Homotypic and Heterotypic Antibodies for Prevention of Experimental Rotavirus Gastroenteritis

GENEVIEVE A. LOSONSKY, STEVEN L. VONDERFECHT, JOSEPH EIDEN, SIOK-BI WEE, AND ROBERT H. YOLKEN*

Department of Pediatrics, Division of Infectious Diseases, The Johns Hopkins Hospital, Baltimore, Maryland 21205

Received 21 May 1986/Accepted 11 September 1986

We investigated the abilities of homologous and heterologous antibody preparations to neutralize murine rotavirus in a mouse model of rotavirus infection. We found that incubation of virus with murine sera obtained from animals experimentally infected with the homologous epizootic diarrhea of infant mice strain of rotavirus resulted in inability of the virus to cause symptomatic disease in infant mice. Sera from nonimmune mice and mice infected or immunized with a heterotypic strain of rotavirus did not effectively neutralize virus in this system. On the other hand, neutralization was noted after incubation of virus with a number of immunoglobulins from other animal and human sources. The neutralizing activity of the preparations in the murine model correlated partially, but not completely, with the level of *in vitro* neutralizing antibody to the murine strain of rotavirus measured in a tissue culture system. In most cases, asymptomatic infection after feeding of the virus and the antibody preparation resulted in subsequent generation of an active immune response in infected animals.

Rotavirus gastroenteritis is a major cause of morbidity and mortality in infants and children living throughout the world (9). One possible strategy for the prevention of rotavirus infection in high-risk individuals unable to mount an effective endogenous immune response to active infection or immunization would involve introduction of passive immunity by administration of immunoglobulins into the gastrointestinal tract. This approach has been used with success in experimental animals (11, 12, 13, 15) and, to a more limited extent, in human newborns and children with immunodeficiency diseases (1, 10). However, there are a number of important questions which must be answered before the potential efficacy of orally administered immunoglobulins for the prevention of rotavirus diarrhea can be determined. For example, since rotaviruses exhibit antigenic variation based on the heterogeneity of several viral proteins (2, 4, 7), it will be important to determine the potential efficacy of antibodies raised against one serotype of rotavirus for the prevention of infection caused by heterologous rotavirus strains. In addition, the relative efficacy of immunoglobulins derived from human and animal sources needs to be ascertained (18).

We thus used tissue culture systems, as well as a murine model of rotavirus infection (3), to evaluate the ability of a variety of immunoglobulin preparations to inhibit *in vitro* and *in vivo* replication of rotaviruses.

(This work was presented in part at the Annual Meeting of the Society for Pediatric Research, San Francisco, Calif., May 1-4, 1984.)

MATERIALS AND METHODS

Virus preparations. The SA-11 strain of rotavirus was obtained initially from H. Mahlerbe, Salt Lake City, Utah. This virus is serotypically identified as human serotype 3. The WA strain (human, serotype 1) of rotavirus was obtained from Richard Wyatt, National Institutes of Health, Bethesda, Md. (7). Both viruses were propagated in MA-104 cells in the presence of 1 µg of trypsin per ml as previously

described (14). Fluids harvested from tissue culture cells were clarified by centrifugation at $12,000 \times g$ for 30 min, and virus was purified from the supernatant fluids by sedimentation on a 20%/60% (wt/wt) sucrose cushion for 2 h at $100,000 \times g$. The material at the interface was collected and used for the immunizations described below. The epizootic diarrhea of infant mice (EDIM) strain of rotavirus was originally obtained from Michael Collins, Bethesda, Md. The infectivity of the purified, virus preparation was determined by orally inoculating litters of 5 to 7-day-old mice with serial 10-fold dilutions of the viral stock. Three days after inoculation, the mice were evaluated for diarrhea by examining the distal colon for the presence of liquid intestinal contents. The 100% infectious dose was defined as the highest dilution of virus that produced diarrhea in all of the inoculated animals. In these studies, all mice inoculated with EDIM virus received a single 100% infectious dose. We have found that this dosing regimen results in consistent manifestations of infections in mice up to 7 days old (3). A cultivatable strain of EDIM was obtained from Harry Greenberg, Stanford University School of Medicine, Palo Alto, Calif. (5).

