Monocyte-Mediated Antibody-Dependent Cell-Mediated Cytotoxicity: The Role of the Metabolic Burst

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Human monocytes respond to opsonized microorganisms with a "metabolic burst" composed of an increase in oxygen consumption, an increase in hexose monophosphate shunt (HMPS) activity, and the generation of reactive oxygen species (ROS). We investigated the role of the metabolic burst in antibody-dependent cell-mediated cytotoxicity (ADCC) by human monocytes toward anti-D coated erythrocyte target cells because recent studies suggested a role for oxygen-dependent bactericidal mechanisms in ADCC. Normal monocytes, we found that ADCC was nearly halved under hypoxic conditions. Several agents known to impair activation of the burst, such as vincristine, cation chelators, and a sulfhydryl reagent, all decreased cytotoxicity if added before initiation of contact between target and effector cells. Cytotoxicity was inhibited by 2-deoxyglucose but not fluoride, suggesting a nonglycolytic role for glucose in ADCC, perhaps in the HMPS pathway. Although these data suggested a role for the metabolic burst in ADCC, scavengers of ROS did not impair cytotoxicity, and monocytes from chronic granulomatous disease (CGD) patients who had a defective metabolic burst had normal levels of ADCC. We conclude that ADCC toward anti-D coated erythrocyte target cells was the result of at least two independent but closely related cytotoxic pathways. Although one of these pathways appeared to involve the metabolic burst, the potentially cytotoxic reactive oxygen species did not appear to play a role in this system.

NONIMMUNE HUMAN MONOCYTES can bind antibody-sensitized target cells and then destroy them through the process of antibody-dependent cell-mediated cytotoxicity or ADCC. ADCC may be involved in autoimmune hemolytic anemia and has been proposed as a potential method of tumor surveillance in vivo. The ADCC process is initiated when the Fc portion of the antibody directed to target cell antigens is bound to the Fc receptor on the surface of the effector cell. Although the biochemical basis of cytotoxicity in this system is unknown, monocytes have several well defined bactericidal mechanisms that may be involved in target cell destruction. For example, it is well known that monocytes respond to opsonized particles with a "metabolic burst" composed of an integrated series of biochemical events, including increased oxygen consumption and increased hexose monophosphate shunt (HMPS) activity. As a consequence of the metabolic burst, human monocytes mediate the sequential univalent reduction of oxygen to a variety of reactive oxygen species (ROS), including superoxide (O2-), hydrogen peroxide (H2O2), and the hydroxyl radical (OH-). Human leukocytes may also possess the capacity to activate oxygen to the reactive singlet oxygen (O2) state or to other species not yet defined. Such reactive oxygen species can injure the target cell directly or can interact with an enzyme such as myeloperoxidase to generate cytotoxic intermediates. Several recent studies have suggested a role for oxygen-dependent bactericidal mechanisms in ADCC, but this role remains controversial.

In the present study, we investigated the role of the metabolic burst in ADCC by monocytes toward anti-D sensitized human red cells. Our results suggested that ADCC in this system was the result of a contribution from separate oxygen-dependent and oxygen-independent mechanisms. Furthermore, it appeared that products of the metabolic burst other than the reactive oxygen species were responsible for the observed oxygen-dependent cytotoxic activity.

MATERIALS AND METHODS

Monocytes

Venous blood from healthy volunteers or patients with chronic granulomatous disease (CGD) was collected in plastic syringes containing sodium heparin (1 mg/10 ml blood) and subjected to Ficoll-Hyphaque density centrifugation. The mononuclear cells were harvested, washed, resuspended in Seligman's balanced salt solution (SBSS) containing 13% autologous serum, and allowed to adhere to plastic dishes (Falcon, Oxnard, Calif.) for 1 hr. The monolayers were then washed 5 times with SBSS, overlayed with a solution of SBSS with 13% autologous serum and 2.6 mM EDTA, and the adherent cells gently resuspended by scraping with a rubber policeman. The cells were then washed twice and resuspended in complete RPMI (RPMI-1640 with 10% heat-inactivated fetal calf serum, 0.3 mg/ml l-glutamine, 0.1 mg/ml gentamicin, and 100 U/ml penicillin). This technique provided monocytes that were greater than 95% viable by dye exclusion and greater than 95% pure by nonspecific esterase stain.

