INFECTION AND IMMUNITY, Apr. 1982, p. 11-16
0019-9567/82/040011-06$02.00/0

Vol. 36, No. 1

Neutrophil Response and Function During Acute Cytomegalovirus Infection in Guinea Pigs

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Received 9 September 1981/Accepted 13 November 1981

The mobilization and functional characteristics of polymorphonuclear leukocytes (PMNs) at the site of an inflammatory stimulus were studied during acute cytomegalovirus infection in guinea pigs. Weanling Hartley strain guinea pigs were inoculated subcutaneously with approximately 10^6 50% tissue culture infective doses of virulent salivary gland-passaged guinea pig cytomegalovirus. The virus was uniformly present in bone marrow, buffy coat, and casein-elicited peritoneal exudate cells 5 to 7 days after the inoculation. The mean numbers of circulating PMNs in the animals were 2,862/μl in uninfected controls and 880/μl in infected animals. The total peritoneal PMNs recovered 14 h after casein injection were 491×10^6 and 237×10^6 in control and infected animals, respectively. The number of 50% tissue culture infective doses of guinea pig cytomegalovirus per 10^6 purified peritoneal PMNs was $10^{2.17}$. Neutrophil O_2 consumption was similar in infected and control animals in response to either stimulation by a neutrophil activator, phorbol myristate acetate, or phagocytosis of *Staphylococcus aureus*. However, the maximal rate of H_2O_2 release and the percent killing of *S. aureus* by peritoneal exudate cells from infected animals were significantly reduced compared with uninfected controls during acute infection. Granulocytopenia, a decreased mobilization of PMNs to the site of the inflammatory stimulus, a diminished cellular release of H_2O_2 in response to a bacterial stimulus, and a modest reduction in bacterial killing were demonstrated during experimental acute cytomegalovirus infection. Such reductions in granulocyte number and function at inflammatory sites during this type of infection could alter antimicrobial defenses.

Human cytomegalovirus (CMV) infections cause significant morbidity and mortality among renal, cardiac, and bone marrow transplant recipients (6, 10, 15–17, 22). In particular, morbidity is heightened by the development of superinfections with bacterial, fungal, or parasitic pathogens during primary CMV infection in these patients (16, 17).

CMV infections in experimental animal models enhance macrophage chemiluminescence but impair lymphocyte responsiveness and increase susceptibility to bacterial and fungal superinfection (8, 14, 24). CMV has been isolated from both human and guinea pig granulocytes during acute infections (7, 18). In addition, acute CMV mononucleosis has been associated with leukopenia both in humans and in the guinea pig CMV (GPCMV) model (7, 22). Nonetheless, Rinaldo et al. (19) did not find any associated defects in the function of polymorphonuclear leukocytes (PMNs) taken from three patients with mononucleosis during acute human CMV infection.

GPCMV infection closely simulates several aspects of human disease. Acute viremia, transplacental CMV transmission, fetal infection, chronic viruria, blood transmission, and protection by vaccination have been examined in this animal model (2–4, 11). We recently investigated CMV-induced mononucleosis in guinea pigs during acute GPCMV infection (7). Viremia, marrow infection, and hematological changes comparable to those seen in human infection occur acutely and resolve approximately 1 month after virus inoculation. This model appears to be useful for further investigation of phagocytic cell function during acute CMV infection.

The purpose of this study was to investigate both the distribution and the effects of GPCMV in bone marrow, circulating neutrophils, and peritoneal exudate cells during acute GPCMV mononucleosis when viremia and granulocytopenia are present.

We attempted to determine whether acute viremic CMV infection itself was associated with as yet undefined defects in the mobilization

and function of neutrophils. Alterations in the formation of peritoneal exudate, in the release of H_2O_2 by neutrophils, and in the microbicidal activity of this phagocytic cell population are described in this study. Results should prove to be applicable to understanding the consequences of acute CMV infections and the immunosuppressive regimens required for the maintenance of organ transplant recipients.

MATERIALS AND METHODS

Virus strain and animal inoculation. The prototype strain of GPCMV (no. 22122; American Type Culture Collection, Rockville, Md.) was used. The preparation and subcutaneous inoculation of virulent salivary gland-passaged GPCMV suspension (GPCMV-SG) were described previously (3). Infectivity titers ranged from 5.5 to 6.6 log 50% tissue culture infective doses ($TCID_{50}$) per ml at passage level 15 in guinea pigs. Experimental animals received 1 ml of stock virus suspension; age- and sex-matched control animals received 1 ml of uninfected salivary gland suspensions subcutaneously.

