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Two enzyme immunoassays for the detection of antibody to rodent coronaviruses

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Two enzyme immunoassays for detection of antibody to rodent coronaviruses were compared. Mouse hepatitis virus (MHV), strain JHM, antigen was in the form of formalin-fixed, infected 17 C1 1 cells. This antigen detected antibody to the homologous strain of MHV as well as to two heterologous MHV strains and a serologically related rat coronavirus, sialodacryodenititis virus. Antibody titers in assays using horseradish peroxidase (HRP)-conjugated or ureiase-conjugated anti-mouse IgG were substantially higher than in an indirect immunofluorescence assay. The ureiase assay was somewhat more sensitive than the HRP assay. MHV-JHM antigen was stable under a variety of storage conditions for at least two months.

rodent coronavirus, mouse hepatitis virus, sialodacryoadenitis virus, enzyme immunoassay, horseradish peroxidase, ureiase

Introduction

The sentiment was expressed twenty years ago that the complement fixation (CF) test was inadequate for the detection of antibody to mouse hepatitis virus (MHV) among mice infected several months prior to testing (Parker et al., 1966). Despite reports of more sensitive serological tests, it was several years before these methods were generally used in rodent virus serology laboratories. Both the conventional enzyme-linked immunosorbent assay (ELISA) and indirect imunofluorescence (IFA) test were shown to be significantly more sensitive than the CF test for detection of antibody to MHV (Peters et al., 1979; Boorman et al., 1982; Smith, 1983). A commercially available ELISA kit was somewhat less sensitive than IFA for detection of MHV antibody in sera of experimentally infected mice (Smith, 1983).

The close antigenic relationship between MHV and the sialodacryodenitis (SDAV)rat coronavirus (RCV) complex allowed the use of MHV antigen as substrate for detection of antibody to these rat coronaviruses. The ELISA and IFA tests using MHV antigen were more sensitive than the CF or neutralization test

using homologous antigen for detection of antibody to these agents (Peters and Collins, 1983; Smith, 1983). The ability of MHV antigen to detect antibody to SDAV/RCV was fortunate, since growth of SDAV/RCV in cell culture has, until recently, been limited to primary rat kidney cells (Hirano et al., 1985).

IFA has been successfully used in this laboratory for the serodiagnosis of rodent coronavirus infection among both naturally and experimentally infected animals (Smith, 1983; Smith, 1986). IFA is easier to interpret than some other serological tests because cells reactive with antibody are viewed directly. Since MHV induces syncytia formation in infected cells, fluorescence in syncytial cells is one good indicator of specificity of reactions. IFA is, however, labor intensive in the case of pathogenesis experiments or other studies involving large numbers of animals and requiring tests of multiple antibody dilutions to determine titers. For such large scale testing, a more automated assay such as the ELISA is preferable. For the conventional ELISA, we have found it necessary to purify MHV antigen used to coat the solid phase in order to eliminate nonspecific reactivity. Such purification requires equipment that may be beyond the financial means of some laboratories. In addition, it may be difficult to obtain identical antigen concentrations for different batches of antigen. This is an important consideration if sensitivity of a diagnostic test is to be standardized. Lastly, the ability to inactivate the test antigen is desirable for laboratories in close proximity to animal quarters. Compounds such as psoralen and beta propiolactone may be used, or antigen may be irradiated. These procedures may also require expensive equipment.

We describe in this paper a simple, inexpensive method for preparing an inactivated MHV antigen for use in a sensitive enzyme immunoassay (EIA). The stability of the antigen under varied conditions is described. In addition, the sensitivities of a horseradish peroxidase (HRP)-conjugated anti-mouse IgG and a ureiase-conjugated anti-mouse IgG are compared.

Materials and Methods

Virus

MHV, strain JHM, was used as the detecting antigen in all assays. The virus was obtained from the American Type Culture Collection (Rockville, MD) and was passaged twice in NCTC 1469 cells (Hobbs et al., 1957), once in adult BALB/cByJ mouse brain and once in 17 Cl 1 cells (Sturman and Takemoto, 1972). This stock had a titer of 10^5 $TCID_{50}$ per ml.

MHV-S and MHV-A59 were also obtained from the American Type Culture Collection. SDAV, strain 681, was isolated and characterized at Yale University (Bhatt et al., 1972).

Mice

Inbred SJL/J mice (resistant to lethal MHV-JHM infection) were obtained from

the Jackson Laboratory (Bar Harbor, ME). Random-bred Crl:CD1(ICR)BR mice were obtained from Charles River Breeding Laboratories (Portage, MI). These mice were free of antibody to MHV and other conventional rodent viruses on arrival in the facility. They were housed in micro-isolator cages (Lab Products, Maywood, NJ) that were opened only in a class II biological safety cabinet.

