Bovine Respiratory Syncytial Virus
Immunopathology and Vaccine Evaluation

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De aanhouder wint

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Respiratory syncytial virus (RSV) infections are since decades recognised as an important cause of lower respiratory tract disease (LRTD) in both infants and calves. Although human respiratory syncytial virus (hRSV) and bovine respiratory syncytial virus (bRSV) are different [1], the viruses are likely to have a common origin and not surprisingly the diseases they cause share many aspects of pathogenesis, epidemiology, immune responses and clinical manifestations. Also, vaccine failures have been encountered with (candidate) vaccines against both hRSV and bRSV. Despite difficulties in RSV vaccine development, many bRSV vaccines have been licensed. Although bRSV vaccines have been on the market for many years, information on their safety and potency is largely lacking. Experiences with bRSV vaccines may nonetheless be helpful for human RSV vaccine development. Development of a vaccine against hRSV remains one of the top priorities of the Global Program for vaccines of the World Health Organization [2]. Therefore, the natural host-model of bRSV infection in calves could be of benefit in hRSV vaccine development work.

The Virus

Bovine and human respiratory syncytial viruses are closely related members of the genus *Pneumovirus* within the family *Paramyxoviridae* (see Figure 1). Respiratory syncytial viruses are pleomorphic enveloped viruses of variable size. The shape can vary from almost spherical to filamentous. The envelope consists of a lipid bilayer derived from the host cell and spike-like glycoproteins. The main elements of the virus structure are illustrated in Figure 2. The lipid-containing envelope is lined by a matrix (M) protein that surrounds the helical nucleocapsid (N). The genome is negative sense single-stranded RNA. Ten proteins associated with the RSV genome have been described, including two nonstructural proteins. The nucleocapsid comprises the RNA genome protected by the nucleoprotein and associated with clumps of the small P and large L proteins that have RNA-dependent RNA polymerase activity. The two large transmembrane glycoproteins are the attachment protein G and the fusion protein F. Another membrane glycoprotein is the small hydrophobic (SH) protein. Infection is initiated by attachment of the virus to a susceptible cell by the G protein. For penetration into the host cell to occur, the F protein must be cleaved by cellular proteolytic enzymes. The cleaved F proteins (F1 and F2) act to fuse the host cell membrane with the viral lipid envelope that allows the nucleocapsid to enter the cytoplasm, where RSV replicates. The viral polymerases (P and L) transcribe the negative-strand RNA to mRNA for the production of viral proteins. The negative-strand RNA also serves as

template for synthesis of positive-strand RNA. The virion-complementary RNA is then copied into progeny negative-strand RNA. Subsequently structural virus proteins are synthesized and virions are released by budding from the host cell membrane.
By RT-PCR and sequence analyses, but also by using monoclonal antibodies, hRSV can be distinguished from bRSV [3]. There are no reports of infection of humans with bRSV. Conversely, experimental inoculation of calves with hRSV isolated from human beings has resulted in signs of respiratory disease and virus excretion [4]. HRSV isolates are divided in two groups of antigenically distinct viruses, classified as subgroups A and B [5, 6]. BRSV isolates are divided into 3 subgroups (designated A, B and intermediate or AB), exhibiting considerable differences in reactivity with monoclonal antibodies [7].

Family: Paramyxoviridae

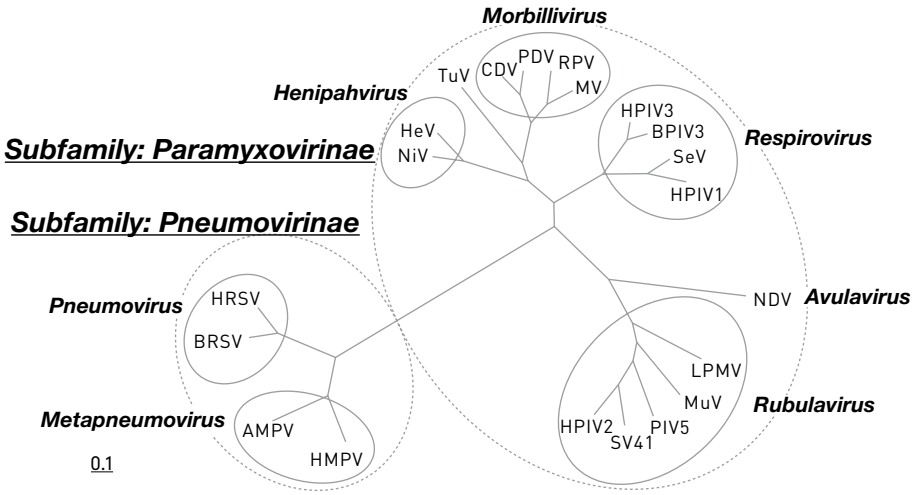

Figure 1. Phylogenetic tree (kindly provided by Dr Ron Fouchier, Erasmus University, Rotterdam, The Netherlands) of the Paramyxoviridae family, showing the inferred evolutionary relationships among genera in the Pneumovirinae and Paramyxovirinae subfamilies. Abbreviations: NiV: nipah virus; HeV: hendra virus; TuV: tupaia virus; CDV: canine distemper virus; PDV: phocine distemper virus; RPV: rinderpest virus; MV: measles virus; HPIV3: human parainfluenza virus type 3; BPIV3: bovine parainfluenza virus type 3; SeV: sendai virus: HPIV1: human parainfluenza virus type 1; NDV: newcastle disease virus; LPMV: la piedad michoacan virus; MuV: mumps virus; PIV5: parainfluenza virus type 5; SV41: simian virus 41; HPIV2: human parainfluenza virus type 2; AMPV: Avian Metapneumovirus; HMPV: Human Metapneumovirus; bRSV: Bovine respiratory syncytial virus; HRSV: Human respiratory syncytial virus.

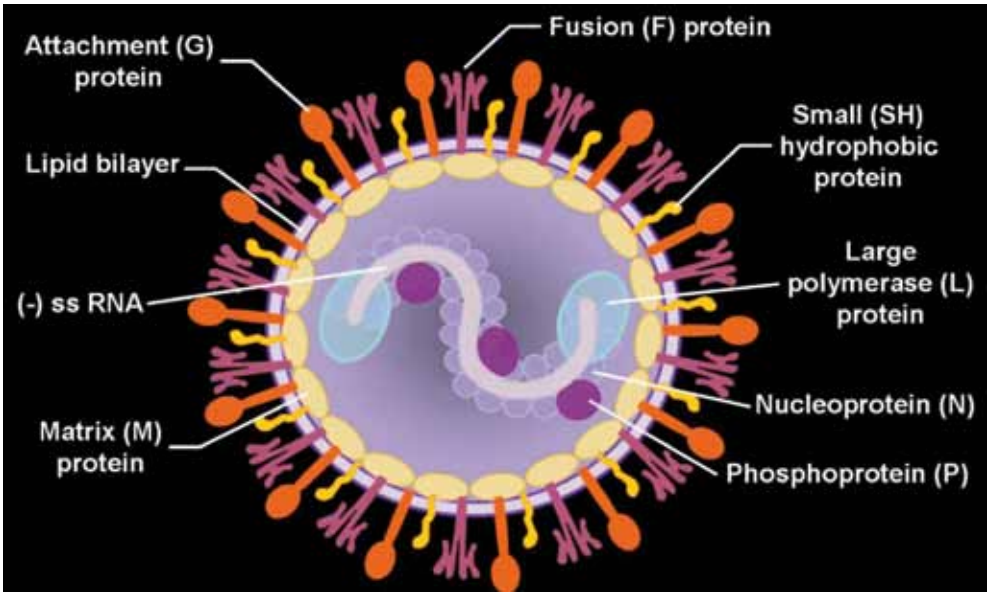

Figure 2. RSV virus structure (www.kuleuven.be/rega/mvr).

RSV history

In 1957, a first report was published about a virus, isolated from infants with lower respiratory tract disease, by Chanock and colleagues [8]. Infections with this agent, which was named RSV, appeared to be prevalent for a three to four month period every year in virtually all paediatric populations in which it has been studied. When it was prevalent in the community, hospitalization for bronchiolitis and bronchopneumonia among infants reached a peak [9]. Subsequently the first candidate vaccines were developed. An effective vaccine against early infection was expected to result in a significant reduction of lower respiratory tract disease and associated hospitalization. The goal of such a vaccine was either to stimulate serum antibody levels higher than those provided by maternal transfer or, alternatively, to induce the development of local respiratory tract antibodies, as it was commonly believed that mainly antibodies were needed for protection. In the 1960s, four field studies were conducted with a formalin-inactivated (FI)-RSV vaccine [10-13]. This FI-RSV vaccine was a crude RSV-monkey kidney cell harvest, precipitated with alum, and concentrated 100-fold. Surprisingly, all four studies reported a dramatic vaccine-mediated enhancement of disease: vaccinated children (usually younger than two years of age) experienced more serious disease upon subsequent natural RSV infection. Early trials with limited material available from these clinical trials suggested that FI-hRSV induced humoral and cellular

responses which were qualitatively and quantitatively different from those induced by a wild type (wt) infection [14, 15].

It was predicted in the 1960s that a virus antigenically related to hRSV was present in cattle [16] and indeed, a few years later, a first case of RSV in cattle was identified by Paccaud & Jacquier in Switzerland [17]. In the years thereafter bRSV was increasingly reported as the cause of severe outbreaks all over the world providing evidence that bRSV was endemic in cattle [18-27]. Strikingly, veterinarians observed many similarities in immunopathology after vaccination with a vaccine inactivated similarly to the hRSV vaccine and subsequent infection [23].

To date, bRSV is still among the leading causes of bovine respiratory disease (BRD) [28-31], a worldwide serious health problem in cattle, causing considerable losses and major animal welfare problems [32]. Currently bRSV is considered to be responsible for more than 60% of the epizootic respiratory diseases observed in dairy herds [30, 33-35] and up to 70% in beef herds [36].

Epidemiology

BRSV has, similar to its human counterpart, a worldwide distribution and has been recognized as one of the major causes of respiratory tract disease in the host species. Similar to hRSV infections which are common and more likely to involve the lower respiratory tract in early childhood [37], bRSV may cause severe and life-threatening lower respiratory tract disease in one to six months-old calves [38, 39].

A seasonal periodicity has been described for human [40, 41] and bovine [42] RSV infections. The highest incidences of RSV infections were found in either the autumn or winter. In other seasons, primary infections are rare.

RSV infections in both humans and in cattle usually start with upper respiratory tract infection characterized by rhinitis, cough and low-grade fever. Two to five days later lower respiratory tract involvement may develop, characterized by tachypnea, chest retractions and sometimes wheezing. Often hypoxemia and hypercapnia is observed. The highest morbidity of hRSV infections is seen in infants between six weeks and six months of age [43]. A high percentage (up to 40%) of young children infected with hRSV for the first time will show signs of lower respiratory tract involvement [44-46], but only some will be so severely affected that hospital admission is necessary. In cattle, both morbidity and mortality seem to be higher in feedlot cattle than in dairy cattle, with disease almost exclusively observed in young animals. Severe, life-threatening lower respiratory tract disease (LRTD) is mostly seen following a primary infection in one to six month-old calves [38, 39].

Many experiments have been conducted to experimentally reproduce respiratory tract disease with bRSV, but this has often been unsuccessful [25-27, 47-50]. This is not surprising, because the clinical presentation of bRSV infections under field conditions is influenced by a multifactor interaction of different stressors, animal susceptibility and simultaneous infections with other respiratory pathogens resulting

in a so called Bovine Respiratory Disease Complex (BRDC). Stressors of relevance are environmental factors as weaning, transport, commingling, crowding, and inadequate ventilation. Clinical outbreaks frequently follow a change of weather and/or a shift from pasture to permanent housing [31, 51]. On the other hand, hosts are not equally susceptible. Genetic heterogeneity can explain differences in immune responses [52]. Age has been identified as an important factor in susceptibility for the development of more severe (lower) respiratory tract disease which at least in part may be attributable immunological immaturity and to the waning of maternal antibodies over time.

Virus spread within a group of animals can be extremely rapid (within 4 to 5 days), which is comparable to the epidemics observed in children [53, 54]. Mortality in bRSV outbreaks generally ranges between 2% and 3%, but can reach up to 20-25% [21].

Most probably cattle are the principal reservoir and usual source of bRSV infection. Introduction of an infected animal into susceptible populations is accompanied by rapid spread accompanied by the appearance of clinical manifestations. Although sheep and goats have been infected experimentally, their role in epidemiology of bRSV infections remains unclear. Re-infections of bovines with bRSV are common like in humans with hRSV. Bovines of all ages can be re-infected, although re-infections are more frequent in younger animals [42]. Cattle can be re-infected more than once within a year. Calves may be re-infected within 3 weeks after primary infection [55]. Re-infections usually run a sub-clinical course.

Pathogenesis: infection and immunity

Susceptible humans are, most probably, infected by direct contact with hRSV containing secretions or by large droplet spread [56-58]. In calves infection with bRSV is presumed to occur predominantly via small particle aerosols [3]. Replication of RSV starts with an infection in the upper part of the respiratory tract, peaks around the 4th day post infection (dpi), and is followed by a variable replication in the bronchiolar and alveolar epithelium (from 4 dpi) peaking around 6 dpi. A major part of the inoculum remains associated with the mucus layer of the upper respiratory tract (URT) without reaching the alveoli. [42]. BRSV only minimally replicates in alveolar macrophages [59]. Downwards spread of the infection along the bronchiolar and alveolar epithelium is presumably a result of destruction of the ciliated tracheo-bronchial epithelium. Neutrophils are important for the clearance of bRSV excreted into the lumen of the respiratory tract [60], but these same neutrophils may also contribute to tissue damage by releasing inflammatory products [61]. A peak virus replication in bronchiolar and alveolar epithelial cells of the lower respiratory tract (LRT) may coincide with more severe clinical signs: tachypnea, dyspnoea and anorexia [60]. Virus replication in the cells of the respiratory lung epithelium may vary significantly, resulting in no obvious cytopathology [62], to cytopathology of lung epithelium cells leading to loss of specialized functions such as cilial motility and epithelial destruction [63]. It has been speculated that direct cytopathology due to virus replication plays a minor role

in clinical symptoms, whereas the host response to the virus infection (immunity) plays a major role in RSV pathogenesis [62, 64, 65].
The case fatality rate in uncomplicated bRSV infections is usually low, but may increase due to secondary infections. The lungs of animals dying in naturally occurring bRSV outbreaks often show atelectasis, interstitial emphysema or pneumonia (which may be caused by secondary bacterial infection). BRSV enhances bacterial colonization and adherence, and alters specific and non-specific defense mechanisms of the respiratory tract. It has been estimated that 90% of all bacterial pneumonias develop after a previous viral infection [66].

Triggered by infection, a variety of cells (among which mast cells, epithelial cells, dendritic cells, macrophages, other antigen presenting cells, T-cells and B-cells) secrete specific cytokines, the regulatory (key) signals of the immune response [67, 68]. Cytokine profiles associated with hRSV disease have been studied extensively in recent years. Tumor necrosis factor-α for example has been shown to induce migration of neutrophils and mononuclear cells to the site of infection. Various mediators of inflammation, e.g. degranulation products of mucosal mast cells and eosinophils have been associated with severity of bronchiolitis. Histamine and leukotrienes are major mast cell mediators responsible for constriction of smooth muscle, increased mucus production, and increased vascular permeability [69, 70].

Cellular immune responses are thought to play an important role in clearance of RSV [65, 71-73]. Cytolytic T-lymphocytes (CTLs) are likely the principal mediators of resolution of the acute virus infection [74]. However, CTLs probably do not contribute significantly to protection against reinfection, because of the short-lived nature of activated CTLs. Nevertheless, a memory CTL response may be important in limiting the severity of disease.

Antibodies appear a principal mediator of resistance to RSV disease and infection. Serum (neutralizing) IgG antibodies protect against lower respiratory tract disease, but do not seem to have a major impact on viral replication in the upper respiratory tract. Humoral immune responses following primary RSV infection initially consist of virus-specific IgM [75]. Virus-specific IgG and IgA antibodies can be detected simultaneously or a few days later [76]. Age and pre-existing virus-specific maternal antibodies may influence the development of antibodies against e.g. the F and G surface glycoproteins, which are the only targets for virus-neutralizing antibodies. Many mechanisms of protection may be afforded by serum antibodies. Antibodies against the G protein probably reduce replication by preventing attachment of virus to cells, while antibodies directed to the F surface glycoprotein reduce replication by preventing fusion of the virus with the cell and inhibiting cell-to-cell spread. Conflicting data have been published on production of virus-specific IgE antibodies, and the association with disease severity. Viral-IgE complexes may attach to mucosal mast cells, which may then induce mast cell degranulation with release of potent mediators of inflammation that are responsible for smooth muscle constriction, increased mucous production, and vascular permeability [77-80].

Immune responses in young individuals are functionally impaired due to multiple factors, including immunological immaturity [81-85], limited B cell repertoire, inefficient mechanisms of antigen presentation and T cell help, lower proinflammatory (in particular TNF-alpha) responses [68] and inhibition by passively acquired maternally derived antibodies (MDA) [83, 86]. It has been suggested that the immune system at birth has a strong tendency to adopt a Th2 profile and that newborns have to acquire the ability to develop a more balanced Th1-Th2 response [83]. An excellent review of the immunologic development in the calf and its impact on vaccine response was recently published [84]. Chase et al. concluded that vaccination of young calves is complicated by the presence of significant levels of maternal antibodies, colostral and neonatal hormonal factors, the lack of full immune competence, and interference in the function of vaccines by the presence of maternal immunity. Results of several studies [87-89] indicate that the relative balance of Th1 and Th2 cytokines influences the outcome of bRSV infection. In general, immunization that leads to a dominant IL-4 and IL-13 response from T cells is associated with an increased disease severity after subsequent challenge infection, whereas immunization that leads to a dominant IFN-γ response is associated with protection [90]. The immature immune system of the newborn therefore may therefore not be capable to develop sufficient protective immune responses against RSV infection.

After primary infection, the peak of disease typically coincides with the development of specific T-and B-cell responses, which therefore may be considered as a factor in the pathogenesis [91]. A previous infection, a vaccination [92] or the presence of maternal antibodies are examples of factors which may change the balance of the local cytokine response and thereby trigger either a protective (Th1-like) response or initiate a cascade that subsequently leads to exacerbated disease (Th2-like immune response) [28, 90, 93]. Vaccine-induced exacerbated disease is characterized by enhanced interleukin (IL)-4, IL-5 and IL-13 responses and tissue eosinophilia [94, 95]. Moghaddam et al. showed that carbonyl groups on formaldehyde-treated vaccine antigens boost Th2 responses and enhance hRSV associated disease in mice, an effect partially reversible by chemical reduction of carbonyl groups [96]. Therefore, the priming effect of a (candidate) RSV vaccine should preferentially be modulated by Th1 driving adjuvants (e.g. ISCOMs, [97]).

Immunopathology

Allergic reactions in children‟s populations, induced by hRSV vaccination and triggered by natural infection have been described extensively in the literature [10-13]. Hypersensitivity related to vaccination with formalin-inactivated vaccines is not a unique phenomenon for RSV; it has also been reported for measles virus (MV), another member of the Paramyxoviridae family. This syndrome was characterized by high fever, pneumonia, abnormal rash, and lymphadenopathy [98]. Also in cattle vaccination with FI-bRSV was shown to predispose calves for enhanced disease upon bRSV challenge infection [87, 99, 100]. These observations initiated many years of study to define the immunopathological mechanisms. Initially it was hypothesized, that vaccinated children had poor fusion inhibiting antibodies due to

alteration of the fusion protein during formalin treatment [101]. It has also been suggested that the altered immune response was a type of delayed hypersensitivity. Delgado et al. demonstrated that nonprotective antibody responses elicited by inactivated hRSV immunogens and ultimately associated with the development of enhanced respiratory disease, resulted from lack of affinity maturation due to deficient TLR activation in B cells [102].

To date, there is still no common understanding of the phenomenon. Vaccine-enhanced disease still continues to hamper vaccine development, whereas its pathogenesis has always remained poorly defined.

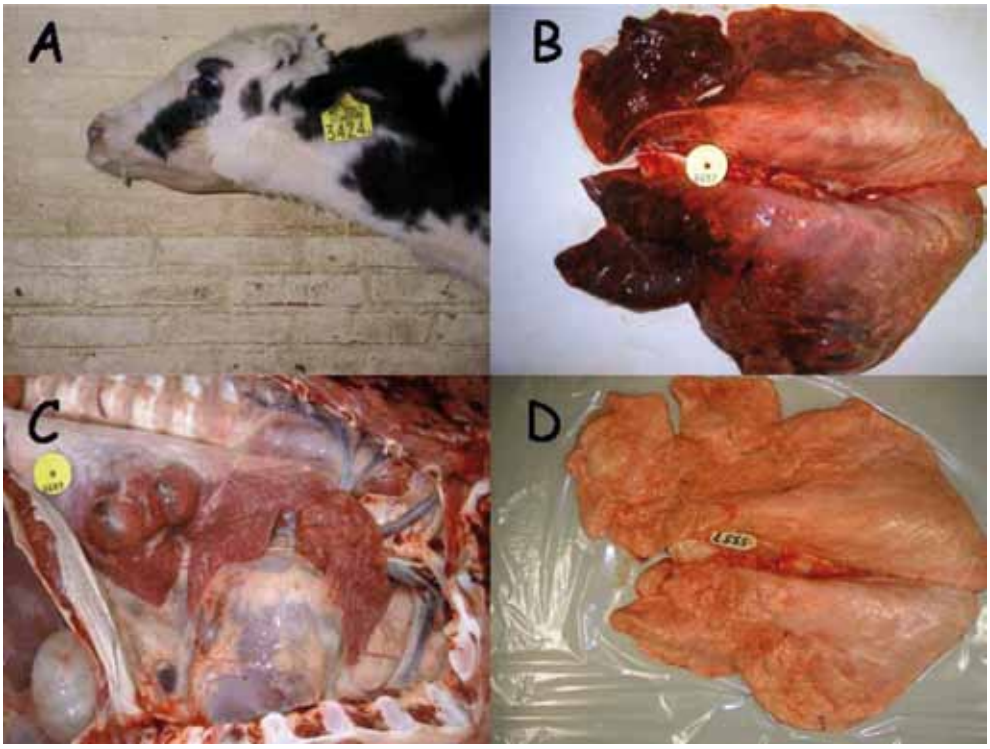

Figure 3. Pathological observations on bovine Respiratory Syncytial Virus infections in calves. (A) Calf #3424 had signs of severe respiratory distress shown by forced breading: stretched neck frothing varied by open mouth breathing and groaning. (B) Macroscopic findings of calf #6697: affected lung tissue is dark-red / consolidated. A large emphysematous bulla is present in the left caudal lung lobe. (C) In situ picture of calf #6689: severe bullous emphysema, note two bullae in right caudal lung lobe and interlobular emphysema and edema in cranial lobes. (D) Macroscopic of Calf #5557: no abnormal lung tissue areas present.

Clinical manifestations

BRSV infections can be associated with a wide spectrum of clinical manifestations, ranging from subclinical infection or mild febrile upper respiratory tract illness to severe and fulminating pneumonia. The clinical presentation is initially the same, including nonspecific upper respiratory tract disease signs: nasal discharge, cough and/or fever. More severe clinical signs may depend on the proportions of the small airways that are involved in the inflammation process. This may result in expiratory wheezing and air trapping, whereas subsegmental atelectasis results from complete occlusion of the small airways. Mildly affected calves exhibit respiratory rates above 40/min, moderately affected calves above 80/min and severely affected calves above 100/min. Complete occlusion results in atelectasis that can be lobular, and usually involves the cranio-ventral part of the lungs [103, 104]. The most severely affected calves may be dyspnoeic and have subcutaneous, emphysematous bullae (Figure 3). Hypoxia is a common finding in calves presented to a veterinary clinic with lower respiratory tract disease. The arterial blood PO_2 is often less than 70mmHg, oxygen saturation may be below 85% [105].

Laboratory diagnosis

Respiratory syncytial virus is labile and requires special and adequate handling when infectious virus should be preserved. The diagnosis of RSV infections therefore depends greatly on the quality of the clinical specimen and its proper handling. Clinical samples that are to be cultured should be transported cooled (melting ice) in the appropriate transport medium (including antibiotics and fetal calf serum) and inoculated onto sensitive cell culture, as embryonic bovine trachea cells or Vero cells, as quickly as possible. Preferred specimen type for diagnosis is nasal wash, nasal-pharyngeal brush sample or lung lavage samples. The most diseased calves may not be the best candidates for sampling since the amount of virus is the highest about 2 to 3 days before severe clinical signs occur [106].

Direct detection of infected cells using immunofluorescence or immunohistochemistry with fluorescein- or otherwise labeled specific antibodies is a reliable method to diagnose bRSV infections in e.g. lung lavage, nasal brush or lung tissue, but also molecular detection (i.e. RT-PCR) has proven to be very useful [107]. For serological diagnosis of RSV infection paired (acute and convalescent) sera are generally required. Blood samples for serology should be collected with an interval of at least two weeks, and preferably three weeks. Because of the presence of maternal antibodies, these classical sero-diagnosis methods are often of limited value in the diagnosis of bRSV infections in calves less than 3 months of age, but have been proven useful in the diagnosis of bRSV infections in older calves [30, 39, 75]. In young calves, the use of isotype-specific ELISAs could provide a suitable diagnostic alternative, as maternally derived antibodies are restricted to the IgG1 isotype in calves [76].

Prevention

The health impact and economic burden attributed to RSV contribute to the urgency for developing safe and effective vaccines. For bRSV several vaccines are commercially available, although safety and potency have not always been demonstrated in established experimental vaccination-challenge models in the natural host under field circumstances. Adequate vaccines must be safe and able to confer protection, also in the presence of maternal antibodies. The regulatory safety requirements for bRSV vaccines are less stringent compared to the hRSV vaccine safety requirements. Safety of RSV vaccines should not only relate to safety upon vaccination, but also to safety upon challenge infection (see below).

Vaccination against hRSV

Nearly four decades after hRSV vaccine development began there is still no licensed vaccine available. James Crowe Jr. summarized nine obstacles in hRSV vaccine development; 1) no fully permissive animal models, 2) the requirement for a bivalent vaccine, 3) Young age of primary vaccine target, 4) specific safety concerns when immunizing neonates, 5) immunological immaturity of neonates, 6) influence of maternal antibodies, 7) disease occurring at portal of entry, 8) short lived and 9) incomplete mucosal immunity, and history of vaccine enhancement [108]. A first generation of modified live-attenuated RSV candidates was generated by isolating mutant viruses generated by passage in cell culture. These initial vaccine candidate viruses were attenuated, infectious and immunogenic in older infants, but exhibited potential problems with genetic stability. A second generation, genetically more stable vaccine candidates, has recently been taken into Phase I clinical trials in seronegative infants. Genetically engineered live-attenuated vaccine candidates have been developed, but require careful evaluation because of the possibility of altered tropism. Also virus vector delivery systems, although looking promising, will always face significant safety concerns.

Vaccination against bRSV

Many bRSV vaccines have been licensed since the first (live-attenuated) bRSV vaccine became available in 1977. Although vaccination against bRSV has been advocated widely and vaccines have been applied intensively for several decades, field studies on the efficacy of the commercially available vaccines remain scarce.

Viral vaccines have been divided traditionally into two broad categories: attenuated or modified live virus (MLV) and inactivated or killed virus (KV) vaccines. MLV vaccines are generally believed to induce a more long-term immunity, but there are safety concerns using MLV vaccines. There is e.g. a potential risk of residual virulence, persistence in the host, shedding into the environment and reversion to more virulent strains. Also, MLV vaccines have the risk of contamination during production procedures with other viruses, such as BVDV. KV vaccines are in general considered to be safer, as there is no residual live virus present, but the duration and the quality of the induced immunity is usually relatively short lived. Potential safety problems with KV vaccines include an increased risk of allergic

reactions e.g. due to large amounts of antigens involved. Inactivation processes can alter functionally important epitopes on e.g. bRSV envelope glycoproteins leading to production of predominantly non-neutralizing antibodies in immunized animals [109].

