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Detection of Antibody to Murine Cytomegalovirus by Enzyme-Linked Immunosorbent and Indirect Immunofluorescence Assays

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We have compared murine cytomegalovirus (MCMV) antibody determination by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay. A comparison of antibody detection with 146 serum samples at a 1:20 dilution showed 100% agreement (60 negatives and 86 positives) between the assays. There was close agreement of endpoint determinations of sera by both methods. After experimental MCMV infection, antibody to MCMV was detected by both assays as early as day 7, and high titers persisted as late as 6 months. In contrast to immunocompetent littermates, athymic nude mice did not develop antibody after infection. Mice lacking antibody detectable by ELISA were susceptible to lethal MCMV challenge. In a survey of animals from five commercial sources, MCMV antibody was not detected unless mice were experimentally infected. MCMV antibody determination by ELISA is a convenient method, comparable to the indirect immunofluorescence assay in sensitivity and specificity.

Cytomegalovirus (CMV) infections continue to play an increasingly important role as a source of human disease. CMV is now the most common infectious cause of congenital abnormalities and is a major cause of disease and death for individuals with alterations in host immune defenses (7, 19). Because of similarities in host-virus interactions, murine CMV (MCMV) infection has provided models of acute, chronic, and latent CMV infections in humans (9, 15) and has been an important source of understanding of CMV infections. Because of this role, a sensitive, reliable, and convenient method of measuring antibody responses to MCMV after virus infection or of screening mice for previous MCMV infection is essential to the understanding and utility of these models.

In the past, antibody responses to MCMV infection have been measured by a number of techniques adapted from the methods used for evaluation of human CMV (8). These include neutralization, complement-enhanced neutralization, and complement fixation assays and variations of indirect immunofluorescence assay (IFA) (1, 4, 11, 14, 17). However, these assays have a number of undesirable aspects. For example, the neutralization assays require the maintenance of tissue cultures for virus propagation. The complementation assay is somewhat cumbersome, while IFAs require specialized equipment. The new generation of enzyme-linked immunosorbent assays (ELISAs) for the detection of antibody has answered many of these problems, providing reliable, convenient, and rapid antibody assays for use with a number of organisms (20). ELISAs have been applied for a number of viruses, including MCMV and human CMV (1, 3, 13, 18). Using an ELISA for MCMV, Anderson and co-workers recently reported that 54.7% of previously uninfected mice obtained from commercial suppliers possessed antibody to MCMV (2). Prompted by this report, we have used an ELISA to examine the antibody response to MCMV infection and to determine the frequency of preexisting antibody in uninfected mice supplied by commercial sources.

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MATERIALS AND METHODS

Mice. For these experiments, male and female BALB/c AnN, female DBA/2N, and homozygous (*nu/nu*) and heterozygous (*nu/+*) athymic nude mice were obtained from Charles River Breeding Laboratories, Inc., Kingston, N.Y. C57BLk/10SCN and B10.A mice were obtained from Harlan Sprague-Dawley Inc., Madison, Wis. BALB/c heterozygous and homozygous nude mice were obtained from the National Cancer Institute, Frederick, Md. Female B10.BR and C57BL/10SNJ (B10) mice were purchased from Jackson Laboratory, Bar Harbor, Maine. In addition, male and female BALB/c AnN and B10.BR mice were bred in our animal care facility.

Animals were maintained in groups of 5 to 10 in isolator units and were fed Laboratory Chow 500 (Ralston Purina, St. Louis, Mo.) and water ad libitum. The colony was monitored for both MCMV infection and other respiratory pathogens. The mice were 6 to 8 weeks of age before use.

Virus. The Smith strain of MCMV was originally obtained from M. C. Jordan, University of Minnesota, Minneapolis. The virus was maintained by serial passage in BALB/c mice and was prepared as a 10% (wt/vol) homogenate of salivary gland tissue. The inoculum for sham-infected controls was prepared from the salivary glands of uninfected BALB/c mice. Virus and control stocks were stored at −70°C with 10% dimethyl sulfoxide as a stabilizer. The virus pool for these experiments, which contained 1.6×10^7 PFU of virus per ml, was screened by an antibody generation test (Microbiological Associates, Bethesda, Md.) and was found to be free of other murine pathogens, including pneumonia virus of mice, reovirus type 3, K virus, polyomavirus, Sendai virus, minute virus of mice, murine adenovirus, lymphocytic

choriomeningitis virus, murine hepatitis virus, ectromelia virus, and lactic dehydrogenase virus.

