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AN ENZYME IMMUNOASSAY FOR IDENTIFICATION AND QUANTIFICATION OF INFECTIOUS MURINE PARVOVIRUS IN CULTURED CELLS

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An enzyme immunoassay was developed to identify and quantify infectious murine parvovirus. The assay was as sensitive as the fluorescent focus test for quantification of virus in clinical material and was less labor-intensive. Viral infectivity could be inactivated with formalin prior to performance of the enzyme immunoassay. Specificity of the reaction product was shown by neutralization of infectivity using a hyperimmune rabbit antiserum to murine parvovirus.

AN INTRODUCTION

Conventionally-housed laboratory rodents commonly sustain infection with one or more viral agents (Foster et al., 1982). Since some of these infections are known or thought to affect the outcome of experimental procedures, it is important to understand their pathogenesis and natural history. The execution of studies designed to elucidate the biology of rodent virus infections requires the ability to detect and quantify infectious virus in large numbers of clinical specimens. Because of the high transmission potential of many of these agents, alternatives to animal inoculation are desirable. Many rodent viruses do not induce morphologic changes in inoculated cell cultures, necessitating the use of immunochemical probes to detect viral antigens. We have used the fluorescent focus test for detection and quantification (Smith, 1983; Smith et al., 1984); however, the procedure is expensive and labor-intensive. It is particularly cumbersome when applied to large numbers of samples.

Smith et al. (1981) reported quantification of herpes, mumps and measles viruses in unfixed cultured cells using the enzyme immunoassay (EIA). Recently, Yong-He et al. (1984) reported the use of EIA to detect several flaviviruses in cultured cells which were formalin-fixed prior to processing. In the case of rodent viruses, viral inactivation prior to EIA is desirable to protect animals housed in the vicinity of the laboratory, as well as personnel in the case of zoonotic viruses.

In this paper, we report the application of EIA methodology to detection and quantification of two allotropic variants of the murine parvovirus, minute virus of mice...
(MVM). The ability of formalin and glutaraldehyde to inactivate infectious virus, while optimizing color development, was also compared.

MATERIALS AND METHODS

Virus strains

The immunosuppressive (MVM(i)) and prototype (MVM(p)) strains of minute virus of mice were obtained from Dr. Peter Tattersall (Department of Human Genetics, Yale University) and were passaged once in BHK21 cells. Virus stocks used for tissue culture inoculation were in the form of crude BHK21 cell lysates. Virus obtained from Dr. Tattersall was also used to inoculate neonatal Crl:CF1Br mice intracerebrally to generate clinical material for testing.

Antisera

Ascitic fluid containing polyclonal antibody to MVM(i) was prepared by injecting multiparous female Crl:CF1Br mice intraperitoneally at weekly intervals with infected infant mouse brain emulsified in Freund's complete adjuvant. Mouse ascitic fluid containing antibody to a mouse brain-adapted rat coronavirus, sialodacryoadenitis virus (SDAV), was similarly prepared for use as a control reagent. Hyperimmune rabbit antiserum to MVM(p) was obtained from Dr. Tattersall.

Cell cultures

C6 rat glioma cells were cultured in Dulbecco's modified essential medium containing 5% heat-inactivated fetal bovine serum and antibiotics (DMEM 95-5). Cells were added in a volume of 0.1 ml to 96-well cluster dishes (Costar, Cambridge, MA) or in a volume of 0.5 ml to 4-chamber Lab Tek slides (Miles Laboratories, Naperville, IL) at densities of 1-2 × 10^3 or 5-10 × 10^3, respectively. Culture vessels were maintained in humidified chambers in a 37°C, 5% CO₂ atmosphere. Cultures were inoculated within 3 h of seeding.

