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The alveolar macrophage: the Trojan horse of *Bacillus anthracis*

Chantal Guidi-Rontani

***Bacillus anthracis*, the causative agent of anthrax, has a particular strategy for invading the host and crossing the alveolar barrier. *B. anthracis* survives within alveolar macrophages, after germination within the phagolysosome, then enters the external medium where it proliferates. Recent data have shown that edema toxin and lethal toxin are the major genetic determinants mediating the survival of germinated spores within macrophages. Here, recent advances in the analysis of *B. anthracis* pathogenesis are summarized and future challenges discussed.**

Chantal Guidi-Rontani
Laboratoire Organismes Photosynthetiques et Environnement, Ecole Normale Superieure, 46 rue d'Ulm, 75005 Paris/CNRS URA2172, France.
e-mail: rontani@biologie.ens.fr

Bacillus anthracis is an endospore-forming [1], aerobic, rod-shaped bacterium. It colonizes its host using a repertoire of virulence determinants that cause bacteremia [2] and toxemia [3,4], and the result is systemic anthrax [5]. The germination of spores and the emergence of vegetative bacilli, in an environment allowing rapid out-growth into the host body, are essential in the early stages of pathogenesis to establish infection. Although anthrax is a well-known disease and was one of the first to be described and linked to its causative organism [1], our understanding of the cellular and molecular interactions between *B. anthracis* and the cells of the host immune system is far from complete, and many important questions remain. For many years, anthrax has been out of the spotlight but has recently been the focus of much attention owing to the emergence of the significant threat of *B. anthracis* being used as a potential agent of bioterrorism [6].

Macrophages as partners of *B. anthracis*

In its vegetative form, *B. anthracis* can survive as an extracellular pathogen and, owing to its anti-phagocytic poly-γ-D-glutamic acid capsule [7–10] and adenylate cyclase activity [11], can avoid ingestion by host phagocytes. However, *in vivo* experiments have demonstrated that, once inhaled, anthrax spores can reach the bronchioles and alveoli of the lung, and most spores are then rapidly and efficiently phagocytosed by alveolar macrophages via recruitment of F-actin [12–14].

In 1999, an article, highlighted by *Trends in Microbiology*, partially revealed the process that follows the phagocytosis of spores [14,15]. It described a novel strategy that *B. anthracis* uses to subvert a host immune cell to its advantage: the efficient germination of *B. anthracis* spores within the phagosomal compartment of bronchoalveolar macrophages following infection by inhalation (Fig. 1) [14]. Moreover, a germination operon (*gerX*) was associated with the germination of *B. anthracis* within macrophages and was shown to encode

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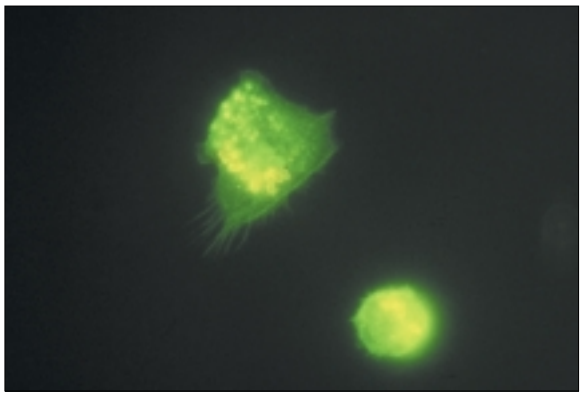

Fig. 1. Germination of *Bacillus anthracis* inside alveolar macrophages. Infected macrophages were detected by staining F-actin with oregon green-488 phalloidin (green). Germinated *B. anthracis* spores were stained with an antibacillus serum and a rhodamine-conjugated secondary antibody (red; co-localization of the two stains shows as yellow).

virulence factors that contribute to the pathogenesis of this microorganism [16]. Indeed, the *gerX*-encoded proteins might be involved in the detection of specific germinants within macrophages. We proposed that variation in the efficiency of germination was responsible for the differences in the susceptibility of different animal species to infection by *B. anthracis*.

