VOLUME 3

PROGRESS IN CLINICAL IMMUNOLOGY

Edited by

Robert S. Schwartz, M.D.

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Preface

During the past decade the clinical and serological features of numerous immunological diseases have been described in detail. As a result there is now an enormous literature that describes immunodeficiency diseases, autoimmune disturbances, various immunopathologic states and allergic conditions. More work of this kind undoubtedly remains. However, investigators of immunologic diseases are turning to molecular mechanisms in increasing numbers. Volume 3 of *Progress in Clinical Immunology* reflects this trend. It is a trend that signals a point of departure for many of us.

The molecular dissection of disease has been applied to the problem of impaired resistance to infection, and several recent discoveries—some of which are dealt with in this volume—have revealed entirely unsuspected mechanisms. For instance, a whole subset of diseases characterized by abnormal biochemical reactions in neutrophils, reviewed here by Cheson, Curnutte and Babior, must now be considered by clinical immunologists in the differential diagnosis of immunodeficiency. This is a complex subject that is not made easier by the conflicting literature. Nevertheless, its importance to immunology may be indicated by the fact that the chemists have already turned their attention to eosinophils and a monocyte biochemistry is now emerging.

The biochemical approach permits us to ask some important questions about lymphocytes. What happens when a lymphocyte is "stimulated" by a mitogen? What signals are transmitted from the plasma membrane to the nucleus that induce proliferation and cell division? How does the lymphocyte come under the control of its environment? These questions are taken up in detail by Strom, Lundin and Carpenter. These authors emphasize the central role of cyclic nucleotides in the life cycle of lymphocytes and their review critically analyzes the burgeoning and sometimes conflicting literature on lymphocyte activation.

Strom et al. deal mainly with events in normal lymphocytes; very little is known about biochemical lesions in abnormal lymphocytes. However, the remarkable observation of a patient with severe combined immunodeficiency whose erythrocytes lacked adenosine deaminase immediately raised the possibility that congenital

biochemical lesions could adversely affect lymphocyte function. This subject is reviewed by Hirschhorn, who identifies three defects involving purine metabolism of lymphocytes. The implication of these very rare diseases may be important. For instance, the effect of adenosine deaminase deficiency is analogous to the effect of azathioprine. Can we learn about better means of immunosuppression from these diseases? And, as Hirschhorn hints, can we generalize about how to correct congenital enzyme deficiencies from therapeutic trials in these patients?

A molecular understanding of suppressor cells seems close. These cells, now very much in vogue, have been implicated in a spectrum of diseases, from immunodeficiency to cancer. Waldmann and Broder, who have devised elegant in vitro systems to analyze the function of human suppressor cells, review this topic. An understanding of modern immunology is incomplete without knowledge of suppressor systems. The review of Waldmann and Broder is strongly recommended to those who have not kept up with the avalanche of papers on this topic.

Two reviews deal with therapy. Schreiber analyzes the mechanism of action of corticosteroids. It seems surprising that corticosteroids have been used for over 30 years, often with dramatic success, yet a comprehensive picture of how they ameliorate immuno-inflammatory disorders still eludes us. Curiously, they are not notably immunosuppressive in human beings, but it is common knowledge that corticosteroid-treated patients can be highly susceptible to certain infectious agents. It seems, as Schreiber emphasizes, that these hormones act particularly on effector mechanisms, thereby frustrating the pathologic (or beneficial) effects of immune responses.

Finally, Parkman deals with the treatment of immunodeficiency by organ transplantation. His main message is that any attempt to correct immunodeficiency by transplantation is useless without a precise diagnosis: the choice of cell or organ, e.g., bone marrow, fetal thymus or fetal liver, depends on the mechanism of the immunodeficiency state. Obviously, the procedures described by Parkman are still experimental and demand a team approach. Their success—or failure—has already taught us much about how the human immune system works.

