Enzyme-Linked Immunosorbent Assays for Snow Mountain and Norwalk Agents of Viral Gastroenteritis

H. PAUL MADORE,* JOHN J. TREANOR, KATHRYN A. PRAY, AND RAPHAEL DOLIN

Infectious Diseases Unit, Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received 30 January 1986/Accepted 19 May 1986

Enzyme-linked immunosorbent assays (ELISAs) for antigen detection and blocking ELISAs for serum antibody rises were developed for the Snow Mountain and Norwalk agents of viral gastroenteritis. The ELISAs were as sensitive as the existing radioimmunoassays and were specific for the Snow Mountain or Norwalk agent. The blocking ELISAs detected the same number of significant rises in antibodies to these agents as did the existing blocking radioimmunoassays.

The Snow Mountain agent (SMA) is a 27- to 32-nm virus that is morphologically similar to but antigenically distinct from the Norwalk agent (5, 7, 19). SMA was the etiologic agent of a large outbreak of gastroenteritis at a resort in Granby, Colo. (22).

The development of a radioimmunoassay (RIA) for the Norwalk virus by Greenberg et al. (14) facilitated large-scale epidemiologic studies of this agent (11, 12, 14, 20). We subsequently developed a similar solid-phase microtiter RIA for SMA antigen detection and a blocking RIA for antibody titration with the stools and sera from the SMA volunteer study (8). This report describes the conversion of our existing SMA and Norwalk agent RIAs to the enzyme-linked immunosorbent assay (ELISA) format by using the biotin-avidin system (10, 15).

Sera and stool specimens were obtained from individuals involved in the SMA outbreak in Granby, Colo. (22), and from the subsequent study of volunteers challenged with SMA (7). Stools containing the Norwalk agent and sera were from a study of volunteers challenged with the Norwalk agent (6). Stools containing rotavirus were from children with acute gastroenteritis in Burlington, Vt. Feline calicivirus (Bolin strain FPL; ATCC VR652) was from the American Type Culture Collection, Rockville, Md. Cocksackie A9, cocksackie B1, echovirus 9, and poliovirus 1 (LSC strain) were obtained from R. Dolin, Rochester, N.Y. Crude 2% (wt/vol) stool homogenates were made up in 0.5% bovine serum albumin-veal infusion broth. The RIAs for the SMA and Norwalk agent were adapted from Greenberg et al. (14) and performed as described previously (8).

For the ELISAs, serum obtained from a volunteer challenged with the SMA or Norwalk agent was used to purify the immunoglobulin G (IgG) indicator antibody by ammonium sulfate precipitation and DEAE-cellulose chromatography, as described previously (14). The purified IgG was biotinylated with biotin-*N*-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, Ill.) as described by Jackson et al. (18).

The ELISAs for antigen detection were performed as follows. Only the 60 inner wells of polystyrene 96-well tissue culture plates (no. 3596; Costar, Cambridge, Mass.) were used. Sera from volunteers at prechallenge and 2 weeks