Various methods of exposure can lead to infection, including intradermal inoculation, ingestion or inhalation of spores [17–19]. The inhalation form of anthrax is the most severe, is associated with rapid progression of the disease and can often be fatal [20–22]. Several lines of evidence indicate that the germination rate has a crucial effect on the outcome of the infection [16]; germination rates can depend on the distribution of various constituents in different tissues. It is clear that to establish disease the vegetative form must emerge rapidly in a niche favorable for survival – *B. anthracis* germinates and multiplies efficiently in the bloodstream. We proposed that in the early stages of pathogenesis, the mechanisms by which *B. anthracis* disseminates from the primary site of infection, concomitant with the germination process, are crucial to the outcome of the infection. Is there a macrophage-cell-dependent molecular mechanism(s) that fits this hypothesis?

'...in the early stages of pathogenesis, the mechanisms by which *B. anthracis* disseminates from the primary site of infection...are crucial to the outcome of the infection.'

The macrophage is a highly versatile and specialized cell type with an impressive repertoire of functions that are expressed according to tissue location and activation status [23,24]. Thus, the fate of *B. anthracis* inside the macrophage can vary greatly, depending upon the host species, anatomical location and state of activation and differentiation. The fact that the outcome of infection can vary with the different routes of infection suggests that the host cells comprising the mucosal barriers might be involved. For any pathogen to cause an infection, it must first circumvent the various host permeability barriers, which include the skin, and the mucosae of the respiratory, gastrointestinal and genitourinary tracts, and which block the entry of virtually all macromolecules and bacteria into the body. The fatal nature of the inhalation form of anthrax indicates that entry through the mucosae of the respiratory tract is a favorable route of infection for *B. anthracis*.

Alveolar macrophages play a central role in the cell-mediated immune response and act as guard cells, clearing invading bacteria from the lung alveoli. *In vitro* experiments have shown that the survival of germinated spores within macrophages is associated with a loss of macrophage cell viability [25]. Recent data lead to conflicting conclusions about the multiplication of *B. anthracis* within macrophages [25–30]. It should be noted that it is difficult to establish whether a microorganism replicates intracellularly *in vitro* and *in vivo* as macrophages are capable of efficient phagocytosis. Part of the disagreement could result from the experimental methods used. Indeed, experiments excluding phagocytosis of germinated *B. anthracis* from the culture medium (involving washing or replacement with medium containing antibiotic) did not detect the multiplication of *B. anthracis* within macrophages [25]. These observations imply that the alveolar macrophages, cells which have an important role in the body's first-line defenses against pathogens, are key cells in *B. anthracis* pathogenesis during the early phase of infection.

Toxins as crucial determinants during the early phase of infection

The expression of the toxin genes closely follows germination within alveolar macrophages [14]. Indeed, image cytometry and expression technology using sensitive fluorescence-based reporter systems have demonstrated rapid onset of the expression of genes encoding virulence factors such as lethal factor (LF), protective antigen (PA), edema factor (EF) and the toxin *trans*-activator, AtxA [14]. Moreover, *in vitro* experiments show that the survival of germinated spores and the loss of macrophage cell viability were associated with the expression of toxins [25]. The lysis of macrophages harboring germinated spores occurs as a result of a cooperative toxin interaction involving edema toxin (EdTx; comprises PA and EF), a calmodulin-dependent adenylate cyclase, and lethal toxin (LeTx; comprises PA and LF), a zinc-dependent protease that cleaves mitogen-activated protein kinase kinase (MAPKK) [25]. Thus, the *B. anthracis* toxins presumably play a key role during early intracellular infection.

The molecular mechanisms involved in LeTx-mediated cytotoxicity are unknown, and the way in which host macrophages are disrupted by intracellular intoxication with LeTx has not been elucidated. Although macrophage-specific cytolysis is

not well understood, several hints led us to hypothesize that the effects of LeTx are a consequence of the accumulation, overloading and diffusion of toxic effectors. Indeed, the modulation of the oxidative burst by LeTx has been described [26] (and is perhaps influenced by the MAPKK cleavage activity attributed to LF [27]), resulting in production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). ROS and RNI can be toxic to the producing cell if they gain access to the cytosol following their overproduction [28]. This possibility received some support with the identification of a protein belonging to the family of transport motor kinesin-like proteins, Kif1C, as a candidate protein involved in determining the susceptibility of mouse macrophages to LeTx [29]. It is therefore likely that the survival of germinated spores in such an environment is a consequence of the presence of virulence factors that contribute to the quenching of free radicals.