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Bruce D. Cheson, John T. Curnutte and Bernard M. Babior

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The Oxidative Killing Mechanisms of the Neutrophil

The neutrophil is one of the class of cells—the phagocytes—which protect the host by ingesting and destroying invading microorganisms. Phagocytes were discovered around the turn of the century by Metchnikoff,[1] and since then a great deal of effort has been directed toward understanding how these cells function. Studies with neutrophils have disclosed three stages in their antimicrobial activity. The first stage, in which the cell locates the microorganisms, is mediated by chemotactic factors.[2] These are chemical messengers which are released in the vicinity of the microorganism and diffuse into the surrounding tissues. The neutrophil senses the concentration gradient of the chemotactic factor[3] and moves toward the source. The second stage is the stage of phagocytosis.[4] It begins when the neutrophil encounters the microorganism and recognizes it as a particle to be ingested. The recognition signal is not the microorganism itself, but rather certain opsonic proteins, derived from plasma, which coat the microorganism and render it ingestible by the phagocyte. The neutrophil flows around the opsonized microorganism, isolating it in an invagination which eventually closes to form a vacuole (the phagocytic vesicle). This vesicle breaks off from the membrane and moves to the cell interior. Enclosed within it are the microorganism and a small amount of the extracellular medium. The third stage, that of killing and degradation, starts at the same time as the second stage, but lasts much longer. It begins with two major events: discharge of the contents of the neutrophil granules into the phagocytic vesicle,[5] and the activation of a specialized oxygen-consuming metabolic pathway.[6] As a result of these events, bactericidal agents and hydrolytic enzymes are released into the phagocytic vesicle and destroy the enclosed microorganism.

In this article the oxidative metabolism of the neutrophil is discussed. Emphasis is placed on the oxygen-consuming metabolic pathway which is activated when the cell encounters microorganisms. This pathway is discussed in terms of its biochemistry as well as its role in the antimicrobial function of the neutrophil. Finally, certain familial disorders involving this pathway are reviewed.

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THE OXIDATIVE METABOLISM OF THE NEUTROPHIL

The Resting Neutrophil

The resting neutrophil is illustrated in Fig. 1-1. It is an unusual cell in many respects, but the feature of most interest in terms of oxidative metabolism is its lack of mitochondria. On the basis of this finding, it would be expected that the neutrophil generates energy (i.e., ATP) without consuming much oxygen. This in fact is