Male and female weanling Hartley strain guinea pigs (1 to 2 months old) were purchased from Camm Research Institute, Wayne, N.J., and tested before virus inoculation for viremia and neutralizing antibodies to GPCMV to identify any previously infected animals. Sterile 12% neutral sodium caseinate (20 ml; Eastman P 914, Eastman Kodak Co., Rochester, N.Y.) was inoculated intraperitoneally into each animal 14 to 16 h before sacrifice on days 3, 5, 7, 14, 21, and 28.

Sampling of blood, tissues, and peritoneal exudate for virus recovery. Preparation of guinea pig embryo monolayer cell cultures, sampling of cardiac blood and bone marrow, inoculation of cell cultures, and identification of virus isolates by neutralization tests were described previously (2, 3). Peritoneal exudates were removed at sacrifice under sterile conditions, and the peritoneum was washed with Hanks balanced salt solution. Exudate cells were removed from the peritoneal supernatant by centrifugation. In some experiments the exudate suspension was centrifuged on a Ficoll-Hypaque gradient at 400 × *g* for 30 min at 4°C to yield a granulocyte-enriched pellet. This neutrophil fraction was then washed in phosphate-buffered saline, and cells were counted. Infectivity titers were determined by serial 10-fold dilutions, and cells were cocultivated with guinea pig embryo monolayer cultures. Hematocrits, leukocyte counts, differential counts, and bone marrow smears were performed by standard techniques described previously (7).

Oxidative function of peritoneal exudate cells. For functional studies, exudate cells were washed in modified Hanks solution, contaminating erythrocytes were removed by hypotonic lysis (20), and cells were resuspended in Hanks balanced salt solution and counted. Oxygen uptake by casein-elicited peritoneal cells was measured with a Clark oxygen electrode as described previously (21). Cells at a final concentration of 5×10^6 PMNs/ml were stimulated either with phorbol myristate acetate (PMA) at a final concentration of 100 ng/ml or with a maximal phagocytic stimulus. PMA is a strong stimulant of neutrophil function which is independent of phagocytic events (5). The phagocytic stimulus was provided by heat-killed *Staphylococcus aureus*, which was preopsonized in normal guinea pig serum and added to neutrophils at a final bacterium-to-PMN ratio of 500:1.

Hydrogen peroxide release from exudate cells was measured by the scopoletin-horseradish peroxidase technique (20). Cells at a final concentration of 2.5×10^6 PMNs/ml were stimulated as described above, and H_2O_2 release was measured as the loss of fluorescence of reduced scopoletin.

Phagocytosis and bacterial killing by peritoneal exudate cells. Phagocytic uptake of radiolabeled bacteria was performed as previously described (9). Heat-killed *S. aureus* grown in mixed ^{14}C-amino acids was added to 2.5×10^6 PMNs/ml in a final bacterium-to-PMN ratio of 500:1 in the presence of normal guinea pig serum as an opsonin. These conditions were chosen to correspond to the maximal stimulus used for oxidative studies. Additionally, phagocytosis was evaluated using live, radiolabeled *S. aureus* 502A preopsonized with normal guinea pig serum, with 2.5×10^6 PMNs/ml at a final bacterium-to-PMN ratio of 10:1, to correspond to the conditions used to assess bactericidal activity. Binding of bacteria to neutrophils (phagocytosis plus adherence) after 20 min of incubation was determined by the association of radioactive counts with the neutrophil pellet after washing and expressed as the percentage of counts present in the initial bacterial inoculum.

Bacterial killing of *S. aureus* 502A was performed with staphylococci in the log phase of growth, preopsonized in normal guinea pig serum, and mixed with 2.5×10^6 PMNs/ml at a final bacterium-to-PMN ratio of 10:1 for optimal bactericidal activity. After 20 min of incubation, surviving bacteria were quantitated by a pour plate technique described previously (9). Percent killing was calculated as 100 minus the percentage of the initial bacterial inoculum surviving. Statistical comparisons were performed for paired data with the signed rank test or the paired *t* test when samples were sufficiently large.

