2. Literature survey

2.1. Anthrax

2.1.1. History

Anthrax is an acute disease primarily affecting herbivores but also found in other mammals including humans and even birds. The word anthrax is derived from the Greek word *anthrakos*, meaning coal and referring to the characteristic eschar found in the human cutaneous form of the disease (Dixon et al., 1999).

Anthrax is an antique disease. The earliest historical reports are thought to be of the fifth and perhaps sixth plaque of Egypt in the time of Moses (Exodus 9:6). Both Greeks and Romans were well acquainted with the disease as described in writings of Homer, Hippocrates and many others (Turnbull, 2002). During this time the occurrence of anthrax in humans was associated with the consumption of contaminated meat for the first time. Throughout the past millennium many major episodes were recorded throughout Europe and later in America (Sternbach, 2003).
The nineteenth century saw anthrax as the first disease of man and animal shown to be caused by a microorganism. Robert Koch formulated his famous postulates in proving that *Bacillus anthracis* is the cause of anthrax and Pasteur did his pioneering work on vaccines using *Bacillus anthracis* (Turnbull, 1991). During the beginning of the last century anthrax still played an important public health role in Europe. Especially people working in the wool industry, curing hides or working directly with animals (e.g. veterinarians) were affected**.** In 1937 an attenuated live spore vaccine came on the market and through the combined application of this livestock vaccine and improved veterinary and public health controls, the disease has declined drastically in the industrialized world. This is not true for third world countries where both human and animal cases are regularly reported. In 2004 there was a major outbreak in Zimbabwe, more than 200 people were infected and showed signs of cutaneous anthrax; approximately 3% died (National Institute for Communicable Dieseases, South Africa, http://www.nicd.ac.za/)

In the past years *B. anthracis* has gained new interest as a potential agent of biological warfare. During World War I, Germany might have inoculated horses and mules, which were shipped to the allies, in an effort to destroy animals used during the war (Bhalla and Warheit, 2004). Further, a German spy was caught in Norway with 18 sugars cubes, each containing a small vial of *B. anthracis* solution (Lamb,

2001). There are indications that Germany planned to drop plaque-infected rats over England during World War II. In retaliation Britain had planned to drop *B. anthracis*-contaminated cattle cakes in the German countryside to affect the beef supplies (Hawley and Eitzen, 2001). In 1941, the British conducted limited experiments by releasing spores on Gruinard Island near Scotland. Viable spores persisted until the island was decontaminated with formaldehyde and seawater in 1986 (Christopher et al., 1997). In the 1960's the emphasis of the research in Porton Down in the UK and Fort Detrick in the USA officially switched from biological warfare potential to elucidating the bacterial pathogenesis and developing an effective prophylaxis. After the Sverdlovsk incident in 1979, when weaponised spores were accidentally released into the atmosphere infecting 79 people of which 86% died (Abramova et al., 1993, Meselson et al., 1994), the potential of *B. anthracis* as a weapon gained new weight. The Gulf wars and the anthrax postal attacks in the USA in 2001 (Matsumoto, 2003) has induced an enormous boost in research activity.

2.1.2. Ecology and Epidemiology

B. anthracis spores are highly resistant to heat, radiation, chemical assault and are known to survive for decades in the environment (Swartz, 2001). Animals are infected when grazing on contaminated pastures. Outbreaks tend to occur in association with marked climatic or ecologic change, such as heavy rainfall, flooding or drought. Even in endemic areas anthrax usually occurs sporadically, often with many years between occurrences (Coetzer et al., 1994). During epidemics, flies and biting insects may play a minor role in mechanical transmission. Contaminated feeding stuff such as hay or meat are a infection source for animal and man (Keim and Smith, 2002).

Within the host the spores germinate (Guidi-Rontani et al., 1999b) to produce the vegetative forms that multiply, eventually killing the host. Sporulation and subsequent dissemination of the spores are dependent on the carcass being opened and coming in contact with oxygen (Keim and Smith, 2002), as the vegetative bacteria are rapidly killed by putrefaction. Sporulation is slow under 20°C and even in opened carcasses *B. anthracis* is rapidly killed at low temperatures. Warm climate favours contamination of soil and environment by anthrax spores, in cold countries anthrax tends to be self-limiting (Keim and Smith, 2002).

It is not clear if vegetative cells survive in the environment. It is a topic of debate to which extent a cycle of germination, multiplication of vegetative cells and re-sporulation occurs. The nutrient level required for survival of the vegetative cells in soil and the low tenacity of the vegetative form makes it seem unlikely that such a life cycle occurs (Coetzer et al., 1994).

Anthrax still is a great problem for animals and humans in most countries of sub-Saharan Africa and Asia, in several southern European countries, in the Americas, and certain areas of Australia. Disease outbreaks in animals also occur sporadically in other countries (World Organisation for Animal Health (OIE) http://www.oie.int/hs2/report.asp?lang=en) (World Anthrax Data Site http://www.vetmed.lsu.edu/whocc/mp_world.htm). Human cases are zoonotic in origin, with no convincing data to suggest that human-to-human transmissions occur or animal-to-animal transmission ever take place.

The disease is found in three forms (World Health Organisation (WHO) http://www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/). Cutaneous anthrax, the most common in humans, results from contact with infected animals or animal products. The pulmonary form is much less common and a result of spore deposition in the lungs, while the gastrointestinal disease is due to ingestion of infected meat**.** Most literature cites cutaneous disease as constituting 95% of cases, with inhalational anthrax responsible for 5% and the gastrointestinal form for 1% to 5% (Dixon et al., 1999).

Three different manifestations of anthrax are recognized in animals. Cattle, sheep, goats and some wild ruminants mainly manifest the peracute form. Horses, donkeys and zebra suffer from the acute form and omnivores and carnivores from the subacute to chronic form (Aiello, 1998). Pigs and dogs are known to be very resistant against infection (Gleiser et al., 1968). Birds are most resistant against anthrax infections, yet outbreaks have occured on ostrich farms and in ducks and cranes (Hugh-Jones and de Vos, 2002).

