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Bacteria–phagocyte interactions: emerging tactics in an ancient rivalry

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1. SUMMARY

Although phagocytes appear to have a redundancy of both oxidative and non-oxidative killing mechanisms, nevertheless, bacterial pathogens are still able to evade these defenses in vivo and cause lethal infection. As the mechanisms by which phagocytes function have become detailed at the molecular level, both the recognition of specific bacterial virulence determinants and their effects at specific sites in the phagocyte are also being identified. Knowledge of these interactions may permit the use of immunomodulators either to neutralize these virulence determinants or to enhance the bactericidal capabilities of the phagocyte.

2. INTRODUCTION

Professional phagocytes (neutrophils, macrophages and eosinophils) are important components of a host's defenses against invading microbes. The outcome of the encounter between phagocytes and the invading microorganism often determines whether disseminated infection, and perhaps host death, will occur following penetration of the host's first line of defense, the mucocutaneous barrier. It is now appreciated that over the millenia the host defenses and the invading microbe have been involved in a deadly minuet by which the microbes alter their own character to take advantage of perceived weaknesses in these defenses. In turn, the phagocytes have acquired an apparent redundancy of effector mechanisms to dispose of these microbes.

During the last decade considerable progress has been made in defining at the molecular level the mechanisms employed by phagocytes to meet and overcome invading microbes. These include the identification of components of the oxidase system and their assembly, description of ligand receptor interactions and their signal transduction (often through the action of phospholipases and GTP-binding proteins), the cloning of effector enzymes, such as protein kinase C, identification of granular cationic proteins and the role of calcium ion fluxes, to name but a few recent developments. Similarly, determinants of potential microbial virulence have also been defined at the molecular level. Many of these molecules, such as bacterial exotoxins, have been used as probes to investigate phagocyte mechanisms. Other cell-associated constituents, such as the sialic acid-containing K1 capsule of *Escherichia coli*, have enabled the microorganism to evade host defense mechanisms by mimicking important host cell constituents. De-

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spite these advances in defining phagocytic cell and microbial mechanisms, our understanding of which microbial moieties and what phagocytic mechanisms are most important in determining the outcome of this encounter in vivo is relatively limited.

In this review we shall describe recent advances in our understanding of bacteria–phagocyte interactions which builds on the descriptions made in earlier, excellent reviews of this subject [1,2]. Since many of the mechanisms used by neutrophils and mononuclear phagocytes are similar, we shall apply the knowledge acquired in one cell to both phagocytes unless otherwise indicated. Through a better understanding of these mechanisms it may be possible to enhance some of the phagocytic defenses, or alternatively, to neutralize microbial virulence factors in order to shift the balance in favor of the host.

3. NORMAL PHAGOCYTE FUNCTION

In order to seek an invading microorganism and kill it, the phagocyte must perform a series of complex functions in a coordinated manner. First, it must sense the presence of the invader and translate this awareness into directional motion. The presence of a microbe may be heralded (1) by moieties present on or extruded into the environment by the microbe, (2) by the generation of chemoattractants through the microbial activation of the complement system (primarily C_5a), or (3) by the generation of chemoattractants from other phagocytes already present at the site of invasion. Upon sensing these chemoattractants, often present at nanomolar concentrations, the phagocyte must then move through the circulation along this gradient, adhere to the vascular endothelia, and exit the vascular compartment to the local tissue (diapedesis). In the invaded tissue the phagocyte must then recognize, ingest and kill the microbe. The recognition of both chemoattractant gradient as well as the targeted microbe occurs primarily through a wide array of phagocyte cell surface receptors whose behavior (number, affinity and location on the plasma membrane) is under careful cellular regulation.

When a phagocyte recognizes its microbial target at the site of invasion, it ingests the organism into a compartment (phagosome or endosome) which will fuse with cellular granular compartments that are rich in both cationic proteins and enzymes capable of generating reactive oxygen species (ROS) as well as degradative enzymes. In these newly formed phagolysosomes the ingested bacteria are killed and digested. Microbes in the immediate vicinity of an activated phagocyte may be killed by the diffusion of granular contents and ROS out of the phagocyte into the local environment, although the relative importance of this extracellular killing is unclear. A variety of cationic proteins have been identified within the granular compartment that vary in size and type of microbial target [3–5]. Although a number of ROS with antimicrobial activity have been identified, hypochlorous acid whose formation is mediated by myeloperoxidase (MPO), and oxidizing radical production, appear to be the most potent [6]. Despite this seeming redundancy of microbicidal effectors, microbes are able to evade and even multiply in these seemingly hostile environments.

