5. Discussion

5.1. In vitro data

5.1.1. Neutrophil / spore interaction

In the present study we found that human neutrophils engulf *B. anthracis* spores and induce them to germinate. A major concern during this study was that the spores had germinated outside the neutrophils and that the cells had phagocytosed vegetative bacteria. This was difficult to determine experimentally, as it proved impossible to distinguishing intracellular spores from germinated spores with heat treatment (60°C, 45 min). This treatment kills vegetative bacteria while spores survive. The reason could be that the neutrophils appear to kill any newly germinated spore in a very short period of time. Interestingly, the engulfed *B. anthracis* spores began to germinate within the neutrophils after approximately 90 min. This was the first time point where germinated cells were found in the transmission EM pictures. In the prior two time points (30 and 60 min) only spores were observed inside the neutrophils. The fact that the first vegetative cells were seen inside the cells only after a 90 min infection period proved a good control that the germination process actually took place inside the neutrophils.

As seen in the EM pictures, the *B. anthracis* spores were found in membrane bound vacuoles, most probably phagosomes. In neutrophils, phagosomes lyse with the neutrophil granules resulting in the formation of phagolysosomes. The structures encompass the antimicrobially active proteins and peptides, the reactive oxygen species produced by the NADPH oxidase, some extracellular components taken up during phagocytosis and plasma proteins from the secretory vesicles (Borregaard and Cowland, 1997). What triggers intracellular germination is unclear. The germination process clearly takes place inside the phagolysosome (see EM pictures), indicating that cytosolic components are not relevant. The hNGE approximately mirrors the components found in the neutrophil phagolysosomes, yet incubation of the spores with the human neutrophil granule extract (hNGE) did not result in spore germination. There are bound to be differences between the phagolysosomal milieu and the hNGE. Firstly, the isolation of the neutrophil granules during the preparation is certain to be contaminated with some cytosolic and nuclear elements. More importantly, the neutrophil granule extract lacks components of the granule contents, which cannot be extracted by acid (e.g. anionic proteins and peptides). Also, ROS

are not present in the hNGE. They theoretically could have an impact on spore germination, but this is unlikely as all known natural germinants are nutrients (Setlow, 2003). Further the pH of the granule extract and the phagolysosome are different. The granule extract was buffered to a pH of 4, the phagolysosomal pH varies between pH 8 and pH 2 depending on stage of phagocytosis (Segal et al., 1981).

Why *B. anthracis* spores germinate at a later time point in neutrophils in comparison to macrophages is unknown. Germination of spores in macrophages has been described after 30 min (Guidi-Rontani et al., 1999b). Intriguingly, macrophage associated germination was not seen in the non-pathogenic *B. subtilis* and *B. megaterium* species, implying that the pathogen *B. anthracis* has evolved specialized abilities to sense its entry into the host and initiate vegetative growth (Ireland and Hanna, 2002). The interaction of these non-pathogens with neutrophils has not been studied.

The germinant responsible for the germination in both neutrophils and macrophages has not been identified. As in neutrophils, the macrophage phagosomes entail extracellular components, but significantly less antimicrobial proteins, reactive oxygen species and large amounts of reactive nitrogen species. Whether the diverse phagosomal contents of the two immune cells is responsible for the different germination induction needs to be examined.

As described, electron microscopic observations showed intracellular spores alongside germinated spores throughout the infection period. The reason for this unsynchronised germination process is not known, but has also been described in macrophages (Guidi-Rontani *et al.*, 2001). Possible explanations are that some spores remain unresponsive to cell specific germinants, or that the neutrophils life span in the tissue is too short to induce complete germination. But as the life span of macrophages in tissue (approximately 2 weeks) is much longer than that of neutrophils (a few hours), and yet the same phenomenon is seen, the second explanation seems unlikely.

Interestingly, spores can remain dormant in vivo for up to 60 days (Friedlander *et al.*, 1993). This phenomenon could explain why only 70% of the spores where killed by the neutrophils independent of the infection period. This could also explain why the eschar resolution takes more than 6 weeks (Tutrone *et al.*, 2002). Potentially the ‘germination-resistant’ spores keep the infectious and inflammatory process alive. This obviously does not pose a problem in vivo as cutaneous anthrax is usually

contained. Neutrophils surround the centre of the eschar necrotic area, similar to abscess formation, and destroy the bacteria bit by bit. Dead neutrophils are constantly replaced by new neutrophils from the blood.