postchallenge with the SMA or Norwalk agent (capture antibody) were diluted 1/5,000 in phosphate-buffered saline (PBS). Samples (100 μ l) of each serum were added to duplicate wells, and the plates were incubated for 6 h at 20 to 22°C. The plates were rinsed two times in washing buffer (0.5 M NaCl, PBS, 0.05% Tween 20), and the wells were filled with blocking buffer (0.5 M NaCl, PBS, 0.5% [wt/vol] gelatin, 1% fetal bovine serum). The plates were then incubated for 16 h at 4°C. The wells were rinsed two times with washing buffer, and 25 μ l of 100% fetal bovine serum was added to all wells. For the test wells, 25 μ l of a stool homogenate was added to each of the duplicate pre- and postchallenge serum wells. For the control wells, 25 μ l of a stool homogenate (positive control, antigen present) or dilution buffer (negative control, antigen absent; 0.5 M NaCl, PBS, 0.5% [wt/vol] gelatin, 10% fetal bovine serum) was added to the duplicate pre- and postchallenge serum wells. The plates were incubated for 2 h at 37°C, excess antigen was suctioned off, and the plates were rinsed six times with washing buffer. The biotinylated IgG (indicator antibody) was diluted 1/800 in dilution buffer, and 50- μ l portions were added to all wells. The plates were then incubated for 2 h at 37°C. The plates were rinsed six times with washing buffer, and 50 μ l of avidin-horseradish peroxidase (Vector Laboratories, Burlingame, Calif.), diluted 1/4,000 in dilution buffer, was added per well. The plates were incubated for 30 min at 37°C and then rinsed six times with washing buffer, and 100 μ l of a freshly made, 3,3',5,5'-tetramethylbenzidine (TMB; Aldrich Chemical Co., Inc., Milwaukee, Wis.) reaction mixture (0.1 mg of TMB per ml, 0.1 M citrate buffer [pH 5.0], 0.006% hydrogen peroxide) was added to all wells. The plates were incubated for 30 min at 20 to 22°C in the dark. To stop the reaction, 50 μ l of 1 N HCl was added per well, and the plates were read visually or at 450 nm in the Microelisa Minireader MR590 (Dynatech Instruments, Santa Monica, Calif.). The reader was blanked with wells containing the TMB reaction mixture alone. A positive/negative (P/N) ratio of ≥ 2 was considered positive. This value represented the optical density at 450 nm (OD₄₅₀) of the duplicate postcapture antibody wells divided by the OD₄₅₀ of the duplicate precapture antibody wells for each test specimen after the OD₄₅₀ for the negative control wells had been subtracted. Borderline positives (P/N ratios of ≥ 2 to 3) were

* Corresponding author.

TABLE 1. Detection of SMA and Norwalk agent antigens by RIA and ELISA^a

Antigen	No. of specimens with indicated reaction			
	RIA positive		RIA negative	
	ELISA positive	ELISA negative	ELISA positive	ELISA negative
SMA	12	0	0	45
Norwalk agent	5	1	1	12

^a Stool specimens from volunteers challenged with the SMA (7) or Norwalk agent (6) were assayed by RIA and the ELISA as described in the text. For both assays, a P/N ratio of ≥ 2 was considered positive.

repeated and had to be repeat positive. The OD₄₅₀ of positives had to have been at least 0.1 OD unit.

The blocking ELISAs for serum antibody were performed as follows. The test wells were coated with serum (capture antibody), blocking buffer, and SMA or Norwalk antigen, as described for the ELISA for antigen detection, except that only postchallenge (positive) serum was used as capture antibody and the antigen-positive stool homogenate was prepared in dilution buffer to give a P/N ratio of ≥ 4 . Negative control (buffer, antigen absent) wells of postchallenge capture antibody were included.

For the blocking antibody step, excess antigen was removed from all wells by rinsing six times with washing buffer. For the test wells, a pair(s) of sera for which the titers were to be determined was diluted 1/100 in dilution buffer. Duplicate samples (100 μ l) were added to the appropriate wells, followed by serial twofold dilutions of 50- μ l samples into wells containing 50 μ l of dilution buffer. Unblocked antigen wells (buffer only) and negative control wells (buffer, antigen absent) were included. The plates were incubated for 2 h at 37°C and rinsed six times with washing buffer. The addition of biotinylated IgG (indicator antibody), avidin-horseradish peroxidase, and TMB reaction mixture was as described for the ELISA for antigen. The endpoint was defined as the highest dilution of serum that reduced the OD₄₅₀ of the wells by 40% in comparison with that of the unblocked antigen wells (100% = OD₄₅₀ of unblocked antigen wells minus OD₄₅₀ of the negative control wells; this value ranged from 0.6 to 1.2 OD₄₅₀ units).

The substrate TMB was previously used by Beards et al. (2) and was not found to be carcinogenic in rats or positive in the Ames test (17).

To develop the ELISA for detection of SMA antigen, stool specimens from the SMA volunteer study previously described (7) that had already been tested by RIA for the presence of SMA antigen, were retested by ELISA for SMA antigen. The ELISA detected the same number of positive and negative specimens as did the RIA (Table 1). Of the 12 positive stools, 7 had initial P/N values of ≥ 2 to 3 and repeated positive. Of the 45 negative stools, 3 were initially low positive and repeated negative. The specificity of the ELISA for SMA is depicted in Table 2 in which it is shown that specimens containing Norwalk virus, human rotavirus, feline calicivirus, and several enteroviruses did not react in the assay.