The mechanisms by which some microbial invaders are able to escape, or even take advantage of, host defenses have been carefully studied. While the purpose of this review is not to provide an encyclopedic enumeration of these microbial–phagocyte interactions, a few selected instances will be provided.

3.1. Phagocytic mobilization

The importance of phagocyte mobilization from the circulation has been demonstrated both clinically and experimentally. Neutrophils from kindreds deficient in the complement receptor type 3 (CR3) membrane glycoprotein family are unable to adhere to surfaces and have defects in chemotactic and phagocytic function [7]. Individuals with this genetic disorder are subject to severe recurrent bacterial infections. Experimentally, the administration of a monoclonal antibody to this glycoprotein inhibits the migration of blood monocytes from the circulation to infective foci, thereby converting a sublethal infection with *Listeria* into a lethal one [8].

Table 1

Mechanisms by which microbes might evade mobilization of phagocytes

Alter phagocyte motility	Example. Exoproduct of *Capnocytophaga*
Inhibit actin formation	Example. Botulinus C2 toxin
"Surreptitious entry"	Example. *Leptospira*

Relatively few microorganisms have been shown to develop strategies to alter this aspect of phagocyte function (Table 1). Some organisms, like leptospira, are able to invade host tissue and disseminate before there is evidence of phagocyte mobilization, but the basis for this "surreptitous entry", or the extent of this mechanism among microorganisms, has not been fully determined. A dialyzable product of a dental pathogen, *Capnocytophaga*, has been shown to alter neutrophil motility [9]. This molecule may play a role in dental infections caused by this organism. Botulinus C2 toxin, a non-neurotoxin that inhibits non-muscle actin polymerization, inhibits cellular migration; however, its role in infection with the intact organism is not known [10].

3.2. Phagocytosis

Phagocytes recognize their microbial targets primarily through surface receptors for the complement components, C_3bi (CR3) and C_3b (CR1) and the Fc portion of immunoglobulin. Microbes that are opsonized, or coated, with immunoglobulin and/or complement attach to these receptors on the plasma membrane of the phagocyte. Engagement of these receptors signals the phagocyte to engulf the microbe into a phagosome [11]. The mechanisms by which microbes evade the deposition of immunoglobulin and complement on their surfaces have been well-studied (Table 2). For example, the composition of the outer membrane of Gram-negative bacteria, particularly *E. coli*, is an important determinant of opsonization. The virulence of extra-intestinally invasive *E. coli* has been correlated with its ability to resist lysis by components in the serum, especially complement (for review see [12]).

3.2.1. Complement is deposited onto the surface of bacteria through the activation of either the classical or the alternative complement pathways. In the former instance, the first component of complement, C1, usually in cooperation with immunoglobulin, is fixed onto its microbial target. This initiates the sequential deposition of C4 and C2 into a surface complex, called C3 convertase. The C3 convertase then acts on fluid phase C3, resulting in the deposition of C3b and other C3 breakdown products, particularly the important opsonin, C3bi, on the bacterial surface. The stability of the C3 convertase is regulated by complement regulatory proteins.

Many bacteria may activate the complement cascade in an antibody-independent manner. This alternate complement pathway, like the classical pathway, leads to the deposition of C3b and its products on the bacterial surface. Through the alternate pathway, there may be amplification in the amount of C3 products deposited on the microbial surface by either complement activating pathway. Once the C3b and C3bi are deposited on the microbial surface, they may serve as ligands

Table 2

Mechanisms by which microbes might escape phagocyte recognition

A. Complement component	
	Failure to activate alternate pathway
	Example. K5-encapsulated *E. coli*
	Lack of antibody induction to initiate classical pathway
	Example. K1-encapsulated *E. coli*
	Surface-related instability of C3 convertase complex
	Example. Sialic acid coated surface
	Cleavage of complement receptor
	Example. Elastase of *P. aeruginosa*
	Diversion of complement deposition via mimicry
	Example. *C. albicans*
	Mimicry of decay-accelerating factor
	Example. *T. cruzi*
	Possible lysis of decay accelerating factor on host cells
	Example. Bacterial phospholipase C
B. Antibody component	
	Poor induction of antibody
	Example. K1-encapsulated *E. coli*
	Possible lysis of Fc receptor$_{III}$ (CD16) anchor
	Example. Bacterial phospholipase C
C. Other	
	Alteration of microbial surface
	Example. Phase variation by *N. gonorrhoeae* or *Borrelia* sp.
	Plasmid acquisition of smooth LPS-*Salmonella*
	Appropriation of host enzymes and substrates-*N. gonorrhoeae*

for CR1 and CR3 receptors on phagocytes, or the deposition of complement may continue to the formation of the membrane attack complex, involving the terminal complement components, by which lysis of bacteria may occur (independent of phagocytes).

