Evidence for Both Oxygen and Non-Oxygen Dependent Mechanisms of Antibody Sensitized Target Cell Lysis by Human Monocytes

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Recent studies suggest that the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$) or superoxide (O$_2^-$) may play a role in monocyte antibody-dependent cytotoxicity (ADCC). We studied ADCC by normal human monocytes, and monocytes from chronic granulomatous disease (CGD) patients, cells unable to generate ROS, toward anti-D sensitized human red cells (RBC) and an antibody sensitized lymphoblastoid cell line (CEM) by $^{51}$Cr release. The effects of hypoxia, scavengers of ROS, and the activity of the hexose monophosphate shunt pathway (HMPS) were examined. We found that monocyte HMPS activity increased two to threefold during ADCC toward RBC but was unchanged during ADCC toward CEM cells. Hypoxia decreased lysis of RBC targets by 80% but did not affect lysis of CEM cells even though hypoxia markedly decreased monocyte HMPS activity. Monocytes from CGD patients had impaired lysis of RBC but lysed CEM cells normally. We could not, however, demonstrate protection by scavengers of ROS. We conclude that monocyte ADCC involves two independent mechanisms: a nonoxidative mechanism active in the lysis of CEM cells, and an oxidative mechanism that may involve an unidentified ROS activated during ADCC toward RBC. The activation and possible interaction of these two mechanisms is determined by the nature of the target cell and sensitizing antibody.

MATERIALS AND METHODS

Monocyte Preparation

Venous blood was collected in EDTA from normal healthy volunteers or from CGD patients. The diagnosis of CGD was established by the failure of these cells to generate ROS in response to opsonized zymosan particles or phorbol myristate acetate (PMA) using criteria previously reported from our laboratory. Mononuclear cells were isolated by Ficoll-Hypaque density centrifugation. Mononuclear interface cells were harvested and washed with Seligmann's balanced salt solution. These cells were resuspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamicin, and 200 mM l-glutamine to a concentration of 4 x 10$^6$/ml. This cell suspension was placed in 15 mm x 100 mm plastic Petri dishes (VWR Scientific Inc., San Francisco, Calif.) at 10–15 ml per dish. After incubating for 60 min at 37°C in a humidified 95% air, 5% CO$_2$ atmosphere, the dishes were washed 5 times (5 ml/wash) with supplemented RPMI and the adherent monocytes removed with a rubber policeman and resuspended in supplemented RPMI at the desired concentration. These preparations were more...
than 95% viable (trypan blue exclusion) and contained greater than 95% monocytes as judged by morphology and nonspecific esterase staining.22,23

**Immune Serum**

A New Zealand white rabbit was immunized on days 1, 14, 21, and 28 with CEM lymphoblasts by intraperitoneal injection of 150 x 10⁶ cells suspended in 10 ml of Hank's balanced salt solution. Pooled sera collected after day 28 was heat inactivated and stored at −70°C. Antibody dependent lysis was maximal at a 1:16 serum dilution. Lysis could be detected with dilutions as low as 1:128. Type O Rh− red blood cells were sensitized with an IgG anti-D concentrate (RhoGAM, Ortho Pharmaceutical Corp., Raritan, N.J.).

**Target Cells**

CEM T-lymphoblasts were grown in supplemented RPMI suspension cultures.29 Radioactive labeling was done by incubating 15 x 10⁶ cells in 0.5 ml Tris buffer solution containing 100 µCi ⁵¹Cr (sodium chromate, New England Nuclear, Boston, Mass.) for 60–90 min at 37°C with gentle agitation every 15 min. The cells were washed once with supplemented RPMI and then antibody coated by resuspending 7.5 x 10⁶ cells in 0.4 ml of supplemented RPMI to which was added 0.1 ml of immune rabbit serum diluted 1:16 with RPMI and then incubating at 37°C for 30 min. The cells were then washed 4 times with supplemented RPMI (5 ml/wash) and resuspended in the same medium at the desired concentration. Control nonantibody coated cells were carried through an identical procedure without immune serum.

