JOURNAL OF VIROLOGY, Dec. 1986, p. 1107–1112
0022-538X/86/121107-06$02.00/0

Vol. 60, No. 3

Diarrheal Response of Gnotobiotic Pigs after Fetal Infection and Neonatal Challenge with Homologous and Heterologous Human Rotavirus Strains

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Received 23 June 1986/Accepted 26 August 1986

Pigs exposed in utero to human rotavirus (HRV) strain Wa serotype 1 from 15 to 36 days prior to birth responded immunologically by modifying their clinical response to neonatal oral challenge with a pathogenic dose of homologous Wa or heterologous M serotype 3 HRV. In these cases, diarrhea was prevented in 12 of 14 pigs and greatly reduced in the other two. However, fecal virus shedding was not significantly modified, since it was detected in 12 of 14 pigs. These results suggest the existence of a closer antigenic relationship between these two different HRV serotypes which may only be expressed in an in vivo test system. Exposure of fetal pigs to HRV DS-1 serotype 2 failed to cause infection or to induce any protection when pigs were challenged at birth with HRV Wa. This model for cross-protection studies in gnotobiotic piglets offers good possibilities for the evaluation of potential HRV vaccine candidates, for the in vivo study of antigenic similarities between rotavirus serotypes, and for the understanding of protective immune responses against diarrhea and virus shedding.

Rotavirus diarrheal disease is a major health problem of children and many young animals (14, 15). The development of a human rotavirus (HRV) vaccine has received a great deal of attention during the last few years (8, 14, 15, 18, 30). The search for potential vaccine candidates has been helped by (i) the recent ability to propagate a variety of human and animal rotaviruses in cell cultures with the help of proteolytic enzymes (4, 27); (ii) the finding that several human and animal rotavirus strains share a common group antigen (5, 10, 16), with some sharing serotype-specific antigens (16); and (iii) the ability to create laboratory rotavirus reassortant strains (8, 14). One of the remaining needs in the development of an HRV vaccine is the availability of an animal model in which cross-protection challenge studies may be conducted. Among a variety of animal models used for HRV research, the neonatal pig has been one of the more useful. Colostrum-deprived (30) and gnotobiotic neonatal pigs (4, 22, 27) have been experimentally inoculated with several HRV strains, resulting in all cases in intestinal infection, virus shedding in the feces, and seroconversion. However, diarrhea can only be induced by HRV infection of gnotobiotic pigs during their first 12 h of life (13, 22). Recently, we reported that the addition of proteolytic enzyme to diets of rotavirus-infected neonatal pigs resulted in a more uniform onset of diarrhea of greater severity (22). This improved animal model provides a better system for the evaluation of clinical diarrhea than did previous models. However, when dealing with HRV strains, the necessity to infect newborn gnotobiotic pigs to induce diarrhea demands a different approach for the design and conduct of cross-protection studies to retain the value of clinical diarrhea as a major evaluating criterion. The purpose of this report is the presentation of the results of cross-protection trials conducted with gnotobiotic pigs by exposing them in utero to HRV strains and subsequently challenging them at birth with homologous or heterologous HRV strains while they were fed a diet supplemented with exogenous proteolytic enzymes.

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MATERIALS AND METHODS

Experimental animals. Six pregnant gilts were obtained from a specific-pathogen-free herd. Gnotobiotic pigs were aseptically derived by hysterotomy and housed in germfree isolators as previously described (23).

Rotaviruses. HRV strains Wa serotype 1 (subgroup II), DS-1 serotype 2 (subgroup I), and M serotype 3 (subgroup II) (1), were obtained from R. G. Wyatt, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md. HRV strains Wa and DS-1 were available as cell-culture-propagated virus (in MA-104 cells) (26, 27). HRV strains Wa and M used for oral challenge were available as pools of homogenized intestinal contents of infected gnotobiotic pigs (unpublished data).

Experimental design. Laparotomies were performed on the six gilts under local anesthesia on days 76 to 95 of gestation. One gravid uterine horn was exteriorized, and all fetuses in the horn were inoculated with HRV preparations (1.0 ml of cell-culture-propagated HRV Wa or HRV DS-1 containing 10^5 50% tissue culture infective doses of virus). Inoculations were done through the uterine wall into the amniotic fluid. The HRV-exposed uterine horn was then marked and returned to the abdominal cavity, and the laparotomy was closed by conventional surgical procedures. Neonatal pigs were delivered by sterile hysterotomy under germfree conditions on day 112 of gestation, as previously described (23). Pigs in the unexposed uterine horn (control pigs) were delivered and placed in sealed transport boxes before the HRV-exposed uterine horn was cut for the removal of the HRV-infected pigs. The infected pigs were placed in a separate transport box. All piglets were ear notched for positive identification and bled from their umbilical cords at birth. Each of the six litters was subdivided into four treatment groups, each of which was housed in an individual