In general, the capsular polysaccharides of *E. coli* impart to the bacteria the ability to resist complement-mediated lysis [13]. The phenotypic characteristics of another cell wall constituent, the lipopolysaccharide (LPS), are another determinant of bacteriolysis: the more complete (or smooth) the LPS, the more resistant is the organism to such killing [14]. These cell-associated moieties critical to serum-mediated lysis are also important determinants of bacterial killing by phagocytes.

In a study of over 200 consecutive *E. coli* strains isolated from the blood and spinal fluid of patients, over 95% were found to be serum-resistant [15]. These strains were, however, susceptible to killing by neutrophils in the presence of normal human serum: strains that had a smooth LPS were less susceptible to opsonophagocytic killing than were those possessing a rough or part-rough LPS. In addition, those strains that had a K1 capsule (which is composed of sialic acid) were more resistant than those that had non-K1 capsules, even if the K1-encapsulated *E. coli* did not have a smooth LPS phenotype.

The mechanism by which bacteria that contain sialic acid in their capsules, such as K1 *E. coli*, group B meningococcus or group B streptococcus, evade complement deposition on their surface has been described [16]. Sialic acid on the bacterial surface favors the deposition of one complement regulatory protein, factor H, over another, factor B. Thus the degradation of the nascent C3 convertase occurs instead of its amplification, and the deposition of this important opsonin on the bacterial surface is limited. Fries et al. have suggested that C3b bound to IgG, but not when bound to other serum glycoproteins, has a reduced affinity for factor H. This may explain the ability of IgG to enhance the activity of the alternate pathway [16a]. Other polysaccharide capsules of *E. coli* are, like the K1 capsule, poor activators of the alternate complement pathway [17].

In addition to their effects on complement deposition, many polysaccharide capsules, both sialated and non-sialated, are poorly immunogenic such that relatively little antibody is generated and this antibody is of low affinity [18]. The presumed basis for the poor immunogenicity of these capsules is the similarity of these capsular polysaccharides to structures on the surface of mammalian cells such that the generation of antibody to the bacteria might induce cross-reacting antibody to the host's own tissue. One of these capsules, the K5 capsule, has a structure similar to desulfoheparin, a precursor to an important mammalian cell polysaccharide, while another capsule, K4, is similar to chondroitin (for a complete review, see [19]). These examples of bacterial mimicry of host tissue have been likened to "wolves hiding in sheep's clothing" [20].

Microbes have developed other strategies to evade recognition and ingestion via complement receptors. For example, elastase produced in inflammatory sites by either *Pseudomonas aeruginosa* or by neutrophils can cleave the CR1 (C3b) receptor on neutrophils obtained from the lungs of cystic fibrosis patients with chronic infection. This proteolytic cleavage of the C3b receptor might interfere with the efficient killing of *Pseudomonas* by neutrophils [21]. Furthermore, neutrophil elastase can remove the C3bi deposited on the surface of opsonized *Pseudomonas* such that there is a functionally important opsonin-receptor "mismatch" by which there is less C3bi ligand on the opsonized organism (for binding to the CR3 receptor) and less CR1 receptor on the phagocyte to bind the C3b ligand [22]. In another strategy, both *Candida* and herpes simplex virus produce surface structures that mimic the complement receptors on phagocytes. Thus, the C3bi opsonin may be diverted away from phagocytes onto *Candida* expressing CR3 [23,24]. Alternatively, this functionally active CR3 receptor on *Candida albicans*, as a member of the integrin receptor superfamily, may mediate the attachment of *Candida* to mammalian cells [25].

Metacyclic trypomastigotes of *Trypanosoma cruzi* exhibit a developmentally regulated surface protein that mimics the action of decay accelerating factor (DAF) [26]. This protein is part of a new family of surface molecules that includes al-

kaline phosphatase and acetylcholinesterase, which is bound by a phospholipase-sensitive phosphatidylinositol anchor to the surface of mammalian cells, including human erythrocytes, neutrophils, monocytes and endothelial cells. DAF inhibits amplification of the complement cascade by accelerating the decay of the C3 convertases through the dissociation of C2a from C4b and Bb from C3b [27]. DAF and a similarly anchored protein, C8-binding protein, are complement regulatory proteins that protect the cell from damage by autologous complement. Patients with paroxysmal nocturnal hemoglobinuria, a condition characterized by hemolytic anemia, lack these regulatory proteins. Microbial phospholipases, such as those produced by *S. aureus* [28], *Pseudomonas* and other microbes could conceivably cleave this anchor, thereby overcoming the regulatory effect of DAF on the developing complement cascade deposited on the cell.

