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Passive protection against bovine rotavirus-induced diarrhea in murine model by specific immunoglobulins from chicken egg yolk

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ABSTRACT

Chicken egg yolk immunoglobulins (yIg) specific against bovine rotavirus (BRV) serotypes 6 (strain Shimane) and 10 (strain KK-3) were used for oral passive immunization of suckling mice against experimental BRV challenge. The protective capacity of the antibody preparation was tested using different concentrations of yIg against a challenge dose of $10^{7.5}$ $TCID_{50}$ for Shimane and $10^{7.0}$ $TCID_{50}$ for KK-3 strain. There was a significant homotypic ($P<0.05$) and heterotypic ($P<0.01$) protection using 160 anti-Shimane or 160 anti-KK-3 neutralizing antibody titer (NAT) compared to control mice given yIg derived from eggs of mock-immunized (control) hens. The titer of infectious BRV recovered from intestinal tissue or luminal chyme decreased with increasing homotypic yIg NAT. A decrease in degree and duration of BRV antigen localization in the villus epithelial lining was observed in mice treated with homotypic yIg at optimum dose for prevention of diarrhea. The NAT in sera of challenged mice increased with decreasing NAT in the yIg given before challenge suggesting that protection was dose-dependent. The present findings indicate that a passive protection could be achieved by the use of yIg against BRV-induced diarrhea in this murine model.

INTRODUCTION

Protection against severe group A rotavirus-induced diarrhea remains an important concern in a wide range of susceptible host species both mammalian and avian (Fukusho et al., 1981; Murakami et al., 1983; Starkey et al., 1986; Takehara et al., 1991). Rotavirus serotypes 6 and 10 predominate in cattle (Snodgrass et al., 1990).

The potential for effective passive immunization in the control of bovine rotavirus (BRV) infection in calves is being explored by various means. Lactogenic immunity depending on immunized cows or continuous feeding of

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immune-colostrum (Saif et al., 1983; Tsunemitsu et al., 1989) have been reported. However, both approaches require expensive technical costs in maintaining cows. More recently, in neonatal calves, protection against homologous challenge of enterotoxigenic *Escherichia coli* was observed using orally administered crude immunoglobulin powder derived from spray-dried chicken egg yolk (Ikemori et al., 1992). The chicken egg yolk immunoglobulin (yIg) is easily purified and readily produced for large-scale use (Polson et al., 1985; Hamada et al., 1991; Yokoyama et al., 1992; Ikemori et al., 1992). To our knowledge, the potential prophylactic and therapeutic value of yIg against BRV infections has not been investigated. The practical benefits derived from their use may have far-reaching importance and implications in the control of diarrhea by BRV infection in calves.

To evaluate the efficacy of passive immunization requires the use of BRV-seronegative neonatal calves (Saif et al., 1983; Tsunemitsu et al., 1989). However, seronegative calves are difficult to obtain and expensive to maintain in an isolated environment for testing. It would be advantageous to establish a laboratory animal model for testing anti-BRV yIg. The availability of genetically well-defined strains of mice provides opportunities for studying the disease produced by non-murine strains of rotavirus and elucidating the passive immune mechanisms (Offit et al., 1984, 1986; Gouvea et al., 1986).

The purpose of the study reported herein was to establish a standardized murine model for testing anti-BRV yIg preparations against either of two distinct BRV serotypes (strain Shimane or KK-3 representing BRV serotypes 6 and 10). To determine the efficacy of yIg in vivo using passively transferred yIg in mice may provide a useful and convenient model for preliminary screening of several anti-BRV yIg preparations.

MATERIALS AND METHODS

Animals

Pregnant CD-1 SPF mice (Charles River Japan, Inc., Kanagawa, Japan) were maintained in individual cages until parturition. The dams were checked for anti-BRV antibody by neutralization test by a method previously described (Fukusho et al., 1981). Suckling mice remained with their dam for the whole duration of the protection trials after birth.

