The Cytotoxic Response to Murine Cytomegalovirus. I. Parameters in vivo

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SUMMARY

Combined in vivo and in vitro protocols for the generation of anti-murine cytomegalovirus (anti-MCMV) cytotoxic responses were investigated using BALB/c mice and syngeneic mouse embryo fibroblast target cells. Injections of doses of MCMV from $10^{2.0}$ up to $10^{3.5}$ p.f.u. into the hind footpad, harvest of draining popliteal lymph node cells after 6 to 8 days, followed by 4 days culture of these cells gave similar and optimal cytotoxic activity against MCMV-infected target cells. After injection of $10^{3.5}$ p.f.u. into the hind footpad, MCMV was detectable at about $10^{3.0}$ to $10^{4.0}$ p.f.u. per popliteal node after 3 days, but became undetectable in most animals by 6 days. Cell numbers in the draining popliteal lymph node increased following MCMV inoculation into the hind footpad, but the extent of the increase was inversely related to virus dose and bore no relationship to the anti-MCMV cytotoxic potential of the cells. Lymph node cells applied directly to target cells upon harvest from MCMV-infected mice never gave detectable anti-MCMV cytotoxic activity at 2, 4, 6, 8, 10, 12, 17 or 19 days post-infection, but lysis of uninfected syngeneic targets was obtained 4 to 8 days post-infection.

INTRODUCTION

The importance of T cells in recovery from acute viral infections by the poxvirus, ectromelia (Blanden, 1974), and the myxovirus, influenza (Ada et al., 1981), has been firmly established. The role of T cells in viral diseases associated with persistent infection is less well defined. How T cell responses relate to the phenomena of chronic infection, latency and virus reactivation has not been clearly elucidated. The family Herpesviridae contains human and animal viruses that exhibit such clinical and subclinical phenomena during infection of natural hosts. Murid herpesvirus type 1, or murine cytomegalovirus (MCMV), a natural mouse pathogen, is an example of such a member of this family. The murine infection also has many of the clinical and subclinical manifestations found in humans who contract infection with human herpesvirus type 5 (human cytomegalovirus), making the mouse model a possible analogue of human infection.

That T cells play a role in protection from fatal infection with MCMV has been demonstrated using T cell transfer into T cell-deficient nude mice (Starr & Allison, 1977). In non-fatal MCMV infections of normal mice, transfer of MCMV immune T cells resulted in viral clearance from spleens (Ho, 1980). This phenomenon was class I. H-2-restricted, implying that the cells responsible for viral elimination were anti-MCMV cytotoxic T (Tc) cells (Blanden et al., 1975), though this cannot be proven without obtaining anti-MCMV Tc clones and transferring them into infected mice. The study of the inductive requirements for such T cell responses and their regulation during primary MCMV infection are necessary prerequisites for an understanding of the latent state and how reactivation of infection may occur.

In the present studies, a method reported for human herpesvirus type 1 (HSV) (Pfizenmaier et al., 1977) was adapted to MCMV. It was demonstrated that after hind footpad (f.p.) inoculation of MCMV there was an increase in viable cell numbers within the draining popliteal lymph nodes (LN). Upon direct in vitro assay of these LN cells no anti-MCMV cytotoxic activity was detected, but anti-MCMV cytotoxicity was generated after several days in culture of the LN cells.
METHODS

Mouse. BALB/c, CBA/H and WEHI-3 mice, 6 to 12 weeks of age, were obtained from the Animal Breeding Establishment of The John Curtin School of Medical Research.

Viruses. The Smith strain of MCMV was obtained from Dr G. Shellam, University of Western Australia, Nedlands. Salivary gland virus stock was prepared by intraperitoneal (i.p.) inoculation of 10^4.3 plaque-forming units (p.f.u.)/0.2 ml into 4-week-old BALB/c female mice. At day 17 post-inoculation, salivary glands were harvested and a 50% (w/v) homogenate prepared in Dulbecco's modified Eagle's medium (cat. no. H16, Gibco), supplemented with 5% foetal calf serum (FCS) (Flow Laboratories), 200 μg/ml streptomycin, 200 U/ml penicillin G and 125 μg/ml neomycin sulphate. (The complete medium is referred to hereafter as DMEM.) Aliquots were stored at -70 °C. For dilution of virus stock 0.2 M-borate-buffered gelatin saline (pH 7.2 to 7.4) was used. Normal salivary gland homogenate was prepared from normal 6-week-old-female BALB/c mice using the same methods and diluents.

