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Characterization of Monoclonal Antibodies to Human Group B Rotavirus and Their Use in an Antigen Detection Enzyme-Linked Immunosorbent Assay

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Three monoclonal antibodies (MAbs)—B5C9, B5E4, and B10G10—to human group B rotavirus, an agent implicated in epidemic outbreaks of diarrhea in the People's Republic of China, primarily in adults, were prepared. MAb reactivity was decreased when virus preparations were treated with EDTA, suggesting reactivity with the outer-capsid protein(s). Competition experiments suggested that these MAbs recognize overlapping epitopes within a single antigenic site. A simple antigen detection enzyme-linked immunosorbent assay (ELISA) specific for the human group B rotavirus was established by using these MAbs as capture antibodies. Fifteen clinical samples obtained from three epidemic areas in the People's Republic of China and previously shown by Chinese scientists to contain group B virus were all positive in the MAb capture antigen detection ELISA, whereas none of the 57 samples lacking the group B virus reacted in the test. The results suggest that this MAb capture antigen detection ELISA will be useful to identify outbreaks caused by the human group B rotavirus and to monitor possible spread of the virus.

Rotaviruses have long been recognized as a major cause of diarrhea in animal and human neonates. The characteristic morphology of double-shelled particles and a genome of 11 segments of double-stranded RNA identify the rotaviruses as members of the family *Reoviridae*. The rotavirus genus is divided into six different groups or serogroups, A to F, and there is evidence of a possible seventh group (1, 24, 25). Groups are identified by serologic assays, with viruses in each group containing cross-reactive or common antigens. The group A rotaviruses are the best characterized and include most of the viruses studied since 1973. The non-group A rotaviruses were first recognized in 1979. The number of reports of non-group A rotaviruses is increasing (25). These viruses have been isolated from humans (2, 10), pigs (27, 34), chickens (18), sheep (5), rats (35), and cows (30).

In the early to mid-1980s, a series of epidemics of adult diarrhea disease was reported in many widely separated parts of the People's Republic of China (15, 31, 36). This disease is unique among rotavirus-induced diarrheas in that it regularly causes severe disease in adults. The disease is characterized by explosive onset and rapid spread. Deaths, usually of older patients suffering from severe dehydration, have been reported. Normally, the disease is self limiting and lasts 3 to 7 days. In two of the reported epidemics, the disease appears to have been spread via contaminated drinking water (15, 36), but in a third report there was no evidence for waterborne infection (31). This virus, or one that is presently indistinguishable from it, has also been shown to cause disease among neonates in the People's Republic of China (8). Further analyses indicated that adult diarrhea rotaviruses were group B rotaviruses (6, 20).

Seroepidemiologic surveys indicate that few people outside the People's Republic of China possess antibodies to human group B rotavirus (3, 14, 21, 26). As a result, human group B virus(es) may pose a major threat to world health. There are no surveillance programs to monitor the possible spread of the human group B virus. Because of the epidemic nature and severity of this disease and the large number of susceptible individuals, establishment of a surveillance program with an assay capable of rapid detection would be valuable to public health officials. Although an enzyme-linked immunosorbent assay (ELISA) for detection of human group B virus has been established (20, 21), the lack of sufficient quantities of characterized reagents for use in this assay system is a major problem in initiating such a monitoring program.

In this report, we describe the generation and characterization of monoclonal antibodies (MAbs) reactive to the human group B rotavirus from China. Using our MAbs, we established a screening ELISA that is specific for the human group B virus in clinical specimens.

MATERIALS AND METHODS

Viruses. Clinical samples (16) of human group B rotavirus were obtained from epidemics that occurred in Jinzhou (15), Guangxi (36; Chengqin Su [Heifei, People's Republic of China], personal communication), and Anhui (31), People's Republic of China. Other clinical samples (n = 57; negative controls) lacking human group B virus were obtained from Texas Children's Hospital, Houston, as described previously (11). All clinical samples were tested in the group B ELISA as previously described, with 10 to 20% Genetron extracts (20). These extracts were prepared by mixing crude stool suspensions with Tris-buffered saline and then with an equal volume of Genetron (trifluorotrichloroethane). After vortexing and centrifugation for 5 min in an Eppendorf microcentrifuge, the virus-containing aqueous phase (extract) was carefully removed. The organic-aqueous interface was re-extracted twice with small volumes of Tris-buffered saline, and the resulting aqueous phase was pooled with the previous extract.

