Differentiation between Type 1 and Type 2 Strains of Herpes Simplex Virus by an Indirect Immunofluorescent Technique

(Accepted 21 April 1971)

The cytoplasmic membranes of cells infected with herpes simplex virus develop altered antigenic specificities (O'Dea & Dineen, 1957; Roizman & Spring, 1967). Strain-specific differences in these surface antigens have been reported (Roizman & Roane, 1963; Peterknecht, Bitter-Suerman & Falke, 1968). In the course of a study of antigenic specificities in BHK cells infected with several herpes simplex viruses (cf. Watson et al. 1966), we have found that the use of appropriately absorbed antisera permits the detection of type-specific membrane antigens. This forms the basis of the simple and rapid immunofluorescent method for typing strains of herpes simplex virus which we report in this note.

Exploratory work was done with two established type 1 strains (HFEM which originated from the Rockefeller strain HF and WAL, a recurrent oral isolate) and two established type 2 strains (LOV, kindly given us by Dr A. J. Nahmias and BRY, a recurrent genital isolate). The viruses were propagated and stored as described by Watson et al. (1966) and assayed by means of plaque counts under carboxymethyl cellulose (Russell, 1962). Antisera were prepared against each in rabbits by a prolonged course of immunization with freeze-dried extracts of disrupted infected RK 13 cells (Watson et al. 1966). In immunodiffusion tests these antisera gave multiple lines with extracts of herpes simplex virus-infected cells.

Absorbed sera were prepared by treatment first with uninfected BHK cells (10⁸/ml. of serum) for 1 hr at 37° and then shaken at 4° overnight. They were further absorbed with cells infected at about 0.1 p.f.u./cell and incubated at 33° for 48 hr (type 1 viruses) or 24 hr (type 2 viruses) and collected by scraping off the glass with a silicone policeman. These cells were washed thrice in buffered saline (Dulbecco & Vogt, 1954) before use. To 3 × 10⁸ washed and sedimented infected cells 1 ml. of antiserum in dilution 1/8 was added, and incubated for 1 hr at 37° in a water bath. The antiserum was separated by centrifugation and added to another similar sample of infected cells. The suspension was incubated at 4° overnight with shaking, then the serum was separated by centrifugation at 4,000 rev./min. for 10 min. and stored at −20° until use.

Tests for immunofluorescence were made by the indirect method using antiserum to rabbit γ-globulin prepared in sheep and kindly provided by Dr D. Catty. The γ-globulin fraction of this serum was obtained by repeated precipitation with ammonium sulphate and conjugated to fluorescein isothiocyanate (Nairn, 1969). Non-specific staining components were removed by absorption with BHK cells as above.

Coverslip cultures were prepared in the bottom of 50 mm. diameter Petri dishes seeded with 10⁸ BHK cells in 5 ml. of growth medium, and incubated at 37° for about 12 hr. These were inoculated with 10 p.f.u. virus/cell. After an adsorption period of 1 hr, the cells were washed thrice with phosphate-buffered saline and incubated under 5 ml. growth medium for 3.5 hr at 37° and then again washed in buffered saline. Tests for cell membrane fluorescence were made with unfixed preparations. The indirect surface-immunofluorescence test was done on the washed coverslip preparation by treating with one drop of anti-herpes serum for 30 min. at 37°. After washing thrice with buffered saline it was drained and treated with one drop of conjugated anti-rabbit γ-globulin for the same time, and after three further
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washes the preparation was mounted in glycerol and buffered saline (Nairn, 1969). Tests for intracellular antigens were done on coverslip cultures, air-dried at room temperature for 30 min. and then fixed for 3 min. in acetone.

The antibody titre of antisera against herpes simplex virus-specific cell membrane and intracellular antigens was determined, using twofold dilutions (starting point 1/8). The reactivity of the serum dilution with the virus-specific antigens was considered positive when well-defined outlines of fluorescing formations were seen. The same filters, ocular and objective lenses were used in every test. All the antisera were also tested simultaneously against uninfected BHK cells. All the coverslip preparations were reacted with pre-immune sera. None of the controls showed any fluorescence.

Table 1. Antibody titres of antisera against herpes simplex virus-specific cell-membrane and intracellular antigens, determined by the indirect immunofluorescent technique

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Absorbed with cells infected by strain</th>
<th>Cell-membrane antigen induced by strain</th>
<th>Intracellular antigen induced by strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepared against herpes simplex virus strain</td>
<td>HFEM</td>
<td>WAL</td>
<td>BRY</td>
</tr>
<tr>
<td>Type 1 HFEM</td>
<td>None</td>
<td>512*</td>
<td>512</td>
</tr>
<tr>
<td>Type 1 WAL</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Type 2 BRY</td>
<td>256</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td>Type 2 LOV</td>
<td>128</td>
<td>64</td>
<td>0</td>
</tr>
<tr>
<td>Type 2 BRY</td>
<td>128</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td>Type 2 LOV</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Type 1 HFEM</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Type 1 WAL</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

* Values are reciprocals of the last dilution at which surface fluorescence was observed.