TABLE 1. Distribution of littermate pigs among the treatment groups

Trial	Litter no.	No. of pigs in treatment group[a]:			
		1	2	3	4
A	A1	4	4	2	1
A	A2	3	4	1	2
B	B1	4	4	2	1
B	B2	3	4	2	2
C	C1	3	3	1	1
C	C2	3	4	1	2

[a] Details of treatments for trials A, B, and C are indicated in Tables 2, 3, and 4, respectively.

germfree isolator. The groups had the following compositions: group 1 and 3 animals were exposed to HRV Wa or HRV DS-1 in utero; group 2 and 4 animals were controls. All piglets from groups 1 and 2 were orally challenged at 2 to 4 h of age with 1.0 ml of a 10% bacterium-free filtrate of a pool of feces containing approximately $10^{5.5}$ virions of either HRV Wa or HRV M. Piglets from groups 3 and 4 received no oral inoculation at birth. All pigs were fed a commercial milk diet plus a proteolytic diet supplementation (Pancrease; Johnson & Johnson, Piscataway, N.J.) as described previously (22). A summary of the number of piglets from each litter that were allocated to each of the treatment groups is given in Table 1.

Measurements and observations. All experimental pigs were observed three times a day for 20 days. Body temperature and weight were recorded daily before the morning feeding. For comparative purposes, birth weights were subtracted from each daily weight to calculate daily weight gain. Rectal swabs were collected in the afternoons and used for virus shedding determinations by an indirect enzyme-linked immunosorbent assay as previously described (16, 22). The diarrheal responses were recorded by using a numerical scale (22) as follows: 0, no feces observed; 1, pasty feces; 2, semiliquid homogeneous feces; 3, semiliquid feces with undigested material; 4, liquid feces with undigested material; and 5, liquid, watery, homogeneous feces. A fecal score of 3 or more was considered diarrhea, and a score of 5 was considered severe diarrhea. All observations and scores were done by the same person throughout the experimental trial. The fecal scores were not done in a completely blind fashion, since the observer knew which were groups containing control animals (groups 3 and 4) owing to the small number of pigs. Blood serum samples were collected from all pigs at the time of necropsy at day 21 postinfection (p.i.). The presence of serum antibodies to HRV common-group antigen was determined by indirect immunofluorescence on HRV Wa-infected MA-104 cell monolayers (22). The statistical significance was calculated by using Wilcoxon's rank sum test tables for small sample sizes (11).

RESULTS

General. All pregnant gilts remained healthy after the experimental inoculation of all the fetuses in one of their uterine horns. The exposure of fetal pigs to HRV strains did not induce any fetal deaths, abortions, or stillbirths or any gross abnormalities that were noticeable after birth. None of the piglets developed fever or vomiting during the observation period. The shedding of HRV in infected piglets was irregular, with breaks of one or more days' duration and with occasional late episodes after several days during which no shedding was detected. All the pigs exposed in utero to HRV strains Wa and M but not DS-1 developed serum antibodies that were detectable at birth. Seroconversion was observed for all piglets challenged with HRV strains Wa and M at birth. Complete results for each of the experimental trials are summarized below.

(i) Trial A. The results of trial A are presented in a

TABLE 2. Summary of results of trial A, comprising pigs from litters A1 and A2 inoculated in utero at 17 and 15 days, respectively, prior to birth

Parameter	Results[a] for treatment group:			
	1	2	3	4
Rotavirus administered in utero/at birth	Wa/Wa	None/Wa	Wa/none	None/none
No. of pigs/no. with diarrhea	7/2	8/8	3/0	3/0
Median (min/max) days with fecal score[b] of				
≥3	0a (0/2)	7 (6/10)	0 (0/0)	0 (0/0)
5	0a (0/0)	5 (2/6)	0 (0/0)	0 (0/0)
No. of pigs shedding rotavirus	5	8	2	0
Median (min/max) days of rotavirus shedding	3b (0/13)	8 (4/14)	4b (0/8)	0 (0/0)
Median (min/max) wt (g) gained by day p.i.				
5	29a (0/198)	−57 (−255/29)	85a (85/227)	57 (28/85)
10	283a (114/396)	57 (−198/142)	284b (199/454)	255 (170/255)
20	595c (369/623)	369 (142/822)	595 (482/822)	482 (397/624)
No. of pigs dead after birth	0	1	0	0
Median antibody titer[c] at				
birth	1:57	≤1:2	1:10	≤1:2
day 20	1:18,513	1:3,673	1:1,040	≤1:2

[a] P values were calculated by Wilcoxon's rank sum test (11), comparing treatment groups 1 with 2 and 3 with 4: a, $P \leq 0.01$; b, $0.01 < P \leq 0.02$; c, $0.02 < P \leq 0.2$.

