

Cytotoxic Activity of Human Monocytes Towards Sensitized Red Cells is not Dependent on the Generation of Reactive Oxygen Species

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The purpose of this investigation was to test whether the generation of oxygen radicals is required for the cytotoxic activity of human monocytes. The cytotoxic activity of human monocytes was measured as the capacity to lyse, in vitro, human red cells sensitized with non-complement-binding IgG anti-D alloantibodies. Lysis was determined by measuring ^{51}Cr release from the red cells. Studies were performed with monocytes from patients with chronic granulomatous disease (CGD). In contrast with normal neutrophils, phagocytosing neutrophils from these patients are unable to generate oxygen radicals, hydrogen peroxide, or singlet oxygen. We found that monocytes from CGD patients were similarly incapable

of generating these reactive oxygen species during phagocytosis. This indicates that, like neutrophils, monocytes from CGD patients are defective in the generation of activated oxygen products. Despite this defect, monocytes from CGD patients displayed a normal cytotoxic activity towards sensitized red cells. In addition, neither scavengers of superoxide, hydrogen peroxide, hydroxyl radicals, or singlet oxygen, nor inhibitors of myeloperoxidase, had any effect on the cytotoxic activity of normal monocytes. Together, these results indicate that the cytotoxic activity of human monocytes towards sensitized red cells is not dependent on the generation of reactive oxygen derivatives.

MONONUCLEAR PHAGOCYTES are capable of several effector functions, such as phagocytosis followed by intracellular killing of bacteria and cytotoxicity by an extracellular mechanism. It has been well established that the first process is accompanied by a series of metabolic changes, generally designated as the "respiratory burst." In this process, various oxygen derivatives, such as superoxide and hydrogen peroxide,¹ are generated. Actual ingestion is not required for the generation of these molecules, since it has been shown that human monocytes treated with antimycin-A generate superoxide and hydrogen peroxide upon incubation with opsonized zymosan, although these cells are severely inhibited in their ingestion capacity.²

The formation of reactive oxygen species is an essential prerequisite for the killing of certain bacteria by phagocytic cells.¹ The purpose of this study was to examine whether generation of oxygen products was also necessary for the second function mentioned above, namely, the extracellular lysis of sensitized red cells by human monocytes. To this end, studies were performed with monocytes from patients with chronic granulomatous disease (CGD). Granulocytes from CGD

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patients are defective in bacterial killing³ and incapable of generating oxygen radicals.⁴⁻⁷ There is evidence⁸⁻¹³ that monocytes from CGD patients share these defects.

MATERIALS AND METHODS

Subjects

Venous blood was obtained from healthy donors, and was collected from five patients with CGD. The criteria for diagnosis of CGD were those described by Johnston and Newman.¹⁴

Preparation of Monocytes and Red Cells

Details of the methods used for the preparation of monocytes and red cells have been described previously.¹⁵ In short, mononuclear cells were isolated from defibrinated blood by spinning over Ficoll-Isopaque and freed from most of the lymphocytes by adherence to plastic Petri dishes. The adherent cells, which contained $71\% \pm 13\%$ monocytes (mean \pm SD, $n = 20$), were suspended in minimal essential medium (MEM) plus 10% (v/v) fetal calf serum (FCS) to a concentration of 10^6 monocytes/ml. Red cells were labeled with ⁵¹Cr and sensitized with non-complement-binding anti-D alloantiserum by standard methods.¹⁵ The concentration of the red cell suspension was adjusted to 1×10^6 – 20×10^6 /ml, in MEM–10% FCS.

Metabolic Activity of Neutrophils and Monocytes

Oxygen consumption,¹⁶ hydrogen peroxide production,⁶ killing capacity toward *Staphylococcus aureus*,¹⁷ and activity of hexose monophosphate shunt enzymes¹⁸ were determined according to previously published methods.

Cytotoxicity Assay

The cytotoxic activity of the monocytes was assessed by determining the capacity to lyse anti-D-sensitized red cells (EAIgG).¹⁵ For this purpose, 5×10^4 monocytes were incubated with 5×10^4 – 1×10^6 EAIgG in a total volume of 0.15 ml MEM–10% FCS. The incubations were carried out for 16 hr at 37°C in wells of round-bottom microtiter plates. Afterwards, the plates were centrifuged, and a sample was taken from the supernatant to determine ⁵¹Cr release. The cytotoxic activity of the monocytes was calculated as [(E-S)/total amount of radioactivity/well] \times number of added EAIgG, in which E is the experimental ⁵¹Cr release (monocytes with EAIgG), and S is spontaneous ⁵¹Cr release (monocytes with nonsensitized erythrocytes). The spontaneous ⁵¹Cr release was always less than 5% of the total amount of radioactivity per well.

Reagents

Superoxide dismutase, isolated from bovine red cells as described by McCord and Fridovich,¹⁹ was a gift from Dr. R. Wever, University of Amsterdam. Catalase (65,000 U/mg) was a product of Boehringer, Mannheim, West Germany. MEM was obtained from Gibco, Grand Island, N.Y. and FCS from Gibco-Biocult, Glasgow, Scotland.

RESULTS

Generation of Hydrogen Peroxide by Monocytes From CGD Patients

CGD was diagnosed with purified neutrophils. Phagocytosis of serum-treated zymosan resulted neither in a stimulation of the oxygen uptake nor in the generation of hydrogen peroxide. Moreover, the killing of ingested *Staphylococcus aureus* in vitro was severely disturbed. The activities of glucose-6-phosphate dehydrogenase, 6-phospho-gluconate dehydrogenase, glutathione reductase, and glutathione peroxidase were normal.

From four of the five patients, the capacity of the monocytes to generate hydrogen peroxide was tested. This capacity was found to be severely depressed (Table 1).

