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A comparison of oral and parenteral routes for therapeutic vaccination with HSV-2 ISCOMs in mice; cytokine profiles, antibody responses and protection

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Abstract

It is likely that recurrent infections with HSV-2 (or HSV-1) are influenced by local levels of immunity at mucosal surfaces, when virus reactivated from the latent state is infecting mucosal epithelial cells. Increasing the levels of cellular and humoral immunity through immunisation and maintaining such increased levels, may reduce establishment and spread of reactivated virus at the local site, thereby ameliorating recurrent disease symptoms. The use of HSV-2 antigens incorporated into immunostimulating complexes (ISCOMs) for immunisation of mice previously infected with HSV-2 was investigated in the present study. Prophylactic administration of HSV-2 ISCOM vaccine to mice elicits local antibody detectable in nasal washings, serum antibody and the presence of cytokines IL-2, IFN-γ and IL-4 in supernatants from spleen cell cultures stimulated in vitro with HSV-2 antigens. Use of the same vaccine in mice infected previously with HSV-2, results in increased levels of total and subclass serum ELISA antibody and also increased levels of serum neutralising antibody. Treatment of HSV-2 infected mice with the HSV-2 ISCOM vaccine also induces higher levels of the cytokines IL-2, IFN-γ and IL-4, in in vitro stimulated spleen cell cultures. Challenge with a lethal dose of HSV-1 showed that mice previously infected with HSV-2 and subsequently given two doses of HSV-2 ISCOMs vaccine were protected.

Keywords: Therapeutic immunisation; Cytokine responses; Antibody responses; HSV-2

1. Introduction

Genital herpes infection is life-long and may result in painful and recurrent genital lesions, systemic complications, serious psychological morbidity and rare but serious outcomes in neonates born to infected women. HSV-2 is the principal cause, with an increasing proportion of first-episode disease caused by HSV-1 (Minson, 1998). Except for long-term administration of antiviral compounds there is no effective therapy to prevent or reduce the incidence of recurrent disease predominantly associated with HSV-2 infec-

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tion, so an effective vaccine designed for prophylaxis against HSV-2, or for therapy in individuals with pre-existing disease, would be of major benefit.

There have been reports that stimulation of the immune system of the latently-infected host may result in improved control of recurrent HSV infections, suggesting that vaccination may augment existing host immunity and ameliorate the frequency or the severity of recurrent genital herpes episodes in humans (Stanberry, 1996). Thus, infection by HSV-1 decreases the incidence and severity of subsequent HSV-2 genital infections (Mertz et al., 1992); animal studies have shown that a variety of immunogens, including killed virus, glycoprotein subunits, viral antigens expressed by live vectors, and attenuated or disabled HSV, can protect against challenge infection (Ashley et al., 1998), and may also modify the incidence and severity of recurrent infections (Jennings et al., 1997; Boursnell et al., 1997).

The immune mechanisms involved in controlling or reducing primary and recurrent HSV disease are not fully understood, although recent studies suggest that cellular immune mechanisms, particularly cytotoxic T lymphocyte (CTL) activity, plays an important role (Rinaldo and Torpey, 1993; Carmack et al., 1996; Posavad et al., 1996; Mikloska and Cunningham, 1998). A role for $CD4^+$ T cells is identified by their clearance of infection from the skin and mucosa (Nash and Gell, 1983; Schmid, 1988). During recurrences in humans, $CD4^+$ T cells localise to sites of infection where IFN-γ production is evident (Cunningham and Merigan, 1983). There is also evidence of a correlation between raised IFN-γ and IL-2 levels and protection against recurrent HSV disease in man (Torseth and Merigan, 1986; Spruance et al., 1993).

The affinity of herpes simplex viruses for cells of ectodermal origin, such as those found in the oral or genitourinary mucosa permits primary HSV infection at mucosal surfaces. To achieve effective immunity against virus transmitted by mucosal routes, such as HSV, it may be necessary to immunise at these surfaces (McGhee et al., 1992; Kuklin et al., 1997). It has been shown that oral immunisation can elicit immunity in mucosal tissues outside the intestine, and that some delivery systems may further raise both mucosal and systemic immunity levels (Walker et al., 1992). However, oral administration of soluble proteins, including subunit vaccines, may induce the phenomenon of systemic, antigen-specific, immunological hyporesponsiveness, termed oral tolerance (Strober et al., 1998), and if such a route for immunisation with an HSV-subunit vaccine is deemed desirable, then some form of delivery system that avoids this problem will be necessary.

If an HSV glycoprotein subunit vaccine is used, the immune response may be influenced by the choice of adjuvant or carrier employed, and a number of groups have reported that the immunostimulating complex (ISCOM) delivery system (Morein et al., 1984) can elicit a range of immune responses to various antigens (Takahashi et al., 1990; Villacres-Eriksson et al., 1992; Hassan et al., 1996), exert immunomodulatory effects including the induction of MHC class-I restricted CTLs in lymph nodes and spleen, and specific IgA responses abrogating tolerance (Mowat et al., 1991, 1993), when administered orally. The use of such a carrier has been proposed for HSV vaccines in humans (Erturk et al., 1992; Hassan et al., 1996).

The current studies were conducted to evaluate the efficacy of an ISCOM-formulated HSV-2 subunit antigen preparation to elicit cytokine and antibody responses, and to assess the impact of immunisation with an HSV-2 ISCOM vaccine given by different routes, on cytokine and antibody levels in mice previously infected with a sublethal dose of HSV-2. The protection afforded against subsequent heterotype HSV-1 challenge infection was also determined in one of these experiments.

2. Materials and methods

2.1. HSV strains and cell cultures

Antigens and vaccines were prepared using HSV-2 strain 333, while challenge infection of mice was carried out using HSV-1 strain WAL. Both HSV-1 and HSV-2 viruses were originally

supplied by Dr F. Rapp, Milton Hershey Medical Centre, Pennsylvania State University, and were grown and assayed by plaque-forming titration in Vero cell cultures (Flow Laboratories, Irvine, Scotland).

