

Age-dependent differences in the pathogenesis of bovine respiratory syncytial virus infections related to the development of natural immunocompetence

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The severity of respiratory syncytial virus (RSV) infections appears to differ with age in both humans and bovines. A primary RSV infection in naïve infants and in young calves runs a more severe course when they are 1–6 months old than in their first month of life. The relative lack of clinical signs in the first month of age may be due to high levels of maternally derived neutralizing antibodies or low exposure to infectious virus. This study examined whether age-dependent differences in the pathogenesis of bovine RSV (bRSV) between neonatal and young calves may be due to differences in age-dependent immunocompetence. To study the effect of age and immune parameters on bRSV disease in neonatal and young calves, neonatal (1-day-old) calves without maternally derived antibodies were infected experimentally with bRSV and the severity of disease and immune responses were evaluated in comparison with disease in similar 6-week-old infected calves. Neonatal calves had more extensive virus replication and lung consolidation, but lower pro-inflammatory [in particular tumour necrosis factor alpha (TNF- α)] responses, specific humoral immune responses, lung neutrophilic infiltration and clinical signs of disease than 6-week-old calves. The lack of correlation between virus replication and clinical signs suggests an important role of pro-inflammatory cytokines, in particular TNF- α , in the disease. The capacity to produce pro-inflammatory TNF- α appeared to increase with age, and may explain the age-dependent differences in RSV pathogenesis.

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INTRODUCTION

Bovine respiratory syncytial virus (bRSV) is one of the major causes of bovine respiratory disease (BRD). BRD results from a complex, multifactorial interaction of stressors, animal susceptibility and respiratory pathogens, and is probably one of the most common and costly diseases of feedlot and dairy cattle worldwide (Snowder *et al.*, 2006). Apparently healthy calves harbour potentially pathogenic bacteria in their respiratory tract waiting for viruses to pave their way to invasive infection, causing BRD (Angen *et al.*, 2009; Babiuk *et al.*, 1988). bRSV is considered endemic in Europe, and infections with bRSV are one of the most common diseases in the first years of life of cattle, with the highest incidence in autumn and winter (Van der Poel *et al.*, 1994). In fact, by

2 years of age, the seroprevalence of antibodies against bRSV is >70%. Nevertheless, bRSV reinfection can occur throughout life, although becoming progressively less severe with the increase in age.

In vivo, bRSV primarily targets the respiratory epithelium in the nasopharynx, bronchi, bronchioles and alveolar spaces. Clinical signs are characterized by general illness (pyrexia, anorexia and depression), upper respiratory tract disease (URTD; nasal and ocular discharge and cough) and lower respiratory tract disease (LRTD; tachypnoea and, in severe cases, dyspnoea). Although in many cases the lower respiratory tract is involved in infection, the clinical signs may remain limited to the upper respiratory tract (Verhoeff *et al.*, 1984). Age has always been identified as an important risk factor for development of the more severe (lower) respiratory tract disease. Severe, life-threatening LRTD is seen mostly during (primary) infection in 1–6-month-old

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calves (Bryson *et al.*, 1978; Kimman *et al.*, 1988). Strikingly, newborns are generally not severely affected. These observations have led to the hypothesis that these very young calves are well protected by, for example, passively acquired maternally derived antibodies (Kimman *et al.*, 1988). Kimman *et al.* (1988) showed that the severity of disease was inversely related to the level of maternal antibodies in calves from 1 to 3 months old, suggestive of a role of immunopathogenic mechanisms. In contrast, Tjørnehøj *et al.* (2003) showed that there was no correlation between the neutralizing antibody titre at inoculation and the peak respiratory rate/severity of elicited pneumonia in calves of 2–5 months of age.

Thus, severe disease appears to be observed less often in older calves, but on the other hand seems to be practically absent in calves younger than 2 weeks, irrespective of the maternal antibody titre (Baker *et al.*, 1986). Kimman *et al.* (1988) suggested that, in these newborn calves, exposure to the virus is lower during the first 10–14 days of their lives due to their individual housing and to them not experiencing the entire period of BRSV circulation on the farm.

In the present study, we hypothesized that age-dependent differences in the pathogenesis of BRSV in neonatal and young calves are due to differences in immunocompetence. We therefore examined the effect of BRSV inoculation in neonatal (1-day-old) and young (6-week-old) calves in the absence of maternal antibodies and evaluated the severity of disease and BRSV immune responses in both groups.

RESULTS

BRSV shedding and specific humoral immune responses after inoculation

To assess the impact of age on severity of disease, 1-day-old neonates were infected by intranasal inoculation of $10^{3.4}$ TCID₅₀ BRSV strain Odijk and compared with 6-week-old ('young') calves. As expected, BRSV was isolated from the upper respiratory tract in all calves, but the neonates shed virus for significantly longer than the young calves [mean \pm SEM, 5.6 ± 0.5 vs 3.8 ± 0.8 days post-infection (p.i.), $P=0.0238$; Fig. 1]. The magnitude of virus excretion was calculated as the area under the curve. The mean virus excretion in young calves was 12.14 ± 2.03 versus 14.26 ± 2.75 TCID₅₀ ml⁻¹ in neonates. Infectious BRSV was isolated from day 6 lung washes in three (two young and one neonatal) calves. All young calves seroconverted by 13 days p.i., whereas three of the neonates remained serologically negative (mean virus neutralizing antibody titre ≤ 2) for the duration of the experiment. Young calves developed significantly more neutralizing antibodies than the neonates (Fig. 2; $P=0.0087$).

