Delayed Hypersensitivity to Murine Cytomegalovirus and Its Depression During Pregnancy

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A delayed hypersensitivity skin test for murine cytomegalovirus is described, in which ear swelling is measured after injection of heat-killed virus. The response appeared within 4 days of infection, peaked at 8 days, and remained at high levels for at least 100 days. When live virus was inoculated into the ear of previously uninfected mice, a much greater degree of ear swelling was seen, maximal at 7 days, but without growth of virus in the inoculated ear. Mice infected with avirulent (cell culture-passaged) virus gave greater delayed hypersensitivity responses than with those infected with virulent (salivary gland-passaged) virus. One of the strains of mice that is susceptible to murine cytomegalovirus (C57BL) developed greater delayed hypersensitivity responses than did the resistant strain (C3H). The delayed hypersensitivity response of neonatally infected mice was greatly depressed during pregnancy and lactation.

Although cytomegalovirus (CMV)-specific cell-mediated immunity (CMI) has been reported before, this was based exclusively on in vitro studies. Thurman et al. (18) reported in vitro blast transformation of peripheral blood lymphocytes from patients with CMV infection when lymphocytes were cultured with CMV-infected WI 37 cells. Thong et al. (17) and Rola-Plesczynski et al. (14) demonstrated CMI to human CMV by using a lymphocyte cytotoxicity assay. For murine CMV (MCMV) Howard et al. (6) and Kelsey et al. (7) have detected virus-specific CMI by in vitro lymphocyte proliferation assays, and Quinnan et al. (12) reported the presence of virus-specific cytotoxic T-cells which was H-2 restricted. Very recently Griffith et al. (B. P. Griffith, P. W. Askenase, and G. D. Hsiung, Fifth International Congress of Virology, Strasbourg, 1981) have shown that guinea pig CMV can induce in guinea pigs a delayed virus-specific cutaneous reaction which appeared to be basophil mediated.

Resistance to CMV infection does not appear to be antibody dependent because CMV can both persist in vivo and replicate in vitro (Chong, unpublished data) despite the presence of high concentrations of neutralizing antibodies. Experimental suppression of the CMI response, however, makes mice more susceptible to MCMV infection (1, 8, 9)—suggesting that CMI plays an important role in defense against CMV infection. Furthermore, MCMV infections are much more severe in congenitally athymic mice (15, 16). In this study, we report MCMV-specific CMI by an in vivo test for delayed-type hypersensitivity (DTH). The response was depressed during late pregnancy and throughout lactation, and this can be related to reactivation of CMV during pregnancy.

MATERIALS AND METHODS

Cell cultures. Primary mouse embryo fibroblasts (MEF) were prepared by the trypsinization of fetuses from 17-day-pregnant CD1 mice. Cells were grown in Eagle minimal essential medium supplemented with 5% fetal bovine serum, 0.11% sodium bicarbonate, 100 U of penicillin, and 100 μg of streptomycin. For both virus growth and plaque assay, secondary cell cultures were used.

Virus. Unless otherwise stated, the strain of MCMV used throughout this study was received from June Osborn (Department of Medical Microbiology, University of Wisconsin, Madison, Wis.), who had developed it from the Smith strain after many passages in mouse salivary gland. The Smith (American Type Culture Collection) strain passaged in salivary glands was used in one experiment. To obtain high-titer stock salivary gland virus (SGV) containing >10⁷ PFU/ml, 3-week-old mice were infected intraperitoneally with 10⁵ PFU of virus. This produced 10% mortality at 5 to 7 days, and the salivary glands of surviving mice were harvested at 2 to 3 weeks after infection. Tissues were homogenized in phosphate-buffered saline with an M.S.E. homogenizer to give 10% tissue suspensions. This was clarified by low-speed centrifugation and stored at -80°C.

