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Transcutaneous immunisation with herpes simplex virus stimulates immunity in mice

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Abstract

Herpes simplex virus (HSV) is common throughout the world and is a target for vaccine development. Transcutaneous immunisation is a novel technique that uses the application of vaccine antigens in solution on the skin in the presence of cholera toxin (CT) as an adjuvant. This study investigated the potential of transcutaneous immunisation in C3H mice, using CT co-administered with whole inactivated HSV-1 (CT+HSVi) or HSV-1 antigens extracted from infected Vero cells (CT+HSVag) or a control protein (CT+BSA). The application of any of the three vaccines on to bare mouse skin resulted in the migration of Langerhans cells from the epidermis and in the production of serum antibodies to CT. Both HSV preparations generated serum and mucosal (faecal) antibodies to HSV, with the CT+HSVi vaccine being a more potent stimulator of humoral immunity. The CT+HSVag vaccine, however, was the more potent stimulator of cell-mediated immunity, giving rise to a strong delayed type hypersensitivity response and lymphocyte proliferation in vitro. When the mice were challenged by epidermal inoculation of HSV, the CT+HSVag vaccine induced a higher level of protection than the CT+HSVi vaccine, a result which may indicate that the efficacy of HSV vaccines depends on stimulation of cell-mediated rather than humoral responses. The success of topical vaccination suggests that the transcutaneous route may offer a promising potential for novel vaccine delivery which merits further investigation.

Keywords: Transcutaneous immunisation; Herpes simplex virus; Mouse; T- and B-cell response to herpes simplex virus

1. Introduction

Herpes simplex virus (HSV) infects a large proportion of the human population: the oral and genital mucosa are the sites most commonly targeted by type 1 and 2, respectively. Both HSV types can cause serious complications in a significant number of cases, such as oedema, localised lymphadenopathy, corneal inflammation and life threatening encephalitis [1,2]. Following a primary infection, the virus becomes latent in the cell bodies of peripheral nerves innervating the site of inoculation. Subsequent reactivation may be triggered by stimuli such as stress, UV light or immunosuppression and may result in recrudescent episodes.

Host genetic background, macrophages, natural killer cells, specific T-cell populations, virus-specific antibodies and lymphokine responses have been implicated as important host defences against HSV infections. In the mouse, delayed type hypersensitivity (DTH) responses can be identified within a few days after disease onset, followed by a cytotoxic T-lymphocyte response and by the appearance of both IgM and IgG specific antibodies. Host responses in humans tend to be more delayed, developing around 7–10 days post-infection.

A successful HSV vaccine needs to be capable of either preventing the primary infection, or preventing recrudescent episodes and virus shedding (when administered to subjects who already harbour latent virus). The induction of a broad and durable 'effective immunity' at all points of entry, e.g. the genital tract, nasal and oro-pharyngeal mucosa, and the eye would be desirable. Animal studies indicate that whilst prophylactic vaccines cannot stimulate effective immunity at the mucosal surface, they can prevent disease and significantly reduce the magnitude of the latent infection and frequency of recurrent disease [3]. Using a murine model that mimics genital herpes in humans,

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Abbreviations: BSA, bovine serum albumin; CT, cholera toxin; DTH, delayed type hypersensitivity; HSV, herpes simplex virus; LC, Langerhans cells; PBS, phosphate-buffered saline; PFU, plaque forming units; PMSF, phenylmethanesulfonyl fluoride; T_H1, T helper 1

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investigators demonstrated that administration of HSV glycoproteins into the footpad, with potent adjuvants, could reduce recurrent HSV disease [4].

Transcutaneous immunisation, the introduction of antigens by topical application to intact skin, without physical penetration by needles, is a novel method of vaccination which has recently been shown to induce mucosal immunity and systemic protection against bacterial toxins. When cholera toxin (CT) was applied to the skin of mice, an immune response was stimulated to co-administered antigens, such as tetanus or diphtheria toxoids [5]. The CT acted as a mucosal adjuvant without systemic toxicity. Protective immunity to toxin-mediated mucosal disease was induced transcutaneously [6] and more recent work detected IgA antibodies to CT and co-administered antigens in the stools and lung washes of immunised mice [7]. Since transcutaneous immunisation induced both systemic and mucosal antibodies to the co-administered antigens, this approach may be beneficial for diseases stemming from mucosal pathogens.

