Neutrophil-Mediated Antibody-Dependent Killing of Herpes-Simplex-Virus-Infected Cells

By Hilary Siebens, Satvir S. Tevethia, and Bernard M. Babior

The ability of neutrophils to serve as effectors of antibody-dependent cell-mediated cytotoxicity (ADCC) was studied using herpes-simplex-virus-infected (HSV-infected) human embryonic lung cells as targets. Killing of infected cells by neutrophils required HSV-immune serum and did not take place in the presence of nonimmune serum or in the absence of serum. Uninfected cells were not killed. ADCC was evident by 30 min and reached a value of 66% by 4 hr at an effector:target ratio of 100:1. Cells from patients with chronic granulomatous disease were as effective as normal cells, and this indicates that ADCC could be mediated by processes that are independent of the respiratory burst.

THE DESTRUCTION of herpes-simplex-virus-infected (HSV-infected) cells is believed to play a major role in host defense against infections by these viruses. Several immune mechanisms have been shown to be involved in the lysis of HSV-infected cells. For example, these cells can be destroyed by a soluble system consisting of complement plus an appropriate antibody. Several cellular cytotoxic systems have also been shown to destroy HSV-infected cells. In one such system, T cells serve as the cytotoxic effectors, and virus-infected cells are lysed in the absence of antibody. The other cellular cytotoxic systems employ antibodies to mark the infected cells for destruction by the effectors. Cell lysis by the latter systems is termed antibody-dependent cellular cytotoxicity (ADCC).

It has been known for some time that certain mononuclear cells (monocytes and K cells) serve as effectors of antibody-dependent cytotoxicity against virus-infected cells. However, it is only recently that neutrophils have been shown to act in this capacity. The first studies reporting an interaction between neutrophils and cells infected with a herpesvirus were those of Rouse and his associates, who showed that these phagocytes were able to lyse bovine kidney cells infected with bovine rhinotracheitis virus. Later studies from this group showed that virus dissemination could also be ablated by neutrophils. Subsequently, Oleske et al., and later Fujiyama et al. and Russell and Miller, demonstrated cytotoxicity of neutrophils against several HSV-infected cell lines. We report here that neutrophils are very potent cytotoxic effectors against human embryonic lung cells infected with HSV. In this report, the properties of this system are described, and certain observations concerning the mechanism of cytotoxicity are presented.
NEUTROPHIL-MEDIATED CYTOTOXICITY

MATERIALS AND METHODS

Patients

Blood from 2 patients with chronic granulomatous disease was used for these studies. The first patient has been reported previously (case 1 of Curnutte et al.15). The diagnosis was established in the second patient, a 7-yr-old male, by a history of recurrent bacterial abscesses resistant to antibiotic therapy, together with laboratory tests showing that the patient's neutrophils were unable to reduce NBT (formazan was seen in 95% of control neutrophils, but in none of the patient's cells) and also showing a marked defect in their ability to kill Staphylococcus aureus (97% survival at 2 hr versus 5% for control cells). In addition, the particles centrifuged (27,000 g) from homogenates of zymosan-activated patient neutrophils made no O2 in the presence of NADPH, whereas control particles produced O2 at a rate of 10.9 ± 1.0 nmoles/min/mg.15 Informed consent was obtained from the mothers of both patients prior to withdrawal of blood.

Sera

Selected convalescent sera from 1 individual who experienced apparent clinical infection with HSV-1 were used to study the ADCC mediated by human polymorphonuclear leukocytes (PMN). The sera were shown to possess neutralizing activity against HSV-1 (titer 1:512 by plaque-reduction neutralization using an end point of 50%). Both HSV-positive and HSV-negative sera were provided by Dr. Gordon Dreesman of Baylor College of Medicine. The sera were heated at 56°C for 30 min prior to their use in the ADCC reaction.

Cells and Virus

The KOS wild-type HSV-1 virus was grown in primary rabbit kidney cells. The virus-infected cells were incubated at 34°C until clear cytopathic effects could be observed, at which time the virus was harvested by freezing and thawing the infected cultures twice and centrifuging the suspension at 400 g at 4°C. The virus assays were performed by the plaque method according to a procedure described previously.14 The stock virus had a titer of 106 PFU/ml.

Human embryonic lung (HEL) cells obtained from Flow Laboratories were used as target cells for infection with HSV-1 for use in the ADCC assay. HEL cells were infected with HSV-1 at a multiplicity of infection (MOI) of 5 and incubated for 20-22 hr at 37°C in a CO2 incubator.

