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Toxicity of pneumolysin and rDNA derived pneumolysin mutants in rats

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Abstract

Pneumolysin is the major cytotoxin of *Streptococcus pneumoniae* and determines bacterial virulence during early pathogenesis of invasive pneumococcal pneumonia through membrane toxicity and complement activation. Pneumolysin is considered as candidate protein vaccine and also as carrier in pneumococcal polysaccharide conjugate vaccines with an additional contribution to the induction of protective immunity by its own.
However, the pneumolysin toxicity may limit its application. Therefore, single and double mutants of pneumolysin (e.g. PdB and PdBD, respectively) were produced to reduce toxicity with conservation of protective immunity against the wild type pneumolysin. In order to compare the toxicity of these rDNA mutants with the original toxin, and to obtain information on the toxicological profile of pneumolysin itself, single and repeated dose toxicity studies were initiated.
Single dose (acute) toxicity studies in male rats (n=4 per group) indicated an for pneumolysin after intravenous administration (IV) between 30 and 45 µg/kg. (IV)LD_{50} values for both mutant products were approximately 300 µg/kg. After formaldehyde treatment as additional detoxification the LD_{50} of both mutant toxins exceeded 900 µg/kg body weight. With regard to the suspected primary targets of toxicity (i.e. blood cells and endothelium), no differences were observed between pneumolysin, PdB and PdBD. At 1 and 7 days after a single (IV or SC) administration of 15µg pneumolysin/kg no effects on haematology or clinical chemistry were observed (male rats; n=12 per group).
In a **repeated dose (sub-acute) toxicity study** with native pneumolysin, five groups - each consisting of 6 male and 6 female animals - were treated once a week for 5 consecutive weeks as follows: 0.5% glycerol (solvent), 5 µg/kg IV, 15 µg/kg IV, 5 µg/kg SC or 15 µg/kg SC. Pneumolysin treatment had no effect on organ weights, haematology, bone marrow cell counts, clinical chemistry and immunological assays. Serum levels of IgM antibodies after the high dose IV pneumolysin treatment were increased ($p<0.05$) in the females only. FACS-analysis of the spleen indicated a relative increase for B cells after SC treatment and a relative and absolute decrease in T cell counts after both IV and SC treatment. Histopathology revealed major kidney damage (glomerular as well as tubular) and minimal inflammatory reactions at the SC injection sites. Female rats were found to be more susceptible to pneumolysin nephrotoxicity than male rats.
The toxic effects of pneumolysin and the rDNA-derived can be ascribed to the membrane toxicity for erythrocytes and bloodvessels. Therefore, when using rDNA-derived pneumolysin toxoids in a vaccine, it seems to be warranted to use a product with a minimum of haemolytic activity with conservation of protective immunity. It is concluded that the acute *in vivo* toxicity of the mutant toxins was approximately 10 % compared to the wildtype, which was more severe than expected on the base of *in vitro* studies (haemolytic activity < 1% of the wild type).

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Samenvatting

Pneumolysine is het belangrijkste (cyto-)toxine van *Streptococcus pneumoniae*. Het is toxisch voor celmembranen, activeert het complement-systeem en bepaalt mede de bacteriële virulentie tijdens de vroege pathogenese van pneumonie door pneumococcen. Pneumolysine is zowel kandidaat als vaccin-eiwit en als een carrier in pneumococcen polysaccharide-conjugaat vaccins. Als zodanig heeft het een eigen additionele bijdrage in de inductie van beschermende immuniteit.
De toxiciteit van pneumolysine zelf beperkt echter de toepasbaarheid. Daarom werden er enkele mutanten en een dubbele mutant (o.a. respectievelijk PdB en PdBD) van pneumolysine gemaakt om de toxiciteit te verminderen met behoud van de beschermende immuniteit tegen het wild type pneumolysine. Om de toxiciteit van deze rDNA mutanten te vergelijken met het originele pneumolysine en om het toxicologisch profiel van pneumolysine zelf te bepalen werden acute en subacute studies verricht.
Op basis van **acute toxiciteitonderzoek** ("single dose toxicity") in mannelijke ratten (n=4 per groep) werd de LD_{50} waarde na intraveneuze toediening (IV) berekend tussen 30 en 45 µg/kg voor pneumolysine en op ongeveer 300 µg/kg voor beide mutant producten. Na formaldehyde behandeling als mogelijke additionele detoxificatie steeg de LD_{50} van beide mutanten boven 900 µg/kg lichaamsgewicht. Er werden geen verschillen waargenomen tussen pneumolysine, PdB en PdBD wat betreft de primaire doelorganen van toxiciteit (bloedcellen en -vaten).
Er werden op 1 en 7 dagen na enkelvoudige (IV of SC) toediening van pneumolysine (15 µg/kg) geen effecten waargenomen op hematologische en klinisch chemische parameters (mannelijke ratten; n= 12/groep).
In een **sub-acute toxiciteitonderzoek** ("repeated dose toxicity") met pneumolysine werden 5 groepen, elk bestaand uit 6 ratten van beide sexen, gedurende 5 weken 1 maal per week behandeld met 0.5% glycerol (groep 1), 5 µg pneumolysine /kg IV (groep 2), 15 µg pneumolysine /kg IV (groep 3), 5 µg pneumolysine /kg SC (groep 4) of 15 µg pneumolysine /kg SC (groep 5). Pneumolysine behandeling had geen effect op orgaan gewichten, bloed- en beenmergparameters, klinische chemie en immunologische testen. Serumspiegels van IgM antistoffen waren verhoogd in vrouwelijke dieren na toediening van 15 µg pneumolysine /kg IV ($p<0.05$). In de milt werd met behulp van FACS-analyse een relatieve toename van B cellen waargenomen na SC toediening en een relatieve en absolute afname van T cellen na zowel IV als SC behandeling. Histopathologisch onderzoek wees uit dat pneumolysine toediening leidt tot ernstige nierschade (glomerulair en tubulair) en een minimale ontstekingsreactie op de SC injectieplaats. Vrouwelijke ratten bleken gevoeliger voor de nefrotoxiciteit van pneumolysine dan mannelijke dieren.
De toxische effecten blijken bijna volledig verklaarbaar op grond van de haemolytische activieit of de membraantoxiciteit van pneumolysine. Daarom is het raadzaam om bij gebruik van een rDNA geproduceerde pneumolysine mutant in een vaccin te streven naar een product met minimale haemolytische activiteit waarbij de beschermende immuniteit gehandhaafd blijft. De acute *in vivo* toxiciteit van de gemuteerde toxines was groter (ongeveer 10% van het wildtype) dan verwacht op grond van *in vitro* vooronderzoek naar haemolytische activiteit (< 1% van het wildtype).

1. Introduction

Pneumolysin is the major cytotoxin of *Streptococcus pneumoniae* and plays an important role in the early pathogenesis of invasive pneumococcal pneumonia by facilitating intrapulmonary bacterial growth and invasion to the blood. It has multiple toxic actions with distinct cytotoxic (haemolytic) and complement activation properties, that have been mapped to several regions of the molecule (1), (2). For membrane toxicity different regions of pneumolysin - expressing binding or lytic activity - could be established (3). Especially for pulmonary infection, pneumolysin was found to exert facilitating activities (1, 4). The virulence of the bacteria during pulmonary infection is diminished when either the cytolytic or complement activities of pneumolysin are absent (1). In contrast to pulmonary infection, for the induction of otitis media the activity of pneumolysin seems less important (5). Also in the development of meningitis pneumolysin seems to be of no importance (6)
As capsular cell wall material and degradation products have an impact on the virulence of *Streptococcus pneumoniae*, accessory proteinaceous virulence factors (including pneumolysin) might be considered as candidates for protein vaccines and carriers in protein-polysaccharide conjugate vaccines (7, 8). It has been suggested that conjugate vaccines should at least contain some of these products, not only to function as mere carrier for the polysaccharides, but also having their own contribution to the induction of protection (7, 9).
However, the membrane toxicity of pneumolysin itself may limit the application of the protein. Several mutants of pneumolysin were produced via the rDNA technique for creating new vaccine candidates (7). Yet, some attenuations did not abolish *in vitro* toxicity and virulence (7). In order to obtain information on the toxicological profile of pneumolysin itself and to compare the *in vivo* toxicity of some rDNA mutants with that of the original toxin, animal studies were initiated. This report describes the results of single dose studies to compare pneumolysin with rDNA-attenuated pneumolysin mutants PdB and PdBD and formaldehyde-treated PdB and PdBD. PdB and PdBD showed a marked decreased activity in an *in vitro* haemolytic assay. Both rDNA-attenuated mutants and toxoids were tested in as feasible alternatives for pneumolysin in future vaccines. In addition the toxicological profile of pneumolysin was determined in a repeated dose study to be used as reference for comparison with future pneumolysin mutants.

2. Materials and methods

2.1 Materials

2.1.1 Animals

Specified Pathogen Free (SPF) male and female WU rats, age 6-8 weeks, were obtained from the Central Animal Facility of the Institute. The animals were randomly distributed into various treatment groups and individually marked in their ear by tattoo. They were housed pair-wise under SPF conditions in macrolon type III cages and provided commercial SSP-Tox or RMH-GS pellets (Hope Farms, Woerden, The Netherlands) and water *ad libitum*. A 12-hour light/dark cycle was operational and the animal room underwent 15 fresh air changes per hour. All other husbandry conditions were maintained according to the national legislation.

2.1.2 Test material

The pneumolysin solution, batch PL105 rDNA produced, containing 1.94 mg/ml, and stored in 50% glycerol at -20^{0}C, was provided by the Laboratory of Vaccine Research of our Institute. The rDNA-produced pneumolysin single mutant PdB: Trp433-Phe, batch 36, containing 2.94 mg/ml and the double mutant PdBD: Trp433-Phe/Asp385-Asn, containing 10.0 mg/ml were kindly donated by J. Paton, Adelaide, Australia, stored and provided the same way as the wild type pneumolysin.
Before administration test materials were thawed and diluted to the various test concentrations in physiological saline (PS). Control animals were injected with solvent only, 0.5% glycerol in PS. The materials were injected either intravenously (IV) or subcutaneously (SC). Formaldehyde-treated PdB and PdBD were prepared using a 0.3 (wt/wt) protein/formalin ratio comparable with 0.035% wt/vol formaldehyde ratio, as described by Nencioni et al (10).

