Resistance to Infection: Prospects for Therapeutic Manipulation

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I have been charged to discuss some aspect of contemporary science in the general area of infectious diseases and to bridge that contemporary science to the future. The contemporary element is easy. Projecting into the future is not. Scientists and scholars have been notoriously bad at extrapolating from their present state of knowledge. For example, consider the following comments from established experts, all made around the time of the founding of the American Pediatric Society 100 years ago.

Note this clear-cut judgment on vaccination by an authority of the day: "Cowpox does not exercise any specific protective power against human smallpox." (E. M. Crookshank, Professor of Comparative Pathology, Kings College, London, 1889) (1).

Consider this comment by the inventor of the phonograph: "The phonograph is not of any commercial value." (Thomas A. Edison, 1880) (1).

The New York Times, then as now an expert on everything, had this to say about the art of Degas: "Degas is repulsive." (*The New York Times*, April 10, 1886) (1).

And finally, consider how dated is this particular comment of 100 years ago: "When a woman becomes a scholar there is usually something wrong with her sexual organs." (Friedrich W. Nietzsche, 1888) (1). This one would not go over particularly well in my household or at Grand Rounds.

So I hope you will forgive me, then, if I am rather cautious as I try to predict the future and if I focus more on present knowledge that should be the basis for future development.

The subject of this general section of the symposium is infection. This particular presentation will consider the host's part in that phenomenon, that is, resistance to infection, or what the host does back to the infecting organism.

To date, the most effective control of infectious diseases has come through their prevention, especially through immunization. Certainly, further efforts must be made with vaccines to achieve broader protection and easier dissemination to developing countries. But I wish to suggest that, in the unfortunate but common circumstance in which prevention is not possible and antibiotics are ineffective, treatment can be achieved by manipulation of the immune system. I will try to trace for you in summary form the evolution of current knowledge of the therapeutic "up-regulation" of one component of that system, namely, phagocytic cells.

The professional phagocytes, neutrophils, monocytes, and tissue macrophages, constitute the best-defined cells that act in the effector limb of the immune response. Cytotoxic lymphocytes and natural killer cells are other constituents of this system but are less well understood. One reason for the better definition of the phagocytic cells is the existence of experiments of nature within these cells lines, for example, chronic granulomatous disease (CGD) (2) and the leukocyte adhesion defect resulting

from deficiency of the CD11-CD18 family of membrane glycoproteins (3). Members of the American Pediatric Society and the Society for Pediatric Research have been leaders in elucidating the relationship between the molecular defects and the predisposition to infection in these disorders, and the combined annual national meeting of these societies has been perhaps the single most important forum for presenting that work.

My hypothesis and the theme of this presentation can be summarized as follows: Treatment of infectious diseases in the future, at least under certain circumstances, will involve a capacity to manipulate the function of phagocytic cells. Full achievement of this capacity will depend upon our understanding the biochemical basis of the functions that we hope to modify.

There is a caveat, however. Inflammation is a double-edged sword. The phagocytic functions necessary for survival against infection can also damage tissue. Because this is the case, however, understanding the biochemical basis of phagocyte function also offers hope for the control of inflammation.

I would like to develop this story by showing you examples, both examples of the manipulation of the function of phagocytic cells and examples of attempts to understand the biochemical basis for the changes induced. I will focus on one particular aspect of phagocyte function, the so-called respiratory burst. That phenomenon is summarized in simplified form in Figure 1.

In this process, a microorganism coated with antibody, and usually also complement, interacts with receptors on the plasma membrane of the phagocytic cell and stimulates an enzyme complex associated with the plasma membrane. This enzyme system takes oxygen from the surrounding medium into the plasma membrane. It inspires, if you will, and reduces the oxygen by addition of one electron to yield superoxide anion (O_2^-). Two molecules of O_2^- interact with each other, in a dismutation reaction, to give hydrogen peroxide. Peroxide and O_2^- interact in the presence of heavy metal, especially iron, to yield a particularly potent oxidant, said to be the most potent oxidant recognized yet in nature, generally schematized as $\cdot OH$ and termed hydroxyl radical (4).

The respiratory burst enzyme complex is presently under intense study. The complex is conceptualized to consist of two to four components that assemble as an active enzyme in the plasma membrane after the cell has been stimulated (4). The best defined constituent is a unique cytochrome, so-called cytochrome b, which is felt to make the final transfer of the electron onto oxygen to give O_2^- (5). Phagocyte cytochrome b is missing in the classic x-linked form of CGD. Data also support NADPH as the source of electrons and the presence of a flavin-dependent enzyme in the system.