The cell culture-grown virus (CCV) was used after 10 to 15 passages in MEF cells at a low multiplicity of infection (approximately 0.01 PFU per cell). The virus growth medium used was Eagle minimal essential medium supplemented with 0.11% sodium bicarbonate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2% fetal bovine serum. For stock virus preparations, the infected cultures were frozen and thawed once, and cell debris was removed by low-
speed centrifugation. Virus titers, as determined by plaque assay in MEF, were between \(5 \times 10^3\) and \(2 \times 10^9\) PFU/ml.

Mice. An outbred, specific-pathogen-free strain of CD1 mice were used for all experiments. Specific-pathogen-free C57BL and C3H strain mice were obtained from Bantan and Kingman, The Field Station, Grimston, Aldbrough, Hull.

**Infectivity assay.** Samples were assayed for virus content in secondary MEF by a plaque-forming technique under carboxymethyl cellulose overlay in which 0.1 ml of virus dilutions were inoculated onto confluent MEF monolayers in Multiwell dishes (Falcon Plastics, Oxnard, Calif.). After 60 min of adsorption at 36.5°C, cultures were overlaid with Eagle medium containing a final concentration of 0.7% carboxymethyl cellulose (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire). The cultures were drained after 5 days and stained with 0.05% crystal violet dissolved in 20% alcohol in water and containing 10% Formalin. Plaques were counted on a Carl- Zeiss Jena Dokumator projection microscope.

**Ear swelling.** Heat-killed (56°C for 30 min) SGV was inoculated (at a dose equivalent to \(5 \times 10^5\) PFU) in 10 µl of phosphate-buffered saline into the pinna of the left ear of anesthetized mice with a Unimetric 5100R microsyringe with a 28-gauge needle. Routinely the swelling was measured 24 to 36 h after challenge by using a Mitotoya engineer’s micrometer. Mean values from 5 or 6 mice were used to calculate the percent increase in thickness of the challenge ear in comparison with that of the uninoculated ear.

**Histology.** Tissues were fixed in formal saline, embedded in wax, sectioned, and stained with hematoxylin-eosin and azure A.

**RESULTS**

**Experiments with heat-killed virus challenge.** CD1 mice infected 11 days earlier with a single intraperitoneal (i.p.) inoculation of \(10^6\) PFU of live SGV were challenged in the ear with killed virus as described above. The increase in ear thickness as measured at 24, 48, and 72 h after challenge was 51, 59, and 39%, respectively. Nonimmune mice given the same dose of heat-killed virus gave a variable response of up to 8% increase in ear thickness. Heated, uninoculated salivary gland extract produced no response in either immune or uninoculated mice. Although DTH responses were readily induced in the ear, very thorough tests failed to show DTH responses to footpad inoculation of challenge virus.

The histological picture in the swollen ear 48 h after challenge with heat-inactivated virus showed moderate to extensive mononuclear cell infiltration. This consisted of predominantly small mononuclear cells, presumably lymphocytes, and some larger cells, presumably macrophages. Large, spread-out, metachromatically staining cells were seen evenly distributed in the dermis, but these were also present in normal mouse ears. However, there were fewer of these cells in infiltrated areas, and it was clear that 99% of the infiltrating cells were not metachromatically stained and therefore not basophils.

To measure the time course of development of DTH, the left ear was injected with \(5 \times 10^6\) PFU of heat-killed virus at various times after i.p. infection of mice with \(10^7\) PFU of MCMV SGV, and the ear response was measured. Significant ear swelling was first observed in mice challenged on day 4 after i.p. infection and was maximal on day 8 postinfection (Fig. 1). The ear swelling could still be induced 100 days after infection.

**Experiments with live virus challenge.** Other mice also infected 11 days earlier were challenged in the pinna with \(5 \times 10^5\) PFU of live SGV, and ear swelling was measured at different times. As with the killed virus, peak swelling was seen at 48 h (115%), with 101% at 24 h and 85% at 72 h. Smaller doses (\(10^4\) PFU) of live virus induced a smaller response or no response (\(10^3\) PFU) in previously infected mice. The larger dose (\(5 \times 10^5\) PFU) of virus was necessary for consistent and reproducible results. However, live virus was not used for routine DTH challenge because it produced marked ear swelling in previously uninfected mice (control animals). The primary ear swelling was compared with DTH ear swellings in groups of five CD1 mice after subcutaneous infection with \(5 \times 10^5\) PFU of live SGV (Fig. 2). It can be seen from Fig. 2 that the primary ear swelling was greater and also of longer duration than the DTH response, with a peak occurring 7 days after infection. The inoculated ear of these mice contained no detectable virus (<5 PFU per ear) at 2 and 8 days. Previously infected mice also showed no virus in the inoculated ear 2 days after live virus challenge.

