Detection of Group B Rotavirus in Fecal Specimens by Dot Hybridization with a Cloned cDNA Probe

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Cloned cDNA copies were synthesized from the genomic RNA of the IDIR strain of group B rotavirus (GBR) isolated in Baltimore, Md. These clones were screened for hybridization with heterologous GBR to identify cDNA for use in dot hybridization experiments. In multiple screening experiments, cDNA clones derived from gene segment 3 provided the most intense hybridization signals. ³²P-labeled probes were produced from one of the gene 3 clones, and these were employed in dot hybridization assays. Purified preparations of bovine GBR were detected in concentrations of ≥ 0.5 ng, and GBR was detected in fecal specimens obtained from five of six infected calves. Four of six human fecal specimens containing the Baltimore strain of GBR were also positive in the hybridization assay, while GBR was identified in only one of the six specimens by means of immunoelectron microscopy. A fecal specimen obtained from a patient infected with the adult diarrhea rotavirus strain of GBR was also positive in the dot hybridization assay. Fecal specimens from uninfected humans, calves, and rats, as well as specimens containing group A rotaviruses, did not hybridize with the cloned cDNA probe.

Group B rotaviruses (GBRs) have recently been recognized as etiologic agents of gastroenteritis in humans and several animal species, including pigs, calves, rats, and lambs (2, 14, 17-19). One strain of GBR, adult diarrhea rotavirus (ADRV), is associated with epidemics of gastroenteritis among the human population of China (9). In addition, a GBR (IDIR strain), initially isolated from rat pups, has been found in fecal specimens from infants and adults in Baltimore, Md. (5). These GBRs are antigenically and genetically distinct from the group A rotaviruses which have previously been associated with wintertime epidemics of gastroenteritis among infants (4, 12). Consequently, the GBRs are not detectable by immunoassays which are directed at the common, group A-specific antigen (4, 12). While immunoassays for the detection of GBRs have been developed (20), the assays have not yet become widely available as a result of the short supply of specific antisera. GBRs have also been detected by dot hybridization with ³²P-labeled probes prepared directly from RNA templates (4). However, these templates were purified from preparations of noncultivable GBR, making standardization difficult. We therefore synthesized cDNA clones of the Baltimore strain of GBR and identified clones with cross-reactivity with heterologous GBR. One of these clones was then employed to develop a dot hybridization assay for the detection of GBR in fecal specimens from animals and humans.

MATERIALS AND METHODS

Viruses. The IDIR strain of GBR from Baltimore, Md., was propagated in infant CD-1 rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) as previously de-

scribed (19). Calf fecal specimens containing GBRs were kindly provided by Alfonso Torres (New York State College of Veterinary Medicine, Cornell University, Ithaca). These viruses had originally been isolated from calf fecal specimens during epizootics of diarrhea in three different geographic locations in Nebraska and Colorado (11). The specimens supplied to us were obtained after passage of the original virus isolates in gnotobiotic calves (18). Human fecal specimens from Baltimore which contained GBR were identified by passage in infant rats as previously described (5). A human fecal specimen containing the ADRV strain of GBR was obtained from an ill patient in China. Fecal specimens containing group A rotaviruses were also obtained from an infected gnotobiotic calf and from a child with acute rotavirus gastroenteritis in Baltimore. The SA11 strain (serotype 3) of group A rotavirus was obtained from H. Malherbe (Salt Lake City, Utah) and passaged in MA-104 tissue culture cells. Fecal specimens were also obtained from uninfected Holstein and Angus calves, aged 1 day to 8 weeks, from a farm in Maryland. In addition, fecal specimens from uninfected infants and infants infected with group A rotaviruses were obtained at the Johns Hopkins Hospital Children's Center.

Extraction of nucleic acids. The purification of rotavirus genomic RNA and the extraction of whole nucleic acids from fecal specimens for dotting onto nitrocellulose has previously been described in detail (4).