The present study was conducted in order to determine whether mice could be transcutaneously immunised with CT and HSV antigens with the aim of identifying any mucosal and systemic humoral immune responses. DTH and in vitro lymphoproliferation assays were carried out to determine whether T-cell responses were stimulated, and mice were challenged epidermally with live HSV to ascertain whether transcutaneous immunisation offered protective immunity.

2. Materials and methods

2.1. Mice

C3H/HeN mice of both sexes, 7–16 weeks old, were used in this study and were kept as previously described [8]. The mice were housed in a licenced facility and all procedures were approved by the Home Office.

2.2. Virus

A plaque-purified strain of HSV-1, isolated from a clinical case and passaged four times in Vero cells at a multiplicity of infection of 0.2, was used throughout [9]. The virus was cultured and titrated as plaque forming units (PFU) on monolayers of Vero cells as previously described [10]. Inactivated HSV was prepared by UV irradiating a virus stock with 12 000 J m^{-2} UV-B. A glycine extract of HSV-1 antigens was prepared as previously outlined [11]. Briefly, monolayers of Vero cells were infected with HSV-1 at a multiplicity of 3 PFU per cell and incubated at 37°C for 24 h. The cells were harvested using a cell scraper, washed three times with phosphate-buffered saline (PBS) and resuspended in buffer prepared from five parts 0.1 M aqueous solution of glycine and three parts 0.1 M aqueous sodium hydroxide. The cells were disrupted by exposure to three freeze-thaw cycles and ultrasonicated for 1 min, then vigorously agitated. The total protein content was established using a Sigma (Poole, UK) protein estimation kit.

2.3. The three vaccines

CT+BSA: 185 μg ml^{-1} CT (Sigma, UK) and 500 μg ml^{-1} bovine serum albumin (BSA) (Sigma, UK) made up in PBS.

CT+HSVi: 185 μg ml^{-1} CT (Sigma, UK) and 1.5×10^7 PFU ml^{-1} of UV-B inactivated HSV-1 in PBS.

CT+HSVag: 185 μg ml^{-1} CT (Sigma, UK) and 500 μg ml^{-1} protein of HSV-1 glycine antigen extract in PBS.

Each vaccine was given to groups of mice on three occasions (days 0, 14 and 35). On each occasion, the mice were anaesthetised using 100 μl intraperitoneal Hypnorm (Janssen, UK) to prevent them from licking the vaccinated area. A 100-μl volume of each vaccine was applied to the shaved back skin and 2 h was allowed for absorption. The backs of the mice were washed with warm tap-water to remove excess vaccine and patted dry with tissues. Separate groups of mice were used in the different experiments.

The schedule for collecting samples was as follows: 10 days after the third vaccination, the sera from three mice per group were pooled and stored at −20°C until used. Faecal samples were collected on days 32 and 42 and pooled. The DTH assay was also performed 10 days after the final vaccination on five mice per group. Different groups of vaccinated mice were challenged epidermally with HSV, again 10 days after the third vaccination. Seven days after this challenge, three mice per group were killed by cervical dislocation and the inguinal lymph nodes removed for the lymphoproliferation assay.

2.4. Langerhans cells (LC) count

In order to investigate the effect of the vaccination on LC numbers in the epidermis, groups of mice were anaesthetised and vaccinated on a single occasion with 20 μl of the above preparations, on the dorsal side of each ear. Two mice were allocated to each vaccine group and a negative control group received 20 μl PBS per ear. Twenty-four hours later, the ears were collected and the dorsal epidermis removed and stained for ATPase activity as previously described [12]. The number of LC in 20 fields per sample was counted.