2.2. HSV-2 vaccine preparation

HSV-2 subunit antigen preparation was obtained and characterised using standard procedures (Erturk et al., 1992; Hassan et al., 1996). The preparations were checked for both protein concentration, and for the presence and quantity of 4 HSV-specific glycoproteins, gB_2, gD_1, gE_1, and gG_2, by ELISA using appropriate monoclonal antibodies, kindly supplied by Dr Stig Jeansson, University of Goteborg, Sweden. HSV-2 ISCOMs were prepared by mixing the appropriate amounts of HSV-2 antigens with Quil A (kindly donated by Dr Bror Morein, National Veterinary Institute, Division of Vaccine Research, Uppsala, Sweden) as described elsewhere (Erturk et al., 1991). The presence of HSV-2 ISCOMs was determined by electron microscopy to visualise the typical cage-like structures.

2.3. Mice

Female Balb/C mice, 8–10 weeks of age, were obtained from a closed randomly-bred colony at the University of Sheffield. All animals were maintained in accordance with Guidelines for the Housing and Care of Laboratory Animals used in Scientific Procedures (Home Office, UK, 1989) and experimental procedures were performed in accordance with UK Home Office Guidelines.

2.4. Experimental designs

The present work comprised two separate experimental designs.

2.4.1. Immunoprophylactic

An experiment was designed to assess the capability of HSV-2 ISCOM vaccine to elicit local and systemic antibody responses, together with a range of cytokine responses, in antigen stimulated, cultured spleen cells, and to determine the duration of these immune responses, following vaccine administration to naive mice in a two-dose oral immunisation regime. Mice, caged randomly in four groups of 12, received two immunisations at two dosage levels, 5 and 15 μg of HSV-2 antigen, 2 weeks apart. The vaccine was delivered in a volume of 0.5 ml by oral gavage.

At both 2 and 20 weeks following the second immunisation spleens were removed from three animals in each group; nasal washings were collected from four mice in each group, and all mice were bled.

2.4.2. Immunotherapeutic

A preliminary experiment (data not shown) was conducted to titrate HSV-2 in vivo by different routes. Mice received footpad or intraperitoneal (i.p.) inocula of HSV-2 (strain 333) ranging from 0.1 to 100 pfu to assess the effects of both route of inoculation and dosage on production of cytokines (IL-4 and IFN-γ) from cultured splenocytes stimulated in vitro with live HSV-2 or HSV-2 antigen preparation. Mice primed with 1 pfu of HSV-2 by the i.p. route showed no HSV-2-associated illness and detectable levels of cytokines could be elicited by restimulation of splenocytes from these animals in vitro.

The experiment described here was to assess the immunomodulatory effects of HSV-2 ISCOM vaccine when given by different routes to mice previously infected with HSV-2. Sixty-six mice were initially infected with a sublethal dose of HSV-2 by the intraperitoneal (i.p.) route. A further 15 mice were infected by oral gavage to determine possible differences in immune responses and subsequent challenge infection, as compared with i.p. infected animals. Spleens were collected 1 week, and bloods 2 weeks following infection. Three of the groups of 15 i.p.-infected mice were now vaccinated twice, 2 weeks apart, with HSV-2 ISCOMs containing 3 μg of HSV-2 antigens, each group by a different route, intraperitoneal (i.p.), subcutaneous (s.c.) or oral. A fourth group of 15, i.p.-infected mice received PBS by the s.c. route. The group of 15 orally-infected mice, received PBS by the s.c. route. A group of 10 non-infected mice received PBS (i.p.) at the commencement, and PBS (s.c.) at all other immunising stages throughout the experiment.

One week following the second and final vaccination, spleens were removed from representative animals in each group; 1 week later all mice were bled, and 24 h following this all previously infected animals challenged with 60 mouse lethal doses (MLD_{50}) of HSV-1, while the uninfected (PBS) group was challenged with 6 MLD_{50} of HSV-1.

2.5. Enzyme-linked immunosorbent assays (ELISA)

Sera separated from individual mouse bloods were tested for total and subclass IgG antibodies by an antibody sandwich technique (Erturk et al., 1992; Hassan et al., 1996) using a crude HSV-2 antigen preparation at a concentration of 1.8 μg/ml, and polyclonal rabbit antiserum against HSV-2 (Dako Laboratories, Copenhagen, Denmark) at a one in 1000 dilution, as coating antibody. Rabbit anti-mouse IgG linked to horseradish peroxidase (Dako Laboratories) was employed as conjugate at a dilution of one in 1000. For determination of IgG_1 and IgG_{2a} mouse subclass antibodies, the same reagents and procedures were used, but the appropriate horseradish peroxidase (HRP) conjugates (Hassan et al., 1996) were supplied by Nordic Laboratories Ltd., Maidenhead. All sera were tested at a single, 1 in 100 dilution for total IgG, IgG_1 and IgG_{2a} subclass antibodies, and at each stage as indicated, a number of sera from each group, selected at random, were tested for total and subclass IgG antibodies by ELISA endpoint titration. The mean ELISA endpoint titres for total and subclass IgG antibody were shown to correlate with the mean ELISA absorbance values determined using a single, 1:100, serum dilution (data not presented).

HSV-specific IgA antibodies in mouse nasal washings were determined by modifications of ELISA procedures described elsewhere (Ben-Ahmeida et al., 1992; McLean et al., 1996). Briefly, microplates were coated overnight with a crude extract of HSV-2 infected Vero cells, and blocked using PBS containing 1% BSA and 0.05 Tween-20. Control plates were coated with an extract from uninfected Vero cells. Nasal wash samples from immunised mice were added, undiluted, to appropriate wells, and following incubation for 1 h at RT and three washes in PBS/Tween-20, rabbit anti-mouse IgA, Fc-specific, diluted 1:1000 in PBS/Tween-20 + 1% BSA, was added. After overnight incubation at RT and three washes, goat anti-rabbit IgG conjugated to HRP was added, and the test completed as described earlier. The plates were read spectrophotometrically at 492 nm.

2.6. Neutralisation tests (NT)

These were carried out in 96 well sterile tissue culture microplates (Costar, Cambridge) on individual mouse sera using procedures described in detail elsewhere (Mathiesen et al., 1988), with appropriate controls. Neutralisation antibody titres for each serum were expressed as the reciprocal value of the highest serum dilution at which the HSV-2 antibody-induced ELISA absorbance value was reduced by 50% of the value shown by the positive control wells.

2.7. Splenocyte cultures and lymphoproliferation assays

Spleens were removed from mice (3–6 per group), subsequent to sublethal HSV-2 infection and final immunisation. Disrupted spleens were washed, finally resuspended in RPMI 1640 medium (Life Technologies, Paisley, Scotland) plus 10% foetal calf serum (FCS), penicillin (40 units/ml), streptomycin (150 units/ml), and amphotericin B (0.5 μg/ml) and the lymphocytes separated using J prep (J Bio, France).

