Protection against ocular and cutaneous infection with herpes simplex virus type 1 by intragastric immunization with live virus

Hiroshi Irie,1 Carolyn Shimeld,2 Neil Williams3 and Terry Hill3*

1 First department of Pathology, University of Teikyo, Tokyo, Japan, 2 Department of Ophthalmology and 3 Department of Pathology and Microbiology, The Medical School, University of Bristol, Bristol BS8 1TD, U.K.

Intragastric administration of live herpes simplex virus type 1 (HSV-1) was assessed for the induction of humoral immune responses and for protection against ocular and cutaneous challenge with virus. Mice showed no clinical abnormalities following intragastric inoculation with three different strains of virus (Miyama +GC, SC16, and P2C6, a thymidine kinase-defective mutant). Replication of virus was not detected in the oesophagus, superior cervical ganglia or coeliac ganglia of such animals and latent infection was not detected in these ganglia at later times after inoculation. Induction of a mucosal immune response was indicated by the presence of antibody (mainly IgG or IgA)-secreting cells in Peyer's patches. Intragastric immunization gave protection to some extent against ocular challenge and to a greater extent against cutaneous challenge with HSV-1. Following the latter challenge, particularly after intragastric immunization with strains SC16 and Miyama, the establishment of latency was almost completely prevented.

Introduction

In recent years, particularly with developments in molecular biology and genetic engineering many strategies have been devised for vaccination against infections with herpes simplex virus (HSV) (Long et al., 1984; Mertz et al., 1984; Paoletti et al., 1984; Roizman et al., 1984; Cremer et al., 1985; Eisenberg et al., 1985; Meignier et al., 1987; Blacklaws et al., 1990). Most have concentrated on induction of systemic immune responses but the primary infection most often occurs at mucosal surfaces such as those of the mouth, genital tract or eye. In the case of the eye, local immune effectors such as IgA in tears (Allansmith & Whitney, 1973; Allansmith et al., 1983; Montgomery & Ayylidiz, 1983) may be particularly important in protection from primary infection since the cornea is avascular. It is clear that induction of mucosal immunity is more effectively stimulated by local application of antigen than by systemic immunization (Ernst et al., 1988; Mestecky et al., 1978; Liew et al., 1984; Chen et al., 1987; Peppard et al., 1991). With respect to HSV, McDermott et al. (1984) have shown that intravaginal administration of viral antigens induces protective immunity against HSV type 2 infection in mice and similar results were obtained by intravaginal immunization with an HSV-2 subunit vaccine in guineapigs (Bowen et al., 1992). Furthermore, following the successful example of oral immunization with poliovirus, administration by mouth of live adenovirus as a vector for HSV genes is being explored (Johnson, 1991). In the same context, Irie et al. (1992) have shown recently that intragastric administration of live HSV induces low levels of serum neutralizing antibody and protects mice against intracerebral challenge. We now report that such immunization induces a mucosal immune response against HSV type 1, protects to some degree against ocular challenge and to a greater extent against cutaneous challenge with the virus and, following cutaneous challenge, gives significant protection against the establishment of latency.

Methods

**Virus.** Suspensions of the following strains of HSV-1 were prepared from infected Vero cells: SC16 (Hill et al., 1975), P2C6 (a mutant defective in thymidine kinase (TK) derived from strain CI(101); Field & Darby, 1980); Miyama +GC (Nii & Kamahora, 1961). Animals. NIH/Ola inbred mice were originally obtained from Harlan-Olac. They were maintained as a breeding colony in the Department of Pathology and Microbiology at the University of Bristol. All were 7 to 10 weeks old when first inoculated. Male mice were used for experiments involving ocular studies since production of lachrymal IgA is higher in this sex (Sullivan & Allansmith, 1985). Females were used for cutaneous challenge because the damage caused by fighting between males interferes with observation of erythema of the pinna following zosteriform spread of virus.