Human red blood cells were routinely obtained from a normal type O Rh− donor and stored in ACD at 4°C for up to 2 wk. For a few experiments the RBC of a patient with documented G6PD deficiency were used. The diagnosis was established by previously reported criteria.30 For individual experiments a subsample of RBC (0.15 ml containing 5–6 x 10⁶ cells) was removed from the stock suspension and washed once with 10 ml Hank's balanced solution without Phenol Red (GIBCO, Grand Island, N.Y.). From the washed RBC suspension an aliquot containing 50–100 x 10⁶ RBC was removed and centrifuged at 1200 rpm (300 g) for 5–7 min. After all but 0.1–0.3 ml of supernatant was removed from the packed RBC the cells were labeled with 100 µCi of Na₂¹⁴C₃O₄ solution at 37°C for 60 min with gentle agitation at 15-min intervals. The ¹⁴C-RBC were then washed once with supplemented RPMI 1640. The ¹⁴C-RBC were further treated by sensitizing the cells with an IgG anti-D concentrate. The ratio of antibody to RBC used was 10 µl of undiluted RhoGAM/10 x 10⁶ RBC. An appropriate control, i.e., RBC without antibody, was included. Both erythrocyte samples were incubated at 37°C for 45 min. The RBC targets were washed 4 times with supplemented RPMI 1640 and resuspended to the desired concentration for the individual experiments.

**ADCC Assay**

The ⁵¹Cr release ADCC assay was carried out in 96 well microtissue culture plates (3040 Falcon Plastics, Oxnard, Calif.) by adding 0.1 ml supplemented RPMI containing any drug to be studied, followed by adding 3 x 10⁴ target CEM cells in 0.1 ml supplemented RPMI, and lastly adding a varying number of monocytes also in 0.1 ml supplemented RPMI. Effector to target ratio was varied by changing the number of effector cells. The plates were centrifuged at 1000 rpm (200 g) (Beckman model TJ-6 centrifuge) for 5 min to initiate cell contact and then incubated at 37°C in a humidified 95% air, 5% CO₂ atmosphere. After 4 hr the plates were centrifuged again and 0.1 ml of supernatant removed from each well to determine the amount of ⁵¹Cr released by counting on a Packard Auto Gamma Spectrometer model 3003. ADCC toward RBC targets was performed in an identical manner except that 1 x 10⁵ target cells were used in each well.

Target cell lysis, expressed as percentage of ⁵¹Cr release, was calculated as follows: % lysis = [(A-B)/A] x 100% where A is the mean counts per minute in the supernatant from wells containing targets and monocytes, B is the mean counts per minute in the supernatant from wells containing targets alone (representing spontaneous release of ⁵¹Cr) and C is the total number of counts per minute added to each well. All ADCC experiments were done in triplicate and performed in the above manner, except for oxygen depletion experiments.

**Oxygen Depletion ADCC Experiments**

Monocyte ADCC under oxygen depleted conditions was determined in the following manner. Monocytes, CEM targets and RBC targets prepared as above, were suspended in supplemented RPMI at 1 x 10⁶, 1 x 10⁵, and 1 x 10⁴ cells/ml, respectively. One ml of monocyte suspension and 1.5 ml of target cell suspension were placed in separate tightly stopped 25 ml triple headed distilling flasks. The side arms of the flasks served as inflow and outflow connections via 18-gauge needles to gas cylinders containing 95% N₂ and 5% CO₂. The center arm served for addition or removal of cell suspensions through the rubber stopper with a needle and syringe. This allowed the N₂ atmosphere within the flask to remain undisturbed. Needles and syringes were flushed with the N₂ gas mixture immediately before use. The gas mixture was humidified and circulated continuously through the flasks for 1 hr while the suspensions were gently stirred with 8 mm magnetic stirrers. One ml of the target suspension was then removed through the central stopper and added to the flask containing monocytes. This resulted in an effector to target ratio of 10:1 for CEM experiments and 1:1 for RBC experiments. The needle connections to the gas cylinders were withdrawn and the still tightly stopped flasks were placed in a 37°C incubator. After 4 hr, 1 ml of supernatant was removed without disturbing the cells settled on the bottom via a long spinal tap needle (20-gauge, 3.5 inches) for determination of ⁵¹Cr release. To assure that the supernatant was devoid of cells, it was centrifuged at 1000 rpm (200 g) for 5 min and 0.5 ml removed for actual measurement of ⁵¹Cr release. In a similar manner, targets were added to flasks containing only supplemented RPMI to determine the spontaneous ⁵¹Cr release from targets alone. Control flasks gassed with 95% air, 5% CO₂ were processed in an identical fashion. Percent lysis was calculated as described above.

