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Solid-Phase Antibody Capture Hemadsorption Assay for Detection of Hepatitis A Virus Immunoglobulin M Antibodies

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A solid-phase antibody capture hemadsorption (SPACH) assay was developed to detect hepatitis A virus (HAV)-specific immunoglobulin M (IgM) antibodies in sera from humans recently infected with hepatitis. The assay is performed with microtiter plates coated with anti-human IgM antibodies to capture IgM antibodies from the test sera. HAV-specific IgM antibody is detected by the addition of HAV hemagglutinating antigen and goose erythrocytes. Hemadsorption of erythrocytes to antigen-antibody complexes attached to the solid phase indicate the presence of IgM antibodies. The SPACH assay was compared to a commercial radioimmunoassay and was found to be equally or more sensitive and specific for the detection of HAV IgM antibodies. The SPACH assay is an alternative, rapid assay that doesn't require hazardous substrates or radioactivity for the detection of HAV-specific antibodies.

A hemagglutination-inhibition (HAI) test for the detection of hepatitis A virus (HAV) antibodies has recently been developed (5). Although the HAI test is a simple test to measure HAV antibodies, it does not distinguish between recent and past HAV infections; this distinction is necessary for serodiagnosis. The demonstration of specific HAV immunoglobulin M (IgM) antibodies in an individual indicates a recent exposure to HAV. IgM antibodies usually are present in patients with clinical symptoms, reach peak levels in a few weeks, and start to decline gradually over the next 4 to 5 months (8).

One of the best ways to detect virus-specific IgM antibodies employs antibody capture methodology. First described by Duermeyer and van der Veen to detect HAV-specific IgM antibodies in an enzyme-linked immunosorbent assay (ELISA) (4), it has since been incorporated in numerous solid-phase radioimmunoassays (RIAs) and ELISAs to detect IgM antibodies to a variety of infectious agents (1, 8, 11). Antibody capture techniques have also incorporated hemagglutination as a means to detect IgM antibodies (2, 6, 7, 9, 10, 12). This study describes a solid-phase antibody capture hemadsorption (SPACH) assay for the specific detection of HAV IgM antibodies and its application for the rapid diagnosis of HAV.

MATERIALS AND METHODS

Preparation of HAV hemagglutinating (HA) antigen. The HM175 strain of HAV adapted to grow in MRC-5 cells was used as the source of antigen for the HAI and SPACH tests. MRC-5 cells grown in 490-cm^2 roller bottles at 35°C were inoculated with HAV at a multiplicity of infection ranging between 0.05 and 0.1. Infected cell cultures were refed weekly with Eagle minimal essential medium containing 2% fetal bovine serum and antibiotics. At 28 days postinoculation, the cell monolayers were washed three times with 100 ml of Hanks balanced salt solution followed by the addition of 50 ml of Eagle's suspension medium containing no fetal bovine serum. After 3 days of incubation at 35°C, the cells were removed from the flasks by agitation. The cell suspension was then centrifuged at 11,600 × *g* for 30 min. The supernatant was further clarified by filtration through a 0.45-μm-pore-size filter and then concentrated by centrifugation at 63,000 × *g* for 2 h. The virus pellets were resuspended in 6.7 mM phosphate-buffered saline (PBS), pH 7.4, to 1:100 of the original volume. The HAV antigen was stored at 4°C until it was used for SPACH and HAI testing.

HAI. HAI assays were performed in microtiter plates by the method described by Eckels et al. (5). Goose erythrocytes were used at a concentration of 0.3% in adjusting diluents.

RIA. Tests for total HAV antibody and IgM-specific antibodies were performed with HAVAB and HAVAB-M RIA kits supplied by Abbott Laboratories, North Chicago, Ill.