The rises in serum antibody to the SMA detected by the ELISA were compared with rises detected by RIA in the volunteers challenged with the SMA (Table 3). All significant rises (fourfold or greater) detected by RIA were also detected by the ELISA. The magnitudes of the rises detected by each method were generally similar, although variation

TABLE 2. Specificity of SMA and Norwalk agent ELISAs

Agent ^a	No. of specimens negative in ELISA ^b :	
	SMA	Norwalk agent
Norwalk	7	
SMA		4
Human rotavirus	2	2
Feline calicivirus	1	1
Coxsackie A9	1	1
Coxsackie B1	1	1
Echovirus 9	1	1
Poliovirus 1	1	1

^a P/N ratios for stools containing the Norwalk agent, as measured by the Norwalk agent RIA, were 4 to 11, and P/N ratios for stools containing the SMA, as measured by the SMA RIA, were 10 to 13. Stools containing rotavirus were strongly positive by Rotazyme (Abbott Laboratories, Diagnostics Div., North Chicago, Ill.). The feline calicivirus titer was 10⁸ PFU/ml; the coxsackie A9, coxsackie B1, and echovirus 9 titers were 10³ 50% tissue culture infective doses per ml; and the poliovirus 1 titer was 10⁹ 50% tissue culture infective doses per ml.

^b The ELISAs were performed as described in the text. None of the specimens tested was positive.

between individuals was noted. The endpoint titers were also similar in both tests.

We subsequently converted our RIA for the Norwalk agent to the same ELISA format described for the SMA. Sera and stool specimens from volunteers challenged with the Norwalk agent (6) were used in these assays. The sensitivity of the ELISA for the Norwalk antigen in stool specimens was similar to that of the RIA (Table 1). Of the six positive stools, only one low-positive stool (P/N ratio of ≥ 2 to 3) was repeat positive, and none of the negative stools was initially positive. The Norwalk ELISA was specific for Norwalk antigen; it did not detect SMA, human rotavirus, feline calicivirus, or enterovirus antigens (Table 2).

The blocking RIA for Norwalk antibody was also converted to the ELISA format. The numbers of significant antibody rises in paired sera from eight volunteers challenged with the Norwalk agent were the same (six of eight) in both assays, and the extents of the rises and endpoint titers of the sera were also similar (Table 4).

In summary, we converted existing RIAs for the detection of the SMA and Norwalk agents to biotin-avidin ELISAs but

TABLE 3. Comparison of blocking assay for SMA antibody by RIA and ELISA^a

Volunteer	RIA titer			ELISA titer		
	S ₁	S ₂	Rise (fold)	S ₁	S ₂	Rise (fold)
1	100	6,400	64	<100	3,200	64
2	200	800	4	<100	800	16
3	100	800	8	100	400	4
4	3,200	1,600	-2	200	400	2
5	100	1,600	16	<100	800	16
6	<100	200	4	<100	3,200	64
7	200	200	0	200	200	0
8	<100	1,600	32	<100	6,400	128
9	<100	800	16	<100	800	16
10	100	3,200	32	<100	400	8
11	<100	<100	0	<100	<100	0
12	200	200	0	200	200	0

^a As described previously (7), pre- and 3-week-postchallenge sera (S₁ and S₂, respectively) from volunteers previously examined by blocking RIA were tested by blocking ELISA as described in the text. The endpoint titer was the highest dilution of serum that reduced the 100% OD₄₅₀ readings by $\leq 40\%$.

TABLE 4. Comparison of blocking assay for Norwalk agent antibody by RIA and ELISA^a

Volunteer	RIA titer			ELISA titer		
	S ₁	S ₂	Rise (fold)	S ₁	S ₂	Rise (fold)
1	200	12,800	64	100	25,600	256
2	<100	12,800	256	<100	6,400	128
3	400	12,800	32	100	6,400	64
4	200	6,400	32	<100	3,200	64
5	3,200	12,800	4	800	25,600	32
6	<100	<100	0	<100	<100	0
7	1,600	1,600	0	200	200	0
8	<100	200	4	<100	200	4

^a Volunteers were challenged with the Norwalk agent as described previously (6). Pre- and 3-week-postchallenge sera (S₁ and S₂, respectively) from volunteers previously examined by blocking RIA were tested by blocking ELISA as described in the text. The endpoint titer was the highest dilution of serum that reduced the 100% OD₄₅₀ readings by $\leq 40\%$.