2.5. Collection of serum and faecal samples

Blood was collected by cardiac puncture under anaesthetic and allowed to clot. The serum was separated by centrifugation at $2000\times g$ for 10 min and stored at −20°C until used in the enzyme-linked immunosorbent assay (ELISA). Faecal pellets were collected from each group of vaccinated mice on two separate occasions, on days

32 and 42. The samples were stored at −80°C until used. Throughout the remainder of the procedure, all reagents were kept on ice. A volume of 1 ml proteinase inhibitor solution (2 mg ml^{-1} trypsin inhibitor from soybean (Sigma, UK) and 0.1 M ethylenediamine–tetraacetic acid in PBS containing 0.1% Tween 20) was added to 0.5 g of faeces [13]. The mixture was vortexed for 30–60 s and centrifuged at $850\times g$ for 10 min. The supernate was removed and phenylmethanesulfonyl fluoride (PMSF) (0.1 M in 100% ethanol) (Sigma, UK) was added to a final concentration of 1%, followed by mixing and centrifugation at $10\,000\times g$ for 10 min. The supernate was again kept and PMSF was added to a final concentration of 2%, and sodium azide to a final concentration of 1%. The sample was mixed well and left to stand for 15 min on ice. Heat-inactivated foetal bovine serum was added to a final concentration of 4%. The samples were then centrifuged at $10\,000\times g$ for 5 min. The supernatant was removed and used in the ELISA.

2.6. ELISA

A HSV-specific antibody ELISA was used as previously described [14] with the following modifications. HSV antigen or uninfected Vero cells (negative control) antigen was used to coat Immunoplates (Nunc, UK) at 2 μg protein per well. After an overnight incubation at 4°C, the pooled sera from each vaccine group were diluted in PBS containing 0.1% Tween 20 (PBS/T) and 100 μl was added to duplicate wells. The plate was incubated at room temperature for 3 h, washed three times with PBS/T and incubated for a further 1 h with anti-mouse immunoglobulins (a single preparation containing antibodies against IgA, IgG and IgM) conjugated to alkaline phosphatase (Sigma, UK, Product A-0162) diluted 1/1000 in pH 9.8 diethanolamine buffer. The plate was then washed three times and *p*-nitrophenyl phosphate substrate (Sigma, UK) was added. The reaction was stopped after 20 min by adding 50 μl per well 3 M aqueous sodium hydroxide. The absorbance of the wells was then determined on a spectrophotometer at 405 nm. The absorbance obtained with the Vero cells-coated plates was subtracted from the absorbance of the HSV-coated plates to give the HSV-specific data presented in Section 3. Positive and a negative control mouse sera were prepared as previously described [14]. The same ELISA procedure was used to detect the presence of faecal antibodies to HSV (presumed IgA). For the measurement of CT antibody levels, 1 μg per well of CT was used to coat the immunoplates. The rest of the procedure was as described above.

2.7. DTH assay

Ten days after the final vaccination (day 45), five mice per group were anaesthetised with halothane gas, and the thickness of each ear was measured using a micrometer. Each ear was injected with 10 μl containing 3×10^5 PFU of UV-B-inactivated HSV. Ear swelling was then measured 24 h later. The mean ear swelling per mouse was calculated first, then the mean ear swelling for each group of mice was determined as previously described [15].

2.8. Lymphoproliferation assay

Individual cell suspensions were prepared by forcing the lymph nodes through a cell strainer, washing the cells twice and culturing in RPMI 1640 medium (Gibco, UK) containing 10% foetal bovine serum, 100 IU ml^{-1} penicillin, 200 μg ml^{-1} streptomycin and 2 mM L-glutamine (Sigma, UK). A concentration of 2×10^5 cells per well was incubated in round-bottomed 96 well plates in the presence of either 1 μg per well concanavalin A (ConA), 5×10^4 PFU per well of inactivated HSV-1 or medium alone [16]. Ten replicate wells were used for each sample. The plates were incubated at 37°C in the presence of 5% CO_2 for 2 days (ConA) or 4 days (HSV) before being radioactively pulsed for 24 h with 0.7 μCi [^{3}H]thymidine (Amersham, UK). Cells were harvested onto filter paper using an automatic cell harvester and the incorporated radioactivity measured in a liquid scintillation counter (Canberra Packard, Switzerland).