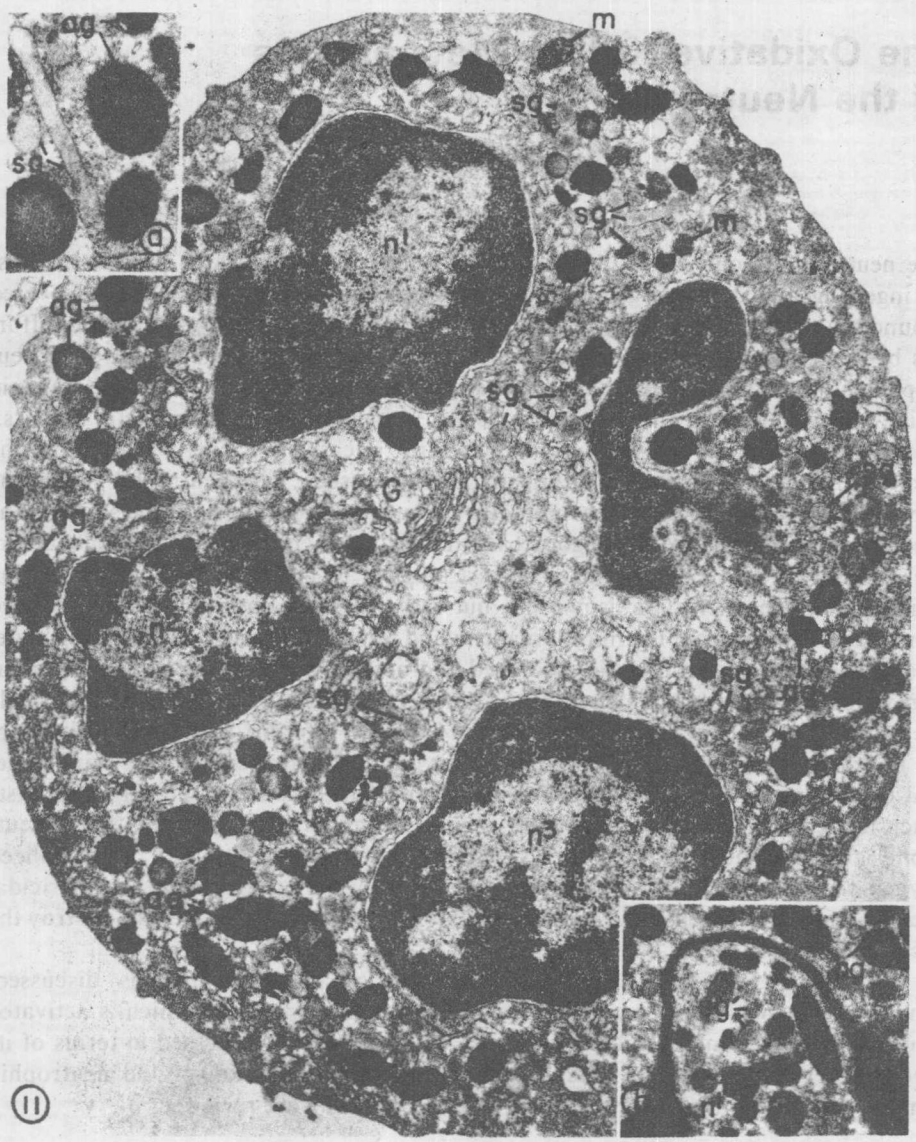

Fig. 1-1. The resting neutrophil.

Table 1-1
Metabolism of Resting Neutrophils

Conditions		Metabolism (nmol/10^7 cells/hr.)			
Source	*Serum*	*O_2 Uptake*	*Lactate Production*	*Glucose Consumption*	*Ref.*
Guinea pig[a]	Absent	140	820	600	11
"	"	85	720		12
"	"	145			13
"	"	260	1080		14
"	"	165	1160		15
"	Present	140	760	480	6
"	"	240	660		16
"	"	100			17
Rabbit[a]	Present	80		310	18
Rat[a]	Absent	120	620	340	19
Human[b]	Absent	90			20
"	"	65			13
"	Present	90	740	200	10
"	"	15			21
"	"	15			22

For purposes of comparison, data taken from the references cited were normalized to the units shown here.

a = peritoneal cavity.

b = peripheral blood.

the case, many studies having shown that the neutrophil obtains most of its energy through aerobic glycolysis.[6-11] Table 1-1 shows representative values for oxygen consumption and lactic acid production by neutrophils from various species. Assuming that the complete oxidation of a mole of glucose by way of the Krebs cycle requires 6 moles of oxygen, while a mole of glucose metabolized by the glycolytic path yields 2 moles of lactic acid,[7] it can be calculated that only 5% of the glucose consumed by the resting neutrophil is oxidized by way of the Krebs cycle. (This calculation ignores the small fraction of glucose that is converted to glycogen, fatty acids, etc., by various anabolic pathways in the cell.)[23,24]

Studies with radio-labeled glucose have in general confirmed this result. These studies have also shown, however, that a portion of the glucose consumed by the resting cell is metabolized via the hexosemonophosphate shunt.[6,23-27] This pathway involves the phosphorylation of glucose to glucose-6-phosphate, followed by the decarboxylation of this product to xylulose-5-phosphate and CO_2, a process accomplished by the sequential action of the two NADP-requiring enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.[23,25] Through a series of subsequent reactions, the xylulose-5-phosphate is converted to fructose-6-phosphate and glyceraldehyde-3-phosphate,[28,29] both of which are also intermediates in the Embden-Meyerhoff (glycolytic) pathway. Moreover, fructose-6-phosphate is in equilibrium with glucose-6-phosphate, the starting material for the hexosemonophosphate shunt, so it is possible for a fragment of a glucose-6-phosphate molecule that was metabolized via the shunt to be incorporated into another glucose-6-phosphate molecule and cycle through the shunt again. The complex interconnections between the glycolytic pathway and the hexosemonophosphate shunt make calculations of the

fraction of glucose metabolized by one or the other pathway difficult and uncertain (for a discussion of this problem, see Ref. 30). Nonetheless, such calculations have been made. Two groups have estimated the fraction of glucose metabolized by way of the hexosemonophosphate shunt in resting neutrophils to be of the order of 2–3%.[24,25] On the other hand, the validity of such estimates has been questioned on technical grounds[6] as well as on the basis of the recycling problem discussed above.[26,27] In any case, the fraction of glucose passing through the shunt in resting neutrophils is small.

