Cell-mediated Immunity in Herpes Simplex Virus-infected Mice: Induction, Characterization and Antiviral Effects of Delayed Type Hypersensitivity

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SUMMARY

Delayed type hypersensitivity (DTH) was induced in mice sensitized with an intradermal inoculation of herpes simplex virus type 1 (HSV-1). The reaction was observed 4 to 5 days p.i. and could still be induced up to 18 months later. In contrast, the adoptive transfer of DTH using draining lymph node cells was only possible during the period 6 to 10 days p.i. The cells taken at these times also contained mediators of antiviral immunity, as determined by a marked reduction of virus titres in the ears of infected animals 1 to 3 days after transfer. Draining lymph node cells taken at later times contained mediators of virus immunity, but titres were not reduced until day 5 after the transfer. The cell type involved in both the DTH and antiviral activity was a T lymphocyte, although the particular T cell subsets involved have yet to be determined.

INTRODUCTION

It is generally recognized that impairment of cell-mediated immunity (CMI) results in an increased susceptibility to virus infections. This is particularly true for herpes simplex virus type 1 (HSV-1) which spreads directly from cell to cell thus avoiding neutralization by antibodies (Lodmell et al. 1973). HSV-1-infected cells are effectively destroyed by cytotoxic T cells before virus spread takes place (Pfizenmaier et al. 1977; Sethi & Brandis, 1977). In addition, other effector cells, e.g. macrophages, are recruited into areas of infection by lymphokine-releasing T cells, where they probably mediate antiviral activity (Blanden, 1971). This latter mechanism is a central feature of the delayed type hypersensitivity (DTH) reaction. DTH is a widely used assay of CMI which is thought to reflect pathological processes involved in infectious diseases most directly. DTH to HSV has been demonstrated in seropositive humans (Anderson & Kilbourne, 1961) and in guinea-pigs (Lausch et al. 1966; Rogers et al. 1972), but hitherto no study has been carried out using mice.

In order to understand more about the induction and control of DTH reactions to HSV-1, a mouse ear model was used in which the primary infection has been extensively characterized (Field et al. unpublished data). The same model has also been used to study HSV-1 latency and recurrences (Hill et al. 1975). In this paper the induction and characterization of DTH to HSV-1 is reported and in particular the antiviral effects of this reaction using an adoptive transfer system have been investigated.
METHODS

**Mice.** Female Balb/c mice were obtained at 4 to 5 weeks old from Bantin and Kingman Ltd. (Grimstone, Hull, England) and injected at 5 to 6 weeks. In some experiments Balb/c mice were obtained from the Department of Pathology Animal House, University of Cambridge. Mice used as recipients in the transfer studies were 6 to 7 weeks old.

**Virus strains.** Most of the work was done using the clone SC16 of HSV-1 (Field *et al.* 1979). For comparative purposes pseudorabies virus (PRV) strain NIA-2 (Field & Hill, 1974) and vaccinia virus (Lister Institute strain) were used. All stocks were propagated in BHK-21 cells except the vaccinia stock which had been grown in RK13 cells. Cells infected at low multiplicity were disrupted by ultrasound and stored at -70 °C until required.

**Inoculation of virus and infectivity assay.** HSV-1 was inoculated at a dose of 10⁵ p.f.u. in 20 μl of Eagle's Glasgow modified medium (EGM) into the pinna of the left ear of anaesthetized mice (Hill *et al.* 1975). To assay infective virus titre in the ear, the pinna was removed and homogenized in 1 ml of EGM supplemented with 10% calf serum and 10% tryptose phosphate broth (ETC). The suspensions were then diluted for independent assay in BHK-21 cells (Russell, 1962).

**Lymph node cell suspensions.** The cervical and auricular lymph nodes were removed aseptically and gently homogenized with forceps in 0.2 ml of heat-inactivated foetal calf serum (FCS). Large clumps of cells were removed by passage through a muslin gauze-covered container and the filtered cell suspension was washed twice in HEPES-buffered EGM containing 3% glutamine, 2 × 10⁵ units penicillin and 10⁵ units streptomycin (H-EGM) supplemented with 2 to 5% FCS.

**Adoptive transfers and measurement of ear swelling.** Lymph node cells (see above) obtained from HSV-1-infected donors were injected intravenously in 0.3 ml of H-EGM into normal syngeneic recipients. Approx. 60 min after the cell transfer 10⁴ p.f.u. HSV-1 was inoculated into the pinna of the left ear. Control mice received either no cells or cells from normal mice, followed by the virus. After 24 h and on successive days thereafter, the ear thickness was measured using a Mitotoyo engineers' micrometer. This has been shown to provide a useful indicator of cell-mediated infiltration at early times into the infected ear. In some experiments the pinna was removed to determine the virus titre.