2.9. Challenge experiments

Ten days after the final vaccination (day 45), under halothane gas anaesthetic, the backs of eight mice per group were tape-stripped eight times and infected by applying 10 μl of 5×10^4 PFU live HSV-1 and gently massaging this liquid into the skin with a pipette tip. The size of the lesions that formed on the backs of the mice was then measured with a ruler in two dimensions, each day for the next 10 days. This procedure has previously been shown to mimic a natural epidermal HSV infection and result in the formation of moderate vesicular lesions on the infected skin [10].

2.10. Statistical analysis

The mean, range and the standard error of the mean are presented in the figures as appropriate. The Student's *t*-test was used to calculate the statistical significance of differences between the data sets. A '*P*' value of less than 0.05 was considered statistically significant.

3. Results

3.1. LC count

LC represent the major antigen presenting cells of the epidermis and therefore, it was of interest to determine whether vaccination by the transcutaneous route resulted

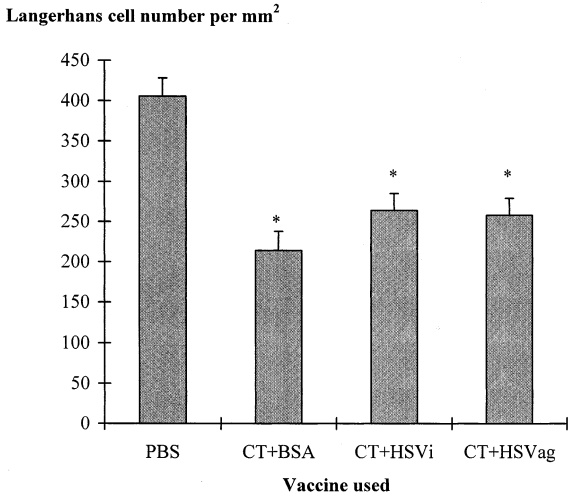

Fig. 1. The mean number of LC per mm^2 of ear epidermis 24 h following transcutaneous vaccination with PBS, CT+BSA, CT+HSVi or CT+HSVag. Twenty fields were counted from four ears for each sample. Bars represent the standard error of the mean and '*' indicates a $P < 0.05$ in comparison with the PBS control using the Student's *t*-test.

in their migration from the skin. Epidermal sheets were collected from the dorsal side of the ears of mice treated 24 h previously with either PBS, CT+BSA, CT+HSVi or CT+HSVag. The epidermal sheets were stained for ATPase which specifically indicates LC. The mean number of LC was around 400 LC mm^{-2} in control mice. This was reduced to around 250 LC mm^{-2} in all the vaccine groups, a statistically significant reduction (Fig. 1). It is likely therefore that the LC had taken up the adjuvant and the vaccine components, and migrated to draining lymph nodes to initiate the immune response.

3.2. Serum and faecal antibody levels

Sera collected 10 days after the final vaccination were tested by ELISA for antibody to HSV. The CT+HSVi vaccine group generated the highest level of serum anti-HSV antibodies (Fig. 2a). The CT+HSVag vaccine group produced a small amount of antibody to HSV while the CT+BSA control vaccine group produced none. A hyper-immune positive control mouse serum was also tested at a dilution of 1/100 and this gave an absorbance of 1.105 U while a negative control serum gave an absorbance of 0.007.

All three vaccines resulted in equal and high serum antibody levels against CT, indicating that the CT was antigenic and that no adverse interaction between the vaccine components took place (absorbance around 0.650 in all cases at a dilution of 1/40).

Faecal samples were collected from the mice on day 32, a few days before the third vaccination. Mucosal antibody to HSV was detected in the CT+HSVi and CT+HSVag vaccine groups (Fig. 2b). A small amount of background colour was observed in the CT+BSA group, at 1/5 dilution, which was probably due to the complex contents of the faecal extract. Mucosal antibody and serum antibody to HSV followed the same pattern in that the CT+HSVi group of mice produced higher levels of both types of antibody than the CT+HSVag vaccine group. The same pattern of results was obtained with faecal samples taken on day 42, 1 week after the third vaccination (data not shown).

