

Monoclonal Antibodies for Detection of Norwalk Virus Antigen in Stools

JOHN E. HERRMANN,^{1*} NEIL R. BLACKLOW,¹ SUZANNE M. MATSUI,² TERRY L. LEWIS,²
MARY K. ESTES,³ JUDITH M. BALL,³ AND JAMES P. BRINKER¹

Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, Massachusetts 01655¹; Department of Medicine (Gastroenterology), Stanford University, Stanford, California 94304²; and Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030³

Received 19 January 1995/Returned for modification 30 March 1995/Accepted 1 June 1995

Monoclonal antibodies against the prototype 8FIIa strain of Norwalk virus were prepared and applied to an enzyme immunoassay (EIA) for detecting Norwalk virus in stool specimens. The monoclonal antibodies immunoprecipitated a 58-kDa protein which had been produced by in vitro transcription-translation of Norwalk virus cloned cDNA, and they reacted by EIA with recombinant Norwalk virus capsid protein at a sensitivity level of 1 ng/ml. The EIA detected virus in all tested samples from 15 different Norwalk virus-infected volunteers. No cross-reactions were seen in stools containing other caliciviruses or in stools containing rotaviruses, astroviruses, or enteric adenoviruses.

Norwalk virus, the prototype human calicivirus, is a major cause of outbreaks of gastroenteritis, affecting primarily older children and adults (1). Norwalk virus and other human caliciviruses have not been serially propagated in cell cultures, which has inhibited the study of these viruses.

Diagnosis of Norwalk virus infections by serological means or by antigen detection has relied for the most part on the use of clinical materials obtained through human volunteer studies. Recent advances in the cloning of the Norwalk virus genome and the expression of Norwalk virus capsid protein (11, 12) have provided a simplified and reproducible means for detection of Norwalk virus antibodies in serological studies. Antisera to recombinant Norwalk virus (rNV) capsid protein have also been prepared and used in an enzyme-linked immunosorbent assay (ELISA) for antigen detection. The ELISA used was highly specific for Norwalk virus and showed that some virus isolates that had been previously identified as Norwalk-related viruses by tests using human reagents were distinct from the prototype Norwalk virus (5). In this report we describe the development of monoclonal antibodies to stool-derived Norwalk virus and their reactivities with clinical stool samples from Norwalk virus-infected volunteers, with rNV capsid protein, and with Norwalk virus protein expressed by in vitro transcription-translation.

Norwalk virus was obtained from the diarrheal stool of a volunteer who had been inoculated with Norwalk virus strain 8FIIa. Virus was partially purified from stool material by differential centrifugation and banding in CsCl gradients, essentially by the procedures described by Greenberg et al. (7). Fractions were collected, assayed for density, and tested by ELISA for Norwalk virus antigen. Fractions which had densities of 1.34 to 1.40 g/ml and showed high-level reactivity by ELISA (described below) were pooled, dialyzed against 0.01 M phosphate-buffered saline (pH 7.2), and used as the virus inoculum for hybridoma production. Stools containing Norwalk

virus antigen obtained from other volunteers were used for screening hybridomas.

Stool samples containing other viruses were used for specificity testing of the monoclonal antibodies obtained. These samples included stools containing various United Kingdom strains of human calicivirus (3), samples containing astroviruses, enteric adenoviruses, rotaviruses, Hawaii virus, Snow Mountain virus, unclassified "small round" viruses, and additional samples from Norwalk virus-infected volunteers (8-10).

For hybridoma production, BALB/c mice (8-week-old females) were inoculated subcutaneously with 0.1 ml of stool-derived Norwalk virus emulsified in an equal volume of Titer-Max adjuvant (Vaxcel, Inc., Norcross, Ga.). The mice were given a second subcutaneous inoculation 3 weeks later. The mice were given two more inoculations (intraperitoneally) of Norwalk virus in Freund's incomplete adjuvant and one intraperitoneal inoculation of virus without adjuvant, all 2 weeks apart. Five days after the last inoculation, mouse spleens were fused to SP 2/0 myeloma cells with Kodak polyethylene glycol 1450 plus dimethyl sulfoxide, according to the procedures described by Lane (13). Hybrid cells were seeded onto 24-well plates in hypoxanthine-aminopterin-thymidine medium containing 10% Hybridoma Cloning Factor (IGEN, Inc., Rockville, Md.). After 7 days, hybridomas were screened for antibodies which reacted with Norwalk virus antigen. Hybridomas that secreted such antibodies were cloned twice by use of the limiting dilution technique. Ascitic fluids for all clones were prepared in BALB/c mice.