Cell culture methods. Mouse embryo cells (MEC) were prepared from embryos of late-term pregnant CD-1 mice (Charles River Breeding Laboratories) by trypsin-EDTA disaggregation and were maintained in tissue culture with Eagle minimum essential medium containing 10% fetal calf serum, 100 U of penicillin per ml, and 50 μg of gentamicin per ml. Quantitation of virus was performed by a plaque assay of MEC with a semisolid overlay containing minimum essential medium, 1% methylcellulose, 10% fetal calf serum, 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and antibiotics.

Virus inoculation of mice. To infect animals, 6- to 8-week-old BALB/c mice were inoculated intraperitoneally (i.p.) with 0.2 ml of a solution of 10^3 PFU of stock virus diluted in phosphate-buffered normal saline (PBS). Homozygous and heterozygous athymic nude mice were infected with 10^3 PFU injected subcutaneously. Control animals were sham inoculated in parallel with an equivalent volume of normal salivary gland homogenate diluted in PBS.

The virus dose of the virus pool producing 50% mortality was also determined. Groups of 10 6-week-old BALB/c mice were inoculated i.p. with 0.2 ml of serial 10-fold dilutions of virus, and mortality over 14 days was assessed. The 50% lethal dose of virus was then calculated by the Reed-Muench method (16).

Serum collection. Serum samples were collected from uninfected mice at 6 to 8 weeks of age, within 1 week of delivery from the supplier. Serum samples were also collected from uninfected mice reared at our animal care facility when the mice were 6 to 10 weeks old. Sera from MCMV- or sham-infected mice were also collected at various times after inoculation as indicated below.

To collect serum, mice were killed by using chloroform or CO_2 narcosis. The thoraxes were opened, the right ventricles were incised, and blood was collected. Serum was stored at −20°C until assay.

Indirect IFA for MCMV antibody. Serum samples were evaluated for antibody to MCMV by a modification of the indirect IFA of Griffith et al. (6). MCMV antigen targets were prepared by infecting MEC monolayers with virus at a multiplicity of infection of 0.1. Sham-infected cells were used as negative controls. When the cytopathic effect involved at least 80% of the MEC monolayer, the cells were mobilized with 0.25% trypsin, washed twice in PBS, and suspended in PBS containing 2.5% fetal calf serum. The MCMV or control cell suspension (25 μl) was placed on the target area of a clean, premarked toxoplasmosis microscope slide (Bellco Glass, Inc., Vineland, N.J.) and air dried. After fixation in acetone at 4°C for 10 min, the slides were stored in a desiccator chamber at 4°C until use.

The serum samples to be tested were initially diluted 1:20 with PBS. For endpoint titration, a twofold dilution series of each serum was prepared. The slides containing MCMV-infected and control MEC were rehydrated in PBS at room temperature for 5 min; then 25 μl of the serum dilution was added to the cell targets and incubated for 30 min at 37°C in a humidity chamber. The slides were washed for 10 min in PBS with 0.1% Tween 20 (PBS-T). Fluorescein-labeled rabbit anti-mouse immunoglobulin G (25 μl; heavy and light chain specific; Cooper Biomedical, Inc., West Chester, Pa.) diluted 1:40 in PBS-T was added to the cells and incubated at 37°C for 30 min. After being washed in PBS-T, the cells were mounted in barbital-buffered glycerol (pH 9.6). The cells were then examined for distinct nuclear immunofluorescence. The titer of a serum sample was defined as the last dilution of serum producing distinct nuclear staining. All tests were run with both a control serum with a known titer for antibody to MCMV and sera from uninfected mice.

