4. Results

4.1. Bacteria

During this thesis the interaction of human neutrophils and *B. anthracis* was examined. *B. anthracis* has two forms, the environmentally resistant and infectious spores and the vegetative bacteria. The interaction of neutrophils with both forms was examined. To elucidate the effects of the anthrax toxin and the capsule separately, two different *B. anthracis* strain were used. 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. The cultivation of *B. anthracis* at 42-43 °C results in the selective curing of the cells of the pXO1 plasmid, which encodes the toxin (Pasteur strain). The capsule encoding plasmid pXO2 can be spontaneously lost. In 1939 Max Sterne isolated a pXO1+ pXO2- *B. anthracis* variant, which has been named Sterne or Weybridge strain since (Friedlander et al., 2002).

Wild type *B. anthracis* strains harbour both virulence plasmids and express both the toxin and the capsule. During this study fully virulent *B. anthracis* was not examined due to biosecurity issues. Through the recent postal attacks in the United States and the concern of bioterroristic potential raised since then, we considered the storage of such strains as needless risk.

4.1.1.Spores

Before infecting neutrophils the purity of the spore samples was tested by heat-treating the spores for 45 min at 60°C. Only spores survive this treatment, vegetative cells do not (Figure 1). Clearly the spore samples in these experiments were of high purity as also assessed by light miscroscopy, where the spores are easily recognised by their refractile properties.

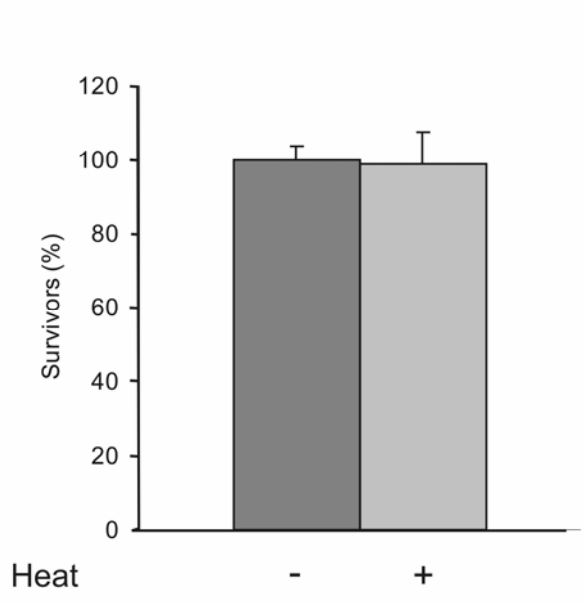

Figure 1: Elucidation of purity of spore preparation. Pasteur strain spores (1 x 10^6/ ml) were heated for 45 min at 60°C or left untreated, serial dilutions made in PBS and the samples plated. Spores survive this treatment whereas the vegetative bacteria do not. Error bars indicate the s.d. from three experiments.

4.1.2. Vegetative bacteria

The purity of the vegetative bacteria was more difficult to assess. When studied under the microscope it was apparent that approximately 5% of the bacteria were spores even in such complex media as brain heart infusion (BHI). In the scanning electron micrograph the spores (S) are ovoid with a rough surface, while vegetative *B. anthracis* (V) are rod shaped, smooth and large (Figure 2). As described before vegetative bacilli tend to form chains (C) in vitro. The bar depicts 2µm.

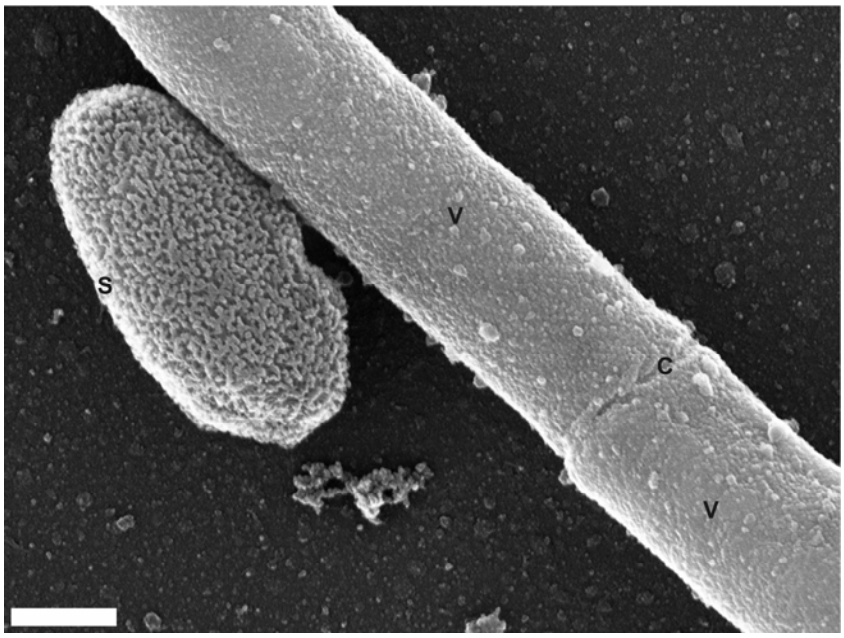

Figure 2: Scanning electron micrograph of *B. anthracis* vegetative cell preparation.
B. anthracis was cultured overnight in BHI supplemented with 0.8% sodium bicarbonate. **S** depicts spore, **V** vegetative bacteria and **C** the chain formation seen in vitro.
Bar indicates 2 µm.

As both the anthrax toxin and the capsule have been described to play a major role in anthrax virulence towards human neutrophils (described in the introduction), it was of importance to verify virulence factor expression. Special growth conditions were devised for toxin and capsule expression (described in materials and methods) and the supernatant of both the Pasteur and the Sterne strains were examined by Western Blot. As seen in Figures 3- 5 only the Sterne strain expresses the oedema factor, lethal factor and the protective antigen, never the Pasteur strain.
The capsule was expressed under the described growth conditions in the Pasteur but not in the Sterne strain. This was examined by India ink stain, but could not be documented due to the restrictions concerning the export of unfixed material from the BSL3 facility.

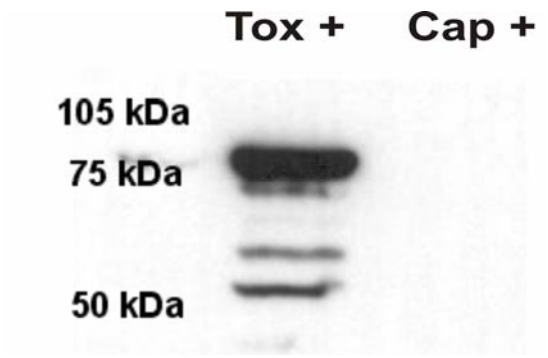

Figure 3: Protective antigen is expressed by the Sterne strain (Tox +) but not by the Pasteur strain (Cap +). Western Blot of TCA precipitated bacterial supernatant.

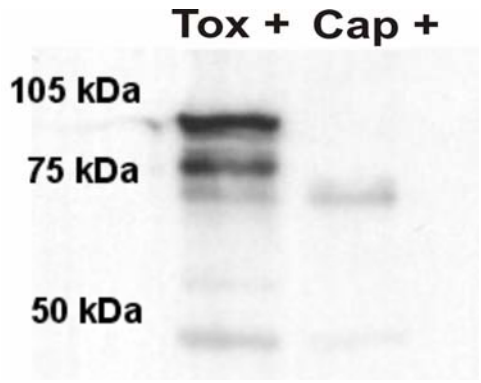

Figure 4: Oedema factor (88 kDa) is expressed by the Sterne strain (Tox+) and not by the Pasteur strain (Cap+). Western Blot of TCA precipitated bacterial supernatant.

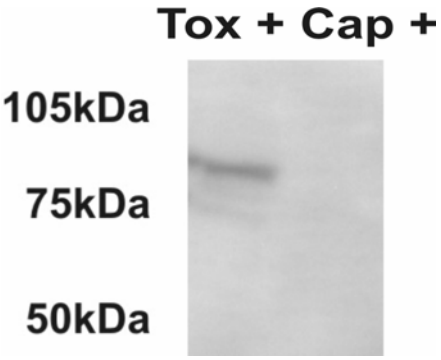

Figure 5: Lethal factor is expressed by the Sterne strain (Tox+) and not by the Pasteur strain (Cap+). Western Blot of TCA precipitated bacterial supernatant.

4.2. Neutrophils

Human neutrophils were isolated as described in materials and methods section. This protocol has been used in Arturo Zychlinsky's laboratory for years and is well established. The neutrophil samples are > 95% pure; contaminants are erythrocytes, lymphocytes and eosinophils (Weinrauch et al., 2002, Brinkmann et al., 2004). Neutrophils require some form of activation, this can be achieved by the addition of PMA (phorbol 12-myristate 13-acetate), or the more physiological chemokine IL-8 (interleukine 8). Both molecules activate the neutrophil protein kinase C, yet the activation status of neutrophils treated with PMA is higher than in IL-8 treated cells. This has the advantage that the cells adhere more efficiently to the surface of the cell culture plates therefore less cells are lost during handling.

Neutrophils are short-lived cells. Once in the cirulation they have an average half-life of 7h. The neutrophils used in these experiments were infected three hours after blood withdrawal. The neutrophil population gained was always heterogeneous as there are different development stages of neutrophils in the blood at all times. Upon activation neutrophils die within a very short period of time. Upon PMA activation this can be accelerated, therefore cell death was routinely monitored by the release of lactate dehydrogenase (LDH). LDH is a stable cytoplasmatic enzyme present in all cells, which is rapidly released into the cell culture supernatant upon damage of the plasma membrane (Weyermann et al., 2005). The higher the concentration of PMA the sooner cell death is induced (Takei et al., 1996). With 25nM PMA the LDH release is increased after four hours as seen in Figure 6. When neutrophils are activated with 5nM PMA LDH release is increased only after six to seven hours incubation time (Figure 7).