Target cell culture. Mouse embryo fibroblast (MEF) cultures were prepared from 16 to 18 day BALB/c or CBA/H embryos by trypsin dispersion and grown in DMEM. Tissue culture flasks (75 cm², Nunc) were seeded at 10⁵-6 cells/flask and incubated at 37 °C for 4 days in 10% CO₂, 7% O₂ and 83% N₂ (special gas) (Commonwealth Industrial Gases, Alexandria, N.S.W., Australia), after which time the cells were either subcultured immediately or kept at room temperature for up to 2 weeks prior to subculture. Conditions of subculture were the same as described for the primary cultures, except that these secondary MEF cultures were always used by 4 days at 37 °C as the source for tertiary MEF target cells in cytotoxicity assays.

Generation of anti-MCMV effector cells. Anti-MCMV effector cells were generated as follows. Seven days after inoculation of 40 μl of MCMV stock (10^5 p.f.u.) into the hind leg, popliteal LN were collected in Hanks' balanced salt solution (HBSS), and passed through a stainless steel mesh to produce a single-cell suspension. The LN cells were cultured in Eagle's MEM (EMEM) (cat. no. F-12, Gibco) supplemented with 10% FCS, 200 μg/ml streptomycin, 100 U/ml penicillin and 125 μg/ml neomycin sulphate (antibiotics), 10⁻⁴ M-2-mercaptoethanol (2-ME) and usually 5% (v/v) concanavalin-A-activated cell supernatant. The LN cells were cultured at 10⁶-8 cells/ml in 5 ml Costar wells (cat. no. 3512) and incubated at 34 °C in an atmosphere containing special gas.

Removal of immunoglobulin-positive (Ig⁺) cells. Popliteal LN cells were depleted of Ig⁺ cells by a one-step procedure (Parish et al., 1974). Briefly, rosettes containing Ig⁺ cells were obtained by reacting LN cells with sensitized sheep red blood cells coated with sheep anti-mouse immunoglobulin. The rosetted cells were separated from non-rosetting lymphocytes by sedimentation on Ficoll-Hypaque.

Cytotoxicity assay. For MEF target cells, 96-well flat-bottomed microtitre trays (Nunc) were seeded with tertiary MEF at 10⁴-6 cells/ml, 0.2 ml/well, 3 days prior to assay. After 2 days in culture, each well was drained and the cells were infected with 25 μl of MCMV stock in DMEM at 37 °C in special gas mixture, providing a multiplicity of infection (m.o.i.) of 5 p.f.u./cell. Subsequent to viral adsorption for 1 h, 6 μCi Na₂¹⁵CrO₄ (¹⁵Cr) (Amersham) in 150 μl of DMEM was added to each well. At 16 h incubation, the targets were washed twice with DMEM and 100 μl of effector cells was added immediately. Triplicate cultures were set up for each dilution of effector cells and incubated at 37 °C in a humidified atmosphere containing special gas.

¹⁵Cr release from the targets was assayed by gamma emissions, and lysis calculated using the following formula:

\[ % \text{lysis} = \left( \frac{\text{cpm experimental} - \text{cpm spontaneous}}{\text{cpm maximum} - \text{cpm spontaneous}} \right) \times 100 \]

Preparation of concanavalin A-activated spleen cell supernatant (CSS). CSS was prepared using a modification of a published method (Pick & Kotkes, 1977). Spleen cells from WEHI-3 mice were cultured in serum-free EMEM with 10⁻⁴ M-2-ME and concanavalin A (Pharmacia) at a final concentration of 5 μg/ml. All CSS preparation was carried out in the absence of FCS. Cultures were set up in 75 cm² plastic tissue culture flasks and maintained at 37 °C in an atmosphere of special gas for 2 h. The cell monolayer was then washed gently three times with warm HBSS and replenished with 30 ml fresh serum-free EMEM containing 10⁻⁴ M-2-ME. The flasks were reincubated at 37 °C in a humidified atmosphere of special gas for 17 h. The supernatant was harvested, centrifuged to remove any cells and concentrated 10-fold on an Amicon PM-10 membrane. The concentrated CSS preparation was sterilized by filtration and stored at -20 °C. The preparation was added to cultures at a concentration of 3% (v/v).