Another distinction can be made between vaccines with single or multiple antigens. Calves are often vaccinated against a wide range of infectious organisms. A practical way to overcome constraints of multiple injections, reducing the costs of stocking and administrating separate vaccines, and reducing the distress to the recipients, could be to use combination vaccines containing different antigens. Many combinations are reportedly as efficacious as the separate vaccines, but combining many antigens might reduce immunogenicity of the vaccine against individual components. The safety, efficacy, and immunogenicity of a combined vaccine in fact may be affected by interactions, not only between the antigens but also between these and other components such as adjuvants, stabilizers, and preservatives. Van Donkersgoed et al. for example showed that an additional antigen (bRSV) to an otherwise identical combination vaccine (Cattlemaster 3 / without bRSV and Cattlemaster 4 / with bRSV) resulted in a significant ($p<0.05$) higher anti-PI3V virus neutralizing antibody (VNA) titers and significant ($p<0.05$) lower anti-BVDV VNA titers in calves vaccinated with CattleMaster 4 (with bRSV) compared to CattleMaster 3 (as CattleMaster 4, but without bRSV) [110]. Antigenic competition in combination vaccines has been described for other vaccines too, e.g. sheep inoculated with a multivalent foot rot vaccine were not as well protected as those vaccinated with a monovalent vaccine [111].

BRSV vaccine requirements

As most severe bRSV infections occur in the first six months of life, appropriate vaccines should stimulate an effective immune response during these first months, comprising mucosal (IgA), serum neutralizing antibodies and cytotoxic T lymphocytes. Thus vaccines must be able to confer clinical protection at young age, usually in the presence of MDA, and must be able to overcome possible immunological immaturity of the recipient. In addition, the vaccines should not lead to vaccine-induced disease exacerbation upon challenge infection. BRSV vaccines should comply for example with vaccine quality criteria as described by Babiuk et al. [112]. The most important criteria in this list are the absence of adverse reaction and high efficacy.

For marketing authorization in Europe, it is required that vaccines have not been associated with an abnormal incidence of immediate hypersensitivity reactions and a significant reduction of virus excretion should be demonstrated. A reduction of clinical disease is desirable but is not a restrictive condition for legislation. Both immunogenicity and efficacy studies are to be conducted in calves of the minimum age for which the vaccine is recommended, which do not have antibodies against bRSV, and for each recommended route of administration. It is remarkable that marketing authorization does not require demonstration of safety data concerning the risk of vaccine-enhanced disease, because bRSV vaccines have been

withdrawn from the market that may have been associated with vaccine-enhanced disease [113].

Vaccine safety

Safety of live vaccines includes essentially the safety of the live pathogen(s) in the vaccine, and of the other accessory ingredients. Key criteria are the level of attenuation of the vaccine strain, the risk of reversion to virulence (to be checked by a reversion to virulence test), and the risk of contamination with extraneous agents (to be checked by tests for extraneous viruses, bacterial, fungal and mycoplasma contamination). Key criteria for safety of the accessory ingredients are absence of any hypersensitivity reactions. The European Pharmacopoeia (EP) further requires a safety test in which a ten-fold overdose for (modified) live virus vaccines or a two-fold overdose for inactivated virus vaccines is required. No abnormal local or systemic (vaccine associated hypersensitivity) reactions should occur in a twenty-one-days observation period [113].

According to the EP, a vaccine is satisfactory if no abnormal effects on the body temperature and no abnormal local or systemic reactions are noted and if vaccinees are not associated with an abnormal incidence of immediate hypersensitivity reactions. It is important to note that, according to these guidelines, it is not required to test for hypersensitivity reactions. In general, altered reactivity (allergic) reactions have been reported in association with the antigenic component of the vaccine, more common causes are the stabilizers, vaccine additives or other vaccine components (residues of antibiotics, egg protein). Gelatin is an example of a potent vaccine stabilizer: it has been reported to cause IgE-mediated hypersensitivity and symptoms of anaphylaxis. Thiomerosal is an example of a preservative, commonly used as a preservative to prevent bacterial contamination of multidose vaccine vials after they have been opened [114], and in too high concentrations can cause adverse reactions [115]. Several bRSV vaccine-associated hypersensitivities have been reported. In 1994 e.g. different batches of Torvac® RSV were withdrawn from the UK market, when a hypersensitivity reaction was observed after antibiotics were given to vaccinated calves. The cause of sensitization was found in the adjuvant and which was similar to an excipient in some antibiotic formulations [116]. In 1996 practitioners in The Netherlands were warned for anaphylactic reactions in cattle treated with different antibiotics and previously vaccinated with Bar Vac® RS. The most probable cause of sensitization in these cases, was poly vinyl pyrrolidone (PVP), used to treat saponine (used as immunostimulator) during the production process. PVP was also present in particular antibiotic formulations [117].

In order to obtain a marketing authorization, the EP requires no abnormal effects on the body temperature and no abnormal local or systemic reactions and no association with an abnormal incidence of immediate hypersensitivity reactions after vaccination.

Potency tests

The immunogenicity and the efficacy of a vaccine is evaluated in potency tests. For a marketing authorization, vaccinees and controls must be infected with a suitable quantity of a low-passage virulent bRSV strain. The vaccine complies if there is a significant ($p<0.05$) reduction in mean titer and in mean duration of virus excretion in vaccinates compared to controls, and a notable reduction in general and local clinical signs in vaccinated animals (if the challenge virus used produces such signs) [118]. Because there is no standard for the challenge virus to be used, results of these studies cannot be compared. Low-passage is not specified, although it has been demonstrated that animal–passaged bRSV results in relatively high virulence compared to cell-passaged bRSV [119]. Due to lack of clinical signs in most experimental bRSV models, only demonstration of reduction of virus is required for the marketing authorization. However, in this thesis the development of a model will be described in which we induce clinical signs, paving the way for studies that can demonstrate reduction of clinical signs in the natural host.

Experiences with currently available bRSV vaccines

Several MLV and KV bRSV vaccines are commercially available in Europe. Unfortunately, limited peer reviewed publications on the efficacy and safety of these vaccines are available. Where absent information was used provided by the corresponding pharmaceutical company: presented on congresses, in non-reviewed literature or manufactures‟ leaflets. There are two single-bRSV (both MLV) vaccines on the European market available and twelve combination vaccines.

Although criteria for vaccine efficacy are defined in the EP, the absence of comparative data on different vaccines makes it difficult for veterinarians and livestock producers to choose the best possible vaccine. Selection of vaccines should ideally be based on efficacy and safety data from controlled field trials [120]. However, this information is often not available, because these comparative studies are expensive and logistically difficult to conduct or have remained un-published. Despite the observations of respiratory disease enhancement after infections in cattle previously vaccinated with inactivated virus [117, 121], inactivated bRSV vaccines based on whole virus have now been used for nearly four decades.

The immunogenicity of a killed virus (KV) combination vaccine (BoviPast RSP, equal to Bovigrip) was evaluated and compared with a modified live virus (MLV) vaccine (Rispoval RS BVD) in five to seven months old (seronegative for BRSV) calves in a study by Kerkhofs et al. [122]. The KV vaccine induced higher ELISA antibody titers than the MLV vaccine, but VNA titers were comparable. Significant cell mediated immune responses specific for bRSV were not detected. The authors concluded that the KV vaccine gave at least as good response as the MLV vaccine, without presenting results of a challenge infection. Mawhinney & Burrows vaccinated approximately four-week-old calves once with either a KV combination vaccine (BoviPast RSP) or a MLV, single antigen, vaccine (Rispoval RS) and challenged these calves three weeks after vaccination. They did not observe any significant clinical differences between both vaccinated groups. None of the

vaccines, after single dose administration, was able to reduce the already moderate clinical signs induced by the challenge virus, compared to the control group [123]. The challenge model was only able to produce mild clinical signs and thus not suitable for evaluation of clinical protection purposes. Significantly less (two out of seven) KV combination vaccine-vaccinated calves shed virus, compared to the control (six out of seven) group. No differences in mean number of days of virus excretion and mean virus excretion titers were observed between both vaccinated groups. It is important to note that there was no comparison with calves after two doses of the vaccine (as required according to the instructions of the manufacturers), as to the authors" experience (unpublished observations) the immunity following a single dose is neither as robust nor as long as that following a full course of vaccinations [123]. Van der Sluijs et al. also evaluated the efficacy of a single dose of the KV combination vaccine. They challenged four six-week-old control calves and compared the post challenge results with the results of five vaccinated calves (single dose at two-weeks-old). They concluded that a two-dose vaccination (4 weeks interval) was required to induce long lasting protection [124]. Only mild clinical signs were observed post challenge infection, and no significant reduction in virus excretion and lung pathology was shown. It was hypothesized that this was due to the presence of MDA [125].

The use of live virus vaccines administered topically (intranasally) for prophylaxis against disease caused by RSV looks promising for two reasons. First, inoculation using a live vaccine virus would induce a more balanced immune response comprising mucosal (IgA) and serum antibodies and cytotoxic T lymphocytes. Second, primary infection of calves with live attenuated vaccine candidates or naturally acquired wild-type virus is not associated with enhanced disease upon subsequent natural reinfection, a major concern for RSV vaccines. However, Kimman et al. have described a severe outbreak with BRSV in five- to seven month-old calves previously vaccinated with a live BRSV vaccine, which remained absent in eight-month-old and older non-vaccinated calves [126]. These calves were intramuscularly vaccinated with a MLV single antigen vaccine (Rispoval®) and the authors suggested that the parenteral vaccination with the live vaccine during a subclinical course of the infection may have enhanced the disease by boosting the immune response. Vaccination might have altered the IgM and IgG1 responses quantitatively or qualitatively and enhanced complement activation and immunopathology. They further suggested that the reason for not having been reported before could have been due to the fact that good controls are normally lacking under field conditions or that calves with apparent disease usually not would have been vaccinated. Thus enhanced disease is incidentally also reported after MLV vaccination.

A commonly known disadvantage of MLV vaccines is the possibility that vaccine virus may spread from the recipients. Recently, as study was published in which vaccinated calves shed vaccine virus from 4 to 11 days post-vaccination and infected negative-control animals [127].

Debouck et al. evaluated the efficacy of a MLV bRSV (single antigen) vaccine (Rispoval® RS) in MDA positive calves younger than four months old. VNA antibody titers and ELISA antibody titers between groups vaccinated at 1, 4 and 17 weeks old calves (n=10); 3, 6 and 17 weeks old (n=10) and at 17 weeks old (n=10) were compared. Intramuscular vaccination in the presence of MDA primed the immune system: VNA antibody titers were significantly higher at 10 and 13 weeks of age in early vaccinated animals compared to controls. ELISA antibody responses were significantly higher after the booster vaccination at 17 weeks [128]. No challenge data were reported in this study.

The efficacy of a quadrivalent combination vaccine (Rispoval^{TM}4) has been evaluated in several studies [129-132]. The combination vaccine was administered as two doses (three week interval). A significant reduction in viral shedding was shown three [131] and five [130] weeks after vaccination and in a long-term (twelve months) duration of immunity study [129]. In two studies [130, 131], a challenge model based on the Snook strain, an European isolate [133], was used and for the third study a challenge model based on strain 165 (origin unknown) was used. None of the models appeared to be suitable for clinical evaluation of the vaccine, as control calves showed hardly any clinical signs and no differences between vaccinees and control calves could be observed. Whether the statement that there was no enhancement of respiratory disease in the vaccinees after challenge infection is correct can be doubted, as the used challenge model apparently was not suitable for this purpose and the proper FI-BRSV-vaccinated control group showing enhanced disease after challenge infection was missing.

The immunogenicity of two other quadrivalent combination vaccines (Cattlemaster4® and Triangle 4) were evaluated by Ellis et al. Vaccination with a quadrivalent combination vaccine including MLV bRSV induced significantly higher ratios of neutralizing and non-neutralizing antibodies than the quadrivalent combination vaccine including KV bRSV [109]. The immunogenicity of a quadrivalent combination vaccine (Cattlemaster4®) was also evaluated by Fulton et al. and compared with a killed virus and two MLV vaccines. The quadrivalent combination vaccine including MLV bRSV induced a more rapid and higher antibody titer in seronegative calves than an inactivated BRSV vaccine [134]. The value of this observation is not clear, as proper T cell responses are required for viral clearance and antibody responses are not always positively correlated with protection. Efficacy and safety evaluations from challenge studies were not presented (Table 1).

	Efficacy				Safety
	Calves < 6 months old		Calves > 6 months old		Absence of vaccine-enhanced disease
	Virus excr.	Clinical symp.	Virus excr.	Clinical symp.	
Nr. of vaccines for which information is publicly available	2 out of 12	1 out of 12	1 out of 12	0 out of 12	0 out of 12

Table 1. Summary of vaccine studies regarding efficacy and safety of 14 commercially available BRSV vaccines. We have used all peer-reviewed publications on the efficacy and safety of BRSVvaccines [109, 122-132, 134], and information presented on congresses and non-peer-reviewed publications, provided by the corresponding companies. Reduction in virus excretion (duration and/or amount) was shown for two (14%) vaccines and clinical protection was shown for one (7%) vaccine in the most permissive age range (< 6 months). One vaccine was shown to be effective in reducing virus excretion in calves older than 6 months. To our knowledge no information is published on absence of vaccine-enhanced disease from safety studies for any of the vaccines. Serological data were available for all vaccines.

Outline of this thesis

There is a need for safe and effective vaccines against both hRSV and bRSV. Appropriate vaccines must have proven clinical protection and should lack disease exacerbating side effects upon exposure to infection. Despite many years of research and vaccine development efforts, there are still no RSV vaccines available for humans. For cattle, on the other hand, several inactivated and modified live bRSV vaccines are on the market, although proper assessment of protection and safety has been hampered by the lack of suitable experimental models [106]. In chapter 2, development of a bRSV challenge model is described in which both classical bRSV respiratory infection and vaccine-enhanced immunopathology is reproduced in the natural host. To study the immunopathology, we have studied pathophysiological mechanisms in calves vaccinated with the classical, formalin-inactivated (FI) vaccine and subsequently challenged with virulent virus. In addition, it is shown that in this challenge model mock-vaccinated control animals develop well-detectable and reproducible clinical sign after BRSV challenge infection. In chapter 3 studies are described on the cellular immune response. We describe the kinetics of antiviral CD8 T cells responses in bRSV infected calves that had been immunized with either formalin-inactivated (FI) or live-attenuated (L) bRSV, with evidence of immunopathology following challenge of calves vaccinated with FI-bRSV. In chapter 4, a study on the IgE responses is described to further characterize immunopathological responses and vaccine safety in a homologous experimental infection model of RSV disease in the natural host. In chapter 5 a study on alpha4- and beta2-integrins describes their role in adhesion of bRSV to activated pulmonary artery endothelial cells and migration by neutrophils in the course of a bRSV infection. In chapter 6 a study is described on the effect of age and immune parameters on bRSV disease in neonatal and young calves. In chapter 7 the evaluation of modified vaccinia virus Ankara (MVA)-based vaccine candidates expressing the bovine RSV-F is described, either or not in combination with the bovine RSV-G protein. Both vaccine candidates induced protective and

safe immune responses. Candidate vaccine constructs were produced simultaneously with human RSV-F and RSV-G proteins and tested in mice [135]. In chapter 8, recent findings on immune and immunopathological responses to bRSV and hRSV vaccines are summarized and the implications for both bovine and human RSV vaccine development are discussed.

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Chapter 2

Vaccine-Induced Immunopathology during Bovine Respiratory Syncytial Virus Infection: Exploring the Parameters of Pathogenesis

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Abstract

The bovine and human respiratory syncytial viruses cause severe lower respiratory tract infections. Effective vaccines against the respiratory syncytial viruses have been lacking since vaccine failures in the 1960s and 1970s. In this report, we describe a bovine respiratory syncytial virus (bRSV) challenge model in which both classical bRSV respiratory infection and vaccine-enhanced immune pathology were reproduced. The classical, formalin-inactivated (FI) bRSV vaccine that has been associated with vaccine failure was efficient in inducing high antibody titers and reducing viral loads but also primed calves for a far more serious enhanced respiratory disease after a bRSV challenge, thereby mimicking the enhanced clinical situation in FI human RSV (hRSV)-immunized and hRSV-infected infants in the 1960s. We show that immunization with FI-bRSV mainly primes a Th2-like inflammatory response that is characterized by a significant eosinophilic influx in the bronchial alveolar lung fluid and lung tissues and high levels of immunoglobulin E serum antibodies. The current model may be useful in the evaluation of new bRSV candidate vaccines for potency and safety.

Introduction

The paramyxovirus bovine respiratory syncytial virus (bRSV) is, like its human counterpart human RSV (hRSV), a major cause of respiratory disease (23). Primary bRSV infection can result in severe lower respiratory tract disease in susceptible cattle, although asymptomatic infections also occur. The virus causes an acute interstitial pneumonia with alveolitis and bronchiololitis, especially in calves and yearlings (24).

bRSV causes a range of clinical symptoms. Mild respiratory disease is characterized by coughing, serous or mucopurulent nasal discharge, slight to moderately increased respiratory rates (RRs), and abnormal breath sounds. Tachypnea, harsh lung sounds, and profound coughing characterize moderately affected calves. The most severely affected calves may be dyspneic and may have subpleural and interstitial emphysema. Emphysematous bullae may be present between lung lobules. Generalized symptoms range from a slightly elevated rectal temperature, mild depression, and anorexia to a high fever, deep depression, and coma (2, 4, 14).

Vaccine development against hRSV and bRSV has been hampered by the dramatic hRSV vaccine failure in the 1960s: vaccination with formalin-inactivated (FI), alum-adjuvanted virus predisposed children to a far more serious, and sometimes lethal, form of RSV infection (13). Subsequently, it was found in the

1970s that a similarly inactivated bRSV vaccine could induce strikingly similar immunopathology in bRSV-infected calves (28). In fact, some inactivated veterinary vaccines were withdrawn from the market after safety problems were discovered (R. S. Schrijver, personal communication).

Studies with murine models of hRSV have demonstrated that alum-adjuvanted FI-hRSV is a strong inducer of Th2 cells, which proved to be the key mediators of immunological hypersensitivity reactions (20). In fact, immunopathogenesis in BALB/c mice can be attributed completely to an oligoclonal response of interleukin-5 (IL-5)-producing CD4 T cells that are specific for the viral attachment protein (G) (26). On the basis of these results, it is evident that further vaccine development depends on a better understanding of the immune mechanisms of this enhanced disease and that these parameters are defined in models that allow evaluation of the safety of candidate RSV vaccines, such as the bRSV model.

Experimental bRSV infection resulting in severe respiratory disease in cattle has been described in only a few reports (3, 5, 6). However, a potential drawback of these studies is that it was unclear in these studies whether other pathogenic microorganisms might also have been involved in pathogenesis. For instance, severe respiratory disease after bRSV infection was reported by Ciszewski et al. (6), but the calves used were not specific pathogen free (SPF) and pathogenic microorganisms were in fact cultured from several animals in the experiment. Evidently, for further study of the (immuno)pathogenesis of bRSV infection and for evaluation of vaccine safety and efficacy, development of a bRSV infection model is urgently needed. In the present study, we have developed such a bRSV challenge model. The impact of prior vaccination with FI or live virus on the outcome of subsequent bRSV infection was analyzed by using a panel of clinical and cellular parameters.

Materials & methods

Vaccine preparation

bRSV, strain Lelystad, sixth passage, was grown in Earle‟s minimal essential medium (MEM; GIBCO) supplemented with 10% fetal bovine serum (FBS) and 0.5% antibiotic cocktail (ABC) on embryonic bovine trachea (EBTr) cells to a titer of 105.5 50% tissue culture infective doses (TCID50) per ml and harvested after 7 days. Supernatant (440 ml in total) was centrifuged (15 min, 1,000 x g) to remove cellular debris. The virus preparation was inactivated by addition of a 37% formaldehyde solution (1:4,000 at 37°C, 72 h), followed by ultracentrifugation (Beckman SW28, 110,000 x g, 60 min, 4°C). The virus pellet was resuspended in phosphate-buffered saline (PBS) and adjusted to a protein concentration of 0.75 mg/ml. A vaccine dose was prepared by mixing FI-bRSV (1:1) with 2% Al(OH)3 and incubating it for 20 min at room temperature.

Study design

Three groups of six SPF calves obtained by caesarean section, deprived of colostrum, and reared in isolation were housed in separate isolation rooms. The calves were free of bovine viral diarrhea virus (BVDV), as shown by BVDV antigen enzyme-linked immunosorbent assay (ELISA) (SERELISA BVD/MD/BD Ag; SYNBIOTICS EUROPE).

After the calves were found to be free of BVDV, they were loose housed in isolation units in groups of three. Starting at an age of 6 weeks, all of the calves received two intramuscular immunizations, with a 3-week interval, with either PBS, FI-bRSV adsorbed to Al(OH)3, or live bRSV (strain Lelystad, $10^{4.3}$ $TCID_{50}$/ml).

All calves were challenged approximately 5 months after the first immunization. bRSV field strain Odijk was used to prepare the challenge virus stock (25). This strain was obtained during an outbreak of acute respiratory disease in a herd of 3- to 5-month-old calves in December 1991 in Odijk, The Netherlands (25), and has never been passaged on cell culture. The virus stock contained $10^{3.9}$ $TCID_{50}$ of bRSV per ml and was found to be free of bacteria, mycoplasmas, BVDV, bovine herpesvirus 1, and parainfluenza 3 virus. For the challenge, all calves were inoculated intranasally with a 2-ml bRSV challenge inoculum with an air jet nebulizer and a jet stream of 0.2 mm, producing 10% droplets <26µm in section, 50% droplets <50 µm in section, and 90% droplets <99 µm in section. Bronchoalveolar lung fluid (BALF), heparinized blood samples, and nasal swabs were obtained from all calves at 3 days prior to challenge and at 1, 4, 7, and 9 days after challenge. All calves were bled weekly and 3 days before challenge and daily thereafter for serology. All animal experiments were conducted in accordance with the Act on Experimental Animals of The Netherlands regulated by the Ethical Review Committee of ID-Lelystad.

Clinical examinations

Calves were clinically examined once a day before challenge (from day 3 to day 0, the day of challenge infection) and then twice a day after challenge (from day 0 until day 9 postinfection). Calves were examined for signs of respiratory disease, ocular and nasal discharge, coughing, breathing, and lung sounds. Rectal temperatures and RRs were recorded. The mean of the prechallenge clinical scores was used as a baseline value (100%). Half-daily scores were expressed as a percentage of this baseline. Standardization was obtained by having all examinations performed by the same veterinarian. The severity of the clinical disease was expressed by allocating a weighting factor for each observation (Table 1).

TABLE 1. **Weighing factors for each clinical observation**[a]

Clinical signs of disease				
Rectal temperature (RT) measurements		RT<39.5°C	39.5<RT≤40.5°C	RT>40.5°C
Clinical signs of respiratory disease				
Ocular discharge / runny eyes	L R	No discharge observed	Serous or mucous discharge observed	Purulent or haemorragic discharge observed
Nasal discharge	L R	No discharge observed	Serous or mucous discharge observed	Purulent discharge observed
Coughing		No coughing observed	Spontaneous coughing	Spontaneous and productive cough present
Respiratory Rate (RR)		RR ≤ 60 / minute	60<RR≤100	RR>100
Respiratory distress		No abnormalities	Dyspnoea	
		Costo-abdominal	Exaggerated intercostal and/or abdominal effort	
		Regular	Irregular	
Respiratory noise		No abnormal respiratory sound	Abnormal / Intense respiratory sound (stridor)	
Trachea sensitivity		No induced cough after moderate pressure on the trachea	Induced cough after moderate pressure on the trachea on one particular place	Induced cough after moderate pressure on the trachea on more places
Lung auscultation		No abnormalities	Abnormal one (left or right) lung side	Abnormal both right and left lung side
			Increased intensity	Vesicular lung sounds
Scoring[b]		**0**	**1**	**2**

[a] the severity of the clinical disease was expressed by allocating a weighing factor (0, 1, or 2) for each mentioned observation (first column). For example: clinical observation: Rectal Temperature (RT), first column; weighing factor: if RT<39.5°C = 0, if RT>39.5 but RT≤40.5°C = 1, and if RT>40.5°C = 2.

[b] scoring values per observed observation. total score was calculated by editing the weighing factors for each observation.

Bronchoalveolar lavages
BALF samples were obtained as described by Fogarty et al. (8). Approximately 100 ml of BALF was obtained from each calf after instillation of approximately 120 ml of PBS. BALF samples were centrifuged (200 x g, 10 min, 4°C). Lavage cells were resuspended in fluorescenceactivated cell sorter buffer (PBS containing 25% FBS, 0.5% bovine serum albumin, and 0.01% Na azide), counted, further diluted to 5 x 106 viable cells per ml, and used for further analysis. Droplets of resuspended cells were put on slides and air dried. A total of 100 cells were counted (Hema-Tek slide stainer), and the lymphocytes, monocytes, and neutrophils (mature and immature) were differentiated on the basis of morphology. Viral antigen was visualized by staining acetone-fixed cells with fluorescein isothiocyanate-conjugated monoclonal antibodies (MAbs) directed against the F protein of bRSV (30 min at 37°C). BALF cell supernatants were stored at -70°C for virus isolation, virus titration, and PCR analyses.

Virological examination
Lavage fluid samples were centrifuged (200 x g,10 min, 4°C), and supernatant was stored at -70°C until virus isolation was done. Virus isolations were performed in quadruplicate on EBTr cells as described previously (25). Viral RNA was isolated with a viral RNA isolation kit (Qiagen, Valencia, Calif.). Reverse transcription (RT)-PCR to detect viral RNA was performed as described by Kuno (16). Briefly, an RT-PCR mixture was prepared that contained 0.5 U of RAV-2 polymerase (Amersham, Pharmacia Biotech, Roosendaal, The Netherlands),1 U of Tth polymerase (Roche), 1 x PCR buffer, 10 U of RNAguard (Amersham), primers, and a 3 µl RNA sample. The primers designed for bRSV-N and bRSV-P were N 5' (GTTTAAACCATGGCTCTYAG CAAGGTC),N 3' (CARTTCCACATCATTRTCTTT), P 5' (GAAATTTCCATGGAAAAATTTGCACCTG), and P 3' (GAAATCTTCAAGTGATAGATC ATTG) (Y = C/T, R = A/G; degenerate because the Odijk sequence was unknown). Water was added to make the total volume 50 µl. RNA was reverse transcribed at 50°C for 45 min, followed by 12 cycles of touchdown PCR (94°C for 1 min; 40 to 34°C in 0.5°C increments for 1 min; 72°C for 2 min) and 30 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min. Positive controls included plasmids containing the bRSV strain Odijk N and P genes, as well as cDNA prepared from bRSV strain Lelystad-infected cells. The N PCR generated a 1.1-kb product, and the P PCR generated a 0.7-kb product.
Lung tissue samples were stored at -70°C until virus isolation was performed. Tissues were homogenized in a mortar with sterile sea sand (Merck) in 5 ml of Dulbecco"s MEM supplemented with 2% ABC and 5% FBS (PAN, 1302P221701; Biotech GmbH). The tissue suspensions were centrifuged for 10 min at 2,000 x g, and the supernatant was collected. Virus isolation was performed on 24-well tissue culture plates. Each well contained a 200µl tissue suspension and 2 ml of an EBTr cell suspension (± 60,000 cells) in Earle"s MEM supplemented with 10% FBS and 0.5% ABC. After 7 days of incubation at 37°C in 5% CO_2,an immunoperoxidase monolayer assay for bRSV was performed (25).

Humoral immune response
A double-antibody sandwich (DAS) ELISA, as described by Westenbrink et al. (30), was used to determine bRSV-specific IgG antibody titers. This ELISA detects antibodies directed against the fusion protein of bRSV (F-ELISA, Ceditest ELISA for bRSV-Ab; Cedi Diagnostics, Lelystad, The Netherlands).

