viral replication occurs, such as after parenteral immunization, the VP7 response may dominate because VP7 is the major constituent of the virus outer capsid. Conversely, efficient viral replication in the gut may allow more-efficient expression of VP4 (31). However, there

TABLE 2. Response of mature cows to parenteral vaccination with UK virus

Virus	Mean VNT titer		No. of animals with fourfold increase
	Prevaccination	Postvaccination	
Wa	117	467	8
DS-1	341	1,452	9
RRV	386	2,559	9
ST-3	282	933	5
OSU	282	1,202	8
UK	564	5,117	10
NCDV	601	5,808	11
ch2	21	55	4
69M	773	3,981	8
678	412	1,990	7
WI-61	301	877	4
B223	341	1,279	6
DS-1 × UK	193	640	7
B223 × UK	412	2,404	10

TABLE 3. Magnitude of increase of VNT titer in mature cows after vaccination with UK virus

Virus	Increase in VNT titer (fold)	Comparison (*P*) with:	
		UK	Unrelated viruses[a]
UK	9.1	—[b]	<0.01
Wa/DS-1/OSU	4.2	<0.01	—
Wa	3.9	<0.05	—
DS-1	4.3	<0.05	—
RRV	6.6	<0.05	NS[c]
ST3	3.3	<0.01	NS
OSU	4.3	<0.05	—
NCDV	9.7	NS	<0.01
ch2	2.6	<0.01	NS
69M	5.2	<0.01	NS
678	4.8	<0.05	NS
WI-61	2.9	<0.01	NS
B223	3.8	<0.05	NS
DS-1 × UK	3.3	<0.05	NS
B223 × UK	5.8	NS	NS

[a] Wa, DS-1, and OSU were shown by the reactions of the naive animals to share no neutralizing epitopes with UK virus. The mean increase in titer to these three viruses was calculated and used as a basis for comparing the responses to other viruses.
[b] —, homologous reaction, no comparison made.
[c] NS, not significant ($P > 0.05$).

TABLE 4. Response of mature cows to parenteral vaccination with UK virus: epitope-blocking assays

Antibody (virus)	Mean EBA titer			No. of animals with fourfold increase
	Prevaccination	Postvaccination	Increase (fold)	
4F8 (RRV)	412	1,128	2.7	5
UK/7 (UK)	160	823	5.1	9
B223/3 (B223)	61	226	3.7	7[a]

[a] Only 10 cows were assayed by B223/3 EBA.

was no evidence in these primary infections that either the VP4 or VP7 produced a greater response, but rather there was evidence that they were weighted equally.

These results in rotavirus-free animals help interpretation of vaccine trials in human infants. The occurrence of asymptomatic rotavirus infections (5) complicates the study of the immune response to vaccination, but our results support the contention that the largely homotypic response of infants under 6 months of age is due to a lack of prior exposure to rotavirus (10). Similarly, the failure of rotavirus vaccines derived from bovine strains to produce antibody to the major human serotypes G1 to G4 (5, 12) is not surprising given the similar lack of response in this study. However, the consistent heterotypic response to the minor human serotypes G8 and G9 may indicate that bovine rotavirus vaccine can protect infants against these strains.

The adult cows represented a population with extensive natural rotavirus exposure. Rotavirus serotypes 6 and 10 occur commonly in cattle, serotype 8 occurs less commonly, and so far no other serotypes have been isolated from cattle in the United Kingdom (28). Genogrouping results suggest that bovine rotaviruses are epidemiologically distinct from those strains infecting humans (18). Although earlier studies indicated that even repeated immunizations produced serotype-specific responses (29), it is assumed that the prevaccination occurrence of antibody to all rotavirus serotypes in these cows indicates a gradual broadening of the immune response after repeated exposure. This may be mediated by minor common neutralizing epitopes, which do not stimulate significant antibody responses in naive animals but become more dominant in animals which have experienced numerous infections. This seems more likely epidemiologically than the alternative that each animal had been exposed to all known mammalian and avian rotavirus serotypes.

The data generated in this study show clearly that vaccinating with a single rotavirus serotype will produce in many experienced animals significant increases in antibody to mammalian and even distantly related avian serotypes. However, in this case the titer increase was greatest to those rotaviruses sharing VP7 but not VP4 with the vaccine virus. Indeed, the possession of the UK VP4 by the DS-1 × UK reassortant did not appear to increase the response to this virus above that to wholly unrelated viruses.

The EBA demonstrated significant increases in antibody responses to epitopes not present in the vaccine, i.e., the serotype-specific epitopes on VP7 of serotypes 3 and 10. Thus, the increases in titer to unrelated viruses shown by the VNT are a specific heterotypic immune response.

Heterotypic immunity as described in this study is a broad term covering two distinct phenomena. The response to heterotypic serotypes in naive animals may be simply explained by possession of shared neutralization epitopes, most of which relate to already-defined major typing epitopes on VP7 and VP4. In adult animals with wide rotavirus experience, we have used the term heterotypic immunity to describe the phenomenon in which single-serotype vaccination produces significant increases in neutralizing antibody to all rotavirus serotypes, even distantly related avian strains. The mechanisms underlying this are more difficult to explain.

(i) The concept of original antigenic sin as derived from human influenza describes the situation in which vaccination in later life stimulates the greatest increase in antibody to the first childhood virus strain encountered, even if that strain is absent from the vaccine (8). As the greatest increases in titer were experienced to viruses with VP7s of serotype G6, which is likely to be the predominant calfhood strain encountered, this concept may be applicable to these experiments. On the other hand, serotype G6 was also the vaccine strain used, and this does not explain the increase to all other serotypes.

(ii) Minor shared epitopes may occur more commonly than has been recognized and may underlie the heterotypic responses. In addition to those demonstrated between UK and several other viruses in the naive animals in this study, several MAbs recognize epitopes shared between distinct VP4 and VP7 types (6, 17, 25). Previous failure to define many cross-reactive MAbs may be due to the use of naive mice in which the serotype-specific response predominates. Assuming that common T-helper epitopes are also present, the heterotypic responses may result from the more frequent presentation of the minor shared epitopes to the immune system rather than from the less frequent exposure to the immunodominant epitopes.

The mechanism underlying the heterotypic response in mature animals therefore remains a matter for conjecture.

The relevance of these observations to vaccine design and interpretation of vaccine trials in humans and other animals is clear. Firstly, serological responses in previously unexposed young children are predictable and will occur to a high titer only to rotaviruses with related VP4s or VP7s. Secondly, the responses to VP4 and VP7 are similar in magnitude. As protection in children is usually associated with serotype-specific antibody (3), the case for multivalent vaccines appears strong.

The results are also relevant to vaccinating older children or adults or vaccinating adult cows for passive immunization of calves through milk (27). In these cases, while a heterotypic response to all serotypes is likely to occur, a maximal response only to immunodominant VP7 epitopes, i.e., the same G serotype, can be expected.

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