SPACH. Wells of Immulon II microtiter plates (Dynatech Laboratories, Alexandria, Va.) were coated overnight at 35°C with 100 μl of affinity-purified goat anti-human IgM (Kirkegaard and Perry, Inc., Gaithersburg, Md.) diluted 1:500 in carbonate buffer. Following washing, 50 μl of test sera diluted 1:200 in PBS was added. Plates were incubated for 1.5 h at 35°C and washed three times, a 50-μl sample of HAV HA antigen (containing 50 HA units) diluted in PBS was added to each well, and the wells were incubated 1.5 h at 35°C. Plates were washed five times, 100 μl of a 0.3% suspension of goose erythrocytes diluted in pH 5.8 adjusting diluent was added, and the mixture was incubated at 4°C. Hemadsorption was read 2 h later. Figure 1 shows a schematic of the SPACH assay.

Sera. A panel of sera from known HAV; hepatitis B virus (HBV); and non-A, non-B hepatitis virus (NANB) cases was obtained from Siriraj Hospital in Bangkok, Thailand, and Teku Hospital in Kathmandu, Nepal, during the years 1987 to 1988.

Separation of IgM and IgG by rate-zonal centrifugation. Sera were diluted 1:3 in PBS to a volume of 0.3 ml and layered on top of a 4.8-ml continuous 10 to 40% linear sucrose gradient. Samples were centrifuged at 114,000 × *g*

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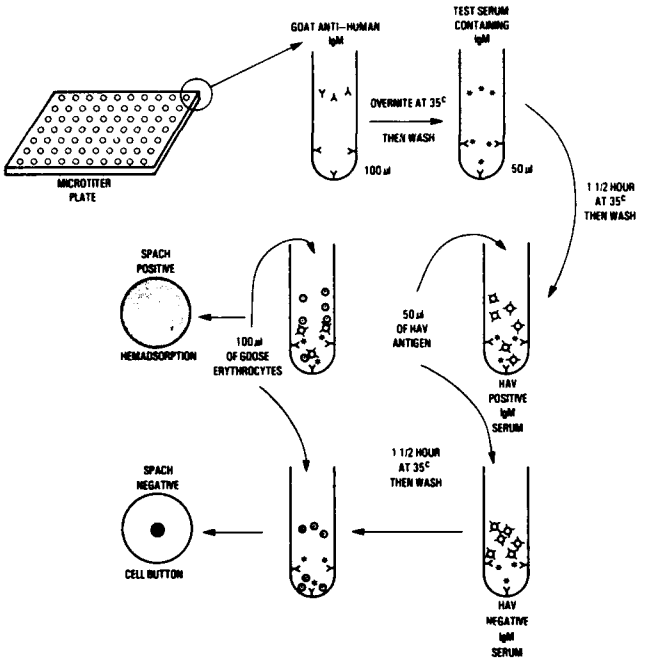

FIG. 1. Schematic of SPACH procedure.

for 18 h in a Beckman SW50.1 rotor. Eleven fractions, each approximately 0.5 ml, were collected. Each fraction was tested for HAV IgM antibodies by SPACH and for human IgM and IgG with radial immunodiffusion (RID) plates (Calbiochem Behring, La Jolla, Calif.).

RESULTS

Optimal SPACH assay parameters. Optimization of the SPACH assay required the assessment of several test parameters. Optimal results were obtained when the capture antibody (goat anti-human IgM) was diluted 1:500 or 1:1,000 in carbonate buffer. We also found we could dilute test sera 1:100 or 1:1,000 without any loss of sensitivity in detecting HAV IgM or any false positives among the negative controls. Lower dilutions of sera (1:10) sometimes gave a false-positive reaction or seemed to inhibit hemadsorption. All sera tested in this paper were diluted at least 1:100 or 1:200.

Another parameter studied was the concentration effect of HAV HA antigen used in the SPACH assay. At least 32 HA units of HAV antigen were necessary for detectable and consistent hemadsorption patterns by positive HAV IgM-containing sera. The sensitivity of the SPACH assay to detect HAV IgM could be increased further by using more HA units (data not shown). Because the sensitivity of the assay seemed more than adequate and in order to conserve HA antigen, we chose 50 HA units for use in the standardized SPACH assay. Figure 2 illustrates a typical SPACH assay using the optimal test parameters described to differentiate HAV infection from other viral hepatitis infections.

