Protection of Mice from Fatal Herpes Simplex Virus Type 1 Infection by Adoptive Transfer of Cloned Virus-specific and H-2-restricted Cytotoxic T Lymphocytes

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SUMMARY

A cloned culture of secondary anti-herpes simplex virus (anti-HSV) cytotoxic T lymphocytes (CTL) generated in vitro when adoptively transferred to intact or cyclophosphamide (CP) pretreated syngeneic mice protected the recipients from death following intraperitoneal infection with HSV-1. This in vivo protective effect conferred by anti-HSV CTL was virus-specific and H-2K/D-restricted. Twenty-four h after HSV-1 infection of BALB/c mice (intact or CP-pretreated) relatively high levels of serum interferon-γ were observed in the recipients of syngeneic anti-HSV CTL and this event may explain, at least in part, the CTL-mediated protective effect.

Human infections with herpes simplex virus (HSV) may assume a fatal course in neonates or immunosuppressed patients (for review, see Nahmias & Roizman, 1973). Additionally, this virus may enter a latent state with frequent recurrences in certain individuals but not in others (Baringer, 1975). Extensive evidence has accumulated which suggests that cell-mediated immune mechanism(s) are critical in recovery and protection against HSV infections (Notkins, 1974). It has become evident that HSV-specific cytotoxic T lymphocytes (CTL), which are restricted by the major histocompatibility complex (H-2 in mouse and HLA in man), although difficult to demonstrate during primary infection (often weak response), can be generated to high levels when primed lymphocytes are secondarily stimulated in vitro, both in murine (Pfizenmaier et al., 1977; Sethi & Brandis, 1977; Lawman et al., 1980; Nash et al., 1980; Kastrukoff et al., 1981; Sonoda et al., 1981) and human (Sethi et al., 1980) models. The present study demonstrates that cloned cultures of in vitro generated HSV-specific and H-2-restricted CTL which are expanded in the presence of T cell growth factor (TCGF) when transferred intraperitoneally (i.p.) to syngeneic mice can effectively protect the recipients against death due to i.p. HSV-1 infection.

HSV-1 strain kos, when injected i.p. into 5- to 6-week-old normal female BALB/c (H-2d) mice at a dose of 5 × 10⁵ p.f.u., routinely killed 75% of animals (mean survival time 14 days), whereas mice pretreated with 100 mg (per kg) cyclophosphamide (CP) showed 100% mortality (mean survival time 8 days) with 10³ p.f.u. of HSV-1. The infected animals showed signs of illness (posterior paralysis, encephalitis) and virus could be consistently recovered from their brains. Experiments were performed to evaluate the effect of HSV-1-specific and H-2-restricted CTL on the course of i.p. HSV-1 infection in untreated and CP-treated BALB/c mice.

The data (Table 1) show that a cloned culture of secondarily stimulated splenic T cells derived from HSV-1-immune BALB/c mice had substantial lytic activity (28% specific ⁵¹Cr release) towards HSV-1-infected P815 (H-2a) cells in a 3 h ⁵¹Cr release assay. The specificity controls show that these CTL were HSV-specific and H-2-restricted since significant amounts of specific ⁵¹Cr were not released from HSV-1-infected H-2 incompatible L-929 cells (H-2k) or from vesicular stomatitis virus serotype New Jersey (VSVNJ)-infected or uninfected syngeneic P815 targets. HSV-specific CTL from A.TL (K⁺/D⁻) mice induced high levels of specific ⁵¹Cr release (19%) from HSV-infected P815 cells (sharing H-2 D⁻) but not L-929 cells that shared I region with CTL. On the other hand, HSV-specific CTL of C3H (H-2k) origin lysed HSV-1-infected L-929 (H-2k) but not P815 (H-2d) cells. As expected, cloned cultures of VSVNJ-specific CTL lysed VSVNJ-infected but not HSV-1-infected or uninfected syngeneic targets. The NK-like cell population(s) induced following i.p. VSVNJ infection and expanded in vitro in the
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of 96-well microtitre plates, preseeded with irradiated (200 rad from a 137cobalt source; Gammator-M; 1800 R/

containing medium. At the time of assay 80 to 85% of the cloned CTL reacted with monoclonal anti-Lyt ÷2

over a monolayer of L-929 or 3T3 cells (2 x 106 to 10 × 106 cells/monolayer) respectively preincubated (37 °C for

a distance of 7-2 cm); before use as stimulators, virus-coated 3T3 or L-929 cells were washed and treated for 1 h

of GM-TCGF and mixed with 0.5 % agarose medium. This mixture was spread over the presolidified agarose in

appeared (usually 8 to 12 days). The clones were picked up and subjected to limiting dilution cloning step. The

preparations (purified by nylon wool columns) from HSV-l-immunized C3H or BALB/c mice were co-cultured