Virus strains

The strain Shimane (serotype 6) (Sato et al., 1978) of BRV was donated by Dr. Sato of the National Institute of Animal Health, Tsukuba, Japan, and the strain KK-3 (serotype 10) (Murakami et al., 1983) of BRV was donated by Dr. Murakami of the same institution. Both viruses were grown in confluent rhesus monkey kidney (MA-104) cells with trypsin treatment and were used for challenge exposure of mice as well as for production of antibody in

rabbit and chicken eggs. NCDV-Lincoln strain (serotype 6) was used as a reference strain in preliminary serological typing by neutralization test.

Preparation of challenge virus

Inocula of Shimane and KK-3 were partially purified as follows: Each virus grown in MA-104 cells was partially purified by centrifugation in a SW 40 Ti rotor (Beckman Instruments, Inc., Palo Alto, Calif.) through a 40% sucrose gradient at 100 000$\times g$ for 3 h at 4°C. The viral pellet was resuspended in phosphate-buffered saline (PBS) overnight after which it was passed through a 0.22 μm filter, apportioned into aliquots (0.5 ml) and stored at −80°C until use.

Titration for infective viruses and neutralization test

Titration for infective virus and neutralization test were done by the method previously described (Fukusho et al., 1981). The infective titer was expressed as the median tissue culture infective dose ($TCID_{50}$) based on observed cytopathic effect. Neutralizing antibody titer (NAT) was expressed as the reciprocal of the highest yIg dilution that showed inhibition of cytophatic effect.

Preparation of yIg specific against BRV

A half milliliter of Shimane or KK-3 viral suspension containing $10^{9.0}$ $TCID_{50}$ per ml, was mixed with an equal volume of Arlacel (Maine Biological Laboratories, Maine) (5% final concentration for Arlacel) and 1.0 ml of the mixture was injected intramuscularly into each five-month-old White Leghorn hen (strain Hyline W36). Six weeks later, the same hens were injected again with the same volume of the material used for priming. Control-antigen was prepared from the same concentrated supernatant without virus, and was used to inject hens in the same manner as the viral antigen. Two weeks after the second immunization, eggs were harvested and the yolks were mixed with an equal volume of PBS. The water-soluble protein rich fraction in the yolk-PBS solution was extracted as previously described (Faith et al., 1973). Purification of yIg from the supernatant was done by a three-step precipitation procedure using saturated ammonium sulfate (50%, 33%, 33%, respectively). The final precipitate was then resuspended in 50 mM NaCl and dialysed against PBS, inactivated for 30 minutes at 56°C, and stored at −20°C until use. The NAT level of such samples were determined by neutralization test using either homologous or heterologous BRV strain as indicator virus. The class of yIg was identified by the Ouchterlony diffusion test.

Passive immunization with yIg against challenge

Three hours before challenge, 25 μl of each dilution of yIg against Shimane or KK-3 was orally administered as a single dose to seven-day-old mice (12

mice per dilution). Median diarrheogenic dose (DD_{50}) determined by the method of Reed and Muench (1938) for Shimane was $10^{6.95}$ $TCID_{50}$ and that of KK-3 was $10^{6.40}$ $TCID_{50}$. The challenge dose per 25 μl per mouse was 3.55 DD_{50} ($10^{7.5}$ $TCID_{50}$) for Shimane strain, and 3.98 DD_{50} ($10^{7.0}$ $TCID_{50}$) for KK-3 strain. The challenge dose of each virus resulted in diarrhea in more than 90% of seven-day-old mice and the onset of diarrhea was observed clinically from 24 to 96 hours post-challenge.