Antibody

SJL/J mice were inoculated intranasally with 10^3 $TCID_{50}$ of MHV-JHM. Groups of mice were killed with CO_2 and exsanguinated on days 5, 10 and 20 post-inoculation. The serum fraction was stored at −20°C prior to testing.

Crl:CD1(ICR)BR mice were inoculated intraperitoneally with 10^4 $TCID_{50}$ of MHV-JHM, MHV-S or MHV-A59 in the form of mouse brain suspensions from infected neonatal mice. These mice were killed with CO_2 ten days later and exsanguinated. Sera from ten mice per virus strain were pooled and stored at −20°C.

A hyperimmune ascitic fluid containing SDAV (strain 681) antibody was prepared by weekly intraperitoneal injections of multiparous Crl:CD1(ICR)BR mice with SDAV-infected infant mouse brain emulsified with Freund's complete adjuvant. The resulting fluid was clarified by centrifugation and stored at −20°C. A hyperimmune ascitic fluid containing antibody to minute virus of mice (parvovirus) was similarly prepared and was used as a negative control during the course of these experiments.

IFA test

The indirect immunofluorescence test was performed as described previously (Smith, 1983). Briefly, teflon-coated slides containing MHV-JHM-infected L cells were exposed to mouse serum or ascitic fluid for 20 minutes, washed with phosphate buffered saline (PBS), treated for 20 minutes with fluorescein-conjugated goat anti-mouse immunoglobulin (Antibodies, Inc., Davis, CA) diluted 1:100 and containing 0.01% Evan's blue counterstain and washed again with PBS. Slides were viewed with an Olympus BH2 microscope after mounting with PBS:glycerol (1:9).

Enzyme immunoassay

Approximately 10^5 17 C1 1 cells, growing in 96-well cluster dishes (Falcon, Oxnard, CA), were infected with 10^3 $TCID_{50}$ per well of MHV-JHM. Twenty-four hours later, 100 μl of 10% neutral buffered formalin were added to 200 μl of existing medium in each well. At this time, 50–75% of each infected monolayer consisted of syncytial cells. In most experiments, the cells were washed with PBS after 24 h in formalin. In one experiment, the cells were washed after 6.5, 24 or 48 h in formalin to determine whether exposure time affected the ability of the antigen to react with antibody. For the EIAs, the cells were washed one additional time with PBS and then saturated with PBS containing 3% gelatin (blocking buffer). After 1 h at 37°C, antiserum diluted in PBS containing 0.5% bovine serum albumin

(BSA) was added for 1 h at 37°C. Following 2 washes with PBS, HRP-conjugated goat anti-mouse IgG (BioRad, Richmond, CA) diluted 1:3000 or ureiase™-conjugated rabbit anti-mouse IgG (Allelix, Mississauga, Ontario, Canada) diluted 1:1000 was added for 1 h at 37°C. Conjugates were diluted in PBS containing 0.5% BSA. All plates were then washed three times with PBS. The ureiase assays were washed three additional times with distilled, demineralized water according to instructions in the product insert. ABTS (HRP substrate, Kirkegaard and Perry, Gaithersburg, MD) or ureiase substrate solution (Allelix) was then added to the appropriate plates which were read spectrophotometrically after 45 min at 37°C. HRP plates were read at 410 nm, and ureiase plates were read at 570 nm. Negative wells in the ureiase assay were bright yellow, whereas positive wells were deep purple.

Antigen stability

After formalin fixation, some plates were stored in sterile PBS at 4°C for 1, 2, 4, 6, or 10 wk prior to testing with HRP conjugate. Other plates were air-dried and stored at room temperature, 4°C, −20°C or −70°C for 4 or 8 wk prior to testing in the HRP and ureiase assays.

Calculations

All sera were tested on infected and uninfected cells. Optical density (OD) values for uninfected cells were subtracted from OD values for infected cells. Corrected OD values were considered significant it they exceeded by 3 SD the mean value for appropriate negative control antisera in the same assay. Antibody titers are expressed as the reciprocal of the highest antibody dilutions resulting in significant corrected OD values. Geometric mean antibody titers were calculated according to the method described by White (1973).

Results

Antibody detection

Initially, the effect of formalin fixation time on the ability of MHV-JHM antigen to detect antibody was studied. Uninfected and infected 17 C1 1 cells were exposed to a final concentration of 3.3% formalin for 6.5, 24 or 48 h. All of these fixation times resulted in inactivation of infectious virus in the supernates of infected cultures (data not shown). In EIAs using HRP-conjugated anti-mouse IgG, OD values were unaffected by fixation time. Representative results with two of the five antibody preparations tested are shown in Table 1.