Regardless of the path traversed by a given molecule of glucose on its way to lactic acid or carbon dioxide, the fact remains that the resting neutrophil, though in an oxygen-rich environment, consumes very little oxygen. Why should this be so? The following may provide at least a partial explanation for this metabolic pattern.

As will be discussed in detail below, the neutrophil, when activated, generates large quantities of O_2^- and H_2O_2 which it uses to kill bacteria. O_2^- and H_2O_2, however, are also harmful to eukaryotic cells, probably including the neutrophil itself.[31,32] Superoxide dismutase,[33] an enzyme which converts O_2^- to oxygen and H_2O_2, and catalase[34] and the glutathione peroxidase-glutathione reductase system,[35] both of which convert H_2O_2 to water (in the case of catalase, oxygen is produced as well), are used by eukaryotic cells to defend themselves against injury by these components. In the case of the neutrophil, large concentrations of these enzymes would be expected to diminish the effectiveness of the cell by destroying some of its bactericidal agents. In accord with this notion, the concentration of superoxide dismutase in the neutrophil has been shown to be only 10% of its concentration in the red blood cell.[36] This low concentration of enzyme would be expected to make the neutrophil unusually susceptible to damage by O_2^- and H_2O_2. It may be speculated that, to compensate for this vulnerability, the neutrophil has evolved in such a way that in its resting metabolism it consumes very little oxygen, so that the amounts of O_2^- and H_2O_2 accidentally formed during its metabolic activities are small enough to be handled by the reduced quantities of protective enzymes contained in the cell.

The ability of the resting granulocyte to produce ATP in the absence of oxygen confers another advantage on this cell. It is often involved in defense against anerobic organisms, and the character of its energy metabolism permits it to function in areas of extremely low oxygen tension such as are found in the vicinity of an anerobic infection. To defend against organisms under these conditions, it employs oxygen-independent bactericidal mechanisms, the nature of which is beyond the scope of this review.

The Activated Neutrophil

THE RESPIRATORY BURST

When neutrophils are exposed to bacteria or other suitable stimuli, they undergo a series of changes in oxidative metabolism which together are termed the "respiratory burst." The first change to be recognized was an increase in oxygen consumption observed by Baldridge and Gerard to occur when the cells were incubated with *Sarcina lutea*.[37] In that experiment, respiration by activated neutrophils was stimulated by a factor of four over resting levels, but more recent experiments have shown stimulation as high as 10–15-fold.[15,20-22] This increase in oxygen uptake is not inhibited by cyanide or other heme enzyme poisons.[6,18,31,38,39] For many

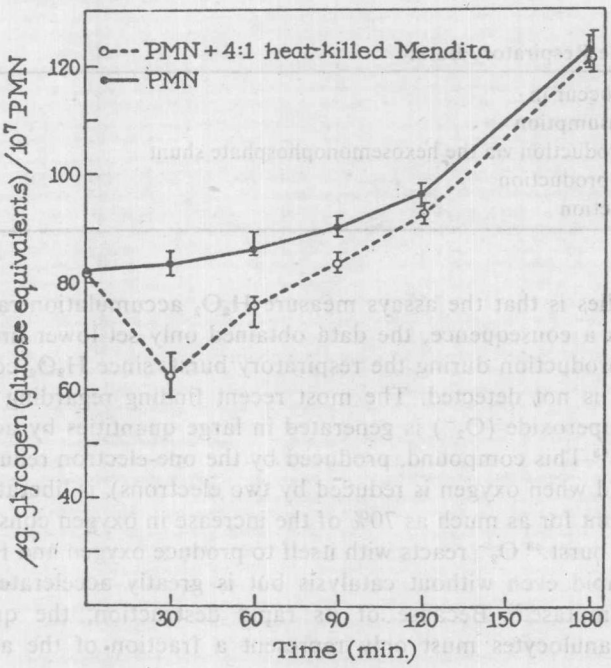

Fig. 1-2. Glycogen consumption by the activated neutrophil.

