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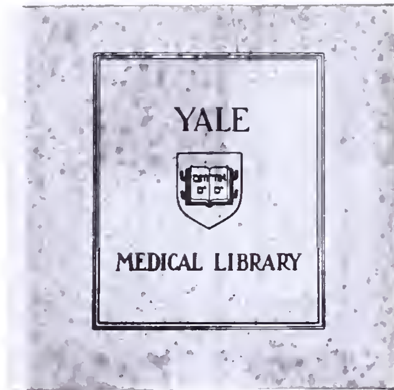
CYTOTOXIC MECHANISMS OF CULTURED HUMAN PERIPHERAL BLOOD MONOCYTES

DEBORAH ELIZABETH DYETT

1980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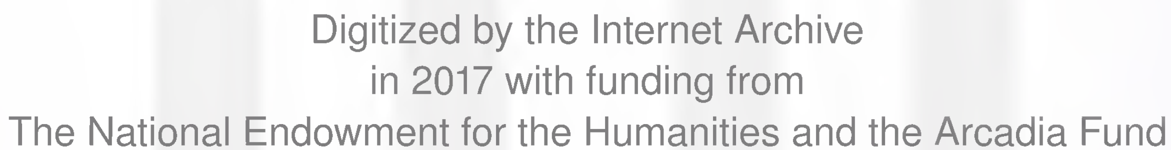
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Cytotoxic Mechanisms of
Cultured Human Peripheral
Blood Monocytes

Deborah Elizabeth Dyett

B.A., Radcliffe College, 1976

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirement for the degree of

Doctor of Medicine

1980

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To my Grandmother

Elva Gertrude Johnston, in loving memory.

ACKNOWLDEGEMENTS

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To Dr. William Greene for advice and guidance.

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Introduction

Mononuclear phagocytes, including peripheral blood monocytes, peritoneal and alveolar macrophages, and the fixed tissue cells of the liver, lymph nodes and spleen, play an important role in host defenses. Some of their well-defined roles include defense against facultative and obligate intracellular parasites, removal of damaged or dying cells and cell debris, and interaction with lymphocytes in the expression of cell-mediated immunity (30).

Mononuclear phagocytes have also been implicated in other important functions. They may play an important role in the regulation of granulopoiesis, in controlling neoplastic cells and in the synthesis of important proteins such as pyrogen, the second and fourth complement components, transferrin, colony stimulating factor, interferon, and plasminogen activator (30,73).

Despite their important role in host defense, the metabolism and microbial activity of human mononuclear phagocytes have not been extensively studied due to problems with procurement and methods. At the present time, it can be said that they are partly dependent on oxidative and peroxidative metabolism for their anti-microbial effect, but may also possess oxygen independent microbiocidal pathways.

The microbiocidal system of polymorphonuclear leukocytes (PMN) has been more extensively studied and is more clearly understood than that of the mononuclear phagocyte.

As it seems that the two systems have much in common, an understanding of the PMN cidal system is essential to the framing of a hypothesis for further study of the mononuclear phagocyte system.

The major functionalunit of the mononuclear phagocyte system is the tissue macrophage (30). Major problems exist in investigating the macrophages of human subjects. One approach to this problem is to place human peripheral blood monoyctes in *in vitro* culture until they mature into macrophages.

The following work was aimed at developing a system for long term culture of human blood monocytes, and at studying the relationship between their oxidative metabolism and their cytotoxic and microbiocidal activities.

I. Antimicrobial and Cytotoxic Mechanisms In Polymorphonuclear Leukocytes

The antimicrobial systems of polymorphonuclear leukocytes (PMNs) have been classified by Klebanoff as follows:

I. Oxygen-dependent

- A. Myeloperoxidase-mediated
- B. Myeloperoxidase-independent
  1. Hydrogen Peroxide (H_2O_2)
  2. Superoxide Anion ($O_2^{\bar{\cdot}}$)
  3. Hydroxyl Radical (.OH)
  4. Singlet Oxygen ($'O_2$)

II. Oxygen-independent

- A. Acid
- B. Lysozyme
- C. Lactoferrin
- D. Granular Cationic Proteins

(84)

These microbicidal systems all require some form of "triggering" for activation, the most common form of such "triggering" being phagocytosis.

Phagocytosis

The antimicrobial activity of polymorphonuclear leukocytes (PMN) begins with phagocytosis. The normal sequence of events of phagocytosis includes 1) Chemotaxis, 2) Recog-

nition of particle, 3) Binding of particle to PMN plasma membrane, 4) Ingestion of particle with formation of membrane bound phagocytic vacuole (phagosome), 5) "degranulation" - fusion of cytoplasmic granules with phagosome with discharge of granule contents into the phagosome, 6) Killing of ingested particles (when they are microbes), 7) Digestion of ingested particles (159, 160, 161).

Metabolism of Phagocytosis

Within seconds of phagocytosis a number of metabolic changes occur in the PMN. These include increased oxygen utilization, increased glucose utilization with stimulation of the hexose monophosphate shunt (HMP) and the production of H_2O_2, superoxide (O_2^-), singlet oxygen ($'O_2$) and hydroxyl radical (.OH) (4,57,83,108,115,140,145,167,178).

Increased oxygen utilization during phagocytosis was first demonstrated by Karnovsky and colleagues (156,157,158). It was later shown that phagocytosis was followed by increased lactate production, increased oxygen uptake and stimulation of the HMP as evidenced by the increased appearance of C-1 glucose as CO_2 relative to C-6 glucose (145) (with the rates of Cl glucose to C6 glucose appearing as CO_2 increasing from 8 in the resting cell to 22 during phagocytosis) (57,145).

Membrane stimulation alone without actual phagocytosis can initiate these metabolic changes. Rossi _et al_ have shown that increased oxygen utilization and the increased $^{14}CO_2$

production from 1-^{14}C began 60 seconds after adding killed opsonized bacteria to PMN's, well before ingestion and degranulation could occur (140,178,180). They proposed that mere contact between cell and bacteria could lead to this increased metabolism. This increased metabolism can be triggered by a large number of substances including endotoxin, plant lectins, complement (c5a), immunoglobulin bound to antigen and cationic ionophore antibiotics (49,50, 129,135). Further evidence that the stimulation of PMN oxidative metabolism is indeed independent of phagocytosis is provided by the fact that pretreatment of PMN's with agents (such as cytochalasin B) that block phagocytosis does not interfere with the "respiratory burst" (97). Rossi has proposed the existence of membrane bound "phagocytic receptors" that play a role in initiating H_2O_2 and O_2^- production by cellular oxidase (128).

PMN's are capable of both intracellular and extracellular killing. The microbicidal and cytotoxic function of neutrophils has been studied more extensively than that of any other white blood cell, and therefore its mechanisms may serve as the basis for initial exploration of similar functions by other white cells, including the monocyte and macrophage.

Oxygen Dependent Antimicrobial and Cytotoxic Systems

Although phagocytosis is unaffected by anaerobiosis, oxygen is required for optimal antimicrobial activity of PMN's (84,103). Human PMN's under anerobic conditions show decreased ability to kill certain bacteria (98). PMN's have recently been shown to employ oxygen-dependent mechanisms in the killing of various mammalian tumor cell lines (24,26,27,45,52). Hypoxic conditions have been shown to inhibit cytotoxicity of PMN's toward tumor cells by 29-73% (52).

That oxygen is required for optimal microbial function of PMN's is supported by the fact that patients with congenital defects in leukocyte metabolism have leukocytes with defective antimicrobial function (69,81). PMN's from patients with chronic granulomatous disease (CGD) exhibit normal chemotaxis and phagocytosis; however, phagocytosis is not accompanied by increased oxygen utilization and increased HMP activity (5,69). These PMN's do not produce H_2O_2 and superoxide anion and fail to kill bacteria that do not themselves produce H_2O_2 (34,69,100).

Myeloperoxidase-mediated System

Myeloperoxidase (MPO), H_2O_2 and a halide constitute a potent _in vitro_ antimicrobial (15,58,76,91,104) and cytotoxic system (24,27,28,40). In the intact cells, after particle

ingestion, MPO is released into the phagocytic vacuole and H_2O_2 is produced in association with the post-phagocytic metabolic burst.

In 1968, Klebanoff reported that myeloperoxidase has an anti-bacterial effect when combined with certain halides and H_2O_2 (76). Iodide was shown to be the most effective halide, followed by bromide then chloride. Phagocytosis is associated with a burst of leukocyte metabolic activity with H_2O_2 being generated as a consequence (76,104). The requirement for H_2O_2 in the MPO-mediated system may be met by H_2O_2 formed in leukocytes or by H_2O_2 produced by microbial metabolism (76).

The MPO-H_2O_2-halide system has also been shown to be effective against mycoplasma, fungi and mycobacteria (15,58, 91).

MPO and H_2O_2 are also released by PMN's into the extracelluar fluid. A mechanism of PMN-mediated tumor cell cytotoxicity has been proposed whereby secreted MPO and H_2O_2 combine with extracellular halides to form a cytotoxic system (24,28,40). Tumor cell killing by human PMN's in the presence of Concanavalin A has been shown to require halides and to be inhibited by agents that block heme enzymes (azide, cyanide) or degrade H_2O_2 (catalase) (27). PMN's deficient in MPO (hereditary MPO deficiency) or H_2O_2 formation (CGD) had impaired Concanavalin A-induced cytotoxic activity and these defects were corrected by the addition of purified

MPO or H_2O_2 generating system respectively (12,126,127). However, approximately one third of cytotoxic activity persisted under conditions that could be expected to preclude a MPO-mediated effect, suggesting that MPO-mediated systems did not account for all of the cytotoxicity observed.

The MPO system contains the following components:

a. Myeloperoxidase

b. H_2O_2

  Leukocyte metabolism

  Microbial metabolism

c. Co-factors

  Halide

  Thiocyanate, Thyroxine

  Tri-iodo thyronine

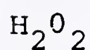
d. Acid

e. Inhibitors (84)

Myeloperoxidase

Peroxidases are enzymes that catalyze the oxidation of a number of substances by H_2O_2. They tend to be heme proteins and are heat and acid labile. There are many distinct peroxidases that differ in structure, nature of heme prosthetic group and in reactions catalyzed. Myeloperoxidase is the name of the peroxidase of neutrophils. It appears to be identical to the proxidase of monocytes, but differs from

that of milk, saliva and eosinophils (84,91). It is a basic protein (76) with a molecular weight of approximately 150,000 and contains 2 heme prosthetic groups per molecule. It is present in neutrophils in high concentrations, being responsible for approximately 5% of their dry weight (40). It is located in the primary granules of the resting cell (14) and is released into the phagocytic vacuole during degranulation (8).



Phagocytosis by PMN's is associated with a burst of oxidative metabolism. Increased oxygen consumption can be detected within seconds of contact between cells and particles. Almost all of the oxygen consumed is converted to H_2O_2. This production of H_2O_2 by phagocytosing leukocytes was first demonstrated by Iyer et al (57), and has since been detected by a number of techniques including the oxidation of formate by catalase (57), inhibition of catalase by aminotriazole (183), oxidation of scopolitin by horseradish peroxidase (132) and the release of O_2 by catalase (181).

The enzyme system required for the respiratory burst in PMN's has been the subject of much study and controversy. Increased H_2O_2 production is accompanied by increased oxygen utilization and increased HMP activity. Any enzyme system model must account for these three events. One proposed

scheme is shown in Table 1, and involves an oxidase that reduces 0_2 to H_2O_2 and also results in increased generation of $NADP^+$ that, in turn, may cause the observed stimulation of the HMP.

The Oxidase

The early metabolic changes induced by phagocytosis are associated with the oxidation of reduced pyridine nucleotides (178). The NADP+/NADPH ratio is increased by phagocytosis while the NAD+/NADH ratio is unaffected (178,147) suggesting that the oxidation of reduced pyridine nucleotide reflects NADPH rather than NADH oxidation. The phagocytosis-induced respiratory burst is largely unaffected by ImM cyanide (140,145). Thus a cyanide-insensitive NADPH or NADH oxidase is likely to be the primary oxidase for converting 0_2 to H_2O_2.

NADH oxidase, which has been proposed as the primary oxidase (23,41,67), is a flavoprotein which reduces 0_2 to H_2O_2 and is insensitive to ImM cyanide. Its activity increased during the respiratory burst following phagocytosis and its activity has been found to be sufficient to account for the respiratory burst (6). However, the formation of O_2^- is not an intermediary in the formation of NAD+ and H_2O_2 by the NADH oxidase and O_2^- seems to be a necessary intermediate in the formation of H_2O_2.

The formation of H_2O_2 by NADPH oxidase is not inhibited by cyanide (141,179). In addition, NADPH oxidase forms O_2^-, a necessary intermediate in the formation of H_2O_2.

Rossi _et al_ have shown that the activity of NADPH oxidase during phagocytosis is sufficient to account for the respiratory burst while that of the NADH oxidase is not (139). They also showed a three-fold increase in the NADP+/NADPH ratio following phagocytosis, while the NAD+/NADH ratio remained unchanged.