2.1.3. Bacteriology

B. anthracis is a large (1-2 µm by 4-10 µm), rod shaped, non-motile, facultative anaerobic, Gram-positive bacterium. The organism grows well on blood agar, the colonies are grey-white and large (4-5 mm after 18-24 h incubation at 37°C). The colonies are rough, have ground-glass appearance and a very tacky, butyrous consistency, with an irregular border described as a 'medusa head' appearance (Dixon et al., 1999).

In vitro *B. anthracis* grows as long chains, but in the host it appears as single organisms or chains of two or three bacilli, which are square ended (WHO http://www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/).

Demonstration of the capsule is achieved by staining the bacteria with polychrome methylene blue (M'Fadyean's reaction) (World Organisation for Animal Health (OIE) http://www.oie.int/hs2/report.asp?lang=en). *B. anthracis* does not produce a capsule when grown on ordinary culture media. Capsule growth can be induced in vitro by high CO_2 pressure, which is one way of distinguishing *B. cereus* from *B. anthracis*. Capsulated *B. anthracis* on solid media are thick, smooth and very slimy.

In the past *B. anthracis* was distinguished from other *Bacilli* species by the absence of haemolysis on sheep blood agar and lack of motility. Additional useful laboratory tests were the susceptibility to the specific diagnostic 'gamma' bacteriophage (Brown and Cherry, 1955) and sensitivity to penicillin (string-of-pearls appearance). Importantly, some genotypes are resistant to the gamma phage and penicillin. To date primers for polymerase chain reaction are now available confirming the presence or absence of pXO1 and/or pXO2 in pure cultures of isolates from hosts or environmental samples (recommended primers found on the OIE website (World Organisation for Animal Health (OIE) http://www.oie.int/hs2/report.asp?lang=en).

B. anthracis is very closely related to *B. cereus,* which is an almost ubiquitous component of the environmental microflora (Jensen et al., 2003). The only unshared antigens that lend themselves to differentiating these two species by immunological approaches are the anthrax toxin antigens, produced during the exponential phase of growth, and the capsule of *B. anthracis*.

Antibody detection in serum from infected animals is rarely used for diagnostic purposes and is essentially a research tool. Currently accepted as the best serological procedure is the enzyme-linked immunosorbent assay (ELISA) in

microtitre plates coated with the protective antigen (PA) (World Organisation for Animal Health (OIE) http://www.oie.int/hs2/report.asp?lang=en).
Animal inoculation may be considered for recovery of *B. anthracis* if all other methods fail. Examples of when this might be needed are specimens from animals that received antibiotic therapy before death or environmental samples containing sporostatic chemicals.

2.1.4. *B. anthracis* spores

In response to nutrient deprivation and sufficient oxygen supply environmentally highly resistant spores are formed. Spores are highly resistant to drying, boiling for 10 min and most disinfectants. A temperature of 120°C for at least 15 min is normally used to inactivate the spores (World Health Organisation (WHO) http://www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/).

2.1.4.1. Spore coat

The coat is fundamental to spore resistance. It is a tough proteinaceous shield that provides mechanical strength yet retains the ability to monitor its environment and allows small molecules to access its germination receptors beneath the coat (Driks, 2002a, 2002b). The core compartment houses the chromosome and is surrounded by two membrane layers. Between these membranes the is a specialised peptidoglycan layer, the cortex. The outermost membrane acts as a site of coat protein deposition. The coat is made up of approximately 30 unique protein species. In *Bacillus subtilis* the coat is the outermost layer of the spore (Henriques and Moran, 2000). In others, such as *B. anthracis* and *Bacillus cereus*, an additional layer, the exosporium exists. The exosporium is a glycoprotein shell, which is separated from the coat by a significant gap. The composition of the exosporium remains to be fully elucidated, yet components are beginning to be identified (Redmond et al., 2004, Kim et al., 2004, Steichen et al., 2003). Spores devoid of the exosporium are as infectious as those without therefore the role of the exosporium remains unknown (Chada et al., 2003).

2.1.4.2. Spore germination

When conditions are favourable for growth the spores germinate and are converted back into a growing vegetative cell. Spores germinate once they enter the body of a host, but can also germinate in the environment if conditions are appropriate, i.e. temperature between 8 and 45°C, a pH between 5 and 9, a relative humidity greater than 95% and the presence of adequate nutrients. These germinants are generally single amino acids, sugars or purine nucleosides (Setlow, 2003). These molecules are recognized by a family of *gerA*-like sensor operons whose activation is the initiating event of germination (Cabrera-Martinez et al., 2003). Once in contact with germinants the spores become committed to germination and the process will proceed even if the germinants are removed. Processes that follow are first the release of spore H+, which elevates the core pH from ~6.5 to 7.7. Secondly, the dipicolinic acid and the associated divalent cations (especially Ca2+) are released and are replaced by water. The peptidoglycan spore cortex is then hydrolysed and finally the core is further hydrated (Moir et al., 2002). With a neutral pH and the increased hydration levels the protein mobility in the core returns, thus allowing enzyme action. Germination takes place without a detectable energy metabolism.

2.1.5. Vegetative cells

Once the spores are taken up by the host they germinate forming vegetative bacteria.

Fully virulent strains of *B. anthracis* carry two large plasmids, pXO1 and pXO2, that encode the primary virulence factors (Mock and Fouet, 2001). pXO1 is 182 kb large and carries the structural toxin genes, *pagA*, *lef* and *cya*; the regulatory elements *atxA* and *pagR*; a resolvase and a transposase; and *gerX*, a three gene germination operon. pXO2 (96 kb) carries *capB*, *capC*, *capA* and *dep*, all known to encode capsule synthesis and degradation. Both plasmids and the entire chromosome (5.3 Mb) have been sequenced (Read et al., 2003).

2.1.5.1. S-layer and capsule

The surface of *B. anthracis* is unique (Fouet et al., 1999). In addition to the classic cytoplasmic membrane and peptidoglycan layer of Gram-positive bacteria, *B. anthracis* has two further structures very few bacteria posses simultaneously: the S-

layer (surface layer) and the capsule (Fouet et al., 1999). When *B. anthracis* does not produce a capsule the cell wall appears layered. The layer surrounding the peptidoglycan is composed of proteinaceous paracrystalline sheaths. The two S-layer components are Sap (surface array protein) and EA1 (extractable antigen 1) (Etienne-Toumelin et al., 1995, Mesnage et al., 1997). EA1 is nearly exclusively cell associated, whereas Sap is also released into the supernatant. Antibodies against both proteins have been detected (Baillie et al., 2003), indicating they are synthesised in vivo and are major surface antigens. Sap is found on the surface predominantly during exponential growth and EA1 during stationary phase. S-layers from *Bacillaceae* were found to function as adhesion sites for cell associated enzymes (Bahl et al., 1997). The S-layer in *B. cereus* contributes to virulence, if this is the case in *B. anthracis* remains to be elucidated (Sara and Sleytr, 2000).

