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DETECTION OF ANTIBODIES TO SENDAI VIRUS IN MOUSE SERA BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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SUMMARY: Enzyme-linked immunosorbent assay (ELISA) for diagnosis of Sendai virus infection in mice was evaluated. A large-scale survey of infected mice showed that ELISA is approximately 100 times more sensitive than the hemagglutination-inhibition or complement-fixation test. Although a few SPF mice showed false-positive reactions at a serum dilution of 1:40, further dilution to 1:80 eliminated the non-specific reaction. It was shown that ELISA is a highly satisfactory method for examination of Sendai virus infection in mice.

INTRODUCTION

Natural infection with Sendai virus occurs in a variety of laboratory rodents (1-4). Sendai virus is the agent of the most important respiratory disease in mice. Hemagglutination-inhibition (HI) and complement-fixation (CF) tests have been used for diagnosis of Sendai virus infection since its finding (5). Enzyme-linked immunosorbent assay (ELISA) is being widely used for diagnosis of numerous infectious diseases for its reliability and high sensitivity (6,7). The choice of the enzyme and its substrate is critical for the assay.

The present study evaluates the ELISA method using horse radish peroxidase (HRP) and 2,2'-azinobis(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS) as its substrate (8) for detection of serum antibody of mice to Sendai virus.

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MATERIALS AND METHODS

Viral antigen: Sendai virus, strain M-73, was prepared by the method described (9). Briefly, the egg-grown virus was used for infection of mice. The virus concentrated by centrifugation at 20,000 rpm for 1 hr was layered onto a 20-60% (w/v) linear sucrose gradient, which was centrifuged at 25,000 rpm for 3 hr. The virus banded between 29 and 38% (w/w) sucrose was collected, sonicated and used for ELISA. Uninfected allantoic fluid treated in the same manner was used as a control antigen. The protein content was determined by the method of Lowry et al (10).

Mouse sera: 4-week-old female mice (Specific pathogen-free: SPF) of C57BL/6 (Shizuoka Laboratory Animal Center, SLAC, Shizuoka) were used. Sendai virus-infected 4-week-old female mice of ICR strain (from SLAC) were used for contact infection as described (9). Healthy mice (C57BL/6) were housed in a cage together with two mice (ICR) infected by nasal instillation with $5 \times 10^{6.0}$ ID₅₀ of Sendai virus 3 day earlier. Three mice (C57BL/6) were killed every 2 days and examined for serum antibody to Sendai virus.

SPF mice of BALB/c, GPC, DD and C57BL/6 were housed in the same room. C57BL/6 mice infected naturally with Sendai virus were accidentally introduced into the room and the viral infection seemed to spread among the mice in the room. The infection was monitored for 3 months, and all 37 mice in the room were killed for detection of antibody to Sendai virus.

Forty-seven sera from SPF mice of different strains free from Sendai virus were also employed in the present study as negative controls.

To investigate a regression line between ELISA titers and ELISA values, 45 sera from mice infected with Sendai virus at an early stage of infection (day 6 to 12) were also used.

Procedures of ELISA: A modification of the ELISA method of Valler et al. (11) was used. From the results of preliminary experiments on the conditions affecting ELISA, the following procedures and conditions were employed. Each well of polystyrene flat-bottomed microtiter plates (Ts-Flat, Toyoshima Co., Tokyo) was coated with 50 μ l of virus antigen suspended in carbonate-bicarbonate buffer (pH 9.6) at a concentration of 4 μ g protein per ml overnight at 4 C and washed four times with 100 μ l of phosphate-buffered saline (PBS, pH 7.4) containing 0.05% (w/v) Tween 20 (PBS-Tween). All the subsequent steps were done in a volume of 50 μ l per well at room temperature, except the substrate reaction in 200 μ l. To avoid non-specific adsorption to the wells, buffer (PBS-Tween) containing 0.1% bovine serum albumin (BSA-buffer) was added to the antigen-coated well for 30 min. After removal of the BSA-buffer, a serum sample diluted in BSA-buffer was added to the wells and allowed to stand for 1 hr. After washing, HRP-conjugated goat anti-mouse immunoglobulin G (heavy- and light-chain specific: Cappel Laboratories, Cochranville, PA) diluted 1:1,000 was added and kept standing for 1 hr. After washing, a 200- μ l volume of freshly prepared substrate (0.3 mg per ml of ABTS, Nakarai Chemicals, Tokyo, plus 70 μ l of hydrogen peroxide per 100 ml of

citrate-phosphate buffer, pH 5.0) was added. The reaction giving an emerald green color was allowed to proceed for 45 min in the light before addition of 10 μ l of 1.25% sodium fluoride (Sigma Chemicals, St. Louis, MO) as a stopper. Absorbance at 415 nm (A415) was measured. Positive and negative control sera were always included and the difference larger than 0.20 in A415 was considered positive. The ELISA titer, therefore, was the reciprocals of the highest serum dilution giving an A415 larger than 0.20.