Enzyme immunoassays (EIAs) were used for screening hybridoma supernatant fluids and for testing virus-specific reactivity. For these tests, both stool extracts known to contain Norwalk virus antigen and rNV capsid protein were tested by a modification of an EIA which used polyclonal human sera for detection of Norwalk virus (9). For the indirect EIA used to screen hybridomas, wells of polyvinyl chloride microtiter plates were coated with pre- and postchallenge sera (4-week convalescent phase) from a volunteer infected with Norwalk virus. The wells were coated for 24 h at room temperature (20 to 22°C) and postcoated with 1% (wt/vol) bovine serum albumin in 0.01 M phosphate-buffered saline (PBS) for 24 h at 4°C. Suspensions of stool-derived Norwalk virus or rNV capsid pro-

* Corresponding author. Mailing address: Division of Infectious Diseases, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655. Phone: (508) 856-2155. Fax: (508) 856-5981.

tein were added to the wells and incubated for 18 to 24 h at 20 to 22°C. The plates were washed with PBS, and 0.05-ml portions of hybridoma supernatant fluids diluted in 50% fetal calf serum–50% 0.025 M Tris-HCl buffer (pH 7.2) with 0.015% Tween 20 were added and incubated for 1 h at 37°C. Peroxidase-labeled goat antibody specific for mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), at 1 µg/ml in the Tris buffer described above, was added and incubated for 1 h at 37°C. The plates were washed five times with PBS, soaked for 30 s with PBS containing 0.05% Tween 20, and washed again. Substrate for peroxidase (0.05 ml of *o*-phenylenediamine-H₂O₂; Abbott Laboratories, North Chicago, Ill.) was added and left for 10 min, and the reaction was stopped with 0.1 ml of 1 N H₂SO₄. The A_{492} of the solution was measured in a plate-reader spectrophotometer. After monoclonal antibodies were obtained, a direct EIA was used for testing stool samples. The same monoclonal antibodies were used for coating plates and for antigen detection. The antibodies were purified from ascitic fluid by ammonium sulfate precipitation and either used directly for coating plates or labeled with peroxidase for use as detector antibodies. The antibodies were labeled with peroxidase by the periodate method of Wilson and Nakane (15). For the EIA, wells were coated with monoclonal antibodies (2 µg/ml) for 18 to 24 h at 20 to 22°C, washed, and postcoated with Superblock (Pierce Chemical Co., Rockford, Ill.) for 4 to 6 h. The plates were washed, and stool samples or controls (known positive and negative stool samples) were added and incubated for 18 h at 20 to 22°C. The plates were washed, and the peroxidase-conjugated antibodies were added and incubated at 37°C for 2 h. The remaining procedures were as described for the indirect EIA. Samples were considered positive for monoclonal antibody to Norwalk virus in the screening tests, or for Norwalk virus antigen in the virus specificity tests, if the absorbance value was both ≥ 0.1 and three or more times that of the negative control (wells coated with preimmune serum in the screening tests and wells coated with an unrelated monoclonal antibody in the virus specificity tests).

Radioimmunoprecipitation tests were done to determine the reactivities of the monoclonal antibodies with Norwalk virus capsid proteins produced *in vitro*. These proteins were produced from full-length open reading frame (ORF)-2 clones, which are known to code for the Norwalk virus capsid protein (12). Reverse transcriptase-PCR was used to obtain full-length ORF-2 clones from Norwalk virus RNA isolated by silica extraction (2, 6) from a fecal sample containing intact virus particles. Oligo(dT) was used for reverse transcription to produce first-strand cDNA. Specific Norwalk virus primers were used in first-round PCRs. PCR products were amplified by using a "half-nested" approach (same 5'-end primers as in the first round, with a unique 3'-end primer internal to the first-round 3'-end primer). Second-round PCR products were made blunt ended with T4 DNA polymerase and digested with *Cla*I. The product (with a staggered 5' end and a blunt 3' end) was ligated into *Cla*I- and *Sma*I-digested pBluescript KS-. ORF-2 was expressed *in vitro* in a coupled transcription-translation system (Promega Corp., Madison, Wis.), and products were metabolically labeled with [³⁵S]methionine. Immunoprecipitations were performed as we previously described for astrovirus capsid proteins (14).

The selected hybridomas which produced antibodies reactive with both stool-derived Norwalk virus and rNV capsid protein were designated 1C9 and 1D8. No hybridomas were found which reacted only with stool-derived virus or only with rNV. The antibody isotype was immunoglobulin G1 for both antibodies. The antibodies appeared to be directed against

