The Interaction of Murine Cytomegalovirus With Murine Neutrophils: Effect on Migratory and Phagocytic Activities

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To investigate the interaction of cytomegalovirus (CMV) with neutrophils, we studied the effects of in vitro incubation of murine neutrophils with murine CMV (MCMV). Neutrophils incubated with MCMV for 4 h had depressed chemotactic activity, mean chemotactic index of 1.34 ± 1.43 for MCMV-treated neutrophils vs 3.22 ± 2.11 for controls. Engulfment of latex spheres by MCMV-treated neutrophils was also reduced. These differences were not attributable to loss of cell viability. UV-inactivation of MCMV pools abolished the inhibitory effects of MCMV, indicating that these effects were related to infectivity of the virus. Electron microscopic studies at 4 h demonstrated virus particles within the phagosomes of occasional neutrophils. These studies demonstrate that neutrophil functions can be altered by an in vitro interaction with infectious CMV and suggest that a direct effect of CMV on neutrophils could account for the abnormal neutrophil functions observed during animal CMV infections.

Key words: cytomegalovirus, neutrophils, chemotaxis, phagocytosis

INTRODUCTION

Cytomegalovirus (CMV) infections of humans and animals have been associated with widespread alterations of host defense mechanisms [2,4,9,11,15,19]. Although macrophage and neutrophil functions do not appear to be abnormal during CMV infections of humans [21,25], several investigators have identified altered function of both macrophages and neutrophils during animal CMV infections [1,13,23,26,27]. For example, diminished neutrophil migration has been observed during CMV infec-
sions of guinea pigs and mice [1,13,27], and murine macrophages infected in vitro with murine CMV (MCMV) have exhibited diminished phagocytic activity [23]. Such defects have been suspected to be factors that contribute to the enhanced susceptibility to secondary infection that occurs during CMV infections of both humans and animals [2,16].

In previous experiments we observed that the inflammatory response of neutrophils was impaired markedly during the course of acute MCMV infection [2]. We subsequently found that neutrophils harvested from MCMV-infected mice had reduced chemotactic activity on days 1 and 3 of MCMV infection [1]. Because MCMV was recovered from the neutrophil-rich fraction of peripheral blood during acute infection, we postulated that altered neutrophil function could be the result of a direct effect of infectious virus on neutrophils [1].

The current experiments were designed to investigate the in vitro interaction of MCMV with murine neutrophils. Our objectives included: 1) to determine the effect of MCMV on the chemotactic and phagocytic activity of neutrophils, and 2) to determine whether MCMV could be detected in neutrophils.

MATERIALS AND METHODS

Virus

Using techniques described previously [2], the Smith strain of MCMV was prepared as a 10% wt/vol homogenate of salivary glands harvested from 3-wk-old female Swiss-Webster mice infected with MCMV 14 days earlier. MCMV prepared in this fashion regularly titered 10^7 plaque-forming units (pfu) of MCMV per milliliter when assayed on confluent monolayers of mouse embryo fibroblast (MEF) cells [1,11]. Salivary gland homogenates were used as the source of MCMV for two reasons: 1) high-titer pools could be readily prepared, and 2) salivary gland-derived MCMV has been shown to be highly virulent [10]. A normal salivary gland pool was prepared from uninfected 3-wk-old mice using similar techniques. As an additional control, aliquots of the MCMV pool were UV-inactivated using techniques described by Shanley [22]. MCMV-containing suspensions on ice were exposed to a GTE 15T8 germicidal ultraviolet light at 10 cm for 10 min. Pools treated in this fashion contained less than 10^3 pfu of MCMV per milliliter.

Preparation of Neutrophils

Murine neutrophils were collected from polyvinyl sponges implanted in 3-wk-old Swiss-Webster mice [1,3]. In brief, sterile 5 × 7 mm sponges (Ivalon, Unipoint Industries, High Point, NC) were placed in the dorsal neck region of anesthetized mice, and the sponges were wet with 0.1 ml of sterile phosphate-buffered saline (PBS). Twenty hours later, the sponges were removed and the fluid collected into microcentrifuge tubes. This procedure typically yielded between 0.5 and 1.5 × 10^6 neutrophils per mouse. Viability was assessed by trypan blue exclusion, and the concentration of neutrophils was adjusted to 1 × 10^6 viable neutrophils/ml by the addition of Eagle’s minimum essential medium containing potassium penicillin 100 units/ml and streptomycin sulfate 50 μg/ml without calf serum (MEM).