Specificity of the SPACH assay for HAV-specific IgM antibodies. The specificity of the SPACH assay for the detection of HAV IgM antibodies was demonstrated in two ways. First, serum immunoglobulins from acute HAV-infected individuals were separated by rate-zonal centrifugation. A quantitative RID assay was used to identify human IgG and IgM in the gradient fractions. Fractions from the gradients

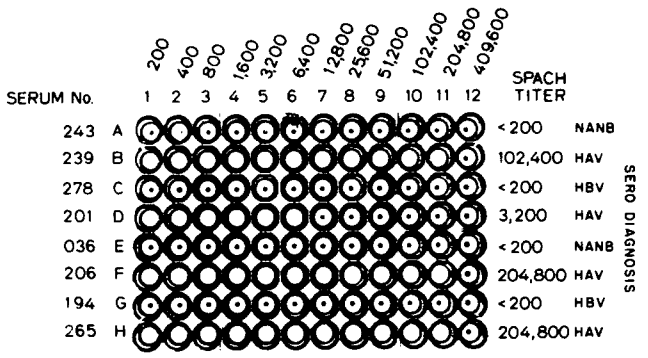

FIG. 2. Photograph of a solid-phase antibody capture hemadsorption assay done in a microtiter plate. Rows A and E, sera from NANB cases; rows B, D, F, and H, sera from HAV infections; rows C and G, sera from HBV cases. All sera were diluted twofold beginning at a 1:200 dilution in column 1. Note that only the sera from the HAV cases have positive hemadsorption titers.

were analyzed for HAV-specific IgM antibodies by the SPACH assay. The results of one gradient separation of a human acute serum specimen is presented in Table 1. The majority of the detectable SPACH activity is associated with those fractions containing IgM. Fraction four, which has the highest SPACH titer (2,048), also had the highest concentration of IgM.

Secondly, a purified IgG preparation of high-titered human anti-HAV serum which is used to detect HAV antigen in our ELISAs was negative in our SPACH assay for HAV IgM antibody despite having a HAV HAI titer of 5,120.

These results suggest there is very little if any cross-reactivity of HAV-specific IgG with the SPACH IgM assay we have designed.

Comparison of SPACH, HAI, and RIA. A total of 18 paired acute- and convalescent-phase serum samples from known cases of HAV and 6 samples from HBV and NANB hepatitis cases were tested by SPACH, HAI, and RIAs (HAVAB, HAVAB-M) for the detection of antibodies to HAV (Table 2).

There was a 100% agreement between the HAI and HAVAB in the ability to detect antibody to HAV. HAI titers were generally higher in convalescent-phase sera than in

TABLE 1. Specificity of SPACH assay to detect IgM antibodies to HAV[a]

Gradient fraction	% Sucrose	Immunoglobulin		SPACH titer
		G	M	
1	39.4	−	−	<2
2	37.4	−	+	2
3	35.0	−	+	1,024
4	32.0	−	+	2,048[b]
5	28.4	+	+	512
6	24.6	+/−	+	256
7	21.0	−	−	64
8	17.8	−	−	32
9	14.6	+	−	128
10	12.6	+	−	32
11	12.2	+	+	1,024

[a] Shown are results of a sucrose density gradient separation of HAV acute-phase serum sample 1008 (HAI titer, 2,560).

[b] Corresponds to the fraction containing the highest IgM concentration as determined by RID.