2.2 Methods

2.2.1 Experimental design

Single dose (acute) toxicity studies
Pneumolysin and two rDNA derived mutant toxins were evaluated for their acute toxicity after administration in the tail vein of male rats (n=4 per group), facilitated by provoked vasodilatation after warming the tail in a waterbath of approximately 40^{0}C. Increasing dosages were administered - starting at a 5µg pneumolysin/kg and 100 µg PdB or PdBD/kg - in order to determine a possible reduction in toxicity of the rDNA mutants based on an estimated LD_{50} value. In these LD_{50} studies the animals were closely observed for clinical signs of toxicity, at day 0 once every hour, days 1 and 2 once every two hours and from days 3 to 10 once or twice daily, depending on the condition of the animals. Autopsy was performed after death of the animals or for the surviving animals at day 10 after the injection. Similar studies were performed for formalin-treated mutant toxins.
Additionally three groups of male rats (n=12 per group) were treated once at day 0 with 0.5% glycerol solution as control, 15 µg/kg pneumolysin IV or 15µg/kg pneumolysin SC,

respectively. At day 1 and day 7 after treatment 6 animals of each group were autopsied and blood was collected for determination of haematological and clinical chemistry parameters.

Repeated dose (sub-acute) toxicity study
Five groups of animals, each consisting of 6 male and 6 female animals, were injected once a week for 5 consecutive weeks as follows: Group 1: solvent only (0.5% glycerol), Group 2: 5 µg pneumolysin/kg IV, Group 3: 15 µg pneumolysin /kg IV, Group 4: 5 µg pneumolysin /kg SC and Group 5: 15 µg pneumolysin /kg SC. Autopsy was performed the day after the last treatment (29-30 days after start of the experiment). Before autopsy blood was collected under ether anaesthesia by orbital puncture. The animals were killed by exsanguination from the aorta under CO_2/O_2 anaesthesia.

2.2.2 Clinical Pathology

Haematological and clinical chemistry parameters were determined at days 1 and 7 in the single dose toxicity study to compare the effects of IV and SC treatment with pneumolysin, and at the end of the repeated dose toxicity study. The blood was collected in EDTA coated tubes for haematology and in glass tubes for serum collection for clinical chemistry.

Haematology
Haematological parameters included WBC (white blood cell count), RBC (red blood cell count), Hb (haemoglobin), Ht (haematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular haemoglobin), MCHC (mean corpuscular haemoglobin concentration), and PLT (platelet count). All haematology parameters in the blood samples were determined in a Multispecies Hematology Analyser Technicon H*1E System (Bayer Diagnostics, Miles Inc., Tarrytown NY, USA). In addition, blood smears were prepared for microscopic evaluation depending on the results obtained with the H*1E analyser.

Clinical Chemistry
Clinical chemistry parameters included:
Glucose, Urea, Creatinin, Total Protein, Albumin, Globulin (calculated), Calcium, Sodium, Potassium, Cholesterol, Bilirubin, Alanin aminotransferase (ALAT), Aspartate Aminotransferase (ASAT), Alkaline Phosphatase (AP), Gamma Glutamyltransferase (γGT).
Clinical chemistry was performed on a COBAS BIO and COBAS FARA analyser (Hoffmann-La Roche & Co, AG, Basel, Switzerland).

2.2.3 Pathology

Single dose (acute) toxicity studies
In the LD_{50} studies gross examination was performed and adrenals, heart, kidneys, liver, lung, spleen, thymus, and organs showing alterations at macroscopic examination, were sampled and evaluated histopathologically.

Repeated dose (sub-acute) toxicity study
At gross inspection the following organs were examined and sampled: adrenals, bladder, brain, bone marrow, heart, kidney, liver, lung, lymph nodes (mandibular, mesenteric, and

popliteal), male accessory sex glands (prostate, seminal vesicle), muscle, oesophagus, ovaries, pancreas, peripheral nerve (*N. ischiadicus*), pituitary, skin including mammary gland, spleen, small and large intestines, stomach, testis, thymus and thyroid. When feasible organ weights were determined (see Results section).
After fixation in 4% neutral buffered formaldehyde (10% formalin) all tissue samples were routinely processed to 5 μm thick Haematoxylin & Eosin (H&E) stained sections. When indicated by the histopathological results, additional recuts were prepared and stained with Perl's for the detection of Iron and a combined Haemoglobin/Haemosiderin staining.
Histopathology was documented using the PATHOS data-entry and reporting system (Pathology Operating Systems Ltd, Harrogate, England).

2.2.4 Immunotoxicity: Splenic FACS analysis and immunoglobin levels

Immunotoxicity was assessed in the repeated dose toxicity study only.
Subpopulations of spleen cell phenotypes were analysed as described by de Waal et al. (11), using a single laser FACScan (Becton and Dickinson Immunocytometry Systems, Mountain View CA, USA). Half of the spleen was collected in tissue culture medium for determination of B-cells, CD4+ (T-helper/inducer), CD8+ (T-cytotoxic/suppressor) and CD3+ (all T)-cells using Fluorescein Isothiocynate (FITC)-conjugated monoclonal antibodies MARK-1, ER-2, OX-8 and OX-19 respectively.
Serum was collected for determination of immunoglobulin (IgG, IgM, IgE, and IgA) levels. These immunological assays are described in detail by Vos et al. (12) and van Loveren et al (13).

Bone marrow cells were collected by flushing 4 ml Impulse Cytometer Fluid through the femur. The count of nucleated cells was determined in a Sysmex E4000 hematology analyser (Toa Medical Electronics Co., Ltd., Kobe, Japan). May-Grünwald Giemsa stained cytospin preparations were made for possible additional microscopic evaluation of the cells.

2.2.5 Quality aspects

The research presented in this report has been carried out according to the OECD principles of Good Laboratory Practice (GLP).

3. Results

3.1 Single dose (acute) toxicity studies

3.1.1 Clinical observations

The results of the initial dose range finding studies with pneumolysin and two rDNA derived mutant toxins are presented in Table 1. Compared to the wild-type (LD_{50} = 30-45 μg/kg) there was a 7- to tenfold increase in LD_{50} value for the rDNA produced PdB and PdBD pneumolysin toxin mutants up to approximately 300 μg/kg. A further decrease in toxicity was obtained by an additional treatment of both mutants with formaldehyde: up to 900 μg/kg could be administered without any sign of toxicity, so the LD_{50} is considered to be greater than 900 μg/kg body weight.
Clinical signs of toxicity included: respiratory distress (dyspneu, superficial and laboured respiration), reduced activity, inactive/stuporous behaviour, ataxia, haematuria and nasal discharge. This reaction was transient, but dose related. Death of the animals occurred generally within 10 minutes after the IV injection of a high toxic dose of either pneumolysin, PdB or the PdBD mutant. One animal was killed at 7 hours after the administration for severe clinical signs.

3.1.2 Macroscopy

The macroscopic observations were as follows: haemorrhagic and discoloured lungs, dark coloured urine in bladder, enlarged adrenals, dark kidneys, haemorrhages and petechiae in thymus. Other observations, including observations in the 10 days surviving animals were: slightly pronounced white pulp in the spleen, minimal lung oedema, discoloured lungs, hydrocephalus, enlarged lymph nodes (iliac), dilatation of the renal pelvis, enlarged thyroid, and skin lesions. These latter lesions were considered background lesions mainly, and were not further investigated. The lesions in the animals acutely dying indicate the occurrence of severe haemolysis.

3.1.3 Histopathology

Group incidences of lesions observed after IV pneumolysin injection are presented in Table 2. Treatment related lesions were observed in intercurrently deceased animals, all animals treated with 45 μg/kg and one animal treated with 30 μg/kg pneumolysin.
In the adrenals (Figure 1) severe necrosis of the *zona fasciculata* was observed including absence of the endothelium. In animals treated with lower dosages vacuolation and focal degeneration of the *zona fasciculata* was observed.
In the kidneys of animals that had died intercurrently (Figure 2), severe degeneration and necrosis was present mainly in distal tubules, but also extending to collecting tubules. Tubular hyalin droplets and haemoglobin casts were observed predominantly in proximal and in distal tubules, respectively. The glomerular damage consisted of severe protein (including haemoglobin) leakage to the Bowman's' space, endothelial swelling, collapse and necrosis.
In the liver (Figure 3) focal and bridging necrosis was observed in one animal, treated with 45 and 30 μg/kg body weight, respectively. Endothelial lining of the sinusoids was severely affected: observations varied from complete absence to swollen or pyknotic nuclei, probably of

endothelium and/or Kupffer cell origin. Also liver cells showed signs of degeneration, indicated by the presence of eosinophilic inclusions which are likely associated with early necrosis. These phenomena were mainly located in zone 2-3 of Rappaport. Perl's staining for haemoglobin revealed negative results in the liver cells. In the larger blood vessels swollen slightly basophilic (H&E staining) erythrocytes are present. (Figure 4).
In the lung alveolar and septal oedema, necrosis of endothelium was observed (Figures 5 and 6).
In the heart the presence of eosinophilic fibers indicated hypercontraction of muscle fibers. An increase in severity from minimal to moderate was found in the intercurrently deceased animals (data not shown). This could not be explained by the treatment with pneumolysin, but might be related to the rigor mortis.
In one animal also lymphoid depletion/degeneration was observed in the spleen and thymus.
All other observed lesions are in line with normal background histopathology of the rat strain used.
After administration of the rDNA modified mutant pneumolysin PdB histopathological examination was limited to the two animals surviving for ten days. The observed changes were limited to the kidney in which an increase in basophilic tubules was observed. After PdBD administration hydropic degeneration of the renal tubules was seen while in the spleen an increase in secondary follicles was observed.