Oxidation of NADPH by a primary oxidase would meet the above stated requirements (increased O_2 consumption, H_2O_2 formation and NADPH oxidation). A primary oxidase other than an NADPH oxidase requires some sort of linkage to NADPH oxidation. The glutathione cycle has been proposed as a possible link. In this cycle H_2O_2 oxidizes reduced glutathione (GSN) in the presence of GSH peroxidase. Oxidized glutathione (GSSG) is then reduced by NADPH and GSSG reductase making NADP+ available for the HMP (120). Human leukocytes contain GSSG reductase (6,55). However, GSH peroxidase has been detected in human PMN's by some investigators (90, 91) but not by others (6). These investigators suggest the nonenzymatic oxidation of GSH by H_2O_2 occurs at a rate sufficient to account for the stimulation of HMP activity by H_2O_2 (6), however this hypothesis is not widely accepted (139).

In summary, the controversy as to whether the critical

enzyme is an NADPH, NADH or primary oxidase is still unsettled. It seems, however, that the weight of the evidence suggests the former.

Microbial Metabolism

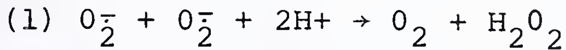
Microbial organisms can provide a source of H_2O_2 for the MPO-mediated anti-microbial system. Certain bacteria (classified as lactic acid bacteria) contain flavoproteins which reduce O_2 to H_2O_2. These organisms may lack catalase which then results in the accumulation of H_2O_2 (176). The H_2O_2 of microbial origin may be toxic to the organisms which formed it or to another microbial species. This toxicity is enhanced by MPO and a halide (84).

The H_2O_2 formed by microorganisms may contribute significantly to the microbicidal activity of the PMN. This is particularly evident in leukocytes that are defective in generating H_2O_2 such as in CGD. H_2O_2 generating bacteria that lack catalase are readily killed by the leukocytes of patients with CGD suggesting that H_2O_2 or microbial origin can compensate for a defective leukocyte H_2O_2 generating system (69,89).

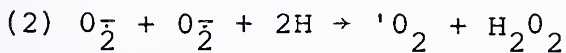
Super Oxide Anion As An Intermediate in H_2O_2 Production

Hydroperoxy radical (HO_2) and its conjugate base are formed by the univalent reduction of oxygen. The pka of the reaction $HO_2^{\cdot} \rightleftarrows H + O_2^-$ is 4.8. Therefore the radical

exists almost entirely as the superoxide anion at neutral pH. Two molecules of superoxide may interact spontaneously in a dismutation reaction resulting in the formation of H_2O_2 (equation 1) the O_2 of which may be in the form of singlet oxygen (equation 2).



(1) $O_2^- + O_2^- + 2H+ \rightarrow O_2 + H_2O_2$



(2) $O_2^- + O_2^- + 2H \rightarrow {}'O_2 + H_2O_2$

This reaction is also catalyzed by the enzyme superoxide dismutase.

That PMN's produce superoxide anion is suggested by the fact that intact PMN's reduce cytochrome C and nitroblue tetrazolium (NBT) and this reduction can be inhibited with superoxide dismutase (9). The formation of superoxide anion is increased by phagocytosis (9) and is low in PMN's of patients with CGD (34). Superoxide anion may be toxic to microorganisms and mammalian cells directly or indirectly by acting as an intermediate in hydroxyl radical, singlet oxygen, or H_2O_2 formation (63).

In addition, there are stoichiometric measurements suggesting that virtually all PMN-generated H_2O_2 is via an O_2^- intermediate. This is supported by the work of Weening et al (172). Klebanoff has suggested that an O_2^- generating system (Xanthine and Xanthine oxidase) (figure 1) can be utilized as the source of H_2O_2 for the MPO-mediated antimicrobial system (82).

Superoxide Dismutase (SOD)

The reactive free radical $O_2^{\bar{}}$ is quite toxic to mammalian cells (44,144). It has been postulated that SOD acts to protect organisms from the toxic effects of $O_2^{\bar{}}$ (44,102). Two types of this enzyme have been shown to be present in eukaryotic cells. One containing manganese is located in mitochondria and one containing copper is found in the cytosol (173). Cells normally contain approximately 10 times more of the cytosol-located enzyme than the mitochondrial enzyme. The presence of SOD in high quantities in leukocytes has been taken as evidence that they produce superoxide in sufficient quantity to play a significant role in the bactericidal process. DeChatelet _et al_ have demonstrated SOD in extracts of PMN's (36), contrary to the findings of Salin and McCord (144), providing further evidence for the production of $O_2^{\bar{}}$ by PMN's.

Co-Factors

The MPO-mediated system has been demonstrated with thiocyanate, iodide, bromide, chloride and the thyroid hormones as co-factors (23,75,76).

Chloride is present in PMN's at a concentration in excess of that which is required as a component of the MPO-mediated anti-microbial system. Klabanoff found maximal killing by the MPO-Halide-H_2O_2 system at chloride concentrations

of 0.5 mEq/liter (76). Extrapolations from studies with rabbit PMN's suggest that the concentration of chloride in human PMN's may be 75 mEq/liter (84), sufficient for the MPO-H_2O_2 system.

On a molar basis iodide is more effective than chloride as the halide component of the MPO-mediated antimicrobial system (76). The thyroid hormones thyroxine (T_4) and triiodo thyronine (T_3) can also replace iodide as the cofactor in the isolated MPO-mediated anti-microbial system (75). Iodide is present in very low concentrations in plasma (less than 1 μg/100 ml). However, the uptake of iodide by normal human leukocytes has been reported to be 1200 times the uptake by erythrocytes (75). T_4 and T_3 are also preferentially taken up by leukocytes (75). These hormones are deiodinated by intact phagocytosing leukocytes (86,87,177) and may serve as an additional source of iodide for the cell.

Mechanism of Action

The mechanism of action of the MPO-mediated antimicrobial system is not completely known. H_2O_2 reacts with the heme prosthetic groups of MPO to form an enzyme-substrate complex or complexes with strong oxidative capacity. The halides are oxidized by this complex resulting in the formation of strong antimicrobial agents (Figure 1), possibly including singlet oxygen or other reactive oxygen products.

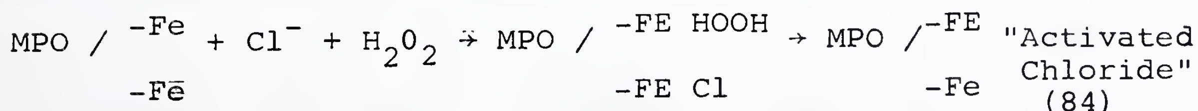
The major intracellular operational site of the MPO-H_2O_2-Halide system has been shown to be the phagocytic vacuole (134)..

Iodide

When iodine is employed as the halide co-factor, death of organisms is associated with the iodination of bacterial proteins (75). The toxicity of the iodide system is not due solely to the iodination of proteins, since iodination can be prevented without impairing bactericidal capacity. However, the products of iodide oxidation are strong oxidizing agents and may contribute to toxicity by oxidizing essential components of cell (20).

Chloride

The reaction between MPO, chloride and H_2O_2 can be depicted as follows:



$$MPO \Big/ \begin{matrix} -Fe \\ -Fe \end{matrix} + Cl^- + H_2O_2 \rightarrow MPO \Big/ \begin{matrix} -FE\ HOOH \\ -FE\ Cl \end{matrix} \rightarrow MPO \Big/ \begin{matrix} -FE \\ -Fe \end{matrix} \text{"Activated Chloride"}$$

(84)

The "activated chloride" (a highly reactive product of chloride activation) may affect organisms in a number of ways. Chlorine derivatives inhibit enzymes which are dependent on sulfhydryl groups for optimum activity. It has been

proposed that chloride derivatives cause death by oxidation of sulfhydryl groups, which leads to the disruption of essential metabolic systems (84).

The iodination of bacteria by MPO, H_2O_2 and iodide is stimulated by chloride when iodine concentrations are low. The presumed sequence of events is that chloride is activated by the peroxidase system followed by the oxidation of iodide by "activated chloride" (84).

Chlorine derivatives react with nitrogenous compounds to form unstable inorganic and organic chloramines, which are hydrolyzed with the release of activated chloride and bactericidal aldehydes (146,164,182).

Singlet Oxygen

Intact PMN's emit light during phagocytosis. It has been proposed that singlet oxygen is responsible for this chemiluminescence (72). Singlet oxygen may be formed by the MPO-H_2O_2-chloride system (72) or by the spontaneous dismutation of the superoxide anion (138). Singlet oxygen is a highly reactive, electronically excited state of oxygen (72) that may react with essential groups in microbes and mammalian cells to cause death (84).

In summary, the mechanism of the MPO-mediated antimicrobial system is complex. It involves a number of cofactors each with a number of possible mechanisms. That the MPO-mediated antimicrobial system plays a role in intact

cells is clear from a number of observations. 1. The components of the MPO-mediated antimicrobial system are present in PMN's and their formation and/or release occurs at a time appropriate to the microbial killing. 2. The components are capable of reaction in the phagocytic vacuole (75,134). 3. PMN's deficient in H_2O_2 formation have decreased microbicidal activity (5,69,81,127). 4. PMN's deficient in MPO have decreased microbicidal activity (79, 93).

Oxygen Dependent MPO Independent Antimicrobial Systems

It is clear that MPO-independent antimicrobial systems exist in PMN's, since PMN's deficient in MPO retain microbicidal activity (127). Antimicrobial systems in MPO-deficient PMN's are partly dependent on oxygen. Anaerobiosis inhibits the staphylocidal activity of MPO-deficient leukocytes (87). MPO deficient leukocytes exhibit delayed killing, having been shown to kill certain bacteria in 3-4 hours while normal cells required 30-40 minutes (93).

There is evidence that MPO-independent systems appear to be "hyperactive" in MPO-deficient cells, even at rest. The addition of azide (which inhibits MPO) to populations of normal and MPO-deficient cells results in greater microbial activity by the MPO-deficient cells (79) suggesting increased activity of MPO-independent antimicrobial systems. The micro-

bicidal defect in MPO-deficient PMN's is not as severe as in CGD PMN's as affected individuals are relatively free of recurrent infections (127). The phagocytosis-dependent increase in oxygen, metabolism, and HMP activity is greater in MPO-deficient than normal PMN's (87,88). These parameters are markedly decreased in CGD leukocytes (69). To a lesser degree, a similar increase in metabolic activity can be demonstrated when normal PMN's are treated with a peroxidase inhibitor (87,88). These studies suggest that MPO-deficiency is associated with increased activity of oxygen-dependent MPO-independent antimicrobial systems which may include H_2O_2, superoxide anion, hydroxyl radicals or singlet oxygen.

H_2O_2

High concentrations of H_2O_2 are microbicidal in the absence of MPO. This activity is increased by certain substances such as iodide (75) and ascorbic acid (106).

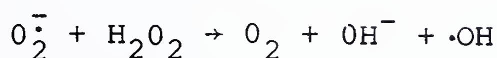
Superoxide Anion

Superoxide (a reactive oxygen anion) has been shown to be produced by phagocytosing but not resting PMN's (9, 33,37,130) and was once felt to be a major bactericidal agent of PMN's. However, O_2^- alone has been shown to be only weakly bactericidal (13,82,99). O_2^- in combination with H_2O_2 has been found to be highly toxic to a number of bacterial

species (13,63), suggesting that a potent antimicrobial agent is formed by a reaction between $O_2^{\overline{\cdot}}$ and H_2O_2.

Hydroxyl Radicals

Hydroxyl radicals ($\cdot OH$) are formed by the interaction of H_2O_2 and $O_2^{\overline{\cdot}}$.



$$O_2^{\overline{\cdot}} + H_2O_2 \rightarrow O_2 + OH^- + \cdot OH$$

Tauber and Babior have shown that PMN's produce hydroxyl radical (167). As mentioned above $O_2^{\overline{\cdot}}$ combined with H_2O_2 has been shown to be highly toxic to bacteria, much more so than either agent alone. .OH scavengers have been shown to inhibit bactericidal activity of $O_2^{\overline{\cdot}}$ generating systems (51) presumably through their reaction with OH.

Singlet Oxygen

That singlet oxygen can be formed by the MPO system has been discussed. It can also be formed by reactions independent of MPO (72) and may contribute to MPO-independent oxygen dependent microbial killing.

Oxygen Independent Antimicrobial Systems

Exposure of PMN's to an atmosphere of nitrogen reduces but does not abolish antimicrobial activity (98). This

suggests that oxygen-independent antimicrobial mechanisms are present in intact PMN's. These mechanisms include acid, lysozyme, lactoferrin and granular cationic proteins.

Acid

Estimates of the pH of the phagocytic vacuole vary from pH 3.0 to 6.5. The source of this acid is completely not known. However, low phagosomal pH may be microbicidal for certain bacteria (84).

Lysozyme

Lysozyme is a basic protein of relatively low molecular weight ($\sim$ 14,500) which is bacteriolytic due to its ability to hydrolyze cell wall components. Although some organisms are killed by lysozyme (21), most are resistant to it (100) and it seems probable that lysozyme plays a digestive rather than a microbial role.

