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Characterization of Serum Antibody Responses to Natural Rotavirus Infections in Children by VP7-Specific Epitope-Blocking Assays

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Knowledge of the immune response to rotavirus is crucial for vaccine development. We compared an epitope-blocking assay (EBA) that uses VP7-specific monoclonal antibodies with neutralization assays (NAs) with polyclonal antisera for detecting serum antibody responses after natural rotavirus infection in children. Twenty-six serum pairs from children living in an orphanage with and without symptoms during two rotavirus outbreaks were evaluated for VP7 type 1-, 2-, 3-, and 4-specific antibody responses. In the first outbreak, which was caused by a VP7 type 3 strain, homotypic antibody responses were detected in 11 of 11 symptomatic children by NA and in 10 of 11 symptomatic children by EBA. Heterotypic antibody responses were detected more frequently (12 of 15 children) by NA than by EBA, and the heterotypic epitope-blocking antibody responses occurred in children older than 14 months of age. Antibody responses in asymptomatic children were more commonly detected by EBA than by NA. EBA results from the sera of children in the second outbreak indicated that it was caused by VP7 type 4, whereas NA results suggested it was caused by VP7 type 3. Our results confirm that EBA is a sensitive and specific method for determining VP7 type-specific immune responses after natural rotavirus infections.

Rotavirus is the most significant cause of diarrhea in young children in both developed and developing nations (1, 2, 10, 11, 17, 18). Infections with rotavirus account for nearly 1 million diarrheal deaths per year, most of which are among children in less developed countries (16). In the United States, rotavirus diarrhea is the most common cause of hospitalization because of gastrointestinal disease in children, and this illness results in as many as 300 deaths per year (15). The annual cost only for a hospital bed for children with rotavirus gastroenteritis in the United States has been estimated to be near $350 million (20). The development of a safe and effective rotavirus vaccine is an important public health priority (16).

Understanding of the immunologic mechanisms involved in protection against rotavirus infection is necessary for effective vaccine development. Clinical observations suggest that the immune response to an initial rotavirus infection reduces the severity of illness from subsequent exposures, even if the first exposure is asymptomatic (6). Protective immunity to rotavirus is thought to be associated with responses to the two outer capsid viral proteins VP7 and VP4 (9, 23, 32). Changes of a VP7 type result in changes of the serotype, as determined by neutralization assays (NAs) (9). Viruses with seven VP7 types have been identified in humans (4, 9, 17), and at least six of these are widespread (5, 12, 21, 24, 30, 31). Although several antigenic types of VP4 have been proposed, the importance of different VP4 types is uncertain (13).

A serologic study of children exposed to rotavirus during outbreaks in an orphanage indicated that high titers (>1:128) of homotypic neutralizing (NT) antibodies were protective against clinical illness (7). Most of these children also had NT antibodies against serotypes other than the serotype that caused the outbreak. These heterotypic antibodies did not appear to be protective against serotype 3 symptomatic infections. A more recent study, which was designed to monitor children with natural rotavirus infections longitudinally, failed to demonstrate that preexisting VP7 type-specific NT serum antibodies were protective (33).

The standard method used to measure serotype-specific antibodies to rotavirus has been NA (7, 33). NAs detect antibody directed against both VP4 and VP7 and against more than one epitope on each protein, and rotavirus neutralization antigens have both type-specific and type-common neutralization epitopes (8, 19, 22, 26). An epitope-blocking enzyme-linked immunosorbent assay (EBA) developed by Shaw et al. (25), using VP7-specific monoclonal antibodies, may prove to be more useful than NA in identifying the VP7 and VP4 epitopes involved in protective immunity. Recent studies by EBA have shown that epitope-blocking (EB) antibody titers correlate well with NT antibody titers among rotavirus vaccine recipients (3, 25, 29). Results of one study of a small number of children suggest that this correlation may also be observed for natural rotavirus infections (29).

We were interested in defining the immune response following natural rotavirus infections. We used paired sera collected from asymptomatic and symptomatic children exposed to rotavirus in outbreaks of diarrhea to validate the EBA for detecting serum humoral immune responses to VP7 of rotavirus. These sera were originally used to demonstrate the protective effect of homotypic NT antibodies (7).

