Antiviral Properties of Neonatal and Adult Human Neutrophils

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ABSTRACT

The antiviral properties of neonatal and adult human neutrophils were investigated by their ability to inhibit the formation of herpes simplex virus (HSV) plaques using an extrinsic viral resistance (EVR) assay. The EVR assay was performed by incubating neutrophils or mononuclear cells (MNC) with HSV-infected Vero or CEM tumor cells for 48 h. The cells were then frozen and viable HSV was quantitated by the ability of the lysate to form viral plaques. Activation of neutrophils from normal adults with phorbol myristate acetate increased their ability to inhibit HSV plaque formation more than 10-fold. The EVR response of neutrophils from newborn infants was much lower, and no significant inhibition occurred using activated neutrophils from patients with chronic granulomatous disease. The presence of rabbit anti-HSV antiserum slightly increased the EVR response of neutrophils but produced a greater increase in the response of the MNC in both adults and newborns. However, the combination of antiserum plus cytokines (granulocyte-macrophage colony-stimulating

factor, IL-2, and interferon- γ) greatly augmented the neutrophil EVR response to the level of the MNC response. Thus, neutrophils are capable of exerting a strong antiviral response comparable to that of MNC that may be important as a second line of defense in the immunocompromised patient. (*Pediatr Res* 36: 792-798, 1994)

Abbreviations

HSV, herpes simplex virus
EVR, extrinsic viral resistance
MNC, mononuclear cells
PMA, phorbol myristate acetate
CGD, chronic granulomatous disease
ROI, reactive oxygen intermediate
GM-CSF, granulocyte-macrophage colony-stimulating factor
IFN- γ , interferon- γ
PFU, plaque-forming unit
LR, log reduction

Neutrophils are essential in combatting bacterial pathogens, but they also may play an integral role in viral infections. Several viruses, including rhinovirus, influenza A, and vaccinia, will activate neutrophils directly in the absence of antibody (1-3). Neutrophils are the predominant cell in respiratory tract secretions of respiratory syncytial virus infection, and their damaging effects on respiratory epithelium may account for much of the pathology associated with this infection (4).

In vitro studies have shown that neutrophils are much more adherent to cells infected with a variety of viruses including herpes simplex and influenza (5, 6). Viruses may also activate neutrophils indirectly by stimulating release of cytokines such as interferon- γ from mononuclear cells

(7). Viral immune complexes that may occur in measles, rubella, and hepatitis B will initiate oxidative burst activity and arachidonic acid metabolism in neutrophils (8, 9).

The activation of neutrophils by viruses or other stimuli may elicit an antiviral response from these phagocytic cells. Viruses may be inactivated *in vitro* by ROI released by neutrophils, but the significance of this viricidal activity *in vivo* is not well understood. Neutrophils also contain large amounts of defensins, which can inactivate certain enveloped viruses and inhibit HIV replication (10). In the presence of specific antiviral antibodies, neutrophils are able to lyse virally infected cells, although lymphocytes are more effective (11).

In the present study, we investigated the role of neutrophils in inhibiting viral production by infected cells. We have compared the antiviral responses of neutrophils from adults with those from newborn infants and from patients with CGD whose cells lack the ability to generate ROI. We have also compared the responses of neutrophils with those of MNC and tested the effects of antibody and various cytokines.

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The method used to study these antiviral responses was the EVR assay, in which the effector cells and virally infected cells are incubated in contact with each other (12). The ability of the effector cells to decrease the number of viable viral particles, as measured by plaque formation, is indicative of the effector cells' antiviral properties or EVR.

METHODS

Human subjects. Peripheral blood was obtained from normal volunteers in good health at the time of the study. CGD patients were recruited from the outpatient population followed by the Division of Immunology/Allergy of the Department of Pediatrics at UCLA. Studies were done in accordance with the UCLA Human Subjects Protection Committee. The ages of the CGD patients ranged from 6 to 21 y, and all were taking oral antibiotics (cotrimoxazole or dicloxacillin) and had no serious illness when studied. None were taking IFN- γ at the time of the study. This group of CGD patients included both X-linked and autosomal forms of the disease, but none had measurable oxidative burst activity.

The cord blood was obtained from detached placentas after normal spontaneous vaginal delivery after uncomplicated births. The peritoneal eosinophils were obtained from the peritoneal fluid of patients on peritoneal dialysis (13). Peritoneal eosinophilia often occurs in these patients, although no known parasite or allergen can be identified to account for it.