ELISA for antibody to MCMV. For this study, the ELISA for antibody to MCMV was a previously reported modification of the assay that Castellano and co-workers used for evaluation of human CMV (3, 18). MCMV antigens were prepared by the alkaline-glycine extraction method of Kettering et al. (10), with some modifications. MEC monolayers in 75-cm^2 tissue culture flasks were infected with MCMV at a multiplicity of infection of 0.1. Sham-inoculated MEC were processed in parallel. When cytopathic effect involved 30 to 50% of the cells, the cell monolayers were washed three times with sterile PBS and 20 ml of minimum essential medium containing antibiotics, but no fetal calf serum was replaced. When the cytopathic effect was confluent, the cells were scraped off of the monolayer surface, pelleted at 700 × *g*, and suspended in 0.1 M glycine buffer (pH 9.5) at 1/20 the original culture volume. The suspension was sonicated three times for 10 s each time with a sonicator (Branson Sonic Power Co., Danbury, Conn.). The suspension was then clarified by centrifugation at 700 × *g*, and the supernatants were stored in 0.5-ml portions at −70°C until use.

Antigen was also prepared by partially purifying MCMV virions from tissue culture supernatant of maximally infected MEC (12, 13, 18). Briefly, MEC monolayers were prepared as described above for the glycine extraction. However, virus infection was allowed to proceed until the monolayers had been extensively destroyed. After one cycle of freezing and thawing, the supernatants were clarified by centrifugation at 700 × *g*. The virions in the supernatants were pelleted by centrifugation at 130,000 × *g* for 1.5 h at 4°C. The virus pellet was suspended in 0.06 M sodium carbonate buffer (pH 9.6) at 1/40 the original volume and stored at −70°C in 0.5-ml portions. Control antigen was prepared in parallel with uninfected MEC.

The optimal working dilutions of MCMV and control antigens were determined by standard checkerboard titration with known MCMV immune serum and sera from uninfected mice.

For measurement of antibody to MCMV by the ELISA, 0.2 ml of MCMV or control antigen diluted in 0.06 M carbonate buffer, pH 9.6, was added to each well of a Linbro enzyme immunoassay plate (Flow Laboratories, Inc., McLean, Va.) and incubated overnight at 4°C. Wells incubated with only carbonate buffer served as an additional negative control. The wells were washed three times with PBS-T, and 0.2 ml of the serum samples diluted in PBS-T was added to wells labeled with MCMV antigen, control antigen, or carbonate buffer. The serum samples were screened for antibody at a 1:20 dilution. The plates were then incubated for 45 min at 37°C. After four washes of plates with PBS-T, 0.2 ml of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (heavy and light chain specific; Cooper Biomedical) diluted 1:200 in PBS-T was added to each well at 37°C for 45 min. After the plates were washed, 0.2 ml of color substrate (100 μg of *o*-phenylenediamine containing 0.3% H_2O_2 per ml) was added to each well, and the plates were incubated at room temperature for 30 min. The plates were then visually inspected and read with an ELISA reader (Artek) at 488 nm. Each assay included endpoint titration of both an MCMV-immune serum of known titer and a negative control serum from an uninfected animal.

Statistical analysis. Both ELISA and IFA were performed

with encoded samples. Comparison of assay methods was performed by least-mean-square analysis, and the correlation coefficient of the titers was determined by linear regression.

RESULTS

Reaction in ELISA of serum samples from uninfected animals. To establish criteria for the identification by ELISA of sera containing antibody to MCMV, the variation in colorimetric reactivity of sera derived from uninfected animals was assessed. Serum samples from 28 uninfected BALB/c mice, which were nonreactive by IFA, were evaluated at a 1:20 dilution in the ELISA by using the MCMV and control antigens prepared by alkaline-glycine extraction. The A_{488} (mean ± standard deviation) for serum samples reacted with MCMV antigen was 0.038 ± 0.020 (range, 0.005 to 0.088), and the A_{488} for serum samples reacted with control antigen was 0.032 ± 0.019 (range, 0.010 to 0.084). Therefore, we selected an absorbance value for MCMV antigen of ≥0.098, which is 3 standard deviations from the mean optical density of the negative sera, to identify sera with significant reactions to MCMV antigen. We also wished to identify sera with significant cross-reactions to control antigen to ensure that the reaction to MCMV antigen was specific. Thus, the corrected absorbance, i.e., absorbance of MCMV antigen − absorbance of control antigen, was determined for the 28 negative serum samples. The mean corrected absorbance (± standard deviation) was 0.0048 ± 0.012. A corrected absorbance of ≥0.041, a value 3 standard deviations from the mean, was selected to identify sera which lacked significant cross-reactions to the control antigen. Based on these criteria, none of the 28 serum samples which were from animals not infected in the laboratory and which were nonreactive by IFA would have been identified as containing antibody to MCMV.

