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Heterologous Protection against Rotavirus-Induced Disease in Gnotobiotic Piglets

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Administration per os of 2×10^6 fluorescent cell-forming units of a human serotype 3 rotavirus (RV-3) protected all of nine gnotobiotic piglets against severe diarrheal disease when they were challenged 10 to 14 days later with 8×10^3 fluorescent cell-forming units of virulent wild-type porcine rotavirus (AT/76). The porcine virus was similar antigenically to porcine prototype strain OSU, previously described as antigenically distinct from all four recognized human serotypes. Administration of RV-3 was associated with the development of serum-neutralizing antibody to both RV-3 and AT/76 in piglets that excreted RV-3. Neutralizing antibody levels to RV-3 and AT/76 increased rapidly postchallenge. Vaccinated piglets were not immune to infection with AT/76 but showed no or minimal gastrointestinal symptoms after challenge. Control nonvaccinated piglets that were fed AT/76 developed severe dehydrating diarrhea and low levels of neutralizing antibody to AT/76 alone. The apparent heterologous clinical protection observed in this study could have been predicted from results of in vitro assays. Neutralization tests with reduction of fluorescence focus indicated a one-way cross-reaction between RV-3 and AT/76 such that hyperimmune antiserum to RV-3 neutralized porcine virus to moderate titer, but not vice versa. The results emphasize the importance of neutralizing antibody in protection against disease and the need to determine reciprocal cross-neutralization titers, rather than serotype alone, in order to predict the ability of rotavirus strains to cross protect.

Rotaviruses are ubiquitous in nature and have been identified in the intestinal tract of the young of most mammalian and avian species. Interspecies infections have not been observed to occur naturally but have been achieved experimentally, usually in gnotobiotic newborn animals (4, 13, 19).

Cross-protection studies have been performed by using a variety of rotavirus strains to infect calves in utero (26), newborn calves (23, 24), or pigs (3, 7, 19, 27). The results of these studies are difficult to interpret. Some have clearly demonstrated cross-protection between rotavirus strains from different animal sources, judged by modification of disease symptoms (19, 26) or decreased excretion of challenge virus (26, 27). These experiments were considered to show heterologous cross protection. They were performed mainly before reliable techniques to cultivate rotavirus strains became available (16). These techniques facilitated the development of neutralization assays that have demonstrated serotypic relationships between human and animal rotavirus strains (10).

Most recent animal studies have concluded that cross-protection exists only between rotavirus strains of the same serotype (3, 7, 24). However, a bovine strain not belonging to any existing human serotype has been shown to protect against a human strain in calves (26) and piglets (27). The same bovine strain has been used to vaccinate young children (21). The basis for heterologous protection with this strain is unexplained.

The ability to infect gnotobiotic piglets with rotavirus strains from a variety of animal sources, together with the relatively long period after birth (>1 month) when piglets are clinically susceptible to challenge with porcine rotavirus (2), has encouraged the use of this animal model for in vivo cross-protection studies. In a limited number of experiments reported by Tzipori et al. in 1980 (19) three fecally derived human rotavirus strains were shown to protect gnotobiotic piglets from clinical illness after challenge with virulent wild-type porcine rotavirus. The basis for this heterologous protection was not then clear. We have now cultivated these strains and identified their serotypes. We decided to repeat the earlier cross-protection studies in gnotobiotic piglets with one human rotavirus strain that is under consideration as a candidate vaccine for use in humans. The results help to explain the apparent heterologous protection observed between human and porcine rotavirus strains in our previous study.

MATERIALS AND METHODS

Rotavirus isolates. Human rotavirus strain Hu/Australia/10-25-10/77/L (RV-3) was adapted to growth in MA-104 cells (1) from a stool obtained from a 4-day-old infant infected asymptomatically. By using reference antisera, RV-3 was shown to belong to serotype 3 (5). Gel electrophoresis of genome RNA of RV-3 performed according to the technique described by Dyall-Smith and Holmes (6) revealed the characteristic pattern shown in Fig. 1. Standard inocula of RV-3 for administration to gnotobiotic piglets were prepared by ultracentrifugation at 80,000 × *g* of fluorocarbon-extracted (Arklone, I.C.I.) and clarified MA-104 cell culture supernatants. Pellets were suspended in Tris-buffered saline (pH 7.2) containing 10 mM calcium chloride. The presence of intact double-shelled virions was checked by electron microscopy (EM). The preparation was divided into 1-ml portions, each representing a single dose, and stored at −70°C until use. Titration of a single dose by fluorescence focus assay indicated that it contained 2×10^6 fluorescent cell-forming units per ml.