Clinical signs after BRSV infection

The calves were monitored clinically for 13 days after infection, at which point the experiment was terminated.

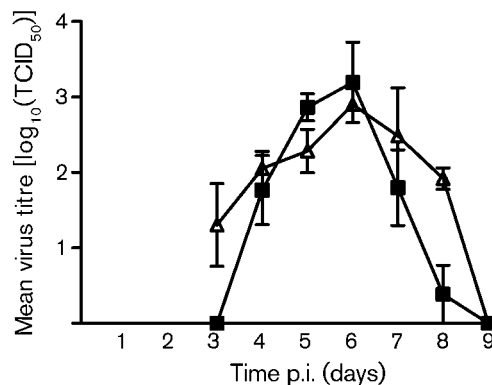


Fig. 1. Mean (\pm SEM) virus titre in nasopharyngeal fluid samples per group from day 0 (prior to infection) up to 10 days p.i. ■, Six-week-old young calves; Δ , 1-day-old neonates. Neonates shed virus for significantly longer than the young calves (5.6 ± 0.5 vs 3.8 ± 0.8 days, $P=0.0238$).

Parameters evaluated were categorized as general illness, URTD and LRTD (Fig. 3). None of the neonates was scored as generally ill, whereas three of five young calves were scored as generally ill for 1–3 days. This was confirmed by assessment of rectal temperatures. Whilst four of five young calves had fever (rectal temperature >39.6 °C) for 1–4 days, none of the neonates had fever. These differences were statistically significant ($P<0.05$).

Signs of URTD were observed in all calves in both age groups. A moderate URTD (score 2) was observed in all neonates from day 5 p.i. for 2–4 days (mean 2.8 ± 0.8 days) and in all young calves from day 6 p.i. for 2–6 days (mean

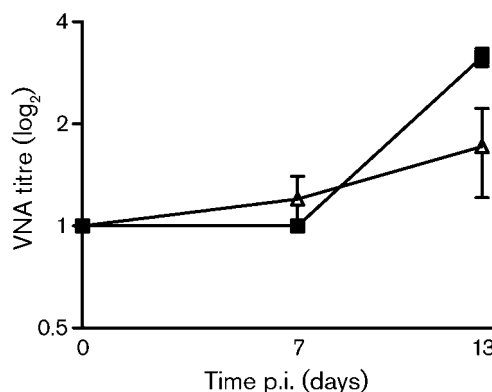


Fig. 2. Mean virus-neutralizing antibody titres (\pm SEM) per group, measured at day 0 (prior to infection), day 7 p.i. and at the end of the study (day 13 p.i.). ■, Six-week-old young calves; Δ , 1-day-old neonates. Antibody titres are expressed as reciprocal values of the highest neutralizing dilution. Starting at a 1:4 dilution, ≤ 2 ($1 \log_2$) was considered to be negative. Six-week-old calves had significantly more neutralizing antibodies than 1-day-old calves ($P=0.0087$) at 13 days p.i.