The capsule is a polymer of D-glutamic acid (Smith, 1956). Both in vivo and in vitro the glutamic acid residues are mainly bound by peptide bonds between γ-carboxyl and α-amino groups. The size of the glutamic acid chains differs between growth conditions. In vitro they are 20-55 kDa, whereas in vivo they have an estimated molecular mass of 215 kDa. The capsule has been described to have antiphagocytic properties (Ezzell and Welkos, 1999).

2.1.5.2. Anthrax toxin

The existence of an anthrax toxin was suspected when a late bacteraemia in *B. anthracis* infected guinea pigs was terminated by antibiotic treatment and the animals did not survive (Smith et al., 1955a Keppie et al., 1955). Also, sterile filtered serum of infected guinea pigs induced oedema and death in healthy animals (Smith et al., 1955a). A toxin composed of three proteins acting in binary combinations was isolated (Stanley and Smith, 1961, Beall et al., 1962) The components are termed protective antigen (PA), for its ability to elicit a protective immune response, lethal factor (LF) and oedema factor (EF). The combination of EF and PA produces a skin oedema, whereas LF and PA together are lethal (Mourez et al., 2002). Any one of the components does not show any activity when injected alone (Moayeri and Leppla, 2004). Both the oedema toxin (PA + EF) and the lethal toxin (PA + LF) fall into the class of A-B toxins (Lacy and Collier, 2002). PA is the common cell-binding domain (B component) able to interact with two different enzyme domains, LF and EF (A component) and translocate them into the cell cytosol. The genes for PA, LF

and EF are all located on the pXO1 plasmid and each encodes a secreted protein (Brossier and Mock, 2001).

2.1.5.2.1. Protective antigen

The mature protein (82kDa) mediates the attachment and entry into the cytosol of both lethal and oedema factor. PA has four domains containing regions involved in binding to the cell receptor, binding LF and EF, for membrane insertion, and translocation of the anthrax toxin (Ascenzi et al., 2002). PA binds to the ubiquitously expressed, single transmembrane, 370 aa long cellular receptor (TEM8) (Bradley et al., 2001). Recently a second major anthrax toxin receptor has been identified (CMG2) (Scobie et al., 2003). Both receptors have a von-Willebrand factor type A domain in their extracellular N-termini, which are highly similar to domain I found in integrins (Abrami et al., 2005).

After binding to its receptor PA is cleaved by a furin or furin-like protease (Scobie and Young, 2005). The resulting amino-terminal 20 kDa fragment (PA20) dissociates from the receptor-bound 63 kDa carboxy-terminal end (PA63) and is released into the extracellular milieu. PA63 then oligomerizes into a heptamer, which then binds LF or EF competitively. The assembled toxin complexes are endocytosed via membrane lipid rafts (Abrami et al., 2003). After fusion with endosomes the low pH induces a conformational change in the PA63 heptamer resulting in the formation of a cation-selective channel (Mogridge et al., 2002). LF and EF are translocated across the endosome, where LF unlike EF dissociates from the endosomal membrane (Guidi-Rontani et al., 2000).

2.1.5.2.2. Oedema factor

EF (88 kDa) is an adenylate cyclase, which converts intracellular ATP into cAMP, an activity dependent on the eukaryotic protein calmodulin and calcium (Ahuja et al., 2004). The physiopathological details of the mechanisms by which EF causes oedema are still unknown. The oedema factor contributes to *B. anthracis* virulence expression in vivo. Bacterial strains lacking oedema factor are still lethal, yet the LD_{50} is higher than of the wild type strain (Pezard et al., 1991). The amino terminus of EF shares five regions of similarity with the amino terminus of the LF suggesting that the amino terminus is the PA binding domain. The carboxy terminus is involved in the catalytic activity (Abrami et al., 2005).

2.1.5.2.3. Lethal factor

LF is a zinc metalloprotease, which cleaves the N-terminus of mitogen activated protein kinases (MAPKK) (Duesbery et al., 1998). Although it is likely that the activity of LF on MAPKK is important in anthrax pathogenesis the cascade of events after this cleavage still remain unclear. The lethal toxin induces apoptosis in macrophages (Guidi-Rontani, 2002) and endothelial cells (Kirby, 2004). Whether this effect is specific for only these cells remains unclear, as is the role of macrophage apoptosis in the course of the disease.

2.1.6. Regulation of virulence factors

The structural genes for the anthrax toxin proteins are located non-contiguously on a 46.6 kb pathogenicity island on the virulence plasmid pXO1 and entail the genes *pagA* (encodes PA), *lef* (encodes LF) and *cya* (encodes EF). Regulators of gene expression are also encoded on the plasmid, namely *pagR* and *atxA*. *pagR* represses expression of the *pag* operon, yet does not seem to play a role in *B. anthracis* virulence (Koehler, 2002).

AtxA (anthrax toxin activator) is a global regulator of virulence gene expression (Hoffmaster and Koehler, 1999). *atxA*-deleted strains produce no detectable anthrax toxin components and exhibit reduced capsule synthesis, which means it does not only control virulence expression on its own plasmid, but also has an effect on capsule synthesis genes on pXO2. *B. anthracis* lacking the pXO2 plasmid display a capsule reduced in thickness (Koehler, 2002). *atxA* also indirectly seems to control the expression of the chromosomal S-layer genes *sap* and *eag* (Mignot et al., 2003).

The second virulence plasmid encodes genes for capsule synthesis, *capA*, *B* and *C*. *dep* is responsible for depolarisation of the capsule. *acpA* (anthrax capsule activator) is a regulator on pXO2 that appears to be specific for the expression of the capsule biosynthetic genes (Bourgogne et al., 2003). As is true for *atxA,* the mechanism by which *acpA* controls gene expression is not known.