Plaque assay for MCMV. A modification of an MCMV plaque assay (Mims & Gould, 1978) was utilized. Briefly, secondary BALB/c cells were seeded into 24-well Linbro tissue culture trays (cat. no. 76-033-05, Flow Laboratories) at 10⁵-6 cells/well/2 ml in DMEM and incubated at 37 °C in special gas. After 24 h the medium was aspirated and 50 μl volumes of virus suspension in DMEM was added to the cell monolayer. Adsorption was carried out at 37 °C for 1 h, after which time 2 ml of 0.5% CM-cellulose in DMEM was added as an overlay and incubation continued for 5 days at 37 °C in special gas. The overlay was then aspirated and a staining solution composed of 0.005% crystal violet in formol saline added for 30 min. Plaque counting was performed using a binocular microscope with 10× magnification. All virus suspensions were sonicated for 30 s using a Branson B12 Sonifier at 50 cycles/s prior to plaque assay.
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Fig. 1. Lysis of uninfected BALB/c MEF target cells by effector cells generated from six BALB/c popliteal LN removed on days 0 (a), 2 (b), 4 (c), 6 (d) and 8 (e) after f.p. inoculation of $10^{4.6}$ p.f.u. of MCMV and cultured as single-cell suspensions for various times (indicated by days in vitro). Percent lysis values are the means of three replicates obtained by addition of a 1/12 aliquot of cultured cells from six popliteal LN onto targets. The standard errors of the mean were <4% and are not shown.

Fig. 2. Specific lysis of MCMV-infected BALB/c MEF target cells by effector cells generated from six BALB/c popliteal LN removed on days 0 (a), 2 (b), 4 (c), 6 (d) and 8 (e) as described for Fig. 1. Percent lysis values were obtained by addition of a 1/12 aliquot of cultured cells from six popliteal LN onto MCMV-infected and uninfected target cells. The % specific lysis values are the result of subtraction of the lysis obtained on uninfected targets from lysis on MCMV-infected targets. Each point represents the mean of three replicates. The standard errors of the mean were <4% and are not shown.

RESULTS

The experimental programme investigated modifications of the method reported by Pfizenmaier et al. (1977), to determine if anti-MCMV Tc cell responses could be generated in cells from popliteal LN draining the site of MCMV inoculation if they were removed from the animal and maintained in vitro. The initial experiments were designed to determine the optimal time intervals in vivo and in vitro for anti-MCMV cytotoxic cell generation. For each in vivo and in vitro time point combination, three BALB/c female mice were inoculated into both hind f.p. with $10^{4.6}$ p.f.u. of MCMV. The six popliteal LN were removed on day 0, 2, 4, 6 or 8 post-inoculation. The LN cells were pooled, cultured for 0, 2, 4, 6 or 8 days without addition of viral antigen and then assayed for cytotoxic activity against MCMV-infected and uninfected control BALB/c MEF target cells. The assays for a particular time point in vivo coupled with variable times in vitro were performed as one experiment, e.g. the assays for day 6 in vivo with days 0, 2, 4, 6, or 8 in vitro were performed on one day.

