Monoclonal Antibody Assay for Detection of Double-Stranded RNA and Application for Detection of Group A and Non-Group A Rotaviruses

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Fastidious viruses are generally detected in human body fluids by means of immunoassay or nucleic acid hybridization systems. These approaches can be difficult to apply to the detection of viruses which display variations in antigenic or genetic composition. Rotaviruses are examples of viruses which can display such variations. Recently identified antigenic variants, designated as non-group A rotaviruses, cannot be detected by immunoassays or nucleic acid hybridization assays which utilize reagents directed at group A rotavirus strains. The incomplete understanding of the extent of antigenic and genetic variation has inhibited the development of assay systems for all of the non-group A rotaviruses and has limited the study of their role in human disease. While rotaviruses display genetic variation, they all contain a genome which consists of double-stranded RNA. We utilized a monoclonal antibody to devise a sensitive assay for the measurement of double-stranded RNA and applied it to the detection of a wide range of rotaviruses. We found that the assay could detect double-stranded RNA from as few as 10 PFU of standard strains of group A rotaviruses. The assay system was also capable of detecting double-stranded RNA from several strains of group B rotaviruses isolated from calves, rats, and pigs at levels below those at which viral RNA could be visualized by means of polyacrylamide gel electrophoresis. When applied to the detection of double-stranded RNA in serial stools shed by rotavirus-infected children, the assay system was capable of detecting double-stranded RNA in samples in which antigen could not be detected by immunoassay. The specific nature of the double-stranded RNA detected by this assay system could be determined by the elution of the nucleic acids from the monoclonal antibody and the reaction of the RNA with specific nucleotide probes. The measurement of double-stranded RNA offers a potential method for the sensitive detection of a wide range of rotaviruses and other members of the family *Reoviridae*.

Rotaviruses are members of the viral family *Reoviridae* which contain a genome consisting of 11 segments of double-stranded RNA (dsRNA). Rotaviruses have been identified as major etiological agents of gastroenteritis in humans and other animals (17). Until recently, it was thought that all rotaviruses share group-reactive antigens localized to the VP6 polypeptide of the inner capsid (14). The development of sensitive immunoassays for the detection of these group-reactive antigens has allowed for the efficient diagnosis of patients with rotavirus infection and for the study of the epidemiology of disease transmission (40). However, there are a number of limitations to currently available assay systems for the detection of rotavirus infections. For example, the limited sensitivity of immunoassay systems makes it difficult to detect viral antigens early in the course of infection when the antigenic levels in the stool are below the detection limits of the immunoassay (generally 0.1 to 1 ng/ml) (38). Furthermore, most immunoassay systems cannot detect antigen which is localized within immune complexes formed by the interaction of viruses with endogenously produced antiviral antibodies. This factor precludes the efficient detection of viral antigens late in the course of infection when antibodies are being generated by intestinal B cells in response to infection (18, 20). The efficiency of immunoassay systems for viral diagnosis is further limited by the antigenic nature of the infecting virus. Thus, immunoassays for the detection of rotaviruses cannot detect viral

strains which do not contain the rotavirus group-reactive antigen. In fact, rotavirus strains have recently been identified which are morphologically identical to group A rotaviruses and which contain a genome consisting of 11 segments of dsRNA, but which do not display antigenic cross-reactivity with standard strains of rotaviruses (24). These agents have been termed non-group A rotaviruses (NGAR) to distinguish them from the group A agents which have been defined as the rotaviruses which contain the group-reacting antigen. NGAR have been identified as agents of diarrheal disease in a number of animals including calves (28, 32), pigs (2, 4-6, 8, 27, 28, 33), sheep (7, 28), fowls (19, 32), and rats (35). While such agents have also been found in the stools of humans living in China (9, 15, 37), the United States (12), Europe (10, 23, 26, 28), Chile (16), Brazil (25), Mexico City (13), and Australia (26), the role of these agents in human disease has not been well defined. The agents are generally fastidious, and few of the viral strains have been adapted to serial passage in tissue culture systems. Furthermore, their immunological cross-relationships have not been completely defined, and immunoassays for their detection are not widely available. While these agents can be detected by electron microscopy (22, 29, 30), nucleic acid hybridization (11), or polyacrylamide gel electrophoresis, such techniques are not generally applicable to the management of patients with acute disease or to large-scale epidemiological studies.