[b] Fecal scores: 0 to 2, Normal; 3 and 4, diarrhea; 5, severe diarrhea.

[c] Determined by indirect immunofluorescence against HRV-infected cells.

TABLE 3. Summary of results of trial B, comprising pigs from litters B1 and B2 inoculated in utero at 15 and 36 days, respectively, prior to birth

Parameter	Results[a] for treatment group:			
	1	2	3	4
Rotavirus administered in utero/at birth	Wa/M	None/M	Wa/none	None/none
No. of pigs/no. with diarrhea	7/0	8/8	4/0	3/0
Median (min/max) days with fecal score of				
≥3	0a (0/0)	6 (3/7)	0 (0/0)	0 (0/0)
5	0a (0/0)	4 (0/6)	0 (0/0)	0 (0/0)
No. of pigs shedding rotavirus	7	8	1	0
Median (min/max) days of rotavirus shedding	10 (6/16)	9 (3/18)	0 (0/1)	0 (0/0)
Median (min/max) wt (g) gained by day p.i.				
5	57a (28/85)	−14 (−141/85)	113a (85/198)	57 (50/142)
10	284a (198/341)	85 (0/316)	298b (283/510)	312 (227/312)
20	598c (494/611)	435 (255/595)	559b (551/708)	497 (488/596)
No. of pigs dead after birth	0	1	0	0
Median antibody titer at				
birth	1:25	≤1:2	1:32	≤1:2
day 20	1:1,024	1:1,802	1:1,324	≤1:2

[a] For explanations, see footnotes to Table 2.

combined form for litters A1 and A2 in Table 2. In utero inoculations were performed 17 and 15 days prior to birth for litters A1 and A2, respectively. Two of seven pigs from group 1 (exposed in utero to HRV Wa and challenged at birth with the same HRV strain) developed mild diarrhea for 2 days. However, five of the seven pigs shed HRV in their feces for a mean of 3 days, with one animal shedding virus for up to 13 days. All of the eight pigs from group 2 (kept unexposed to HRV in utero and challenged at birth with HRV Wa) developed diarrhea for a mean of 7 days. Severe diarrhea was observed for a mean of 5 days. One pig in this treatment group died as a result of diarrhea and dehydration. All pigs shed HRV in their feces for a mean of 8 days. None of the pigs from group 3 (exposed in utero to HRV Wa but not challenged at birth) developed diarrhea. However, two pigs from this group shed HRV for a mean of 4 days. Diarrhea and shedding were not observed for the three pigs from control group 4. In relation to weight gain, pigs from treatment groups 1 and 4 had similar growth patterns. Growth of the pigs from treatment group 2 was severely affected by the diarrheal disease during the first 10 days of life; however, some final weights at day 20 were comparable to those found for pigs in the other treatment groups. Pigs in treatment group 3 had the highest rates of weight gain during the first 10 days of life.

(ii) Trial B. The results for litters B1 and B2 are summarized in Table 3. In utero inoculations were performed 15 and 36 days prior to birth for litters B1 and B2, respectively.

TABLE 4. Summary of results of trial C, comprising pigs from litters C1 and C2, both inoculated in utero 27 days prior to birth

Parameter	Results[a] for treatment group:			
	1	2	3	4
Rotavirus administered in utero/at birth	DS-1/Wa	None/Wa	DS-1/none	None/none
No. of pigs/no. with diarrhea	6/6	7/7	2/0	3/0
Median days with fecal score of				
≥3	5 (3/9)	6 (2/7)	0 (0/0)	0 (0/0)
5	3.5 (3/4)	4 (2/5)	0 (0/0)	0 (0/0)
No. of pigs shedding rotavirus	6	7	0	0
Median days of rotavirus shedding	2 (1/4)	3 (2/7)	0 (0/0)	0 (0/0)
Median wt (g) gained by day p.i.				
5	−100 (−227/−85)	−142 (−198/0)	100a (86/114)	29 (28/85)
10	−58 (−170/28)	56 (0/142)	298b (284/312)	141 (141/142)
20	198 (169/228)	441 (430/539)	539 (511/567)	510 (510/511)
No. of pigs dead after birth	3	3	0	0
Median antibody titer at				
birth	≤1:2	≤1:2	≤1:2	≤1:2
day 20	1:1,040	1:723	≤1:2	≤1:2

[a] For explanations, see footnotes to Table 2.

