Arch Virol (1996) [Suppl] 12: 153–161

Archives of Virology

© Springer-Verlag 1996

The gnotobiotic piglet as a model for studies of disease pathogenesis and immunity to human rotaviruses

L. J. Saif, L. A. Ward, L. Yuan, B. I. Rosen, and **T. L. To**

Food Animal Health Research Program,
Ohio Agricultural Research and Development Center,
The Ohio State University, Wooster, Ohio, U.S.A.

Summary. Gnotobiotic piglets serve as a useful animal model for studies of human rotavirus infections, including disease pathogenesis and immunity. An advantage of piglets over laboratory animal models is their prolonged susceptibility to human rotavirus-induced disease, permitting cross-protection studies and an analysis of active immunity. Major advances in rotavirus research resulting from gnotobiotic piglet studies include: 1) the adaptation of the first human rotavirus to cell culture after passage and amplification in piglets; 2) delineation of the independent roles of the two rotavirus outer capsid proteins (VP4 and VP7) in induction of neutralizing antibodies and cross-protection; and 3) recognition of a potential role for a nonstructural protein (NSP4) in addition to VP4 and VP7, in rotavirus virulence. Current studies of the pathogenesis of group A human rotavirus infections in gnotobiotic piglets in our laboratory have confirmed that villous atrophy is induced in piglets given virulent but not cell culture attenuated human rotavirus (G1, P1A, Wa strain) and have revealed that factors other than villous atrophy may contribute to the early diarrhea induced. A comprehensive examination of these factors, including a proposed role for NSP4 in viral-induced cytopathology, may reveal new mechanisms for induction of viral diarrhea. Finally, to facilitate and improve rotavirus vaccination strategies, our current emphasis is on the identification of correlates of protective active immunity in the piglet model of human rotavirus-induced diarrhea. Comparison of cell-mediated and antibody immune responses induced by infection with a virulent human rotavirus (to mimic host response to natural infection) with those induced by a live attenuated human rotavirus (to mimic attenuated oral vaccines) in the context of homotypic protection has permitted an analysis of correlates of protective immunity. Results of these studies have indicated that the magnitude of the immune response is greatest in lymphoid tissues adjacent to the local site of viral replication (small intestine). Secondly, there was a direct correlation between the degree of protection induced and the level of the intestinal immune response, with significantly higher local immune responses and complete protection induced only after primary exposure to

virulent human rotavirus. These studies thus have established basic parameters related to immune protection in the piglet model of human rotavirus-induced disease, verifying the usefulness of this model to examine new strategies for the design and improvement of human rotavirus vaccines.

Introduction

Group A rotaviruses are a leading cause of dehydrating diarrheal infections in infants and young children worldwide [8]. Public health problems posed by rotaviruses have stimulated research on vaccination strategies. Vaccine development has focused on the use of live attenuated oral vaccines in a "Jennerian" approach using heterologous animal strains or human/animal reassortants as candidate vaccines [5, 8]. Unfortunately, these candidate vaccines have often failed in various aspects of safety, immunogenicity or efficacy, especially when tested in developing countries [8]. To facilitate and improve vaccine development, a more comprehensive understanding of rotavirus pathogenesis and mucosal immunity to rotaviruses is needed. These studies are most readily accomplished by the use of animal models to study disease pathogenesis and immunity *in vivo*.

Advantages of gnotobiotic piglets as models for studies of rotavirus pathogenesis and immunity

Although laboratory mice and rabbits serve as useful models for evaluating immune responses to rotaviruses, especially host-specific strains, these animal models are not conducive for studies of active immunity to clinical infections induced by human rotaviruses because human rotavirus infections in these species are usually subclinical [3, 13]. Moreover, older mice and rabbits are refractory to rotavirus disease and permit evaluation of active protection only against infection.

In comparison, gnotobiotic piglets remain susceptible to infection and disease induced by several human rotavirus strains for as long as 6 weeks of age (14, 22, 24, Saif, LJ, unpublished). Other advantages of the gnotobiotic piglet model include: 1) they closely resemble humans in gastrointestinal physiology (monogastrics) and mucosal immune development [9, 12]; 2) the placenta of pigs acts as a barrier to the transfer of maternal antibodies; hence colostrum-deprived gnotobiotic pigs are devoid of rotavirus maternal antibodies and are immunologically virgin but immunocompetent at birth, permitting analysis of true primary immune responses [9]; and 3) the derivation and maintenance of piglets in a gnotobiotic environment assures that exposure to extraneous rotaviruses or other enteric pathogens is eliminated as a confounding variable [1, 23].

