Typing of Herpes Simplex Virus with Type-Specific Human Immunoglobulin M in an Indirect Immunofluorescence Assay

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Sera from nine individuals with suspected primary herpes simplex virus type 1 (HSV-1) or type 2 (HSV-2) infection were screened to identify those containing HSV type-specific immunoglobulin M (IgM). Selected sera were then utilized in an IgM-specific indirect immunofluorescent-antibody HSV-typing assay (patent pending). To evaluate the procedure, 29 HSV isolates were grown in cultures of continuous human amnion cells, fixed, and used as substrates for indirect immunofluorescence. Determination of virus type was based on intensity of fluorescence of the substrate with HSV-1 and HSV-2-specific antisera after staining with fluorescein-conjugated anti-human IgM. Typing of the isolates by restriction endonuclease digestion showed that of 29, 18 were HSV-2 and 11 were HSV-1. Results by IgM-specific indirect immunofluorescent-antibody assay were identical to those by restriction endonuclease digestion for 27 of the isolates; 2 isolates failed to replicate adequately in the test cells. The IgM-specific indirect immunofluorescent-antibody procedure appears to be a simple, rapid, and accurate technique which could be of use to clinical virology laboratories.

A variety of techniques have been used for typing herpes simplex virus (HSV) isolates, including replication in chicken embryo cells, kinetics of neutralization or endpoint neutralization tests, immunoperoxidase- or fluorescein-labeled antibody procedures, indirect hemagglutination, solid-phase radioimmunoassay, and digestion of isolates with a restriction endonuclease (4, 5). These procedures are complicated, may require adsorption of sera to remove cross-reacting antibodies, and are available only in research laboratories.

Human immunoglobulin from patients with recurrent skin lesions due to HSV type 1 (HSV-1) or HSV type 2 (HSV-2) reacts readily with both virus types because of common antigens (9). In 1975, Schmidt et al. (10) reported that seven of seven patients undergoing primary HSV-1 infection and one of eight patients with primary HSV-2 disease had only type-specific HSV-neutralizing antibody in the immunoglobulin M (IgM) fraction of immunoglobulin. These observations led us to consider that individuals producing HSV-type-specific IgM could be identified and bled and a large pool of sera could be created which could be used to type HSV in an IgM-specific indirect immunofluorescent-antibody (IgM-IFA) assay. We report here our success in finding individuals with type-specific IgM and the results of typing 29 HSV isolates by an IgM-IFA assay.

MATERIALS AND METHODS

Patient population. Serum samples were obtained from one adult with herpes gingivostomatitis and from eight adults with their first episode of herpes simplex genitalis. Samples were assayed by IgM-IFA (see below) to identify IgM directed exclusively against HSV-1 or HSV-2. After documentation of informed consent, subjects producing type-specific IgM were plasmapheresed, and an inventory of sera was acquired. Virus isolates utilized in the evaluation of the IgM-IFA assay were originally acquired in our herpetic clinic from patients with herpes simplex genitalis or labialis.

IgM-IFA assay. Cells for the IgM-IFA assay were prepared by infecting AV 1 cells with test or reference HSV. AV 2 cells, a line of continuous human amnion cells (ATCC), were propagated in medium 199 with 20% calf serum, HEPES buffer (N-2-hydroxyethylpipеразине-N'-2-етансульфонная кислота), and antibiotics and used to inoculate four-chambered tissue culture slides (Lab-Tek Products, Div. Miles Laboratories Inc., Westmont, Ill.) at a cell concentration of 1.5 × 10^5 per chamber. The cells were incubated at 37°C in a 5% CO 2 atmosphere for 48 h and then exposed to virus at a multiplicity of infection of approximately 1. After incubation for an additional 16 to 18 h, at which point scattered foci of rounded cells first became apparent, the chambers were removed, and the slides were rinsed twice in 0.85% saline and twice in 0.85% saline–0.1% NaHCO 3 . The cells were then fixed by exposure to 80% isopropyl alcohol for 20 min at room temperature, rinsed once in saline, and stored in 0.01 M phosphate-buffered saline (pH 7.4) until use.