3.3. DTH assay

A DTH response is a measure of T helper 1 (T_H1) cell-mediated immunity [17], and the extent of ear swelling is proportional to the strength of the immune response. Ten days after the third vaccination, a DTH assay to HSV-1 was performed. The mice which were vaccinated with CT+BSA produced an ear swelling response of around 3 U (Fig. 3). This is considered a negative response, due to

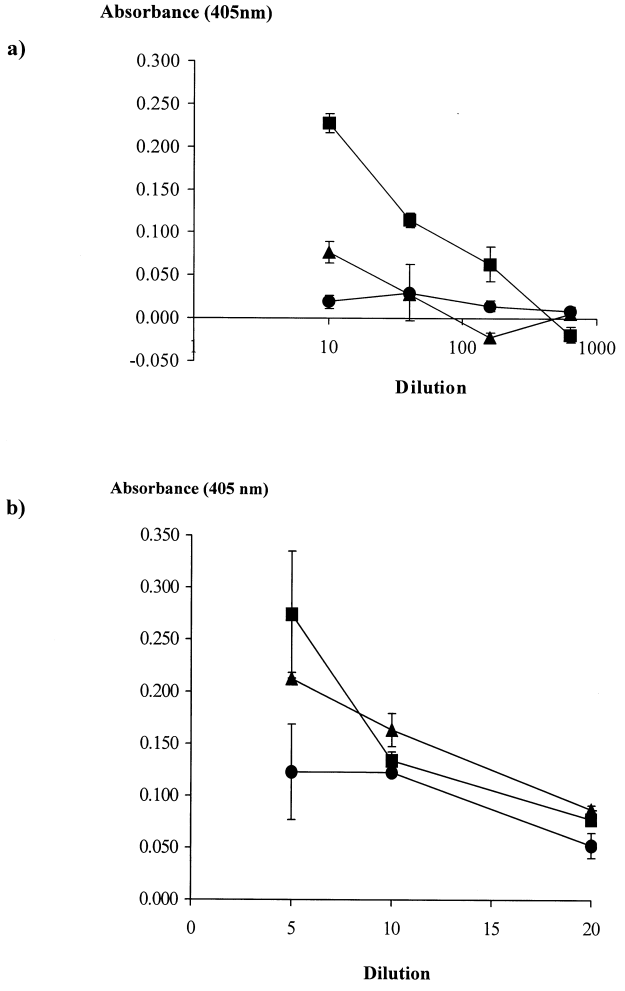

Fig. 2. The ELISA results for serum and faecal antibodies to HSV. Three mice per group were immunised with each vaccine on days 0, 14 and 35. Faecal pellets were collected on day 32 and serum was collected on day 45. The specific mean absorbance of duplicate wells, as a measure of serum antibodies to HSV, is represented in (a) and of faecal antibodies in (b). Solid squares represent the mice vaccinated with CT+HSVi, solid triangles CT+HSVag and solid circles CT+BSA. The error bars represent the range.

the intrapinnal injection procedure itself. The CT+HSVi group produced a response of 7 U, indicating that a DTH cell-mediated immune response to HSV was generated by this vaccination protocol, which used whole inactivated virions. An even stronger cell-mediated immune response was generated by the CT+HSVag vaccine, which utilised a glycine extract of HSV proteins (Fig. 3). Both HSV vaccines induced a statistically significant DTH response to HSV, demonstrating that a T_H1 response was generated by both HSV vaccines. The same result was obtained when this experiment was repeated on a separate occasion.

3.4. Lymphoproliferative response

Seventeen days after the third vaccination, and 7 days post-HSV challenge, lymph node cells from vaccinated animals were cultured in vitro in the presence of ConA or HSV. All cells proliferated to give a response of around 90 000 cpm with ConA, indicating that healthy populations of lymphocytes were isolated in all cases (Fig. 4a). A proliferative response to HSV of just over 1000 cpm was detected only in the CT+HSVag group (Fig. 4b). The other groups showed no proliferation above the background. This HSV-specific proliferative response was low but was of a similar magnitude to that previously obtained from mice, 14 days after a secondary epidermal infection with HSV-1 [16].