The pathogenesis of a group A human rotavirus in neonatal gnotobiotic piglets

In spite of the host-specificity of rotaviruses, several researchers have found that gnotobiotic piglets or gnotobiotic calves were susceptible to infection and

disease by heterologous rotaviruses, including human rotaviruses, under experimental conditions [10, 11, 14–16, 18, 21–24]. This observation agrees with antigenic and genetic data showing a close relationship between certain human and animal rotaviruses, suggesting that interspecies transmission of rotaviruses may occur under certain poorly defined circumstances in nature [19]. Gnotobiotic piglets were invaluable for initial studies of human rotaviruses. Passage of the Wa strain of human rotavirus (G1, P1A) from stool filtrates of infected infants into gnotobiotic piglets provided an amplified source of viable rotavirus, free of maternal or actively induced (in the early stages of infection) antibodies, and resulted in the first successful adaptation of a human rotavirus to serial propagation in cell culture [23].

In subsequent studies, the pathogenesis of the infant stool-passaged, virulent Wa strain of human rotavirus was analyzed in gnotobiotic piglets [21]. Piglets orally inoculated with 10^5 focus-forming units (FFU) (or 10^5 median infectious doses) of Wa rotavirus developed diarrhea within 13 post-inoculation (PI) hours, which correlated with the presence of rotavirus antigen within villous epithelial cells (Table 1). Mild to moderate villous atrophy was observed at PI hours 24–48 coincident with the peak of virus replication. Diarrhea and rotavirus shedding persisted between 4 to 7 PI days (PID). Recovery correlated with the presence of morphologically normal villi by PID 7. Thus the Wa human rotavirus induced lesions in gnotobiotic pigs, similar but less severe than those seen after infection with some (but not all) homologous porcine rotavirus strains [17]. Moreover, factors other than villous atrophy may contribute to the early diarrhea induced by PI hour 13, preceding the detection of villous atrophy.

Gnotobiotic piglets also proved useful in a recent study to identify the rotavirus genes associated with virulence and host range restriction [6]. The response of gnotobiotic piglets was analyzed after oral administration of a porcine X human reassortant rotavirus derived from a parental porcine rotavirus (G4, P9, SB1A strain) which caused diarrhea in piglets and a parental human rotavirus (G2, 1B, DS-1 strain) which was attenuated for piglets. The major conclusions were that replacing the VP3, VP4, VP7 or NSP4 genes of the attenuated human strain with the corresponding genes of the virulent porcine rotavirus yielded viral reassortants that failed to induce diarrhea. Similarly, reassortants possessing only one, two or three of these porcine rotavirus genes on the human rotavirus genetic background failed to induce diarrhea. These results suggest that replacement of any one of these four genes of a human rotavirus with that of an avirulent animal rotavirus could attenuate the human rotavirus, leading to a new rotavirus vaccine strategy.

Passive immunity to human rotaviruses in a gnotobiotic piglet model of disease

Gnotobiotic piglets have been used to evaluate the efficacy of passively administered bovine antibody to a human rotavirus for preventing human rotavirus-

Table 1. Summary of clinical signs, lesions, rotavirus detection and seroconversion after oral inoculation and challenge of gnotobiotic piglets[a] with Wa human rotavirus

Primary virus inoculum[a]	Primary inoculation[a]					Challenge (virulent Wa)[a]	
	Moderate to severe diarrhea	Fecal virus shedding	Viral antigen in gut	Villous atrophy	Sero-conversion	Moderate to severe diarrhea	Virus shedding
Virulent	Yes	Yes	Yes	Yes	Yes	No[b]	No
Attenuated	No[b]	Yes[c]	No[c]	No[c]	Yes	Partial[d]	Partial[d]
None	No[b]	No	No	No	No	Yes	Yes

[a] n = 8–18 piglets used for analysis of each response; piglets were orally inoculated with virulent, attenuated or no rotavirus (controls) and orally challenged with virulent Wa rotavirus at post-inoculation day 21