The virulent strain of porcine rotavirus (AT/76) used in this experiment was originally obtained from a diarrheic piglet (20). The strain was passaged in gnotobiotic piglets

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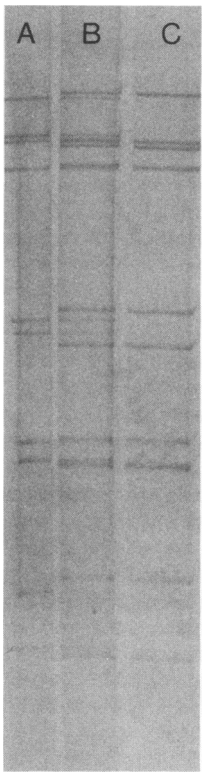

FIG. 1. RNA patterns of RV-3 (A), RV-3 and AT/76 (B), and AT/76 (C). The bands were separated by polyacrylamide gel electrophoresis and visualized by silver staining. The direction of migration is from top to bottom.

and administered as wild-type virus. This was prepared as an extract of particles obtained from stool and gut scrapings from an infected gnotobiotic piglet. These were homogenized in phosphate-buffered saline (20%, vol/vol) and centrifuged at 10,000 *g* for 30 min. The supernatant was filtered through a 0.45-μm-pore membrane filter. Samples of 1 ml representing a single dose were stored at −70°C until use. Purity of the challenge dose was checked by gel electrophoresis of genome RNA, which revealed only the characteristic 11 bands (Fig. 1). Titration of a single dose by fluorescence focus assay indicated that it contained 8×10^3 fluorescent cell-forming units per ml. The wild-type virus was later adapted for growth in MA-104 cells. Gel electrophoresis of genome RNA revealed the long pattern shown in Fig. 1.

Human and porcine rotavirus strains were compared by coelectrophoresis of genome RNA (Fig. 1) and by neutralization assays to determine homologous and heterologous titers with hyperimmune sera prepared against each virus in outbred New Zealand White rabbits.

Preparation of hyperimmune sera. Outbred New Zealand White rabbits showing either no or negligible amounts of serum antibody to rotavirus were used for production of hyperimmune antisera. Viruses grown in MA-104 cells were fluorocarbon extracted and purified by cesium chloride gradient centrifugation as described previously (15). The double-shelled particles were harvested and mixed with Freund complete adjuvant, and animals were immunized with a preparation containing 10^8 particles per ml by the subcutaneous route. At 2 weeks later, the animals were inoculated with the same preparation in Freund incomplete adjuvant, followed by a further subcutaneous inoculation 2 weeks later still with purified virus particles suspended in normal saline. Rabbits were exsanguinated 10 days later.

Subgroup assay. Monoclonal antibodies (8) specific for subgroup I and subgroup II antigens of human rotaviruses (supplied by T. H. Flewett, WHO Collaborating Centre for Rotavirus Research, Birmingham, United Kingdom) were used in an enzyme immunoassay (EIA). Microtiter plates were coated with a hyperimmune rabbit antiserum to a serotype 2 human rotavirus (RV-5) previously isolated in this laboratory (1). This antiserum binds both subgroups of rotaviruses equally well. Antigen in the form of tissue culture fluid was then added, followed by the addition of monoclonal antibodies and peroxidase-labeled rabbit anti-mouse immunoglobulin conjugate (Dakopatts, Copenhagen, Denmark). Optical densities of the wells were read with a Titertek Multiscan spectrophotometer (Flow Laboratories, Melbourne, Australia). The ratio of optical density of the antigen with subgroup II monoclonal antibody to the optical density of antigen with subgroup I monoclonal antibody was determined. Samples giving a ratio of >3.0 were assigned to subgroup II, and those giving a ratio of <0.5 were assigned to subgroup I (8). Known rotaviruses belonging to both subgroups were included in the assay as positive controls.