A blocking G peptide ELISA, as described by Langedijk et al. (17), was used to determine bRSV G-specific antibody titers and is based on blocking of the binding of a bRSV G-specific MAb (MAb 20) with the coated peptide by peptide-specific antibodies that may be present in the serum.

Neutralizing antibodies were determined in a virus neutralization assay (VNA). VNAs were carried out as previously described (17).

A sandwich ELISA, as described by Kooyman et al. (15), was used to detect total serum IgE responses. The value of the blank (PBS-GT) was set at 0. The corrected optical density (OD) value of the undiluted standard serum was set at 100%. On the basis of this standard, the OD values of the test sera were transformed to percentages, similar to the arbitrary units described by Kooyman et al. (15).

Haptoglobin assays
Serum haptoglobin was determined (on days postimmunization [dpi] 139 and 146 and daily from dpi 146 to 156) with a haptoglobin assay from Tridelta Ltd. that is based on the preservation of hemoglobin peroxidase activity by haptoglobin in the serum samples. Haptoglobin concentrations were read from a standard curve.

Postmortem
Calves were anaesthetized with a pentobarbital overdose and euthanized by exsanguination. The lungs were immediately removed, and dorsal and ventral photographs were taken, from which the extent of macroscopic lesions (consolidated lung areas) was rated as a percentage of the lung tissue area. The extent of consolidated lung tissue was scored on a scale of 0 to 5 as described by Viuff et al. (27) and used as an indication of the pneumonic lung tissue surface area.

Necropsy samples of 10 predetermined sites were stored in 10% neutral buffered formalin or snap-frozen in liquid nitrogen and/or frozen at -70°C. Formalinized samples were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin for histologic examination. Snap-frozen samples were examined for the presence of bRSV by immunohistochemistry. Long tissue samples stored at -70°C were also used for virus isolation.

Statistical analysis
For statistical analyses, the average temperature, the average respiratory scores, and the average clinical scores of the calves before challenge (morning measurements on dpi 143, 144, 145, and 146) were taken as the reference (baseline) values. For each calf, the severity of the clinical symptoms was

expressed as the area under the curve (AUC) for dpi 146 to 155 of the experiment. Significance of correlation was calculated with the Kruskal-Wallis nonparametric test and the Wilcoxon-Mann-Whitney test. Correlations and differences were considered significant when P was <0.05.

Results

Development of a bRSV infection model

The primary aim of our study was to evaluate the (immuno)pathogenesis of bRSV infection after prior immunization with an FI-bRSV vaccine. Therefore, it was essential to first set up a bRSV vaccination-challenge model. To achieve this, we used the bRSV field strain Odijk. We first sought to establish infection conditions that would produce clinical symptoms. After optimizing the inoculation method, we found that intranasal inoculation with an air jet nebulizer ($10^{4.2}$ $TCID_{50}$) consistently resulted in productive bRSV infections and clinical symptoms (data not shown).

bRSV infection after vaccination with L-bRSV or FI-bRSV

To assess the impact and efficacy of prior immunization with live attenuated bRSV (L-bRSV) or FI-bRSV, SPF calves were subjected to three different immunization regimens. Animals were immunized with FI-bRSV or L-bRSV or received a mock immunization with PBS. Humoral immune responses after vaccination were measured with commercially available F-ELISA kits, a G peptide ELISA, and the virus-neutralizing activity was tested in a neutralization assay. As expected, we found that none of the mock-immunized calves had bRSV-specific antibodies before inoculation. Immunization with either FI-bRSV or L-bRSV resulted in seroconversion in all animals. Figure 1 shows the average bRSV antibody titers for each group of calves. The first animal to test positive in the F-ELISA had received the FI-bRSV vaccine. F-specific antibodies were detected at 20 dpi. Three weeks later, all L-bRSV-and FI-bRSVimmunized calves were positive in the F-ELISA. Interestingly, a significantly greater antibody response (P = 0.0022) was observed in the FI-bRSV-immunized group. In contrast, the neutralizing antibody response was stronger in the L-bRSVimmunized group: Neutralizing antibodies were detected in three out of six FI-bRSV-immunized calves from dpi 41 onward, whereas all L-bRSV-immunized calves had neutralizing antibody responses starting from dpi 20 (Fig. 1).

All calves were challenged at dpi 146 by intranasal inoculation of $10^{4.2}$ $TCID_{50}$ of bRSV strain Odijk. The kinetics of viral replication in lung washes was analyzed by virus isolation and by RT-PCR. Unexpectedly, bRSV was detected by virus isolation in only two of the animals (animals 5539 and 5550, mock immunized and L-bRSV immunized, respectively) on days post challenge infection (dpci) 4 and 7, respectively. Next, the presence of viral RNA was measured by RT-PCR. Viral RNA was detected in BALF samples of four (out of six) calves at dpci 4 and in those of all six PBS-immunized calves at dpci 7. No viral RNA was detected in BALF samples from any of the FIbRSV-immunized calves, whereas a weak RT-PCR signal was detected at dpci 7 in one L-bRSV-immunized animal (data not shown).

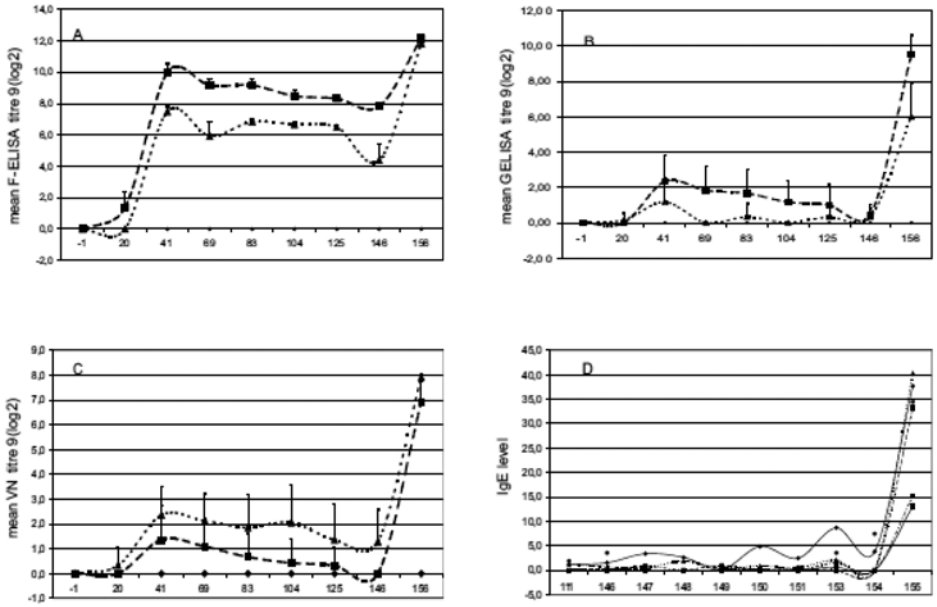

Figure 1 Humoral immune responses Average BRSV antibody titres (A, B, and C) measured on a three weekly interval (dpi –0, 20, 41, 69, 83, 104, and 125), on day of challenge (dpi 146), and end of experiment (dpi 156), with standard deviations. Group PBS (♦): six calves mock-immunised, group FI-BRSV (ν): six calves immunised with formalin-inactivated BRSV, and group L-BRSV (σ): 6 calves immunised with live-BRSV. Figure 1D: IgE antibody responses detected in the IgE double antibody sandwich ELISA. Corrected OD values were transformed to percentages of a standard positive serum. Only FI-BRSV immunized calves were found to be IgE positive. Each line in this figure represents a calf immunized with FI-BRSV vaccine.

Thus, both immunization protocols resulted in strongly decreased viral loads after challenge. Moreover, all FI-bRSV-and L-bRSV-immunized calves seroconverted in the F-ELISA, G-ELISA, and VNA after challenge. Interestingly, elevated levels of total IgE were detected in serum samples of all FI-bRSV-immunized calves at dpci 9 (Fig. 1D). None of the other animals showed any IgE responses.

In conclusion, both vaccines appear to be highly effective on the basis of the reduced viral loads and induction of and/or increases in (neutralizing) antibodies after challenge.

Clinical symptoms after challenge of FI-bRSV-or L-bRSVimmunized or mock-immunized animals.
The clinical symptoms after challenge were expressed as the AUC. AUC values were calculated for 9 dpci. The parameters that were included in the AUC calculation were increased RR, increased body temperature, nasal discharge, coughing, sensitivity of the larynx and/or trachea, abnormal breathing, and lung

sounds. Interestingly, clinical signs were first observed in the FI-bRSVimmunized group. Following this trend, we found the greatest increases in clinical scores after bRSV challenge administration in the FI-bRSV-immunized group (Fig. 2). Whereas some of the FI-bRSV-immunized calves presented severe clinical signs as early as 2 days after the challenge administration (> 200% compared to the baseline), calves immunized with either L-bRSV or PBS displayed clinical signs no earlier than 5 days after challenge administration. More pronounced clinical signs were observed in the PBS-immunized group than in the L-bRSV-immunized group (Fig. 2). Differences in the average AUC values of the three different groups were significant (P = 0.0041). Compared to the AUC values of calves immunized with L-bRSV, both the PBS and FI-bRSV-immunized animals showed significantly more severe clinical symptoms (two-sided P values of 0.0216 and 0.0087, respectively, with the Wilcoxon-Mann-Whitney test).

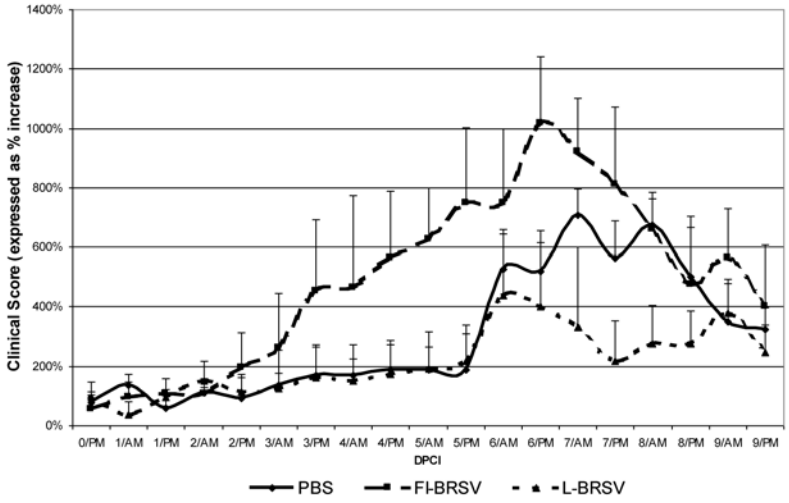

Figure 2 Clinical observations. Average clinical scores per group in percentages regarding to the baseline, daily twice (AM and PM) from challenge (dpi 146) till end of study (dpi 155) with standard deviations. Group PBS: six calves mock-immunized, group FI-BRSV: six calves immunized with a formalin-inactivated BRSV vaccine, group L-BRSV: six calves immunized with a live-attenuated BRSV vaccine.

Our assessment of clinical symptoms was confirmed by the analysis of acute-phase responses (haptoglobin). Acute-phase responses were detected, and they peaked around 8 to 9 days after inoculation of bRSV (Fig. 3). The AUC was determined per calf and compared within groups. The mock-immunized group showed a significantly more severe response (P = 0.0476) than the L-bRSV-immunized group. The magnitude of the haptoglobin response correlated well with the severity of clinical signs and with the extent of lung consolidation in the PBS- and L-bRSV-immunized groups.

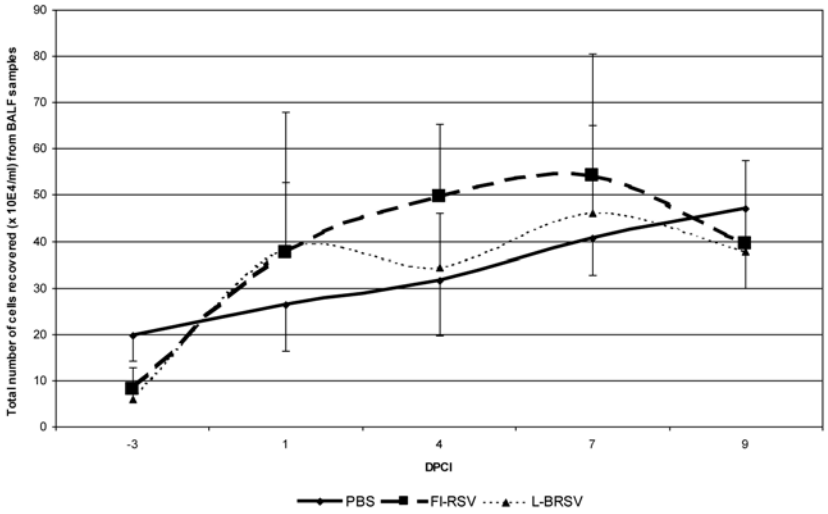

Figure 3 Total number of cells recovered from the Bronchoalveolar lung fluid samples (BALC) (Y-axis) on the particular days post challenge infection (DPCI) (X-axis) per group. Significant more BALC were recovered from FI-bRSV immunized calves (p=0.0152), and L-bRSV immunized calves (p=0.0043) compared to mock-immunized calves.

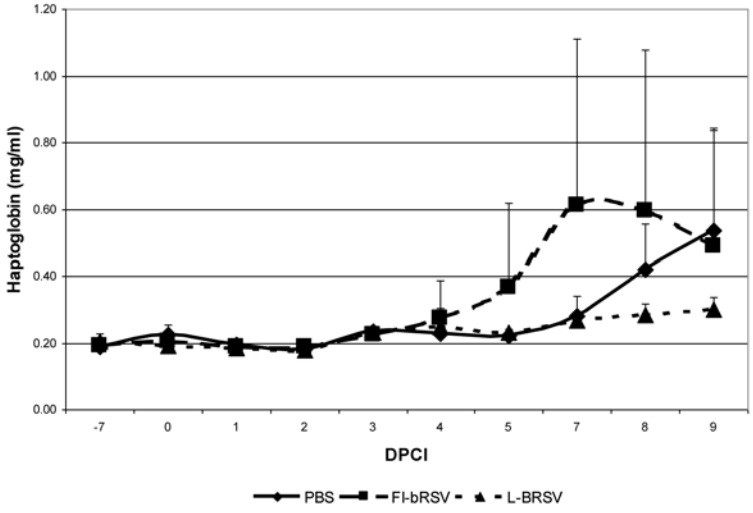

Figure 4 Haptoglobin analyses. Acute phase responses were detected, and peaked around 8-9 days post challenge infection (DPCI). The AUC was determined per calf and compared within groups. The mock immunized group showed a significantly more severe response (p=0.0476) compared to the L-bRSV immunized group. The magnitude of the haptoglobin response was found to correlate well with the severity of clinical signs and with the extent of lung consolidation in the PBS and L-bRSV immunized groups.

Thus, on the basis of clinical appearances, we have been able to model severe bRSV infection and, importantly, the enhanced disease after prior immunization with FI-bRSV. It is striking that enhanced disease in the FI-bRSV-vaccinated group coincided with a very strong reduction of viral loads compared to the PBS-immunized control group.

Cellular immune responses in the lungs

Respiratory infection is usually accompanied by an influx of lymphocytes and granulocytes into the lungs (Fig. 4). To obtain a better insight into the nature of the cell populations migrating into the infected lung, we performed phenotypic analyses of cells from BALF samples. BALF samples were collected at different time points before and after a bRSV challenge. Analysis of these samples revealed a strong eosinophil infiltration in FI-bRSVimmunized animals, starting at day 1 after challenge administration (Table 2). Four out of six FI-bRSV-immunized calves showed an eosinophilic infiltration (7% ± 10%) at day 4 after challenge administration. Eosinophil infiltration was observed in all calves at days 7 (19% ± 13%) and 9 (28% ± 19%) after challenge. The influx of eosinophils was slower in the L-bRSVimmunized group: only two BALF cell samples showed an eosinophilic infiltration at 7 days after challenge administration (5% ± 8%), and all animals showed eosinophil infiltration by day 9 after challenge administration (10% ± 7%). The eosinophilic infiltration observed at 7 days after the challenge infection was significantly greater ($P = 0.0346$) in the FI-bRSV group. No eosinophil infiltration was observed in the PBS immunized group.

Pathology

All animals were euthanized at dpci 9 to allow a detailed analysis of virus-and/or immune response-induced pathology. At necropsy, the percentage of abnormal lung surface area ranged from 0.2 to 12.3% within the entire group of 18 animals. Mean percentages (± the standard error of the mean) in the PBS-, FI-bRSV-, and L-bRSV-immunized groups were 8.2% ± 2.6%, 3.6% ± 2.1%, and 0.8% ± 0.7%, respectively. When extended, consolidated lung tissue areas were scored as described by Viuff et al. (27), five out of six PBSimmunized calves and one FI-bRSV-immunized calf were scored 2 (5 to 15% consolidated lungtissue), whereas all of the others were scored 0 or 1. Differences between the PBS-immunized group and both of the other groups were significant (one-sided P values of 0.0390 for the PBS group versus the FI-bRSV group and 0.0076 for the PBS group versus the LbRSV group).

Calves of all three groups had developed multifocal bronchointerstitial pneumonia, often with characteristic multinucleated syncytial cells, a marked proliferation of bronchiolar epithelium, alveolar epithelialization, alveolar edema, and hyaline membrane formation. The lumina of many bronchioles were narrow and were often filled with secretion. The superficial bronchial epithelial cells were swollen. In the bronchiolar walls, a variably dense lymphocytic infiltrate was observed. Lymphocytic aggregates and follicles were visible, especially in the calves that were immunized

with PBS. The lung parenchyma showed atelectasis next to parts with alveolar emphysema.

TABLE 2. total number if cells recovered in bronchoalveolar lung fluid samples and percentages eosinophilic granulocytes (EO) before and after the viral challenge.

DPCI[a]		-3		1		4		7		9	
		BALC[b] (10E4/mL)	EO[c] (%)	BALC (10E4/mL)	EO (%)	BALC (10E4/mL)	EO (%)	BALC (10E4/mL)	EO (%)	BALC (10E4/mL)	EO (%)
Mock immunized group		13	0	5	0	48	0	49	0	11	0
		24	0	29	0	13	0	39	0	56	0
		28	0	31	0	32	0	26	0	43	0
		14	0	27	0	44	0	37	0	53	0
		17	0	36	0	29	0	48	0	58	0
		23	0	30	0	23	0	44	0	60	0
Fl-bRSV immunized group		13	0	28	1	49	6	49	18	36	18
		6	0	41	0	60	27	31	22	74	54
		13	0	29	0	53	7	38	5	20	4
		3	0	52	0	16	3	107	42	21	14
		12	0	59	0	59	0	33	13	37	35
		2	0	15	0	61	0	67	12	48	40
L-bRSV immunized group		11	0	18	0	17	0	80	20	41	18
		5	0	13	0	44	0	54	0	48	5
		9	0	99	1	31	0	53	8	56	17
		0	0	40	0	53	0	34	0	23	6
		5	0	43	0	34	0	23	0	37	15
		7	0	16	0	27	0	31	0	21	1

a days post challenge infection (DPCI)
b Bronchoalveolar lung cells (BALC)
c eosinophilic granulocyts (EO)

The pathology data confirmed our findings on eosinophilic infiltrations in the lungs. In the calves immunized with FI-bRSV vaccine, we found marked eosinophilic tracheobronchitis and bronchiolitis characterized by diffuse infiltration of mainly eosinophils in the submucosa and in the interstitium surrounding the bronchi and bronchioles. In addition, a focal-to-diffuse eosinophilic periarteritis of the pulmonary arteries was observed. Quantification of eosinophilic densities in the respiratory tract showed no significant differences, although an indication of a difference ($P = 0.0649$) between the numbers of eosinophils in the FI-bRSV-primed animals compared to those in the live-bRSV-vaccinated calves was found. The highest numbers of eosinophils were found surrounding the walls of bronchi and bronchioles, and hardly any were found in the alveolar tissue and alveolar lumina. The calves immunized with PBS showed the most severe bronchointerstitial pneumonia but had no eosinophilic infiltration in the respiratory system. Finally, three predetermined site samples, chosen on the basis of gross pathology findings, were tested for bRSV by immunoperoxidase monolayer assay. In none of the samples tested was infectious bRSV detected.

Discussion

In this report, we describe a bRSV challenge model in which both the classical bRSV respiratory infection and vaccine-enhanced immune pathology were reproduced. We have analyzed the kinetics of viral replication in the lungs, the humoral immune response, and the clinical symptoms in bRSV-infected calves that had been immunized with either L-bRSV or FI-bRSV or mock immunized with PBS.

One of the most striking findings in our study is the discrepancy between viral loads and humoral immune responses on the one hand and pathogenesis and clinical symptoms on the other hand. Three different patterns of infection and subsequent pathogenesis can be distinguished. First, in PBS-immunized and bRSV-infected animals, we have observed viral replication in the lungs and concurrent pathogenesis. In this situation, clearance of the viral infection coincided with a rise in (neutralizing) IgG antibody titers. Second, immunization with L-bRSV provides protective immunity: viral RNA was virtually undetectable, only mild clinical symptoms were observed, and (neutralizing) antibody titers increased rapidly after a challenge infection. The third, and most striking, pattern of infection was observed in the animals that had received the FI-bRSV vaccine. Immunization with FI-bRSV resulted in strong IgG antibody responses against F and G. Neutralizing antibodies could also be detected, but titers were lower than those of L-bRSV-immunized calves. Strikingly, these strong antibody responses did not prevent the early onset of severe clinical symptoms. Immune responses in these animals were characterized by high IgE antibody titers accompanied by marked eosinophilia. Preliminary data show that this IgE response is, at least in part, bRSV specific (unpublished results). Combined, these results are indicative of a type I hypersensitivity reaction. It should be noted, however, that challenge infection of L-bRSV-immunized calves was also associated with eosinophilia, albeit in a milder form (Table 2). Combined, we feel that our data are most consistent with a model in which pathogenesis in FI-bRSV-immunized calves has an immunological basis,

whereas infection of mock-immunized animals leads to a more conventional virus-induced pathogenesis.

It is evident that accurate quantitation of clinical symptoms was essential for the interpretation of our data. The parameters that we have used include (i) the duration of fever, (ii) the extent of consolidated lung areas, and (iii) the analysis of acute-phase responses (haptoglobin). As shown by Heegaard and coworkers (10), the magnitude and duration of the haptoglobin response correlate with the severity of clinical signs (fever) and with the extent of lung consolidation. In our model, fever was recorded in PBS-immunized calves for 2 to 3 days and was associated with significant consolidated lung areas (and lack of eosinophilia). In contrast, less extensive acute phase responses, shorter duration of fever, and less consolidated lung areas were found in L-bRSV-immunized group calves. Thus, viral loads, as well as clinical signs, were clearly reduced in L-bRSV-immunized calves. The situation was radically different in FI-bRSV-immunized animals: a pronounced acute-phase response was observed, peaking 6 to 7 days postchallenge, and fever was found in all six calves for 1 to 5 days. In contrast, pathological, consolidated lung areas were less extensive than in mock-immunized calves. Thus, for the FIbRSV-immunized calves, we found no correlation between the haptoglobin response, eosinophilia, and clinical signs on the one hand and the extent of lung consolidation on the other hand. However, it should be noted that the calculated consolidated red lung areas, used as an indication of the pneumonic surface area, may not provide an accurate estimate of the total pneumonic involvement since we found many deep parenchymal lesions that did not have any surface involvement.

Our results obtained with FI-bRSV-immunized calves mimic the enhanced clinical situation in FI-hRSV-immunized and hRSV-infected infants, as reported by Kapikian et al. (12). So far, enhanced pathogenesis after immunization with FI virus has been difficult to reproduce in cattle. In one early study, similar adverse effects could be induced in calves after immunization with FI-bRSV vaccines (28), whereas West and coworkers only reported an earlier onset and resolution of clinical disease after immunization with FI-bRSV vaccine (29). In the field, Schreiber et al. reported high mortality rates associated with bRSV infection in Belgian Blue calves in the field previously vaccinated with (β-propiolactone-)inactivated bRSV vaccine (22). In contrast, Mohanty et al. observed both neutralizing antibodies and protection in calves after immunization with an FI-bRSV vaccine (18). Only Gershwin et al. have been able to demonstrate that immunization with an FI vaccine led to more severe clinical signs after a challenge compared to those of nonvaccinated control calves (9). However, in contrast to the findings of Gershwin et al., we found virus-neutralizing antibodies in FI-bRSV-immunized calves and found indications of a deregulated T-cell response (IgE), correlating with a hypersensitivity reaction (see below).

There has been extensive speculation on the nature of enhanced RSV pathogenesis after immunization (1, 19). So-called “sub-neutralizing” antibodies were the first potential culprits to be identified (1, 19). This hypothesis implies that the antibodies primed by immunization are insufficiently neutralizing, with the

predicted result of higher virus titers after challenge. Consistent with this, Polack et al. (21) found that enhanced pathogenesis in the mouse model of RSV infection is associated with low neutralizing antibody titers, high viral titers, and the deposition of immune complexes. In contrast, weobserved the opposite in our model: viral loads in FI-bRSVimmunized animals were strongly reduced, since no viral RNA was detected, and the FI-bRSV vaccine did induce virus-neutralizing antibody titers. Thus, our data are inconsistent with a model of enhancement that implies inadequate antibody function and an increased viral load.

The hypothesis that has attracted the most attention recently states that immunization with FI vaccines mainly primes a Th2-like inflammatory response (20). This vaccine-induced Th2-biased memory response would then set the stage for the expansion of Th2-polarized CD4 T cells and, indirectly, the enhanced pulmonary lesions found after a subsequent challenge. The Th2 cytokines IL-4, IL-13, and IL-5 would play major roles in this scheme. This model has received experimental support from the well-studied murine RSV infection model (11, 20, 26) and from an experimental RSV infection model involving macaques (7). Interestingly, these aberrant Th2-like responses are related to a hyperactive IgE response. Our findings of significant eosinophil infiltrations in BALF samples and high titers of IgE serum antibodies after a challenge infection in all FI-bRSV-immunized calves reflect an underlying Th2-biased CD4 T-cell response. It is of interest that a deregulated (Th2 biased) T-cell response has been hypothesized to play a key role in airway hypersensitivity reactions as asthma and airway hyperresponsiveness (11).

A deeper understanding of the role of vaccine-primed and T-cell-mediated immune dysregulation, as reflected by IgE responses, is a prerequisite for future rational vaccine design, and the bovine RSV infection model described in this study could be instrumental in understanding disease augmentation.

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Chapter 3

Kinetics of antiviral CD8 T cell responses during primary and post-vaccination secondary bovine Respiratory Syncytial virus infection

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Kinetics of antiviral CD8 T cell responses during primary and post-vaccination secondary respiratory syncytial virus infection.

Abstract

We have measured antiviral CD8 T cells responses in bovine respiratory syncytial virus (bRSV) infected calves that had been immunized with either formalin-inactivated (FI) or live-attenuated (L) bRSV, with evidence of immunopathology following challenge of calves vaccinated with FI-bRSV. In all cases, bRSV infection induced potent pulmonary CD8 T cell responses. The kinetics of the post-challenge response in L-bRSV immunized animals was accelerated compared to the FI-bRSV and PBS groups, suggesting that only the L-bRSV vaccine, and not the FI-bRSV vaccine, had primed memory T cells. The differences between primary and post-vaccination secondary infection were very minor, in terms of the proliferation status of pulmonary CD8 T cells. Functional IFN-Ɣ+ CD8 responses were slightly higher in the FI-bRSV vaccinated animals. Furthermore, the existence of strong IFN-Ɣ+ CD8 responses in FI-bRSV vaccinated animals after challenge suggests (i) that these IFN-Ɣ+ responses in FI-bRSV immunized animals do not protect against immunopathology, and (ii) that Th-2 biased responses during bRSV challenge after vaccination with FI-bRSV have a limited impact on the CD8 responses in the bronchoalveolar lavage fluid. Thus, several response patterns (Th-1/Th-2) seem to co-exist during bRSV-infection.