3.5. Epidermal challenge with live HSV

Ten days after the third vaccination, the three immunised groups were infected on the epidermis of the back with live HSV, and the subsequent development of herpetic lesions was recorded. Lesions started to appear by 2 days post-infection and resolved over the next 8 days (Fig. 5). Lesion size was consistently and significantly larger on CT+BSA-immunised mice compared to lesions on the backs of mice immunised with CT+HSVi or CT+HSVag. Lesion size was smallest on CT+HSVag-immunised mice, indicating that this vaccine provided superior protective immunity from an epidermal challenge in comparison with the CT+HSVi vaccine. This result was confirmed when this experiment was repeated (data not shown).

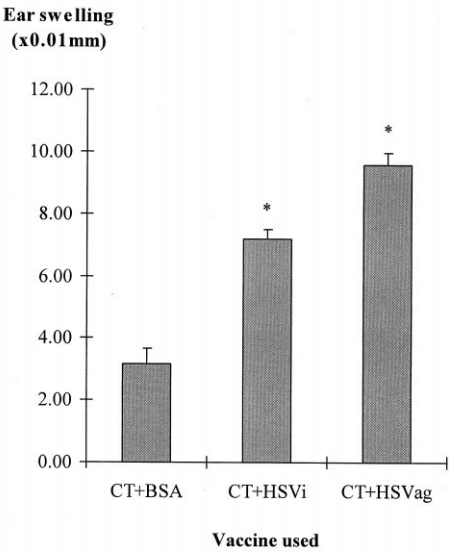

Fig. 3. The DTH response of vaccinated mice to HSV. Five mice per group were immunised with CT+BSA, CT+HSVi or CT+HSVag on days 0, 14 and 35. All mice were injected with 3×10^5 PFU of inactivated HSV-1 per ear on day 45. The mean ear swelling response was measured on day 46. Bars represent the standard error of the mean and '*' indicates a $P<0.05$ in comparison with the CT+BSA control.