Viral infectivity assays

For the fluorescent focus test, 10% tissue homogenates or 10-fold virus dilutions in volumes of 0.3 ml were added to chambers of Lab Tek slides containing C6 cells. After a 1 h absorption period at 37°C, the virus inocula were removed, and the cells were washed once with DMEM 95-5. Medium (1 ml per chamber) was added, and the cultures were maintained for periods outlined in the results. Acetone-fixed cells were processed for indirect immunofluorescence using hyperimmune mouse ascitic fluid to MVM(i) diluted 1:50 and fluorescein-conjugated goat anti-mouse immunoglobulin (Antibodies Inc., Davis, CA) diluted 1:50 and containing 0.01% Evans blue (Roboz Surgical Instruments Co., Washington, DC). Glycerol-mounted slides were viewed with an Olympus BH-2 transmission fluorescence microscope, and single cells or foci containing viral antigen were counted at 100 or 40 diameters, respectively.
For the EIA, 10% tissue homogenates or 10-fold virus dilutions were added in volumes of 0.1 ml to existing medium (0.1 ml per well) in 96-well cluster dishes. After incubation periods specified in the results, 0.1 ml of varying concentrations of E.M.-grade glutaraldehyde (Taab Laboratories, Reading, England) or neutral buffered formalin was added to each well (1:3 dilution of fixative). Within 48 h of fixation, the cells were washed once with phosphate buffered saline (PBS) and saturated with PBS containing 3% gelatin (blocking buffer). After 1 h at 37°C or 18 h at 23°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, horseradish peroxidase conjugated goat anti-mouse IgG (BioRad, Richmond, CA; 1:3,000 in PBS containing 0.5% BSA) was added for 1 h at 37°C. Results were read spectrophotometrically (414 nm) 20-30 min after the addition of ABTS substrate (Kirkegaard and Perry, Gaithersburg, MD) to thrice-washed wells.

**Virus neutralization**

Log dilutions of hyperimmune rabbit antiserum to MVM(p) were mixed in 96-well transfer plates (Dynatech Laboratories, Inc., Alexandria, VA) with equal volumes of virus suspensions containing either 100 or 1,000 median infectious doses of MVM(p). After 1 h at 37°C, the mixtures were added to newly seeded C6 cells in cluster dishes. After 4 days, the cells were fixed and processed through the EIA protocol outlined above. The hyperimmune rabbit antibody reduced infectivity of MVM(p) or MVM(i) in plaque assays by 90% when diluted 1:10,000 (Tattersall and Bratton, 1983).

**Calculations**

Optical density (O.D.) values were considered significant if they exceeded by 3 SD the mean value for control wells in the same assay. Virus titers measured by the EIA were calculated by the method of Reed and Muench (1938). Fluorescent focus titers were calculated by the method described by Lorenz and Bogel (1973).

**RESULTS**

In an early experiment, C6 cells inoculated with log dilutions of MVM(p) and held for 4 days were treated with (final concentrations) 2% or 0.02% glutaraldehyde and processed for EIA. O.D. values for cells treated with 2% glutaraldehyde and dilutions of SDAV antibody (1:100–1:400) ranged from 0.7 to 1.4 and were equivalent to values for cells treated with the same dilutions of MVM antibody. There was no gradient in response to varying concentrations of virus inocula. In contrast, there was a dose-response for cells fixed with 0.02% glutaraldehyde and treated with dilutions of MVM antibody (Table 1). O.D. values for cells inoculated with virus diluent and treated with MVM antibody and for cells inoculated with virus and treated with heterologous antibody or antibody diluent were similar. In further experiments, MVM antibody was used at a 1:100 dilution for detection.
### TABLE 1

O.D. values for C6 cells inoculated with dilutions of MVM(p), fixed after 4 days with 0.02% glutaraldehyde and processed for EIA using dilutions of MVM antibody

| MVM dilution | Antibody and dilution | 1:50 | 1:100 | 1:200 | 1:400 | SDAV Diluent |
|--------------|-----------------------|------|-------|-------|-------|--------------|
| 10^{-1}      |                       | 1.51 | 1.45  | 1.24  | 1.16  | 0.31         |
|              | (0.04)                | (0.03)| (0.03)| (0.04)| (0.01)| (0.00)       |
| 10^{-2}      |                       | 1.54 | 1.34  | 1.12  | 1.04  | 0.33         |
|              | (0.08)                | (0.06)| (0.08)| (0.08)| (0.02)| (0.00)       |
| 10^{-3}      |                       | 0.96 | 0.70  | 0.68  | 0.63  | 0.32         |
|              | (0.16)                | (0.01)| (0.05)| (0.11)| (0.01)| (0.00)       |
| 10^{-4}      |                       | 0.61 | 0.76  | 0.34  | 0.38  | 0.33         |
|              | (0.01)                | (0.13)| (0.00)| (0.07)| (0.04)| (0.01)       |
| 10^{-5}      |                       | 0.45 | 0.42  | 0.36  | 0.35  | 0.36         |
|              | (0.03)                | (0.04)| (0.08)| (0.01)| (0.01)| (0.01)       |
| No virus     |                       | 0.41 | 0.33  | 0.29  | 0.30  | 0.34         |
|              | (0.01)                | (0.03)| (0.02)| (0.02)| (0.01)| (0.01)       |

Values are mean O.D., (SD for 2 replicates).