3.1.4 Clinical pathology

There was no effect on the various haematological (Table 3) or clinical chemistry (Table 4) parameters as measured after single SC or IV administration of 15 μg pneumolysin/kg.

3.2 Repeated dose (sub-acute) toxicity study

3.2.1 Clinical observations and macroscopy

In the repeated dose study no clinical signs of toxicity were observed. At autopsy occasionally the following alterations were observed: hydronephrosis, hydrometra, foam in trachea, minimal haemorrhages in various organs (thymus, adrenals and lymph nodes), bladder concrements (data not shown). These changes are considered either euthanasia-induced or as background pathology. There was no effect of pneumolysin treatment on organ weights (Table 5).

3.2.2 Clinical pathology

There were no effects on the various haematological or clinical chemistry parameters (Tables 6 and 7).

3.2.3 Histopathology

General
The group incidences of histopathology of the organs investigated after repeated IV or SC administration of pneumolysin are presented in Table 8. For most organs only control and high dose group (15 μg/kg IV and SC) were investigated. Examination of the low dose group (5 μg/kg body weight) was performed if possible treatment related effects were found in the

high dose group. Some alterations were observed made in pneumolysin-treated animals only e.g. adrenal ectopic bone formation and vacuolation, pigment in the mandibular lymph node, pancreatic inflammation, ovarian cysts and mammary gland hyperplasia. Still, these lesions were not considered to be treatment related: they can be commonly found as background histopathology in the rat strain used and occurred in a low incidence as usually.
SC injection sites: in general only a minimal inflammatory reaction, composed of a few leukocytes, was observed.

Liver
The parenchymal cells contained microvacuoles containing a varying amount of pale to lightly eosinophilic (in H&E staining) amorphous product. Kupffer cells were swollen and an (minimally) increased number of mononuclear cells in sinusoids was observed. Additional Perl's staining showed the presence of a minimal amount of Fe in parenchymal cells, generally associated with Fe containing Kupffer cells in the central and portal areas. As the vacuolation, Kupffer cells swelling and increased numbers of sinusoidal mononuclear cells was seen in 0.5% glycerol treated controls as well as pneumolysin-treated animals, in approximately equal severity and incidences, these changes were considered not pneumolysin treatment related (data not shown).

Kidney
Major pneumolysin effects were observed in the kidney. Lesions are presented in Table 9. Female rats were found to be more susceptible for pneumolysin nephrotoxicity than males and therefore the present description is focused on female animals. A treatment and dose related effect was only noted for the IV treatment with regard to the haemosiderin/Fe deposition in the proximal tubular epithelium (Table 10). The effect was most prominent in the IV treated females of the high dose group. For the low dose group similar observations were found but with lower severity. After repeated IV administration of a high dose (15 µg/kg) of pneumolysin an increase in occurrence of basophilic (regenerative) tubules was observed. In the same dose group tubular changes as pigment storage (probably haemosiderin) and haemoglobin cast's as well as associated interstitial nephritis were increased in both incidence and severity. Occasionally the presence of cellular debris in the basophilic tubules, and tubular degeneration/necrosis with haemoglobin casts was noted. The haemoglobin and haemosiderin staining and Perl's staining for iron (Fe) confirmed the suspected origin of the pigment.
Occasionally, mineralisation was present at corticomedullary junction, more frequently in females, as usually. In addition also dystrophic calcification was observed in morphologically altered tubules.
In contrast to the female animals, all treated (glycerol control and toxin) male animals had protein droplets in the tubular epithelium. These droplets could not be identified as reabsorbed haemoglobin and haemosiderin.

Thymus, Lymph nodes and Spleen
Pneumolysin treatment induced no obvious lesions in the immunological organs examined (Table 11).
Also, no difference of iron (Fe) storage was noted after pneumolysin treatment but the spleen of female animals contained more Fe, both in control and high dose treated animals.

3.2.4 Splenic FACS analysis and immunoglobin levels

In the spleen both the IV and SC treatment resulted in an effect on the relative spleen cell distribution (Table 12b). For the T cells a decrease was observed after both IV and SC treatment, while for B cells after SC treatment an increase was noted. For the absolute number of T cells a tendency for a reduction in cell number was present (Table 12a).
Repeated high dose pneumolysin IV treatment resulted in a significant increase in total serum IgM level ($p<0.05$)(Table 13). However, the increase was only present in female and not in male animals (data not shown).

4. Discussion

In the acute toxicity studies the major clinical symptoms (dyspneu and somnolence/ inactivity) and histopathology (haemoglobin leakage into the Bowman's space and renal tubules) indicated severe haemolysis and damage to blood vessels and alveoli in the lung.after IV administration of high doses of pneumolysin or both mutants. In the lung and kidney necrosis seemed to be limited to the blood vessels, resulting in additional tissue damage such as necrosis of lung alveolar and renal tubular epithelium. The haemoglobin released from the erythrocytes can be considered responsible for the adverse effects on the kidneys.

With regard to the suspected target organs of toxicity, no differences were observed between pneumolysin and the two rDNA mutants PdB and PdBD. Severe haemolysis leads to a reduced oxygen availability due to loss of oxygen binding haemoglobin in the urine (haemoglobinuria) but also to a dramatic shift in oxygen binding capacity of the free haemoglobin compared to cellular haemoglobin.

In experiments with the pneumolysoid mutant toxin PdBD also red coloured urine was observed, which was scored as haematuria. However, this was more likely haemoglobinuria. The histopathology revealed in the acute deceased animals the presence of severe necrosis of endothelium, especially of the endothelium of small blood vessels (capillaries/sinusoids) in the adrenals, renal glomeruli, lungs and liver.

The ratios between observed LD_{50} values of the mutant toxins (300 μg/kg) on one hand and pneumolysin (approximately 30-45 μg/kg) on the other hand are approximately 10. These *in vivo* results were lower than expected on the base of the results from *in vitro* studies on erythrolysis (ED_{50}mutants was less than 1% of the ED_{50}native pneumolysin, unpublished data). For the formaldehyde-inactivated mutant toxins the LD_{50} was more than 900 μg/kg body weight. However, such formaldehyde treatment must be thoroughly validated, both in terms of inactivation and remaining antigenicity of the toxin. A rather severe formaldehyde treatment, resulting in complete loss of toxicity, can be accompanied by a loss of protective immunogenicity as well, as indicated by several experiments (unpublished data).

Pneumolysin was found to exert two major toxic effects, erythrolysis and endothelial necrosis, both considered to be a result of the membrane toxicity of oligomerised pneumolysin and can possibly be enhanced by complement activation. Pneumolysin is a protein with a Fe-like domain capable of activation complement via of the classical pathway (14). This complement activation is known to be relevant in humans and animal models of pneumococcal induced diseases. Additionally, in the repeated dose study - with possible induction of anti-pneumolysin antibodies - complement activation through the classical pathway on the base of antibody-antigen reaction can not be excluded. Especially in IV treated animals the bioavailability of pneumolysin and complement are probably so optimal, that blood cells and the endothelium (i.e. the nearest possible target cells for complement mediated cytolysis) are the first target cells of toxicity. On the other hand, repeated SC administration led to a very mild inflammatory reaction at the injection site thus indicating only a minimal complement reactivity/availability after repeated pneumolysin administration, at least after SC dosing. Since antibodies against pneumolysin are supposed to be absent in

the SPF animals, complement activation via the classical pathway seems to be irrelevant in the outcome of the single dose toxicity studies.

Alexander et al. (15) demonstrated that reduction in either cytotoxic activity or complement activation by pneumolysin decreases the virulence of the mutant pneumococci. Still, the haemolytic activity and complement activation of pneumolysin contribute specifically to the pathogenesis of pneumococcal pneumonia at different stages of infection and by different mechanisms (1). The first correlates with acute lung injury and bacterial growth at 3 and 6 h after endotracheal instillation, the latter correlates with bacterial growth and bacteremia at 24 h after pulmonary infection (15; 16). Pneumolysin-induced complement activation is not associated with the degree of alveolar-capillary injury or recruitment of leukocytes during initial pulmonary infection. However, pneumolysin-induced complement activation inhibits killing of mutant bacteria in an *in vitro* complement-dependent neutrophilic granulocyte killing assay.

Since the acute toxicity of both pneumolysin mutants are comparable and the double mutant has a mutation in the complement activating region of the pneumolysin, leading to a 99 % decrease of complement activation, the acute toxicity of the two mutant toxins is likely not related to complement activation only. Other additional mechanisms of toxicity - like through enhanced NO synthesis (17) or tumor necrosis factor alpha and interleukin-1 (18) - may play a substantial role in the pathogenesis of pneumococcal disease at least for pneumonia since decreased complement activating capacity is still associated with pneumonia (19).

Surprisingly, despite the significant haemolysis indicating pathology in the repeated dose toxicity study, haematology did not show significant toxic effects of pneumolysin on red blood cell parameters at lower levels. Haemolysis was not observed. However, intra-luminal tubular and intracellular haemoglobin in combination with intracellular (iron containing) pigment accumulation, was found in the affected female animals. Both phenomena indicate haemolysis. Apparently, the lower dosages of pneumolysin induce minimal to slight haemolysis, which was not detected clinically at 24 hours and onwards after toxin administration. The limited haemolysis can, however, be regarded responsible for the release of a sufficient amount of haemoglobin to induce marked nephrotoxicity in the repeated dose toxicity study.

For the non-functional immune parameters of the spleen a decrease in the relative amount of T cells was found both after IV and SC administration. A relative increase in B cells was only observed after SC administration of pneumolysin. Although not significantly, also the absolute number of T cells was diminished. So, pneumolysin seems to have a minimal effect on T cells. An in increase in serum IgM was found after IV pneumolysin, notably in females. This increase is likely to be caused by anti-pneumolysin IgM antibody production. Besides, it may be related to a blockade of isotype switch during immunoglobulin synthesis. Some of the other immunoglobulin levels show a tendency to decrease, which supports the latter hypothesis. The limited changes in lymphoid organs (weights, histopathology, splenic FACS analysis and serum immunoglobin levels) do not indicate biologically relevant toxic effects on the immune systeme, buth rather slightly reflect expected immunological activation upon IV administration of the immunogenic pneumolysin.