To identify individuals with type-specific IgM,
slides of AV3 cells were infected with reference HSV-1 strain McIntrye (ATCC) or reference HSV-2 strain MS (ATCC). Fixed cells infected with the McIntrye strain and cells infected with MS were overlaid with serial dilutions of the test serum, reference serum containing IgG reactive with both HSV-1 and HSV-2 (positive control), or reference serum lacking antibody against HSV-1 or HSV-2 (negative control) and incubated at 37°C for 1 h. The slides were then rinsed once with phosphate-buffered saline and overlaid with either fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgM (test sera and negative control) or FITC-conjugated goat anti-human immunoglobulin (positive control). Infected cells reacted with anti-HSV IgG were also stained with FITC-conjugated anti-IgM to control for the specificity of the anti-IgM.

All reference sera and FITC conjugates were obtained from the Microbiological Research Corp., Bountiful, Utah. The slides were incubated at 37°C for 30 min, rinsed once with phosphate-buffered saline, covered with buffered glycerol mounting fluid (pH 7.8) and a glass cover slip, and examined by fluorescent microscopy. Intensity of fluorescence was judged by assigning the positive control well a value of 4+ and the negative control well a value of 0. The serum titer of anti-HSV antibody was defined as the reciprocal of the last dilution to give 2+ fluorescence.

Patient sera containing anti-HSV IgM specific for type 1 and type 2 were used in the typing of the clinical HSV isolates. The working dilutions of the sera were the highest dilutions that still showed a 3+ fluorescence intensity when reacted with cells infected with the homologous virus. Typing of test viruses was performed by the IgM-IFA assay, as illustrated in Fig. 1. AV3 cells on four-chambered tissue culture slides were infected with the test virus, as described above. The first well was overlaid with a 1:10 dilution of serum containing IgM specific for HSV-1 (patient A), the second well was overlaid with a 1:40 dilution of serum with IgM specific for HSV-2 (patient B), the third well was overlaid with a 1:10 dilution of serum containing IgG reactive with HSV-1 and HSV-2 (positive control), and the fourth well was overlaid with a 1:10 dilution of serum lacking HSV antibody (negative control). Wells 1 and 2 were then stained with FITC-conjugated anti-human IgM, and wells 3 and 4 were stained with FITC-conjugated anti-human immunoglobulin. A judgment was made on the virus type only when there was a 2+ difference in the intensity of fluorescence between wells 1 and 2. If neither well 1 nor well 2 attained a fluorescence intensity of 2+ or greater, the cell infection was considered inadequate.

Restriction endonuclease typing assay. The procedure for restriction endonuclease typing of HSV was a modification of procedures described by Buchman et al. and by Lonsdale (1, 5). Petri dishes (35 mm) were seeded with Vero cells (Flow Laboratories, Rockville, Md.) or with a continuous line of human melanoma cells (Charles Grose, University of Texas Health Sciences Center, San Antonio). The cells were fed with Eagle minimal essential medium ( Gibco Laboratories, Grand Island, N.Y.) supplemented to contain 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, and 100 µg of gentamicin per ml, and incubated in 5% CO2 at 37°C. Phosphate-free medium, similar to the above except for the omission of inorganic phosphate and the substitution of 1% for 10% fetal calf serum, was prepared in our laboratories. At 50 to 70% confluency, the cell culture medium was changed to phosphate-free medium, and the cells were incubated for an additional 24 to 48 h. The medium was removed by aspiration, and the cells were exposed to high-titered HSV in a volume of 0.2 ml for 1 h. The virus inoculum was then removed by aspiration, and the monolayer was washed twice with phosphate-free medium. The cells were incubated in 1 ml of phosphate-free medium, and 50 to 100 µCi of [32P]orthophosphate (New England Nuclear Corp., Boston, Mass.) was added. With 70 to 80% cytopathic effect was apparent, the cells were frozen and thawed three times. Cell debris was precipitated by centrifugation at 1,000 × g for 10 min, and the supernatant fluid was added to a polypropylene tube (Falcon Plastics, Oxnard, Calif.) containing 2.625 ml of distilled water, 0.250 ml of a heat-treated tronate solution (20 mg/ml; Cal-Biochem, La Jolla, Calif.), and 0.125 ml of 0.4 M EDTA (Sigma Chemical Co., St. Louis, Mo.). The solution was mixed by swirling, and 1 ml of 10% sodium dodecyl sulfate (BDH Chemicals, Poole, England) was added. Nucleic acid was then extracted once with phenol and precipitated with 2 volumes of 95% ethanol. The precipitate was collected by centrifugation at 20,000 × g for 20 min and suspended in 150 µl of distilled water containing 2 U of RNase T1 and 10 µg of RNase A (heat-treated; Millipore Corp., Bedford, Mass.) Acid-precipitable radioactivity was then measured in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