years it was thought that the increase in oxygen uptake was necessary to provide energy for phagocytosis, but this view was proven to be incorrect when Sbarra and Karnovsky showed in 1959 that particle uptake occurred under nitrogen.[6]

Hexosemonophosphate shunt activity also increases during the respiratory burst. Using $^{14}CO_2$ production from [1-^{14}C]-glucose as an indicator, Sbarra and Karnovsky showed that stimulation of the neutrophil resulted in a tenfold rise in glucose metabolism via the shunt, a finding that has been confirmed many times.[6,11,12,14,17,21,22,24,27,40-42] In contrast, measurements of rates of lactate production and of [6-^{14}C]glucose oxidation indicate that there is little change in the rate of glycolysis. Total glucose consumption increases only slightly, a result to be expected since the change in glucose metabolism during the respiratory burst involves the hexosemonophosphate shunt, a pathway which even in the stimulated neutrophil accounts for only a relatively small fraction of glucose consumption. Some glycogen is also consumed during the respiratory burst[6,18,39] (Fig. 1-2). Glycogen consumption is somewhat greater in the absence of exogenous glucose than when glucose is present in the medium.

An indication as to the fate of the oxygen consumed in the respiratory burst was provided in 1961, when Iyer, Islam, and Quastel showed that H_2O_2 was released into the medium by activated neutrophils.[38] Several other groups have subsequently made similar observations.[13,43-45,47,50,51] Although the amount of H_2O_2 detected in the original experiment accounted for only 0.05% of the increment in oxygen uptake during the respiratory burst, more recent studies have reported amounts of H_2O_2 accounting for 30[51] to 70[44] percent of the oxygen consumed. Part of the problem

Table 1-2
The Events of the Respiratory Burst

Large increases occur in
Oxygen consumption
NADPH production via the hexosemonophosphate shunt
Superoxide production
H_2O_2 production

with these studies is that the assays measure H_2O_2 accumulation rather than H_2O_2 production. As a consequence, the data obtained only set lower limits on the true rate of H_2O_2 production during the respiratory burst, since H_2O_2 consumed during the incubation is not detected. The most recent finding regarding the respiratory burst is that superoxide (O_2^-) is generated in large quantities by activated neutrophils.[45,46,48,49,52,53] This compound, produced by the one-electron reduction of oxygen (H_2O_2 is formed when oxygen is reduced by two electrons), is liberated in quantities that may account for as much as 70% of the increase in oxygen consumption during the respiratory burst.[48] O_2^- reacts with itself to produce oxygen and H_2O_2, a reaction that is very rapid even without catalysis but is greatly accelerated in tissues by superoxide dismutase.[33] Because of its rapid destruction, the quantity of O_2^- released by granulocytes must only represent a fraction of the amount actually produced.

The formation of H_2O_2 by the dismutation of O_2^- raises the question as to what fraction of the H_2O_2 generated in the respiratory burst arises by way of O_2^-. On the basis of some recent studies on H_2O_2 production by activated neutrophils in the presence and absence of ferricytochrome *c*, a reagent that traps O_2^-, Root has estimated that 40–60% of the H_2O_2 produced by neutrophils is derived from O_2^-.[54] It is thought likely that all the H_2O_2 generated by neutrophils is formed in this way.

The events of the respiratory burst are listed in Table 1-2, and typical values for the rates of these events in resting and phagocytosing cells are presented in Table 1-3. The burst is currently explained on the basis of the following sequence:

1. Exposure of the neutrophil to a suitable stimulus activates an enzyme which reduces oxygen to O_2^-. The activity of this enzyme explains the increase in oxygen uptake and O_2^- production seen in the burst.
2. At least a portion of the O_2^- dismutes, either spontaneously or under the influence of superoxide dismutase, to produce the H_2O_2 liberated by activated neutrophils.
3. The formation of O_2^- and H_2O_2 requires the consumption of a reducing agent which has to be regenerated. The increase in hexosemonophosphate shunt activity, which is thought to be a manifestation of this regeneration process, indicates that NADPH has been oxidized to NADP within the cell, since it is the concentration of NADP which limits the rate of the hexosemonophosphate shunt.[25] Several explanations have been offered for the connection between NADPH consumption and the respiratory burst. Some have postulated that NADPH reacts directly with oxygen in the O_2^--forming reaction, while others have proposed different roles for NADPH, as discussed further below.

