Involvement of Natural Killer Cells in the Pathogenesis of Murine Cytomegalovirus Interstitial Pneumonitis and the Immune Response to Infection

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SUMMARY

The significance of the natural killer (NK) cell response to murine cytomegalovirus (MCMV) infection was evaluated in C3H/HeN mice. This strain was selected for study after preliminary demonstration that the NK cell response, occurring between 3 and 6 days post-infection was relatively high in comparison to other mouse strains studied. A dose-response effect of hydrocortisone treatment on suppression of this response was found. A dose of hydrocortisone, given subcutaneously on two successive days, which was found to markedly inhibit the NK cell response, had no effect on development of serum interferon or antibody levels, or spleen cytotoxic T cell activity under the conditions studied. Suppression of the NK cell response by this treatment, however, was accompanied by enhanced spleen and pulmonary virus replication in vivo and increased susceptibility of mice to lethal infection. MCMV interstitial pneumonitis was characterized histologically and lung lymphocytes studied at 4 days post-infection were found to have increased NK cell activity. Treatment of mice with hydrocortisone was found to inhibit development of gross and histological evidence of pneumonitis. These findings indicate that NK cells are involved in the pathogenesis of MCMV interstitial pneumonitis and may function early in infection to restrict the extent of virus replication.

INTRODUCTION

Increased natural killer (NK) cell activity develops in response to murine cytomegalovirus (MCMV) (Quinnan & Manischewitz, 1979) and lymphocytic choriomeningitis virus (LCMV) (Welsh & Zinkernagel, 1977) infections in mice. In a preliminary report, a similar response was noted during murine influenza virus infection (Daisy et al., 1980). In each case the response begins within 3 days of onset of infection and then wanes as an H-2-restricted cytotoxic T cell response begins, about 6 days after initiation of infection (Quinnan et al., 1978; Quinnan et al., 1980; Ennis et al., 1978; Zinkernagel & Doherty, 1974). In these infections the NK cell response is, thus, one of the earliest components of the immune response. The possibility that these effector cells constitute a first or early line of defence against the pathological effects of virus infections has not been studied. The mechanism by which these effector cells might preferentially recognize virus-infected cells and participate in restriction of virus replication is unclear. Since NK cells induced by MCMV and LCMV infection recognize both virus-infected cells and tumour cells, similar to spontaneously occurring cytotoxicity, they are not strictly virus-specific in the manner of cytotoxic T cells.
Nevertheless, some evidence that NK cells may possess unique specificities for different tumour cell lines and for some virus-infected cells has been reported (Blair, 1980; Minato et al., 1979). These observations are consistent with the possibility that cells infected by these viruses are induced to express specificities which can be recognized preferentially by NK cells. This preliminary evidence for the existence of unique receptors on NK cells for different antigenic specificities provides a theoretical basis for suggesting that they function as a defence mechanism against virus infections. The present study was performed to examine the role of NK cells in the pathogenesis and immune control of CMV infection.

**METHODS**

**Viruses.** The source and preparation of the Smith strain MCMV used in these studies have been previously described (Manischewitz & Quinnan, 1980). Salivary gland virus was used for mouse inoculation and tissue culture-passaged virus was used for infection of target cells. Viruses were assayed by plaque titration in monolayer cultures of primary mouse embryo cells (MEC) (Wise et al., 1979).

**Mice.** C3H/HeN mice were obtained from the Animal Production Unit, National Institutes of Health, Bethesda, Md., U.S.A. Mice were routinely inoculated with $1 \times 10^5$ p.f.u. of MCMV intranasally (i.n.) or intraperitoneally (i.p.) at 4 weeks of age. Embryos were removed from pregnant females 5 days before term for preparation of MEC cultures. In some cases mice were inoculated subcutaneously (s.c.) with cortisone acetate (Sigma) or hydrocortisone sodium succinate (HC, Sigma) in 20% (v/v) ethanol in distilled water at intervals after virus inoculation.

**Histological examinations of lungs.** Mice were sacrificed by cervical dislocation. Lungs were removed, dissected free of mediastinal tissues, and placed in Bowen's fixative. After fixation, lungs were embedded in paraffin, sectioned, and stained with haematoxylin and eosin.

