Differentiation Between Herpes Simplex Virus Type 1 and Type 2 Strains by Immunoelectroosmophoresis

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A method has been elaborated to differentiate between herpes simplex type 1 and type 2 viruses by immunoelectroosmophoresis. With rabbit immune sera cross-absorbed with heterologous virus antigen, a distinct difference was shown between the two virus types. Herpes simplex type 1 virus tested against cross-absorbed type 1 antiserum gave two precipitin lines. Herpes simplex type 2 virus gave one precipitin line when tested against cross-absorbed homologous serum. When the viral antigens were tested against cross-absorbed heterologous immune sera, no or only very weak precipitin reactions were observed. The test is easy and rapid, requires relatively small quantities of antigen and antibody, and is suitable for typing of herpes simplex virus in diagnostic routine work.

Herpes simplex virus (HSV) has been classified into two distinct antigenic groups, type 1 and type 2 (18, 19). Differentiation between HSV type 1 and type 2 can be achieved by serological and biological procedures (7, 14, 16). Some serological methods, such as various neutralization tests (5, 18, 23), are laborious. The fluorescent-antibody technique (8, 17) requires reagents of considerable purity and specificity. Recently, indirect hemagglutination inhibition has been suggested as a rapid method for typing of HSV isolates (3), and so has a temperature marker test (14). Determination of pock size on the chorioallantoic membrane has been used as a typing procedure (16). Amstey (1) typed HSV isolates by using primary chicken embryo cells, in which type 2 forms plaque but type 1 does not.

Recently Schneweis and Nahmias (22), using immunodiffusion, found evidence of antigens specific for HSV type 1 and type 2. They found two type-specific antigens for HSV type 1, one type-specific antigen for HSV type 2, and three other antigens common to both types. Ordinary immunodiffusion requires relatively large quantities of antigen and antibody. Immunoelectroosmophoresis (IEOP) has been shown to be about 20 times as sensitive (11) as conventional immunodiffusion (20) in detecting Australia antigen. Because it is a rapid, simple, and a relatively sensitive method, IEOP was tried in typing of HSV strains.

MATERIALS AND METHODS

Tissue culture. BHK-21 clone 13 (15) was kindly supplied by Flow Laboratories Ltd., Scotland, and was grown in 1-liter roller bottles. Eagle minimum essential medium (MEM) with 5% noninactivated fetal bovine serum was employed for outgrowth, and with 0.3% bovine serum albumin for maintenance medium.

GMK-AH 1 cells (10) were grown in Eagle MEM with 10% inactivated calf serum and for maintenance the same medium without serum.

Chicken embryo cell cultures were produced as follows. Ten-day-old chicken embryos were trypsinized. The cells were seeded into Roux bottles with the following growth medium: Eagle MEM with 5% inactivated fetal bovine serum, and 2% 0.1 M L-glutamine. After removal of growth medium the monolayers were washed with phosphate-buffered saline (PBS), and Parker’s medium 199 was used as the maintenance medium. All tissue culture media were supplemented with 100 units of penicillin per ml and 100 μg of streptomycin per ml.

Virus strains. The following virus strains (i) Maclntyre (VR), HSV type 1, (ii) Tyler cornea, HSV type 1, (iii) MS, HSV type 2, and (iv) Curtis thigh lesion, HSV type 2, were kindly provided by A. J. Nahmias, Emory University, Atlanta, Ga.

(v) Herpes simplex virus Z, HSV type 1, has a long passage history on the chorioallantoic membrane. It was originally obtained from D. C. Gajdusek, National Institute of Neurological Disease, Bethesda, Md.

(vi) HSV type 2 was received from the National Bacteriological Laboratory, Stockholm, 2nd passage on RK 13 (2). It was originally obtained from G.
Plummer, Loyola University of Chicago. These six strains were all passed once on GMK-AH 1 cells and kept at -65 C.