2.1.6.1. CO_2 effect

Synthesis of the above discussed major virulence factors is enhanced when *B. anthracis* is grown in media containing bicarbonate or dissolved CO_2 (Thorne et al.,

1952). The three toxin genes and the capsule operon require CO_2 for activation of transcription (Sirard et al., 1994). AtxA expression is not enhanced through CO_2, the *atxA* gene is expressed constitutively throughout growth. The response to CO_2 is specific and not due to its buffering capacity, yet the mechanism is poorly understood.

2.1.6.2. Growth phase dependent toxin expression

Steady state levels of toxin proteins are highest during the transition from exponential growth phase to stationary phase. This phenomenon has been associated with *abrB*, a transition state regulator, as it activates and represses numerous genes during transition in growth phase. The chromosome encoded AbrB represses the toxin genes and when deleted PA, LF and EF synthesis increases (Koehler, 2002).

2.1.7. Pathogenesis

B. anthracis infections occur only when spores enter the body from the external environment. There are no well-documented cases of vegetative cells occurring in the environment and no natural examples of live animal to live animal transmissions. The environmentally resistant spores can enter the body via the skin, the gut or the lung. In vivo experiments have demonstrated that once inhaled, the anthrax spores reach the alveoli of the lung and are then rapidly phagocytosed by the alveolar macrophages (Guidi-Rontani et al., 1999b) (Ross, 1957). The spore containing phagosomes then fuse with lysosomes (Guidi-Rontani et al., 1999a). The spores germinate within the alveolar macrophages, they survive (Guidi-Rontani et al., 2001) and potentially multiply (Dixon et al., 2000) within the phagolysosome, ultimately escaping the macrophages. The anthrax toxin is expressed by newly germinated spores within macrophages and play a role in lysing the cells (Guidi-Rontani et al., 2001). The spores are transported to the regional lymph nodes by macrophages and after leaving these cells enter the blood stream and the lymphatic system (Ross, 1957). At time of death animals can harbour up to 1×10^8 vegetative bacteria/ml of blood (Dixon et al., 1999). Cause of death is not the resulting bacteraemia but the effect of the lethal toxin (Smith et al., 1955b). The role of the toxin is a matter of debate and will be further elucidated in the discussions section.

2.1.8. Clinical Features

2.1.8.1. Human anthrax

2.1.8.1.1. Cutaneous anthrax

Ninety five percent of the worldwide human anthrax cases are due to the cutaneous form (World Health Organisation (WHO) http://www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/).
This form is initiated when spores of *B. anthracis* are introduced into the skin through cuts or abrasions or by biting flies. The signs and symptoms become apparent within 5 days after exposure. The initial cutaneous manifestation is a small erythematous painless macule, which develops into a papula approximately 48-72 h later. Within the following 24-48 h multiple vesicles and oedema surround the lesion. If the patient has not been treated, bacilli can be cultured from the fluid. The surrounding oedema can be extensive but is not painful. 1-3 days after the vesicles appear, the papule ruptures and ulcerates. This leaves the characteristic black eschar, which classically measures 1-5 cm in diameter. Fever, malaise and regional adenopathy can often be associated features. The healing process can take up to 6 weeks. If left untreated 80-90% of all patients survive (Tutrone et al., 2002).

2.1.8.1.2. Gastrointestinal anthrax

The gastrointestinal form is rare and is most frequent in the developing world. This form occurs after the ingestion of contaminated and undercooked meat (Kanafani et al., 2003). The symptoms appear two to five days after spore-containing meat is ingested. It is presumed that the spores enter the body via a breach in the mucosal lining. The disease begins with fainting spells, low-grade fever and headache. This is followed by abdominal pain and nausea. In the last stage ascites, gastrointestinal bleeding and shock occurs, followed by death in most cases. On pathological examination the bacilli can be found in the mucosal and submucosal lymphatic tissue (Dutz and Kohout, 1971). There are marked signs of oedema of the mucosa with numerous small necrotic ulcers.

2.1.8.1.3. Pulmonary anthrax

This disease was until recently a disease mainly of historical interest, with sporadic cases reported mostly among wool handlers and persons in close contact with infected animals (Shafazand et al., 1999). But when dispersed as an aerosole during

accidental or intentional release, *B. anthracis* spores can present a hazard even far downwind from the point of release (Abramova et al., 1993). *B. anthracis* spores are <3µm in diameter, a size that is optimal for reaching the alveolar ducts and alveoli.
The incubation period is approximately 5-6 days, the LD_{50} for humans is estimated to be ~10000 spores (Franz et al., 2001). The initial stage is characterised by malaise, fatigue, myalgia, a non-productive cough and fever. This stage lasts for approximately 4 days and can be easily confused with a common cold. Within 24 h patients develop acute respiratory stress, hypoxemia and cyanosis, which culminate in the death of nearly 100% of untreated patients. Typical for pulmonary anthrax is the massive oedematous haemorrhagic mediastinitis (Albrink, 1961).

2.1.8.2. Livestock/domesticated animals

2.1.8.2.1. Peracute form

This form is found prominently in ruminants and is characterised by a very short course of disease. After usually less than 24 h, the majority of animals are found dead without having shown signs of illness. If clinical signs occur they are staggering, dyspnea, trembling and a few convulsive movements (Aiello, 1998; Coetzer et al., 1994).

2.1.8.2.2. Acute form

This course is usually less than 72 h. There is an abrupt rise in body temperature and a period of excitement followed by depression, respiratory and cardiac distress, convulsions and death. Bloody discharges may be seen from natural body openings (Aiello, 1998; Coetzer et al., 1994).

2.1.8.2.3. Subacute or chronic form

In this form of the disease the course usually extends for more than three days before recovery or death occurs. The most frequent sign is the oedematous swelling of the throat and neck, which may result in suffocation. The infection can remain localized or progress to septicaemia, which is often fatal. If the primary infection site is in the lower intestinal tract, clinical signs are not always obvious, although vomiting and haemorrhagic diarrhoea may occur (Aiello, 1998; Coetzer et al., 1994).