As a functional indication of the degree of oxygen depletion obtained under these conditions, HMPS activity of the monocytes was measured by ¹⁴C-1-glucose oxidation. 1 x 10⁶ monocytes suspended in 2 ml of Earle's balanced salt solution with 50 mg% glucose (MEBSS) were placed in flasks and gassed with either the N₂ mixture or the air mixture while being gently stirred. After 1 hr, 0.2 ml of ¹⁴C-1-glucose (10 µCi/ml, specific activity 4.0 µCi/μM Amersham Corp., Arlington Heights, Ill.) was added to the flasks. The stopped flasks were incubated at 37°C for 2 hr then placed in a −10°C freezer to stop the reaction. ¹⁴CO₂ derived from the metabolism of the labeled glucose was measured on an ionization chamber-electrometer apparatus described below.

**Glucose Metabolism**

HMPS activity of monocytes and target cells, both individually and in varying effector to target ratios, was determined by measuring the ¹⁴CO₂ production from ¹⁴C-1-glucose substrate.31 Monocytes and target cells were suspended in varying concentration in MEBSS. One ml of these suspensions were placed both separately and in combination in triple headed distilling flasks. Total volume of
all flasks was kept at 2.0 ml by addition of MEBSS where necessary. Two-tenths of a milliliter of \( ^{14} \text{C}-1\)-glucose was added to each flask which was then incubated at 37°C for 2 hr, after which the flasks were placed in a -10°C freezer to stop the reaction. Total \( ^{14} \text{CO}_2 \) production was measured with a 275 ml Cary-Tolbert ionization chamber and a Cary model 401 vibrating reed electrometer (Cary Instruments, Fairfield, N.J.) apparatus previously described. In some experiments, the CEM cells were poisoned with N-Ethylmaleimide (NEM-Eastman Kodak, Rochester, N.Y.) by incubating 5 x 10^4 cells in 2 ml of 0.1 mM NEM in supplemented RPMI at 37°C for 30 min. The poisoned cells were washed twice with fresh supplemented RPMI and resuspended to the desired concentration.

**Materials**

Drugs tested for effects on monocyte ADCC include: sodium benzoate, catalase (13,000 U/mg), superoxide dismutase (SOD, 2900 U/mg), sodium azide (all Sigma Chemical Co., St. Louis, Mo.), mannitol (Merck Sharp and Dohme, West Point, Penn.), phenol (Dept. of Pharmacy, OSU Hospitals, Columbus, Ohio).

**Statistical Analysis**

Statistical analysis was performed using Student’s t test. Values are expressed as ± 1 SD.

**RESULTS**

**Normal Monocyte ADCC**

Normal human monocytes were found to be actively cytotoxic for CEM targets and for antibody sensitized human red cell targets. Results of a typical CEM microtiter experiment done at an effector to target ratio of 10:1 are shown in Table 1. Monocytes produced significant increases in \( ^{51} \text{Cr} \) release from both antibody sensitized and nonantibody sensitized CEM cells \( (p < 0.001) \) compared to the spontaneous \( ^{51} \text{Cr} \) release from target cells alone. Spontaneous \( ^{51} \text{Cr} \) release from CEM targets was 5%-10% during a 4-hr assay. Kinetic experiments indicated that the \( ^{51} \text{Cr} \) release at 2 hr was approximately 85% of the 4-hr value. Figure 1 shows the lysis of CEM targets by monocytes at various effector to target ratios. For these experiments the number of effector cells was increased while the number of target cells was kept constant. Lysis of antibody sensitized CEM targets increased rapidly as the effector to target ratio was increased to 10:1 after which only modest increases could be detected. Maximum lysis obtainable in this system was approximately 60%. The mean value for 25 experiments at an effector to target ratio of 10:1 was 43% ± 13%. Lysis of nonantibody sensitized CEM cells, the lower line in Fig. 1, rose only slightly as the number of effector cells was increased. The mean value at a 10:1 ratio was 5.1% ± 3.3% \( (n = 25) \).