Lymphoproliferation assays were carried out using procedures described in detail previously (Hassan et al., 1996). Briefly, splenocytes were plated in 96-well cell culture plates at a concentration of 4×10^5 viable cells in 100 μl per well. The cells were then re-stimulated in vitro with live or heat inactivated HSV-2, HSV-2 subunit antigen preparation, phytohaemagglutinin (PHA) as positive control, or left unstimulated as a negative control. After 72 h supernatant fluids were removed to −70°C for subsequent cytokine assays, and the remaining cell cultures pulsed with 50 μl (0.5 μci/well) of (^{3}H)-thymidine at 1 mci mmol^{-1}

(Amersham International, Amersham, UK). The amount of incorporated label was determined in a scintillation counter. Cell proliferation was expressed as counts per minute (c.p.m.) in the presence of antigen, minus c.p.m. in the absence of antigen. In representative experiments, a mock-infected Vero cell control preparation was incorporated to assess background levels of proliferation to this material. Results were essentially identical to those obtained using controls consisting of media alone.

2.8. Cytokine assays

Assays for IFN-γ, IL-2 and IL-4 were performed using ELISA procedures described elsewhere (Hassan et al., 1996). Briefly, for IFN-γ and IL-2, assays were set up in triplicate in 96-well, flat-bottomed tissue culture plates (Costar, Cambridge), coated with purified rat anti-mouse IFN-γ (R4-6A2) or IL-2 (JES6-1A12) obtained from Pharmingen, Cambridge Bioscience Ltd., Cambridge. After overnight incubation at 4°C and subsequent washing, wells were blocked (1 h/37°C) using 10% heat-inactivated FCS in PBS, pH 7.2. After further washing, dilutions of standard recombinant mouse IFN-γ or IL-2 (Cambridge Bioscience) or undiluted supernatants were added to appropriate test wells. Following incubation for 2 h at 37°C, wells were washed and biotin-labelled rat anti-mouse IFN-γ or IL-2 antibody (XMG 1.2 or JES 6-5H4; Pharmingen) added. After a further 45 min at 37°C and further washes, streptavidin-HRP conjugate (Southern Biotechnology Association) was added, and the plates incubated at 37°C for 30 min. Following addition of buffered TMB/DMSO substrate (Sigma) and incubation for 30–60 min at 37°C, the reaction was stopped using 10% sulphuric acid, absorbance values read at 405 nm and the results expressed as picograms of IFN-γ/ml or units of IL-2/ml.

Similar procedures were used for IL-4 assays except that reagents related to IL-4 (purified rat anti-mouse IL-4, 11B11, standard recombinant mouse IL-4, and biotin-labelled rat anti-mouse IL-4, BVD6-24G2), were used. These were kindly donated by DNAX Research Institute, Palo Alto. The results are expressed as units of IL-4/ml.

The sensitivity of the cytokine assays was determined from the standard deviations of the values from six control wells for each cytokine assay, and calculating three standard deviations from these values. ELISA readings at or below the level of the three SD values for control tests, were considered negative. The baseline values thus obtained were ≤ 0.3 units/ml for IL-2; ≤ 0.18 units/ml for IL-4 and ≤ 0.26 pg/ml for IFN-γ.

2.9. Statistical analysis

The Mann-Whitney U test was used to statistically analyse antibody levels in mouse sera or nasal washings, lymphoproliferative responses and cytokine levels in splenocyte cultures supernatants. Values of $P \leq 0.05$ were taken as significant.

3. Results

3.1. Immunoprophylactic experiment

The induction of serum and nasal antibody, and cytokine responses in mice administered HSV-2 ISCOM vaccine by the oral route were determined using the experimental design described earlier. Fig. 1(a–b) shows the total and subclass serum IgG antibody levels (as indicated by mean ELISA absorbance values) at 2 and 20 weeks subsequent to the second dose of HSV-2 ISCOMs vaccine containing either 5 or 15 μg of HSV-2 subunit antigen preparation. For total IgG, as well as for the IgG1 and IgG2a subclass antibodies, marginally greater levels, at 2 weeks subsequent to the second vaccine dose, were elicited by the 15, as opposed to the 5 μg HSV-2 ISCOM vaccine dose, but these differences, as well as those between the levels of IgG1 and IgG2a antibody, were not significant (Fig. 1(a)). There was also no significant difference between the levels of both total and subclass IgG antibodies at 20 weeks post-immunisation, compared to the levels detected at 2 weeks (Fig. 1(a–b)), although the titres had demonstrably decreased at the latter, as compared to the former time-point.

The levels of IgA in mouse nasal washings are also shown (Fig. 1(c–d)), and indicate a some-

what different pattern in local, as compared to systemic, immune responses. Thus, a significantly ($P < 0.05$) higher level of IgA antibody was detected in nasal washings from mice given 2×5 μg of ISCOM vaccine preparation by oral gavage than was detected in animals given 2×15μg of the same vaccine, at 2 weeks following immunisation (Fig. 1(c)). At 20 weeks, the IgA levels in nasal washings were found to have changed marginally with respect to their level at 2 weeks post-immunisation. Thus, at the former timepoint greater levels of IgA were detectable in mouse nasal washings collected from animals immunised with two 5μg doses of the HSV-2 ISCOM vaccine preparation, than that seen at 2 weeks (Fig. 1(d)); this increase was not significant. In addition, a small but not significant rise in mean nasal wash IgA levels was detectable at 20 weeks in mice receiving 2×15 μg of HSV-2 ISCOMs (Fig. 1(c–d)).

The results of the cytokine levels and lymphoproliferation responses of immunised mice at both 2 and 20 weeks post-immunisation are shown in Fig. 2, and indicate that, as with the humoral immune responses at 2 weeks post-immunisation, the greatest levels of cytokines IL-2, IL-4 and IFN-γ, as well as the lymphoproliferation levels at 2 weeks post-immunisation (Fig. 2(a–d)) were detected from mice immunised by oral gavage with 2×15 μg of HSV-2 ISCOM vaccine. However, in general, the levels of all cytokines stimulated under these in vitro conditions were low, although in most instances, slightly higher levels were detected using live HSV-2 virus as the in vitro stimulant, as opposed to heat inactivated HSV-2 virus or the HSV-2 subunit antigen preparation.

