Rapid Herpes Simplex Virus Susceptibility Testing Using an Enzyme-Linked Immunosorbert Assay Performed In Situ on Fixed Virus-Infected Monolayers

GERARD P. RABALAI$*$, MYRON J. LEVIN, AND FRANK E. BERKOWITZ†

Department of Pediatrics, Infectious Diseases Section, University of Colorado School of Medicine, Denver, Colorado 80262

Received 3 November 1986/Accepted 13 March 1987

An enzyme-linked immunosorbert assay was performed directly on fixed, virus-infected cell monolayers to rapidly determine the susceptibility of herpes simplex virus to antiviral drugs. Susceptibility determinations by this method correlated well with those obtained by plaque reduction assay and avoided some of the drawbacks of other methods currently available.

The susceptibility of herpes simplex virus (HSV) to antiviral drugs can be determined by several methods (3, 5–10). Each of these assays has one or more drawbacks, such as the need for titer determinations for virus, large inoculum effects, subjective endpoints, or a format incompatible with a routine viral diagnostic laboratory. The need for a rapid, reproducible assay that avoids these problems prompted us to modify a previously described enzyme-linked immunosorbent assay (ELISA) which was used to test the activity of antiviral drugs against varicella-zoster virus (1).

Confluent monolayers of human embryonic lung fibroblasts in Falcon microtiter plates (Minitext III; Becton Dickinson Labware, Oxnard, Calif.) were infected with HSV isolates. The HSV inoculum varied from 25 to 3,000 PFU per well in 0.1 ml of Dulbecco minimal essential medium with 2% fetal calf serum and antibiotics. Triplicate wells received acyclovir (acyclovir; 9-[2-hydroxyethoxy]methyl guanine) or 9-(1,3-hydroxy-2-propoxymethyl)guanine (DHPG; Burroughs Wellcome Co., Research Triangle Park, N.C.) diluted in 0.1 ml of Dulbecco minimal essential medium with supplements at twice the desired final concentrations (micrograms per milliliter) of 0.06, 0.125, 0.25, 0.5, 1.0, and 10.0. Uninfected wells and infected wells without antiviral agents served as tissue and virus controls, respectively. The ELISA was performed at 24 or 48 h after infection when three or more plaques per well were seen on a brief examination of the virus control wells.

The assay was initiated by washing the monolayers with phosphate buffered saline containing 1% bovine serum albumin and fixing them in situ with 0.2 ml of 0.05% glutaraldehyde for 20 min at 4°C. We then sequentially added polyclonal rabbit anti-HSV immunoglobulin (1/2,000) (Accurate Chemicals, Westbury, N.Y.) for 1 h, peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody (1/2,000) (TAGO, Inc., Burlingame, CA) for 2 h, and the substrate. Intervening washes were done with phosphate buffered saline containing 0.05% Tween 20 and 1% bovine serum albumin. The substrate was 1.0 mg of the diammonium salt of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co., St. Louis, Mo.) per ml in phosphate-citrate buffer (pH 4.8) with 0.005% hydrogen peroxide. In the presence of peroxidase, the substrate changes from light to dark green. The A405 of the wells was read at room temperature with a Bio-Tek ELISA reader (model EL 307; Bio-Tek Instruments, Inc., Burlington, Vt.) when the A405 of the virus control was 1.0 to 1.4. The susceptibility to antiviral agents was expressed as the 50% inhibitory dose (ID50), i.e., the concentration of drug that reduced the A405 to 50% of that of the virus control.

Because the sensitivity of an ELISA used to determine antiviral susceptibility is dependent on the antigen generated in the virus control wells, preliminary experiments were performed to determine the relationship at various times after infection between virus inoculum and the A405. With a laboratory strain of HSV type 2 (HSV-2) (434D), HSV replication could be reliably detected at 24 h with an inoculum as low as 200 PFU per well and at 48 h with an inoculum of 25 PFU per well (Fig. 1). HSV-1 and HSV-2 could be detected by using rabbit antibody to either serotype.

The effect of inoculum size and duration of incubation on ID50 was assessed by infecting duplicate sets of plates with 30, 300, or 3,000 PFU of HSV 434D per well in the presence of acyclovir. The results were similar at each inoculum regardless of whether the ELISA was performed at 24 or 48 h after infection. At 48 h, a fourfold difference in ID50 (0.05

* Corresponding author.
† Present address: Department of Pediatrics, Baragwanath Hospital, Johannesburg, Republic of South Africa.