In the next experiment, cells in cluster dishes and Lab Tek slides were inoculated in parallel with MVM(p) dilutions and fixed for EIA and IFA, respectively, on day 2, 4, 5 or 6 (cluster dishes only on day 6). ID_{50} and fluorescent focus unit (FFU) titers were calculated for each day (Table 2). The virus titer as determined by fluorescent focus test did not change with time, although the size of countable foci did increase. The titer calculated from the EIA increased significantly between day 2 and day 4, plateauing on day 5 at the same level as the fluorescent focus titer.

We next wished to determine whether MVM infectivity was being inactivated by the glutaraldehyde fixation, since processing of large numbers of plates in a biological safety cabinet was cumbersome. Cells were inoculated with 3,000 or 30,000 ID_{50} of either MVM(p) or MVM(i) and held for 4 days. Supernatant culture fluid was collected from wells prior to treatment with 0.02% glutaraldehyde and at 2, 6, and 24 h after fixation. Viral infectivity was measured by fluorescent focus assay. Infectivity of MVM(i) was reduced by about 70% at 24 h post-fixation. Infectivity of MVM(p) was unaltered by treatment with this concentration of glutaraldehyde, even at 24 h.

Since viral inactivation was not achieved with 0.02% glutaraldehyde and since a higher concentration had resulted in unacceptable background signal, we studied the ability of neutral buffered formalin to inactivate virus in the supernates of wells inoculated with 30,000 ID_{50} of MVM(p). The experiment was performed only with MVM(p), since this strain had been more resistant to inactivation. Formalin at final
TABLE 2

Comparison of EIA and fluorescent focus titer (log_{10} per ml) for MVM(p) tissue culture stock

| PID-a | Fluorescent focus units | Median infectious doses |
|-------|-------------------------|------------------------|
| 2     | 5.7 (single cells)      | <2.0                   |
| 4     | 5.6 (foci)              | 4.5                    |
| 5     | 5.7 (foci)              | 5.5                    |
| 6     | not done                | 5.5                    |

a Day post-inoculation of cell fixation.

concentrations of 0.3% and 1.0% reduced somewhat the infectivity of MVM(p). Inactivation was complete with 2% formalin at 6 h post-fixation and with 3% formalin at 2 h. O.D. values for titrations treated with any of the four formalin concentrations and MVM antibody were similar, and background signal (wells treated with SDAV antibody) was not affected by fixative concentration. Future experiments were conducted with cells fixed with 3% formalin and held overnight at 4°C prior to processing for EIA.

To confirm that the reaction product detected in the EIA was specific for MVM antigen, a neutralization test using rabbit hyperimmune MVM antibody was performed (Table 3). Using a challenge dose of 100 ID_{50} of MVM(p), a marginal mean O.D. value was obtained only at the highest antibody dilution tested (1:10,000). With 10-fold more virus, there was a significant mean O.D. reading (0.97) for this antibody dilution, but the value was lower than the mean for wells inoculated with virus plus diluent (1.51).

Having confirmed the specificity of the EIA results, we wished to determine whether the assay could be applied to clinical material (infected animal tissue). Homogenates of

TABLE 3

O.D. values for neutralization test using hyperimmune rabbit antiserum containing MVM antibody

| Virus dose | Rabbit anti-MVM dilution |
|------------|--------------------------|
|            | 1 : 10 | 1 : 100 | 1 : 1,000 | 1 : 10,000 | Diluent |
| 100 ID_{50} | 0.00  | 0.02   | 0.01     | 0.17       | 1.24    |
|            | (0.02) | (0.01) | (0.01)   | (0.02)     | (0.07)  |
| 1,000 ID_{50} | -0.01 | 0.01   | 0.09     | 0.97       | 1.51    |
|            | (0.06) | (0.01) | (0.02)   | (0.03)     | (0.07)  |

a Mean values (SD for 2 replicates) corrected for mean value of wells incubated with rabbit antibody plus diluent in place of virus = 0.27 (0.02).
Table 4