**Preparation of lymphocyte suspensions.** Spleens were removed aseptically, placed in 60 mm plastic Petri dishes (Falcon, Oxnard, Ca., U.S.A.) containing 2 ml minimum essential medium supplemented with 10% foetal bovine serum, penicillin and streptomycin (medium), and then disrupted by grinding with the top of an inverted 50 ml plastic centrifuge tube (Falcon). Single cell suspensions were prepared by repeated pipetting and filtration through sterile gauze pads. Aliquots were removed for plaque assay infectivity titration, and erythrocytes in the remaining suspension were lysed by treatment with ammonium chloride (Roos & Loos, 1970). Spleen cells were then washed and resuspended to a final concentration of $2.5 \times 10^6$ to $5 \times 10^6$ cells/ml.

Lungs were disrupted in a similar fashion except that a sterile gauze sponge was placed on the bottom of the Petri dish. After suspension, filtration and removal of an aliquot for plaque assay, the cells were centrifuged at 400 g for 10 min and resuspended in media. Lymphocytes were obtained by Ficol-hypaque (Pharmacia) gradient centrifugation, washed three times and suspended as were the spleen cells for use as effector cells.

**Cytotoxicity assay.** MCMV-infected MEC were prepared as target cells for assays of virus-specific cytotoxicity as previously described (Manischewitz & Quinnan, 1980). Natural killer cell activity was measured using the continuous lymphoid line RBL5 cells, for target cells (Quinnan & Manischewitz, 1979). Assays were routinely performed in plastic round-bottomed Linbro 96-well plates at a 50:1 effector-to-target ratio, using either $5 \times 10^3$ or $1 \times 10^4$ target cells per well and an 18 h incubation at 37 °C in a 5% CO$_2$ atmosphere. The methods used for harvesting and determination of the specific lysis (SL) percentage have been described previously (Quinnan & Manischewitz, 1979).

**Neutralizing antibody and serum interferon assays.** Neutralizing antibody was assayed by
NK cell response in MCMV pneumonitis

NK cell and CTL responses to intraperitoneal infection

C3H/HeN mice were chosen for study since, in preliminary experiments, they were found to develop higher spleen NK cell responses than several other strains studied. The cytotoxic responses were, in other respects, similar to those described previously in Balb/c mice (Quinnan & Manischewitz, 1979; Quinnan et al., 1978; Quinnan et al., 1980). Two components of the virus-specific cytotoxic response could be distinguished after i.p. inoculation of a sublethal dose of MCMV. The NK cell response, which occurred between days 3 and 6 post-infection, was characterized by increased spleen cell cytotoxicity for uninfected RBL5 cells and syngeneic and allogeneic MCMV-infected MEC target cells. These cells did not adhere to nylon wool and were resistant to treatment with anti-theta serum and complement. The cytotoxic T cell responses developed later, between days 6 and 21. We have previously demonstrated that the CTL response of C3H/HeN mice is H-2-restricted (Quinnan et al., 1978). Experiments were also performed which confirmed that these CTL were non-adherent and were killed by treatment with anti-theta serum and complement. Thus, the cytotoxic cells measured on day 3 or 4, using either uninfected tumour cells or MCMV-infected MEC for target cells, were NK cells, whereas cytotoxic T cells could be detected after day 6 using infected MEC for target cells.

Effect of hydrocortisone on spleen NK cell response

Mice inoculated i.p. with 1 x 10^5 p.f.u. of MCMV were given various doses of hydrocortisone (HC) subcutaneously on days 1 and 2 post-infection and spleens were harvested on day 3 for NK cell assays. Suppression of the NK cell response was anticipated as previously reported by Djeu et al. (1979). Inhibition of cytotoxicity against MCMV-infected MEC was seen when mice were given doses of HC as low as 25 mg/kg (Table 1), while marked inhibition of lysis of both infected MEC and uninfected tumour cell targets occurred at doses of 50 and 100 mg/kg. Similar results were obtained by treating mice with cortisone acetate (results not shown). In subsequent experiments the HC dosage used was 50 mg/kg.