DNA digestion was performed with 1 µl of KpnI or

**FIG. 1.** Diagram of a four-chambered tissue culture slide showing the contents and significance of each well for an IgM-IFA typing assay.
**RESULTS**

Table 1 shows the results of IgM-IFA testing of sera from nine patients with herpetic disease, using goat anti-human gamma globulin or goat anti-human IgM and HSV-1 or HSV-2-infected AV3 cell monolayers. The patient with herpes gingivostomatitis and two of the patients with herpes genitalis had type-specific IgM class antibody against HSV. These three sera were negative when assayed for rheumatoid factor at a dilution of 1:20. Tests for HSV complement-fixing antibodies were performed on the three patients with type-specific antibody, and low titers of complement-fixing antibody (8 to 32) were found in each case, although it is noteworthy that the sera tested were collected 10 to 30 days after the onset of illness, too late in the course of infection to help establish whether these cases were primary or initial herpetic disease (8).

The results of typing 29 clinical isolates by the IgM-IFA assay, restriction endonuclease digestion, and replication in chicken embryo cells are shown in Table 2. The results by the IgM-IFA procedure matched with the results obtained by restriction endonuclease digestion. Two isolates, both HSV-2, failed to replicate adequately in the AV3 cells used in the IgM-IFA assay. IgM-IFA correctly typed four HSV-2 isolates that grew poorly and showed high background 32P in the restriction endonuclease assay (Fig. 2). In contrast, typing by the chicken embryo cell procedure was erroneous for one of these four isolates (HG-14), indeterminate for two others (HG-9 and HG-10), and was not performed on the fourth (HG-7).

**DISCUSSION**

Our results confirm the findings of Schmidt et al. (10), showing that some persons undergoing HSV-1 or HSV-2 infection will produce only type-specific IgM against the infecting virus. We utilized the sera from two such subjects for an IgM-IFA HSV-typing assay and have shown among a small number of isolates that the IgM-IFA assay type determinations matched those obtained by restriction endonuclease digestion. Gerna et al. (2) have also successfully typed HSV isolates with human sera, employing peroxidase-labeled antiglobulin.

The type specificity of the IgM antibody re-
response in HSV infection has been the subject of conflicting reports in the literature. Several investigators have found that serum IgM in response to primary HSV-2 infection is often reactive with an HSV-1 substrate, assayed either by membrane fluorescence (7), IgM-IFA (3, 6) virus neutralization (10), or indirect peroxidase-labeled antibody (2). However, with the indirect hemagglutination procedure, Farris et al. (W. A. Farris, J. A. Stewart, and J. R. Evrard, Abstr. Annu. Meet. Am. Soc. Microbiol., 1974, V337, p. 256) found that patients with primary HSV-2 disease produced IgM that was mostly reactive with the homologous virus.

After primary HSV-1 infection, the type specificity of serum IgM appears also to depend on the assay used. Sera from seven cases assayed by neutralization had IgM directed only against HSV-1 (10), whereas eight cases studied by the indirect hemagglutination procedure all had IgM which reacted with both HSV-1 and HSV-2 substrates (Farris et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, V337, p. 256). The basis for these different results remains to be determined.

Our ability to accurately type HSV isolates by the IgM-IFA assay without adsorption of sera with the heterologous strain suggests that this technique might be a simple and rapid typing procedure for investigators and clinical virology laboratories. We are presently extending our experience with the IgM-IFA assay by typing additional isolates and testing a different cell substrate.

LITERATURE CITED