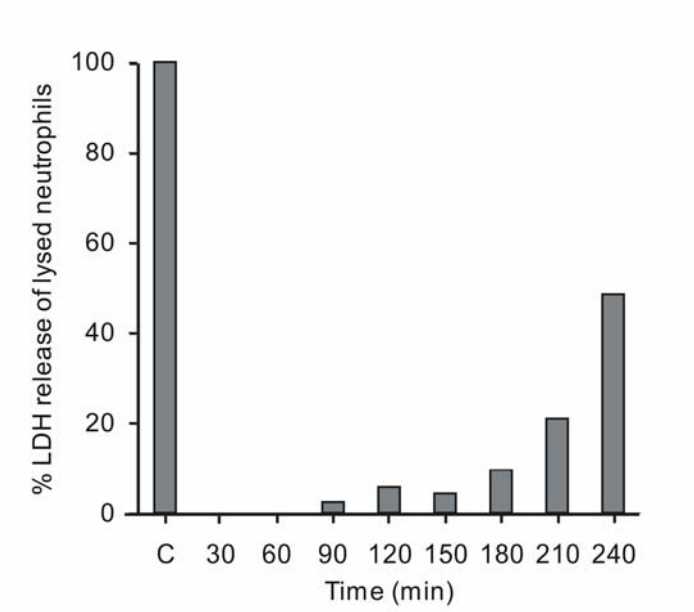

Figure 6: LDH release of PMA (25nM) activated neutrophils infected with Sterne strain spores. Neutrophils were infected at a MOI of 1:1 and the supernatant examined for LDH release at the given time points. Positive control was the supernatant of neutrophils lysed with 1% Triton.

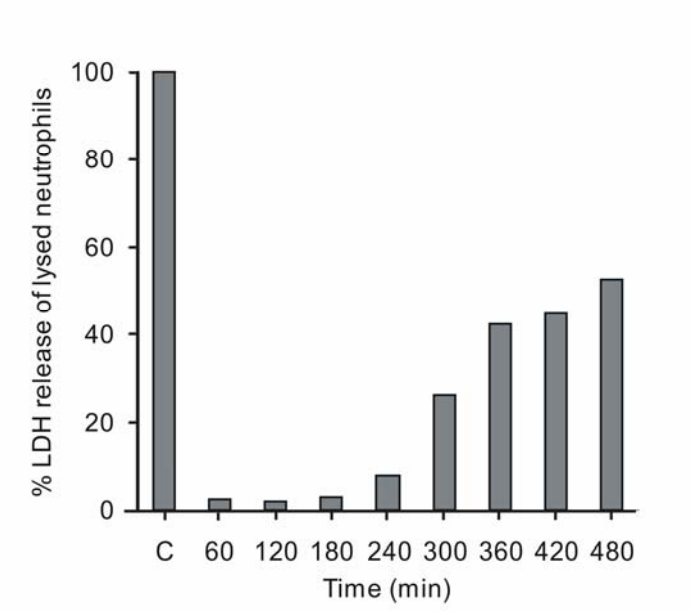

Figure 7: LDH release of PMA (5nM) activated neutrophils infected with Sterne strain spores. Neutrophils were infected at a MOI of 1:1 and the supernatant examined for LDH release at the given time points. Positive control was the supernatant of neutrophils lysed with 1% Triton.

4.3. Neutrophil / *B. anthracis* interaction

4.3.1. Neutrophil / spore interaction

4.3.1.1. Neutrophil killing and phagocytosis

To examine the interaction of human neutrophils with *B. anthracis* spores, the neutrophils were incubated with either the toxin- or capsule-producing strain. Different multiplicities of infection (MOI, spores: neutrophils) were assessed (1:100, 1:10, 1:1 and 10:1). The first two showed similar results as the MOI of 1:1, which was subsequently used in all spore experiments. At an MOI of 10:1 spore killing could still be seen to a slightly lesser extent. Neutrophils were activated with 25nM PMA (phorbol 12-myristate 13-acetate), which results in the activation of protein kinase C and ultimately leads to the induction of the respiratory burst (Liu and Heckman, 1998). These neutrophils killed 50% of spores from either strain after 2.5 h and at four hours only 35% of the spores survived (Figure 8).

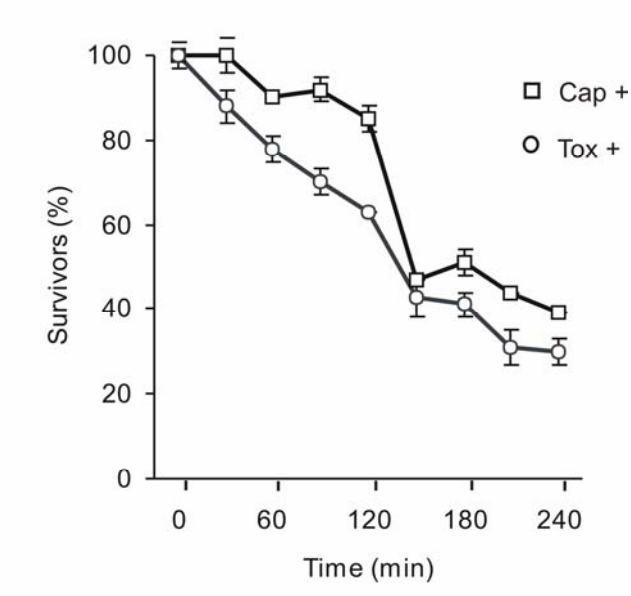

Figure 8: Survival of Pasteur (Cap+) and Sterne (Tox +) strain spores incubated with human neutrophils. Activated neutrophils (25 nM PMA) were infected with spores generated either from toxin (○) or capsule (□) producing strains at an MOI of 1:1 (spore: neutrophil). Colony forming units were counted at indicated time points. Error bars indicate the s.d. from three experiments.

To potentially increase maximum killing, the spores were incubated with human neutrophils for eight hours instead of four. The neutrophils during this experiment were activated with 5nM PMA, instead of the usual 25nM. For this experiment the

neutrophils could not be activated with the higher concentration of PMA as this concentration induces cell death at too early a time point. Under these differing activation statuses the number of spores killed still did not exceed 70% (Figure 9). The reason for this incomplete spore killing is not clear. A possibility is that at the end of the incubation period the neutrophils are in the process of dying and can no longer efficiently kill the spores. In vivo this should not pose a problem, as during inflammation many neutrophils are constantly recruited to the site of infection and new waves of neutrophils can target the spores.

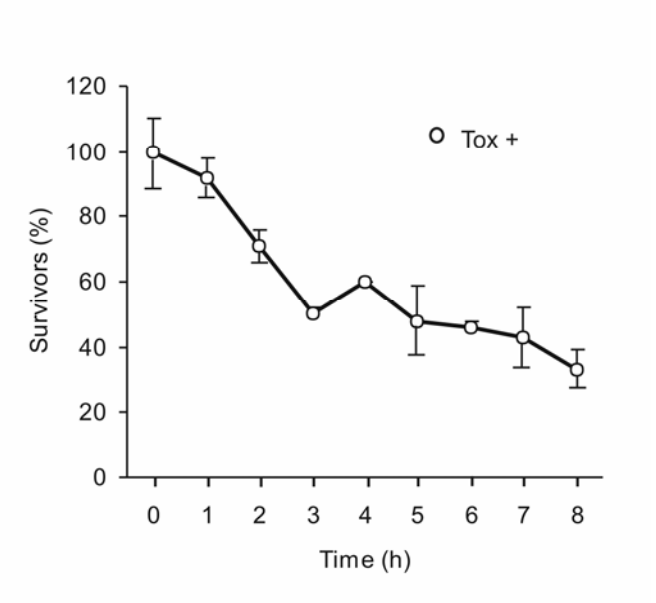

Figure 9: Survival of Sterne (Tox+) strain spores exposed to human neutrophils. Activated neutrophils (5 nM PMA) were infected with spores generated from toxin (○) producing strain at an MOI of 1:1. Colony forming units were counted at indicated time points, maximum incubation time was 8 h. Error bars indicate the s.d. from three experiments.

To assess phagocytosis of *B. anthracis* spores by neutrophils, the human cells were activated with 25nM PMA and infected with spores of the Pasteur or Sterne strain at an MOI of 1:1. As examined by transmission electron microscopy, the spores were taken up by the cells as early as 30 min after infection (Figure 10). The arrows depict *B. anthracis* spores in a membrane bound vacuole, the size bar indicates 10 µm.

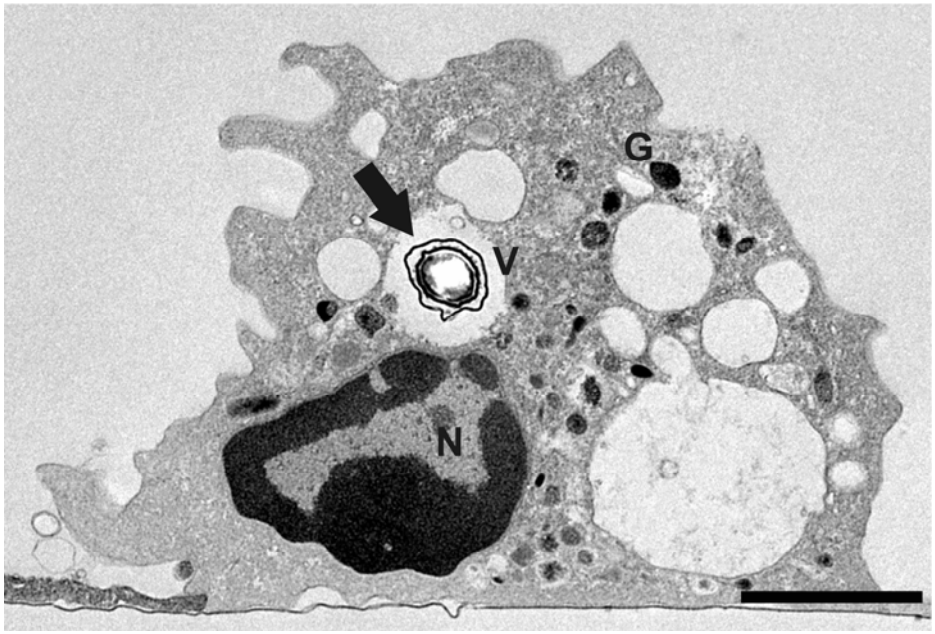

Figure 10: Transmission electron micrograph of Sterne strain spore phagocytosed by neutrophil. Activated (25 nM PMA) neutrophils were infected with spores of the toxin producing strain for 30 min at an MOI of 1:1. At this time point only spores (arrow) were present. **N** depicts the nucleus, **V** a membrane bound vacuole and **G** the neutrophil granules. The bar indicates 10 µm.