All members of the *Reoviridae* contain genomes which consist of dsRNA. An assay system directed at the detection of dsRNA would thus allow for the detection of all members

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of the *Reoviridae* family. We report here that a monoclonal antibody generated to DNA-RNA hybrids has a high affinity for dsRNA. We have utilized this monoclonal antibody to devise a sensitive immunoassay for the measurement of dsRNA and have applied this assay to the detection of group A and B rotaviruses in the stools of infected humans and other animals.

MATERIALS AND METHODS

Reagents. The infectious diarrhea of infant rat (IDIR) strain of group B rotavirus was prepared by the experimental infection of infant rats, using previously described methods (34). The bovine enteric syncytial virus (BESV) strain of group B rotavirus was prepared by the experimental infection of gnotobiotic calves as described previously. (Specimens were originally provided by Alfonso Torres, New York State College of Veterinary Medicine, Cornell University, Ithaca.) Direct extraction and polyacrylamide gel electrophoresis of viral RNA from serial dilutions of this fecal specimen indicated that the concentration of viral RNA in the BESV specimen was approximately 10 to 15 $\mu\text{g/ml}$ of feces. Fecal specimens from pigs infected with porcine group B rotavirus (Ohio isolate, porcine rotavirus-like virus) were obtained from Ken Thiel, Ohio Research and Development Center, Ohio State University, Wooster. The SA-11, Wa, and NCDV strains of group A rotavirus were propagated in MA-104 cells by previously described methods (33). The level of antigen in the SA-11 specimen was quantitated by means of an enzyme-linked fluorescence assay, using previously described methods (39). Reovirus type 1 was obtained from the American Type Culture Collection, Rockville, Md., and also propagated in MA-104 cells.

Purified immunoglobulin G subclass 2a of a hybridoma culture (monoclonal S9.6) obtained from a mouse immunized with a DNA-RNA heteropolymer duplex and the β -D-galactosidase-labeled Fab' fragments of the antibody were the generous gift of Robert Carrico, Ames Division, Miles Laboratories, Inc., Elkhart, Ind. The production of the monoclonal antibody and the preparation of the labeled Fab' fragments have been described previously (3, 37). *Escherichia coli* rRNA, salmon sperm DNA, and poly(C) were obtained from Sigma Chemical Co., St. Louis, Mo. Poly(dC), poly(I) · poly(C), poly(A) · poly(U), poly(dA) · poly(dT), poly(dG) · poly(dC), poly(rA) · poly(dT), and poly(I) · poly(dC) were obtained from Pharmacia Fine Chemicals, Piscataway, N.J.

Specimens. Serial specimens were obtained from rats infected with IDIR, using previously described techniques (34). Four- to 6-day-old rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were inoculated orally with 10 μl each of either IDIR virus-containing intestinal extracts or uninfected intestinal extract. Five animals were sacrificed on days 1, 2, 4, 7, and 10 postinfection, and nucleic acids were extracted from their intestinal contents and tested for the level of dsRNA as described below. Specimens were also obtained from control rats fed intestinal extracts prepared from uninfected rats. The specimens from the IDIR-infected and control rats were stored at -70°C until testing.

Serial fecal specimens were obtained from children with rotavirus gastroenteritis as defined by standard enzyme immunoassay procedures. These children were inpatients at the Johns Hopkins Hospital and were participants in a prospective study of the etiology of diarrheal disease. This study was approved by the Joint Committee on Clinical Investigation, Johns Hopkins Medical Institutions. The hu-

man stools were stored at -70°C following collection from the patients and were subsequently processed for the presence of dsRNA by the methods described below.