The work of Stuart Orkin, who is a member of the APS, with Mary Dinauer and associates, has located the gene for the major piece of cytochrome b (there are two chains) on the short arm of the x chromosome (Xp21.1) (6). This protein exists primarily in the membrane of specific granules in resting human neutrophils. When the cell is stimulated, the cytochrome is transferred to the

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Fig. 1. The phagocytosis-associated respiratory burst.

Fig. 2. Extent of O_2^- release by mouse peritoneal macrophages plotted as a function of time, with PMA as stimulus. Cells were elicited by intraperitoneal injection of LPS or thioglycollate, or obtained from animals infected with the BCG strain of mycobacterium, without or with the further injection of heat-killed (hk) BCG. (Modified from Ref. 8, with permission)

plasma membrane as the specific granules discharge their contents (5). There are one, two, or maybe even three additional factors present in the cytosol of the resting cell. One of these could be the flavin-dependent enzyme identified in cell membranes from stimulated cells, but this relationship is unclear. In this system, then, an electron is removed from NADPH and transferred to oxygen. The enzyme system is generally referred to as the NADPH oxidase or the respiratory burst oxidase.

Let us move next in the story to activated macrophages. By definition, a macrophage is activated if it has an increased capacity to kill microorganisms or tumor cells (7). About 10 years ago, the following question arose: Could the increased killing capacity of activated macrophages be ascribed at least in part to an increase in the respiratory burst? The experiment shown in Figure 2 suggested that it might.

The figure indicates that macrophages obtained from mice infected with the bacille Calmette-Guerin (BCG) strain of mycobacteria or injected with an inflammatory agent [endotoxin (lipopolysaccharide, LPS) or thioglycollate] have a markedly greater capacity to release O_2^- than do resident (normal) macrophages when the cells are stimulated. In this experiment, the stimulus was the phorbol diester, phorbol myristate acetate (PMA). Unstimulated macrophages of any sort do not release O_2^- .

For the sake of clarity, this up-regulation has been defined precisely by the term "priming." Webster's fourth definition of the transitive verb "to prime" is, "to put into working order by filling or charging with something," as for example, in charging a pump. As applied to phagocytes, "primed" refers to the state or condition of a phagocyte in which the cell is modified so that it responds to phagocytosis or membrane perturbation, particularly from PMA, with a marked increase in the respiratory burst. This phenomenon is demonstrable only upon stimulation. It is a manifestation of macrophage activation but is not the same thing as macrophage activation, which includes many other biochemical and functional changes (7).

It seemed important to reproduce priming with a defined priming molecule *in vitro* to be able to study the phenomenon under controlled conditions. In the system shown in Figure 3, normal resident macrophages were preincubated overnight with LPS or muramyl dipeptide (MDP), a constituent of the cell wall of most bacteria, in the concentrations shown. Exposure to ng/mL concentrations of either LPS or MDP primed the macrophages for a much greater respiratory burst, measured as release of O_2^- .

Priming of macrophages has been achieved with other agents, which are summarized in Table 1. Some of these agents have therapeutic potential, particularly interferon- γ , and perhaps also colony-stimulating factor-granulocyte, monocyte (CSF-GM) (7, 10-13). Those in the second category may be active at local sites

Fig. 3. Effect of concentration of MDP or LPS in the medium on enhancement of the O_2^- -generating capacity of resident mouse macrophages cultured overnight. Values shown represent the mean O_2^- produced in response to stimulation with PMA after priming with LPS or MDP ($n = 6$, SEM < 15). The O_2^- response of untreated control cultures was 84 ± 7 ($n = 6$). (From ref. 9, with permission)

Table 1. Factors capable of priming macrophages for an enhanced respiratory burst

I. With possible therapeutic potential:	
Interferon- γ	
Colony-stimulating factor-granulocyte, monocyte	
Somatotropin	
Muramyl peptides	
Vitamin D ₃	
II. Possibly active at local sites:	
Tumor necrosis factor- α	
Platelet-activating factor	
Serotonin	
Proteases	

of inflammation, as judged primarily by studies *in vitro* (7, 13–15).

Macrophages are not the only phagocytic cells whose activity can be up-regulated. Neutrophils can also be primed. This capacity to be primed has demonstrated that neutrophils are not an end-stage cell in the functional sense, as had been thought previously (16). Agents that can prime neutrophils for an enhanced respiratory burst include bacterial products (LPS, MDP), chemotactic factors, platelet-activating factor, tumor necrosis factor- α , CSF-GM, and interferon- γ (17).