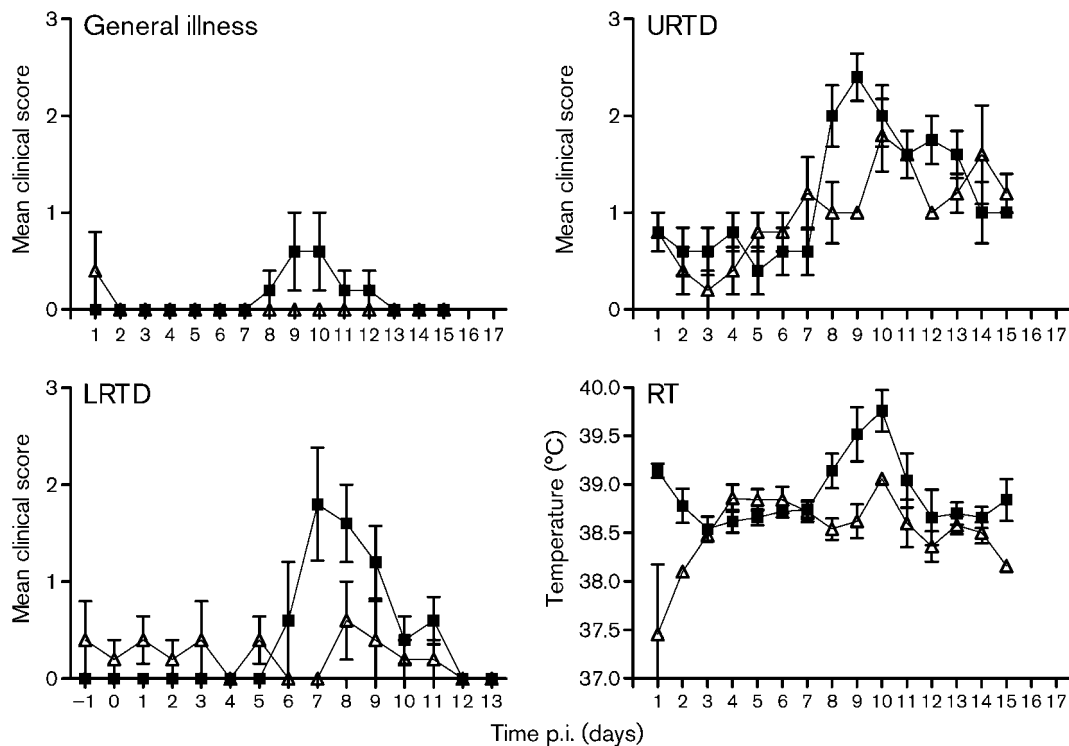


Fig. 3. Clinical observations. Mean clinical scores (\pm SEM) per group from day -1 (prior to infection on day 0) until the end of the study (day 13). ■, Six-week-old young calves; △, 1-day-old neonates. RT, Rectal temperature.

4.6 \pm 1.5 days). Severe URTD (score 3) was scored four times in three calves in the young calves group, and twice in two calves in the neonates group. LRTD was scored in all young calves and in four of five neonates. Severe LRTD was observed in only two young calves for 1–3 days, resulting in a significant difference ($P < 0.001$) on day 7 p.i. between the groups. A significantly higher number of days with tachypnoea, defined as >40 breaths min^{-1} , was observed in the young calves group compared with the neonates (mean 4.8 \pm 1.6 vs 2.0 \pm 1.3 days, $P < 0.05$).

Characteristics of inflammatory responses in peripheral blood and in the lungs

Phenotypic analysis of the cells migrating into the infected lungs revealed a strong neutrophilic infiltration starting from day 3 in the young calves, which coincided with the peak of clinical infection around day 8 and decreased thereafter (Fig. 4). Neonates revealed significantly more neutrophilic granulocytes at day 0, which decreased thereafter and peaked again at day 13 (as a result of one calf, #9069). Higher percentages of neutrophilic granulocytes were found in bronchoalveolar lung fluid (BALF) samples in the young calves at days 6 ($P = 0.0714$) and 9 ($P = 0.0317$) p.i. compared with neonates. Blood smear analyses showed overall significantly higher percentages of neutrophils in neonates compared with young calves (Fig. 5; $P < 0.001$). In young calves, a clear drop in peripheral blood

neutrophils was observed on day 10 p.i. compared with day 9 p.i. (8.2 \pm 2.7 vs 16.4 \pm 7.0 %, respectively; $P = 0.06$). The bronchoalveolar lung cell (BALC) counts and numbers of macrophages and lymphocytes in lung lavage samples collected on study days 0, 3, 6, 9 and 13 are summarized in Fig. 4.

BALF samples were analysed using a SearchLight Bovine Cytokine Array for interleukin-4 (IL-4), IL-6, tumour necrosis factor alpha (TNF- α) and gamma interferon (IFN- γ) (Fig. 6). Significantly higher levels of TNF- α were measured in young calves versus neonates (overall mean 705.6 \pm 304.4 vs 303.9 \pm 109.1 pg ml^{-1} , respectively; $P < 0.05$). Significantly higher levels of IL-6 were measured in neonates versus young calves (overall mean 958.5 \pm 231.4 vs 612.7 \pm 169.1 pg ml^{-1} , respectively; $P < 0.05$). For IL-4, a significant difference ($P < 0.05$) was measured on study day 9. For IFN- γ , a significant difference was observed, but only on day 6 p.i. ($P < 0.05$). Higher IFN- γ BALF levels (mean 2391 pg ml^{-1}) were measured in young calves compared with neonates (mean 74.12 pg ml^{-1}).

The partial pressure of O_2 (pO_2) in arterial blood samples directly reflects the lung's ability to oxygenate blood and thus low levels are indicative of lung damage. A clear drop in the supply of oxygen was observed around the peak of infection, corresponding to the observed clinical signs of LRTD and increased respiratory rates (Fig. 7). This drop (mean values below 70 mmHg, reference/normal arterial