Preparation of antibodies. Hyperimmune guinea pig and murine antisera to SA-11 and EDIM were prepared by the following method. Virus purified as described above was mixed with an equal volume of complete Freund adjuvant and injected subcutaneously. Two weeks later, purified virus was administered in a similar way but with incomplete Freund adjuvant. The antiserum was harvested on day 37 to 41 after initial immunization and stored at -20°C until use. Murine antibodies to SA-11 were also prepared by oral inoculation. A 0.1-ml portion of clarified virus was administered on three successive days to 4 to 5-week-old seronegative CD-1 mice. Serum was harvested from the mice 39 days after inoculation. Murine antisera to EDIM were also prepared by oral inoculation of EDIM into seronegative CD-1 mice in a manner similar to that described above for SA-11 inoculation.

Guinea pig and gnotobiotic calf sera to serotype 1 strains of rotavirus and rotavirus seronegative calf sera were ob-

* Corresponding author.

FIG. 1. Antigen excretion in mice experimentally infected with the EDIM strain of murine rotavirus incubated with murine serum containing anti-EDIM antibodies (○, ●), and incubated with nonimmune mouse serum (□, ■). Samples were obtained 3 and 5 days after infection as indicated on the horizontal axis, and prepared and analyzed as described in the text. The values indicated on the vertical axis are optical density activities as measured by enzyme-linked immunoassay for rotavirus antigen. Closed symbols indicate the occurrence of liquid intestinal contents.

tained from Richard Wyatt, National Institutes of Health. Chicken yolk-derived antibodies to the WA and SA-11 strains of rotavirus were prepared as previously described (8). Human immunoglobulins for intravenous use (gamma-immune) was obtained from Cutter Laboratories, Berkeley, Calif.

Neutralization assays. Neutralization assays for in vitro measurement of antibody to rotaviruses were performed by a modification of previously described techniques (16). The antibody preparation was diluted log 10-fold (with a starting dilution of 1:40) and incubated with approximately 10^5 50% tissue culture infective doses of virus per ml for 2 h at 37°C. The virus-antibody mixture were then inoculated onto monolayers of MA-104 cells grown in 24-well plastic plates. After incubation for 4 h at 37°C, the virus-antibody mixture was decanted and replaced with minimum essential medium containing 1 µg of porcine trypsin per ml, and the cells were incubated for 3 to 5 days at 37°C in a CO₂ incubator. Supernatant fluids were removed from the cells and tested for generation of rotavirus antigen by a previously described enzyme immunoassay (3). A dilution of the antibody preparation was considered to contain rotavirus antibody if it resulted in at least a 50% decrease in generation of rotavirus antigen as determined by enzyme immunoassay for EDIM antigen compared with virus grown in the absence of added antibody.

In vivo neutralization experiments. EDIM virus prepared as described above was incubated in 10-µl portions with an equal volume of undiluted test serum or antibody for 1 h at 37°C. After incubation, the virus-antibody mixture was orally administered to 3 to 7-day-old rotavirus seronegative mice by previously described techniques. Control mouse groups were fed the EDIM virus preparation incubated only with phosphate-buffered saline (pH 7.4). Each litter was used for only a single antibody preparation. Litters were housed separately to prevent cross-infection. In each case, the entire intestinal tract was collected from two to four mice in each litter immediately before inoculation and from the

remainder of the litter at 3 to 5 days postinoculation. Diarrhea was determined by examination of the intestinal tract for the presence of liquid stools and gaseous distention. In addition, the entire intestine was homogenized and evaluated for the presence of EDIM antigen by enzyme immunoassay (3). In some cases, serum was also obtained from animals at 30 days postinfection and assayed for the presence of rotavirus antibody by a previously described enzyme immunoassay blocking system (19).