The J-1 human group B isolate (previously called adult diarrhea rotavirus) was purified by using previously de-

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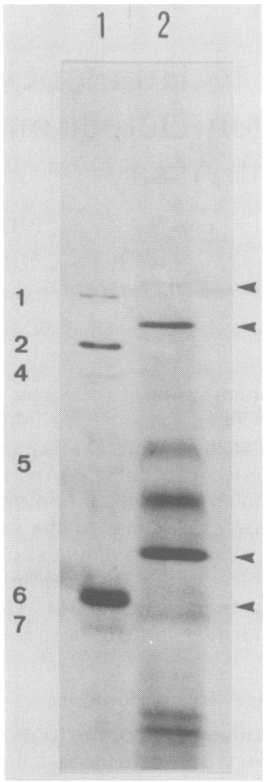

FIG. 1. Polypeptide patterns of CsCl-purified human group B rotavirus proteins. Following electrophoresis on a 10% polyacrylamide gel, gradient-purified double-shelled SA11 (lane 1) or group B (lane 2) rotavirus particles were stained with silver. The SA11 particles were generated in the presence of trypsin. The numbers at the left indicate the viral polypeptides of SA11 as designated by Liu et al. (17). Arrows highlight the major bands of the group B virus that probably correspond to VP1, VP2, VP6, and VP7.

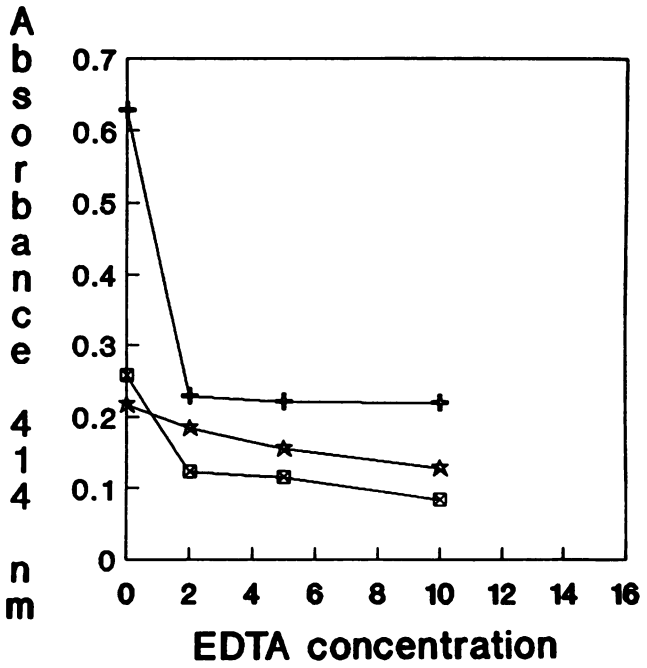

FIG. 2. Effect of EDTA treatment on MAb binding. Genetron-extracted material was incubated in 2, 5, or 10 mM EDTA for 15 min at room temperature as described in Materials and Methods. Before EDTA treatment, this sample (S2) contained >90% double-shelled particles. The EDTA-treated material was diluted and tested in the antigen detection ELISA using the capture MAb procedure. The capture MAbs were B5C9 (☆) B5E4 (☒), and B10G10 (✚).

scribed procedures and used to immunize mice (20). After Genetron extraction, virus was pelleted through a sucrose cushion and banded in a self-forming CsCl gradient (starting refractive index, 1.369). The banded material was collected, extensively dialyzed against Tris-buffered saline, and found to contain primarily double-shelled particles when stained with uranyl acetate and examined by electron microscopy (22).