It has been proposed that PA is involved in the delivery of EF and LF to the cytosol, allowing them to bypass the phagosomal membrane [25]. Transmission electron microscopy reveals a tight interaction between the exosporium, the outermost integument of the spore, and the phagolysosomal membrane [25]. Disruption of the phagolysosomal vacuole and release of nascent bacilli into the cytosol has been described [C. Guidi-Rontani (2001) 4th International Conference on Anthrax, Annapolis, MD, USA]. Therefore, the germinated spore might express virulence factors with membrane-damaging activity. The disruption of the phagolysosome membrane would allow nascent bacilli to move from the phagolysosome into the macrophage cytoplasm. This suggests that the mature vegetative form can escape and emerge from alveolar macrophages into site(s), such as the circulation, where it can multiply rapidly. Encapsulated vegetative cells have not been detected following germination within macrophages (C. Guidi-Rontani, unpublished) but they do appear within 30 min of germination *in vitro* [J.W. Ezzell and T.G. Abshire (1995) 3rd International Workshop on Anthrax, Winchester, England); these results led us to hypothesize that the function of the capsule is mainly to facilitate systemic invasion and dissemination within the bloodstream.

To exploit its alveolar niche, *B. anthracis* must use and adapt the alveolar macrophage appropriately. Alveolar macrophages, which are found within the lumen of alveoli, are strategically localized to filter foreign material. They have an unique position within the body, being in intimate contact with both air- and blood-borne materials, and are responsible for clearance of the lungs during inhalation. It is clear that *B. anthracis* has to co-opt the alveolar macrophage migration machinery to cross the bilayer of the mucosal barrier and gain access to lymph nodes. Interestingly, bronchoalveolar lavage has shown that *B. anthracis* spores cause a significant increase in lung permeability and strongly suggested infiltration of the site by inflammatory neutrophils (C. Guidi-Rontani, unpublished). The contribution of dendritic cells, the lung's other phagocytes, to the migration of spores across the mucosal barrier must be taken into account and addressed in the future.

Transmigration, a double-edged sword

Leukocyte extravasation from the blood across the endothelium might be mediated by the toxins causing dysregulation of the immune response. Interestingly, EdTx seems to influence the production of cytokines by human monocytes: the increased synthesis of intracellular cAMP induces the production of the pro-inflammatory cytokine interleukin (IL)-6 [31]. EdTx stimulates chemotaxis of human polymorphonuclear neutrophils [32] and, as a secreted toxin, it can exert its effects at sites distant from the site of infection. EdTx stimulation can interfere with the normal modulation of chemotaxis by disrupting the chemoattractant gradient elicited during infection. This could interfere with leukocyte recruitment at the site of acute inflammation. EdTx increases host susceptibility to infection by suppressing polymorphonuclear neutrophil function and thereby impairing host resistance. Could the macrophage-derived free radical gas, nitric oxide (NO), a mediator of the vascular and inflammatory response, be involved? Indeed, sub-lytic doses of LF reduce the release of NO induced by lipopolysaccharide (LPS)/interferon γ (IFN-γ) [33]. Possibly, the reduction in the release of NO might contribute to the early phase of infection by preventing microvasculature: as the release of NO is inhibited, the vessel cannot dilate, inhibiting the reduction of blood velocity and so interfering with the dynamics of lymphocyte attachment to the endothelium – tethering and rolling.

The clinical correlate of this finding is that, following inhalation of *B. anthracis*, bronchoalveolar macrophages initially produce pro-inflammatory cytokines but, subsequently, produce cytokines, including IL- 10, that downregulate the release of tumor necrosis factor (TNF)-α and interleukin (IL)-1b. Interestingly, sub-lytic concentrations of LeTx, orders of magnitude lower than those required to induce lysis of macrophages, induce these cells to express IL-1b and TNF-α *in vitro* [34]. By contrast, Pellizzari and co-workers and Erwin and co-workers observed that LeTx inhibited the production of cytokines induced by LPS, suggesting that LF might suppress rather than induce cytokine production in macrophages [33–35]. As macrophages are very heterogeneous, it is plausible that the dysregulation of cytokine networks in macrophages, resulting from the actions of LeTx, varies greatly. Death from anthrax might require the dysregulation of cytokine networks in macrophages mediated by pro-inflammatory cytokines, such as IL-1b and TNF-α, produced by macrophages after stimulation by LeTx. Thus, the disrupting effects of LeTx on specialized cells can be highly diverse, depending upon the species, the anatomical location (and associated host factors such as surfactant and