Cytotoxicity against uninfected control MEF target cells was maximal in LN cell populations harvested 4 days after viral inoculation and cultured for 2 to 6 days (Fig. 1). Lysis of MCMV-
Table 1. **Infectious MCMV titres in lymph nodes at different times after infection***

<table>
<thead>
<tr>
<th>Time after infection (days)</th>
<th>Popliteal LN†</th>
<th>Iliac LN§</th>
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<tbody>
<tr>
<td>0-04</td>
<td>3.54</td>
<td>2.45</td>
</tr>
<tr>
<td>3</td>
<td>3.51</td>
<td>2.96</td>
</tr>
<tr>
<td>6</td>
<td>3.51</td>
<td>2.45</td>
</tr>
</tbody>
</table>

* Mice were infected with 10^4.6 p.f.u. salivary gland preparation of MCMV into both hind footpads.
† Viral p.f.u. values are shown for individual mice. (−) indicates no infectious virus detected.
‡ Two popliteal LN/animal are treated as one lymphoid organ.
§ Four iliac LN/animal are treated as one lymphoid organ.

Infected MEF targets over and above the lysis of uninfected controls required minimum intervals of 4 days *in vivo* post-inoculation of virus plus 2 days *in vitro* prior to the cytotoxicity assay (Fig. 2). Maximum antiviral cytotoxic responses required 6 to 8 days *in vivo* post-inoculation of MCMV plus at least 4 days of culture. The culture of LN cells from animals at day 0 and day 2 after f.p. inoculation of MCMV did not produce cytotoxic activity even after 6 to 8 days *in vitro* (Fig. 2).

**Infectious MCMV titres in lymph nodes on different days after f.p. inoculation**

Twenty BALB/c female mice, 6 to 12 weeks of age, were inoculated into both hind f.p. with 10^4.6 p.f.u. MCMV. At 1 h, 3 days, 6 days or 9 days after inoculation, draining popliteal and iliac LN were removed from five animals into DMEM and stored at −70 °C until assayed for p.f.u. On the day of assay, the LN were thawed and sonicated for 30 s. The results (Table 1) showed that infectious virus could be detected in draining popliteal LN of all five mice on day 3, in 1/5 LN on day 6, and in no LN on day 9. The iliac LN revealed a similar temporal profile for virus titres except that not all the iliac LN were infected on day 3.

**Kinetics of anti-MCMV cytotoxic activity with different viral doses**

Four groups of 15 BALB/c female mice were inoculated into both hind f.p. with 10^6.1, 10^5.6, 10^4.6 or 10^3.6 p.f.u. of MCMV. On days 0, 2, 4, 6 and 8 post-inoculation the draining popliteal LN from three mice of each group were collected and single cell suspensions were prepared. The viable cells were counted, cultured for 4 days at 10^5.8 cells/ml and then assayed for cytotoxic activity against MCMV-infected and uninfected BALB/c MEF. The f.p. inoculations for a particular viral dose were staggered at 2-day intervals such that LN harvest, cell culture and subsequent cytotoxicity assays could be performed at the one time. The assays for the different viral doses were performed on different days for logistic reasons. Hence, the absolute values for % lysis induced by different viral doses are not comparable; only the temporal profiles can be compared.

The results (Fig. 3) showed the development of anti-MCMV cytotoxic activity in all cell pools with maximum activity at days 6 to 10 after MCMV inoculation. Cytotoxic activity did not relate to the viable cell numbers in the popliteal LN at the time of harvest (Fig. 4). Cytotoxic activity against uninfected targets was maximal on day 4 for all viral doses (data not shown).

Because cell numbers in the popliteal LN did not correlate with the anti-MCMV cytotoxic activity generated *in vitro* from these cells, it was of interest to determine if the salivary gland homogenate in the stock virus preparations rather than MCMV caused the changes in LN cell numbers. Therefore, on days 0, 2, 4, 6 and 8 after bilateral hind f.p. inoculation of 10^4.6 p.f.u. of MCMV in 40 μl of diluted, infected salivary gland homogenate, both popliteal LN of three BALB/c mice were obtained, cells pooled and viable cell counts performed. All mice were injected on the same day with the subsequent LN collections staggered 2 days apart. A similar protocol was used for control mice that were injected with normal salivary gland homogenate.

