In vivo immunofluorescence to diagnose herpes simplex virus keratitis in mice

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Abstract

Background/aims—Herpes simplex virus keratitis (HSK) is the most common cause of corneal blindness in the Western world. Delay in the treatment of HSK can lead to a more significant corneal scar and topical steroid treatment in unsuspected active HSK can lead to corneal melting. Current culture techniques for herpes simplex virus (HSV) take several days and commercially available HSV laboratory based diagnostic techniques such as Herpchek vary in sensitivity. This study was conducted to assess the viability of a new, quicker, and simpler method to diagnose HSK.

Methods—Direct immunofluorescence was used in vivo in a masked study to diagnose HSK in mice using a standard slit lamp with cobalt blue illumination. Murine monoclonal fluorescently labelled antibody was applied to the cornea for 10 or 20 minutes and then washed off with phosphate buffered solution. Mice with HSK were stained with either fluorescently labelled monoclonal antibody against HSV or fluorescently labelled monoclonal antibody against cytomegalovirus. Mice with corneal abrasions of non-viral origin were given fluorescently labelled monoclonal antibody against HSV.

Results—Fluorescence was seen only in the mice with HSK given fluorescently labelled monoclonal antibody against HSV. This observation was confirmed upon microscopic immunofluorescent imaging of the corneal epithelial sheets. Conclusion—In vivo immunofluorescence may be useful in the clinical diagnosis of HSK.

Materials and methods

Animals
Female, 8-week-old, albino, inbred NIH mice were obtained from Harlan/Olac (Bicester, Oxford). Before inoculation both eyes were examined by a slit lamp (Zeiss USL) and any with abnormal eyes were rejected. After inoculation the mice were changed from sawdust to shredded newspaper bedding to avoid risk of ocular damage.

Anaesthesia
Midazolam (Hypnovel) (Roche) and fentanyl citrate/fluanisone (Hynnorm) from Chemicals Co Ltd were administered in the scruff of the neck, as two separate subcutaneous 0.1 ml injections (midazolam hydrochloride 2.4 mg/kg, fluanisone 4.8 mg/kg, and fentanyl citrate 0.15 mg/kg).

Inoculation
Mice were anaesthetised and while viewing through a binocular microscope (Vickers) they were inoculated by scarification of the left cornea through a 5 µl drop of maintenance medium containing HSV type 1 (HSV-1) strain McKrae. The dose was 1 x 10⁸ plaque forming units (pfu). The scarification was done immediately after the inoculum was instilled.
Five linear corneal scarifications and a further five perpendicular to the first, were made with a 26 gauge × 3/8 inch needle (Becton Dickinson).

On the day of examination, lesions were made in the corneal epithelium of control mice with a 26 gauge needle.

**ANTIBODIES**

Three different murine monoclonal antibodies (MoAb) labelled with fluorescein isothiocyanate (MoAb-F) class IgG1 were used (obtained from Biogenesis Ltd, Poole); these antibodies were dialysed to remove preservative and stored at 4°C. The fluorescein isothiocyanate (FITC) bound to antibodies has the same absorption peak (approximately 495 nm) as fluorescein used clinically to stain corneal epithelial defects. The first MoAb was directed against HSV-1 envelope glycoprotein gB, the second MoAb recognised a cytoplasmic protein in HSV-1 infected cells, and the third MoAb was directed against cytomegalovirus (CMV). The MoAbs were labelled with FITC to a molar fluorescein to IgG ratio of 8:1 for the MoAb against HSV-1 envelope glycoprotein gB, 2:1 for the MoAb against a cytoplasmic protein in HSV-1 infected cells, and 5:1 for the MoAb against CMV. A combination of the MoAbs against HSV-1 envelope glycoprotein gB and MoAb against a cytoplasmic protein in HSV-1 infected cells was used at a concentration of 2 mg/ml. These two combined MoAb-Fs will be referred to as combined HSV-1 MoAb-Fs. Both of the above MoAb-Fs, comprising the combined HSV-1 MoAb-Fs, showed very good staining of virus infected cells at a dilution of 1:20 in vitro. The CMV MoAb-F placed on their eye, two mice for 10 minutes and the others for 20 minutes; four mice had corneal abrasions with combined HSV-1 MoAb-Fs placed on their eye, two mice for 10 minutes and the others for 20 minutes. The normal (right) corneas of two of the above mice had combined HSV-1 MoAb-Fs placed on them, one mouse for 10 minutes and the other for 20 minutes. Mice were placed in a dark cage (at room temperature) during the incubation time with the antibody. The HSK and abrasions were produced by, and the MoAb-Fs placed on the eye by, a different observer from the one examining the eyes with the slit lamp.

**Results**

All the HSV-1 infected corneas had significant ulceration at the time of application of the MoAb-Fs. There was no evidence of fluorescence, when the corneas were viewed with the slit lamp, in the following control mice; mice with corneal abrasions or mice with normal corneas which had combined HSV-1 MoAb-Fs applied or animals with HSK that had CMV MoAb-F applied.