TABLE 2. Comparison of HAI, SPACH, and RIA for the detection of HAV antibodies

Serum no. (days postillness)	HAI titer[a]	RIA result[b]		SPACH titer
		HAVAB	HAVAB-M	
HAV acute- and convalescent-phase sera				
196				
6	1,280	90.5	36.7	256,000
188	2,480	98.7	5.0	4,000
239				
4	640	89.4	34.8	160,000
122	10,240	98.0	6.6	16,000
242				
5	160	65.5	9.5[c]	640,000
34	1,280	96.1	33.6	160,000
265				
5	2,560	89.6	39.0	40,000
35	1,280	95.8	25.8	320,000
269				
8	2,560	96.6	35.6	40,000
37	2,560	97.5	15.9	40,000
273				
5	320	88.6	23.4	320,000
36	2,560	96.7	28.1	320,000
277				
7	2,560	91.6	30.8	80,000
37	1,280	96.1	27.5	160,000
285				
4	5,120	92.8	39.1	320,000
35	2,560	95.9	23.5	320,000
286				
1	640	91.4	32.2	128,000
30	5,120	96.0	20.1	32,000
002				
4	320	91.9	38.6	512,000
11	5,120	95.3	40.7	512,000
008				
3	2,560	95.0	44.1	128,000
12	2,560	96.4	39.5	512,000
009				
4	10,240	95.9	48.3	>4,096,000
11	10,240	96.6	44.1	>4,096,000
014				
8	10,240	92.1	35.1	256,000
16	1,280	96.5	42.0	512,000
180				
6	1,280	90.3	39.8	256,000
135	40,960	97.9	3.5	8,000
201				
5	20,480	81.7	32.1	128,000
157	5,120	97.1	2.0	4,000
251				
6	5,120	94.9	45.4	2,480,000
59	40,960	98.3	8.3	8,000
257				
6	10,240	96.2	42.7	>4,096,000
43	10,240	97.7	35.4	256,000
264				
9	160	86.3	35.2	512,000
92	5,120	96.8	6.1	2,000
Negative control (HBV acute-phase) sera				
194 (14)	2,560	97.7	1.3	<100
210 (5)	10,240	97.5	1.5	<100
216 (14)	<10	1.6	1.1	<100
244 (5)	<10	−12.2	0.4	<100
259 (5)	<10	−10.6	1.2	<100
278 (5)	<10	−2.9	1.2	<100
Negative control (NANB acute-phase) sera				
036 (14)	320	94.5	1.1	<100
128 (2)	1,280	92.6	1.1	<100
195 (4)	5,120	96.1	1.1	<100
204 (3)	<10	−14.0	1.0	<100
243 (2)	2,560	97.8	1.4	<100
268 (8)	640	ND[d]	ND	<100

[a] HAI test results are given as a reciprocal dilution of test serum that inhibited 8 units of HAV HA antigen.
[b] HAVAB measures total antibodies to HAV; values above 50% are positive. HAVAB-M measures IgM antibodies to HAV; values above 10 (positive/negative) are positive.
[c] 242 acute sera was diluted and therefore probably positive.
[d] ND, not done.

acute-phase sera. The exceptions were serum no. 265, 277, 285, 014, and 201, which showed reduced titers whereas the HAVAB showed elevated inhibition values. Higher HAI titers were also associated with higher inhibition percentages of the HAVAB test.

Although the HAI and HAVAB compared well, their usefulness for serodiagnosis is limited since both tests measure IgG and IgM antibodies. On the other hand, the HAVAB-M and SPACH tests can be used as serodiagnostic tests since they detect only IgM and can differentiate between acute- and convalescent-phase HAV sera. Figure 3 shows a scatterplot comparing the SPACH assay with the HAVAB-M in detecting IgM from the sera listed in Table 2. The mean SPACH titer for acute sera taken less than 9 days from illness was 802,666. HAVAB-M assay results were negative in 6 HAV convalescent-phase serum samples that were positive by HAI. These were late-convalescent-phase sera ranging from 59 to 188 days postillness. These same convalescent-phase sera were positive by the SPACH assay, with titers ranging from 2,000 to 16,000. We also tested acute- and convalescent-phase sera from other forms of viral hepatitis. The HAI assay and HAVAB detected HAV antibodies in two of six HBV acute-phase serum samples and five of six NANB acute-phase serum samples. All of the sera from the non-HAV cases of hepatitis were found negative by the SPACH and HAVAB-M. These results show conclusively that only the IgM-based tests such as the SPACH and HAVAB-M assays can be of any diagnostic value in determining recent HAV infections by using single serum specimens.

