Identification of a Group-Reactive Epitope of Group B Rotaviruses Recognized by Monoclonal Antibody and Application to the Development of a Sensitive Immunoassay for Viral Characterization

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Group B rotaviruses (GBRs) are fastidious agents which cause enteric disease in humans and a number of other animal species. Detailed study of the role of GBRs in human disease has been hampered by the lack of immunoreagents suitable for large-scale studies. We developed a monoclonal antibody which recognizes a group-reactive antigen contained in a number of strains of GBRs. When utilized in conjunction with a hyperimmune guinea pig antiserum to GBR, this monoclonal antibody can be used in an enzyme immunoassay system to detect a wide range of GBRs. Alternatively, this monoclonal antibody can be combined with sera obtained from GBR-infected animals to devise assays which are largely specific for the homologous strain of GBR. Reactivity was not noted in either system with strains of group A or group C rotaviruses or with other members of the family *Reoviridae*. These results indicate that GBRs contain both group-reactive and species-specific antigens which are distinct from those found in group A rotaviruses. The availability of well-defined immunoreagents will facilitate detailed studies of GBR infections in humans and animals.

Rotaviruses are members of the viral family *Reoviridae* which contain 11 segments of double-stranded RNA. Epidemiological studies have identified rotaviruses as major etiological agents of gastrointestinal disease in humans and animals (3, 14, 33). Until recently, it was thought that all rotaviruses contain a group-reactive antigen localized to the VP6 peptide in the inner viral capsid (11, 31). The use of immunoreagents directed at this antigenic determinant has allowed the development of immunoassays for the detection of rotaviruses in fecal and environmental samples (35).

However, a number of viral agents have recently been identified which are morphologically identical to rotaviruses and which contain 11 segments of double-stranded RNA but which do not display any immunological cross-reactivity with the standard rotavirus strains. These agents, which have been termed rotaviruslike agents, pararotaviruses, or atypical rotaviruses, have been further subdivided into groups B, C, D, and E, in distinction to the typical rotaviruses, which have been classified as group A (4, 15, 19, 21, 28). These classifications have been based on the electrophoretic mobility of RNA segments, nucleic acid hybridization analyses, and, in some cases, immunological cross-relationships (7). Group B rotaviruses (GBRs) are of particular interest due to the fact that rotaviruses of this type have been shown to infect a wide range of animals, including cows, pigs, lambs, and rats (17, 18, 27, 28). Furthermore, large-scale outbreaks of human disease caused by this agent in children living in China have been documented (6, 13, 30).

The ability to perform large-scale studies of the role of GBRs in enteric diseases has been hampered by the limited availability of immunoreagents suitable for the detection of GBRs. Since they have not been successfully adapted to serial propagation in tissue culture, GBRs must, for the present, be purified from the feces of infected animals. Immunization of animals with such virus preparations may result in the synthesis of polyclonal antibodies which are not

sufficiently specific for use in diagnostic immunoassays (32). We have thus developed a monoclonal antibody to the GBRs and combined this monoclonal antibody with additional anti-GBR antibodies to devise immunoassays for the detection and characterization of GBRs.

MATERIALS AND METHODS

Antigens. Rat intestinal contents containing the infectious diarrhea of infant rat (IDIR) strain of GBR were prepared by the oral infection of suckling rats by previously described methods (28). Specimens containing the bovine enteric syncytial virus (BESV) strain of bovine GBR were prepared by the feeding of germ-free calves with this virus as previously described (16, 27). Standard preparations of IDIR and BESV were prepared for use in the immunoassay analyses indicated below. Viral RNA was visualized following separation by polyacrylamide gel electrophoresis and staining with silver nitrate (12) in a 1:20 dilution of the standard preparations of BESV and IDIR but not in a 1:40 dilution of these preparations. Bovine strains of GBR isolated in the United Kingdom were kindly provided by David Snodgrass, Animal Diseases Research Association, Moredun Research Institute, Edinburgh, Scotland.

Rat intestinal contents containing human strains of GBR were prepared by feeding stools obtained in Baltimore, Md., from humans with diarrheal illness (8) to suckling rats and harvesting the intestinal contents as previously described. Porcine group B and porcine group C rotaviruses were kindly provided by Ken Thiel and Linda Saif, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, Ohio. Standard strains of group A rotaviruses and reoviruses were maintained in MA-104 cells following previously established methods.