MATERIALS AND METHODS

Study subjects. Two outbreaks of rotavirus gastroenteritis occurred in March and October 1982 among infants and children less than 2 years of age living in the Hokkaido Central Infant Home in Japan. Sera were obtained from the

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children before and after each outbreak. Rotavirus was detected by electron microscopy in 16 of 39 children in the first outbreak and in 9 of 45 children in the second outbreak. One isolate from each outbreak was propagated in MA104 cells and was characterized as a subgroup II, VP7 type 3 virus by electrophoresis and neutralization. RNA electrophoresis of other stool samples from children involved in the outbreaks suggested that each outbreak was caused by a single rotavirus strain.

Serum specimens. The paired pre- and postoutbreak serum specimens from all children involved in the two outbreaks were tested previously for NA titers against human rotavirus VP7 types 1, 2, 3, and 4 by using a fluorescent focus reduction assay (4). The results of NA analysis of serum specimens collected for the study described here were reported previously (7). Serum specimens for testing by EBA were available from a subset of children involved in each outbreak. During the first outbreak, serum specimens were collected within 1 week before and 40 days after the outbreak. During the second outbreak, specimens were collected within 2 weeks before and 70 days after the outbreak. The virus strains used as antigens for NA were KU (type 1), S2 (type 2), MK (type 3, derived from the first outbreak), and Hochi (type 4).

EBA. The EBA was modified from the assay previously described by Shaw et al. (25). Polyvinyl chloride 96-well microtiter plates were coated with 50 μl of an optimal dilution of hyperimmune guinea pig antiserum to one of the human rotavirus VP7 types, 1, 2, 3, or 4 (21), and were incubated overnight at 4°C. After two washing steps with 10 mM phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween (PBST), plates were blocked with 200 μl of PBS containing 1% bovine serum albumin (BSA) and were incubated for 2 h at 37°C. Next, 50 μl of homologous virus WA (VP7 type 1), S2 (type 2), SA11 (type 3), or St. Thomas 3 (type 4) was used as antigen. Each antigen was diluted in PBS-BSA with 10% fetal calf serum (FCS) to give an optical density reading of greater than 0.25 when it was tested in the absence of blocking antibodies. After an overnight incubation at 4°C, the plates were washed five times with PBST and 50 μl of serum diluted 1:10 and 1:40 in PBS-BSA-FCS was added. Control wells received PBS-BSA-FCS only (no blocking), homologous serum from hyperimmunized guinea pigs (complete blocking), or serum from a patient with a known virus titer (internal standard). After a 1-h incubation at 37°C, the plates were washed five times with PBST; this was followed by the addition of 50 μl of an optimal dilution of competing monoclonal antibody to the homologous viral VP7 type diluted in PBS-BSA-FCS. The plates were then incubated for 1 h at 37°C. The VP7 epitope-specific monoclonal antibodies used were KU-4 (VP7 type 1), S2-2G10 (type 2), YO-1E2 (type 3), and ST-2G7 (type 4) (27, 28). After the incubation period, the plates were washed five times and 50 μl of horseradish peroxidase-conjugated, goat anti-mouse immunoglobulin G diluted 1:3,000 in PBS-BSA-FCS plus 2.5% normal guinea pig serum was added, and the solution was left to incubate for 1 h at 37°C. The plates were then washed five times with PBST, and 100 μl of substrate 2,2′-azino-bis(3-ethyl benzthazioline sulfonic acid) (Sigma Chemical, St. Louis, Mo.) plus 0.012% hydrogen peroxide was added to each well. The plates were read after 30 min on a Titertek Multiskan enzyme-linked immunosorbent assay reader (Flow Laboratories, McLean, Va.). The titer was expressed as the reciprocal of the highest dilution of serum which gave an optical density value that was less than or equal to 50% of the value of the nonblocking controls. Samples positive for blocking at a dilution of 1:40 were further diluted twofold to 1:1,280 and were retested.

Statistical methods. Fisher's exact test (two-sided), was used for comparison of groups.