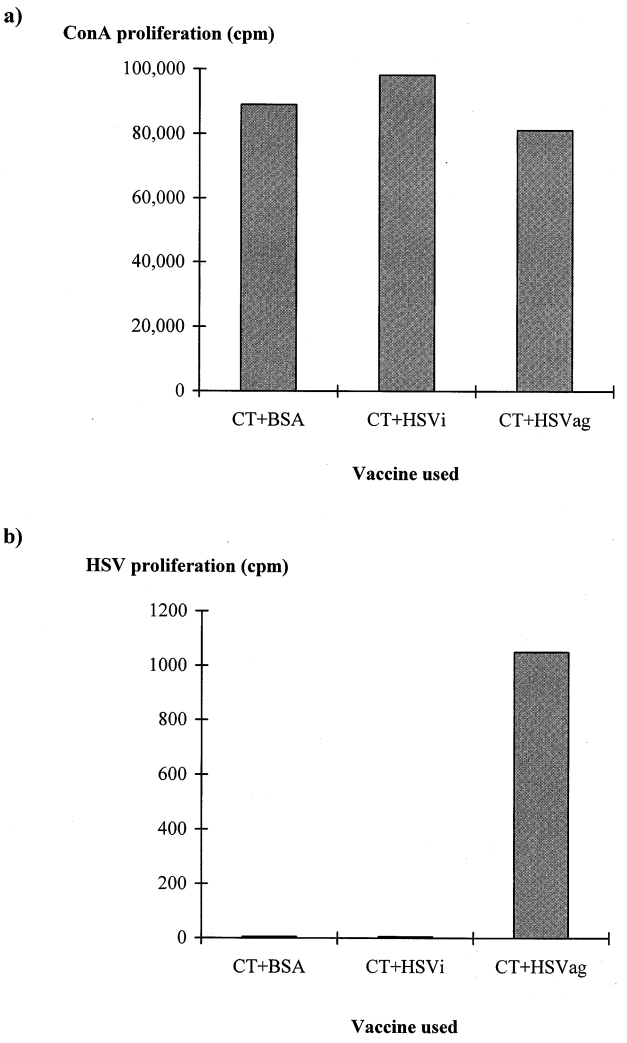

Fig. 4. The specific proliferative response of lymph node cells to 1 μg per well ConA (a) or 5×10^4 PFU per well HSV (b). Three mice per group were immunised with CT+BSA, CT+HSVi or CT+HSVag on days 0, 14 and 35. All mice were each infected epidermally with 5×10^4 PFU of HSV-1 on day 45. On day 52, the mice were killed and the inguinal lymph nodes removed. The mean specific response of 10 replicate wells is shown after subtracting the appropriate background proliferation of pulsed cells in the presence of medium alone.

4. Discussion

The ability of HSV to cause primary infection, followed

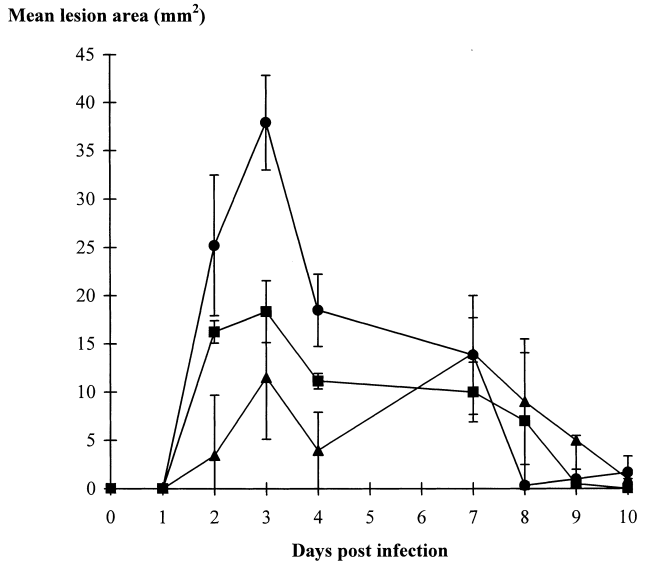

Fig. 5. The size of herpetic HSV-1 lesions on the backs of vaccinated mice. Seven mice per group were immunised with CT+BSA (circles), CT+HSVi (squares) or CT+HSVag (triangles) on days 0, 14 and 35. All mice were then infected epidermally with 5×10^4 PFU of HSV-1 per mouse on day 45. The mean lesion area per mouse and the standard error of the mean are shown. The lesion size was significantly lower in the mice vaccinated with CT+HSVag on days 2, 3 and 4 post-infection ($P<0.05$) when compared with the CT+BSA group. The CT+HSVi group also presented a less severe infection in comparison with the control CT+BSA group but this was statistically significant only on day 3 post-infection.

by latency and recurrent infections throughout an individual's lifetime, makes this virus an important target for vaccine development, particularly for immunocompromised subjects. Whilst intense research has revealed potential experimental vaccines, there is no effective HSV vaccine currently available. Past vaccines against HSV have all been administered systemically, and it is possible that they failed to prevent infection because of inadequate mucosal immunity [18] which is difficult to elicit [19].

