Comparison of the Hemagglutination Inhibition Procedure and an Enzyme-Linked Immunosorbent Assay for Detection of Specific Antibodies to Pneumonia Virus of Mice in Experimentally Infected Laboratory Rats

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An enzyme-linked immunosorbent assay (ELISA) and the hemagglutination inhibition (HI) test were used to evaluate the response of laboratory rats to experimental infection with pneumonia virus of mice. The ELISA procedure was more sensitive than the HI test and detected very low levels of antibodies early in the course of infection. At 5 days postinfection, ELISA detected antibody increases in five of five animals, whereas only two of five increases were detected by the HI test. At 9 days postinfection, the HI test failed to detect one titer increase measured by ELISA. Later during the course of infection, increases were detected by both tests. The ELISA procedure was, in general, more sensitive for detecting low levels of antibody than was the HI test, but was equal in sensitivity when high titers of antibody were measured.

The enzyme-linked immunosorbent assay (ELISA) has been shown to be a very valuable procedure for detecting antibodies to various antigens in humans (4, 9) as well as in animals (1, 7, 10). Recently, we adapted this procedure for detecting specific antibody to pneumonia virus of mice (PVM) in the serum of laboratory rats (8). The results showed that ELISA was more sensitive than the hemagglutination inhibition (HI) test, which is currently used to detect the presence of antibody to PVM in the sera of laboratory animals.

To demonstrate the specificity of the PVM antibody titers detected by the ELISA-PVM test, a group of laboratory rats without demonstrable HI antibody titers to the virus were infected intranasally. The serological response of the animals was studied at different times postinfection (p.i.), and the results obtained by ELISA-PVM were compared to HI titers. This paper reports the results of this study.

MATERIAL AND METHODS

Virus and cell culture. PVM and Vero cells were obtained originally from the American Type Culture Collection (Rockville, Md.) and maintained in our laboratory as previously described (8). Minimal essential medium (Earle base) was used with 10% fetal calf serum for cell growth and 1% fetal calf serum for virus production.

Animals. Sixty Sprague-Dawley female rats weighing approximately 200 g were obtained from a commercial supplier. On arrival, the animals were individually housed in suspended cages. They were fed and watered ad libitum, and all contacts with other laboratory animal species were avoided during the course of the experiment.

The animals were divided into two groups. The experimental group (30 animals) received intranasally 200 μl of a preparation containing 10^11 50% tissue culture infectious doses per 0.05 ml of PVM. The control group (30 animals) received intranasally 200 μl of supernatant fluid from uninfected Vero cells. Preinfection serum was obtained from each animal of both groups by venous orbital sinus bleeding. Serum samples from five animals per group were also collected by heart puncture on days 9, 13, 19, 22, and 26 p.i.

HI test. The HI test was performed as described elsewhere (6). We used a commercial preparation of hemagglutinating antigen (Microbiological Associates, Bethesda, Md.), mouse erythrocytes (1%), and 0.01 M phosphate-buffered saline (without calcium and magnesium) containing 0.2% bovine serum albumin. The test was performed by the microtechnique, and the sera were tested without any treatment other than inactivation at 56°C for 30 min.

ELISA. PVM was produced in Vero cells in TM-4 modules of the Corbeil-Bellco system (2). The medium was changed every 3 days until a maximum cytopathic effect was observed. Two liters of infectious supernatant fluid was produced. The viral antigen obtained was sonicated for 5 min with a ¾-in. (0.95-cm) probe on a Braunsonic model 1500 sonicator at 300 W/cm². It was then centrifuged at 3,000 × g for 20 min, and the supernatant fluid was adjusted to 1.0 M NaCl and 8% polyethylene glycol 6000. After 2 h at 4°C, the precipitate was centrifuged at 3,000 × g for 30 min, and the sediment was suspended in phosphate-buffered saline to 1/50 of the original volume. This material was centrifuged in a discontinuous sucrose density gradient: 20 ml was layered on 10 ml of 50% (wt/wt) and 10 ml of 30% (wt/wt) sucrose in a cellulose nitrate tube in an SW27 rotor, and the gradient was
centrifuged for 90 min at 25,000 rpm. Two-milliliter fractions were collected from the bottom and evaluated as antigen for ELISA-PVM. The hemagglutination activity of those fractions was also evaluated. A control antigen was also prepared in the same manner with uninfected Vero cell cultures.

The ELISA procedure has been described (7, 8). Briefly, the antigens were diluted in 0.05 M glycine buffer at pH 10 and allowed to adsorb to polystyrene 96-well plates overnight at 4°C. Washing fluid was distilled water with 0.05% Tween 80. A pool of 10 HI-positive sera and a pool of 10 HI-negative sera were used at a final dilution of 1:50 to evaluate the antigens. Sera were diluted in phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 80. The adsorbed immunoglobulin G was detected by using a commercial conjugate (peroxidase) anti-rat immunoglobulin G (Miles Laboratories, Inc., Elkhart, Ind.). Peroxidase activity was detected with 2 mg of 5-aminosalicylic acid per ml and 0.005 M hydrogen peroxide at pH 6 as the substrate. The results were evaluated visually, and any reaction above the background was considered positive. The titers were expressed as the last dilution with a positive reaction.