Lactoferrin

Lactoferrin is a protein that inhibits bacterial growth by binding to the iron that is required by microbes as an essential nutrient (22,74,14). It is present in PMN granules (90,101) and may be released into phagosomes following phagocytosis. It is, however, microbiostatic rather than microbiocidal and is unlikely to contribute significantly to microbicidal activity of PMN's.

Granular Cationic Proteins

Phagocytin and leukin were first extracted from leukocytes in 1956 (53,154). They are heat-stable, resistant to strong acid and strongly cationic. They are released into phagosomes and exhibit antimicrobial activity (84). Recently cationic proteins from PMN's have been shown to be cytotoxic for tumor cells (25); however, their role in the intact cell is as yet undetermined.

In summary, the antimicrobial and cytotoxic mechanisms of PMN's comprise a "formidable armamentarium." A review of these mechanisms in monuclear phagocytes follows.

II. Anti-Bacterial and Cytotoxic Mechanisms In Mononuclear Phagocytes

The Mononuclear Phagocytes

The mononuclear phagocytes are composed of many different cells with many functions. They include the peripheral blood monocyte, the tissue macrophages of the lung and peritoneal cavity and the "reticuloendothelial" cells of the liver and spleen. The cells take part in the host's defense system against intracellular bacterial, viral and fungal pathogens. They also participate in the immunological defense systems of the host, having been implicated in the cell-mediated immune response including tumor cell destruction, transplant rejection, and hypersensitivity. Proteins synthesized by mononuclear phagocytes may include endogenous pyrogen, the second and fourth complement components, transferrin, colony stimulating factor, interferon and plasminogen activator (29,73,168). The mononuclear phagosome may also serve as the "scavenger" of the body, removing dead and defective cells and foreign matter.

Although it is clear that the mononuclear phagocyte has a ubiquitous role in host defense, its functions are not completely understood. The following is a review of current knowledge of the mononuclear phagocyte beginning with a review of its origins, maturation and morphology.

The precursor cells of the blood monocyte and tissue macrophage are the monoblast and promonocyte which are

located in the bone marrow (29,169). The concept of a continuum of cells from the bone marrow monoblast and promonocyte through the peripheral blood monocyte to the tissue macrophage is crucial to an understanding of mononuclear phagocytes. Figure 2 diagrams the cells along this continuum. Mononuclear phagocytes at different levels of maturation have different metabolic, functional and structural characteristics which are discussed below.

ORIGINS

Bone Marrow

Monocytes are produced in the bone marrow (73). The myelogenous origins of the mononuclear phagocyte has been demonstrated by (1) labeling studies in x-irradiated animals with partially shielded bone marrow, (2) infusion of labeled cells from various sources into syngeneic recipients and (3) culture of bone marrow cells (31,169). The spleen has been ruled out as a major source of monocyte precursors by labeling studies after splenectomy (169). In the bone marrow, a stem cell gives rise to a monoblast which undergoes mitosis and differentiates into a promonocyte (31). It has been postulated that the promonocytes then divide giving rise to daughter cells half of which remain promonocytes, half of which give rise to monocytes (29,169). These newly formed monocytes then enter the peripheral blood stream without lingering in the bone marrow. After an injection of ^{3}H-

thymidine, labeled monocytes can be detected in the peripheral blood within two hours (169).

Peripheral Blood

The monocytes of the peripheral blood form a population of cells on the way from their place of origin, the bone marrow, to their ultimate location, the tissue. Monocytes were first demonstrated to transform into macrophages in *in vitro* culture in 1925 (29). In 1939, Ebert and Florey demonstrated the transformation from monocyte to macrophage *in vivo*. Using rabbit ear chambers, they observed that monocytes containing phagocytized carbon particles or stained supravital dyes migrate from blood vessels into the connective tissue and there transform into cells indistinguishable from tissue macrophages (29).

Once in the peripheral blood stream, circulating monocytes leave it in a random manner, by a mechanism similar to that used by polymorphonuclear leukocytes (31,169). Monocytes spend only a short time in the peripheral blood as they disappear with t-1/2 of 22 hours (169).

Tissue

Once in the tissues, monocytes transform into macrophages. The number of tissue macrophages is thought to be much greater than that of blood monocytes with estimates that the ratio in man is 400:1 (169). Macrophages differ from

tissue to tissue. It has been suggested that several enzyme systems in the macrophage may be induced or suppressed by environmental factors. This is supported by the fact that rabbit alveolar macrophages have a higher content of lysozyme than rabbit peritoneal macrophages (29,80,112). There is also evidence that macrophages adapt their metabolism to their environment. Alveolar macrophages are exposed to high pO_2 environments and they derive the energy needed for phagocytosis primarily from oxidative metabolism. Other tissue macrophages, however, derive this energy primarily from glycolysis (29).

Macrophages also have the capacity to fuse with each other to form multi-nucleate giant cells. These giant cells are extremely rich in granules, hydrolytic enzymes and mitochondria. (29).

The "Activated" Macrophage

The macrophage responds to infection and inflammation with an adaptive increase in its defense capacities. It has been shown that this enhanced macrophage function has an immunological basis and involves the lymphocyte (95). These "activated" macrophages spread out in glass more extensively, are more heavily endowed with mitochondria and lysosomes, are more phagocytic, have higher basal metabolic rates and are better able to kill bacteria than their normal counterparts (70,71,165). Activation of macrophages varies with time

and extent of infection. For example, a small intravenous dose of Mycobacterium bovis in mice produces only minor activation, while a large dose produces a rapid effect (19). This activation of macrophages is non-specific in that macrophage activation by any infection produces an activated macrophage with enhanced activity toward a wide variety of targets (29).

Cline has drawn the following conclusions about mononuclear phagocytes:

(1) The peripheral blood monocyte is derived from a rapidly proliferating cell in the bone marrow.

(2) Within 24 hours of a pulse of tritiated thymidine, labeled monocytes appear in the peripheral blood.

(3) The immediate progenitor of the monocyte remains in the bone marrow about 18 hours after the last DNA synthesis.

(4) The number of divisions between stem cell and monocyte is 3 or possibly 4.

(5) Monocytes seem to leave the circulation in a random fashion, that is, independent of age. The half survival time in the rat is between 13 and 74 hours.

(6) Once the monocyte leaves the circulation, it does not return.

(7) In the tissues, the monocyte undergoes transformation to a macrophage.

(8) The macrophage may live many months, even years (29).

Structure and Function

Monoblasts

Monoblasts are extremely rare in normal bone marrow. They are seen with frequency only in monocytic leukemia. The monoblast is a small round non-motile cell (10 microns in diameter) that is not phagocytic and does not adhere to glass (29). It is esterase, peroxidase and lsyozyme positive and has both complement and IgG receptors (73). The monoblast has a large nucleus with minimal nuclear indentation, loosely dispersed chromatin granules and prominent nucleoli. It has a small Golgi complex and a few scattered vesicles (43).

Promonocytes

The promonocyte is a relatively large (10-20 micron) cell capable of adhering to glass. It is characterized by a high nuclear/cytoplasmic ratio, peroxidase activity, a conspicuous Golgi complex and many immature and mature azurophil (Wrights Stain) granules (29,73). They are capable of endocytosis, but show little phagocytosis (181,458).

Monocytes

The blood monocyte is approximately 10-18 microns in size with a centrally located indented nucleus and small numbers of faint azurophilic granules. It is slowly motile

by means of blunt pseudopodia. By electron microscopy, the monocyte is characterized by numerous mitochondria, a well-developed Golgi complex and numerous lysosomes. It adheres to glass with cytoplasmic spreading and active membrane ruffling (31,43).

Macrophages

Macrophages are large cells, 20-80 microns in diameter, with one or more nuclei and numerous cytoplasmic granules and inclusions. By electron microscopy, the macrophage is characterized by large vesicular nuclei with multiple nucleoli. The cytoplasm is filled with granules, mitochondria, and digestive vacuoles. The golgi apparatus and endoplastic reticulum are much more highly developed than that of the monocyte (29,31,43).

Anti-microbial and Cytotoxic Mechanisms In Mononuclear Phagocytes

Phagocytosis

Phagocytosis leads to metabolic alterations in mononuclear phagocytes similar to those that occur in PMN's. As in the PMN, phagocytosis leads to increased respiration, increased glucose oxidation via the HMP shunt and increased production in H_2O_2, superoxide radical and other reactive oxygen products (37,64,115,116,125,143).

These events may be initiated by membrane active agents

as in the PMN. Johnston *et al* showed that mononuclear phagocytes generated superoxide anion both during phagocytosis and upon exposure to fixed aggregated IgG (64). Romeo *et al* observed increased respiration, glucose oxidation and H_2O_2 production by rabbit alveolar macrophages exposed to concanavalin A (135).

Oxygen Dependent Anti-Microbial and Cytotoxic Systems

Oxygen is required for the optimal anti-microbial activity of mononuclear phagocytes. This is supported by the fact that monocytes from patients with CGD have been reported to be defective in killing bacteria and fungi (35,92,123) and by the fact that macrophages demonstrate increased oxygen consumption following phagocytosis.

Myeloperoxidase-mediated system

The presence of a MPO-H_2O_2-halide system in mononuclear phagocytes is controversial. As discussed above, as monocytes mature into macrophages, they lose the cytoplasmic granules known to contain peroxidase activity (113). Macrophages from inflammatory infiltrates have been reported to lack peroxidase-positive granules (43,113). Some investigators report that mature mononuclear phagocytes completely lack peroxidase activity (113), while others have concluded that peroxidase mediated anti-microbial mechanisms are relatively important in macrophages (17).

Paul <u>et al</u>, however, found significant MPO activity in mouse peritoneal macrophages but not rabbit alveolar macrophages (117). Laer, Romeo <u>et al</u> demonstrated peroxidase-like activity in rabbit alveolar macrophages (124) and the 20,000 g pellet obtained by centrifugation of rabbit alveolar macrophage homogenate was shown to contain peroxidase activity and to kill E. coli in the presence of H_2O_2 and I- (118). Lehrer has implicated MPO-linked mechanisms in the fungicidal activity of human monocytes (92).

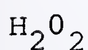
The nature of the peroxidase involved is not well defined. It may be identical to that of the PMN as patients with hereditary MPO-deficiency have both peroxidase negative neutrophils and peroxidase negative monocytes (84) and monocytes from these patients show decreased anti-microbial activity (92).

While Paul has isolated a peroxidase from rabbit alveolar macrophages that forms a potent <u>in vitro</u> anti-microbial system in combination with H_2O_2 and I- (118), Bigger <u>et al</u> failed to detect iodination of bacteria by intact rabbit alveolar macrophages before or after supplying exogenous peroxidase in the form of MPO-coated ingestible particles (17), raising questions about the role of the MPO-H_2O_2-halide system in intact macrophages. However, this iodination has been demonstrated using monocytes (80).

It has been proposed that catalase may serve as the

peroxidase in macrophages (80). Catalase exhibits anti-microbial activity in the presence of iodide ions (77) and is delivered into the phagocytic vesicle (162). Biggar *et al* have shown that inhibitors of catalase activity, such as aminotriazole and sodium nitrate, fail to inhibit bacterial killing by rabbit alveolar macrophages suggesting that catalase is not the peroxidase involved (17).

Although the identity of the peroxidase involved is not clear, the inhibition of anti-microbial activity of mononuclear phagocytes by heme enzyme inhibitors (azide, cyanide) (80) suggests a role for peroxidase mediated mechanisms in anti-microbial activities of mononuclear phagocytes.



H_20_2

H_20_2 production has been noted in several species of mononuclear phagocytes. These include human monocytes (7, 121), and rat, guinea pig and mouse peritoneal macrophages (117). If H_20_2 has a significant role in bacterial killing, an increase in its production should accompany phagocytosis. Paul *et al* have reported increased H_20_2 production upon phagocytosis by mouse peritoneal macrophages but not rabbit alveolar macrophages (117). However, Klebanoff and Hamon observed the formation of H_20_2 by rabbit alveolar macrophages and its stimulation by phagocytosis, using both the inhibition of catalase by aminotriazole and formate oxidation (80).

More evidence of a role for H_2O_2 in anti-microbial activities of mononuclear phagocytes is provided by the fact that peripheral blood monocytes of patients with CGD have a microbicidal defect similar to that of their neutrophils (80).

The formation of H_2O_2 by intact mononuclear phagocytes, the increase in its production accompanying phagocytosis and the microbicidal defect noted in the monocytes of patients with CGD suggest that H_2O_2 is involved in the microbicidal activities of the mononuclear phagocyte.

Superoxide, Hydroxyl Radical and Singlet Oxygen

The production of superoxide, hydroxyl radical and singlet oxygen have been demonstrated in mononuclear phagocytes (38,64,143,174). Reiss and Ross studied phagocytosing monocytes and neutrophils and found that both showed an increased release of superoxide anions (121). Johnston _et al_ showed human monocytes produced superoxide anions and chemiluminescence on stimulation with fixed aggregated IgG (64). Sagone _et al_ noted that monocytes generated chemiluminescence that could be inhibited with superoxide dismutase (143) suggesting that mononuclear phagocytes are capable of production of both superoxide anion and singlet oxygen. Adler _et al_ have demonstrated that H_2O_2 produced by human monocytes is derived exclusively from superoxide anion precursors, with H_2O_2 pro-

duction completely inhibited in the presence of superoxide scavengers (1).