**Effect of virus strain and dose on the development of DTH.** Groups of 6 mice were infected i.p. with either CCV or SGV, giving either \(10^4\) or \(10^5\) PFU per mouse. At 11 days after infection mice were challenged with a standard dose of heat-killed SGV, and ear swelling was measured as usual. It can be seen from Table 1 with the smaller dose that the avirulent CCV induced a greater ear response than the virulent SGV (\(P < 0.01\)). The smaller dose of CCV (\(10^4\) PFU) induced a significantly greater ear response than the larger dose (\(10^5\) PFU) (\(P < 0.02\)).

**DTH responses in different strains of mice.** Three different strains of 4-week-old mice were injected i.p. with different doses of Osborne or Smith MCMV, and the mortality is shown in Table 2. The Smith strain was more virulent than the Osborne strain, and it can be seen that although C3H mice were more resistant than C57BL and CD1 mice to the Osborne strain of virus, they were not significantly more resistant to the Smith strain of virus. Hypersensitive ear
FIG. 1. Time course for the induction of DTH ear swelling to MCMV after ear injection of heat-killed virus. Each point represents the mean of five mice, and the vertical bars represent 95% confidence limits.

FIG. 2. Comparison of primary ear swelling (■) in previously uninfected mice with DTH ear swelling (●) after ear injection of $5 \times 10^5$ PFU of MCMV SGV. Each point represents mean of five mice, and the vertical bars represent 95% confidence limits.
responses were compared in mice of the same three strains after infection i.p. with $10^4$ PFU of SGV (Table 3). When tested at 5 days postinfection, the ear swelling was much greater in the C57BL mice than in C3H mice ($P < 0.001$). CD1 mice were not significantly different from C3H mice at this time, and by 11 days all strains of mice showed similar maximum ear responses. By 90 days postinfection, the response of C57BL and C3H mice had diminished, but that of CD1 mice was not significantly changed.

**Effect of pregnancy.** Neonatal CD1 mice were infected i.p. with $10^5$ PFU of CCV and when 21 days old were weaned and caged separately according to sex. At 7 months after infection, the females were mated with uninfected males, and the presence of vaginal plugs was considered the first day of pregnancy. Mice were challenged with the standard dose of heat-killed virus at various times during pregnancy and lactation. Nonmated neonatally infected females were used as controls and were challenged similarly. The delayed hypersensitivity ear response of the pregnant and nonpregnant mice were as shown on Fig. 3. The ear response was markedly suppressed at all times tested during pregnancy and lactation.

**DISCUSSION**

We have demonstrated a DTH skin reaction that appeared in mice infected with MCMV. It could be elicited 4 to 5 days after infection, reached a peak at 8 days, and showed no signs of decreasing in strength 100 days after infection. It was not a basophil (Jones-Mote type) response and is therefore distinct from that described for guinea pig CMV (Griffith et al., Fifth International Congress of Virology, Strasbourg, 1981). Large doses of heat-killed virus were needed to elicit the response, which was not seen after the more conventional DTH test in the mouse footpad. The ear has been successfully used to demonstrate herpes simplex-specific DTH skin reactions (11), and we were thus encouraged to try the ear. When live virus was used in our test, there was no replication in the inoculated ear, but in previously uninfected mice the ear gradually increased in thickness to reach a maximum at 7 days. MCMV has been shown to replicate in dermal cells in newborn mouse skin (10).