Materials. The GM-CSF was a gift from Dr. Gayle Baldwin of the Division of Hematology/Oncology, UCLA; the IFN- γ was from Genentech, South San Francisco, CA; and the IL-2 was from Cetus Corporation, Emeryville, CA. The anti-HSV type I antiserum was from Dako of Accurate Chemical and Scientific Corporation, San Diego, CA, and Lee Biomolecular Research, Inc., San Diego, CA. Most other reagents were obtained from Sigma Chemical Co., St. Louis, MO.

Effector cells. MNC and neutrophils were isolated from whole blood to act as effector cells in the assays (14). One part of heparinized blood was mixed with 0.15 parts of Plasmagel (Roger Bellon Laboratories, Neuilly sur Seine, France) in a syringe and incubated at 37°C to sediment the erythrocytes. The supernatant was collected, and MNC were separated from neutrophils by Ficoll-Hypaque buoyant density centrifugation. In some assays, MNC and neutrophils were not separated. Erythrocytes were lysed with hypotonic saline, washed in PBS, and resuspended in RPMI 1640 with penicillin (100 U/mL), streptomycin (100 μ g/mL), L-glutamine (2 mM), and 10% heat-inactivated FCS (complete medium will be referred to as RPMI). Cell samples were adhered to glass slides by cytocentrifugation for differential cell counts.

Treatment of effector cells. Neutrophils were activated in some experiments by incubating them in 100 ng/mL of PMA for 10 min at 4°C (15). The cells were then washed in cold PBS and resuspended in cold RPMI. The cell

suspensions were kept in an ice bath until the start of the assay.

The concentrations of cytokines, GM-CSF (250 pM), IL-2 (1000 U/mL), and IFN- γ (500 U/mL) were determined by previous cytotoxicity studies done in our laboratory in which these concentrations were found to be effective (13–15). The cytokines were added at the start of the 48-h incubation and remained in contact with the target and effector cells for the entire assay. Preliminary experiments showed greater EVR activity if the rabbit antiserum to HSV was not washed out after an initial incubation with the target cells; therefore, the antiserum was also allowed to remain in for the entire assay. Neither the cytokines nor the antiserum caused any significant reduction of viral plaques without the presence of effector cells.

EVR assay (Vero method). The initial assay used to measure EVR was an adaptation of that described by Morahan *et al.* (12) to assess macrophage antiviral activity. The Vero cells, grown adherent in 75-cm² flasks, were removed by treatment with trypsin and EDTA (16). The cells were washed and resuspended in RPMI and adjusted to a concentration of 5×10^4 cells/mL. The cells were then placed into a 96-well, flat-bottom plate (5×10^3 cells per well) and allowed to adhere at 37°C for 2 to 3 h.

To infect the Vero cells with HSV, the medium was removed from the Vero cells and replaced with medium containing 5×10^5 PFU of viral stock prepared from supernatants of infected Vero cells per well (16). The cells were then incubated another 90 min, the nonabsorbed HSV was washed off by rinsing with buffer three times, and fresh medium was added. When effector cells were to be present, the medium was again removed and 5×10^5 effector cells in RPMI were added (for a 100:1 effector:target ratio) to each well. The total volume per well was brought up to 0.2 mL, and the cells (effectors and targets) were incubated together for 48 h. The cells were then scraped off the bottom of the well with a pipette tip and the contents aspirated. The cell mixture was then frozen and stored at -70°C until the plaque assay (which is the same using either the Vero or CEM method).

EVR assay (CEM method). We further adapted the EVR assay to use CEM cells grown in suspension as targets. This adaptation, by using CEM cells in suspension, eliminated the steps of adhering and then removing the target cells from multiwell plates. On the day of the assay, the CEM cells grown in suspension were resuspended in fresh medium and HSV was added at a ratio of 40 HSV PFU per CEM cell. The HSV and cells were incubated together for 90 min in a 1-mL volume, and the nonabsorbed HSV was then removed by washing three times with buffer. The infected cells were resuspended in RPMI at a concentration of 1×10^5 /mL. The infected CEM cells (2.5×10^4) and effector cells (2.5×10^6) were placed in a 5-mL polystyrene culture tube in 1 mL of RPMI for a resulting effector:target cell ratio of 100:1 based on preliminary experiments. The cell mixture was incubated at

37°C for 48 h and then frozen in the tube and stored at -70°C.