RESULTS

Study subjects. Sera were available from 15 of the 39 children involved in the March outbreak and 11 of the 45 children involved in the October outbreak. The means and ranges of the ages of these children were similar to those described for all children in the outbreak (for the study children from the March outbreak, mean, 11 months; range, 3 to 23 months; for children from the October outbreak, mean, 8 months; range, 1 to 17 months). Sera were available from 11 symptomatic and 4 asymptomatic children and from 6 symptomatic and 5 asymptomatic children from the March and October outbreaks, respectively.

Homotypic antibody responses of children in the March outbreak. The EBA and NA titers to VP7 type 3 (considered to be the infecting serotype in the outbreak) before and after infection are portrayed in Fig. 1 and 2, respectively. Children who did not have symptoms and those in whom virus was detected in stool specimens are indicated in Fig. 1 and 2. VP7 type 3 antibody titer rises (fourfold or greater increase in titer or a titer that went from negative to positive) were detected in 11 and 10 symptomatic children by NA and EBA, respectively (Table 1). The only child who had an NA response without an EBA response was a 3-month-old who had a decrease in EB antibodies. Of the four asymptomatic children, antibody titer rises were found in the oldest child by both EBA and NA, a rise was demonstrated in two children only by EBA, and in one child a detectable rise was not found by either method.

Heterotypic antibody response of children in the March outbreak. The heterotypic VP7-specific antibody response differed significantly when measured by NA and EBA (Table 1). In all 11 symptomatic children in the March outbreak, an antibody rise to at least one of the VP7 types other than type 3 was found when the antibody titers were measured by NA. The heterotypic responses ranged from fourfold lower to fourfold higher than the corresponding homotypic responses. In contrast to these 11 symptomatic children who had a heterotypic response demonstrated by NA, only two children had a heterotypic response demonstrated by EBA ($P < 0.001$). In asymptomatic children, a heterotypic rise was detected in one child by NA and in two children by EBA. The four children (two symptomatic and two asymptomatic) in whom a heterotypic antibody rise was demonstrated by EBA were older than 14 months of age and all had detectable VP7 type 3 antibody titers before the infection. In those children, the homotypic response was 16-fold or greater than the heterotypic response.

Serologic responses of children in the October outbreak. Table 2 summarizes the antibody responses in the 11 children from the October outbreak. Antibody responses by NA and/or EBA were detected only in the six youngest children. The five older children were asymptomatic, and an antibody response was not demonstrated in any of the children by either method, suggesting that they escaped infection. There was a marked discordance among the antibody responses detected by NA and EBA in the October outbreak. VP7 type 3 antibody responses were detected in all six children by NA and in only one child by EBA. In contrast, VP7 type 4 responses were detected in five of the six children by EBA and in only two children by NA. Of the five children with