RESULTS

The effect of feeding the EDIM strain of rotavirus to seronegative 5-day-old mice along with immune and nonimmune serum preparations is depicted in Fig. 1. No animal fed rotavirus concomitantly with immune murine sera displayed symptomatic diarrhea or rotavirus antigen shedding 3 or 5 days after infection. On the other hand, 7 of 10 animals fed virus along with nonimmune sera developed diarrhea, and 9 shed detectable levels of rotavirus antigen ($P < 0.01$). The rates of diarrhea and antigenic shedding in animals fed nonimmune sera did not differ from those observed in animals fed virus alone (Table 1). In addition, feeding of murine sera obtained from mice which had been orally infected or parenterally immunized with the heterotypic SA-11 strain of rotavirus failed to provide significant protection against experimental EDIM infection (Table 1). Failure of anti-SA-11 antisera to prevent infection was confirmed by testing multiple litters of mice (data not shown).

We also investigated the ability of immunoglobulins derived from other animals to neutralize murine rotavirus in the mouse model. Guinea pig sera derived from animals parenterally immunized with EDIM provided complete protection against diarrhea and antigen shedding. No neutralization was observed after incubation with guinea pig sera obtained from animals parenterally immunized with the heterotypic WA (human serotype 1) or SA-11 (antigenetically identical to human serotype 3) strain of virus nor with nonimmune guinea pig sera. However, bovine sera obtained from gnotobiotic animals naturally infected with a human serotype 1 (D strain) of rotavirus did offer protection against EDIM diarrhea and antigen excretion. No protection was provided by rotavirus seronegative bovine sera. Protection from symptomatic EDIM infection was also provided by immunoglobulin derived from yolks of eggs laid by chickens immunized with the WA or SA-11 virus strains. In addition, protection was provided by incubation of virus with a commercially available pool of human immunoglobulin derived from normal adults. The ability of the antibody preparations to inhibit in vivo replication of the EDIM strain of rotavirus in the animal model system correlated partially, but not completely, with the in vitro neutralizing activity directed against EDIM as measured in a tissue culture growth system (Table 1).

We also investigated the ability of animals infected with rotavirus and antibody preparations to develop an active immune response to rotavirus. We thus obtained serum 21 days after infection from 13 animals fed rotavirus along with murine serum (6 mice), human immunoglobulin (4 mice) or bovine serum (3 mice) preparations. None of these animals had developed symptomatic gastroenteritis after infection, and only two had detectable levels of rotavirus antigen in their intestinal fluids. Sera from these animals were tested for the presence of antibody by means of enzyme immunoassay neutralization analysis (19). The analysis revealed that 11 (84.6%) of the 13 animals developed an immune response

TABLE 1. Efficacy of antibody preparations for prevention of experimental murine rotavirus infection

Prepn	Virus strain (route of administration) ^a	Titer of neutralizing antibody to:			No. of animals positive/no. tested	
		EDIM	SA-11	WA	Diarrhea	Rotavirus antigen excretion
Mouse serum	None	<40	<40	<40	7/10	9/10
	EDIM (oral)	>40,000	400	400	0/13 ^b	0/13 ^b
	SA-11 (oral)	40	40,000	4,000	9/9	
	SA-11 (par)	400	>40,000	40,000	10/10	
Guinea pig serum	EDIM (par)	4,000	40	400	0/9 ^b	0/9 ^b
	SA-11 (par)	400	4,000	4,000	10/10	9/10
	WA (par)	400	40	4,000	10/15	11/15
Calf serum	None	<40	<40	<40	6/8	6/8
	D (oral)	40	40	400	0/11 ^b	2/11 ^b
Chicken yolk	WA (par)	4,000	400	4,000	0/8 ^b	0/8 ^b
	SA-11 (par)	4,000	4,000	400	0/11 ^b	0/11 ^b
Human immunoglobulin G	(NI)	400	4,000	400	0/11 ^b	0/11 ^b
None		— ^c	—	—	9/12	12/12

^a par, Parenteral immunization; NI, natural infection.^b Ratio significantly different than that of controls fed virus without antibody ($P < 0.05$ by Fisher's exact test).^c —, Not tested.

to rotavirus despite the lack of development of clinical symptoms.