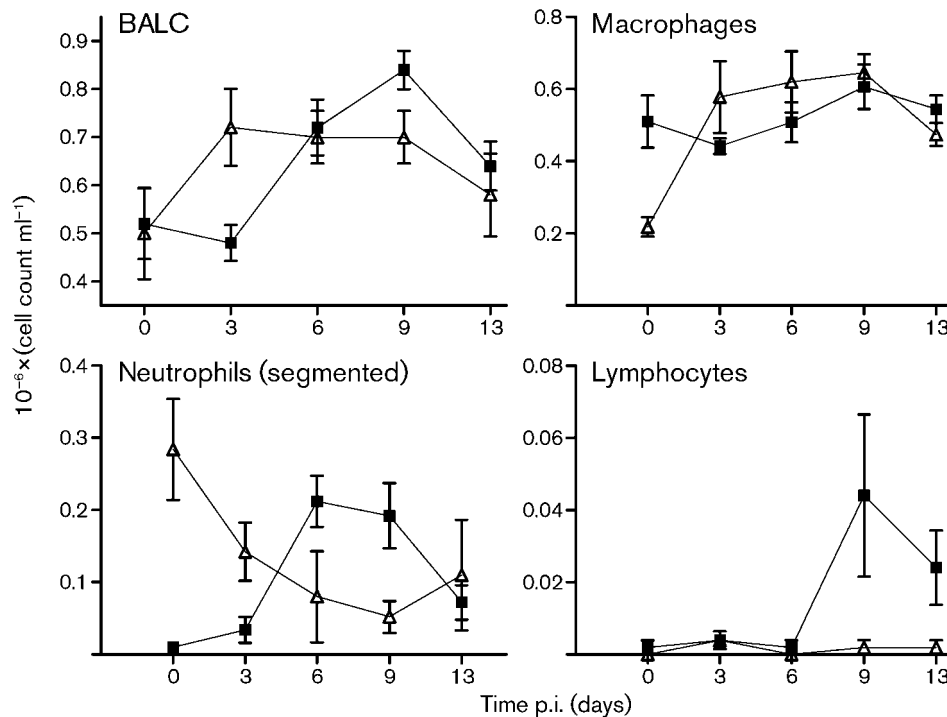


Fig. 4. Mean numbers of cells (\pm SEM) recovered (expressed as cells ml^{-1}) from BALF samples per group from day -1 (prior to infection on day 0) until the end of the study period (day 13). ■, Six-week-old young calves; \triangle , 1-day-old neonates. BALC, Bronchoalveolar lung cells.

pO_2 value is >85 mmHg; Coghe *et al.*, 2000) between days 7 and 9 was observed in both age groups. CO_2 retention was not observed.

Haptoglobin is a major acute-phase protein in cattle and is considered a good indicator of the host response to BRSV (Heegaard *et al.*, 2000). Plasma lactate is widely used as an indicator of anaerobic metabolism, which reflects the

severity of disease (Coghe *et al.*, 2000). Mean concentrations are shown in Fig. 7. Prior to inoculation, plasma lactate concentrations (mean \pm SEM) were significantly higher in neonates than in young calves (4.04 ± 0.79 vs 0.94 ± 0.14 mmol l^{-1} , respectively; $P < 0.0001$; Fig. 7). Following infection, no significant differences in plasma lactate concentrations were observed between neonates and young calves.

Upon inoculation, significantly higher haptoglobin concentrations ($\mu\text{g ml}^{-1}$) were detected in young calves compared with neonates (overall mean \pm SD 123.5 ± 46.63 vs 79.27 ± 31.73 $\mu\text{g ml}^{-1}$, respectively; $P < 0.05$). A positive correlation ($r = 0.2833$, $P < 0.001$) between rectal body temperature and haptoglobin concentrations was observed.

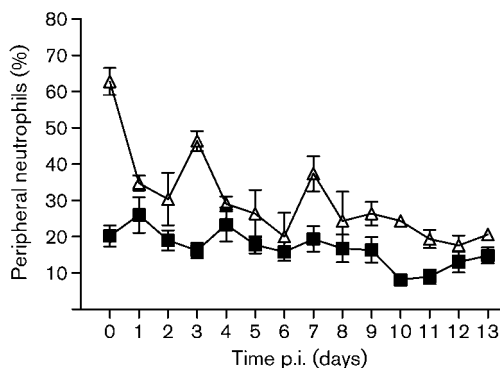


Fig. 5. Mean percentages of peripheral neutrophils (\pm SEM) per group from day 0 (prior to infection) until the end of the study period (day 13). ■, Six-week-old young calves; \triangle , 1-day-old neonates.

Pathology

At necropsy, the overall percentages of consolidated lung tissue area ranged from 0 to 5.1%. The mean percentage (\pm SD) of consolidated lung tissue area in neonates was significantly higher compared with young calves (2.35 ± 1.51 vs 0.35 ± 0.36 %, respectively; $P < 0.05$). When consolidated lung areas were scored as described by Viuff *et al.* (2002), four of five neonates scored 1, whilst all young calves scored 0. Calves of both groups developed multifocal bronchointerstitial pneumonia, with a marked proliferation

