Antigen-presenting Capacity of Epidermal Cells Infected with Vaccinia Virus Recombinants Containing the Herpes Simplex Virus Glycoprotein D, and Protective Immunity

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SUMMARY

We studied the association of herpes simplex type 1 (HSV-1) glycoprotein D (gD-1) expression in epidermal cells (EC) with virus-specific immunity and protection of mice from fatal HSV-2 challenge. Vaccinia virus recombinants containing gD-1 under the control of an early (VP176) or late (VP254) vaccinia virus promoter were used. Mature gD-1 protein was expressed in VP176-infected EC and they had accessory cell function for HSV-2-induced T cell proliferation of immune lymph node cells (LNC). It was not expressed in VP254-infected EC and they did not act as accessory cells. LNC from VP176- but not VP254-immunized mice proliferated in response to HSV antigen and only VP176-immunized mice had complete long-term protection from HSV-2 challenge.

The significant rise in the incidence of herpes simplex virus type 2 (HSV-2) infections has evoked public desire for vaccine development (Watson & Enquist, 1985). However, progress has been impeded by our relatively poor understanding of the role of virus-specific immunity in protection against infection. Recent studies have shown that a vaccinia virus recombinant (VgD52) in which HSV-1 glycoprotein D (gD-1) production is driven by a late vaccinia virus promoter induces virus-specific neutralizing antibody and protects mice from fatal HSV-1 challenge (Cremer et al., 1985). However, it fails to induce HSV-specific cytotoxic T cells and does not protect from cutaneous disease due to high dose HSV-1 challenge (Martin & Rouse, 1987). On the other hand, we found that a recombinant (VP176) in which gD-1 is driven by an early vaccinia virus promoter protects guinea-pigs from cutaneous disease due to high dose HSV-2 challenge, a protection associated with the induction of HSV-specific T cell responses (Wachsman et al., 1987).

To address this apparent contradiction we sought to determine the role of temporal regulation of gD-1 expression in the induction of protective immunity in the same animal model of HSV infection. We found that VP254 and VP176, two recombinants in which gD-1 is driven by a late (11K) or early (Pi) vaccinia virus promoter, respectively, induce equal titres of HSV-specific neutralizing antibodies and protect mice from HSV-2 challenge at 14 days post-immunization (p.i.) (Wachsman et al., 1988). However, the effect that temporal regulation of gD-1 expression may have on the induction of long-term protective immunity, and the potential involvement of immunologically active elements in the epidermis (Stingl et al., 1980) remained unclear.

Standard molecular cloning techniques were used to construct the recombinants used in these studies. VP176 contains a 1-4 kb HindIII/NruI fragment of HSV-1 (KOS) DNA containing the
gD-1-coding sequences flanked by 70 bp untranslated HSV DNA at the 5′ end of the gene and 160 bp at the 3′ end of the gene (Watson et al., 1982). In VP176 the gD-1 gene is under the control of a 0-4 kb transposed fragment containing an early vaccinia virus promoter (Pi) derived from a Sau3AI subclone of AvaI fragment H. The Pi promoter was mapped by analysis of early vaccinia virus transcription (A. Piccini, unpublished data) and corresponds to the region upstream from an open reading frame coding for a 5K glycine-rich protein (Kotwal & Moss, 1988). It was selected because it represents an abundant early message (A. Piccini, unpublished). The VTK-79 mutant of the vaccinia virus S variant (Panicali et al., 1981) was used as the rescuing virus. To construct VP254, the same gD-1 gene was placed under the control of the 11K late vaccinia virus promoter (Bertholet et al., 1985). VP254 contains the transposed 11K promoter (0-1 kb, ATG removed) linked to the 1-4 kb HindIII/NruI gD-1-containing fragment using the S variant as the rescuing virus. In both cases insertion was at the thymidine kinase locus of vaccinia virus (Hruby & Ball, 1982). No rearrangements of the inserted sequences were detected.