Virus titers (log₁₀ per gram) for brain homogenates from MVM(p)- or MVM(i)-infected mice determined by enzyme immunoassay and fluorescent focus test

| PID-b | MVM(p) | MVM(i) |
|-------|--------|--------|
|       | ID₅₀  | FFU    | ID₅₀  | FFU    |
| 3     | 4.5   | 5.2    | 4.0   | 4.2    |
|       | (4.5, 4.5) | (4.0, 4.5) |
| 4.5   | 5.3    | 3.5    | 4.2   |
|       | (4.0, 3.5)    |        |
| 7     | 5.0    | 6.4    | 2.5   | 2.5    |
|       | (4.5, 5.5)    | (4.5, 5.5) |
| 4.0   | 4.6    | 3.5    | 4.3   |
|       | (4.0, 3.5)    | (3.5, 3.5) |
| 10    | 4.5    | 5.5    | 2.5   | < 2.5  |
|       | (4.5, 4.0)    | (2.5, 2.5) |
| 4.5   | 5.5    | 2.5    | < 2.5 |
|       | (2.5, 2.5)    |        |

* EIA and fluorescent focus titrations were inoculated in parallel except for results in parentheses which represent EIA titrations performed independently.

b Day post-inoculation of brain collections from neonatal mice infected intracerebrally.

brains from neonatal mice inoculated intracerebrally with MVM(p) or MVM(i) and collected on day 3, 7 or 10 post-inoculation were assayed by fluorescent focus test and EIA (Table 4). Selected homogenates were assayed repeatedly by EIA to assess reproducibility. Virus titers determined from the two types of infectivity assays were generally similar. In some cases, fluorescent focus titers were about ten-fold higher than EIA titers. The greatest variability among independent assays was 1.0 log₁₀ for a brain sample collected from MVM(p)-infected mice 7 days post-inoculation.

DISCUSSION

The application of EIA methodology to rodent virus quantification as described here has several advantages over previously used techniques. EIA uses a microtiter format and therefore requires small volumes of reagents and facilitates processing of large numbers of specimens. Results may be obtained without the eye fatigue induced by long periods of microscopic examination required for immunofluorescence assays. Formalin fixation prior to assay eliminates the risk of infecting animals which might be housed in the vicinity of the laboratory. Yong-He et al. (1984) reported that mosquito cells fixed with 10% formalin detached during the course of the EIA. In contrast, we found that C6 rat glioma cells fixed with 3% formalin adhered to the plastic surface, while those fixed with 2% formalin did not. However, in both studies, viral antigen
liberated from the fixed cells and subsequently bound to the solid phase was apparently detected. Additionally, we have shown that neutralization tests may be performed using this method. Antibody preparations used for neutralization and detection must, of course, each be made in a different species.

Fluorescent focus titers tended to be higher than EIA titers obtained for brain homogenates from infected infant mice. The most likely explanation for this observation is that the assays were terminated 4 days post-inoculation of C6 cells. This was necessitated by the fact that the cells had reached confluency and could not be held for an additional day. As shown in Table 2, optimum virus titers determined by EIA are not reached until day 5. Thus, it was not surprising that EIA titers were, in some cases, about 10-fold lower than fluorescent focus titers. This problem may be rectified in the future by the use of cells seeded at lower densities. The absolute association between parvovirus replication and cell division dictates that conditions for an assay such as the one described here be fully standardized. Such standardization may be necessarily incomplete in the clinical setting. Toxicity induced by tissue-associated enzymes may result in the death of a proportion of inoculated cells, and tissue-associated growth factors may induce cell division at a rate faster than that of control, uninoculated cells.

Based on this experience with EIA applied to a murine parvovirus, we feel that the method is worthy of further development and application to other rodent viruses. The ability to assay virus in formalin-fixed cells renders the method especially attractive for the assay of agents which are known to be zoonotic, such as lymphocytic choriomeningitis virus and Hantaviruses.

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