Recovery of viable infective virus

Individual mice treated or non-treated with anti-Shimane or anti-KK-3 yIg were sacrified at 8, 14, 24, 48, 72 and 96 hours post-challenge. Collection of gut washing from the entire small intestine of individual mouse was done as follows: An incision in duodenum, 0.5 cm from the gastric-duodenal junction was made. The ileum was cut 0.5 cm from the ileal-cecal junction and the whole small intestine was gently pulled from the mesenteric tissue. A plastic tube connected to a one ml syringe was placed in the intestinal lumen at the site of the duodenal incision. The tube was held in place with one hand while one ml of cold PBS was gently infused into the intestines. The gut washings (including intestinal chyme) were collected in small tubes. One ml of air was then injected to remove the remaining fluid. The amount of fluid recovered ranged between 0.95 and 1.05 ml. Samples were kept on ice at all times and were homogenized. The emptied small intestines were also homogenized in one ml of PBS. Both samples were centrifuged for 40 min at $5000 \times g$. The supernatants were removed and were kept frozen at $-80°C$ until titration. Viral infectivity in intestinal tissue or intestinal fluid was expressed as log $TCID_{50}$/g of intestinal tissue, or log $TCID_{50}$/ml of intestinal chyme-PBS solution, respectively.

Detection of rotavirus antigen by immunoperoxidase stain

For the demonstration of BRV antigen in the intestinal tissue, 4 μm sections of upper (U), middle (M), and lower (L) segments of the mouse small intestine were deparaffinized and stained by the Avidin-Biotin-Complex method (ABC) using immunoperoxidase (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, Calif.) with a hyperimmune rabbit anti-Shimane or anti-KK-3 serum or mock-immunized (control) rabbit serum. Peroxidase activity was revealed by 0.1% diaminobenzidine-4HCl in 0.1 M Tris-HCl buffer (pH 7.2) mixed with 0.02% H_2O_2.

Humoral immune response post-challenge

Twelve mice were treated with homotypic yIg or yIg from mock-immunized (control) hens before challenge with Shimane or KK-3 strain. At 3 and 6 weeks post-challenge, 6 mice were sacrificed and pooled sera from 6 mice derived from the same treatment group were assayed for humoral immune

response by neutralization test as circumstantial evidence for the presence of subclinical infection.

RESULTS

Production of yIg from immunized hen egg and class of yIg

The NAT of egg yolks from chickens immunized with Shimane virus or KK-3 virus ranged from 5120 to 10 240 NAT, and the persistence of these titer in the chickens after the second immunization was over a half year past.

TABLE 1

In vitro cross-reactivity between Shimane and KK-3 strains using yIg from immunized hens

BRV strain	(serotype)	yIg NAT/0.1 ml		
		anti-Shimane	anti-KK-3	control
NCDV-Lincoln	(6)	40960	640	<10
Shimane	(6)	81920	1280	<10
KK-3	(10)	1280	81920	<10

TABLE 2

Passive protection of suckling mice from BRV-induced diarrhea with homotypic or heterotypic yIg treatment

Challenge virus (dose)[a]	yIg specificity	yIg NAT[b]/mouse		No. of mice with diarrhea/total no. of mice (%)	
		Shimane	KK-3		
Shimane (7.5)	anti-Shimane	640	<10	0/12**	(0)
		160	<10	1/12**	(8.3)
		40	<10	7/12	(58.3)
	anti-KK-3	160	10240	2/12**	(16.7)
		40	2560	8/12	(66.7)
	control	<10	<10	11/12	(91.7)
KK-3 (7.0)	anti-KK-3	<10	2560	1/12**	(8.3)
		<10	640	2/12**	(16.7)
		<10	160	5/12*	(41.7)
		<10	40	9/12	(75.0)
	anti-Shimane	10240	160	2/12**	(16.7)
		2560	40	8/12	(66.7)
	control	<10	<10	11/12	(91.7)

[a] Log_{10} $TCID_{50}$/mouse.
[b] yIg NAT level against in vitro indicator virus.
** $P<0.01$ (Fisher exact test).
* $P<0.05$ (Fisher exact test).

