Indirect micro-immunofluorescence test for detecting type-specific antibodies to herpes simplex virus

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SUMMARY A rapid indirect micro-immunofluorescence test capable of detecting and differentiating type-specific antibodies to herpes simplex virus is described. The test proved highly sensitive and, in 80 patients with active herpes ocular infection, antibody was detected in 94%. No anti-herpes antibody was detected in a control group of 20 patients with adenovirus infections.

Testing of animal sera prepared against herpes simplex virus types 1 and 2 and of human sera from cases of ocular and genital herpes infections showed that the test can differentiate antibodies to the infecting serotypes. Specimens of whole blood, taken by fingerprick, and eye secretions, both collected on cellulose sponges, could be tested by indirect micro-immunofluorescence. Anti-herpes IgG, IgM, and IgA can also be detected.

Various techniques have been used to identify and differentiate antibodies to herpes simplex virus type 1 (HSV1) and type 2 (HSV2). The microneutralisation test,\(^1\) a plaque reduction test,\(^2\) measurement of kinetics of neutralisation,\(^3\) and the detection of antibody-mediated cytolysis\(^4\) have all been shown to be sensitive but are too complex for routine use. A simpler technique, the modified passive haemagglutination test,\(^5\) can also differentiate HSV1 and HSV2 antibodies but requires prior cross-absorption of the test sera with antigens to achieve a type-specific result.

Indirect fluorescent antibody techniques have found favour for the study of serum responses to various viruses.\(^6\) These techniques generally utilise type-specific antigens, which appear in the membranes of infected cells and have been used successfully by Nahmias\(^7\) and Smith\(^8\) to study anti-herpes antibodies. However, the requirement for prior absorption of sera, the nature of the antigens in infected cells, and the skill required to recognise specific membrane fluorescence make these techniques difficult to apply routinely.

A microtitre fluorescent antibody technique using purified antigens has been developed by Wang and Grayston\(^9\) and modified by Treharne\(^10\) to detect antibodies to chlamydia. This test is capable of differentiating immunoglobulin classes of antibody to all known chlamydial serotypes and can detect antibody in serum, whole blood, and in ocular and genital secretions. This test is used routinely in our laboratory for the diagnosis of chlamydial infections of the eye\(^11\) and cervix.\(^12\) Because of its simplicity, we have adapted this technique for the detection and differentiation of antibodies to herpes simplex virus.

In this paper we present details of an indirect micro-immunofluorescence (IF) test developed for detecting different immunoglobulin classes of type-specific anti-herpes antibodies, and we report the results of testing sera and eye secretions using this new technique.

Material and methods

Antigens

Herpes simplex virus type 1 (VRL/10711) and type 2 (Bryan strain) were obtained from the Central Public Health Laboratory, London. Each type was propagated in HeLa 229 cell culture (Flow Laboratories, Scotland) and passed to produce virus stocks. It was necessary to passage each type into four 2-litre roller bottles of cells to produce sufficient quantities of virus. Infected cells were harvested when 90-100%
of cells showed a cytopathic effect. Medium was
removed from the bottles and mixed with poly-
ethylene glycol (final concentration 8% w/v in
the presence of 0-5m NaCl) to precipitate free protein,
including virus.13 The precipitate was pelleted by
centrifugation at 1000 g for 10 minutes at +4°C and
then resuspended in approximately 10 ml phosphate
buffered saline (PBS), pH 7-4.
Infected cells were stripped from the bottles by
adding approximately 20 ml distilled water to each
and shaking with small glass beads for 2 minutes.
Cell harvest and media precipitates were pooled (four
roller bottles yielded approximately 100 ml) and
freeze-thawed three times to disrupt the cells. This
harvest was then centrifuged at 1000 g for 10 minutes
at +4°C to remove gross debris. The supernatant
then appeared opalescent and free from large
particles and was centrifuged at 48000 g for 2 hours
at +4°C to pellet virus particles. This pellet was
resuspended in one-tenth the original volume of
PBS, pH 7-4, by vigorous shaking with small glass
beads.
The infectivity titres of these preparations were
estimated using a plaque assay technique,14 and each
virus was found to contain approximately 10⁸
plaque-forming units per millilitre (PFU/ml).
Each virus type suspension was then mixed with
uninfected fertile hen’s egg yolk to achieve a final
concentration of 5% v/v yolk in order to enhance the
adhesion of antigens to the slides and to provide a
background to the antigen dots, which aided location
during microscopy. These antigens were then dis-
dispensed into 0-3 ml aliquots and stored at −70°C.
Uninfected HeLa cells were similarly processed to
prepare a control antigen.