0.5% agarose medium (Pearson

limiting dilution technique in the presence of TCGF (Lotze

et al., 1980) for soft agarose cloning, 4 ml amounts of

0-5% agarose medium (Pearson et al., 1980) containing 20%, TCGF were plated into 35 mm Petri dishes, and the

plates incubated at 4 °C for 10 min. The cells from bulk CTL cultures, usually 103 to 104, were suspended in 0-1 ml

of GM–TCGF and mixed with 0-5% agarose medium. This mixture was spread over the presolidified agarose in

Petri dishes, allowed to gel, and incubated at 37 °C in a humidified CO2 atmosphere until individual colonies

appeared (usually 8 to 12 days). The clones were picked up and subjected to limiting dilution cloning step. The

cultures were diluted (50, 10 and 5 cells per 200 gl) in GM–TCGF and distributed in individual wells (200 μl/well)
of 96-well microtiter plates, preseeded with irradiated (200 rad from a 137cobalt source; Gammatron-M; 1800 R/ 

min) syngeneic or allogeneic peritoneal cells (PC) (104 PC/well). The cultures were incubated at 37 °C in a CO2

atmosphere and kept under constant observation (cloning efficiency was 60 to 80%). Cells from wells showing

sufficient growth were removed and expanded in the presence of TCGF-containing GM. Supernatants from

cocanaevalin A-stimulated normal BALB/c spleen cells obtained by the described procedure (Woolnough & 

Lafferty, 1979) served as the source of TCGF. Secondary VSVNj-specific CTL were obtained by culturing

leukocytes (105 cells/2.5 ml) harvested from spleens of VSVNj-immunized BALB/c mice with purified G protein

preparation (15 μg/culture) of VSVNj. After 5 to 6 days of culture, T cells were expanded and cloned in TCGF-

containing medium. At the time of assay 80 to 85% of the cloned CTL reacted with monoclonal anti-Lyt2

antibody. Splenic lymphocytes from mice infected (i.p.) 3 days earlier with VSVNj and cultured 4 to 5 days in the

presence of 20% TCGF served as the source of NK-like cells. Splenic T cell-enriched populations from unsensitized

BALB/c mice were incubated overnight in GM–TCGF before use in assays.

† Cytolysis assays were carried out as described previously (Sethi & Brandis, 1980). Briefly, target cells (L-929, 
P815) were infected with HSV-1 at an m.o. of 5 or with VSVNj (m.o.i. 20). After virus adsorption (37 °C for 1 h),
cultures were washed and 105 cells incubated with 1 mCi Na251CrO4; mock-infected (including NK-sensitive 
YAC-1) cells were treated with 51Cr as usual. After 2 h (HSV-l-infected) or 8 h (VSVNj-infected), cells were washed and suspended in GM. Aliquots (100 μl, 105 cells) of test targets were mixed in round-bottomed 
plastic tubes with 100 μl of appropriate effectors to give an effector-to-target cell ratio of 30:1. After centrifugation
(50 g for 10 min) the tubes were incubated for 3 h at 37 °C. The tubes were centrifuged (400 g for 10 min) in the 
cold and, from each tube, 100 μl of supernatant was counted in a gamma counter. The results are expressed as
percentage specific 51Cr release according to the formula: 100 x (E - S)/(M - S), where E = release in test, S = 
spontaneous release in medium alone, and M = maximum release (ct/min) in 1% Triton X-100. The tabulated
values are means of quadruplicate assays in a typical experiment with standard errors less than 8 to 10%. The

values are means of quadruplicate assays in a typical experiment with standard errors less than 8 to 10% and are

omitted. Similar data were obtained in repeated experiments. Spontaneous 51Cr release (3 h) values in the

presence of medium alone: L/HSV-1 = <12%, P815/HSV-1 = <27%, L/VSVNj = <12%, P815/VSVNj =

<16%; L-929 = <14%; P815 = <10%; YAC-1 = <8%.

presence of TCGF could lyse HSV-infected P815 of L-929 cells to the same extent as uninfected cells; these effectors were sufficiently potent to lyse the NK-sensitive YAC-1 (H-2a) cells.