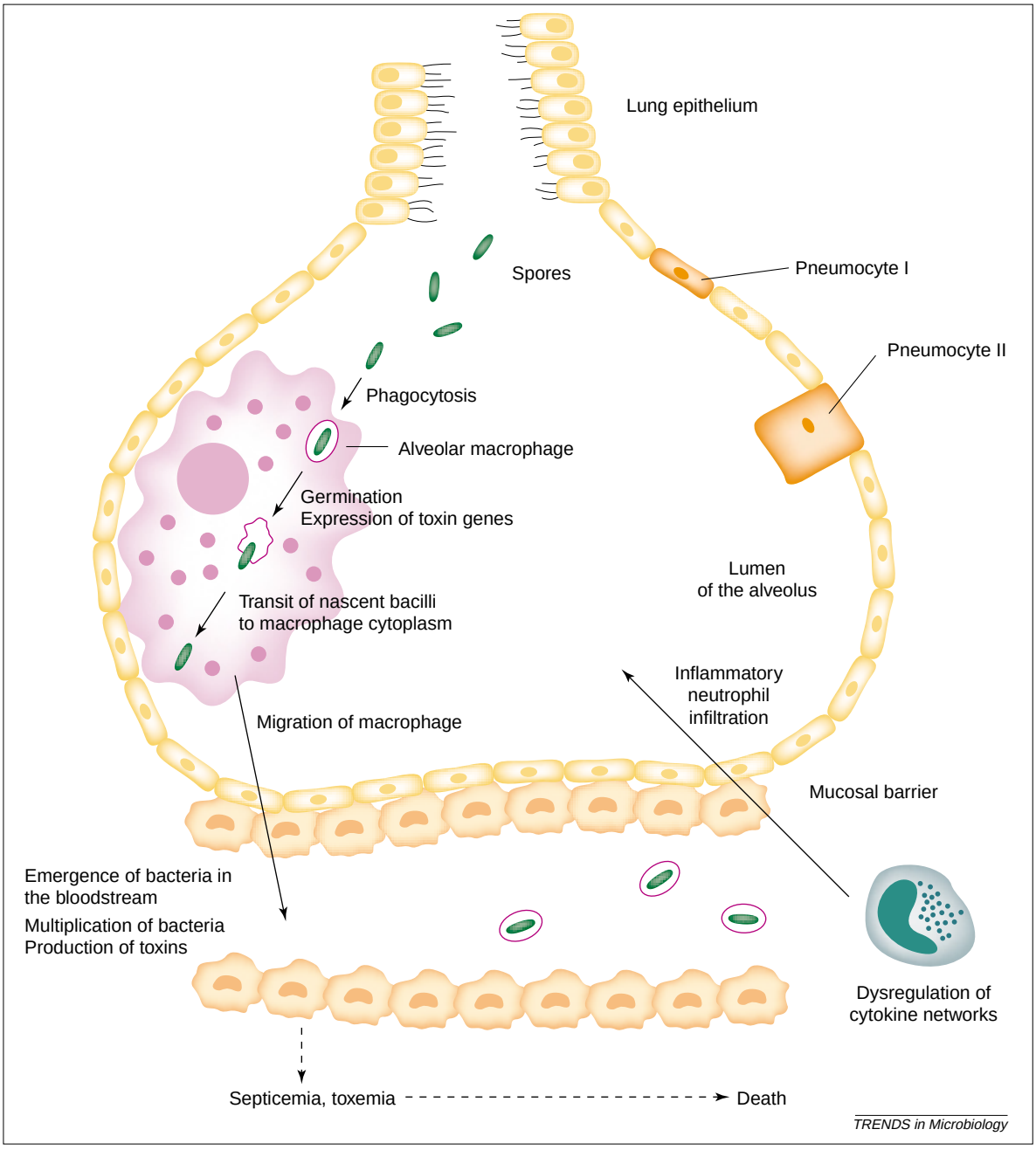

Fig. 2. The molecular and cellular mechanisms involved in the early phase of *Bacillus anthracis* infection. *B. anthracis* spores are shown reaching the lumen of the alveolus. Once they reach the phagolysosome, after phagocytosis by alveolar macrophages, the spores germinate and produce toxins [edema toxin (EdTx) and lethal toxin (LeTx)]. Disruption of the phagolysosome membrane (promoted by virulence factors with membrane-damaging activities) would allow the transit of nascent bacilli into the macrophage cytoplasm. Bacteria that have escaped from the macrophage phagosome are carried from the primary site of infection by the migrating macrophage. The survival of nascent bacteria is associated with a loss of macrophage cell viability. Once the bacteria emerge in the bloodstream, they can multiply. As the infection progresses, bacteria spread through the blood and lymph causing the bacillemia and the toxemia characteristics of fatal anthrax infection.

plasma in the fluid environment), and the states of activation and differentiation. For example, resident peritoneal macrophages in mice, in contrast to bronchoalveolar macrophages, do not inhibit or kill *Cryptococcus neoformans* [36].

Modulation of cytokine expression is a complex phenomenon involving a multiplicity of substrates, each belonging to complex and unresolved pathways that have apparently redundant and interacting signal-transfer functions. The mechanism of action of LeTx might be dependent on the concentrations of the various signaling components (threshold stimuli required to trigger a response), their accessibility to each other (compartmentalization and/or simple diffusion), and the kinetics of the control of (kinase) activation. Different types of events can occur simultaneously in tissue or cell cultures exposed to the same initial insult, and they depend on physiological specialization. Possibly, a downstream controller is required to direct cells towards necrosis. An over-vigorous inflammatory response can lead to host tissue destruction, whereas a weak response can result in failure to control infection. It is possible that monocytes harboring germinated spores can re-enter the lymphatic system. It should be noted that a large overall decrease in the number of spores in bronchial alveolar fluids was observed as soon as 24 h following mice intranasal infection [14]. Moreover, no dormant spores were detected in bronchial alveolar fluids 5 d after mice intranasal infection (C. Guidi-Rontani, unpublished). These data strongly suggest ungerminated spores do not persist in the lungs.

