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Aerosol challenge of calves with *Haemophilus somnus* and *Mycoplasma dispar*

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Abstract

The aim of the study was to examine the ability of *Haemophilus somnus* and *Mycoplasma dispar* to induce pneumonia in healthy calves under conditions closely resembling the supposed natural way of infection, viz. by inhalation of aerosol droplets containing the microorganisms. The infections were investigated by recording clinical data, cytokine expression of peripheral blood cells and pathology. Twelve calves were included in the study: Three animals were exposed to *H. somnus* only, and two to *M. dispar* only, whereas five were challenged to *M. dispar* followed by exposure to *H. somnus* 11–14 days later. Also, one calf was exposed to *M. dispar* followed by exposure to a sterile saline solution 11 days later, and one calf was only exposed to a sterile saline solution. Just one animal, only challenged with *H. somnus*, developed a focal necrotizing pneumonia, from which *H. somnus* was isolated. Thus, the ability of *H. somnus* and *M. dispar* to act as primary pathogens under these conditions were minimal and inconsistent.

However, a transient rise in body temperature, a marked granulocytosis and increased levels of interleukin-8 in peripheral blood after inoculation with *H. somnus* indicated a clear systemic response, probably as a consequence of the natural non-specific local and systemic defence mechanisms acting in healthy calves.

Keywords: *Haemophilus somnus*; *Mycoplasma dispar*; Aerosol infection model; Cattle; Bacteria; Pneumonia; Interleukin-8

1. Introduction

Diseases of the respiratory tract are one of the primary sources of losses in the Danish beef and dairy cattle industries. It represents a complex problem as the development and

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severity of the disease are influenced by a number of infectious agents including bacteria, virus and mycoplasmas, as well as factors related to host and environmental conditions, such as immunity, stress, stocking rate, etc. Though intensively studied, the relative significance of several of the infectious agents by themselves remains to be demonstrated conclusively. In Denmark, *Haemophilus somnus* is one of the most common bacteria isolated from fatal cases of calf pneumonia (Tegtmeier et al., 1999b). Therefore, the present study was focused on *H. somnus* in order to clarify its potential for inducing pneumonia in healthy unstressed animals under experimental conditions closely resembling the presumed natural route of infection, viz. by aerosol exposure. *Mycoplasma dispar* is another microorganism often isolated from pneumonic calf lungs (Tegtmeier et al., 1999b), and this microorganism was used for studying concomitant infection with *M. dispar* and *H. somnus*.

Most previous experimental studies of *H. somnus* pneumonia have been based on intratracheal or intrabronchial deposition of bacterial suspensions (Krogh et al., 1986; Gogolewski et al., 1987; Jackson et al., 1987; Potgieter et al., 1988; Tegtmeier et al., 1999a). This way of introducing the bacterium to the respiratory tract has yielded valuable information on the pathogenesis of the disease. It is, however, not a design that closely mimics the natural route of infection. One can speculate that the introduction of such a huge number of bacteria in a viscous suspension might overload the physical barriers leading to a vicious circle of exacerbated cytokine release, thereby, compromizing the natural defence mechanisms of the lung to a degree uncompatible with the natural pathogenesis of the disease. In that case, the lung lesions might partly be explained as an unbalanced reaction independent of the viability of *H. somnus* organisms, that is, the lesions could be induced by inoculation of dead bacteria or bacterial components in itself. In line with this, Whiteley et al. (1991) found that inoculation of killed *Mannheimia (Pasteurella) haemolytica* bacteria were capable of causing fibrin exudation, platelet aggregation and alveolar epithelial damage in a degree similar to live bacteria (The name *Pasteurella haemolytica* has been changed to *Mannheimia haemolytica*, Angen et al., 1999).

Only a limited number of papers report on calves that have been exposed to aerosols of *H. somnus*. Nayar et al. (1977) aerosol-inoculated three calves with *H. somnus*. Two of these calves developed clinical signs of respiratory disease 24 hrs post inoculation and *H. somnus* was cultured from blood samples. However, none of these animals were necropsied, thus no information on pulmonary pathology or concomitant infection with other pathogens was available. Krogh et al. (1986) inoculated four calves with aerosols containing *H. somnus* and/or *M. dispar* through the nostrils. When *H. somnus* was inoculated alone (one animal), no pulmonary lesions were present at necropsy, whereas inoculation with *M. dispar* prior to the *H. somnus* inoculation (three animals) lead to scanty to more extensive consolidation in the lungs.