Groups of 10 to 20 untreated BALB/C mice or CP-pretreated mice (24 h before adoptive transfer) were given intravenously 107 cloned BALB/c HSV-specific CTL per mouse in 0-1 ml of

Table 1. Comparative lytic activities of different effector cell types grown in the presence of TCGF for virus-infected and uninfected targets

<table>
<thead>
<tr>
<th>Effector cell type*</th>
<th>HSV-1-infected targets†</th>
<th>VSVNj-infected targets</th>
<th>Uninfected targets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P815 (H-2a)</td>
<td>L-929 (H-2a)</td>
<td>YAC-1 (H-2a)</td>
</tr>
<tr>
<td>BALB/c anti-HSV-1 CTL (cloned)</td>
<td>28 0 2 1</td>
<td>0 0 1</td>
<td></td>
</tr>
<tr>
<td>C3H anti-HSV-1 CTL (cloned)</td>
<td>1 35 1 0</td>
<td>0 1 2</td>
<td></td>
</tr>
<tr>
<td>A.TL anti-HSV-1 CTL (cloned)</td>
<td>19 0 1 2</td>
<td>0 0 2</td>
<td></td>
</tr>
<tr>
<td>BALB/c anti-VSVNj CTL (cloned)</td>
<td>0 1 42 2</td>
<td>0 1 3</td>
<td></td>
</tr>
<tr>
<td>BALB/c VSVNj-induced splenic NK cells (bulk culture)</td>
<td>25 36 32 42</td>
<td>22 40 52</td>
<td></td>
</tr>
<tr>
<td>BALB/c normal (unsensitized) splenic T cells</td>
<td>1 2 3 4</td>
<td>2 2 19</td>
<td></td>
</tr>
</tbody>
</table>

* The in vitro generation of secondarily stimulated HSV- or VSVNj-specific and H-2-restricted CTL has been described previously (Sethi & Brandis, 1977, 1980; Sethi & Wolff, 1980). In brief, 105 splenic T cell-enriched preparations (purified by nylon wool columns) from HSV-1-immunized C3H or BALB/c mice were co-cultured over a monolayer of L-929 or 3T3 cells (2 x 106 to 10 × 106 cells/monolayer) respectively preincubated (37 °C for 1 h) with HSV-1 (106 p.f.u./culture) completely inactivated with u.v. light (12 min exposure to a germicidal lamp at a distance of 7-2 cm); before use as stimulators, virus-coated 3T3 or L-929 cells were washed and treated for 1 h with mitomycin C (30 μg/ml). After 3 to 5 days of incubation in growth medium (GM) containing RPMI 1640 (Flow Laboratories), 10% heat-inactivated foetal calf serum, 20 mM-HEPES buffer, 20 mM-glutamine, 5 x 10⁻⁵ M-2-mercaptoethanol, antibiotics and 20% T cell growth factor (TCGF), the cultures were assayed for lytic activities, and active bulk CTL cultures expanded for 4 weeks and then cloned first by soft agarose plating and then by the limiting dilution technique in the presence of TCGF (Lotze et al., 1980).
**Table 2. Adoptive transfer of cloned HSV-1-specific and H-2-restricted CTL protects the recipients against HSV-1 infection**

<table>
<thead>
<tr>
<th>Cells transferred</th>
<th>Untreated BALB/c mice</th>
<th>CP-treated BALB/c mice†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survivors/total</td>
<td>Serum IFN-γ* titre (range)</td>
</tr>
<tr>
<td>None</td>
<td>2/10 (20)‡</td>
<td>40</td>
</tr>
<tr>
<td>BALB/c anti-HSV-1 CTL (cloned)</td>
<td>19/20 (95)</td>
<td>1250-2500</td>
</tr>
<tr>
<td>C3H anti-HSV-1 CTL (cloned)</td>
<td>2/10 (20)</td>
<td>40</td>
</tr>
<tr>
<td>BALB/c anti-VSV$_{NJ}$ CTL (cloned)</td>
<td>3/10 (30)</td>
<td>40</td>
</tr>
<tr>
<td>BALB/c VSV$_{NJ}$-induced NK cells (bulk)</td>
<td>4/10 (40)</td>
<td>80</td>
</tr>
<tr>
<td>BALB/c normal (unsensitized) splenic T cells</td>
<td>3/10 (30)</td>
<td>40</td>
</tr>
</tbody>
</table>

* The presence of serum IFN-γ (pH 2-labile) was monitored by the neutralization of challenge VSV$_{NJ}$ infectivity in L-929 target cells (Stringfellow & Glasgow, 1972). The titres are the reciprocal dilution of serum samples that caused < 50% reduction in plaques.
† Mice were pretreated with 100 mg (per kg) of CP 2 days before i.p. infection (24 h before adoptive transfer) with HSV-1 (10$^5$ p.f.u./mouse). Intact (not treated with CP) mice were inoculated i.p. with 10$^5$ p.f.u./mouse of HSV-1.
‡ The numbers represent survivors observed up to days 65 to 80 post-infection. The figures in parentheses denote percentage mortality.