The number of spores found within and outside the neutrophils was counted in transmission electron micrographs. 57% of all spores were found intracellularly, whereas 43% were in the extracellular space (n (number of spores) = 104). This relative efficient phagocytosis was surprising, as the spores had not been opsonised prior to infection. Despite neutrophile activation, opsonization usually increases the phagocytosis rate. Opsonization is a process where foreign particles, such as bacteria, are coated with self-derived substances, which signal phagocytes that the particle should be ingested. Important opsonins are complement factors, plasma-derived factors such as LPS binding proteins (LBP) and IgG antibodies, which are all found in serum (Janeway et al., 2001). The spore killing efficiency of neutrophils needed to be tested in serum free media, i.e. without opsonization, since some sera induce germination (Ireland and Hanna, 2002) of spores.

Neutrophils can kill microbes through reactive oxygen species (ROS) or antimicrobial peptides or proteins. ROS are generated by the NADPH oxidase and are important in some bacterial infections. To test the role of ROS in *B. anthracis* killing, the NADPH oxidase was inhibited with diphenyleneiodonium (DPI) (Cross and Jones, 1986), an irreversible inhibitor of flavoproteins. The efficiency of the NADPH inhibition

could be assessed with a chemiluminescence assay, which monitors the amount of superoxide anion production. When neutrophils were activated with PMA, the superoxide anion production increased dramatically. But when the cells were pretreated with DPI, the radical production was greatly reduced. PMA (25 nM) activated neutrophils were incubated in the presence or absence of DPI and chemiluminescence was monitored in a BD Pharmagen TM, Monolight 3096 microplate luminometer in the presence of luminol (Figure 11).

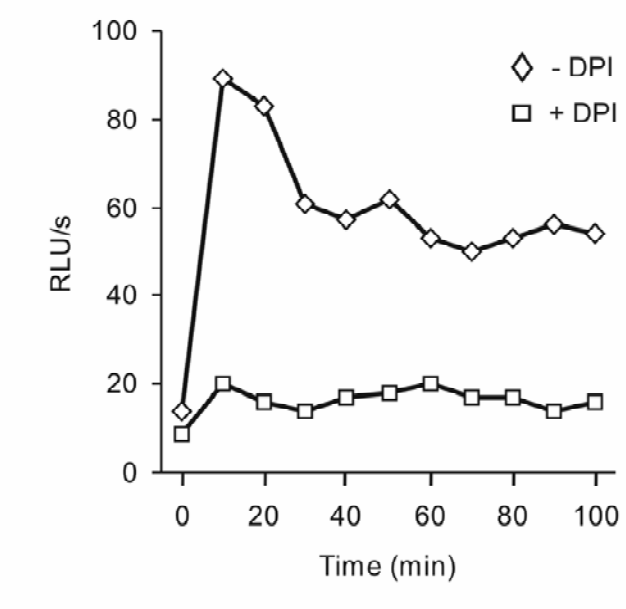

Figure 11: Inhibition of respiratory burst in neutrophils by DPI measured in relative light units per second. PMA (25 nM) activated neutrophils were incubated in the presence (□) or absence (◊) of DPI and chemiluminescence monitored in a BD Pharmagen TM, Monolight 3096 microplate luminometer in the presence of luminol.

Neutrophils pretreated with DPI and then activated with PMA (25nM) were infected with either Pasteur or Sterne strain. Independent of the production of ROS 70% of all spores were killed by the neutrophils after 4h (Figure 12, data not shown). This indicates that the antimicrobial activity is independent of the NADPH oxidase and that reactive oxygen species are not required for the killing of *B. anthracis* spores.

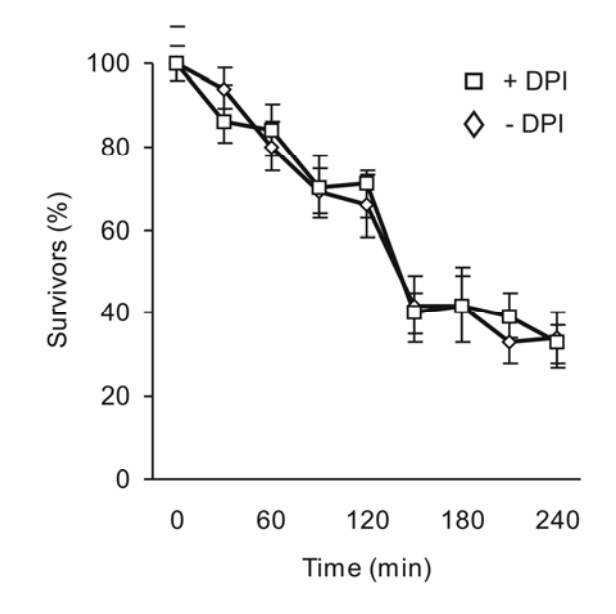

Figure 12: Reactive oxygen species (ROS) are not required for killing of *B. anthracis* spores. Activated neutrophils (25 nM PMA) were infected with spores (capsule producer) at an MOI of 1:1 in the presence (□) or absence (◊) of NADPH oxidase inhibitor DPI. Colony forming units were counted at indicated time points. Error bars indicate the s.d. from three experiments.

4.3.1.2. Human neutrophil granule extract killing

Since ROS were irrelevant, we tested whether the second arm of the neutrophil arsenal, the antimicrobial proteins, are effective against *B. anthracis*. Spores were incubated with a human neutrophil granule extract enriched in granule proteins (hNGE). This extract is made by isolating neutrophils, isolating and destroying the granules and with sulphuric acid extracting proteins from the sample. This leads to the production of a highly complex protein and peptide mixture, which encompasses all the antimicrobially active proteins, proteases and cationic peptides found in all three neutrophil granule subsets.

Spores from the toxin-producing strain were incubated in 50% hNGE or in PBS for the indicated time points (Figure 12). One hundred percent survival refers to the number of bacteria present in the buffer control. Even at these extremely high concentrations of hNGE, the spores remained viable throughout a four-hour incubation period. The spores did not germinate during this time as was examined under the light microscope.

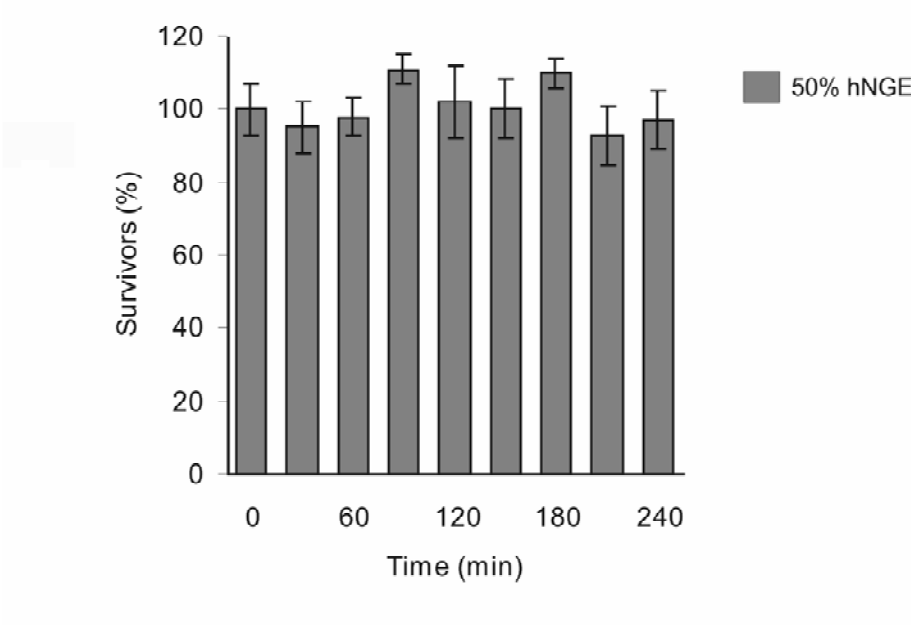

Figure 12: Human neutrophil granule extract (hNGE) does not kill *B. anthracis* spores. Spores from the toxin-producing strain were incubated in 50% hNGE or in buffer for the indicated time points. The CFU was determined by serial dilution. One hundred percent survival refers to the number of bacteria present in the buffer control. Error bars indicate the s.d. from three experiments.

4.3.1.3. Germination of spores in neutrophils

As neither the oxygen dependent nor the oxygen independent neutrophil killing mechanism had any effect on *B. anthracis* spore viability the following hypothesis was tested. As described in the introduction, *B. anthracis* spores can germinate inside macrophages within 30 min after infection. This and the findings described above, prompted us to test whether spores also germinate in neutrophils and whether the vegetative forms are then susceptible to neutrophil killing. Germination of *B. anthracis* in neutrophils was analysed by transmission electron microscopy. Neutrophils were infected with spores at an MOI of 1:1 and transmission electron micrographs made after every half an hour. After a 90 min infection period, a new structure could be observed in the cells. In Figure 13 a neutrophil infected with *B. anthracis* spores can be seen. The nucleus (N) in neutrophils is usually multilobulated, but due to the cutting of the section the lobes cannot be identified. The morphologically distinct granule subsets can be identified (G), as can the cell membrane (M). Within the cytoplasm the arrow depicts a spore, the arrowhead the newly germinated cell.

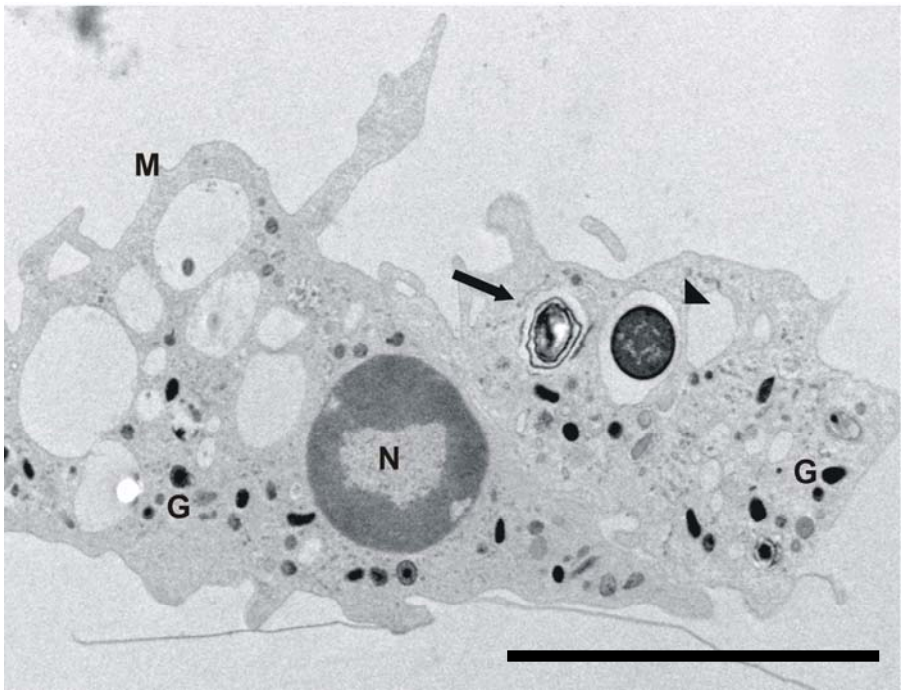

Figure 13: *Bacillus anthracis* spores germinate within human neutrophils. TEM of neutrophil infected with spores of the toxin producing strain for 90 min at an MOI of 1:1. At this time point both spores (arrow) and germinated bacteria (arrowhead) were present. **N** depicts the nucleus, **G** the neutrophil granules and **M** the cell membrane. The bar indicates 5 µm.