**Separation of T and B lymphocytes.** The method described by Nash (1976) was used. Briefly, a 1/50 dilution of rabbit anti-mouse poly(Ig) sera (donated by Dr D. Catty, Department of Immunology, University of Birmingham, England) was added to *Staphylococcus* protein A-coated dishes. The dishes were washed and 8 × 10⁶ lymph node cells were added and left for 60 min at room temperature. Non-adherent cells were removed by swirling the dish and the adherent cells were recovered by displacement with jets of medium from a Pasteur pipette. The Ig-positive and Ig-negative (85 to 90% thy-1-positive cells) cell fractions were used in cell transfer experiments.

**Anti-thy-1.2 serum.** A monoclonal anti-thy-1.2 serum was a gift from Dr H. Waldmann, Department of Pathology, Cambridge. The antiserum was known to abolish T helper cell functions at dilutions of 1/1000. Cells (5 × 10⁷) were mixed with 0.5 ml of anti-thy-1.2 (1/1000 dilution) on ice for 30 min. The cells were washed and resuspended in 1 ml of 1 in 6 dilution of fresh guinea-pig serum (absorbed with mouse spleen cells) for 30 min at 37 °C.

RESULTS

**Induction of DTH in mice with HSV-1**

Balb/c mice inoculated in the pinna (intradermally) with HSV-1 undergo a local cell-mediated reaction with peak virus titres on day 5 to 6; the fall in virus titre is preceded by a large infiltration of mononuclear cells which is largely under T lymphocyte control (H. J.
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Table I. Dose of virus required to elicit a DTH response and the specificity of the reaction in pre-sensitized mice*

(a) Dose of virus required

<table>
<thead>
<tr>
<th>Challenge dose of HSV-1 (p.f.u./ear)</th>
<th>Increased ear thickness after 24 h (mm × 10^{-2})†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>10^{8}</td>
<td>7.5 ± 2 (8 ± 3)</td>
</tr>
<tr>
<td>10^{9}</td>
<td>11 ± 3.5 (12.5 ± 4)</td>
</tr>
<tr>
<td>10^{6} (heat killed)</td>
<td>22 ± 5 (22.5 ± 6)</td>
</tr>
</tbody>
</table>

(b) Specificity of response

<table>
<thead>
<tr>
<th>Challenge virus‡</th>
<th>Increased ear thickness after 24 h (mm × 10^{-2})†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>18.0 ± 4</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>2.0 ± 2</td>
</tr>
<tr>
<td>Pseudorabies</td>
<td>4.6 ± 1</td>
</tr>
<tr>
<td>BHK cells</td>
<td>4.3 ± 1</td>
</tr>
</tbody>
</table>

* Pre-sensitized mice were inoculated with 10^6 p.f.u. HSV-1 6 weeks previously.
† Mean ± s.D. of four to five mice/group. Figures in parentheses indicate the day 5 primary ear thickness response.
‡ All viruses were heat inactivated (HSV-1 and PRV at 56 °C for 40 min and vaccinia at 60 °C for 40 min) and 10^6 p.f.u. inoculated into the ear pinna. Vaccinia produced an increase of 20 units when tested for DTH activity in vaccinia pre-sensitized mice.

Fig. 1. Time course for the induction of the DTH response to HSV-1 (SC16). Groups of mice, infected with 10^8 p.f.u. HSV-1 (SC16) were challenged in the contralateral ear pinna with 10^4 p.f.u. heat-killed virus, and ear thickness measurements taken 24 h later. Each point represents the mean ± s.d. of increased ear thickness in groups of three to four mice.

Field et al. unpublished data). The infiltrating cells at day 5 to 6 are largely mononuclear cells characteristic of a DTH reaction. The dose of live virus required to elicit a DTH response is shown in Table 1(a). Note that the magnitude of the DTH reaction was equivalent to the magnitude of the 5 day reaction in the primary response for any given dose.