DISCUSSION

These studies indicate that a variety of immunoglobulin preparations can neutralize intestinal replication of a pathogenic strain of murine rotavirus and prevent development of symptomatic rotavirus infection and viral shedding in infected mice. In the case of antisera derived from mice or guinea pig, such neutralizing activity was noted after incubation of virus with sera obtained from animals infected with the homotypic EDIM strain of rotavirus but not after infection or immunization with heterotypic strains. These findings are consistent with several previous studies which have suggested that neutralizing antibody against rotavirus is highly serotype specific and that heterotypic antibodies do not provide significant degrees of in vitro cross neutralization (4, 7, 15). However, we also found that some animal immunoglobulins derived from infection with heterotypic strains of rotaviruses did appear to provide immune protection against rotavirus infection when incubated with virus before animal inoculation. Thus, antibodies purified from yolks of eggs from chickens immunized with heterologous strains of rotavirus provided efficient protection against EDIM infection. Similarly, serum from a gnotobiotic calf infected with the heterotypic serotype 1 strain of human rotavirus provided protection against infection, as did serum obtained from immunoglobulin derived from healthy donors. In addition, we have previously shown that milk obtained from cows with evidence of natural infection with bovine rotaviruses can provide protection against murine rotavirus infection (18). These findings are consistent with those of Wyatt et al. (17), who demonstrated that in utero infection of calves with one strain of rotavirus provides protection against subsequent infection with heterotypic rotavirus strains. Why infected calves and the immunized chickens generated heterotypic antibody whereas immunized guinea pigs and mice did not generate such antibody cannot be

determined with certainty but might be related to the doses or strains of viruses used, the route of administration, different levels of previous natural exposure to rotaviruses, or inherent differences in the immune responses of the animal species.

We also found that the level of in vitro neutralizing antibody to EDIM was not totally predictive of in vivo protection against EDIM infection. These findings might be related to the antigenic complexity of rotavirus proteins involved in viral neutralization (2) and the fact that the epitopes involved in in vitro neutralization might not be identical to those involved in in vivo protection or to factors related to the stability of different immunoglobulin species within the gastrointestinal tract. The factors determining in vivo and in vitro viral neutralization should be addressed by more specific techniques such as those making use of antibodies to specific viral proteins and peptides likely to be involved in neutralization such as VP3 and VP7 (6). In addition, since the mouse model used antibodies which were incubated with virus before administration to the animals, we did not address the question of the pharmacokinetics of immunoglobulin in the gastrointestinal tract or the ability of immunoglobulin to neutralize virus within the intestinal environment. These parameters should also be the subject of future investigations. The availability of practical means for providing effective antirotavirus antibodies might result in a significant decrease in the morbidity and mortality associated with severe rotavirus infections in human populations unable to mount an effective immune response to natural infection or to rotavirus immunization.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant 5R01-AM-33089 from the National Institutes of Health and by the Thrasher Research Fund.