3.2.2. Microbes have also developed mechanisms which may affect their interaction with the Fc receptor. Recently, the expression on an anaerobic streptococcus of protein L, which binds the light chain of all isotypes of immunoglobulin, was correlated with the development of bacterial vaginosis [29]. How this protein contributes to the virulence of these anaerobic streptococci is unclear. The previously described protein A of *S. aureus* and protein G of groups C and G streptococci which bind to the Fc regions of IgG and IgA have not been found to contribute to the virulence of these organisms.

A form of the low affinity Fc receptor, CD16, is known to be bound to the neutrophil surface by a phosphatidylinositol-glycan anchor. As in the case of DAF, this anchor is susceptible to cleavage by a phosphatidylinositol-specific phospholipase C [30], which may be elaborated by some bacteria. At this time the possible effect of bacterial phospholipases on this receptor is entirely speculative.

Another, perhaps primordial, form of phagocytosis involves the non-opsonic recognition of bacteria by phagocytes. This type of phagocytosis, used by amoebae, may have evolved as a means of ingesting bacteria that do not activate the alternate complement pathway. Recognition between phagocyte and its target occurs by carbohydrate biding proteins with a complementary sugar on the surface of the second cell. This interaction, called "lectinophagocytosis", most typically occurs between bacterial pili or fimbriae and their receptors on phagocytes [31]. It is speculated that this form of phagocytosis may be important in serum-poor sites of infection. Bacteria may escape lectinophagocytosis by phase variation by which pili are no longer expressed. Opsonin-mediated phagocytosis may have developed in mammals as a means by which they ingest organisms that evaded lectinophagocytosis [31].

3.2.3. Microorganisms may escape opsonization by alteration of their surface antigens. For example, *Salmonella* may rapidly alter its LPS phenotype through the acquisition of a plasmid [32]. As noted above, bacteria may affect their interaction with phagocytes by altering the expression of their pili or fimbriae. The effect of variation of surface antigens in *Neisseria gonorrhoeae* (protein II and pilin), African trypanosomes and *Borrelia* sp. (relapsing fever) on phagocyte interaction has been well detailed [33].

3.2.4. Phagocytes also play a role in altering microbial surfaces. Recently, groups in England and the United States have described a novel situation in which neutrophils have been shown to alter the lipooligosaccharide (LOS) of a bacterial pathogen, *N. gonorrhoeae* [34,35]. While strains of *N. gonorrhoeae* that cause disseminated infection remain serum resistant, those isolated from the urethra upon subculture in the laboratory become sensitive to lysis by normal human serum. Incubation of these serum-sensitive strains in human serum, red cells and white cells induces these strains to become serum resistant. This induced resistance to complement is accompanied by a shift to higher molecular weight bands in the LOS pattern on SDS PAGE [35]. Gonococci have been found which contain in their LOS a structure that accepts sialic acid, a molecule previously associated with complement-mediated serum resistance in *E. coli* (see section 3.2.1). Sialic acid (neuraminic acid), which is widespread in mammalian tissues, as cytidine 5′-monophosphate-*N*-acetyl neuraminic acid (CMP-NANA), can be added to the LOS by a sialyltransferase. This reaction is accompanied by a demonstrable change in the

LOS pattern and induction of serum resistance, presumably by inhibiting the attachment of both complement and bactericidal antibody to the organism [34]. Mandrell et al. [35] present experimental evidence supporting the idea that the gonococcus may effect this transfer of sialic acid to the LOS using its own sialyltransferase or they may perhaps borrow, as a "thieving magpie", the sialyltransferase that is present in neutrophils which are abundant during ureteral infection. Thus, phagocytes present at the site of infection increase the ability of gonococci to resist a major defense mechanism and permit their ability to maintain an infection. This phenomenon of alteration of LOS was shown to occur during infection of two human volunteers with a well-defined isolate of gonococcus (H. Schneider, pers. comm.). Whether this phenomenon occurs with other LOS or LPS-occurring strains, particularly common Gram-negative bacilli, is not known.

Neutrophils have also been shown to alter the toxicity of the LPS moiety of Gram-negative bacteria [36]. Munford and Hall have described an enzyme, acyloxyacyl hydrolase, within granules that detoxifies LPS by removing fatty acyl chains in the lipid A moiety of the LPS. This appeared to detoxify LPS 100-fold as measured by the dermal Shwartzman reaction. Since the ability of this detoxified LPS to stimulate B lymphocytes was reduced only 12-fold, however, they concluded that this may be a host defense-mechanism that reduces the toxic effects of LPS while preserving potentially beneficial effects on the immune system.