VP176, VP254 and VTK-79 had similar growth characteristics (kinetics and maximal virus titres) in HEp-2 and Vero cells (50 p.f.u./cell at 72 h.p.i.) as well as in intradermally (i.d.) inoculated mice (1 × 10^3 to 2 × 10^3, 1 × 10^3 and 3 × 10^3 to 5 × 10^3 p.f.u./ml on days 1, 3 and 5 p.i. respectively). Essentially identical results were previously reported for vaccinia virus in i.d. infected guinea-pigs (Friedman et al., 1962). However, gD-1 expression as determined by Western blot assays with antiserum to total HSV-2 proteins (anti-HSV-2) (Wachsmann et al., 1987, 1988) or by immunoprecipitation with gD-specific monoclonal antibody (MAb) 18βB3 (Balachandran et al., 1982), depended on the vaccinia virus promoter located 5′ to the inserted gD-1 gene. As shown in Fig. 1, two bands with M_r values of 50K and 57K, consistent with those of the pgD and gD forms of native gD-1 (Eisenberg et al., 1982) were recognized by anti-HSV-2 serum in Western blots of VP176-infected HEp-2 cells (10 p.f.u./cell) at 2 to 48 h.p.i. (Fig. 1a, lanes 1, 3 and 7). Their synthesis did not depend on viral DNA replication (Fig. 1a, lane 5). In cells infected with VP254 (10 p.f.u./cell), pgD was first seen at 10 to 14 h.p.i. (Fig. 1a, lane 2), consistent with previous reports for a chimeric construct in which the chloramphenicol acetyltransferase gene is driven by a vaccinia virus late promoter (Weir & Moss, 1984). gD was first detected at 24 h.p.i. (Fig. 1a, lane 4). Expression required viral DNA synthesis (Fig. 1a, lane 6).

Similar results were obtained in immunoprecipitation with MAb 18βB3. Both pgD and gD were observed in cells labelled with [35S]methionine at 0 to 12 h.p.i. with VP176 (Fig. 1d, lane 1) while in VP254-infected cells, gD expression was first seen at 12 to 24 h.p.i. (Fig. 1d, lane 7). However, quantitative estimation by densitometric scanning (with a Gilford Response UV-Vis Spectrophotometer) indicated that the levels of gD-1 expressed by the two recombinants at 24 h.p.i. were virtually identical (peak areas of integration were 1.14 ± 0.29 and 1.83 ± 0.92 for VP176 and VP254 respectively). Proteins were not identified in VTK-79-infected cells reacted with the anti-HSV-2 serum (Fig. 1d, lanes 4 and 9) and there was no difference in the expression of vaccinia virus proteins in cells infected with VP176, VP254 or VTK-79 (b). Identical results were obtained in Vero cells (data not shown).

Processing of pgD was studied in HEp-2 cells labelled with [35S]methionine (100 μCi/ml in MEM without methionine) at 12 h.p.i. with VP176 and at 16 and 24 h.p.i. with VP254. Cells were harvested immediately (pulse) or replenished with MEM containing 400 μg/ml cycloheximide (to inhibit protein synthesis) and incubated for an additional 0-5 to 10 h (chase). Cytoplasmic extracts from the pulse and chase samples were immunoprecipitated with MAb 18βB3 and analysed by SDS–PAGE (Fig. 1c). The 30 min pulse with [35S]methionine indicated that pgD was synthesized equally well in VP176- and VP254-infected cells (lane 7 and lanes 1, 4, respectively). However, the processing steps involved in the conversion of pgD to gD (Cohen et al., 1980) occurred within 30 min (lane 8) after the beginning of the chase period in VP176-infected cells, whereas they required 4 to 8 h in cells infected with VP254 for 16 h (lanes 2 and 3). The apparent defect in pgD processing in VP254-infected cells presumably reflects decreased levels of glycosylating enzymes that result from early vaccinia virus-mediated host shutoff (Bablanian, 1984). The total failure to observe pgD processing in cells infected with VP254 for 24 h (lanes 5 and 6) is consistent with this interpretation. However, the levels of gD-1 detected following a long pulse (12 to 24 h) were similar for both recombinants.
We considered the potential involvement of immunologically active elements in the epidermis (Stingl et al., 1980) in the induction of HSV-specific immunity following immunization with vaccinia virus recombinants, since Ia+ epidermal cells (EC) act as antigen-presenting cells (APC) in HSV-2-induced T cell proliferation and impairment of this function is associated with immune suppression and increased disease severity (Hayashi & Aurelian, 1986; Yasumoto et al., 1986, 1987; Aurelian et al., 1988). EC, prepared as described (Hayashi & Aurelian, 1986) from non-immune mouse ear skin (2 to 10% Ia+), were cultured (12 h; 37 °C) under conditions that favour cell attachment [polylysine-treated polystyrene microtitre plates (Falcon 3072); heat-inactivated (56 °C, 30 min) foetal calf serum], infected with VTK−79, VP176 or VP254 and assayed for gD-1 expression by Western blot assays. To address the possibility that our previous failure to detect gD-1 expression in VP254-infected EC (Wachsman et al., 1988) reflects suboptimal infection, EC were infected at a higher multiplicity of infection (25 rather than 10) and extracts were electroblotted on nitrocellulose (rather than GeneScreen) transfer membranes.