ANIMAL SERA
Antisera against HSV1 and HSV2 were prepared in
mice. Ten 3-4-week-old albino Swiss female mice
were used for each virus type. Each mouse was
inoculated intravenously via the tail vein⁹ with 0-5
ml of prepared antigen without yolk diluted to 10⁸
PFU/ml in PBS, pH 7-4 (day 0). A further inoculation
was given on day 7, and mice were exsanguinated
three days later (day 10). Exsanguination was carried
out by anaesthetising the mice with ether, opening
the ventral skin layer, and cutting the axillary plexus.
Arterial blood drained into the pocket formed
between the skin and the thorax and was withdrawn
with a pipette. Blood was pooled for each virus type,
and the serum was separated and stored at −20°C.
Sera were absorbed with equal volumes of control
antigen for 1 hour at 37°C and then overnight at
+4°C before testing.
Commercially available rabbit anti-HSV1 and
anti-HSV2 sera prepared from antigens produced in
rabbit cornea cells (Mercia Brocades Ltd, UK) were
also used in the test without prior absorption.

HUMAN SERA
Blood from 92 patients, 70 with herpetic ocular
infections, two with herpetic genital infection, and 20
with adenovirus keratoconjunctivitis, was collected
by venepuncture. Sera were separated after clotting
and stored at −20°C.
In a further 10 patients with herpetic ocular
infection, eye secretions (tears) and whole blood,
taken by finger-tip pricking, were collected using
cellophane sponges, as described by Darougar and
colleagues.11

INDIRECT MICRO-IMMUNOFLUORESCENCE
(IF) TEST
Microscope slides were thoroughly cleaned by
scrubbing with detergent, and rinsed three times in
tap water, three times in distilled water, and finally
in absolute ethanol. Slides were stored under alcohol
until used. Antigen micro-dots were applied to slides
using sterile mapping pen nibs (Cumberland
Graphics Ltd, UK) previously degreased with ether.
Separate nibs were used for each of the three anti-
gens (HSV1, HSV2, and control). Antigens were
arranged in nine clusters on each slide using a
template to aid location. Slides were then air-dried
for 30 minutes and fixed in acetone for 10 minutes at
room temperature. Fixed slides were stored at −20°C
until used.

Dilutions of sera, blood, and tears were prepared
with PBS, pH 7-4, as diluent, and appropriate dilutions
were overlaid onto the antigen clusters fixed on
slides using a bacteriological loop. Slides were then
incubated in a humid chamber for 30 minutes at
35°C and then thoroughly washed for 10 minutes in
PBS, pH 7-4, using staining troughs and a magnetic
stirrer. They were then air-dried, and the antigens
were covered with the appropriate antiserum con-
jugated with fluorescein isothiocyanate (FITC) as a
second layer. Incubation, washing, and drying were
repeated as before.

Swine anti-mouse and anti-rabbit whole globulin
FITC conjugates (Nordic Immunological Labora-
tories, UK) were used to stain the animal sera, and
swine anti-human IgG, IgM, and IgA FITC con-
jugates (Hyland Laboratories, UK) were used for
the human sera. Rhodamine bovine albumin (Difco
Laboratories, UK) was used as counterstain, and all
conjugates were previously titrated to determine their
optimum working dilutions.

Stained slides were mounted in buffered glycerol
(9 parts glycerol: 1 part PBS, pH 8-5) and examined
at a magnification of × 400 using a Zeiss standard 18
microscope. The microscope was fitted with an HBO
Table 1  Criteria of positivity by the IF test

<table>
<thead>
<tr>
<th>Appearance of antigen</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC filter</td>
<td>FITC and Rhodamine filter</td>
</tr>
<tr>
<td>Particles bright fluorescent green</td>
<td>Particles dull green +</td>
</tr>
<tr>
<td>Particles dull green</td>
<td>Particles orange/yellow -</td>
</tr>
<tr>
<td>Antigen dot dull green (no particles visible)</td>
<td>Antigen dot orange -</td>
</tr>
</tbody>
</table>

50 mercury-vapour bulb using an epi-illumination system and a Zeiss filter set 10H.