B. anthracis spores can be recognized by the exosporium (E), the 'empty 'space (S) between the exosporium and the coat (C) and the typical mother of pearl appearance in the electron microscope (Figure 14). Germinated cells no longer show these features, but instead there is DNA (D) apparent in cells bound by a thick cell membrane. Both spores and vegetative bacteria were found in membrane bound vacuoles (M), most likely phagosomes. The bar depicts 5µm in Figure 13 and 500nm in Figure 14.

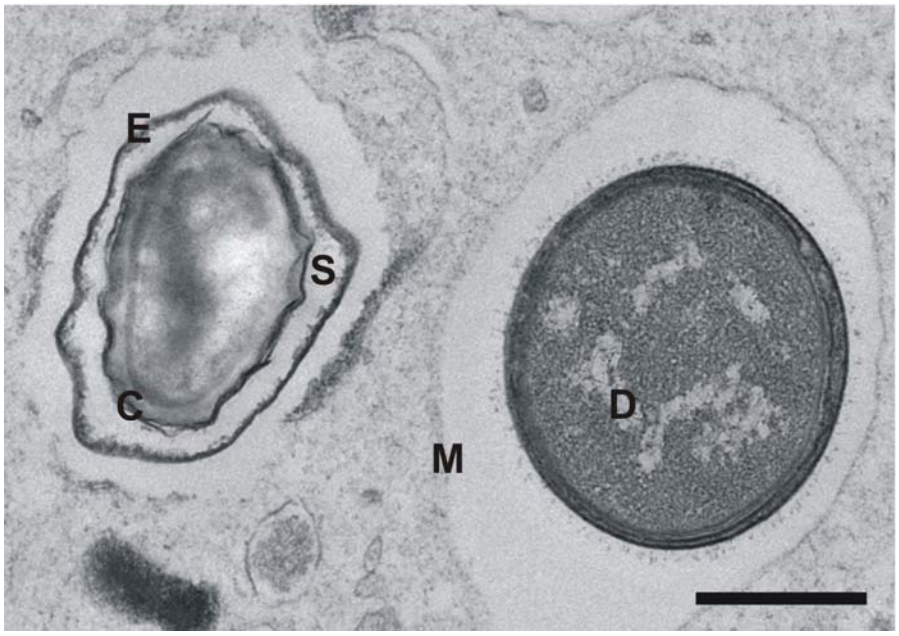

Figure 14: *B. anthracis* spores germinate in neutrophils after a 90 min incubation period. Amplification of the spore and vegetative bacterium. **E** depicts the exosporium of the spore, **S** the empty space between the exposporium and the coat (**C**). The bar indicates 500 nm.

By 180 min neutrophils contained mostly germinated bacteria as depicted by the arrow heads in Figure 15. Intriguingly, the germination process does not seem synchronised, as spores were found within the neutrophils throughout the infection period of 240 min. Whether the spores remain unresponsive to cell specific germinants, or whether the neutrophils life span is too short to induce complete germination remains to be examined. This observation could also explain the occurrence of the incomplete spore killing described in Figures 8 and 9. Whether the vegetative cells resporulate within the neutrophils, a phenomenon seen in the gut of animals infected with *B. cereus*, is discussed in the Discussion section.

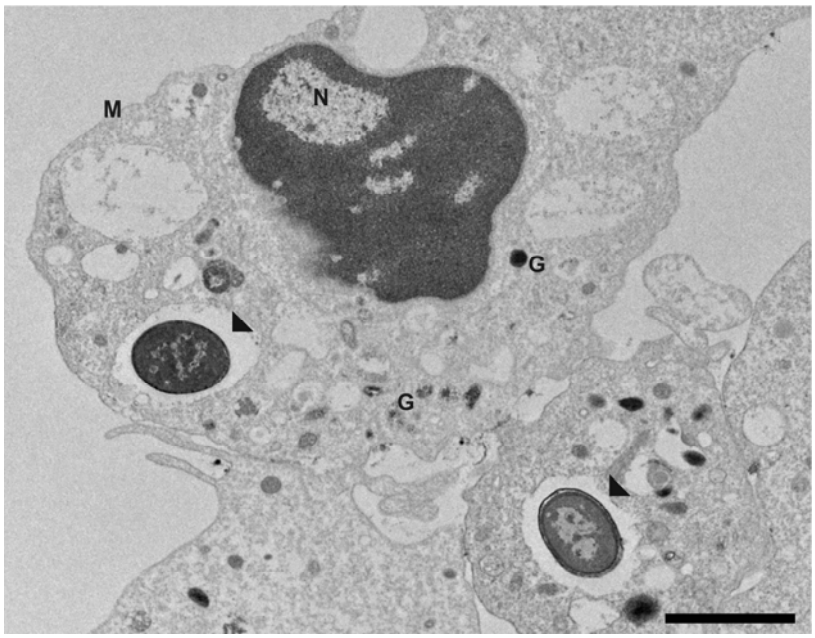

Figure 15: *B. anthracis* spores germinate in neutrophils after a 180 min incubation period. TEM of neutrophil infected with spores of the toxin producing strain for 180 min at an MOI of 1:1. At this time point neutrophils contained mostly germinated bacteria (arrowheads). **N** depicts the nucleus, **G** the neutrophil granules and **M** the cell membrane.
The bar indicates 2 µm.

4.3.2. Neutrophil/ vegetative bacteria interaction

As the *B. anthracis* spores germinated and formed vegetative cells within the neutrophils, it was of interest to determine whether vegetative *B. anthracis* are killed by neutrophils. Activated neutrophils were infected with *B. anthracis* expressing either the toxin or the capsule. At an MOI (vegetative bacteria: neutrophil) of 1:100, 1:10 and 1:1, more than 95% of the toxin producing strain and > 90% of the capsule producers were killed. Time course experiments were done at an MOI of 10:1 in order to see time dependent killing. All 30 min experiments were done at an MOI of 1:1.

At an MOI of 10:1, the vegetative bacteria (V) were phagocytosed in spite of their large size after 30 min infection time (Figure 16). The neutrophils (N) show great plasticity to be able to phagocytose multiple large bacteria (arrow). It is to be noted that the vegetative bacteria can be as long as a human lymphocyte cell. The round structure in the picture is a lymphocyte (L). The bar indicates 5µm.

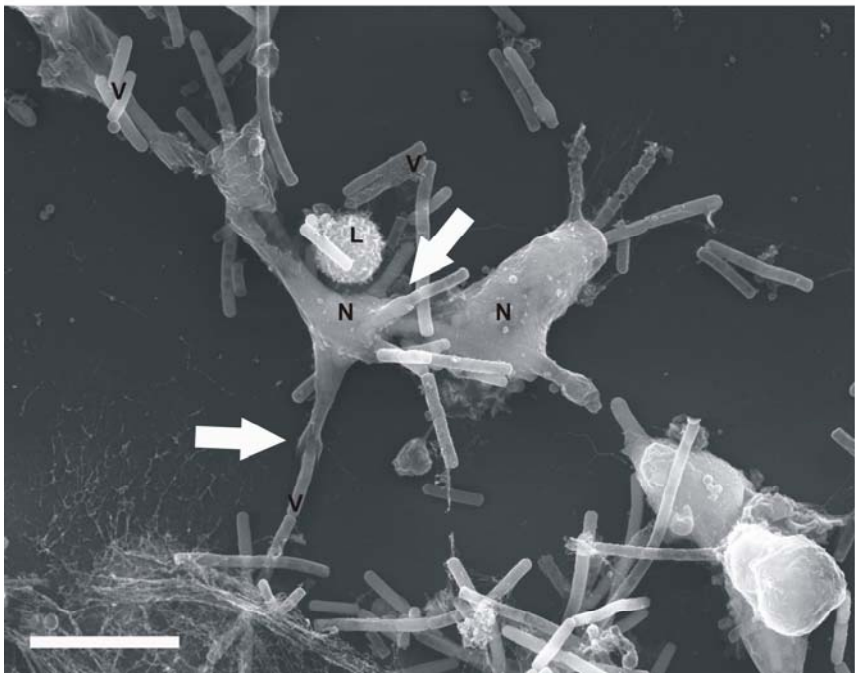

Figure 16: Scanning electron micrograph of neutrophils infected with vegetative form of the toxin producing strain for 30min. Neutrophils efficiently engulf *B. anthracis* despite their size. **N** depicts neutrophil, **L** lymphocyte and **V** vegetative bacteria. Bar = 5µm.

PMA activated neutrophils were infected with capsule or toxin expressing vegetative bacteria for indicated time points at an MOI of 10:1. Three hours post infection the activated neutrophils killed more than 90% of the toxin producing bacteria and 80% of the capsule producers (Figure 17). When the neutrophils were activated with IL-8, both strains were killed as efficiently as with PMA activated cells (Figure 18). The expression of the poly D-glutamic acid capsule does seem to have a minimal effect on neutrophil killing efficiency. Complete killing of all bacteria was never seen, probably due to spore contaminants of the vegetative sample.