Both pgD and gD (50K and 57K respectively) were observed in EC infected with 10 or 25 p.f.u./cell of VP176 (Fig. 1 e, lane 3), but only pgD was detectable in VP254-infected EC and only at an m.o.i. of 25 (Fig. 1 e, lane 4). Extracts of VTK−79-infected EC were negative (Fig. 1 e, lanes 1, 2). We cannot exclude the possibility that the failure to detect gD-1 in VP254-infected EC is due to a technical problem. However, this seems rather unlikely since gD-1 was also not observed in VP254-infected macrophages (data not shown) but it was detected in EC infected with 10 p.f.u./cell of VP176 and assayed in parallel. Although the EC subpopulation that supports pgD expression is unknown, the failure to detect gD-1 in VP254-infected EC is consistent with the previous finding that late vaccinia virus genes are not expressed in infected macrophages (Natuk & Holowczak, 1985) and our observation that their expression is inefficient in VP254-infected cells.

The role that the temporal regulation of gD-1 expression may play in the induction of HSV-specific protective immunity was studied in female BALB/c (H-2d) mice (6 to 8 weeks old; Jackson Laboratory) immunized with VP176, VP254 or VTK−79 (1 x 10⁶ p.f.u.) by footpad inoculation. Animals were challenged with HSV-2 (5 x 10⁶ p.f.u.) by i.d. inoculation in the ipsilateral footpad at 50 days post-inoculation and followed for 40 days in a blind trial for the development of severe cutaneous (zosteriform) lesions, neurological symptoms (paralysis) and death. The development of HSV-specific immunity was established by lymphoproliferative assays using lymph node (LNC) and spleen (SC) cells obtained after primary (1°) or secondary (2°) immunization. Briefly, cells (4 x 10⁵/well) were cultured (4 days; 37 °C) under previously established optimal conditions [antigen dose, time of in vitro culture, duration of tritiated thymidine (3H[TdR]) pulse] (Hayashi & Aurelian, 1986; Yasumoto et al., 1987; Aurelian et al., 1988) with 20 μg protein/ml of u.v.-inactivated viral or 'mock' antigen (prepared in Vero cells) in flat-bottomed 96-well microtitre plates in RPMI 1640 medium with 10%, heat-inactivated horse serum (Gibco), 25 mM-HEPES, 5 x 10⁻⁵ M-2-mercaptoethanol. They were pulse-labelled (6 h) with 1 μCi/well of 3H[TdR] (New England Nuclear) and the results are expressed as (experimental mean c.p.m.) – (control mean c.p.m.) (Hayashi & Aurelian, 1986; Yasumoto et al., 1987; Aurelian et al., 1988).

HSV-specific proliferation was observed in LNC from VP176-immunized animals after 1° and 2° immunization. SC were studied only after 1° VP176 immunization and they proliferated in the presence of u.v.-inactivated HSV-2 and VTK−79 antigen. However, neither LNC nor SC from mice immunized with VP254 responded to u.v.-inactivated HSV-2 antigen. Vaccinia virus-specific lymphoproliferative responses were similar in VP176-, VP254- or VTK−79-immunized mice (Table 1).