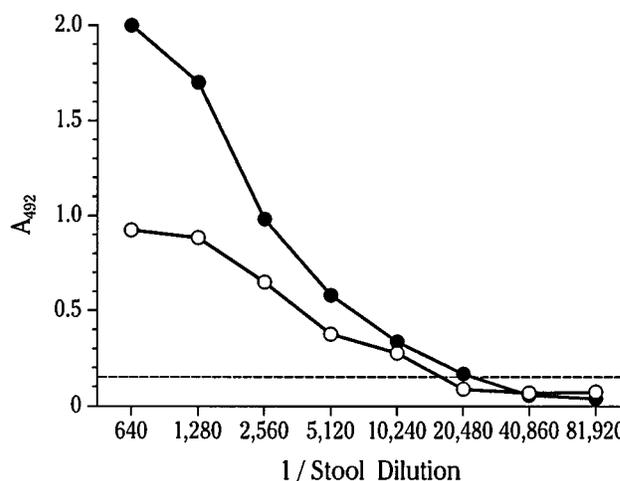


FIG. 1. Titration of Norwalk virus antigen in a stool specimen from a volunteer by monoclonal antibody ELISA (closed circles) and polyclonal antibody ELISA (open circles). Points above the dashed line represent positive values for the immunoassays.

different epitopes on the basis of blocking ELISA tests and the finding that a mixture of the two gave higher ELISA values than either one tested singly. This was confirmed by the finding that only monoclonal antibody 1D8 reacted in an ELISA with a synthetic peptide to the N terminus of the capsid (data not shown). On the basis of these findings, a mixture of the two antibodies was used both for coating plates and as detector antibodies. To determine the sensitivity of the monoclonal antibody ELISA relative to that of our previously developed polyclonal ELISA, a stool sample from a volunteer was diluted in PBS and tested by both immunoassays. The results are shown in Fig. 1. The monoclonal antibody ELISA showed greater reactivity (higher A_{492} values) and a twofold-higher sensitivity in detecting Norwalk virus in a diluted stool specimen than the polyclonal antibody ELISA. Both of the ELISAs are of the direct type. The detection limit for the monoclonal antibody ELISA, as determined by end point titration of rNV capsid antigen, was 0.05 ng per microwell, or 1 ng/ml (data not shown).

The specificity of the monoclonal antibody ELISA for Norwalk virus was examined by ELISA tests with representative types of gastroenteritis viruses in stool samples. The results,

TABLE 1. Specificity of the monoclonal antibody ELISA for detecting Norwalk virus in stools

Patient group	No. of patients	No. ELISA positive
Volunteers inoculated with:		
Norwalk virus ^a	15	15
Hawaii virus	9	0
Patients with:		
Rotavirus infection	20	0
Astrovirus infection	7	0
Enteric adenovirus infection	7	0
United Kingdom calicivirus infection	5	0
Snow Mountain virus infection	1	0
"Small round" virus infection	9	0
Diarrhea of unknown etiology	30	0
No diarrhea	25	0

^a Stools previously found to contain Norwalk virus by a polyclonal ELISA (9).

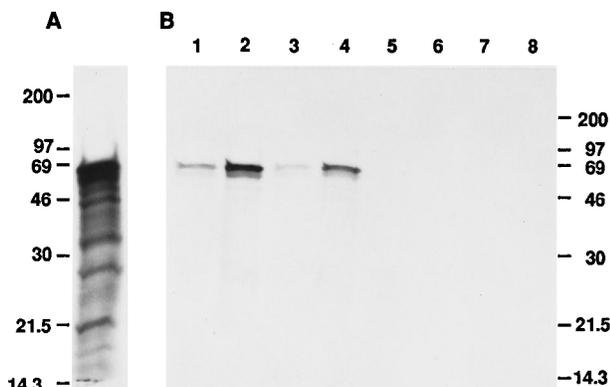


FIG. 2. (A) In vitro transcription and translation of Norwalk virus ORF-2. Untreated, radiolabeled translation products are shown. (B) Immunoprecipitation of in vitro transcription-translation products. Immunoprecipitation of radiolabeled Norwalk virus ORF-2 translation products with Norwalk virus-specific monoclonal antibodies 1D8 (lane 1, 3 μ l of reaction product immunoprecipitated; lane 2, 15 μ l) and 1C9 (lane 3, 3 μ l; lane 4, 15 μ l) was compared with immunoprecipitation with astrovirus-specific monoclonal antibody 8E7 (lane 5, 3 μ l; lane 6, 15 μ l). Norwalk virus-specific monoclonal antibodies 1D8 (lane 7) and 1C9 (lane 8) were also tested for reactivity with radiolabeled astrovirus ORF-2 translation products (capsid). Numbers on the left and right are molecular masses in kilodaltons.

presented in Table 1, indicate that antibody reactivity did not occur with normal stool samples or with samples containing viruses other than Norwalk virus. All 15 Norwalk virus samples tested which were previously found to be positive in a polyclonal antibody ELISA were also positive in the monoclonal antibody ELISA.