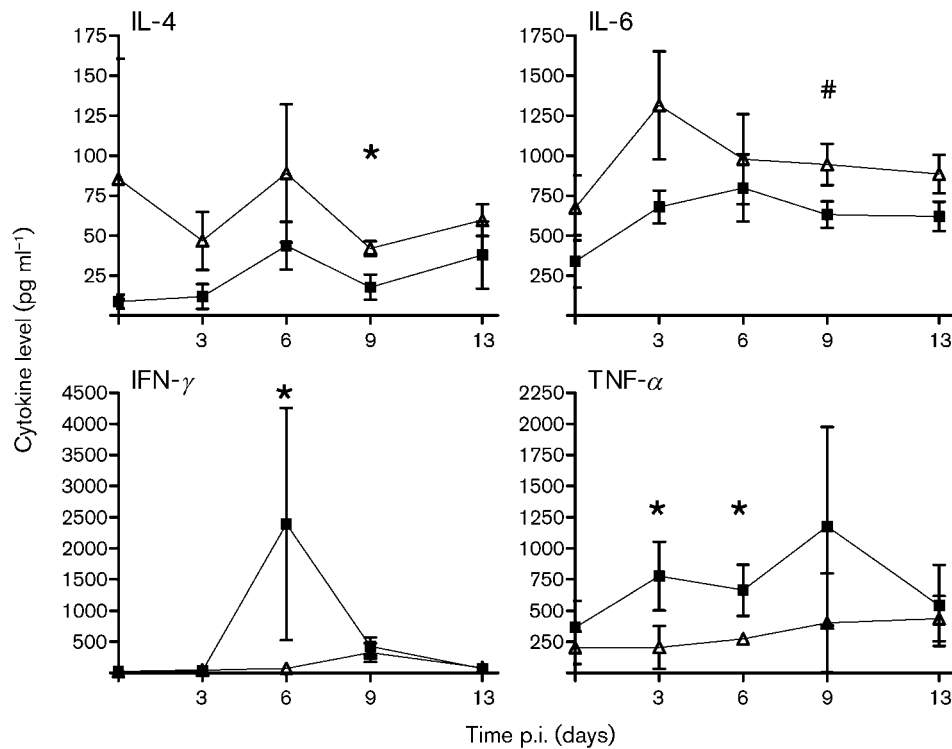


Fig. 6. Mean cytokine measurements (+SEM) in BALF samples per group from day 0 (prior to infection) until the end of the study period (day 13). Statistically significant time points ($P < 0.05$) are indicated by an asterisk. The calculated P value for IL-6 on day 9 was 0.07 (#). ■, Six-week-old young calves; △, 1-day-old neonates.

of bronchiolar epithelium. Bronchioles were narrow and often filled with secretion. It should be noted that calf #9069 did not have any consolidated lung area.

DISCUSSION

To study the effect of age on the pathogenesis of bRSV infection in the absence of maternal antibodies, we compared the clinical representation of disease in colostrum-deprived neonatal (1-day-old) and young (6-week-old) calves. To our knowledge, the study reported here is the first to describe differences in bRSV infection in neonates and young calves of these ages.

We observed no severe disease in the neonates following experimental bRSV infection. Kimman *et al.* (1988) observed similar effects after natural infection (no clinical disease in calves irrespective of the maternal antibody titre), but hypothesized that this was due to a low virus exposure. Here, we have provided evidence that this hypothesis is not correct. In the current study, neonates were clinically less affected than the young calves, despite a longer period of virus replication and increased lung pathology. Thus, direct cytopathology plays a minor role in bRSV pathogenesis, and a role of immune mechanisms in causing clinical symptoms is suggested.

Most cases of severe RSV disease in the field are observed in 1–3-month-old calves, when the calves possess maternally derived neutralizing antibodies but at lower levels than at birth. The possible influence of maternally derived antibodies (MDAs) on the outcome of disease remains a controversial issue. Whilst high MDA levels are considered to mitigate the disease, moderate to low MDA levels might aggravate the disease by immunopathological mechanisms. Because the highest neutralizing antibody titres are found in the youngest individuals, one may suggest a relationship between MDA titres and RTD severity. Our findings do not support this notion, but instead suggest a role of differences in immunocompetence between the different age groups.

Although still a controversial issue, it has been concluded from a number of studies that immunological factors play a major role in the most severe type of pulmonary damage associated with RSV infection (Coomer *et al.*, 2001; Heegaard *et al.*, 2000; Rontved *et al.*, 2000). This view has evolved from unusual epidemiological patterns of RSV disease and from attempts to interpret the altered reactivity to RSV infections observed after primary vaccination. Apparently, the virus in the respiratory tract by itself does not explain the severe disease manifestations. Tregoning *et al.* (2008) demonstrated in BALB/c mice that a neonatal (aged 4 days) human RSV (hRSV) infection was associated