FIG. 1. Effect of virus inoculum on ΔA405 for HSV 434D when tested by ELISA at 24 and 48 h after infection. The ΔA405 was calculated as the mean A405 of the uninfected control wells (usually 0.15 to 0.20) subtracted from the mean A405 of the virus-infected wells. The inoculum of 500 PFU per well corresponds to a multiplicity of infection of 8 × 10^(-2).

946
versus 0.23 μg/ml) was noted between the monolayers infected with 30 and 3,000 PFU per well, respectively. Testing at 24 h was not possible with an inoculum of 30 PFU per well, but inocula of 300 and 3,000 PFU per well gave ID₉₀ results of 0.10 and 0.36 μg/ml, respectively. Furthermore, the ID₉₀ of acyclovir for strain 434D was obtained in 18 additional experiments in which the inoculum was varied from 30 to 3,000 PFU per well (data not shown). These ID₉₀ results varied only from 0.05 to 0.42 μg/ml, suggesting a minimal inoculum effect (r = 0.35 on linear regression analysis). The mean ID₉₀ of acyclovir for these 18 separate determinations was 0.20 ± 0.11 μg/ml.

Plate-to-plate variability was studied for strain 434D and two clinical HSV-2 isolates (strains 1402 and 771) by performing triplicate assays on the same day with an inoculum of 300 PFU per well. This test resulted in a plate-to-plate variation in ID₉₀ of 27, 18, and 14%, respectively. At any acyclovir concentration, the mean well-to-well percent coefficient of variation of ID₉₀ for these three isolates was 11, 10, and 5%, respectively (data not shown).

Acyclovir ID₉₀ results for 15 additional stock HSV isolates for which titers had been determined (nine isolates of HSV-2 and six isolates of HSV-1, provided by S. Lehman, Burroughs Wellcome) were then determined by both ELISA and plaque reduction assay (PRA) (Table 1). Three of the four isolates with an ID₉₀ by ELISA of >10.0 μg/ml also had an ID₉₀ by PRA of >10.0 μg/ml. The fourth isolate had an ID₉₀ by PRA of 3.0 μg/ml. These four isolates were considered resistant to acyclovir. Excluding these four isolates, values determined by the two methods correlated well (correlation coefficient, 0.80). The ID₉₀ of DHPG for strain 434D was 0.05 μg/ml by ELISA and 0.06 μg/ml by PRA.

The ID₉₀ of six clinical HSV isolates (all type 2, for which titers were not known) were determined by infecting human embryonic lung fibroblast monolayers with 10-, 100-, and 1,000-fold dilutions of fluid taken from the primary isolation tube after one freeze-thaw cycle. ID₉₀s determined by ELISA were available within 24 h for both the 10-fold and 100-fold dilutions; the mean ID₉₀ of acyclovir by ELISA were 0.36 and 0.24 μg/ml, respectively. These six isolates were subsequently determined to contain from 2.8 × 10³ to 2.9 × 10⁵ PFU/ml, and the ID₉₀s determined by PRA were compared with those determined by ELISA (Table 1).

This ELISA method for determining the ID₉₀ of anti-HSV drugs has several advantages over the commonly used assays, i.e., PRA and the neutral red-dye uptake assay. The gold standard, PRA, requires 3 days and virus for which titers have been determined and has a subjective endpoint, whereas the ELISA avoids the need for such virus and has an objective endpoint. The neutral red test can be performed with virus for which titers have not been determined (6, 7), but ID₉₀ results obtained in this manner have not been compared with those obtained by PRA and appear to be hampered by large-inoculum effects. We did not see the large-inoculum effects on ID₉₀ noted by others (2, 4). Our ELISA also has advantages over nucleic acid hybridization (3) and [³²P]iododeoxyoxycytidine uptake (9) by avoiding the need for nucleic acid probes and radioisotopes.

Two previous works describe the application of ELISA methods to HSV susceptibility testing. In the first report (5), only a single HSV-1 isolate for which titers had been determined was studied, and no ID₉₀ was determined. The second report (10) describes the testing of 41 HSV isolates. The assay was not performed in situ; rather, the contents of each well were solubilized and viral antigen was determined in a separate plate. In addition, in that report, ID₉₀s are not compared with results by PRA, the reproducibility of the test is not clearly presented, and the researchers use their own high-titer rabbit anti-HSV antibody. In contrast, our in situ ELISA avoids these problems and uses commercially available antiserum. It facilitates simple and reproducible HSV susceptibility testing and is suitable for the rapid evaluation of large numbers of isolates should such evaluation become necessary because of the emergence of widespread resistance.

LITERATURE CITED


ric assay for the measurement of the sensitivity of herpes simplex viruses to antiviral agents. Antiviral Res. 3:223–234.