Jadamus *et al.* (2000) described that *Bacillus cereus* spores taken up orally by chicken and piglets, can germinate within the gut but also resporulate inside the intestine. The question is if this could explain that spores were found intracellularly throughout the infection period. *Bacillus cereus* is a good non-lethal model, as some believe that a plasmidless *B. anthracis* strain is indistinguishable from *B. cereus* (Rasko et al., 2005). Yet, the taxonomic separation of *B. anthracis* and *B. cereus* is still a cause of controversy and distinguishing these species is rather difficult even with molecular tools. The markers generally used for distinguishing *B. cereus* from *B. anthracis* are all encoded on the two virulence plasmids of *B. anthracis* (Turnbull, 1999).

The phenomenon described by Jadamus et al. (2000) is unlikely to explain our data as the resporulation in the gut seems to be induced by an increase in bacterial metabolites after bacterial multiplication (Jadamus et al., 2001). Inside the cells there was never any sign of bacterial growth. Also, the germinated bacteria were killed very quickly by the neutrophils, as a) in none of the EM picture long rod-shaped bacteria were found, implying there was no time for growth and b) heat treatment (45 min, 60°C) of neutrophils infected with spores never differentiated between a spore and a vegetative bacteria population. This indicates that the vegetative cells were killed within 45 min and judging by the killing assays performed with neutrophils and vegetative bacteria at an MOI of 1:1 probably much earlier.

Inside macrophages this phenomenon is also not likely to occur. Macrophages do not kill vegetative *B. anthracis* as efficiently as neutrophils (discussed below), they escape the macrophage phagosome and survive in the cytoplasm (Dixon et al., 1999). This environment is a nutrient pool, making resporulation irrelevant. Whether the vegetative bacteria multiply within the macrophages is still a matter of debate (Dixon et al., 2000, Guidi-Rontani et al., 1999b).

5.1.2. Neutrophil / vegetative bacteria interaction

5.1.2.1. Effect of anthrax toxin

From a pathophysiological viewpoint, *B. anthracis* is expected to use some strategy to avoid being detected and destroyed by the innate immune system. This is likely as

B. anthracis, at least during a pulmonary infection, can spread throughout the body in a very short period of time. The dogma in the anthrax field is that the toxin and the capsule expressed by the vegetative bacteria have antiphagocytic properties. O'Brien et al. (1985) showed that when neutrophils were pre-treated with the anthrax toxin for 60 min phagocytosis of vegetative bacteria was inhibited. The concentration of toxin used during this study seems very high (1µg/ml), as the LC_{50} of lethal toxin inducing cell death in macrophages is 30 ng (Mohamed et al., 2004). It is difficult to imagine such high concentrations in vivo over long periods of time. In the same study, when neutrophils were exposed to the toxin and the bacteria at the same time, the anthrax toxin did not inhibit phagocytosis. These data are in agreement with our observations, as the toxin did not affect the killing capacity of the neutrophils. To the best of our knowledge further studies examining the effect of the anthrax toxin on neutrophil phagocytic or killing activity have not been published.

The effect of the anthrax toxin on the reactive oxygen species production in neutrophils has been examined. Two in vitro studies postulated that the oedema toxin (O'Brien et al., 1985) and the lethal toxin (Wright and Mandell, 1986) reduce the levels of ROS production released after LPS priming. Alexeyev *et al.* (1994) examined neutrophils from human cutaneous anthrax cases from the Sverdlovsk incidence and found that the patient cells produced less ROS in comparison to healthy donors. We clearly showed that the neutrophil killing mechanism of both the spores and the vegetative bacteria is independent of ROS production, so the relevance of reduced ROS for killing activity during infection is questionable. The role ROS play in bacterial killing is controversial. It is not clear if reactive oxygen species are produced in high enough concentrations in neutrophils to kill bacteria (Roos and Winterbourn, 2002), or if they play a role in the activation and release of granule proteins from their highly organized intra-granular structure (Reeves et al., 2002). Most bacteria respond to oxygen independent killing mechanisms, yet some Gram-positive bacteria, such as *Staphylococcus aureus* are no longer killed by neutrophils once the NADPH oxidase is inhibited (Kristian et al., 2003).

