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GUEST COMMENTARY

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 exerting their effector functions primarily 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 the killing of microorganisms by both oxidative and nonoxidative mechanisms. Oxidative mechanisms involve the generation of reactive oxygen species by activated neutrophils via the multicomponent phagocyte NADPH oxidase, which generates superoxide and also myeloperoxidase, which catalyzes the conversion of hydrogen peroxide that forms from superoxide to hypochlorous acid and hydroxyl radicals (reviewed in reference 21). Neutrophil granule proteins, including cationic peptides, comprise the nonoxidative mechanisms for direct microbial killing. In addition to having 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 the stimulation of adaptive responses (reviewed in references 5 and 22). Neutrophil serine proteases can also function to both activate and degrade cytokines, resulting in either enhancement or dampening of inflammatory responses (reviewed in reference 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 on both epithelial and immune cells, with proinflammatory or regulatory effects.

Neutrophil-*A. fumigatus* interactions: the prevailing wisdom. The statement that the 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 during the period from 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. that examined the pathogenicity of *A. fumigatus* in models using mice immunosuppressed in various 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 with both resting and swollen conidia. Nitrogen mustard-treated mice could withstand disease when infected intravenously 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 with 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, reexamination in light of subsequent investigations suggests that the conclusions of this study may have been overgeneralized, as experiments were performed at least partly with models having low physiological relevance. A second limitation considered at the time is that these models are not truly selective for a single immune defect.

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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-conidium 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 the viability of resting conidia, but the 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). An inhibitory effect of iodine-derived halides that is relatively more potent than the effect of halides derived from chloride suggests 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 the 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 the loss of green fluorescent protein 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). The mouse strains used differed from those in the present study, which may be of relevance. 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 the administration of Tween, in 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 that may result in neutrophil recruitment in this model may be exposed.

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 (3). As acknowledged by the authors, this idea remains a 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 an oversimplification.

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