Mononuclear Phagocyte Antimicrobial and Antitumor Activity: The Role of Oxygen Intermediates

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Although considerable information has accumulated to indicate a key role for mononuclear phagocytes in the effector limb of cell-mediated immunity [1-5], relatively little is known about the biochemical mechanisms which underlie the broad range of activities displayed by these cells. Monocytes, macrophages, thymus-derived (T) lymphocytes, and especially their interactions have all been implicated in host responses to a variety of immunologic stimuli including microbial pathogens and malignant neoplasms [5]. In addition, this cellular immune system also appears to interact in a complex fashion with humoral components (immunoglobulins, complement proteins, immune complexes, coagulation factors) in both microbe and tumor cell killing and in related processes such as inflammation and graft rejection. Efforts to characterize the mechanisms subtending the protean activities of mononuclear phagocytes have explored a variety of cellular functions, and increasing attention is now being focused on oxygen-dependent systems. Recent work in this laboratory has been directed at examining mononuclear phagocyte generation of reactive oxygen intermediates and the role of these products in macrophage antimicrobial and antitumor activity. Although our observations are derived primarily from the mouse peritoneal macrophage model, similar findings are currently being made with human monocytes as well.

OXIDATIVE CAPACITY OF MACROPHAGES

While both the capacity of polymorphonuclear leukocytes (PMN) to generate oxidative metabolites and the importance of these toxic products in PMN bacterial killing have been apparent for over a decade [6-8], only recently has it become clear that mononuclear phagocytes share similar oxidative powers. After appropriate activation and subsequent plasma membrane perturbation, monocytes and macrophages consume oxygen and promptly generate superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH·) and probably singlet oxygen (O_2) [9-12]. This respiratory metabolic burst may be triggered in vitro by a variety of stimuli including soluble membrane-active agents (phorbol myristate acetate (PMA) and endotoxin), contact with complement proteins or nonphagocytosable surfaces coated with immunoglobulin or immune complexes, or phagocytosis of microbes or inert test particles [9,10,13]. O_2^- is formed by the partial reduction of molecular oxygen and H_2O_2 arises by the spontaneous or superoxide dismutase (SOD) catalyzed dismutation of O_2-. Although the pathways(s) by which OH- and 'O_2 are produced have not been firmly established, it appears likely that these latter more distal intermediates are formed by the interaction of O_2^- and H_2O_2 [14].

Current techniques permit quantitation of only the extracellularly released portion of the O_2-, H_2O_2, and OH- generated by phagocytic cells, and thus, have not allowed characterization of oxidative events within phagocytic vacuoles. It is probable, however, that the O_2- generating system is sufficiently proximate to the plasma membrane to result in simultaneous extracellular release and intravacuolar delivery of O_2-. H_2O_2 detected extracellularly appears to be primarily derived from O_2- initially released into the surrounding medium [10]. No doubt some of the intracellularly generated H_2O_2 also diffuses outside the cell as well [15]. Extracellular OH· and 'O_2 are presumably formed by similar mechanisms. No matter the original site of production, once delivered these toxic oxidative metabolites may injure or kill a variety of both intra and extracellular targets by disrupting membranes via lipid peroxidation, protein denaturation, or by interacting with target cell constituents to produce still other potentially deleterious products [17].

It should also be pointed out that unlike macrophages monocytes contain myeloperoxidase. Thus, similar to PMN's, monocytes possess an additional oxygen-dependent system which in the presence of an oxidizable halide cofactor may serve to augment the toxicity of H_2O_2 [17].

Oxygen Intermediates in Mononuclear Phagocyte Antimicrobial Activity

Evidence suggesting a role for oxygen-dependent systems in mononuclear cell microbialidal activity first stemmed from several previous observations. Monocytes from patients with chronic granulomatous disease (CGD) exhibit oxidative and bactericidal defects similar to those seen with CGD PMN's [17], and exposing normal human monocytes to an O_2- scavenger (SOD) at the time of S. aureus infection reduces staphylococcal killing [18]. Monocytes from patients with hereditary myeloperoxidase deficiency also fail to kill certain Candida species normally [17]. Depriving macrophages of oxygen, however, has yielded conflicting results in terms of inhibiting bacterial activity [19]. Since macrophages are of particular importance in host resistance to intracellular pathogens, we have explored the role of oxygen intermediates in both a cell-free system and a macrophage model using two such organisms, Toxoplasma gondii [20,21] and Trypanosoma cruzi [22].