5.1.2.2. Effect of anthrax capsule

The in vivo role of the capsule as a virulence factor has been clearly shown. *B. anthracis* strains lacking the capsule encoding plasmid or deletion mutants of the *cap* genes are avirulent in most animals or highly attenuated in mice (Welkos, 1991),

(Drysdale et al., 2005). Yet the direct interaction of the capsule with host cells is not well understood and is often inferred from studying other capsulated microorganisms (Makino et al., 1989). Smith *et al. (1955)* examined the interaction of 'young' and 'old' *B. anthracis* organisms from infected guinea pigs expressing different capsule thickness. When the capsule was better developed the rate of phagocytosis through leucocytes was decreased. Human leucocytes also could not phagocytose Pasteur strain *B. anthracis* as efficiently as Sterne strain bacteria (Makino et al., 1989). As in the above study, neutrophils were not isolated from other leucocytes and the experiment was done with unactivated and unprimed host cells and the bacteria were not opsonized.

Dep is a depolymerase which catalyses the hydrolysis of poly –D-glutamic acid (sole capsule component) into lower molecular weight polyglutamates. *Dep* null mutants are avirulent in mouse infections and therefore *dep* is essential for the virulence of *B. anthracis* in mice. During this study (Makino et al., 2002), the phagocytosis of the mutant strain and a fully virulent parent strain was examined in macrophages. Dep mutants were phagocytosed more efficiently than the parent strain by macrophages, but notably the parent strain was phagocytosed.

Taking these publications into account, the direct interaction of capsulated *B. anthracis* with isolated human neutrophils has never been conclusively studied and documented. Our data clearly show that the capsule producing Pasteur strain is killed by human neutrophils.

Due to bio-safety concerns we decided not to work with a fully virulent *B. anthracis* strain. Although the use of the Pasteur and the Sterne strain is an elegant system for studying the role of both major virulence factors this decision has its drawbacks. The most critical and far-reaching regulator is *atxA*, a pXO1 gene that appears to be unique to *B. anthracis*. a*txA* deletion mutants exhibit no toxin production and reduced capsule synthesis (Koehler, 2002). This means that the Pasteur strain under the right growth conditions can express the poly-D-glutamic acid capsule, but the capsule of the wild type strain is thicker (Uchida et al., 1997). This could have an effect on the efficiency with which the wild type is phagocytosed and killed.

5.1.2.3. Comparison of intracellular and extracellular killing

The cytochalasin D treated neutrophils, when activated with IL-8, kill 40% of the toxin-producers and 30% of the capsule-producers intracellularly. The capsule does have an effect on the efficiency of neutrophil killing, which could be explained by reduced phagocytosis. This is consistent with the different neutrophil killing mechanisms. Neutrophils phagocytose microbes, the phagosome fuses with the neutrophile granules and the antimicrobially active contents of the granules exerts its effect (Burg and Pillinger, 2001). The granules of activated neutrophils can also fuse with the cell plasma membrane and exocytose their granule contents into the extracellular space, where bacteria are then killed (Faurschou et al., 2002). Brinkmann *et al* (2004) showed that neutrophils also release extracellular fibers, the neutrophil extracellular traps (NETs). They are composed of DNA, histones and granule proteins, which can kill bacteria extracellularly. An interesting aspect could be that during *B. anthracis* infection, when sufficient neutrophils are recruited to the site of infection, the bacteria are trapped in these NETs, prevented from further spreading and killed before the bacteria are engulfed by the neutrophils. To date defensins have not been described as constituents of the NETs, but other azurophil granule proteins such as neutrophil elastase and cathepsin G have (Brinkmann personal communications).

