THE ROLE OF NEUTROPHILS IN INVASIVE ASPERGILLOSIS

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Invasive aspergillosis is a disease of immune compromised hosts. Most commonly, disease follows inhalation of airborne conidia (spores), which germinate in the lung and then grow as hyphae (9). The increasing incidence of this disease over the past several decades has enhanced interest in understanding the mechanisms by which disease is prevented in the normal host and methods for immune modulation that can protect susceptible hosts (18). Neutrophils long have been considered a key cell population for host defense against *Aspergillus fumigatus*. Their relevance for protection against invasive aspergillosis was inferred in early descriptions of this disease because of its occurrence in neutropenic patients (27), as well as in those with defective neutrophil function, such as those with chronic granulomatous disease (6). The ability of neutrophils to contribute to damaging host responses also has been recognized in patients with immune reconstitution inflammatory syndromes and in animal models (1, 4, 25). Neutrophils have been viewed as primarily exerting their effector functions against hyphae, the form in which the organism grows in tissue, resulting in direct destruction, with additional damage caused by vascular invasion and resulting infarction. In this issue, Bonnett, et al., offer intriguing evidence that neutrophils also may have a novel effect on conidia during invasive pulmonary disease (3).

**Neutrophil Effector Functions.** Neutrophils function in the innate immune response through killing of microorganisms by both oxidative and non-oxidative mechanisms. Oxidative mechanisms involve the generation of reactive oxygen species by activated neutrophils via the multi-component phagocyte NADPH oxidase, which generates superoxide and myeloperoxidase, which catalyzes the conversion of hydrogen peroxide that forms from superoxide to hypochlorous acid and hydroxyl radicals (reviewed in (21)). Neutrophil granule proteins, including cationic peptides, comprise the non-oxidative mechanisms for direct microbial killing.
In addition to these two traditional microbicidal mechanisms, neutrophils more recently have been recognized as playing additional roles in the host response. Neutrophils produce a variety of chemokines and cytokines and some granule proteins are chemotactic not only for neutrophils, but for monocytes, immature dendritic cells and T cells, providing a role for neutrophils in stimulation of adaptive responses (reviewed in (5, 22)). Neutrophil serine proteases can also function both to activate and degrade cytokines, resulting in either enhancement or dampening of inflammatory responses (reviewed in (19)). Additional actions of these molecules include the induction of chemokine receptors in bronchial epithelial cells (26). Thus, neutrophils can influence the local milieu through actions both on epithelial and immune cells, with pro-inflammatory or regulatory effects.

**Neutrophil – A. fumigatus interactions: the prevailing wisdom.** The statement that control of infecting conidia in the lung is performed by alveolar macrophages, while neutrophils mediate hyphal killing once germination has occurred often appears in the literature. Studies published in the late 1970s to early 1990s, which sought to dissect the relative roles of macrophages and neutrophils *in vivo* and *in vitro*, form the basis for this belief. A key contribution to this perception was the work by Schaffner, *et al.*, which examined the pathogenicity of *A. fumigatus* in models using mice immunosuppressed in varying ways (24). Thus, nitrogen mustard-treated mice served as the model for neutropenic hosts, while cortisone acetate treatment was used to induce macrophage dysfunction. As germination is a two stage process, consisting of conidial swelling followed by the emergence of a germ tube from which hyphal growth ensues, experiments were performed both with resting and swollen conidia. Nitrogen mustard-treated mice could withstand disease when infected i.v. with resting conidia, supporting the view that macrophages are sufficient for host defense against conidia. Their
inability to prevent hyphal growth after infection with swollen conidia supports the importance of neutrophils for hyphal killing (24), though one could argue that these data may support a role for neutrophils in controlling the later stages of germination. In contrast, the enhanced susceptibility of cortisone acetate-treated mice to disease after infection with resting conidia establishes a role for macrophages in the initial steps of host defense (24). More limited experiments in an inhalation model found that nitrogen mustard-treated mice clear conidia at the same rate as control mice, while cortisone acetate administration results in impaired clearance and hyphae are observed in the lungs (24). Though few would argue the ongoing importance of this work, re-examination in light of subsequent investigations suggests that the conclusions of this study may have been over-generalized, as experiments were performed at least partly in models with low physiological relevance. A second limitation considered at the time is that these models are not truly selective for a single immune defect. Unfortunately, the problem of model remains one of significance.