Hydroxyl radical and perhaps other reactive oxygen products, are thought to be the products of the reaction between $O_2^{\bar{\cdot}}$ and H_2O_2. Using an assay system of ethylene generation from methional, cell preparations of human monocytes have been demonstrated to generate hydroxyl radical (174). The ethylene generation was dependent on $O_2^{\bar{\cdot}}$ and H_2O_2 (reaction was inhibited by SOD and catalase) suggesting that the Haber-Weiss reaction ($O_2^{\bar{\cdot}} + H_2O_2 + H+ \rightarrow .OH + H_2O + O_2$) may describe the scheme responsible for .OH production in these cell preparations.

These results demonstrate that the active oxygen radicals $O_2^{\bar{\cdot}}$, .OH, $^1O_2$ and perhaps others are produced by mononuclear phagocytes and may play a role in anti-microbial activity.

Coupling Enzyme

Mononuclear phagocytes share many phagocytosis associated events (e.g. respiratory burst, increased glucose utilization via HMP shunt and increased release of H_2O_2 and active oxygen products) in common with PMN's. The close association between the respiratory burst and HMP shunt stimulation suggests that a coupling enzyme system linking respiration and HMP shunt activity may be present in mononuclear phagocytes as well as PMN's.

An NADPH-oxidase, which catalyzes the reduction of oxygen

with formation of H_2O_2 and NADP+ (the rate-limiting step of the HMP shunt) is a suitable candidate for the coupling enzyme. An NADPH-oxidase, that is activated during phagocytosis, has been demonstrated in rabbit and guinea pig alveolar and peritoneal macrophages (136). This enzyme can also be reversibly activated with conconavilin A and its activation is proportional to the extent of stimulation of respiration (135). The NADPH-oxidase of macrophages has been compared to that of the PMN. It has been found to have an activity comparable to that of the PMN's and to show increased activation proportional to the incremental increase of respiration as in the PMN. However, it is more sensitive to KCN than that of the PMN and unlike that of the PMN its K_m does not change with phagocytosis (125,139).

The above evidence suggests that the coupling enzyme of macrophages is a cyanide-sensitive NADPH-oxidase. Its exact subcellular location and other characteristics are still unknown.

O_2-independent Anti Microbial Mechanisms

Oxygen independent antimicrobial mechanisms have been proposed for mononuclear phagocytes. These include acid, lysozyme, lactoferrin and cationic proteins.

Acid

Particle ingestion by mononuclear phagocytes is followed

by a fall in intravesicular pH (80). The intravesicular pH has been estimated to be between 3.5-5.5 (59,80). This fall in pH may serve several functions. The acid conditions may be fatal to certain organisms while halting growth in others. It may also create conditions favorable to the activity of other antimicrobial systems such as catalase or peroxidase-mediated systems or to the activity of the digestive enzymes of the phagosome.

Lysozyme

Lysozyme has been detected in rat and human monocytes, mouse peritoneal macrophages and rabbit alveolar macrophages (80,112). Little or no lysozyme activity has been detected in oil-induced rabbit peritoneal macrophages (112). Brumfitt and Glynn have provided evidence that lysozyme-sensitive bacteria are killed by lysozyme in the intact macrophage (21, 48). However, lysozyme-sensitive bacteria are limited to a small number of saprophytic bacteria and in general bacterial death precedes the action of lysozyme (80). Although this does not rule out a synergism between other anti-microbial systems and lysozyme it suggests that the main function of lysozyme may be digestive rather than microbicidal.

Lactoferrin

Lactoferrin, as discussed earlier, is an iron binding

protein which has microbiostatic properties when not fully saturated with iron, that is present in high concentrations in the PMN. Lactoferrin, however, is not present in mononuclear phagocytes and, therefore, cannot be implicated in their anti-microbial activities (74,101).

Cationic Proteins

Heat stabile, strongly cationic proteins with anti-microbial activity have been extracted from PMN's. Similar proteins have not been extracted from mononuclear phagocytes (54,80,155). Furthermore, bacteria ingested by macrophages do not become cationic, in contrast to the finding in PMN's (155). These results suggest that mononuclear phagocytes do not contain detectable amounts of cationic protein and that these agents cannot be implicated in the microbicidal activity of these cells.

Experimental Rationale

As it is difficult to obtain large numbers of human macrophages, the knowledge of cytotoxic mechanisms in human macrophage-mediated cytotoxicity is derived largely from studies of human PMN's and animal macrophages.

In this work culture derived human (CDH) macrophages are employed as they are available in large numbers. CDH macrophage-mediated chicken red blood cell lysis is used as an analogue of macrophage-mediated tumor cell killing.

With the current knowledge of the cytotoxic mechanisms of PMN's and animal macrophages as a foundation, this work aims to examine the role of various aspects of oxidative metabolism in CDH macrophage-mediated cytotoxicity.

Materials and Methods

Preparation of Cells (61,107,110)

Monocytes

200 ml of heparinized blood was drawn from normal controls and separated on a Ficoll-Hypaque gradient. The interface which was composed of 20-35% monocytes, 65-80% lymphocytes, 0-2% polys was washed twice in HBSS with Penicillin (Pen) and Streptomycin (Strep), and once in RPMI 1640 with Pen, Strep and 2% FCS. Viability at this stage was 95-98% as assessed by counting in Trypan Blue. The cells were adjusted to 3.5×10^6/ml in RPMI 1640 with Pen, Strep, 5-F-C,* Gentamycin. Aliquots of 10 ml were added to 75 mm^2 Corning Culture Plates and incubated at 37^oC in a humidified 5% CO_2 room air atmosphere for 1-7 days.

At the end of the culture the cells were scraped off culture plates with rubber scrapers and resuspended in RPMI 1640 with Pen, Strep, and 4% FCS. They were counted in Trypan Blue and incubated with Neutral Red, with 85-95% viability and 40-80% cells taking up neutral red in 10 min. respectively. Cells were adjusted to concentrations of 3×10^6/ml in RPMI 1640 with Pen, Strep and 4% FCS.

Chicken red blood cells (RBC) were collected under sterile conditions in Alsever's solution and stored at 4^oC for

*5-Fluorocytosine

1-7 days. The CRBC's were prepared for use by washing three times in HBSS with Pen, Strep and 10% FCS. 4×10^7 RBC's were then incubated with 100 μCi ^{51}Cr in volume of .2 ml at 37° for 1 hour. They were then washed twice in HBSS with 10% FCS and once in RPMI with 4% FCS before use.

Cytotoxicity Assay

4×10^5 (number varied depending upon investigational goals) viable mononuclear phagocytes (as determined by incubation with Trypan Blue and uptake of neutral red respectively) in 0.1 ml of RMPI 1640 with Pen, Strep and 4% FCS were added to Corning culture tubes. 3×10^4 ^{51}Cr-labeled CRBC in 0.1 ml of RPMI 1640 in Pen, Strep and 4% FCS were added.

In inhibition experiments, Na Azide, SOD, Na Benzoate and catalase in varying concentrations were added in 0.1 ml of RPMI. The plates were centrifuged at 100 x g for 5 min. The plates were then incubated for 18 hr. in humidified 5% CO_2 atmosphere at 4°, 25° or 37^oC after which they were centrifuged at 750 x g for 10 mins. and 1/2 the supernatant was withdrawn.

^{51}Cr was calculated as (108) described by Muchmore and Blaise, as follows:

$$\frac{\text{Supernatant Counts}}{\text{Pellet counts + Supernatant Counts}} \times 100 = \%\ \text{lysis}$$

Background lysis

To determine background lysis, tubes were prepared in the manner described above. However, effector cells were replaced by unlabeled CRBC.

Materials

Ficoll 400	Pharmacia Piscataway, N.J.
Hypaque	Winthrop N.Y., N.Y.
HBSS (modified) Hanks Balanced Salt Solution	Flow Laboratories Rockville, MD
RPMI 1540 with Glutamine	Flow Laboratories Rockville, MD
Trypan Blue 0.5% in 0.85% saline	Flow Laboratories Rockville, MD
FCS (Fetal Calf Serum)	Flow Laboratories Rockville, MD
Penicillin	Flow Laboratories Rockville, MD
Streptomycin	Flow Laboratories Rockville, MD
Gentamycin	Schering Corporation Kenworth, N.J.
5-FC (Fluorocytosine)	Hoffman-LaRoche, Inc. Nutley, N.J.
Culture Tubes	Corning Bowenlicates Culture Tubes
Neutral Red	Sigma Laboratories

^{51}cr as NA Chromate	New England Nuclear
Culture Dishes	Corning 75 cm^2/tissue Culture Flasks

Results

Spontaneous Cytotoxicity-Time Course

Human peripheral blood monocytes were incubated on plastic, alone, for periods up to 7 days, then removed from plastic and incubated with chicken red blood cell targets for 18 hours to determine the development of "spontaneous" cytotoxicity. An effector target cell ratio of 10:1 was arbitrarily chosen for this set of experiments. No cytotoxicity was observed on day 1-3 of culture. However, the cultured monocytes began to show cytotoxicity towards labeled chicken red blood cell targets after 5 days of culture (mean % lysis, 4 experiments, 24 ± 3.5), exhibiting increasing cytotoxicity on days 6 and 7 (mean % lysis for 4 experiments 42 ± 2.2 and 47.1 ± 3.3 respectively). These values were all significantly greater than background lysis ($p < .002$, day 5; $p < .001$, day 6; $p < .001$ day 7). These results are depicted in Table 2 and Figure 3.

Spontaneous Cytotoxicity Dose Response

The optimal effector: target cell ratio for cell lysis was examined by performing a series of assays with different effector:target cell ratios. Experiments were carried out with monocytes after 7 days of culture.

Cytotoxicity, as measured by ^{51}Cr released from ^{51}Cr labeled chicken red blood cells, was not present at effector: target ratios of .1:1 and increased progressively at ratios of .5:1, 1:1, 5:1. There was no statistical difference

between cytotoxicy observed at ratios of 5:1 and 10:1 and there was a marked decrease in cytotoxicity at ratios of 20:1. These results are found in Table 3 and Figure 4.

High background lysis was observed at low effector ratios (.5:1, 1:1). This occurrence is explained by the high rate of spontaneous lysis observed in CRBC stored at concentrations $< 10^5$/ml.

Temperature Dependence

Assays performed at 4°, 25°, 37°C were compared. Significant cytotoxicity was observed only at 37° C. Results are depicted in Figure 5.

Cytotoxic Activity of Media post 18 hour Incubation with Effector Cells.

An assay was performed to determine if cultured monocytes lyse CRBC by releasing toxic substances into the environment. Effector cells were incubated under assay conditions for 18 hours without the addition of target cells. They were then killed by rapid freezing followed by thawing. The supernatants were then incubated with target cells under standard assay conditions. These media exhibited no cytotoxic activity. Results are depicted in figure 6.

Inhibition Studies

Effector and target cells were incubated with various inhibitors of the process or products of oxidative metabolism in an attempt to generate information relevant to the cytotoxic mechanisms in mononuclear phagocytes.

Sodium Azide

Effector and target cells were incubated with the inhibitor of MPO, sodium azide. In 4 such experiments a concentration of sodium azide of 1mM resulted in complete inhibition of cytotoxic activity with cytotoxicity reduced from 38.5% to 4.15% lysis (a value equivalent to control lysis) representing 100% inhibition of cytotoxicity (see figure 7, table 4). In one experiment, effector and target cells were incubated with azide at concentrations of 0, .1, 1 and 10 mM. Results of 42, 17.8, 8.2, and 5% respectively were obtained.

Catalase

To examine the role of H_2O_2 in the monocyte mediated cytotoxicity 6 assays were carried out in the presence of catalase at the concentration of 2000 μ/ml. Cytotoxicity was reduced from 38.85% to 8.6% lysis representing a 78% reduction (see figure 8, table 5).

In an attempt to determine if this effect was enzymatic or due to a non-specific effect of the protein, the experiment was repeated with boiled catalase. The results (see Table 5) showed that cytotoxic activity of monocytes incubated with target cells and boiled catalase was equal to that of monocytes incubated with target cells alone. In one experiment, effector and target cells were incubated with catalase in increasing concentrations, (0, 200, 2,000, 20,000 μ/ml) resulting in decreasing cytotoxicity (42, 27, 17, 4%

lysis respectively).

Superoxide Dismutase (SOD)

To examine the role of $O_2^{\bar{\cdot}}$ in monocyte mediated cytotoxicity 6 assays were carried out in the presence of 20 μg/ml SOD. Cytotoxicity was reduced from 38.85 to 10.58% lysis a reduction of 73%. (See figure 9, table 6). Active but not autoclaved SOD was effective at inhibiting cytotoxicity (see table 6). In one experiment, effector and target cells were incubated with SOD at increasing concentrations (0, 2, 20 and 200 μg/ml). Cytotoxicity was found to decrease with increasing concentrations of SOD (42, 22.4, 18.9, 8.9% lysis respectively).