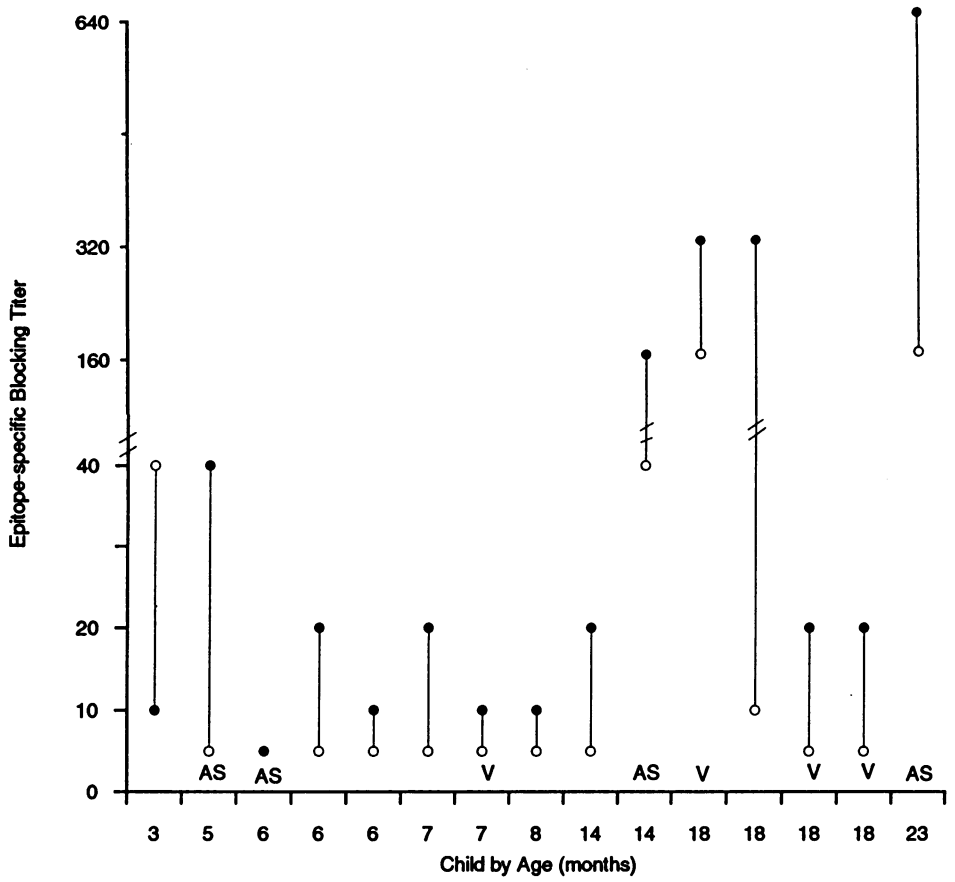

FIG. 1. Serum epitope-specific blocking antibody responses to VP7 type 3 rotavirus among children exposed to rotavirus in the March outbreak. The children are arranged on the x axis from youngest to oldest. The antibody titers before (○) and after (●) the occurrence of the outbreak are portrayed for each child separately. Note that the scale of the y axis below the break in the line is linear and that the scale above the break is logarithmic. Some children were asymptomatic (AS) during the outbreak period. V, children in whose stools rotavirus was detected by electron microscopy.

VP7 type 4 responses by EBA, four had a weak response (from less than 1:10 to 1:10) and one had a strong response (from less than 1:10 to 1:80). The child with the strong EB antibody response to type 4 (a 4-month-old child) had a greater response to type 4 than to VP7 type 3 by NA (1:32 to 1:256 and 1:64 to 1:256, respectively). By comparing the RNA electropherotypes of rotaviruses in outbreak stool specimens with that of one serotyped, cultivated isolate from the outbreak, the outbreak was initially considered to have been caused by a type 3 virus (7). In light of the EB titers, we could not exclude the possibility that the outbreak was caused by mixed types or that the outbreak was caused by a VP7 type 4 virus.

Responses to more than one VP7 type were found in all six children by NA and in only one child (a 6-month-old child without preexisting EB antibody titers) by EBA.

Correlation of preexisting serum antibody titers to rotavirus and protection against clinical illness. The correlation of preexposure NT antibody titers and protection in the population described here was reported previously (7). EB antibody to VP7 type 3 (1:10 or more) prior to the March outbreak was found in 2 of 4 children who were asymptomatic in the outbreak and in 3 of 11 symptomatic children (P = 0.56). None of the children had detectable preoutbreak antibody to the other VP7 types by EBA. In the October outbreak, because the rotavirus VP7 type(s) that caused the outbreak was in doubt after the serologic findings detected by EBA, we analyzed the results by considering VP7 type 3 or type 4 to be the outbreak strain. For this outbreak, two of six symptomatic children and four of five asymptomatic children had preoutbreak antibodies to VP7 type 3. One of six symptomatic children and two of five asymptomatic children had antibodies to VP7 type 4 prior to the second outbreak. Two of the asymptomatic children also had preoutbreak EB antibodies to VP7 type 1 virus. If type 3 strains caused both the March and October outbreaks, preexisting VP7 type 3 antibody demonstrated by EBA correlated with protection as follows: 5 of 17 symptomatic children and 6 of 9 asymptomatic children had preexisting homotypic antibody (P = 0.10). If VP7 type 3 caused the first outbreak and VP7 type 4 caused the second outbreak, then 4 of 17 symptomatic children and 4 of 9 asymptomatic children had preexisting homotypic antibodies (P = 0.38). If the second outbreak was caused by both VP7 type 3 and type 4 rotaviruses, 5 of 17 symptomatic and 7 of 9 asymptomatic children had preexisting homotypic antibodies (P = 0.037).