**Intragastric immunization.** Mice were anaesthetized by subcutaneous injection of fluanisone in a fentanyl citrate base and were then given 106 p.f.u. HSV-1, in 0.2 ml medium 199 with 2% fetal calf serum (FCS), into the stomach through a stainless steel cannula (0.8 × 70 mm).
Challenge infection. At least 4 weeks after intragastric immunization, mice were challenged by infection with HSV-1 strain SC16 either by (i) scarification of the left cornea with a 26-gauge needle through a 5 μl drop of medium containing 10^5 p.f.u. of virus (Tullo et al., 1983) or (ii) scarification of the right side of the ventral surface of the neck through a 10 μl drop containing 10^5 p.f.u. (Blyth et al., 1984).

Isolation of infectious virus from tissues. Mice were killed with an intraperitoneal (i.p.) injection of sodium pentobarbitone and the following tissues removed: the left and right trigeminal ganglia (TG), left and right superior cervical ganglia (SCG), the coeliac ganglion and the oesophagus (divided into the upper and lower halves). The tissues were each ground in 0.45 ml of medium 199 containing 2% FCS, frozen and thawed three times and 50 μl samples were inoculated in triplicate onto Vero cells for assay of infectious virus.

Examination of clinical disease following challenge and isolation of virus from eye-washings. After corneal challenge, the cornea, eyelids and periorcular skin were examined daily for signs of disease using a slit lamp microscope. Corneal disease was scored as follows: 1, small ulcers with mild exudate; 2, dendritic ulceration with mild exudate; 3, large ulcers with marked exudate or stromal involvement. Skin disease was scored: 1, vesicles on the ipsilateral eyelid or adjacent skin; 2, vesicles or ulcers on the ipsilateral eyelids, periorbital skin and forehead; 3, as in 2 but extending to the nose tip; 4, vesicles or ulcers extending to the contralateral side.

Following cutaneous challenge, the incidence of zosteriform spread of infection to the ipsilateral pinna was recorded and the severity of erythema of the pinna was scored (Blyth et al., 1984). During the assessment of ocular and cutaneous disease, groups of mice were coded so that the observations were made ‘blind’.

Eye-washings for isolation of virus were performed by the method of Tullo et al. (1983).

Detection of latent infection. Two methods were used. First, at least 1 month after challenge by neck scarification with virus, the right-hand side second and third cervical ganglia were removed, cultured for 5 days at 35 °C in 0.5 ml tissue culture growth medium, ground in a tissue grinder and 50 μl samples put in triplicate onto monolayers of Vero cells to detect the presence of HSV (Harbour et al., 1983). Second, at least 1 month after intragastric inoculation, both TG, both SCG and the coeliac ganglion were removed and placed separately in glass tubes (1 x 10 cm) already containing a monolayer of Vero cells. The tubes were incubated at 35 °C in a roller drum apparatus (10 rotations/h). The monolayers were examined daily for 2 weeks for development of cytopathic effects due to reactivated HSV-1. The medium was changed every 2 to 3 days and special care was taken to avoid cross-contamination. In particular, new pipettes were used for each tube and tubes were discarded once they were positive. Use of the roller drum was intended to increase the oxygenation of the tissue fragments and thereby aid tissue survival and increase the chance of virus reactivation. It has been found to give better detection of latent HSV than by using the first method (T. Hill, unpublished observations).

Preparation of lymphocyte populations. Mice were killed by cervical dislocation and the spleen and Peyer’s patches were removed. Single-cell suspensions were prepared from the spleen and Peyer’s patches by forcing the tissue through stainless steel mesh. The cells were washed in Hanks’ balanced salt solution and suspended in the alpha modification of Eagles’ MEM with l-glutamine, 10% FCS, penicillin/streptomycin and 2-mercaptoethanol (Flow Laboratories).

Enzyme-linked immunospot (ELISPO T) assay. The number of spleen and Peyer’s patch cells producing different anti-HSV antibody isotypes was measured by ELISPO T assay as described by Wilson et al. (1991). In brief, 96-well nitrocellulose bottom plates (Millipore) were coated with HSV-1 antigen by overnight incubation at 4 °C. After blocking with RPMI/10% FCS, spleen or Peyer’s patch cells were added in 100 μl and incubated for 6 h at 37 °C. Plates were then washed with PBS and PBS–TWEEN, and incubated overnight with goat anti-mouse IgG (1:1000), or anti-IgM (1:500) or anti-IgA (1:500) peroxidase conjugate. After further washing, the spots were developed using 3-amo-9-ethylcarbazole (AEC) substrate (Sigma) in citrate buffer pH 5.

