Microneutralization Test in PK(15) Cells for Assay of Antibodies to Louping Ill Virus

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A microneutralization test in PK(15) cells was developed to measure the neutralizing antibody response of a group of ponies experimentally challenged with louping ill virus. Viral cytopathic effect was maximal after 6 days of incubation, at which point titration endpoints were clear-cut and readily determinable. The assay compared favorably with the mouse neutralization test for accuracy and ease of performance.

Various in vivo systems have been used for the detection of serum neutralizing (SN) antibodies to louping ill (LI) virus, a flavivirus belonging to the Togaviridae family. Neutralization testing was, for many years, carried out in weanling or adult mice. Initial attempts to develop a neutralization assay in cell culture were hindered by the fact that LI and the other viruses of the Russian spring-summer encephalitis complex produced only a partial cytopathic effect (CPE) in a variety of cell systems (7). To overcome this problem, Porterfield (7) devised a simple plaque inhibition test, using chicken embryo fibroblasts under an agar overlay. This technique, though lacking somewhat in accuracy, was easy to perform and could be used as a qualitative test for the detection of SN antibodies. Kaariainen and Halonen (4) developed a metabolic inhibition test in HeLa cells with cell-adapted LI virus, which although having certain limitations (3), could be successfully applied to large-scale epidemiological investigations.

The neutralization assay was further improved by the development of a simplified macroplate method by de Madrid and Porterfield (2) based on plaque neutralization in a pig kidney cell line (PS cells) under a carboxymethyl cellulose overlay. Shortly afterwards, Reid and Doherty (9) described a plaque reduction assay for LI virus with the IB/RS2 clone 60 pig kidney cell line that had equivalent sensitivity to the mouse neutralization test (H. W. Reid, personal communication). However, it was less suitable than the technique of de Madrid and Porterfield (2) for large-scale epidemiological studies.

A major limitation to the unrestricted availability of either the IB/RS2 clone 60 or PS pig kidney cell line for conducting neutralization assays for LI virus is that both cell systems are considered contaminated with hog cholera virus (H. W. Reid, personal communication). In this report we describe a simple microneutralization assay, using the commercially available PK(15) cell line, which compared favorably with the mouse protection test for accuracy and ease of performance.

The microneutralization test was based on the methods of Pantuwatana et al. (7) and Rosenbaum et al. (10), and assays were performed in 96-well flat-bottom microtest plates (Nunc, Roskilde, Denmark). The PK(15) cell line (GIBCO Biocult, Ltd., Paisley, Renfrewshire, Scotland) grown on Eagle minimal essential medium supplemented with 5% fetal bovine serum was used at serial passage level 170 throughout this study. The cells had been screened previously for latent bovine viral diarrhea virus by direct immunofluorescence examination (6), with negative results. The test strain of virus was an equine isolate LI/74/E/61 recovered from a fatal case of encephalitis (13) and passed once in suckling mice and twice in PK(15) cells. Second-passage tissue culture fluid had an infectivity titer of $10^{7.8}$ 50% tissue culture infective doses per ml. Reference mouse hyperimmune ascitic fluid against this strain of virus was prepared as described by Brandt et al. (1). Neutralization assays were performed on serum samples collected from a group of ponies at various intervals after experimental challenge with LI virus (12). The sera were stored at −20°C until shortly before testing, when they were heat inactivated at 56°C for 30 min.

Inactivated sera were transferred to the test plates with Micropette pipettes (Scientific Manufacturing Industries, Emeryville, Calif.), and serial twofold dilutions were made with a Titertek multidiluter (Flow Laboratories, Irvine, Scotland). Each sample was tested in duplicate according to the constant virus–varying serum technique, and all assays were performed by the same technologist. After addition of test virus (200 50% tissue culture infective doses in 0.025 ml), the plates were covered, gently shaken, and incubated at 37°C for 1 h. After incubation, 0.1 ml of a suspension of PK(15) cells in Eagle minimal essential medium with 5% fetal bovine serum adjusted to contain 6 × 10⁵ cells per ml was added to each well, the plates were reshaken, and the wells were sealed by the addition of ca. 0.1 ml of liquid paraffin. Plates were incubated at 37°C and examined under a microscope after 4, 5, and 6 days for evidence of CPE. CPE could be visualized more readily when the cell sheets were stained with 0.1% Formalin-buffered crystal violet solution. The titer of a serum was taken as the highest dilution that completely neutralized the challenge dose of virus.

For comparison, each serum sample was also assayed for neutralizing antibodies in mice by the method of Smithburn et al. (12). The technique employed was the varying virus–constant serum method, and tests were performed by intracerebral inoculation of serum–virus mixtures into adult Swiss mice (Porton Down, Wiltshire, England). Interpretation of the test results was based on the standards of Smithburn et al. (11), and log neutralization indices were calculated according to the formula of Karber (5).

The results obtained from the two neutralization assay procedures were compared by linear correlation analysis, and Pearson's product moment coefficient was determined. The microneutralization test in PK(15) cells proved effec-
FIG. 1. Microneutralization assay for antibodies to LI (strain LI/74/E/61) virus in PK(15) cells. Cell sheet after 6 days stained with Formalin-buffered crystal violet. 1, 2, 3, and 4 represent pony sera with SN antibody titers of 64, 1,024, 64 and 8, respectively. Test controls included serum controls (SC), reference positive serum (+S), negative serum (−S), virus (VC), and cell controls (CC).

tive for the detection and estimation of SN antibodies to LI virus. A specific CPE consisting of rounding up and sloughing of infected cells off the monolayer was initially observed in affected wells on day 2 or 3 of incubation. By day 5 or 6, these changes were very clear-cut. The cell sheet became almost completely disrupted and consisted of an open meshwork of distorted, refractile cells (Fig. 1). When virus was neutralized, there was a marked decrease in pH of the cell contents, and endpoints could be easily determined on the basis of the color reactions that had taken place.

Mean (±1 standard error) values of the SN antibody responses of the ponies experimentally challenged with LI virus as determined by the two neutralization assay procedures are presented in Fig. 2. There was evidence of a positive linear correlation between the quantitative data derived from the two test systems \((r = 0.91)\). Respective antibody profiles were comparable except for a disparity in the rates of increase in antibody titer during the first 3 to 4 weeks after challenge. The rise in antibody level measured by the mouse neutralization test was more gradual than that

FIG. 2. The pattern of the SN antibody response of seven ponies challenged with LI (strain LI/74/E/61) virus. Values expressed as mean (±1 standard error) serum antibody levels by microneutralization test titers in PK(15) cells (○) or mouse neutralization indices (★). \(r = 0.91\).
estimated in the microneutralization test in PK(15) cells. Persistence of antibody titers was similar, irrespective of the assay procedure used.

The microneutralization test for LI virus was simple and easy to perform and offered significant advantage over other currently available cell culture assay procedures (6, 8) in that the pig kidney line used was not contaminated with hog cholera virus. The CPE associated with LI virus infection of PK(15) cells was clearly discernible after incubation of cultures for 4 to 6 days and very similar to that previously reported in pig kidney secondary monolayers (14). Titration endpoints were clear-cut and could be readily determined. In addition to easy reading of test results, the microneutralization assay provides a rapid and relatively inexpensive means of detection and estimation of SN antibody levels to LI virus with satisfactory accuracy. It has considerable advantages over the mouse neutralization test and is less laborious than alternative cell culture procedures (7, 8).

The microneutralization test described in this report should be of value not only as a diagnostic technique but also as a research procedure for conducting pathogenesis or large-scale epidemiological studies with LI virus.

LITERATURE CITED