Sodium Benzoate

To examine the role of the hydroxyl radical (.OH) in these cytotoxic events, 4 assays were conducted in the presence of 1mM sodium benzoate, a scavenger of .OH. Results (figure 10, table 7) show a reduction in cytotoxicity of 44% (38.5 to 2. .8 lysis). Sodium benzoate itself appears to cause some lysis of red blood cell targets. Correction for the phenomenon (subtraction of control lysis) yields a value of 65% reduction in lysis.

Discussion

Human peripheral blood monocytes became spontaneously cytotoxic toward chicken red blood cell targets after 5 to 7 days of *in vitro* culture. The system described in this paper represents a unique opportunity for studying large numbers of culture derived human macrophages. In the past, the acquisition of knowledge about human macrophages has been hampered by difficulties in acquiring large numbers for study. This system circumvents this problem by using monocyte-derived macrophages, enabling investigators to study large numbers of human macrophages without pooling cells from different donors. It is well documented that human monocytes are transformed into macrophages after several days of *in vitro* culture (29, 152, 169).

Muchmore, *et al* have shown that the mononuclear leukocyte responsible for the cytotoxicity observed in this system is the peripheral blood monocyte (110). Nelson and Seljelid found that mouse peritoneal macrophages become cytotoxic towards autologous red blood cells after 18 hours of *in vitro* culture but that freshly seeded macrophages did not exhibit this cytotoxicity (105). Apparently the effector cells in both of these systems require some sort of "activation" that is provided by *in vitro* culture. It has been suggested by Muchmore *et al* that this requirement for *in vitro* culture can be explained by the time depen-

dent loss of suppressive regulator cell activity (107).

This spontaneous cytotoxicity has been observed without lectin or antibody stimulation, and has raised questions about how the effector cells recognize the target cells. The oligosaccharide cellobiose has been found to inhibit this cytotoxicity without affecting effector cell viability or PHA-induced cytotoxicity (109), suggesting that human monocytes-macrophages express receptors on their surfaces which may recognize targets by interaction with specific cell surface carbohydrates.

The effector cells involved were found to lyse cells at physiologic temperatures but not at 25° or 4°C just as human PMN have been shown to exhibit maximal phagocytic activity between 38°-40°C (16), the usual range in clinical fever. The relative activity of these cells at temperatures of 38°, 39°, and 40°C thus represents an area for further study.

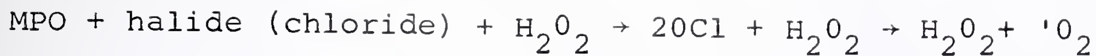
Observed cytotoxicity increased steadily from effector: target ratios of .5:1 to 5:1. At this level a "plateau region" was reached as cytotoxicity at effector:target cell ratios of 10:1 equaled that observed at ratios of 5:1. At effector:target cell ratios of 20:1 a marked fall-off in cytotoxicity was noted. This fall-off in cytotoxicity at high ratios can possibly be explained by macrophage "crowding," perhaps blocking access to target cells or exerting their toxic effect on fellow phagocytic cells.

Although the mononuclear phagocyte is known to exhibit cytotoxic activity, there is a scarcity of knowledge about the biochemical basis of this activity. Mononuclear phagocytes have been shown to produce H_2O_2, $O_2^{\bar{\cdot}}$, .OH and $'O_2$ but dating linking the production of these substances with cytotoxic and antimicrobial activity is lacking. The results obtained during this study represent such a link between the production of highly reactive oxygen products and spontaneous cytotoxic activity in culture derived human macrophages.

The addition of azide, an inhibitor of MPO, in concentrations of 1mM completely inhibited all cytotoxic activity. It thus seems that the MPO-halide-H_2O_2 system may have a major role in the observed cytotoxicity.

In the past macrophages have generally been reported to have little or no significant peroxidase activity (117). Recently, a fraction prepared from a homogenate of alveolar macrophages centrifuged at 20,000g has been found to contain peroxidase activity and to kill E. coli in the presence of H_2O_2 and I- suggesting that the MPO-H_2O_2-halide system is operative in macrophages (118). Although the exact mechanisms of action of the MPO-halide-H_2O_2 system are not clear, current evidence suggests that the generation of hihgly reactive products of oxygen reduction (singlet oxygen, hydroxyl radical) play a major role (119).

It has been proposed that the MPO system can generate singlet oxygen ($'O_2$) as follows

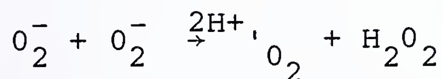

MPO + halide (chloride) + H_2O_2 → 2OCl + H_2O_2 → H_2O_2+ $'O_2$

The inhibition seen with the addition of azide can be attributed to prevention of the formation of highly reactive oxygen compounds. The fact that 100% inhibition was observed with the addition of azide remains unexplained. Azide quenches singlet oxygen (185). Perhaps this effect, in addition to inhibition of the MPO system, is responsible for the high level of inhibition observed. Despite this, these cells appear to have other cytocidal mechanisms so that inhibition with azide would be expected to be partial rather than complete.

Catalase catalyzes the reaction H_2O_2 → O_2 → H_2O_2, thereby acting to remove H_2O_2 from the cell's environs. When added to effector cells in concentrations of 2000 μ/ml and target cells, catalase reduced cytotoxicity by 78%. It can be postulated that this inhibition was due to 1) a reduction in the amount of H_2O_2 available for the MPO-H_2O_2-halide system, 2) a reduction in the amount of H_2O_2 available for the formation of singlet oxygen and hydroxyl radical by the Haber-Weiss Reaction (see below), 3) a reduction in the amount of H_2O_2 available for direct toxicity. The last of these, that H_2O_2 acting alone exhibits major cytotoxic effects in this system, is not supported by the fact that SOD which increases the amount of H_2O_2 available (11), was found

to inhibit cytotoxicity. Catalase did not completely inhibit cytotoxicity suggesting that 1) mechanisms not involving H_2O_2 were also operative or 2) that the catalase did not have access to all of the H_2O_2 produced. That this inhibition was due to the enzymatic activity of catalase is demonstrated by the absence of inhibition with boiled catalase.

Superoxide anion ($O_2^{\dot{-}}$) is believed to be bacterio- and cytocidal by its interactions with itself and other compounds to form highly reactive oxygen products. It is highly unstable and spontaneously dismutates to generate singlet oxygen ($'O_2$) in the reaction:

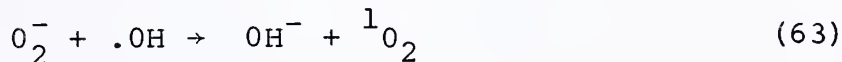


$$O_2^- + O_2^- \xrightarrow{2H+} {'O_2} + H_2O_2$$

It is also converted to hydroxyl radical (.OH) in the Haber-Weiss Reaction.

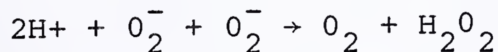
$$O_2^- + H_2O_2 + H+ \rightarrow .OH + H_2O + O_2 \qquad (119)$$

Hydroxyl radical may also react with $O_2^{\dot{-}}$ to generate $'O_2$



$$O_2^- + .OH \rightarrow OH^- + {}^1O_2 \qquad (63)$$

The reaction catalyzed by superoxide dismutase (SOD)



$$2H+ + O_2^- + O_2^- \rightarrow O_2 + H_2O_2$$

decreases the amount of superoxide available to react with H_2O_2 and .OH and does not generate singlet oxygen (63).

The addition of SOD in concentrations of 20 μg/ml was found to reduce cytotoxicity by 73%. No inhibition was seen

with autoclaved SOD suggesting that this effect was enzymatic rather than nonspecific. The inhibition observed with SOD may be due to the suppression of $'O_2$ formation (from .OH or spontaneous dismutation) or from the suppression of the formation of .OH. Suppression of cytotoxicity with both catalase and SOD supports the hypothesis that $O_2^{\bar{\cdot}}$ and H_2O_2 interact to form toxic products.

Sodium benzoate in concentrations of 1mM inhibited cytotoxicity by 65%. As sodium benzoate is a scavenger of hydroxyl radical (.OH) this finding perhaps implicates at least a partial role for hydroxyl radical either directly or through the generation of singlet oxygen as described above.

Recently, Nathan _et al_ (184,185) have studied extracellular cytolysis by activated mouse peritoneal macrophages. They found that phorbol myristate acetate (PMA) activated mouse peritoneal macrophages were capable of rapidly lysing lymphoma cells and that this activity correlated well with the production of H_2O_2. This cytotoxic effect could be completely eliminated by catalase. In contrast to the findings described in this paper, Nathan _et al_ did not demonstrate decreased cytolysis with the addition of inhibitors of MPO (azide, cyanide), scavengers of superoxide anion (superoxide dismutase) or scavengers of hydroxyl radical (histidine, ethanol, mannitol, benzoate, tocopherol and butylated hydroxytoluene). Cytolysis was also unaffected by agents used to quench singlet oxygen (DABCO, diphenylforan, bilirubin,

histidine, tocophenol and azide). Their findings suggested that in a system of activated mouse peritoneal macrophages directed against lymphoma cells, H_2O_2 was necessary and sufficient for cytolysis. They did not implicate $O_2^{\cdot -}$, .OH of $^1O_2$.

The results described herein suggest a role for the MPO-H_2O_2-halide system in spontaneous cytotoxicity by cultured human macrophages. They also suggest that other systems that generate reactive oxygen products are active in these cells. These data presented here expose many areas for further study. The activity of these macrophages in an anaerobic environment should be examined. Attempts should be made to measure production of H_2O_2, $O_2^{\cdot -}$, .OH and chemiluminescence and to correlate this production with cytolysis. Further experiments with other scavengers of hydroxyl radical and quenchers of singlet might be worthwhile. Lastly, the examination of cytotoxicity towards a broad range of target cells (bacteria, tumor cell lines) should be examined.

Summary

Culture derived human macrophages became spontaneously cytotoxic towards CRBC after several days in culture. This cytotoxicity was completely abolished by the inhibitor of MPO and quencher of singlet oxygen, azide, and partially inhibited by catalase, superoxide dismutase, and sodium benzoate. These results raised the possibility of a role for the MPO-H_2O_2-halide system in human culture derived macrophages. They also provided evidence for the role of highly reactive products of oxygen reduction in spontaneous cytotoxic activity of human mononuclear phagocytes.

APPENDIX

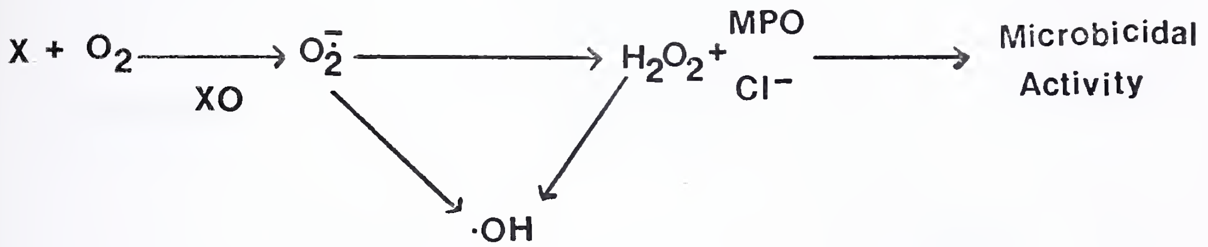
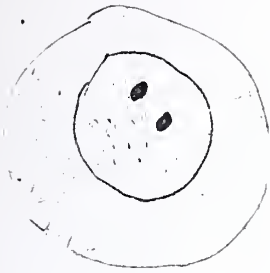
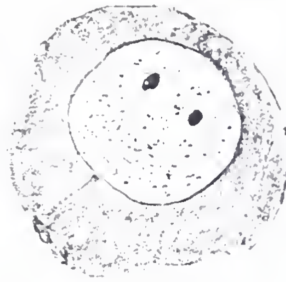
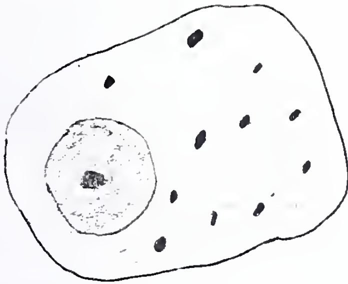


Figure 1. The Xanthine (X) - Xanthine oxidase (XO) - Chloride - myeloperoxidase (MPO) microbicidal system.
(82)







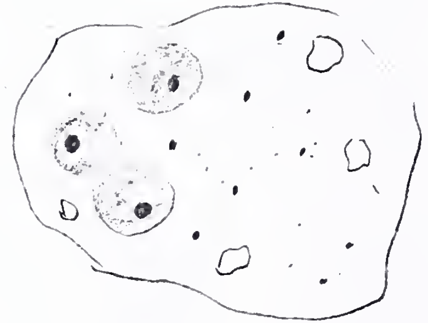


Figure 2. Sequence of development of cells of the mononuclear phagocyte series.