At 20 weeks post-immunisation (Fig. 2(e–h)), lymphoproliferation responses and the levels of cytokines detectable in spleen cell cultures stimulated in vitro were markedly decreased or below baseline values in both groups compared to the levels recorded at 2 weeks post-immunisation. In-

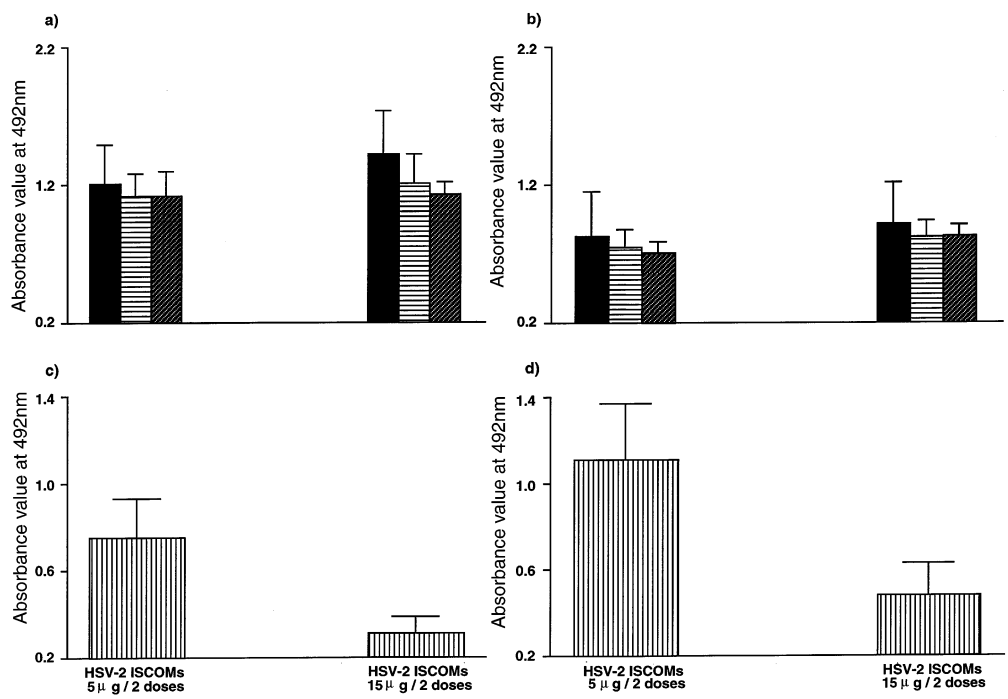

Fig. 1. Serum and nasal wash antibody levels in mice received HSV-2 ISCOMs by the oral route, at 2 (a, c) or 20 (b, d) weeks postimmunisation. ■, Total IgG; ▤, IgG-1; ▨, IG6-2a; ▥, Nasal IgA.

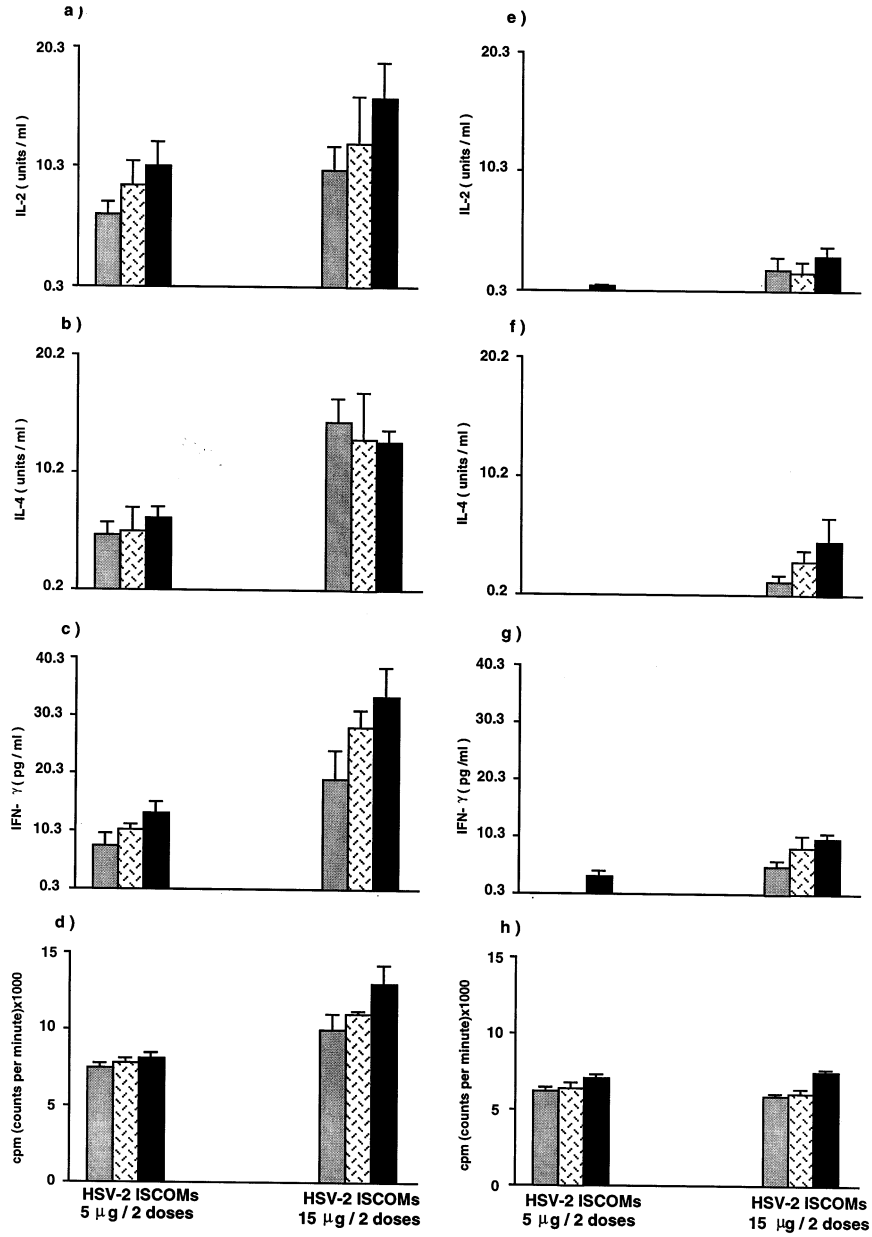

Fig. 2. Levels of IL-2 (a, e), IL-4 (b, f), IFN-γ (c, g) and lymphoproliferation responses (d, h) detected in spleen cell cultures at 2 (a–d) or 20 (e–h) weeks following immunisation with HSV-2 ISCOMs by the oral route. The in vitro stimulants used were HSV-2 subunits ▭ ; heat inactivated HSV-2 ▨ ; or live HSV-2 ■.

deed, at 20 weeks post-immunisation in only one of the immunised groups of mice, that given 2 doses of HSV-2 ISCOM vaccine at 15 µg antigen concentration, were all 3 cytokines IL-2, IL-4 and IFN-γ detectable in the presence of all in vitro stimulants (Fig. 2(e–h)).