In pigs, *Actinobacillus pleuropneumoniae* is a common cause of pneumonia and an experimental infection model has recently been developed for aerosol inoculation of pigs with *A. pleuropneumoniae* (Jacobsen et al., 1996). In this model, pigs are placed in a closed chamber and subjected to bacterial aerosols generated by a nebulizer. This model was used in the present study to expose calves to aerosols containing *M. dispar* and/or *H. somnus*.

The aim of the present study was (1) to examine the ability of viable *H. somnus* bacteria to act as the primary causative organism in development of calf pneumonia in healthy animals under conditions closely resembling the proposed natural way of infection, that is, by inhalation of aerosol droplets containing *H. somnus*, and (2) to examine the possible effect of infection with *M. dispar* prior to inoculation with *H. somnus*. The infections were investigated by recording clinical data, post-mortem pathology and changes in the composition of and cytokine expression of peripheral blood cells.

2. Materials and methods

2.1. Animals

Twelve calves (No. 1–12) were purchased from a closed Red Danish Dairy herd free of *Salmonella* and bovine viral diarrhea virus, as monitored by the Danish Veterinary Laboratory (DVL). The calves were removed immediately after birth without any contact to the mother cow or the surroundings, transported to DVL and placed two and two in isolation units. The calves were fed with milk substitute and in addition, each calf was given 100 ml bovine hyperimmune serum orally three times a day for the first 8 days of life. The serum was obtained from a donor cow that had been kept in isolation its entire life without episodes of respiratory disease. The cow had previously been vaccinated and boosted with Trivacton 6 (Rhône Merieux), a vaccine containing antigens against *Escherichia coli*, rota- and coronavirus. Also, intramuscular antibiotic treatment (Ampivet, Boehringer Ingelheim), 10 mg/kg was given twice a day for the first 8 days of life.

The calves were challenged when approximately 4 weeks old. During the whole life span, the health status was monitored by clinical examination.

To exclude the presence of pneumonic lesions before challenge, all calves were radiographed approximately 1 week prior to the first aerosol exposure as described elsewhere (Tegtmeier and Arnbjerg, 1999).

Heparin stabilized peripheral blood samples were collected from the jugular vein before and after challenge of animal number 6, 7, 8 and 11 for determination of cytokine expression and cellular parameters in the peripheral blood cells.

As described elsewhere (Grell et al., 2000), cytokine expression (interleukin-8 (IL-8)) was determined by reverse transcribed polymerase chain reaction with specific primers. Briefly, total RNA was extracted by guanidine thiocyanate (Promega) and dissolved in RNAse-free water (diethylpyrocarbonate treated). Contaminating DNA was digested with DNAse. A standardized amount of total RNA (measured by absorbance at 260 nm) was reverse transcribed to cDNA using oligo dT as a primer and reverse transcriptase from GibcoBRL (Superscript II). Specific primers for bovine IL-8 were: TTC ACA GCA CTC GGA ATC CTG and ATG ACT TCC AAA CTG GCT GTT. The identity of the RT-PCR product was verified by sequencing.

Two housekeeping genes (β-actin and GAPDH) visualized after PCR by agarose gele electrophoresis were used to calibrate samples. The relative concentration of specific

cytokine transcripts in the samples was estimated by the amount of RT-PCR product obtained, as judged from product band density after agarose gel electrophoresis. All cytokine mRNA levels were estimated relative to the levels observed after the *M. dispar* challenge but before the *H. somnus* aerosol challenge.

Differential leukocyte counts were performed by a combination of automated leukocyte counting (AUTOCounter AC 900) and FACS analysis (FACScan, Becton Dickinson).