Neutralization assays. The fluorescence focus reduction neutralization assay used for titration of neutralizing antibodies in sera has been described previously (5). Briefly, serial dilutions of sera were incubated with 100 fluorescent cell-forming units of the appropriate cell-culture propagated rotavirus. MA-104 cell monolayers were infected with the mixture overnight, and then the cells were fixed and stained by using indirect immunofluorescence. The neutralization titer was the reciprocal of the highest serum dilution giving a 50% reduction in the number of fluorescing cells.

Procedure for cross-protection experiments. Piglets were delivered by cesarian section and maintained under gnotobiotic condition throughout the study (12). A total of 15 piglets was derived from five litters. Twelve piglets were given 1 ml of RV-3 preparation per os on day 2 or 3 of life. Three piglets from two of the litters served as controls and were not given RV-3. After 10 to 16 days, nine surviving piglets previously given RV-3 and three uninoculated piglets were given 1 ml of wild-type porcine rotavirus (AT/76) preparation per os. Feces were collected daily throughout the course of each experiment and were examined for rotaviruses by EM or EIA or both. Daily observations were made of the general condition of each piglet. Frequency and character of feces were recorded. Clinical status was assessed daily for development of anorexia, vomiting, depression, emaciation, or dehydration. Piglets were killed 2 to 4 days after challenge with porcine rotavirus, depending on clinical status. Serum was obtained on the day of challenge before administration of AT/76 and at death.

Necropsy procedure. All piglets were necropsied and examined for gross pathological changes in intestinal tract and other organs. Specimens were taken from five equally spaced sites in the small intestine and from cecum and colon. After fixation in neutral-buffered Formalin, all samples were sectioned and stained with hematoxylin and eosin. In addition, some specimens were stained by the peroxidase-antiperoxidase technique (18), by using hyperimmune rabbit antiserum to SA11 to identify viral particles in the mucosal cells.

Detection of rotavirus in gut contents. Intestinal contents (or feces) were homogenized in phosphate-buffered saline (10%), divided into equal portions, and stored at 4°C until processed (usually within 2 weeks of collection). The ho-

TABLE 1. Neutralization titers of polyclonal and monoclonal antibody reacted with rotavirus strains of human and porcine origin

Virus	Titer of polyclonal antibody against:					Titer of monoclonal antibody against:	
	RV-3	AT/76	OSU[a]	Gottfried N-926[a]	Gottfried K-998[a]	RV-3:1[b]	RV-3:2[b]
Human RV-3 (serotype 3)	800,000	<200	520	3,200	250	200,000	200,000
Porcine AT/76	46,000	60,000	9,800	580	100	<100	400

[a] Serum supplied by Linda Saif, Ohio State University, Wooster. Homologous titers determined by L. Saif by using plaque assay are 560 (versus OSU), 32,000 (versus N-926), 1,850 (versus K-998).
[b] Derived to RV-3 and recognizing VP7 of SA11 rotavirus (5).

mogenate was clarified by centrifugation at 600 × *g* for 10 min, and the supernatant was concentrated with polyacrylamide hydrogel (22) before negative staining with ammonium molybdate and examination by EM. The supernatant was also examined for rotavirus by EIA with guinea pig and rabbit anti-SA11 sera (14). Genome RNA from rotavirus-positive specimens were subjected to polyacrylamide gel electrophoresis by the method of Dyall-Smith and Holmes (6).

RESULTS

Comparison of human and porcine rotavirus strains. In vitro cross-neutralization tests of inhibition of fluorescence foci with hyperimmune antisera showed one-way reaction between human (RV-3) and porcine (AT/76) rotaviruses (Table 1). Antiserum to RV-3 neutralized both the homologous virus and AT/76 to high titer. Antiserum to AT/76 showed a high homologous titer but did not neutralize RV-3. Cross-neutralization reactions showed no significant relationship between AT/76 and human serotypes 1, 2, and 4. Table 1 shows neutralization titers of AT/76 against antisera raised to porcine strains OSU and Gottfried (supplied by L. Saif, Ohio State University, Wooster). These results indicated that AT/76 is closely related to the OSU strain that has been classified as serotype 5 (10). There was minimal neutralization with two antisera raised to the Gottfried strain. This strain has previously been classified as serotype 4 (10). It was not possible to conduct neutralization assays with anti-AT/76 sera and OSU and Gottfried strains of rotavirus since importation of these strains into Australia is not permitted.