Lymphocytes and Granulocytes

Lymphocytes and granulocytes of greater than 95% purity and viability were isolated by previously published methods.
Target Cells

Human red cells, type O Rh+ (CDe) were donated weekly by a single donor and stored at 4°C in acid citrate dextrose. Target cells were radioactively labeled by adding 50 μCi 51Cr (sodium chromium, New England Nuclear Co., Boston, Mass.) to 500 x 10^6 cells for 60 min at 37°C. After two washes in Hank's balanced salt solution (HBSS), the targets were antibody coated by incubating them for 30 min at 37°C in a 1:4 dilution of a high titer human anti-D antiserum. This resulted in the attachment of 2.5-5 x 10^7 antibody molecules per cell using an 125I-staphylococcal protein A technique. An aliquot of cells was similarly incubated in HBSS alone. After five more washes in HBSS, the targets were resuspended in complete RPMI.

Chemical Reagents

Dimethylfuran was obtained from Aldrich Chemical Co., Milwaukee, Wisc., and was passed twice over an aluminum oxide (Woolm Pharma, Eschwege, W. Germany) column. All other chemical reagents were obtained from Sigma Chemical Co., St. Louis, Mo. All reagents were diluted in complete media and the pH adjusted to 7.4.

Microtoxicity Assay

The standard ADCC assay was carried out in 96 flat-bottom well tissue culture plates (Costar, Cambridge, Mass.). Inhibitors and scavengers were preincubated with effector cells for 15 min prior to the addition of target cells, unless otherwise noted. Except for experiments correlating effector cell number to ADCC, 10^6 target cells were routinely added to 0.25 x 10^6 effector cells so as to provide an effector:target (E:T) ratio of 1:4. Spontaneous release of 51Cr was determined by omitting effector cells from otherwise complete microwells. The microtest plates were centrifuged at 50 g for 3 min to initiate cell contact and then incubated at 37°C in a humidified atmosphere of 95% air, 5% CO2. After a 4-hr incubation, the plates were again centrifuged at 50 g for 3 min, following which 0.1 ml of supernatant from the 0.3 ml total volume of each well was sampled to determine the amount of 51Cr released.

Target cell lysis, expressed as percent 51Cr release, was calculated simply by dividing the total cpm release into the supernatant by the total cpm added to each well, assuming that the total counts per minute added to each well were constant between samples. In order to calculate 51Cr release due specifically to antibody-dependent cytotoxicity, we subtracted 51Cr release in wells with uncoated targets from 51Cr release in wells with antibody-coated targets otherwise treated identically. Percent 51Cr release from nonantibody-coated erythrocyte targets did not exceed spontaneous release, and averaged 0.5%/hr. All experiments were performed in triplicate. Statistical analysis was performed with the Student's t test for nonpaired samples.

ADCC Under Hypoxic Conditions

One-hundred percent carbon monoxide was slowly bubbled through a target cell suspension for 15 min in a capped but vented tube. Target cells were then similarly bubbled with a mixture of 95% N2-5% CO2 for an additional 20 min. The first step was designed to substitute carbon monoxide for hemoglobin-bound oxygen in the red cells and the second to remove residual oxygen and carbon monoxide dissolved in the media. Target cells (10^6) and 0.25 x 10^6 monocytes (total volume, 0.3 ml) were added to a 12 x 75 mm polypropylene tube (Falcon 2005), which was then stoppered and gassed with a mixture of either 95% air-5% CO2 or 95% N2-5% CO2 for 20 min. The residual PO2 was not measured. The stopped tubes were then incubated for 4 hr at 37°C, following which the stoppers were removed. A quantity of 2.7 ml of fresh complete RPMI was then added, and the tubes centrifuged at 500 g for 5 min. One milliliter of supernatant was sampled and ADCC calculated according to the standard formula.

