

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 cytolysis⁴ 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 *et al.*⁷ and Smith *et al.*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 Grayston⁹ and modified by Treharne *et al.*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, in whole blood, and in ocular and genital secretions. This test is used routinely in our laboratory for the diagnosis of chlamydial infections of the eye¹¹ 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 passaged 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%

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of cells showed a cytopathic effect. Medium was removed from the bottles and mixed with polyethylene 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 10⁹ 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 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 cellulose 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 antigens (HSV1, HSV2, and control). Antigens were arranged in nine clusters on each slide using 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 conjugated 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 Laboratories, UK) were used to stain the animal sera, and swine anti-human IgG, IgM, and IgA FITC conjugates (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

Appearance of antigen		Result
FITC filter	FITC and Rhodamine filter	
Particles bright fluorescent green	Particles dull green	+
Particles dull green	Particles orange/yellow	-
Antigen dot dull green (no particles visible)	Antigen dot orange (no particles visible)	-

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

Serum	Antigen		
	HSV1	HSV2	Control
Mouse			
Anti HSV1	320*	10	0
Anti HSV2	80	80	0
Normal	0	0	0
Rabbit			
Anti HSV1	2048	256	0
Anti HSV2	512	512	0

*Reciprocal end-point titre.

Table 3 Levels of type-specific anti-herpes IgG and IgM antibody detected by IF test in sera from four patients with proven herpes ocular and genital infections

Patient	Antigen					
	HSV1		HSV2		Control	
	IgG	IgM	IgG	IgM	IgG	IgM
1 Ocular	128*	8	0	0	0	0
2 Ocular	256	0	8	0	0	0
3 Genital	128	0	128	0	0	0
4 Genital	32	0	32	0	0	0

*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

Clinical diagnosis	No. of patients	HSV1		HSV2		Total no. of patients positive
		IgG	IgM	IgG	IgM	
Ocular herpes	80	75*	31†	12	0	75‡
Isolation positive	68	63	27	10	0	63
Isolation not done	12	12	4	2	0	12
Genital herpes	2	2	0	2	0	2§
Ocular adenovirus (control group)	20	0	0	0	0	0

*IgG \geq 1/16.†IgM \geq 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

Antigen	Ig class	Number of sera						Total	
		Reciprocal herpes virus antibody titre							
		8	16	32	64	128	256	512	
HSV1	IgG	0	3	4	19	16	14	7	63
	IgM	10	9	3	5	0	0	0	27
HSV2	IgG	0	7	3	0	0	0	0	10
	IgM	0	0	0	0	0	0	0	0

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

Clinical diagnosis	Blood				Tears			
	HSV1		HSV2		HSV1		HSV2	
	IgG	IgM	IgG	IgM	IgG	IgA	IgG	IgA
Primary herpes simplex	128*	8	0	0	0	0	0	0
Dendritic ulcer	64	8	0	0	8	0	0	0
Dendritic ulcer	32	0	0	0	8	0	0	0
Dendritic ulcer	128	0	0	0	16	8	0	0
Dendritic ulcer	16	0	0	0	0	0	0	0
Herpetic keratitis	64	0	0	0	0	0	0	0
Herpetic keratitis	64	0	0	0	0	0	0	0
Chronic corneal ulcer	128	0	16	0	64	16	0	0
Recurrent herpetic keratitis	128	8	0	0	32	32	0	0
Resolving herpetic keratitis	256	0	16	0	0	0	0	0

*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

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 antiserum 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.*¹⁵

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.⁶ 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 eye¹¹ or genital tract.¹² 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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