Experimental Infection

Treatment of neutrophils from individual mice consisted of incubation of 2 × 10^5 neutrophils with MCMV at multiplicities of 0.1 or 10 pfu of MCMV per
neutrophil or with equivalent dilutions of normal salivary gland homogenate. Prior to use, MCMV pools and normal salivary gland homogenates were filtered through a 1.2 μm Millipore filter. Cell suspensions were incubated on a rotator at 37°C in 5% CO₂. After incubation neutrophils were removed, washed once with MEM by centrifugation at 300 × g for 10 min, and resuspended in MEM. Cell concentration and viability were determined immediately before each assay of neutrophil function.

Chemotaxis Assay

Using techniques described previously, the chemotactic activity of paired control neutrophils and neutrophils incubated with MCMV was assayed in a 48-well microchemotaxis apparatus (Neuroprobe, Inc., Bethesda, MD) [1,3,7]. These studies used a 5-μm-pore size polycarbonate filter and a chemotactic factor prepared as a culture filtrate of Escherichia coli grown in medium 199 for 24 h [1,3]. Random migration was determined by incubating cells against MEM in place of bacterial factor. The filled chemotaxis apparatus was incubated for 60 min at 37°C in 5% CO₂. After incubation, the filter was washed with PBS, stained, and the numbers of neutrophils in ten random fields were counted. The chemotactic index was then determined by the formula: (number of neutrophils migrating to the bacterial factor minus random migration of neutrophils) × 100 divided by the number of viable neutrophils added per well. Random migration was less than 10% of the directed migration in all assays. Assays were run in duplicate and results expressed as the average of replicates for each animal.

Phagocytosis (Engulfment) Assay

In additional experiments, the phagocytic activity of neutrophils was determined by assaying engulfment of fluorescent latex particles [5]. Cell suspensions, containing 5 × 10⁴ viable neutrophils from control or MCMV-treated specimens, were incubated for 1 h at 37°C with normal mouse serum (10% final concentration) and fluoresbrite microspheres (1.78 μm diameter, Polyscience, Inc., Warrington, PA) at a ratio of 100 spheres per cell. This ratio was determined by preliminary experiments using ratios ranging from 0.1 to 1,000 spheres per cell [3]. After incubation, cells were washed twice with PBS by centrifugation and fixed with 0.1 ml of 1% glutaraldehyde. Phagocytic activity was determined by examining wet preparations of cell suspensions using a fluorescence microscope with transmitted light (Olympus Optica, Ltd., Tokyo, Japan). Only spheres within phagocytes were counted. One hundred phagocytic and nonphagocytic cells were counted, and the phagocytic activity of the leukocytes was expressed as a percentage of the total cells. The number of fluorescent spheres per phagocytic cell was also determined.

Adsorption of MCMV to Neutrophils

Adsorption of MCMV to neutrophils was assessed by incubating cells with MCMV at a multiplicity of 10 pfu of MCMV per leukocyte and by assaying residual MCMV in culture supernatants. Neutrophils were harvested from sponges, suspended in MEM to a concentration of 8 × 10⁴ cells/ml, and incubated with MCMV at 37°C on a rotator. At 0, 1, 2, and 4 h aliquots of supernatant fluids were removed, centrifuged at 200 × g for 10 min, and collected for assay of MCMV. As a control, adsorption of MCMV to MEF cells was also determined. MEF cells were harvested by trypsinization from confluent monolayers, washed with MEM, and incubated with
MCMV at a ratio of 10 pfu MCMV/MEF cell. At 0, 1, 2, and 4 h, supernatant fluids were removed and assayed in similar fashion for MCMV.

Electron Microscopic Studies

Transmission electron microscopic studies were performed on cells harvested 4 h after incubation with either normal salivary gland homogenates or MCMV. Cells were washed once with PBS by centrifugation, fixed with one-fourth strength Karnovsky fixative [1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4)] and post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide in cacodylate buffer (pH 7.2). Samples were dehydrated in graded alcohols, embedded in Spurr's plastic, and sectioned. Thin sections were stained with 5% uranyl acetate and examined using a Hitachi 600 transmission electron microscope. Controls for virus identification included salivary glands from MCMV-infected mice and mouse embryo fibroblast cell monolayers infected with MCMV.

Statistical Methods

Chemotactic and phagocytic activities of neutrophils were compared using paired or independent two-tailed t tests.

RESULTS

Chemotactic Activity of Neutrophils Incubated with MCMV

In preliminary experiments, we studied the chemotactic activity of neutrophils after 2, 4, 8, or 16-h incubations with either MCMV or normal salivary gland homogenates. Chemotaxis of neutrophils incubated with MCMV was not altered after a 2-h incubation. After 8 h, cell viability decreased significantly (mean of 60% at 8 h vs 87% at 4 h and 94% at 2 h), and neutrophil migration as negligible. Subsequent experiments therefore focused on 4-h incubations. We first compared the chemotactic activity of neutrophils incubated with normal salivary gland homogenates.