3.2. Immunotherapeutic experiment

As described in Section 2, this experiment was designed to investigate the influence of prior HSV-2 infection on the antibody and cytokine responses to HSV-2 vaccination.

It was demonstrated in the preliminary experiment that i.p. inoculation of mice with 1.0 pfu of live HSV-2, could elicit detectable immune responses with no severe associated illness. Accordingly, this dosage of HSV-2 was used to establish a group of infected mice in which the effects of therapeutic vaccination on the immune responses and protection against heterologous infection could be evaluated.

The results for total and subclass IgG serum antibody responses, and the NT antibody responses of mice following oral or i.p. inoculation of sublethal HSV-2 and two subsequent vaccinations with HSV-2 ISCOMs by different routes, are presented in Fig. 3; Table 1. The results show that infection of mice with HSV-2 by the oral route promoted essentially similar levels of antibodies to those infected intraperitoneally, and that, inoculation of PBS to mice previously infected with a sublethal dose of HSV-2, by either route did not significantly alter the antibody levels. It can also be seen (Fig. 3; Table 1) that HSV-2 infected mice receiving two subsequent inoculations, each of 3 μg, with HSV-2 ISCOMs by different routes, all responded with increased levels of total IgG, IgG_1 and IgG_{2a} over those present post-HSV-2 infection, although these increases were not statistically significant. The levels of IgG_{2a} antibody in all groups of mice post-vaccination, were also higher, although not statistically so, than those of IgG_1 subclass antibody, a situation reflecting that seen following HSV-2 infection.

The greatest ELISA antibody responses at 2 weeks post-second vaccination, were observed in the group of mice receiving HSV-2 ISCOMs by the oral route, a situation also indicated by the ELISA endpoint antibody titres (Table 1).

The results for the geometric mean NT antibody titres from mice post-infection and post-vaccination are also presented in Table 1 and indicate that administration of two doses of HSV-2 ISCOMs by different routes significantly ($P < 0.05$) increased NT antibody titres over those present post-infection, with the highest levels seen in groups of mice vaccinated orally followed by the s.c. and then the i.p. route, a situation essentially

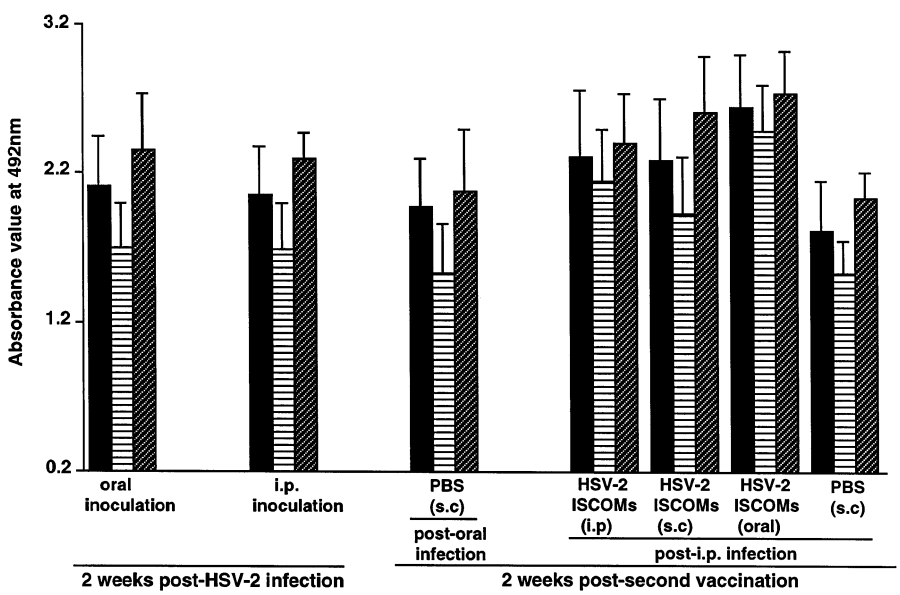

Fig. 3. ELISA absorbance values for total and subclass specific IgG antibody in sera of HSV-2 infected mice subsequently vaccinated with HSV-2 ISCOMs by different routes. ■, total IgG; ▤, IgG-1; ▨, IgG-2a.

Table 1
ELISA antibody and NT antibody levels detected in sera from HSV-2 infected mice, subsequently vaccinated with HSV-2 ISCOMs by different routes

Inoculated material (Route)	No. of sera tested	Geometric mean antibody titres 2 weeks post-infection				Inoculated material (Route)	No. of sera tested	Geometric mean antibody titres 2 weeks post-second vaccination			
		Total IgG	IgG_1	IgG_{2a}	NT			Total IgG	IgG_1	IgG_{2a}	NT
Sublethal HSV-2 (oral)	15	17 800[a]	12 900	18 700	256[a]	PBS (s.c.)[b] × 2	15	16 250	11 200	17 000	234
						PBS (s.c.) × 2	10	16 600	11 800	17 800	223
Sublethal HSV-2 (i.p.)[c]	15	18 800	12 750	18 000	268	HSV-2 ISCOMs (i.p.) (2 × 3 μg)	10	21 000	17 000	21 500	549
						HSV-2 ISCOMs (s.c.) (2 × 3 μg)	10	21 750	15 750	22 800	588
						HSV-2 ISCOMs (oral) (2 × 3 μg)	10	23 000	20 500	24 500	630
PBS (i.p.)	10	< 200	< 200	< 200	< 10	PBS (s.c.) × 2	10	< 200	< 200	< 200	< 10

[a] Reciprocal of endpoint dilution for ELISA or NT (neutralising) antibody.
[b] s.c., subcutaneous.
[c] i.p., intraperitoneal.