Nucleic acid extraction. Nucleic acids were extracted from the tissue culture fluids, fecal specimens, and gut washings by a modification of previously developed methods for nucleic acid preparation. Porcine and bovine fecal specimens and rat intestinal washing specimens were diluted 1:10 in phosphate-buffered saline (PBS; pH 7.4). All specimens were digested at 37°C for 30 min with 50 μg of proteinase K per ml in the presence of 0.5% sodium dodecyl sulfate. The samples were extracted in equal volumes of phenol and chloroform-isoamyl alcohol (24:1). Three volumes of 95% ethanol were added to the final aqueous layer. Nucleic acids were precipitated at -70°C for 1 h and then recovered by centrifugation at $10,000 \times g$ for 10 min. The pellet was dried by evaporation at room temperature and suspended to the initial volume with PBS containing 0.05% Tween 20 (PBST) and 0.5% gelatin prior to analysis in the assay system described below.

Enzyme immunoassay system for dsRNA and DNA-RNA hybrids. Enzyme immunoassay for the immunodetection of viral nucleic acids was performed by methods modified from those we have utilized for the detection of viral antigens in fecal samples (40). All volumes were 100 μl unless otherwise noted. The optimal dilution of all reagents was determined by checkerboard titration. Briefly, black polystyrene 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with the S9.6 monoclonal antibody diluted to 1.3 $\mu\text{g/ml}$ in 0.06 M carbonate buffer (pH 9.6). Following incubation overnight at 4°C , the plates were washed five times with PBST (pH 7.4) by means of an automated microplate washer (Skatron Microwash). Samples prepared as described above were added to duplicate wells and incubated overnight at room temperature. The plates were then washed five times with PBST as above, and a 100- μl aliquot of β -galactoside-labeled S9.6 antibody diluted to 0.065 $\mu\text{g/ml}$ in PBST with 0.5% gelatin was added to the wells. The plate was incubated for 2 h at 37°C and then washed again with PBST. The wells were filled with 100 μl of substrate solution, and the plate was incubated for 40 min at room temperature. The substrate was prepared as follows. A 10-mg portion of 4-methylumbelliferyl β -D-galactoside was dissolved in 2 ml of *N,N*-dimethylformamide and added to 98 ml of distilled water. Just prior to use, 1 part substrate stock was diluted with 2 parts 10 mM phosphate buffer (pH 7.4) containing 100 mM NaCl, 1 mM MgCl_2 , and 50 μg of bovine serum albumin per ml. The amount of fluorescent umbelliferone generated by reaction of the nonfluorescent galactoside substrate with enzyme-linked monoclonal antibody bound to the solid phase was quantitated in a microplate fluorometer (Dynatech Microfluor). A dilution of a specimen was considered to be positive for the presence of immunoreactive nucleic acid if it yielded a fluorescence activity which was 2 standard deviations greater than that generated by known negative control samples.

For some experiments, virus was incubated with antiviral antibody for 16 h at 37°C prior to processing as described above and was analyzed in the enzyme immunoassay for dsRNA. The virus preparation before and after incubation with viral antibody was also tested directly in an enzyme-linked fluorescence immunoassay for rotavirus antigen. The antigen assay was performed as described previously except that β -galactosidase-avidin was used as the conjugate and the tests were quantitated by reaction with a fluorescent substrate (40). These experiments were performed to deter-

mine the effect of immune complex formation on the sensitivity of the assay systems.