In another experiment, the sensitivities of HRP-conjugated and ureiase-conjugated anti-mouse IgGs were compared (Table 2). Results are shown for two similarly prepared hyperimmune mouse ascitic fluids. OD values for the ureiase con-

TABLE 1

Effect of formalin exposure time on antibody detection by MHV-JHM antigen in EIA using HRP-conjugated anti-mouse IgG.

Time of fixation	Antibody dilution	Antiserum					
		MHV-JHM			MVM		
		U	I	I–U	U	I	I–U
6.5 h	1:160	0.11	0.49	0.38	0.11	0.14	0.03
	1:320	0.11	0.46	0.35	0.10	0.10	0.00
	1:640	0.09	0.34	0.25	0.08	0.08	0.00
	1:1280	0.07	0.26	0.19	0.07	0.07	0.00
	1:2560	0.07	0.18	0.11	0.07	0.06	–0.01
24 h	1:160	0.12	0.52	0.40	0.13	0.12	–0.01
	1:320	0.11	0.42	0.31	0.13	0.13	0.00
	1:640	0.10	0.36	0.26	0.08	0.13	0.05
	1:1280	0.08	0.28	0.20	0.07	0.07	0.00
	1:2560	0.07	0.19	0.12	0.11	0.06	–0.05
48 h	1:160	0.13	0.50	0.37	0.12	0.14	0.02
	1:320	0.12	0.42	0.30	0.10	0.11	0.01
	1:640	0.09	0.30	0.21	0.08	0.09	0.01
	1:1280	0.08	0.21	0.13	0.07	0.08	0.01
	1:2560	0.07	0.18	0.11	0.06	0.07	0.01

Results are given as $O.D._{410}$ for uninfected (U) and MHV-JHM-infected (I) 17 Cl 1 cells.

jugate with uninfected cells were consistently higher than for the HRP conjugate with uninfected cells, irrespective of specific antibody content in the ascitic fluid. However, the corrected OD values ($OD_{infected}$–$OD_{uninfected}$) were also substantially higher for the ureiase conjugate. Both conjugates were used in parallel for further studies.

Earlier studies with BALB/cByJ mice (genetically susceptible to lethal MHV-JHM infection) and SJL/J mice (genetically resistant to lethal MHV-JHM infection) had shown that BALB/cByJ mice are uniformly seropositive by 10 days post-

TABLE 2

Comparison of HRP-conjugated and ureiase-conjugated anti-mouse IgGs for detection of SDAV antibody in hyperimmune mouse ascitic fluid.

Antibody dilutions	Antibody											
	SDAV						MVM					
	HRP			ureiase			HRP			ureiase		
	U	I	I–U	U	I	I–U	U	I	I–U	U	I	I–U
1:640	0.12	0.71	0.59	0.31	1.19	0.88	0.09	0.06	–0.03	0.29	0.23	–0.06
1:1280	0.10	0.63	0.53	0.30	1.24	0.94	0.10	0.09	–0.01	0.28	0.19	–0.09
1:2560	0.09	0.43	0.34	0.26	0.98	0.72	0.08	0.10	0.02	0.36	0.21	–0.15
1:5120	0.08	0.11	0.03	0.26	0.66	0.40						
1:10240				0.24	0.44	0.20						

Results are given as $O.D._{410}$ (HRP) and $O.D._{570}$ (ureiase) for uninfected (U) and MHV-JHM-infected (I) 17 Cl 1 cells.

TABLE 3

Detection of antibody to MHV-JHM in sera of experimentally infected SJL/J mice.

Post-inoculation day	Indirect immunofluorescence	Horseradish peroxidase EIA	Ureiase EIA
5	0/8	0/8	0/8
10	0/8	2/8 (20)	2/8 (30)
20	5/7 (10)	5/7 (480)	5/7 (1120)

Sera were screened by immunofluorescence at a dilution of 1:10 and by EIA at a dilution of 1:20. Results are given as number positive/number tested (geometric mean titer for positive sera).

inoculation, whereas SJL/J mice are still seronegative at this time (manuscript in preparation). These data were based on IFA testing with sera diluted 1:10. One reason for developing the assays reported here was to determine whether EIA would be a more sensitive means of detecting antibody in sera of MHV-infected SJL/J mice.

Sequentially collected sera from SJL/J mice infected intranasally with 10^3 $TCID_{50}$ of MHV-JHM were tested by IFA and the HRP and ureiase EIAs (Table 3). Both EIAs were somewhat more sensitive than IFA in their ability to detect antibody in sera of SJL/J mice. Two of eight mice tested on day 10 were antibody-positive in both EIAs, whereas all eight were seronegative based on the IFA test. The EIAs detected the same seropositive mice as IFA on post-inoculation day 20, but antibody titers were significantly higher in the HRP EIA than in the IFA test.