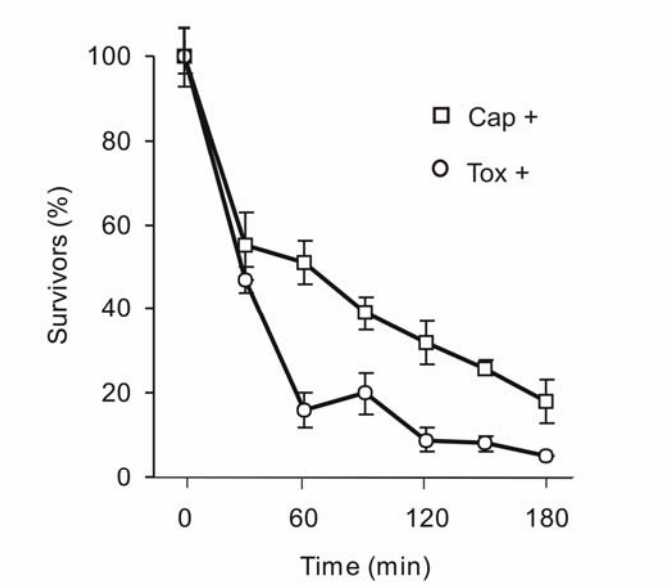

Figure 17: Neutrophils kill both *B. anthracis* strains efficiently. Activated neutrophils (25 nM PMA) were infected with capsule (◊) or toxin (○) expressing vegetative bacteria for indicated time points at an MOI of 10:1. The CFU was determined by serial dilution Error bars indicate the s.d. from three experiments.

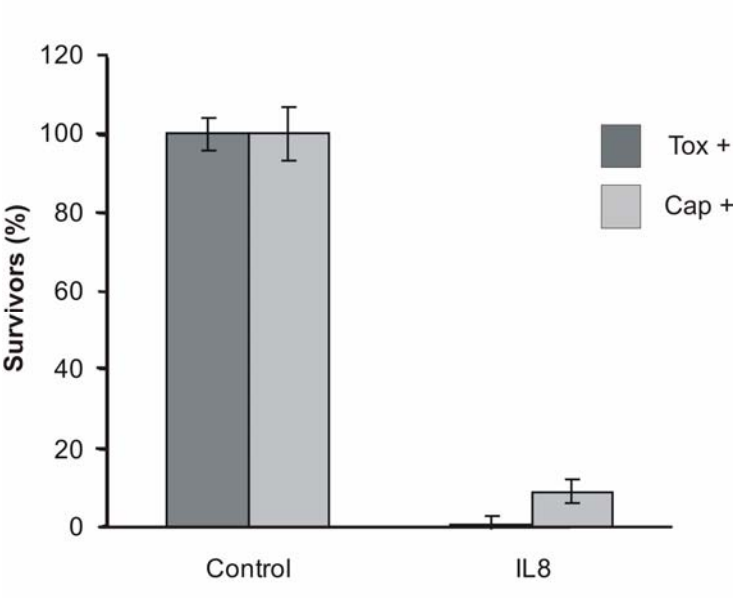

Figure 18: Neutrophils kill both *B. anthracis* strains efficiently when activated with IL-8 (10 ng/ml). Activated neutrophils were infected with capsule (light grey) or toxin (dark grey) expressing vegetative bacteria for indicated time points at an MOI of 1:1. Controls are bacteria in media alone. The CFU was determined by serial dilution. Error bars indicate the s.d. from three experiments.

To quantify the number of bacteria phagocytosed and killed intracellulary, the neutrophils were treated with cytochalasin D prior to infection. Cytochalasin D induces depolymerization of the actin cytoskeleton and thus inhibits the ability of the neutrophils to phagocytose particles (Cross and Jones, 1986). Neutrophils activated either with PMA or IL-8 were infected at an MOI of 1:1 for 30 min. When the neutrophils were pre-treated with cytochalasin D it became clear that only approximately 20% of the bacteria were killed intracellulary in the PMA activated neutrophils, yet approximately 40% in the IL-8 activated cells (Figure 19). This could be explained by the fact that PMA is a more potent activator, which also leads to an increased release of granule proteins and ROS into the extracellular space.

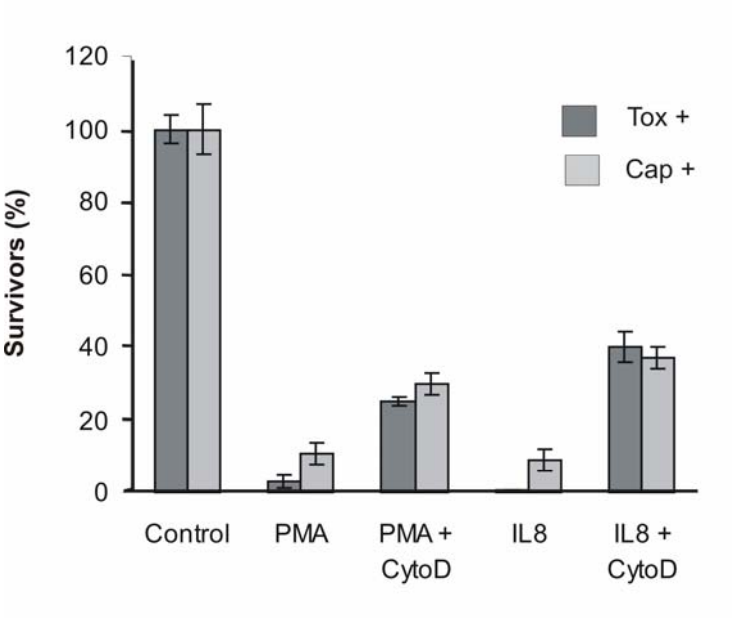

Figure 19: *B. anthracis* is killed by both extracellular and intracellular mechanisms. Neutrophils were pretreated with cytochalasin D and either activated with PMA (25 mM) or IL-8 (10 ng/ ml). The cells were infected for 30 min at an MOI of 1:1. Controls are bacteria in media alone. The CFU was determined by serial dilution. Error bars indicate the s.d. from three experiments

Neutrophils were infected with capsule and toxin producing strains at an MOI of 1:1 in the presence or absence of serum for 30 min. Interestingly, opsonisation did not affect the rate of killing of either capsule- or toxin-expressing vegetative bacteria (Figure 20), indicating that activated neutrophils potentially recognize *B. anthracis* directly. The other possibilities are that the PMA treated neutrophils are highly activated and no longer need any stimuli for phagocytosis. Further, some of the

neutrophil granule proteins such as BPI have opsonic properties and are exocytosed into the extracellular space upon activation, potentially rendering serum addition useless.

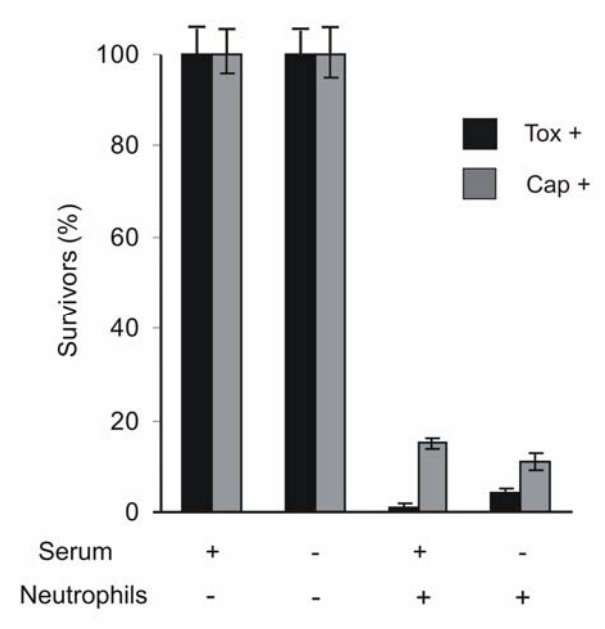

Figure 20: Opsonisation is not required for neutrophil anti-*B. anthracis* activity. Neutrophils were infected with capsule (gray bar) and toxin (black bar) producing strains at an MOI of 1:1 in the presence or absence of serum for 30 min. Controls are bacteria in media alone. Error bars indicate the s.d. from three experiments.

To elucidate the mechanism of how neutrophils kill vegetative bacteria the NADPH oxidase activity was inhibited. Neutrophils kill both capsule and toxin producing *B. anthracis* independently of NADPH oxidase activity. Activated neutrophils were incubated with DPI (10 µM) before infection. Bacterial counts were determined 30 min post infection at an MOI of 1:1. In contrast to the Gram-positive bacterium *Staphylococcus aureus* which is killed in a ROS dependent manner (Kristian et al., 2003), both toxin- and capsule-producing strains were killed in the absence of ROS (Figure 21).

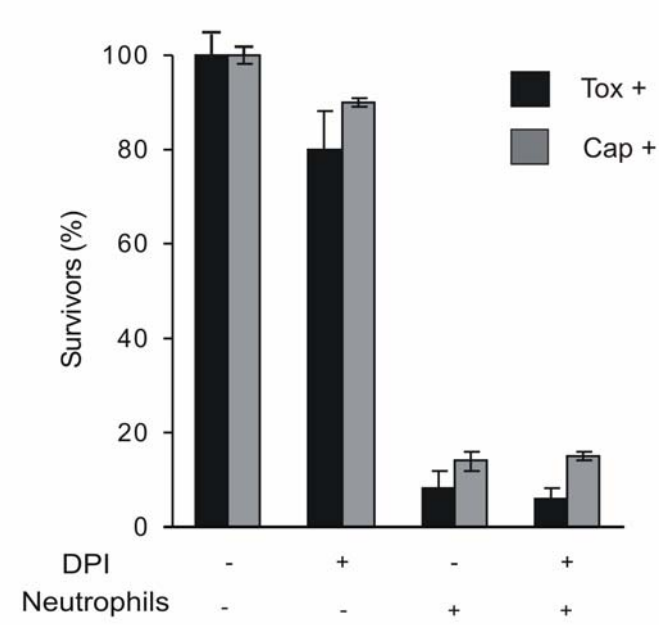

Figure 21: Neutrophils kill both capsule (gray bar) and toxin (black bar) producing *B. anthracis* independently of NADPH oxidase activity. Activated neutrophils (25 nM PMA) were incubated with DPI (10 µM, 30 min) before infection. Bacterial counts were determined 30 min post infection (MOI 1:1). Error bars indicate the s.d. from three experiments.

Therefore, we investigated whether *B. anthracis* is killed by granule antimicrobials. The toxin and capsule producing strain were incubated with 1 and 3% hNGE. At the indicated time points, remaining CFU were determined and referred to the number of bacteria in controls of bacteria incubated in buffer. Both *B. anthracis* strains were surprisingly susceptible to low concentrations of hNGE (Figure 22). The *B. anthracis* culture was never completely killed by the hNGE, presumably due to the contamination of the bacterial culture with spores.

To compare the susceptibility of *B. anthracis* to hNGE, another Gram-positive and a Gram-negative bacterium were subjected to hNGE treatment. 10% hNGE only killed 50% of the Gram-positive *Staphylococcus aureus* bacteria as seen in Figure 23. Even the Gram-negative *Shigella flexneri* were not as susceptible to the neutrophil granule proteins (Figure 24).