To measure the time course of the DTH response the contralateral ear was injected with 10^6 p.f.u. of heat-killed virus (equivalent in ear swelling responses to 10^4 p.f.u. "live" virus) at various times after infection and the ear response measured 24 h later. DTH was first observed 4 to 5 days after sensitization and could still be induced up to 9 months later (Fig. 1). This reaction was specific for HSV-1. Heat-killed pseudorabies virus, vaccinia virus or BHK cell sonicate did not elicit a significant response [Table 1(b)].
Fig. 2. Transfer of DTH with draining lymph node cells taken 8 days after infection with 10⁶ p.f.u. HSV-1 (SC16). Donor lymph node cells were transferred to normal syngeneic recipients simultaneously infected with 10⁴ p.f.u. HSV-1 (SC16) and ear thickness measured on successive days (■—■). Control mice received no cells (●—●). Each point represents the mean ± s.d. of four mice/group.

Table 2. Detection of DTH cells in the draining lymph node at various times after infection

<table>
<thead>
<tr>
<th>Time p.i. (days)†</th>
<th>Transfer (DLN cells)‡</th>
<th>Control (no cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>2x ± 4§</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>8</td>
<td>2x ± 3</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>9</td>
<td>25 ± 1</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>13</td>
<td>13.5 ± 2</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>28</td>
<td>14 ± 4</td>
<td>12.5 ± 6</td>
</tr>
<tr>
<td>56</td>
<td>13.6 ± 2</td>
<td>11.7 ± 2</td>
</tr>
</tbody>
</table>

* Mean ± s.d. of three to four mice/group.
† Donor mice received 10⁶ p.f.u. HSV-1 (SC16) into left ear.
‡ 2 × 10⁷ DLN cells transferred to each recipient.
§ Numbers in bold type indicate significant differences when compared to control values (*P < 0.001*).

Transfer of DTH response with draining lymph node cells (DLN)

Lymph nodes regional to the site of HSV-1 inoculation (10⁵ p.f.u.) were taken at various times after infection and washed cell suspensions transferred to normal syngeneic recipients which were inoculated with virus 60 min later. The ear swelling response was then measured on successive days. An example of a transfer of DTH with lymph node cells taken 8 days p.i. is shown in Fig. 2. Increased ear swelling was pronounced 48 to 72 h after transfer compared to control mice which received non-immune or no cells. DTH was transferable with DLN cells 6 to 9 days p.i. but was not transferred from day 13 onwards (Table 2).

The transfer of serum obtained from HSV-1-infected Balb/c mice (neutralization titre 1/40) did not produce any increased ear swelling above control values 48 h later.

Virus titres in the ears of recipients following cell transfer

The HSV-1 titres were measured in the ears of recipient mice following the transfer of DLN cells from 9 day or 28 day infected donor mice. Table 3 shows that day 9 DLN cells reduced the virus titres by 1·6 logs at day 3 and 2·6 logs on day 5 after transfer. Control
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Table 3. Titres of infectious HSV-1 in the ears of recipient mice receiving DLN cells from 9 day or 28 day infected donor mice

<table>
<thead>
<tr>
<th>Time after infection and cell transfer (days)</th>
<th>HSV-1 titres (log₁₀ p.f.u./ear)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 9†</td>
<td>Day 28‡</td>
</tr>
<tr>
<td>Control (no cells)</td>
<td>Transfer (no cells)</td>
</tr>
<tr>
<td>1</td>
<td>3·27 (3·00–3·36)</td>
</tr>
<tr>
<td>3</td>
<td>4·00 (2·43–2·60)</td>
</tr>
<tr>
<td>5</td>
<td>4·15 (2·30–2·38)</td>
</tr>
</tbody>
</table>

* Geometric mean and range of three to four mice/group.
† Ear swelling at 48 h: transfer = 18 units, control = 11 units.
‡ Ear swelling at 48 h: transfer = 15·2 units, control = 18 units.

Table 4. Effect of anti-thy 1.2 plus complement on the transfer of DTH and the HSV-1 titres in the ears of infected mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Increased ear thickness* (mm × 10⁻² at 48 h)</th>
<th>HSV-1 titres† (log₁₀ p.f.u./ear)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no cells)</td>
<td>13 ± 2</td>
<td>4·45 (4·15–4·56)</td>
</tr>
<tr>
<td>Untreated DLN (complement only)</td>
<td>25 ± 1</td>
<td>1·84 (1·78–1·90)</td>
</tr>
<tr>
<td>Anti-thy 1.2 + c'–treated DLN</td>
<td>13 ± 1·5</td>
<td>3·32 (3·00–3·69)</td>
</tr>
</tbody>
</table>

* Mean ± s.d. of three mice/group.
† Geometric mean and range of three ears/group. Ears taken 5 days after cell transfer and infection with 10⁴ p.f.u. HSV-1 (SC16).

mice normally show a sharp drop in virus titres on day 6 to 7. In comparison, day 28 DLN cells were not effective on day 3 but by day 5 of the transfer had reduced the titre by 2·5 logs. Interestingly, one day after transferring the day 28 DLN cells there was a small increase in the HSV-1 titres. This observation was reproducible with increases up to 1 log being observed.