Regarding pneumococcal vaccine development, it seems to be warranted to use a rDNA derived toxoid with a minimum of haemolytic activity with conservation of protective

immunity. However, systemic availability and toxicity of both pneumolysin and the two mutants will probably be markedly decreased if administered via the intended routes of vaccination (sc or im). Studies to compare local reactions as well as systemic toxicity of (mutant) pneumolysin vaccine candidates with those of reference vaccines as DPT-polio should be performed to shed light on this issue.

5. Deviations from the protocol

The animals that died immediately after IV injection of PdB, were not examined by mistake. In case of missing organs that are usually symmetrically present, such as adrenals, it was considered acceptable for histopathological examination that one of the two was missing or incompletely present. Evaluation of these organs was considered complete if all tissue compartments were found in one organ sample. When a tissue compartment was not present, the samples were diagnosed as “Sample Inadequate”. For these samples at least one additional recut was prepared in order to look for the missing tissue compartment. Two untreated male and one untreated female rat were added to the repeated dose study; analyses of these animals revealed the liver changes (e.g. Kupffer cell swelling) to be the only significant glycerol-related. Therefore other data were excluded from analyses and presentation.

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Appendix 1 Mailing list

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Appendix 2 Tables and figures

Table 1: Acute toxicity finding studies (IV, single administration) with pneumolysin and pneumolysin rDNA derivatives PdB (a single mutant) and PdBD (a double mutant).

Pneumolysin[a]		PdB		PdBD		PdB-toxoid[b]		PdBD- toxoid[b]	
µg/kg	Death	µg/kg	Death	µg/kg	Death	µg/kg	Death	µg/kg	Death
5	0/4	100	0/4	100	0/4	100	0/4	100	0/4
15	0/4	200	0/4	200	0/4	300	0/4	300	0/4
30	1/4	300	2/4	250	0/4	900	0/4	900	0/4
45	4/4			300	5/6				

a) Estimated LD50 values:

Pneumolysin	30-45 µg/kg
PdB	300 µg/kg
PdBD	250-300 µg/kg
PdB-toxoid	> 900 µg/kg
PdBD-toxoid	> 900 µg/kg

b) formaldehyde-inactivated

Table 2: Histopathology after IV administration of a single dose of native pneumolysin, group incidences

	Dose (µg/kg):	5	15	30	45
	Number:	4	4	4	4
Adrenal					
	Number examined	4	4	4	4
	Not remarkable	0	1	0	0
	Vacuolation	4	3	3	0
	Necrosis	0	0	1	4
	Focal degeneration	0	0	2	0
Heart					
	Number examined	4	3	4	4
	No sample	0	1	0	0
	Haemorrhage	0	0	1	0
	Eosinophilic fibers	4	3	4	4
Kidney					
	Number examined	4	4	4	4
	Not remarkable	1	3	0	0
	Tubular dilatation	1	0	0	0
	Basophilic tubules	3	1	2	0
	Hyaline droplets	0	0	1	1
	Dilated pyelum	1	0	1	0
	Tubular haemoglobin	0	0	1	1
	Tubular degeneration	0	0	1	4
	Glomerular damage	0	0	1	4
Liver					
	Number examined	4	4	4	4
	Necrosis	0	0	1	1
	Inflammatory cell foci	4	4	4	4
	Liver cell degeneration	0	0	0	4
	Endothelial necrosis	0	0	1	4
Lung					
	Number examined	4	4	4	4
	Not remarkable	0	0	0	1
	Alveolar haemorrhages	4	4	3	0
	Alveolar edema	0	0	0	3
	Septal degen/necrosis	0	0	1	3
Spleen					
	Number examined	4	4	4	3
	Not remarkable	2	1	1	3
	Dev sec follicles	2	3	2	0
	Lymphodepletion	0	0	1	0
Thymus					
	Number examined	4	4	4	3
	Not remarkable	4	4	3	1
	Haemorrhage	0	0	1	2
	Lymphoid degeneration	0	0	1	2

Table 3: Effect of pneumolysin (15 μg/kg) on haematological parameters after a single IV or SC administration.

MALES	Blood values at day 1 after pneumolysin administration			Blood values at day 7 after pneumolysin administration		
Treatment	0.0 μg/kg 0.5% glycerol	15 μg/kg IV	15 μg/kg SC	0.0 μg/kg 0.5% gly	15 μg/kg IV	15 μg/kg SC
WBC (G/l)	12.1 ± 1.2	9.8 ± 1.6	11.2 ± 1.7	9.6 ± 1.8	8.8 ± 0.8	9.6 ± 1.2
RBC (T/l)	7.7 ± 0.1	7.4 ± 0.6	7.4 ± 0.5	7.7 ± 0.3	7.6 ± 0.3	7.6 ± 0.3
Hb (mmol/l)	9.6 ± 0.2	9.3 ± 0.7	9.2 ± 0.4	9.5 ± 0.3	9.4 ± 0.3	9.4 ± 0.3
Ht (l/l)	0.49 ± 0.01	0.47 ± 0.04	0.46 ± 0.02	0.48 ± 0.02	0.47 ± 0.02	0.47 ± 0.02
MCV (fl)	63 ± 2	64 ± 2	62 ± 2	62 ± 4	62 ± 1	63 ± 2
MCH (fmol)	1.24 ± 0.04	1.25 ± 0.02	1.24 ± 0.04	1.24 ± 0.06	1.22 ± 0.01	1.25 ± 0.04
MCHC (mmol/l)	19.7 ± 0.2	19.7 ± 0.3	20.0 ± 0.3	20.0 ± 0.3	19.8 ± 0.2	19.9 ± 0.3
RDW %	13.1 ± 0.6	13.2 ± 0.5	12.8 ± 0.7	12.7 ± 0.8	13.0 ± 0.9	12.4 ± 0.3
PLT (G/l)	1072 ± 86	1114 ± 87	1196 ± 119	1146 ± 91	1098 ± 67	1081 ± 59
WBC (G/l)	12.1 ± 1.2	9.8 ± 1.6	11.2 ± 1.7	9.6 ± 1.8	8.8 ± 0.8	9.6 ± 1.2
Eosin. gran.	0.14 ± 0.04	0.07 ± 0.03	0.10 ± 0.03	0.09 ± 0.03	0.07 ± 0.02	0.08 ± 0.02
Basoph. gran.	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Neutroph. gran.	0.77 ± 0.2	0.89 ± 0.2	0.85 ± 0.4	0.70 ± 0.2	0.73 ± 0.1	0.74 ± 0.3
Lymphocytes	10.8 ± 1.1	8.29 ± 1.3	9.73 ± 1.3	8.46 ± 1.6	7.72 ± 0.8	8.42 ± 1.1
Monocytes	0.31 ± 0.06	0.38 ± 0.18	0.37 ± 0.09	0.26 ± 0.04	0.21 ± 0.05	0.27 ± 0.12
Other cells	0.10 ± 0.02	0.13 ± 0.06	0.14 ± 0.03	0.08 ± 0.03	0.07 ± 0.02	0.07 ± 0.02

For all groups n=6, except for the 0.5% glycerol control at day 1 n=5.

Table 4: Effect of a single pneumolysin administration on clinical chemistry parameters

	DAY 1 after administration			DAY 7 after administration		
	Glycerol 0.5% IV	Pneumolysin[a] 15 µg/kg IV	Pneumolysin 15 µg/kg SC	Glycerol 0.5% IV	Pneumolysin 15 µg/kg IV	Pneumolysin 15 µg/kg SC
AP (U/l)	247 ± 32	296 ± 42	250 ± 33	219 ± 21	216 ± 24	220 ± 31
ALAT (U/l)	33 ± 7	73 ± 60	40 ± 4	43 ± 6	48 ± 18	42 ± 3
ASAT (U/l)	67 ± 12	145 ± 151	66 ± 5	65 ± 7	80 ± 24	71 ± 3
Cholesterol (mmol/l)	2.9 ± 0.4	2.9 ± 0.4	2.6 ± 0.4	2.6 ± 0.2	2.6 ± 0.5	2.6 ± 0.3
Glucose (mmol/l)	10.0 ± 2.0	13.0 ± 2.3	12.7 ± 3.1	12.3 ± 2.0	14.6 ± 1.5	13.6 ± 1.9
γGT (U/l)	0.33 ± 0.08	0.74 ± 0.21	0.42 ± 0.08	0.47 ± 0.07	0.41 ± 0.14	0.54 ± 0.12
Creatinin (µmol/l)	47 ± 3	50 ± 4	48 ± 1	50 ± 2	50 ± 3	49 ± 4
Total bilirubin (µmol/l)	3.6 ± 0.3	3.8 ± 0.3	4.2 ± 0.5	3.8 ± 0.3	3.6 ± 0.2	3.9 ± 0.2
Urea (mmol/l)	6.6 ± 0.7	6.5 ± 0.8	6.9 ± 1.0	7.0 ± 1.2	6.4 ± 0.5	6.4 ± 0.6
Potassium (K, mmol/l)	0.73 ± 0.08	0.77 ± 0.08	0.75 ± 0.05	0.72 ± 0.08	0.70 ± 0.06	0.72 ± 0.08
Sodium (Na, mmol/l)	148 ± 7	146 ± 8	150 ± 11	144 ± 1	146 ± 1	147 ± 3
Calcium (Ca, mmol/l)	3.4 ± 0.0	3.6 ± 0.0	3.6 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.3 ± 0.0
Albumin (g/l)	34 ± 0.4	35 ± 0.9	33 ± 0.8	34 ± 0.6	34 ± 0.6	34 ± 0.8
Total protein (g/l)	66 ± 2	68 ± 2	64 ± 2	66 ± 2	65 ± 1	66 ± 3

For all groups n=6

[a] One animal (#15) had for all enzymatic parameters (AP, ALAT, ASAT, and γGT) rather high values resulting in unequal distribution of the data. For these parameters this animal can be considered an outliner. Excluding this animal leads to the following data: AP 293 ± 46, ALAT 48 ± 5, ASAT 84 ± 15, and γGT 0.67 ± 0.14.