DISCUSSION

Rapid and specific serodiagnosis of HAV is essential in order to identify HAV and to contain outbreaks of this disease. At the present time, commercial RIAs or ELISAs are being used for HAV serodiagnosis. This paper describes an alternative assay for HAV diagnosis.

The advantages of the SPACH assay for HAV diagnosis are several. Reagents are relatively inexpensive; the antigen utilized in the SPACH assays described in this paper was a crude, concentrated antigen. A newer and simpler procedure is available for the preparation of HAV HA antigen (3). No special equipment is required to read the test. No special serum treatments (extraction or absorption) are required.

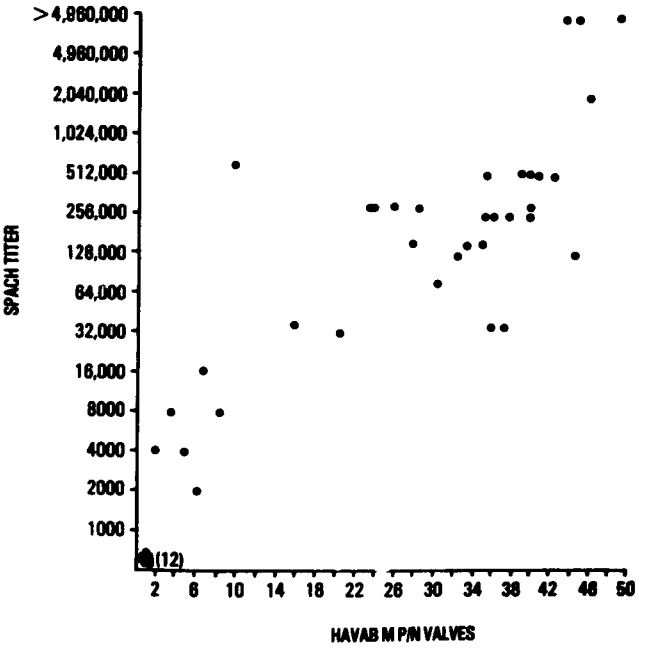

FIG. 3. Scatterplot comparing SPACH titers with corresponding positive/negative (P/N) values of HAVAB-M RIA.

When precoated anti-IgM plates are available, results can be obtained in less than 6 h, compared with the 2-day RIA or ELISA. Also, the SPACH is safer to perform because no hazardous isotopes or carcinogenic substrates are used.

The SPACH assay can also be adapted to measure other serum immunoglobulins besides IgM. Using different antibody capture specificities, we have measured IgG and IgA HAV-specific antibodies.

Another inherent advantage to the assay is that false positives due to rheumatoid factor would be reduced if not eliminated. Most rheumatoid factors bind to the Fc portion of IgG antibodies which are commonly radiolabeled or conjugated with enzymes to detect the antigen bound by the captured IgM. No labeled secondary IgG antibodies are used in the SPACH assay. The bound HAV antigen is detected instead by hemadsorption when goose erythrocytes are added at acidic pH.

The specificity and the sensitivity of the SPACH compared well with those of the HAVAB-M RIA. The specificity of the SPACH to measure HAV-specific IgM was confirmed by the lack of cross-reactivity from acute- and convalescent-phase sera from other non-A hepatitis cases. In addition, the results from the sucrose density gradient serum separation demonstrated that HAV-specific IgG did not interfere with the SPACH assay ability to measure HAV-specific IgM antibodies. The increased sensitivity of the SPACH assay to measure HAV-specific IgM antibodies in 6 of 18 late-convalescent-phase sera (greater than 59 days of illness) that were negative by HAVAB-M is probably explained by a high HAVAB-M positive cutoff value. The SPACH assay could be made more comparable to the HAVAB-M in distinguishing acute- from convalescent-phase sera by raising the positive cutoff titer to 1:10,000 or greater.

The SPACH assay as described in this paper can be used to determine whether a patient has an acute or subclinical HAV infection. Our results indicate the SPACH assay is as sensitive and specific as the commercial RIA assays tested and offers a new approach in the serological diagnosis of HAV.

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