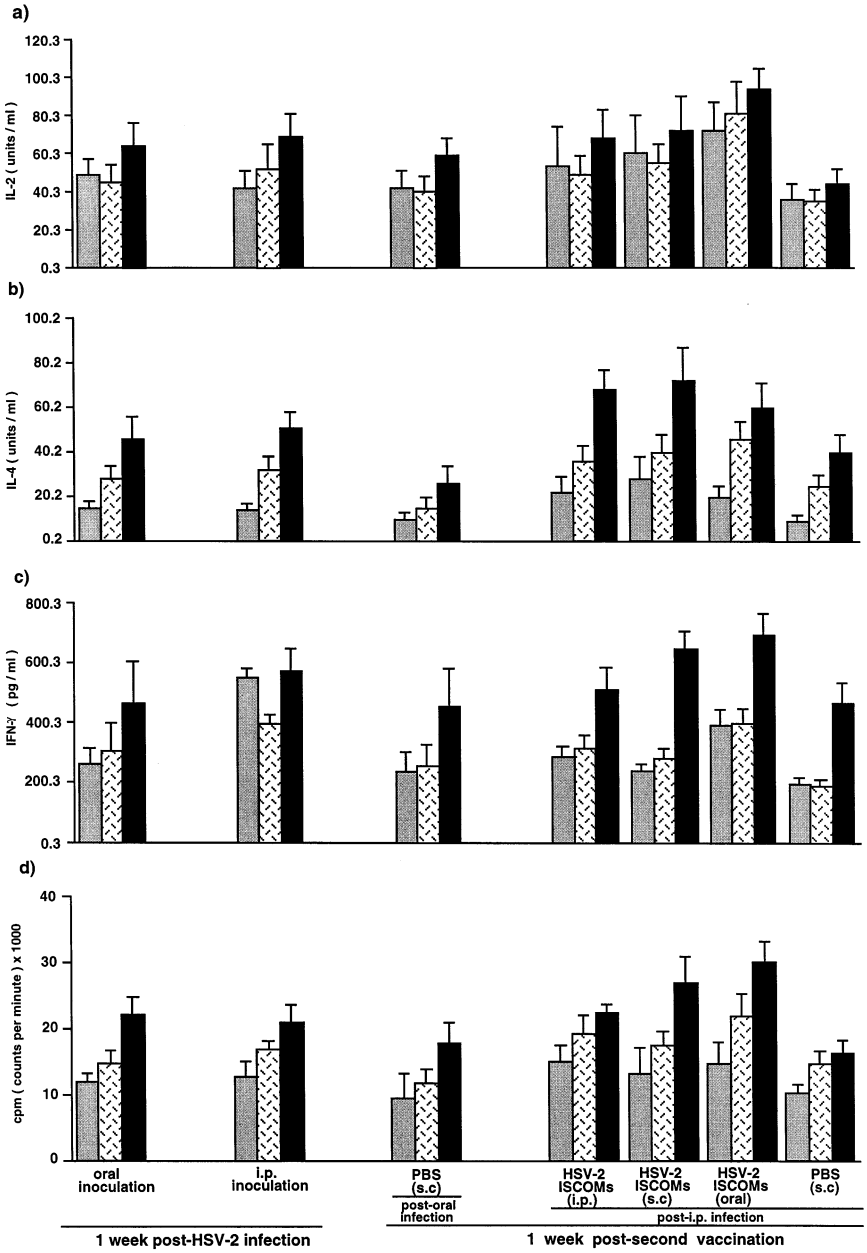

Fig. 4. Levels of IL-2, IL-4, IFN-γ and lymphoproliferation responses detected in spleen cell cultures from HSV-2 infected mice subsequently vaccinated with HSV-2 ISCOMs by different routes. IL-2 (a), IL-4 (b), IFN-γ (c) levels and lymphoproliferation responses (d), in mouse spleen cell cultures obtained from HSV-2 infected mice 7 days subsequent to the second vaccination with HSV-2 ISCOMs by different routes. The in vitro stimulants used were HSV-2 subunits ▭; heat inactivated HSV-2 ▨; or live HSV-2 ■.

reflecting the findings with respect to ELISA antibodies (Fig. 3; Table 1).

Fig. 4 shows the results of the lymphoproliferative responses, and the levels of the cytokines IL-2, IL-4 and IFN-γ detected following in vitro stimulation of mouse splenocyte cultures from

animals infected with HSV-2, and subsequently given two vaccinations with HSV-2 ISCOMs or PBS. The mouse splenocyte cultures were restimulated in vitro with live or heat-inactivated HSV-2 virus, or with HSV-2 antigen preparation. The results for lymphoproliferation (Fig. 4(d)) indicate that essentially similar levels of cell proliferation were observed in splenocyte cultures established from the spleens of all HSV-2 infected mice (although the highest levels were seen following in vitro re-stimulation with live HSV-2). Marginally decreased lymphoproliferative levels were observed following the administration of PBS to mice previously infected with HSV-2 by either the oral or i.p. routes. However, there was a slightly greater decrease, although this was not significant, in those animals previously infected by the i.p. route.

Lymphoproliferative response levels of HSV-2-infected mice subsequently immunised with HSV-2 ISCOMs by different routes, were increased over those observed post-infection, but these increases were not significant, with one exception, that of the group of infected mice subsequently receiving two doses of HSV-2 ISCOMs orally and splenocyte culture restimulation with live HSV-2, which induced significantly higher levels of proliferation ($P < 0.05$) than those observed in similarly restimulated cultures established 1 week post-infection (Fig. 4(d)).

The results for in vitro IL-2 secretion in restimulated spleen cell cultures are presented in Fig. 4(a) and indicate similar patterns to those of proliferation. Although mice infected by the oral route induced similar levels of IL-2 to i.p.-infected mice, they showed higher levels of IL-2 following subsequent inoculation with PBS as compared to the i.p.-infected mice receiving PBS. The patterns of IL-2 secretion of spleen cell cultures also show (Fig. 4(a)) that marginally higher IL-2 levels were induced in restimulated spleen cell culture supernatants from infected mice receiving two doses of HSV-2 ISCOMs by different routes compared to the levels detected post-infection, with the highest levels detected in mice vaccinated orally; this group induced significantly higher ($P < 0.05$) IL-2 levels than those detected in HSV-2 infected animals subsequently receiving two doses of PBS, a situation reflecting the lymphoproliferative responses (Fig. 4(a,d)).