DISCUSSION

The ability to identify the rotavirus epitopes involved in protective immunity could be invaluable in the development of effective vaccines. Previous studies in vaccine recipients

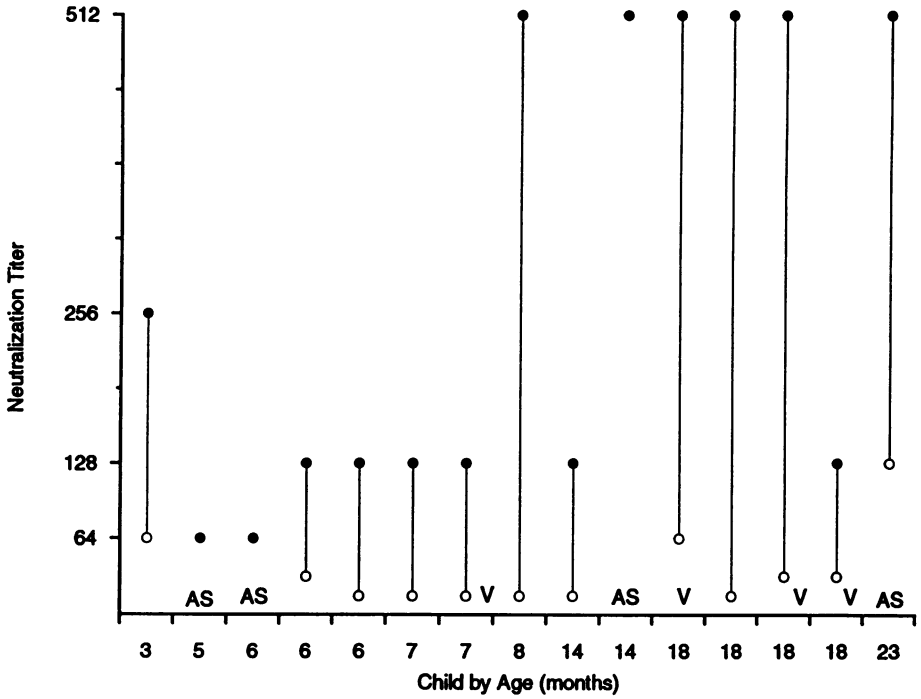

FIG. 2. Serum NT antibody responses to VP7 type 3 rotavirus among children exposed to rotavirus in the March outbreak. The data are for the same children as in Fig. 1 and are arranged on the x axis in the same order as in Fig. 1. The antibody titers before (○) and after (●) the occurrence of the rotavirus outbreak are portrayed. Asymptomatic children (AS) and children with proven rotavirus infections during the outbreak (V) are the same as those whose data are given in Fig. 1.

have shown that EBA can efficiently detect epitope-specific serum antibodies and that the measured response seems to be more VP7 type specific than that measured by NA (14).

In this study, we compared EB antibody and NT antibody responses to natural infection among children involved in sequential rotavirus outbreaks in an orphanage. The results confirm that EBA can be as sensitive as NA in detecting an overall immune response in children after a natural rotavirus infection. In contrast, Shaw et al. (25) reported that EBA detected only a few immune responses after vaccination. This may have reflected a poor vaccine "take" in that study population. In our study, five children had no response by NA or EBA. There were only two children who had a response by NA who did not have a response to any specific VP7 type by EBA. These two children were 3 months and 1 month of age, and one of them had a decrease in EB antibody titers, suggesting that the measured EB antibody titers were maternally acquired. On the other hand, there were two exposed asymptomatic children in the March outbreak who did not have a serum NT antibody response but who did have a response demonstrated by EBA. A rise in VP7 EB antibody titers without a rise in NT antibody

TABLE 1. VP7 type 3 and non-type 3 NT and EB antibody responses among children involved in the March 1982 rotavirus outbreak

Clinical status	No. of children with antibody responses to the following VP7 type by the indicated assay[a]:			
	Type 3		Type 1, 2, and/or 4	
	NA	EBA	NA	EBA
Symptomatic (n = 11)	11	10	11[b]	2[b]
Asymptomatic (n = 4)	1	3	1	2
Total	12	13	12	4[c]

[a] A positive response was a fourfold rise in titer for NA or conversion from a negative to a positive titer or a fourfold rise in titer for EBA.
[b] Differences between NT and EB antibody responses ($P < 0.001$, by Fisher's exact test, two-sided).
[c] The four children were 14, 18, 18, and 23 months old.