Purification of viruses. Fecal suspension (10 ml) containing either BESV or IDIR virus was diluted in 20 ml of buffer (0.05 M Tris, pH 7.4, 0.1 M NaCl, 10 mM CaCl₂) containing 0.5% Triton X-100, and homogenized with a hand-held tissue grinder (Tenbroeck). The suspension was centrifuged at

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5,000 $\times$ g for 5 min at room temperature. The supernatant was placed on a cushion of 40% sucrose in the buffer described above and centrifuged for 2 h at 90,000 $\times$ g in an SW28 rotor at 5°C. The resulting pellet was suspended in 0.5% sodium deoxycholate, placed on a 40% sucrose cushion in water, and centrifuged at 150,000 $\times$ g for 2 h in an SW40.1 rotor at 5°C. The resulting pellet was suspended in 10 mM dithiothreitol in 1% sodium *N*-lauroylsarcosinate and again placed on a cushion of 40% sucrose containing 1% sodium *N*-lauroylsarcosinate and centrifuged for 2 h at 150,000 $\times$ g in an SW40.1 rotor at 5°C. The final pellet was suspended in phosphate-buffered saline (PBS) containing 0.01% CaCl₂ and 0.01% MgCl₂ · 6H₂O (Ca²⁺-Mg²⁺-PBS).

Monoclonal antibodies. The IDIR virus preparation described above was further purified on a potassium tartrate-glycerol gradient by previously described procedures (1). After centrifugation of the gradient for 60 h at 150,000 $\times$ g in an SW40.1 rotor at 5°C, 0.5-ml fractions were collected and analyzed for IDIR virus antigens by enzyme immunoassay and for intact IDIR virus particles by negative-stain electron microscopy. The fraction having a density of 1.26 g/ml was found to have the highest IDIR virus antigen level and was the only fraction to contain large numbers of intact IDIR virus particles. This fraction was used to immunize mice for monoclonal antibody production.

BALB/c mice were subcutaneously immunized with 0.1 ml of a 1/10 dilution (in Ca²⁺-Mg²⁺-PBS) of the gradient fraction described above that had been emulsified in 0.3 ml of Freund complete adjuvant. This injected material contained approximately 35 μ g of protein. One month later, the mice were boosted subcutaneously with a similar preparation, with the exception that Freund incomplete adjuvant was used in place of complete adjuvant. Ten weeks after the initial immunization, each mouse was boosted with an intraperitoneal injection of 0.2 ml of a 1/10 dilution (in Ca²⁺-Mg²⁺-PBS) of the appropriate gradient fraction. Three days later, the mice were exsanguinated and the splenocytes were collected in preparation for fusion with myeloma cells.

Equal numbers of splenocytes and Sp2/0-Ag-14 myeloma cells were fused by using previously described procedures (10). Fusion cultures were grown in medium containing hypoxanthine, aminopterin, and thymidine to select for successful hybrids as previously described (10). The hybridomas were grown in Dulbecco modified Eagle medium containing 100 mg of glucose per liter. Each liter of medium also contained 100 ml of virus-free fetal bovine serum, 2.38 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 0.36 ml of 8 M KOH, 0.05 g of sodium pyruvate, 200 U of bovine insulin, and 0.15 g of oxaloacetic acid.

Supernatants from hybridoma cultures were screened for antibodies to GBRs by using the enzyme immunoassay described below. Since we were interested primarily in cross-reactive GBR antibodies, all supernatants were screened for the presence of antibodies to both rat and bovine GBRs. Hybridomas producing antibodies to both viruses were subcloned twice by limiting dilution (5). A control monoclonal antibody was selected which contained an equivalent amount of immunoglobulin G (IgG) but which did not display reactivity to the standard preparations of GBR in the assay systems described below. The immunoglobulin type and subtype of the cloned antibody was determined by reaction with monospecific anti-murine immunoglobulins (Bio-Rad Laboratories, Richmond, Calif.) in a solid-phase enzyme immunoassay format. For the immunization analyses, the monoclonal antibody was fur-

ther purified by protein A column chromatography (Affigel; BioRad). A control monoclonal antibody was also selected which did not display reactivity to the standard preparations of GBR in the assay systems described below.