Included in Table 2 are results of experiments performed to examine the comparative effects of HC treatment on aspects of the response to and the extent of virus replication in vivo. HC treatment on days 1 and 2 consistently inhibited the NK cell response, but did not effect the development of the virus-induced rise in serum interferon. In contrast, treatment with HC on days 5 and 6 did not reduce CTL activity measured on day 7. Although the results included here are representative of results of other similar experiments and suggest that an increase in CTL activity occurred after HC treatment, such was probably not the case. A consistent reduction in spleen size was produced by HC treatment; a decrease in total spleen cell number without a change in total number of CTL may have accounted for increased CTL activity when measured on a per cell basis. No effect of HC treatment on development of serum antibody was seen post-infection (results not shown). Thus, the NK cell response was the only component of the immune response found in these experiments to be inhibited by HC treatment. Associated with this suppressed NK cell response, a twofold or greater increase in virus replication in spleens and lungs was consistently found (Table 2). The possibility that this association was a manifestation of a protective effect of NK cells was further evaluated by studying the effect of HC treatment on resistance to lethal MCMV infection. Using the Reed-Muench method of calculation (Hawkes, 1979), the dose of salivary gland MCMV lethal for 50% of mice was found to be 1 x 10^6.0 for untreated mice and 1 x
Table 1. Dose-dependent inhibitory effect of hydrocortisone sodium succinate on splenic NK response to MCMV infection

<table>
<thead>
<tr>
<th>Time post-infection (days)</th>
<th>HC dosage* (mg/kg)</th>
<th>MCMV-infected MEC SL (%)</th>
<th>RBL5 cells SL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 None</td>
<td>&lt;0.0</td>
<td>&lt;0.0</td>
<td>&lt;0.0</td>
</tr>
<tr>
<td>3 None</td>
<td>71.0</td>
<td>60.3</td>
<td>33.9</td>
</tr>
<tr>
<td>3 25</td>
<td>51.8</td>
<td>39.4</td>
<td>32.2</td>
</tr>
<tr>
<td>3 50</td>
<td>41.6</td>
<td>22.2</td>
<td>24.8</td>
</tr>
<tr>
<td>3 100</td>
<td>35.8</td>
<td>8.1</td>
<td>17.8</td>
</tr>
</tbody>
</table>

* Hydrocortisone (HC) was administered on days 1 and 2.
† Effector: target ratio; each spleen cell suspension was prepared by pooling cells from spleens of three mice.

10^5.3 for HC-treated mice. Moreover, lethally infected mice treated on days 1 and 2 with HC began dying 2 days earlier (on days 4 and 5 post-infection) than untreated mice. This difference in susceptibility to lethal infection was also seen in numerous experiments. Thus, HC treatment of mice infected i.p. decreased their resistance to lethal infection, resulted in increased virus replication in vivo and suppressed the NK cell response to this infection.

**NK cell response to MCMV pneumonitis**

Previous work reported by Jordan (1978) demonstrated that interstitial pneumonitis could be consistently induced by i.n. inoculation of salivary gland MCMV. Experiments were performed to further characterize the pathogenesis of, and the potential role of NK cells in this infection. Thirty-six sections of 12 lungs removed from mice 4 days after i.n. inoculation of 1 x 10^5 p.f.u. of MCMV were examined after haematoxylin and eosin staining. Evidence of interstitial pneumonitis was present in all sections examined. The most consistent abnormality found was a peribronchial lymphocytic infiltration extending to the level of the terminal bronchiole. In many cases this infiltrate extended into the interstitium of the alveolar septae. In a few sections the infiltrate was extensive, obliterating the normal architecture of large sections of lung tissue. Lymphocytic infiltration was not present in lungs from normal mice.

Pulmonary lymphocytes were assayed for NK cell activity in cytotoxicity assays using uninfected RBL5 cells for target cells. The results obtained at various times after onset of infection are presented in Fig. 1. Each assay of lung lymphocyte cytotoxicity was performed using pooled lymphocytes obtained from 15 to 30 lungs. The SL percentage values expressed in Fig. 1 are those obtained using 50:1 effector-to-target ratio. The NK cell activity present in equivalent numbers of lymphocytes increased progressively from day 2 to a peak on day 4. In parallel, there was a progressive increase in the number of lymphocytes recovered from an average of 1 x 10^7/lung on day 0 to 6.1 x 10^7 on day 4. Thus, there was a significant increase in pulmonary NK cell activity at this early stage of infection.