maintained the sensitivity of the RIAs. Gary et al. (9) previously developed a biotin-avidin ELISA for Norwalk agent detection and also observed a sensitivity similar to or greater than that of their Norwalk RIA. Our ELISAs are specific for the SMA or Norwalk agent and do not cross-react with human rotavirus, feline calicivirus, or several enteroviruses. Gary et al. (9) also indicated a lack of cross-reaction between their Norwalk ELISA and stools from volunteers inoculated with the SMA. These observations help to confirm the antigenic distinctiveness of the two agents (5, 7). Herrmann et al. (16) converted their RIA for Norwalk antigen detection to an ELISA by directly linking the indicator IgG to peroxidase. This ELISA was more sensitive for antigen detection than was the RIA and did not react with stools from volunteers inoculated with the antigenically distinct Hawaii agent (5, 19).

Although the Norwalk agent (13) and SMA (21), like the caliciviruses (24), possess a single virion structural protein, our SMA and Norwalk ELISAs did not cross-react with feline caliciviruses. In an earlier study, human calicivirus-like particles did not appear to be serologically related to the Norwalk virus (13).

The blocking ELISAs to SMA and Norwalk antibody detected a number of significant antibody rises in volunteers challenged with those agents equal to those detected by the RIAs. Gary et al. (9) previously converted their Norwalk blocking RIA to an ELISA.

Norwalk virus, the prototype for the Norwalk-like viruses (1, 4, 5, 23, 25), plays a significant role in the etiology of acute gastroenteritis; 34 to 42% of the outbreaks of acute infectious nonbacterial gastroenteritis have been attributed to Norwalk virus (12, 20). Similar extensive seroepidemiologic studies with the SMA have not been performed. In addition to the original outbreak in Granby, Colo. (22), the SMA has been linked to a small outbreak in Chittenden County, Vt., by RIA serology (3). A more recent outbreak in Rochester, N.Y., associated with the ingestion of clams, has been attributed to the SMA by ELISA serology and antigen detection (B. I. Truman, H. P. Madore, M. Menegus, J. L. Nitzkin, and R. Dolin, Annu. Epidemic Intelligence Serv. Conf., p. 54, 1985). With the SMA ELISA, we can now define more clearly the role of the SMA in outbreaks of gastroenteritis.

We thank Grace Ann Duttweiler for her excellent technical assistance.

This work was supported, in part, by Public Health Service grant R22 AI20070 from the National Institutes of Health.