Quantitation of Erythropagocytosis and EA Rosettes

Erythropagocytosis and rosette formation between monocytes and antibody-coated erythrocytes (EA) using anti-D coated target cells were quantitated in liquid suspension following a 2-hr incubation as previously described. An E:T of 1:10 was used. Rosettes were stained with 1% new methylene blue in isotonc saline and were defined as having greater than 3 erythrocytes attached to a leukocyte.

Further Metabolic Studies

Oxygen consumption and superoxide production were measured using standard techniques.

RESULTS

ADCC by Normal Effector Cells

Figure 1 shows that monocytes are the sole effector cells under the conditions of this assay. The effector:target ratio of 0.25:1 was on a linear portion of the dose-response curve and was used for subsequent experiments. Figure 2 illustrates that cytotoxicity was substantial at 2 hr and neared completion by 4 hr. Phagocytosis of erythrocyte targets was a rare event under the conditions of this assay and was noted in a maximum of 3% of effector cells after 4 hr of incubation.

Effect of Hypoxia on ADCC

Figure 3 shows that a reversible decrease in cytotoxic capacity was seen upon deprivation of O2. In 6
experiments, oxygen deprivation caused a mean 41% \( \pm \) 18% decrease in ADCC \((p < 0.02\) from control), which could be restored to 83% \( \pm \) 14% of normal by supplying oxygen back to the system for 4 hr following the 4 hr of hypoxia. No subsequent increment in cytotoxicity was seen in the oxygen deprived tubes if oxygen was not restored.

**Effect of Vincristine**

The antitubulin, vincristine, caused a dose-dependent reduction in cytotoxicity following addition to the effector cells up to 15 mm following initiation of the incubation by addition of antibody coated red cells (Fig. 4A). However, addition at 60 mm had little deleterious effect on cytotoxicity. These concentrations of vincristine had no effect on rosette formation (Table I).

**Effect of Cation Chelators**

Cytotoxicity was modestly reduced by EDTA and by Mg-EGTA, which more specifically chelates Ca\(^{++}\), if these agents were added up to 15 min after the beginning of incubation (Fig. 4B). Neither chelating agent had an effect on rosette formation (Table I).

**Effect of N-ethyl Maleimide (NEM)**

NEM freely diffuses into cells and irreversibly inactivates sulphydryl moieties. One millimolar NEM

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Percent Rosettes</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>92</td>
</tr>
<tr>
<td>120 ( \mu M ) vincristine</td>
<td>88</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>97</td>
</tr>
<tr>
<td>10 mM EGTA</td>
<td>97</td>
</tr>
<tr>
<td>1 mM NEM</td>
<td>4</td>
</tr>
<tr>
<td>1 mM NEM added after 15 min</td>
<td>13</td>
</tr>
<tr>
<td>1 mM NEM added after 60 min</td>
<td>94</td>
</tr>
</tbody>
</table>

Mean of two experiments.

*Inhibitors and effectors were incubated for 15 min before target cells were added, unless otherwise noted.
Table 2. Role of Glucose Metabolism in ADCC

<table>
<thead>
<tr>
<th>Addition to Glucose-Free Medium</th>
<th>Percent Control ADCC</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>11.1 mM glucose</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>10 mM 2-deoxyglucose</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>11.1 mM glucose + 10 mM 2-deoxy-glucose</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>55.5 mM glucose + 10 mM 2-deoxy-glucose</td>
<td>97 ± 12</td>
</tr>
<tr>
<td>11.1 mM glucose + 1 mM NaF</td>
<td>113 ± 9</td>
</tr>
</tbody>
</table>

Results of 4 experiments with each agent. Control ADCC performed in complete medium.

*Standard concentration of glucose in complete medium.

We completely inhibited cytotoxicity when added 15 min prior to the addition of target cells (see Table 4). Some activity was retained when NEM was added 15 min into the incubation, and full activity was seen when this agent was added 60 min following the initiation of the target:effector interaction. These reductions in cytotoxicity were paralleled by a reduction in rosette formation (Table 1).