Plaque assay for HSV. Vero cells from stock were grown on tissue culture dishes (60 mm) until confluent (16). The samples previously prepared (using either the Vero or CEM methods) were thawed and whirled rapidly, and appropriate dilutions were made based on previous experiments. Dilutions of the thawed samples were added to the plates, which were then incubated for 2 h at 37°C in a CO₂ incubator. The plates were then overlaid with 1.0% methylcellulose in minimum essential medium with 2% FCS and incubated another 72 h at 37°C. The cells were then fixed by adding 3 mL of 4% formalin to each plate for at least 20 min. The formalin was then aspirated, and the plates were stained with 0.5% aqueous crystal violet for 10–15 min. The plates were then rinsed and allowed to dry overnight. The number of viral plaques on those plates with aliquots from the frozen samples (either Vero or CEM) containing no effector cells were compared with those samples with effector cells. The decrease in the number of plaques due to the effector cells reflects the effector cells' EVR response. The EVR response was expressed as the LR in the number of viral plaques.

Statistics. *t* test (two-tailed, unpaired unless otherwise stated) was used to compare the reported means (with SEM) for the different variables.

RESULTS

EVR by activated neutrophils. We initially assessed the EVR response of neutrophils by two different methods. The Vero method (Fig. 1) uses HSV-infected Vero cells adherent to a plastic culture dish. In the CEM method (Fig. 2), the HSV-infected target cells are in suspension with the neutrophils. In both assays, the purified neutrophils had been activated by incubation in PMA (100 ng/mL) in the cold followed by washing before the assay. We have previously used this method for activating neu-

trophils for cytotoxicity assays (13) and found it to be effective.

In Figure 1, we compared neutrophils from normal adults, newborn infants (cord blood), and patients with CGD, whose phagocytic cells lack the ability to form ROI (15). The EVR response was assessed by the LR of viral plaques using the plaque assay for HSV (see Methods). When the neutrophils had been exposed to buffer alone (no activation), the EVR was low (<0.4 LR) for all three groups at a 100:1 effector:target ratio. After PMA activation, the EVR response of the adult neutrophils increased to 1.71 ± 1.6 LR ($n = 15$, $p < 0.005$) at an effector:target ratio of 100:1. At an effector:target ratio of 50:1, the adult neutrophil response after PMA activation was 1.04 ± 0.26 LR ($n = 5$, not shown in Fig. 1). The EVR response of the activated cord neutrophils was only 0.71 ± 0.22 LR ($n = 6$) and significantly lower ($p < 0.05$) than the adult response. When activated CGD neutrophils were used, the response was also lower than that of normal adult cells (0.65 ± 0.21 LR, $n = 5$). These results are analogous to results obtained in cytotoxicity studies in which cytolysis by PMA-activated cord and CGD neutrophils was defective compared with cytolysis in adult cells (15).

When the CEM method with cells in suspension was used, similar results were obtained (Fig. 2). The EVR response of the adult neutrophils increased from 0.27 ± 0.07 LR with buffer alone to 2.12 ± 0.33 LR after PMA stimulation ($n = 17$, $p < 0.01$). The responses of the cord (1.31 ± 0.61 LR, $n = 9$) and CGD (0.18 ± 0.06 LR, $n = 2$) neutrophils were again considerably lower than that of the adults.

Also shown in Figure 2 is the EVR response of purified (>95%) eosinophils from the peritoneal fluid of individuals on peritoneal dialysis ($n = 3$). The response of the eosinophils (4.0 ± 0.72 LR) was about twice that of the normal adult neutrophils. We have previously shown that eosinophils, whether from the blood or peritoneal fluid, are much more cytotoxic than neutrophils after activation

Figure 1. The EVR response of neutrophils with buffer treatment and after activation with PMA using the Vero method. The response of the neutrophils from newborn infants (cord) and from CGD patients was lower ($p < 0.05$) than that of adult cells.

Figure 2. The EVR response of granulocytes with buffer treatment and after activation with PMA using the CEM method. The *asterisk* indicates that the responses of the CGD neutrophils (0.18 ± 0.06 LR) and peritoneal eosinophils (4.00 ± 0.72 LR) were significantly different from that of the adult neutrophils (2.12 ± 0.33 LR).

with PMA (13). It was also noted that some of the cord cell preparations had an increased percentage of eosinophils (up to 38%). We separated the responses of the cord cells on the basis of eosinophil count (above or below 15%) and found that, after PMA activation, the cord cell preparations with >15% eosinophils (16 to 38% eosinophils, $n = 6$) increased their response to 2.03 ± 0.78 LR, whereas the cord cells with <15% eosinophils ($n = 13$) increased only to 0.46 ± 0.11 LR. Thus, the presence of increased numbers of eosinophils in the cord preparations was also associated with a much higher EVR response.