TABLE 2. Type-specific antibody responses measured by NA and EBA in children exposed to the October 1982 rotavirus outbreak

Age (mo)	Clinical status[a]	Antibody responses to the following VP7 type by the indicated assay:					
		VP7 type 3		VP7 type 4		VP7 type 1 and/or 2	
		NA	EBA	NA	EBA	NA	EBA
1	Sym	+	−	−	+	+	−
1	Sym	+	−	−	−	+	−
4	Sym	+	−	+	+	+	−
4	Sym	+	−	−	+	+	−
5	Sym	+	−	−	+	+	−
6	Sym	+	+	+	+	+	+
10	Asym	−	−	−	−	−	−
12	Asym	−	−	−	−	−	−
13	Asym	−	−	−	−	−	−
14	Asym	−	−	−	−	−	−
17	Asym	−	−	−	−	−	−

[a] Sym, symptomatic; Asym, asymptomatic. Virus excretion was detected in all symptomatic patients described here.

titers also was detected in a few children in a previous study (14). It is possible that in the presence of preexisting antibodies, EBA may discriminate a VP7 antibody response more efficiently than NA does.

The type specificity of the responses, in contrast, differed significantly between the tests. Heterotypic and homotypic NT antibody responses were of similar magnitude in 75% of the children. All the responses detected by EBA were against a single VP7 type except in five children. Four of these five children were older than 1 year of age, had detectable preexisting antibodies, and had homotypic responses that were at least 16-fold greater than the heterotypic responses. Heterotypic responses in older children with preexisting antibodies have been reported for EBA (14, 29). The mechanism of this heterotypic response in an assay that uses type-specific monoclonal antibodies is uncertain, but it probably represents broadening of the response to heterotypic epitopes on VP7 following multiple infections. Longitudinal studies in which the antigens of the infecting viruses are characterized in detail may be required to explain this phenomenon. Further studies are necessary to determine whether this pattern of response in older children is common and whether these heterotypic antibodies play a role in heterotypic protection. The higher VP7 type specificity of EBA is not surprising and is in accordance with our hypothesis that a test that measures antibodies against specific epitopes would be more specific than one that measures NT antibodies involving many epitopes.

The results observed in the October outbreak were somewhat unexpected. We found a marked disagreement in the type-specific responses detected by NA and EBA. We do not have a clear explanation of why NA detected mainly VP7 type 3 and type 1 responses while EBA detected type 4 responses. Because the stool samples were consumed by the multiple assays that we performed, we can only speculate as to what may be the cause of the differences that we noted. In one of the children, the results of the EBA and NA suggested that the child actually had a VP7 type 4 infection. In the other young children, the EB VP7 type 3 responses were absent and the type 4 responses were weak. It is possible that the NT epitopes on MK (the antigen used for the VP7 type 3 NA) do not cross-react with the SA11 epitope detected by YO-1E2. This possibility alone would not explain why these children had a weak VP7 type 4 EB antibody response but no NT antibody type 4 response. On the other hand, the MK strain may be VP7 type 3 but may contain an epitope detected by ST-2G7. Further studies are under way to resolve this question.

Because of the number of children examined in this study, we cannot make a conclusive statement regarding the correlation of serum EB antibodies and protection against rotavirus infection or disease. There is a suggestion that children with higher titers of epitope-specific antibody competing with YO-1E2 and/or ST2G7 may have been protected, but this was not always the case. Future studies are necessary to establish whether the use of VP7- and VP4-specific monoclonal antibodies will allow the differentiation of epitopes involved in protective immunity. This study confirms that EBA is sensitive and epitope specific, and it will likely prove to be a useful tool in the analysis of humoral immune responses following natural rotavirus infections.

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