RESULTS

Acute CMV infection. Acute guinea pig infection with virulent GPCMV-SG consistently caused viremia in animals 5 to 7 days after virus inoculation (Table 1). During this time, virus was also isolated from buffy coat of infected animals, from bone marrow, and from elicited peritoneal cells, but it was not recovered from the cell-free supernatants of peritoneal exudates. CMV was generally not recovered from any of these sites in 11 additional animals sacrificed on day 3 or from days 14 to 28 after virus inoculation. Even in animals sacrificed 5 to 7 days after virus inoculation, relatively few cells in peritoneal exudate were infected: 2.25 log $TCID_{50}$ of GPCMV per 10^6 cells were recovered from the unseparated population of peritoneal exudate cells, and 2.17 log $TCID_{50}$ of GPCMV per 10^6 cells were recovered from the purified PMN fraction (Table 1).

Effects of CMV infection on neutrophil counts.

TABLE 1. Recovery of GPCMV from Hartley strain guinea pigs during acute GPCMV-SG infection[a]

Animal group	Material inoculated	No. inoculated	No. of animals from which GPCMV was isolated				Log $TCID_{50}$ of GPCMV per 10^6 cells[b]	
			Bone marrow	Buffy coat	Peritoneal cells	Peritoneal supernatant	All exudate cells	Exudate PMNs
Controls	Uninfected salivary gland	32	0	0	0	0	0	0
CMV infected	GPCMV-SG	38	35	36	35	0	2.25	2.17

[a] All animals tested 5 to 7 days after inoculation of uninfected salivary gland or GPCMV-SG.
[b] Mean values for 14 animals in each group.

The effects of acute CMV infection on bone marrow, peripheral blood, and peritoneal exudate are shown in Table 2. Infected animals were leukopenic on days 5 to 7 of infection, with a pronounced decrease in the percentage of circulating PMNs resulting in significant granulocytopenia. Mean circulating PMN counts in CMV-infected animals were approximately 30% of mean counts from matched control animals (880 versus 2,863 PMNs/μl, $P < 0.005$). CMV-infected guinea pigs had lower mean hematocrits (42.3 versus 46.3, $P < 0.005$) and lower myeloid/erythroid ratios in bone marrows (0.89 versus 1.77, $P < 0.005$) when compared with uninfected controls. However, the decrease in the myeloid/erythroid ratio appeared to be largely attributable to a relative increase in the erythroid series.

Concomitant with viremia and granulocytopenia, there was a decrease in the total number of neutrophils mobilized into the peritoneum of CMV-infected animals in response to the casein inflammatory stimulus (Table 2). The mean total number of leukocytes mobilized was approximately half that of uninfected controls (287×10^6 versus 549×10^6, $P < 0.005$), with a slightly lower percentage of neutrophils present. Hence, there was a greater than 50% reduction in total neutrophils mobilized into the inflammatory exudate in acutely CMV-infected guinea pigs when compared with controls (237×10^6 versus 491×10^6, $P < 0.005$).

Neutrophil function in CMV-infected animals. Oxygen consumption by normal neutrophils increases after phagocytosis of opsonized staphylococci, or after exposure to PMA, the action of which is independent of phagocytosis. Peritoneal neutrophils from CMV-infected guinea pigs and from control animals did not differ in their O_2 consumption in response to either of these stimuli (Table 3).

The release of H_2O_2 after neutrophil activation by PMA was equivalent in CMV-infected and uninfected control exudate cells (Fig. 1A). However, H_2O_2 release after maximal phagocytic stimulation with opsonized staphylococci was significantly decreased in the cells from infected animals (Fig. 1B). This did not appear to be a consequence of decreased phagocytic uptake, since binding of opsonized staphylococci to exudate cells under similar conditions (a 500:1 bacterium-to-PMN ratio) was not altered in cells from infected animals (Table 3).