From 100 eggs, 50 ml of yIg solutions were collected. The class of yIg preparations was IgG. The NAT contained in this precipitate as determined by neutralization test was 81 920/0.1 ml for both yIg preparations against strain Shimane or KK-3 (Table 1). The NAT of the precipitate derived from mock-immunized (control) hens was $<10/0.1$ ml against strain Shimane or KK-3.

In vitro cross-reactivity between BRV serotypes

A cross-reactivity test using immunized hen yIg preparation (Table 1) revealed a very low serological relationship between the Shimane and KK-3 strain.

In vivo protection by homotypic or heterotypic yIg in suckling mice

Table 2 summarizes the percentage protection against diarrhea by yIg after homologous or heterologous challenge. Compared to control mice given yIg derived from mock-immunized hens, the use of yIg with 160 NAT/dose/

TABLE 3

Mean virus titer in intestinal wash fluid and tissue from mice given oral homotypic yIg before challenge with Shimane virus

yIg specificity	yIg NAT[a]/mouse	Time post-challenge (hours)	Viral titer[b]	
			Tissue[c]	Fluid[d]
anti-Shimane	640	8	<1.25	<1.50
		14	<1.25	<1.50
		24	<1.25	<1.50
		48	<1.25	<1.50
		72	<1.25	<1.50
		96	<1.25	<1.50
	160	8	1.76	2.08
		14	2.79	2.16
		24	<1.25	<1.50
		48	<1.25	<1.50
		72	<1.25	<1.50
		96	<1.25	<1.50
control	<10	8	1.64	2.13
		14	3.63	2.16
		24	2.46	2.08
		48	2.03	1.75
		72	1.84	1.88
		96	<1.25	<1.50

[a]yIg NAT level against challenge virus strain.
[b]Geometric mean titer ($n=6$).
[c]Log_{10} $TCID_{50}$/g of intestinal tissue.
[d]Log_{10} $TCID_{50}$/ml of intestinal fluid.

mouse or higher resulted in statistically significant protection of suckling mice ($P<0.05$, Fisher exact test) against rotavirus-induced diarrhea using anti-Shimane or anti-KK-3 yIg upon homologous or heterologous challenge. The 160 NAT was the minimum effective antibody titer when the indicator virus used was the same as the BRV strain used for mouse challenge tests. The 3.55- or 3.98-fold DD_{50} for Shimane and KK-3 strains, respectively, induced diarrhea consistently in more than 90% (11/12) of control mice given yIg derived from mock-immunized hens.

Recovery of infective virus from the gut

The mice treated with homotypic yIg did not yield detectable infective virus when 640 and 2560 NAT/dose/mouse against Shimane and KK-3 respectively were administered (Table 3, 4). This was observed from 8 up to 96 hours post-homologous challenge. At a dose of yIg which gave >80% protection against clinical diarrhea using either strain for homologous challenge, isolation of infective virus from the gut of suckling mice was observed until 14 hours post-challenge, with almost no detection at 24 hours and no detec-

TABLE 4

Mean virus titer in intestinal wash fluid and tissue from mice given oral homotypic yIg before challenge with KK-3 virus

yIg specificity	yIg NAT[a]/mouse	Time post-challenge (hours)	Viral titer[b]	
			Tissue[c]	Fluid[d]
anti-KK-3	2560	8	<1.25	<1.50
		14	<1.25	<1.50
		24	<1.25	<1.50
		48	<1.25	<1.50
		72	<1.25	<1.50
		96	<1.25	<1.50
	640	8	1.39	1.50
		14	2.14	2.04
		24	<1.25	1.75
		48	<1.25	<1.50
		72	<1.25	<1.50
		96	<1.25	<1.50
control	<10	8	1.71	1.92
		14	2.80	2.83
		24	2.58	2.04
		48	2.13	1.67
		72	1.44	1.85
		96	<1.25	<1.50

[a,b,c,d]Same as in Table 3.

tion from 48 to 96 hours. The above results indicate a dose-dependent pattern of inhibition of BRV replication in the mouse gut according to the NAT of yIg given pre-challenge.