Because the number of target cells in the assay could be more easily assessed using the CEM method and results were similar with both methods, all additional EVR assays were done using the CEM method with the cells in suspension.

Enhancement of EVR by antibody. The effect of anti-HSV antibody on the EVR response is shown in Figure 3. The responses of neutrophils (referred to as PMN on graph) and MNC from both adults and newborns (cord)

are also compared. The MNC were obtained from the same donors and consisted of lymphocytes (60 to 80%) and monocytes (20 to 40%). The EVR responses of the adult and cord neutrophils were not significantly different, and there was only a slight enhancement by antibody. The antibody did increase the EVR of both the adult ($n = 14$) and cord ($n = 6$) MNC, although the adult response was much higher (1.51 ± 0.12 LR for adult MNC, 0.74 ± 0.17 LR for cord). The anti-HSV antibody alone (at a 1:10 000 dilution) did not significantly affect the EVR response.

Although the EVR activity of neutrophils was much lower than that of the MNC, the possibility that neutrophils and MNC may interact to influence the EVR response was next investigated. In this series of experiments (not shown), in addition to isolating adult neutrophils and MNC, we obtained another sample of blood from the same individual and isolated the total leukocyte population or "buffy coat" (MNC and neutrophils together). No significant difference was noted between the responses of MNC and buffy coat cells in the

Figure 3. The EVR response of neutrophils (PMN) and MNC from both adults and newborn infants (cord blood) in the absence and presence of anti-HSV rabbit antiserum. The p values are comparing the response with antibody to the response in the absence of antibody.

presence or absence of anti-HSV antibody. Thus, the neutrophils did not appear to influence the EVR response of the MNC.

In other studies, neutrophils and MNC were combined at the same ratio as present *in vivo*, but these reconstituted buffy coat cells did not respond differently in EVR assays from cells that had not been fractionated (1.44 ± 0.23 LR for reconstituted cells with antibody; 1.45 ± 0.23 LR for unfractionated cells; $n = 6$). This would indicate that it was not likely that the cell isolation procedures altered the responses of the different cell populations.

Enhancement of EVR by cytokines. The effects of cytokines (GM-CSF, IL-2, IFN- γ) and cytokine combinations on the EVR response of normal adult neutrophils is shown in Figure 4. The assays were also done in the presence and absence of anti-HSV antibody. The responses with cytokine(s) are compared with the response without cytokines; the responses with antibody and cytokines are compared with the response with antibody alone. The addition of the cytokines greatly augmented the EVR response of neutrophils, and it also appeared that the combinations of cytokines had a synergistic or at least additive effect. In the absence of cytokines, the neutrophil response was 0.40 ± 0.08 LR without antibody and 0.56 ± 0.08 LR with antibody. This increased to 0.79 ± 0.11 LR without antibody when all three cytokines, GM-CSF, IFN- γ , and IL-2, were present and to 1.72 ± 0.24 LR with antibody plus the three cytokines ($n = 11$, $p < 0.001$).

The EVR response of MNC and buffy coat cells from normal adults was also tested with these same cytokines and cytokine combinations. The cytokines produced only slight increases in the EVR response of these cells. The EVR response of buffy coat cells with antibody was initially 1.77 ± 0.16 LR and increased to 2.19 ± 0.19 LR with all three cytokines present ($n = 11$, not significant). Thus, the neutrophils appeared to be much more responsive to cytokine treatment than the MNC, although the

response of neutrophils in the absence of cytokines was much lower (Fig. 3).

DISCUSSION

Mononuclear cells, lymphocytes, and monocytes provide the major defenses against viruses, but in this study we have shown that neutrophils, under the appropriate conditions, can exert comparable antiviral activity as measured by the EVR assay.

The EVR response, as described by Morahan *et al.* (12), refers to the ability of leukocytes, notably macrophages, to reduce the number of viral PFU when cultured with virally infected cells. It differs from "intrinsic viral resistance," which refers to the ability of the macrophage to inhibit viral replication within itself. This EVR response peaked at 3 to 4 d of culture before a specific immune response developed. In some of our assays, specific antibody was added to augment the response, although the effector cells themselves were not sensitized to the virus. Therefore, we will refer to the EVR response in the present study as simply the ability of noninfected effector cells to decrease the number of viral PFU after culture with HSV-infected cells.