In the study presented here, mice were vaccinated on three occasions with either CT+BSA, CT+HSVi or CT+HSVag in order to investigate the potential benefit of transcutaneous immunisation in raising mucosal and serum antibody responses as well as cell-mediated immune responses and protection against viral challenge. Initial experiments showed that the application of all three vaccines on mouse skin resulted in the migration of LC from the epidermis. This is an essential first step in the priming of the immune response where transport of antigens to draining lymph nodes occurs, followed by processing and presentation to T-lymphocytes in the paracortical area. Antigen presentation was indicated by the detection of serum antibody to CT in all cases and serum antibody to HSV-1 in mice vaccinated with CT+HSVi. The latter vaccine also induced the secretion of mucosal antibody in faeces. It is likely that the faecal antibody comprises mainly IgA. The CT+HSVag mice produced a lower amount of serum and mucosal antibody to HSV-1. The CT+HSVi vaccine was therefore considered more potent than the CT+HSVag vaccine at generating a humoral immune response. Whilst stimulation of an antibody response to an antigen may confer some protective immunity in many cases, studies have revealed that antibody plays little role in protection against HSV infection [20]. It has been noted that some human infants born to HSV-infected mothers suffering recurrent disease were protected from infection during birth by their maternal antibody, although not in all cases [21]. One study showed that, in spite of robust vaginal antibody responses at the time of challenge, these were often insufficient to preclude viral invasion even by a modest dose of virus [18]. These results appear to indicate that antibody, at least against gB or gD, the currently favoured candidates for subunit vaccines, fails to prevent virus infection. Our data agree with these findings although it should be noted that antibody plays a role in preventing the spread of the virus to the nervous system and occasionally in protecting against lethal infections [22–25].

When cell-mediated immunity was investigated, the efficacy of the two HSV vaccines was reversed. For example, the CT+HSVag vaccine induced a stronger DTH response to HSV-1 than CT+HSVi. It is known that $CD4^+$ T_H1 cells are responsible for generating DTH responses [17], therefore the CT+HSVag-immunised mice showed a more vigorous T_H1 response and a stronger cell-mediated immunity than the CT+HSVi group. Further confirmation of this was obtained with the lymphoproliferation assay which verified the presence of lymphocytes capable of recognising HSV-1 in vitro, though this was only detected in the CT+HSVag group and was a relatively weak response. Our previous experiments indicate that this level of response is equivalent to the proliferation obtained from mice which were immunised with 3×10^6 PFU per mouse of inactivated HSV-1, 6 days prior to epidermal challenge and the lymph node cells collected 14 days after this secondary challenge [16].

To test the efficacy of the vaccines at protecting against virus infection, the mice were challenged with live HSV-1. An epidermal challenge was chosen in order to mimic the natural route of infection and this leads to the development of lesions on the back which are similar to herpetic lesions in human subjects [10]. Both HSV vaccines offered significant protection over the negative control vaccine. The CT+HSVag vaccine induced a higher level of protection than the CT+HSVi vaccine against a primary HSV-1 infection. This corresponds with the pattern observed for the generation of cell-mediated immunity but not humoral immunity and indicates that cell-mediated responses are primarily responsible for resistance against re-infection. Manickan et al. [26] have shown that immunisation with dendritic cells transfected with HSV DNA leads to significant enhancement in protective immunity which has been attributed to enhanced HSV-specific $CD4^+$ T_H1 responses. It is not known however, if either of the transcutaneous

vaccines will prevent the emergence of HSV from latency. The difficulty of determining this lies in the lack of a suitable model system in mice.

It is interesting to note that the composition of the vaccine directed the outcome of the immune response. The CT+HSVi vaccine, which contained whole inactivated virions, tended to generate a stronger humoral response while the glycine-extracted HSV vaccine generated a stronger cell-mediated immune response and offered better protection from live virus challenge. It is likely that whole inactivated virions are taken up and processed by the LC via a different mechanism from the extracted protein antigens. The results shown here suggest that extracted antigens mimic the virus infection more closely than whole inactivated virus.

Whilst the role of CT as a powerful adjuvant was confirmed, the mechanism of its effect on the immune system is still not fully understood [27]. The reduction in LC numbers in the epidermis following transcutaneous vaccination indicates the involvement of these potent antigen presenting cells in initiating the systemic immune response, even in the mucosa. The mechanism of immune stimulation merits further investigation.

The CT+HSVag vaccine offers some potential for the production of a novel HSV vaccine which will not require the use of needles or other complex/expensive delivery systems. This would decrease the risk of needle-born diseases, eliminate the need for trained personnel and sterile equipment and improve vaccine compliance in the developing world. The prospect of using a patch, applied to the skin, to deliver a vaccine deserves further scrutiny, particularly as cell-mediated, mucosal and protective immunity can be stimulated by this technique.

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