Detection of virus antigen

The percentages of positive areas stained with ABC method were tabulated (Table 5). In the mice treated with control yIg the percentage of positive epithelial cells by ABC staining was highest (>50% of total per villus) at 48 hours for both BRV challenge groups with <20% to about 50% stained at all the other observation times. In groups treated with homotypic yIg, ABC staining in the U and M segments detected positive areas at 72 hours only (<20%) (Table 5). Positive areas were observed in the L segment at 24, 48, and 72 hours but not at 96-hours.

TABLE 5

Detection of BRV-antigen by immunoperoxidase staining in intestinal segments from mice orally administered homotypic yIg before challenge

Challenge virus (dose)[a]	yIg specificity (NAT[b]/mouse)	IS[c]	Extent of antigen localization (Hours post-challenge)			
			24	48	72	96
Shimane (7.5)	anti-Shimane (160)	U	–[d]	–	+	–
		M	–	–	+	–
		L	+	+	+	–
	control (<10)	U	+	++	++	+
		M	+	+++	+	+
		L	++	+++	+	–
KK-3 (7.0)	anti-KK-3 (640)	U	–	–	+	–
		M	–	–	+	–
		L	+	+	+	–
	control (<10)	U	+	+++	+	+
		M	+	+++	+	+
		L	+	+++	+	+
not challenged	no treatment	U	–	–	–	–
		M	–	–	–	–
		L	–	–	–	–

[a]$LogTCID_{50}$/mouse.
[b]yIg NAT level against challenge virus strain.
[c]IS = intestinal segment, U = upper, M = middle, and L = lower small intestine.
[d]Non-detection (–) and detection of BRV antigen by immunoperoxidase stain in <20% (+), 20%~50% (++), and >50% (+++) respectively of epithelial cells in each intestinal segment.

TABLE 6

Antibody response of mice orally administered homotypic yIgb

yIg specificity	yIg NAT[a]/mouse	Diarrhea (%)[b]	Pooled serum NAT post-challenge ($n=6$)	
			3 weeks	6 weeks
anti-Shimane	640	0	<10	<10
	160	8.3	80	40
control	<10	91.7	160	160
anti-KK-3	2560	8.3	<10	<10
	640	16.7	160	80
control	<10	91.7	160	160

[a]yIg NAT level against challenge virus strain.
[b]Mice used same as in Table 2.

Humoral immune response post-challenge

Pooled sera from each treatment group of 6 mice at 3 and 6 weeks post-challenge demonstrated neutralizing antibodies against the challenge virus used for each litter (Table 6). The observed NAT in sera were dependent on the yIg dose given to their respective litters. Groups of suckling mice given 160 NAT/mouse against Shimane challenge or 640 NAT/mouse against KK-3 challenge resulted in higher NAT (40 to 160) in their sera at 3 and 6 weeks post-challenge compared to litters given 640 NAT/mouse against Shimane challenge or 2560 NAT/mouse against KK-3 challenge (<10). Control mice given yIg from mock-immunized hens yielded a pooled serum NAT of 160 in both challenge groups. The above data showed a degree of inhibition of subclinical infection in suckling mice correlative to the NAT of yIg administered pre-challenge.

DISCUSSION

The concept of immune protection by passive transfer of specific yIg derived from immunized hens was applied in this study to the murine system as a preliminary step towards assessment of protection conferred by yIg preparations against BRV-induced diarrhea in calves. Our data showed that diarrhea induced by BRV serotypes 6 or 10 in mice could be prevented by orally administered yIg preparations under homologous or heterologous challenge conditions with respect to these two serotypes. The rate of protection afforded by the present yIg preparations was dose-dependent and reached up to 100% at the highest concentration used. Under heterologous challenge con-

dition using the two BRV serotypes, the minimum dose of neutralizing antibody which gave a significant level of protection ($P<0.05$) was the same as in homologous challenge (160 NAT) when the NAT was determined using the heterologous BRV serotypes as indicator virus, that is, the same as challenge virus. Therefore, the dose of neutralizing antibody specific for the BRV challenge virus serotypes in the case of yIg given before homologous or heterologous challenge in mice would determine the protective capacity of a given yIg preparation.