Failure to detect HSV-specific lymphoproliferative responses in VP254-immunized mice correlates with the observation that 10 of 12 (83%) animals developed zosteriform skin lesions and paralysis following HSV-2 challenge. This proportion of symptomatic animals is similar to that (14 of 15; 93%) seen following HSV-2 challenge of VTK−79-immunized mice. All the VTK−79-immunized mice died within 20 days post-challenge but seven of 12 (58%) of those immunized with VP254 survived. Significantly, all 11 mice immunized with VP176 remained completely free of clinical symptoms following HSV-2 challenge.
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Fig. 1. (a) Western blot with anti-HSV-2 serum. HEp-2 cells infected with VP176 at 6 h p.i. (lane 1); VP254 14 h p.i. (lane 2); VP176 24 h p.i. (lane 3); VP254 24 h p.i. (lane 4); VP176 24 h p.i. with cytosine β-D-arabinofuranoside (Ara-C) (40 μg/ml) (lane 5); VP254, 24 h p.i. with Ara-C (40 μg/ml) (lane 6); VP176 48 h p.i. (lane 7); VP254 48 h p.i. (lane 8). (b) Western blot with anti-vaccinia virus serum.
We interpret these data as confirming the conclusion of Coupar et al. (1986) that recombinants expressing a foreign gene under the control of an early vaccinia virus promoter elicit a better T cell response. Thus, the two recombinants were constructed from the S variant of vaccinia virus (VP254) and its TK- mutant (VP176). The TK gene, which may be involved in vaccinia virus virulence (Buller & Moss, 1985), is absent in VP176 and is non-functional in VP254, where it is interrupted by the chimeric promoter-gD-I coding sequence. Indeed, VP176 and VP254 grew equally well in cultured cells and in i.d. infected mice. They induced virtually identical titres of HSV-specific neutralizing antibody (Wachsman et al., 1988). However, only VP176 induced long-term protective immunity and it correlated with HSV-specific proliferative responses. Animals immunized with VP254 were not equally protected and their LNC and SC failed to proliferate in the presence of u.v.-inactivated HSV-2 antigen. This was not due to improper experimental conditions because (i) LNC from VP176-, VP254- and VTK-79-immunized animals responded equally well to u.v.-inactivated VTK-79 antigen, (ii) proliferation was under previously established optimal conditions including antigen dose, time of in vitro culture and duration of [3H]TdR pulse (Hayashi & Aurelian, 1986; Yasumoto et al., 1987; Aurelian et al., 1988) and (iii) LNC obtained on days 20 to 270 p.i. with VP254 were still negative for HSV-specific proliferation (unpublished data). Indeed, we have previously shown that HSV-specific delayed-type hypersensitivity responses are significantly lower in animals immunized with VP254 than in those immunized with VP176 (Wachsman et al., 1988) and HSV-specific cytotoxic T cells were not observed in animals immunized with VgD52, another recombinant in which gD-1 is driven by a late vaccinia virus promoter (Martin & Rouse, 1987).

Two series of experiments were done in order to address the possibility that the failure to detect HSV-specific lymphoproliferation in VP254-immunized mice is due to impaired

### Table 1. [3H]TdR incorporation in cultures of lymphoid cells from VP176-immunized mice

<table>
<thead>
<tr>
<th>Immunizing virus</th>
<th>Cells</th>
<th>Antigen expression (c.p.m.)</th>
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<tbody>
<tr>
<td>VP176</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1°</td>
<td>LNC</td>
<td>37269 ± 8714</td>
</tr>
<tr>
<td>2°</td>
<td>LNC</td>
<td>19996 ± 616</td>
</tr>
<tr>
<td>1°</td>
<td>SC</td>
<td>8148 ± 2036</td>
</tr>
<tr>
<td>VP254</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1°</td>
<td>LNC</td>
<td>13138 ± 4167</td>
</tr>
<tr>
<td>2°</td>
<td>LNC</td>
<td>6679 ± 3040</td>
</tr>
<tr>
<td>1°</td>
<td>SC</td>
<td>2179 ± 1547</td>
</tr>
<tr>
<td>2°</td>
<td>SC</td>
<td>5094 ± 311</td>
</tr>
</tbody>
</table>

* Groups of three BALB/c mice were given a 1° and a 2° immunization with VP176 or VP254 (1 x 10⁶ p.f.u.) by footpad inoculation. The 2° immunization was 14 days after the 1°.

† LNC and SC were obtained at 10 days after 1° and 6 days after 2° immunization. They were cultured with 20 μg of antigen per ml (prepared in Vero cells) and assayed for [3H]TdR incorporation on the fourth day of culture. Data are presented as mean ± S.E.M.

‡ ND, Not done.
Table 2. Accessory cell function of VP176- and VP254-infected EC for HSV-2-induced T cell proliferation of HSV-2 immune LNC

<table>
<thead>
<tr>
<th>U.v.-HSV-2 antigen</th>
<th>[3H]TdR incorporation (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia⁺ immune LNC*</td>
<td>+</td>
</tr>
<tr>
<td>Ia⁻ immune LNC</td>
<td>+</td>
</tr>
<tr>
<td>Ia⁺ immune LNC + EC</td>
<td>+</td>
</tr>
<tr>
<td>Ia⁻ immune LNC + uninfected EC†</td>
<td>-</td>
</tr>
<tr>
<td>Ia⁻ immune LNC + VTK⁻ 79/EC</td>
<td>-</td>
</tr>
<tr>
<td>Ia⁻ immune LNC + VP254/EC</td>
<td>-</td>
</tr>
<tr>
<td>Ia⁻ immune LNC + VP176/EC</td>
<td>-</td>
</tr>
</tbody>
</table>

* Immune LNC non-depleted (Ia⁺ immune LNC) or depleted (Ia⁻ immune LNC) of Ia⁺ cells by complement-mediated lysis with MAb anti-I-A<sup>d</sup> (hybridoma MK-D6, Becton Dickinson) were cultured (4 x 10⁵ cells/well) alone or with non-immune EC (5 x 10⁴ cells/well) in the presence of HSV-2 antigen (20 µg protein/ml). They were assayed for [3H]TdR incorporation on the fourth day in culture.