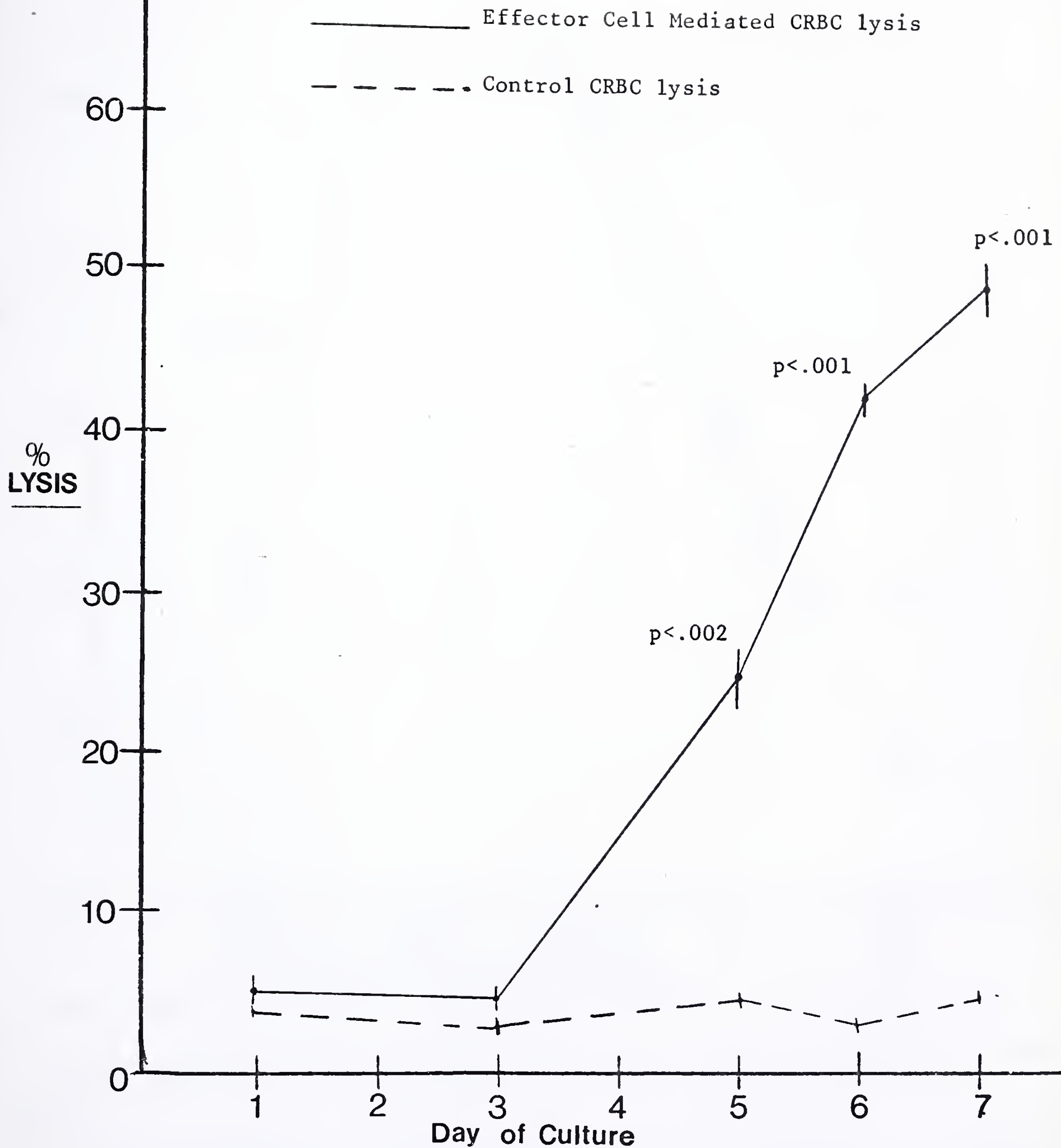


Figure 3. Development of Spontaneous Cytotoxicity Over 7 Days of Culture

Values are mean ± SEM for four experiments. Means were compared to control means using Student's t test. Experimental conditions are as described in Table 2.

Cultured monocytes became cytotoxic towards CRBC targets after 5 days of culture, exhibiting increasing activity through day 7.

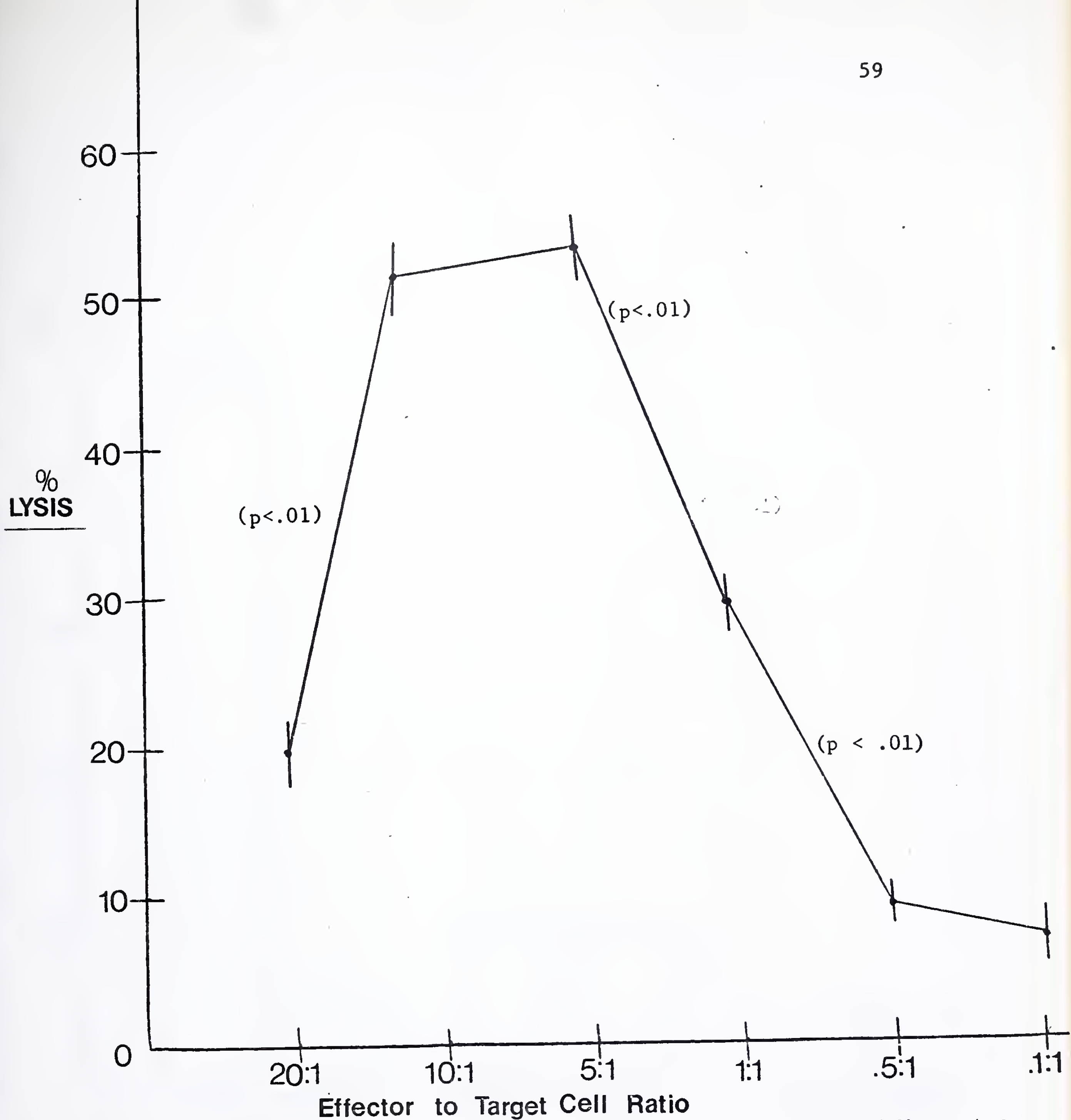


Figure 4. Effect of Effector:Target Cell Ratio on Cytotoxicity of Cultured Monocytes tward CRBC.

Values are mean ± SEM for four experiments minus mean lysis for four experiments. Value a each ratio was compared to that for the preceeding ratio using student's t test for dpendent variables.

Cytotoxicity (% lysis) increased progressively with increasing E:T ratio at ratio of .5:1 11, 5:1. No significant difference was observed between ratios of 5:1 and 10:1. A marked dcrease in cytotoxicity was observed at ratios of 20:1.

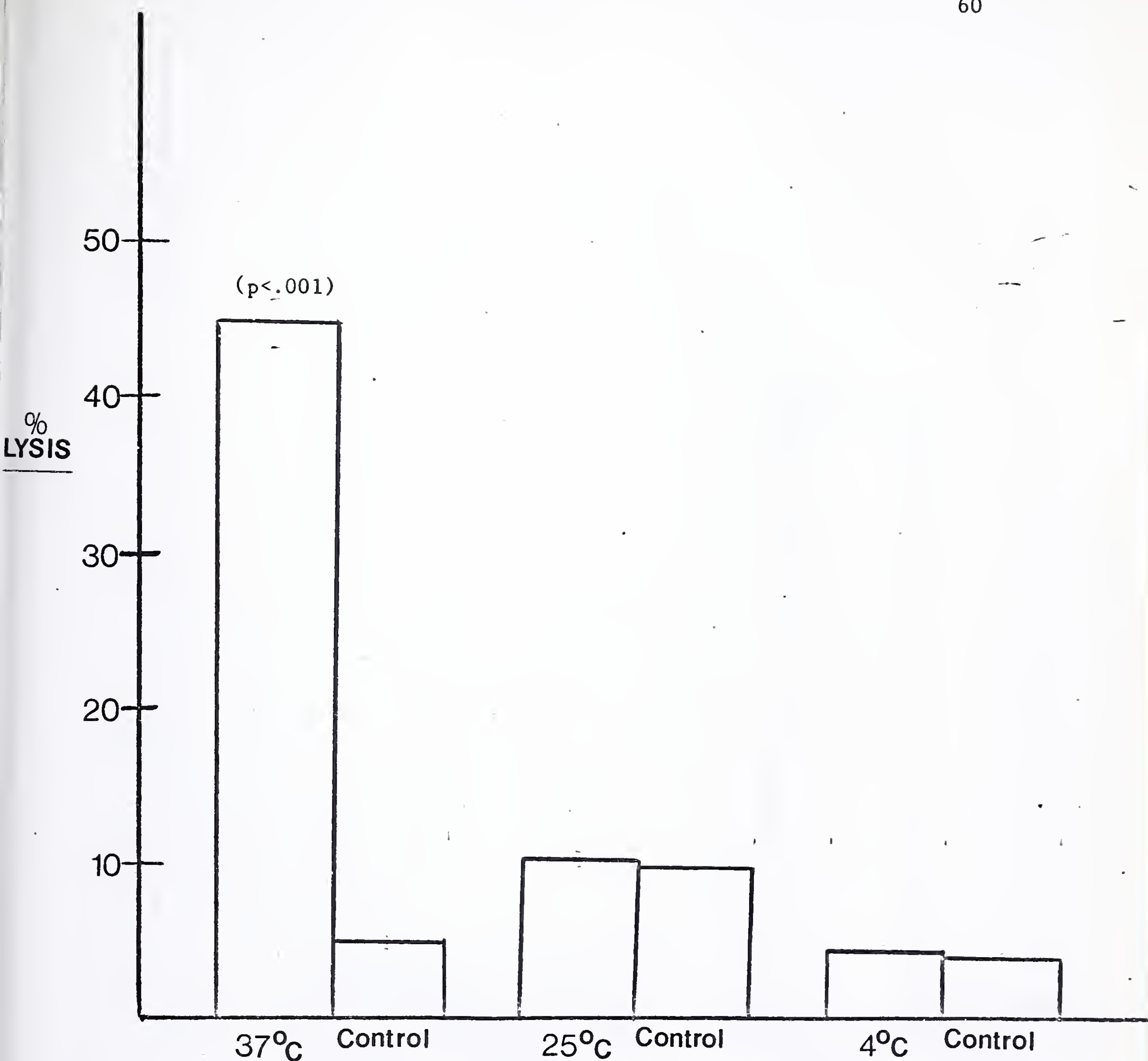


Figure 5. Effect of Temperature on Cultured Monocyte Mediated Cytotoxicity. Values are mean ± SEM for 4 experiments. Experiments were carried out as described in Table 2, but incubated at 37°, 25° or 40° C.

Mean lysis was compared to control lysis using student's t test for independent variables.

Mean lysis significantly greater than control lysis was noted only at 37°C.