Antigens. For use in immunoelectrophoresis and absorption studies, roller bottle cultures of BHK-21 were inoculated with 10 ml of HSV type 1 (v; numerals i-vi indicate the prototype strain as defined above) [10\(^{+4.5}\) median tissue culture infective dose (TCID\(_{50}\))/0.10 ml] and type 2 (vi) virus [10\(^{+4.0}\) TCID\(_{50}\)/0.10 ml]. After one or two days, a complete cytopathic effect (CPE) had developed. The infected cells were shaken off the glass and separated from the medium by centrifugation at 1,500 \(\times\) g for 15 min. The packed cells, approximately 10\(^4\) cells from one roller bottle, were suspended in 2 ml of Eagle MEM and stored at -65 C. For use in booster immunization of rabbits, HSV type 2 was grown in chicken fibroblasts (6, 7). Monolayers of chicken fibroblasts in Roux bottles were infected with HSV type 2. After two or three days, the CPE was complete, and the infected cells were shaken off the glass and separated by centrifugation at 1,500 \(\times\) g for 15 min. The packed cells from one Roux bottle were suspended in 2.5 ml of Eagle MEM and stored at -65 C. Each of the virus strains used in IEOP was inoculated into one tissue culture tube of GMK-AH 1 cells. After one or two days a complete CPE had developed, and the infected cells were shaken off the glass. Each tube was centrifuged at 1,500 \(\times\) g for 15 min, all medium was withdrawn, and 0.10 ml of Eagle MEM was added to the cell pellet. This antigen was frozen and thawed three times before use in IEOP.

Antiserum. Rabbits were immunized by infecting the scarified corneas with 0.05 ml of virus suspension. The following virus strains were used. HSV type 1 (v) grown on GMK-AH 1 for several passages was used in a dilution giving 10\(^{+4.5}\) TCID\(_{50}\)/0.10 ml. HSV type 2 (vi), first passage on GMK-AH 1, was used undiluted with a titer 10\(^{+9.5}\) TCID\(_{50}\)/0.10 ml. When the keratoconjunctivitis had healed, the rabbits infected with HSV type 2 were given an intravenous booster of virus grown in chicken fibroblasts. Rabbits infected with HSV type 1 received no booster injection. The rabbits were bled two weeks after healing of the corneal lesions or two weeks after the intravenous booster injection. Sera were checked for precipitating activity in IEOP, and the most potent type 1 and type 2 sera were selected for absorption.

Absorption. Before absorption, BHK-21 cells infected with HSV type 1 (v) or type 2 (vi) were thawed, dialyzed against 0.075 m barbital buffer, pH 8.6, and disrupted by ultrasonic treatment. A MSE (Measuring and Scientific Equipment Ltd., London) 60 w ultrasonic disintegrator was used. The cells were treated at 20 kc/sec at 1.8 to 2.0 amp during five 1-min periods in an ice bath. Equal volumes of antisera [HSV type 1 (v) or type 2 (vi) antisera] and heterologous, BHK-21-grown, virus antigen [HSV type 1 (v) or type 2 (vi) antigen] were mixed and incubated for 1 hr in a water bath at 37 C and overnight at +4 C and were then centrifuged for 1 hr at 80,000 \(\times\) g in a Spinco model L ultracentrifuge, Beckman Instruments, Inc. After centrifugation the mixture was concentrated to a third of the original volume of serum with "Lyphogel" (Gelman Instruments, Ann Arbor, Mich.). This procedure was repeated until no or only weak activity could be detected in IEOP of the serum against the heterologous antigen while the serum still showed significant precipitin activity against the homologous antigen. For typing of HSV strains in IEOP, these cross-absorbed sera were used in a dilution experimentally determined as optimal for differentiation between HSV type 1 and type 2—highest dilution giving + (see Table 1) precipitation. In the present study sera were used in dilution 1/4 (Table 1).