**Effect of hydrocortisone treatment on MCMV pneumonitis**

Thirty-six sections of 12 lungs from mice inoculated with HC on days 2 and 3 post- i.n. inoculation of MCMV were examined on day 4. Most were normal in appearance. Only two of these 36 sections were found to have minimal peribronchitis. NK cell activity and pulmonary virus titres in lungs of mice treated in this fashion were compared to those of mice not treated with HC. Results of a typical experiment are shown in Table 3. In parallel with the greater inflammation seen by histology, the average weight of lungs and the total number of lymphocytes recovered per lung from untreated mice were significantly greater than those from HC-treated mice. NK cell activity, measured at a 50:1 effector-to-target ratio, was also
Table 2. **Effect of hydrocortisone treatment on immune responses and virus replication during MCMV infection**

<table>
<thead>
<tr>
<th>Day post-infection of assay</th>
<th>HC administration*</th>
<th>SL (%) ± standard error†</th>
<th>Serum interferon titre‡</th>
<th>Geometric mean virus infectivity titres§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MCMV-infected MEC</td>
<td>RBL5 cells</td>
<td>Spleen (p.f.u./10⁸ cells)</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>45.6 ± 3.0</td>
<td>63.7 ± 4.7</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Day 1, 2</td>
<td>23.1 ± 2.8 (P &lt; 0.001)</td>
<td>48.2 ± 2.0 (P &lt; 0.01)</td>
<td>2.4</td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>16.6 ± 3.6</td>
<td>10.7 ± 4.0</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>Day 5, 6</td>
<td>39.8 ± 3.1 (P &lt; 0.001)</td>
<td>8.6 ± 2.5</td>
<td>ND§</td>
</tr>
</tbody>
</table>

* Hydrocortisone sodium succinate was administered in dose of 50 mg/kg s.c. on days indicated.
† Assays were performed at 50:1 effector-to-target ratios.
‡ Results expressed as log₁₀ of the reciprocal of the dilution inhibiting 50% of virus infectivity. No interferon was detected in sera of uninfected mice. Serum interferon titres on days 1, 2 and 4 post-infection were 2.6, 3.1 and 2.4 respectively, in mice not treated with HC, and were not significantly different in HC-treated mice.
§ Results are means of three experiments, each with 5 spleens or 15 lungs pooled for assay (results ± standard error).
‖ Significance of differences between SL percentage values were determined by Student’s t test.
ND, Not done.
Fig. 1. Pulmonary NK cell response during MCMV interstitial pneumonitis: lysis of RBL5 cells at a 50:1 effector-to-target ratio.

Table 3. Effect of HC treatment on splenic and pulmonary NK activity during MCMV pneumonitis after intranasal virus inoculation

<table>
<thead>
<tr>
<th>Lymphocyte source*</th>
<th>HC dosage</th>
<th>Lymphocyte yield per lung</th>
<th>Average lymphocyte weight (mg)</th>
<th>MCMV titre (p.f.u./g)†</th>
<th>Lysis of RBL5 cells SL(%) (50:1 E/T)</th>
<th>Relative lytic activity‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>None</td>
<td>$6.1 \times 10^5$</td>
<td>108.4</td>
<td>$23.4 \pm 1.2$</td>
<td>$25.8 \pm 2.1$</td>
<td>14.8</td>
</tr>
<tr>
<td>Lung</td>
<td>50 mg/kg</td>
<td>$0.6 \times 10^5$</td>
<td>77.3</td>
<td>$1122.0 \pm 1.2$</td>
<td>$17.7 \pm 1.3$</td>
<td>1.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td>$11.0 \pm 1.0$</td>
<td>‡</td>
</tr>
<tr>
<td>Spleen</td>
<td>50 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td>$2.5 \pm 1.7$</td>
<td>§</td>
</tr>
</tbody>
</table>

* Lungs and spleens were harvested on day 4 after i.n. inoculation of $1 \times 10^5$ p.f.u. of MCMV; HC was administered on days 2 and 3.
† Results are geometric mean titres obtained from lungs of 15 mice in each group (results ± standard error).
‡ Relative lytic activity = [SL percentage (untreated mice) x average number of lymphocytes/lung in untreated mice]/[SL percentage (HC-treated mice) x average number of lymphocytes/lung in HC-treated mice].
§ Significant difference by Student's t test (results ± standard error).

significantly greater in lungs of untreated mice. The relative difference in the total number of NK cells present per lung was estimated by multiplying the ratio of SL percentage values by the ratio of numbers of lymphocytes recovered per lung from untreated and HC-treated mice respectively. The total amount of NK cell activity recovered per lung was, thus, about 14-fold greater in lungs of untreated mice. In contrast to the evidence of decreased inflammation and NK cell activity, pulmonary virus titres were approx. 10- to 1000-fold greater in repeated experiments, in lungs of HC-treated mice.