Generation of hybridomas. Hybridomas secreting MAbs reactive to the human group B rotavirus were generated as previously described (4). Female BALB/c mice were hyperimmunized with the J-1 virus purified on CsCl gradients as previously described. Splenocytes were harvested and fused with P3X63 Ag8.653 murine myeloma cells with polyethylene glycol 4000. The fusion mixture was plated in 96-well tissue culture plates in Dulbecco minimal essential medium containing 2% Ewing sarcoma growth factor (Costar Europe Ltd., Badhoevedorp, The Netherlands), 20% fetal bovine serum, and hypoxanthine-aminopterin-thymidine (Sigma Chemical Co., St. Louis, Mo.). Following 2 weeks of growth, hypoxanthine-aminopterin-thymidine was gradually removed from the medium. The viable hybrids were screened with an ELISA (20). Hybridoma culture supernatants were added to coated and blocked microtiter plates containing CsCl gradient-purified virus. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G and M serum (Tago, Inc., Burlingame, Calif.) was used as the detector serum. Positive cultures were expanded and subcloned twice by limiting dilution, and ascites fluids were produced in pristane-primed BALB/c mice. MAbs were purified from ascites fluid by high-pressure liquid chromatography with the Bakerbond ABx column as described previously (4, 23). The isotype of each individual MAb was determined with the MonoAb-ID enzyme immunoassay isotyping kit (Zymed Laboratories, South San Francisco, Calif.).

MAb capture antigen detection ELISA (MCADE). An ELISA used for detecting virus in Genetron extracts was performed by using a MAb capture technique. Polyvinyl chloride microtiter plates were coated with either guinea pig hyperimmune serum or purified immunoglobulin (diluted in carbonate-bicarbonate buffer, pH 9.6) for 1 h at 37°C and then overnight at room temperature. As a control, preimmune guinea pig serum or an anti-simian virus 40 T antigen immunoglobulin G2a, MAb, PAb101 (12), was included. Coated plates were blocked with bovine lacto transfer technique optimizer for 2 h at 37°C (16). Genetron-extracted stool material, diluted 1:5 in 1% nonfat dry milk (bovine lacto transfer technique optimizer diluted 1:5) and 10% fetal bovine serum, was added to each well and incubated for at least 2 h at 37°C. Plates were washed three times with 0.01 M phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20 (PBS-T). Rabbit hyperimmune serum to human group B rotavirus was added, and the plates were incubated for at least 1 h at 37°C. After three additional washes in PBS-T, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (HyClone Laboratories, Logan, Utah) was added, and the plates were incubated for 1 h at 37°C. After a final three washes in PBS-T, 2,2′-azino-bis(3-ethylbenzthiazoline-

sulfonic acid (Sigma Chemical Co.) and H_2O_2 substrate were added. After 30 min of incubation, the plates were read at 414 nm with a Titertek Multiskan plate reader (Flow Laboratories, Inc., McLean, Va.).

To determine whether the MAbs reacted with the outer or inner capsid, we pretreated virus preparations with EDTA. Genetron-extracted material was incubated at room temperature for 15 min in various concentrations of EDTA before being diluted in 1% nonfat dry milk and 10% fetal bovine serum. The treated preparation was then tested for reactivity in the MCADE.

Epitope mapping. Epitope mapping was performed with a competitive MAb capture ELISA (CMCE) as described previously (4, 13). Polyvinyl chloride microtiter plates were coated with 60 μl (2 μg/ml) of purified immunoglobulin in carbonate-bicarbonate buffer, pH 9.6, and blocked with bovine lacto transfer technique optimizer. Simultaneously, limiting concentrations of antigen obtained from a single source were incubated overnight at room temperature in glass tubes containing a homologous or heterologous competitor MAb. The antigen-antibody complexes were added to coated and blocked microtiter plates and incubated for at least 3 h at 37°C. The rest of the assay was performed as described above, with rabbit hyperimmune serum to human group B rotavirus (20) as the detector antibody.