Immunoelectrophoresis. The viral antigens for immunoelectrophoresis were grown in BHK-21 cells as described above. The infected cells were thawed, sonically treated, and centrifuged at 3,000 \(\times\) g for 60 min. The supernatant fluid was concentrated five times with "Lyphogel" and stored at -65 C. Immunoelectrophoresis was performed as described by Grabar, Uriel, and Couron (9). Agarose was dissolved in 0.075 m barbital buffer, pH 8.6. Gels of 0.75% (w/v) were used. Samples of 5 »lters were applied and separated by electrophoresis (100 V, 16 ma, for 60 min) in a water-cooled chamber followed by immunodiffusion for 1 day at room temperature and 10 days at +4 C. The precipitates were photographed directly without staining.

IEOP. IEOP was performed according to Hanson and Johnsson (11) on a glass plate (20 by 10 by 0.15

**TABLE 1. Comparative antigen and antiserum titrations in immunoelectrophoresis**

| Antigen | Cross-absorbed antisera |
|---------|------------------------|
|         | Herpes simplex type 1 (v) serum | Herpes simplex type 2 (vi) serum |
|         | Dilution | Dilution | Dilution | Dilution |
| GMK AH 1 uninfected control antigen | 1/1 | 1/2 | 1/4 | 1/2 | 1/4 |
| Dilution | 1/1 | 0* | 0 | 0 | 0 | 0 |
| 1/4 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1/16 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1/64 | 0 | 0 | 0 | 0 | 0 | 0 |
| Herpes simplex type 1 (v) antigen | 1/1 | ++ | ++ | + | (+) | (+) |
| 1/4 | (+) | (+) | (+) | + | 0 | 0 |
| 1/16 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1/64 | 0 | 0 | 0 | 0 | 0 | 0 |
| Herpes simplex type 2 (vi) antigen | 1/1 | 0 | 0 | + | + | + |
| 1/4 | 0 | 0 | + | + | + | + |
| 1/16 | 0 | 0 | + | + | + | + |
| 1/64 | 0 | 0 | 0 | 0 | 0 | 0 |

* Symbols: 0, no precipitate, not even after staining; +, faint precipitate visible only after staining; (+), very faint precipitate visible only after staining; ++, strong precipitate visible without staining.
cm) with a 1-mm thick gel. The gel consisted of 0.75% agarose (w/v) in 0.075 M barbital buffer, pH 8.6. Parallel rows of wells 4 mm in diameter were cut in the gel 6 mm apart. In each double row, the wells close to the anode were filled with antiserum and the wells close to the cathode with antigen. The electrophoresis was carried out in a water-cooled chamber. The electrophoresis was run for 50 min at a field strength of 7 v/cm. After the electrophoretic run, the plates were incubated at room temperature for 24 hr and examined for precipitin lines. Additional precipitin reactions were observed after washing in saline overnight (4). After washing, the plates were dried and stained with 0.2% Coomassie brilliant blue in acetic acid-methanol-water for 15 min and destained in acetic acid-methanol-water for 20 min. Staining revealed a few further positive reactions (4, 11).

RESULTS

Immunoelectrophoresis. Immunoelectrophoresis of HSV type 1 (v) and type 2 (vi) was tried as a method to demonstrate type-specific antigens and evaluate their electrophoretic mobility. The immunoelectrophoretic precipitin pattern is shown in Fig. 1. With HSV type 1 (v) serum, two precipitin lines develop against homologous antigen and one precipitin line against heterologous antigen. HSV type 2 (vi) serum gives two distinct precipitin lines against homologous antigen and two lines, one distinct and one very faint, against heterologous antigen. The results confirm the presence of type-specific antigens (22) and indicate that the majority of the antigens move towards the anode.