2.2. Bacterial and mycoplasma strains

A Danish β-haemolytic field strain of *H. somnus* (DVL 939/90), originally isolated from a case of bovine pneumonia, was used throughout the study. This strain has previously been used as inoculum for experimental pulmonary infection of calves (Tegtmeier et al., 1999a). Also, the strain has earlier been characterized by biotyping, plasmid profiling, REA-patterns and ribotyping, and represents the dominant group of Danish *H. somnus* strains isolated from cases of calf pneumonia (Fussing and Wegener, 1993).

Four Danish *M. dispar* strains isolated from cases of calf pneumonia (MK 332, MK 341, MK 358 and MK 372) were used for inoculation. A pool of four isolates was used, in an attempt to ensure an optimal potentiating effect, as heterogenesity of *M. dispar* has previously been described (Friis, 1978).

2.3. Preparation of inoculum

The *H. somnus* strain, kept at −80°C, was resuscitated on 5% bovine blood agar plates (Columbia agar base, Oxoid), incubated overnight in an atmosphere of 10% CO_2 and 90% air at 37°C, subcultivated and re-incubated for 16 h prior to use. Immediately before use, the bacteria were harvested by washing each agar plate with 1 ml of a 37°C sterile 0.9% NaCl solution, giving an inoculum with approximately 10^9 colony forming units per ml (CFU/ml). The purity of the inoculum was verified by bacteriological examination (as described under 2.5).

The *M. dispar* strains were all kept at −80°C until use. The inoculum was prepared as a pool of broth cultures of the four different strains which had been filtered through a 0.45 μm membrane and cloned once from solid medium. The cultures were used in the 5th or 6th passage in artificial medium, representing a 10^{12}–10^{15} dilution of the original lung tissue. A medium described by Kobisch and Friis (1996) was used for cultivation, and identification was performed by the disc growth inhibition test by using antiserum for the type strain NCTC 10125 (462/2). The titer was estimated to 10^8–10^9 viable units per ml in the *M. dispar* containing medium, used for inoculation.

2.4. Aerosol exposure

Equipment for an aerosol infection model previously developed for inoculation of pigs with *A. pleuropneumoniae* (Jacobsen et al., 1996) was used in the present study, where calves were exposed to *M. dispar* and/or *H. somnus*. Briefly, the equipment consisted of a

Table 1
Results of necropsy and microbiological examinations of lungs of calves inoculated with *Mycoplasma dispar* and *Haemophilus somnus*

Calf No.	Inoculation with[a]			Euthanasia	Necropsy findings[b]	Microbiology	
	M. dispar	*H. somnus*	Sterile saline			Bacterial-, viral-, & mycoplasma examination	
						Lung[c]	Nasal svab[d]
1	–	Day 0	–	Day 4	Acute focal necrotizing pneumonia	*H. somnus*	*H. somnus*
2	–	Day 0	–	Day 4	None	None	*H. somnus*
3	–	Day 0	–	Day 4	None	None	*H. somnus*
4	Day −11	–	–	Day 0	None	*M. dispar*	Negative
5	Day −11	–	–	Day 0	None	*M. dispar*	Negative
6	Day −11	Day 0	–	Day 4	None	*M. dispar*	*H. somnus*
7	Day −11	Day 0	–	Day 4	None	*M. dispar*	*H. somnus*
8	Day −11	Day 0	–	Day 4	None	*M. dispar*	*H. somnus*
9	Day −14	Day 0	–	Day 4	None	*M. dispar*	*H. somnus*
10	Day −14	Day 0	–	Day 4	None	*M. dispar* *Pasteurella* spp.	*H. somnus*
11	Day −11	–	Day 0	Day 4	None	*M. dispar*	Negative
12	–	–	Day 0	Day 4	None	None	Negative

[a] The time of challenge with *H. somnus* was considered as Day 0.
[b] In some animals, a few atelectatic lobules were present at necropsy, these are not mentioned in the table.
[c] Lung tissue was examined for the presence of pathogenic bacteria, virus and mycoplasmas.
[d] Nasal svabs were examined only for bacteria.

closed wooden chamber into which aerosols were introduced by an ultrasonic nebulizer (Model 99, DeVilbriss Company, Somerset, PA).