4. INTRACELLULAR KILLING

Upon ingestion of a pathogen into a phagosome, the phagocyte mobilizes its granules to fuse with the phagosome into a phagolysosome. It is here that the phagocyte is capable of killing the organism by both oxygen-independent and oxygen-dependent mechanisms. The metabolic burst is accompanied by increased oxygen consumption by the phagocyte, production of superoxide anion and a marked increase in the activity of the hexose monophosphate shunt. Superoxide is rapidly dismutated to hydrogen peroxide, whose toxicity is enhanced upon the myeloperoxidase-mediated conversion to the potent oxidant, hypochlorous acid. Chloramines and hydroxyl radicals are also formed from superoxide anions and are probably important microbicidal molecules. Recently, activated macrophages have been shown to synthesize nitrogen oxides from guanido nitrogens of arginine [37]. This mechanism may be important in the microbistatic activity of macrophages [38] and affect their function at sites of inflammation [39]. The importance of the oxygen-independent cidal mechanisms was also demonstrated by the ability of neutrophils to efficiently kill bacteria in an anaerobic environment [40].

4.1. Phagolysosomal events

One strategy employed by pathogens to escape killing is the prevention of phagosome-lysosome fusion. *Mycobacterium tuberculosis* can inhibit such fusion through the elaboration of strongly acidic sulfatides [41]. Similar abnormalities of lysosome–phagosome fusion have been demonstrated with *Toxoplasma gondii*, *Histoplasma capsulatum*, chlamydia, ehrlichia and legionella. Pathogen viability is necessary for this inhibition to occur, suggesting that the elaboration of as yet unidentified microbial molecules may prevent this fusion. Recently, a protein, considered to be part of the annexin family of proteins that bind to membrane phospholipids in the presence of calcium, was isolated in abundance in the cytosol of human neutrophils and considered to be an important mediator of phagolysosomal fusion [41a]. Such a protein might serve as an important target for modulation by products of microbial pathogens.

Alterations in the intraphagolysosomal pH may also affect the ability of phagocytes to kill intracellular organisms. Virulent *Nocardia asteroides* was shown to prevent the acidification of phagosomes, whereas a nonvirulent strain and *Saccharomyces* did not have this effect [42]. More recently, proteins derived from *M. tuberculosis* were shown to also prevent acidification [43]. The hydrolytic activity of lysosomal enzymes as well as the bactericidal capability of cationic granular peptides are dependent on an acidic pH. Some pathogens, like the amastigote stage of *Leishmania*

donovani or *L. amazonensis*, do not inhibit phagosome–lysosome fusion, but have adapted to the acid environment of this compartment such that they actually multiply [44]. *Coxiella burnetii*, the causative agent of Q fever, requires the low pH of the phagolysosome to multiply [33].

Perhaps the best studied example of adaptation to the phagosomal environment is that of Legionella infection, which in nature is found within protozoa such as amoebae. Such an environment may permit the *Legionella* to avoid being killed, and may explain the difficulty in eradicating the organism during water purification treatments. This organism cannot replicate within an animal host unless it is in an intracellular environment. Following adherence to the monocyte via the CR1 and CR3 complement receptors, *Legionella* enter the monocyte by a process of "coiling phagocytosis" around which mitochondria and ribosomes cluster [45]. This is a novel mechanism of engulfment by which the phagocytic pseudopod ingests live and killed, but not antibody-coated *L. pneumophila*. A surface component of this organism may mediate this unusual form of phagocytic ingestion. Live but not dead or avirulent *Legionella* prevent phagosome–lysosome fusion, and the organisms multiply within the phagosome where they are resistant to antibody, complement, levels of antimicrobial agents that are lethal in vitro, and to disinfectants.

Cell-mediated immune responses resulting in activated mononuclear cells are able to control *Legionella* infection and play a major role in host defenses. The induction of cellular immunity may be initiated by the elaboration of a secretory protein by the *Legionella* in both the phagosome and onto the surface of the phagocyte [47]. Activation of cells by gamma interferon decreases both bacterial entry and intracellular replication. The decreased entry may be due to the demonstrable down-regulation of the complement-receptors by which *Legionella* enter the cell, while the interferon-mediated decrease in intracellular replication may in part be due to the effect on iron metabolism. Cellular activation by interferon gamma down-regulates mononuclear phagocytic surface transferrin receptors by which the intracellular organisms obtain the iron necessary for their growth (neutrophils at the site of inflammation may even provide the transferrin) as well as the intracellular concentration of ferritin, which is the major intracellular store of iron within mononuclear phagocytes [48,49].