HI and CF test: The HI and CF tests were performed by the methods of Sever (12) and Suzuki et al. (1), respectively.

RESULTS

Conditions for ELISA for the Detection of Antibodies to Sendai Virus

Our aim was focused first at controlling the reaction-conditions for ELISA for serodiagnosis of Sendai virus infection. Although the adsorption of antigen to wells arrived at a peak in 30 min at 4 C (13), overnight adsorption at 4 C was adopted for routine use. To determine the optimal concentration of the viral antigen, the wells were coated with various concentrations of the antigen. They were washed and allowed to react with serum from infected mice and the specific reactions were determined by reading A415. From the standard curve, it was evident that ELISA value reached a plateau of approx. 1.6 OD at an antigen concentration of 6 μ g/ml (data not shown). This showed that an antigen concentration of 3 μ g/ml would give the highest antibody titer, but for routine assay, we selected an antigen concentration of 4 μ g/ml (0.2 μ g/well). ELISA with different concentrations of the conjugate showed that the 1,000-fold dilution would be suitable for use in the test (data not shown).

Further experiments were performed to seek the optimal temperature by which the serum, conjugate and substrate reactions are induced. From a series of experiments, we arrived at the following conclusion: all the reactions should be done at 25 C.

To know occurrence false-positive reactions, sera from 47 mice free from Sendai virus were examined with uninfected allantoic fluid (negative control antigen) by ELISA. Although two (4.3%) were found to react at a serum dilution of 1:40, an additional twofold dilution to 1:80 eliminated the non-specific reaction.

The reciprocal of the highest serum dilution with A415 larger than 0.20 was used as the antibody titer. Value smaller than 0.20 at a serum dilution of 1:80 were considered negative.

Parallel Titrations of Serum Antibody by ELISA, HI and CF Tests

ELISA was evaluated in the following two ways: One was the time course of antibody rise in C57BL/6 mice infected by contact with infected mice through nasal instillation. Thirty-three mice 4-week-old were used and three mice were randomly killed every 2 days for antibody titration by ELISA, HI and CF. As can be seen in Fig. 1, the antibody was first detected on day 6 by ELISA and all mice became antibody-positive two days later. HI and CF tests, however, failed to detect antibody on day 6 after infection, indicating a 2-day delay in seroconversion by these tests. Mice were found to possess the highest ELISA titers of 10,240 from 16 to 22 days post infection.

Fig. 1. The time course of antibody response determined by ELISA, HI and CF in sera of C57BL/6 mice infected by contact with Sendai virus-infected mice. Results are expressed as the mean ELISA antibody titers (●—●) with the standard deviation (vertical lines). HI (○-----○) and CF titers (■—■) are expressed in the same manner.

Table I. Titration of sera from mice naturally infected with Sendai virus¹⁾

Mouse No.	Strain	Titer to Sendai virus by		
		ELISA	HI	CF
1	ICR	1:160	— ²⁾	—
2		40	—	—
3		160	—	—
4		— ³⁾	—	—
5		—	—	—
6		320	—	—
7		320	1:10	—
8		320	10	1:20
9		320	20	10
10		320	10	10
11		640	20	10
12		320	20	5
13		320	20	10
14		640	40	20
15		1,280	40	10
16		320	10	10
17		2,560	20	20
18		320	20	10
19		1,280	20	10
20	GPC	1,280	20	40
21		1,280	20	40
22		2,560	40	40
23		5,120	40	40
24		40	10	20
25	C57BL/6	1,280	20	80
26		640	20	80
27		—	—	—
28		—	—	—
29	BALB/c	—	—	—
30		—	—	—
31		—	—	—
32		320	—	—
33	DD	640	—	10
34		—	—	—
35		—	—	—
36	DD	—	—	—
37		—	—	—

1) The infection of mice is explained in Materials and Methods.

2) Negative at 1:40 serum dilution by ELISA.

3) Negative at 1:5 serum dilutions by HI and CF.

The antibody titer was defined as the reciprocal of the highest serum dilution giving positive reaction in ELISA, complete inhibition in HI and positive reaction (no or less than 25% hemolysis) in CF.

Secondly, 37 SPF mice comprising BALB/c, GPC, DD and C57BL/6 strains were kept in the room where accidental infection with Sendai virus occurred. The infection was monitored for about 3 months after the possible introduction of the virus and all 37 mice were killed to examine for the antibody to Sendai virus by the three methods (Table I). Many mice were infected with Sendai virus and the antibody titer was markedly higher in ELISA than in HI or CF test. The antibodies with titers of 40 to 320 by ELISA were not detectable by HI or CF, showing again that ELISA is more sensitive than the others.