As summarized in Table 1, neutrophils incubated for 4 h with MCMV (10 pfu of MCMV per neutrophil) had reduced chemotactic activity—chemotactic index of 1.34 ± 1.43 for MCMV-treated cells vs 3.22 ± 2.11 for normal salivary gland treated cells (P < .01). Reduced chemotactic activity of MCMV-treated neutrophils at 4 h was not due to decreased neutrophil viability (mean viability of 87.1 ± 12% for MCMV-treated vs 87.4 ± 8.6% for controls). Neutrophils incubated with 0.1 pfu of MCMV per neutrophil had normal chemotactic activity (Table 1), suggesting that the effects were related to virus dose.

To control the effects of salivary gland cell lysates, present in both MCMV-containing and normal salivary gland pools, we also assayed additional neutrophils incubated only with MEM (Table 1). These neutrophils had greater chemotactic activity, indicating that the soluble materials in salivary gland lysates had a detrimental effect on chemotactic activity of neutrophils.

To determine whether the reduced chemotactic activity of neutrophils incubated with MCMV was attributable to infectious virus, we next studied the effects of UV-inactivated MCMV pools on neutrophil chemotaxis. In these experiments, paired samples of neutrophils were incubated for 4 h with either infectious MCMV (10 pfu MCMV per neutrophil) or UV-inactivated MCMV. As shown in Figure 1, neutrophils incubated with UV-exposed MCMV had greater chemotactic activity than cells
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TABLE 1. Chemotactic Activity of Neutrophils Incubated for 4 h With MEM, MCMV Pools, or Normal Salivary Gland Homogenate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemotactic activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MOI 10 pfu MCMV/neutrophil</th>
<th>MOI 0.1 pfu MCMV/neutrophil</th>
</tr>
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<tbody>
<tr>
<td>MCMV</td>
<td>1.34 ± 1.43&lt;sup&gt;b&lt;/sup&gt; (n = 14)</td>
<td>5.17 ± 3.13 (n = 11)</td>
<td></td>
</tr>
<tr>
<td>Normal salivary gland</td>
<td>3.22 ± 2.11 (n = 14)</td>
<td>5.32 ± 3.08 (n = 11)</td>
<td></td>
</tr>
<tr>
<td>MEM</td>
<td>6.78 ± 3.06&lt;sup&gt;c&lt;/sup&gt; (n = 8)</td>
<td>-</td>
<td></td>
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</table>

<sup>a</sup>Mean ± SD.

<sup>b</sup>Significantly less than the activity of neutrophils incubated with either normal salivary gland homogenate or MEM (P < .01).

<sup>c</sup>Significantly greater than the activity of neutrophils incubated with either MCMV-containing or normal salivary gland homogenate (P < .01).

MOI = Multiplicity of infection.

n = Number of individual animals.

incubated with infectious virus—mean chemotactic index of 3.23 ± 1.82 for neutrophils incubated with UV-exposed MCMV vs 1.85 ± 1.25 for infectious MCMV-treated cells (P < .01). Thus, the chemotactic activity of neutrophils incubated with UV-inactivated MCMV was nearly identical to the activity of control cells incubated with normal salivary gland homogenates—mean chemotactic index of 3.23 ± 1.82 for UV-inactivated MCMV vs 3.22 ± 2.11 for cells incubated with normal salivary gland. These studies strongly suggest that reduced chemotactic activity of neutrophils was the result of an interaction of infectious MCMV particles with neutrophils.

Phagocytic Activity

The phagocytic activity of neutrophils was expressed in two manners: 1) the percentage of cells ingesting latex spheres, and 2) the number of latex spheres per phagocytic cell. At 2 h the percentage of phagocytic cells did not differ significantly for neutrophils incubated with either normal salivary gland or MCMV. These values were significantly lower than the mean activity for neutrophils incubated with MEM, 23.8 ± 16.7%, indicating that factors in salivary gland homogenates reduced phagocytic activity. At 4 h, however, the phagocytic activity of neutrophils incubated with infectious MCMV was significantly diminished, when compared with either normal salivary gland or MEM-treated neutrophils (Table 2).