Table 1-3
Changes in Neutrophil Metabolism Induced by Phagocytosis

Conditions			Metabolism (nmol/10^7 cells/hr)										Stimulated rate/resting rate		
Source	*Particle*	*Opsonin*	*Oxygen Uptake*		*H_2O_2 Production*		*O_2^- Production*		*Glucose Consumption*		*Lactate Production*		*Glucose-1-^{14}C → $^{14}CO_2$*	*Glucose-6-^{14}C → $^{14}CO_2$*	*Ref.*
			R*	ϕ	R	ϕ	R	ϕ	R	ϕ	R	ϕ			
Guinea pig	Latex	Absent	140	150					600	650	820	985	6.3	5.2	11
Guinea pig	"	"	150	365	4.3	11.6							7.0		13
Human	"	"	65	150	0.9	4.2							4.3		13
Guinea pig	*E. coli*	"			1.1	4.8									43
Guinea pig	*S. aureus*	"	90	235							720	760	9.5		12
Human	Latex	Present											5.6		42
Guinea pig	"	"	140	350					480	440	760	1040	7.0	2.5	6
Human	"	"	95	1350	10	970	150	1000							20,44,45
Guinea pig	*M. tuberculosis*	"	245	390								660			16
Human	*Propionibacterium shermanii*	"											11.4	2.4	24
Guinea pig	*B. subtilis*	"	260	650							1090	1395	3.8		14
	S. aureus	"	15	180									12.3		21
Guinea pig	*M. tuberculosis*	"	100	215									4.5	0.9	17
Human	*S. aureus*	"	15	130									8.5		22
Guinea pig	*B. subtilis*	"	165	1750							1160	1240	16.7		15
Human	*E. coli*	"	1460	3940			35	760							46
Human	Zymosan	"		2040	2.4	70									47
Human	"	"					55	480							48
Human	"	"					45	790							49

* R = Resting.
ϕ = Phagocytosing.
For purposes of comparison, data from the references cited were normalized to the units shown here.

ACTIVATING AGENTS

Neutrophils are activated by exposure to any of several types of particles. As expected on functional grounds, a variety of microorganisms, including bacteria,[12,14-18,21,22,24,43,46,55-57] fungi[58] and mycoplasma,[59] can activate the respiratory burst, provided they first have been opsonized by incubation with plasma or serum. Zymosan, a preparation of yeast cell walls, also stimulates the respiratory burst,[6,47-49,53] but like bacteria must be opsonized to be effective. In contrast, polystyrene latex particles are able to stimulate the respiratory burst without opsonization.[6,13,47,60,61]

The initiation of the respiratory burst does not occur immediately on mixing the particles with the neutrophils, but shows a consistent lag of about 15–30 seconds.[47,55,56,62] The extent of the burst—i.e., the increment over resting values—varies from particle to particle, being greatest with zymosan and live bacteria, less with killed bacteria, and least with latex.[57,63,64] In addition, it has been shown with latex beads that the extent of the burst is roughly proportional to the mass of particles taken up by the neutrophils during the incubation.[60,61]

Phagocytosis occurs with all the particulate stimuli, and one of the questions which has been raised concerning the activation of the neutrophil is whether or not phagocytosis is essential for the initiation of the respiratory burst. Several lines of evidence suggest that it is not. The first indication that this was the case came from experiments showing that certain inhibitors, in particular colchicine,[58,65-67] chloramphenicol[58,68] and hydrocortisone[69] were able to dissociate phagocytosis from the respiratory burst. Specifically, these compounds were shown to partially inhibit the respiratory burst at concentrations where phagocytosis was unaffected. The discovery of a number of soluble activating agents has confirmed this notion (Table 1-4).