Nucleic acid hybridization assay. For selected experiments, the dsRNA bound to the solid phase by the above protocol was eluted and probed for specific sequences by means of nucleic acid hybridization procedures. For these experiments, 100- μ l aliquots of extracted nucleic acids (prepared as described above) were added to each of six wells of a microtiter plate coated with S9.6 monoclonal antibody. After an overnight incubation at room temperature, the plates were washed five times with PBST. This was followed by a second extraction procedure in which each well was filled with 100 μ l of PBS containing 50 μ g of proteinase K per ml and 0.5% sodium dodecyl sulfate. The plates were incubated for 1 h at 37°C, and the contents from the six wells for each sample were pooled in a microcentrifuge tube (Eppendorf). Nucleic acids were then reextracted with phenol and chloroform-isoamyl alcohol as described above and precipitated by the addition of 3 equal volumes of 95% ethanol. The samples were then centrifuged at $10,000 \times g$ for 10 min, and the dried pellet was suspended in 4 μ l of TE (10 mM Tris hydrochloride [pH 7.4], 0.1 mM EDTA) and 12 μ l of $10\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)—6.15 M formaldehyde. The suspended pellets were heated to 65°C for 15 min and rapidly cooled on ice. Aliquots (4 μ l) were spotted onto nitrocellulose filters in duplicate. Hybridization was performed to 32 P-labeled cDNA probes at 42°C with 50% formamide. Probes were prepared from either the SA-11 strain of group A rotavirus or the IDIR strain of the NGAR to a specific activity of $>10^7$ cpm/ μ g by reaction with reverse transcriptase and random oligonucleotide primers by the use of previously described methods (11). Papers were exposed to Kodak X-O-mat AR X-ray film, using a Cronex Lightning-Plus intensifying screen (Du Pont Co., Wilmington, Del.) at -70°C for 5 days.

RESULTS

Initial studies were directed at determining the sensitivity of the assay system for the detection of synthetic polynucleotides and natural nucleic acids. The assay system was capable of detecting polynucleotides consisting of dsRNA or DNA-RNA heteroduplexes at concentrations as low as 10^{-8} g/liter (10 pg/ml) (Fig. 1). For most of these polynucleotides, a dose-response relationship between concentration and fluorescent signal was observed at concentrations ranging from 10^{-5} to 10^{-8} g/liter. Polynucleotides consisting of double-stranded DNA, single-stranded deoxynucleotides, or single-stranded ribonucleotide did not generate detectable signals at concentrations of $<10^{-3}$ g/liter (10^6 pg/ml).

We then utilized the assay system to quantify dsRNA extracted from the SA-11 strain of rotavirus. The assay system was capable of detecting dsRNA in a 2^{-17} ($10^{-5.1}$) dilution of virus (Fig. 2). Since this virus preparation had a titer of 10^6 PFU/0.1 ml (the volume used for the anti-dsRNA assay), the assay could pick up approximately 10 PFU per well. The same preparation could be detected at a dilution of 2^{-13} in a solid-phase enzyme immunoassay system for the detection of rotavirus antigen. The assay for the detection of dsRNA was thus approximately 16-fold more sensitive than the antigen detection assay for the detection of this virus. The assay for the detection of dsRNA could detect equivalently low concentrations of other group A rotaviruses such as the Wa and NCDV strains, as well as reovirus type 1 (Fig. 3). The assay system was capable of the detection of viral dsRNA even when the virus was complexed with antiviral

FIG. 1. Detection of polynucleotides and natural nucleic acids in an enzyme immunoassay for dsRNA and DNA-RNA hybrids. Symbols: ▲, poly(A) · poly(dT); △, poly(I) · poly(dC); ●, poly(I) · poly(C); ○, poly(A) · poly(U); ■, poly(dC); □, poly(C); ◆, poly(dC) · poly(dG); ◇, *E. coli* rRNA; *, salmon sperm DNA.

antibody prior to the extraction and measurement of the dsRNA. Viral antigens could not be detected in viruses subjected to these conditions (Fig. 4). We then investigated the sensitivity of the assay system for the detection of dsRNA extracted from NGAR. These agents also have a segmented dsRNA genome but do not share any detectable antigenic relationship with the group A rotaviruses. The assay was capable of detecting nucleic acids in samples derived from rats infected with the IDIR strain of NGAR, calves infected with the BESV strain of NGAR, and a pig infected with the Ohio State University porcine strain of NGAR (Fig. 5). Reactivity was not noted with stools or intestinal contents obtained from uninfected rats and calves.