[b] 11–13% of piglets developed transient mild diarrhea

[c] Only 6% of piglets shed virus after inoculation with attenuated Wa rotavirus; none of the piglets examined for viral antigen or villous atrophy shed rotavirus prior to euthanasia

[d] 56% of piglets developed diarrhea (compared to 83% of controls) and 81% shed virus (compared to 100% of controls) after virulent Wa rotavirus challenge

induced diarrhea [15]. Cows were immunized with inactivated human rotavirus serotypes G1, P1A (Wa strain) and G2, P1B (S2 strain) and simian rotavirus (G3, [P2], SA11 strain) and the (immune) colostrum collected. Antibody concentrates from colostrum were fed three times daily to gnotobiotic piglets subsequently challenged with virulent Wa rotavirus. The immune colostrum feeding effectively reduced or eliminated both rotavirus shedding and diarrhea in a dose-dependent manner, confirming that a quantitative relationship exists between the protective antibody dose and the diarrheal disease response. Furthermore, piglets fed the immune colostrum and therefore protected against human rotavirus-induced disease, seroconverted to Wa rotavirus, indicative of the development of active immune responses in the presence of protective levels of passive colostral antibodies.

Active immunity to human rotaviruses in a gnotobiotic piglet model of disease

Previous studies of porcine rotavirus infections in gnotobiotic piglets confirmed that rotaviruses that share common VP4 (P) and VP7 (G) serotypes induced a high degree, or complete cross-protection against challenge with rotavirus strains bearing the common P or G types [7]. Little or no cross-protection was evident in the piglets inoculated and challenged with heterotypic (in both G and P type) serotypes [1].

We have expanded these studies to identify correlates of homotypic (common G and P types) protection in the gnotobiotic piglet model of human rotavirus-induced diarrhea [14, 22, 24]. In these studies, 3- to 5-day-old piglets were orally inoculated with the virulent (stood-passaged) or attenuated (cell culture-passaged) Wa strain of human rotavirus and challenged at PID 21 with the homologous virulent Wa rotavirus. These viruses were selected to mimic natural infection with virulent rotavirus or oral inoculation with a live attenuated candidate rotavirus vaccine. Piglets were examined for clinical signs of illness and for rotavirus shedding (by ELISA [4]and cell culture immunofluorescence assays [1]) after inoculation and challenge and intestinal lesions were evaluated in selected pigs [14, 21, 22, 24; Table 1). Correlates of protective immunity were determined by ELISPOT (20, 24, B cell responses) and lymphoroliferative assays (2, 22, LPA, T cell responses) using intestinal (gut lamina propria; mesenteric lymph node) and systemic (blood; spleen) lymphoid tissues collected at various PID or post-challenge days (Table 2).

Piglets inoculated with virulent Wa rotavirus developed diarrhea and villous atrophy was evident within 24–72 PI hours [14, 21, 22, 24, Table 1]. All piglets shed virus in feces and seroconverted with neutralizing antibodies to Wa rotavirus. Upon challenge with homologus virulent Wa rotavirus, all piglets were protected from virus shedding and severe to moderate diarrhea. Piglets given attenuated Wa rotavirus developed transient mild or no diarrhea (like controls) and no villous atrophy was evident (Table 1). Fecal shedding was detected in only 6% of the pigs, but 96% of the pigs seroconverted to Wa

Table 2. Peak immune responses to Wa human rotavirus in intestinal lamina propria lymphoid tissues 21 days after primary inoculation or 4 days after challenge with Wa human rotavirus

Virus Inoculation	Primary inoculation (PID 21)[a]				Challenge (PCD 4)[a]			
	Mean (±SEM)[b] No. ASC[b]/5 × 10^5 MNC[b]			LPA[b]	Mean (±SEM)[b] No. ASC[b]/5 × 10^5 MNC[b]			LPA[b]
	IgG	IgA	IgG/IgA[c]	Mean (±SEM) CPM[b]	IgG	IgA	IgG/IgA[c]	Mean (±SEM) CPM[b]
Virulent	64 (±26)	53*[d] (±28)	1.2	3.2 × 10^4*[d] (±5.6 × 10^3)	108 (±43)	47 (±19)	2.3	2.7 × 10^4 (4.8 × 10^3)
Attenuated	41 (±26)	6* (±4)	6.8	1.8 × 10^4* (±3.8 × 10^3)	270 (±52)	46 (±10)	5.9	2.6 × 10^4 (2.6 × 10^3)