Bacterial infection itself can prime neutrophils (18). The priming molecule is not yet understood but might be a product of the infecting organism or any of the other byproducts of inflammation just mentioned. In fact, multiple sources are likely, perhaps depending upon the type of infection. LPS, which is a particularly potent priming agent for neutrophils, also stimulates the release of platelet activating factor and tumor necrosis factor- α .

The nitroblue tetrazolium (NBT) test, popular a few years ago as a means of distinguishing bacterial infection from viral infection (19), is a direct manifestation of priming. In that test, the increased reduction of NBT to form blue formazan is a result of the increased production of O_2^- by primed neutrophils. Unfortunately, the relationship between a positive NBT test and bacterial infection is not consistent enough to aid substantially in the resolution of the common problem of distinguishing between these two classes of infection.

If the molecular basis for the priming phenomenon could be understood, then we might manipulate it intelligently for the purposes of therapy. Any of several possible biochemical changes might underlie the priming or activation of phagocytes (20). It is possible that priming causes an increase in the number or affinity of receptors for the stimuli. This has been studied with several stimuli, but there is no evidence to date that a change in number or affinity of receptors can explain priming. Theoretically, there could be an increase in the content or efficiency of the NADPH oxidase. This appears not to be the case in the primed but unstimulated cell. There is possibly a down-regulator of the NADPH oxidase present on the plasma membrane of monocytes and macrophages, as suggested by the observation that brief protease treatment of these cells can prime them (15).

A fourth possibility, that there might be modification of stimulus-response coupling, or signal transduction, does seem likely. There are reasonably good data to indicate that transduction of the signal induced by stimulus-receptor interaction is faster in a primed cell (16, 17, 20). This transduction process appears to be greased or "charged" so that it works more efficiently.

There are many steps in the transduction process. None is fully understood. The process might be conceptualized currently as a mosaic. There are pieces of the mosaic that are appreciated at least partially, but understanding of the interactions between the pieces is vague if it exists at all. Currently the fundamental science of cell biology focuses most of its efforts at understanding this process across a variety of cell types. As examples, I will show you experiments relating to only two of these steps.

Figure 4 analyzes the change in electrical potential across the plasma membrane of human neutrophils after stimulation by chemotactic peptide. Membrane potential in this system is measured indirectly by a change in fluorescence of a fluorescent dye, oxonol 5, that partitions into the plasma membrane and does not accumulate in the interior of the cell (17). After stimulation at time zero, normal cells had a very weak hyperpolarization and a very slow depolarization. Cells primed by prior exposure to LPS responded with a vigorous hyperpolarization followed by a pronounced depolarization. The precise basis for this difference is not understood, but the result indicates a much greater capacity of the primed cell for ion flux across the plasma membrane. The consequences to the cell of this difference are likely to be profound.

Figure 5 illustrates that free, unbound Ca^{2+} inside neutrophils, shown on the vertical axis as fluorescence of the Ca^{2+} -binding

Fig. 4. Time course of change in membrane potential in control and LPS-primed human neutrophils, measured as change of fluorescence intensity of oxonol V. The graph shows superimposed tracings for control and LPS-primed cells stimulated at time 0 by exposure to chemotactic peptide (f-met-leu-phe, 1 μ M). (From ref. 17, with permission)

Fig. 5. The effect of LPS priming of human neutrophils on baseline (unstimulated) and poststimulation concentrations of intracellular free Ca^{2+} , measured as fluorescence of fura-2. The chemotactic peptide f-met-leu-phe (FMLP) was added, as shown by the arrow, at concentrations of 1 μ M (upper panel) and 1 nM (lower panel). (From Ref. 17, with permission)

dye, fura-2, was higher in unstimulated cells that had been primed with LPS than in control cells. This represents Fura-2 that penetrates the cell and fluoresces in proportion to the amount of Ca^{2+} bound. Stimulation with chemotactic peptide induced a greater increase in this free Ca^{2+} in the primed cells.

Table 2. *Treatment of mice infected with M. intracellulare with agents that promote macrophage activation**

Treatment	CFU/spleen (millions)
None	14.1 ± 1.7
Saline	13.3 ± 1.3
Indomethacin (600 µg)	2.0 ± 0.4
Muramyl dipeptide (50 µg)	0.4 ± 0.01
Interferon-γ (500 U)	0.2 ± 0.02
Interferon-γ (2500 U)	0.3 ± 0.01

* Mice were given bacteria i.v.; 4 wk later, 8–15 mice were given the indicated agent i.v. daily for 7 d. Colony-forming units (CFU) are expressed as mean ± SEM, $n = 8$ –15. (Data from ref. 22).