Table 5: Effect of pneumolysin on organ weight after repeated (five times) IV or SC administration.

Treatment	0.0 µg/kg	5 µg/kg	15 µg/kg	5 µg/kg	15 µg/kg
FEMALES	0.5% glycerol	IV	IV	SC	SC
Animal (g)	236 ± 21	234 ± 22	230 ± 16	227 ± 20	238 ± 24
Brain (mg)	1867 ± 145	1773 ± 80	1858 ± 59	1805 ± 88	1809 ± 73
Thymus	360 ± 45	344 ± 78	359 ± 57	350 ± 73	355 ± 74
Heart	1025 ± 86	1059 ± 101	1024 ± 104	969 ± 125	992 ± 177
Lung	1779 ± 268	1793 ± 265	1799 ± 377	1818 ± 336	1963 ± 493
Liver	5840 ± 596	5540 ± 676	5696 ± 575	5564 ± 651	5752 ± 746
Spleen	475 ± 48	451 ± 52	498 ± 55	462 ± 55	444 ± 71
Kidneys (L+R)	1732 ± 214	1565 ± 134	1677 ± 200	1604 ± 133	1544 ± 160
Adrenals (L+R)	62 ± 8	57 ± 6	71 ± 6	64 ± 6	59 ± 3
Ovaries (L+R)	73 ± 6	81 ± 11	72 ± 12	81 ± 16	84 ± 13
MALES					
Animal (g)	361 ± 32[a]	343 ± 17	347 ± 17	343 ± 13	363 ± 25
Brain (mg)	2032 ± 70	1956 ± 36	1947 ± 43	1955 ± 86	1983 ± 36
Thymus	653 ± 153	587 ± 87	624 ± 67	534 ± 125	597 ± 115
Heart	1438 ± 175	1368 ± 113	1339 ± 92	1299 ± 68	1448 ± 122
Lung	3138 ± 698	2863 ± 756	2399 ± 486	2506 ± 609[c]	2732 ± 915
Liver	10712 ± 1194	10175 ± 486	9938 ± 632	9908 ± 645	10625 ± 763
Spleen	703 ± 131	676 ± 66	660 ± 96	609 ± 58	659 ± 114
Kidneys (L+R)	2444 ± 331	2320 ± 151	2333 ± 222	2284 ± 195	2300 ± 212
Adrenals (L+R)	63 ± 8	58 ± 5	59 ± 8	57 ± 4	62 ± 6
Testes (L+R)	3548 ± 342	3261 ± 189	3253 ± 130	241 ± 203	3337 ± 172

For all groups n=6, except for [a] n=5.

Table 6a: Effect of pneumolysin on haematological parameters after repeated (five times) IV or SC administration, female animals.

Treatment FEMALES	0.0 µg/kg 0.5% glycerol	5 µg/kg IV	15 µg/kg IV	5 µg/kg SC	15 µg/kg SC
WBC (G/l)	8.5 ± 1.1	7.4 ± 1.3	6.9 ± 2.0	6.3 ± 1.2	5.7 ± 0.9
RBC (T/l)	8.8 ± 0.3	8.8 ± 0.3	8.7 ± 0.3	8.7 ± 0.3	8.6 ± 0.2
Hb (mmol/l)	10.1 ± 0.3	10.0 ± 0.2	9.9 ± 0.2	9.8 ± 0.2	9.6 ± 0.2
Ht (l/l)	0.46 ± 0.02	0.46 ± 0.01	0.46 ± 0.01	0.45 ± 0.01	0.44 ± 0.01
MCV (fl)	52 ± 1	52 ± 2	53 ± 2	51 ± 1	52 ± 1
MCH (fmol)	1.14 ± 0.03	1.13 ± 0.02	1.14 ± 0.05	1.12 ± 0.02	1.13 ± 0.03
MCHC (mmol/l)	22.1 ± 0.3	21.6 ± 0.3	21.6 ± 0.2	21.8 ± 0.3	21.8 ± 0.2
RDW %	11.5 ± 0.4	11.2 ± 0.3	11.4 ± 0.5	11.1 ± 0.4	11.3 ± 0.4
Reticulocytes	18.2 ± 5.0	10.0 ± 4.7	13.2 ± 6.8	11.3 ± 4.3	12.3 ± 7.3
PLT (G/l)	1052 ± 81	981 ± 69	1084 ± 149	962 ± 79	1027 ± 60
WBC (G/l)	8.5 ± 1.1	7.4 ± 1.3	6.9 ± 2.0	6.3 ± 1.2	5.7 ± 0.9
Eosin. gran.	0.12 ± 0.08	0.12 ± 0.03	0.12 ± 0.04	0.11 ± 0.05	0.10 ± 0.03
Basoph. gran.	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.0	0.01 ± 0.01	0.01 ± 0.01
Neutroph. gran.	0.73 ± 0.4	0.52 ± 0.1	0.46 ± 0.1	0.54 ± 0.2	0.47 ± 0.1
Lymphocytes	7.39 ± 1.1	6.48 ± 1.4	6.00 ± 1.7	5.36 ± 1.2	4.87 ± 0.9
Monocytes	0.21 ± 0.05	0.22 ± 0.14	0.23 ± 0.16	0.18 ± 0.08	0.20 ± 0.14
Other cells	0.07 ± 0.01	0.05 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.02
Bone marrow cells	19.0 ± 4.4	17.8 ± 5.0	21.2 ± 5.6	17.9 ± 4.0	18.2 ± 4.4

For all groups n=6.

Table 6b: Effect of pneumolysin on haematological parameters after repeated (five times) IV or SC administration, male animals.

Treatment MALES	0.0 µg/kg 0.5% glycerol	5 µg/kg IV	15 µg/kg IV	5 µg/kg SC	15 µg/kg SC
WBC (G/l)	11.7 ± 2.1	10.1 ± 1.7	9.6 ± 1.6	10.3 ± 1.6	9.3 ± 1.5
RBC (T/l)	8.3 ± 0.4	8.3 ± 0.2	8.4 ± 0.4	8.5 ± 0.1	8.3 ± 0.3
Hb (mmol/l)	9.7 ± 0.3	9.6 ± 0.2	10.0 ± 0.3	9.8 ± 0.2	9.8 ± 0.2
Ht (l/l)	0.44 ± 0.02	0.44 ± 0.01	0.46 ± 0.02	0.46 ± 0.01	0.45 ± 0.01
MCV (fl)	54 ± 1	54 ± 1	55 ± 1	54 ± 1	54 ± 1
MCH (fmol)	1.18 ± 0.04	1.17 ± 0.01	1.19 ± 0.02	1.16 ± 0.03	1.17 ± 0.03
MCHC (mmol/l)	22.0 ± 0.3	21.7 ± 0.3	21.7 ± 0.4	21.4 ± 0.2	21.7 ± 0.4
RDW %	11.5 ± 0.5	11.4 ± 0.1	11.5 ± 0.1	11.9 ± 0.6	12.1 ± 0.8
Reticulocytes	19.0 ± 6.6	15.3 ± 4.5	13.2 ± 2.6	16.8 ± 5.9	18.7 ± 5.5
PLT (G/l)	1073 ± 51	993 ± 50	996 ± 44	1072 ± 27	1050 ± 65
WBC (G/l)	11.7 ± 2.1	10.1 ± 1.7	9.6 ± 1.6	10.3 ± 1.6	9.3 ± 1.5
Eosin. gran.	0.15 ± 0.05	0.12 ± 0.04	0.10 ± 0.03	0.15 ± 0.02	0.15 ± 0.04
Basoph. gran.	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Neutroph. gran.	0.91 ± 0.2	0.98 ± 0.2	0.93 ± 0.3	1.03 ± 0.2	1.07 ± 0.2
Lymphocytes	10.19 ± 1.7	8.65 ± 1.5	8.24 ± 1.5	8.68 ± 1.4	7.71 ± 1.5
Monocytes	0.32 ± 0.17	0.27 ± 0.18	0.28 ± 0.12	0.33 ± 0.18	0.31 ± 0.17
Other cells	0.09 ± 0.02	0.05 ± 0.03	0.08 ± 0.03	0.07 ± 0.02	0.06 ± 0.02
Bone marrow cells	38.9 ± 5.6	29.3 ± 6.5	33.8 ± 4.6	31.1 ± 6.9	31.7 ± 4.4

For all groups n=6.

Table 7a: Effect of repeated pneumolysin administration on clinical chemistry parameters, female animals.

FEMALES	Glycerol 0.5% IV	Pneumolysin 5 µg/kg IV	Pneumolysin 15 µg/kg IV	Pneumolysin 5 µg/kg SC	Pneumolysin 15 µg/kg SC
AP (U/l)	88 ± 24	76 ± 25	91 ± 10	88 ± 10	78 ± 14
ALAT (U/l)	28 ± 4	32 ± 10	32 ± 8	31 ± 6	26 ± 7
ASAT (U/l)	77 ± 10	74 ± 15	81 ± 17	104 ± 26	80 ± 9
Cholesterol (mmol/l)	2.0 ± 0.3	1.8 ± 0.3	2.0 ± 0.3	1.8 ± 0.5	2.2 ± 0.4
Glucose (mmol/l)	7.6 ± 1.1	6.4 ± 0.6	6.6 ± 2.4	6.9 ± 1.2	6.3 ± 1.0
γGT (U/l)	0.24 ± 0.08	0.29 ± 0.16	0.40 ± 0.12	0.26 ± 0.18	0.31 ± 0.21
Creatinin (µmol/l)	59 ± 9	56 ± 2	66 ± 5	63 ± 8	60 ± 6
Total bilirubin (µmol/)	3.6 ± 0.5	3.9 ± 0.6	3.8 ± 0.4	3.2 ± 0.6	3.8 ± 0.6
Urea (mmol/l)	8.0 ± 1.2	7.2 ± 1.2	9.4 ± 0.9	7.8 ± 0.9	6.8 ± 0.5
Potassium (K, mmol/l)	0.65 ± 0.12	0.64 ± 0.08	0.65 ± 0.08	0.62 ± 0.08	0.63 ± 0.05
Sodium (Na, mmol/l)	146 ± 2	147 ± 4	146 ± 2	146 ± 3	148 ± 2
Calcium (Ca, mmol/l)	3.0 ± 0.2	3.0 ± 0.1	3.0 ± 0.1	3.0 ± 0.3	3.0 ± 0.2
Albumin (g/l)	33 ± 1.2	33 ± 1.0	33 ± 1.3	32 ± 1.6	34 ± 2.4
Total protein (g/l)	65 ± 3	65 ± 1	68 ± 2	62 ± 3	67 ± 6

N=6, except for Pneumolysin 5 µg/kg IV and Pneumolysin 5 µg/kg SC n=5.