Introduction

The human and bovine respiratory syncytial viruses (hRSV and bRSV) are major causes of lower respiratory tract infections. Both with respect to epidemiology and pathogenesis, bRSV infection in calves closely resembles hRSV infection in infants and bRSV therefore provides a valuable animal model for hRSV [1]. Vaccine development has been hampered by the fact that immunization with formalin-inactivated (FI) virus primes for a much more severe, and sometimes even lethal, form of respiratory disease [2-6]. This phenomenon was first observed in a human vaccine trial in the 1960‟s [4] and was later found to also occur in cattle immunized with formalin- or beta-propriolactone-inactivated bRSV [5,6]. Enhanced disease resulting from immunization with FI-virus has an immunopathological basis and has now been modeled in hRSV-infected mice [2,7-9] and monkeys [10] and in bRSV-infected cattle [1,11-13]. In mice, immunization with inactivated virus evokes a Th-2 biased CD4 T cell response, which is associated with eosinophilia and clinical symptoms upon challenge [2]. In cattle, enhanced clinical symptoms post immunization were associated with pulmonary eosinophilia and increased IgE titers [1].

RSV-specific CD8 T cell responses play a key role in the control of infection and in the maintenance of antiviral immunity [2,3,14]. This was most convincingly shown by T cell subset depletion experiments in cattle and mice [14,15]. Depletion of CD8 T cells in gnotobiotic calves resulted in more prolonged virus shedding and more severe pathologic lesions (i.e., more extensive lung consolidation), after bRSV infection. CD4 T cell depletion did not affect the kinetics of viral clearance, but did result in more serious symptoms. Consistent with a key role of the CD8 T cell response, McInnes and coworkers showed that acute bRSV infection in cattle is associated with an influx of activated CD8 T cells into the lungs and the trachea, peaking at day 10 post infection [16]. On the other hand, CD8+ cytotoxic T lymphocytes also have the capacity to contribute to lung pathogenesis, as shown in hRSV-infected mice [14,17,18]. Th-1 and Th-2 responses seem to co-exist in a delicate balance during RSV infection. Several studies in the mouse model have provided evidence for mutual down-regulation of CD8 and Th-2-biased CD4 T cell responses [9,19,20], whereas other studies have shown that antiviral CD8 T cell responses are essential in the development of pulmonary eosinophilia [21]. In addition, IL-5 and IFN-Ϋ producing G-specific CD4 T cells co-exist in RSV-infected BALB/c mice [22,23]. Thus, the combined literature indicates that in murine RSV-induced immune responses, Th-1, and/or CD8, and Th-2-biased CD4 T cell responses could either co-exist or down-regulate each other.

The role of CD8+ CTLs in the control of infection and in enhancement of lung pathology [17,18], combined with the pathogenic potential of a Th-2-skewed CD4 T cell response [23-25] and the limited longevity of cellular immunity [26], provides a challenge for vaccine development [27]. Most pneumoviruses, including bRSV and hRSV, are species-specific. Thus, these specific virus-host relationships provide an argument to study natural host-pathogen interactions, in parallel to the murine hRSV model. The bRSV challenge model in calves is promising because FI-bRSV associated enhanced pathogenesis can be experimentally reproduced in bRSV-infected animals [1,13]: after challenge, FI-bRSV immunized calves presented with severe symptoms, eosinophilia and high IgE titers, with specificity for the F protein (Antonis et al., unpublished observations).

In the present study, we asked how antiviral CD8 T cell responses develop during primary bRSV infection, and how vaccination with FI-bRSV or L-bRSV would affect this response, both in terms of kinetics and magnitude. We focused on CD8 T cell responses because their role in pulmonary eosinophilia has been ambiguous [28]. Our data indicate that post-challenge T cell responses in PBS- and in FI-bRSV immunized animals display similar kinetics, whereas those responses in L-bRSV immunized animals peaked earlier. Because accelerated recall T cell responses, indicative of the recruitment of memory cells, were observed only in L-bRSV animals, it appears that the FI-bRSV vaccine was not very efficient in priming long term T cell memory. However, IFN-Ϋ T cell responses were readily detectable in FI-bRSV-immunized and challenged animals. This suggests that Th-1 CD8 responses per se do not protect against severe symptoms. In our model, IFN-Ϋ+ CD8 T cell

responses appear to co-exist with Th-2 associated phenomena such as eosinophilia and elevated IgE levels.

Materials & methods

Viruses and animals

BRSV field strain Odijk [29] was used for viral challenge. Vaccination and challenge experiments have been described in detail previously [1]. Briefly, bRSV was cultured on embryonic bovine trachea (EBTr) cells. After clarification, supernatant was treated with formalin (37% formaldehyde solution, 1:4,000 at 37°C for 72 hours), followed by ultracentrifugation. A vaccine dose consisted of 1ml sedimented virus material, equivalent to 0.75mg protein or 106.7 TCID50, mixed with 1ml 2% Al(OH)3. In the case of the live-attenuated bRSV vaccine, we used a dose of 104,6 TCID50 of bRSV strain Lelystad in 2ml EMEM, supplemented with 10% FBS. SPF- and bRSV-naïve calves (6 per group), which had been obtained by ceasarean section [1], received 2 intramuscular vaccinations with FI-bRSV, L-bRSV (strain Lelystad) or PBS, and were challenged with bRSV Odijk [29] (at $10^{5.5}$ $TCID_{50}$ /ml, 2ml dose) approximately 4 months later. Intranasal virus inoculation was done using a nebulizer. Bronchoalveolar lavage fluids (BALF) were obtained as described [1]. Approximately 100 ml of BALF was obtained from each animal after instillation of 120 ml PBS. BALF samples were centrifuged (200 x g, 10', 4°C) and lavage cells were resuspended in PBS containing 25% FCS, 0.5% BSA and 0.01% NaN3, counted and used for further analysis. Lymph node and spleen samples were obtained during postmortem analysis as described [1]. PBMC were isolated by Ficoll centrifugation of heparinized blood samples. bRSV Lelystad strain was grown in embryonic bovine trachea cells (EBTr), as described [1].

Vaccinia virus recombinants

To construct recombinant vaccinia viruses expressing bRSV genes, viral RNA was isolated from BALF from a bRSV Odijk-infected animal or from bRSV Lelystad-infected EBTr cells [1], using RNA isolation kits from Qiagen (Valencia, CA). RNA preparations were subjected to RT-PCR amplification, using Pfu polymerase (Promega) and primer sets designed to amplify the complete open reading frames for the N, P, M, M2 and G genes, as described previously [1]. cDNAs encompassing the N. P, M and M2 sequences were amplified from viral RNA derived from the Odijk strain, whereas the G-protein cDNA was generated from the Lelystad RNA. PCR-amplicons were subjected to sequence analysis to confirm the absence of unwanted mutations, and inserted into pCRII (Invitrogen). Inserts were then subcloned into the vaccinia virus transfer vector pSC11. Recombinant vaccinia viruses (strain Western Reserve) were generated using standard methodology, as described [30]. The MVA-F recombinant was provided by Dr. Gerd Sutter (Institute of Molecular Virology, GSF-Research Center and Technical University Munich, Munich, Germany) as part of the EU IMPRESSUVAC project. Details on the construction of the MVA-F recombinant will be described elsewhere. Expression of the inserted bRSV genes was confirmed using (i) an immuno-peroxidase

monolayer assay (IPMA) and (ii) by metabolic 35S-Met labeling of vaccinia virus recombinant infected cells followed by radioimmunoprecipitation either with a polyclonal goat anti-hRSV (A+B) serum (Biodesign International) or with monoclonal antibodies directed against the bRSV N, P, G, and F proteins (Animal Sciences Group, Lelystad).

Antibodies and flow cytometry

Cells harvested from BALF were incubated with the appropriate antibodies for 30' on ice. FITC- or R-PE-labeled antibodies against CD4 (clone CC8), CD8 (clone CC63), and IFN-γ (clone CC302, IgG1) were purchased from Serotec (Oxford, UK). Allophycocyanin (APC)-labeled anti-bovine CD8 antibody was prepared by labeling antibody, purified from the supernatants of hybridoma cultures (clone CC63, obtained from the ATCC), with the APC labeling kit from Prozyme (San Leandro, CA). R-PE-labeled Ki-67 antibody (clone B56, IgG1), and the isotype control (MOPC-21), were obtained from BD Biosciences (Mountain View, CA). The B56 antibody is cross-reactive with the bovine protein [31]. Surface stainings were done in FACS buffer (PBS supplemented with 1% BSA and 0.01% NaN3). For intracellular Ki-67 staining, cells were fixed and permeabilized using the FACS lysing and FACS permeabilization reagents from BD Biosciences. After surface and/or intracellular staining, cells were fixed in 1% paraformaldehyde and acquired on a FACScalibur flow cytometer. Data were analyzed using CellQuest (BD Biosciences, Moutain View, CA) and FlowJo (Tree Star, San Carlos, CA) software.

Intracellular cytokine staining

Lymphocytes were stimulated with bRSV Lelystad (m.o.i = 0.1) (or medium from uninfected EBTr cells as the negative control), or with recombinant vaccinia or MVA viruses expressing bRSV genes, or control recombinants (wt MVA and T7 RNA polymerase expressing vaccinia virus) (m.o.i. = 1). After overnight incubation (37˚C, 16 hours), brefeldin A was added and incubation was continued for another 6 hours at 37˚C. As a positive control, cells were stimulated with SEB (Sigma) in the presence of brefeldin A. Cells were then fixed using FACS lysing solution, permeabilized using FACS permeabilization solution, stained with APC- and FITC-labeled antibodies against CD8 and IFN-γ, and acquired using a FACScalibur flow cytometer. The MOPC-21 IgG1 monoclonal antibody was used as an isotype control.

Statistics.

Statistical analysis was done with Prism and InStat3 software (Graphpad Software), using the Mann-Whitney test and the Kruskal-Wallis nonparametric test). Differences were considered significant when P was <0.05.

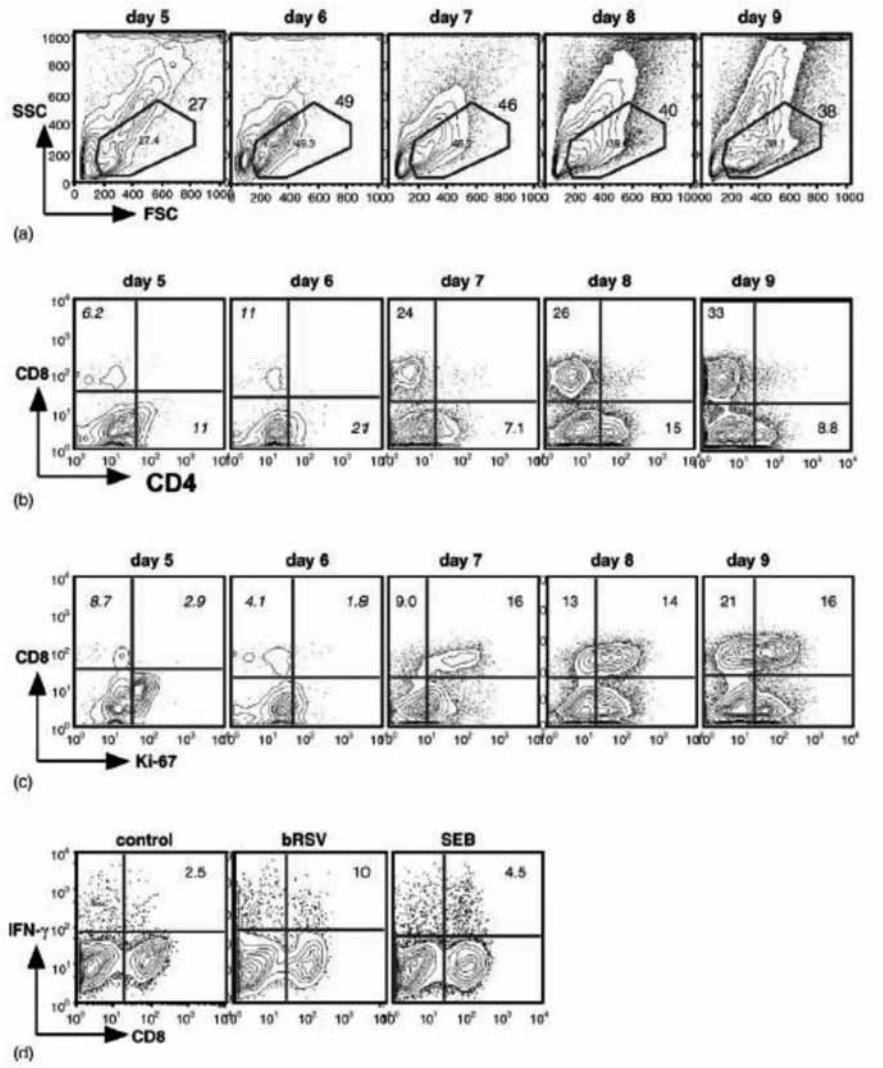

Figure 1. Kinetics and specificity of pulmonary CD8 T cell responses during acute bRSV infection. Two animals were infected with bRSV Odijk and BALF were collected daily from day 5 through 9 post infection. All data shown are from one of the two animals and are representative. (a) Percentage of lymphocytes of BALF and gating strategy based on forward (FSC) and side scatter (SSC) parameters. (b) Influx of predominantly CD8 T cells at day 7 post infection. Percentages of the relevant cell populations are indicated in the quadrants. (c) Infiltrating CD8 T cells at days 7-9 are proliferating (Ki-67^{high}). Percentages of Ki-67^{+} CD8 T cell populations are indicated in the upper right quadrant. Percentages in italics (days 5 and 6) indicate that a very small of cells were analyzed. (d) Specificity of infiltrating lymphocytes. Day 9 BALF were stimulated with bRSV (m.o.i.=1), SEB or control medium from uninfected EBTr cells, and analyzed by intracellular cytokine staining. Percentages IFN-γ^{+} $CD8^{+}$ cells of total $CD8^{+}$ cell numbers are shown. Note that the SEB-stimulated sample appears to have more IFN-γ^{+} cells than the bRSV-stimulated sample; this is, however, solely the result of different total cell numbers in the two plots.

Results

Primary bRSV infection coincides with CD8 T cell proliferation and migration into the lungs

Acute bRSV infection is associated with an influx of activated CD8 T cell lymphocytes into the lung [16]. To further study the dynamics of this response and the proliferation status of the infiltrating T cells, we analyzed the lymphocyte populations isolated from brochoalveolar lavage fluid (BALF). For this experiment, two calves were infected with bRSV strain Odijk [1,29]. This strain was selected because it induces a symptomatic infection in calves [29]. BALF samples were collected daily from days 5 through 9 after infection, and cells were stained with monoclonal antibodies against CD4 and CD8 and, after permeabilization, with an antibody against the proliferation marker Ki-67. Lymphocytes were gated using forward and side scatter parameters (Fig.1a). As shown in Fig. 1b, infiltrating CD4 and CD8 T cells were first detected at day 7 post infection (p.i.). Apparently, an influx of T cells starts between days 6 and 7 p.i. At day 8 post infection, approximately 16% of all BALF cells consisted of CD4 and CD8 T cells (Fig.1a and 1b). The data shown in Fig.1 are representative for the two animals. These kinetic data are consistent with results described by McInnes et al. [16]. The majority (60-65%) of the infiltrating CD8 T cells were proliferating (Ki-67high) at day 7 p.i. (Fig.1c). From day 7 through 9, the numbers of CD8 T cells in the lungs increased, but expression of Ki-67 slightly decreased (40-45% at day 9), suggesting that fewer cells were cycling. Since CD8 T cell proliferation is usually a function of TCR stimulation after antigen encounter in the local lymph nodes, it seems likely that the dividing CD8 T cells observed in the lungs are predominantly bRSV-specific. However, non-specific recruitment of CD8 T cells also occurs during respiratory infections [32]. Thus, to assess the bRSV-specificity, we stimulated BALF cells (obtained at 9 days post infection) with virus (m.o.i. = 0.1, virus harvested from EBTr cells) and visualized responding cells using intracellular IFN-γ staining and flow cytometry. The cells were incubated overnight with bRSV, after which the Golgi-inhibitor brefeldin A was added, followed by another 6 hour incubation. As controls, cells were stimulated with the superantigen SEB or were incubated in the presence of medium from uninfected EBTr cells. Cells were then permeabilized and stained with monoclonal antibodies against CD8 and IFN-γ. We found that up to 10-15% of all pulmonary CD8 T cells produced IFN-γ upon bRSV stimulation (Fig.1d), suggesting that at least a significant fraction of the infiltrating T cell population is bRSV-specific. It is possible that this underestimates the fraction of the response that is bRSV-specific, since stimulation with whole (live) virus may not result in optimal antigen presentation. The control SEB-stimulation yielded a 4-7% response of IFN-γ+ CD8 T cells. Thus, quantitative analysis of T cell proliferation through Ki-67 staining provides us with a rough estimate of the dynamics and magnitude of the antiviral responses. Such an estimate is particularly useful in situations in which few T cell epitopes have been identified, as is the case for bRSV.

CD8 T cell proliferation in bRSV-infected calves: impact of immunization and immunopathogenesis

We have recently described a bRSV challenge model for Th-2-driven FI-vaccine-induced immunopathology [1]. We now extended these studies by analyzing the CD8 T cell responses after bRSV challenge. Eighteen SPF calves, immunized intramuscularly with FI-bRSV, L-bRSV, or mock-immunized with PBS, and challenged 4 months later, were analyzed for T cell responses. The clinical and serological results of this study have been described previously [1]. The extent of viral replication was assessed by measuring RNA levels in the BALF by RT-PCR, as reported previously [1]. Briefly, viral RNA was detected in the BALF of PBS-immunized animals in 4/6 animals at day 4 p.i. and in all 6/6 animals at day 7 p.i. [1]. No viral RNA was detected in the BALF samples from the immunized animals at either day 4 or 7 p.i., with the exception of a weak PCR signal in one L-bRSV immunized animal at day 7 [1].Thus, both vaccines led to reduced levels of viral RNA in the BALF, but only the L-bRSV vaccine provided partial protection against symptoms. Immunization with FI-bRSV primed animals for immunopathogenesis. Thus, this experimental setting allowed a comparison of primary bRSV infection with two modes of secondary infection, i.e., semi-protective (L-bRSV) and immunopathogenic (FI-bRSV).

BALF samples were collected at 3 days before challenge and at days 1, 4, 7 and 9 p.i., and were first used to visualize the kinetics of CD4 and CD8 T cell influx into the lungs. During primary infection, i.e., in PBS-immunized animals, we found that the dynamics of T cell influx were consistent with the data shown in Fig. 1: infiltrating T lymphocytes were first detectable at day 7 p.i. (Fig. 2), and were present at slightly increased levels at day 9 after bRSV infection. Post-challenge CD8 T cell responses in FI-bRSV immunized animals were very similar in kinetics but smaller in magnitude (Fig.2a). In contrast, we observed accelerated post-challenge responses in L-bRSV immunized animals, with the response peaking at day 7 post challenge instead of day 9 (Fig.2a). The CD8 T cell responses in L-bRSV immunized animals at day 7 were significantly different from those in PBS- or FI-bRSV immunized animals ($P = 0.041$ and $P = 0.004$, respectively). The largest responses were observed in the PBS-immunized group (i.e., the animals undergoing primary infection) at day 9. These responses differed significantly from the number of CD8 T cells in L-bRSV-immunized animals ($P = 0.026$) and there was a trend towards significance when compared with FI-bRSV-immunized animals ($P = 0.065$). A similar trend was seen for the numbers of CD4 T cells in the BALF (Fig.2b). Responses in L-bRSV-immunized animals appeared to peak earlier, at day 7, and this was significantly different from the response in FI-bRSV-immunized animals ($P = 0.04$). The differences between numbers of CD4 T cells at day 9 were not significant.

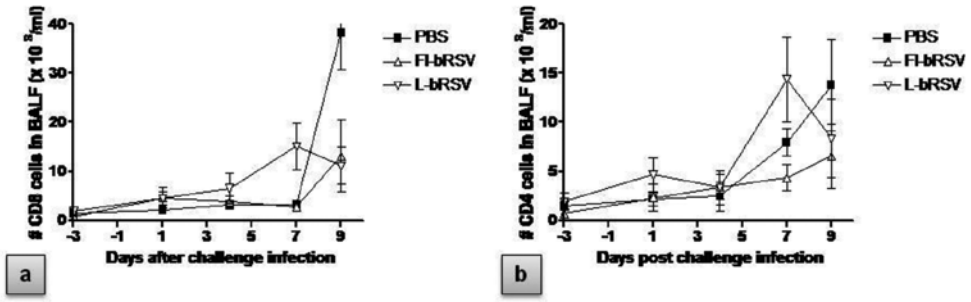

Figure 2. Total numbers of $CD8^+$ (a) and $CD4^+$ (b) cells in the BALF, at 3 days before infection and days 1, 4, 7 and 9 post challenge infection. Cell numbers were calculated using the total numbers of cells recovered from BALF [1] and the frequencies of $CD4^+$ and $CD8^+$ cells determined by flow cytometry, using the ungated population of cells. Error bars indicate the standard error of the mean as determined using Graphpad Prism software. Significance was determined using the -Mann-Whitney and Kruskal-Wallis tests (InStat3 software).

We then tested whether the CD8 T cell populations in the three different groups displayed the same proliferation characteristics, by staining for the Ki-67 antigen. Based on the results shown in Figure 1, we analyzed day 7 BALF samples for Ki-67 expression. Again, we found only minor differences between the three groups (Fig.3), and these were not significant ($P > 0.05$ for all comparisons). In all animals, we found that high numbers of the CD8 cells in the BALF stained positive for Ki-67+, indicating a high level of proliferation (Fig.3).

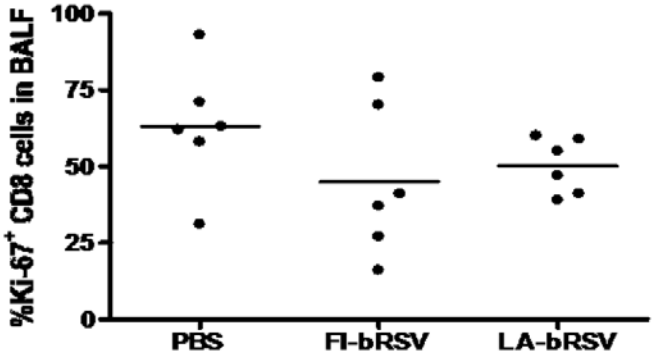

Figure 3. Proliferation of $CD8^+$ cells in the lungs at day 7 post infection. Percentages Ki-67^+ cells of total CD8+ cells are indicated for all animals. Vaccination history and average percentages Ki-67^+ cells for each group are indicated. Significance was determined using the Wilcoxon-Mann-Whitney test (InStat3 software). No significant differences were observed. PBS versus FI-bRSV, P = 0.3; PBS versus L-bRSV, P = 0.13; FI-bRSV versus L-bRSV, P = 0.5).

Antiviral peripheral and pulmonary CD8 T cell responses

Although staining for the Ki-67 antigen shows the extent of T cell proliferation, it does not reveal functionality or antigenic specificity. To assess the bRSV-specificity of the T lymphocytes infiltrating the lungs, we stimulated BALF cells with an MVA-recombinant expressing the viral F protein. As shown by Nixon and coworkers, infection of human or simian lymphocyte preparations with vaccinia virus recombinants results in effective stimulation of CD8 T cells, after which responses can be quantitated by IFN-γ ELISPOT or by intracellular cytokine staining [33,34]. Gaddum et al. recently validated this approach for bRSV-specific memory T cell analysis [35].

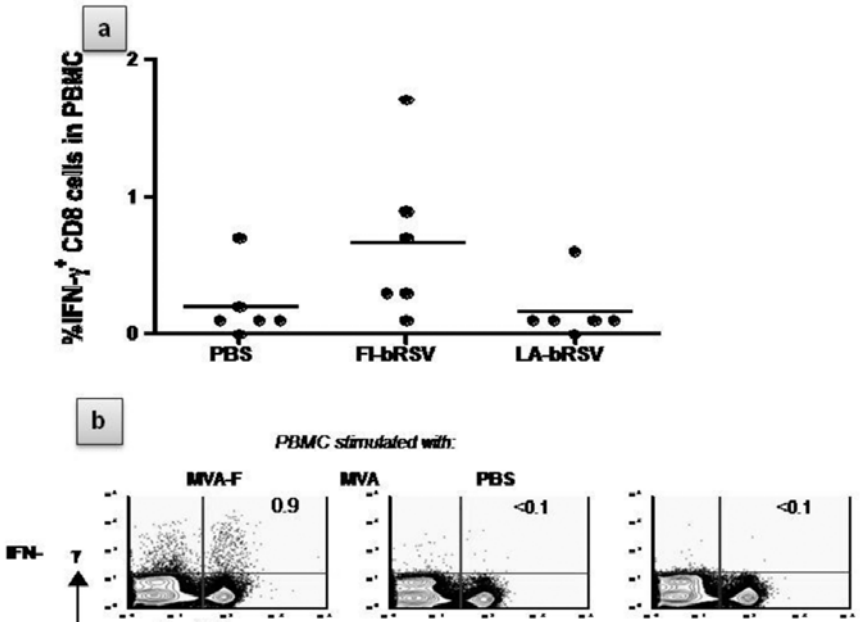

Figure 4. (a) Peripheral IFN-Y+ CD8 T cell responses during acute bRSV infection in all animals, after in vitro stimulation with MVA-F. Stimulation with wtMVA yielded responses <0.1%. Vaccination history and average percentages IFN-Y+ cells for each group are shown; (b) PBMC from animal #48 (vaccinated with FI-bRSV, as indicated) were stimulated with MVA-F, MVA or PBS, and stained for IFN-Y expression. Frequencies are expressed as percentages IFN-Y+ CD8+ cells of total CD8+ cells. Significance was determined using the Wilcoxon–Mann–Whitney test (InStat3 software).

To assess the specificity of antiviral responses, we analyzed F-specific CD8 T cell responses before and after vaccination and after the challenge in the peripheral blood. Pre-challenge and post-challenge responses were also analyzed in BALF samples. Lymphocytes were isolated from heparinized blood before vaccination, on day 14 post vaccination, on day 3 before challenge infection and on days 1, 4, 7 and 9 post infection. Lymphocytes were also isolated from BALF samples on day 3 before infection and on days 1, 4, 7 and 9 post infection. In all cases, lymphocyte samples were infected with the MVA-F recombinant, or, as a control, with wild type MVA virus. Uninfected lymphocytes were used as an additional specificity control. Cells were

stained with monoclonal antibodies against CD8 and IFN-γ. We found no F-specific CD8 T cell responses in the periphery before challenge and at days 1, 4 and 7 post challenge (data not shown). However, F-specific responses were detected in PBMC in 5 out of 18 animals at day 9 p.i., (Fig.4a). Interestingly, three of these five animals had received the FI-bRSV vaccine (Fig.4). This could suggest that somewhat stronger F-specific IFN-γ+ responses existed in the six FI-bRSV immunized animals. However, the differences were not significant ($P > 0.05$ for all comparisons). Thus, in 10 out of 12 other animals, no or very weak IFN-γ-producing F-specific CD8 T cells were detected in PBMC samples, indicating that there was little systemic involvement in these animals. In the BALF samples, we found IFN-γ+ responses in most of the animals at day 7 p.i. (Fig.5a), and no significant differences were observed between the groups ($P > 0.05$ for all comparisons). No responses were measured before challenge and at days 1 and 4 p.i. (not shown). Note that these samples did not contain CD8 T cells (Fig.2). Unfortunately however, as illustrated in Fig.5b, the responses in BALF samples measured at day 7 p.i. were observed not only after stimulation with the MVA-F recombinant but also after stimulation with wild type, non-recombinant MVA virus (although the percentage of IFN-γ+ CD8 cells was ~40% lower). No such background responses were measured in unstimulated cells (Fig.5b), or in MVA-stimulated PMBC or lymph node cells (Figs.4 and 6).