Antigen for coating the plates was prepared by the method of Ertuck et al. (1992). Briefly, Vero cells infected with HSV-1 strain PSC6 were disrupted using the non-ionic detergent NP40 (Sigma) (Jennings et al., 1988). After sonication for 30 s using an MSE 150 W ultrasonic disintegrator at full power, the cell–detergent mixture was centrifuged at 32000 r.p.m. for 1 h, dialysed with PBS for 4 days and stored in aliquots at −70 °C. Before use, the optimal concentration of antigen was determined by titration. Control preparations were made from mock-infected Vero cells by the same method.

Statistical analysis. The χ^2 test was used for analysis of death after ocular challenge and incidence of latency after cutaneous challenge. Friedman rank sums (Hollander & Wolf, 1973) were used to analyse incidence and severity of erythema of the pinna following cutaneous challenge and the Kaplan-Mayer method was used to analyse the incidence of virus-shedding in eye-washings after ocular challenge.

Results

Development of clinical disease and isolation of virus from mice after intragastric inoculation

In two experiments each strain of virus was inoculated intragastrically into a group of 15 to 18 mice and groups of similar size were given PBS alone. None of the animals exhibited clinical abnormalities such as loss of appetite or paralysis. With the exception of two animals in the first experiment (one of 18 given SC16 and one of 16 given PSC6 on 20 and 18 days after inoculation respectively), none of the animals died. Virus was not isolated from the brain, spinal cord, oesophagus, adrenal glands, SCG, TG and coeliac ganglion of these two mice. To examine infection of the most likely target organs following intragastric inoculation with the Miyama +GC strain, samples of both SCG, both TG, the coeliac ganglion and the oesophagus (examined as the upper and lower halves) were assayed for infectious virus from four animals on each of days 1 to 4 after inoculation. No virus was isolated.

At more than 4 months after intragastric inoculation with HSV-1 SC-16 or Miyama +GC, the left and right SCG and coeliac ganglion, from eight and six mice respectively, were examined for latent infection by the roller tube method. None was detected.

ELISPO T assay

At 84 days after intragastric inoculation with the three virus strains, mice were given a second intragastric inoculation with the same dose and strain of virus. Significant numbers of anti-HSV antibody-forming cells
Intragastric immunization with HSV-1

Immunogen

PBS
Positive control
SC16
Miyama
P2C6

(a)

No. of spot-forming cells/100 μl

0 10 20 30 40

(b)

PBS
Positive control
SC16
Miyama
P2C6

0 2 4 6

Fig. 1. Numbers of spleen (a) and Peyer’s patch cells (b) producing different anti-HSV antibody isotypes (□ immunoglobulin, □ IgG, ■ IgM, □ IgA) following intragastric inoculation with different strains of HSV-1. Positive controls were given i.p. inoculations of strain Miyama and negative controls were given intragastric PBS alone. For the spleen, counts were done on pooled preparations from three animals. The same animals were used for preparation of Peyer’s patch cells but here the results are the means of counts from each individual and the s.d. is less than 1.2 in all cases.

No spots were detected in wells without coating antigen or which contained cells from unimmunized mice.

were found in the spleen 5 days later by using the ELISPOT method (Fig. 1a). Total number of such cells were about threefold higher in the positive control mice (killed 81 days after an initial inoculum of 10^6 p.f.u. strain Miyama into the peritoneum and booster i.p. inoculations of 10^4 p.f.u. 53 and 7 days before the assay). In all cases the predominant cells were IgG-producing. Lower levels of IgM and IgA were detected when Miyama or P2C6 were used, but only IgG was detected following inoculation with SC16. In Peyer’s patch cells from the same animals, the total number of anti-HSV immunoglobulin-producing cells was approximately sixfold lower than in the spleen following intragastric inoculation (Fig. 1b). However, it is noteworthy that, with the exception of the positive controls, IgA-producing cells were found in all groups given intragastric virus. Moreover, in such animals, numbers of IgA-producing cells were higher than those producing other isotypes.

No spots were detected in wells without coating antigen or which contained cells from unimmunized mice.