Cell numbers in the popliteal LN of MCMV-infected mice increased to a maximum at 6 to 8 days after viral inoculation (Fig. 5). No significant cellular increase occurred after normal
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Fig. 3. Kinetics of anti-MCMV cytotoxic activity with different viral doses. Effector cells were generated from six BALB/c popliteal LN removed at various days after f.p. inoculation of (a) $10^2$, (b) $10^4$, (c) $10^5$ or (d) $10^6$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days. Percent specific lysis values were obtained as described in Fig. 2. The standard errors of the mean were <4% and are not shown.

Fig. 4. Viable cell numbers in draining popliteal LN after hind f.p. inoculation of (a) $10^6$, (b) $10^5$, (c) $10^4$ or (d) $10^3$ p.f.u. MCMV. Each point represents the mean numbers of viable cells per LN obtained from six pooled popliteal LN at the indicated times post-inoculation.

salivary gland injection. A subsequent experiment which utilized a similar sampling protocol was performed on days 0, 1, 2, 5, 6, 8, 10, 14 and 19 after bilateral hind f.p. inoculation. There was an increase in cell numbers in the popliteal LN of MCMV-infected mice with a peak in cell numbers at 6 to 8 days after MCMV inoculation and a decrease to normal cell numbers by day 19 (data not shown). Similar responses to MCMV were obtained using CBA/H mice (data not shown).
Fig. 5. Cell numbers in the draining popliteal LN following hind f.p. inoculation with $10^9$ p.f.u. of MCMV (---) and normal salivary gland suspension (---). Each point represents the mean numbers of viable cells per popliteal LN ± standard deviation of the mean (in groups of five).

Table 2. Effect of viral dose on cytotoxicity mediated by MCMV-immune cultured LN cells* against MCMV-infected target cells

<table>
<thead>
<tr>
<th>Viral dose (p.f.u./f.p.)</th>
<th>% Lysis of BALB/c MEF targets†</th>
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<tbody>
<tr>
<td></td>
<td>MCMV-infected</td>
</tr>
<tr>
<td>$10^3$</td>
<td>48.5 ± 2.6</td>
</tr>
<tr>
<td>$10^4$</td>
<td>41.9 ± 3.0</td>
</tr>
<tr>
<td>$10^6$</td>
<td>49.8 ± 2.4</td>
</tr>
</tbody>
</table>

* Effector cells were generated from three groups of six BALB/c popliteal LN removed 7 days after f.p. inoculation of $10^3$, $10^4$, or $10^6$ p.f.u. MCMV and cultured as single-cell suspensions for 4 days.

† The % lysis values are the means ± standard errors of the mean of three replicates obtained by addition onto targets of a 1/36 aliquot of cultured cells from six BALB/c popliteal LN.

The data in Fig. 3 suggested that viral doses from $10^2$ to $10^6$ p.f.u. up to $10^6$ p.f.u. stimulated anti-MCMV cytotoxic responses provided that 6 to 10 days elapsed between viral inoculation and LN cell harvest followed by 4 days in culture. On this basis, 7 days post-inoculation in vivo and 4 days in vitro were selected as the standard interval for further studies. An example of such an experiment using different viral doses is shown in Table 2. Viral doses of $10^2$ to $10^6$ p.f.u. were then used in further experiments using this protocol as described in this and the accompanying paper (Sinickas et al., 1985).

Direct lymph node assay

Tests were conducted to ascertain if anti-MCMV cytotoxic activity could be detected in draining popliteal LN of MCMV-infected mice without the 4 days in culture. Groups of three BALB/c mice were inoculated with $10^4$ p.f.u. into both hind f.p. at intervals of 2 to 5 days. On days 0, 2, 4, 6, 8, 10, 12, 17 and 19 post-inoculation, the six popliteal LN of each group were harvested and a 1/12 aliquot of each group's pooled LN cells was assayed for cytotoxic activity on MCMV-infected and uninfected BALB/c MEF targets. Positive control anti-MCMV cytotoxic cells (to ascertain MCMV-infected MEF target sensitivity to lysis) were obtained as
Table 3. Cytotoxic activity of LN cells from BALB/c mice 7 days after MCMV infection assayed directly or after a further 4 days in culture