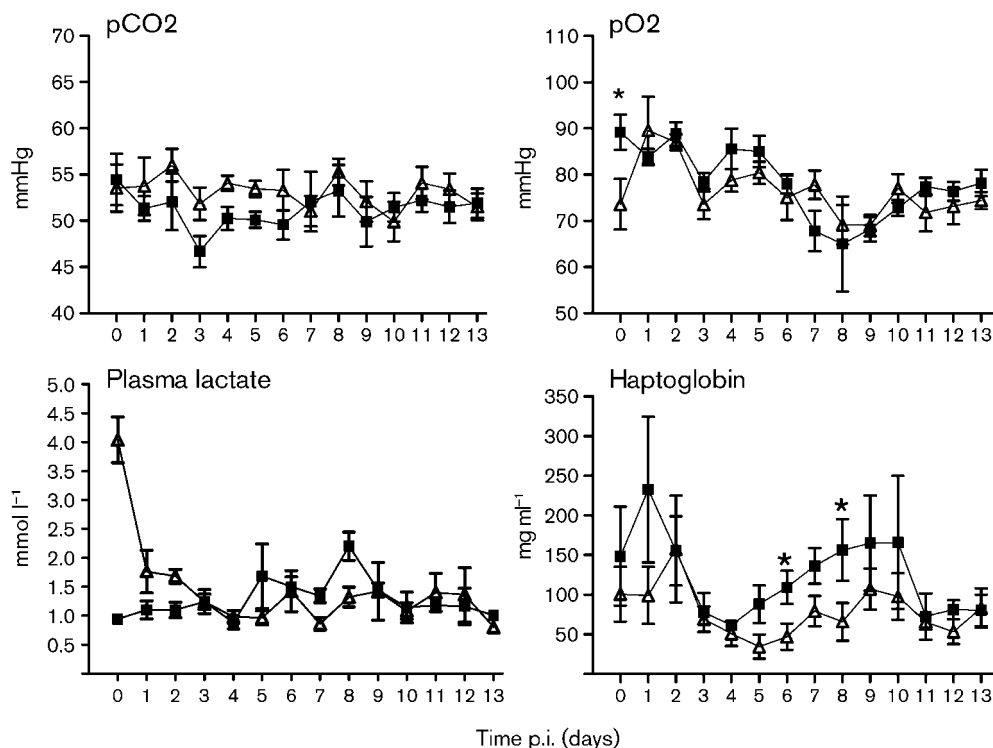


Fig. 7. Mean arterial pO₂ and pCO₂ values and mean concentrations of plasma lactate and haptoglobin (\pm SEM) per group from day -1 (prior to infection on day 0) until the end of the study period (day 13). ■, Six-week-old young calves; Δ , 1-day-old neonates. Haptoglobin concentrations were significantly higher on days 6 and 8 p.i. (* $P < 0.05$). The blood gas status of newborn calves differed significantly from that of 6-week-old calves; pO₂ was significantly lower in 1-day-old than in 6-week-old calves (73.6 ± 11 vs 89.2 ± 7.5 mmHg; $P < 0.05$). There was a significant increase ($P < 0.01$) in pO₂ on the second day of life compared with the first day of life (89.6 ± 14.6 vs 73.6 ± 11 mmHg), which was considered to be caused by adaptation of the neonatal mechanics of breathing to extra-uterine life in the newborn calves (Varga *et al.*, 1998). A clear drop in the supply of oxygen was observed around day 8 p.i., corresponding to the observed peak in the LRTD clinical signs and respiratory rates (not shown). This drop was observed in both age groups, but was more profound in the 6-week-old calves (days 7–9, mean values < 70 mmHg). CO₂ retention was not observed.

with less inflammation and disease than in immature adults (aged 4–6 weeks) and suggested that this was due to different cytokine production profiles of cells recruited to the site of infection.

To control, as far as possible, endogenous flora such as *Pasteurella* spp., *Haemophilus somnus* and *Mycoplasma* spp., we decided to use Caesarean-derived rather than conventionally raised calves in this study. After birth, the calves were kept in our own highly controlled animal facilities (different hygiene barriers and HEPA-filtered ingoing air), to maintain animals free of major bovine pathogens. In this way, the pathological bRSV infection was not complicated by pathogenic secondary bacterial infections. Considering this, there is a possibility that clinical signs noted in our study may have been less severe than would have been the case under field conditions, where bacterial superinfections are considered to have a major effect on the course of the disease (Angen *et al.*, 2009; Babiuk *et al.*, 1988).

It was concluded previously by others that an influx of neutrophils in the lung tissue of animals experiencing bovine respiratory disease resulted from secondary bacterial infections (Haslett, 1999; Slocombe *et al.*, 1985; Soethout *et al.*, 2004). However, in this study we observed a profound influx of neutrophils in the absence of a secondary bacterial superinfection. Moreover, we observed intriguing different neutrophil infiltrations in the lungs of neonatal and young calves. It is tempting to speculate that this difference was caused by the different activation of pro-inflammatory cytokines, in particular TNF- α .

Young calves showed a clear drop in peripheral blood neutrophils on day 10 p.i. (Fig. 5). Lukens *et al.* (2010) described an increase in neutrophil precursors for hRSV patients between days 7 and 9 after the onset of symptoms and this subsided a few days later (Lukens *et al.*, 2010). All of their patients required mechanical ventilation because of respiratory failure due to an LRTD, which could have induced an enhanced neutrophil activation. Lukens *et al.*

(2010) started measuring cell levels 3–8 days after the onset of symptoms, which would have been approximately days 10–15 p.i. in our study.

The pattern of cytokine and chemokine expression induced during the innate immune response activates migration of immune cells (macrophages, eosinophils, basophils, neutrophils and natural killer cells) to the sites of infection, regulates B- and T-cell activation and may enhance viral clearance or exacerbate disease (Decleva *et al.*, 2002; Janssen *et al.*, 2007; Rutigliano & Graham, 2004). We evaluated expression of four cytokines in our study: IL-4, IL-6, IFN- γ and TNF- α . No overall significant differences in expression of IL-4 and IFN- γ were observed, although a significant increase in IFN- γ expression after infection was observed in young calves, with a significantly higher expression ($P < 0.05$) observed on day 6 p.i. compared with neonates. On the other hand, IL-4 expression appeared to be higher in the neonates. TNF- α expression was overall significantly higher in young calves and IL-6 overall in the neonates.