The specific nature of the dsRNA species bound to the solid-phase monoclonal antibody was examined by eluting the bound nucleic acid from the antibody-coated wells of the microtiter plate and hybridizing the nucleic acids with 32 P-labeled cDNA probes complementary to the group A and B rotaviruses. The eluted nucleic acids displayed specific hybridization with homologous cDNA probes to either the SA-11 strain of group A rotavirus or the IDIR strain of NGAR (Fig. 6). There was no cross-hybridization of the probes to the nucleic acids from the uninfected animals or the uninfected tissue culture specimens. We also did not observe hybridization in this experiment between the IDIR probe and the heterologous group B rotavirus strain from the calf. This was most likely the result of insufficient RNA from the bovine specimen, since we previously observed that hybridization with heterologous rotaviruses requires 10- to 100-fold more nucleic acid than homologous reactions (11).

The assay was also utilized for the detection of intestinal shedding in rats experimentally infected with the IDIR strain of NGAR. Rats were found to shed dsRNA for 7 days following infection (Fig. 7). The levels were highest 1 day following infection and fell to undetectable levels by day 10 postinoculation. The duration of time of detectable dsRNA is similar to that observed in IDIR-infected rats by means of antigen detection techniques (34). Significant levels of dsRNA were not found in the intestinal contents of sham-infected animals.

FIG. 2. Detection of SA-11 strain of group A rotavirus in enzyme immunoassay for viral antigens (○) and in immunoassay for dsRNA (●).

We then performed an investigation to determine the potential utility of the dsRNA method for the detection of rotaviruses in human stools. For these experiments, we quantitated the levels of dsRNA in serial stools obtained from five hospitalized infants with rotavirus gastroenteritis.

We were able to detect dsRNA in the stools of all infants. dsRNA was not detected in the stools of children without diarrheal disease or other evidence of rotavirus gastroenteritis. The shedding of dsRNA generally correlated temporally with the detection of rotavirus antigen by means of a standard enzyme immunoassay system (Fig. 8). However, in some cases the dsRNA was detected in samples obtained before (patient E) or after (patients D and E) virus was detectable by the enzyme immunoassay. In one case, a patient who had only a single stool positive for rotavirus antigen by enzyme immunoassay was found to be excreting dsRNA in his stool over a 5-day period.

FIG. 3. Detection of dsRNA viruses in enzyme immunoassay for dsRNA. Symbols: ▲, SA-11; ○, NCDV; and △, Wa strains of group A rotavirus; ●, reovirus type 1; ■, MA-104 culture control.

FIG. 4. Comparison of enzyme immunoassays for viral antigens for genomic dsRNA in the detection of rotavirus in complexes. Serial dilutions of the SA-11 strain of rotavirus were incubated overnight at 37°C with chicken anti-rotavirus antibody (▲, ●) or with normal egg immunoglobulins (△, ○). The samples were then tested in the enzyme immunoassay for viral antigens (▲, △) or dsRNA (●, ○). The results are expressed as percentage of maximal activity.

FIG. 5. Enzyme immunoassay for dsRNA to detect non-group A rotavirus genomes from fecal or intestinal washings from an infected calf, pig, and rat and control calf and rat.