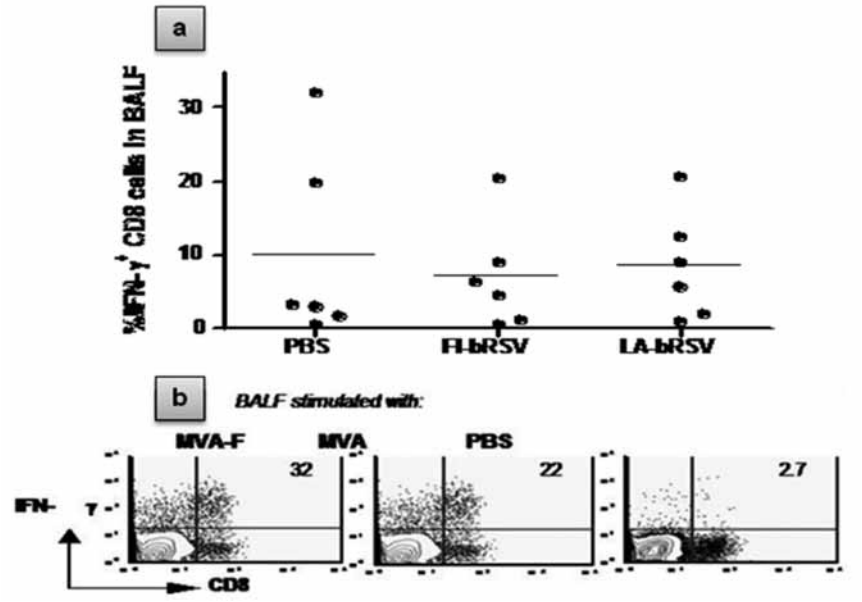

Figure 5. (a) IFN-γ responses in day 7 BALF from all animals, after in vitro stimulation with MVA-F. Stimulation with wt MVA yielded responses of ~60% of MVA-F induced responses. Vaccination history and average percentages IFN-γ^+ cells for each group are shown. (b). BALF from animal #43 (vaccinated with PBS) were stimulated with MVA-F, control MVA or PBS. Frequencies are expressed as percentages IFN- γ^+ $CD8^+$ cells of total $CD8^+$ cells. Significance was determined using the Wilcoxon-Mann-Whitney test (InStat3 software).

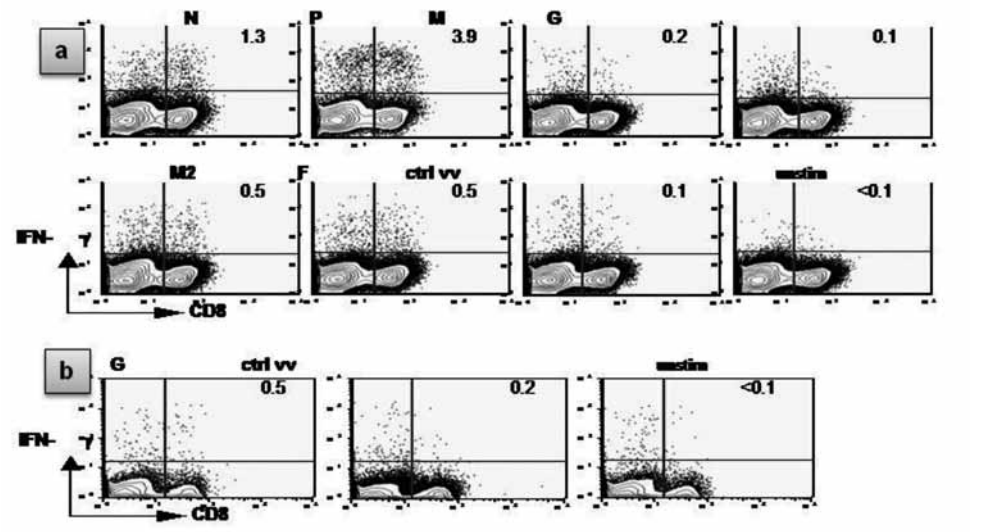

Figure 6. Specificity and breadth of the response in animal #47 (vaccinated with FI-bRSV) (a) and illustration of G-specific responses in animal #42 (vaccinated with PBS) (b). Lymphocytes from the draining lymph nodes from animals #47 (tracheobronchial lymph nodes) and #42 (mediastinal lymph nodes) were stimulated with recombinant viruses and stained for IFN-γ expression. The bRSV or control genes expressed by the recombinant viruses are indicated. Frequencies are expressed as percentages IFN-γ^+ $CD8^+$ cells of total $CD8^+$ cells.

Table 1. *% IFN-γ^+ CD8 cells in draining lymph nodes after stimulation with recombinant vaccinia viruses*

	PBS immunized animals						FI-bRSV immunized animals						L-bRSV	
Stimulation	38	39	40	41	42	43	44	45	46	47	48	49	50	51
N	**1.2**[1]	0.2	0.1	0.1	0.3	0.1	0.2	**0.7**	**0.7**	**1.3**	**1.1**	**1.2**	0.1	0.1
P	**0.9**	0.3	0.1	0.2	**1.1**	0.1	0.1	0.2	0.1	**3.9**	0.2	**0.6**	0.1	0.1
M	0.4	0.1	0.2	0.2	0.2	0.3	0.1	**0.7**	0.1	0.2	0.2	**1.2**	0.2	0.2
G	**1.4**	0.1	0.2	**0.5**	**0.5**	0.1	**0.6**	**0.4**	0.1	0.1	**0.6**	0.4	0.2	0.1
M2	**0.8**	**0.7**	0.1	**0.7**	**0.9**	0.1	0.3	0.3	0.2	**0.5**	**0.6**	**0.9**	0.4	0.2
F	0.5	**1.2**	0.2	0.1	**0.9**	0.1	**0.7**	**0.4**	0.2	**0.5**	0.4	0.4	**0.5**	0.3
ctrl vv	0.5	0.3	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
unstim	0.3	0.4	0.2	0.2	0.1	0.1	0.2	0.1	0.1	0.3	0.3	0.3	0.3	0.2

-γ+ CD8 cells responding to vaccinia viruses expressing bRSV genes (N, P, M, G, M2, F). 'Ctrl vv' indicates stimulation w ith a control vaccinia virus expressing T7 polymerase. 'Unstim' indicates unstimulated lymphocytes. Shaded boxes indicate where responses above background levels have been measured. 'Above background' was operationally defined as average of all ctrl vv values (0.16) + 2 x SD (2x0.12=0.24), leading to a cut-off of 0.4.

Antiviral CD8 T lymphocytes recognize multiple bRSV proteins

To further determine the antigenic specificity of the response, we measured antiviral responses in the draining lymph nodes using intracellular IFN-γ staining. In these experiments, we focused on the breadth of the CD8 T cell response and the potential differences in specificity between the groups, focusing on the difference between the mock-immunized and the FI-bRSV-immunized groups. To identify the relevant antigens, we constructed a set of vaccinia virus recombinants expressing the viral N, P, M, G and M2 proteins, in addition to the MVA-F recombinant. Lymphocytes were isolated from the tracheobronchial and mediastinal lymph nodes at day 10 after infection and were stimulated by infection with the recombinant viruses expressing the different bRSV proteins, including the MVA-F recombinant. After overnight incubation, cells were permeabilized and stained with monoclonal antibodies against CD8 and IFN-γ. Within the group of 14 bRSV-infected animals that we analyzed, we found responses against all proteins tested (Table 1). The results showed that whereas similar numbers of PBS immunized and FI-bRSV immunized calves recognized the P, M, G, M2 and F proteins, there was a bias towards N protein recognition in FI-bRSV immunized calves compared with PBS controls. Thus, 5 out of 6 FI-bRSV immunized calves recognized the N protein compared with only 1 out of 6 PBS immunized calves ($P = 0.065$). A representative animal from the FI-bRSV immunized group (#47) with strong bRSV-specific responses (including N) is shown in Figure 6a. It is interesting that CD8 T cell responses against the G protein were observed in several animals (Table 1 and Figure 6b). From these experiments we conclude that immunization did not result in a strong specificity bias towards individual viral proteins, with the possible exception of the N protein, and that most viral proteins are potential targets for CD8 T cells.

Discussion

In the present study, we have analyzed antiviral CD8 T cell responses in a symptomatic bRSV infection model [1]. In particular, we have compared T cell responses in infected calves undergoing primary infection and in calves undergoing secondary infection after FI-bRSV or L-bRSV immunization. Both immunizations result in reduced viral replication, but only the L-bRSV vaccine provides partial protection against symptoms [1]. FI-bRSV vaccination primes for enhanced pathogenesis that is associated with eosinophilia and increased IgE titers. Recent data indicate that these IgE antibodies are specific for the bRSV F protein (Antonis et al., unpublished observations). Antiviral T cell responses were quantitated by measuring (i) the kinetics of T cell influx and proliferation status in the lungs, and (ii) the specificity and frequencies of IFN-γ producing CD8 T cells in the lungs, draining lymph nodes and periphery. IFN-γ producing cells were visualized by stimulating lymphocytes using bRSV antigens produced by recombinant vaccinia or recombinant MVA vectors. Since it is possible that not all bRSV-specific CD8 T cells respond to recombinant vaccinia-produced antigens by IFN-γ production [36], we cannot exclude that our assay underestimates the true magnitude of the response. Furthermore, a particular technical problem that we encountered with our

intracellular IFN-γ staining assay was that stimulation of BALF samples with the control MVA virus yielded a strong background response. These results are consistent with a recently published study by Sandbulte et al.[37] and, as suggested by these authors, could reflect superantigenic properties of MVA, or stimulation of innate pathways. An alternative explanation is that the CD8 T cells in the lung have a low activation threshold, with the result that viral (i.e. MVA) replication could trigger IFN-γ production. It is obvious that this complicates the interpretation of these data: although it is clear that the BALF samples from infected animals contain considerable numbers of CD8 T cells that can be activated to produce IFN-γ (and therefore display a Th-1-like phenotype), their specificity cannot be determined in detail using this method.

Our study makes three points. First, we found potent virus-induced pulmonary CD8 T cell responses in all three experimental groups, although the magnitudes, expressed as total numbers of T cells in the BALF, displayed different kinetics. Accelerated responses, indicative of the recruitment of memory T cells, were observed in the L-bRSV-immunized group, but not in the FI-bRSV-immunized group, as compared to the PBS group. The largest responses, in terms of the numbers of T cells in the BALF, were seen in PBS-immunized calves. The levels of IFN-γ+ T cells were similar in the three groups, although IFN-γ+ CD8 T responses were slightly higher and more focused to the N protein in the FI-bRSV group. Because recalled memory T cell responses in the immunized animals should result in an accelerated recruitment of antiviral T cells as compared to naïve animals, our data suggest that the L-bRSV vaccine has the capacity to prime bRSV-specific memory cells. In contrast, no such accelerated recruitment was observed for the FI-bRSV immunized group, leading to the conclusion that this vaccine did not prime very high levels of long-term T cell memory. It cannot be excluded that the FI-bRSV vaccine induced a weak N-specific memory response. The conclusion that FI-bRSV vaccination fails to prime CD8 T cell responses conclusion is supported by recent work from Woolums et al. [38]. In contrast, West and coworkers reported that vaccination with modified-live bRSV resulted in somewhat accelerated antiviral CTL responses post bRSV challenge, suggesting that the modified-live vaccine did prime CD8 memory in calves [39], similar to our data. The greater magnitude of the T cell response (in terms of numbers of T cells in the BALF) in PBS-immunized animals compared to vaccinated animals could be explained by different levels of antigen: both vaccines were very efficient in reducing viral replication [1]. Second, Th-1 (IFN-γ+) and Th-2 (IgE) [1] associated responses co-exist in FI-bRSV immunized and bRSV-challenged animals. BRSV-specific T cells were readily identified by IFN-γ staining. In a separate study, we analyzed production of the Th-2 cytokine IL-4 by bRSV-induced T cells, but we did not find any IL-4 secreting T cells after in vitro stimulation with bRSV

antigens (Antonis and van der Most, unpublished data). We are currently developing IFN-γ, IL-4 and IL-5 Q-PCR assays to better assess the Th-2 bias of the response. Combined, the data suggests that IFN-γ+ pulmonary CD8 T cell responses do not necessarily protect against severe symptoms (which were only observed in the FI-bRSV group) [1]. A similar Th1/Th2 co-existence has been demonstrated in RSV-infected BALB/c mice, in which the pathogenic Vβ14+ population of G-specific CD4 T cells is characterized by the simultaneous production of the both IFN-γ and IL-5 [23]. Third, the antigenic targets recognized by the antiviral CD8 T cells in the draining lymph nodes are diverse: the N, P, M, M2, F and G proteins are all recognized in different animals, indicating that immunization did not result in a strong specificity bias, with the possible exception of N, which is more often recognized in FI-bRSV immunized animals. Salient detail is the detection of CD8 T cell responses specific for the G protein in several animals, especially since a lack of G-specific CD8 T cell responses in RSV-infected BALB/c mice has been proposed as the key factor in enhanced pathogenesis [20]. Clearly this is not the case in bRSV-infected calves, since even FI-bRSV immunized animals with severe symptoms harbored anti-G CD8 T cell responses (see animals 44 and 48). Consistent with our data, showing a broad response, Gaddum et al. recently identified the N, F and M2 proteins as antigenic targets of memory CD8 T cell responses in bRSV-infected MHC-I homozygous animals [35].

Our previous study revealed that vaccination with either the FI-bRSV or L-bRSV vaccines resulted in reduced levels of viral RNA in the lungs [1]. Assuming that the FI-bRSV vaccine is indeed a poor inducer of long-lived CD8 memory responses, it appears that the main protective component of this vaccine is the level of antibodies. The efficacy of the L-bRSV vaccine, which reduces viral replication in the absence of immunopathology, could perhaps be explained by its capacity to induce bRSV-specific memory T cells. If this is indeed the case, then it seems plausible that RSV vaccination strategies can be optimized by further enhancing the capacity of such vaccines to induce CD8 T cell memory.

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Chapter 4

Bovine Respiratory Syncytial Virus (bRSV) related immunopathology in calves primed by vaccination is associated with a bRSV specific IgE response

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Bovine Respiratory Syncytial Virus (bRSV) related immunopathology in calves primed by vaccination is associated with a bRSV specific IgE response

Abstract

Severe respiratory syncytial virus (RSV) disease is associated with an imbalanced immune response rather than massive viral replication. This Th2-biased immune-response is characterized by an enhanced release of specific soluble mediators, such as immunoglobulin E (IgE), and cellular infiltration (eosinophils) into the lower respiratory tract. Enhanced disease after natural infection can be primed by vaccination with inactivated virus, and has been modelled in bovine RSV (bRSV) infected calves. To study the role of IgE in bRSV disease enhancement we have developed a bRSV specific IgE capture ELISA and used this test to demonstrate in our natural host model that calves vaccinated with alum absorbed formalin inactivated bovine RSV (FI-bRSV) antigens, responded with bRSV-specific IgE production. These vaccinated calves developed an exaggerated airway hypersensitivity response after challenge infection. Thus, calves were primed for an IgE mediated, Th2-biased, immune response by vaccination, presumably generating Th2-biased memory. Upon challenge infection, calves developed an IgE booster response. The authenticity of the bRSV natural host model implies that these results can be interpreted without the bias of a heterologous animal model. Thus, the bRSV-specific IgE ELISA reported herein can be used to evaluate vaccine safety.

Introduction

Human and bovine respiratory syncytial viruses (hRSV and bRSV) are closely related members of the Pneumovirus genus (Paramyxoviridae family). RSV infections in humans (predominantly children and elderly) and cattle are associated with upper and lower respiratory tract diseases (URTD and LRTD). Clinical signs of URTD infections are predominantly limited to rhinitis, conjunctivitis and a mild fever, whereas LRTD symptoms include bronchitis, bronchiolitis, pneumonia and high fever. Children and calves infected with their respective viruses have remarkably similar dichotomy of clinical signs. It is increasingly appreciated that severe forms of LRTD after RSV infection result from an imbalanced antiviral immune response rather than from massive viral replication in the lungs.

A particular problem with RSV vaccine development is the altered, exaggerated clinical response seen in RSV-infected infants in the 1960s after vaccination with formalin-inactivated (FI) -hRSV vaccine. A similar pattern of enhanced disease was observed in the 1970"s in vaccinated calves. We recently described a model of

vaccine-induced immunopathology in bRSV-infected cattle [1] in which calves immunized with formalin-inactivated (FI) virus presented with far more serious (enhanced) respiratory disease after bRSV challenge compared to their non-vaccinated controls. Starting two days after the challenge infection, calves became dyspnoeic and started wheezing. Moreover, respiratory rates increased up to >100 per minute. Similar to previously reported data by Jolly et al, these primed calves developed a paroxystic respiratory distress syndrome [2]. Airway hypersensitivity reactions (AHR) involve a diversity of cell types and a multitude of chemical mediators that are associated with clinical symptoms. Numerous studies have revealed that an orchestrated continuum of cellular activities lead to these airway allergic inflammations in predisposed individuals. This sensitization step likely depends on the development of a Th2-biased immune response, mediated by Th2 cytokines. Among Th2-derived cytokines, IL-5 and IL-13 stimulate an eosinophilic response and IL-4 potently enhances of the production of IgE antibodies [3,4]. IgE (or IgE-antigen complexes) binds to the high affinity Fcε receptor (FcεRI) on airway mast cells, which sets the stage for an acute inflammatory response on subsequent antigen exposure [5-7], and to the low affinity Fcε receptor (FcεRII) on B cells (prior to isotype switching), monocytes, eosinophils and platelets. IgE binding to the FcεRI triggers the release of mediators, such as histamine and tryptase, which are associated with the production of IgE-dependent allergic reactions. These mast cell-derived mediators collectively produce acute-phase clinical symptoms by enhancing vascular leakage and venoconstriction, resulting in pulmonary oedema and spasm of the bronchi and bronchioles [8,9]. Simultaneously, some mast cell mediators up-regulate expression of adhesion molecules on endothelial cells, facilitating interactions with eosinophils, basophils and lymphocytes. This is one of the key elements in the late-phase allergic response [3]. There is strong evidence that this response pattern is associated with enhanced disease after RSV infections. Autopsies and histological examination of the lungs performed on the fatal cases of the FI-hRSV vaccine tragedy, revealed peribronchiolar infiltration and increased levels of eosinophils [10]. In our experiments with vaccinated and challenged calves we observed a remarkable influx of eosinophils into the bronchoalveolar lung (BAL) fluids, a marked eosinophilic tracheobronchitis and bronchiolitis characterized by a diffuse infiltration of eosinophils in the submucosa and in the interstitium surrounding the bronchi and bronchioles [1].

The concept that antiviral IgE might be involved in the pathogenesis of RSV disease has received support from studies in which free RSV specific IgE was measured in secretions of infants during convalescence [11,12]. Welliver and colleagues reported RSV specific IgE antibodies, as well as high concentrations of histamine, in nasopharyngeal secretions of children suffering from the most severe form of the disease. In contrast, children with only URTD did not have detectable IgE responses to the virus in their nasopharyngal secretions [12]. Similar findings have been reported in calves [4,13]. In experimentally infected calves, severity of disease correlated with the level of bRSV specific IgE suggesting that a hypersensitivity response plays an important role in the bRSV pathogenesis. Indeed, RSV infections in general are associated with strong T helper (Th)2 responses [14-19], that seem to co-exist with potent Th1 responses [20,21].

Experiences with the FI-RSV vaccines in both humans [22] and cattle [23,24], showed that these predilections can be further driven to the Th2 side of the response. These observations make RSV an unusual, perhaps even unique, viral agent, in that it triggers an immune response that resembles an allergic reaction or immune responses against parasitic infections [25,26]. The central role of IgE in this pathogenesis model warrants further study of the kinetics of the IgE response after vaccination and challenge. To address this issue, we have developed a bRSV-specific IgE ELISA, which has allowed us to detect bRSV-specific IgE antibodies in the serum. Our data also establish a link between post-challenge serum IgE levels and severity of disease. Combined, our data imply that the vaccine itself primes the Th2 response, and that this response is then amplified by the subsequent viral infection.

Materials & methods

Experimental design

Two vaccination/challenge experiments, conducted within the context of an EU-funded project to study RSV immunopathogenesis and develop novel vaccines, were performed in 2001 and 2003. In total, forty-two caesarean derived and colostrum deprived (CD-CD) calves were obtained and tested negative for blood-borne Bovine Viral Diarrhoea Virus (BVDV) infections by ELISA (SERELISA BVD/MD/BD Ag; SYNBIOTICS EUROPE) and for serum antibodies directed against BVDV [27] and bRSV [28]. The calves were vaccinated twice, see Table 1, intramuscularly with a three-week interval and were challenged with bRSV strain Odijk [1,29]. Blood samples for serology were collected weekly until four days before bRSV challenge, and were collected daily after challenge. All animal experiments were conducted in accordance with the Act on Experimental Animals in The Netherlands regulated by the Ethical Review Committee of the Animal Sciences Group (ID-Lelystad).

Table 1 Study design animal experiment

Group	*Experiment*	*Test Item*	*N=*	*Test Item Administration (age in weeks)*		*(challenge) infection (age in weeks)*
				First	*Second*	
1	I	PBS	6	6	9	27
2	I	FI-bRSV	6	6*	9*	27
3	I	LA-bRSV	6	6	9	27
4	II	PBS	6	4	7	23
5	II	FI-bRSV	6	4*	7*#	23
6	II	MVA-F	6	4	7	23
7	II	MVA-F&G	6	4	7	23

* Vaccine batch produced in 2001 (Formalin treatment before ultra centrifugation)
# Vaccine batch produced in 2003 (ultracentrifugation before formalin treatment)

Vaccine preparation

Two vaccine batches were prepared and were used as described [1,30]. Briefly, bRSV was cultured in embryonic bovine trachea (EBTr) cells. After clarification (15 minutes, 1,000 x g), the supernatant was formalin treated (37% formaldehyde solution, 1:4,000 at 37°C for 72 hours), followed by ultracentrifugation (Beckman

SW28, 110,000 x g for 60 minutes at 4°C). A second vaccine batch was produced, and used for the second vaccination in the second animal experiment, in which formalin treatment was done after ultracentrifugation. Inactivated vaccine-materials were further diluted in PBS to a final protein concentration of 0.75 mg/ml and mixed (1:1) with 2% $Al(OH)_3$.

To detect IgE in cattle we used a sandwich ELISA for the detection of total IgE. An antibody capture ELISA was used to detect bRSV specific IgE antibodies.

Total IgE ELISA

To detect total IgE in serum we used an indirect double antibody sandwich (IDAS) ELISA previously described by Kooyman et al. [31]. Briefly, microtitre plates were coated for 2 hours (h) at room temperature (RT) with, protein G purified, anti-sheep-IgE (IE7). Coating was stabilized with Stabilcoat®, dried and stored at 4°C until use. Sera were decomplementized (30 minutes at 56°C) and diluted in phosphate buffered saline (PBS), pH 7.3, supplemented with 0.1% gelatine and 0.05% Tween 80 (PBS-GT) and incubated for one hour at RT. Bound IgE was detected with a polyclonal protein G purified rabbit anti-bovine-IgE with horseradish peroxidase (HRPO) goat anti-rabbit immunoglobulin as conjugate. The conjugate was diluted in PBS-GT with 10% Foetal Bovine Serum (FBS) and incubated for 1 hour at RT. Subsequently, the plates were incubated with a chromogen/substrate solution (tetra-methyl-benzidine –TMB-) for 10-15 minutes at RT. Colour development was stopped by adding 0.5M H2SO4. The optical density (OD) at 450 nm was measured by an ELISA reader. After each incubation step, plates were rinsed twice five times with deionised water containing 0.05% Tween80. To evaluate our modified assay [31], we tested 558 IgE negative serum samples. Analysis of the mean percentages positivity (PP) values of these negative samples, revealed that the observed specificity of the total-IgE ELISA was 92.1% at a cut-off value of 10 PP, 99.1% at a cut-off value of 20 PP and 100% at a cut-off value of 30 PP. Based on these results, we set the cut-off value at 20 PP (99.1% specificity). To assess the sensitivity of our IDAS assay, we used sera from calves that had been infected with *Dictyocaulus viviparus* (lungworm), as these should be IgE positive after infection. When comparing the results with our modified ELISA with the original ELISA, using a set cut-off-value of 15PP, we concluded that the sensitivity of the assay was improved (data not shown). To evaluate the reproducibility of the IgE ELISA, reference serum samples (n=4) were included in duplicate in each test run on each plate (n=11), and eleven positive (n=3) and negative (n=8) sera were tested in replicates of eight on three different days. Based upon the coefficients of variations (CV) for repeatability, the intra-plate and inter-plate variation were evaluated. The intra-plate variation of the four reference sera varied from 10-18% and the inter-plate repeatability varied from 1.7 to 4.1% (Data not shown). All CV percentages were considered to be acceptable at this stage of assay development.

IgE purification from serum by affinity chromatography

In order to confirm the presence of bRSV-specific IgE, IgE was batch-wise purified from 6 positive serum samples by affinity chromatography and then further analyzed.

A monoclonal mouse anti-sheep IgE, cross-reactive with bovine IgE, was coupled to a solid matrix (CNBr-activated Sepharose 4B, Amersham Biosciences) following the instructions of the manufacturer. The Sepharose-anti IgE suspension in PBS was aliquoted into several 15ml tubes and the six serum samples were incubated overnight at 4ºC on a roller bank: approx. 3ml swollen gel (Sepharose-anti IgE in PBS) was incubated with 1ml serum. Flow-through fraction (FTF) was collected. After centrifugation (5 min.100xg at RT), the supernatants were collected. Gels were washed with aliquots of 1ml PBS until the supernatants had an OD_{280} lower than 0.1. Bound IgE antibodies were then eluted. Bound IgE was eluted with PBS containing 1M NaCl, several times until the OD at 280nm was < 0.1. Eluates with the highest OD were dialyzed against PBS before testing. Sepharose-anti IgE gel was re-equilibrated by washing with PBS. Sera, FTF with the highest absorbance (see Table 2), and IgE eluates were further analyzed in the bRSV-antibody ELISA (Ceditest® BRSV, CediDiagnostics B.V. Lelystad, The Netherlands) and later in the bRSV-specific IgE ELISA. Percentages positivity, in the bRSV-antibody ELISA were significantly (p=0.0411) higher in the late post-challenge sera as compared to the pre-vaccination sera. No bRSV-specific IgE was detected in the pre-vaccination sera, but in all late post-challenge sera bRSV-specific IgE was detected. These results were confirmed in the bRSV-specific IgE ELISA.

Table 2 Results of the total IgE ELISA

Percentages positivity after FI-bRSV vaccination (weeks post vaccination)												
	First animal experiment Calves vaccinated at 6 and 9 weeks age, day 0 and 21 respectively						Second animal experiment Calves vaccinated at 4 and 7 weeks age, day 0 and 21 respectively					
Week	**5544**	**5545**	**5546**	**5547**	**5548**	**5549**	**6920**	**6921**	**6923**	**6924**	**6925**	**6926**
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	**36**
2	**25**	**27**	**26**	**97**	**24**	**21**	-	**24**	**29**	**27**	**43**	**69**
3	-	**30**	**20**	-	-	**32**	-	-	-	-	**32**	**22**
4	**81**	**78**	**32**	**23**	-	**86**	-	-	-	-	**58**	**31**
5	**28**	**28**	-	-	-	**86**	-	-	-	**72**	**46**	**36**
6	-	-	-	-	-	**24**	-	-	-	**54**	**31**	**22**
7	-	-	-	-	-	**23**	-	-	-	**41**	-	-
8	-	-	-	-	-	-	-	-	-	**28**	-	-
	All calves were tested negative from week 8 till day of challenge infection						All calves were tested negative from week 8 till day of challenge infection					
Percentages positivity after challenge infection (days post challenge infection)												
	First animal experiment Calves were challenged when they were 27 weeks old on day 0 (day of challenge infection)						Second animal experiment Calves were challenged when they were 23 weeks old on day 0 (day of challenge infection)					
Day	**5544**	**5545**	**5546**	**5547**	**5548**	**5549**	**6920**	**6921**	**6923**	**6924**	**6925**	**6926**
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	**20**	-	-	-	-	-	-	-	-	-
6	Na	Na	Na	Na	Na	Na	-	-	-	-	-	-
7	**34**	-	**87**	-	-	**52**	-	-	-	-	-	-
8	**55**	**95**	**140**	**40**	-	**143**	-	-	-	-	-	-
9	**64**	**79**	**143**	**66**	**34**	**100**	-	-	-	**34**	-	-
10	†	†	†	†	†	†	-	-	-	**76**	**34**	-

Results of the IgE ELISA: negative (-) means percentage positivity is <20%. In case the percentage positivity was ≥20%, the actual PP value is expressed. Na means not available. In life phases ended at day 9 and 10 in respectively the first and second animal experiment. Calves were vaccinated twice with a three-weeks-interval. Blood samples for serology were collected on a weekly base till the day of challenge infection. After challenge infection (day 0), blood samples were collected on a daily base.