Electropherotypes. Purified viral double-stranded RNA segments were separated by electrophoresis through 7.5% polyacrylamide gels as previously described (4). Before transfer to diazobenzyloxymethyl (DBM) paper, gels were stained with 1 μ g of ethidium bromide per ml and photographed under UV light. After the electrophoretic transfer of RNA from the gel to the paper (as described below), residual

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RNA was stained with silver by using the technique described by Herring et al. (8).

cDNA probes prepared from RNA templates. Avian myeloblastosis virus reverse transcriptase (Molecular Genetics Resources, Tampa, Fla.) was used in the rapid primer method of Taylor et al. (16) to synthesize mixed cDNA probes from viral double-stranded RNA. The specific activity of these probes was 2×10^7 to 2×10^8 cpm/ μ g of cDNA when [α - 32 P]dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was used in the reaction mixtures. The primer for the reaction was a synthetic hexadeoxyribonucleic acid (Pharmacia, Piscataway, N.J.). After alkaline digestion of RNA templates, cDNA was separated from unincorporated deoxyribonucleoside triphosphates on a Sephadex G50 column (Pharmacia).

Synthesis of rotavirus cDNA clones. First-strand cDNA synthesis was performed as described above for production of cDNA probes, but the reaction mixtures were phenol extracted and ethanol precipitated, rather than alkaline digested. Second-strand synthesis then proceeded as detailed by Watson and Jackson (21), using RNase H and *Escherichia coli* DNA polymerase I. Poly(dC) tails were added to the 3' ends of the resulting double-stranded cDNA, and this was annealed to *Pst*I-digested, poly(dG)-tailed pBR322 plasmid. Competent RR1 *E. coli* cells were transformed by the recombinant plasmids by the technique of Hanahan (7). Tetracycline-resistant colonies were then transferred to nylon filters (Amersham) (6). These filters were then hybridized with 32 P-labeled cDNA probes in a manner similar to that described below for dot hybridization. Colonies which exhibited hybridization to cDNA probes were then grown in small-scale plasmid preparations (10), and the size of the insert DNA was determined by electrophoresis of the *Pst*I-digested plasmids on 1% agarose gels.

Preparation of probes from cDNA clones. Insert DNA was excised from recombinant plasmids and separated on low-melting-point agarose gels. 32 P-labeled probes were synthesized from cloned cDNA inserts with *E. coli* DNA polymerase I and DNase I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The specific activity of the resulting probes was approximately 10^8 cpm/ μ g of DNA.

Dot hybridization. Hybridizations with specimens dotted onto nitrocellulose were performed with 32 P-labeled cDNA probes essentially as described previously (4). Briefly, nitrocellulose papers were prehybridized at 42°C for 2 h in a solution containing $5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7]), 50 mM sodium phosphate [pH 6.5], 0.2% sodium dodecyl sulfate, $1 \times$ Denhardt solution, and 50% formamide. After the addition of denatured salmon sperm DNA to a final concentration of 100 μ g/ml, prehybridization was continued for another 2 h. 32 P-labeled probe (10⁶ cpm/ml) was added to the solution along with 1/5 the volume of 50% dextran sulfate. Hybridization was performed at 42°C for 16 to 20 h. The hybridization papers were then washed twice with $2 \times$ SSC for 5 min each at room temperature, followed by two additional washes of 15 min each at 37°C with $0.1 \times$ SSC. Two final washes with $2 \times$ SSC were performed at 37°C for 5 min. The papers were then exposed to X-Omat AR X-ray film (Eastman Kodak Co., Rochester, N.Y.) with a Cronex intensifying screen (E. I. du Pont de Nemours & Co., Wilmington, Del.) at -70°C for 24 to 48 h.

Northern (RNA) blot hybridization. Viral RNA was transferred to DBM paper by the method of Stellwag and Dahlberg (15). Prehybridization and hybridization buffers, as well as washing conditions, were as described by Alwine et al.

(1). Except where indicated in Results, all hybridizations were performed in 10% formamide for 4 to 6 h at 52°C. These conditions permitted the hybridization of nucleic acids possessing no more than 30% base mismatch (4).