DISCUSSION

These studies indicate that an immunoassay for the detection of dsRNA can be utilized for the direct detection of viral nucleic acids in tissue culture or body fluid samples. This assay system differs from traditional nucleic acid hybridization assay systems in that it detects dsRNA regardless of the base-pair composition (Fig. 1). Thus, it would not be expected that this assay would be specific for a particular viral species. In fact, the assay system proved to be capable of detecting low concentrations of a number of antigenically diverse viruses with dsRNA genomes. This assay system proved to be substantially more sensitive than available enzyme immunoassay systems for the detection of the viruses. Furthermore, since the epitope recognized by the monoclonal antibody is resistant to proteolytic digestion, the assay was capable of detecting the presence of viruses bound in immune complexes under conditions in which the virus

FIG. 7. Shedding of dsRNA virus from IDIR-infected and control rats. Intestinal washings were obtained from animals on days 1, 2, 7, and 10 postinoculation. Each point represents assay results for a pool of 5 to 12 animals.

could not be detected by means of assay systems based on antigen detection.

Since this assay system can be expected to display a broad range of reactivity, care must be taken in the interpretation of assay results. For example, it is likely that the detection of dsRNA in the stool or intestinal contents of a human or animal will be complemented by the performance of additional assays directed at determining the specific nature of the infecting pathogen. For example, group A rotaviruses, which would be expected to constitute a common source of dsRNA in fecal samples, can be specifically identified by means of standard immunoassay procedures. Similarly, group B rotaviruses might be identified by specific immunoassays for the detection of that rotaviral group (21, 34). Cultivable dsRNA viruses which might be present in stools, such as reoviruses, can be identified by cultivation techniques. Samples which have dsRNA but which are negative in these assays can be further analyzed by means of more specific nucleic acid analytical methods such as nucleic acid hybridization. We have found that hybridization procedures can be facilitated by the elution of dsRNA from the microtiter plates prior to reaction with labeled probe (Fig. 6). Since the solid-phase monoclonal antibody does not bind to double-stranded DNA, the use of this technique might minimize nonspecific reactions which can occur due to interactions between labeled probes and DNA derived from fecal bacteria (1). Additional analyses might also be performed by means of electron microscopy or polyacrylamide gel electrophoretic analysis of viral RNA.

The sensitivity and specificity of this assay system for the detection of enteric viruses with dsRNA genomes in human body fluids collected under a range of clinical conditions should be the subject of future investigations. Since the assay for the measurement of dsRNA is sensitive, broadly reactive, and simple to perform, it is possible that this assay can be utilized as an initial screening test for the detection of RNA viruses in the stools of humans and other animals. Specimens which have detectable dsRNA can be further analyzed by means of more specific but more time-consuming and laborious assay systems. The use of the dsRNA assay might allow for the early institution of appropriate therapy and infection control measures pending the results of the other assays. The efficient application of these assays would result in the earlier containment of viral transmission within hospitals and other closed environments. It is also possible that the widespread usage of this assay system will allow for performance of detailed epidemiological studies of

FIG. 6. Autoradiographs following dot hybridization with ^{32}P -labeled DNA probe complementary to the SA-11 strain of group A rotavirus (group A probe) (A) or the IDIR strain of group B rotavirus (group B probe) (B). As discussed in Materials and Methods, duplicate nucleic acid extracts were hybridized from the following specimens: A1, feces of calf infected with bovine group B rotavirus; B1, feces from uninfected calf; A2, intestinal washings from rat infected with IDIR; B2, intestinal washings from uninfected rat; A3, tissue culture fluid from SA-11-infected MA-104 cells; B3, tissue culture fluid from uninfected MA-104 cells.

FIG. 8. Detection of dsRNA in human stools. Fecal samples were collected from hospitalized children with rotavirus gastroenteritis and analyzed for the presence of group A rotavirus antigen and dsRNA by the methods presented in the text. The "+" symbol above the point indicates that the stool specimen was positive for group A rotavirus antigen, while the "-" symbol indicates that the sample was negative. Bar represents the mean + 3 standard deviations of dsRNA determinations without evidence of rotavirus infection.

NGAR and other members of the *Reoviridae* which cannot be identified by currently available enzyme immunoassay systems. Such studies might lead to the identification of additional pathogenic *Reoviridae* members and might result in an improved understanding of the epidemiology of human gastroenteritis caused by these agents.

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