5.1.2.4. Comparison of neutrophil and macrophage killing activity

Macrophages do not kill vegetative *B. anthracis* as efficiently as neutrophils. Instead they escape the macrophage phagosome and survive in the cytoplasm (Dixon et al., 1999) and through the anthrax toxin production ultimately induce macrophage cell death (Popov et al., 2002b). Guidi-Rontani et al. (2001) showed that after a three-hour incubation period the macrophage cell line RawB only killed approximately 10% of the Sterne spores (MOI 1:1). Dixon et al. (2000) on the other hand showed 80% killing after 4 hours of Sterne strain spores by RawB macrophages at an MOI of 1:1. In this case a gentamycin protection assay was used and Dixon et al explained the high killing rate by an increased macrophage cell membrane permeability and subsequent entry of the antibiotic into the cell, which resulted in germinated bacteria killing.

Macrophages are the differentiated form of monocytes. These cells are most closely related to neutrophils. They too are professional phagocytes, they express reactive

oxygen species and reactive nitrogen species and they contain granules (Forman and Torres, 2001). Importantly, phagocytes of the monocytic lineage tend to lose the antimicrobially active factors (defensins, elastase, cathepsinG), which are packaged in granules in the course of differentiation. This could explain the different killing efficiencies of the two cell types. We showed that *B. anthracis* killing was independent of reactive oxygen species but dependent on the contents of the neutrophil granules. As macrophages do not have any defensins and lack many other antimicrobial proteins, this could explain why they cannot cope with a *B. anthracis* infection.

B. anthracis are by no means the only bacteria which can manipulate macrophages after phagocytosis, and yet are killed efficiently by neutrophils. *Shigella flexneri* infections are such an example. During *Shigella* infection, bacteria invade the colonic mucosal layer, are engulfed by resident macrophages from which they escape and then enter epithelial cells from the basolateral side. *Shigella* can escape the phagosome of macrophages using an invasion complex comprised of the virulence factors IpaB and IpaC (Tran Van Nhieu et al., 2000). The bacteria ultimately induce macrophage apoptosis (Zychlinsky et al., 1992). Unlike macrophages, neutrophils prevent the escape of *Shigella* from the phagocytic vacuoles in which they are finally killed. Weinrauch et al. (2002) demonstrated that nanomolar concentrations of neutrophil elastase (NE) degrade virulence factors that are necessary for *Shigella* escape from phagocytic vacuoles, thus enabling neutrophils to effectively kill the bacteria. Proteins not involved in virulence are not targeted.

Staphylococcus aureus on the other hand are not easily killed by neutrophils. The capsule, a slime layer composed of capsular polysaccharides (CP), is important for bacteria to evade neutrophil phagocytosis (Peterson et al., 1978). The anti-phagocytic activity of the capsule is well established and recent evidence suggests that the quantity of capsule is decisive for *S. aureus* virulence.

S. aureus, like many Gram-positive bacteria, is also resistant to defensins (Peschel and Collins, 2001, Harder et al., 1997) probably due to the composition of the cell wall and the cytoplasmic membrane. Its cell wall consists of a thick proteoglycan layer containing teichoic acid (TA) that incorporate D-alanine. Bacterial cytolysis is mediated by the interaction of the anionic phospholipids with the defensin leading to pore formation and thus permeabilization of the microbial membrane (Zasloff, 2002).

The decreased negative charge on the bacterial surface, renders the bacteria more resistant to the cationic-driven attack of defensins (Peschel et al., 1999)

5.1.2.5. Defensins

α-defensins are a group of small antimicrobial peptides found in human neutrophil granules (Ganz, 2003). The three major human α-defensins differ by one amino acid and kill viruses, fungi and bacteria (Ganz and Lehrer, 1995) effectively. The three defensins show differences in potency against some bacteria such as *B. cereus*, *Enterobacter aerogenes* and *E. coli* (Ericksen et al., 2005). During our study we did not examine the relative potency of the α-defensins towards *B. anthracis*

The results presented do not imply that α-defensins are the sole anti-*B. anthracis* molecules present in the human neutrophil granule extract or in the neutrophil granules. As discussed, the granule extract is a good method for studying the oxygen independent neutrophil killing mechanisms, but due to the isolation technique, constituents of the granules can be lacking. Furthermore, the isolation method via RP-HPLC is only one potential approach. During RP-HPLC, harsh organic solvents are used, which can easily denature large proteins with a complex tertiary structure, resulting in loss of function. To date, the only neutrophil factor described as relevant for *B. anthracis* killing is the secretory phospholipase A2 group IIa enzyme found in the neutrophil cytoplasm (Gimenez et al., 2004).