RESULTS

Characterization of MAbs to human group B rotavirus. Three MAbs reactive to human group B rotavirus were generated with CsCl gradient-purified virus particles. Analysis of the protein profile of the group B virus showed major polypeptide bands similar to those of the group A virus, SA11 (Fig. 1). The apparent molecular weights of the group B polypeptides appeared to be higher than those of their probable group A counterparts.

Characterization of the MAbs included determination of their reactivities with double- and single-shelled particles and epitope mapping with CMCE. The three MAbs (B5C9, B5E4, and B10G10) appeared to recognize outer-capsid proteins, since their reactivities were decreased after EDTA treatment of the virus (Fig. 2). Immune electron microscopy verified this result by showing that the antibody bound to double-shelled but not single-shelled particles (data not shown).

To determine whether the MAbs recognized the same epitopes, competitive binding experiments were performed with a CMCE. The B5E4 MAb was able to compete with MAb B5C9 in a one-way manner (Fig. 3a and b), and B5C9 did not compete with MAb B10G10 for antigen binding (Fig. 3b and c). However, MAbs B5E4 and B10G10 reciprocally competed (Fig. 3b and c). This suggests that MAb B5E4 binds between the B5C9 and B10G10 epitopes, competing with both of these MAbs.

MCADE. We previously developed a group B ELISA that used hyperimmune serum as the capture reagent (20). The group B MAbs were tested by using the previous format and were able to detect virus captured by the polyclonal antiserum (data not shown). The individual MAbs and a mixture of B5C9 and B10G10 were also evaluated as capture antibodies in the MCADE. This format was chosen because greater ELISA specificity and sensitivity had been reported when group A rotaviruses were detected by MAbs (7, 28, 33). To increase the probability of detecting virus in positive samples, the mixture of B5C9 and B10G10 was evaluated because of the distinguishable reactivities of these two MAbs