By prior treatment of mice with homotypic yIg, the course of detection of virus antigen post-challenge was modified by specifically reducing the extent of epithelial cell infection and consequently viral replication as can be seen from reduced antigen detection by ABC staining. Antigen was detected in spite of oral dosing with protective yIg concentrations indicating that protection did not necessarily mean prevention of infection but rather reduction in the amount of infective virus. The serological reactions of pooled sera from suckling mice given different concentrations of yIg before challenge also indicated a yIg dose-dependent susceptibility to BRV infection.

From the above results, it seems possible to compare the differences between several anti-BRV yIg preparations in a murine model. In this study, we adopted a challenge dose which could cause diarrhea in over 90% of control mice (11/12), which is about the same level of virulence obtained in previous reports (Offit et al., 1984, 1986). However, although BRV caused diarrhea and a clear immune response was elicited (Table 2, 6), BRV did not replicate well in the mice, even if a high dose of challenge virus ($>10^7$ $TCID_{50}$ per mouse) was inoculated. This result corresponds to previous studies with non-murine strains of rotavirus infections in mice (Offit et al., 1984; Gouvea et al., 1986). Therefore, this mouse model with BRV may not be an exact model for rotavirus disease as it occurs in nature. Exact evaluation of anti-BRV yIg should be done with susceptible neonatal calves by feeding of yIg twice or three times a day. The murine model may be useful for preliminary characterization of several anti-BRV yIg preparations.

Hen egg contains as much as 200 mg of immunoglobulin, which is found almost exclusively in the yolk (Rose et al., 1974). The immunoglobulin is described as being similar to mammalian IgG, although recently, evidence has emerged to suggest that this avian immunoglobulin, called either IgG and IgY, is antigenically similar to mammalian IgA (Hadge et al., 1984). The avian IgG (200 to 220-kDa) (Hamada et al., 1991; Tizard, 1987) is a monomer with molecular weight slightly higher than that of the mammalian IgG (180-kDa) (Tizard, 1987). One immunized chicken produced one egg per day in this study. The antibody titers of concentrated yolk IgG (81 920 NAT) and the initial yolk-water soluble protein fraction (>5120 NAT) remained stable over a half year period; a long duration of a high immune response in the hen against BRV antigen was noted in this study. As infective titers of

harvest supernatant of Shimane virus is usually over $10^{8.5}$ $TCID_{50}$ per ml, mass production of antigen is easily achieved. The maintenance of a large flock of layers is more economical than keeping large numbers of cows for antibody production. This makes egg yolk antibody more feasible for large scale-production (Hamada et al., 1991). A high degree of purity of yolk IgG may be achieved by conventional precipitation with ammonium sulfate although the degree of purity obtained by this method is not an essential requirement for oral dosing, which makes the use of easily prepared crude preparations highly attractive. Another advantage of yolk antibody over colostrum is that a high neutralizing antibody titer remains stable for a long period as compared to that of colostrum which decreases a few days after initial harvest (Tsunemitsu, et al., 1989). Further studies of differences between the duration of the immune response to BRV in cows and hens should be undertaken.

Recently, for large-scale production of yIg, methods of separation of yolk-water-soluble fraction containing rich yIg by enteric coating polymers have been developed and the protective efficacy against enterotoxigenic *E. coli* infection in suckling piglets (Yokoyama et al., 1992) and in neonatal calves (Ikemori et al., 1992) has been recognized. The efficacy of anti-BRV yIg purified by the coating polymers is currently being investigated in susceptible neonatal calves. The murine model may be useful for the study of cross-protection against challenge with several anti-BRV yIg purified by different methods.

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