5.2. In vivo data

The data in this thesis support our hypothesis that human neutrophils can phagocytose and kill *B. anthracis* spores and vegetative cells and therefore have a dramatic impact on the outcome of the disease. The timely recruitment of neutrophils to the site of a cutaneous anthrax infection could be the reason why these infections resolve spontaneously. The missing neutrophil infiltration in lung infections could be the reason that the infection becomes systemic.

It is difficult to study this question in an in vivo animal model. As mentioned it was not possible to further study the role of neutrophil defensin in a α-defensin knock-out mouse model. Mice do not possess any neutrophil α-defensins (Eisenhauer and Lehrer, 1992), and are thus imperfect experimental models in scenarios in which neutrophil function is significant. Further, mice, as most laboratory animals, do not show the cutaneous form of the disease.

Due to obvious ethical reasons the findings in this study also could not be verified in humans. Yet literature from human cases and animal studies support our findings and will be discussed here.

5.2.1. Human cases

In 1943 the histopathology of three untreated, fully developed cutaneous anthrax cases was examined (Lebowich et al., 1943). The epithelial cell layer of the lesion was necrotic and ulcerative and filled with bacilli. The adjoining intact epithelial cell layer was oedematous and invaded by neutrophils. There was a marked, yet diffuse neutrophil infiltration without any abscess formation, and a heavy fibrinous network was found with large numbers of bacteria enmeshed. Shieh et al. (2003) described the histopathological features of bio-terrorism related cutaneous anthrax cases of 2001. They found various degrees of inflammation in the lower epidermis and the dermis of all case specimens.

In contrast, the description of the necropsies of three mill workers who had died of pulmonary anthrax showed that the lungs of the patients exhibited oedema but no haemorrhage or leukocytic infiltration (Albrink et al., 1960). On the other hand, studies such as the case reports made after the Sverdlovsk incidence, suggest that primary lung anthrax does occur, meaning that there is inflammation of the lung tissue. In a report of the Sverdlovsk incidence the most predominant and consistent lesions in pulmonary anthrax patients are haemorrhagic thoracic lymphadenitis and a haemorrhagic mediastinitis (Abramova et al., 1993). But in 25% of all human cases a focal haemorrhagic necrotizing pneumonia could be seen. Brachman (1980) was convinced that without pre-existing pulmonary disease no true primary respiratory infection develops during a pulmonary anthrax infection. Meselson et al. (1994) also hypothesized that the existence of such pulmonary inflammation among humans might be influenced by the presence of pre-existing pulmonary lesions. The incidence of pre-existing pulmonary lesions was not specifically addressed for these cases. However, epidemiological data reported suggests that a significant part of the population affected during the Sverdlovsk incidence engaged in activities such as smoking or occupational hazards that could lead to impaired pulmonary function (Meselson et al., 1994). The relevance of these observations has not been studied.

5.2.2. Animal studies

In one of the few studies examining infected animals prior to their anthrax mediated death Lyons et al. (2004) looked at the histology of mice infected i.n. or i.t. with the fully virulent Ames strain 3, 5, 24 and 48h after exposure. After the first two time points no changes were detected in the lungs. After 24h signs of airway epithelium necrosis and a mild increase in intravascular neutrophils became apparent. After 48h epithelial cell death was reduced, vegetative bacteria were distributed within alveolar capillaries and alveolar inflammation was typically infrequent to nonexistent.

Besides mice, other animal models are also difficult to compare to human *B. anthracis* infection, as most animals do not show the cutaneous from of the disease. Yet, as in humans, the pulmonary form is not accompanied by neutrophil infiltrations in the lung. In a comparison of aerosol and subcutaneous exposure of rabbits to *B. anthracis* spores, (Zaucha et al., 1998) demonstrated that the most consistent histopathologic findings occurred in lymphoid tissues, including lymph nodes, spleen and gut-associated lymphoid tissues independent of the route of administration. The pattern of lymph node involvement and the relative severity of the lesions depended on the route of exposure. In both animal groups but only minimal to mild perivascular and peribronchiolar infiltration of neutrophils in the lung could be detected. An acute dermatitis was a consistent feature at the s.c. inoculation site, accompanied by some neutrophil infiltration and a marked expansion of the dermis by oedema. In rabbits the dermal lesions were more oedematous and the cellular infiltration less intense than those seen in humans (Gleiser, 1967). Ulceration and eschar formation characteristic of cutaneous anthrax in humans was also not observed.