Table 1. Generation of Hydrogen Peroxide by Monocytes From CGD Patients

Patients	H ₂ O ₂ Produced (nmole/min/10 ⁶ monocytes)
K.B. (female)	0.09
H.V. (female)	0.09
J.P. (male)	0
M.J. (male)	0
M.V. (female)	ND*
Controls (n = 11)	0.8 ± 0.3 (Mean ± SD)

*Not done.

Cytotoxic Activity of Monocytes From CGD Patients

The cytotoxic activity of monocytes from CGD patients towards anti-D-sensitized red cells was studied in comparison with monocytes from healthy individuals. Figure 1 shows that there was no appreciable difference in cytotoxic activity.

The Effect of Scavengers and Inhibitors on the Cytotoxic Activity of Normal Monocytes

Scavengers of superoxide (superoxide dismutase), hydrogen peroxide (catalase), hydroxyl radicals (mannitol, benzoate), or singlet oxygen (histidine, azide) had no effect on the cytotoxic activity of normal monocytes towards anti-D-sensitized red cells (Table 2). Since azide also inhibits myeloperoxidase, this enzyme, too, is not involved in the cytotoxic process. This was confirmed with cyanide. Only with 40 mM ethanol was a strong inhibition of the cytotoxicity found; however, this was shown to be due to extensive damage to the monocytes (fluorescein diacetate splitting was strongly inhibited). The other agents had no effect on the viability of the monocytes or the spontaneous lysis of the erythrocytes.

DISCUSSION

These studies show that monocytes from CGD patients are, like neutrophils from these patients, incapable of generating hydrogen peroxide during phagocytosis. Recently, Weiss et al.¹² showed that monocytes from a CGD patient were unable to produce hydroxyl radicals during phagocytosis. Together with the present data, this

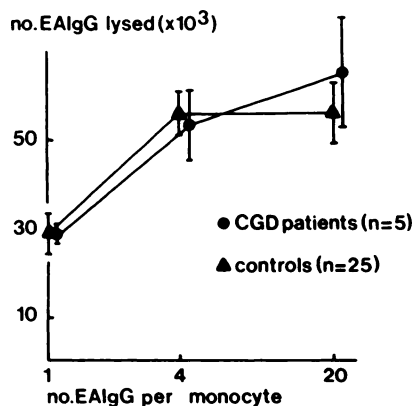


Fig. 1 Cytotoxic activity of monocytes from patients with chronic granulomatous disease (CGD). Abscissa: number of EAIG per monocyte. Ordinate: number of EAIG lysed ($\times 10^3$). Means \pm SEM are shown.

Table 2. Effects of Scavengers of Oxygen Radicals on the Cytotoxic Activity of Normal Monocytes

Scavenger	Cytotoxic Activity*
Superoxide dismutase (150 µg/ml)	102 ± 6.5
Catalase (250 µg/ml)	98 ± 3.0
Mannitol (10 mM)	106 ± 4.5
Sodium benzoate (20 mM)	82 ± 8.0
Histidine (50 mM)	124 ± 7.9
Sodium azide (10 mM)	133 ± 9.4
Potassium cyanide (1 mM)	121 ± 8.3

*Expressed as percentage of control (mean ± SEM, *n* = 6).

indicates that monocytes from CGD patients have a similar defect in the metabolic response to phagocytosis, as do the neutrophils from these patients.

Despite this defect, the monocytes from these patients displayed a normal cytotoxic activity towards anti-D-sensitized red cells. This indicates that the generation of reactive oxygen species is not essential for the lysis of these red cells by monocytes. Previously, it has been shown^{20,21} that the cytotoxic activity of mononuclear leukocyte suspensions towards red cells sensitized with the same anti-D serum that was used for the present investigations is due to the activity of the monocytes: among the cells that did not adhere to plastic, we found 5% monocytes and 95% lymphocytes; this cell suspension gave less than 10% of the cytotoxicity of suspensions with 70% monocytes and 30% lymphocytes. Therefore, we may safely conclude that the 30% lymphocytes in the CGD cell suspensions cannot be responsible for the normal cytotoxicity towards anti-D-sensitized red cells.

The other results presented in this report support the conclusion that monocytes do not lyse red cells by oxygen radicals. Neither scavengers of superoxide, hydrogen peroxide, hydroxyl radicals, or singlet oxygen,^{12,22} nor inhibitors of myeloperoxidase, had any effect on the cytotoxic activity of normal monocytes. Similar concentrations of these agents, as used here, effectively inhibit the bacterial killing by granulocytes,^{17,23} a process dependent on the generation of activated oxygen derivatives.¹ Moreover, granulocytes produce larger amounts of these products than do monocytes.^{2,23} Thus, it is unlikely that the lack of effect of these substances was due to too low concentrations.

In another communication²⁰ we have shown that monocytes release lysosomal enzymes upon incubation with anti-D-sensitized red cells. We have presented evidence that these enzymes are responsible for the damage leading to lysis of the red cells. Taken together, the data of the latter report and this one support the conclusion that the release of lysosomal enzymes, and not the generation of oxygen radicals, is responsible for the lysis of sensitized red cells by human monocytes. In this regard, human monocytes appear to differ from human neutrophils. Recently, Clark and Klebanoff²⁴ showed that neutrophils from CGD patients had a reduced antibody-dependent cytotoxic activity towards tumor cells, indicating that the generation of oxygen radicals is required for the full expression of the cytotoxic activity of human neutrophils. An alternative possibility is that this apparent difference in cytotoxic activity of human monocytes and neutrophils is due to the different antibody-dependent cytotoxicity assays used. This possibility remains to be investigated.

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