LITERATURE CITED

1. Barnes G. L., L. W. Doyle, and P. H. Hewsen. 1982. A randomized trial of oral gammaglobulin in low birth weight

- infants infected with rota-virus. *Lancet* **i**:1371-1374.
2. Estes, M. K., D. Y. Graham, and B. L. Petrie. 1985. Antigenic structure of rotaviruses, p. 389-406. *In* M. Van Regenmortel and A. R. Neurath (ed.), *Immunochemistry of viruses—the basis for serodiagnosis and vaccines*. Elsevier Biomedical Press, Amsterdam.
 3. Eydeloth, R. S., S. L. Valdesuso, J. F. Sheridan, L. D. Enders, and R. H. Yolken. 1984. Kinetics of viral replication and local and systemic immune responses in experimental rotavirus infection. *J. Virol.* **50**:947-950.
 4. Greenberg, H. B., J. Valdesuso, K. Van Wyke, K. Midthun, M. Walsh, V. McAuliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino. 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. *J. Virol.* **47**:267-275.
 5. Greenberg, H. B., P. T. Vo, and R. Jones. 1986. Cultivation and characterization of three strains of murine rotavirus. *J. Virol.* **57**:585-590.
 6. Gunn, P. R., F. Sato, K. F. H. Powell, A. R. Bellamy, J. R. Napier, P. K. Harding, W. S. Hancock, L. T. Siegman, and G. W. Both. 1985. Rotavirus neutralizing protein VP7: antigenic determinants investigated by sequence analysis and peptide synthesis. *J. Virol.* **54**:791-797.
 7. Hoshino Y., R. G. Wyatt, H. B. Greenberg, J. Flores, and A. Z. Kapikian. 1984. Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque reduction neutralization. *J. Infect. Dis.* **149**:694-702.
 8. Jensenius, J. C., I. Anderson, and J. Hau. 1981. Eggs: conveniently packaged antibodies. *Methods for purification of yolk IgG*. *J. Immunol. Methods* **46**:63-68.
 9. Kapikian, A. Z., H. W. Kim, and R. G. Wyatt. 1976. Human reo-like agent as a major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. *N. Engl. J. Med.* **294**:965-971.
 10. Losonsky, G. A., J. P. Johnson, J. A. Winkelstein, and R. H. Yolken. 1985. The oral administration of human serum immunoglobulin in immunodeficiency patients with viral gastroenteritis: a pharmacokinetic and functional analysis. *J. Clin. Invest.* **76**:2362-2367.
 11. Offit, P. A., and H. F. Clark. 1985. Protection against rotavirus-induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. *J. Virol.* **54**:58-64.
 12. Saif, L. J., D. R. Redman, K. L. Smith, and K. W. Theil. 1983. Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from immunized or nonimmunized cows. *Infect. Immun.* **41**:1118-1131.
 13. Snodgrass, D. R., C. R. Madeley, P. W. Wells, and K. W. Angus. 1977. Human rotavirus in lambs: infection and passive protection. *Infect. Immun.* **16**:268-270.
 14. Urasawa, T., S. Urasawa, and K. Taniguchi. 1981. Sequential passages of human rotavirus in MA-104 cells. *Microbiol. Immunol.* **25**:1025-1029.
 15. Woode, G. N., N. E. Kelso, T. F. Simpson, S. K. Gaul, L. E. Evans, and L. Babiuk. 1983. Antigenic relationships among some bovine rotaviruses: serum neutralization and cross-protection in gnotobiotic calves. *J. Clin. Microbiol.* **18**:358-364.
 16. Wyatt, R. G., H. D. James, Jr., A. L. Pittman, Y. Hoshino, H. B. Greenberg, A. R. Kalica, J. Flores, and A. Z. Kapikian. 1983. Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes. *J. Clin. Microbiol.* **18**:310-317.
 17. Wyatt, R. G., C. A. Mebus, R. H. Yolken, A. R. Kalica, H. D. James, A. Z. Kapikian, and R. M. Chanock. 1979. Rotavirus immunity in gnotobiotic calves, heterologous resistance in human virus induced by bovine virus. *Science* **203**:548-550.
 18. Yolken, R. H., G. A. Losonsky, S. Vonderfecht, F. J. Leister, and S. B. Wee. 1985. Antibody to human rotavirus in cows milk. *New Engl. J. Med.* **312**:605-610.
 19. Yolken, R. H., R. Viscidi, F. Leister, C. Harris, and S.-B. Wee. 1986. Enzyme immunoassay for the detection of rotavirus antigen and antibody, p. 573-581. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), *Manual of clinical laboratory immunology*, 3rd ed. American Society for Microbiology, Washington, D.C.