TNF- α is a pro-inflammatory cytokine produced by activated cells of the monocyte–macrophage lineage that provides a rapid form of host defence against various infectious agents. TNF- α is known as a potent mediator of inflammation and plays a key role during the inflammatory responses of hRSV bronchiolitis (Becnel *et al.*, 2005; McNamara *et al.*, 2004; Morrison *et al.*, 2007). TNF- α may play a protective role in RSV infection (Neuzil *et al.*, 1996). However, when in excess, TNF- α can provoke chronic inflammatory disorders. You *et al.* (2006) recently published the results of a study in a neonatal-mouse model and suggested that TNF- α plays a key role in mediating chronic inflammation (e.g. asthma) of the bronchi after RSV infection. Tight regulation of TNF- α levels might therefore be essential to separate its beneficial from its harmful effects. Indeed, our data also showed that young calves with more severe clinical disease had significantly higher TNF- α levels than newborn calves, indicating that alveolar macrophages from young calves produce more TNF- α than alveolar macrophages from newborn calves. Neutrophils respond to TNF- α with a respiratory burst after a β_2 -integrin-dependent adherence to extracellular matrix proteins (Decleva *et al.*, 2002). TNF- α has been proposed as an ideal drug target for the therapy of RSV-induced bronchiolitis (Rutigliano & Graham, 2004; You *et al.*, 2006). Overall, our study points to a role for innate immunity in RSV disease, a conclusion that was also drawn from a genetic-association study in children (Janssen *et al.*, 2007).

Why alveolar macrophages in young calves produce more TNF- α remains a subject for further study. Mallard *et al.* (1998) published substantial evidence that both innate and acquired defence mechanisms are lowest from 3 weeks pre-calving to 3 weeks post-calving. Mallard *et al.* (1998) concluded that calves are born with a functional immune system and are capable of responding to certain antigenic

stimuli, but that the system does not yet operate at the optimum response capacity. Chase *et al.* (2008) reported that the innate response mediated by phagocytic cells (neutrophils and macrophages) declines in functional capacity around birth, due to an increase in fetal cortisol levels. However, there is conflicting evidence about the bovine neonatal innate response. For example, other papers have shown that phagocytosis, respiratory burst and bactericidal activity of calf neutrophils are intact and functional from the first week of life (Kampen *et al.*, 2006); a reduced percentage of phagocytosing cells has been reported in neonatal calves compared with older calves (Menge *et al.*, 1998); and neonatal macrophages have been reported to exhibit enhanced phagocytic activity and oxidative burst activity (Menge *et al.*, 1998). Such conflicting data might result from differences in experimental protocols and read-out parameters. Further studies are clearly required to address these important issues in more detail.

Another interesting finding in our study was the absence of detectable neutralizing antibody responses in some of the neonatal calves. Significantly lower neutralizing serum antibody titres ($P < 0.05$) appeared by 13 days p.i. in neonates compared with young calves. These findings were largely confirmed by ELISA (data not shown). The ability to respond to viral surface glycoproteins with a protective antibody response appears to be acquired gradually over the first months of life. It is thus likely that the limited antibody response is explained by an inefficient antigen presentation and T-helper-cell response due to immunological immaturity (Delespesse *et al.*, 1998; Siegrist, 2001). Another explanation could be a limited number of circulating B cells in neonates (Chase *et al.*, 2008). However, despite lower numbers of B cells, such calves may have the capacity to respond to an antigen (Kampen *et al.*, 2006).

Although further studies are needed, it appears that differences in immune responses, in particular of the natural and pro-inflammatory immune response, are responsible for the differences in clinical appearance of BRSV infection in newborn versus young calves. Despite the presence or absence of maternally derived antibodies, neonates do not appear to become severely ill, probably due to immune incompetence. This is despite the fact that the virus replicates to higher levels in neonates than in 6-week-old (young) calves. During the process of maturation of the immune system, disease can even be aggravated in the first few months of life, but, at an older age when the immune system is fully functioning, healthy cattle appear to easily resist (severe) BRSV disease.

METHODS

Calves. Two groups of five specific-pathogen-free (SPF) calves were obtained by Caesarean section at full term with 6 weeks in between to reach an identical age difference. All calves were kept colostrum-deprived and were housed in one large isolation room. All calves were

tested to be free of bovine viral diarrhoea virus (BVDV), as shown by a BVDV antigen ELISA test kit (IDEXX Herdchek), and free of antibodies directed against bRSV (Ceditest BRSV; Cedi Diagnostics BV).

Inoculation. Calves were inoculated as described previously (Antonis *et al.*, 2003). Briefly, calves were inoculated intranasally with an air-jet nebulizer with bRSV strain Odijk on the same day when they were 6 weeks of age (young calves) or 1 day old (neonates). bRSV strain Odijk was obtained during a field outbreak in Odijk, the Netherlands, in 1991 (van der Poel *et al.*, 1996). The inoculum (2 ml per calf) used in this study contained $10^{3.1}$ TCID₅₀ bRSV ml⁻¹, after being passaged four times *in vivo* in SPF calves.

Clinical investigations. Calves were clinically examined daily by the same veterinarian and scored for signs of general illness (reduced responsiveness, decreased appetite, depression and/or retreats), URTD (coughing, nasal/ocular discharge) and LRTD (hyperpnoea and dyspnoea). Rectal temperatures and respiratory rates were also recorded. The severity of the clinical disease was expressed by allocating a weighing factor for each observation, varying from 1 (mild) to 3 (severe).