Effect of anti-thy-1.2 treatment on the DTH response

Draining lymph node cells taken 9 days p.i. were treated with anti-thy 1.2 and complement (c'). This treatment produced 50 to 60 % cell death as measured by trypan blue uptake. Eight × 10⁶ thy 1.2-negative or 2 × 10⁷ cells treated with c' only, were transferred to normal syngeneic recipients and the ear thickness measured on successive days. In this experiment the virus titres on day 5 were also measured (Table 4). Anti-thy-1.2 treatment abolished the DTH reaction measured at 48 h and these cells reduced the virus titres by approx. 1 log on day 5, compared to a 2·6 log reduction in preparations treated with complement only.

DISCUSSION

Delayed type hypersensitivity (DTH) to HSV has previously been reported in the guineapig (Lausch et al. 1966; Rogers et al. 1972) and man (Anderson & Kilbourne, 1961). In this report DTH was readily induced in the ear pinna of Balb/c mice previously sensitized with a single intradermal inoculation of live HSV-1. This particular mouse model has several
advantages for studying DTH responses to virus infections, namely, that measurements of
the ear are easily and reproducibly carried out; the ear tissue is rapidly processed for
histological investigations and virus titres can readily be determined. In addition, the
mouse has been extensively studied immunologically and specific antisera are available to
make possible a detailed investigation of the lymphocyte subpopulations involved in the
DTH reaction.

In mice infected with a dose of $10^5$ p.f.u. of HSV-1 the DTH response was first detected
4 to 5 days p.i. and remained inducible for at least 2 years. The reaction was maximum
between 24 and 48 h after challenge. In contrast to the induction of DTH responses over
long periods, the ability to transfer DTH adoptively using draining lymph node cells was
only demonstrable between 6 to 10 days after primary infection. In this case the reaction
was maximum between 48 and 72 h after challenge. The failure to transfer DTH beyond
day 10 suggests that either DTH-producing cells are absent from the draining lymph node
at 'late' times or that such cells are under the control of specific suppressor lymphocytes.
The latter possibility can be tested since suppressor cells with B lymphocyte characteristics
have been observed that suppress the DTH response to HSV-1 (Nash & Gell, 1980).

Following the adoptive transfer of DTH the virus titre in the ears of recipients was
reduced from day 1 onwards. In contrast, DLN cells taken at later times (day 28) were
only effective in reducing virus titres 5 days after transfer. One difference between these two
groups is that during the period when DTH transfers are effective, cytotoxic T cells are
present in the DLN, but these cells are absent from the DLN from day 13 onwards (A. A.
Nash, R. Quartey-Papafio & P. Wildy, unpublished data). It is also evident from these
studies that T cells are responsible for the DTH response and antiviral effect. Other workers
have highlighted the importance of T cell function in protective immunity against HSV-1
infection (Oakes, 1975; Rager-Zisman & Allison, 1976). However, no attempt was made
in these reports to correlate antiviral or protective immunity with a particular T cell
subpopulation.

The nature of the T cell subpopulation involved in the antiviral effect has yet to be deter-
dined. There are at least three possibilities: first, T cells involved in the induction of DTH
($T_{DTH}$) recruit and arm macrophages - these cells are known to be important in the
regression of infectious foci (Blanden, 1971); second, cytotoxic T cells (Tc) contribute to
the antiviral effect in vivo, similar to the role of the effector cells in lymphocytic chorio-
meningitis and influenza virus infections (Doherty & Zinkernagel, 1974; Yap et al. 1978)
and, finally, a combination of $T_{DTH}$ and Tc cells are essential for the reduction of virus
titres. To investigate these possibilities further would require a means of discriminating
between $T_{DTH}$ and Tc cells. This would be possible in the mouse using anti-Lyt antisera
(Cantor & Boyse, 1975; Huber et al. 1976).

In this report we have concentrated our attention on the regional lymph node for the
site of virus infection. However, preliminary investigations show that the contralateral
lymph node and spleen obtained from latently infected donors (later than 1 month p.i.,)
contain cells that transfer a weak DTH response. This contrasts with the DLN taken at
these times which contains suppressors of DTH. Whether the tendency for suppressors of
DTH to predominate in the DLN is in any way related to the recurrences of virus at the
original inoculation site is under further investigation.

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REFERENCES


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