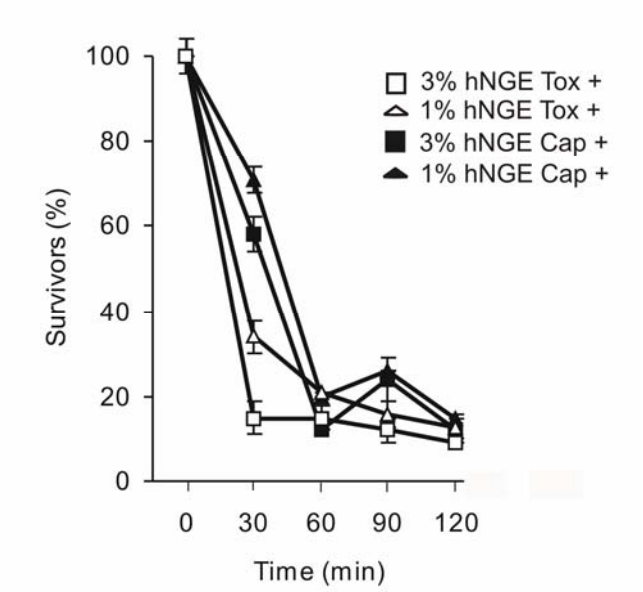

Figure 22: hNGE kills both *B. anthracis* strains. Toxin (closed symbols) and capsule producing strain (open symbols) were incubated with 1 (△) and 3 (□)% hNGE. At the indicated time points, remaining CFU were determined and referred to the number of bacteria in controls of bacteria incubated in buffer. Error bars indicate the s.d. from three experiments.

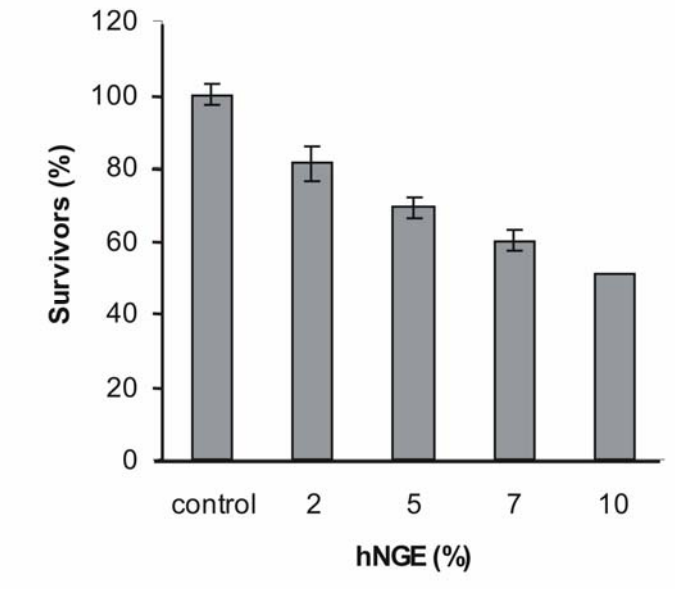

Figure 23: High hNGE concentrations kill *Staphylococcus aureus. Staphylococcus aureus* were incubated with different hNGE concentrations. After 30 min, remaining CFU were determined and referred to the number of bacteria in controls of bacteria incubated in buffer. Error bars indicate the s.d. from three experiments.

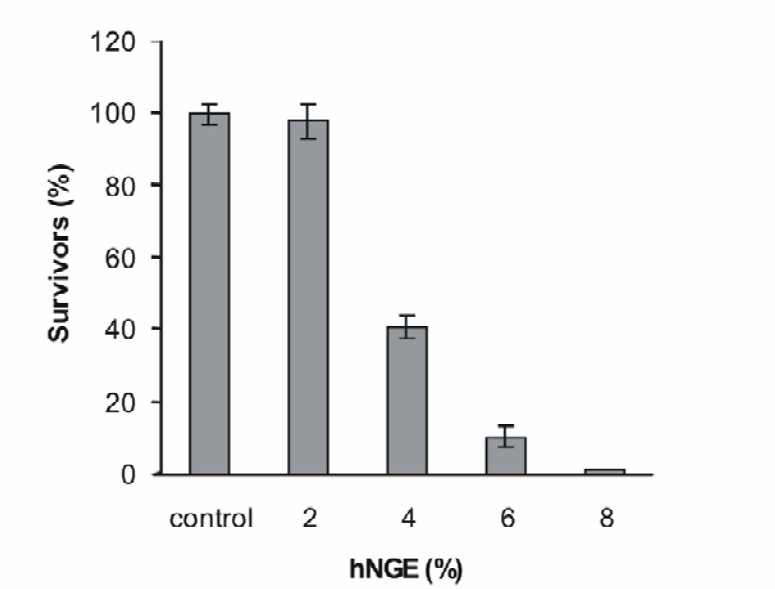

Figure 24: hNGE kill *Shigella flexneri* efficiently. *Shigella flexneri* were incubated with different hNGE concentrations. After 30 min, remaining CFU were determined and referred to the number of bacteria in controls of bacteria incubated in buffer. Error bars indicate the s.d. from three experiments.

4.4. Analysis of anti-*B. anthracis* activity

As described the neutrophil granule extract is a complex mixture of proteins and peptides found in the azurophil, specific and gelatinase granules of the neutrophils. When run on a 12% SDS Page gel and stained with silver the complexity becomes apparent as seen in Figure 32. When run on a 2D gel it became obvious that a great number of proteins and peptides are highly cationic, having an isoelectric point of above 8 (Figure 25).

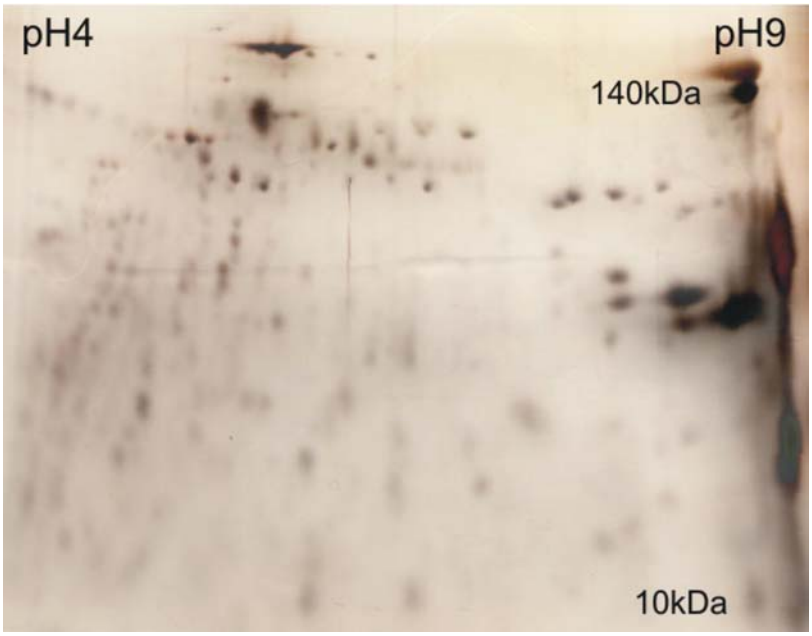

Figure 25: 2D gel of human neutrophil granule extract.

In order to isolate the component or components of the neutrophil granule extract which is responsible for the *B. anthracis* killing, the hNGE was fractionated using high performance liquid chromatography (RP-HPLC). The idea was to test the *B. anthracis* killing activity of each fraction and if anti-*B. anthracis* was found, re-emit this fraction onto another column and so purify the killing activity. This was done until the large number of proteins of the granule extract was reduced to a few bands on a silver 1 D gel. The remaining bands could then be identified by mass spectrometry.

As many of the granule extract proteins are cationic and most of the known antimicrobially active proteins, such as BPI, lysozyme and Cap18 have a high isoelectric point, the isolation began with a cationic HPLC column. Figure 26 shows a superimposition of the chromatogram of the neutrophil granule extract run on a cation exchange HPLC column and the antimicrobial activity of each HPLC fraction. The fractionation of the hNGE was promising, yet when the fractions were tested for anti-*B. anthracis* activity it became apparent that the salt used to elute the proteins off the HPLC column posed a problem. The increasing antimicrobial activity corresponded to the increasing salt concentration.

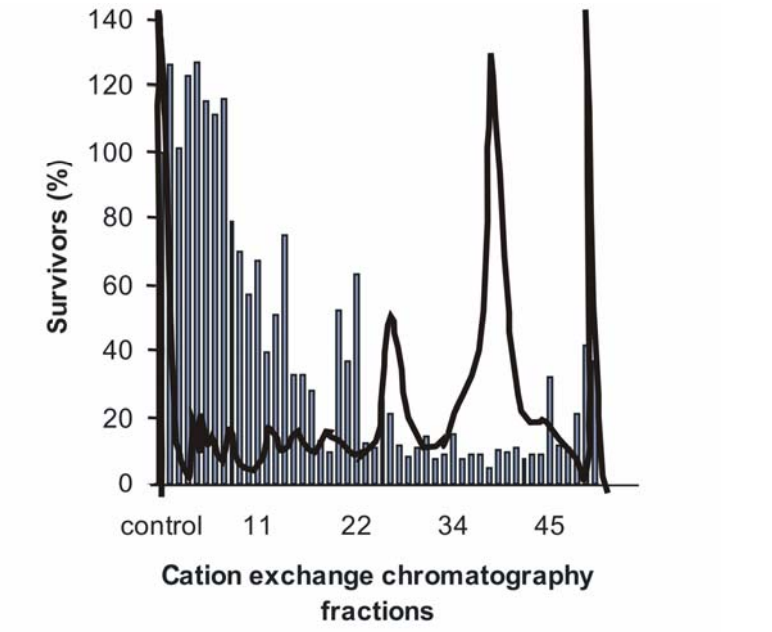

Figure 26: Purification of the anti-*B. anthracis* activity in hNGE. Superimposition of cation exchange HPLC chromatogram and killing activity of HPLC fractions. hNGE was resolved in a cation exhange HPLC column and the fractions were tested for their killing activity of *B. anthracis* (bars).

When tested, the vegetative form of *B. anthracis* was found to be very salt sensitive, even 100mM NaCl affected viability by more than 20% (Figure 27).