The results of the immunoprecipitation studies are given in Fig. 2. A predominant 58-kDa band, indicative of Norwalk virus protein (12), was seen in crude lysates. Norwalk virus-specific proteins were immunoprecipitated with monoclonal antibodies 1D8 (Fig. 2B, lanes 1 and 2) and 1C9 (Fig. 2B, lanes 3 and 4). The predominant product seen in lanes 1 to 4 of Fig. 2B is a 58-kDa protein. An astrovirus-specific monoclonal antibody (8E7) failed to immunoprecipitate any Norwalk virus ORF-2 translation products (Fig. 2B, lanes 5 and 6). Conversely, Norwalk virus-specific monoclonal antibodies 1D8 and 1C9 did not immunoprecipitate any astrovirus capsid proteins (Fig. 2B, lanes 7 and 8).

The reactivities of monoclonal antibodies with Norwalk virus in stools were determined primarily with stools obtained from volunteers infected with a strain of Norwalk virus circulating in 1968 (4). Because sera prepared against rNV capsid protein appeared to be highly specific for the prototype Norwalk virus (5), we had expected that monoclonal antibodies prepared against native virus would be more broadly reactive

than rNV-generated antibodies with Norwalk virus in naturally occurring outbreaks. However, preliminary results with the few stools we have from a documented Norwalk virus outbreak have been inconclusive (eight stool samples positive by polyclonal antibody ELISA and five stool samples positive by monoclonal antibody ELISA). Strains from additional Norwalk virus outbreaks, especially recent ones, will need to be obtained and tested to determine the utility of the monoclonal antibodies for the diagnosis of Norwalk virus gastroenteritis.

This work was supported by The Thrasher Research Fund and Public Health Service Award AI 30448.

REFERENCES

- Blacklow, N. R., and H. B. Greenberg. 1990. Viral gastroenteritis. *N. Engl. J. Med.* **325**:252-264.
- Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Nooraa. 1990. Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**:495-503.
- Cubitt, W. D., N. R. Blacklow, J. E. Herrmann, N. A. Nowak, S. Nakata, and S. Chiba. 1987. Antigenic relationships between human caliciviruses and Norwalk virus. *J. Infect. Dis.* **156**:806-814.
- Dolin, R., N. R. Blacklow, H. DuPont, S. Formal, R. Buscho, J. Kasel, R. Chames, R. Hornick, and R. M. Chanock. 1971. Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates. *J. Infect. Dis.* **123**:307-312.
- Graham, D. Y., X. Jiang, T. Tanaka, A. R. Opekun, H. P. Madore, and M. K. Estes. 1994. Norwalk virus infection of volunteers: new insights based on improved assays. *J. Infect. Dis.* **170**:34-43.
- Green, J., J. P. Norcott, D. Lewis, C. Arnold, and D. W. G. Brown. 1993. Norwalk-like viruses: demonstration of genomic diversity by polymerase chain reaction. *J. Clin. Microbiol.* **31**:3007-3012.
- Greenberg, H. B., J. R. Valdesuso, A. R. Kalica, R. G. Wyatt, V. J. McAuliffe, A. Z. Kapikian, and R. M. Chanock. 1981. Proteins of Norwalk virus. *J. Virol.* **37**:994-999.
- Herrmann, J. E., N. R. Blacklow, D. N. Taylor, and P. Echeverria. 1991. Astroviruses as a cause of gastroenteritis in children. *N. Engl. J. Med.* **324**:1757-1760.
- Herrmann, J. E., N. A. Nowak, and N. R. Blacklow. 1985. Detection of Norwalk virus in stools by enzyme immunoassay. *J. Med. Virol.* **17**:127-133.
- Herrmann, J. E., D. M. Perron-Henry, and N. R. Blacklow. 1987. Antigen detection with monoclonal antibodies for the diagnosis of adenovirus gastroenteritis. *J. Infect. Dis.* **155**:1167-1171.
- Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes. 1990. Norwalk virus genome cloning and characterization. *Science* **250**:1580-1583.
- Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes. 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. *J. Virol.* **66**:6527-6532.
- Lane, R. D. 1985. A short-duration polyethylene glycol fusion technique for increasing production of monoclonal antibody-secreting hybridomas. *J. Immunol. Methods* **181**:223-228.
- Lewis, T. L., H. B. Greenberg, J. E. Herrmann, L. S. Smith, and S. M. Matsui. 1994. Analysis of astrovirus serotypes 1 RNA, identification of the viral RNA-dependent RNA polymerase motif, and expression of a viral structural protein. *J. Virol.* **68**:77-83.
- Wilson, M. B., and P. K. Nakane. 1978. Recent developments in the peroxidase method of conjugating horseradish peroxidase (HRPO) to antibodies, p. 215-224. *In* W. Knapp, K. Holubar, and G. Wick (ed.), *Immunofluorescence and related staining techniques*. Elsevier/North-Holland Biomedical Press, Amsterdam.