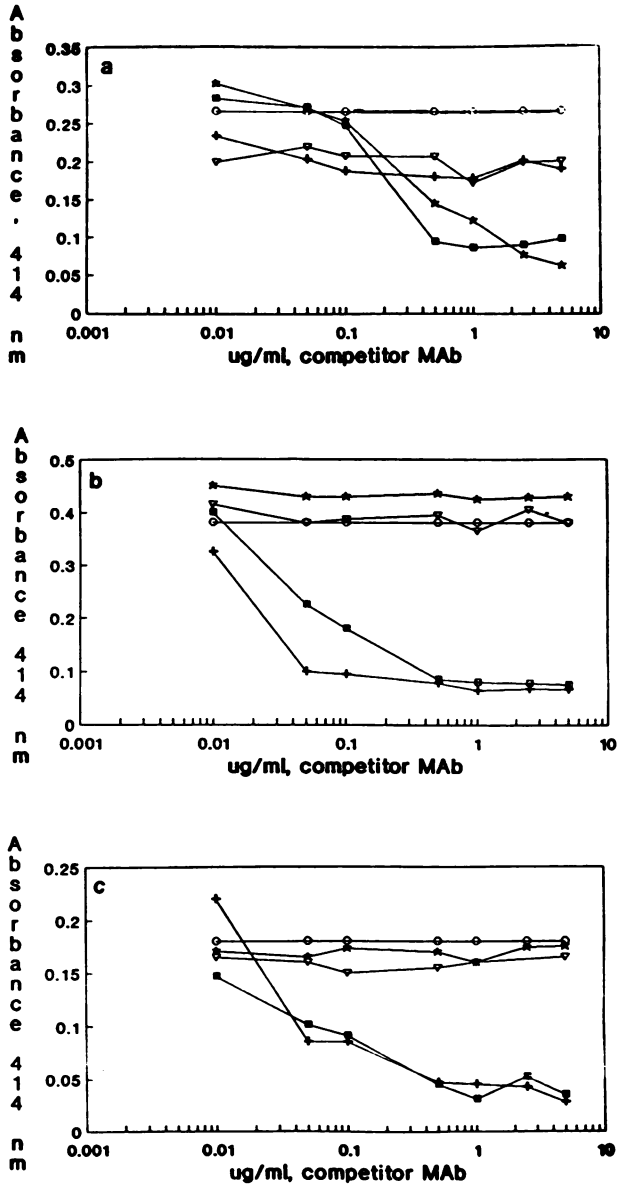

FIG. 3. Typical competitive binding curves generated from CMCE data. Increasing concentrations of a purified competitor MAb were used to determine the extent of competition with a purified capture MAb bound to the wells of a microtiter plate (see Materials and Methods). The capture MAb was B5C9 in top panel a, B5E4 in panel b, and B10G10 in panel c. The competitor MAbs were B5C9 (☆), B5E4 (☒), B10G10 (✚), PAB101 (▽), and no MAb (○). No MAb indicates the average of results obtained with phosphate-buffered saline instead of a competitor MAb. PAb101 is a MAb reactive to simian virus 40 T antigen.

in the competition experiments. The results obtained with the MAbs as capture antibodies were compared with those obtained with pre- and hyperimmune guinea pig sera (Table 1). The mixture of group B MAbs (B5C9 and B10G10) was very effective in detecting virus in clinical specimens obtained from patients during outbreaks of group B virus infection. In most cases, the mixture of MAbs used as the capture reagent reacted as well as the guinea pig hyperimmune serum. To verify the specificity of the assay system, 57 negative stool samples obtained from Texas Children's Hos-

TABLE 1. Human group B rotavirus MAb capture ELISA

Group B sample	A_{414} (positive/negative ratio) with the indicated capture antibody[a]				
	Hyperimmune guinea pig serum	B5C9	B5E4	B10G10	B5C9 + B10G10
J1	0.587 (31.7)	0.041 (2.2)	0.180 (9.7)	0.486 (26.3)	0.555 (30.0)
F1	0.132 (3.3)	0.126 (3.15)	0.049 (1.2)	0.104 (2.6)	0.135 (3.4)
G1	0.354 (30.8)	0.307 (26.7)	0.061 (5.3)	0.223 (19.4)	0.321 (27.9)
S2	0.224 (3.3)	0.199 (2.95)	0.087 (1.3)	0.211 (3.1)	0.268 (4.0)
S3	0.375 (11.2)	0.202 (6.0)	0.073 (2.18)	0.149 (4.45)	0.232 (6.9)
S4	0.215 (2.6)	0.170 (2.0)	0.094 (1.1)	0.129 (1.5)	0.206 (2.5)
S9	0.257 (3.3)	0.228 (2.9)	0.095 (1.2)	0.199 (2.6)	0.227 (2.9)
S14	0.153 (14.0)	0.045 (4.0)	0.166 (15.0)	0.123 (11.2)	0.175 (15.9)
S15	0.170 (7.7)	0.183 (8.3)	0.052 (2.4)	0.092 (4.2)	0.112 (5.1)

[a] The negative value was the A_{414} value obtained with preimmune guinea pig serum as the capture antibody. All samples were tested simultaneously and were received at the same time, except for sample J1, which was used throughout this and previous studies as the positive control (20).

pital were tested. None of the 57 negative control specimens, including 37 known to contain group A rotavirus (by ELISA or electron microscopy or both; data not shown) and 1 containing bacteriophage (by electron microscopy), were incorrectly identified. A summary of all of the test results is shown in Table 2.

DISCUSSION

The severity of disease produced by the human group B rotavirus(es) isolated from the People's Republic of China and the apparently large numbers of susceptible individuals worldwide indicate the need for methods for rapid detection. Routine testing of clinical samples from areas with outbreaks of diarrhea of unknown etiology would be one method to monitor the possible spread of the virus. Such testing requires hyperimmune sera, usually prepared in two different animal species. Because of the limited availability of the human group B virus, which has not been successfully adapted to growth in tissue culture, generation of high-titer antisera has been difficult. The development and use of MAbs to detect virus, as reported here, offers an ability to rapidly and routinely detect human group B rotaviruses. Modification of the MCADE by use of a biotinylated or enzyme-labeled MAb as the second antibody could alleviate the need for hyperimmune serum altogether and further simplify the assay.

