Enzyme-Linked Immunosorbent Assay for Immunoglobulin G Antibody to Encephalomyocarditis Virus

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An enzyme-linked immunosorbent assay for immunoglobulin G antibody to encephalomyocarditis virus was developed. This assay was comparable to antibody assay by neutralization. Its adaptability should be useful for laboratory and epidemiological studies of infections due to encephalomyocarditis virus.

Infections due to encephalomyocarditis (EMC) virus are widespread in nature (11). Although this virus is considered to be primarily an infectious agent of rodents, infections of other vertebrates, including humans, occur (1, 9-11). EMC virus has been isolated from humans, non-human primates, and other mammals and has been associated with a number of epizootics in domestic animals (5, 6, 8). Several strains of EMC virus have been isolated which are closely related antigenically, but differ in the pathogenesis of infection that they produce (4, 11). One of these strains, the M strain of EMC virus (EMC-M), has been the focus of recent interest because of its propensity to cause diabetes in certain strains of mice (3). During recent studies of murine infection due to EMC-M we developed a simple enzyme-linked immunosorbent assay (ELISA) to detect immunoglobulin G (IgG) antibody to this agent.

MATERIALS AND METHODS

Mice. BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used throughout these studies.

Cell culture methods. Primary mouse embryo fibroblast (MEF) monolayers prepared by trypsin-ethylene diamine tetraacetic acid dissociation of embryos of late-term pregnant mice were maintained in Eagle minimum essential medium containing 10% newborn calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Quantitation of virus was performed by plaque assay in MEF monolayers under tracagancium overlay (10).

Virus. For these studies, EMC-M was kindly supplied by A. Notkins (National Institutes of Health, Bethesda, Md.) Another strain of EMC virus, the Helwig prototype strain, was obtained from the American Type Culture Collection (Rockville, Md.) (ATCC VR 129). Virus stocks were prepared by infecting MEF monolayers. After 48 h, cell lysates prepared by three freeze-thaw cycles were clarified by centrifugation at 10,000 × g for 30 min at 4°C and stored in Eagle minimum essential medium plus 5% newborn calf serum at -70°C. The titers of the stock virus pools for both EMC-M and the prototype strain were 2 × 10⁶ plaque-forming units per ml.

Experimental infection of mice. Mice inoculated with 500 plaque-forming units of EMC-M intraperitoneally were killed and exsanguinated at various intervals after infection. Uninfected mice served as controls. Serum was collected, heated at 56°C for 30 min, and stored at -20°C. The globulin fraction of normal mouse serum was prepared by two cycles of (NH₄)₂SO₄ precipitation followed by dialysis using 0.01 M phosphate-buffered 0.15 M saline (PBS), pH 7.4 (7).

PRNT assay. Antibody to EMC was determined by plaque reduction neutralization (PRNT) assay as described by Tesh and Wallace (10). The highest dilution of serum producing a ≥50% plaque reduction was recorded as the titer endpoint.

Preparation of EMC antigens for the ELISA. Viral antigens for both the M and prototype strains for the ELISA were prepared by inoculating 75-cm² MEF monolayers with a 10⁻⁴ dilution of stock virus. Control antigens were simultaneously prepared from mock-infected cell monolayers. After adsorption and washing, the cells were maintained at 37°C in Eagle minimum essential medium containing no newborn calf serum until complete destruction of the cells had occurred, usually 2 days. After three freeze-thaw cycles and clarification by centrifugation at 10,000 × g for 30 min, supernatant fluids were then centrifuged for 4 h at 130,000 × g at 4°C in a Beckman L5-50 ultracentrifuge. The virus pellet was suspended in 0.06 M carbonate buffer (pH 9.6) at 1/20 the original volume and stored at -20°C in glass vials. The viral antigens were stable, despite freeze-thawing, for up to 6 months.

To prepare the solid-phase antigens for the ELISA, 0.2-ml samples of viral or control antigen diluted in 0.06 M carbonate buffer (pH 9.6) were incubated overnight at 4°C in 96-well round-bottom polystyrene Cooke microtiter plates (Dynatech Laboratories, Alexandria, Va.). The optimal dilution of each antigen preparation was determined by checkerboard titration using known EMC antibody positive and negative sera.

Conjugate and substrate. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was purchased from Cappel Laboratories (Cochranville, Pa.). The optimum dilution of conjugated antiserum was determined by testing various conjugate dilutions against
the globulin fraction of murine serum by the method of Bidwell et al. (2).

O-Phenylenediamine was used as the enzyme color substrate. A fresh solution containing 1 mg of O-phenylenediamine per ml and 0.003% H$_2$O$_2$ in distilled water was prepared on the day of testing.

**ELISA procedure.** The ELISA procedure followed the basic format of Bidwell et al. (2). At the initiation of the antibody assay, the antigen was removed, and the plates were washed three times in PBS (pH 7.4) with 0.05% Tween-20 (PBS-T). Serum samples of 200 µl diluted in PBS-T, were added to wells and were incubated for 45 min at 37°C. After removal of the serum samples and three wash cycles with PBS-T, 200 µl of enzyme conjugate diluted in PBS-T was then added and incubated for 30 min at 37°C. The plates were again washed, and 200 µl of O-phenylenediamine substrate was incubated in each well for 30 min at room temperature in the dark. The reaction was terminated by adding 20 µl of 8 N H$_2$SO$_4$. The serum titers could be evaluated visually as the last well of each series to give a distinct color reaction. In these tests the contents of each well were diluted into 0.8 ml of PBS-T, and the absorbance was read at 490 nm. All tests included EMC-positive and -negative sera of known titer as controls.