The 12 calves were exposed for 15 min to aerosol droplets of *H. somnus* and/or *M. dispar* and/or a sterile saline solution (Table 1). Three calves were exposed to *H. somnus* only (No. 1–3), two animals were exposed to *M. dispar* only (No. 4–5), five animals were exposed to *M. dispar* followed by exposure to *H. somnus* 11–14 days later (No. 6–10). One animal was exposed to *M. dispar* followed by exposure to a sterile saline solution 11 days later (No. 11), and one animal was only exposed to a sterile saline solution (No. 12). All calves were placed singly in the aerosol chamber and exposed to approximately 25 ml of the inoculum. For determination of airborne viable *H. somnus* or *M. dispar* organisms in the chamber, a two step Andersen Sampler (Andersen, 1958), was connected. The Andersen Sampler allows 23 l air per minute to pass, and the lowest step of sampler only allows the respirable fraction, that is, particles with an aerodynamic diameter <5 μm to pass (Jacobsen et al., 1996). During inoculation, blood agar plates were placed in the Andersen Sampler for periods of 5 min, followed by incubation as described under post-mortem examination (2.5). Animals exposed to *H. somnus* or sterile saline (No. 1–3 and 6–12) were euthanized 4 days after inoculation, whereas the two calves only exposed to *M. dispar* (No. 4 and 5) were euthanized 11 days after inoculation.

2.5. Post-mortem examination

The calves were euthanized with an overdose of sodium pentobarbital and necropsied.

Samples from 5 to 10 areas of the lung, and the brain, liver, spleen and jejunum were examined bacteriologically. Cultivation attempts for bacteria were performed as described elsewhere (Tegtmeier et al., 1999b). In brief, samples were cultured on two 5% bovine blood agar plates of which one plate was incubated in a normal atmosphere at 37°C and the other in an 37°C atmosphere consisting of 10% CO_2 and 90% air. All plates were inspected for growth after 16 h and 40 h. The identification of *H. somnus* was based on growth of tiny white or yellow-white colonies on plates incubated with 10% CO_2 and no or feeble growth in normal air, by being Gram-negative, oxidase positive, catalase negative and by production of indol and fermentation of glucose.

Other bacterial pathogens were identified according to standard laboratory procedures.

The *H. somnus* isolates were ribotyped in order to compare and verify that the isolate was identical to the strain used for inoculation. Ribotyping was performed by DNA digestion by *Hind*III and hybridization at 56°C as described by Fussing and Wegener, (1993).

In addition, nasal swabs and 4–6 lung samples were examined for *H. somnus* by a species specific PCR test based on the 16s rDNA gene (Angen et al., 1998). DNA was obtained from the nasal svabs by chloroform/phenol extraction, precipitation with 96% ethanol and washing twice in 70% ethanol and dissolved in dH_2O. 2 μl of the solution was used for PCR. Lung samples were incubated on agar plates as described above and each plate was washed with 2 ml of dH_2O. After lysis, 2 μl was used for PCR.

Mycoplasma examination of lungs was performed by cultivation as described elsewhere (Tegtmeier et al., 1999b). Furthermore, lung tissue was examined for relevant respiratory virus (bovine respiratory syncytial virus (BRS virus), parainfluenza-3 virus (PI-3 virus), bovine coronavirus, bovine virus diarrhea virus) according to (Tegtmeier et al., 1999b).

3. Results

3.1. Animals

All calves had normal rectal temperature, normal respiratory rate and were without clinical signs of disease before challenge. The body weight varied from 37–63 kg at the time of euthanazia (mean 47.5 kg).

By radiography, an approximate 2 cm × 4 cm area of consolidated tissue was detected in the cranio-ventral lung region of calf No. 4. No radiographic abnormalities were determined in the other animals.

After exposure, most animals inoculated with *H. somnus* showed a transient rise in rectal temperature within the first 24 hrs (data not shown), whereas no rise in temperature was observed in the animals when inoculated with *M. dispar* or sterile saline.