Polyclonal antibodies. To prepare guinea pig antiserum to BESV, 0.4 ml of the BESV preparation described above was emulsified in 0.8 ml of Freund complete adjuvant and injected subcutaneously into an adult guinea pig. One month later, the guinea pig was boosted with a similar preparation, with the exception that Freund incomplete adjuvant was used in place of complete adjuvant. Seventeen days after the booster immunization, the guinea pig was exsanguinated and the serum was collected and stored at -20°C.

Rat and bovine antisera to GBRs were obtained from animals experimentally infected with the indicated strains by the oral route as previously described. Guinea pig antiserum to the AEDRV strain of human GBR was kindly provided by Hung Tao, Institute of Virology, Chinese Academy of Medical Sciences, Beijing, People's Republic of China.

Assay systems. The assays for the detection of antigens from GBRs were performed by modifications of enzyme immunoassays which we have previously devised for the detection of group A and group B rotaviruses (29, 35). The optimal dilutions of the individual reagents were determined by checkerboard titrations.

Polyvinyl microdilution plates (Dynatech Laboratories, Alexandria, Va.) were coated with goat anti-mouse IgG (Antibodies Inc., Davis, Calif.) diluted 1:10,000 in PBS. After incubation for at least 14 h at 37°C, the plates were washed five times in PB1 (pH 7.4) containing 0.05% Tween 20 (PBST) and were incubated with the anti-GBR monoclonal antibody optimally diluted in PBST containing 1% gelatin. In the case of the testing of fecal or intestinal samples, duplicate wells were also coated with the control monoclonal antibody selected as described above to serve as control wells. After incubation for 2 h at 37°C and overnight at 4°C, the plates were washed five times with PBST and incubated with nonimmune mouse serum diluted 1:1,000 in PBST containing 1% gelatin. The nonimmune mouse serum was added to saturate binding sites of the solid-phase anti-murine reagent which were not occupied by the monoclonal immunoglobulin. After incubation for 30 min at 37°C, the plates were washed and antigen, diluted 1:2 or greater in PBST containing 20% nonimmune horse serum, was added to the wells coated with the anti-GBR and control monoclonal antibodies. Antigens were diluted in the horse serum to minimize the effects of intestinal proteases on the ability of the immunoreagents to react with fecal antigens as previously described (25). After incubation for 2 h at 37°C or overnight at 4°C, the plates were again washed five times with PBST and guinea pig anti-GBR, prepared as described above, was diluted 1:400 in PBST containing 1% gelatin, 0.2% nonimmune mouse serum, and 1% nonimmune horse serum and added to the wells. After an incubation for 1 h at 37°C, the plates were washed and the reaction was completed by the addition of alkaline phosphatase-labeled goat anti-guinea pig IgG (Miles Laboratories, Elkhart, Ind.) and nitrophenyl phosphate substrate prepared by previously described methods. For some assays (see below), rat anti-IDIR or bovine anti-BESV antiserum was substituted for the guinea pig anti-GBR antiserum. In such cases, the reactions were completed by the addition of peroxidase-labeled affinity-purified goat anti-rat and anti-bovine antisera (K&P Laboratories, Gaithersburg, Md.), respectively, and *o*-phenylenediamine-peroxide substrate prepared by previously described methods. In all cases, the reactions were

quantitated by means of a microplate colorimeter with filters of the appropriate wavelengths. In the case of the fecal samples, a specific activity was calculated for each sample by subtracting the mean measured absorbance value recorded in wells coated with the irrelevant monoclonal antibody from the mean value measured in the wells coated with the anti-GBR monoclonal antibody (34).

The reactivity of the monoclonal antibody to GBR antigens was also investigated by the performance of indirect immunofluorescence assays by the following method. Three-day-old infant rats were infected with the IDIR strain of GBR. After the development of clinically apparent disease, the intestines were harvested, fixed with alcohol, embedded in paraffin, sectioned, and applied to microscope slides by standard techniques (28). The sections were reacted with the monoclonal antibody and fluorescein-labeled anti-mouse IgG, both for 1 h at 37°C. After the sections were washed, the cells were examined by standard fluorescence microscopic techniques.