Effector Cells

Human PMN were prepared according to a procedure described previously.15 Briefly, 28.5 ml of blood obtained from healthy human volunteers were mixed with 1.5 ml of 27% EDTA and 90 ml of normal saline at room temperature and subjected to centrifugation in a Hypaque-Ficoll gradient.16 The pellet containing red cells and neutrophils was mixed with dextran (6% dextran 70 in normal saline) and allowed to stand at room temperature for 1 hr. The PMN in the top layer were pelleted at 4°C, and the remaining red cells were removed by hypotonic lysis. The PMN (>99% pure) were suspended in Eagle's medium containing 10% heat-inactivated fetal calf serum (FCS). Pure PMN were similarly prepared from patients suffering from chronic granulomatous disease (CGD).

ADCC Assay

HSV-1-infected and uninfected target cells in 75-sq-cm tissue culture flasks were labeled with 250 μCi of sterile sodium chromate (New England Nuclear, Boston, Mass.). After overnight incubation, the cultures were washed three times with serum-free Eagle's minimal essential medium (EMEM), and the cells were brought into single-cell suspension by treating the monolayers with 0.25% trypsin. The resulting cell suspension was washed four times with EMEM in the cold. After the final wash, the cell pellet was resuspended in EMEM containing 10% heated FCS. The cell concentration was adjusted to contain 2 x 10⁶ cells/ml. For the ADCC assay, 2 x 10⁵ labeled target cells in 0.1 ml were added to each of 16 125-mm tissue culture tubes, followed by 0.1 ml of appropriately diluted HSV-1-immune or -nonimmune serum and 0.3 ml of effector cells. Controls were included in which serum or effector cells or both were replaced with EMEM. The mixture was then incubated at 37°C in a CO2 incubator for various time periods, after which 0.5 ml of EMEM with 10% FCS was added to each tube to bring the
total volume to 1 ml. The cultures were centrifuged at 400 g at 4°C, and 0.5 ml of supernatant was carefully withdrawn. Both the tubes containing 0.5 ml of supernatant and those containing the remaining supernatant plus the pellet cells were counted for radioactivity in a Beckman gamma counter. Maximum release of $^{51}$Cr from the labeled target cells alone was determined by subjecting $2 \times 10^4$ labeled target cells to sonication for 1 min in a Branson sonifier. The results were expressed as percentage release of radioactivity, which was calculated according to the following formula:

$$\text{release of radioactivity (\%)} = \frac{R - R_s}{R_m - R_s} \times 100$$

where $R$ is release under the conditions indicated, $R_s$ is release in the absence of serum and effector cells (spontaneous release), and $R_m$ is maximum release.

RESULTS

Neutrophils as Mediators of ADCC

HSV-infected HEL cells were found to be efficiently lysed by human neutrophils in the presence of antibody. The requirements for lysis are shown in Table 1. Antibody is required, since lysis of targets was not seen when the antibody (in the form of HSV-immune serum) was replaced with buffer or with serum free of anti-HSV antibodies. Lysis was also abolished by the omission of neutrophils and by the use of uninfected targets. Lysis could not be accounted for by mononuclear cell contamination of the neutrophil preparation, since mononuclear cells from the same preparations did not release label from the target cells when added at concentrations exceeding those present in the purified neutrophils. These experiments establish a requirement for neutrophils, antibody, and infected targets in the cytotoxic system under study. A requirement for complement can be excluded, since heated serum was used as the source of antibody in every case.

The results in Fig. 1 show the kinetics of ADCC mediated by human PMN against HSV-1-infected human cells. Lysis was apparent as early as 30 min after mixing and by 4 hr had resulted in the destruction of more than two-thirds of the infected targets. However, uninfected targets were almost untouched under these conditions. These results clearly show that the ADCC mediated by human PMN was a rapid and efficient process.

Figure 2 shows the effect of neutrophil concentration on target lysis during a 2-hr

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$^{51}$Cr Release (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected Cells</td>
</tr>
<tr>
<td>Neutrophil (50:1)</td>
<td>Immune</td>
</tr>
<tr>
<td>Neutrophil (50:1)</td>
<td>Nonimmune</td>
</tr>
<tr>
<td>Neutrophil (50:1)</td>
<td>None</td>
</tr>
<tr>
<td>Mononuclear (1:2)</td>
<td>Immune</td>
</tr>
<tr>
<td>Mononuclear (2:1)</td>
<td>Immune</td>
</tr>
<tr>
<td>None</td>
<td>Immune</td>
</tr>
</tbody>
</table>

*Incubations were conducted for 1 hr using effector cells at the concentrations shown and serum at a final dilution of 1:100. When serum or effector cells were omitted, the volumes were made up with EMEM. Duplicates agreed to within 3%. The data were corrected by subtracting from each observed value the value for spontaneous release (i.e., release in the absence of effectors or serum), which for infected cells was 9.1% and for uninfected cells was 10.5%. The results shown are representative of three experiments.
Fig. 1. Release of \(^{51}\text{Cr}\) from target cells as a function of time. Incubations were conducted for the times noted using neutrophils at an effector:target ratio of 100:1 and serum at a final dilution of 1:400. Duplicates agreed to within 3%. The data were not corrected for spontaneous release, which at 4 hr amounted to 13.8% for infected cells and 17.3% for uninfected cells. The results shown are representative of two experiments. Filled symbols indicate infected cells; open symbols indicate uninfected cells; circles: with neutrophils; triangles: without neutrophils.

incubation. Lysis was clearly evident at effector:target ratios as low as 10:1. Lysis increased monotonically, as expected, with increasing numbers of neutrophils.