Before inoculation, leukocyte counts were 4–9 × 10^6/ml but increased up to 3–4 times 2–3 days post inoculation (p.i.) with *H. somnus* (No. 6–10). For the control calf (No. 11)

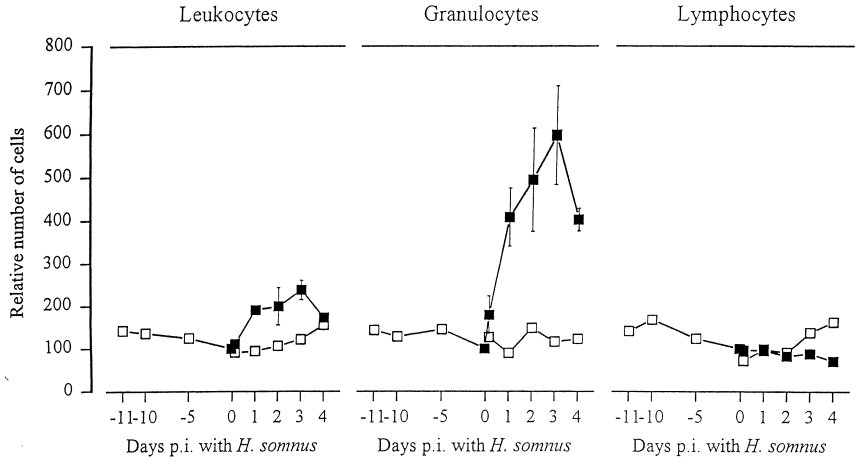

Fig. 1. Leukocyte counts of peripheral blood in calves infected with *Haemophilus somnus*. All calves were aerosol inoculated with *H. somnus* at Day 0. ■: Mean ±SEM of inoculated calves (No. 6–10), (the latter two were only sampled at −1 h, 3 h, 24 h p.i.). □: One calf (No. 11) infected with *M. dispar* on Day −11 and saline on Day 0. Measurements were performed in duplicate.

only inoculated with *M. dispar*, the leukocyte counts were 4–9 × 10^6/ml throughout the experiment. In those calves inoculated with *H. somnus*, a pronounced increase in granulocytes accounted for the distinct increase in peripheral leukocytes (Fig. 1). No significant differences between control and inoculated animals were found for monocytes and lymphocytes.

Semiquantitative RT-PCR on PBMC showed an increase in IL-8 expression as early as 3 h after challenge, compared to pre-challenge levels; there was, however, individual differences in the rapidity of the IL-8 response after challenge. Among a number of other cytokines investigated (IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFNγ, TNFα and GM-CSF) no consistent changes were found before and after challenge with *H. somnus*.

3.2. Aerosol exposure

The estimation of viable airborne microorganisms during the inoculation process revealed an uncountable number (>10^3 CFU) of *H. somnus* on both detection plates of the Andersen Sampler and also widespread growth of *M. dispar*. In all samplings, a small number (5–50 CFU) of non-pathogenic bacteria were identified by cultivation.

3.3. Post-mortem examination

The results of necropsy and microbiology are summarized in Table 1.

An approximate 1 cm × 2 cm acute focal necrotizing pneumonic lesion surrounded by a rim of red consolidated tissue, was present in the right cranial lobe of calf No. 1 inoculated with *H. somnus*. In this animal, *H. somnus* was isolated from the pneumonic lesion in pure culture, whereas no other pathogens were determined. The ribotype of the

isolate was identical to that of the inoculation strain (data not shown). A few areas of atelectasis scattered in a lobular, primarily subpleural pattern, were observed in some animals (including calf No. 4 where pre-inoculation radiography revealed a small area of consolidation). All such atelectatic areas were bacteriologically negative by cultivation and PCR, in addition to the 5–10 other investigated areas of the lung.

H. somnus was only recovered from calf No. 1, whereas *M. dispar* was isolated in pure culture from the lungs of all calves inoculated with this microorganism. A few *Pasteurella* spp. were isolated from one area of the lungs in calf No. 10 (inoculated with both *M. dispar* and *H. somnus*), where no lesions were present. Virus was not detected in any case.

Pathological and microbiological examinations of other organs revealed no significant findings.

PCR examination of lung tissue for detection of *H. somnus* was negative, except for the necrotic area in calf No. 1. At necropsy, all nasal swabs from calves inoculated with *H. somnus* were postive in the PCR test, whereas nasal swabs from calves inoculated with only *M. dispar* or sterile saline were negative.