The use of MAbs for virus identification, although becoming increasingly popular, should be evaluated carefully. Because of antigenic variation and genetic reassortment that can occur among rotaviruses, use of an individual MAb to screen for the presence of a virus could lead to problems. Although the degree of antigenic variation that occurs with human group B rotaviruses remains unclear, analysis of RNA electropherotypes suggests that some variation among human group B rotavirus samples isolated at different times and locations occurs (9, 14, 20, 36). The use of the more sensitive oligonucleotide mapping technique has demonstrated a high degree of conservation in some genome segments, with considerable variation in others (9). The variable reactivities seen with different human group B rotavirus isolates when the individual MAbs were used in the MCADE also suggest that antigenic variation occurs. Because of this, a mixture of two noncompeting MAbs, B5C9 and B10G10, was used in the MCADE. In this format, all 15 clinical samples obtained during disease outbreaks were positively identified, whereas none of the negative samples reacted. Some of the positive clinical samples had low ELISA reactivities with both the MAbs and hyperimmune sera. This may reflect low virus concentration, particle degradation during long periods of storage, protease activity in the sample, or a combination of these factors. Further testing with additional samples (currently not available) is required to determine whether all human group B viruses can be detected with this assay.

The decrease in binding following EDTA treatment strongly suggests that the epitopes recognized by the MAbs are located on the outer capsid of the virus particle. Furthermore, the competition data suggest that the MAbs recognize three different epitopes within a single antigenic site or domain. Possibly the epitope recognized by MAb B5E4 is situated between and overlaps the epitopes recognized by MAbs B5C9 and B10G10. Similar findings have been reported with MAbs to the outer-capsid glycoprotein of rhesus rotavirus and human group A rotavirus (19, 29, 32). Identification of the protein specificities of the MAbs is difficult because these viruses are noncultivatable; attempts using immunoblots with heat-denatured and reduced proteins have been unsuccessful (unpublished data; Z. Fang [Centers for Disease Control, Atlanta, Ga.], personal communication). The immunoblot results suggest that group B MAbs recog-

TABLE 2. Reactivities of Genetron-extracted clinical samples in the human group B MCADE

No. of samples	Geographic origin	No. of samples positive with indicated capture antibody[a]				
		Hyperimmune guinea pig serum	B5C9 (IgG1)	B5E4 (IgG1)	B10G10 (IgG2a)	B5C9 + B10G10
15	PRC[b]	15	10	9	14	15
57	Houston, Tex.	0	0	0	0	0

[a] Positive reactivity was determined to be an A_{414} of at least 0.100 and a ratio of >2.0 compared with preimmune guinea pig serum or MAb PAb101 (anti-simian virus 40 T antigen).

[b] These samples were obtained from T. Hung, C. Su, and S. Wang, Beijing, Heifei, and Nanning, respectively, People's Republic of China (PRC).

nize conformation-dependent epitopes. By continued analogy with the group A rotaviruses, conformation-dependent epitopes found on the outer-capsid proteins are often associated with neutralizing activity. Should the group B MAbs prove to be neutralizing, they may have a possible application in passive therapy.

Because of the increasing number of animal group B rotaviruses being isolated and the speculation that human group B rotaviruses may be of animal origin, it will be of interest to determine the reactivities of the MAbs described here to other group B viruses. Preliminary results suggest that MAb B5E4 may cross-react with both porcine and bovine group B rotavirus, whereas MAbs B5C9 and B10G10 appear to be specific for human group B rotaviruses (data not shown). If testing of additional isolates from animals confirms this cross-reactivity pattern, these MAbs could be useful in monitoring the postulated transmission of group B rotaviruses between animals and humans.

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