Multiple Assessment and Serum Neutralization of Arbovirus Mixtures

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Mixtures of Venezuelan equine encephalomyelitis, Rift Valley fever, and chikungunya viruses may be assayed by selective immunofluorescence staining of infected cell monolayers. A multiple serum neutralization test is described for quantifying reactions of these viruses with mixtures of serum antibodies.

Selective immunofluorescence staining of infected cell monolayers in conjunction with fluorescent cell counting has been used to assay quantitatively mixtures of three or more viral agents (1). This assessment procedure is specific, rapid (less than 24 hr), and versatile in application for conducting experimental investigations that involve more than one virus. It was successfully used to characterize and to compare the survival of three viruses in an airborne mixture (5) and to confirm the existence of dual arbovirus infections in primates by detection of the etiologic agents in blood (N. L. Pollok and G. P. Briggs, unpublished data). This present communication describes an extension of the technique for the assessment of the three arboviruses, Venezuelan equine encephalomyelitis (VEE), chikungunya (Chik), and Rift Valley fever (RVF) in mixtures and for estimating specific antibodies for these viruses by using a multiple serum neutralization test.

Virus strains used in this study were the Trinidad (VEE), Banganike (Chik), and van Wyk (RVF). To demonstrate that the three viruses in mixture could be assayed individually, suspensions of each were mixed in equal volumes and in an approximate virus to cell ratio of 1:100. The mixture was inoculated in 0.2-ml volumes onto cover-slip cultures of L-929 cells. Individual suspensions of virus, in comparable volumes, served as controls. Centrifugation (19,000 to 29,000 × g, 15 min) was employed to attach inoculum onto cell monolayers. After removal of residual inoculum and the addition of cell maintenance medium (mixture 199 plus 5% fetal calf serum), cell monolayers were incubated at 35 C for 1 hr to effect penetration of viruses into cells. To prevent a secondary cycle of infection by extracellular viruses, an overlay consisting of equal volumes of a 1:4 dilution of VEE, Chik, and RVF antisera was added in 1-ml volumes onto each cell monolayer. These were then incubated at 35 C from 16 to 22 hr. After being fixed with acetone (−60 C), cover-slip cell monolayers were separated into three groups and selectively stained with one of three viral immune sera previously conjugated with fluorescein isothiocyanate. Preparation of antiviral sera, details of established immuno-fluorescence assays for each virus, staining procedures, and fluorescence microscopy equipment have been described elsewhere (2-4).

The titer of each virus in mixture was comparable to that of the corresponding titer of the virus control (Table 1). The simultaneous introduction of the viruses in mixture onto cell cultures did not result in interference among the viruses. Each virus, therefore, could be independently and quantitatively assayed in the presence of the others.

An attempt was made to carry out a multiple serum neutralization test by reacting a mixture consisting of a constant quantity (2 × 10^4 cell-infecting units) of each of the three viruses with serial dilutions of a mixture of three corresponding antiviral monkey sera. In a comparable volume relationship, individual virus suspensions were mixed with dilutions of corresponding anti-viral serum. The latter protocol was that of a conventional serum neutralization test. Virus mixtures or individual virus suspensions were also reacted with dilutions of normal monkey serum for control purposes. The diluent for all reactants was phosphate-buffered saline (pH 7.1) free from calcium and magnesium ions. Test mixtures were incubated at 35 C for 90 min and then assayed for unneutralized viruses in the manner described earlier. To determine the 50% serum-neutralizing titer, the per cent reduction of fluorescent cell counts for each antiserum dilution was computed from normal serum (control) counts. Reduction percentages were then plotted against the logarithm of the corresponding final dilutions of antiserum on probability paper. A linear relationship...
TABLE 1. Multiple assessment of arbovirus mixtures

| Inoculum                          | Virus titera |
|-----------------------------------|--------------|
|                                   | VEE         | RFV        | Chik       |
| Virus mixture                     | $9.0 \times 10^8$ | $2.7 \times 10^7$ | $3.1 \times 10^6$ |
| Individual virus                  | $9.3 \times 10^8$ | $2.2 \times 10^7$ | $4.4 \times 10^6$ |

a Cell-infecting units of virus per milliliter; determined by selective immunofluorescence staining and counting of infected L-929 cells.

TABLE 2. Multiple serum neutralization of arbovirus mixtures

| Inoculum                           | Antiviral serum neutralizing titera |
|-------------------------------------|-------------------------------------|
|                                     | VEE      | RFV      | Chik     |
| Mixture of viruses and specific antisera | 1,600   | 2,000   | 3,000   |
| Individual virus and specific antisera | 2,000   | 1,800   | 3,200   |

a Reciprocal of 50% serum-neutralizing titer.

b Mixture of VEE, RFV, and Chik viruses, each in a constant quantity ($2 \times 10^4$ cell-infecting units), was reacted with serial dilutions of a mixture of three corresponding antiviral monkey sera or normal serum (control) and incubated at 35°C for 90 min; unneutralized viruses were assayed by selective immunofluorescence staining and counting of infected L-929 cells.

was obtained over a critical range; the 50% serum-neutralizing titer was determined by interpolation.

Results (Table 2) show that the 50% serum-neutralizing titer of each antiviral serum determined by the multiple serum neutralization test was comparable to the titer of the corresponding antiviral serum obtained by the conventional tests. These data indicate that multiple neutralization of virus mixtures can be measured quantitatively without loss of specificity or sensitivity.

The assessment technique described in this report, by obviating time-consuming and intricate procedures usually required to assay mixtures of viral agents, can facilitate studies on viral interference and mixed infections, particularly with viruses that evoke similar or indistinct cellular responses. That the technique is adaptable for performing a multiple serum neutralization test further demonstrates its versatility. With the capability of quantifying the reactions of several viral agents with mixtures of serum antibodies, the multiple serum neutralization test may prove highly applicable for expediting the identification of different viruses and specific antibodies, establishing antigenic relationships among virus strains, and for characterizing and comparing kinetic neutralization rates.

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