2.1.9. Therapy

2.1.9.1. Human therapy

Currently there are three types of antibiotics approved for anthrax: ciprofloxacin, tetracyclins and penicillins. For exposed patients without symptoms the current antibiotic regime lasts for 60 days, as spores have the ability to persist in tissue (Friedlander et al., 1993). An alternative post exposure prophylaxis is a four weekly use of ciprofloxacin combined with three doses of vaccine administered during the four week period (Centres for Disease Control and Prevention, Atlanta, USA http://www.bt.cdc.gov/agent/anthrax/anthrax-hcp-factsheet.asp). The response to treatment of cutaneous anthrax cases is excellent if started promptly, however eschar formation may still occur. Treatment of pulmonary and gastrointestinal anthrax is usually ineffective, as the early stages of the disease are often mistaken with influenza and the therapy thus administered too late. The antibiotic treatment is very effective if given early enough (World Health Organisation (WHO) http://www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/).

2.1.9.2. Livestock therapy

Treatment of infected livestock animals is also usually ineffective as the course of the disease is peracute to acute. If an outbreak occurs it is best to treat all sick animals with the above mentioned antibiotics and immunized the apparently non-infected animals of the herd (World Health Organisation (WHO) http://www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/).
Importantly animals are to be removed from contaminated pastures and dead animals, manure and all potentially contaminated materials cremated or deeply buried) (World Organisation for Animal Health (OIE) http://www.oie.int/hs2/report.asp?lang=en). Disinfection procedure guidelines are laid down by the WHO.
In Germany the treatment of anthrax in livestock is specified by the ‘Verordnung zum Schutze gegen den Milzbrand und den Rauschbrand’ from 23. May, 1991. Therein it is degreed that only veterinarians are authorized to treat animals infected by *B. anthracis*. Further infected animals or those, which are suspected of being infected, are to be killed without any withdrawal of blood and are not to be skinned.

2.1.10. Vaccines

Immunity after human cutaneous anthrax most probably occurs, as re-infections are reported very rarely and tend to be much less severe. Data on animals also suggests that animals, which survived an infection are less susceptible to subsequent challenge (Friedlander et al., 2002).

Serum from animals vaccinated with PA against anthrax can confer protection to non-vaccinated animals, suggesting that antibodies are the main mechanism of vaccine induced immunity (Little et al., 1997). Natural immunity after a *B. anthracis* infection is believed to induce immunity against the protective antigen, as well as against the germination process and vegetative cells, resulting in greater protection (Beyer, 2004). Antibody protection against anthrax in the susceptible host is almost completely dependent on the immune response to the protective antigen. Measurable anti-PA antibodies do not guarantee that the patient is protected, but they must be present for protection to occur. It has become apparent that the relationship between PA and protection is not straightforward. A live spore vaccine can result in better protection, but a lower antibody titer in comparison to PA vaccines (Ivins and Welkos, 1988). The anti-PA antibodies were also associated with enhanced phagocytosis and an increased rate of germination in macrophages, which could potentially result in increased killing of the vegetative bacteria (Welkos et al., 2001). Immune responses to the other two toxin components may contribute to protection (Ivins and Welkos, 1988).

Whether cellular immunity plays a role in *B. anthracis* infection is still insufficiently understood (Brey, 2005). Treatment of peripheral blood lymphocytes with protective antigen in combination with either LF or EF effectively blocked T cell signalling, the production of cytokines, and cell proliferation (Paccani et al., 2005). As *B. anthracis* can be associated with sub-acute infections (such as cutaneous anthrax) the inhibition of T cell function may provide some advantages to the pathogen.

2.1.10.1. Livestock vaccine

Louis Pasteur demonstrated protective immunization against anthrax in 1881 using a heat-attenuated strain. The primary inoculums were subcultures of *B. anthracis,* which had been incubated at 42-43°C for 15-20 days, after which the organism was cured of the temperature sensitive toxin encoding plasmid. The second inoculation was with cultures of *B. anthracis*, which had been heated for only 10-12 days

(Hambleton et al., 1984). Today it is clear that this vaccine was a mixture of the attenuated strain and the fully virulent bacteria, which conferred the protection through the protective antigen.

In 1939, Max Sterne isolated a non-capsulated *B. anthracis* strain (34F2 strain) from which a live attenuated spore vaccine was developed. The strain used was an avirulent dissociant derived from a subculture of an isolate from a case of bovine anthrax. The bacteria were grown on horse serum nutrient agar in a 30% CO_2 atmosphere for 24 h. The final formulation consisted of approximately 10^6 spores/ml suspended in 0.5% saponin in 50% glycerine saline. Apart from the fact that todays preparation entails 10^7 spores/ml, it is unaltered. The strain is avirulent for domestic animals and is still used for routine immunization of livestock in many countries worldwide. A single inoculation provides protection for nine months, but a yearly boost is considered efficient (Coetzer et al., 1994). Effective immunity develops within a week, in horses this may take up to four weeks (Coetzer et al., 1994). Animals should be vaccinated at least two months prior to slaughter. The live spore vaccine seems to retain some virulence in goats and llamas, but can also cause occasional losses in laboratory animals. The LD_{50} is as low as 10^3 spores in some inbred strains of mice (Welkos et al., 1986). In Germany it is prohibited to vaccinate animals against anthrax (Verordnung zum Schutze gegen den Milzbrand und den Rauschbrand).

2.1.10.2. Human vaccines

A live attenuated spore vaccine resembling the Sterne vaccine is used in Russia and China as a human vaccine, unfortunately there is little information on the efficacy of the preparation (Baillie, 2001).

As there is always some concern about the use of live attenuated vaccines in humans both the current British and the American vaccine consist of a sterile culture filtrate of a toxin expressing attenuated strain adsorbed to an aluminium hydroxide adjuvant. The British vaccine consists of a cell-free filtrate of the 34F2 Sterne strain. The US vaccine is derived from a non-capsulated, non-proteolytic derivative of strain V770 from a case of bovine anthrax in Florida in 1951. The vaccines both contain PA, the essential protective immunogen, small amounts of EF, LF and other undefined bacteria and media derived proteins. These elements probably contribute to the relative high rate of local and systemic reactions. The PA content seems to be

higher and the LF and EF content much lower in the American vaccine (Turnbull, 1991). In respect to protective efficacy in animal trials both vaccines behave similarly (Turnbull, 1991). There is only one study examining the efficacy of the human vaccine in a human trial (Brachman et al., 1962) stating a 92.5% degree efficacy.
The protection given by both described vaccines is considered insufficient. In addition frequent complaints of unpleasant side reactions, such as massive oedema induction, are heard. Therefore in the past years a significant amount of work has gone into the development of a second-generation anthrax vaccine.