When first tested 5 days after infection the DTH response in a susceptible strain of mice (C57BL) was significantly greater than in resistant C3H mice (Table 3). The greater susceptibility of C57BL than C3H mice confirms the findings of Chalmer et al. (2). Susceptible CD1 mice, however, did not develop significantly different DTH responses from resistant C3H mice. Perhaps DTH has immunopathological as well as protective roles. Attenuated cell culture virus induced strikingly greater DTH responses than virulent salivary gland virus. It is possible that virulent strains of virus are able to suppress DTH to MCMV, a property that would contribute to their virulence.

The DTH response was markedly depressed at all stages tested throughout pregnancy and lactation in mice that had been infected neonatally. Neonatally infected mice have also been shown to reactivate MCMV during pregnancy (5). Depression of CMV-specific CMI in association with pregnancy has not previously been reported in mice, but there have been several reports of impairment of CMV-specific CMI in mothers of infants with congenital CMV infection (4, 13, 14, 17). Furthermore, Gehrz and coworkers (3) very recently showed that CMV-induced lymphocyte proliferation was markedly

**TABLE 1. Comparison of the DTH ear response of mice infected with CCV or SGV**

<table>
<thead>
<tr>
<th>Virus</th>
<th>% Increase at virus dose:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁴ PFU</td>
</tr>
<tr>
<td>CCV</td>
<td>84.4 (76–96)</td>
</tr>
<tr>
<td>SGV</td>
<td>54.9 (40–74)</td>
</tr>
</tbody>
</table>

*Mean percent increase in ear thickness of six mice. The range of readings is within parentheses. Student's t test showed that the response to 10⁴ PFU of CCV was significantly greater than to SGV ($P < 0.01$). Also, the response to the smaller dose of CCV was significantly greater than to the larger dose ($P < 0.02$).

**TABLE 2. Mortality of different strains of mice to i.p. inoculation of two strains of MCMV**

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>No. dead/no. inoculated with the following dose of SGV:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Osborn strain*</td>
</tr>
<tr>
<td></td>
<td>10⁵ PFU</td>
</tr>
<tr>
<td>C57BL</td>
<td>0/6</td>
</tr>
<tr>
<td>CD1</td>
<td>0/7</td>
</tr>
<tr>
<td>C3H</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Similar results were obtained in two other experiments with the Osborn strain of MCMV.

*Numbers within parentheses indicate average survival time in days.

*ND, Not done.
FIG. 3. Effect of pregnancy and lactation on expression of hypersensitive ear swelling to MCMV. Each point represents increase in ear thickness of 6 or 7 (14- and 18-day-pregnant) or 4 or 5 (4-day-pregnant and 5- and 10-day-postpartum) mice. The vertical bars represent 95% confidence limits. Symbols: ear swelling of pregnant mice (○) and nonpregnant mice (●).

TABLE 3. Hypersensitive ear swelling to MCMV in different strains of mice

<table>
<thead>
<tr>
<th>Strain of mice</th>
<th>% Increasea on day after infection:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>C57BL (susceptible)</td>
<td>77b (65–80)</td>
</tr>
<tr>
<td>CD1 (susceptible)</td>
<td>57 (40–71)</td>
</tr>
<tr>
<td>C3H (resistant)</td>
<td>43b (33–50)</td>
</tr>
</tbody>
</table>

a Mean percent increase in ear thickness in groups of six mice. Those tested at 90 days were the same individuals previously tested at 5 or 11 days, but challenged in the opposite ear. The range of readings is within parentheses.
b At 5 days postinfection, the ear swelling was much greater in the C57BL mice than in the C3H mice (P < 0.001)

depressed during the second and third trimesters of normal pregnancy in seropositive women, CMV antibody titers remaining unchanged. The depression, which lasted for nearly a year after delivery, appeared to be selective for CMV because T-cell counts and mitogen-induced proliferation were unaffected.

Further studies are in progress using the mouse model described here, to elucidate the role of depressed CMI and of hormonal changes in the reactivation of CMV infection during pregnancy.

ACKNOWLEDGMENTS

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LITERATURE CITED