Table 1. Incidence of deaths following corneal inoculation* with HSV-1 in mice orally immunized with different strains of live virus

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Mean survival (days ± s.d.)</th>
<th>No. of deaths/total no. of mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS alone</td>
<td>8.85 ± 2.79</td>
<td>13/15 (86)</td>
</tr>
<tr>
<td>SC16</td>
<td>10.87 ± 3.46</td>
<td>9/15 (60)</td>
</tr>
<tr>
<td>Miyama + GC</td>
<td>12.47 ± 3.39</td>
<td>5/15 (33)</td>
</tr>
<tr>
<td>P2C6</td>
<td>9.13 ± 3.07</td>
<td>12/15 (80)</td>
</tr>
</tbody>
</table>

* Challenged in cornea with 10^6 p.f.u. HSV-1 strain SC16, 7 weeks after intragastric inoculation.

‡ \( \chi^2 = 6.8, P = < 0.001 \) compared with controls of PBS alone.

Ocular challenge

At 49 days after intragastric inoculation, mice were challenged by corneal inoculation with HSV strain SC16. The incidence of deaths in the control group (given intragastric PBS alone) was 86% (Table 1). This incidence was lower in groups given intragastric virus but the decrease (to 33%) was only significant in the group given Miyama virus (\( \chi^2 = 6.8, P = < 0.01 \)).

No significant differences were seen in the incidence or severity of corneal disease (including epithelial and stromal disease) or associated skin disease (including lesions on the eyelid), between the control and immunized groups.

In comparison with the control group, mice immunized by mouth, with either strain SC16 or Miyama, cleared virus from their eye-washings 1 day earlier and 5 days after corneal challenge the incidence of virus in the washings from these groups was significantly lower than that in the control (\( P = < 0.01 \)) (Table 2).

Cutaneous challenge

At 32 days after intragastric inoculation, mice were challenged by scarification of the neck with strain SC16. The severity of erythema of the right pinna groups given intragastric virus was significantly less than that in the controls (\( P = < 0.02 \); Fig. 2a). Moreover, in all groups given intragastric virus the incidence of zosteriform spread, as judged by the development of erythema of the ipsilateral pinna, was significantly lower (maximum of
Table 2. Incidence of virus in eye washings following corneal inoculation* with HSV-1 in mice orally immunized with different strains of live virus

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Time after inoculation (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>PBS</td>
<td>15/15 (100)†</td>
</tr>
<tr>
<td>SC16‡</td>
<td>13/15 (87)</td>
</tr>
<tr>
<td>Miyama + GC‡</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td>PzC₆</td>
<td>15/15 (100)</td>
</tr>
</tbody>
</table>

* Challenged in cornea with 10⁵ p.f.u. HSV-1 strain SC16, 7 weeks after intragastric inoculation.
† No. with virus/total tested; % is given in parentheses. Eye-washings taken on days 8, 9 and 10 yielded no virus.
‡ More rapid clearance compared with controls, \( P = < 0.01 \).

One month after cutaneous challenge the highest incidence (80%) of latent infection was found in the cervical ganglia from the control group (Table 3). Significantly lower percentages were found in all three groups immunized with intragastric virus, 38%, 10% and 8% in the groups given PzC₆, SC16 and Miyama respectively.

Table 3. Incidence of latent infection in cervical ganglia following inoculation* by scarification of the neck in mice orally immunized with different strains of live virus

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>No. with latent infection/ P compared to controls†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mice tested (%)</td>
</tr>
<tr>
<td>PBS alone</td>
<td>12/15 (80%)</td>
</tr>
<tr>
<td>SC16</td>
<td>1/10 (10%)</td>
</tr>
<tr>
<td>Miyama + GC</td>
<td>1/13 (76%)</td>
</tr>
<tr>
<td>PzC₆</td>
<td>5/13 (38.5%)</td>
</tr>
</tbody>
</table>

* Challenged in skin of neck with 10⁵ p.f.u. HSV-1 strain SC16 32 days after intragastric immunization.
† \( P \) values calculated by \( \chi^2 \) test.