It was clear that by absorbing anti-herpes sera with cells infected with heterotypic virus strains the sera became type specific for these antigens on the surfaces of cells infected for 5.5 hr (Table 1). This therefore formed a sound basis for a typing test. Less satisfactory discrimination resulted when intracellular antigens were revealed in fixed preparations, possibly because some common antigens are not represented at the surface. These tests were repeated using antisera prepared against BRY and WAL, with similar results.

The cell-membrane antigens induced by type 1 herpes simplex virus appeared as very fine granules equally distributed along the cytoplasmic membrane (Fig. 1a). The cell-membrane antigens induced by type 2 herpes simplex virus appeared as intermittent large granules (Fig. 1b). Type-specific antisera reacted with less densely distributed antigenic formations showing weaker fluorescence than unabsorbed sera.

These results suggested that cell-membrane immunofluorescence with absorbed sera would be a reliable means of type differentiation. This was verified in a series of tests with eight freshly isolated virus strains. These strains were typed by conventional methods employing kinetic neutralization tests with standard antisera inactivated at 56° for 30 min. Appropriate concentrations of virus were mixed with an equal volume of serum diluted 1/10 in buffered saline. After 10, 20 and 30 min. at room temperature, the mixtures were diluted 1/100 in cold growth medium and the surviving plaques assayed. The neutralization rate constant $k$ was calculated according to the formula: $k = \frac{(2 \cdot 3) \log (V_0/V_i)}{C_t}$, where $C = concen$.
tration of antiserum, \( t = \) time in min. \( V_0 = \) original virus infectivity and \( V_t = \) surviving virus infectivity at time \( t \).

The immunofluorescence tests were made without knowing the results of the neutralization tests. The results (Table 2) were in every case clear cut and showed complete agreement between the two methods.

![Fig. 1. Cell membrane immunofluorescence by indirect method done 5½ hr after infection (a) with type 1 herpes simplex virus using type 1 specific antiserum, (b) with type 2 herpes simplex virus using type 2 specific antiserum.](image)

### Table 2. Typing of herpes simplex virus isolates by means of type-specific antisera using the indirect cell-membrane immunofluorescent technique

<table>
<thead>
<tr>
<th>Herpes simplex virus strains tested</th>
<th>Reaction of surfaces of the infected cells with antisera</th>
<th>( k )-values determined with antiserum against</th>
<th>( k )-value ratios (Type 1/Type 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type 1 specific*</td>
<td>Type 2 specific†</td>
<td>Unabsorbed anti-type 1‡</td>
</tr>
<tr>
<td>64179</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>1716</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>5516</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>IL</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Type 1 HFEM</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>2017</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5289</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2248</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3345</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Type 2 BRY</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* 32 Antibody units.  † 2 antibody units.  ‡ 32 antibody units against type 1, 8 antibody units against type 2 herpes simplex virus.

Antibody unit = titre of antiserum working dilution.

The indirect cell-membrane immunofluorescent test made with ‘type-specific’ antisera establishes a new method of typing of new herpes simplex virus isolates. It has the advantage of simplicity and rapidity over the other methods of typing (Nahmias et al. 1969; Goodheart,
Plummer & Waner, 1968; Munk & Donner, 1963; Figueroa & Rawls, 1969; Nahmias & Dowdle, 1968) and gives highly specific results. Our tests clearly demonstrate the presence of type 1 and type 2 specific virus antigens in the cell membrane, the high type 1 specific antibody titres in type 1 antisera and small amounts of type-specific antibodies in antisera against type 2 herpes simplex virus. All the herpes simplex virus strains could be typed without finding any intermediate types.

We should like to thank Dr Constance A. C. Ross (Ruchill Hospital, Glasgow), Dr F. O. MacCallum (Radcliffe Infirmary, Oxford) and Dr J. E. M. Whitehead (Public Health Laboratory, Coventry) for providing fresh virus isolates, and Dr D. H. Watson for one of the antisera. We are also indebted to Professor P. Wildy for his valuable advice and criticism. The work was supported by grants from the Wellcome Trust and the Birmingham United Hospitals Endowment Fund.

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(Received 22 March 1971)