Some organisms, like those of the genus *Rickettsia* and *T. cruzi* [44] survive intracellularly by escaping from the phagolysosome into the cytoplasm. Recently, a mechanism by which a pathogen can evade killing inside the phagolysosome and then subvert the use of phagocyte structures for its own purposes has been demonstrated. *Listeria monocytogenes* that contain hemolysin have been shown to lyse the phagosomal membrane that surround it and escape into the cytoplasm. In contrast, bacteria lacking the hemolysin are killed and digested within that cellular organelle [50,51] Remarkably, the hemolysin-positive *Listeria* in the cytoplasm are then able to subvert the actin cytoskeletal protein of the phagocyte towards its own ends. Intracytoplasmic *Listeria* become surrounded by actin which orients (presumably at the direction of a listerial product) around the bacterium in a polar fashion, causing a "rocket" tail of actin to be formed. Thus propelled, the organism moves to the surface of the phagocyte, and surrounds itself in a finger-like projection from the cell. This bacteria-containing projection is in turn engulfed by an adjacent phagocyte where the bacteria then breaks out of this second phagosome into the cytoplasm. The process is repeated in this manner such that there is phagocyte-to-phagocyte spread of *Listeria* without exposure to serum elements [51]. Mononuclear phagocytes activated in vitro by interferon gamma appear to limit infection by *Listeria* by inhibiting their ability to escape from the phagolysosome [52].

4.2. Evasion of oxygen-dependent killing

Pathogens have developed methods for blunting the effectiveness of the respiratory burst. For example, virulent *Salmonella typhi* are better able to suppress the generation of superoxide, lessen oxygen uptake and decrease iodination of protein (a measure of phagocytic oxidative metabolism and myeloperoxidase activity) compared to an avirulent strain of *S. typhi* [53]. Microorganisms

such as *Toxoplasma gondii*, *Leishmania*, *Y. pestis* and *Histoplasma capsulatum* appear to avoid the toxic effects of superoxide by either failing to trigger its release [54] or inhibit its formation. The prior ingestion of *Histoplasma* also prevents the primed state of the phagocyte [55]. An acid phosphatase on the surface of *L. donovani* promastigotes has been shown to block the formation of superoxide anion by neutrophils following stimulation with the agonist, FMLP [56].

Bacteria can produce their own superoxide dismutase as well as catalase which destroys hydrogen peroxide and aborts the production of the myeloperoxidase-mediated generation of toxic halides; however, the role of these bacterial products in the pathogenesis of infection requires better definition. In the case of *Shigella flexneri* pathogenesis, experiments with bacterial mutants suggested the production of superoxide dismutase, but not catalase, may contribute importantly to virulence [57]. In addition, the growth phase of bacteria may be an important determinant of their ability to resist oxidative agents. Log-phase *L. monocytogenes* were better able to resist oxidative antibacterial agents than stationary phase bacteria. This was correlated with a 2.5-fold higher amount of bacteria-associated catalase activity in the log phase bacteria [58].

It is now appreciated that the generation of oxidants following the encounter between pathogen and phagocyte is a more complex event than originally realized. Bacteria may utilize oxygen for their own cellular processes, and the result of this competition may limit oxygen availability to the phagocyte [59]. Moreover, gonococci can also use host-derived lactate, the principal product of resting glucose metabolism by the neutrophil, to enhance its own rate of oxygen metabolism. Oxidants generated by the phagocytes are able to kill microorganisms. Bacteria are able to rapidly adapt (10- to 100-fold increase in synthesis within 10 min), however, by producing detoxifying enzymes and free-radical scavenging molecules, as well as DNA and protein repair systems in a manner similar to that demonstrable when bacteria are exposed to heat shock, low pH and glucose starvation. This interaction is reviewed in detail by Hassett and Cohen [60]. Interestingly, similar heat shock proteins are produced by bacteria in order to protect themselves from oxygen stress and by the phagocytosing macrophages [61,62]. The role of these heat shock proteins in macrophages is unclear, but they may protect the cell from oxidants that it generates to kill pathogens [60].

4.3. Oxygen-independent microbicidal systems

Once inside the phagolysosome, a pathogen is exposed not only to ROS but also to a variety of cationic proteins. Some of these proteins, like defensins [3,63] have a broad range of target specificity, while others, such as bactericidal/permeability-increasing protein (BPI) or the more recently described bactenecins (in bovine neutrophils), have a narrower spectrum of activity [4,64]. Susceptibility of some strains of Gram-negative bacilli to cationic proteins has been related to the composition of the LPS in their outer membrane: smooth strains of *Salmonella* were more resistant to killing in an anaerobic environment than were rough strains, and this was later shown to be dependent on the glycosyl groups of O antigens and the phosphoryl groups on the lipid A [5,65]. *Salmonellae* may harbor plasmids that determine the O antigen composition [32]. More recently, a locus on the *Salmonella* chromosome has been identified which controls the ability of these organisms to resist the bactericidal action of defensins [66]. Interestingly, this *phoP* locus is also associated with bacterial hypersensitivity to serum complement.