There is evidence in favor of a model in which germinated spores are transported from the lumenal surface of the bronchiolar epithelium across the epithelium via the intercellular space to the basement region by macrophages, thus allowing vegetative cells to be delivered to the bloodstream.

Conclusions and future directions

The dialogue between macrophages and LeTx is central to the pathogenesis of *B. anthracis* (Fig. 2). In any bacterial disease, the pathogen must first circumvent the host permeability barrier. Although *B. anthracis* is not an intracellular pathogen, it can use alveolar macrophages as a sanctuary during the early phase of infection both to get started and to cross the host permeability barriers. For the disease to become established, the spore-bearing macrophages must allow the spores to germinate, and the bacilli must bypass the barrier formed by the alveolar epithelium to gain access to the bloodstream. They migrate along the lymphatic channels to reach the regional lymph nodes and the local mediastinal lymph nodes. As infection progresses, toxins are synthesized and this is associated with cellular disruption. The bacteria then emerge in the bloodstream causing the bacillemia and toxemia that are characteristic of fatal anthrax infection. There are associated complications including septic shock, respiratory distress and multi-organ failure; the lung serves only as a point of invasion.

'These data strongly suggest ungerminated spores do not persist in the lungs.'

Cultures of tissue-specific cell types are of particular interest as model systems to elucidate the mechanisms responsible for LeTx intoxication. Validation of the transmigration process and identification of the molecular mechanisms could reveal possibilities for new therapeutic and preventative strategies, leading to the design of drugs to inactivate *B. anthracis* targets essential for the early stages of infection. There is no doubt that tissue-culture models and exploiting developments in high-resolution visualization techniques, such as confocal microscopy and image reconstruction, are likely to make valuable contributions to clarifying the complexities of the relationships between the intrinsic functions of host resident macrophages and *B. anthracis*. An important goal for anthrax research is to identify the germinant specifically responsible for germination within macrophages.

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