In another study, chimpanzees were subcutaneously infected with *B. anthracis* spores and observed for changes at the site of inoculation (Berdjis and Gleiser, 1964). Frequently an oedematous, elevated, erythematous lesion developed during the first 2 days, which either regressed in survivors (which had been infected with a very low dose) or became extensive and infiltrative. Microscopically the epithelium and corium were undamaged but the subcutaneous tissue was characterized by massive oedema, some mononuclear and neutrophil cell infiltration especially around the blood vessels. But the number of neutrophil infiltrates was not comparable to human infection and the typical dermal changes were missing.

As in humans there are cases in *B. anthracis* infected monkeys that show pulmonary lesions. Fritz et al infected Rhesus monkeys with *B. anthracis* via the lung. In this

study 2 of 13 monkeys showed an anthrax related pneumonia characterized by mild infiltrates of neutrophils. Vasconcelos et al. (2003) described pulmonary neutrophil infiltration in 29% of Cynomolgus monkeys infected with *B. anthracis* via the lungs.
An important factor, which might correlate to neutrophil infiltration, is the relative host susceptibility. It is known that there are highly susceptible and resistant animal species. Highly susceptible hosts develop mild responses, in contrast to intense inflammation seen in hosts more resistant to *B. anthracis* infection. Mice, rabbits and herbivores are highly susceptible, humans and monkeys are relatively resistant and dogs and pigs highly resistant.
In a very interesting in vivo study Gleiser et al. (1968) infected pigs and dogs with very high doses of aerosolised *B. anthracis* spores. In contrast to any other animal model significant lesion were found in the lungs. The lesions were haemorrhagic and fibrinous with an intense cellular response. There was a dense accumulation of neutrophils, plasma cells and macrophages. The lesion was discrete but without any sign of encapsulation. Bacteria could be recovered from the lung for the first thirteen days of infection, but not at a later time point. All animals survived the infection. Whether pre-existing lung lesions were involved was not examined, importantly nearly all of the animals showed neutrophil infiltration and all survived.
In another communication a histopathological study of the evolution of dermal lesions of *B. anthracis* in susceptible (mice, rabbits, guinea pigs) and relatively resistant animals (swine, dogs) was recorded (Cromartie et al., 1947). The susceptible animals exhibited acute disease, which was terminated by death in 48 to 96 hours. The resistant animals developed a more chronic type of infection from which they appeared to be recovering at the time of sacrifice (10 p.i.). Characteristic lesions of the susceptible animals were swelling of the skin and subcutaneous tissue. Germinated bacteria were detected until time of death, as were small numbers of leucocytes. In contrast, the skin lesion, which developed in dogs and pigs, consisted of an area of induration, with the central portion developing necrosis and ulcerations. After 26h the lesions were infiltrated by large numbers of neutrophils and mononuclear cells. The infiltration was associated with degeneration and disappearance of the bacteria. After 3 days the epidermis in the central part of the lesions was necrotic and the dermis and adjacent subcutaneous tissue was infiltrated by dense masses of leucocytes, surrounded by fibrinous material. The lesions are

reminiscent of the cutaneous lesion found in humans. The bacteria seemed to be contained by the inflammatory infiltrates.
Another intriguing point is that at the beginning of last century the live and slightly attenuated vaccine Pasteur introduced in 1881 (see introduction) was co-injected with 1-10% saponin. As this vaccine still contained many fully virulent live spores, systemic infections could result after administration. Saponin at the above mentioned concentrations provokes a violent inflammation at the site of infection, which limited generalization of the *B. anthracis* infection (Turnbull, 1991). This again strengthens the hypothesis that neutrophils at the site of infection can kill *B. anthracis* and contain the infection.