**Oxygen-Dependent Killing Mechanisms in Neutrophil-Mediated ADCC**

The killing of bacteria by neutrophils is accomplished in part by means of oxygen-dependent microbicidal systems that use as antimicrobial agents certain compounds formed by the partial reduction of oxygen\(^{17-19}\). These compounds are generated by a metabolic process generally referred to as the respiratory burst. There is an inherited abnormality of phagocyte function (CGD) in which the respiratory burst fails to take place. Neutrophils in this disease cannot use the oxygen-dependent microbicidal systems for killing, since the oxygen-derived antimicrobial agents normally generated by neutrophils are not produced. These cells therefore provide a useful tool for investigating the role of oxygen-dependent killing systems in neutrophil function.

To determine if oxygen-dependent killing systems are essential for the neutrophil-mediated cytotoxicity observed in these experiments, we examined the ability of CGD neutrophils to lyse the target cells. Table 2 shows that the release of label
Table 2. Release of \(^{51}\text{Cr}\) From Target Cells by Neutrophils From Patients With CGD

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>(^{51}\text{Cr}) Release (% of total)</th>
<th>Serum Present</th>
<th>Serum Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>32.8</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>37.3</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>CGD</td>
<td>34.3</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>CGD</td>
<td>31.3</td>
<td>8.2</td>
<td></td>
</tr>
</tbody>
</table>

*Incubations were conducted for 2 hr using neutrophils at an effector:target ratio of 50:1 and serum at a final dilution of 1:100. The entire experiment was conducted in a single day using neutrophils from 2 CGD patients and 2 normal controls. The data were corrected for spontaneous release, which in the presence and absence of serum was 33% and 27%, respectively.

from the targets by neutrophils from 2 patients with CGD was similar to control values obtained with normal neutrophils. These results indicate that the oxygen-dependent killing systems are not necessary for the expression of antibody-dependent cytotoxicity by neutrophils.

**DISCUSSION**

Although they are best known for their ability to ingest and destroy invading microorganisms, neutrophils have recently been recognized as cytotoxic effector cells. Gale and Zighelboim have demonstrated that human neutrophils can destroy antibody-coated leukemic cells. Clark and Klebanoff have shown that zymosan-stimulated neutrophils kill Moloney-virus-induced lymphoma cells in the absence of antibody, and Sacks et al. have similarly shown antibody-independent killing of endothelial cells in culture by complement-activated neutrophils. Antibody-dependent destruction of herpesvirus-infected cells by neutrophils was first demonstrated by Rouse and his colleagues using bovine rhinotracheitis-virus-infected cells. Later, several groups, beginning with Nahmias and his associates, showed that cells infected with HSV were killed by neutrophils. Our own studies have confirmed these findings, showing that human neutrophils are efficient mediators of cytotoxicity against HSV-infected HEL cells.

A number of mechanisms may be postulated as being responsible for the destruction of target cells by neutrophils. These may be divided into two classes: those that require oxygen (i.e., those for which respiratory burst activity is necessary) and those that do not. In those studies in which killing has been accomplished by activated neutrophils in the absence of antibody, the oxygen-requiring mechanisms (the myeloperoxidase-peroxide-halide system, or oxidizing radicals) appear to have been primarily responsible for the cytotoxic action of the effectors. However, in the present study, cytotoxicity was observed only in the presence of antibody. Under these conditions, oxygen-independent mechanisms were sufficient for killing to take place. It seems reasonable to postulate that because of the antibody on the surfaces of the HSV-infected cells, the neutrophils could attach directly to the targets through their Fc receptors and that this proximity between the effector and the target permitted the oxygen-independent killing mechanisms to be used effectively.

The foregoing studies have shown that under suitable circumstances, neutrophils can act in vitro as anti-HSV effectors. Clinical observations have suggested that
neutrophils may play a similar role in vivo. In a study of HSV infections in patients with hematologic malignancies, Muller et al.\(^2^3\) reported that patients with disorders of either the lymphoid or the myeloid series were unusually susceptible to protracted and widespread infections with this virus. Of particular interest was the inclusion in their report of several patients with chronic myelogenous leukemia and a patient with myelofibrosis, both conditions in which lymphocyte-mediated host defenses are believed to be relatively intact. The fact that diseases affecting neutrophil number and function are associated with unusually severe HSV infections is indirect but suggestive evidence that neutrophils are among the cells that participate in the host defense against infections by HSV.

REFERENCES


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