Positive reactions appeared as bright green, uniformly sized, ‘pin-point’ particles. These particles were mostly evenly distributed throughout the antigen dot, but some loose aggregates were present. Non-specific staining did occur because extraneous material present in the antigens sometimes also stained green. However, we found no difficulty in differentiating the pleomorphic debris from true particulate reactions. Our criteria for positive and negative reactions by the IF test using both the FITC excitation filter and the FITC and Rhodamine excitation filter are shown in Table 1. The end-point readings were taken as the highest dilution of serum or secretion producing a positive result.

Results

Animal sera

The results of testing mouse and rabbit antiserum prepared against HSV1 and HSV2 by the IF test against homologous, heterologous, and control antigens are shown in Table 2. These results demonstrate that antiserum prepared against HSV1 reacts to the greatest degree with the homologous antigen whereas antiserum prepared against HSV2 reacts to equal titres with both HSV1 and HSV2 antigens.

Repeat tests over the course of six months using

Table 2  Levels of type-specific anti-herpes virus antibody detected by IF test in animal sera prepared against HSV1 and HSV2

<table>
<thead>
<tr>
<th>Serum</th>
<th>Antigen</th>
<th>HSV1</th>
<th>HSV2</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Anti HSV1</td>
<td>320*</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti HSV2</td>
<td>80</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Anti HSV1</td>
<td>2048</td>
<td>256</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Anti HSV2</td>
<td>512</td>
<td>512</td>
<td>0</td>
</tr>
</tbody>
</table>

*Reciprocal end-point titre.

the same antigens stored at −70°C and mouse antisera stored at −20°C showed no marked difference in the levels of anti-herpes antibodies detected.

Human sera

Initially, sera from four patients, two with ocular herpes infections and two with genital herpes infections, were screened against HSV1, HSV2, and control antigens using the IF test. Herpes simplex virus was isolated in cell culture from these four patients and was typed as HSV type 1 from the ocular infections and as HSV type 2 from the genital infections. Sera from patients with ocular infection reacted most strongly with HSV1 antigen whereas sera from patients with genital infection showed equal titres to both HSV1 and HSV2 antigens (Table 3).

Sera or whole blood from a total of 100 patients were tested by the IF test. Eighty patients had ocular herpes infection and 20 had microbiologically proven ocular adenovirus infections (controls). The sera from the control group were all negative for anti-herpes antibodies by the complement fixation test. In those patients with ocular herpes infections anti-herpes IgG at a dilution of 1/16 or higher was detected in 75 (94%) and anti-herpes IgM at a dilution of 1/8 or higher in 31 (39%) (Table 4), all reactions being at least four times higher against HSV1 antigen than against HSV2 antigen. No anti-herpes antibodies were detected in patients with ocular adenovirus infections. The levels of anti-herpes IgG and IgM detected in the blood of 68 patients with microbiologically proven herpes virus infections are shown in Table 5.

In 10 patients with ocular herpes infection, eye secretions (tears) were collected by sponges. The levels of anti-herpes IgG and IgA detected in the tears of these patients (Table 6) are compared to the levels of anti-herpes IgG and IgM detected in the blood, also collected by sponges.
Table 4  Detection of type-specific anti-herpes IgG and IgM antibodies by IF test in blood from 102 patients

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>No. of patients</th>
<th>HSV1 IgG</th>
<th>HSV1 IgM</th>
<th>HSV2 IgG</th>
<th>HSV2 IgM</th>
<th>Total no. of patients positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ocular herpes</td>
<td>80</td>
<td>75*</td>
<td>31†</td>
<td>12</td>
<td>0</td>
<td>75†</td>
</tr>
<tr>
<td>Isolation positive</td>
<td>68</td>
<td>63</td>
<td>27</td>
<td>10</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>Isolation not done</td>
<td>12</td>
<td>12</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Genital herpes</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2†</td>
</tr>
<tr>
<td>Ocular adenovirus (control group)</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* IgG ≥ 1/16.
† IgM ≥ 1/8.
‡ All titres highest against HSV1.
§ All titres equal against HSV1 and HSV2.