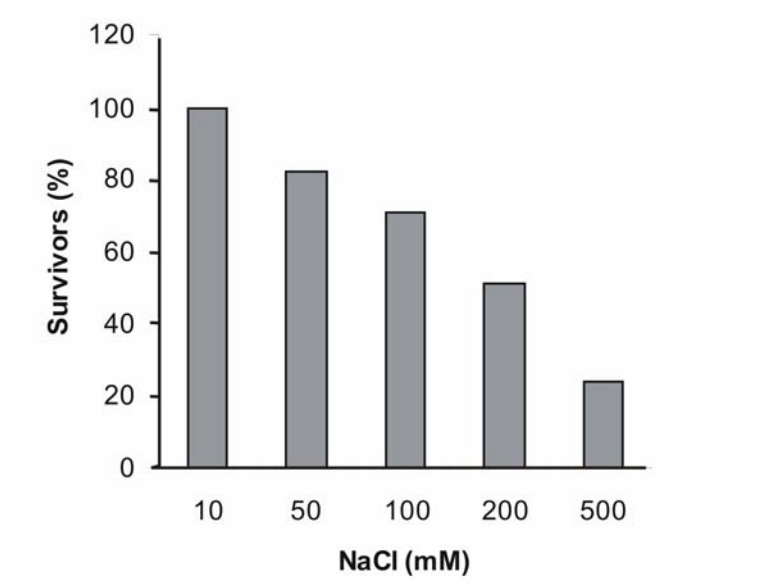

Figure 27: Vegetative *B. anthracis* are susceptible to killing with NaCl. The Pasteur strain (1 x 10^6/ml) was incubated with different NaCl concentrations. After 30 min, remaining CFU were determined and referred to the number of bacteria in controls of bacteria incubated in buffer.

The fractions were thus subjected to a concentration and desalting step using Microcon spin columns with a cut off level of 3 kDa. When the protein content was compared before and after the spin column step it became apparent that the proteins stuck to the spin column.

Another option for reducing salt concentration was to subject all fractions of the first cationic column to a C4 reverse phase column. The fractions containing protein were tested for antimicrobial activity, yet no sufficient activity could be found. This was put down to a low protein concentration after the two isolation steps.

Table 1 shows the different columns and conditions and possible combinations used to isolate the protein(s) responsible for the *B. anthracis* killing.

Table 1: HPLC conditions used for isolation of anti- *B. anthracis* activity

1. column/ mobile phase	**2. column**
Cation exhange/ 0.5M NaCl	
Cation exchange/ 1M ammonium acetate	
Cation exchange/ 0.5M NaCl	Reverse phase C4
Cation exchange/ 0.5M NaCl	Reverse phase C18
Reverse phase C4 (pH2, 4, 5, 6)	
Reverse phase C4 (varying acetonitril gradients	
Reverse phase C4	Cation exchange
Reverse phase C4	Reverse phase C18
Reverse phase C4	Reverse phase C4
Reverse phase C18	Reverse phase C4

In the end the most successful HPLC column combination was a C4 column (mobile phase of pH2) followed by a C4 column with a different mobile phase (pH5) followed by a C18 RP-HPLC column (Figures 28-30).

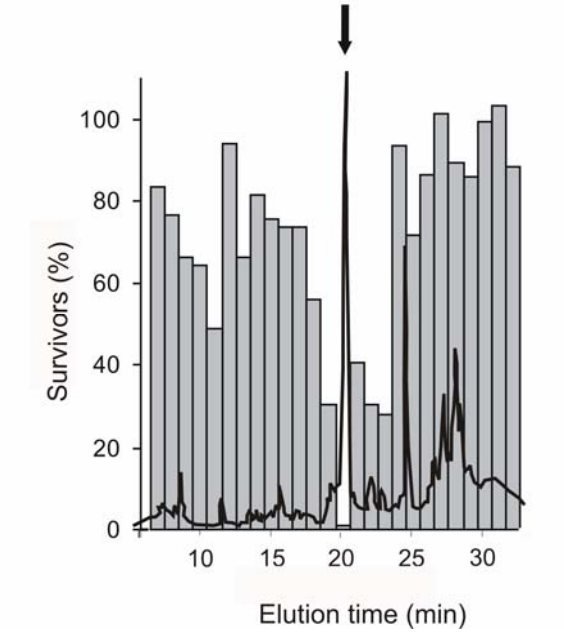

Figure 28: Purification of the anti-*B. anthracis* activity in hNGE. Superimposition of C4 RP-HPLC (pH2) chromatogram and killing activity of HPLC fractions. hNGE was resolved in a C4 RP-HPLC (pH2) column and the fractions were tested for their killing activity of *B. anthracis* (bars). The main chromatographic peak coincides with the *B. anthracis* killing activity (arrow).

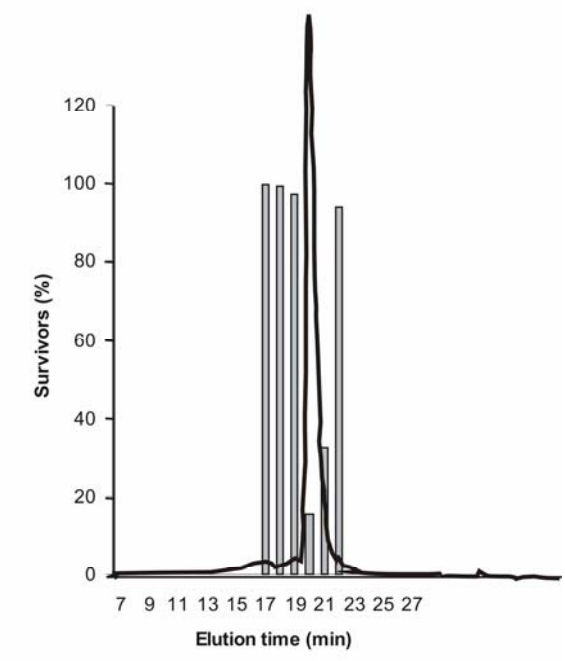

Figure 29: Purification of the anti-*B. anthracis* activity in hNGE. Superimposition of C4 RP-HPLC (pH5) chromatogram and killing activity of HPLC fractions. hNGE was resolved in a C4 RP-HPLC (pH5) column and the fractions were tested for their killing activity of *B. anthracis* (bars). The large chromatographic peak coincides with the *B. anthracis* killing activity.

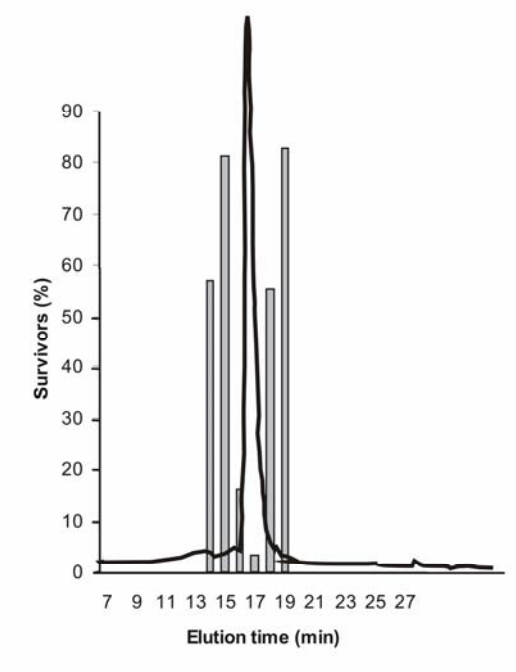

Figure 30: Purification of the anti-*B. anthracis* activity in hNGE. Superimposition of C4 RP-HPLC (pH5) chromatogram and killing activity of HPLC fractions. hNGE was resolved in a C4 RP-HPLC (pH5) column and the fractions were tested for their killing activity of *B. anthracis* (bars). The main chromatographic peak coincides with the *B. anthracis* killing activity.

Tricine SDS-PAGE analysis of the antimicrobially active fraction after the last isolation step showed a single peptide migrating at 3-4kDa (Figure 31)

Figure 31: Silver stained SDS Page gel of human neutrophil granule extract and *B. anthracis* killing fraction after C4/C4/C18 RP-HPLC isolation

The active component was identified by MALDI MS peptide mass fingerprinting (PMF) and MS/MS data of five peptides as human neutrophil α-defensin (Figure 32). Peak heights of molecules not belonging to the identified protein were below 3%, implying high purity of the sample. Only the one-fold and the two-fold charged molecule peaks were detected (Figure 33) in the MS spectrum of the non-trypsinised peptide, confirming purity of the sample.

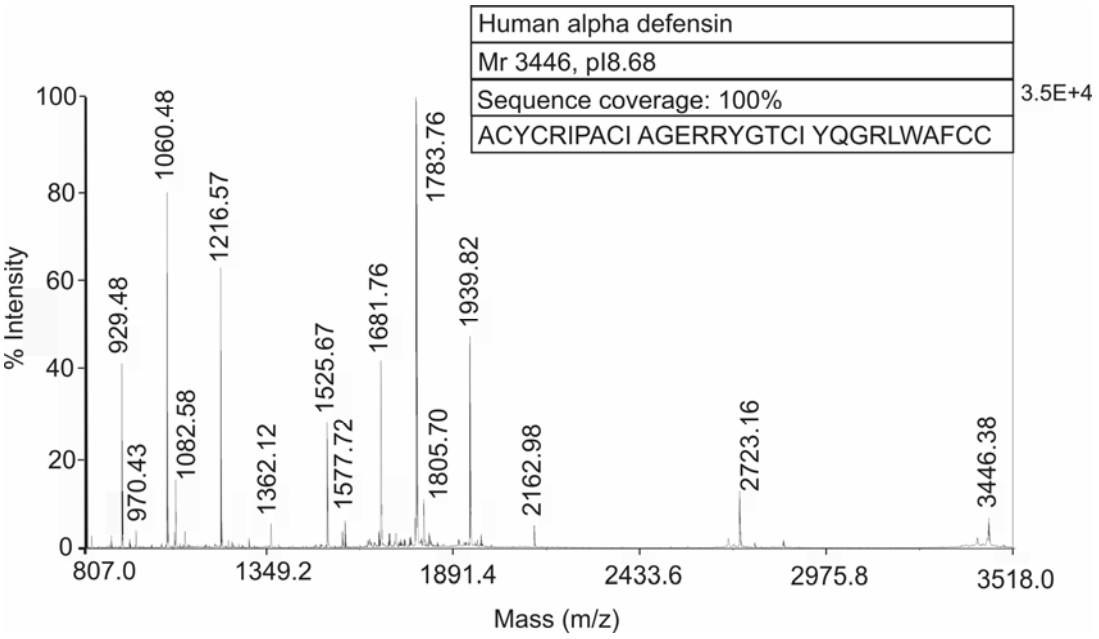

Figure 32: MALDI analysis of C4/C4/C18 fraction. The active component was identified by MALDI MS (mass spectrometry) peptide mass fingerprinting (PMF) as human neutrophil α-defensins. Combined MASCOT search of PMF and MS/MS data (five MS/MS spectra) resulted in a Score value of 313. Peak heights of molecules not belonging to the identified protein were below 3%.