<table>
<thead>
<tr>
<th>Effector LN cells</th>
<th>MCMV-infected</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct LN assay†</td>
<td>13.5 ± 1.9</td>
<td>9.8 ± 0.7</td>
</tr>
<tr>
<td>Ig⁺ cells removed‡</td>
<td>12.4 ± 1.0</td>
<td>14.6 ± 1.0</td>
</tr>
<tr>
<td>Positive control§</td>
<td>72.8 ± 1.3</td>
<td>15.6 ± 0.5</td>
</tr>
</tbody>
</table>

* % Lysis values are means ± standard error of the mean of three replicates obtained by addition of a 1/12 aliquot of cells from six BALB/c popliteal LN.
† Popliteal LN cells were obtained from three BALB/c mice infected 7 days previously with 10⁴.8 p.f.u. of MCMV and assayed directly.
‡ LN cells obtained as in † above were treated as described in Methods to remove Ig⁺ cells and assayed directly.
§ The positive control effector cells were obtained as in † above, but were cultured for an additional 4 days.

described above by harvesting LN on day 7 post-inoculation of MCMV, pooling LN cells and culturing for 4 days.

To test the possibility that infected B cells in the LN cell pool were acting as unlabelled competitor targets in the cytotoxicity assay, a cell suspension was prepared from another group of six LN 7 days after MCMV inoculation and the cell population was depleted of Ig⁺ cells prior to cytotoxic assay of a 1/12 aliquot of the remaining cells. Anti-MCMV cytotoxicity was not detected by direct assay of popliteal LN cell pools at any time post-infection (data not shown). The removal of Ig⁺ cells from the day 7 cell pool did not unmask the presence of cytotoxic cells (Table 3).

DISCUSSION

This paper describes experimental procedures leading to the generation of murine cytotoxic cells that lyse MCMV-infected MEF target cells. The system chosen for study was similar to that described for HSV (Pfizenmaier et al., 1977) and utilized bilateral hind f.p. inoculation of salivary gland MCMV, and harvest of draining popliteal LN followed by culture of single-cell suspensions. This and the accompanying paper (Sinickas et al., 1985) report the detailed characterization of the individual steps of the experimental protocol in order to study the kinetics, specificity and other relevant parameters of the cytotoxic response. As with HSV, the expression of cytotoxic potential against MCMV-infected MEF target cells was dependent upon a further period of time in vitro after the LN cells were removed from the mouse. Four days in vitro was consistently sufficient for this purpose.

MCMV doses from 10⁴⁻⁶ to 10⁶⁻¹ p.f.u. resulted in cells with anti-MCMV cytotoxic potential being present in the popliteal node from 4 to 10 days after infection with maximal levels at about 8 days. After a dose of 10²⁻⁶ p.f.u., potential cytotoxicity did not appear until 6 days after infection and then persisted at maximal levels until day 10. Time points after day 10 were not studied but it would be of interest to investigate the temporal decline of cytotoxicity. MCMV was detected in the draining popliteal LN (> 10³⁻⁶ p.f.u.) 3 days after inoculation of 10⁴⁻⁶ p.f.u. into the f.p., was barely detectable on day 6, and was undetectable by day 9. These results suggest that several days may be needed for MCMV to multiply in some cells of the tissues of the foot and/or of the LN, resulting in display of MCMV-dependent cell surface antigens recognized by precursors of cytotoxic lymphocytes. However, even the lowest dose of virus used (10²⁻⁶ p.f.u.) was sufficient to stimulate an anti-MCMV response.

Numbers of viable cells in the draining popliteal LN increased after MCMV inoculation in the f.p. and the extent of increase was inversely related to the dose of virus. Thus, up to 10-fold increases in cell numbers were found 6 to 8 days after 10²⁻⁶ or 10⁴⁻⁶ p.f.u. of MCMV, but 10⁶⁻¹ p.f.u. caused only an initial increase in cell numbers that was not sustained beyond the second day after inoculation. These data imply that high doses of MCMV can interfere with processes that would lead to increases in LN cell numbers such as local cell proliferation or migration of cells into the LN via the blood or afferent lymph, but we have not evaluated this
aspect further. The majority of the cells contributing to the increase in cell numbers were not directly relevant to the anti-MCMV cytotoxic response since the anti-MCMV cytotoxic potential was unrelated to the numbers of cells in the LN at time of removal for culture.