BRSV-specific IgE ELISA

The presence of virus-specific IgE antibodies in post-vaccination sera could be an important indicator for vaccine safety. However, IgE purification by affinity chromatography is too laborious for routine use. An isotype-specific ELISA would be less laborious, more sensitive, easier to standardize and a useful tool to evaluate the safety of a candidate vaccine. An inherent problem in isotype-specific ELISA is inter- and intra-isotype competition. Inter-isotype competition may occur when anti-Ig isotype-specific reagents are used as conjugate, whereas intra-isotype competition may occur when anti-Ig isotype-specific reagents are used as the capturing antibodies. We developed an antibody capture assay (ACA), based on the conclusion of Van Zaane and IJzerman that inter-isotype competition does not occur in the ACA [32], to detect bRSV-specific IgE in serum, nasal fluid and bronchoalveolar lung fluid samples. Briefly, microtiter plates were coated overnight at RT with saturated ammonium sulphate (SAS) precipitated monoclonal antibody IE7. Sera were diluted 1:2 in buffer that constituted of 0.35M NaCl, 1mM EDTA and 0.05% Tween80 in phosphate buffered saline and was supplemented with 4% horse serum. Samples were incubated for two hours at RT. In the subsequent incubation (overnight at RT) a 1:5 dilution of bRSV in low salt buffer diluted NP40-treated bRSV (strain Lelystad) was added. Next day, HRPO conjugated anti bRSV-F proteins were diluted in low-salt buffer and are incubated for one hour at RT. Incubation with chromogen/substrate, colour development interruption was done as described for the total IgE determination. Evaluating the mean percentages positivity of negative sera (n=173) the cut-off value for negativity was set at 5PP (specificity 100%).

Reading of the IgE ELISAs

To account for inter-assay variables [33], the raw data (OD values) were converted to percentage positivity (PP) values. For this purpose, a positive reference serum was selected to supply the maximum OD signal (100 PP) and a negative reference serum, to supply the background signal. The PP value for tested sample was calculated according to the following formula:

$$PP = (OD_{sample} - OD_{negative\ reference}) / (OD_{positive\ reference} - OD_{negative\ reference}) \times 100\%.$$

The positive (bRSV-specific IgE) reference serum originated from a calf (#5549) vaccinated with formalin inactivated bRSV [1]. The OD_{450} of this positive serum constituted the 100 PP level ($OD_{positive\ reference}$) of both assays. The negative reference serum used in the total IgE ELISA originated from a batch of commercially available foetal bovine serum. The negative reference serum used in the bRSV-specific IgE ELISA originated from calf #5549, collected nine days prior to its first vaccination.

Results

We measured serum IgE levels after vaccination in samples collected in two studies. Details of these studies are shown in Table 1. Serum samples collected from mock immunized calves, from calves vaccinated with live-attenuated bRSV and from calves vaccinated with recombinant MVA vaccines (expressing either the bRSV-F or bRSV-F and bRSV-G proteins) were tested negative for IgE in total IgE assay (data not shown). However, IgE antibodies were detected in calves that had received two intramuscular vaccinations with a formalin-inactivated and alum adsorbed bRSV vaccine (Figure 1a). A rapid increase in IgE was observed from the second week after the first vaccination (Table 2). Marked biphasic IgE responses were observed in most calves. After the second immunization on day 21, a booster effect (seroconvalescence) was observed in some (8 out of 12) calves. IgE was detectable in serum samples of these calves for 1 to 7 (mean 3.7) weeks. Thereafter, all calves were tested IgE negative for at least ten subsequent weeks before challenge infection.

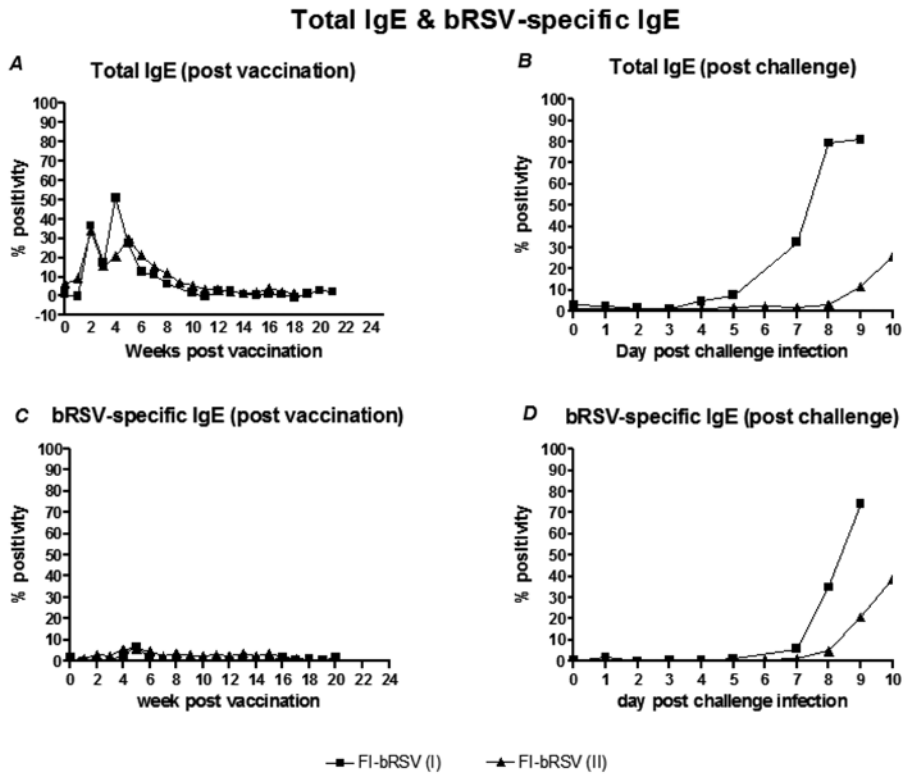

Figure 1 Mean percentages positivity (y axis) in the Total IgE ELISA (A and B) and the bRSV-specific IgE ELISA (C and D), of calves immunized with a formalin-inactivated vaccine in two different animal experiments (I and II), for the time post vaccination untill the challenge (A and C) and after challenge infection (B and D).

Table 2 Results of the bRSV-specific IgE ELISA

	Percentages positivity after FI-bRSV vaccination (weeks post vaccination)											
	First animal experiment Calves vaccinated at 6 and 9 weeks age, day 0 and 21 respectively						Second animal experiment Calves vaccinated at 4 and 7 weeks age, day 0 and 21 respectively					
Week	5544	5545	5546	5547	5548	5549	6920	6921	6923	6924	6925	6926
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	5.0	-	-	-	-	-
4	-	5.3	-	-	-	8.5	-	5.6	-	4.1	10.4	7.2
5	6.4	16.9	-	-	-	16.4	-	-	-	7.9	9.5	8.2
6	-	-	-	-	-	7.1	-	-	-	6.5	7.1	5.0
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
	All calves were tested negative from week 8 till day of challenge infection						All calves were tested negative from week 8 till day of challenge infection					
	Percentages positivity after challenge infection (days post challenge infection)											
	First animal experiment Calves were challenged when they were 27 weeks old on day 0 (day of challenge infection)						Second animal experiment Calves were challenged when they were 23 weeks old on day 0 (day of challenge infection)					
Day	5544	5545	5546	5547	5548	5549	6920	6921	6923	6924	6925	6926
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
6	Na	Na	Na	Na	Na	Na	-	-	-	-	-	-
7	6.4	-	14.7	-	-	9.2	-	-	-	-	-	-
8	30.5	43.0	48.3	16.9	-	68.7	-	-	-	7.8	12.5	
9	49.4	86.3	96.1	76.0	39.7	97.5	15.6	20.4	10.3	34.8	34.8	9.8
10	†	†	†	†	†	†	10.5	43.8	24.8	68.4	70.7	16.2

Results of the bRSV-specific IgE ELISA: negative (-) means percentage positivity is <5%. In case the percentage positivity was ≥5%, the actual PP value is expressed. Na means not available. In life phases ended at day 9 and 10 in respectively the first and second animal experiment. Calves were vaccinated twice with a three-weeks-interval. Blood samples for serology were collected on a weekly base till the day of challenge infection. After challenge infection (day 0), blood samples were collected on a daily base

Using this bRSV-specific IgE ELISA, we showed that 8 (out of 12) calves vaccinated with the formalin-inactivated bRSV vaccine seroconverted in the bRSV-specific IgE ELISA within 3-5 weeks after initial immunization (Table 3). Serum samples collected five weeks after immunization from mock immunized calves, calves vaccinated with live-attenuated bRSV and calves vaccinated with recombinant MVA vaccines were all negative for bRSV specific IgE antibodies (data not shown). However, bRSV-specific IgE antibodies samples were detected after challenge infection (end-sera) in 2/6 calves vaccinated with live attenuated vaccine and 3/6 calves vaccinated with the rMVA-F/G candidate vaccine.

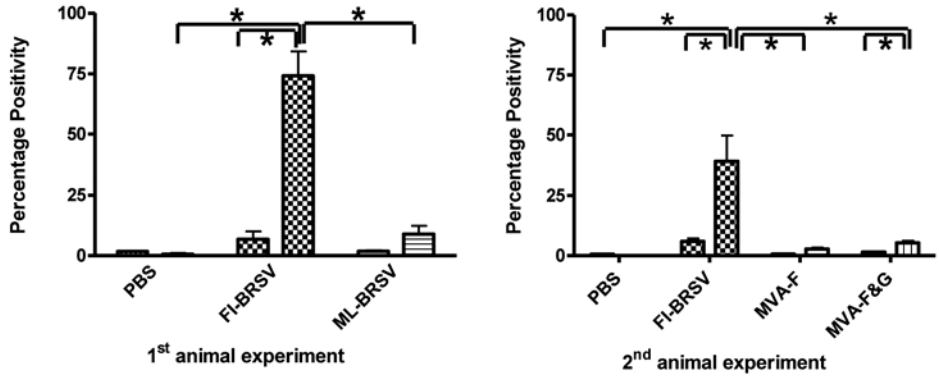

Figure 2 mean percentages positivity per treatment group. Sera collected on day 0 were all tested negative (data not shown), first bar = mean PP of sera collected two weeks after the second vaccination, 2nd bar, are mean PP in sera collected at the end the in vivo phase

Mean percentages positivity are shown in figure 2. These results indicate that even a very limited viral replication in these animals could trigger an IgE response, albeit without the associated symptoms. In contrast, immunization with FI-BRSV did result in an IgE response. Calves were positive for IgE for 2 (1 to 3) weeks after vaccination. After challenge virus infection, all calves seroconverted in bRSV-specific IgE ELISA.

Discussion

In the present study, we have analyzed total and bRSV specific IgE responses in vaccinated and bRSV challenged cattle. The key question addressed in this study was whether vaccination with FI-bRSV vaccine would prime the immune system for Th2-biased IgE responses. To address this issue, we have first optimized an ELISA for total unbound bovine IgE, based on an existing IDAS ELISA [31]. We then refined this assay to allow detection of bRSV-specific IgE.

The total IgE ELISA allowed us to monitor IgE seroconversion in calves that were immunized with a formalin-inactivated (alum adjuvanted) bRSV vaccine (FI-bRSV). An enhanced response was observed in these animals after challenge infection with a virulent field isolate. This prompted the question whether these IgE antibodies were indeed specific for bRSV. To address this question, we tested affinity-purified IgE antibodies from positive tested serum samples in a standard bRSV antibody ELISA and could indeed measure IgE responses against bRSV. However, the relatively low reproducibility and labour-intensiveness of this combination procedure prompted us to further refine the analysis of bRSV specificity. To this end, we developed an assay to detect bRSV-specific IgE antibodies. The bRSV-specific IgE ELISA was very specific (100%, using a cut-off value of 5 PP) and highly reproducible. Thus, this ELISA is an ideal tool to assess

the kinetics of post-vaccination IgE responses. Moreover, using this ELISA we showed that FI-bRSV vaccinated calves produced bRSV-specific IgE antibodies. This suggests the presence of an underlying Th2-biased CD4 T cell response specific for bRSV. In our study, the IgE response was characterized by an increase from undetectable amounts to peak levels of free (unbound) RSV-IgE in serum, three to five weeks post vaccination. In the context of bRSV, such T cell responses are undesirable as they would prime vaccinated animals/individuals for a type I hypersensitivity mechanism leading to clinical and pathological manifestations of bRSV infection after challenge virus exposure. It is clear from various studies, including our own work, that IgE responses are a normal component of the host antiviral response, i.e., increased IgE titres were also observed in calves that had been vaccinated with live-attenuated bRSV or MVA-recombinants, after bRSV challenge infection. In these cases, no enhanced disease occurred, indicating that the presence of IgE per se does not also predispose to enhanced disease. Dakhama et al. [34] reported similar findings in their RSV infected BALB/c mice studies, concluding that, consistent with clinical reports from human studies, RSV-IgE antibodies were developed as a component of a “normal” host response. Similar findings were reported for calves [4] and guinea-pigs [35]. However, when Th2-biased CD4 T cell responses are primed by the vaccine, i.e. in the case of the FI-bRSV vaccine, the outcome is one in which a pathogenic Th2-driven response dominates, with clear adverse results. The unanswered question, therefore, is how the vaccine primes this Th2-biased response. Clearly, bRSV itself has the capacity to stimulate such responses (1), but it is unclear whether this requires viral replication (i.e. live virus) or undefined properties of the viral structure. In addition, the formalin treatment (2), but also the adsorption to aluminium hydroxide (3), could have affected the anti-vaccine response and the resulting IgE induction. Nonetheless, it seems most likely that FI-bRSV provides a strong Th-2 skewing context to the dendritic cells that processed the vaccine. If this priming event is not balanced by the Th-1 bias of replicating virus, a pathogenic Th-2 response follows. From this, one would predict that combination of the FI-bRSV vaccine with analogues of viral replication, such as Th-1 inducing Toll-like receptor ligands (e.g., poly-I:C, imiquimod), should counteract the pathogenic response.

In summary, the key finding of this study, which is supported by two independent ELISA tests, is that vaccination with FI-bRSV, but not with other vaccines, results in the generation of bRSV specific IgE responses. These transient post-vaccination IgE responses are predictive for enhanced disease after viral challenge, even when serum IgE levels are reduced to undetectable levels at the time of challenge. Possible explanations for this include the continued presence of IgE on mast cells or the persistence of a Th2-biased CD4 memory T cells. Increased titres of IgE antibodies are associated with the development of an exaggerated airway hypersensitivity reaction, consistent with the previously reported undesired side effects of this vaccine. Immunization with IgE-priming vaccines clearly led to a dramatic increase in the risk for developing airway hypersensitivity reactions. The history of the FI-inactivated vaccines for hRSV and bRSV in the 1960‟s and 70‟s has made it very clear that such adverse side effects should be avoided at all cost. As the absence of post-vaccination Th2 responses may be one of the key safety

criteria for future vaccines, it appears that the bRSV-IgE ELISA is a useful extension of the available tools to evaluate the spectrum of immune responses induced by candidate bRSV vaccines.

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Chapter 5

Bovine respiratory syncytial virus infection influences the impact of alpha- and beta-integrin-mediated adhesion of peripheral blood neutrophils.

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Bovine respiratory syncytial virus infection influences the impact of α4- and β2-integrin-mediated adhesion of peripheral blood neutrophils

Abstract

Neutrophil migration into the airways and pulmonary tissue is a common finding in bovine respiratory syncytial virus (BRSV) infections. Although neutrophil trans-endothelial migration in general depends on β_2-integrins, alternative integrins such as the α_4-integrins have been implicated. In this study, rolling and firm adhesion of peripheral blood neutrophils isolated from healthy and BRSV-infected calves to tumour necrosis factor (TNF)-α activated pulmonary endothelium was investigated under flow conditions *in vitro*. For neutrophils obtained from healthy animals, inhibition of the β_2-integrin reduced firm adhesion to 63% and inhibition of α_4-integrin to 73% compared with untreated controls. Inhibition of both integrins reduced firm adhesion to 25%. Rolling velocity, which is used as a parameter for integrin involvement in neutrophil rolling, increased 1·7-fold by blocking β_2-integrin and was significantly augmented to 2·5-fold by blocking both α_4- and β_2-integrins. For neutrophils obtained from BRSV-infected animals, however, rolling velocities at 10 days after infection (p.i.) were not influenced by blocking adhesion of α4- and β_2-integrins, indicating that these integrins did not support neutrophil rolling. In addition, the inhibition of firm adhesion by blocking both α_4- and β_2-integrins was reduced significantly 9 days post-infection, resulting in a residual 68% neutrophil binding at 9 days p.i. Non-blocked firm adherence was not reduced, indicating that binding was achieved by other mechanisms than through α_4- and β_2-integrins. These results demonstrate an important function for α_4- and β_2-integrins in rolling and firm adherence of bovine neutrophils, to TNF-α-activated endothelium and show the dynamic use of these integrins for adhesion and migration by neutrophils in the course of BRSV infection.

Introduction

Neutrophils are early emigrating leucocytes in response to proinflammatory signals and changes in the vasculature [1]. Neutrophil migration is accomplished in general by a sequence of steps. First, the leucocyte forms initial tethers and rolls along the postcapillary venules. This process is mediated largely by L-selectin, expressed on the neutrophil membrane. Then, the cell firmly adheres to and finally migrates through the endothelium [2]. It has long been accepted that for firm adhesion, neutrophils use exclusively β2-(CD18) integrins, whereas other leucocytes such as lymphocytes and eosinophils make use of both α4-(CD49d) and β2-integrins [3]. Organ-specific differences occur, however, as the lung seems to allow neutrophil

migration independent of β2-integrin function [4–7]. These findings are supported by necropsy reports from humans as well as from calves deficient in expression of β2-integrins that revealed extensive neutrophil infiltration into the broncho-alveolar lumen and connective tissue of infected lungs. Other tissues such as the intestines and the oral cavity were largely devoid of neutrophil infiltration, despite signs of extensive chronic inflammation and ulceration [8–11]. In addition to migration from the postcapillary venules, neutrophil migration in the lung appears to occur from the capillaries [12].

While neutrophils were considered originally to be devoid of β1-integrins (including α4β1), to date dynamic surface expression of α2β1–α6β1 and α9β1 has been detected on human and rodent neutrophils, primarily after emigration from the vasculature [13]. Increased expression of the α4-integrin was found on human neutrophils when stimulated *in vitro* [14], and *in vivo* in critically ill septic patients [15]. The α4-integrin on these neutrophils as well as on *in vitro*-stimulated neutrophils is functionally active, as it binds to VCAM-1 [15,16] and tumour necrosis factor (TNF)-α-stimulated endothelium [16]. In particular, the α4β1-integrin may mediate neutrophil extravasation, as shown in mouse and rat [17–22].

Recently, we reported that in calves, severely affected by respiratory inflammation, the α4-integrin expression on peripheral blood neutrophils was increased compared to neutrophils from healthy animals [23]. However, data on the function of α4-integrins in neutrophil emigration are lacking. In this study, we examined the role of α4-integrin and β2-integrin in neutrophil adhesion to pulmonary endothelial cells *ex vivo* and the effect of experimental bovine respiratory syncytial virus (BRSV) infection in this role.

Materials & methods

Animals and experimental design

Specific-pathogen-free (SPF) calves were obtained by caesarean section, deprived of colostrum and reared in isolation units. The calves were found to be free of bovine virus diarrhoea virus (BVDV) and of antibodies against bovine herpes virus 1, BRSV, BVDV and parainfluenza 3 at the start of the experiments.

In this study, we investigated the expression and function of α4- and β2-integrins for neutrophil adhesion. The study was performed using neutrophils from healthy calves (n = 5, 8–10 weeks of age) and neutrophils from BRSV-infected calves. In the infection experiments, we used neutrophils from calves (n = 3, 8–9 weeks) at days −1, 5, 8, 9 and 12 after BRSV infection (p.i.) and neutrophils from calves (n = 4, 28 weeks) 10 days after BRSV infection. Peripheral blood samples were obtained from the jugular vein in vacutainer tubes (Becton-Dickinson, San Jose, CA, USA) containing sodium citrate (0·38% final volume) as anticoagulant.

Virus shedding

BRSV infection was carried out by nebulization of 2 ml 103·9 TCID50/ml of BRSV, Odijk strain. A broncho-alveolar lavage (Bal) was performed at several days p.i. [24] to demonstrate development of infection by reverse transcription-polymerase chain reaction (RT-PCR) following the protocols described by Kuno [25]. Primers were designed for BRSV-N and BRSV-P, generating PCR-products of 1·1 kb and 0·7 kb, respectively, as follows: N 5′: GTTTAAACCATGGCTCTYAGCAAGGTC, N 3′: CARTTCCACATCATTRTCTTT, P 5′: GAAATTTCCATGGA AAAATTTGCACCTG P 3′: GAAATCTTCAAGTGATAGAT CATTG, Y = C/T, R = A/G; degenerate as the BRSV–Odijk sequence was not known. Positive controls included plasmids containing the BRSV–Odijk N and P genes, as well as cDNA prepared from BRSV–Lelystad-infected cells.

Detection of cellular adhesion molecules on neutrophils in peripheral blood

The following monoclonal antibodies (MoAbs) were used for fluorescent staining: interleukin (IL)-A110 (antineutrophil granulocyte, kindly provided by Dr Naessens, ILRI, Nairobi, Kenya) [26], R15·7 (anticanine β2-integrin, cross-reactive to its bovine homologue, kindly provided by Dr Rothlein, Boehringer Ingelheim Pharmaceuticals, Ridgefield, USA) [8] and BII218·1 (cross-reactive antisheep α4-integrin, kindly provided by Dr A. Young, Basel Institute of Immunology, Switzerland) [27]; R73 (IgG1) specific for rat T cell receptor [28] served as isotype control MoAb. HUTS21 was kindly provided by Dr Sanchez-Madrid (Universidad Autonoma de Madrid, Spain). The MoAb recognizes an activation epitope on a regulatory region (355–425) of the human beta-1 chain [29], which is 95% identical to its bovine homologue (CD29 bos taurus, accession code NM_174368) by BLAST comparison.

Peripheral blood (50 *μ*l) was incubated with MoAbs (0·25 *μ*g/ml) for 30 min on melting ice, followed by two washes in FACS buffer (PBS, 0·1% azide, 0·5% bovine serum albumin), and incubated subsequently with saturating amounts of goat-antimouse FITC (Becton-Dickinson, San Jose, CA, USA). Peripheral blood leucocytes were purified by lysis of red blood cells in FACS-brand lysing solution (Becton-Dickinson, San Jose, CA, USA). Flow cytometry was performed on FACSCalibur (Becton-Dickinson, Brussels, Belgium). A minimum of 10 000 events was recorded for each sample. Neutrophils were identified on the basis of IL-A110 (antineutrophil) specific staining and light scatter profile. The expression index (EI) quantified the expression of cell adhesion molecules (CAM) on gated neutrophils and was defined as the quotient of specific MoAb and isotype control MoAb fluorescence.

Isolation and culture of bovine endothelial cells

Endothelial cells from the bovine pulmonary artery (BPAEC) were isolated from fresh lungs, obtained from 4–8-week-old calves at a local abattoir. The pulmonary artery was dissected and perfused with PBS (4°C) to remove blood clots. Both ends were ligated, and the dissected artery was injected with 0·05% (g/v) trypsin (Becton-Dickinson, San Jose, CA, USA) solution at 37°C for 15 s. The injected volume was recovered, pooled with an equal volume of bovine calf serum and

centrifuged (500 g for 10 min at 4°C). Isolated cells were seeded into a 25-cm2 flask, coated with 1% gelatin (Merck, Darmstadt, Germany), and cultured in Iscove's tissue culture medium (GIBCO BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin, 50 *μ*g/ml streptomycin and 2 mM L-glutamine (all from Sigma, Zwijndrecht, the Netherlands). After 2–3 days, single endothelial colonies were identified by typical cobblestone morphology [30] and subcultured. The subcultured endothelial cells were characterized by uptake of acetylated low-density lipoprotein labelled with fluorescent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL, Sigma, Zwijndrecht, the Netherlands) and screened by fluorescence microscopy (data not shown) [31]. Cell-lines were found free of mycoplasma by PCR analysis on genomic DNA and on 2-day culture supernatant (data not shown) [32].

Isolation of neutrophils

During the complete isolation procedure, blood and neutrophils were kept on melting ice and centrifugation steps were performed at 4°C. Neutrophils were isolated by hypotonic lysis [33] and resuspended in neutrophil incubation buffer (20 m M Hepes, 132 m M NaCl, 6 m M KCl, 1 m M MgSO4, 1 m M KH2PO4, 5 m M glucose, 1·0 m M CaCl2, 0·05% (w/v) bovine serum albumin (Sigma, Zwijndrecht, the Netherlands), pH 7·4, before use in perfusion assays. Purity of neutrophils was >95%. The viability (>95%) was determined by trypan blue exclusion.

Perfusion assays

Neutrophil rolling adhesion and firm adhesion on endothelial cells under steady flow was investigated in a modified form of the transparent parallel plate perfusion chamber as described previously [34]. To simulate an immunologically activated pulmonary environment, BPAEC (third to fifth passage) were preactivated by human TNF-α for 7 h (100 U/ml, 37°C; Boehringer Mannheim, Mannheim, Germany) [35]. Activation of endothelial cells induces expression of VCAM-1 and increased expression of ICAM-1, which are major ligands for the leucocyte α4- and β2-integrins, respectively [36,37]. The perfusion equipment was placed in a 37°C temperature box. Neutrophils kept in suspension (2 × 106 cells/ml in neutrophil buffer) on melting ice until start of the experiment were allowed to adjust to 37°C for 20 min with or without blocking MoAbs (10 *μ*g/ml) before the perfusions. The concentration of the blocking MoAbs was saturating as determined by FACS analysis (data not shown). The MoAbs were described as cross-reactive and function-blocking MoAbs specific for the bovine homologues of canine β2-integrin (R15·7) [8,38] and human α4-integrin (HP 2/1) [27,34,39]. Neutrophils were aspirated for 5 min from a reservoir through plastic tubing and the perfusion chamber with a Harvard syringe pump (Harvard Apparatus, South Natick, MA, USA), followed by buffer. The shear stress was set at 2·5 dynes/cm2. Rolling and firmly adherent cells were detected using video recordings of at least 25 randomized high-power fields, representing a total surface of at least 1 mm2 and analysed by customized software [34]. The percentage of rolling cells was detected in a sequence of 50 frames covering a 2-s period. At least 100 cells per experiment were investigated for rolling velocity. The cut-off speed for distinguishing rolling and

static adherent cells was set at 1 *μ*m/s. As the time-span for the experiments was limited by the functional life-span of the isolated neutrophils, controls were restricted to neutrophils without MoAbs. In a pilot experiment, adhesion of neutrophils (no. of adherent neutrophils/mm2 ± s.e.m.), incubated with the MoAb W6/32 specific for human MHC I and cross-reactive to bovine MHC [40], was not different from adhesion of control neutrophils, 1206 ± 43 and 1232 ± 44, respectively (P = 0·735, paired Student's t-test).