Three of the six eyes with HSK and combined HSV-1 MoAb-Fs showed areas of fluorescence seen with the slit lamp at 32.3 times magnification. In all of these three eyes in which fluorescence was seen with the slit lamp the fluorescence was associated with disease; in one of the three cases (mouse 1, Fig 1) the fluorescence was bifocal at the margin of the HSK ulcer and in the other two mice (mice 3 and 11) the fluorescence was multifocal at the margins of the ulcers and granular in appearance. Figure 1 shows the focal nature of fluorescence that was seen in mouse 1. The focal fluorescence in all of these three mice (mice 1, 3, and 11) remained when reviewed half an hour later with the slit lamp using cobalt blue light.

On fluorescent microscopy of the corneal epithelial sheets only non-specific diffuse fluorescence (Fig 2) or no fluorescence was seen in the following control mice; mice with corneal abrasions or mice with normal corneas that had combined HSV-1 MoAb-Fs applied or animals with HSK that had CMV MoAb-F applied.

Specific microscopic fluorescence was seen in the epithelial sheets from three of six mice with HSK treated with combined HSV-1 MoAb-Fs (mice 1, 3, and 11). These were the same mice that showed positive fluorescence in...
vivo. Mice 1 and 3 showed linear fluorescence outlining an ulcer (Figs 3 and 4). Mouse 11 showed focal fluorescence.

The durations of application of antibody in the three mice that showed fluorescence in vivo and specific microscopic fluorescence of their corneal epithelial sheets were, in mice 1 and 11, 10 minutes, and in mouse 3, 20 minutes.

**Discussion**

HSV-1 replicates in host cells and expresses envelope glycoprotein in the infected host cell membrane. We assumed that a MoAb-F against a HSV-1 envelope glycoprotein would be an important MoAb-F in vivo to detect HSV-1 infection, as it would bind to the corresponding HSV-1 envelope glycoprotein found in infected cell surface membranes. MoAb-F against HSV-1 antigen present in the cytoplasm of infected cells was also used to attempt to increase the number of virus infected cells detected, assuming some infected cells would be damaged and allow antibody to penetrate. Our study used a higher fluorescein to antibody ratio than is conventionally used for in vitro staining. This may have resulted in non-specific staining; but this was not the case in this study.

These results show that the in vivo immunofluorescent technique correctly detected HSK in three out of six cases, giving a sensitivity of 50%, though the number of eyes examined was too small to evaluate the true sensitivity of this test. The specificity was 100% (n=10) as none of our controls—mice with corneal abrasions or mice with normal corneas which had combined HSV-1 MoAb-Fs applied or animals with HSK that had CMV MoAb-F applied—showed either fluorescence when viewed with the slit lamp or specific fluorescence of their corneal epithelial sheets under direct fluorescent microscopy. Again, the number of eyes examined was too small to evaluate the true specificity of this test. The sensitivity of our technique may be a result of different rates of virus antigen expression with time after inoculation. However, it has been shown that up to 3 days after infection the virus antigen in the corneal epithelium is maximum.9 The specific fluorescence, seen with the slit lamp and with microscopy, appears to be at the margins of the HSK ulcer where the HSV-1 antigen is expressed.9 Enhancement or improved observation of the fluorescein signal from the bound MoAb-F could improve the sensitivity of this technique. The fluorescein signal could be increased by maximising the binding of antibody to the HSV-1 infected cells by the use of a pool of MoAb-Fs targeted to different HSV-1 antigens or by using fluorescein labelled...
polyclonal antibody. Increased concentration of the MoAb-Fs and a higher fluorescein to antibody ratio than that used in this study could also improve the fluorescein signal. Although the indirect fluorescence method may improve the fluorescein signal we felt that this would be less applicable in the clinical situation and may also reduce the specificity by non-specific binding of the secondary fluorescein labelled antibody. Observation of the fluorescein signal could be improved by using higher magnification than that used in this study, or confocal microscopy.

In vivo immunofluorescence has been used to detect toxoplasmic chorioretinitis in rabbits 2 to 4 hours after the intravenous injection of 20–30 mg of anti-toxoplasma fluorescently labelled IgG antibodies. Our study used up to 10 µg of MoAb-Fs applied topically in a masked study and the specific fluorescence was visible 10 or 20 minutes after application of the antibody. The ocular surface appears to be an ideal place to study disease with in vivo immunofluorescence, as a high concentration of antibody can be delivered topically to the area of interest with less dilution as would occur with intravenous application of fluorescently labelled antibody, and it can be viewed readily. Furthermore, foreign fluorescently labelled antibodies applied topically should induce far less of an immune response in the host than intravenous foreign antibodies. The authors feel that in vivo immunofluorescence may have a role to play in the clinical setting to diagnose ocular surface disease such as HSK, but further work needs to be done to investigate the host immune response to topical foreign antibodies.

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