TCGF-containing medium. As controls, similar groups of mice received either 10$^7$ unsensitized BALB/c splenic T cells, cloned HSV-specific CTL of C3H origin, cloned BALB/c VSV$_{NJ}$-specific CTL, or bulk cultures of NK-like cells induced by VSV$_{NJ}$ in BALB/c mice. On day 1 after the transfer of cells, mice in different groups were inoculated i.p. with 10$^5$ p.f.u./mouse of HSV-1 except the CP-pretreated groups which received 10$^3$ p.f.u./mouse. The results recorded in Table 2 show that adoptive transfer of BALB/c HSV-specific CTL to groups of mice both with or without CP pretreatment protected the recipients from i.p. HSV-1 infection (15/20 of CP-treated and 19/20 of untreated group survived at day 80 post-infection). The survivors revealed no clinical abnormalities and attempts to isolate HSV from their brains by co-culture (Price & Schmitz, 1978) failed. The transfer of syngeneic normal splenic T cells or VSV$_{NJ}$-specific CTL had no effect on the survival of HSV-infected mice both in the untreated as well as CP-pretreated groups. Note that the mice receiving HSV-specific CTL of C3H origin had no protective effect against i.p. HSV-1 infection in BALB/c mice. The following relevant data (not shown in Table 2) has been obtained. Passive transfer of 10$^7$ C3H anti-HSV CTL to homologous C3H mice protected all the recipients (10/10 survivors on day 70 post-infection) against HSV infection. On the other hand, transfer of equal numbers of A.TL anti-HSV CTL to C3H mice (compatibility only at I region) failed to confer protection (2/10 survivors on day 65 post-infection) but could protect BALB/c recipients (8/10 survivors on day 70 post-infection) which showed compatibility with CTL donors at the H-2D$^d$ region. The sera collected from mice in different experimental groups on day 2 post-infection were assayed for the presence of murine immune interferon (IFN-γ) (Interferon Nomenclature, 1980). Strikingly, high levels of serum IFN-γ were detected in the recipients of HSV-specific and H-2K/D-compatible CTL when compared to other groups. The cloned CTL used here spontaneously produced very low titres of IFN-γ (titre < 16) when maintained in vitro culture with TCGF. Whether or not the transfer of cloned CTL to recipients with 1 region compatibility alone can mediate the production of elevated serum IFN-γ levels in the present system is under investigation. In this respect, it is of special interest that Morris et al. (1982) have reported in vitro production of IFN-γ by a cloned influenza virus-specific T cell line on interaction with H-2-compatible virus-infected targets. Earlier studies (Ennis, 1973; Rager-Zisman & Allison, 1976; Mogensen & Andersen, 1981) have shown that adoptively transferred spleen cells from HSV-sensitized mice can protect the recipients against lethal HSV infection but the nature of cell type involved in the protective
event in these systems was not investigated. The present observations imply strongly that HSV-specific and H-2K/D-restricted CTL mediate in vivo protection against lethal HSV-1 infection.

Two groups of investigators have previously attempted to map the H-2 region involved in the protection conferred by adoptively transferred anti-HSV T cells. Nash et al. (1981) demonstrated that for rapid clearance of infectious HSV from the inoculation site, compatibility is required both for H-2K/D and I-A regions, compatibility at I-A region alone being sufficient for transfer of delayed type hypersensitivity response to HSV but not for virus clearance. Howes et al. (1979) concluded for their adoptive transfer experiments that H-2K/D region compatibility confers short-term protection (up to 14 days) whereas long-term protection is I region-restricted. The above-mentioned conclusions may not contradict our present findings, since the earlier studies are different in methodology from our present study. In our model, intact HSV-1-infected BALB/c mice surviving day 14 post-infection did not die over the 80 day observation period and consequently the data presented herein may not be relevant for interpreting the status of I region in the protection of the type observed by Howes et al. (1979).

Here, it may be mentioned that bulk (uncloned) T cell cultures which displayed in vitro K/D region cytotoxicity for HSV-infected targets when transferred intravenously (2 × 10^6 cells/mouse) to normal (untreated) or CP-treated syngeneic mice could effectively protect the recipients against lethal i.p. HSV-1 infection (data not recorded). However, as will be shown in a subsequent paper (in preparation) these effector bulk T cell cultures contained both Ly 1^-2+3^+ and Ly 1^+2^-3^- subsets and although the adoptive transfer of either one of these two subsets of secondary HSV-specific T cells proved effective in protecting the recipients against lethal HSV-1 infection, but the extent of protection and the mechanism(s) by which they mediated the protective effect were not identical. Potentially, passively transferred anti-HSV CTL may be mediating the in vivo protective effect in unprimed recipients by directly killing infected targets during very early stages of infection. However, such a suggestion would not exclude the possibility that elevated serum IFN-γ levels observed in the recipients of virus-specific and H-2-compatible CTL may also contribute to the protective mechanism either by increasing the susceptibility of infected targets to CTL (Sethi & Brandis, 1978), augmenting NK cell activity (Welsh & Doe, 1980) or by interfering with the assembly of infectious virus per se. The role and relative importance of these CTL-dependent protective mechanisms against HSV-1 infection remains to be elucidated.

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REFERENCES
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