RESULTS

Initial efforts were directed at the development and characterization of the monoclonal antibodies. Extensive evaluation of hybridomas generated by the immunization of BALB/c mice with IDIR and fusion with myeloma cells yielded a single stable hybridoma clone producing immunoglobulin reacting with both the homologous IDIR virus and the heterologous BESV strain of GBR. This immunoglobulin was of the IgG2a subtype as indicated by reaction with monospecific anti-murine antibodies. The specific reactivity of this antibody to GBR was confirmed by immunofluorescence microscopy which demonstrated the binding of the antibody to intestinal epithelial cells of rats infected with IDIR (Fig. 1). The pattern of fluorescence was identical to that which we have observed with rat antibodies generated following oral infection with GBR (28). Fluorescent staining was not noted following testing of intestinal sections from uninfected rats. While additional clones were found to be producing antibodies with similar specificities, all were of the IgM class and proved to be unstable producers of specific antibody. The IgG2a monoclonal antibody was thus further utilized to develop immunoassays for the analysis of the GBRs.

We found that this monoclonal antibody could be used most efficiently in an anti-IgG capture system in which the monoclonal antibody is bound to the solid phase through binding to a polyclonal antibody directed at murine IgG. This system proved to be superior to assay formats which involved the direct attachment of the monoclonal antibody to the solid-phase surface. We used this reaction format to investigate the reaction characteristics of polyclonal anti-GBR antibodies to GBR antigens bound to the monoclonal anti-GBR antibody. We found that when a hyperimmune anti-GBR antiserum prepared by the parenteral administration of guinea pigs with purified BESV was used as the liquid-phase reagent, the assay could detect low concentrations of both IDIR virus and BESV. As depicted in Fig. 2, this system was capable of detecting antigen in a 1:12,800 dilution of a standard strain of BESV and a 1:3,200 of a standard strain of IDIR. These concentrations are approximately 100-fold less than those which could be detected by the visualization of viral RNA following polyacrylamide gel electrophoresis and staining with silver nitrate (12). We also found that guinea pig antisera raised against the AEDRV strain of human GBR in China displayed reactivity with both

FIG. 1. Microscopic immunofluorescence analysis of intestinal cells infected with GBR. Indirect immunofluorescence was performed on alcohol-fixed, paraffin-embedded intestinal tissue obtained from animals orally infected with the IDIR strain of GBR as described in the text. The intestinal tissue was examined by standard fluorescence microscopy. Areas of viral replication are indicated by the presence of the fluorescent dye in the cytoplasm of the intestinal epithelial cells. Fluorescent staining was not noted following reaction with sections obtained from uninfected animals (not shown). Magnification, $\times 400$.

the IDIR and BESV strains of GBR (data not shown). On the other hand, the use of antibodies generated by the oral infection of rats with the IDIR strain of GBR and cows with the BESV strain of GBR yielded reactivities which were largely specific to the strain of the infecting virus, with the homologous GBR strains being detectable at lower concentrations than the heterologous GBR antigens (Fig. 3). None of the GBR antibodies displayed measurable reactivity in the assay system when tested with the following strains of group A rotavirus: WA (serotype 1), S2 (serotype 2), SA-11 (serotype 3), MMU (serotype 3), EDIM (serotype 3), and VA-70 (serotype 4). Nor did these antibodies display reactivity when tested with reoviruses type 1, 2, or 3 or with the porcine strain of group C rotavirus. We used the assay system with the monoclonal antibody and the guinea pig anti-BESV sera to detect virus in additional samples from infected animals. We found that we could use this system to detect GBR antigens in the intestinal contents of rats for up to 7 days following experimental oral infection with IDIR (Fig. 4). Interestingly, the levels of excreted antigens varied as a function of the day following infection, with levels rising on day 1, falling to low levels on day 3, and rising again 5 days after infection. This biphasic pattern of viral excretion is consistent with our previous studies of GBR excretion in infected rats (26).

We also found that this assay system could detect GBR antigen in the intestinal contents of rats experimentally infected with a human strain of GBR isolated in Baltimore, Md. (7). Similarly, the assay was capable of detecting GBR

FIG. 2. Reactivity of group A and group B rotaviruses in the monoclonal antibody enzyme immunoassay system. Plates were coated with anti-mouse IgG and monoclonal anti-GBR monoclonal antibody and reacted with the indicated antigens as described in the text. The reaction was completed by reaction with guinea pig hyperimmune antibodies to GBR and alkaline phosphatase-labeled anti-guinea pig IgG. The antigens tested were as follows: BESV (■), IDIR (+), SA-11 (simian [serotype 3] strain of group A rotaviruses [*]), and NCDV (Nebraska calf diarrhea virus [serotype 6] strain of group A rotavirus [□]). Neg Ctr, Negative control (mean $\pm$ 3 $\times$ the standard deviation of fecal and intestinal contents from uninfected calves and rats).

antigens in the stools of five cows infected with BESV and in the stools of two cows infected with a bovine strain of GBR isolated in the United Kingdom (22) (Fig. 5). We also could detect virus in the stool of a gnotobiotic pig infected with a porcine strain of GBR (24) (data not shown). There was no detectable reactivity noted following the testing of specimens obtained from uninfected rats, pigs, cows, or humans or in specimens obtained from humans or animals infected with group A rotaviruses.