† Ia⁻ immune LNC (4 x 10⁶ cells/well) were cocultured with non-immune EC (5 x 10⁴/well) uninfected or infected (2 h; 37°C) with VP176 (10 p.f.u./cell) or with VTK⁻ 79, or VP254 (25 p.f.u./cell) and assayed for [3H]TdR incorporation on the fourth day in culture.

‡ Significantly different (P < 0.05) by one-way analysis of variance.

The accessory cell function of VP254-infected EC. In the first series, EC were infected with 0.02 to 20 p.f.u./cell of VTK⁻ 79 and cocultured (5 x 10⁴ cells/cell) with LNC (4 x 10⁵ cells/well) obtained from BALB/c mice immunized with VTK⁻ 79 (1 x 10⁶ p.f.u.) and depleted of Ia⁺ cells by passage over a Sephadex G-10 column followed by complement-mediated lysis with MAb anti-I-A<sup>d</sup> as described (Hayashi & Aurelian, 1986). [3H]TdR incorporation was assayed on day 4 in culture. Maximal incorporation was observed in cultures containing EC infected with 20 p.f.u./cell (c.p.m. ± s.e.m. = 10 460 ± 927). It was still evident when EC were infected with 0.2 or 0.02 p.f.u./cell of VTK⁻ 79 (c.p.m. ± s.e.m. = 4778 ± 867 and 3472 ± 375 respectively) but not when the EC were mock-infected (c.p.m. ± s.e.m. = 1109 ± 190).

The second series of experiments sought to determine whether VP176- or VP254-infected EC can restore the HSV-specific proliferation of HSV-2-immune LNC depleted of Ia⁺ adherent cells (Ia⁻ immune LNC). As described (Hayashi & Aurelian, 1986), proliferation was restored by culturing the Ia⁻ immune LNC with non-immune EC in the presence of 20 µg protein/ml of u.v.-inactivated HSV-2 antigen. It was also restored by VP176-infected EC (10 or 25 p.f.u./cell) but not by mock-infected EC, or EC infected with 10 or 25 p.f.u./cell of VP254 or VTK⁻ 79 (Table 2).

Extrapolation from in vitro to in vivo data should be viewed with caution. However, the finding that VP254-infected EC fail to present antigen to HSV-immune LNC correlates with the absence of HSV-specific lymphoproliferative responses in VP254-immunized mice and with the impaired pgD processing in VP254-infected EC. Indeed, EC infected with as little as 0.02 p.f.u./cell of VTK⁻ 79 had accessory cell function for VTK⁻ 79 immune LNC, as did EC infected with 10 and 25 p.f.u./cell of VP176 and VP254, respectively. However, only VP176-infected EC expressing the product form of gD-1 (57K) had accessory cell function for HSV-2-immune LNC, consistent with the interpretation, recently reviewed by Mills (1986), that APC infection and expression of viral glycoproteins on its surface are involved in viral antigen presentation to the T cells.

We do not know whether the failure to detect HSV-specific lymphoproliferation in VP254-immunized mice is due to impaired gD-1 expression and accessory cell function of VP254-infected EC. An alternative interpretation is that restricting major histocompatibility complex antigen must be synthesized simultaneously with the foreign antigen in order to achieve effective T cell recognition, and this does not occur in VP254-infected EC in which cellular protein synthesis is inhibited before pgD synthesis. However, consistent with the importance of HSV-specific T cell responses for long-term protection (Howes et al., 1979; Nash et al., 1987; Wachsman et al., 1987), all mice immunized with VP176 evidenced complete long-term protection from high titre HSV-2 challenge. On the other hand, as previously shown for VgD52
Thus, most (83%) VP254-immunized mice developed severe cutaneous and neurological symptoms when challenged with HSV-2 at 50 days p.i. However, unlike animals immunized with VTK-79 that succumbed to HSV-2 challenge, approximately half (58%) of those immunized with VP254 survived.

Final conclusions pertaining to the regulation of gD-1 expression in infected EC, and their role in the generation of class II-restricted responses and protective immunity must await the results of further studies. However, from the practical standpoint of vaccine development, our findings indicate that random construction of potential vaccines may not be an effective approach to the control of HSV-induced disease.

REFERENCES


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