These observations show that the ability of the host to respond with neutrophil infiltration may play a role in the missing systemic infection and therefore in the relative resistance of the host (Gleiser, 1967). As all mammals can succumb to anthrax, the outcome of an infection is a matter of infectious dose. Neutrophil numbers called to the site of infection need to be high enough to overcome the number of invading bacteria. If this happens early during infection, systemic spread of bacteria in the blood can be prevented and increasing host survival.

5.3. Reasons for different neutrophil recruitment patterns

One of the most interesting questions, which results from these examinations, is why human neutrophils are recruited to the site of *B. anthracis* infection in some cases but not in others. Also, why neutrophils are recruited to lung infection sites in pigs and dogs, but not in other mammals, remains a mystery. The answer must lie in the pro-inflammatory signalling events initiated after *B. anthracis* infection. As only the macrophages and dendritic cells have been implicated in *B. anthracis* pathogenesis and the role of epithelial cells for example has not been examined the discussion will focus on responses of these cells. Also, epithelial cells are not likely to play a role during this infection, as *B. anthracis* is not an invasive pathogen. In other infections, e.g. *Shigella flexneri*, epithelial cell signalling significantly contributes to the recruitment of inflammatory cells (Sansonetti, 2001).

The role of the macrophage during *B. anthracis* infection is controversial. Anthrax spores are phagocytosed and germinate within the cell (Guidi-Rontani et al., 1999b). The vegetative bacteria produce lethal factor, which kills the activated

macrophages by apoptosis (Hsu et al., 2004). It was thought that the lethal factor induces a major release of cytokines, which leads to the typical shock-like symptoms and ultimately death (Hanna et al., 1993). More recent data imply that lethal factor induces hypoxia in the liver, which leads to death (Moayeri et al., 2003). Further, rats killed by a constant lethal factor infusion exhibited shock symptoms, but no elevation of pro-inflammatory cytokines in the blood.

Local cytokine concentrations reflect the signalling events after *B. anthracis* infection more precisely than cytokine levels in blood. Also here the release of proinflammatory cytokines from macrophages in vitro is as controversial.

Hanna et al. (1993) reported that sublytic concentrations of lethal factor triggered the production of tumor necrosis factor α- and interleukin 1ß by a macrophage cell line. Pickering et al (2004), Pickering and Merkel, (2004) also reported a positive stimulation of cytokine expression by *B. anthracis* spore infection. In their study the J774A.1 mouse macrophage cell line released TNF-α and IL-6 in response to infection with *B. anthracis* spores. These results were reproducible in primary mouse macrophages.

Recently conflicting evidence accumulated on the role of lethal factor and cytokine release from macrophages. Erwin et al. (2001) presented evidence that lethal toxin suppresses rather than induces proinflammatory cytokine production in macrophages (RawB) in vitro. Popov et al. (2002a) showed that sublytic concentrations of lethal factor inhibit the secretion of pro-inflammatory cytokines in human monocyte derived macrophages. Further, lethal factor was shown to inhibit the activation of IFN-regulatory factor 3, a crucial transcriptional factor implied in proinflammatory cytokine and chemokine secretion in response to Toll receptor activation.

Recent data was also published concerning the role of dendritic cells in signalling for proinflammatory cells. Pickering et al. (2004) described increased cytokine expression in human dendritic cells infected with *B. anthracis* spores. In contrast, Agrawal et al. (2003) found that the lethal toxin impairs the proinflammatory cytokine production of dendritic cells. They further reported that anthrax lethal toxin exposure also leads to abnormal maturation of the dendritic cells, i.e. there is defective up-regulation of co-stimulatory molecules. Tournier et al. (2005) proposed that not only the lethal factor but also the oedema factor severely impairs dendritic cell cytokine secretion.

Recent data seems to indicate that proinflammatory signalling events are inhibited after *B. anthracis* phagocytosis by macrophages and dendritic cells. This could explain why neutrophils are not called to the site of infection in some hosts or in some tissue. Yet one must take into account that most studies were performed with lethal toxin only and not by infecting macrophages with spores or vegetative bacteria. Yet the data still does not explain why neutrophils are recruited to the skin in humans but not to the lung. It also does not explain the different host susceptibilities to *B. anthracis* infection.