Table 7b: Effect of repeated pneumolysin administration on clinical chemistry parameters, male animals.

MALES	Glycerol 0.5% IV	Pneumolysin 5 µg/kg IV	Pneumolysin 15 µg/kg IV	Pneumolysin 5 µg/kg SC	Pneumolysin 15 µg/kg SC
AP (U/l)	168 ± 19	163 ± 5	171 ± 41	150 ± 21	167 ± 20
ALAT (U/l)	40 ± 10	35 ± 7	39 ± 14	38 ± 2	39 ± 4
ASAT (U/l)	79 ± 16	76 ± 13	79 ± 23	75 ± 10	81 ± 18
Cholesterol (mmol/l)	2.6 ± 0.4	2.1 ± 0.1	2.1 ± 0.3	2.4 ± 0.5	2.6 ± 0.7
Glucose (mmol/l)	11.1 ± 2.6	11.5 ± 1.8	11.8 ± 2.6	11.9 ± 1.9	11.2 ± 1.8
γGT (U/l)	0.23 ± 0.09	0.35 ± 0.11	0.38 ± 0.12	0.31 ± 0.11	0.30 ± 0.08
Creatinin (µmol/l)	52 ± 6	54 ± 5	51 ± 4	54 ± 4	51 ± 3
Total bilirubin (µmol/)	3.5 ± 0.4	3.6 ± 0.4	3.4 ± 0.4	3.3 ± 0.5	3.3 ± 0.3
Urea (mmol/l)	6.6 ± 0.4	7.3 ± 1.7	6.8 ± 0.4	6.6 ± 0.8	6.6 ± 0.4
Potassium (K, mmol/l)	0.65 ± 0.05	0.63 ± 0.05	0.62 ± 0.04	0.65 ± 0.08	0.65 ± 0.08
Sodium (Na, mmol/l)	146 ± 1	149 ± 2	146 ± 2	147 ± 2	148 ± 2
Calcium (Ca, mmol/l)	3.2 ± 0.2	3.2 ± 0.2	3.1 ± 0.2	3.2 ± 0.2	3.1 ± 0.1
Albumin (g/l)	32 ± 1.0	32 ± 0.3	32 ± 0.8	32 ± 1.0	32 ± 0.6
Total protein (g/l)	66 ± 4	65 ± 1	64 ± 2	66 ± 2	64 ± 2

N=6 for all groups.

Table 8: Histopathology, group incidences in controls and after repeated IV or SC administration of 15 µg pneumolysin/kg (all organs, except for kidney, immune system and bone marrow: see Tables 9, 10 and 11).

Dose µg/kg:	0	15	15	0	15	15
Sex:	M	M	M	F	F	F
Route:	IV	IV	SC	IV	IV	SC
Number:	6	6	6	6	6	6
SC injection site						
Number examined	0	0	6	0	0	6
Not remarkable	0	0	1	0	0	4
Inflammation	0	0	5	0	0	2
Heart						
Number examined	6	6	6	6	6	6
Not remarkable	4	5	6	6	6	5
Inflammation	1	0	0	0	0	0
Myocarditis	1	1	0	0	0	1
Lung						
Number examined	6	6	6	6	6	6
Not remarkable	0	0	1	4	4	2
Inflammatory cell foci	0	0	1	0	0	0
Perivascular infiltrate	0	1	0	1	0	0
Alveolar haemorrhage	6	6	5	1	2	4
Arterial mineralisation	1	0	0	0	0	0
Brain						
Number examined	6	6	6	6	6	6
Not remarkable	5	6	6	5	6	6
Inflammation	1	0	0	1	0	0
Nerve						
Number examined	6	6	6	6	6	6
Not remarkable	6	6	6	6	6	6
Pituitary						
Number examined	6	5	6	6	6	6
Sample inadequate	0	1	0	0	0	0
Not remarkable	4	5	6	6	6	6
Cyst	2	0	0	0	0	0
Adrenal						
Number examined	6	6	6	6	6	6
Not remarkable	5	3	3	6	5	4
Ectopic bone	0	0	2	0	0	0
Focal inflammation	0	1	0	0	0	0
Vacuolation medulla	1	0	0	0	1	2
Lipomatosis	0	0	1	0	0	0
Vacuolation cortex	0	3	2	0	0	0
Thyroid						
Number examined	6	6	6	6	6	6
Not remarkable	6	6	6	6	6	6
Skin + mammary gland						
Number examined	5	6	6	6	6	6
Not remarkable	5	2	0	6	3	1
No sample	1	0	0	0	0	0
Inflammation	0	1	1	0	0	2
Folliculitis	0	0	0	0	1	0
Mammary hyperplasia	0	4	6	0	2	5

Table 8: Histopathology after repeated IV or SC administration of pneumolysin, group incidences (continued).

Dose µg/kg:	0	15	15	0	15	15
Route:	IV	IV	SC	IV	IV	SC
Sex:	M	M	M	F	F	F
Number:	6	6	6	6	6	6
Muscle						
Number examined	6	6	6	6	6	6
Not remarkable	4	5	4	6	6	5
Degeneration	0	0	0	0	0	1
Inflammation	2	1	2	0	0	1
Stomach						
Number examined	6	6	6	6	6	6
Not remarkable	6	6	5	6	6	6
Inflammation	0	0	1	0	0	0
Small intestine						
Number examined	6	6	6	6	6	6
Not remarkable	6	6	5	6	6	6
Mineralisation	0	0	1	0	0	0
Large intestine						
Number examined	6	6	6	6	6	6
Not remarkable	6	6	6	6	6	5
Haemorrhage	0	0	0	0	0	1
Liver						
Number examined	6	6	6	6	6	6
Inflammatory cell foci	6	6	6	6	6	6
Vasculitis	0	0	0	1	2	0
Kupffer cell swelling	6	6	5	6	6	6
Pancreas						
Number examined	6	6	6	6	6	6
Not remarkable	5	5	6	6	6	4
Inflammation	0	1	0	0	0	2
Lipomatosis	1	0	0	0	0	0
Urinary bladder						
Number examined	6	6	6	6	6	6
Not remarkable	5	5	6	6	6	6
Inflammation	1	0	0	0	0	0
Desquamation	0	1	0	0	0	0
Ovary						
Number examined	-	-	-	6	6	6
Not remarkable	-	-	-	6	4	5
Cyst	-	-	-	0	2	1
Lipomatosis	-	-	-	0	0	0
Uterus						
Number examined	-	-	-	1	2	2
Not remarkable	-	-	-	1	2	2
Testis						
Number examined	6	6	6	-	-	-
Not remarkable	6	6	6	-	-	-
Prostate						
Number examined	6	6	6	-	-	-
Not remarkable	5	5	5	-	-	-
Inflammation	1	1	1	-	-	-
Seminal vesicle						
Number examined	6	6	6	-	-	-
Not remarkable	5	6	6	-	-	-
Hyperplasia	1	0	0	-	-	-

Dose: 0 µg = glycerol 0.5% IV.

Table 9: Renal histopathological lesions after repeated IV or SC administration of pneumolysin.

Dose µg/kg:	0	5	15	5	15	0	5	15	5	15
Sex:	M	M	M	M	M	F	F	F	F	F
Route:	IV	IV	IV	SC	SC	IV	IV	IV	SC	SC
Number:	6	6	6	6	6	6	6	6	6	6
Kidney										
Number examined	6	6	6	6	6	6	6	6	6	6
Not remarkable	0	0	0	0	0	3	0	0	0	1
Basophilic tubules	4	5	6	6	6	2	5	6	5	3
minimal	4	4	3	5	5	2	5	0	4	3
slight	0	1	3	0	1	0	0	1	1	0
moderate	0	0	0	1	0	0	0	1	0	0
marked	0	0	0	0	0	0	0	4	0	0
Tubular pigment	0	0	3	0	0	1	5	6	3	2
minimal	0	0	3	0	0	1	5	1	2	2
slight	0	0	0	0	0	0	0	0	1	0
moderate	0	0	0	0	0	0	0	4	0	0
marked	0	0	0	0	0	0	0	1	0	0
Tubular haemoglobin	0	0	1	0	0	0	0	6	0	0
minimal	0	0	1	0	0	0	0	1	0	0
slight	0	0	0	0	0	0	0	2	0	0
moderate	0	0	0	0	0	0	0	2	0	0
marked	0	0	0	0	0	0	0	1	0	0
Tubular protein	1	0	0	0	1	0	1	0	0	1
minimal	1	0	0	0	1	0	1	0	0	1
slight	0	0	0	0	0	0	0	0	0	0
Protein droplets	5	6	4	4	4	0	0	0	0	0
minimal	5	4	2	3	3	0	0	0	0	0
slight	0	2	2	1	0	0	0	0	0	0
moderate	0	0	0	0	1	0	0	0	0	0
Interstitial nephritis	3	2	2	2	3	1	3	6	1	0
minimal	3	2	2	2	3	0	2	1	0	0
slight	0	0	0	0	0	1	1	1	0	0
moderate	0	0	0	0	0	0	0	1	0	0
marked	0	0	0	0	0	0	0	3	1	0
Mineralisation	0	1	1	0	1	2	4	5	2	2
minimal	0	0	1	0	1	1	3	3	2	1
slight	0	0	0	0	0	1	1	2	0	1
moderate	0	1	0	0	0	0	0	0	0	0
Focal fibrosis	0	0	0	0	0	0	0	0	0	0
Medullary atrophy	0	0	0	1	0	1	0	0	1	1
minimal	0	0	0	1	0	0	0	0	0	0
slight	0	0	0	0	0	0	0	0	0	1
moderate	0	0	0	0	0	0	0	0	1	0
marked	0	0	0	0	0	1	0	0	0	0
Papillitis	0	0	0	0	0	0	0	0	1	0
Hydronephrosis	2	1	1	2	0	1	0	1	1	2
minimal	1	0	0	0	0	0	0	1	0	0
slight	1	0	0	2	0	0	0	0	0	2
moderate	0	1	1	0	0	0	0	0	1	0
marked	0	0	0	0	0	1	0	0	0	0

Dose: 0 µg = glycerol 0.5% IV.