Neutrophils incubated with MCMV also had fewer spheres per cell than neutrophils incubated with either normal salivary gland or MEM (Table 2). At 2 h there was a trend toward fewer spheres in neutrophils incubated with MCMV. At 4 h neutrophils incubated with MCMV had significantly few spheres—mean of 1.53 ± 0.33 for MCMV vs 2.39 ± 0.67 for normal salivary gland and 2.39 ± 0.56 for MEM (P < .05). These results indicate that incubation with MCMV for 4 h diminished the number of cells ingesting spheres and reduced the number of spheres ingested by each phagocytic cell.

To study the mechanism by which incubation with MCMV reduced phagocytic activity, we also incubated neutrophils with UV-exposed MCMV and assayed phagocytic activity at 4 h. Both the percentage of phagocytic cells and the number of particles per cell were not significantly different for neutrophils incubated with UV-
exposed MCMV vs cells treated with normal salivary gland homogenates exposed to UV light (mean of 11.7 ± 7.5% and 2.09 ± 0.45 for UV-MCMV vs 11.2 ± 6.4% and 2.33 ± 0.45 for controls). These results suggest that reduced phagocytic activity at 4 h resulted from events associated with a direct interaction of infectious virus with phagocytic leukocytes.

**Adsorption of MCMV**

Adsorption of MCMV to neutrophils did not differ from adsorption of MCMV to MEF cells, a permissive cell for MCMV. The percentage of pfu of MCMV remaining in culture supernatants after the 4 h incubation was 76% for neutrophils vs 73% for MEF cells. For example, titers of MCMV in supernatant fluids of neutrophil incubations dropped from a mean of 7.8 ± 0.88 x 10^5 at time 0 to a mean of 5.7 ± 0.62 x 10^5 (N = 5) after 4 h.

**Detection of MCMV in Phagocytic Leukocytes**

After a 4-h incubation with MCMV, neutrophil suspensions were examined by transmission electron microscopy for viral particles. In occasional cells, herpes-like virions were identified in phagosomes (Fig. 2a, b). These particles were similar in size and morphology to virions observed in control sections of MCMV-infected
### TABLE 2. Phagocytic Activity of Neutrophils Incubated With MCMV, Normal Salivary Gland Homogenate, or MEM

<table>
<thead>
<tr>
<th></th>
<th>2 h</th>
<th>4 h</th>
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<tbody>
<tr>
<td></td>
<td>% Phagocytic cells$^a$</td>
<td>Spheres/cell$^a$</td>
</tr>
<tr>
<td>MCMV (10 pfu/neutrophil)</td>
<td>6.36 ± 4.87 (n = 11)</td>
<td>1.71 ± 0.60 (n = 11)</td>
</tr>
<tr>
<td>Normal salivary gland</td>
<td>8.46 ± 6.16 (n = 11)</td>
<td>2.35 ± 0.97 (n = 11)</td>
</tr>
<tr>
<td>MEM</td>
<td>23.8 ± 16.7$^c$ (n = 12)</td>
<td>2.43 ± 1.06 (n = 12)</td>
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</table>

$^a$Mean ± SD.
$^b$Significantly less than the phagocytic activity of neutrophils incubated with either MEM or normal salivary gland homogenate (P < .05).
$^c$Significantly greater than the phagocytic activity of neutrophils incubated with either MCMV or normal salivary gland homogenate (P < .01).

n = Number of individual animals.
Fig. 2.  a) Electron micrograph of a murine leukocyte containing a herpes-like virion within a phagosome (arrow) (image magnification × 20,000, bar equals 1 μm). b) Higher magnification of the same cell showing an enveloped virion with a dense core (image magnification × 57,000, bar equals 0.1 μm).
Fig. 3. Electron micrograph of MCMV-infected salivary gland tissue, as used to prepare MCMV pools. This section of the nucleus shows multiple viral nucleocapsids (open arrow) and an enveloped virion (solid arrow) similar to particles observed within murine neutrophils incubated in vitro with aliquots of MCMV pools (image magnification × 57,000, bar equals 0.1 μm).

Salivary gland tissues (Fig. 3) and resembled the herpes-like virions identified in neutrophils during human CMV infections [14]. Approximately 1 in every 100 murine neutrophils contained herpes-like particles identifiable by electron microscopy.