4. Discussion

The presumed natural route of pulmonary infection was imitated by an aerosol inhalation model with the purpose to evaluate, whether *H. somnus* was capable of inducing pneumonia under such conditions in healthy animals. The study revealed that the pathogenic potential of *H. somnus* under these circumstances was minimal and inconsistent, as only one (No. 1) out of the eight calves developed a focal necrotizing pneumonic lesion, too small to induce clinical signs of respiratory disease.

Five animals were inoculated with *M. dispar* prior to *H. somnus*, as it was speculated that a previous challenge with *M. dispar* could induce pulmonary lesions, thereby compromizing the respiratory defence mechanisms to a degree severe enough to facilitate the establishment of *H. somnus* within the lung. In a previous experimental study of aerosol-induced *M. dispar*-associated calf pneumonia, a few small lung lesions were present at necropsy (Friis, 1980). Also, Krogh et al. (1986) observed lesions when calves were aerosol-inoculated with *M. dispar* prior to *H. somnus*. However, in the present study, the previous challenge with *M. dispar* did not enhance the ability of *H. somnus* to induce pneumonia, nor did *M. dispar* in itself induce macroscopic pneumonia. Furthermore, the calf (No. 1) that developed the pneumonic lesion had only been challenged with *H. somnus*.

In contrast to *H. somnus*, *M. dispar* became established in the lower respiratory tract, as it was re-isolated from all animals inoculated with this microorganism. The few scattered areas of atelectasis observed in some animals might in part have been induced by *M. dispar*, which is capable of inducing bronchiolitis with secondary atelectasis of the surrounding parenchyma (Friis, 1980). However, such atelectatic aeras were also observed in animals not challenged with *M. dispar.*

The reasons for the discrepancy between our results and the results previously presented by Krogh et al. (1986) might be several. In both studies, the animals were

placed in isolation barns immediately after birth and challenged when approximately 1 month old and in the present study, radiography was performed prior to challenge in order to ensure that no lung lesions were present before inoculation. However, differences in the immune status of the animals, differences in inoculation technique and differences in the virulence of inoculation strains might contribute to the conflicting observations.

In calves, much research has been focused on the presumed pathogenesis of pneumonia caused by *M. haemolytica*. This microorganism and associated diseases have been studied intensively and was reviewed by Frank (1989). According to that review, healthy calves carry small numbers of *M. haemolytica* serotype 1 in the nasopharynx and when, for example, concomitant stress or viral infection compromise the defence mechanisms, the animals get highly susceptible to pneumonic pasteurellosis as the bacteria are able to multiply rapidly in the nasopharynx. Thus, high numbers of bacteria may subsequently be inhaled in aerosol droplets to the lower parts of the respiratory system where a rapid replication in the lungs will result in development of pneumonia. A similar pathogenesis for *H. somnus* induced pneumonia is a natural assumption, although the ability of *H. somnus* to develop disease in the present study was minimal. Even though direct inhalation of high numbers *H. somnus* into the lung parenchyma as well as multiplication of the bacteria on the nasal mucosa were possible (as *H. somnus* was detected from nasal svabs by PCR in all animals inoculated with *H. somnus*), no pneumonic lesions developed in the majority of the cases. This indicates that *H. somnus* should not be considered as a primary pulmonary pathogen capable of inducing pneumonia alone.

Previous studies on aerosol induction of bacterial calf pneumonia have focused on *M. haemolytica* and *Pasteurella multocida*. Jericho and Langford (1978); Jericho et al. (1982); Yates et al. (1983) studied the effects of combinations of aerosols of *bovine herpes virus*-1 (BHV-1), PI-3 virus and *M. haemolytica*. In these studies, exposure of calves to aerosols containing *M. haemolytica* failed to produce respiratory disease, whereas calves previously exposed to BHV-1 or PI-3 virus developed pneumonia when subsequently exposed to *M. haemolytica*. However, Jericho and Carter (1985) reported that pneumonia could be induced by aerosol exposure to *P. multocida* alone.