Table 5  Distribution of levels of type-specific anti-herpes IgG and IgM antibody detected by IF test in sera from 68 patients with ocular herpes infections and positive virus culture

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Ig class</th>
<th>Number of sera</th>
<th>Reciprocal herpes virus antibody titre</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>HSV1</td>
<td>IgG</td>
<td>0</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>10</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>HSV2</td>
<td>IgG</td>
<td>0</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6  Levels of type-specific anti-herpes IgG, IgM, and IgA antibodies detected by IF test in blood and tears from 10 patients with clinically diagnosed herpes ocular infections

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Blood</th>
<th></th>
<th>Tears</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV1 IgG</td>
<td>IgM</td>
<td>HSV1 IgG</td>
</tr>
<tr>
<td>Primary herpes simplex</td>
<td>128†</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Dendritic ulcer</td>
<td>64</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Dendritic ulcer</td>
<td>32</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dendritic ulcer</td>
<td>128</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dendritic ulcer</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Herpetic keratitis</td>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Herpetic keratitis</td>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chronic corneal ulcer</td>
<td>128</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Recurrent herpetic keratitis</td>
<td>128</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Resolving herpetic keratitis</td>
<td>256</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

* Reciprocal end-point titre.

Discussion

The IF test described is shown to be sensitive for the detection of type-specific antibodies against herpes simplex virus. The antigens required for this test can be grown in bulk in tissue culture. In this study we have used HeLa 229 cells for propagation of herpes virus but other cell culture systems, such as HEp 2, WI 38, and HEL, are similarly sensitive. We also found that simple differential centrifugation proved satisfactory for the purification and concentration of antigens. The more involved techniques of sucrose density centrifugation and 'Millipore' molecular separation were also tried but found to have no advantages over differential centrifugation. The amount of antigen required for each test is minimal since one ampoule containing 0·3 ml of purified virus can produce hundreds of slides. Studies on the storage of antigen showed that purified virus can be kept at -70°C for at least six months without obvious deterioration in antigenicity. However, we
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found that repeated freeze-thawing reduced the antigenicity of the virus, and it was necessary to discard an ampoule after four freeze-thaw cycles. To economise on antigen, it is possible to prepare slides of fixed antigen in advance and to store them at -20°C. These slides can be used for periods of up to six months.

In this study anti-herpes antisera prepared in mice were absorbed with control antigen before testing since the same antigens were used to inoculate the mice as were used in the test itself. No prior absorption of the commercially prepared rabbit antisera or the human sera was found to be required.

Because of the small amounts of blood or local secretion required for this test (0-1 ml approximately) we found it feasible to collect these specimens by saturating cellulose sponges. The method of sponge collection of blood after a finger prick is far simpler and more rapid than venepuncture and does not require highly trained staff.

In staining the antigen-antibody complexes with commercially prepared anti-species antisera conjugated with FITC it is important to determine the optimum working dilution for each batch of conjugate before use.

If positive reactions produced bright green uniform particles or clumps of particles. Occasionally some non-specific staining did occur but we found no problem in differentiating this amorphous debris from true particle reactions.

In our hands the IF test proved highly sensitive for detecting antibodies to herpes simplex virus. In 80 patients with clinical evidence of an active herpes ocular infection, antibody was detected in 94% of cases. No antibody was found in a control group of 20 patients with adenovirus infections who had previously been shown to be negative by complement fixation. Testing of animal sera specially prepared against HSV1 and HSV2 and blood from a few cases of herpes ocular and genital infections showed that this test can differentiate antibodies to the herpes virus serotypes responsible for the infection. We found that in HSV1 infection the highest level of antibody is against the homologous antigen whereas in HSV2 an equal antibody titre to both serotypes is seen. This result confirms the findings of Doerr et al. This test was also able to identify anti-herpes IgG, IgM, and IgA classes of antibody. The presence of anti-herpes IgM in the blood may be associated with active herpes virus infections. In studies of chlamydial infections it has been shown that the presence of IgM in the blood and of IgG and IgA in tears or cervical secretions is closely associated with active chlamydial infections of the eye or genital tract.

Therefore it is hoped that the presence of these anti-bodies against herpes simplex virus in blood or local secretions may provide evidence of active herpetic infection.

The results of this study indicate that the IF test is a simple, rapid, and inexpensive method for detecting type-specific anti-herpes antibodies of different immunoglobulin classes. This fact, coupled with the ease of collecting blood and local secretions with cellulose sponges, should make the test a valuable technique for the routine serodiagnosis of herpes simplex virus infections.

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