The Relationship between the ELISA Value and Antibody Titer

The antibody titers obtained by ELISA, HI and CF tests were compared to see if a linear relationship is observed among them. As shown in Table II, the

Table II. Comparison of ELISA to HI and CF titers to Sendai virus of mouse sera

Anti-body	Number of sera ex- amined	Number of sera with ELISA titer of								
		10,240	5,120	2,560	1,280	640	320	160	80	0
HI-titer										
640	2		2							
320	5	2	2	1						
160	8		2	3	2		1			
80	3				3					
40	4		1	1	1	1				
20	11			1	4	2	4			
10	7					1	5			1
5	0									1
0	30						3	2	1	24
CF-titer										
320	6		5	1						
160	6	1	1	3	1					
80	2			1	1					
40	7		1	1	5					
20	6			1	1	2	2			
10	11				2	2	4	3		
5	2						1			1
0	30						3	2	1	24

ELISA titers tended to increase in parallel with the HI and CF titers. The ELISA titers were 100-fold higher than the HI and CF titers. To obtain a regression line, serum samples were grouped according to the levels of ELISA titers. When ELISA values obtained at a single dilution of 1:80 of sera from mice were plotted against the antibody titers, a linear regression curve was obtained (Fig. 2). The linear regression curve of A415 of 0.24 to 1.4 indicates that an ELISA value at a single serum dilution of 1:80 can be converted to the antibody titer.

Fig. 2. The relationship between ELISA value and antibody units. Results are expressed as the mean ELISA value with the standard error (vertical line) versus the antibody units and a regression line is depicted. The regression equation is $y = 0.0891 X + 0.03$, and the correlation coefficient (r) is 1.00.

DISCUSSION

ELISA using HRP-conjugate and substrate-ABTS is useful for detection of antibody to Sendai virus. Conditions for ELISA, such as the concentration of the coating antigen (4 µg/ml), reaction temperature (25 C or room temperature for 1 hr), the concentration of enzyme-conjugated antiglobulin (1:1,000 dilution) and the dilution of the test serum, were determined. Alkaline phosphatase (AP) and its substrate, *p*-nitrophenyl phosphate (NP), have widely been used at 37 C (14) and satisfactory in many assays, whereas the present study revealed that the viral antigen was unstable at 37 C. For this reason, HRP and ABTS, which give an emerald green color in the light at 25 C, were used instead of AP and NP because of unstable antigenicity of Sendai virus. Comparison of the results obtained in ELISA with 70 sera with those in HI and CF led us to the conclusion that ELISA is very sensitive. Moreover, ELISA is very useful for detection of such low levels of antibody that can not be detected by HI or CF, detecting seroconversion at early stages of Sendai virus infection. False-positive reactions (4.3%) observed at a 1:40 serum dilution could be eliminated by an additional single twofold dilution to 1:80 of the test serum. Simple and rapid procedures, in addition to its high sensitivity, make ELISA more useful than HI and CF for serodiagnosis of Sendai virus infection in mice.

REFERENCES

1. Suzuki, E., Matsubara, J., Saito, M., Muto, T., Nakagawa, M. and Imaizumi, K. (1982): Japan. J. Med. Sci. Biol., 35, 249-254.
2. Parker, J. C. and Reynolds, R. K. (1968): Amer. J. Epidemiol., 88, 112-125.
3. Appell, L. H., Kovatch, R. M., Reddecliff, J. M. and Gerone, P. J. (1971): Amer. J. Vet. Res., 32, 1835-1841.
4. Robinson, J. W. E., Cureton, R. J. R. and Hearsh, R. B. (1968): J. Med. Microbiol., 1, 89-95.
5. Parker, J. C., Whiteman, M. D. and Richter, C. B. (1978): Infect. Immun., 19, 123-130.
6. Parker, J. C., Obeirne, A. J. and Collins, M. J. (1979): J. Clin. Microbiol., 9, 444-447.
7. Ertl, H. C. J., Gerlich, W. and Koszinowski, J. H. (1979): J. Immunol., Meth., 28, 163-176.
8. O'sullivan, M. J. and Marks, V. (1981): Meth. Enzymol., 73, 147-166.
9. Suzuki, E., Kinoshita, K., Muto, T., Nakagawa, M. and Imaizumi, K. (1982): Japan. J. Med. Sci. Biol., 35, 81-85.

10. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951): J. Biol. Chem., 193, 265-275.
11. Voller, A., Bidwell, D. and Barlett, A. (1976): *In* N. Rose and H. Friedman [eds.], p.506-512. Manual of clinical immunology American Society for Microbiology, Washington, D. C.
12. Sever, J. L. (1962): J. Immunol., 88, 320-329.
13. Bidwell, D. E., Bartlett, A. and Voller, A. (1977): J. Infect. Dis., 136, S274-278.
14. Gilman, S. C. and Docherty, J. J. (1977): J. Infect. Dis., 136, S286-293.