2.2. Interaction of *B. anthracis* and innate immune cells

B. anthracis causes an acute and fatal disease and can in some form either circumvent the host immune system or avoid being detected by it (Fukao, 2004). This is likely because of the extensive bacterial spread in a short period of time and the lacking inflammatory response in most disease forms. To date the immune evasion mechanisms of *B. anthracis* are not well understood. Innate immune cells potentially capable of eliminating the infectious particles or inducing an adaptive immune response are macrophages, dendritic cells and neutrophils.

2.2.1. Macrophages

B. anthracis spores are phagocytosed by alveolar macrophages and germinate within these cells (Guidi-Rontani et al., 1999b). There is evidence that macrophages can, to a small extent, kill intracellular *B. anthracis* once germinated (Guidi-Rontani et al., 2001), yet the vegetative cells predominantly survive (Cote et al., 2004) inside the macrophages. They express the lethal toxin, which ultimately leads to macrophage death (Park et al., 2002). There is evidence that the vegetative cells escape the phagolysosome of the macrophages and multiply in the macrophage cytoplasm (Dixon et al., 2000). How the vegetative cells survive the harsh phagolysosomal environment and how they escape this cell compartment is unclear. Interestingly, PA antibodies were also associated with enhanced phagocytosis and an increased rate of germination in macrophages, which could potentially result in increased killing of the vegetative bacteria (Welkos et al., 2002, 1989).

2.2.2. Dendritic cells

Dendritic cells bridge the innate and adaptive immune response (Rescigno, 2002). They act as antigen-presenting cells, i.e. they take up antigens, which can be processed and presented in the form of peptides bound to both major histocompatibility complex class I and II molecules. Naïve T-cells are activated through the dendritic cells (Rescigno, 2002).

The anthrax lethal toxin impairs the proinflammatory cytokine production of dendritic cells (Agrawal et al., 2003, Brittingham et al., 2005). Anthrax lethal toxin exposure also leads to abnormal maturation of the dendritic cells, i.e. there is defective up-regulation of co-stimulatory molecules. The dendritic cells can no longer prime naïve T cells and the specific adaptive immunity is impaired. Further, dendritic cells have been implicated in *B. anthracis* dissemination. Anthrax spores are phagocytosed by dendritic cells, the dendritic cells still partially mature and upregulate CCR7 and can thus home to their regional lymph nodes (Brittingham et al., 2005).

2.2.3. Neutrophils

Neutrophils are the first cells recruited from the blood stream to sites of infection. They play a central role in the resolution of bacterial infections (Mayer-Scholl et al., 2004). Surprisingly little is known about the role these cells play in the pathogenesis of anthrax. Both the oedema factor and the lethal factor have been described to inhibit phagocytosis of the vegetative cells by neutrophils (O'Brien et al., 1985, Keppie et al., 1963), but only if the neutrophils were pre-incubated with the toxin over long periods of time. Pre-treatment of neutrophils with the anthrax toxin resulted in the inhibition of superoxide release by the neutrophils after stimulation (Wright and Mandell, 1986, Alexeyev et al., 1994). A Sterne-derived supernatant filtrate was suggested to have chemotactic properties on mouse neutrophils, yet the chemotactic efficiency was dependent on the mouse strain from which the neutrophils were derived (Welkos et al., 1989).

The γ-D-glutamic acid capsule of *B. anthracis* serves as a principle virulence factor during *B. anthracis* infection, as strains lacking the capsule are non-virulent in most animal species (Welkos, 1991, Drysdale et al., 2005). It has been proposed that the capsule allows the anthrax bacilli to grow unimpeded in the infected host (Ezzell and Welkos, 1999) due to its ability to inhibit phagocytosis by neutophils (Keppie et al., 1963), Direct evidence to support this theory is still missing.

2.3. Neutrophils

Polymorphonuclear leucocytes (neutrophils) are essential for innate host defence against invading microorganisms, such as bacteria and fungi. Neutrophils are the first cells recruited from the blood stream to sites of infection. They are terminally differentiated cells, incapable of cell division, and synthesise very low levels of RNA and protein. Neutrophils are generated from the pluripotent haematopoietic stem cells in the bone marrow and are characterised by multi-lobed nuclei and abundant granules in the cytoplasm, which contain host-defence molecules (Mayer-Scholl et al., 2004).

2.3.1. Neutrophil recruitment

Neutrophils are an essential component of the acute inflammatory response and the resolution of microbial infection. Recruitment to inflamed or infected tissue occurs within minutes to hours. Molecules signalling for neutrophil infiltration are predominantly the aminoterminal formylated methionin bacterial peptides (fMLP), IL-8 and TNF-α from macrophages and epithelial cells, and the C5a, C3a and C4a from the complement cascade (Burg and Pillinger, 2001). Vasodilatation through TNF-α results in the reduced velocity of blood flow. The tight junctions of vascular endothelial cells are less tight, which results in an oedema and an accumulation of plasma proteins. Physiologically circulating neutrophils in blood contact the endothelium and transiently interact with it, a phemomenon termed rolling. Molecules mediating this are leucocyte (L), platelet (P) and endothelial (E) selectins, which permit interaction between neutrophils, and neutrophils and endothelial cells (Janeway, 2001). After exposure of circulating neutrophils to chemoattractants (IL-8, fMLP, C5a, LTB4) the mentioned selectins are shed, resulting in the conversion of the rolling state to a state of tight stationary adhesion (Burg and Pillinger, 2001).

Neutrophils adhere to the endothelium and secretory vesicles of the neutrophils are mobilised. This enriches the neutrophils with ß2 integrins and the cells transmigrate either between or through endothelial cells. During migration through the tissue proteases are liberated from the different granule subsets degrading vascular basement membranes and the intercellular matrix (Faurschou and Borregaard, 2003).