The ability of the HRP and ureiase assays to detect antibody to heterologous as well as homologous viruses was then further assessed (Table 4). Both enzyme immunoassays were more sensitive than IFA. Antibody titers were 4- to 64-fold higher in the HRP EIA than in the IFA test. Titers in the ureiase EIA were 4- to 256-fold higher than in the IFA test. For MHV-A59 and SDAV antibody preparations, ureiase assay titers were 4-fold higher than HRP titers.

Antigen stability

One series of plates was fixed with formalin for 6.5 h and stored at 4°C in PBS for up to 10 wk. Serial antibody dilutions were freshly prepared at each assay in-

TABLE 4

Titers of immune reagents prepared against three strains of mouse hepatitis virus and a rat coronavirus, sialodacryoadenitis virus, with MHV-JHM antigen.

Antibody	Test		
	Indirect immunofluorescence	Horseradish peroxidase EIA	Ureiase EIA
MHV-JHM	640	2560	2560
MHV-A59	160	640	2560
MHV-S	20	1280	1280
SDAV (hyperimmune)	40	2560	10240

TABLE 5

Stability of MHV-JHM antigen during storage in PBS at 4°C.

Weeks in PBS	Antibody			
	MHV-JHM	MHV-S	MHV-A59	SDAV
0	1280	320	1280	2560
1	2560	640	2560	2560
2	2560	640	1280	2560
4	2560	320	2560	5120
6	2560	640	2560	5120
10	1280	640	2560	5120

Results are expressed as the antibody titer determined from EIAs using horseradish peroxidase-conjugated antimouse IgG.

terval, and plates were tested only with the HRP conjugate. As shown in Table 5, JHM antigen was stable under these conditions. Titers of four rodent coronavirus antibody preparations were essentially unchanged during the ten week period with no more than two-fold variation for any single antiserum.

A second series of plates was formalin-fixed for 24 h, washed with PBS and air dried prior to storage at room temperature, 4°C, −20°C or −70°C. These plates

TABLE 6

Stability of MHV-JHM antigen after drying and storage at four temperatures.

Antibody	Storage temperature (°C)	Horseradish peroxidase			Ureiase		
		T0	T1	T2	T0	T1	T2
MHV-JHM		1280			2560		
	room		1280	640		2560	1280
	4		640	1280		1280	1280
	−20		2560	1280		5120	2560
	−70		1280	2560		2560	2560
MHV-S		640			1280		
	room		320	<80		640	640
	4		160	160		320	320
	−20		320	<80		640	640
	−70		320	160		320	320
MHV-A59		640			2560		
	room		1280	640		2560	1280
	4		640	320		2560	1280
	−20		640	320		2560	1280
	−70		1280	640		1280	160
SDAV		2560			10240		
	room		2560	1280		5120	2560
	4		2560	2560		5120	5120
	−20		2560	1280		20480	5120
	−70		2560	1280		5120	5120

T0 = plates tested after 24 h in formalin; T1 = plates tested after 24 h in formalin plus 1 month dry storage; T2 = plates tested after 24 h in formalin plus 2 months dry storage.

were tested with both the HRP and ureiase conjugates after one and two months of storage. A single dilution series of each antibody was prepared and stored at −20°C between assays. As shown in Table 6, titers of individual antisera generally did not vary more than four-fold irrespective of storage conditions of the antigen. There were three notable exceptions. With the HRP conjugate, MHV-JHM antigen stored for two months at room temperature or at −20°C did not detect MHV-S antibody. These antigen storage conditions did not affect reactivitiy with MHV-S antibody in the ureiase assay. Additionally, MHV-A59 antibody had a substantially reduced titer with MHV-JHM antigen stored for two months at −70°C and tested with the ureiase conjugate. Antigen stored similarly and tested with MHV-A59 antibody and HRP conjugate was as reactive as freshly prepared antigen.

Discussion

The data presented above support the contention that MHV-JHM antigen in the form of formalin-fixed, infected 17 C1 1 cells is capable of detecting antibody to both the homologous MHV strain as well as to two heterologous MHV strains (MHV-S and MHV-A59) and to an antigenically related rat coronavirus (SDAV) in enzyme immunoassays. Antigen preparation is simpler than for the conventional ELISA and the antigen is readily inactivated. A ureiase conjugate was somewhat more sensitive than an HRP conjugate, since antibody titers were often higher with the ureiase conjugate. The dramatic color development in the ureiase assay may render it quite useful for laboratories without access to spectrophotometric equipment.

MHV-JHM antigen was stable for at least two months under almost all storage conditions tested. The exceptions found in this study require confirmation. In addition, longer term stability studies are required to determine precisely how long the antigen may be stored without deleterious effects.

Both the EIAs described here were more sensitive than the IFA test. Large numbers of plates may be prepared with minimal labor. Antibody titers in large experimental studies, such as pathogenesis studies, may be determined with much less labor than is involved in the IFA test.

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