Cell Free System

When exposed to glucose and glucose oxidase (GO), a reaction which produces no oxygen intermediates other than H_2O_2, trypanosomes are rapidly killed [22]. In contrast, toxoplasmas are resistant to fluxes of H_2O_2 (8-10 mmoles/min) generated by similar amounts of GO and remain viable despite exposure to up to 10^{-3} M reagent H_2O_2 [20]. A likely explanation for varying sensitivity to H_2O_2 is endogenous parasite catalase which trypanosomes lack and toxoplasmas abundantly possess. In the presence of a peroxidase and halide, however, toxoplasmas are promptly killed by as little as 10^{-6} M H_2O_2 [20].

The toxicity of other oxygen intermediates has been further investigated by exposing toxoplasmas to xanthine and xanthine oxidase. This reaction generates O_2-, H_2O_2, OH- and probably 'O_2 [14], and thus, is conveniently analogous to the cellular oxidative events initiated by phagocytosis. After brief exposure to xanthine-xanthine oxidase, the bulk of toxoplasmas in suspension were readily killed. Evidence for the participation of oxygen intermediates in this system was demonstrated by the inhibition of parasite killing by scavengers of O_2- (SOD), H_2O_2 (catalase), OH- (mannitol, benzoate), and 'O_2 (DABCO, histi-
dine). Since both SOD and catalase prevented toxoplasma killing, neither $O_2^\bullet -$ nor $H_2O_2$ could be implicated as toxic agents. The requirement for both metabolites for toxoplasmaclad activity, however, suggested a key role for products of $O_2^\bullet -$ - $H_2O_2$ interaction. Observing that scavengers of $OH^-$ and quenchers of $'O_2^\bullet$ also effectively reversed xanthine oxidase parasite killing indicated that $O_2^\bullet$ and $H_2O_2$ functioned importantly as precursors, but that more distal intermediates in the reduction of oxygen such as $OH^-$ and $'O_2^\bullet$ were toxoplasmaclad [21].

MACROPHAGE-PARASITE MODEL

In Vivo Activated Cells

We have also reported similar data indicating a role for an oxygen-dependent system in the antimicrobial activity of macrophages activated both in vivo and in vitro. Cultivated resident peritoneal macrophages from normal mice allow unrestricted growth of both T. gondii [21] and T. cruzi [22]. In response to phagocytic (zymosan particles) or membrane-activating stimuli such as PMA, these cells release little $O_2^\bullet$ or $H_2O_2$ [9,10]. Macrophages from immune mice, however, exhibit microbicidal activity against the respective organism, and in the case of toxoplasma immune mice, release approximately 4X more $H_2O_2$ than normal cells. In contrast, peritoneal macrophages from immune mice further activated in vivo by boosting with homologous microbe antigen are distinctly microbicidal. In the toxoplasma model, these immune-boosted cells release up to 25X more $H_2O_2$ after PMA triggering than macrophages from normal mice [21]. Thus, there appears to be a direct correlation between the capacity of the macrophage to generate oxidative metabolites (as judged by extracellular release of $H_2O_2$) and their ability to influence the intracellular fate of these pathogens.

Further evidence indicating the importance of an oxygen-dependent system in macrophage antitoxoplasma activity was derived by exposing microbicidal (immune) and microbicidal (immune-boosted) cells to oxygen intermediates scavengers. Treatment with scavengers of $O_2^\bullet$, $H_2O_2$, $OH^-$ and $'O_2^\bullet$ all resulted in clear reversal of macrophage in vitro antitoxoplasma activity, providing firm evidence for the participation of oxidative metabolites [21]. Similar to our findings in the cell-free model, these observations also suggested that radicals more distal to $O_2^\bullet$ and $H_2O_2$ are active against intracellular toxoplasmas. $OH^-$ and $'O_2^\bullet$ appear to be likely agents [21]. Depriving immune and immune-boosted macrophages of glucose, a technique which ablates $H_2O_2$ release [23], also reversed the capacity to inhibit or kill intracellular toxoplasmas. Finally, providing non-immune, resident macrophages from normal mice with an exogenous source of oxygen intermediates other than $H_2O_2$ (by adding xanthine-xanthine oxidase after parasite ingestion to the extracellular medium) also resulted in inhibition of intracellular toxoplasma multiplication [21].