Since H_2O_2 is an important bactericidal product of neutrophils, we measured their bactericidal activity against preopsonized *S. aureus* 502A at a bacterium-to-PMN ratio of 10:1. The bactericidal activity of neutrophils from CMV-infected exudate was slightly but significantly diminished compared with matched control exudate neutrophils (Fig. 1C). Neutrophil uptake of bacteria measured simultaneously at the 10:1 ratio of bacterium-to-PMN was, however, similar to

TABLE 2. Effects of acute GPCMV infection on bone marrow, peripheral blood, and peritoneal exudate formation[a]

Animal group	Bone marrow (myeloid/erythroid ratio)	Peripheral blood				Peritoneal exudate		
		Hematocrit	Leukocytes per μl	% PMNs	PMNs/μl	Total leukocytes (millions)	% PMNs	Total PMNs (millions)
Controls	1.77	46.3	5,562	47.6	2,862	549	88.5	491
CMV infected[b]	0.89	42.3	3,423	26.9	880	287	84.3	237

[a] Mean values for 21 to 37 animals, tested 5 to 7 days after inoculation of uninfected salivary gland or GPCMV-SG.
[b] $P < 0.005$ compared with matched control animals for all parameters listed.

TABLE 3. Effects of acute GPCMV infection on bacterial binding and maximal rate of oxygen consumption by peritoneal exudate cells[a]

Animal group	O_2 consumption (nmol/min)[b]		% Binding[c]	
	PMA	Staphylococci (500:1)	Staphylococci (500:1)	Staphylococci (10:1)
Controls	17.8 ± 1.5 (6)	16.5 ± 1.7 (6)	38.8 ± 3.6 (16)	62.9 ± 2.1 (8)
CMV infected	14.8 ± 1.4 (6)	14.3 ± 1.6 (6)	39.4 ± 3.2 (23)	57.7 ± 2.2 (7)

[a] All animals tested 5 to 7 days after inoculation of uninfected salivary gland or GPCMV-SG. Mean ± standard error of (*n*) animals.

[b] Maximal rate of O_2 consumption by 5×10^6 PMNs after stimulation by PMA or preopsonized staphylococci (see text).

[c] Percentage of opsonized radiolabeled bacteria bound to cells after 20 min of incubation with 2.5×10^6 PMNs/ml at the bacterium-to-PMN ratios noted.

that of cells from control animals (Table 3), indicating that the small difference in bactericidal activity was not attributable to a difference in phagocytosis.

DISCUSSION

The effects of acute CMV infection on human neutrophil functions are, as yet, not well understood. Although several viruses are known to depress neutrophil chemotaxis or bactericidal ability in vitro or in vivo (1, 23), there is no direct evidence of such effects occurring in human CMV infections. CMV can be cultured from the neutrophil fraction of human blood during acute infection, which suggests the opportunity for modification of neutrophil function by CMV (18). Rinaldo et al. (19) examined peripheral blood neutrophils from three patients with acute CMV infections but found no significant alterations in phagocytosis, chemotaxis, or the reduction of Nitro Blue Tetrazolium dye (a measure of oxidative function). Mice acutely infected with murine CMV demonstrate increased mortality rates during bacterial and fungal infections in which PMN host defense is of considerable importance (8).

GPCMV infection allows a detailed examination of PMN function during acute CMV mononucleosis, using age- and sex-matched control animals to assess virus distribution, quantitation in various cell populations, and minimization of the effects of technical variations in functional studies. Using this model, Griffith et al. (7) demonstrated leukopenia during the period of acute viremia occurring in acute GPCMV mononucleosis. The present study demonstrated that GPCMV was present in bone marrow, circulating blood cells, and peritoneal exudate neutro-