**Statistical methods.** Where appropriate, the correlation of data was performed using the least-mean-square method.

**RESULTS**

**Characterization of viral and control antigen preparations.** Before use in the ELISA antibody assay, all viral and control antigen preparations were evaluated for specificity. Employing the ELISA reaction, antigens were tested by checkerboard titration using an EMC-M convalescent (immune) murine serum with a neutralizing titer of ≥1:1280. Normal mouse serum free of neutralizing activity at 1:20 dilution was used as a negative control. Negligible color reaction occurred for the control antigen tested against immune mouse serum or for EMC-M antigen tested against normal mouse serum (Fig. 1). In contrast, reaction of the EMC-M antigen with immune mouse serum resulted in a distinct color reaction which diminished with antigen dilution. For this example, the working antigen dilution selected for the antibody ELISA was 1:20. Activity of antigen preparations held for up to 6 months at −20°C was stable despite repeated thawing and freezing.

Since various strains of EMC virus have been shown to cross-react in both hemagglutination inhibition and neutralization assays, antigen for the prototype strain of EMC virus was tested by ELISA assay for reaction with immune serum to EMC-M. The ELISA reaction of the prototype strain with both immune and normal mouse serum was identical to that of the EMC-M antigen run in parallel. These results indicate that the cross-reaction of strains of EMC virus seen in other assay systems also occurs in this ELISA.

**ELISA reaction of sera lacking antibody to EMC-M virus.** The range of colorimetric reaction of EMC antibody-negative sera was determined in order to establish reliable criteria to identify sera containing antibody to EMC virus. Twenty-one sera with neutralizing antibody titers of <1:20 were evaluated by ELISA reaction, using both EMC-M and control antigens. Figure 2 shows the distribution of absorbance values of these samples against EMC-M antigen. The mean absorbance was 0.077 ± 0.025. For the control antigen the mean value was 0.055 ± 0.020. An absorbance value of 0.127, 2 standard deviations from the mean, was selected as a cut-off value to identify EMC-M antibody-negative sera. Using this value, only 1 of 21 sera would have been incorrectly categorized.

**Comparison of ELISA and PRNT assays for antibody to EMC-M virus.** The ability of the ELISA to identify sera containing antibody to EMC-M virus was compared to that of the PRNT test. A total of 37 sera were evaluated in both systems. In addition to the 21 sera derived from uninfected mice, 16 sera obtained from animals >1 month after infection were tested. The results of these comparative titrations (Fig. 3) indicate a good correlation of these two methods ($r = 0.91$). Of 16 sera from previously infected animals, 14 were antibody positive in both assay systems. Two sera from convalescent mice were reactive by PRNT at 1:40 and 1:80, but nonreactive by ELISA. One of the 21 sera non-reactive by PRNT was reactive in the ELISA. In general, the antibody titers obtained by PRNT were higher than those in the ELISA.

**DISCUSSION**

In the past, serological testing for antibody to EMC virus has relied primarily on either hemagglutination inhibition or virus neutralization assays. In this paper, we have described an ELISA method for determining IgG antibody to the M strain of EMC virus. When compared with existing antibody assays, this ELISA has a number of definite advantages. The reagents for the ELISA either are commercially available or can be prepared and standardized in the laboratory with relative ease. Once prepared, the reagents can be stored at −20°C for long periods of time without loss of activity. The assay itself is relatively uncomplicated and can be performed in a short time with a minimum of laboratory support and equipment. Moreover, the test gives a clear colorimetric endpoint which can be evaluated either with a visible-light spec-
ELISA FOR EMC VIRUS

**Fig. 1.** ELISA reaction (absorbance at 490 nm) of EMC-M antigen dilutions with EMC-M immune mouse serum at 1:20 (●) and 1:160 (○), as well as normal mouse serum at 1:20 (■). The reaction of control antigen dilutions with EMC immune serum at 1:20 (O) is also shown.

**Fig. 2.** Distribution of the ELISA reaction values (absorbance at 490 nm) for 21 sera lacking neutralizing antibody to EMC-M.

**Fig. 3.** Comparative evaluation of murine sera for antibody activity to EMC-M virus by PRNT and ELISA IgG antibody assays. The linear regression line and correlation coefficient (r = 0.91) indicate close agreement of these two systems.

The results of these studies indicate that the ELISA and PRNT methods are comparable. Although the higher antibody titer values obtained by the neutralization test suggest that the PRNT assay may be more sensitive than this ELISA for sera of very low antibody titers, both assay systems correlated in their identification of sera reactive and nonreactive with EMC virus. The titer differences may be due in part to the detection by PRNT of other classes of immunoglobulins, such as IgM, not detected by an ELISA for IgG.

The development of ELISA antibody determinations has introduced a new era of viral serology, and new applications are continually being reported. Although this ELISA for antibody for EMC virus has not yet been evaluated with sera of other animal species, it should prove adaptable in investigating many aspects of the biology of this virus. The stability of the reagents and the ease and rapidity of performance lend
themselves to antibody determinations in unfavorable situations where sophisticated laboratory facilities are not available. Because of its characteristics, this rapid, reliable assay would be a valuable asset for the study of EMC virus both in the laboratory and in the field.

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LITERATURE CITED