Table 10: Semi-quantitative score of haemoglobin and iron (Fe) presence in kidney, spleen and liver after repeated IV or SC administration of pneumolysin.

Dose µg/kg:	0	5	15	5	15	0	5	15	5	15
Route:	IV	IV	IV	SC	SC	IV	IV	IV	SC	SC
Sex:	M	M	M	M	M	F	F	F	F	F
Number:	6	6	6	6	6	6	6	6	6	6
Renal tubular haemosiderin/Fe										
minimal	-	4	6	1	-	5	-	-	2	6
slight	-	1	-	-	-	1	2	-	3	-
moderate	-	-	-	-	-	-	4	1	1	-
marked	-	-	-	-	-	-	-	4	-	-
severe	-	-	-	-	-	-	-	1	-	-
Liver Fe										
minimal	-	-	-	-	-	6	5	5	3	6
slight	-	-	-	-	-	-	1	-	3	-
moderate	-	-	-	-	-	-	-	-	-	-
marked	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-
Spleen Fe										
minimal	4	4	5	3	6	-	-	-	-	-
slight	2	2	1	3	-	-	-	-	-	-
moderate	-	-	-	-	-	6	6	6	6	6
marked	-	-	-	-	-	-	-	-	-	-
severe	-	-	-	-	-	-	-	-	-	-

Table 11: Histopathology of lymphoid organs after repeated IV or SC administration of pneumolysin, group incidences.

Dose µg/kg:	0	5	15	5	15	0	5	15	5	15
Route:	IV	IV	IV	SC	SC	IV	IV	IV	SC	SC
Sex:	M	M	M	M	M	F	F	F	F	F
Number:	6	6	6	6	6	6	6	6	6	6
Spleen										
Number examined	6	0	6	0	6	6	0	6	0	6
Not remarkable	1	0	0	0	0	0	0	0	0	0
Pigment	0	0	0	0	0	6	0	6	0	6
Erythropoiesis	5	0	4	0	6	1	0	0	0	0
Dev sec follicles	2	0	4	0	4	5	0	6	0	5
Thymus										
Number examined	6	0	6	0	6	6	0	6	0	6
Not remarkable	2	0	1	0	2	2	0	2	0	2
Pigment	0	0	0	0	0	0	0	1	0	0
Haemorrhage	4	0	5	0	3	4	0	3	0	4
Other tissue	0	0	0	0	1	0	0	2	0	0
Femur + marrow										
Number examined	6	0	6	0	6	6	5	6	0	6
Sample inadequate	0	0	0	0	0	0	1	0	0	0
Lipomatosis	6	0	6	0	6	6	5	6	0	6
Normoblastosis	0	0	0	0	0	0	1	2	0	0
Axillary lymph node										
Number examined	6	0	6	0	6	6	0	6	0	6
Sinus histiocytosis	6	0	6	0	6	5	0	6	0	5
Dev sec follicles	6	0	5	0	6	4	0	3	0	6
Plasmacytosis	6	0	5	0	3	0	0	2	0	5
Paracortical "starry sky"	5	0	5	0	4	5	0	3	0	3
Inguinal lymph node										
Number examined	5	0	5	0	5	6	0	5	0	6
No sample	1	0	0	0	1	0	0	1	0	0
Sample inadequate	0	0	1	0	0	0	0	0	0	0
Haemorrhage	0	0	1	0	0	0	0	0	0	0
Sinus histiocytosis	4	0	5	0	4	4	0	5	0	5
Dev sec follicles	4	0	3	0	3	3	0	1	0	3
Plasmacytosis	1	0	2	0	1	0	0	0	0	2
Paracortical "starry sky"	3	0	5	0	1	5	0	3	0	4
Mandibular lymph node										
Number examined	6	0	6	0	6	6	0	6	0	6
Pigment	0	0	0	0	0	0	0	2	0	6
Haemorrhage	0	0	0	0	0	2	0	0	0	1
Sinus histiocytosis	6	0	6	0	6	5	0	6	0	6
Other tissue	6	0	6	0	6	6	0	6	0	6
Dev sec follicles	6	0	6	0	6	6	0	6	0	6
Plasmacytosis	5	0	5	0	5	6	0	5	0	6
Paracortical "starry sky"	5	0	6	0	6	5	0	3	0	4
Mesenteric lymph node										
Number examined	6	0	6	0	6	6	0	6	0	6
Sinus histiocytosis	6	0	6	0	6	6	0	6	0	6
Dev sec follicles	4	0	5	0	5	4	0	1	0	3
Plasmacytosis	0	0	0	0	0	0	0	1	0	1
Paracortical "starry sky"	6	0	6	0	6	5	0	6	0	6

0 µg = glycerol 0.5%

Table 12a: Effect of repeated pneumolysin administration on FACS analyses of splenic cell population (absolute numbers [x10^7]; mean ± SD; n= 12 unless indicated otherwise).

	Mark-1 (B-cells)	OX-19 (CD3)	Er-2 (CD4)	OX-8 (CD8)
Controls	2.61 ± 1.01	6.32 ± 2.72	4.36 ± 1.25	1.98 ± 0.69(n=11)
5 µg IV	2.53 ± 1.24	4.86 ± 1.97	4.21 ± 2.00	1.74 ± 0.85
15 µg IV	3.08 ± 1.56	5.47 ± 2.40	4.85 ± 2.29	1.94 ± 0.91
5 µg SC	2.84 ± 1.04	4.72 ± 1.03	4.49 ± 1.07	1.77 ± 0.58
15 µg SC	2.92 ± 0.87	4.54 ± 1.81	4.53 ± 1.90	1.77 ± 0.62

Table 12b: Effect of repeated pneumolysin administration on FACS analyses of splenic cell population (relative numbers [%]; mean ± SD; n= 12 unless indicated otherwise).

	Mark-1 (B-cells)	OX-19 (CD3)	Er-2 (CD4)	OX-8 (CD8)
Controls	29.0 ± 3.9	70.1 ± 10.7	50.5 ± 8.8	21.7 ± 3.1(n=11)
5 µg IV	30.6 ± 4.9	60.2 ± 8.4*†	51.3 ± 7.4	20.8 ± 4.3
15 µg IV	32.6 ± 4.3	59.2 ± 8.9*††	51.8 ± 6.1	20.6 ± 3.6
5 µg SC	34.1 ± 7.5*†	57.9 ± 4.8***‡	55.2 ± 8.7	21.2 ± 4.1
15 µg SC	35.4 ± 3.9*††	53.2 ± 4.5***‡	53.1 ± 8.7	53.2 ± 4.5

- * or † = p<0.05;**or †† = p<0.01 ;***or ‡ = p<0.001 in single factor ANOVA or in student t-test, respectively.

Table 13: Effect of repeated pneumolysin administration on relative serum antibody levels (mean ± SD; n= 12 unless indicated otherwise).

	IgG	IgM	IgA	IgE
Controls	100 ± 75	100.0 ± 37.7	100.0 ± 33.5 (n=11)	100.0 ± 146.9
5 µg IV	67.4 ± 62.7 (n=11)	255.8 ± 211.9*	111.9 ± 25.8 *(n=11)	116.8 ± 165.2
15 µg IV	97.2 ± 71.7	322.3 ± 281.0*†	76.3 ± 22.5 *(n=11)	84.1 ± 84.9
5 µg SC	51.0 ± 35.8	133.8 ± 62.7	107.8 ± 27.3(n=11)	76.8 ± 49.4
15 µg SC	79.9 ± 98.1	125.8 ± 64.6	111.3 ± 32.3(n=11)	69.6 ± 40.6

* or † = $p<0.05$ in single factor ANOVA or in student t-test, respectively.

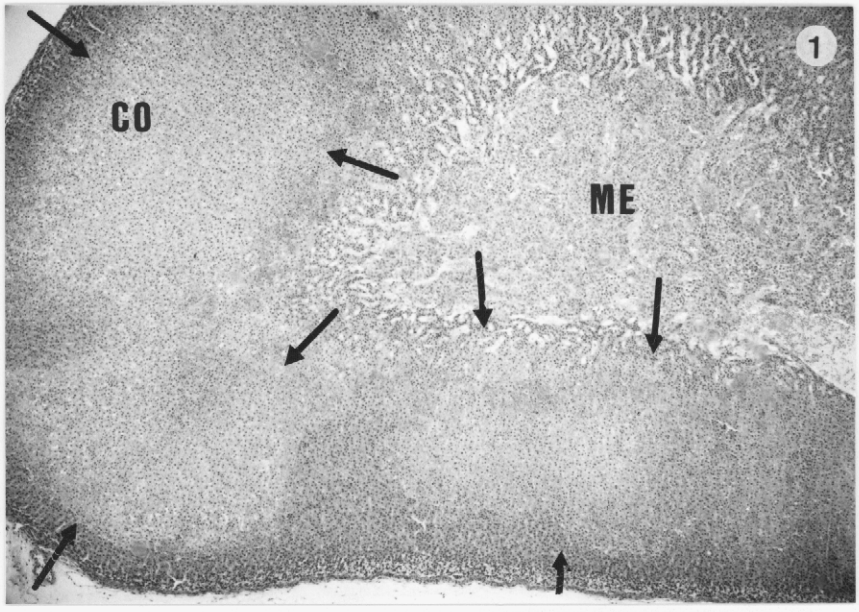

Figure 1: Adrenal of a male rat after single IV application of 30 µg pneumolysin/kg. ME = medulla, CO =cortex. Pale cortical area (indicated with arrows) represents necrosis (H&E, x 45).