LITERATURE CITED

1. Appleton, H., M. Buckley, J. L. Cotton, and S. Henderson. 1977. Virus-like particles in winter vomiting disease. *Lancet* i:409-411.
2. Beards, G. M., A. D. Campbell, N. R. Cottrell, J. S. M. Peiris, N. Rees, R. C. Sanders, J. A. Shirley, H. C. Wood, and T. H. Flewett. 1984. Enzyme-linked immunosorbent assays based on polyclonal and monoclonal antibodies for rotavirus detection. *J. Clin. Microbiol.* 19:248-254.
3. Brondum, J., K. C. Spitalny, R. L. Vogt, K. Godlewski, H. P. Madore, and R. Dolin. 1985. An outbreak of gastroenteritis in Vermont attributed to Snow Mountain agent. *J. Infect. Dis.* 152:834-837.
4. Clarke, S. K. R., G. T. Cook, S. I. Egglestone, T. S. Hall, D. L. Miller, S. E. Reed, D. Rubenstein, A. J. Smith, and D. A. J. Tyrrell. 1972. A virus from epidemic vomiting disease. *Br. Med. J.* 3:86-89.
5. Cukor, G., and N. R. Blacklow. 1984. Human viral gastroenteritis. *Microbiol. Rev.* 48:157-179.
6. Dolin, R., N. R. Blacklow, H. Dupont, R. F. Buscho, R. G. Wyatt, J. A. Kasel, R. Hornick, and R. M. Chanock. 1972. Biological properties of Norwalk agent of acute infectious nonbacterial gastroenteritis. *Proc. Soc. Exp. Biol. Med.* 140:578-583.
7. Dolin, R., R. C. Reichman, K. D. Roessner, T. S. Tralka, R. T. Schooley, W. Gary, and D. Morens. 1982. Detection by immune electron microscopy of the Snow Mountain agent of acute viral gastroenteritis. *J. Infect. Dis.* 146:184-189.
8. Dolin, R., K. M. Roessner, J. J. Treanor, R. C. Reichman, M. Phillips, and H. P. Madore. 1986. Radioimmunoassay for the detection of Snow Mountain agents of viral gastroenteritis. *J. Med. Virol.* 19:11-18.
9. Gary, G. W., Jr., J. E. Kaplan, S. E. Stine, and L. J. Anderson. 1985. Detection of Norwalk virus antibodies and antigen with a biotin-avidin immunoassay. *J. Clin. Microbiol.* 22:274-278.
10. Green, N. M. 1963. Avidin. 1. The use of [¹⁴C]biotin for kinetic studies and for assay. *Biochem. J.* 89:585-591.
11. Greenberg, H. B., J. Valdesuso, A. Z. Kapikian, R. M. Chanock, R. G. Wyatt, W. Szmunn, J. Larrick, J. Kaplan, R. H. Gilman, and D. A. Sack. 1979. Prevalence of antibody to the Norwalk virus in various countries. *Infect. Immun.* 26:270-273.
12. Greenberg, H. B., J. Valdesuso, R. H. Yolken, E. Gangarosa, W. Gary, R. G. Wyatt, T. Konno, H. Suzuki, R. M. Chanock, and A. Z. Kapikian. 1979. Role of Norwalk virus in outbreaks of nonbacterial gastroenteritis. *J. Infect. Dis.* 139:564-568.
13. 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.
14. Greenberg, H. B., R. G. Wyatt, J. Valdesuso, A. R. Kalica, W. T. London, R. M. Chanock, and A. Z. Kapikian. 1978. Solid-phase microtiter radioimmunoassay for detection of the Norwalk strain of acute nonbacterial, epidemic gastroenteritis virus and its antibodies. *J. Med. Virol.* 2:97-108.
15. Guesdon, J.-L., T. Ternynck, and S. Avrameas. 1979. The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* 27:1131-1139.
16. 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.
17. Holland, V. R., B. C. Saunders, F. L. Rose, and A. I. Walpole. 1974. A safer substitute for benzidine in the detection of blood. *Tetrahedron* 30:3299-3302.
18. Jackson, S., J. A. Sogn, and T. J. Kindt. 1982. Microdetermination of rabbit immunoglobulin allotypes by Elisa using specific antibodies conjugated with peroxidase or with biotin. *J. Immunol. Methods* 48:299-309.
19. Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica, and R. M. Chanock. 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J. Virol.* 10:1075-1081.

20. Kaplan, J. E., G. W. Gary, R. C. Baron, N. Singh, L. B. Schonberger, R. Feldman, and H. B. Greenberg. 1982. Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. *Ann. Intern. Med.* **96**:756-761.
21. Madore, H. P., J. J. Treanor, and R. Dolin. 1986. Characterization of the Snow Mountain agent of viral gastroenteritis. *J. Virol.* **58**:487-492.
22. Morens, D. M., R. M. Zweighaft, T. M. Vernon, G. W. Gary, B. T. Wood, R. C. Holman, and R. Dolin. 1979. A waterborne outbreak of gastroenteritis with secondary person-to-person spread: association with a viral agent. *Lancet* **i**:964-966.
23. Oshiro, L. S., C. E. Haley, R. R. Roberto, J. L. Riggs, M. Croughan, H. Greenberg, and A. Z. Kapikian. 1981. A 27-nm virus isolated during an outbreak of acute infectious nonbacterial gastroenteritis in a convalescent hospital: a possible new serotype. *J. Infect. Dis.* **143**:791-795.
24. Schaffer, F. L., H. L. Bachrach, F. Brown, J. H. Gillespie, J. N. Burroughs, S. H. Madin, C. R. Madeley, R. C. Povey, F. Scott, A. W. Smith, and M. J. Studdert. 1980. *Caliciviridae*. *Intervirology* **14**:1-6.
25. Wyatt, R. G., R. Dolin, N. R. Blacklow, H. L. Dupont, R. F. Buscho, T. S. Thornhill, A. Z. Kapikian, and R. M. Chanock. 1974. Comparison of three agents of acute infectious nonbacterial gastroenteritis by cross-challenge in volunteers. *J. Infect. Dis.* **129**:709-714.