The levels of IL-4 (Fig. 4(b)) present the same pattern as that of IL-2 post-infection, but lower levels of IL-4 were detected following oral inoculation with PBS, as compared with i.p. infected mice subsequently receiving PBS. The results for IL-4 levels post-vaccination (Fig. 4(b)), indicate that administration of two doses of HSV-2 ISCOMs by the s.c. route to HSV-2 infected mice induced the highest levels of IL-4 in splenocyte culture supernatants followed, in decreasing order, by the i.p. route and the oral route, although these differences were not statistically significant. There were statistically significant differences ($P < 0.05$) however, between the levels of IL-4 secreted following live HSV-2 restimulation of splenocyte cultures of infected mice vaccinated by either the i.p. or s.c. routes with HSV-2 ISCOMs, and the levels of IL-4 induced following live HSV-2 restimulation of spleen cells of infected mice receiving two doses of PBS (Fig. 4(b)).

The results for IFN-γ levels, presented in Fig. 4(c), indicate that marginally higher levels of IFN-γ were induced in orally, than in i.p.-infected mice and these levels decreased post-inoculation with PBS. The greatest IFN-γ levels were observed in infected mice vaccinated twice orally with HSV-2 ISCOMs, but this difference did not reach statistical significance, although this group induced significantly higher ($P < 0.05$) levels of IFN-γ than those receiving PBS, irrespective of the type of in vitro stimulant used, at 1 week post-second vaccination, immediately prior to challenge infection.

3.3. Protection of mice against heterologous (HSV-1) challenge

The results in Fig. 5 show that complete protection against lethal heterologous, challenge infection with $60MLD_{50}$ of HSV-1 was achieved in infected mice immunised with two doses of the HSV-2 ISCOM preparation by either the oral or s.c. routes, while a 92% protection level was afforded to infected mice vaccinated with HSV-2 ISCOMs by the i.p. route. Mice receiving PBS post-i.p. infection showed 58% degree of protection, while the level of protection afforded to the

group of mice infected initially by the oral route was 50%. No protection was seen in the uninfected unimmunised animals challenged with $6MLD_{50}$ of HSV-1 (Fig. 5).

4. Discussion

Vaccination represents the most successful means yet to protect against infectious diseases. Many human pathogens initiate their infectious processes at mucosal surfaces, but most of the licensed vaccines available against such agents are given parenterally. Oral immunisation represents an attractive vaccination strategy for several reasons, including greater acceptability, cost benefit, and increased potential for promotion of local immunity (Walker, 1994; Jennings et al., 1997).

It is clear from current and previous studies in this laboratory (Ghazi et al., 1995) and elsewhere (Mowat et al., 1991, 1993; Scheepers and Becht, 1994; Kazanji et al., 1994; Ugozzoli et al., 1998; Hu et al., 1998; Morein et al., 1999) that ISCOMs can be used for mucosal delivery of vaccine antigens, and promote superior responses to those seen when protein antigens alone are administered

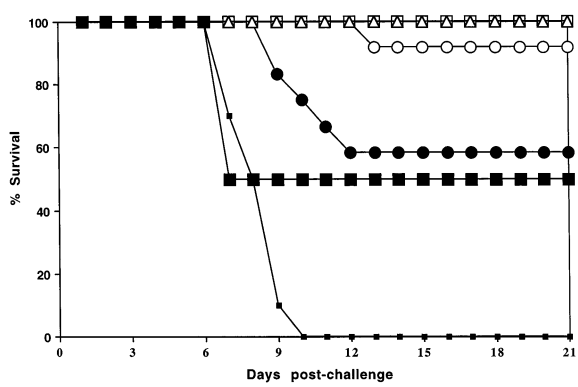

Fig. 5. Protection of HSV-2 infected mice against heterologous HSV-1, strain WAL following challenge at 2 weeks subsequent to the second vaccination. Groups of mice infected by the intraperitoneal route were vaccinated with HSV-2 ISCOMs by the oral (△), subcutaneous (□) or intraperitoneal (○) routes. Further groups of mice were given PBS following infection by either the oral (■) or intraperitoneal (●) routes. One group of mice (■) received PBS intraperitoneally and again subcutaneously on two occasions prior to challenge with HSV-1.

by the mucosal route to mice. In the present studies using HSV-2 antigens incorporated into ISCOMs, oral administration to mice in a two-dose schedule induced IgA in nasal secretions, total and subclass IgG1 and IgG2a antibody responses in serum, and detectable levels of IL-2, IL-4 and IFN-γ in restimulated spleen cell cultures, indicating that this vaccine formulation may elicit cellular, systemic and local humoral immune responses. The ability of HSV-ISCOMs to induce protection against HSV-challenge infection following subcutaneous immunisation of mice has been demonstrated in previous studies from this laboratory (Erturk et al., 1989, 1992; Ghazi et al., 1995; Hassan et al., 1996; Mohamedi, 1999), while the findings of the prophylactic experiment described here indicate that ISCOMs represent an appropriate formulation for oral delivery of HSV antigens to promote immune responses in mice. Such procedures could maximise the efficacy of, and the response to, vaccine delivery by the oral route if translatable to the human situation.

Investigations have revealed that infection with herpes simplex viruses (type 1 and 2) in the mouse can induce a wide range of immune responses, including CD8 + cytotoxic T cells (Bonneau and Jennings, 1990; Rouse et al., 1994), $CD4^+$ T cells (Manickan et al., 1995; Ghiasi et al., 1997) and neutralising antibodies (Nash and Cambouropoulos, 1993; Ghiasi et al., 1994; Whaley et al., 1994; Gallichan and Rosenthal, 1995, 1998). It is possible that a cellular response, mediated, at least in part via IFN-γ and CTLs, may play a crucial role in limiting primary or recurrent HSV infection in man (Cunningham and Merigan, 1983; Rinaldo and Torpey, 1993; Carmack et al., 1996; Posavad et al., 1996; Tigges et al., 1996; Koelle et al., 1998), while antibody has been correlated with protective immunity against HSV-infection (Fujinaga et al., 1987; Ashley et al., 1998). In the present study, sublethal HSV infection was established by a mucosal (oral) route in mice as well as by a systemic route (intraperitoneal) to define if the route of infection induces different immune response patterns which might influence the immune response levels or protection afforded to mice on subsequent mucosal or parenteral immu-

nisation. The results indicate that similar levels of immune responses and protection were observed in both oral or i.p. infected mice, when these were subsequently mock-immunised, indicating that the mucosal route of HSV-2 infection did not significantly change the pattern of immune responses and protection afforded to the animals, and that the experimental i.p.-infected murine model is a surrogate to assess immune responses to oral immunisation.