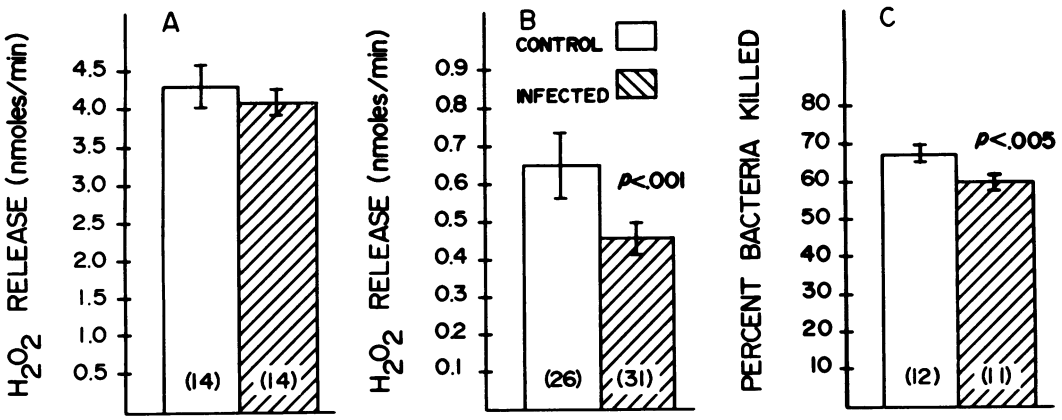

FIG. 1. (A) Maximal rate of H_2O_2 release from 2.5×10^6 peritoneal exudate PMNs per ml after neutrophil activation with PMA. There was no difference between cells from control and infected animals. (B) Maximal rate of H_2O_2 release from 2.5×10^6 peritoneal exudate PMNs per ml after phagocytosis of heat-killed, preopsonized staphylococci. H_2O_2 release from cells of CMV-infected guinea pigs is diminished in comparison with the release from cells of uninfected controls (0.46 versus 0.65, $P < 0.005$). (C) Bactericidal activity of peritoneal exudate cells against preopsonized staphylococci. Percent bacteria killed by cells from CMV-infected animals is decreased in comparison with uninfected controls (59.0 versus 66.9, $P < 0.005$).

phils during this viremic phase. This virus was cell associated and could not be cultivated from cell-free exudate supernatant. After the separation of peritoneal cells, GPCMV was isolated from PMN-enriched fractions, although low virus titers indicated that only a small proportion of cells in peritoneal exudate contained infectious virus.

During this period of viremia, when virus was present in bone marrow and circulating PMNs, we attempted to determine whether acute CMV infection altered PMN function. Infected animals showed pronounced granulocytopenia and a significant depression of their exudative response to intraperitoneal casein. The PMNs from these exudates showed normal phagocytic uptake after a maximal bacterial challenge (a 500:1 bacterium-to-cell ratio), but a decreased release of H_2O_2 when given the same challenge. The normal response to PMA, an agent which is thought to act directly on the neutrophil surface membrane, indicated that PMN oxidative capacity remained intact and suggests a possible defect in the coupling of phagocytosis to postphagocytic oxidative events.

The functional importance of this decreased H_2O_2 release may be reflected in the small but consistent decrease in bacterial killing by CMV-infected exudate neutrophils, despite their normal binding of labeled bacteria under assay conditions. This observation supports the postphagocytic nature of these altered events. Defective intracellular killing of *S. aureus* by alveolar macrophages has been described during Sendai virus pneumonia by Jakab and Green (12), and appears to correlate with a defect in phagosome-lysosome fusion after the ingestion of organisms (13). The mechanism of this effect remains unclear, and it is not evident whether such a defect could cause a normal metabolic burst of oxygen consumption but a decrease in H_2O_2 release. Other mechanisms may also be involved since a dissociation of phagocytosis from oxidative metabolic events has been described after the removal of PMN surface sialic acid with neuraminidase, an enzyme which can be virus associated (25).

The present study, therefore, demonstrates that granulocytopenia was accompanied by a moderate decrease in the peritoneal exudate response to an inflammatory stimulus, intraperitoneal casein, during acute GPCMV mononucleosis. Neutrophils from these exudates demonstrated a consistently diminished release of H_2O_2 in response to a bacterial phagocytic stimulus and a moderate decrease in bactericidal ability. These effects are clearly associated with acute CMV infection despite the fact that only a small proportion of cells present in the inflammatory exudate of CMV-infected animals contained virus in vivo. Whether these alterations associated with acute CMV infection are important in reducing defenses against bacteria is not known. However, GPCMV infection is suitable for such an investigation, as well as for an examination of the possible potentiating role of immunosuppressive therapy.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants 5-RO1 AI-13521 and 5-T32-AI-07033 from the National Institute of Allergy and Infectious Diseases and Public Health Service research grant HD-10609 from the National Institute of Child Health and Human Development. F.J.B. is a George A. Carden Scholar at Yale University School of Medicine. B.P.G. is the recipient of Public Health Service Young Investigator Award AI-16028 from the National Institute of Allergy and Infectious Diseases.

We thank G. D. Hsiung for her continued advice and encouragement and H. Lucia for evaluation of bone marrow smears and gratefully acknowledge the technical assistance of Maureen Tarsio and Julia Metcalf.

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