2.3.2. Bacterial recognition by neutrophils

The concept of bacterial recognition is based on so-called pathogen associated molecular patterns (PAMPs), which are recognised by pattern recognition receptors (PRRs) (Gordon, 2002). PAMPs are microbial structures, which, upon interaction with elements of the host innate immune system, trigger the initiation of host protective responses cumulating in the clearance of the pathogen by phagocytic cells. PAMPs are ideal targets as they allow distinction between self and microbial non-self, are found on all microorganisms (which allows a limited number of receptors to recognize PAMPs), and importantly are essential for microbial survival, therefore no escape mutants can be generated (Mukhopadhyay et al., 2004).

Neutrophils have the following pattern recognition receptors:

- Lectins, e.g. dectin-1, which detect bacterial carbohydrates.
- Scavenger receptors, e.g. MARCO, are structurally unrelated membrane molecules, which bind and internalise modified lipoproteins, lipopolysaccharides, and lipoteichoic acid.
- Complement receptor, e.g. CR1-4, which recognise complement proteins from the serum which have opsonized the microbes
- Fc receptors, which recognize IgG opsonized micoorganisms.
- Toll-like receptors (TLRs). To date eleven different TLRs have been discovered (Akira, 2003). TLR 2 and 4 are expressed by neutrophils, they recognise lipoproteins and lipopolysaccharide respectively (Muzio et al., 2000)

2.3.3. Neutrophil killing mechanisms

Upon encountering bacteria, neutrophils engulf these microbes into a phagosome, which fuses with intracellular granules to form a phagolysosome (Lee et al., 2003). In the phagolysosome the bacteria are killed after exposure to enzymes, antimicrobial peptides and reactive oxygen species (ROS). Traditionally the arsenal of cytotoxic agents is divided into either oxygen- independent or -dependent mechanisms (Figure 2). Both these systems probably collaborate in killing microbes (Roos and Winterbourn, 2002).

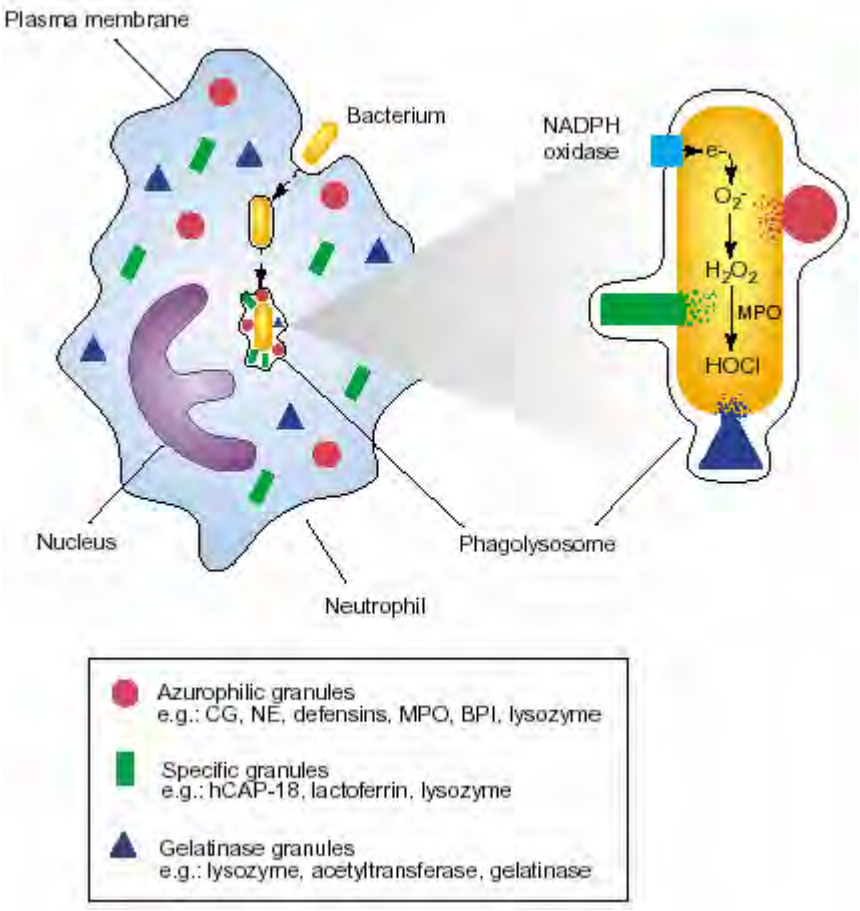

Figure 1. Schematic presentation of the oxygen dependent and independent mechanisms during neutrophil phagocytosis of bacteria. The oxygen-independent mechanisms encompass the contents of the three neutrophil granule subsets: the azurophil, specific and gelatinase granules, which contain characteristic proteases, antimicrobial proteins and peptides, and enzymes. Lysozyme, for instance, disrupts anionic bacterial surfaces, rendering the bacteria more permeable, whereas NE degrades virulence factors. The oxygen-dependent mechanism relies on the NADPH oxidase complex that assembles at the phagosomal membrane and produces O_2^-, which is rapidly converted to hydrogen peroxide. In turn, a constituent of the azurophil granules, myeloperoxidase, generates HOCl from hydrogen peroxide (Mayer-Scholl et al., 2004)

The oxygen-independent mechanisms encompass the contents of the three neutrophil granule subsets: the azurophil, specific and gelatinase granules, which contain characteristic proteases, antimicrobial proteins and peptides, and enzymes (Borregaard and Cowland, 1997). Antimicrobial proteins such as defensins, bactericidal/permeability-increasing protein (BPI) and the enzyme lysozyme, predominantly function by disrupting anionic bacterial surfaces, probably rendering the bacteria more permeable (Kagan et al., 1990). Proteases, such as neutrophil

elastase (NE) and cathepsin G (CG), degrade bacterial proteins, including virulence factors (Weinrauch et al., 2002). The importance of the oxygen-independent mechanism in defence is made clear in two very rare inherited diseases, the Chediak-Higash syndrome (Introne et al., 1999) and Specific Granule Deficiency (Gombart and Koeffler, 2002). Both disorders are characterised by recurrent infections and shortened life expectancy. In Chediak-Higashi syndrome, neutrophils contain giant granules resulting from specific and azurophil granule fusion. Specific Granule Deficiency is characterised by the absence of specific granules and defensins. The severity of the symptoms in these diseases underlines the fundamental role of granule proteins in host defence.