Thus, most previous studies show that aerosol exposure to bacteria alone is not capable of inducing pneumonia. These observations strongly indicate that stress due to, for example, transportation, concomitant infection, damage of the respiratory tract, and/or immunosuppresion is necessary for establishment of a bacterial pneumonia. This is in accordance with our results which furthermore revealed that infection with *M. dispar* alone was not sufficient as predisposing factor. Therefore, one should consider other predisposing factors. In Denmark, BRS virus is a common pathogen detected in pneumonic lungs (Tegtmeier et al., 1999b) and this virus might, as BHV-1, be able to play the triggering role in development of pneumonia.

Another reason for the inconsistent results in the present experiments could be that the particular *H. somnus* strain used throughout the study might represent a low-virulent variety of the species. However, this *H. somnus* strain has previously induced severe pulmonic lesions in calves when inoculated intrabronchially (Tegtmeier et al., 1999a).

Even though no major lesions were observed at necropsy in the present study, except the focal necrotizing pneumonia in one calf and the few small areas of atelectasis, the results of blood cell and cytokine analysis clearly indicate that a systemic response was

provoked after inoculation with *H. somnus* as does the rise in rectal temperature within the first day after inoculation. This response is most probably due to the inhalation of bacteria into the lungs where resident macrophages have been reported, at least in the pig to be particularly sensitive to bacterial LPS (Lin et al., 1994). Peripheral blood cell changes occurred 2–3 days after inoculation with *H. somnus*. The granulocytes were active as shown by phagocytosis of propidium iodide-labelled *Staphylococcus aureus* measured by FACS analysis (data not shown). The changes in peripheral blood cells clearly indicated that a significant expansion of granulocytes took place immediately after challenge and peaked at 2 days after challenge whereas the lymphocyte population remained remarkably constant (Fig. 1). This is consistent with the induction of mRNA for IL-8 peaking from 3 h to between 12 and 72 h after exposure in different animals, all indicating that bacteria were indeed introduced into the lungs giving rise to a systemic granulocyte reponse.

5. Conclusion

In conclusion, the most likely explanation for the results obtained in the present study is that the calves were able to eliminate the inhaled *H. somnus* bacteria by the innate non-specific local and systemic defence responses operating in healthy animals. The amount of bacteria used for inoculation is most probably higher than what is found under natural conditions. Nevertheless, the granulocytosis and the induction of mRNA for IL-8 presumably mimic the response occurring when calves are exposed to *H. somnus* under natural conditions, thus giving valuable information on the cellular mechanisms involved in defeating the development of pneumonia.

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References

Andersen, A.A., 1958. New sampler for the collection, sizing, and enumeration of viable airborne particles. J. Bacteriol. 76, 471–484.

Angen, Ø., Ahrens, P., Tegtmeier, C., 1998. Development of a PCR test for identification of *Haemophilus somnus* in pure and mixed cultures. Vet. Microbiol. 63, 39–48.

Angen, Ø., Mutters, R., Caugant, D.A., Olsen, J.E., Bisgaard, M., 1999. Taxonomic relationships of the [*Pasteurella*] *haemolytica*-complex as evaluated by DNA–DNA hybridizations and 16s rRNA sequencing with proposal of *Mannheimia haemolytica* gen.nov., comb.nov., *Mannheimia granulomatis* comb.nov.,

Mannheimia glucosida sp.nov., *Mannheimia ruminalis* sp.nov. and *Mannheimia varigena* sp.nov. Int. J. Syst. Bacteriol. 49, 67–86.

Frank, G.H., 1989. Pasteurellosis of cattle. In: Adlam, C., Rutter, J.M. (Ed.), Pasteurella and Pasteurellosis. Academic Press, NY ISBN 0-12-044274-4, pp. 197–222.

Friis, N.F., 1978. Heterogeneity of *Mycoplasma dispar*. Acta. Vet. Scand. 19, 407–412.

Friis, N.F., 1980. *Mycoplasma dispar* as a causative agent in pneumonia of calves. Acta. Vet. Scand. 21, 34–42.

Fussing, V., Wegener, H.C., 1993. Characterization of bovine *Haemophilus somnus* by biotyping, plasmid profiling, REA-patterns and ribotyping. Zbl. Bakteriol. 279, 60–74.