In vitro studies of neutrophils and hyphae. The ability of human neutrophils to damage A. fumigatus hyphae by extracellular mechanisms in vitro was demonstrated in a landmark paper by Diamond, et al., which reported that neutrophils attach to hyphae, spread over their surfaces and degranulate (8). Exogenous opsonins are not required for this effect. Killing in this system is prevented by substances that inhibit neutrophil motility and by inhibitors of the myeloperoxidase-peroxide-halide system but not by cationic peptides (8). A. fumigatus hyphae are more resistant to killing by purified preparations of neutrophil cationic proteins or neutrophil lysates enriched for granule contents than are Rhizopus hyphae, underscoring the importance of oxidative mechanisms for hyphal killing of this particular filamentous fungus (7). Singlet
oxygen, but not hydroxyl radical, is directly toxic and, though superoxide dismutase is not needed for killing, the requirement for catalase is controversial (7, 23).

**Neutrophil-conidia interactions.** Early *in vitro* experiments demonstrated a requirement for heat-labile serum opsonins for neutrophil phagocytosis of conidia (10), exposing a potential limitation of these studies in their application to initial interactions in the lung. In systems using serum, phagocytosis by neutrophils does not impair viability of resting conidia, but killing of swollen conidia is significantly enhanced, despite comparable rates of phagocytosis (10, 13). Exposure to resting conidia stimulates the respiratory burst in murine and human neutrophils, but weakly, and degranulation in response to such exposure is minimal (2, 12, 13). Resting conidia are also significantly more resistant to damage from reactive oxygen species (11). Nonetheless, the myeloperoxidase-hydrogen peroxide-halide system can inhibit germination and kill conidia in cell free systems (11). A relatively more potent inhibitory effect of iodine-derived halides compared to those derived from chloride is seen as suggesting a potential reason for the lack of intracellular cytotoxicity, where chloride is the predominant anion (10). Thus, the absence of conidial killing in these systems is attributed to the combined lack of stimulation of neutrophil effector mechanisms and reduced susceptibility.

Nonetheless, the interaction of neutrophils with conidia may proceed differently in the presence of opsonins produced in the lung, such as surfactant proteins (SP). Though SP-A and SP-D do not induce conidial phagocytosis or neutrophil generation of reactive oxygen intermediates as efficiently as does serum, these opsonins may enhance neutrophil killing of conidia *in vitro* (14). Though beneficial effects of exogenous administration of SPs are seen in murine models (15), that such benefits result from enhanced neutrophil conidiocidal activity *in vivo* has not yet been demonstrated directly.
Neutrophil aggregates? In this issue, Bonnett, et al., report the very provocative finding that neutrophil aggregates form around conidia of Aspergillus fumigatus and provide data suggesting that neutrophils indeed participate in control of germination of the organism in vivo (3). In addition to countering the traditional view described above, extracellular inhibition of spore germination by neutrophil aggregates may represent a novel antimicrobial mechanism. Most bacterial spores are resistant to killing by neutrophils, despite phagocytosis. Notably, neutrophils induce Bacillus anthracis to germinate intracellularly and only then can neutrophils kill this bacterium (16). Similarly, alveolar macrophages are not considered to be effective killers of A. fumigatus conidia until swelling occurs intracellularly (20). In contrast, the present study suggests that at least some conidia are prevented from swelling within the aggregates and may even be killed, based upon loss of GFP fluorescence. As for hyphal killing, this germination-inhibitory activity of neutrophils on conidia requires the presence of intact oxidative defense mechanisms (3).

Why have germination-inhibitory neutrophil aggregates not been observed in previous studies? In the study by Schaffner, et al., discussed earlier, data regarding pathology are not presented and bronchoalveolar lavage samples were not studied (24). Different mouse strains were used from those in the present study, which may have relevance. Nonetheless, another major difference between the studies is that Schaffner, et al., used aerosol flasks that do not require suspension of the organism in tween-containing solution, while Bonnett, et al., administered the organism either intranasally or intratracheally, both routes requiring the organism to be in suspension. Though conidia were administered without tween, a relatively high concentration of tween was used to make the initial conidial suspensions. Though administration of tween, itself, in the concentrations used for most studies, induces negligible
neutrophil recruitment (17), we have shown that tween-containing solutions can alter the surface charge of the organism (25). Thus, different surface molecules may be exposed that may result in neutrophil recruitment in this model.

Another interesting finding of the present study relates to susceptibility differences between BALB/c and C57BL/6 mice, the latter strain being somewhat more susceptible to invasive disease (3, 25). Bonnett, et al., propose that this difference results from delayed neutrophil recruitment in C57BL/6 mice, such that germination can proceed. As acknowledged by the authors, this idea remains hypothesis and other explanations remain possible. However, if true, even in part, this result would highlight the value of developing preventive strategies whose goal is to inhibit germination, a key step in the pathogenesis of this organism.

Exactly how neutrophil reactive oxygen species prevent germination in this model is unknown, though data presented in the present study suggest a direct fungicidal effect. Whether or not this mechanism for control of germination occurs in human disease remains to be demonstrated. Nonetheless, the present work suggests that the notion that there is an absolute division of labor in the forms of the organism handled by different populations of innate immune effector cells represents oversimplification.
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