DISCUSSION

Observations in animals indicate that CMV infections can be accompanied by abnormalities in neutrophil functions. Yourtee and co-workers noted neutropenia, diminished neutrophil migration, diminished H₂O₂ release, and reduced bactericidal activity of neutrophils harvested from the peritoneal cavity during CMV infection of guinea pigs [27]. Similarly, Tannous and Myers observed chemotactic defects of peripheral blood neutrophils from CMV-infected guinea pigs [26]. In previous studies of MCMV infection we observed impaired inflammatory responses to bacterial stimuli, as well as diminished migratory and chemotactic activity of neutrophils harvested from MCMV-infected mice [1,2]. These abnormalities occurred on days 2 through 4 of MCMV infection and correlated strongly with the timing of enhanced mortality and impaired clearance of bacteria observed during combined MCMV-Escherichia coli infections [2]. Recently, Lineaweaver and co-workers also observed impaired directed and random migration of neutrophils harvested from peritoneal fluids during days 3, 5, and 8 of a nonlethal MCMV infection [13].
Although the mechanism responsible for these defects in neutrophil function has not been completely defined, a direct interaction of neutrophils by CMV could conceivably contribute to these abnormalities. The defects observed by Yourtée and associates correspond with CMV viremia, and infectious virus could be recovered from guinea pig neutrophils [27]. Similarly, abnormal neutrophil function in MCMV-infected mice correlated with recovery of MCMV from the neutrophil-rich fraction of peripheral blood [1]. However, in both mice and guinea pigs, the amount of infectious virus present in neutrophils was relatively small, on the order of 10^2 pfu of CMV per 10^6 neutrophils [1,27]. Because we observed MCMV antigen in greater numbers of neutrophils, we postulated that abortive infection of neutrophils could also contribute to altered function [1].

As an alternative explanation, Tannous and Myers suggested that abnormal neutrophil function resulted from inhibitory factors that were produced during CMV infections [26]. These investigators observed that plasma from CMV-infected guinea pigs inhibited the chemotactic activity of neutrophils from normal animals. Inhibitors were of two types, one cell-directed and the other directed against C5-derived chemotactic fractions. Inhibitory activity peaked on day 4 of infection and disappeared by day 10. Thus, the time course of plasma inhibitory activity corresponded with the timing of neutrophil abnormalities observed during both guinea pig and murine CMV infections.

The current studies investigated the in vitro interaction of MCMV with neutrophils. After a 4-h incubation with MCMV, neutrophils had impaired chemotaxis and modestly reduced phagocytic functions. These alterations were not due to decreased viability of MCMV-treated neutrophils. UV-inactivation of MCMV pools abolished the inhibitory effect on chemotactic and phagocytic activities, suggesting that a direct interaction of infectious virus with neutrophils accounted for these abnormalities. Adsorption of MCMV to neutrophils, although difficult to quantitate, appeared similar to adsorption of MCMV to MEF cells; electron microscopic studies established the presence of MCMV within an occasional neutrophil.

The relationship of these neutrophil abnormalities in experimental animals to CMV infections of humans has not been fully established. Rinaldo and associates studied neutrophil function in patients with CMV-mononucleosis and found no defects [21]. However, because defects in experimental animals occurred early after infection and were transient, the possibility exists that defects could be missed in humans with CMV mononucleosis. In contrast, Soriano and co-workers described abnormal neutrophil functions and recurrent staphylococcal infections in a young child with chronic CMV infection [25].

Several observations indicate that blood leukocytes, including neutrophils, contain infectious virus during CMV infections of humans [8,9,12,18]. Recently, Martin and co-workersdetected CMV in leukocytes using molecular hybridization techniques in a patient with leukemia who developed fatal CMV pneumonitis [14]. Neutrophils from this patient were estimated to contain approximately ten viral genome copies per cell. In addition, these investigators identified herpes-like virions within phagosomes of approximately 3% of the patient's neutrophils. Functional studies of neutrophils were not performed in this patient.

Productive infection of human leukocytes has not been observed when leukocytes have been incubated with CMV in vitro [20]. Leukocytes adsorb virus, but viral replication does not take place [24]. Recently, Einhorn and Ost, using immunofluo-
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rescence techniques demonstrated that certain human CMV strains induced early antigens in human leukocytes incubated with CMV [6]. Wild strains of CMV, isolated from the urines of congenitally infected infants, induced antigens in between 0.03 and 2.35% of leukocytes. The majority of cells expressing antigens were monocytes, but some neutrophils did contain early antigens. This suggests that CMV-related molecular events can occur within neutrophils, and that such events could alter cellular functions. The recent studies of Rice and co-workers further support this possibility. These investigators, studying in vitro CMV infection of human mononuclear leukocytes, demonstrated that abortively infected cells lost certain immune functions such as the response to phytohemagglutinin [17].

In summary, the current experiments demonstrate that neutrophil chemotactic and phagocytic activities can be impaired by in vitro interaction with infectious MCMV. Such abnormalities in function may be related to early molecular events associated with MCMV infection of leukocytes. These studies thus suggest that a direct effect of CMV on neutrophils could account for abnormal neutrophil functions that occur during CMV infections.

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