Comparison of ELISA and IFA in detection of antibody to MCMV. To compare the performance of ELISA and IFA for detection of antibody to MCMV, 146 serum samples were screened by both assays at a 1:20 dilution. Sixty serum samples were collected from mice after more than 14 days of infection with MCMV, and 86 samples were collected from uninfected mice. All 60 serum samples from the infected animals were positive by both assays. Similarly, no antibody was detected by either assay in sera from uninfected mice.

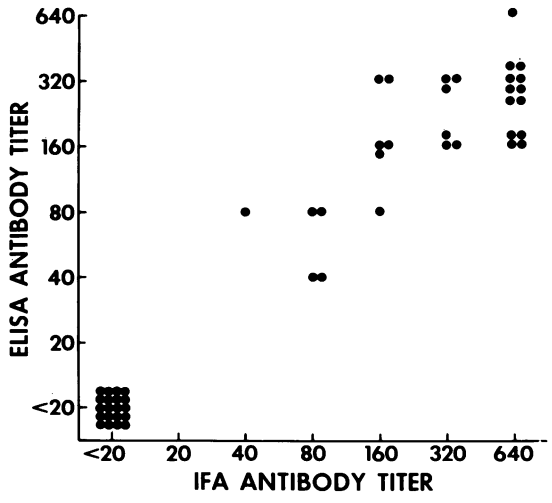

FIG. 1. Comparison of antibody titers as determined by ELISA and IFA. Serum samples were from 30 MCMV-infected mice (>14 days after i.p. inoculation with 10^3 PFU) and 20 uninfected mice.

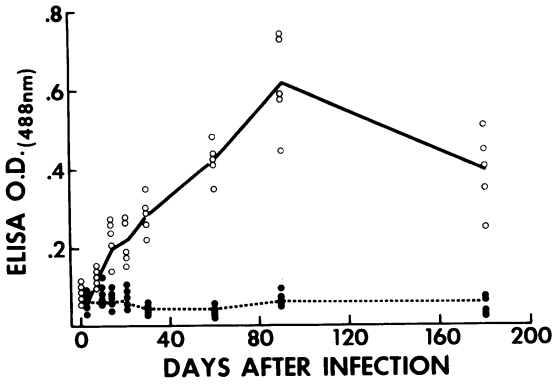

FIG. 2. ELISA colorimetric reactions to MCMV (○) and control (●) antigens from BALB/c mice after i.p. inoculation with 10^3 PFU of MCMV. O.D., Optical density.

Endpoint titrations of sera reactive by both IFA and ELISA were then compared with 30 serum samples containing antibody to MCMV (Fig. 1). Although there was a very close agreement among the titers of seropositive animals as determined by both assays (r = 0.732 by linear regression analysis), the titers determined by the IFA tended to be higher than those determined by the ELISA.

Comparison of MCMV antigens for ELISA. Several authors have reported using partially purified virions as a source of antigen for the ELISA (13, 18). Therefore, we compared the reactivity of the MCMV and control antigens prepared from partially purified virions to the antigens prepared by alkaline-glycine extraction. Of 23 serum samples evaluated for antibody, 14 were from uninfected mice and 9 were from MCMV-infected mice after more than 14 days of infection. There was no difference in the colorimetric reactions of sera from uninfected mice with either MCMV or control antigens prepared by either method. Antibody to MCMV was detected in nine of nine serum samples from MCMV-infected mice. The balance of the studies were performed by using the alkaline-glycine-extracted antigen.