Laboratory investigations. Blood samples from the jugular vein were collected daily from day 0 until the end of the experiment. Full blood samples were collected for bRSV serology and heparinized blood samples for haematological examination. The total numbers of white blood cells, red blood cells and platelets were determined by using a Coulter counter (Sysmex). Additionally, a blood smear analysis was performed for differential counts of leukocytes. Blood smears were stained using a Hema-Tek slide stainer.

Nasopharyngeal brush samples were obtained daily from day 0 until the end of the experiment. Samples were collected using sterile nylon bristle swabs (MW126; Medical Wire and Equipment). Following sampling, swabs were agitated in 2 ml tissue culture medium supplemented with antibiotics and FCS.

BALF samples were obtained on days 0 (day of inoculation, prior to virus administration), 3, 6, 9 and 13. BALF samples were obtained as described by Fogarty *et al.* (1983). Approximately 35–75 ml BALF was obtained from each calf after instillation of 100 ml PBS. Foam, large purulent exudates and blood clots were removed from the BALF samples under aseptic conditions. BALF sample material was inoculated onto sheep blood agar plates and incubated under aerobic conditions at 37 °C. Bacteria were identified using standard laboratory procedures. BALF sample material (2 ml) was centrifuged for 5 min at 25 g in a cytocentrifuge (Shandon Cytospin 2). Slides were air-dried and fixed in methanol for 10 min at room temperature. A total of 400 cells was counted (Hema-Tek slide stainer), and macrophages, lymphocytes, monocytes and neutrophils were differentiated on the basis of morphology. The remaining BALF was centrifuged (200 g, 10 min, 4 °C). Lavage cells were resuspended in 0.5 ml Dulbecco's minimal essential medium (DMEM) containing 1% antibiotic cocktail and 50% FCS, carefully added to 1 ml freeze medium (DMEM containing 1% antibiotic cocktail, 50% FCS and 20% DMSO) and frozen at -70 °C. BALF supernatants were stored at -70 °C for virus isolation and virus titration.

Virus isolations were performed in duplicate from BALF and nasopharyngeal brush samples as described previously (van der Poel *et al.*, 1996) and positive samples were titrated in an end-point titration.

Serum samples collected on days 0, 7 and 14 were tested for the presence of virus-neutralizing antibodies in a virus neutralization assay (Langedijk *et al.*, 1996).

Arterial blood samples were collected daily from the caudal auricular artery of the ear (intermediate or lateral branch) by puncture of the

vessel with a 25 gauge (0.5 × 16 mm) needle after which the running blood was drawn into a heparinized capillary. Rectal temperature was measured simultaneously with blood sampling. After sampling, the capillaries were closed immediately and stored on ice until analysis in a pH/blood gas analyser (IL Synthesis; Instrumentation Laboratory) for measurement of arterial pH, pCO₂ and pO₂ with correction for rectal body temperature.

Venous blood samples were collected once daily from the jugular vein in heparinized vacutainer tubes and stored on ice until determination of plasma lactate concentrations (mmol l⁻¹) using Accutrend Lactate and BM-Lactate test strips (Roche Diagnostics).

Haptoglobin was determined daily in serum with a haptoglobin assay (Tridelta). This assay is based on the preservation of haemoglobin peroxidase activity by haptoglobin in these samples. Haptoglobin concentrations were read from a standard curve.

Bovine cytokines in serum and culture supernatants (IL-4, IL-6, IFN- γ and TNF- α) were determined quantitatively using a SearchLight Bovine Cytokine Array (Pierce Biotechnology). Briefly, each well of a microplate was pre-spotted with cytokine-specific antibodies (antibodies against four cytokines per well). These antibodies capture specific cytokines in each well. After the supernatant was washed away, biotinylated detecting antibodies were added that bound to a second site on the target proteins. After removing excess detecting antibody, streptavidin-horseradish peroxidase was added. Finally, a chemiluminescent substrate was added to produce a luminescent signal that was detected with a cooled CCD camera. The amount of signal produced was proportional to the amount of each cytokine in the original standard or sample.

Post-mortem investigations. Thirteen days after virus inoculation, calves were anaesthetized using pentobarbital and euthanized by exsanguination. The lungs were removed immediately, and photographs of dorsal and ventral parts of the lungs were taken. From these photographs, the extent of macroscopic lesions (consolidated lung area) was scored on a scale of 0 to 5 as described by Viuff *et al.* (2002). Necropsy samples of ten pre-determined sites were stored in 10% neutral-buffered formalin. Formalized samples were embedded in paraffin, and 5 μ m sections were cut and stained with haematoxylin and eosin for histological evaluation. Histological examination was performed on the right middle lobe, the cranial part of the right caudal lobe, the cranial part of the left caudal lobe and the left middle lobe.

Statistical analysis. GraphPad Prism software was used for analysis of the data. A two-way analysis of variance and non-parametric (Mann-Whitney) test were used for comparisons. Correlations and differences were considered significant for $P < 0.05$.

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