In Vitro Activated Cells

The contribution of oxidative metabolism to the antimicrobial activity of macrophages activated in vitro has also been examined by exposing resident or proteose-peptone elicited peritoneal cells from normal mice to lymphocyte products (lymphokines). These soluble products were generated in a standard fashion by cocultivation of sensitized spleen lymphocytes from immunized mice with specific microbial antigen [21,22]. In the T. cruzi model, daily addition of fresh lymphokine to macrophage monolayers before and after infection resulted in a progressive enhancement of $H_2O_2$ release after PMA triggering and simultaneous eradication of intracellular trypanosomes [22]. In the T. gondii system, lymphokine treatment resulted in comparably augmented $H_2O_2$ release, but no induction of antitoxoplasma activity. This discrepancy between stimulation of macrophage oxidative metabolism and no apparent enhancement of antimicrobial capacity by in vitro activating techniques is currently being investigated, and may be related to the varying susceptibilities of these two intracellular parasites to oxygen intermediates.

OXIDATIVE METABOLISM IN MONONUCLEAR PHAGOCYTE ANTITUMOR ACTIVITY

Sufficient evidence has also accumulated from studies of both animal and human cells to suggest that mononuclear phagocytes are important participants in the host response to tumor cell growth [24]. Activation of the monocyte-macrophage-T lymphocyte system by immunologic adjuvants or chronic infections confers not only enhanced in vivo resistance to microbial pathogens but to various tumors as well [4,5]. Cytotoxic macrophages and monocytes can also be induced in vitro by specific and non-specific stimuli such as endotoxin treatment or exposure to soluble mediators (lymphokines) released by antigen- or mitogen-stimulated T lymphocytes [25-27]. As we and others have shown, lymphokine treatment also influences a variety of other cellular and biologic functions including enhancement of antimicrobial activity and release of oxygen intermediates. Recent studies using PMN's have also demonstrated that these phagocytes may be potent cytotoxic cells as well [16,28].

Although phagocytosis does not occur, close contact between effector macrophages and target cells appears to be required for macrophage-mediated tumor cell killing [25]. Antibody-dependent cell-mediated cytotoxicity (ADCC) is one such model by which monocytes bearing Fe receptors may bind to and subsequently lyse immunoglobulin-coated tumor cells [29]. However, the actual biochemical mechanisms which monocytes and activated macrophages utilize in the recognition and destruction of extracellular targets such as mammalian neoplastic cells have until recently been largely unexplored.

Secretion of thymidine and arginine may account for some of the in vitro cytostatic and cytolytic effects of macrophages; however, it is unlikely that these molecules mediate similar effects in vivo [30]. A role for products of the oxidative metabolic burst has been suggested in tumor cell killing since enzymatically generated and reagent $H_2O_2$ (with or without a peroxidase and halide) can effectively lyse mammalian cells in vitro [30,31]. Moreover, the results of several recent studies using human PMN's have indicated that extracellular release of oxygen intermediates such as $O_2^\bullet$ and $H_2O_2$ form an effective system against tumor cells [16,28]. In certain of these in vitro PMN-tumor cell models, myeloperoxidase and an oxidizable halide cofactor are required for effective target cell lysis [28].

In this laboratory, Dr. Carl F. Nathan has demonstrated in an antibody-free system that after in vivo activation and in vitro triggering, macrophages release copious amounts of $H_2O_2$ and that this intermediate alone is sufficient for extracellular cytolyis [23,30]. When incubated with P388 lymphoma cells, cultivated macrophages from BCG-infected mice fail to release $H_2O_2$ or kill the tumor cells in the absence of a triggering agent. Upon the addition of PMA, however, these macrophages promptly released $H_2O_2$ and readily lysed the P388 targets within 4–6 hr. Procedures which ablated the phagocyte oxidative metabolic burst including anaerobiosis and glucose deprivation abrogated the ability of BCG-activated macrophages to lyse the tumor cells. Catalase and ferricytochrome C (which oxidizes $O_2^\bullet$, the precursor of $H_2O_2$) markedly reduced macrophage cytocytotoxicity, while SOD (which scavenges $O_2^\bullet$ by promoting its dismutation to $H_2O_2$) enhanced cytotoxicity. Scavengers of $OH^-$ and quenchers of $'O_2^\bullet$ had no effect on P388 cell killing suggesting that these latter intermediates played no role in this system. Exposing P388 tumor cells to starch particles covalently coupled with glucose oxidase (GO) in amounts that generated fluxes of $H_2O_2$ similar to BCG-activated cells (2-4 nmoles $H_2O_2$/5 min) resulted in cytolyis comparable to that observed with PMA-triggered macrophages. Catalase abolished the cytotoxicity of particle-bound GO. Finally, no evidence for a peroxidase-mediated effect could be demonstrated. Macrophages were negative cytochemically for peroxidase, and peroxidase inhibitors (azide, cyanide) failed to decrease cytotoxic-
ity. Thus, in this macrophage-tumor cell system, H_2O_2 generated enzymatically or released after triggering of activated macrophages was both necessary and sufficient for extracellular cytolytic activity of lymphoma cells.