Neutralization titers with monoclonal antibodies specific for serotype 3 (5) showed positive reactions with RV-3 (Table 1) but no or slight reactions with AT/76. EIA with monoclonal antibodies specific for subgroups I and II showed that AT/76 belonged to subgroup I. Gel electrophoresis of genome RNA showed clearly distinct patterns for RV-3 and AT/76 (Fig. 1).

Excretion of rotavirus after administration of RV-3 and AT/76 per os. Results are listed for 10 test piglets and 3 control piglets in Table 2. Results from two additional test piglets are omitted from this table. One was killed 2 days after administration of RV-3 in an attempt to identify rotavirus particles in intestinal epithelium, and the other was killed after 4 days because of development of intestinal obstruction. Of the 10 piglets given RV-3, 7 excreted the virus in feces obtained on at least one day postinoculation in amounts graded by EM as + (see below), i.e., approximately 10^6 particles per ml. Excretion was detected 4 to 7 days postinoculation. Virus was identified in feces obtained on more than one day in four of the piglets. Gel electrophoresis of rotavirus RNA extracted from fecal material showed the pattern characteristic of RV-3 electropherotype (Fig. 1). Feces collected from piglets after challenge with AT/76 showed excretion of rotavirus in four of six piglets previously vaccinated with RV-3 (Table 2). These piglets excreted rotavirus for 1 to 3 days, beginning on the day following

TABLE 2. Virus excretion and clinical symptoms in gnotobiotic piglets given RV-3 on day 2 to 3 of life, or challenged with porcine rotavirus (AT/76) on day 12 to 15 of life, or both

Group and pig no.	After administration of RV-3 per os		After administration of AT/76 per os	
	Virus excretion (days postinoculation)	Intestinal symptoms	Virus excretion (days postchallenge)	Intestinal symptoms
Test 1	+ (4,7)	−	+ + + (1,2,3)	Moist feces
2	+ (4)	−	+ + (1,2)	None
3	+ (5)	−	+ (3)	Vomiting (once)
4	−	−	+ + (1,2)	Moist feces
5	+ (4,7)	−	−	Moist feces
6	+ (4,7)	−	−	None
7	−	−	NT[a]	Depression, anorexia
8	+ (5)	−	NT	Moist feces
9	+ (5,6)	−	NT	None
10	−	Rectal prolapse (day 12)	—[b]	
Control 11	NA[c]	NA	+ + + (2)	Watery diarrhea, depression, anorexia, dehydration
12	NA	NA	+ + + (1,2)	Watery diarrhea, depression, anorexia, dehydration
13	NA	NA	+ + + (2)	Watery diarrhea, depression, anorexia, dehydration

[a] NT, Not tested.
[b] Killed before challenge.
[c] NA, Not applicable.

TABLE 3. Titer of rotavirus neutralizing antibody in sera from gnotobiotic piglets after administration of human rotavirus on day 2 to 3 of life, or porcine rotavirus on day 12 to 15 of life, or both

Group and pig no.	Serum neutralizing antibody titer (reciprocal of dilution)			
	10 days after RV-3 per os		4 days after AT/76 per os	
	Vs RV-3	Vs AT/76	Vs RV-3	Vs AT/76
Test 1	150	100	1,050	300
2	150	80	1,000	250
3	NT[a]	NT	550	100
4	NT	NT	150	100
5	160	100	640	100
6	380	100	640	100
7	<100	<10	NT	NT
8	100	20	NT	NT
9	176	160	NT	NT
10	<100	10	Not challenged	Not challenged
Control 11	NT	NT	<100	40
12	NT	NT	<10	20
13	NT	NT	<10	10

[a] NT, Not tested.

challenge in three piglets. The amounts excreted were assessed by EM as +, ++, or +++, corresponding to 10^6, 10^7, and $>10^7$ particles per ml, respectively. Three control piglets excreted rotavirus each day after challenge in amounts graded as +++ by EM. Gel electrophoresis identified the characteristic AT/76 electropherotype in feces excreted by test piglets.