The second mechanism of neutrophil killing is oxygen-dependent (Roos et al., 2003). Phagocytosing neutrophils undergo an 'oxidative burst' during which the NADPH oxidase complex assembles at the phagosomal membrane and produces O_2-, which is rapidly converted to hydrogen peroxide by the enzyme superoxide dismutase. In turn, a constituent of the azurophil granules, myeloperoxidase, generates HOCl from hydrogen peroxide. How the bacteria are actually killed is not known. Hydrogen peroxide is bactericidal only at high concentrations, therefore a variety of secondary oxidants have been proposed to account for the destructive capacity of the neutrophils (Hampton et al., 1998). The importance of ROS for antimicrobial activity is validated by the susceptibility to infections of patients suffering from chronic granulomatous disease, a condition where the NADPH oxidase complex is inactive (Dinauer et al., 2000).

In the past, studies often focused on the effects of either the oxygen-dependent or oxygen-independent mechanisms. However, more recently, (Reeves et al., 2002) proposed that ROS might also serve the function of recruiting K^+ to the phagolysosome, allowing NE and CG to go from a highly organized intra-granule structure into solution. The relative contribution of ROS to these two different mechanisms is very intriguing, yet it seems premature to draw conclusions as to whether ROS contribute directly to microbial killing or only as activators of granule proteins (Roos and Winterbourn, 2002). Besides killing bacteria inside the phagolysosomes, neutrophils can also degranulate and release antimicrobial factors into the extracellular space (Faurschou and Borregaard, 2003). The cells can also generate neutrophil extracellular traps (NETs), which are composed of granule and nuclear constituents that kill bacteria extracellularly (Brinkmann et al., 2004).

2.4. Antimicrobial peptides

Antimicrobial peptides are an important element of the innate immune system and are conserved throughout evolution. These peptides are widely distributed among all eukaryotes, including mammals, amphibians, insects, plants and protozoa (Brogden et al., 2003). The two main antimicrobial peptide families are defensins and cathelicidins. Other mammalian antimicrobial peptides such as histatins, protegrins, dermicidin and anionic peptides are restricted to a few animal species and tissue (Lehrer and Ganz, 1999). Most of these molecules have cationic properties, which allow interactions with negatively charged phospholipids of the bacterial membrane. These cationic antimicrobial peptides (CAMP) often contain less than 100 amino acids. They are produced by leucocytes and epithelial cells and are efficient in killing a wide variety of both Gram-positive and -negative bacteria, fungi and viruses.

2.4.1 Defensins

Mammalian defensins are ß-sheet peptides with between 29 and approximately 40 amino acid residues and three intra-molecular disulfide bonds between cysteines. There are α- and ß-defensins which differ in respect to their cysteine pairing (1-6, 2-4, 3-5 in α-defensins and 1-5, 2-4, 3-6 in ß-defensin), the length of the peptide fragments between cysteines, their pattern of expression and their precursor structure (Lehrer et al., 1993).

Defensins are predominantly found in tissue and cells, which are involved in host defence against microorganisms, such as leucocytes, predominantly neutrophils and Paneth cells (specialized host defence cells of the small intestine). Tissue distribution of the defensins varies greatly among species (see Table 1).

Table 1: Defensin expression in neutrophils of different mammals (Ganz, 2003)

Species	Neutrophil defensins
Human	α-defensin 1-4
Rhesus monkey	α-defensin and θ- defensin
Mouse	none
Rat	α-defensin
Pig	None detected in granule extracts
Cow	ß-defensin
Chicken	ß-defensin

In humans there are six different α-defensins. Human α-defensins 1-4 are found in the neutrophil granules, the α-defensins five and six are Paneth cell specific. The human α-defensins 1-3 are approximately 100 times more abundant than human α-defensin 4 (Lehrer and Ganz, 2002).

α-defensins are encoded as a prepropeptide. This sequence contains an amino terminal signal sequence, an anionic propiece (which is lacking in ß-defensins) and a carboxy terminal mature piece. Neutrophil α-defensins are synthesized in the bone marrow in the promyelocytes and are packaged into the azurophil granules during the promyelocytic stage of neutrophil development (Borregaard and Cowland, 1997).

α-defensins permeabilize the target cell membranes by inserting into the negatively charged membrane and forming pores (Kagan et al., 1990). If the pores are stable or only expressed transiently is not conclusively studied (Ganz, 2003), neither is the structure of the defensin complex in the membrane. As defensins permeabilize bacterial cell membranes, but not the membranes of the neutrophil granules the suggestion was made that the selectivity is determined by differences in the lipid composition of the target membrane (Hristova et al., 1997).

Under optimal conditions, i.e. low concentrations of divalent cations or plasma proteins, antimicrobial activity is observed against many bacteria, fungi and enveloped viruses at concentrations as low as 1-10µg/ml. Increasing concentrations of salt and plasma inhibit defensin antimicrobial activity (Ganz and Lehrer, 1995).

2.5. Study aim

As described above pulmonary and gastrointestinal *B. anthracis* infections result in sepsis and death, while cutaneous anthrax almost always remains localized. In the human pulmonary form, no ulcerative lesions of the respiratory tract comparable to those found in the skin are observed. Histologically, neither signs of pneumonia are seen, nor any neutrophil infiltration (Albrink et al., 1960)

In untreated cutaneous cases on the other hand, neutrophils surround the necrotic, bacteria-containing tissue, of the ulcerated cutaneous lesion. The neutrophil infiltration penetrates throughout the subcutaneous tissue but stops at the boundary between subcutaneous fat and muscle. There is no abscess formation (Lebowich et al., 1943).

We therefore made the hypothesis that human neutrophils can kill *B. anthracis* efficiently and potentially have a dramatic impact on the outcome of the disease. We examined the interaction of human neutrophils with the infectious spores and also with the vegetative cells. To examine the effects of the anthrax toxin and the capsule separately, we used two different *B. anthracis* strains. The Pasteur strain, which harbors the capsule encoding virulence plasmid, but does not express the toxin; and the Sterne strain, which expresses the toxin but cannot produce the capsule.