Normal monocytes produced significant lysis of antibody sensitized RBC \( (p < 0.001) \) but not of nonantibody sensitized RBC. Lysis of nonantibody sensitized RBC was less than 2% at all effector to target ratios tested. Spontaneous \( ^{51} \text{Cr} \) release was less than 1% in this RBC system. As in the CEM system, \( ^{51} \text{Cr} \) release at 2 hr was approximately 85% of the 4-hr value. Fig. 2 illustrates the lysis of antibody sensitized RBC at various effector to target ratios. At low ratios the % lysis of antibody sensitized RBC was significantly greater than that of CEM cells \( (p < 0.001) \). In the RBC system the mean % lysis at a 1:1 ratio was 40% ± 9% \( (n = 10) \) but only 7% ± 3% \( (n = 8) \) in the CEM system. At high ratios maximum RBC lysis was approximately 60%, similar to that of CEM cells.

**HMPS Activity of Monocytes During ADCC**

To determine if monocyte HMPS activity increased during ADCC, indicative of a respiratory burst, oxidation of \( ^{14} \text{C}-1\)-glucose substrate by monocytes responding to antibody sensitized target cells was compared to that of monocytes and target cells alone. Monocytes incubated with antibody sensitized RBC, in ratios at which RBC were actively lysed, had a two to threefold increase in HMPS activity compared to that of monocytes alone. Figure 3 shows the results of representative experiments done at an effector to target ratio of

**Table 1. Sample \( ^{51} \text{Cr} \) Release Data From CEM ADCC Experiment**

<table>
<thead>
<tr>
<th>Monocyte Number</th>
<th>Antibody Coated CEM</th>
<th>Uncoated CEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant CPM*</td>
<td>% ( ^{51} \text{Cr} ) Release</td>
</tr>
<tr>
<td>3 x 10^6</td>
<td>4769 ± 130</td>
<td>53.0</td>
</tr>
<tr>
<td>None</td>
<td>650 ± 8</td>
<td>7.2</td>
</tr>
<tr>
<td>Total counts</td>
<td>8997 ± 145</td>
<td>—</td>
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*Gamma counts per minute.
In contrast to RBC targets, monocytes incubated with antibody sensitized CEM cells in a 10:1 ratio, a ratio at which ADCC is optimal, did not have a burst in HMPS activity (Fig. 4). HMPS activity was not increased when monocytes and CEM targets were incubated in a 1:1 ratio. In the antibody sensitized RBC system an effector to target ratio of 10:1 increased monocyte HMPS activity from $2.31 \pm 0.21$ to $3.12 \pm 0.11$ ($n = 3$, $p < 0.05$). To rule out the possibility that the CEM cells were actively inhibiting monocyte function, antibody sensitized CEM cells were metabolically poisoned by preincubation in 0.1 mM NEM. This reduced CEM $^{14}$C-glucose oxidation by approximately 65%. Monocyte HMPS activity was unchanged when incubated with these cells. NEM treated cells were lysed normally in ADCC assays although $^{51}$Cr spontaneous release was approximately doubled.

**Effects of Hypoxia**

To determine if oxygen was required for monocyte ADCC a system was designed that allowed cell suspensions to be incubated under a 95% N$_2$-5% CO$_2$ or similar air-CO$_2$ atmosphere. As a functional indication of the degree of hypoxia obtained the HMPS activity of monocytes under these conditions was measured. Figure 5 shows that monocyte lysis of antibody sensitized CEM cells was unimpaired when incubated in a hypoxic environment, despite the fact that these conditions produced a marked impairment of monocyte HMPS activity. The HMPS activity of monocytes under the N$_2$ atmosphere was only $13 \pm 12\%$ that of monocytes under air. These conditions
would limit the production of potentially cytotoxic oxygen metabolites such as H$_2$O$_2$ or O$_2^-$ which require HMPS activity to generate reduced nicotinamide adenine dinucleotide phosphate (NADPH) needed for the reduction of molecular oxygen. Monocytes incubated under N$_2$ atmosphere and subsequently exposed to air functioned normally in ADCC and metabolic assays.