None of the seven pigs from group 1 (exposed in utero to HRV Wa and challenged at birth with HRV M) developed diarrhea, but all of them shed HRV in their feces for a mean of 10 days. All eight piglets from treatment group 2 (uninoculated in utero and challenged at birth with HRV M) developed severe diarrhea for up to 6 days and shed HRV in the feces for a mean of 9 days, with some of the pigs shedding virus for up to 18 days. One pig from this group died of severe dehydration. None of the four pigs from treatment group 3 (exposed only to HRV Wa in utero) developed diarrhea, but one shed virus for 1 day. All three control piglets (treatment group 4) remained healthy throughout the trial. The pigs from treatment group 1 had a similar rate of weight gain to that of pigs from control treatment group 4. Pigs from treatment group 2 had body weights below birth weight at 5 days p.i. and lower weight gains at day 10 p.i. than those from any other treatment group. However, the final mean weight at day 20 p.i. was comparable to those of controls. All pigs from treatment group 3 had the highest rates of weight gain throughout the experimental period.

(iii) Trial C. A summary of data collected from experimental litters C1 and C2 is presented in Table 4. In utero inoculations of members of litters C1 and C2 were done 27 days prior to birth. All six pigs in treatment group 1 (infected in utero with HRV DS-1 and challenged at birth with HRV Wa) developed severe diarrhea and shed virus, some for up to 4 days. Three pigs from litter C1 died as a result of severe diarrheal disease on day 5 p.i. All seven pigs from treatment group 2 (uninoculated in utero and challenged at birth with HRV Wa) also developed severe diarrhea and shed HRV. As with the previous treatment group, three pigs from litter C1 died on day 5 p.i. None of the pigs from treatment groups 3 or 4 had any diarrhea or shedding during the observation period. The rates of weight gain of the pigs in treatment groups 1 and 2 were severely depressed. In both cases, at day 5 p.i., none of the surviving pigs had gained any weight since birth and many had lost 80 to 227 g, particularly those found dead that day. Pigs from treatment group 3 had the highest rates of weight gain, higher than those of control pigs of group 4.

DISCUSSION

The effect of exposure of fetal animals to enteric pathogens and their response after oral challenge at birth with homologous or heterologous preparations has been studied for calves with rotavirus (29), *Escherichia coli* (7), and coronavirus (19) and for sheep with *E. coli* (7). In all these cases, the fetuses responded immunologically and were protected against the neonatal oral challenge. In studies of in utero fetal inoculations with rotaviruses, neither abortion (17, 20, 28) nor congenital anomalies were observed (17, 20, 29). Studies with calves have also demonstrated that exposure of fetuses to one rotavirus serotype (bovine neonatal calf diarrhea virus) can induce a heterologous resistance to neonatal challenge with HRV strain D (28, 29). The results given in those reports are comparable to the findings of the present study.

In utero exposure of pigs to HRV strain Wa serotype 1 prevented clinical diarrhea after neonatal challenge with the homologous HRV Wa serotype 1 or with the heterologous HRV M serotype 3. With the exception of two pigs in trial A that had a transient mild diarrhea for 2 days (Table 2), none of the pigs developed any clinical diarrhea. This difference was statistically significant ($P \leq 0.01$) compared with the diarrheal response of all pigs in treatment group 2 that were kept as controls until birth and then orally challenged with HRV Wa or M. The protective effect of in utero exposure to HRV Wa upon neonatal challenge with HRV Wa or M was also supported by the statistical differences in the rate of weight gain between the pigs in treatment group 1 (exposed in utero) and those in treatment group 2 (control in utero) (summarized in Tables 2 and 3). However, exposure of fetal pigs to HRV Wa did not significantly modify the shedding of rotavirus in the feces after neonatal challenge with HRV Wa or M. Pigs in treatment group 3 exposed to HRV Wa in utero only and not challenged at birth did not develop diarrhea and showed the highest weight gain during the observation period. However, three of seven pigs in this category (Tables 2 and 3) shed rotavirus in the feces after birth, one for up to 8 days. Because of this finding, it is possible that a portion of the rotavirus shed by the pigs exposed to HRV in utero and challenged at birth was due to residual rotavirus replication from the in utero exposure and not entirely to postnatal intestinal replication after oral challenge. A final determination of the serotype shed was not possible with the enzyme-linked immunosorbent assay procedure used for monitoring fecal virus shedding. It is of interest that the protective effect of in utero exposure of pigs to HRV Wa serotype 1 against challenge at birth with HRV M serotype 3 suggests a possible closer antigenic relationship between these two rotavirus serotypes. The observed cross-protection could be due to the in vivo expression of this antigenic relationship, even though its in vitro cross-neutralization is only very weak or totally absent (13).