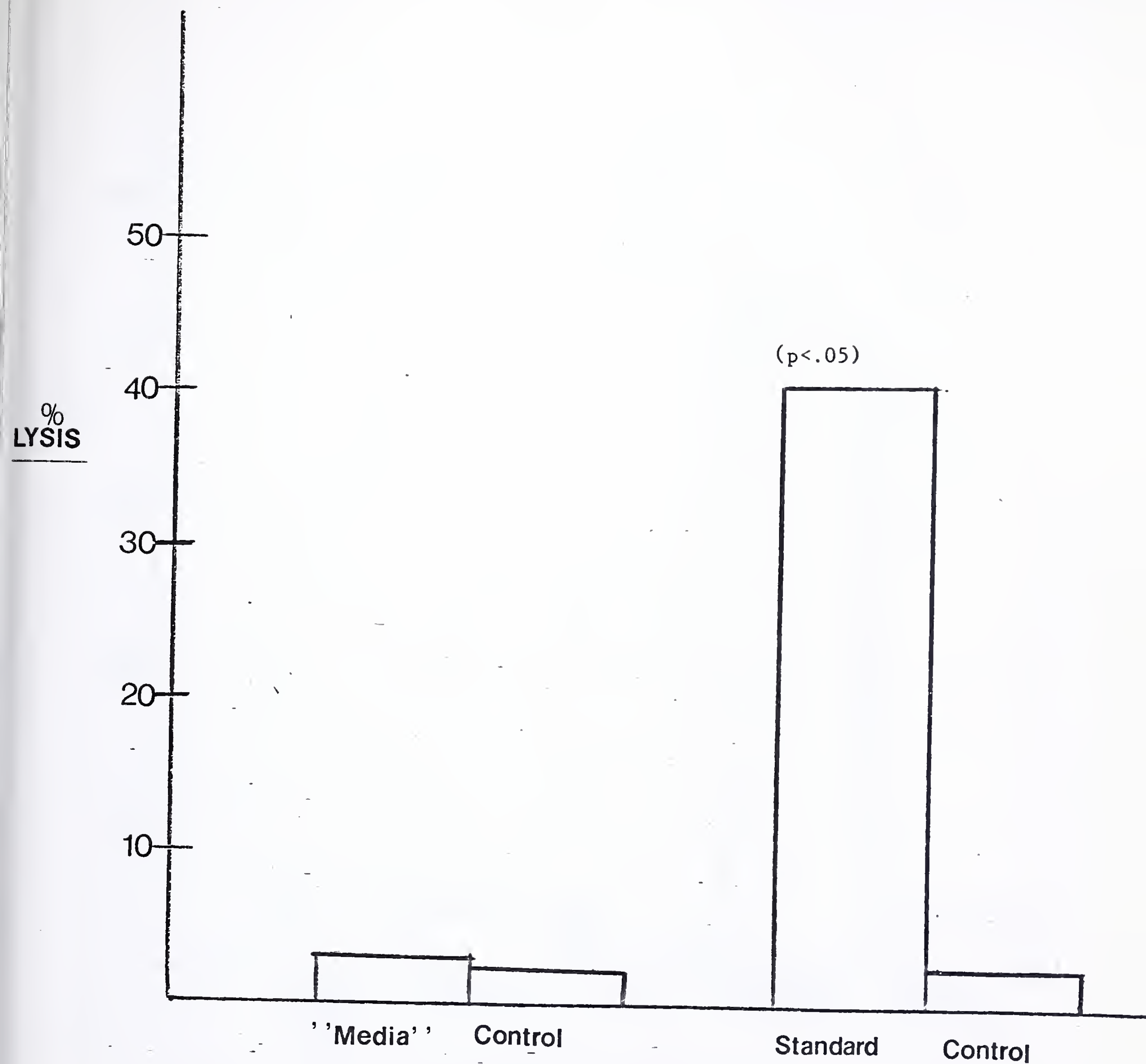


Figure 6. Cytotoxic Activity of Media post 18 hr. Incubation of Cultured Monocytes.

Values are mean ± SEM of three experiments. Mean value was compared to control value using student's t test. In each experiment, effector cells were incubated for 18 hours without target cells at 37°C. They were then killed by rapid freeze-thawing:CRCB were then incubated with resulting solution under conditions described in Table 2. "Standard"experiments are as described in Table 2.

Cytotoxicity (% lysis) in these 3 experiments was not above control values.

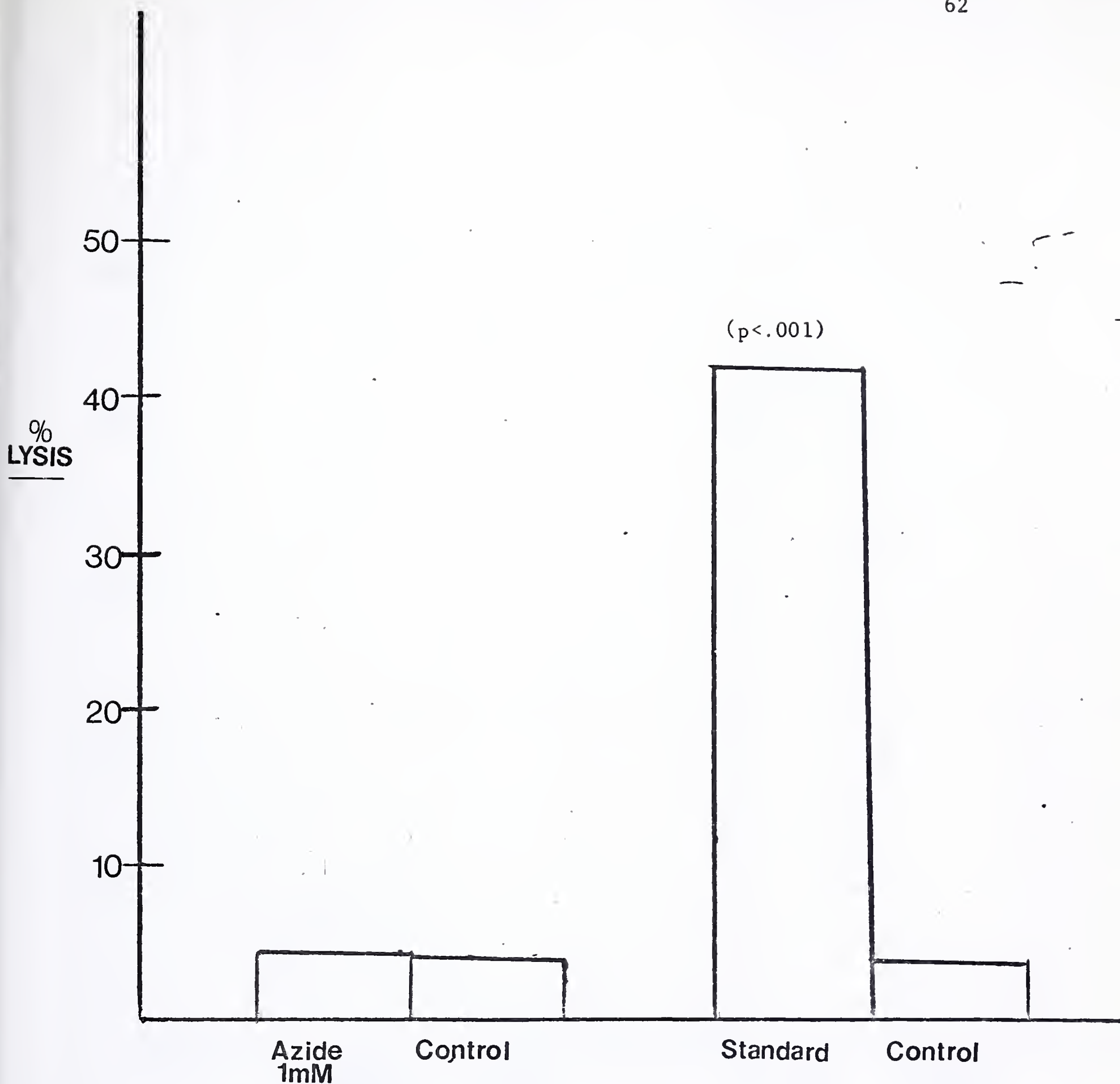


Figure 7. Effect of Sodium Azide on Cytotoxicity.

Values are mean ± SEM for 4 experiments. Experimental conditions were as described in Table 2. Mean was compared to control lysis using student's t test.

Addition of 1mm Azide led to complete inhibition of cytotoxicity.

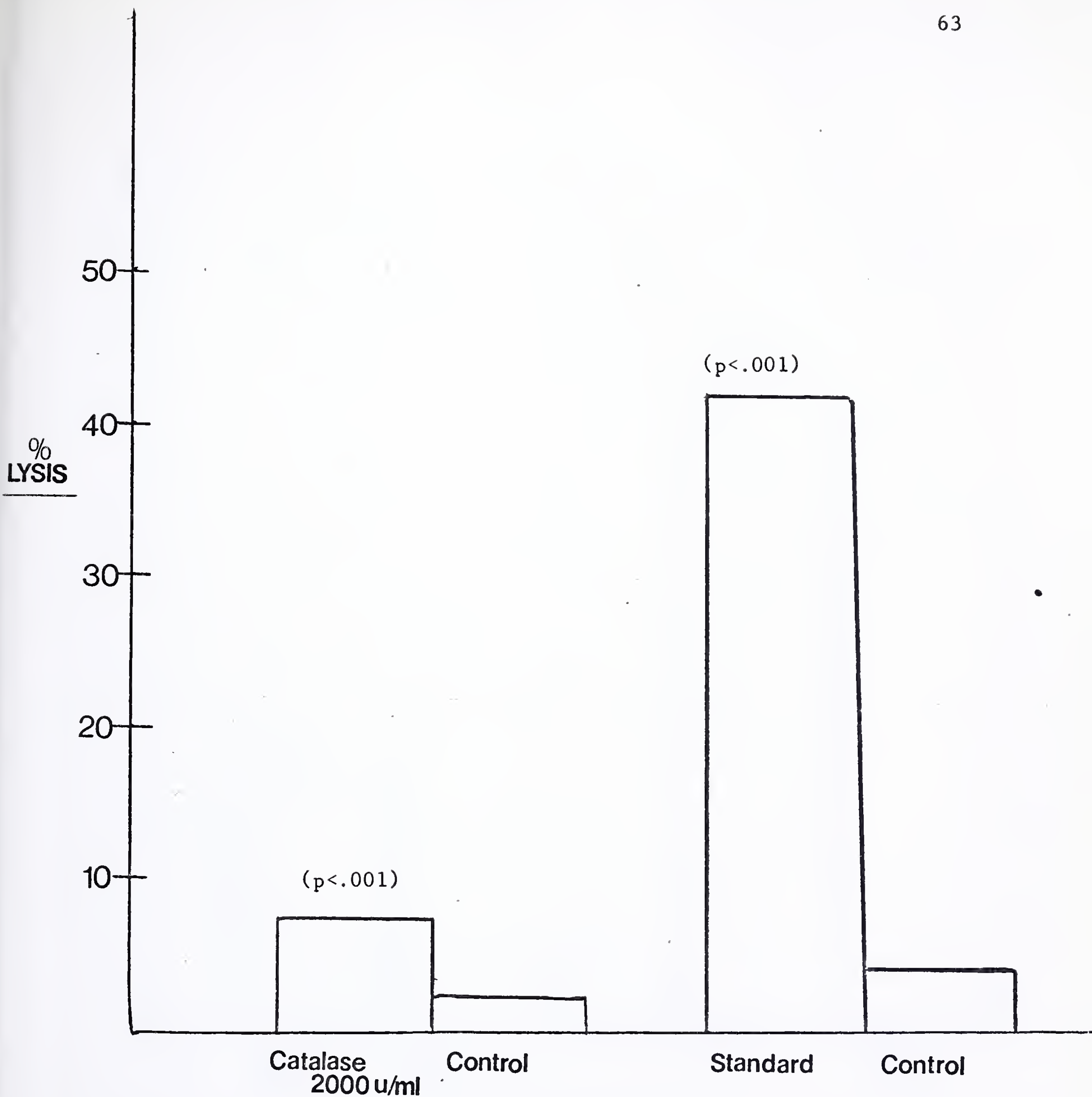


Figure 8. Effects of 2000 μ/ml Catalase on Cultured Monocyte Mediated Cytotoxicity.

Values are mean ± SEM for 6 experiments. Mean lysis was compared to control lysis using student's t test. Addition of 2000 μ/ml catalase reduced cytotoxicity by 78%.