Statistical analysis

Results were compared by ANOVA, with Bonferroni correction for multiple comparison using the statistical software GRAPHPAD PRISM® and GRAPHPAD STATMATETM (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $P < 0{\cdot}05$. Results were expressed as mean ± s.e.m.

Results

Expression and function of CAM on neutrophils from healthy cattle

Integrin expression on neutrophils

CAM on neutrophils, obtained from healthy calves (n = 5), were detected by flow cytometry and expression levels were converted into the EI ± s.e.m., which was 13·9 ± 1·8 for β2-integrin and 2·8 ± 0·3 for α4-integrin.

Perfusion experiments

The total number of adherent neutrophils (rolling and firmly attached) to 7 h TNF-α-activated BPAEC was determined (Fig. 1a). To investigate the role of α4- and β2-integrins, neutrophils were preincubated with blocking MoAbs. Co-administration of α4- and β2-integrin blocking MoAbs inhibited adherence to 47% of the control value ($P < 0{\cdot}001$). The percentage of rolling neutrophils increased by a factor 4·8 ($P < 0{\cdot}001$) by the combination of blocking antibodies compared to control values. Treatment of neutrophils with blocking MoAbs specific for α4-integrin or β2-integrin reduced the number of firmly adherent neutrophils to 73% ($P < 0{\cdot}05$) and 63% ($P < 0{\cdot}01$), respectively (Fig. 1b). When these MoAbs were used in combination, firm adhesion reached up to 25% of control levels ($P < 0{\cdot}001$).

Rolling velocity with and without preincubation of neutrophils with blocking MoAbs was used as a parameter for integrin-mediated rolling. Involvement of integrins in neutrophil rolling resulted in increased neutrophil speed of MoAb pretreated neutrophils. Mean rolling velocities of neutrophils on TNF-α activated endothelium increased by a factor of 1·7 ($P < 0{\cdot}05$) by blocking β2-integrins, whereas blocking of α4-integrins did not significantly enhance neutrophil speed. Co-administration of α4- and β2-integrin-specific MoAbs significantly enhanced neutrophil velocity 2·5-fold compared to unblocked controls ($P < 0{\cdot}001$) and to singly blocked neutrophils (Fig. 1c).

Together, these results indicate that both α4- and β2-integrins mediated firm adhesion and supported rolling adhesion, despite differences in level of integrin expression.

Expression and function of CAM on neutrophils from BRSV-infected cattle

BRSV infection experiments were set up to monitor neutrophil adhesion *in vitro* during pulmonary infection. Infection developed similarly in all animals. In the first infection experiment (*n* = 3), production of viral RNA in BALF was determined daily, from days 5–9 p.i. Viral RNA production peaked at day 7 p.i. (data not shown). Similarly, in the second experiment (*n* = 4), viral RNA production was detected at day 7 p.i.

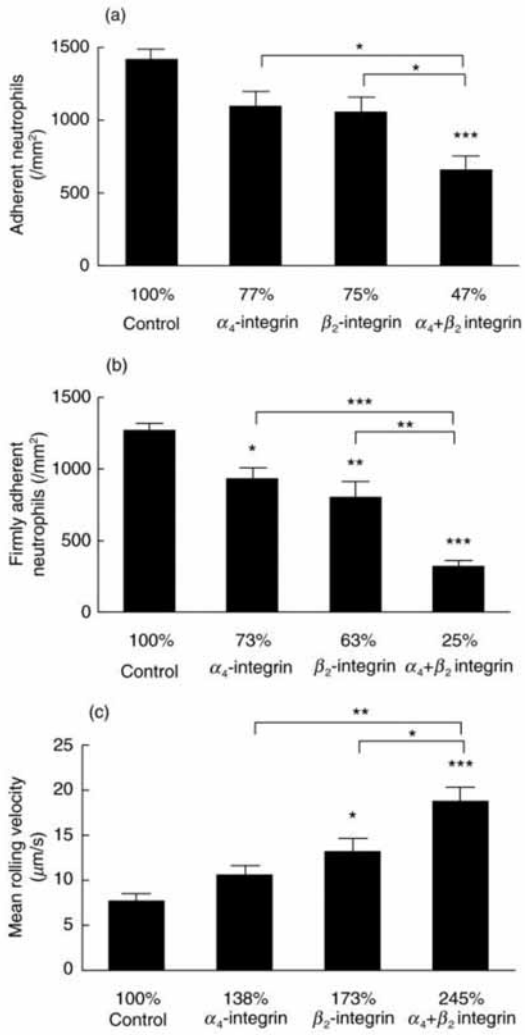

Figure 1. Effect of blocking α_4- and β_2-integrins on the interaction of neutrophils to TNF-α-activated BPAEC. Neutrophils (2×10^6/ml) preincubated with or without blocking MoAbs (10 μg/ml, 20 min, 37°C) were perfused at a shear stress of 2·5 dynes/cm^2. After 5 min, microscopic images were recorded on video and the number of adherent cells − rolling or firmly attached − were determined in at least 25 images per perfusion experiment. (a) The effect of blocking MoAbs specific for α_4- and β_2-integrins on the number of adherent cells (rolling and firmly attached) to BPAEC. (b) The effect of blocking MoAbs on the number of firmly attached cells as percentage of the total number of adherent cells. (c) The effect of blocking MoAbs on mean rolling velocity of neutrophils. The rolling velocities of at least 100 cells per experiment were determined. Means are plotted for five animals per group ± s.e.m. The mean percentages for each group compared to the control group have been indicated. The statistically significant effects of the blocking MoAbs compared to the control situation or between different treatments (as indicated in the figure) were determined by ANOVA with Bonferroni correction for multiple comparison, *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Integrin expression on neutrophils

Expression levels of α4- and β2-integrins were measured by flow cytometry. In the first infection, both the α4- and β2-integrins did not change significantly from day −1 (EI 2·6 ± 0·5 and 13·9 ± 3·4, respectively) until day 10 p.i., but increased at day 12 p.i. (EI 3·7 ± 0·7, $P < 0·05$ and 38·7 ± 5·4, $P < 0·001$, respectively) (Fig. 2). In the second infection, expression levels of α4- and β2-integrins before and 8, 9 and 10 days after infection were similar to the results found in the first infection experiment. In addition, expression of an activation epitope for β1-integrins (HUTS21) was determined. The expression decreased in all animals from 1·27 ± 0·17 at day −1 to a minimum of 1·005 ± 0·01 at day 8 and 1·038 ± 0·05 at day 10 p.i. Mean expression levels of HUTS21 were, however, not significantly different ($P = 0·1053$).

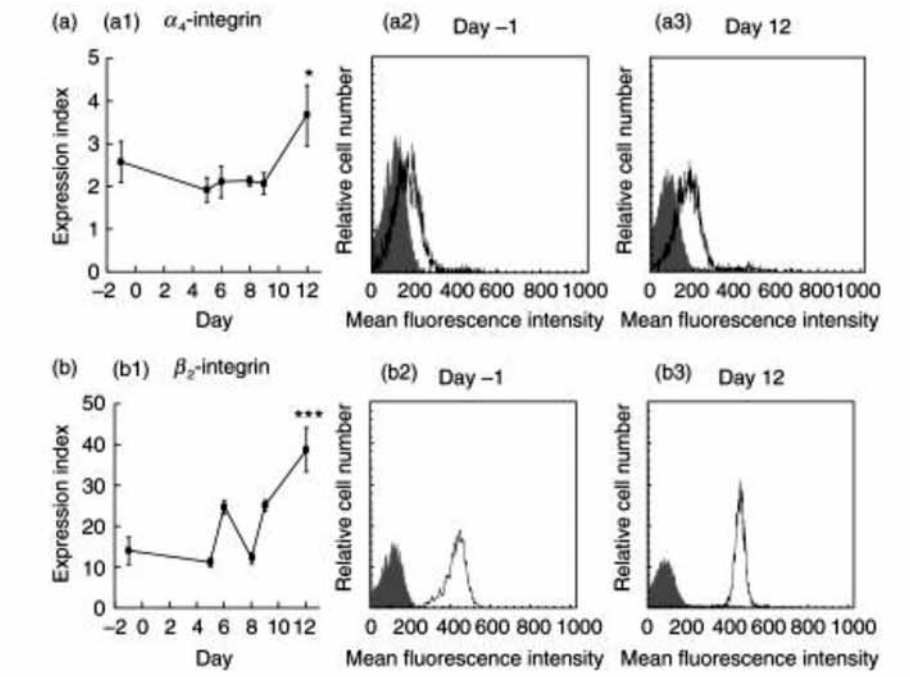

Figure 2 CAM expression on peripheral blood neutrophils of calves (n = 3) in the course of BRSV infection. The mean fluorescence intensities of gated neutrophils stained with CAM-specific MoAbs (BII218·1 for α_4-integrin and R15·7 for β_2-integrin) or isotype control MoAb (R73) were measured. The expression index (EI), defined as the quotient of specific MoAb and isotype control MoAb fluorescence, quantified the expression of CAM. (a) Expression of α_4-integrin: (a1) expression indices before and in the course of infection; (a2) mean fluorescence intensities of α_4-integrin (line) and its isotype control (grey area) at day −1; (a3) mean fluorescence intensity of α_4-integrin (line) and its isotype control (grey area) at day 12. (b) Expression of β_2-integrin: (b1) expression indices, before and in the course of infection; (b2) mean fluorescence intensities of β_2-integrin (line) and its isotype control (grey area) at day −1; (b3) mean fluorescence intensities of β_2-integrin (line) and its isotype control (grey area) at day 12. Means are plotted ± s.e.m. Histograms of one animal, representative of three, are shown. Significant differences in EI levels compared to day −1 are indicated. *$P < 0·05$; ***$P < 0·001$.

Perfusion experiments

During the first infection, neutrophil perfusion experiments were performed before (day −1) and at several days after (days 5, 8, 9 and 12) BRSV infection. The overall frequency of adherent cells (without blocking antibodies) remained constant, whereas the frequency of rolling neutrophils increased (P = 0·0020) from 10·6% ± 3·6 before infection to 36·7% ± 6·0 at day 12 p.i. The frequency of firmly adherent cells tended to decrease (P = 0·092) in the course of infection.

Blocking either α4- or β2-integrins separately did not result in significant differences before and after infection (data not shown). The number of α4- and β2-integrins doubly blocked cells per mm2, adherent to activated endothelial cells (TNF-α 100 U/ml, 7 h), changed significantly in the course of infection and was determined in absolute numbers and as a percentage of adherent, control (untreated) neutrophils on that day. The percentage of adherent (which equals the entire number of firmly adhered and rolling cells), doubly blocked neutrophils increased after BRSV infection. At day −1 it was 52 ± 8% of control values. At day 9 p.i., it was 71 ± 3% ($P < 0·05$). The biological significance of this finding is limited, as the absolute number of doubly blocked adherent neutrophils was not significantly different at this time-point (Figure 3a,b).

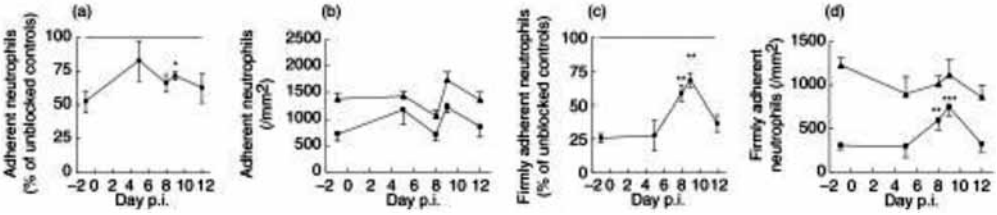

Figure 3. Effect of inhibition of α4- and β2-integrins on the interaction of neutrophils, isolated in the course of BRSV infection, to TNF-α-activated BPAEC. Neutrophils were isolated from calves (n = 3) 1 day before and 5 (n = 2), 8, 9 and 12 days after BRSV infection. Adhesion of isolated neutrophils was investigated in the presence of α_4- and β_2-integrin-blocking MoAbs (Hp2/1 and R15·7, respectively) using neutrophil flow experiments. The numbers of adherent neutrophils on 7-h TNF-stimulated BPAEC were determined by computer-assisted image analysis using at least 25 video recordings. (a) α_4- and β_2-integrin-blocked adherent neutrophils (firm and rolling) as percentage of controls (P = 0·0495). (b) Absolute number of adherent neutrophils (firm and rolling) using α_4- and β_2-integrin-blocked (squares, P = 0·0053) and control (triangles, P = 0·016) neutrophils. (c) α_4- and β_2-integrin-blocked, firmly adherent neutrophils as percentage of controls (P = 0·0022). (d) Absolute number of firmly adherent neutrophils using α_4- and β_2-integrin blocked (squares, P = 0·0006) and control (triangles) neutrophils (P = 0·27). Means are plotted ± s.e.m. Significant differences at one time-point compared to day −1 are indicated. **$P < 0·01$; ***$P < 0·001$.

The percentages of rolling cells, incubated with α4- and β2-integrin blocking antibodies did not change significantly in the course of infection.

The number of firmly adherent, doubly blocked neutrophils increased during infection. Before infection, blocking both α4- and β2-integrins reduced firm adhesion to 25 ± 4% of control values. In the course of infection, blocking the α4- and β2-integrins was less effective, resulting in higher residual binding (P = 0·0022, Fig. 3c). At day 8 p.i., 58 ± 6% ($P < 0·01$, compared to d -1) and at day 9, 68 ± 5% ($P < 0·01$) of the neutrophils still adhered firmly despite the presence of blocking

MoAbs. The rise in firmly adherent cells that had been pretreated with α4- and β2-integrins blocking MoAbs was also significant in absolute numbers. This indicates that the effect was due to an increase in the number of firmly adherent neutrophils and not to a decrease in the total number of adherent cells (Fig. 3d).

A non-significant trend suggesting a reduced influence of α4- and β2-integrins blocking MoAbs on rolling speed in the course of infection was detected (data not shown).

In the second infection experiment, neutrophil perfusions were performed at day 10 p.i. No significant effect was found by blocking α4- and β2-integrins on the total number of rolling and firmly adherent cells at this time-point (Fig. 4a). Co-administration of MoAbs enhanced the percentage of rolling neutrophils 2·3-fold ($P < 0·01$). Firm adherence of neutrophils isolated from BRSV-infected animals was inhibited by co-administration of α4- and β2-integrins blocking MoAbs to 51% of the adherence of control neutrophils ($P < 0·01$) and to 60% of the adherence reached after β2-integrin blocking of neutrophils ($P < 0·05$, Fig. 4b).

Rolling velocities of neutrophils isolated from BRSV infected animals were not affected significantly by blocking MoAbs (Fig. 4c).

These data show that 8, 9 and 10 days after BRSV infection the contribution of α4- and β2-integrins to neutrophil firm adhesion decreased significantly. In addition, neutrophil rolling at day 10 p.i. functioned independently of α4- and β2-integrins. An increase in expression of α4- and β2-integrins was detected at day 12 p.i. Simultaneously, the dependency of neutrophil adhesion to the function of both integrins was restored. The decrease in expression of HUTS21 may indicate that the β1-integrin, which forms a heterodimer with the α4-integrin chain, falls back in the inactive conformation.

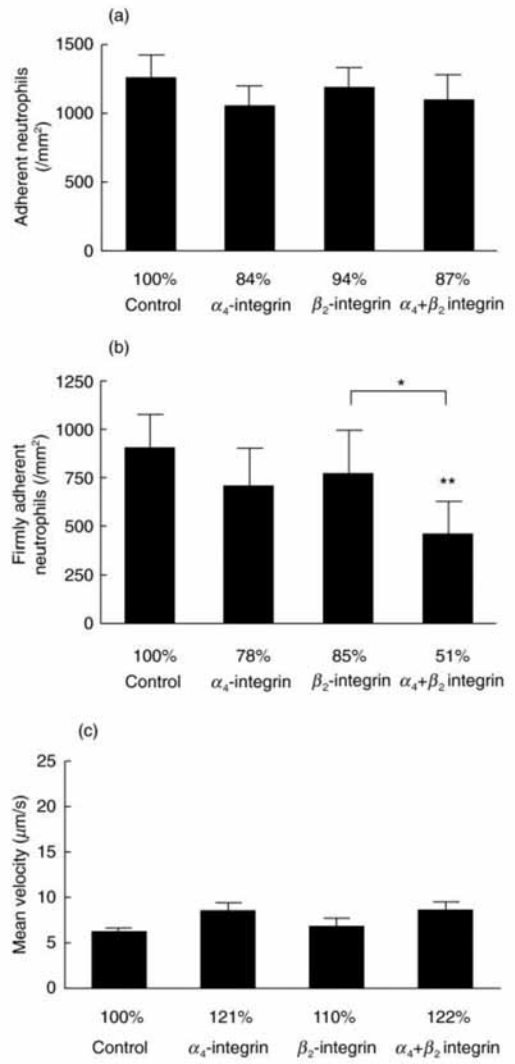

Figure 4 Effect of blocking α4- and β2-integrins on the interaction of neutrophils isolated from 10-day BRSV-infected calves to TNF-α-activated BPAEC. Neutrophils (2×10^6/ml) preincubated with or without blocking MoAbs (10 μg/ml, 20 min, 37°C) were perfused at a shear stress of 2·5 dynes/cm2. After 5 min, microscopic images were recorded on video and the number of adherent cells − rolling or firmly attached − were determined in at least 25 images per perfusion experiment. (a) The effect of blocking integrins on the number of adherent cells (rolling and firmly attached) to BPAEC. (b) The effect of blocking integrins on the number of firmly attached cells as percentage of the total number of adherent cells. (c) The effect of blocking integrins on mean rolling velocities of at least 100 rolling cells. Means are plotted for four animals per group ± s.e.m. The mean percentages for each group compared to the control group have been indicated. The statistically significant effects of the blocking MoAbs compared to the control situation or between different treatments (as indicated in the figure) were determined by ANOVA with Bonferroni correction for multiple comparison, $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

Discussion

In healthy cattle, expression of α4-integrin on neutrophils was relatively low compared to β2-integrin. Despite the differences in expression between α4- and β2-integrins, adhesion to activated pulmonary artery endothelial cells seemed to depend on both integrins. The present experiments illustrate that the α4-integrin, although expressed at a low level on neutrophils, may contribute significantly to leucocyte-endothelial adherence.

The function of α4- and β2-integrins in neutrophil rolling was investigated by measuring rolling speed. This velocity is influenced by differences in the 'on' or 'off' rate of each CAM that binds to its endothelial ligand. Because blocking of β2-integrins increased rolling speed, while the effect was augmented by co-administration of α4-integrin-blocking antibodies, both the α4- and β2-integrins may support neutrophil rolling. These contributions of α4- and β2-integrins in neutrophil rolling were also acknowledged recently by intravital microscopy studies in mice [20]. For integrin αLβ2 (LFA-1), it was reported previously that the inactive (closed) conformation of the integrin dimer supports the rolling phase, while the activated (open) conformation mediates leucocyte firm adhesion [41]. Firm adhesion of neutrophils isolated from healthy cattle was mainly mediated by α4- and β2-integrins. The inability of doubly blocked neutrophils to enter the stage of firm adhesion was shown by a reduced number of firmly adherent neutrophils and a rise in rolling neutrophils.

In experimentally induced BRSV pneumonia, increased integrin expression on neutrophils was detected at 12 days p.i. These findings are consistent with our earlier work that reports higher levels of α4-integrin expressed on neutrophils from severely pneumonic cattle compared to healthy cattle [23]. Similarly, humans with sepsis were found to have elevated levels of α4-integrin expressed on peripheral blood neutrophils [15]. Furthermore, in human cases of RSV infection, increased expression of the β2-integrin Mac1 (αMβ2) was detected [42].

A peak in virus production in the lungs 7 days p.i. preceded a period of 5 days, displaying two remarkable phenomena. First, α4- and β2-integrin expression did not increase up to 10 days p.i. Interestingly, BRSV production at day 7 p.i. is associated with proinflammatory cytokine production in the following days [43]. Despite this proinflammatory environment, increased expression of α4- and β2-integrins was detected several days later, after day 10 p.i.

Secondly, an α4- and β2- integrin-independent type of adhesion occurred. This was marked by the fact that blocking the α4- and β2- integrins did not influence neutrophil rolling 10 days p.i. (Fig. 4c). The numbers of rolling cells and rolling velocities observed in the α4- and β2-integrin-independent adhesion of neutrophils from infected animals at this time-point were less than from uninfected animals. This would suggest that the function of these integrins in supporting rolling is unique for neutrophils from healthy calves and is taken over by other rolling receptors in the course of BRSV infection. A similar phenomenon was detected in neutrophil firm adhesion, i.e. the inhibiting effect of blocking antibodies on firm adhesion decreased significantly 8, 9 and 10 days p.i (Figs 3c,d and and 4b).4b). Together, these findings show that BRSV infection does not

increase integrin expression on neutrophils up to 10 days p.i., but may prime circulating neutrophils from day 8 to day 10 p.i. to start extravasating in a α4- and β2-integrin-independent manner. In this time-frame there seems to be redundancy in the CAM that may be used for neutrophil adherence.

The reduced involvement of α4- and β2-integrins in neutrophil rolling might have been compensated for by increased function of other CAM, such as L-selectin, as the number of rolling neutrophils (non-blocked) increased in the course of infection. Other CAM that may be involved in leucocyte firm adhesion, and possibly mediate α4- and β2-integrin independent neutrophil adhesion, are β3-integrins such as αVβ3. This integrin is expressed constitutively on neutrophils and associated possibly with transendothelial migration [44]. Expression of the activation epitope on the β1-chain tended to decrease, suggesting a shift of the active conformation to the non-active conformation during infection. This would suggest that β1-integrins are not the missing adhesion factor in the infected animals. Alternatively, non-integrins such as ICAM-1 may be induced after BRSV infection. An indication for the possible involvement of ICAM-1 is that its expression was up-regulated on human neutrophils after RSV infection [42].

The use of a β2-integrin-independent type of adhesion may be a general characteristic of pulmonary inflammation [45]. It was reported previously that neutrophils from acutely infected patients used a β2-integrin-independent pathway, whereas neutrophils from chronically infected patients used a β2-integrin-dependent type of migration *in vitro* [45]. Alternatively, α4- and β2-integrin-independent migration may be a specific aspect of syncytial virus infections. Several reports on human RSV indicate a direct or indirect influence of the virus on leucocyte function. Direct incubation of viable or inactivated RSV with human neutrophils seemed to exert an activating effect as it induced production of proinflammatory chemokines [46]. In addition, the RSV G-protein − which is produced in a soluble form by bovine and human RSV [47,48] − was shown to influence IL-8 production by neutrophil granulocytes in a concentration-dependent fashion [49].

In conclusion, the results of the present study show that, to a large extent, neutrophils from healthy calves use α4- and β2-integrins for adhesion to activated pulmonary endothelial cells in vitro. The majority of firm adhesion interactions are mediated by these integrins. In addition, both integrins support neutrophil rolling. BRSV infection of the lungs induces an α4- and β2-integrin-independent type of adhesion in circulating neutrophils. Only at 12 days after infection do α4- and β2-integrins tend to reclaim their prominent role in adhesion, which is associated with an increase in expression of both integrins. These results demonstrate a dynamic use of α4- and β2-integrins by neutrophils for adhesion and migration, which is induced in the course of BRSV infection.

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Chapter 6

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

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Abstract

The severity of respiratory syncytial virus (RSV) infections appears to differ in 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 one-to-six 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. Here we examined whether age-dependent differences in the pathogenesis of bRSV between neonatal and young calves may be due to differences in age-dependent immunocompetency. To study the effect of age and immune parameters on bRSV disease in neonatal and young calves, we experimentally infected neonatal (one-day-old) calves without maternally-derived antibodies and evaluated the severity of disease and bRSV immune responses in comparison with disease in similar six-week-old infected calves. Neonatal calves had more extensive virus replication and lung consolidation, but lower proinflammatory (in particular TNF-alpha) responses, specific humoral immune responses, lung neutrophilic infiltration, and clinical signs of disease than six-week-old calves. The lack of correlation between virus replication and clinical signs, suggests an important role of proinflammatory cytokines, in particular of tumour necrosis factor-alpha (TNF-α) in the disease. The capacity to produce proinflammatory TNF-α appears to increase with age, and may explain age-dependent differences in RSV pathogenesis.

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 all over the world (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 bovine respiratory disease (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 two years of age, seroprevalence of antibodies against bRSV is over 70%. Nevertheless, bRSV reinfection can occur throughout life, becoming progressively less severe with the increase of age.

BRSV *in vivo* primary 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 (nasal and ocular discharge and cough) and lower respiratory tract disease (tachypnea 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 the development of more severe (lower) respiratory tract disease. Severe, life-threatening lower respiratory tract disease (LRTD) is mostly seen during (primary) infection in one to six month-old calves (Bryson et al., 1978, Kimman et al., 1988). Striking is that the newborns, are generally not severely affected. These observations led to the hypotheses that these very young calves are well protected by e.g. passively acquired maternally-derived antibodies (Kimman et al., 1988). Kimman et al. showed that the severity of disease was inversely related to the level of maternal antibodies in calves from one to three months old (Kimman et al., 1988), suggestive of a role of immunopathogenic mechanisms. Tjørnehøj et al. on the contrary showed that there was no correlation between the neutralising antibody titre at inoculation and the peak respiratory rate / severity of elicited pneumonia in calves 2 to 5 months of age (Tjornehoj et al., 2003).

Thus severe disease appears to be less often observed in older calves, but on the other hand seems to be practically absent in calves younger than two weeks, irrespective the maternal antibody titre (Baker et al., 1986). Kimman et al. 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 due to not experiencing the entire period of bRSV circulation on the farm (Kimman et al., 1988).

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 immunocompetency. We therefore examined the effect of bRSV inoculation in neonatal and six-week-old calves in the absence of maternal antibodies and evaluated the severity of disease and bRSV immune responses in both groups.

Materials & methods

Calves

Two groups of five SPF calves were obtained by caesarean section at full term with six 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 BVDV antigen enzyme-linked immunosorbent assay (ELISA) BVDV antigen Test Kit (IDEXX Herdcheck, Sweden) and free of antibodies directed against bRSV (Ceditest BRSV, Cedi Diagnostics BV., Lelystad, The Netherlands).

Inoculation

The calves were inoculated as described previously (Antonis et al., 2003). Briefly, the calves were inoculated intranasally with an air-jet nebulizer with bRSV strain Odijk on the same day when they were six weeks of age (young calves) or one-day-old (neonates). BRSV strain Odijk was obtained during a field outbreak in The Netherlands (Odijk, 1991) (van der Poel et al., 1996). The inoculum (2 ml per calf) used in this study contained 103.1 TCID50 bRSV per ml, after being passaged four times in vivo in SPF calves.

Clinical investigations

Calves were daily clinically examined by the same veterinarian every day 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 (WBC), red blood cells (RBC) and blood platelets were determined using a Coulter counter (Sysmex). Additionally a blood smear analysis was performed for differential count of leucocytes. 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 Co. Ltd). Following sampling, swabs were agitated in 2 mL of tissue culture medium supplemented with antibiotics and foetal bovine serum.

Bronchoalveolar lung fluid (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. (Fogarty et al., 1983). Approximately 35-75 ml of BALF was obtained from each calf after instillation of 100 ml phosphate-buffered saline (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 that were incubated under aerobic conditions at 37oC. Bacteria were identified using standard laboratory procedures. Two ml BALF sample material was centrifuged 5 min at 25 x g in a cytocentrifuge (Shandon Cytospin2, Shandon, Breda). Slides were air dried and fixed in methanol for 10 minutes at room temperature. A total of 400 cells were counted (Hema-Tek slide stainer), and the macrophages, lymphocytes, monocytes and neutrophils were differentiated on basis of morphology. The remaining of the BALF was centrifuged (200 x g, 10 min, 4oC). Lavage cells were resuspended in 0.5ml Dulbecco's

minimal essential medium (DMEM) containing 1% ABC (antibiotic cocktail) and 50% Foetal Calf Serum (FCS), carefully added to 1ml freeze medium (DMEM, containing 1% ABC, 50% FCS and 20% DMSO), and frozen at -70oC. BALF supernatants were stored at -70oC for virus isolation and virus titration.