[a] Gnotobiotic piglets were orally inoculated with virulent or attenuated Wa rotavirus and orally challenged with virulent Wa rotavirus at post-inoculation day (PID) 21; PCD = post-challenge day

[b] SEM = standard error of the mean; ASC = antibody secreting cells; MNC = mononuclear cells; LPA = lymphoproliferative assay; CPM = counts per minute (minus background)

[c] IgG/IgA = ratio of Wa rotavirus-specific IgG ASC to IgA ASC based on mean numbers of ASC per 5 × 10^5 MNC

[d] "*" denotes significantly different ($p < 0.05$) numbers of ASC or LPA responses (following rank transformation of mean CPM) between the virulent and attenuated rotavirus-inoculated groups

rotavirus. Piglets were only partially protected from diarrhea (56% with diarrhea) and virus shedding (81% shed virus) after challenge exposure.

Assessment of the immune responses in these pigs revealed that the highest numbers of antibody secreting cells (ASC) (measured by ELISPOT) [14, 24] and LPA responses [22] [measured by virus-stimulated counts per minute (CPM) minus background CPM] were in intestinal tissues (adjacent to the site of rotaviral replication) of both groups of pigs (Table 2). The number of ASC in intestinal tissues was at least 5-fold higher than the number of ASC in systemic tissues (data not shown) before challenge. The number of IgA ASC and the LPA responses (CPM) were significantly higher ($p < 0.05$) at challenge (PID 21) in the intestinal lymphoid tissues of the virulent-Wa rotavirus-inoculated pigs compared to the attenuated Wa rotavirus-inoculated pigs (Table 2). Moreover the mean IgG/IgA ratios were ~ 1 in the virulent Wa rotavirus-inoculated pigs, but were ~ 7 in the attenuated Wa rotavirus- inoculated pigs, reflecting the predominance of IgG ASC in the latter group of pigs. After challenge of the virulent Wa rotavirus-inoculated pigs, only transient low ($\leqslant 2$ fold) or no increases occurred in numbers of IgA and IgG ASC and LPA responses (Table 2) reflecting the limited viral replication and antigenic stimulation which coincided with complete protection. The lower numbers of ASC (particularly IgA ASC) and LPA immune responses seen in the attenuated Wa rotavirus-inoculated pigs at challenge exposure (Table 2, PID 21) correlated with induction of only partial protection against diarrhea and virus shedding after challenge (Table 1). Furthermore, these pigs developed greatly increased ASC numbers (6–7-fold) and LPA responses (2–4-fold) after challenge, consistent with virus infection. Thus it appears that the magnitude of the immune response is greatest in lymphoid tissues adjacent to the site of rotavirus replication and tissue destruction (small intestine) and that the level (and IgA antibody isotype) of the local immune response may correlate with the degree of protection induced.

Acknowledgements

Salaries and research support provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Supported in part by Grants R01AI33561 and R01AI37111 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health and Grant GPV/V27/181/24 from the World Health Organization.

References

1. Bohl EH, Theil KW, Saif LJ (1984) Isolation and serotypes of porcine rotaviruses and antigenic comparison with other rotaviruses. J Clin Microbiol 19: 105–111
2. Brim TA, Van Cott JL, Lunney JK, Saif LJ (1994) Lymphocyte proliferative responses of pigs inoculated with transmissible gastroenteritis virus or porcine respiratory coronavirus. Am J Vet Res 55: 494–501
3. Conner ME, Gilger MA, Estes MK, Graham DY (1991) Serologic and mucosal immune response to rotavirus infection in the rabbit model. J Virol 65: 2562–2571