The neutrophils were activated with PMA to induce an antiviral response in the first series of experiments (Figs. 1 and 2). PMA is a very potent stimulus for neutrophils, but the response is nonspecific, resulting in damage to any other cell or microorganism in close proximity to the activated cell (17). In previous cytotoxicity studies, we have found that it takes up to 18 h for PMA-activated neutrophils to achieve maximal killing of tumor target cells, although most of the release of ROI, which are responsible for much of the cytotoxicity, is completed within the first hour (13, 15). Resting neutrophils had little EVR activity, but EVR increased several fold (results shown on log scale) after activation with PMA. The responses of the activated neutrophils from CGD patients

Figure 4. The effects of rabbit anti-HSV antisera (antibody) and cytokines on the EVR response of neutrophils from normal adults. The GM-CSF (250 pM), IL-2 (1000 U/mL), and IFN- γ (500 U/mL) were present throughout the entire assay. The responses with cytokines are compared with responses without cytokines (control).

and from cord blood were lower than those of normal adults by two different methods (using either Vero or CEM cells as targets). The decreased EVR activity of the CGD cells is likely due to their inability to make ROI. When CGD neutrophils were activated by PMA in cytotoxicity assays, their ability to lyse tumor cells or erythrocytes was also decreased compared with normal cells (15). Similarly, neutrophils from newborns (cord) have been reported to be defective in ROI production as well as in other assays of neutrophil function (18). We also found that PMA-activated eosinophils have a much greater EVR response than do neutrophils (Fig. 2), which again parallels the findings in cytotoxicity (13). Thus, although activation by PMA may not represent a particularly physiologic stimulus, it does demonstrate that granulocytes can express antiviral activity.

The presence of antiserum with antibody specific to HSV increased the EVR response of neutrophils, although antiserum produced greater enhancement when MNC were used as effector cells (Fig. 3). Others have reported that neutrophils will lyse virally infected cells in an antibody-dependent assay, but this would not necessarily explain how the neutrophil inactivates the virus (11, 14). In our EVR assays, the anti-HSV antibody may simply act to bring the infected target cell in contact with the neutrophil via the neutrophil's Fc receptors. Binding to the Fc receptors may also activate the neutrophil to release defensins and other antiviral agents. Antibody-mediated cytotoxicity, unlike cytotoxicity mediated by PMA activation, does not appear to be dependent on ROI formation (15), which may explain why patients with CGD do not usually have more problems in containing viral infections.

We did not find a significant difference when we compared the EVR response of adult and cord neutrophils in the presence of antibody (Fig. 3). Kohl *et al.* (19) found that the antibody-dependent cell-mediated cytotoxicity response of neonatal neutrophils to HSV-infected cells was not different from that of adults. However, the EVR response of the cord MNC in the presence of antibody was lower than that of adult MNC ($p < 0.01$). Leibson *et al.* (20) found that neonatal MNC, and the natural killer cells in particular, were impaired in their ability to inhibit HSV replication in fibroblast monolayers compared with adult cells. Kohl *et al.* (21) also reported that the IL-2-stimulated natural killer cytotoxicity to HSV-infected cells was decreased in newborns compared with the cytotoxicity of adult cells.

The effects of cytokines, particularly with antibody, were dramatic when neutrophils were used as effectors (Fig. 4). Both GM-CSF and IFN- γ , in addition to activating neutrophils directly (22–25), will also increase the viability of neutrophils *in vitro* (26, 27), which may explain in part the greater EVR response if the neutrophils were living longer. IL-2 will also stimulate neutrophil cytotoxicity and oxidative activity *in vitro* (28, 29). The highest EVR response occurred when all three cytokines plus antibody were present, indicating that the enhancing

effects were additive. By contrast, the cytokines produced only slight increases in the EVR responses of MNC. However, we had selected a high effector:target ratio (100:1) based on neutrophil studies, and it is possible that the MNC were already exhibiting maximal responses at this ratio.

In summary, we have examined the antiviral properties of neutrophils using the EVR assay. The EVR response in neutrophils may be elicited by PMA stimulation, which appears to be dependent on ROI formation. The EVR response of neutrophils may also be enhanced more specifically by anti-HSV antibody, although it is much lower than that of MNC. However, the addition of cytokines plus antibody will greatly enhance the EVR response of neutrophils, approaching the EVR response of MNC.

Although it is unlikely that neutrophils contribute greatly to fighting viral infections in the normal adult, the neutrophil antiviral response, augmented by antibody and cytokines, may be significant in an immunocompromised host. Neutrophils may potentially play an important role in fighting viruses in the HIV-infected patient whose lymphocytes have been depleted or in the very young infant whose lymphocyte function is impaired compared with adults. The increasing availability for clinical use of growth factors and other cytokines may allow the neutrophil's potential as an antiviral cell to be realized in these patients.

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