Discussion

Vaccination against infectious disease by the oral route has a number of advantages including convenience of administration and priming of the mucosal immune system in situations where this may be of importance. In previous work (Irie et al., 1992) intragastric administration of live, virulent HSV-1 to mice was judged to be safe because no adverse clinical signs were seen. This observation was confirmed in the present study in a different strain of mouse and using two virulent and one relatively avirulent TK-defective strain of HSV-1 (PzC₆). Even though HSV is rapidly inactivated in the stomach, intragastric administration of u.v. light-inactivated virus was found to be less effective at inducing immunity than live virus (Irie et al., 1992). This suggests that either some limited replication is necessary for the induction of immunity or inactivation by u.v. light renders virus less immunogenic when given by mouth. If such replication does occur, the present study indicates that it is not in the oesophagus and the rapid inactivation in the stomach makes it unlikely that infection will occur at lower levels of the gut. Additional experiments to examine other sites for infection following intragastric inoculation such as the nasopharynx and respiratory tract, or using other replication-defective mutants will be necessary to examine this further.
ELISPOT assays showed that with all three strains of virus, intragastric inoculation led to the induction of cells in the spleen that produced anti-HSV antibodies. As would be expected at systemic sites, IgG was the predominant class of antibody produced in the spleen.

Evidence for a mucosal immune response against all three strains of virus following intragastric inoculation is provided by the presence of IgA-producing cells in Peyer’s patches. It has been demonstrated that, following antigenic challenge in the gut, specific secreted antibody may be detected not only in the intestine but also in the lacrimal gland, salivary gland and the lactating mammary gland (Brandtzæg et al., 1989; Mestecky et al., 1978). Such observations have led to the concept of a common mucosal immune system, probably resulting from the preferential homing of gut lymphocytes back to mucosal tissues (Mestecky, 1987; Brandtzæg et al., 1989; McDermott & Bienenstock, 1979). In view of the importance of ocular infection with HSV, it will be of interest to determine whether oral immunization with the virus can induce evidence of anti-HSV IgA in tears and/or IgA-producing cells in the lacrimal gland.

Evidence for the protection of intragastrically immunized mice against ocular challenge was provided by the significant increase in survival of mice immunized with strain Miyama and the more rapid clearance of virus from eye-washings of mice immunized with strain Miyama or SC16. Similar increased rates of clearance were also observed in mice immunized with an immune-stimulating complex (ISCOM)-based HSV-1 vaccine (Erturk et al., 1992). Since the cornea is avascular and richly innervated, after corneal scarification virus may rapidly gain access to susceptible cells and thereby to the nervous system before an immune response can be effective. Therefore, demonstration of protection against primary ocular challenge is likely to be more demanding than against cutaneous infection. This may explain the lack of significant difference in ocular disease between the control and intragastrically immunized mice. In natural infections, where the infecting dose may be much lower than that used for experimental challenge, protection against ocular infection may be easier to establish.

Following cutaneous challenge, intragastric immunization gave clear protection against zosteriform spread of virus. This effect may reflect a reduction of replication at the inoculation site and/or a reduction in spread of virus in the nervous system (Shimeld et al., 1990). Evidence for the latter was also provided in the immunized mice by the significantly lower incidence of latent infection in the sensory ganglia that supply the cutaneous inoculation site. Again, these results on latency and zosteriform spread parallel those obtained following cutaneous challenge after subcutaneous immunization with an ISCOM-based HSV-1 vaccine (Erturk et al., 1992).

The immune effector mechanisms underlying the protection against ocular and cutaneous challenge following intragastric immunization of mice are not clear at present. Levels of neutralizing antibody in such animals are low and protection against i.p. challenge with HSV was possible by passive transfer of spleen cells but not serum from intragastrically immunized mice (Irie et al., 1992). Therefore, as has been suggested with an ISCOM vaccine (Erturk et al., 1992), protection may involve mechanisms such as antibody-dependent cell cytotoxicity or cell-mediated effectors.

In summary, the present results provide evidence that intragastric immunization with live strains of HSV-1 can induce mucosal as well as systemic immunity against the virus and significant protection against both ocular and cutaneous challenge. More work will be required to optimize the conditions of immunization and to explore further the use of safer immunogens such as glycoproteins or deletion mutants.

We wish to thank Tsuchiya Ikueikai for financial support for part of this work.

References


(Received 16 December 1992; Accepted 3 March 1993)