4. Hoblet KH, Saif LJ, Kohler EM, Theil KW, Bech-Nielsen S, Stitzlein GA (1986) Efficacy of an orally administered modified-live porcine-origin rotavirus vaccine against post-weaning diarrhea in pigs. Am J Vet Res 47: 1697–1703
5. Hoshino Y, Kapikian AZ (1994) Rotavirus vaccine development for the prevention of severe diarrhea in infants and young children. Trends Microbiol 2: 242–249
6. Hoshino T, Saif LJ, Kang SY, Sereno M, Chen WK, Kapikian AZ (1995) Identification of group A rotavirus genes associated with virulence of a porcine rotavirus and host range restriction of a human rotavirus in the gnotobiotic piglet model. Virology 209: 274–280
7. Hoshino Y, Saif LJ, Sereno MM, Chanock RM, Kapikian AZ (1988) Infection immunity of piglets to either VP3 or VP7 outer capsid protein confers resistance to challenge with a virulent rotavirus bearing the corresponding antigen. J Virol 62: 744–748
8. Kapikian AZ, Chanock RM (1990) Rotaviruses. BN Fields, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, Roizman B, Straus SE (eds) Virology, Raven Press, New York, pp 1353–1403
9. Kim YB (1975) Developmental immunity in the piglet. Birth Defects 11: 549
10. Mebus CA, Wyatt RG, Sharpee RL, Sereno MM, Kalica AR, Kapikian AZ, Twiehaus MJ (1976) Diarrhea in gnotobiotic calves caused by the reovirus-like agent of human infantile gastroenteritis. Infect Immun 14: 471–474
11. Middleton PJ, Petric M, Szymanski MT (1975) Propagation of infantile gastroenteritis virus (orbigroup) in conventional and germfree piglets. Infect Immun 12: 1 276–1 280
12. Phillips RW, Tumbleson ME (1986) Models. In: Tumbleson ME (ed) Swine in Biomedical Research. Plenum Press, New York, pp 437–440
13. Ramig F (1988) The effects of host age, virus dose, and virus strain on heterologous rotavirus infection of suckling mice. Microb Pathog 4: 189–202
14. Saif LJ, Ward L, Yuan L, To TL (1996) Studies of the pathogenesis and immunity to a human rotavirus in a gnotobiotic pig model of enteric disease. Proc First Intl Rushmore Conf on Mechanisms in the Pathogenesis of Enteric Diseases, Rapid City, SD, September 27–30, 1995. Plenum Press, New York
15. Schaller JP, Saif LJ, Cordle CT, Candler E, Winship TR, Smith KL (1992) Prevention of human rotavirus induced diarrhea in gnotobiotic piglets using bovine antibody. J Inf Dis 165: 623–630
16. Steel R, Torres-Medina A (1984) Effects of environmental and dietary factors on human rotavirus infection in gnotobiotic piglets. Infect Immun 43: 906–911
17. Theil KW, Bohl E, Cross R, Kohler E, Agnes A (1978) Pathogenesis of porcine rotaviral infection in experimentally inoculated gnotobiotic pigs. Am J Vet Res 39: 213–220
18. Torres-Medina A, Wyatt RG, Mebus CA, Underdahl NR, Kapikian AZ (1976) Diarrhea in gnotobiotic piglets caused by the reovirus-like agent of human infantile gastroenteritis. J Infect Dis 133: 22–27
19. Urasawa S, Hasegawa A, Urasawa T, Taniguchi K, Wakasugi F, Suzuki H, Inouye S, Pongprot B, Supawadee J, Suprasert S, Rangsiyanond, Tonusin S, Yamazi Y (1992) Antigenic and genetic analysis of human rotaviruses in Chiang Mai Thailand: evidence for a close relationship between human and animal rotaviruses. J Inf Dis 166: 227–234
20. Van Cott J, Brim T, Lunney J, Saif LJ (1994) Contribution of antibody secreting cells induced in mucosal lymphoid tissues of pigs inoculated with respiratory or enteric strains of coronavirus to immunity against enteric coronavirus challenge. J Immunol 152: 3980–3990
21. Ward LA, Rosen BI, Yuan L, Saif LJ (1996) Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs. J Gen Virol 77: 1431–1441

22. Ward LA, Yuan L, Rosen BI, Saif LJ (1996) Local and systemic T cell immunity to human group A rotavirus in neonatal gnotobiotic pigs. Clin Diag Lab Immunol 3: 342–350
23. Wyatt RG, James WD, Bohl EH, Theil KW, Saif LJ, Kalica AR, Greenberg HB, Kapikian AZ, Chanock RM (1980) Human rotavirus type 2: cultivation *in vitro*. Science 207: 189–191
24. Yuan L, Ward LA, Rosen BI, To TL, Saif LJ (1996) Evaluation of systemic and intestinal antibody-secreting cell responses to human rotavirus in a gnotobiotic piglet model of disease. J Virol 70: 3075–3083

Authors' address: Dr. L. J. Saif, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio 44691, U.S.A.