Monoclonal Antibodies for Detection of Norwalk Virus Antigen in Stools

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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 TiterMax 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 polystyrene mircrotiter 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-
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. Peroxi-
dase-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 Chi-
cago, Ill.) was added and left for 10 min, and the reaction was
stopped with 0.1 ml of 1 N H₂SO₄. The A₄₉₂ of the solution was
measured in a plate-reader spectrophotometer. After monoclonal
antibodies were obtained, a direct ELISA was used for
testing stool samples. The same monoclonal antibodies were
used for coating plates and for antigen detection. The antibod-
ies were purified from ascitic fluid by ammonium sulfate preci-
sitation 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 Wil-
son 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
ClaI. The product (with a staggered 5′ end and a blunt 3′ end)
was ligated into ClaI- and Smal-digested pBluescript KS-..
ORF-2 was expressed in vitro in a coupled transcription-trans-
lation system (Promega Corp., Madison, Wis.), and products
were metabolically labeled with [35S]methionine. Immunopreci-
sipitations were performed as we previously described for ast-
rovirus 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
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
direct ELISA, a stool sample from a volunteer was diluted
to 1/25 in PBS and tested by both immunoassays. The results
are shown in Fig. 1. The monoclonal antibody 1D8 showed
greater reactivity (higher A₄₉₂ values) and a twofold-higher
sensitivity in detecting Norwalk virus in a diluted stool speci-
men 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 Nor-
walk virus was examined by ELISA tests with representative
types of gastroenteritis viruses in stool samples. The results,

![](image)

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

<table>
<thead>
<tr>
<th>TABLE 1. Specificity of the monoclonal antibody ELISA for detecting Norwalk virus in stools</th>
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<tbody>
<tr>
<td>Patient group</td>
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<tr>
<td>----------------</td>
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<tr>
<td>Volunteers injected with:</td>
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<tr>
<td>Norwalk virus</td>
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<tr>
<td>Hawaii virus</td>
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<tr>
<td>Patients with:</td>
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<tr>
<td>Rotavirus infection</td>
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<tr>
<td>Astrovirus infection</td>
</tr>
<tr>
<td>Enteric adenovirus infection</td>
</tr>
<tr>
<td>United Kingdom calicivirus infection</td>
</tr>
<tr>
<td>Snow Mountain virus infection</td>
</tr>
<tr>
<td>“Small round” virus infection</td>
</tr>
<tr>
<td>Diarrhea of unknown etiology</td>
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<tr>
<td>No diarrhea</td>
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</tbody>
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* Stools previously found to contain Norwalk virus by a polyclonal ELISA (9).
samples obtained and tested to determine the utility of the monoclonal antibodies for the diagnosis of Norwalk virus gastroenteritis.

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REFERENCES