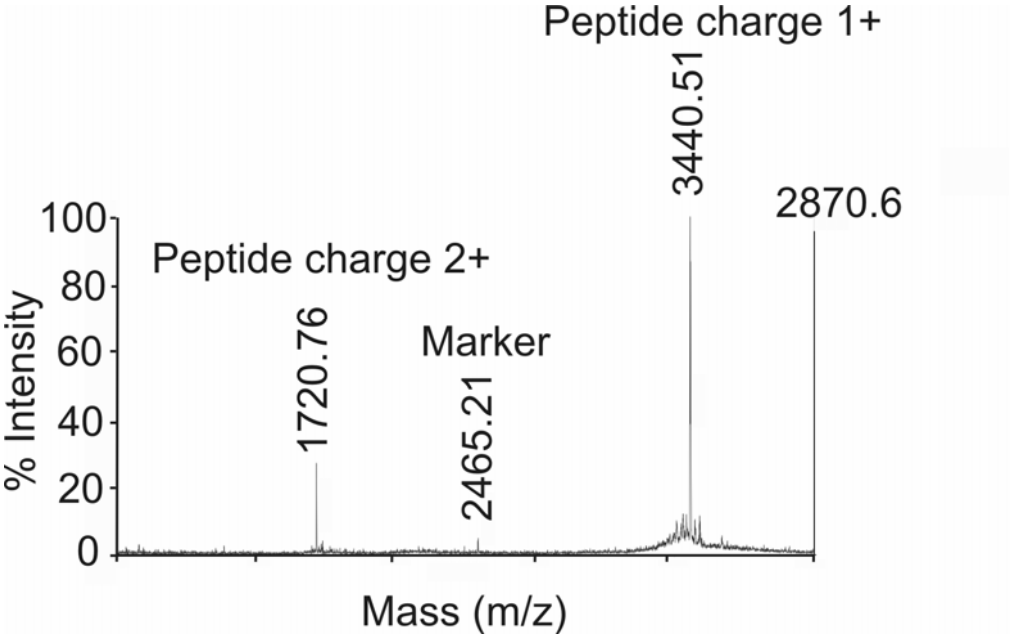

Figure 33: Purity of the C4/C4/C18 sample was confirmed by the analysis of the uncleaved peptide. As the protein was analyzed in non-reducing conditions it is present in its oxidized form reducing the Mr from 3446 to 3440.

To confirm that α-defensins are responsible for neutrophil killing of *B. anthracis*, the neutrophil granule extract was depleted of α-defensins by immunoprecipitation with an α-defensin 1-3 antibody. Again the cationicity of the sample proved a problem, as under the conditions used, the pull down was never specific. 'IP beads' refers to the acrylamide beads after the pull down, where theoretically only the antibody and the defensins should adhere. Yet, beside the heavy and the light chain of the antibody, more than one band can be seen in the silver gel. Further the irrelevant antibody (PA) also pulled down a 3-4 kDa peptide (Figure 34). The neutrophil granule extract, which theoretically should have been specifically depleted of α-defensins (IP) is obviously depleted of many more proteins.

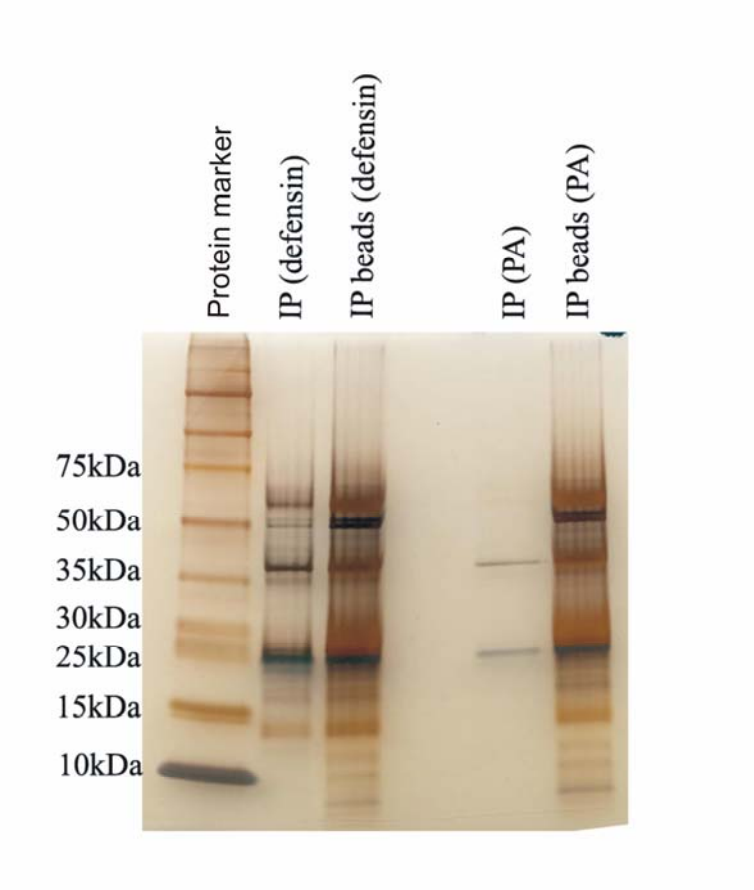

Figure 34: Depletion of α-defensins from hNGE by immunoprecipitation with α-defensin 1-3 antibody. The antibody was coupled to acrylamide beads and incubated with the hNGE. 'IP beads' refers to the acrylamide beads after the pull down, 'IP' to the depleted hNGE. Probably due to cationic properties of hNGE components the reaction was not specific. Control antibody was a protective antigen antibody.

When the immunoprecipitated sample was tested for antimicrobial activity, both the defensin antibody and the irrelevant control antibody reduced the anti *B. anthracis* activity almost completely (Figure 35).

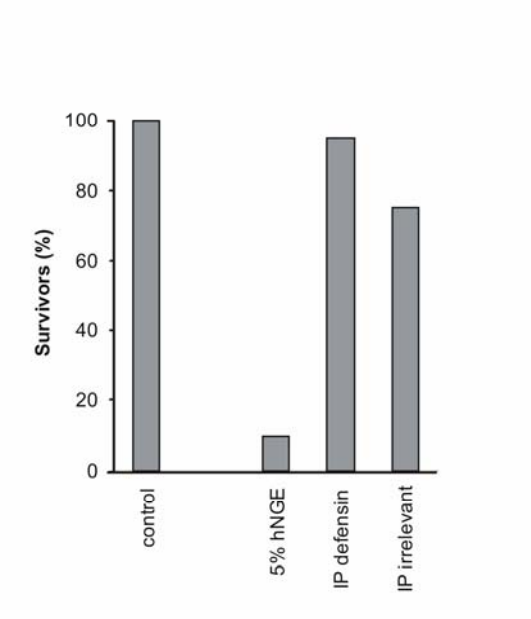

Figure 35: The α-defensin 1-3 antibody depleted hNGE reduces anti-*B. anthracis* activity. Depleted hNGE was incubated with Sterne strain bacteria (1 x 10^6/ ml) for 30 min. Remaining CFU were determined and referred to the number of bacteria in controls of bacteria incubated in buffer.

Unfortunately, the assay conditions could not be rendered more stringent by salt or cationic detergent addition, as these additions alone would have killed the bacteria.

To confirm that α-defensins are responsible for neutrophil killing of *B. anthracis* the activity of the C-18 fraction (the last step in the purification, where only α-defensins were detected) and of a synthetic human α-defensin was compared (Figure 36). Vegetative bacteria were incubated for varying time points with 8µg/ml synthetic or purified peptide and referred to the number of bacteria in buffer only. Both samples had a comparable specific activity confirming human α-defensins as a hNGE component responsible for killing both the capsulated and toxin-producing *B. anthracis* strains. The differences in the killing activity of the samples are difficult to interpret as the protein concentration of the sample was not precisely determined, and the effect of the harsh RP-HPLC conditions on protein integrity and thus activity are difficult to assess.

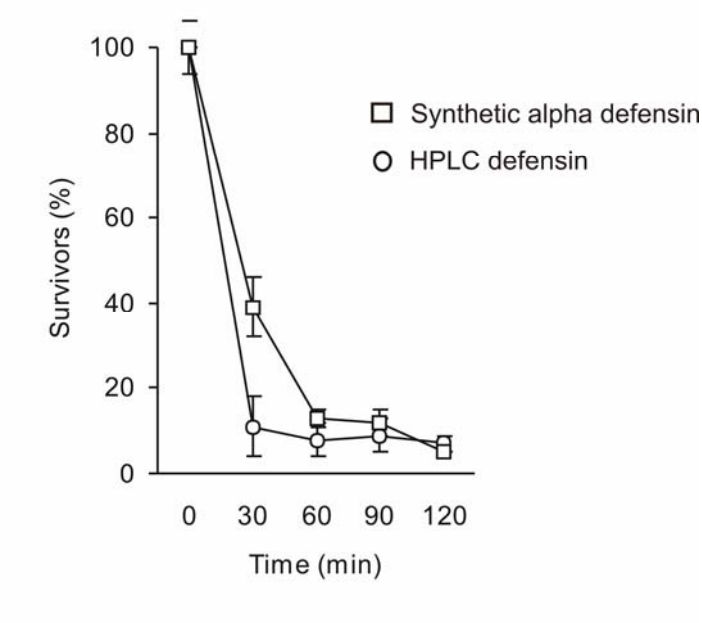

Figure 36: The purified fraction and synthetic α-defensin have comparable specific activity. Vegetative toxin producing bacteria were incubated for varying time points with 8 µg/ml synthetic (□) or purified (○) peptide and referred to the number of bacteria in buffer only. Error bars indicate s.d. of three experiments.

Unfortunately it was not possible to further study the role of neutrophil defensin in an α-defensin knock out mouse model. Mice do not possess any neutrophil α-defensins, also, they do not show the cutaneous form of the disease, making the in vivo examination of the hypothesis difficult.