Gogolewski, R.P., Leathers, C.W., Liggitt, H.D., Corbeil, L.B., 1987. Experimental *Haemophilus somnus* pneumonia in calves and immunoperoxidase localization of bacteria. Vet. Pathol. 24, 250–256.

Grell, S.N., Tegtmeier, C., Riber, U., Angen, Ø., Heegaard, P.M.H., 2000. Characterization of bovine cytokines and cellular responses to subclinical infection with *Haemophilus somnus*, in preparation.

Jackson, J.A., Andrews, J.J., Hargis, J.W., 1987. Experimental *Haemophilus somnus* pneumonia in calves. Vet. Pathol. 24, 129–134.

Jacobsen, M.J., Nielsen, J.P., Nielsen, R., 1996. Comparison of virulence of different *Actinobacillus pleuropneumoniae* serotypes and biotypes using an aerosol infection model. Vet. Microbiol. 49, 159–168.

Jericho, K.W.F., Carter, G.R., 1985. Pneumonia in calves produced with aerosols of *Pasteurella multocida* alone and in combination with bovine herpesvirus 1. Can. J. Comp. Med. 49(2), 138–144.

Jericho, K.W.F., Langford, E.V., 1978. Pneumonia in calves produced with aerosols of bovine herpesvirus 1 and *Pasteurella haemolytica*. Can. J. Comp. Med. 42, 269–277.

Jericho, K.W.F., Darsel, C.leQ., Langford, E.V., 1982. Respiratory disease in calves produced with aerosols of Parainfluenza-3 virus and *Pasteurella haemolytica*. Can. J. Comp. Med. 46, 293–301.

Kobisch, M., Friis, N.F., 1996. Swine mycoplasmoses. Rev. Sci. Tech. Off. Int. Epiz. 15(4), 1569–1605.

Krogh, H.V., Pedersen, K.B., Friis, N.F., 1986. Pneumonia in calves associated with *Haemophilus somnus*. In: Proceedings of the 14th International Congress on Diseases of Cattle. Dublin, pp. 585–589.

Lin, G., Pearson, A.E., Scamurra, R.W., Zhou, Y., Baarsch, M.J., Weiss, D.J., Murtaugh, M.P., 1994. Regulation of Interleukin-8 expression in porcine alveolar macrophages by bacterial lipopolysaccharide. J. Bio. Chem. 269, 77–85.

Nayar, P.S.G., Ward, G.E., Saunders, J.R., MacWilliams, P., 1977. Diagnostic procedures in experimental *Haemophilus somnus* infection in cattle. Can. Vet. J. 18(6), 159–163.

Potgieter, L.N.D., Helman, R.G., Greene, W., Breider, M.A., Thurber, E.T., Peetz, R.H., 1988. Experimental bovine respiratory tract disease with *Haemophilus somnus*. Vet. Pathol. 25, 124–130.

Tegtmeier, C., Arnbjerg, J., 1999. Evaluation of radiology as a tool to diagnose pulmonic lesions in calves i.e., prior to experimental infection studies. J. Vet. Med. B., in press.

Tegtmeier, C., Bloch, B., Jensen, N.E., Jensen, H.E., 1999a. Initial lung lesions in two calves experimentally infected with *Haemophilus somnus*. J. Vet. Med. B. 46, 517–523.

Tegtmeier, C., Uttenthal, Aa., Friis, N.F., Jensen, N.E., Jensen, H.E., 1999b. Pathological and microbiological studies on pneumonic lungs from Danish calves. J. Vet. Med. B. 46, 693–700.

Yates, W.D.G., Jericho, K.W.F., Doige, C.E., 1983. Effect of viral dose on experimental pneumonia caused by aerosol exposure to bovine herpesvirus 1 and *Pasteurella haemolytica*. Can. J. Comp. Med. 47, 57–63.

Whiteley, L.O., Maheswaran, S.K., Weiss, D.J., Ames, T.R., 1991. Alterations in pulmonary morphology and peripheral coagulation profiles caused by intratracheal inoculation of live and ultraviolet light-killed *Pasteurella haemolytica* A1 in calves. Vet. Pathol. 28, 275–285.