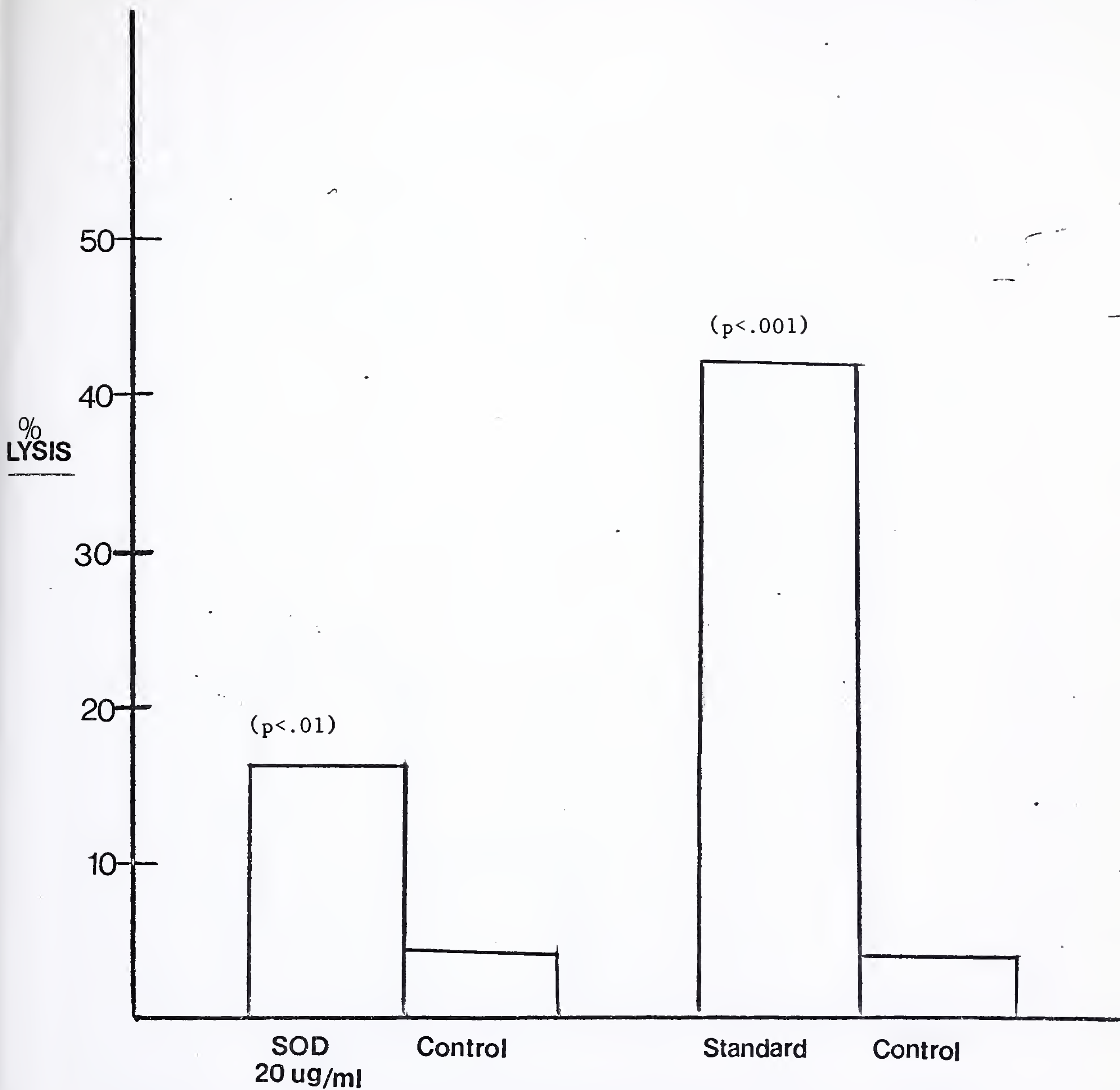


Figure 9. Effects of Superoxide Dismutase on Cytotoxicity.

Values are mean ± SEM for 6 experiments. Mean lysis compared to control lysis using student's t test.

The addition of 20 μg/ml SOD reduced cytotoxicity by 73%.

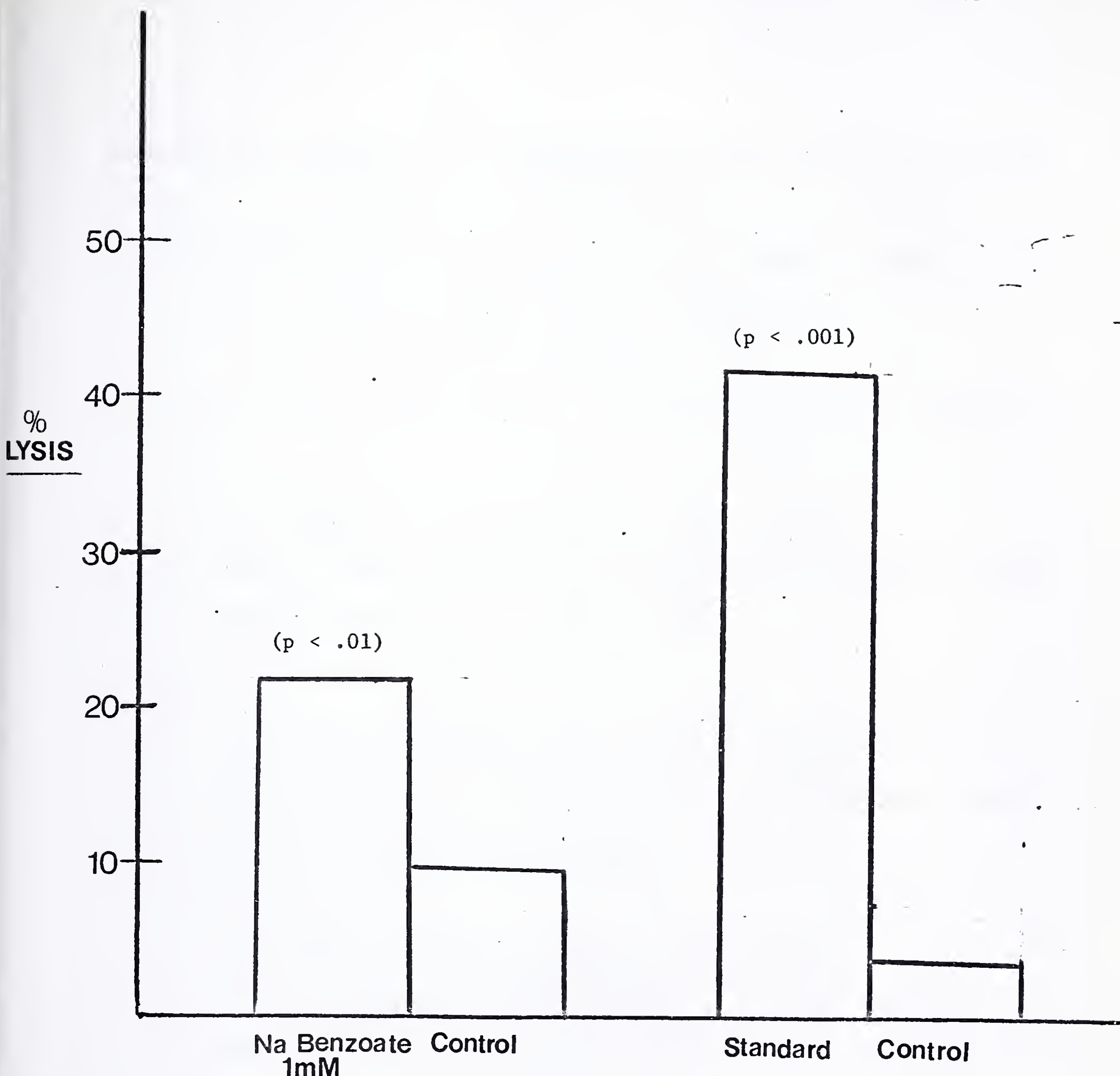


Figure 10. Effects of Sodium Benzoate on Cytotoxicity.

Figures are mean ± SEM for four experiments. Mean lysis was compared to control lysis using student's t test. Experimental conditions were as described in Table 2.

Addition of 1mM Na Benzoate led to a 65% reduction in cytotoxicity. The addition of Na Benzoate increased spontaneous lysis of CRBC. This value has been adjusted for this phenomenon (See Text).

TABLE 1

Mechanisms Proposed for Stimulation of Respiration and of HMP Pathway in PMN Leucocytes

I. Stimulation of HMP pathway directly linked to respiration.

1. Activation of NADPH oxidase → NADP+ H_2O_2

2. NADP+ stimulates HMP pathway

II. Stimulation of HMP pathway indirectly linked to respiration

A)

1. Activation of NADH oxidase → NAD+ + H_2O_2

2. H_2O_2 + 2GSH $\xrightarrow{\text{glutathione peroxidase (?)}}$ 2 H_2O_2 + GSSG

3. GSSG + NADPH + H^+ → 2 GSH + $NADP^+$

4. $NADP^+$ stimulates HMP pathway

B)

1. Activation of NADH oxidase → NAD^+ + H_2O_2

2. Activation of NADPH-linked lactate dehydrogenase-$NADP^+$

3. $NADP^+$ stimulates HMP pathway

C)

1. Activation of NADH oxidase → NAD^+ + H_2O_2

2. NAD^+ + NADPH $\xrightarrow{\text{transhydrogenase}}$ NADH + $NADP^+$

3. $NADP^+$ stimulates HMP pathway

(139)

Table 2

Cytotoxic Activity of Human Monocytes After *in vitro* Culture: Time Course

	Day 1	Day 3	Day 5	Day 6	Day 7
% lysis	4.95 ± 1.3	4.15 ± 1.12	24 ± 3.5 (p→.002)	42 ± 2.2 (p<.001)	47.1 ± 3.3 (p<.001)
Backround lysis	4.21 ± 45	1.85 ± 1.02	5 ± .69	3.2 ± 1.5	4.6 ± .65

Values are mean ± SEM for 4 experiments.

In each experiment effector and target cell were incubated at 37°C for 18 hours at a ratio of 10:1. The mean lysis was compared to backround lysis using the Student's t test for independent variables.

Cultural monocytes became spontaneously cytotoxic toward CRBC targets after 5 days of culture, exhibiting increasing activity through day 7.

Table 3

Effect of effector target cell ratio on cytotoxicity

Effector:Target Cell Ratio	.1:1	.5:1	1:1	5:1	10:1	20:1
%lysis	25.7 ± 4	19.6 ± 3	40.7 ± 4	56.5 ± 4.3	53.2 ± 5	22.7 ± 4
		($p < .05$)	($p < .01$)	($p < .002$)	($p < .002$)	($p < .02$)
Backround lysis	15.8 ± 3	7.6 ± .2	10.8 ± 8	4.1 ± 5	2.175 ± .15	2.55 ± 2

Values are mean ± SEM for four experiments. Experimental conditions were as described in table 2, with varying E:T ratios. Mean lysis was compared to backround lysis using the student's t test for independent variables.

The high backround lysis observed at low E:T ratios is explained by observation that CRBC have high rate of spontaneous lysis at concentrations $< 10^5$/ml.

Significant lysis was first observed at ratios of .5:1 ($p < .05$) and cytotoxicity increased progressively with increasing ratios of 1:1 and 5:1. There was no significant difference between mean % lysis at E:T ratios of 5:1 and 10:1 and a marked decrease was observed at ratios of 20:1

Table 4

Inhibition of cytotoxicity with 1mM Na Azide

% lysis	
Azide	4.15 ± .7
Control	3.85 ± .9
Standard	38.5 ± 2.25 ($p < .001$)
Control	4.7 ± .33

Values are mean ± SEM for 4 experiments. Experimental conditions were as described in Table 2. Mean lysis was compared to control lysis using Student's t test for independent variables. Addition of 1 mM led to 100% inhibition of cytotoxic activity.

Table 5

Inhibition of Cytotoxicity with Catalase 2000 μ/ml

Catalase	8.6 ± .8	($p < .001$)
Control	2.16 ± .22	
Standard	38.85 ± 1.16	($p < .001$)
Control	4.8 ± .17	
Heat-Inactivated Catalase	45*	
Control	4*	

Values are mean ± SEM for 6 experiments. Experimental conditions were as described in Table 2. Mean lysis was compared to control lysis using Student's t test.

Addition of 2000 μ/ml catalase led to 78% reduction in mean lysis.

*Results of a representative experiment with heat-inactivated (HI) catalase showing that HI-catalase does not inhibit cytotoxicity.

Table 6

Inhibition of Cytotoxicity with Superoxide Dismutase 20 μg/ml

SOD	10.58 ± 1.36 ($p < .01$)
Control	3.4 ± .5
Standard	38.85 ± 1.16 ($p <.001$
Control	4.8 ± .17
Heat-Inactivated SOD	34*
Control	1*

Values are mean ± SEM for 6 experiments. Experimental conditions were as described in Table 2. Mean lysis was compared to control lysis using Student's t test.

Addition of 20 μg/ml SOD led to 73% reduction in mean lysis.

*Results of a representative experiment with autoclaved (HI)SOD, showing that HI-SOD does not inhibit cytotoxicity.

Table 7

Inhibition of Cytotoxicity with Na Benzoate 1mM

Na Benzoate	22 ± 1.5	($p < .02$)
Control	10 ± 1.5	
Standard	38.5 ± 2.25	($p < .001$)
Control	4.7 ± .33	

Values are mean ± SEM for 4 experiments. Conditions were as described in Table 2. Mean lysis was compared to control lysis using Student's t test.

Addition of 1 mM Na Benzoate led to a 65%* reduction in cytotoxicity.

*This figure has been corrected for the high background lysis of CRBC (secondary to the Na Benzoate).

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