DISCUSSION

At this time there is only limited evidence that any specific cellular immune function has a direct impact on the outcome of any virus infections. The potential effects of different types of cytotoxic cells are of special interest in this regard, since it is possible that they may lyse infected cells early in the replicative cycle, before the formation of infectious virions (Shore et al., 1976). This mechanism could provide an effective means of limiting virus spread in vitro. One example of a protective effect mediated by cytotoxic cells is the transfer of resistance to
influenza infection in nude mice by CTL (Wells & Ennis, 1981). Similar findings were obtained by Ho (1980), who demonstrated that protection mediated by passive transfer of spleen cells from mice acutely infected with CMV was H-2-restricted, suggesting that the effector cell involved was a cytotoxic T cell. There is no direct evidence that other types of cytotoxic cells serve a protective role in vivo. The possibility that natural killer cells, though generally considered non-specific, may mediate a protective effect is worth considering, since natural killer cell responses to CMV (Quinnan & Manischewitz, 1979), LCMV (Welsh & Zinkernagel, 1977) and influenza virus (Daisy et al., 1980) infections of mice and CMV infection of humans (Quinnan et al., 1981) have been demonstrated, indicating that this type of response may well be a generally occurring phenomenon.

The murine model for CMV infection is particularly well suited for study of the contribution of NK cells to in vivo control of virus infection. The NK cell response occurs early in the course of infection, between days 3 and 6 after virus inoculation, whereas most other immune responses occur at later times (Quinnan & Manischewitz, 1979). The only documented exception is the interferon response which begins on day 1, as demonstrated in the present study. The other specific responses which have been measured include CTL, from days 6 to 21 (Quinnan et al., 1978; Quinnan et al., 1980), serum antibody which mediates antiviral antibody-dependent cell-mediated cytotoxicity (ADCC) beginning about day 10 (Manischewitz & Quinnan, 1980), serum neutralizing antibody which develops about day 21 (Quinnan et al., 1980; Wise et al., 1979) and a virus-specific lymphocyte proliferation response which develops about day 7 (Howard et al., 1977). It is not possible to exclude the possibility that local antibody production at the site of infected tissue begins at an earlier time, but this phenomenon has not been documented during this infection. As a result of the temporal relationships of these responses to each other, it is possible to examine the role of NK cell activity in this model by focusing studies on events occurring on days 3 and 4 post-infection, at which time it is likely that little, if any, contribution to in vivo control of virus replication is made by other types of immune responses.

One aspect of this study was to examine the effect of HC treatment, which reduced NK cell activity of mice, on the course of infection. Efforts were made to determine what effects this might have on other aspects of the immune response. HC treatment on days 1 and 2 did not affect the rise in serum interferon levels or the later development of serum neutralizing antibody. Nor did treatment on days 5 and 6 affect the level of spleen CTL activity which developed by day 7. These results indicated that the suppression of the NK cell response observed was relatively selective. An effect of HC on other immune functions not documented by these techniques cannot be excluded. However, it is reasonable to speculate that the changes in the course of infection which occurred were related to the observed suppression of the NK cell response.

In parallel with suppression of the NK cell response there was enhancement of spleen and pulmonary virus replication which occurred after HC treatment. In addition, this treatment resulted in a significant increase in susceptibility to lethal infection and a shortening of the time from onset of infection to death. These findings are consistent with the possibility that the NK cell response to CMV infection serves to restrict in vivo virus replication.

Hydrocortisone treatment also modified the pathogenesis of MCMV interstitial pneumonitis. As previously described (Jordan, 1978), i.n. inoculation of appropriate amounts of this virus reproducibly results in development of interstitial pneumonitis. As reported here, the pulmonary changes which had taken place within 4 days of i.n. virus inoculation included an increase in lung weight and in numbers of lung lymphocytes. Histological changes consisted of peribronchitis with lymphocytic infiltration extending into the alveolar septae sometimes completely obscuring the normal pulmonary architecture. These changes were accompanied by a marked increase in pulmonary NK cell activity. By contrast, HC treatment blocked all
of the changes normally seen within 4 days of onset of infection, including suppression of the local NK cell response, and also resulted in a marked increase in pulmonary virus replication.

The results of this study clarify the significance of the NK cell response to MCMV infection. They are involved in the local immune response to and pathogenesis of MCMV interstitial pneumonitis. Indirect evidence was obtained that suggests that NK cells may also function, early in infection, to restrict virus replication, and as an initial defence against lethal infection with MCMV.

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REFERENCES


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