Clinical outcome after administration of RV-3 and AT/76 per os. Results from test and control piglets are listed in Table 2. None of the 10 test piglets developed clinical symptoms related to rotavirus infection on any day postinoculation with RV-3. One of these piglets (no. 10) developed symptoms of rectal prolapse on day 12 of life and was killed before challenge with AT/76. The remaining nine piglets were challenged with AT/76 10 to 12 days after administration of RV-3. Four of these piglets showed signs of increased moisture content in the feces 3 to 4 days later but were otherwise healthy, one piglet vomited 2 days postchallenge but showed no other clinical signs, and three piglets remained unaffected. One of the nine piglets (no. 7) became depressed and anorectic 4 days after the challenge but did not develop diarrhea. In contrast, the three control piglets not previously exposed to RV-3 developed profuse watery diarrhea 1 to 2 days after challenge with AT/76. They showed signs of depression, anorexia, emaciation, and dehydration before being killed 3 days postchallenge.

Necropsy findings. Multiple samples of small and large intestine from one test piglet necropsied 2 days after inoculation with RV-3 showed normal mucosal morphology when sections were examined by light microscopy. Peroxidase-antiperoxidase stained sections of gut taken from 20 different locations showed no evidence of viral antigen in enterocytes.

Histological changes in the small intestine obtained at necropsy from test and control piglets after challenge with AT/76 correlated well with clinical manifestations. Seven of nine test piglets showed mild villous atrophy in lower ileum while the proximal small intestine remained intact. The remaining two test piglets (nos. 1 and 3) had moderate but focal villous atrophy in mid and distal ileum. In control piglets with severe disease, there was extensive villous atrophy, edema, and congestion in the lamina propria and the submucosa, and hypertrophy and glandular crypt cells throughout the length of the small intestine.

Serum antibody results. Serum was obtained 10 days after inoculation with RV-3 from eight test piglets. Neutralizing antibodies to RV-3 were found in six of these eight piglets with titers ranging from 1:100 to 1:380 (Table 3). Neutralizing antibodies to AT/76 were detected at the same time in the same six piglets, with titers ranging from 1:20 to 1:160. Two piglets (nos. 7 and 10) showed no or very low levels of neutralizing antibody to RV-3 and AT/76. Titers of neutralizing antibodies to standard strains of human rotaviruses of serotypes 1, 2, and 4 were less than 1:10 in sera from all eight piglets.

Serum was obtained from six test piglets 4 days after challenge with AT/76. Four of these six piglets (piglets 1, 2, 5, and 6) had prechallenge sera available. The postchallenge sera showed boosts in level of neutralizing antibody to RV-3. In two of the four piglets (nos. 1 and 2), there was an associated boost in neutralizing antibody to AT/76. Two piglets (piglets 3 and 4) from which no prechallenge sera were obtained showed postchallenge neutralizing antibody titers to RV-3 of 1:550 and 1:150, respectively, and to AT/76 of 1:100. Serum from the three control piglets killed 3 days after challenge with AT/76 showed low levels of neutralizing antibody to AT/76 and no detectable antibody to RV-3.

All piglets that excreted detectable amounts of RV-3 developed neutralizing antibodies to RV3 and AT/76 (Tables 2 and 3). These titers increased after administration of AT/76. Only low levels of neutralizing antibodies were detected in the three test piglets that did not excrete RV-3 (piglets 4, 7, and 10). It is notable that one of these (piglet 7) developed more severe symptoms postchallenge than did piglets with prechallenge neutralizing antibodies.

DISCUSSION

Our results show that administration per os of a human rotavirus strain (serotype 3) protected gnotobiotic piglets from severe diarrheal disease when they were challenged 10 to 14 days later with a virulent wild-type porcine rotavirus (presumptive serotype 5). Administration of human rotavirus did not protect against infection with porcine rotavirus on challenge, but symptoms of infection were much less severe. These results confirm those previously observed with similar viruses (19) and appear to demonstrate heterologous clinical protection between rotavirus strains of different serotypes from different animal sources. This heterologous protection was different from protection observed when AT/76 was used previously as primary and challenge doses. Such homotypic protection was associated with complete absence of symptoms and failure to excrete detectable quantities of virus after challenge (S. R. Tzipori, unpublished observation). Our results can be compared with published reports of cross-protection studies that have produced contradictory results. Some have shown cross-protection only between rotavirus strains of the same serotype (2, 7, 24). Others have shown heterologous protection that is inexplicable in terms of current serotype classification (26, 27), although protection of calves against a human rotavirus strain, by inoculation of bovine virus, may have been caused by development of heterotypic neutralizing antibodies to human serotypes 1 and 2 in utero (25). It is important to understand the mechanism and extent of cross-protection between strains in order to select effective vaccine strategies.