Detection of antibody after MCMV infection. We examined the appearance of antibody detectable at a 1:20 dilution by IFA and ELISA after laboratory infection of BALB/c mice with 10^3 PFU of MCMV given i.p. Antibody was not detected in the sera of five mice by either assay 3 days after infection. Seven days after inoculation, antibody was detected in five of five mice by IFA and in three of five mice by ELISA. Thereafter, antibody was detectable by both assays in five of five mice at 14, 21, 30, 60, 90, and 120 days after inoculation. Antibody to MCMV was not detected in sham-inoculated control mice by either assay.

The development of antibody to MCMV after virus inoculation was evaluated by both IFA and ELISA. The development of ELISA reactivity to MCMV and control antigens is shown in Fig. 2. In general, the time course and magnitude of antibody responses to MCMV were comparable in both assays, although the titers determined by IFA were slightly higher. Antibody at a high titer (≥1:160) detected by both assays persisted in the sera as late as 6 months after virus inoculation.

MCMV antibody in athymic nude mice. The appearance of antibody to MCMV in T-cell-deficient athymic nude mice (*nu/nu*) and in their immunocompetent heterozygous (*nu/+*)

TABLE 1. Detection by ELISA of antibody to MCMV in the sera of T-cell-deficient athymic nude mice (*nu/nu*) and their immunocompetent heterozygous (*nu/+*) littermates after infection with MCMV[a]

Mouse genotype	No. of mice with MCMV antibody/no. tested at day:					
	0	3	7	10	14	21
nu/+	0/5	0/2	0/2	3/3	3/3	3/3
nu/nu	0/5	0/5	0/5	0/5	0/5	0/5

[a] Serum samples were screened for antibody at a dilution of 1:20. Samples were obtained from mice after intranasal inoculation with 10^3 PFU of MCMV.

littermates after laboratory infection was assessed (Table 1). At a serum dilution of 1:20, antibody to MCMV was not detected in sera of uninfected *nu/+* or *nu/nu* mice or in *nu/+* mice at 3 and 7 days after virus challenge. Antibody to MCMV was detected in the sera of five of five *nu/+* mice at 10, 14, and 21 days after virus inoculation. In contrast, antibody to MCMV was not detected in the sera of *nu/nu* mice challenged with MCMV, even at 21 days after inoculation.

Detection of MCMV antibody in the sera of mice from commercial sources. Sera from mice from a variety of commercial suppliers were screened at a 1:20 dilution for the presence of antibody to MCMV. Individual serum samples from 90 mice obtained from four commercial sources and from 16 mice reared in our facility were screened for antibody (Table 2). In addition, 29 BALB/c mice experimentally infected in our laboratory were used as antibody-positive controls. Unless experimentally infected in the laboratory, none of the mice from commercial suppliers or from our facility were found to possess antibody to MCMV. In contrast, all 29 mice that were infected in the laboratory developed antibody responses to MCMV detectable by ELISA.

As an additional control, BALB/c mice, whose sera were devoid of antibody to MCMV by ELISA, were examined for susceptibility to lethal virus challenge. Ten mice without antibody to MCMV were inoculated with two BALB/c-derived 50% lethal doses given i.p. Mice previously infected with MCMV and possessing antibody to MCMV were also inoculated. After 14 days, none of the 10 seronegative mice survived, while 10 of 10 mice with antibody did survive.

DISCUSSION

The development of ELISAs for antibody detection has provided a convenient, reliable, and sensitive alternative to previous antibody assays (20). However, the utility of an ELISA depends on careful standardization of the assay conditions to permit discrimination of sera which contain or lack specific antibody. We have previously used an ELISA for detection of antibody to MCMV (18). Evaluation of this test system indicates that this measurement by ELISA compares favorably in sensitivity and specificity with the measurement of antibody by IFA. In developing this assay, we also examined the development of antibody after experimental infection. In both assays, antibody to MCMV in serum diluted 1:20 was detectable early in the course of infection. Moreover, antibody in the serum persisted at a relatively high titer for up to 6 months after infection. The absence of antibody correlated with susceptibility to lethal MCMV challenge. These observations suggest that the ELISA would be useful as a means of screening mice for previous infection.