DISCUSSION

We identified a monoclonal antibody which reacted with a wide range of strains of GBRs from different animal species but which did not display any reactivity for group A or group C rotaviruses. We also found that hyperimmune sera gener-

FIG. 3. Reactivity of group B rotaviruses in the monoclonal antibody enzyme immunoassay system utilizing postinfection sera. The assay was performed as described for Fig. 2 except that the indicated antisera obtained from rats and cows infected with the homologous strains of GBR were used in place of the hyperimmune guinea pig antiserum. Reactivity was not noted with either antisera to the SA-11 and NCDV strains of group A rotavirus (data not shown). Symbols: ■, rat antibody reactivity to IDIR; +, bovine antibody reactivity to IDIR; *, rat antibody reactivity to BESV; □, bovine antibody reactivity to BESV.

FIG. 4. Excretion of GBR antigens in rats infected with IDIR. Rats were infected (■) with the IDIR strain of GBR. Intestinal contents were harvested at the indicated time intervals and evaluated in the enzyme immunoassay system as described in the text with the hyperimmune anti-BESV antiserum. Control rats (+) were sham infected with intestinal contents from uninfected rats and were processed in the same manner as the IDIR-infected rats. Each point represents the mean specific activity of two to four animals.

ated by the parenteral administration to animals of purified virus also reacted with a wide range of GBRs but did not react with other viral species. These findings support the classification of the GBRs as a distinct rotaviral group (7, 17). On the other hand, antisera generated in rats orally infected with the IDIR strain of GBR and cows infected with

FIG. 5. Enzyme immunoassay for the detection of GBRs in fecal samples. Specimens were obtained from the feces and intestinal contents of infected animals and reacted in the solid-phase immunoassay with the monoclonal anti-GBR and the guinea pig anti-BESV antibodies as described in the text. The closed circles in column A represent individual samples from rats infected with a strain of GBR originally identified in a human infant (7). Specific activities in individual samples from cows experimentally infected with GBR in the United States (■) (27) and from cows naturally infected with a different strain of GBR in the United Kingdom (▲) (22) are shown in column C. Values in columns B and D represent the mean and standard deviations of optical density values measured in samples from uninfected rats and cows, respectively.

the BESV strain displayed reactivity directed largely at the homologous GBR strain. These data indicate that there are also some antigenic epitopes which are largely specific to individual strains of GBRs. The identification and localization of group-reactive and type-specific epitopes and the elucidation of their relationship to the in vitro and in vivo neutralization of viral infectivity should be the subject of additional investigations.

We were able to use a monoclonal and polyclonal antibody directed at the group-reactive GBR antigen to develop an efficient enzyme immunoassay for the detection of a range of GBR strains. It is of note that we could detect antigens at concentrations which were substantially lower than those which could be detected by means of polyacrylamide gel electrophoresis, a technique which has been widely used for the detection of rotaviruses in fecal samples. It is thus possible that epidemiological studies based on polyacrylamide gel electrophoretic methods might underestimate the incidence of infections due to GBRs (20, 23). We did not have access to a sufficient number of human samples from China to evaluate the efficiency of the immunoassay system for the detection of the AEDRV strain of GBR, which has been implicated as a cause of epidemic and endemic gastroenteritis in that population. However, the fact that guinea pig antisera directed at AEDRV reacted to both the IDIR and BESV strains of GBR in our system suggests that the AEDRV agent is antigenically related to these strains, a finding which is consistent with other analyses of this virus (17). Enzyme immunoassay systems directed at group-reactive GBR antigens should thus be utilized to examine large numbers of clinical samples from this and other populations. The availability of practical assay systems suitable for the efficient detection of GBR in fecal samples should facilitate the study of the role of these viruses as etiological agents of disease in human and animal populations and should provide indications of the need for specific measures for the prevention and control of GBR infection.

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