RESULTS

Experiments were initially performed in order to synthesize cDNA clones from the genomic RNA of the IDIR strain of GBR. These IDIR clones were then screened for strong hybridization with other GBRs in order to identify potential probes for use as diagnostic reagents. First-strand cDNA synthesis was primed with random hexanucleotides in order to ensure that all regions of the genome would be included in the library. The remainder of the cDNA cloning procedure was performed as described in Materials and Methods. Transformed bacterial cells containing recombinant plasmids and IDIR inserts were identified by colony hybridization with 32 P-labeled probes prepared from the IDIR genome. Northern blot hybridizations indicated that sequences from all 11 IDIR genes were synthesized. The lengths of the cDNA inserts ranged from 500 to more than 1,500 nucleotides. These IDIR cDNA clones were screened in order to identify clones which strongly hybridized with other GBRs. A bovine GBR was chosen for this purpose, as it was available in sufficient quantity to permit the production of 32 P-labeled probes. All 11 bovine genomic segments were included in the probe synthesized from the bovine GBR RNA, but the most intense hybridization signals were observed between the bovine probe and IDIR gene 3 clones. This strong hybridization of bovine GBR with IDIR gene 3 clones was consistently observed during three different cloning experiments. Northern blot hybridization demonstrated that the IDIR gene 3 clones were also complementary to gene segment 3 of the bovine GBR strain (Fig. 1). Each of these gene 3 cDNA clones was approximately 1,000 base pairs in length, and one cDNA clone was further evaluated for use as a diagnostic probe.

32 P-labeled probes were prepared from the IDIR gene 3 clone and hybridized with serial dilutions of purified rotavirus genomic RNA (Fig. 2a). Homologous and heterologous GBR RNAs were detected at concentrations of ≥ 0.5 ng, while hybridization was not observed between the GBR clone and group A rotavirus RNA. This assay proved to be more sensitive for the detection of heterologous GBR RNA than a similar assay which employed a cDNA probe synthesized from whole, genomic RNA (Fig. 2b). RNAs from other GBR strains were not available in sufficient quantity to assess hybridization with the gene 3 probe.

The gene 3 clone was next evaluated for hybridization with homologous and heterologous strains of GBRs present in crude nucleic acid extracts of fecal specimens. 32 P-labeled probe hybridized with fecal extracts from infant rats on day 1 postinoculation but not on day 3 postinoculation (Fig. 3a). This result was similar to that obtained with whole genome cDNA probes. The cloned cDNA probe was also evaluated for hybridization with fecal specimens isolated from humans in Baltimore (Fig. 3b). The presence of GBR had previously been identified in six of these specimens by passage in infant rats. Crude nucleic acid extracts from four of the six specimens hybridized with the gene 3 clone probe. Four of the six specimens were also identified by immunoassay, but GBR was not detected in any of the six specimens by polyacrylamide gel electrophoresis. Virus was observed in only one of six specimens by immunoelectron microscopy. No hybridization was noted between the gene 3 probe and 16

FIG. 1. Northern blot hybridizations of bovine GBR genomic RNA with 32 P-labeled probes synthesized from either whole bovine GBR genomic RNA (left) or the cDNA clone of gene 3 of the IDIR strain of GBR (right). Individual double-stranded RNA segments of the bovine GBR genome were separated by polyacrylamide gel electrophoresis and transferred from the gel to DBM paper as described in Materials and Methods. The DBM paper was then sequentially hybridized with the cloned cDNA probe, followed by hybridization with the whole-genome probe. This procedure indicated that the gene 3 cDNA clone synthesized from the IDIR strain also hybridized with genomic segment 3 of the bovine GBR. RNA segments 1 to 11 are indicated to the left of the gels.

human fecal specimens which contained group A rotavirus (data not shown).

Hybridizations were next performed in order to determine if the gene 3 probe could be employed in assays to detect the presence of heterologous GBRs in fecal specimens. Specimens were obtained from six calves which were experimentally infected with viruses originally isolated during outbreaks of GBR infection in herds of beef cattle. Hybridization was observed between the gene 3 probe and fecal specimens obtained from five of the six infected calves (Fig. 3c). GBR was also not detected in the negative specimen when examined by immunoassay, immunoelectron microscopy, or polyacrylamide gel electrophoresis. The presence of GBR in the five positive specimens was also detected by one or more of these confirmatory assays. Fecal specimens from uninfected calves did not exhibit hybridization with the cloned cDNA probe.

A single human fecal specimen containing the ADRV strain of GBR was also available for hybridization with the cloned cDNA probe (Fig. 3d). Hybridization was clearly observed with ADRV, but the small quantity of the specimen did not permit quantitative dilutions or Northern blot hybridization.