Role of Glucose Metabolism and the Hexose Monophosphate Shunt in ADCC

A glucose-free medium was used for these experiments (Table 2). Standard ADCC was only moderately diminished in this medium, despite a lack of glucose. However, when 10 mM 2-deoxyglucose (DOG) was added 15 min prior to incubation, cytotoxicity was markedly decreased. The block by DOG could be superseded by the simultaneous addition of fivefold excess glucose. One millimolar sodium fluoride had no deleterious effect on cytotoxicity.

Effect of Myeloperoxidase Inhibitors and Scavengers of Reactive Oxygen Species on ADCC

Table 3 shows that no effect of the myeloperoxidase and cytochrome oxidase inhibitors, azide and cyanide, could be demonstrated. Similarly, scavengers of H₂O₂ (catalase), superoxide (superoxide dismutase), hydroxyl radicals (mannitol, ethanol, or azide), and singlet oxygen (urate, histidine, tryptophan, or dimethylfuran) did not diminish ADCC.

Cytotoxic Activity of CGD Monocytes

Table 4 demonstrates that monocytes from patients with CGD are able to mediate ADCC normally. These effector cells did not show an increment in oxygen consumption following phagocytosis of zymosan or following addition of phorbol myristate acetate, nor were they able to generate superoxide under these circumstances.

DISCUSSION

The attachment of anti-D coated red cells to Fc receptors on monocytes elicited a potent cytotoxic activity that resulted in the destruction of almost one target cell per effector cell within a 4-hr period. Because phagocytosis was rarely observed under the conditions of our assay, destruction of the target either represented a target cell–effector cell membrane–membrane interaction or was the result of the liberation of a cytotoxic mediator by the effector cells.

We designed the present study to investigate the contribution of monocyte oxygen and glucose metabolism to the generation of the cytotoxic activity in ADCC. There was a 41% reduction in ADCC under hypoxic conditions with cytotoxicity being restored to nearly normal levels if oxygen was subsequently added back to the system. There is, therefore, a role for oxygen metabolism in ADCC by normal monocytes, as there is in bactericidal activity by normal monocytes.29 Borregard et al.21 and Klassen et al.22 also reported that ADCC was impaired under hypoxic conditions, but did not show that cytotoxicity was restored when oxygen was restored. Our data demonstrated that irreversible nonspecific damage to the effector cells was not occurring during the hypoxic incubation. The cytochrome oxidase inhibitors, azide and cyanide, did not reduce cytotoxicity, discounting the role of the oxidative phosphorylation pathway of oxygen metabolism in ADCC. We then postulated a role for the “metabolic burst,” which is the hallmark of an azide- and cyanide-insensitive bactericidal capacity, defined by its absence in monocytes from patients with chronic granulomatous disease.30 The
metabolic burst is an integrated series of metabolic pathways characterized by a large increment in oxygen consumption, in glucose metabolism through the HMPS, and generation of reactive oxygen species, all of which are activated shortly after opsonized particles come in contact with monocytes or other phagocytic leukocytes. Because the molecular basis of the cyanide-insensitive bacterial activity has not yet been confirmed, we investigated several lines of evidence to implicate this mechanism.

Cohen et al. reported that activation of the metabolic burst and the propagation of this activity once activated are under separate control. It has been shown that activation of the burst is sensitive to the antitubulin, vincristine, dependent on divalent cations, primarily Ca++, and inhibited by N-ethylmaleimide, a compound that irreversibly alkylates sulfhydryl groups. Once activated, burst activity does not appear to be sensitive to any of the above inhibitors. Our data show that ADCC was inhibited by all three classes of burst activation inhibitors, including vincristine, cation chelators, and a sulfhydryl reagent, but only if the inhibitors were added to effector cells either with or before the target cells. Although it is well known that each of these compounds has many effects other than the one described, our data are consistent with the involvement of the burst in ADCC.