CONCLUSION

The studies briefly outlined here illustrate the broad range of techniques currently available for investigating cellular oxidative metabolism. The observations from this laboratory provide firm evidence for the participation of an oxygen-dependent system in mononuclear phagocyte antimicrobial and antitumor activity. It appears likely that the generation of toxic oxygen intermediates plays an important role in macrophage resistance to intracellular parasites such as T. cruzi and T. gondii. Our studies with these two micro-organisms also suggest that there may be significant variation in parasite susceptibility to oxidative metabolites. Moreover, persistent intracellular parasitization of certain cells may be related to the inability to generate or deliver either sufficient concentrations of toxic intermediates or particular metabolites required to kill a specific pathogen. Extracellular release of oxygen intermediates also appears to be an important mechanism underlying tumor cell killing by activated macrophages.

REFERENCES


DISCUSSION

GREEN: Can activated macrophages or the degree of macrophage activation be measured in vivo in disease states? This may be the next step in understanding the role of active macrophages in disease etiology.

COHN: It depends upon what you mean by activation. If you mean the increase of a certain enzyme or the absence of a certain enzyme, I am sure you could do this. In terms of some of the more important physiological functions of the activated cell, such as microbicidal and tumoricidal, one can do this in a rather general way by doing organ counts, such as George Mackiness and his colleagues have done for many years. At a more specific level, though, I think it is rather difficult. It brings up another question and that is, do we have any specific marker for the macrophage surface? I would have to answer no, we do not. We know of no specific differentiation antigen in the macrophage and this obviously would be of great importance to know.

BRICKY: Can you amplify the kinds of ratios of activated macrophages and do they require the same ratios to achieve tumor cell killing? More specifically, if one increases the numbers of tumor cells per activated macrophage does one see a decrease in the effectiveness of tumor cell killing? Does one also see a decrease in the state of activity of the macrophages? In other words, is there any evidence that tumor cells produce a material that can disarm the macrophage?

COHN: The usual multiplicities employed in these studies range from 1 to 1, with which one gets appreciable killing to as much as 20 to 1, with which one gets complete destruction. Tumors vary in their sensitivity to both killing and to killing by nascent hydrogen peroxide in the mouse system, and there seems to be a good correlation between these two. Whether this is related to other pathways that utilize hydrogen peroxide in the tumor cell, catalase being an apparent one, peroxide being another, is unclear at this point. These multiplicities, by the way, I think we have somewhat analogous systems in the sheep system.

GIGLI: The finding that you can precipitate iodinated Factor B with antibodies to Factor B would imply that the macrophage makes Factor B. COHN: The usual multiplicity employed in these studies range from 1 to 1, with which one gets appreciable killing to as much as 20 to 1, with which one gets complete destruction. Tumors vary in their sensitivity to both killing and to killing by nascent hydrogen peroxide in the mouse system, and there seems to be a good correlation between these two. Whether this is related to other pathways that utilize hydrogen peroxide in the tumor cell, catalase being an apparent one, peroxide being another, is unclear at this point. These multiplicities, by the way, I think we have somewhat analogous systems in the sheep system.

GIGLI: But wouldn't the iodination imply that it has to be an active component of the cell membrane?
MANNIK: Are activated macrophages indiscriminate killers of adjacent cells or are only selected types of cells killed?
COHN: We have not looked at a great variety of cell types but we know that activated macrophages can kill normal macrophages. They can destroy red cells, granulocytes, and a variety of cell types other than transformed or tumor cells, but only in the very highly activated state. It certainly is true that in the experiments of Hibbs and others there seems to be a predilection for a killing of tumor cells versus a normal counterpart. I am not sure if this is true for all situations. But I know they can kill normal cells under these conditions.

Announcement

The Western Section of the Society for Investigative Dermatology will meet jointly with the Western Section of the American Federation for Clinical Research and the Western Society for Clinical Research in Carmel, California, February 4, 5, and 6, 1981.

Abstracts for the Carmel meeting are due by September 26, 1980 and should be mailed to Charles B. Slack, Inc., 6900 Thorofare Rd., Thorofare, New Jersey 08086.

The Midwest Regional meeting of the SID will meet jointly with the AFCR on Saturday, Nov. 8, 1980. Abstracts are due by July 11, 1980 and should be mailed to Charles B. Slack, Inc., 6900 Thorofare Rd., Thorofare, New Jersey 08086.