5. CYTOKINE-MEDIATED HOST DEFENSES

Cytokines are an essential means of communication within the immune system. They are produced by lymphocytes, macrophages and non-leukocytic cells such as endothelial cells and fibroblast, and, in turn, exert their effects on a similar array of leukocytic and non-leukocytic cells. They include the interleukins (IL1-IL8), tumor necrosis factor (TNF), interferons (IFN), colony stimulating factors (CSF), and cell growth factors. With the availability of recombinant cytokines and, in many instances, monoclonal antibodies directed against these molecules, their role in

host defenses is becoming more clearly defined. These studies are also aided by the availability of mouse strains which are blocked in their ability to produce cytokines. The role of cytokines, especially interferon gamma, in cell-mediated immunity has come under intense scrutiny. Several excellent reviews discuss the role of this and other cytokines in host defenses against parasites (e.g. *Leishmania*) and classical intracellular bacteria such as *Salmonella* and *Listeria* [68]. It has become clear that cytokines also play an important role in host defenses against bacteria that have often been thought to be controlled by humoral defenses (for review see [68,69]).

In keeping with our interest in the immune response to *E. coli*, we wished to examine the cytokine response to *E. coli* strains having different LPS and capsular phenotypes. Our experimental system involved a mouse strain known to be blocked in the LPS-induced production of cytokines, the C3H/HeJ mouse, alongside the cytokine-producing counterpart mouse, C3H/HeN. The results showed that as few as 10 K1-encapsulated, smooth LPS *E. coli* organisms represented the LD_{50} for the C3H/HeJ mice whilst for the C3H/HeN mice the LD_{50} was in the order of 10,000 [70]. Furthermore, the administration of exogenous TNF-alpha and IL-1 alpha to the C3H/HeJ mice protected them from lethal infection with up to 10–20 LD_{50}, while administration of these cytokines to the C3H/HeN mice resulted in a 2- to 3-fold enhancement in protection. We noted that the increased virulence of *E. coli* for the cytokine-blocked C3H/HeJ mice was strictly associated with the presence of certain capsule serotypes (e.g. K1 or K5) in conjunction with a smooth LPS. It was previously determined that the K1-encapsulated LPS smooth phenotype is a particularly resistant phenotype with regard to other aspects of the immune response, such as complement attack and neutrophil phagocytosis (see section, 3.2.1). Our results suggest that certain cytokines, such as TNF and IL-1, may have a crucial role to play in the immune response to serum-resistant *E. coli* phenotypes. We have recently extended these findings to the virulent K2 serotype of *Klebsiella*.

Bacterial clearance studies demonstrated that the K1-encapsulated, LPS smooth *E. coli* phenotype was cleared from the blood of both C3H/HeN and C3H/HeJ mice, however, in the C3H/HeJ mice there was a re-entry of bacteria into the bloodstream with the achievement of high-grade bacteremia. Electron micrographs of the Kupffer cells demonstrated the presence of *E. coli* organisms in the phagolysosomes. In the Kupffer cells of the LPS-responsive C3H/HeN mouse the bacteria were clearly in the process of being degraded; however, multiplication of bacteria was seen inside the phagolysosomes of Kupffer cells isolated from C3H/1HeJ mice. This would seem to tie in with the findings of Weinstein et al. [71] who correlated the increased sensitivity of C3H/HeJ mice to *Salmonella typhimurium* infections with a decreased ability of their macrophages to curtail bacterial multiplication. It is speculated that the IL-1 and TNF, in concert, facilitate the ability of phagocytic cells to more efficiently kill the bacteria in the phagolysosome. The mechanism for this cytokine-enhanced killing by phagocytes is not clear. Interleukin-1 has been shown to increase the clearance of bacteria from the blood [72], while both IL-1 and TNF have been reported to enhance oxidative kill [69,73]. The combined activity of these cytokines may be sufficient to initiate a chain of events that result in the killing of the organism.