A feature of the popliteal LN cell response to MCMV infection was that from 4 days after infection significant cytotoxicity was expressed against uninfected self MEF target cells. This effect was most pronounced with LN cells taken 4 days after infection and cultured for 2 to 6 days, but was also present in uncultured LN cells taken from 4 to 8 days after infection. Autoreactivity has been reported previously as a feature of antiviral responses \textit{in vivo} (Blanden & Gardner, 1976) and \textit{in vitro} (Gardner & Blanden, 1976) and has been attributed to either cytotoxic T cells (Blanden & Gardner 1976; Gardner & Blanden, 1976) or natural killer cells (Welsh & Zinkernagel, 1977). It is a possible complication in the interpretation of recent work involving limiting dilution to isolate clones of Tc cells responding to MCMV infection (Reddehase \textit{et al.}, 1984). Since the clones were not divided and assayed on both MCMV-infected self targets and uninfected self targets, it was not determined with certainty which clones were MCMV-specific and which were autoreactive.

Although autoreactive cytotoxic activity was present in popliteal LN cells taken directly from MCMV-infected mice 4 to 8 days after infection, MCMV-specific cytotoxicity over and above autoreactivity was not directly detectable at any time up to 19 days after infection. The possibility that infected cell populations within the LN pool were acting as unlabelled competitor targets during the direct \textit{in vitro} cytotoxicity assay, thereby decreasing the sensitivity of the assay for MCMV-specific cytotoxic cell detection, was tested by removal of the largest cell pool likely to be MCMV-infected (Ig + cells: Olding \textit{et al.}, 1975) prior to \textit{in vitro} cytotoxicity testing. No anti-MCMV cytotoxic activity was unmasked using this technique on cells obtained from the draining popliteal LN on day 7 post-inoculation of $10^4.6$ p.f.u. MCMV. This does not exclude the presence of other infected cells in the non-Ig + cell fraction, since we made no assessment of viral antigen expression within this cell pool. Nevertheless, since Ig + cells comprise approximately 50\% of the LN cell pool, a major possible infected cell source was removed without effect.

The inability to detect direct cytotoxicity agrees with difficulties reported by other workers for MCMV (Ho, 1980) and HSV (Pfizenmaier \textit{et al.}, 1977), but is in contradistinction to others who were able to detect cytotoxicity to MCMV from two lymphoid populations, spleen and cervical LN after intraperitoneal and intranasal routes of inoculation respectively (Quinnan \textit{et al.}, 1978, 1980). The discrepancy may be accounted for by technical differences in target cell and virus preparations and manipulations of variables such as mouse ages, route of inoculation and viral dose. All of the latter are important parameters for mouse survival against MCMV infection (Mannini & Medearis, 1961), and hence may be important factors affecting the generation of effector cells responsible for viral elimination.

In our hands, 2 to 4 days were always required for anti-MCMV cytotoxicity activity to develop \textit{in vitro} in popliteal LN cells taken from the mouse 6 to 10 days after infection. The presence of CSS in the culture medium was necessary for the consistent expression of this anti-MCMV cytotoxic potential, thus raising the possibility that soluble factors which are necessary for the proliferation and/or differentiation of anti-MCMV cytotoxic cells are limiting or are insufficient to overcome some suppressor mechanism \textit{in vivo}. The lack of a detectable cytotoxic response \textit{in vitro} is an important feature of the immunobiology of herpesvirus infections, since many other virus groups have been shown to provoke primary antiviral Tc cell responses \textit{in vivo} within 4 to 8 days (Blanden & Gardner, 1976; Yap & Ada, 1977). Therefore, we have undertaken a thorough reductionist approach to the separate steps of the experimental protocol required to generate and assay anti-MCMV cytotoxic cells, beginning with the \textit{in vitro} parameters studies here. Future reports will deal with the \textit{in vitro} parameters, characterization of effector cells, and factors affecting the efficiency of their recognition of MCMV-infected target cells.

\textbf{REFERENCES}


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