Considerable interest in the contributions of Th_1 and Th_2 subsets (Mosmann and Coffman, 1989) in resistance to and control of various infections has recently been generated. Th_1 cells, which characteristically secrete IL-2 and IFN-γ, are associated with cell-mediated immunity (CMI), and have been shown to be important in the control of intracellular infections (Karupiah et al., 1996) including those caused by HSV (Rinaldo and Torpey, 1993). In contrast, Th_2 cells produce mainly IL-4 and IL-5 (Mosmann and Coffman, 1989; Mosmann and Sad, 1996) and are associated with high antibody and relatively poor CMI responses, and with susceptibility to infection by intracellular pathogens (Yasumoto et al., 1994; Moran et al., 1996).

Since clearance of a number of virus infections is dependant on the generation of CTLs and the release of cytokines, such as IFN-γ (Chisari and Ferrari, 1995), it is apparent that Th_1will be more effective than Th_2-type immune responses against certain virus diseases.

In the studies described here, splenocytes from HSV-2 infected mice, cultured in vitro showed secondary recall responses in the presence of HSV-2 antigen suggesting that components of T cell recognition are directed to HSV antigens. In addition, presence of IL-2 and IFN-γ in restimulated splenocyte culture supernatants suggest a Th_1-like response following HSV-2 infection of these animals. Recent studies have demonstrated that production of Th_1 cytokines is characteristic of naturally acquired immunity to HSV in healthy adults (Carmack et al., 1996). Amongst antibodies, certain IgG isotypes may be more important than others in conferring protection against viral infection (Coultier et al., 1987; Hocart et al., 1989; Nguyen et al., 1994). Passive immunisation studies in mice have indicated that antibodies of the IgG_{2a} isotype are more important in virus neutralisation than IgG_1 (Richards et al., 1998), and it has been documented that preferential switching to IgG_{2a} can be maintained by IFN-γ (Coffman et al., 1988; Nguyen et al., 1994). Based on these observations it seems likely that both $CD4^+$ and CD8 + T lymphocyte responses and antibody responses are important to optimally control HSV-infection.

In the present study the dominant ELISA IgG subclass antibody in sera of mice post-infection was IgG_{2a}. Moreover, induction of high levels of functional (NT) antibody in sera of HSV-2 infected mice correlated well with the IgG_{2a} predominance, while the presence of IFN-γ as well as IL-4, in restimulated splenocyte culture supernatants correlates with the observation that both IgG_{2a} and IgG_1 subclasses are elicited following HSV infection. The predominance of IgG_{2a} in mouse serum suggests that the IL-2 and IFN-γ cytokine profile demonstrated in splenocyte cultures from these animals, is the dominant in vivo phenotype following HSV-2 infection.

Studies of HSV vaccine therapy have demonstrated that efficacy, at least in guinea-pigs, can be affected by vaccine formulation, including influence of both the immunogen and adjuvant (Sanchez-Pescador et al., 1988; Ho et al., 1989), while the route of vaccine administration can also influence efficacy of immunotherapy (Stanberry, 1994). It is pertinent to note that the nature and formulation of vaccine for use immuno-therapeutically in man to limit or reduce recurrent HSV infections should elicit or enhance those facets of the immune response beneficial to the host. Only aluminium salts are currently licensed world-wide for use as adjuvants in humans, but their relatively poor adjuvanticity, and their promotion of Th_2, rather than Th_1 responses to the accompanying antigen (Gupta et al., 1993; York et al., 1995), make them less than ideal, in this respect.

The aim of vaccination against HSV-2 infection in a therapeutic setting, is to limit recurrent genital infection and neonatal herpes, and this involves limiting the replication of, and the symptoms induced by, virus reactivated from latent state; this was not addressed in the current

studies. However, if a Th_1 profile through which relatively high levels of CTLs, believed to be important in controlling recurrent HSV infections (Cunningham and Merigan, 1983; Torseth and Merigan, 1986; Mikloska and Cunningham, 1998) is to be elicited, then an HSV vaccine containing the appropriate glycoproteins formulated with a suitable adjuvant/carrier or delivery system able to produce this profile, is required. The formulation of antigens into ISCOMs (Morein et al., 1984), is known to promote greater Th_1 than Th_2 responses in mice, although both are induced (Takahashi et al., 1990; Villacres-Eriksson et al., 1992; Hassan et al., 1996; Sjolander et al., 1997), and in the present studies this approach was used for presenting a mixture of HSV-2 antigens to the immune system of infected mice, and the results indicate that therapeutic vaccination of animals with HSV-2 ISCOMs can maintain and reinforce immune parameters, particularly IgG_{2a}/IFN-γ, and significantly increase levels of NT antibodies over those seen post-infection. The high levels of NT antibodies may be of particular relevance to prevention of recurrent infection in view of the recent report that such antibodies can inhibit axonal spread of HSV-1 to epidermal cells in vitro (Mikloska et al., 1999).

A further goal of therapeutic vaccination against HSV is to increase immune mechanisms operating against virus persisting in the host to levels that preclude infection by heterologous strains of HSV. That such cross-immunity is functional in man has already been demonstrated (Mertz et al., 1990), and the current results show that such immunity can be achieved using HSV-2 ISCOMs, providing high levels of protection in animals against subsequent, lethal heterologous HSV-1.

The studies reported here, indicate that HSV-2 ISCOMs administered orally, can promote high systemic, and local, immune responses. Although local immunity was not assessed in the immunotherapy studies, the current and previous work has shown that ISCOMs represent an appropriate formulation for oral delivery of HSV antigens to promote long-lasting immune responses in naive mice, including nasal IgA (Mohamedi; unpublished data). The oral route also affords complete protection against heterologous lethal challenge and such a route of vaccination would be the most acceptable in humans. The data, presented here, together with the increased recent understanding of the precise mechanisms of immunomodulation by ISCOMs (Villacres-Eriksson et al., 1997a,b; Morein et al., 1998), provides real hope for the use of such a vaccine in humans.

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