All the results of trial C indicated a failure of in utero exposure of pigs to HRV DS-1 to modify their response after neonatal challenge with HRV Wa. This failure was based on the inability of HRV DS-1 to replicate in the fetal pigs, with a consequent failure to induce an immune response as demonstrated by the absence of serum antibody responses at birth (Table 4). Other researchers have also reported a failure to infect experimental pigs with other subgroup I HRV strains (30).

The observed presence of rotavirus shedding without diarrhea in neonatal pigs after inoculation with HRV in utero and postnatal oral challenge may have been the result of an immune response that was capable of reducing the intestinal replication of rotavirus to the point at which diarrhea was not induced but not sufficient to eliminate all intestinal virus replication. It is very likely that the inoculation of HRV into the amniotic fluid acts as an oral exposure to the fetus, with the development of an active immune response that may include systemic humoral, local intestinal, and cell-mediated components. There is ample evidence that rotavirus fecal shedding may be present in the absence of diarrhea. This has been observed for experimentally infected animals (22, 24), as well as children under natural conditions (12). This situation may be the result of a balance between the number of enterocytes infected, the amount of rotavirus being produced by the infected enterocytes, and the concentration of antirotavirus intestinal antibodies present. If there is a situation in which the balance of rotavirus production and enterocyte infection exceeds the neutralizing capacity of the intestinal antibodies, both diarrhea and virus shedding will be present. If, on the other hand, the neutralizing capacity of the intestinal antibody exceeds that of the rate of rotavirus replication, a complete cessation of diarrhea and virus shedding will occur. In intermediate cases, it is possible that there is sufficient virus replication to cause detectable rotavirus shedding in the feces but not enough virus replica-

tion and enterocyte damage to produce the pathophysiological alterations that lead to clinical diarrhea. Studies with mice have suggested that low levels of rotavirus replication in the intestinal mucosa are capable of inducing local and systemic immune responses (9). Studies with mice have also indicated that the presence of intestinal antibodies may not be sufficient by itself to prevent rotaviral diarrhea or rotavirus clearance (9, 21). The detected humoral antibody response of the piglets exposed to HRV in utero in the present study indicated that the piglets had a systemic exposure to rotavirus antigens, which also may have resulted in the induction of cell-mediated immunity. It has been suggested that, in rotavirus infection in mice, cell-mediated immunity is related to the clearance of rotaviral diarrhea and infection (21). However, it has been postulated that in the pig, the principal immune response responsible for the clearance of viral enteric infections is that of the active or passive intestinal presence of immunoglobulin A antibodies (2, 3). Further studies are needed to elucidate the mechanisms for rotavirus shedding in the absence of diarrhea in this model, as well as in natural infections of animals and humans. There is no clear explanation for the observation that piglets infected in utero with HRV and not challenged at birth had the highest rates of weight gain. We can only speculate that the in utero intestinal replication of HRV may have stimulated a more mature intestinal mucosa and thus a more efficient digestive process than that of germfree gnotobiotic littermates.

Clarification of the mechanisms responsible for the prevention of diarrhea but not shedding would have important implications for the development of rotavirus vaccines. It could be argued that an effective rotavirus vaccine is one that prevents clinical diarrhea, regardless of shedding. After all, the water and electrolyte disturbances created by the diarrhea are the life-threatening components of rotavirus infections. On the other hand, there will be a valid concern about a rotavirus vaccine that does not prevent virus shedding or that induces virus shedding even with no diarrhea. The experimental model used in the present studies, that of exposing fetal pigs to HRV to study their response after postnatal oral challenge, offers the possibility of evaluating both diarrhea and shedding parameters, as well as the development of humoral, cellular, and intestinal immune responses.

ACKNOWLEDGMENTS

This work was supported in part by Diarrhoeal Disease Control Programme grant C6-181-75 from the World Health Organization and by the Nebraska Agricultural Experiment Station, Lincoln, Neb.

The technical assistance of Ginger Mills and Gene Steppat and the statistical guidance of Hollis N. Erb are greatly appreciated.

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