We examined MCMV antigens prepared by the alkaline-glycine extraction of MCMV-infected cells and by the partial purification of virions. The MCMV proteins present in these preparations have not been defined, although one study has demonstrated that alkaline-glycine antigens of human CMV predominantly contain nucleocapsids with some virions and viral membranes (5). Presumably, the partially purified antigen contains primarily MCMV structural proteins. In this limited evaluation, there were no differences in the detection of sera which contained or lacked antibody to MCMV, although a broader study is needed to confirm this agreement. The preparation for ELISA of antigens from virions provides an alternative to the alkaline-glycine method of antigen preparation.

We also compared the development of antibody to MCMV in T-cell-deficient (*nu/nu*) mice with that of their immunocompetent (*nu/+*) littermates. As with the BALB/c mice, antibody developed rapidly in the *nu/+* mice after experimental infection. In contrast, the *nu/nu* mice failed to generate antibody responses, indicating that production of antibody against MCMV is a T-cell-dependent function. It was also reassuring that there were no false-positive serological tests for the *nu/nu* mice.

Anderson and co-workers previously described a similar ELISA for MCMV, but with some notable differences (1, 2). Their assay used an alkaline phosphatase conjugate, rather than horseradish peroxidase. Additionally, they screened sera at a 1:100 dilution. After experimental infection, their assay compared favorably with both the complement fixation and the nuclear anticomplement immunofluorescence antibody assays for the detection of antibody to MCMV. However, antibody responses to MCMV were not detected by the ELISA for 4 weeks, while antibody was detected by the nuclear anticomplement immunofluorescence antibody assay within 10 days. The late appearance of antibody in their ELISA was probably due to the fact that serum was screened at a 1:100 dilution. In our study, antibody was detectable as early as 7 days after infection in sera screened at a 1:20 dilution, and high antibody titers to MCMV persisted for up to 6 months.

Anderson et al., using their ELISA, recently detected ELISA responses in 54.7% of previously uninfected mice supplied by commercial suppliers (2). Although antibody to

TABLE 2. Detection by ELISA of antibody to MCMV in the sera of 106 uninfected and 29 experimentally infected mice from five commercial sources[a]

Kind of mouse	Source[b]	No. of mice with antibody to MCMV/no. tested by:	
		ELISA	IFA
Uninfected	A	0/28	0/28
	B	0/38	0/28
	C	0/20	0/20
	D	0/4	0/4
	E	0/16	0/16
Experimentally infected	A	25/25	22/22
	D	4/4	ND[c]

[a] Serum samples were screened for antibody at a dilution of 1:20. Samples were obtained from experimentally infected mice at >14 days after inoculation with 10^3 PFU of MCMV.

[b] A, Charles River Breeding Laboratories, Inc.; B, Harlan Sprague-Dawley; C, Jackson Laboratory; D, National Cancer Institute; E, Newington Veterans Administration Medical Center, Newington, Conn.

[c] ND, Not done.

MCMV was not detected by either complement fixation or nuclear anticomplement immunofluorescence antibody assay, we speculated that the ELISA reactivity represented previous subclinical infections in these mice. In contrast, in our ELISA of sera from mice from five U.S. sources, antibody to MCMV was not detectable in mice unless they had been experimentally infected in the laboratory. We also found that the presence of antibody correlated with resistance to lethal virus challenge. The reasons for the discrepancies between the two studies are not clear, but are probably due to technical differences in the conduct of the respective ELISAs. However, based on the susceptibility of mice to MCMV challenge and their subsequent antibody responses after experimental infection, it appears that antibody to MCMV is not common in mice from U.S. commercial suppliers.

The findings of these studies indicate that the ELISA is a simple and convenient method for detection of antibody to MCMV, comparing favorably in sensitivity and specificity with IFA. The ELISA is also a useful way to determine antibody responses to MCMV after experimental infection and to survey animals for previous MCMV infection. However, careful standardization of the ELISA is essential to minimize the problem of false-positive reactions.

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