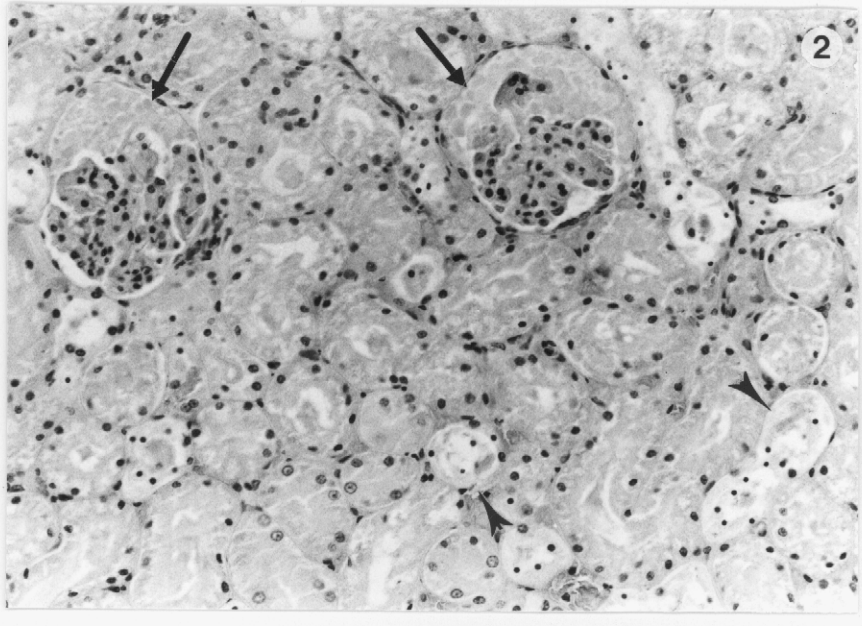

Figure 2: Kidney of a male rat after single IV application of 45 µg pneumolysin/kg. Glomerular leakage into Bowman's space (arrows) and extensive tubular epithelial necrosis with desquamation (indicated with arrowheads) is obvious (H&E, x 220).

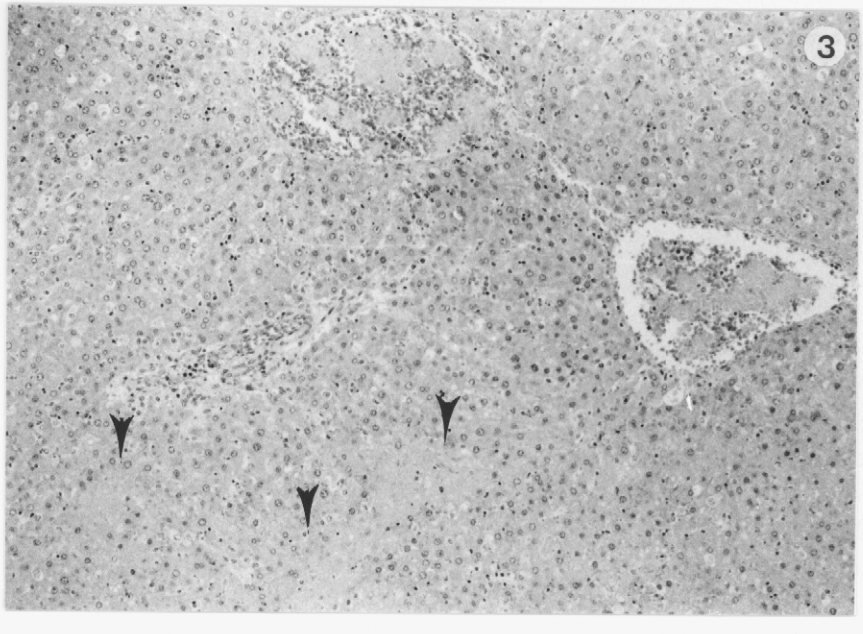

Figure 3: Liver of a male rat that died after single IV application of 45 µg pneumolysin/kg. Necrosis (indicated with arrow-heads) is obvious (H&E, x 110).

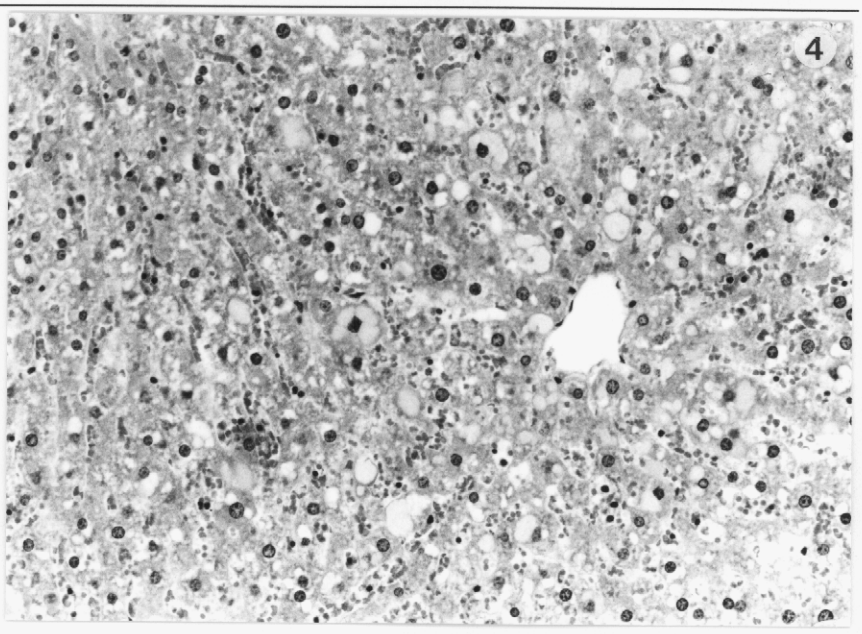

Figure 4: Liver of a male rat 10 days after single IV application of 30 µg pneumolysin/kg. Liver cells are loaded with iron containing pigment granules (H&H staining, x 220).

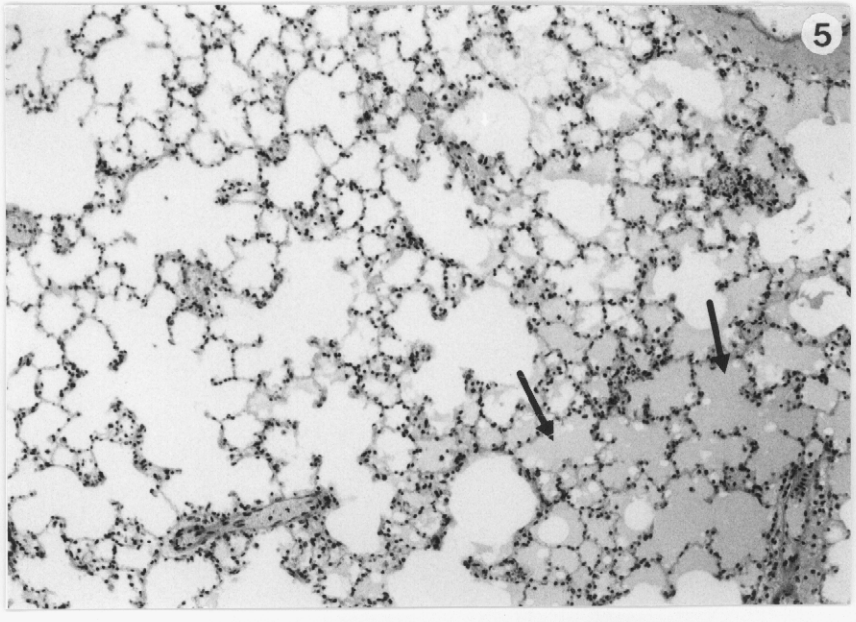

Figure 5: Lung of a male rat that died after single IV application of 30 µg pneumolysin/kg. Marked alveolar oedema (indicated with arrows) is present (H&E, x 110).

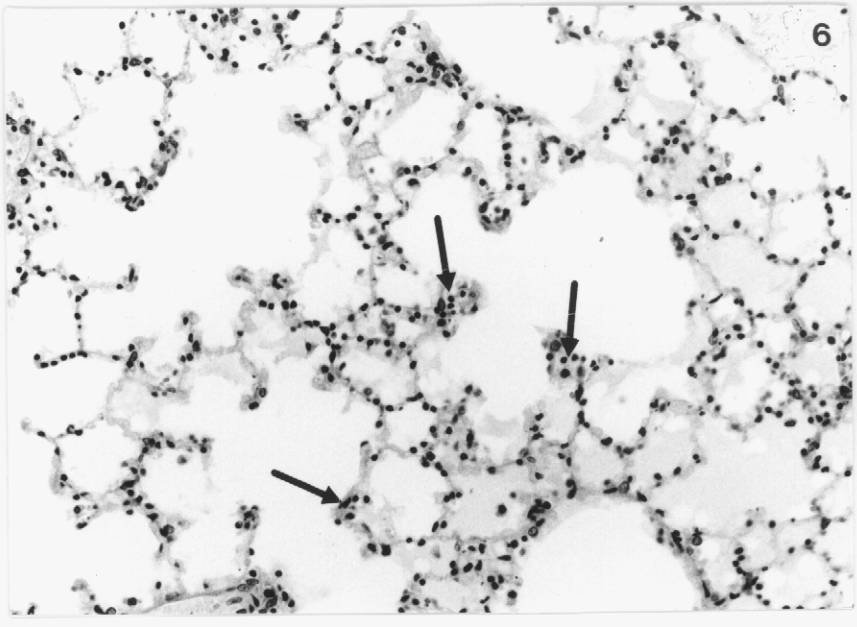

Figure 6: Magnification of the centre of Figure 5; alveolar and interstitial oedema are accompanied by necrosis of numerous septal (alveolar as well as endothelial) cells (indicated with arrows) are present (H&E, x 220).