In contrast to these results, monocyte ADCC toward RBC was strikingly impaired when incubation was carried out under hypoxic conditions. At a 1:1 effector to target ratio, the lysis of RBC was decreased by 80% under a N$_2$ atmosphere (Fig. 6). The lower target lysis in these hypoxia experiments compared to that in the microtiter system is likely due to less than optimal contact between monocytes and target cells.

In these experiments the reaction flasks were not centrifuged to initiate cell contact.

**ADCC by CGD Monocytes**

The ADCC capabilities of monocytes from 2 patients with documented CGD were studied. Table 2 shows that at optimal effector to target ratios, CGD cells lysed antibody sensitized CEM cells normally, but had impaired ADCC toward antibody sensitized RBC. When monocytes from patient 1 were incubated with antibody sensitized RBC, they did not have the burst of HMPS activity characteristic of normal monocytes (data not shown).

**Effects of Scavengers of ROS**

Despite the requirement for activation of oxygen metabolism in the lysis of antibody sensitized RBC, protection by scavengers of ROS could not be demonstrated. Scavengers of hydrogen peroxide (catalase), superoxide (SOD), hydroxyl radical (mannitol, benzoate, phenol), and singlet oxygen (azide, which also inhibits the enzyme myeloperoxidase), in concentrations known to impair leukocyte bacteriocidal capability or to protect against injury by exogenously produced ROS, had no significant effect on monocyte ADCC toward RBC or CEM targets (Table 3).
DISCUSSION

Our studies confirm that human monocytes are capable of substantial cytotoxicity toward both antibody sensitized RBC and tumor cells. The percent lysis of sensitized targets increased to a maximum of approximately 60% for both target cells as the effector to target ratio was increased. This may reflect a limit to the amount of effector-target contact possible in this system. At low effector to target ratios, however, monocytes were able to lyse a significantly greater percentage of RBC than of CEM cells. This may represent the “spontaneous,” non-ADCC, monocyte cytotoxicity reported by other workers, which noted that monocytes lysed a small but significant percentage of nonantibody sensitized CEM cells. This may represent the “spontaneous,” non-ADCC, monocyte cytotoxicity reported by other workers, which takes substantially longer to become optimal in vitro.

When we examined the oxygen metabolism of monocytes in these systems, we found that monocytes responding to antibody sensitized RBC, in effector to target ratios producing substantial lysis, had a two to threefold increase in HMPS activity. Monocytes from a patient with documented CGD did not respond to antibody sensitized RBC with this burst of HMPS activity. This indicates that the increased HMPS activity involves activity of the enzyme NADPH oxidase, a key enzyme in the reduction of molecular oxygen to ROS, an enzyme that is deficient or defective in CGD monocytes. When RBC from a patient with documented G6PD deficiency were used, identical results were obtained, indicating that RBC do not contribute to the increase in HMPS activity. In contrast to these results, when monocytes were incubated with antibody sensitized CEM cells, again in ratios that produced substantial target lysis and that produced a burst of HMPS activity in the RBC system, there was no increase in monocyte HMPS activity. Active inhibition of monocyte response by the CEM cell was ruled out by using CEM cells metabolically poisoned with NEM.

If ROS are involved in the lysis of target cells, protection should be afforded by incubation in a hypoxic environment that would limit the availability of substrate for the generation of ROS. We found that when monocytes and antibody sensitized RBC were incubated in hypoxic conditions, the lysis of RBC was reduced by approximately 80%. Monocyte ADCC toward CEM cells, however, was unimpaired by hypoxia, even in the face of striking impairment of monocyte HMPS activity under these conditions. Monocytes incubated in hypoxic conditions and subsequently re-oxygenated, continued to function normally in ADCC and metabolic studies.