Alongside our interest in the role of LPS and capsule in the sensitivity of *E. coli* to cytokine-mediated host defenses, we have been probing the role of these bacterial components in the induction of the macrophage cytokine response. Our experimental system involved testing a range of *E. coli* strains having different LPS and capsular phenotypes for their ability to induce the production of TNF in the RAW 264.7 murine macrophage cell line, a cell line known to produce cytokines in response to LPS [74]. We found that strains expressing the rough LPS phenotype were superior inducers of TNF, in terms of both the number of organisms required and the time taken to induce detectable TNF in the RAW cell supernate, compared with isogenic strains expressing the smooth LPS phenotype (Kelly, N.M., Young, L. and Cross, A.S., manuscript in prep.). This finding was extended to include rough and smooth LPS phenotypes of *Salmonella*. The superior ability to induce TNF was correlated with a far greater

tendency for rough LPS strains to shed their LPS when compared with the smooth LPS strains. LPS released from Gram-negative bacteria is known to be toxic for man, and therefore has been termed endotoxin. Since the discovery of TNF in the mid-1970's [75], followed by the availability of recombinant TNF and specific antibodies, numerous experiments have demonstrated that TNF is an essential intermediate in the lethal effects of LPS [76]. Like its inducer, LPS, TNF is a molecule having both beneficial and detrimental effects for its host. The working hypothesis is that the local production of TNF at an inflammatory site is beneficial for the host, by virtue of its potent induction of phagocytic bactericidal mechanisms [73], while the overproduction of TNF, leading to its presence in the circulation, is associated with detrimental effects. In the past, Gram-negative bacteria expressing the smooth LPS phenotype were considered to be better pathogens than their counterparts expressing the rough LPS phenotype primarily because of their ability to resist complement killing (see earlier sections of this review). From the point of view of the bacterium the animal host represents a niche which they wish to exploit, e.g. as a nutrient source, rather than to kill. We would like to suggest, therefore, that the decreased tendency towards LPS shedding by the LPS smooth bacteria, with the concomitant decrease in the induction of TNF, represents a selective advantage for this bacterial phenotype in terms of its interaction with the animal host.

As might be expected, microbes have been found to have mechanisms for evading cytokine-mediated defenses. In some instances, bacterial products might directly degrade cytokines as has been shown for alkaline protease of *P. aeruginosa* and gamma interferon [78]. In other instances organisms may develop a reduced sensitivity to the effects of cytokines on cells, as has been shown in the case of *R. prowazekii* [79].

6. EFFECT OF BACTERIAL PRODUCTS ON PHAGOCYTES

In an attempt to identify virulence factors from bacteria, exotoxins have been identified whose effects have long been a subject of study. Recently, the effects of these purified toxins on phagocytes have been studied in detail (see [79]) and many, such as pertussis toxin and botulinus C2 toxin, are now used routinely as probes to study signal transduction pathways. Krause and Lew found that nearly every step of neutrophil function could either be inhibited or mimicked by bacterial toxins [79]. Many bacteria, such as *S. aureus* and *P. aeruginosa*, produce leukocidins which are directly toxic to phagocytes. Others, by way of their proteases, destroy opsonins and cell surface receptors (see section 3.2.1). Another family of bacterial exotoxins alters cellular nucleotide metabolism by ADP-ribosylation of proteins (pertussis toxin [cysteine residues of G proteins], cholera toxin [arginine residues of G proteins], diphtheria [diphthamide] and botulinus C3 [GTP-binding protein] and C2 [G actin] [80], or through an invasive adenylate cyclase, causing an increase in $cAMP_i$ (*Bordetella pertussis*, *Bacillus anthracis*). Bacterial phospholipases, such as that from *P. aeruginosa*, generates arachidonic acid metabolites from phagocytes. These are potent inflammatory molecules [81]. Hemolysin from *E. coli* has been shown to induce leukotriene release from neutrophils with an enhancement in the rate of phagocytosis of bacteria [82]. It should be stressed, however, that until studies are performed in vivo using parental, wild-type strains and isogenic mutants lacking genes for production of these toxins, their role in pathogenesis of infections will be largely unknown.

Metabolic byproducts of bacterial infection may also affect phagocyte function. For example, Rotstein and colleagues have demonstrated that succinic acid, a metabolic byproduct of *Bacteroides*, inhibits a wide array of normal neutrophil functions [83].

7. CONCLUSION

Microorganisms have developed a vast array of mechanisms by which they can evade killing by phagocytes, and these can be rapidly called upon to meet sudden changes in their environment. A seeming extravagance of microbicidal mechanisms

has evolved in phagocytes; however, in dealing with the heterogeneity of microbial targets as well as the ability of each to rapidly adapt to stressful situations, phagocytes would appear to need every effector function they could muster. Immunotherapeutic strategies directed at the neutralization of bacterial factors, such as passive immunization with immunoglobulins, and directed toward the enhancement of phagocyte function, such as the use of adjuvants, are currently being evaluated. The development of a second generation of treatment modalities will depend on an even better appreciation of these microbial–phagocyte interactions.

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