The two panels represent results with two different concentrations of the stimulus. In every experiment, LPS-primed cells had an enhanced respiratory burst (mean of 11-fold).

Priming for an enhanced respiratory burst can be induced in both neutrophils and macrophages by the relatively Ca^{2+} -specific ionophore ionomycin (21), and priming by LPS can be blocked by preloading the cells with agents that chelate the intracellular free Ca^{2+} (17). These combined results raise the possibility that the respiratory burst in phagocytic cells is controlled by regulation of intracellular Ca^{2+} , which, in turn, could achieve its effect through modulation of a Ca^{2+} -dependent enzyme or enzymes.

Two recent examples of the manipulation of phagocytes *in vivo* will illustrate the potential for future development. The first involves a model of chronic mycobacterial infection in mice (22). In this model, infection is induced by injection of *Mycobacterium avium-intracellulare* into the tail vein. There is an initial containment of bacterial replication, followed by accelerated bacterial growth and death of the mice after several months. The primed state of the macrophage, as an index of macrophage activation, increased for about 2 wk, as demonstrated by an increase in the release of O_2^- over that 2-wk period. This was followed by a surprising decline in the primed state, which was especially pronounced if the macrophages were stimulated by the infecting organism.

Splenic and peritoneal macrophages from infected animals, studied at 2 or 3 wk after the onset of infection, released large amounts of prostaglandin E_2 (PGE_2) when stimulated by antigens from the infecting organisms. PGE_2 is known to suppress lymphocyte function. In other experiments, it was found that PGE_2 -containing supernates from macrophages of infected mice suppressed the release of interferon from lymphocytes. Thus, it appeared that the microorganism induced macrophage PGE_2 release, which inhibited release of macrophage-activating lymphokines (22).

Table 2 indicates that this block in lymphokine release could be circumvented or suppressed. In the experiment shown, after 4 wk of infection, 8–15 mice were injected i.v. daily for 1 wk with one of the agents shown in the left column. Only a graduate student would do this project.

Mice given nothing or saline had 13 to 14 million bacteria per spleen on the eighth day. Injection of indomethacin, which inhibits the synthesis of PGE_2 , permitted a significant reduction in the infection. The block in release of macrophage-activating lymphokine(s) was circumvented effectively by injection of the macrophage activators, MDP or interferon-γ. This suppression of the activation process during infection by intracellular parasites may be widespread in nature (22) and may play a role in the chronicity of intracellular infections.

Interferon-γ, the best defined of the macrophage-activating factors, is beginning to be tested experimentally in humans and has been tried in AIDS, lepromatous leprosy, chronic hepatitis B, condyloma acuminata, chronic myelogenous leukemia, renal cell carcinoma, and under particular circumstances, in CGD (23, 24).

My last example involves the use of interferon-γ in CGD.

Interferon-γ has had a surprisingly potent effect in patients with this disorder. Esekowitz *et al.* have shown that interferon-γ, added *in vitro* to human phagocytes from patients with cytochrome b-deficient x-linked CGD, induced synthesis of messenger RNA for the major chain of cytochrome b and modest activity of the respiratory burst (25). Thus, in the type of CGD in which the defect lies in an inability to produce normal amounts of cytochrome b, interferon-γ exhibited a partial capacity to overcome or circumvent the genetic defect *in vitro*.

On the basis of this result, they injected interferon-γ at a dose of 0.1 mg/m² s.c. on two successive days into two patients who had the classic, cytochrome b-negative, most severe form of x-linked CGD, and two patients with a milder variant of this x-linked form (26). By the eighth day after injection, release of O_2^- by stimulated neutrophils was within the normal range in the case of the two patients with the milder disease, and values remained at this level for 30 d. Release of O_2^- by neutrophils from the two patients with severe disease was affected less by injection of interferon-γ, but levels still reached 20–30% of normal at peak on d 15. The patient's phagocytes also demonstrated an increase in messenger RNA transcripts for the heavy chain of cytochrome b and an increased capacity to kill ingested staphylococci. Similar findings to these have been obtained by Gallin *et al.* at the National Institutes of Health (27), and a large clinical efficacy trial of interferon-γ in CGD is now being planned.

Three conclusions appear reasonable. First, pharmacologic up-regulation of phagocyte function will permit enhanced rejection of infection and, perhaps, cancer. The capacity to achieve this effect in patients is at hand. Second, it may also be possible in the future to down-regulate phagocytic function under circumstances in which inflammation is detrimental. What seems most certain is that future success in these endeavors will depend upon our gaining further understanding of molecular mechanisms.

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