IEOP studies. With unabsorbed HSV type 1 (v) and type 2 (vi) sera, no differentiation between the two virus types could be shown in IEOP. These sera gave three or four precipitin lines with each virus type. With absorbed HSV type 1 (v) serum, only HSV type 1 antigen gave distinct precipitin lines in IEOP. After absorption HSV type 2 (vi) serum showed only trace activity against the heterologous antigen (Fig. 2). Comparative two-dimensional antigen and antiserum titrations were made to evaluate the sensitivity and power of IEOP to differentiate between HSV type 1 and HSV type 2. HSV type 1 (v) and type 2 antigens (GMK-AH 1 cell-phase, freeze-thawed antigens) and immune sera were diluted in Eagle MEM. The results of IEOP are shown in Table 1. The test

![Fig. 1. Immunoelectrophoresis of HSV type 1 and type 2 antigen. (A, B) Top well, HSV type 2 (vi) antigen; lower well, HSV type 1 (v) antigen. (A) HSV type 1 (v) unabsorbed antiserum in central trough. (B) HSV type 2 (vi) unabsorbed antiserum in central trough.]

![Fig. 2. Precipitin pattern after immunoelectroosmophoresis of HSV type 1 and type 2 antigen against cross-absorbed homologous and heterologous sera. Both plates were stained with Coomassie brilliant blue. (A, B) Wells close to the cathode contain the following antigens (from left to right): HSV type 1 (i), type 1 (ii), type 2 (iii), type 2 (iv), type 1 (w), type 2 (vi), and uninfected GMK-AH 1 control antigen. (A) HSV type 1 (v) cross-absorbed antiserum in wells close to the anode. (B) HSV type 2 (vi) cross-absorbed antiserum in wells close to the anode.]
shows a clear discrimination between HSV type 1 and type 2, and there is no reaction with uninfected GMK-AH 1 control antigen. The stained plates were initially viewed against an illuminated white background. Later it was found that the sensitivity could be increased by dark-field illumination. The plates were illuminated with a daylight fluorescent lamp (40 w, Luma) and the stained lines showed an intense yellow or red fluorescence. By this method additional precipitin lines could be detected and the sensitivity was increased.

To evaluate the use of IEOP for typing of HSV isolates, six prototype strains of HSV (type 1 strains i, ii, and v, type 2 strains iii, iv, and vi) and fresh strains isolated from patients were used. The virus antigens were used undiluted in IEOP. The amount of antigen produced was reproducible both with prototype strains and with fresh isolates of HSV. The antigens could therefore be used in IEOP in predetermined dilutions. It was observed that all HSV type 1 strains tested against cross-absorbed HSV type 1 (v) serum gave two precipitin lines and that all strains of HSV type 2 tested against cross-absorbed type 2 (vi) serum showed only one precipitin line (Fig. 2).

**Correlation between IEOP and other typing procedures.** To compare IEOP with other methods of typing, five HSV type 1 and five type 2 strains isolated and typed in this laboratory were sent to P. Leinikki, University of Helsinki, Helsinki, Finland. In return, five HSV type 1 and four type 2 strains isolated in Helsinki were obtained from him. For classification, Leinikki used immunofluorescence (17) and kinetic neutralization tests (23) as well as the capacity of the strains to form plaques in chicken embryo fibroblasts (1) and to grow at 39.5 C (14). The typing results of the two laboratories were in full agreement, except for one of the strains classified here as type 2. This strain behaved atypically in the thermostatilability test, growing well at 39.5 C, but on serological investigation Leinikki found it to be a type 2 strain.

**DISCUSSION**

Schnieweis and Nahmias (22) have found that HSV type 1 may have two specific antigens and HSV type 2 one specific antigen. The precipitin patterns observed in IEOP of HSV type 1 and type 2 virus support these findings. According to the present investigation, IEOP performed with cross-absorbed antisera is an easy and rapid technique for typing of HSV. About 30 specimens may be tested in a single run on one glass slide. The method is specific and there is no need for complicated equipment. Antigens for IEOP can easily be made from one tissue culture tube. The proposed method, therefore, seems to be suitable for typing of HSV in routine diagnostic work. Thus far, 176 HSV strains isolated in connection with epidemiological studies on genital HSV infection (12, 13) have been typed by using this method; 166 of these strains were typed simply by IEOP, 10 strains were also typed by other procedures by P. Leinikki. The results were reproducible, and among these strains no intermediate types were found, which is contradictory to Roizman's suggestion (21) that herpes simplex viruses form a continuously variable spectrum of variants.

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