Electron microscopy. For electron microscopy examination, the specimens were placed on carbon-coated grids, negatively stained with a 3% phosphotungstic acid solution at pH 6, and examined with a Philips EM-300 electron microscope.

RESULTS

Antigen preparation. To eliminate nonspecific reactions encountered in ELISA when using whole-cell extracts as antigen, the virus was concentrated and purified by centrifugation in a discontinuous sucrose gradient. Maximum hemagglutination and ELISA-PVM activities were found in different gradient fractions (Fig. 1). The hemagglutination-reactive antigen was of a lighter density, and maximum titers were found at the 0 to 30% sucrose interface (fractions 8 and 9), whereas the ELISA-PVM-reactive antigen was found in fractions 4, 5, and 7. From electron microscopy observations, fractions reactive in ELISA-PVM contained mostly intact viral particles and some membranes, whereas fractions more reactive in the hemagglutination test contained mostly soluble (free) hemagglutinins. Fractions 4, 5, and 7 were pooled and used as antigen in the ELISA-PVM test at dilutions ranging from 1:50 to 1:200, as determined by checkerboard titration. When uninfected Vero cell culture antigen was used, there was no reaction with positive or negative sera.

Experimental infection. The sera of all animals were evaluated by HI and ELISA-PVM tests. None of the animals had any clinical manifestations of respiratory infection during the course of the experiment. Figure 2 illustrates the correlation between HI and ELISA-PVM tests on the sera of the three groups: negative control (preinfection sera), experimental control (noninfectious material), and infected (PVM infected). In the negative control group, all sera were negative by HI, but some had ELISA-PVM titers of up to 1:64. In the experimental control group most sera were HI negative except two

![Fig. 1. Hemagglutination and ELISA-PVM activities in various fractions of PVM-infected supernatant of Vero cell cultures after centrifugation in a discontinuous sucrose gradient. A pool of positive sera diluted 1:50 was used in ELISA to titrate the antigen.](http://jcm.asm.org/Downloaded-from)
with a titer of 1:20 and one with a titer of 1:10. In this group the ELISA-PVM titer distribution was similar to that observed for the negative control group with titers ranging from negative to 1:128.

In the infected group of rats a progressive increase in both HI and ELISA-PVM titers was observed from day 5 p.i. until day 26 p.i. However, on day 5 p.i. one rat already had a titer of 1:32 and three had a titer of 1:64 in the ELISA-PVM test, whereas the HI titers were still negative.

Table 1 shows the results of tests on paired sera of individual rats. In group A (5 days p.i.) ELISA-PVM detected five antibody increases, whereas by HI only two were detected, and at titers of only 1:10. In group B (sacrificed 9 days p.i.) ELISA-PVM detected five increases, but by HI one serum remained negative. In the other groups, representing 13, 19, and 22 days p.i., antibody increases were detected in all animals by both tests.

**DISCUSSION**

The mouse is the natural host of PVM, and in this animal species, the virus induces a fatal pneumonia (5). The virus also seems to exist in laboratory rats (3), but very little is known about rat susceptibility to PVM. Although clinical signs were not observed in the infected animals in this study, laboratory rats appear to be susceptible to PVM, since an antibody response was detected in all animals after an intranasal inoculation with infectious virus. The absence of clinical manifestations might be explained by the fact that the virus was passaged in tissue culture, resulting in a loss of virulence.

The PVM antigen used in the ELISA-PVM test was produced in tissue culture and purified by ultracentrifugation in a discontinuous sucrose density gradient. Two ELISA-reactive peaks of antigens were found, but these two peaks were different from the hemagglutination-reactive peaks, suggesting that different antigens were measured in the two systems. Electron micro-
scop ic observation of the fractions showed that the ELISA peaks contained intact virions and modified cellular membranes, whereas the lighter peak of hemagglutination activity seemed to be due to a soluble hemagglutinin less reactive in ELISA. The ELISA-reactive material was very specific, reacting only with the positive serum and at titers greater than 1:1,000 when tested with a standard positive serum diluted 1:50.

When this assay was used to assess the response of laboratory rats experimentally infected with PVM, its superiority was demonstrated by the early detection of specific antibodies. Later in the course of infection, both techniques were adequate for detecting specific antibodies. The techniques gave similar titers once antibodies reached higher levels. The ratio HI/ELISA-PVM titers was near unity, with slight tendencies in favor of the HI test at 13 days p.i. and in favor of the ELISA-PVM test at 22 and 26 days p.i. This may reflect the type of antibody produced, immunoglobulin G or M, or a difference in avidity of the antibodies for the antigen.

When the results are plotted to show the correlation between the HI and ELISA-PVM tests (Fig. 2), the efficacy of ELISA-PVM for detecting low levels of antibodies is clearly demonstrated by the number of sera negative by HI but with significant ELISA-PVM titers. Only two sera were positive by HI and negative by ELISA-PVM in the control group.

These results confirm a previous study (8) in which we demonstrated the high sensitivity of the ELISA-PVM procedure to detect a low level of antibodies, and they clearly demonstrated that the ELISA assay was more efficient than the HI procedure for detecting low-level antibody titers to PVM in the sera of rats experimentally infected with PVM.

ACKNOWLEDGMENTS
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LITERATURE CITED

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* HI/ELISA-PVM antibody titers.  
ND, Not done.