The first of the soluble activating agents to be described was fluoride ion (F^-). Early studies by Sbarra and Karnovsky,[6] and later by Selvaraj and Sbarra,[12] showed that F^- stimulated both oxygen uptake and hexosemonophosphate shunt activity by neutrophils, although glycolysis was reduced because of the inhibition of enolase. More recently, O_2^- production was also shown to be stimulated by F^-.[70] It is thus clear that the respiratory burst is induced by F^-. What is not clear is whether, when it acts, F^- is acting as a particulate or soluble agent. Though in solution when added to the neutrophils, it is possible, as proposed by Sbarra and Selvaraj, that in the vicinity of the neutrophil F^- is precipitated as the insoluble magnesium fluorophosphate salt. If this is the case, it is conceivable that the precipitate could be taken up by the cells and activate them after phagocytosis. In this manner F^- could be acting as a particulate activating agent.

With other soluble activating agents, such a possibility is not a problem. Five such agents have been described recently: phorbol myristate acetate, an ester of a complex polycyclic alcohol derived from croton oil;[21,22] C5a, a cationic peptide released during the activation of complement;[48,71,72] kallikrein, an enzyme activated by Hageman factor which liberates bradykinin from kininogen;[61] and A23187 and X537A, ionophores which facilitate the transport of cations such as Ca^{++} across cell membranes.[73] All these agents have been shown to stimulate one or another component of the respiratory burst. With C5a, respiratory burst activation takes place in the presence of cytochalasin B,[48] an agent that paralyzes phagocytosis through a mechanism thought to involve an interaction with microfilaments, thus excluding phagocytosis as an obligatory precursor of the respiratory burst.

Table 1-4
Soluble Activating Agents

Fluoride
Phorbol myristate acetate
C5a
Kallikrein
Ca^{++}-transporting ionophores A23187 and X537A
Concanavalin A
Antineutrophil antibodies
Detergents (digitonin, deoxycholate, saponin, etc.)
Phospholipase C

Some of the agents also stimulate fusion of the granules with the cell membrane, a process analogous to the fusion of the granules with the wall of the phagocytic vesicle during phagocytosis. This process results in the discharge of the granule contents into the extracellular medium. An observation of particular interest is that with phorbol myristate acetate, only one of the two classes of neutrophil granules undergoes fusion with the cell membrane.[74,75] After exposure of the cells to this agent, the number of specific granules seen in electron micrographs is decreased, and lysozyme, an enzyme contained within these granules, appears in the medium. The azurophilic granules, which contain a large number of hydrolytic enzymes as well as myeloperoxidase (see below), are unaffected. Granule fusion does not appear to be necessary for the activation of the respiratory burst, however, because with C5a plus cytochalasin B, the burst occurs without release of either β-glucuronidase (azurophilic granules) or lysozyme (specific granules) into the medium.[48]

Agents that affect cell membranes non-specifically have also been shown to activate neutrophils. Exposure of these cells to certain detergents, including digitonin,[76] deoxycholate,[76] saponin[14,77] and fatty acids[78,79] or to concanavalin A,[80] antineutrophil antibodies,[81] phospholipase C,[82,83] or low ionic strength,[84] results in the stimulation of oxygen uptake and the hexosemonophosphate shunt.

Finally, endotoxin has been discussed in terms of the activation of the respiratory burst. Two early studies reported conflicting results. One, describing the effect of endotoxin on oxygen uptake by whole blood, showed a small rise that was attributed to the leukocytes in the blood.[85] The other demonstrated that lactate production (i.e., glycolysis) by purified neutrophils was stimulated by endotoxin, but showed no effect on oxygen uptake.[86] More recently, this question was restudied by Graham et al.[76] They found that oxygen uptake by guinea pig neutrophils incubated in the absence of serum was doubled by endotoxin, but that most of the stimulated oxygen uptake was inhibited by 1mM cyanide. Inhibition by cyanide is a property of mitochondrial respiration, but not of the oxygen uptake associated with the respiratory burst. Together, the data suggest that endotoxin does not activate the burst in neutrophils.

THE OXIDASE

By "the oxidase" is meant the oxygen-consuming enzyme of neutrophils which catalyzes the initial reaction of the respiratory burst. The nature of this enzyme has been the subject of much controversy, and its identity is not yet settled. Because the