These experiments suggest that activation of oxygen metabolism occurs during the lysis of antibody sensitized RBC but not during the lysis of CEM cells. When we examined the ADCC capabilities of monocytes from two patients with documented CGD, cells whose only known defect is an inability to generate significant amounts of ROS, we found that lysis of antibody sensitized RBC targets was impaired but that these monocytes lysed CEM targets normally. These results differ from those of Fleer et al. who found CGD monocytes to have a relatively normal ability to lyse antibody sensitized RBC. This may be due to a monocyte preparation which, in their experiments, contained a greater number of lymphocytes. Lymphocytes have been shown to be capable of lysing antibody sensitized RBC targets and have been suggested to modulate some forms of monocyte/macrophage cytotoxicity. More recently Katz et al. have reported CGD mononuclear cells to have decreased lysis of antibody sensitized RBC which they attributed to a defective postphagocytic burst. They suggested that monocyte ADCC can occur either intracellularly or extracellularly which may determine the mechanism of lysis, a finding supported by our studies.

We were unable to demonstrate protection of antibody sensitized RBC target lysis by the addition of scavengers of ROS in concentrations known to be effective in non-ADCC systems. Scavengers of \( \text{H}_2\text{O}_2 \), \( \text{O}_2^- \), hydroxyl radical, singlet oxygen and inhibition of myeloperoxidase, had no significant effect on the lysis of RBC or CEM targets. These results are similar to those reported by others. Clark and Klebanoff have suggested that damage by ROS may occur in extremely limited areas of membrane contact between target and effector cells and may not be accessible to scavengers. It is also possible that oxidative injury in ADCC involves a ROS not scavenged by these compounds. In fact, we have previously shown that normal human RBC are relatively resistant to injury by \( \text{H}_2\text{O}_2 \) or \( \text{O}_2^- \) generating systems, suggesting that oxidative injury may be due to a ROS produced independently of \( \text{H}_2\text{O}_2 \) or \( \text{O}_2^- \).

These experiments suggest that monocyte ADCC involves two independent, but closely associated mechanisms, whose degree of activation may be determined by the nature of the target cell and sensitizing antibody. Obvious oxygen dependence, a burst of HMPS activity, and impaired lysis by CGD monocytes strongly suggest the involvement of ROS in monocyte ADCC toward RBC. We were, however, unable to identify the ROS using scavengers. Hypoxic conditions did not completely protect RBC from lysis, nor were CGD monocytes completely inactive in RBC target lysis, suggesting the existence of a second,
MECHANISMS OF MONOCYTE ADCC

nonoxidative mechanism of ADCC. This mechanism of monocyte ADCC can be activated independently of oxidative mechanisms, as shown by the experiments with CEM tumor cells. In this system there was no oxygen dependence to ADCC, no burst of HMPS activity, and normal lysis by CGD monocytes. The lower percent lysis of CEM targets compared to RBC targets at most effector to target ratios may reflect only partial activation of monocyte cytotoxic potential by CEM targets.

Binding to Fc receptors may thus trigger more than one mechanism of cytotoxicity. The reason for the inability of antibody sensitized CEM targets to activate monocyte oxidative metabolism is not clear. It may reflect different thresholds of activation for different cytotoxic mechanisms. Factors such as the number of Fc receptors bound, their spatial arrangement, and the immunoglobulin type or subtype may all be important but have not been thoroughly investigated. The nature of the target stimulus does appear to be critical in determining the degree of monocyte activation. Papain treatment of anti-D sensitized RBC, for example, markedly augments lysis by mononuclear effector cells. Anti-A or B sera have been shown to be less effective in ADCC than anti-D and even the source of anti-D serum affects the degree of RBC lysis. Subtle target cell variations in antigen density or distribution or the type or amount of sensitizing antibody may be important in determining the mechanism of monocyte ADCC.

We conclude that human monocytes have two independent mechanisms of ADCC: a nonoxidative mechanism, and a second oxidative mechanism that appears to involve ROS. The activation and possible interactions of these mechanisms are determined by the nature of the target cell and sensitizing antibody. Further studies are required to more fully characterize these mechanisms and factors controlling their activation and interaction.

REFERENCES

Evidence for both oxygen and non-oxygen dependent mechanisms of antibody sensitized target cell lysis by human monocytes

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