On the other hand, in addition to its effects on activation of the burst, the sulfhydryl inhibitor NEM also demonstrated a marked effect on attachment of the antibody-coated targets to the monocytes. The lack of Fc-Fc receptor interaction would sufficiently explain the ability of NEM to inhibit ADCC, because contact is a prerequisite for ADCC. Other investigators have shown that under some circumstances, the binding of antibody-coated target cells to effector cells requires divalent cations. However, cation chelators had no effect on rosette formation in the present system, suggesting that ADCC was inhibited after attachment of targets had occurred.

Inhibition of ADCC by 2-deoxyglucose, which blocks all glucose metabolism beyond the formation of 2-deoxyglucose-6-phosphate, but not sodium fluoride, the hexose monophosphate shunt appeared likely to participate in ADCC.

Since our data demonstrated a role for an oxygen-dependent and HMPS-related pathway in ADCC, we looked for a contribution by the third defined component of the metabolic burst, the reactive oxygen species, but could find no inhibition of cytotoxicity in the presence of scavengers of ROS. Clark and Klebanoft, in reporting similar findings for granulocyte ADCC, pointed out that the large enzymic scavengers catalase and superoxide dismutase may not gain access to the site of target cell damage, which presumably lies at the junction of the closely applied effector and target cell surfaces. Many other investigators have also had difficulty in demonstrating a role for ROS in ADCC, with the exception of Borregand and Kragballe who suggested a role for the hydroxyl radical. Unfortunately, however, the 50 mM concentration of mannitol used in their study may have been sufficient not only to scavenge the hydroxyl radical but also to inhibit target cell lysis through osmotic effects. Therefore, there remains no conclusive evidence that ROS participate in ADCC.

The results of our experiments using monocytes from patients with CGD, combined with the observation of a large residual ADCC under hypoxic conditions in normal monocytes, showed that the requirement for an increase in oxygen metabolism and HMPS activity in normal monocytes was not an absolute requirement for the ADCC process. In the present study, CGD effector cells were able to carry out a normal level of cytotoxicity despite being defective in their ability to generate metabolic burst activity following appropriate stimuli. Even though CGD monocytes have had some level of cytotoxic activity in each study, the ability of CGD monocytes to carry out ADCC normally has been debated in the literature. Although most CGD patients are similar in their inability to generate metabolic burst activity following appropriate stimuli, there is a heterogeneity in clinical expression of the defect that allows some patients to survive into their second and subsequent decades of life. Therefore, the ability to increase non-burst-related oxygen-independent cytotoxic capacities may explain why our CGD patients, who were 12 and 19 yr old, had normal ADCC, and may possibly explain the discrepancies reported in the literature.

The cytotoxic mechanisms operating in one ADCC assay are not necessarily the same as those acting under different assay conditions. Therefore, it may be hazardous to compare data from the present study with data concerning the mechanism of ADCC reported in other studies, especially when the molecu-
lar basis for the observed activity has not been defined. ADCC can be performed by monocytes, granulocytes, and lymphocytes against a variety of different target cells coated with antibody molecules of various specificities and from various sources. It has been shown that a single type of effector, the monocyte, performs ADCC toward tumor cells and red cells via different mechanisms. Major differences in monocyte-mediated ADCC toward red cell targets under various assay conditions are also apparent. For example, depending on the source and specificity of the antibody, ADCC can be optimal at 1 hr, 4 hr, or 16 hr. Phagocytosis has been shown to play an important role in several assays of monocyte-mediated ADCC toward erythrocyte targets but did not contribute to cytotoxicity in the present study or in other studies using human anti-D antibody and a 4-hr incubation.

Our data support the conclusion that ADCC by normal monocytes was partly a consequence of oxygen-dependent metabolic pathways and partly independent of oxidative pathways. Unfortunately, since we were unable to implicate a role for reactive oxygen species in ADCC, the identification of the product(s) of the metabolic burst that resulted in target cell lysis was unsuccessful. Further studies will be necessary to determine the molecular basis of ADCC so that comparison between assay systems will be possible.

REFERENCES

Monocyte-mediated antibody-dependent cell-mediated cytotoxicity: the role of the metabolic burst

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