DISCUSSION

Cloned cDNA copies of genome segments have proven to be sensitive and specific alternatives for the detection of

group A rotavirus in fecal specimens (3, 13). We have now identified a cloned cDNA probe for the detection of GBRs in fecal specimens obtained from calves, rats, and humans. The cloned probe was highly specific for GBRs and appeared to be at least as sensitive as immunoelectron microscopy, polyacrylamide gel electrophoresis, or immunoassay for the detection of GBRs in the specimens tested. The dot hybridization assay described here could be employed as a primary means for the detection of GBRs, and it should also be useful in confirming the presence of GBR in specimens identified by other techniques.

Dot hybridization with the GBR probe appeared to be comparable in sensitivity with those reported for group A rotaviruses. Dimitrov et al. (3) have found that a group A rotavirus cDNA clone of gene 6 could detect as little as 0.27 ng of homologous RNA or 0.5 to 31 ng of RNA from heterologous group A rotavirus strains. Similar sensitivity was observed in the detection of GBRs with the GBR gene 3 cDNA clone. For the detection of homologous RNA, dot hybridizations with the cloned probe appeared as sensitive as hybridization with a probe synthesized from whole genomic RNA. The cloned probe was more sensitive for the detection of the heterologous, bovine GBR. Although hybridization was demonstrated between the gene 3 clone and a fecal extract of ADRV, insufficient quantities of ADRV were available for quantitative analysis. Fecal specimens containing other strains of GBR were also not available for hybridization. When such specimens can be obtained, it will be important to determine their hybridization with the cloned probe. However, the hybridizations observed in this study indicate that the probe is reactive with several GBRs which possess distinct electropherotypes and infect a variety of different hosts.

It is of interest that clones of gene 3 hybridized strongly with RNA of other GBRs. These observations indicate a relative conservation of gene 3 sequences among the GBRs examined in this study. The protein encoded by this GBR gene has not been identified, and no direct comparisons have yet been reported between the gene coding assignments of the group A rotaviruses and GBRs. Thus, the structure, size, function, and antigenic determinants of the GBR gene 3 products are unknown at this time. Our results indicate that

FIG. 2. Dot hybridizations of purified rotavirus genomic RNA with 32 P-labeled cDNA probes synthesized from either a cDNA clone of gene 3 of the IDIR strain of GBR (a) or whole genomic RNA of the Baltimore strain of GBR (b). Serial dilutions of genomic RNA from bovine GBR, the IDIR strain of GBR, and the SA11 strain of group A rotavirus were dotted onto nitrocellulose and hybridized with cDNA probes as described in Materials and Methods. Autoradiographs of the nitrocellulose papers showed the hybridization results.

FIG. 3. Autoradiographs following dot hybridizations of fecal specimens with IDIR gene 3 clone. ^{32}P -labeled probes were synthesized from the IDIR gene 3 clone and hybridized with whole nucleic acids extracted from fecal specimens as described in Materials and Methods. Autoradiographs of the nitrocellulose papers showed the hybridization results. (a) Hybridization with fecal extracts obtained from IDIR-infected rats. Each dot represents a specimen obtained from an individual animal. The postinoculation (p.i.) day on which the specimen was obtained is indicated at the top of each lane. (b) Hybridization of fecal extracts from humans infected with GBR in Baltimore. Specimens obtained from humans infected with GBR are denoted as B. Specimens from an uninfected rat (-) and a human infected with group A rotavirus (A) served as negative controls. A specimen from a rat infected with IDIR (+) was the positive control. (c) Hybridization of fecal extracts from calves infected with bovine GBR. Dotted extracts were obtained from calves infected with bovine GBR (B), a calf infected with group A rotavirus (A), and uninfected calves (U). The negative control was also provided by the fecal sample of an uninfected infant rat (-), while a fecal specimen from an infant rat infected with IDIR (+) served as the positive control. The upper left corner contained no specimen. (d) Hybridization of the IDIR gene 3 clone with a fecal extract obtained from a human infected with the ADRV strain of GBR [human B(ADRV)]. Positive controls included fecal specimens from an infant rat infected with the IDIR strain of GBR (rat B) and a calf infected with bovine GBR (calf B). Negative controls were provided by fecal specimens from an uninfected infant rat (rat -) and a human infant infected with group A rotavirus (human A).

such studies of GBR gene 3 should be valuable in understanding of the GBRs and elucidating their differences with group A rotaviruses.

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