Detailed study of serological cross reactions in vivo and in vitro between the two strains used in this study reveals the

probable mechanism of the cross-protection. Our results also offer a possible explanation of the diverse findings recorded in the literature to date. Administration of the human rotavirus strain RV-3 to newborn piglets resulted in development of various low levels of neutralizing antibody in serum 10 days postinfection in the majority of piglets. This neutralizing antibody showed a higher titer to human virus than to porcine virus, but nevertheless some neutralizing antibody to the heterologous porcine virus was induced. The titers of homologous and heterologous antibody varied from piglet to piglet and seemed related to the amount of virus excreted, e.g, the lowest titers were observed in two piglets that did not excrete detectable quantities of human virus. Development of serum antibody was probably initiated by growth rotavirus in gut epithelial cells, rather than by passive uptake of viral antigen, although there was no direct evidence that RV-3 infected the gut cells. There was indirect evidence of infection since virus genetically similar to the inoculum was excreted 4 to 7 days after administration of the original dose, and the total amount excreted appeared to exceed the amount given. Excretion of virus was intermittent and at the limit of detection by both EM and EIA, implying that replication (if it occurred) was limited.

Challenge with virulent wild-type porcine rotavirus resulted in mild or no symptoms in the majority of piglets, even though the amounts of porcine virus excreted indicated that replication of wild-type virus had occurred in the gut of some piglets. This clinical protection was associated with an increase in neutralizing antibodies in serum taken 3 to 4 days postchallenge. It was not clear whether the increase in neutralizing antibody after challenge resulted from continued production of antibody after the original dose of human rotavirus or from boosting by challenge with porcine virus (or both). Reinfection has been observed to boost neutralizing antibody to heterotypic strains that have previously infected the host animal (17).

The apparent heterotypic immunity conferred by the serotype 3 human rotavirus strain used in this study is explicable as being caused by induction of antibody that neutralized the heterologous (probable serotype 5) porcine strain. These in vivo results could have been predicted from results of in vitro cross-neutralization assays. Thus, hyperimmune serum raised to the human virus neutralized porcine virus to high titer in vitro. Hyperimmune sera to porcine virus had no effect on infectivity of the human virus in the same assay.

It is evident from published data that considerable cross-neutralization occurs between rotaviruses of different serotypes (3, 10). These cross-reactions are thought to have their basis in the existence of two outer capsid proteins, of molecular weights of approximately 34,000 (VP7) or 80,000 to 90,000 (VP3), that provoke neutralizing antibody responses. Classification of rotavirus strains has so far been based on identification of these proteins by using hyperimmune sera. However, this could give confusing results if the genes coding for the two antigens segregate independently (9) or if viruses of different serotypes share immunodominant neutralization sites (B. S. Coulson, J. M. Tursi, W. J. McAdam, and R. F. Bishop, Virology, in press). In fact, one human strain has already been shown to have serological determinants in common with human serotypes 1 and 4 due to sharing one outer capsid protein (gp34 and p84) with each of the two serotypes (9). In addition, an immunodominant determinant shared between human serotypes 1 and 3 has been detected by using a monoclonal antibody (Coulson et al., in press).

In the present study, the porcine virus was not neutralized by two serotype 3-specific monoclonal antibodies that bind to two regions (one of which is immunodominant) of the major (VP7) glycoprotein (11). The observed neutralization of the porcine virus by hyperimmune serotype 3-specific antiserum could have been mediated via another antigenic region on VP7 or by sharing of VP3. However, it is difficult to explain the one-way cross-neutralization observed in our study by either mechanism.

Our results emphasize the importance of neutralizing antibody in protection against clinical symptoms of rotavirus infection. They also highlight the present confusion in classification of rotavirus serotypes based on the use of standard hyperimmune sera. Development of monoclonal antibodies should permit more precise identification of shared antigenic structures. However, it may still be necessary to undertake extensive cross-neutralization titrations in vitro in order to predict the ability of strains to cross protect in vivo.

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