Virus isolations were performed in duplicate on 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 day 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 G (0.5 x 16 mm) needle after which the running blood was absorbed in a heparinized capillary. Rectal temperature was measured simultaneously with blood sampling. After sampling, the capillaries were closed immediately and stored on ice until analysis on a pH/Blood Gas Analyzer (IL Synthesis®, Instrumentation Laboratory) for measurement of arterial pH, pCO2, 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) with Accutrend® Lactate and BM-Lactate test strips (Roche Diagnostics).
Haptoglobin was determined daily in serum with a haptoglobin assay (Tridelta Ltd, Bunde The Netherlands). 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 quantitatively determined using the SearchLight® Bovine Cytokine Array (Pierce Biotechnology, 35A Cabot Road, Woburn, MA 01801, USA). 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 supernatant was washed away, biotinylated detecting antibodies were added that bind to a second site on the target proteins. After removing excess detecting antibody, streptavidin-horseradish peroxidase (SA-HRP) was added. Finally, a chemiluminescent substrate was added to produce a luminescent signal that was detected with a cooled CCD camera. The amount of produced signal is proportional to the amount of each cytokine in the original standard or sample.

Postmortem investigations

Thirteen days after virus inoculation, calves were anaesthetized using pentobarbital and euthanized by exsanguination. The lungs were immediately removed, 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. (Viuff et al., 2002). Necropsy samples of 10 predetermined sites were stored in 10% neutral buffered formalin. Formalized samples were embedded in paraffin, sectioned at 5µm, and stained with hematoxylin and eosin for histologic evaluation. Histologic 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

The GraphPad Prism® software was used for analysis of the data. The two-way ANOVA and non-parametric (Mann-Whitney) test were used for comparisons. Correlations and differences were considered significant when p was <0.05.

Results

BRSV shedding and specific humoral immune responses after inoculation.

To asses the impact of age on severity of disease, one-day-old neonates were infected by intranasal inoculation of $10^{3.4}$ $TCID_{50}$ of bRSV strain Odijk and compared with young, six-week-old, calves. As expected bRSV was isolated from the upper respiratory tract in all calves, but the neonates shed virus significantly (p=0.0238) longer, 5.6 ± 0.5 days, than the young calves, 3.8 ± 0.8 days (Figure 1).

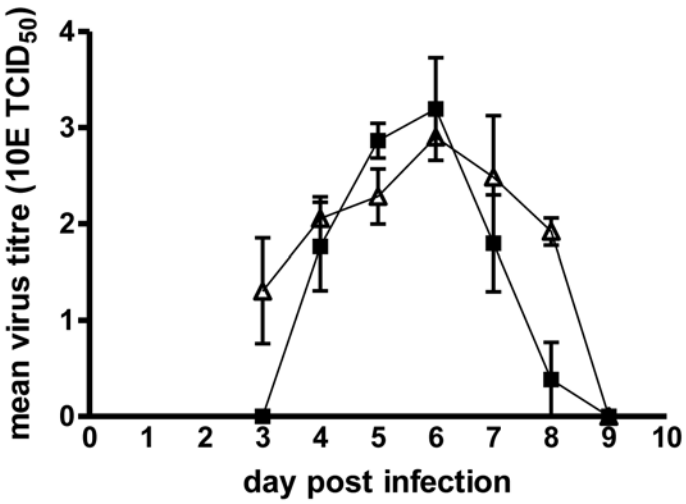

Figure 1 mean (± SEM) virus titer in nasopharyngeal fluid samples per group (six-week-old "young" calves (■) versus one-day-old "neonates" (Δ)) from prior to infection (study day 0) till ten days post infection. Neonates shed virus significantly (p=0.0238) longer, 5.6 ± 0.5 days, than the young calves, 3.8 ± 0.8 days.

The magnitude of the 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)

$TCID_{50}$ / ml in neonates. Infectious bRSV was isolated from day"s 6 lung washes in three (two young calves and one neonatal) calves. All young calves sero-converted 13 days after infection, whereas three of the neonates remained serological negative (VNA titre ≤ 2) for the duration of the experiment. Young calves developed significantly (p=0.0087) more neutralizing antibodies than the neonates (Figure 2).

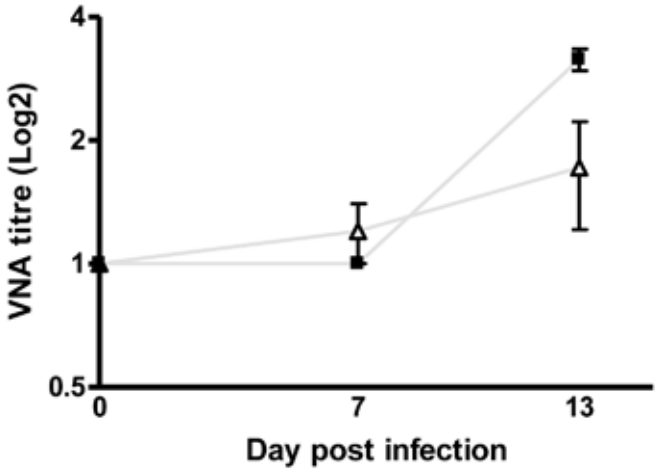

Figure 2 Mean virus neutralizing antibody (VNA) titres per group. Mean VNA (with SEM) titres per group (six-week-old (■) versus one-day-old (Δ) calves) from prior to infection (day 0), seven days post infection (day 7) and at the end of the study (day 13). Antibody titres are expressed as reciprocal values of the highest neutralizing dilution. Starting at a 1:4 dilution, ≤2 (1log2) is considered as being negative. Six-week-old calves had significant (p=0.0087) more (neutralizing antibodies compared to on-day-old calves.

Clinical signs after bRSV infection.

The calves were clinically monitored for 13 days after infection, until the experiment was terminated. Parameters evaluated were categorised in general illness, upper respiratory tract disease and lower respiratory tract disease (Figure 3). None of the neonates was scored as general ill, whereas three (out of five) young calves were scored as general ill, for 1-3 days. This was confirmed by the assessment of the rectal temperatures. Whereas four (out of five) young calves had fever (rectal temperature > 39.6oC) for one to four days, none of the neonates had fever. These differences were statistically significant ($p<0.05$).

Signs of upper respiratory tract disease were observed in all calves, in both age groups. A moderate upper respiratory tract disease (URTD, score 2) was observed in all neonates from day 5, for 2-4 (mean 2.8±0.8) days and in all young calves from day 6, for 2-6 (mean 4.6±1.5) days. Severe URTD (score 3) was scored four times in three calves in the young calves, and twice (in two calves) in the neonates. Lower respiratory tract disease was scored in all young calves and in four (out of five) neonates. Severe LRTD was observed in only two young calves for 1-3 days

resulting in a significant ($p<0.001$) difference on study day 7 between both groups. Significant ($p<0.05$) more number of days with tachypnea, defined as >40 breathings per minute, were observed in the young calves group (mean 4.8 ± 1.6 days) compared to the neonates (2.0 ± 1.3 days).

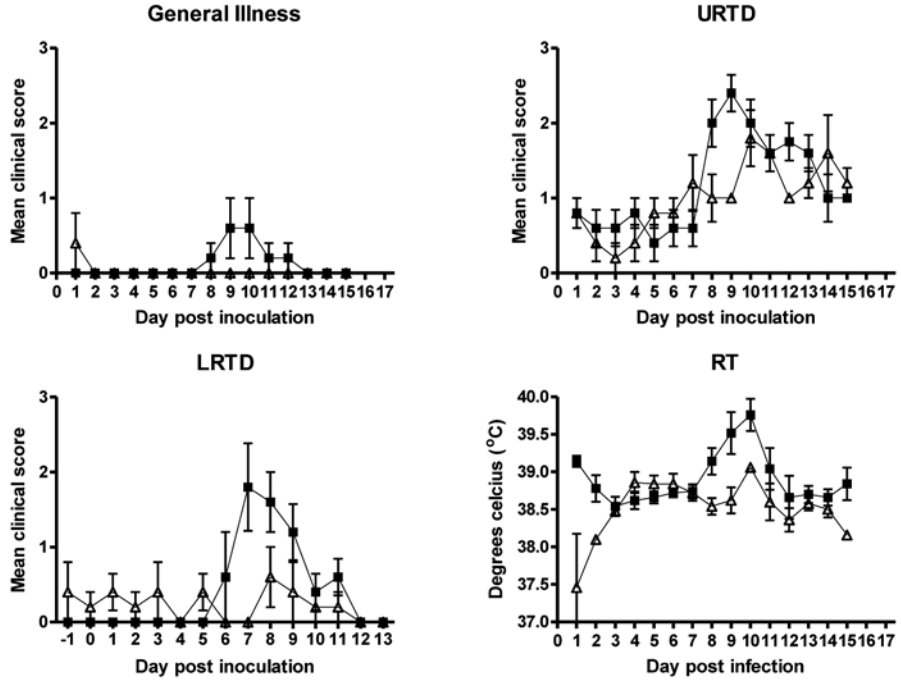

Figure 3 Clinical observations. Mean clinical scores (with SEM) per group (six-week-old (■) versus one-day-old (Δ) calves) from prior to (day -1) infection on day 0 until the end of the study (day 13).

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 the clinical infection around day 8, and decreased thereafter (Figure 4). Neonates revealed significantly more neutrophilic granulocytes at day 0, which decreased in the days thereafter and peaked again at day 13 (caused by one calf, calf #9069). Higher percentages of neutrophilic granulocytes were found in bronchoalveolar lung fluid samples in the young calves at days 6 ($p=0.0714$) and 9 ($p=0.0317$) post infection compared the neonates. Blood smear analyses showed overall significant ($p<0.001$) higher percentages neutrophils in neonates compared to young calves (Figure). In young calves a clear drop ($p=0.06$) in peripheral blood neutrophils was observed on study day 10 (8.2±2.7%) versus 16.4±7.0% on study day 9. BALC counts and number of macrophages and lymphocytes in lung lavage samples collected on study days 0, 3, 6, 9 and 13 are summarized in figure 4.

Bronchoalveolar lung fluid samples were analysed in the SearchLight® Bovine cytokine array for IL-4, IL-6, TNF-α and IFN-γ (Figure 6). Significant ($p<0.05$) higher levels of TNF-α were measured in young calves (overall mean 705.6 ± 304.4 pg / ml) versus neonates (overall mean 303.9 ± 109.1 pg / ml). Significant ($p<0.05$) higher levels of IL-6 were measured in neonates (overall mean 958.5 ± 231.4 pg / ml) versus young calves (overall mean 612.7 ± 169.1 pg / ml). For IL-4 a significant ($p<0.05$) was measured on study day 9. For IFN-γ a significant ($p<0.05$) difference was observed on study day 6. Higher IFN-γ BALF levels (mean: 2391 pg / ml) were measured in young calves compared to neonates (mean: 74.12 pg / ml).

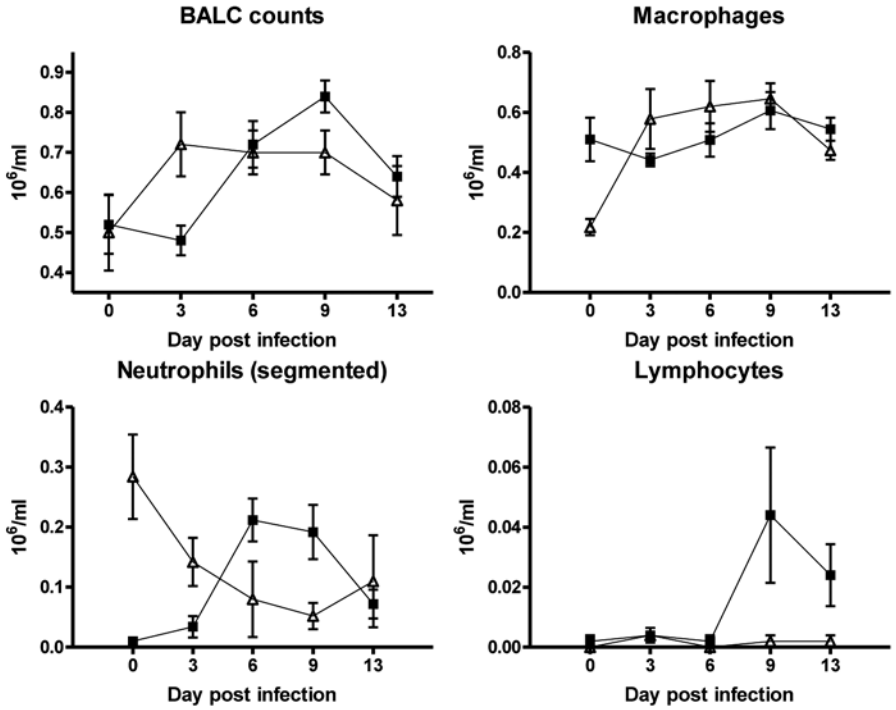

Figure 4 Mean numbers (+SEM) cells recovered (expressed as cells per ml) from BALF samples per group (six-week-old (■) versus one-day-old (Δ) calves) from prior to (day -1) infection on day 0 until the end of the study (day 13).

PaO2 in arterial blood samples directly reflect 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 with the observed clinical signs of lower respiratory tract disease and increased respiratory rates (Figure 6). This drop (mean values below 70 mmHg, reference / normal value

PaO2 is > 85 mmHg (Coghe et al., 2000)) between day 7-9 was observed in both age groups. Carbon dioxide retention was not observed.

Haptoglobin is a major acute phase protein in cattle and is considered as 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 Figure 7. Prior to inoculation, plasma lactate concentrations (mean ± SEM) were significantly ($p<0.0001$) higher in neonates (4.04 ± 0.79 mmol/L) compared with young calves (0.94 ± 0.14 mmol/L) (Figure 7). No significant differences in plasma lactate concentrations are observed between neonates and young calves.

Upon inoculation, significant ($p<0.05$) higher haptoglobin concentrations (microgram/ml) were detected in young calves (overall mean 123.5 ± SD 46.63 µg/ml) compared to neonates (overall mean 79.27 ± SD 31.73 µg/ml). A positive correlation ($r=0.2833$, $p<0.001$) between rectal body temperature and haptoglobin concentrations were observed.

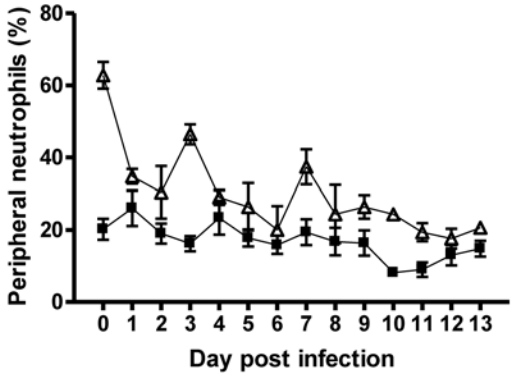

Figure 5 Mean numbers (+SEM) of peripheral neutrophils per group (six-week-old (■) versus one-day-old (Δ) calves) from infection on day 0 until the end of the study (day 13).

Pathology.

At necropsy, the overall percentages of consolidated lung tissue area (CLA) ranged from 0 to 5.1%. Mean percentage (± SD) CLA in neonates (2.35 ± 1.51%) was significantly ($p<0.05$) higher compared with young calves (0.35 ± 0.36%). When consolidated lung areas were scored as described by Viuff et al. (Viuff et al., 2002), four (n=5) neonates scored 1, while all young calves scored 0. Calves of both groups had developed multifocal bronchointerstitial pneumonia, with a marked proliferation of bronchiolar epithelium. Bronchioles were narrow and often filled with secretion. Noteworthy to mention is that calf #9069 did not have any consolidated lung area.

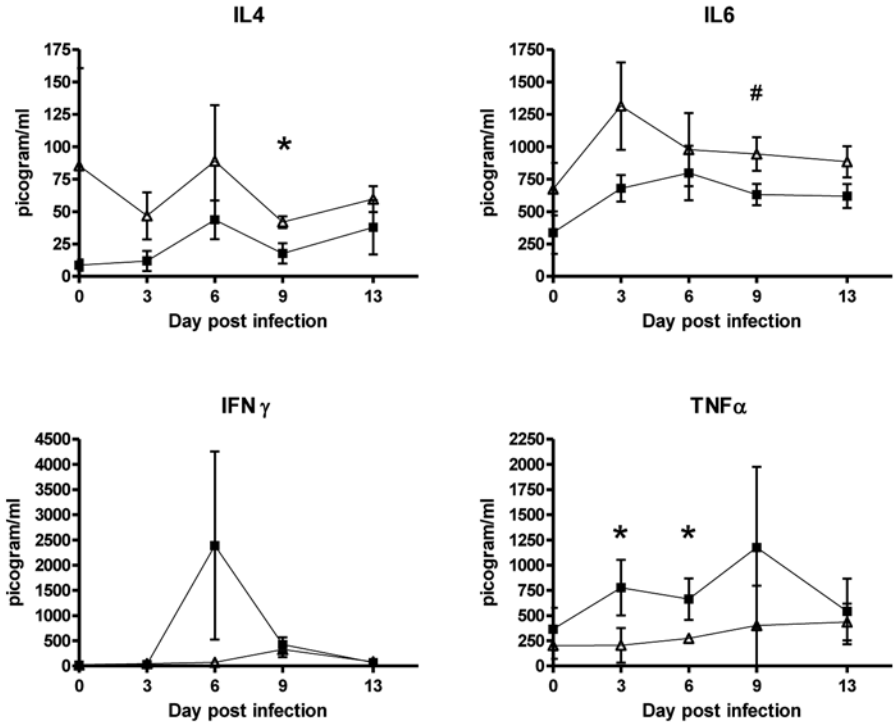

Figure 6 Mean cytokine measurements (+SEM) in BALF samples per group (six-week-old (■) versus one-day-old (Δ) calves) from prior to infection on day 0 until the end of the study (day 13). Statistically significant ($p<0.05$) time points are indicated by an asterisk (*). The calculated p-value for IL6 on study day 9 was 0.07(#).

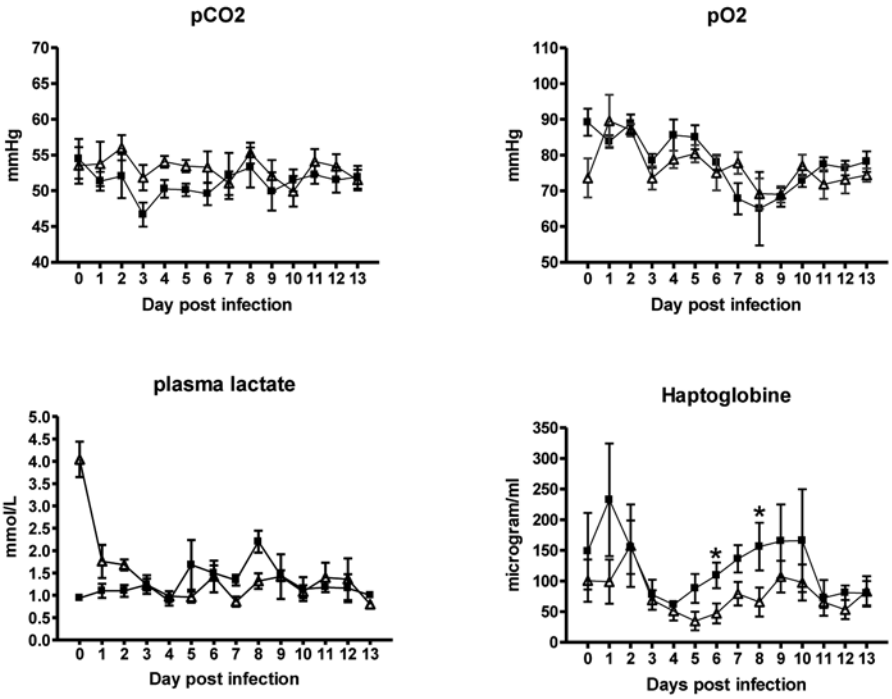

Figure 7 Mean arterial PO2 and PCO2 values, mean concentrations plasma lactate and haptoglobin (with SEM) per group (six-week-old (■) versus one-day-old (Δ) calves) from prior to (day -1) infection on day 0 until the end of the study (day 13). Haptoglobin concentrations were significantly ($p<0.05$) 6 and 8 days post infection (marked with an asterisk) higher. The blood gas status of newborn calves differences significantly from 6-week-old calves; PO2 is significant ($p<0.05$) lower in one-day (73.6±11 mmHg) versus 6-week old (89.2±7.5 mmHg) calves. There is a significant increase ($p<0.01$) in PO2 on the 2nd day of live day (89.6±14.6 mmHg) compared to the 1st day of life (73.6±11 mmHg), which is considered to be caused by the 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 is observed around day 8 post infection corresponding with the observed peak in the LRT clinical signs and respiratory rates (not shown). This drop was observed in both age groups, but was more profound in the six-week-old calves (day 7-9 mean values below 70 mmHg). Carbon dioxide retention was not observed.

Discussion

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

We observed no severe disease in the neonates upon experimental bRSV infection. Kimman et al. observed similar effects after natural infection (no clinical disease in calves irrespective of the maternal antibody titre), but hypothesised that this was due to a low virus exposure (Kimman et al., 1988). We here provide 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 more 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 one to three months-old calves when 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. Whereas 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 relation between MDA titres and RTD severity. Our findings do not support this notion, but instead suggest a role of differences in immunocompetences 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 (Coomber 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. demonstrated in BALB/c mice that a neonatal (age, 4 days) hRSV infection was associated with less inflammation and disease than in immature adults (age, 4 to 6 weeks) and suggested that this was due to different cytokine production profiles of cells recruited to the site of the infection (Tregoning et al., 2008).

To control as much as possible endogenous flora, such as Pasteurella spp., H. 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 pathologic 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 super-infections are considered to have an major effect on the course of the disease (Angen et al., 2009, Babiuk et al., 1988).

It was previously concluded by others that an influx of neutrophils in lung tissues 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 absence of a secondary bacterial super infection. Moreover, we observed an intriguing different neutrophilic infiltration in the lungs of neonatal and young calves. It is tempting to speculate that this difference is caused by different activation of proinflammatory cytokines, in particular TNF-α.

Young calves showed a clear drop in peripheral blood neutrophils on study day 10 (Figure 5). Lukens et al. described an increase in neutrophil precursors for human RSV patients between days 7 and 9 after onset of symptoms and which subsided a few days later (Lukens et al.). All Lukens patients required mechanical ventilation because of respiratory failure due to a LRTD, which could have induced an enhanced neutrophil activation. Lukens et al. started measuring 3-8 days after onset of symptoms, which would have been approx study day 10-15 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 NK-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 and a significantly ($p<0.05$) higher expression was observed on study day 6, 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.

Tumour necrosis factor alpha (TNF-α) is a proinflammatory 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 human RSV 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. for example recently published 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 (You et al., 2006). A tight regulation of TNF-α levels might therefore be essential to separate its beneficial from its harmful effects. Indeed, our data also show that young calves with more severe clinical disease had significantly higher levels TNF-α compared to newborn calves, indicating that alveolar macrophages (AM) from young calves produce more TNF-α than AM cells from newborn calves. Neutrophils respond to TNF-α with a respiratory burst after a β2-integrins-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). Altogether our study points to a role of innate immunity in RSV disease, a conclusion that was also drawn from genetic association studies 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. published substantial evidence that both innate and acquired defence mechanisms are lowest from 3 wk pre-calving to 3 wk post-calving (Mallard et al., 1998). Mallard et al. 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. reported that the innate response mediated by phagocytic cells (neutrophils and macrophages) declines in functional capacity around birth, due to an increase in foetal cortisol levels (Chase et al., 2008). 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 to 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. Significant ($p<0.05$) lower neutralizing serum antibody titres appeared within thirteen days post infection in neonates compared to 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 gradually acquired 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 number 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 proinflammatory immune response, are responsible for the differences in clinical appearances 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 immuno-incompetence. This is despite the fact that virus replicates to higher levels in neonates than in six week older (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.

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Chapter 7

Vaccination with recombinant Modified Vaccinia virus Ankara expressing bovine respiratory syncytial virus (bRSV) proteins protects calves against RSV challenge.

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Vaccination with recombinant Modified Vaccinia virus Ankara expressing bovine respiratory syncytial virus (bRSV) proteins protects calves against RSV challenge.

Abstract

Respiratory syncytial virus (RSV) is a major cause of severe respiratory disease in infants and calves. Bovine RSV (bRSV) is a natural pathogen for cattle, and bRSV infection in calves shares many features with the human infection. Thus, bRSV infection in cattle provides the ideal setting to evaluate the safety and efficacy of novel RSV vaccine strategies. Here, we have evaluated the efficacy and safety of modified vaccinia virus Ankara (rMVA)-based vaccine candidates, expressing the bovine RSV-F protein, either or not in combination with the G protein, in colostrums-deprived SPF calves born by caesarean section. Vaccination induced bRSV-specific IgG and CD8 T cell responses. Importantly, no IgE responses were detected. After bRSV challenge, rMVA vaccinated calves experienced less severe symptoms of lower respiratory tract disease compared to the mock-immunized control group. Immunized animals showed reduced pulmonary virus loads, and no eosinophilic infiltration or enhanced respiratory distress. In conclusion, candidate rMVA/bRSV vaccines induced protective and safe immune responses in calves.

Introduction

Respiratory syncytial virus (RSV) infections are a cause of respiratory tract diseases in both calves and humans. Primary RSV infection in early life, but also re-infections late in life, can cause severe lower respiratory tract disease, manifesting as bronchiolitis or pneumonia. Therefore, a safe and effective vaccine is urgently needed. However, vaccine development against hRSV has been hampered by a dramatic vaccine failure in the 1960s: vaccination with formalin-inactivated (FI), alum-adjuvanted virus predisposed children to an enhanced RSV disease after subsequent infection. Interestingly, the epidemiology and pathogenesis of bovine (b) RSV infection in calves closely resembles the human (h) RSV infection in young infants, and children. Indeed, vaccine-induced immunopathology was reproduced in our natural-host bRSV model [1]. Thus, bRSV models can be used to obtain a better understanding of RSV disease as well as vaccine-induced enhanced disease, but also to test new RSV vaccine candidates.

In general, viral vaccines can be divided into two broad categories: live-attenuated vaccines, and inactivated or ´killed´ vaccines. Live- attenuated vaccines usually induce long-term immunity. Indeed, some of the most successful vaccines known

to medicine (smallpox and yellow fever virus vaccines) are live-attenuated vaccines. However, development of a live-attenuated RSV vaccine has met several obstacles. First, there are safety concerns, including the potential risk of residual virulence, persistence in the host, shedding into the environment, reversion to more virulent strains and contamination with other viruses such as bovine viral diarrhoea virus (BVDV). Second, there may be significant limitations to industrial scale vaccine production as bRSV does not replicate very efficiently *in vitro*, and specific care has to be taken to maintain standardized infectivity of virus preparations. Finally, the development of live-attenuated vaccines is also hampered by the difficulty to find a proper balance between attenuation and immunogenicity [2]. Contrary, inactivated vaccines should be safer in terms of risk due to residual live virus, yet the duration of protective immunity can be limited, and most importantly, immune responses elicited by inactivated vaccines have been associated with a predisposition for enhanced disease upon RSV infection. Safety considerations, together with practical limitations due to poor in vitro replication have prompted the development of new generation RSV vaccines. The latter approaches include live viral vectors expressing RSV candidate genes, e.g. vector vaccines based on modified vaccinia virus Ankara (MVA). MVA was been obtained by serial passage of vaccinia virus (more than 570 passages) in primary chicken embryo fibroblasts (CEF) cells [3] resulting in a severely restricted cellular host range; MVA replicates well in vitro in avian cells, but is largely replication deficient in human and other mammalian cells. MVA vaccines have a favourable safety profile when used for orthopoxvirus-specific immunization [4-7] and can safely be used as a basis for recombinant human vaccines, even in immunocompromised individuals [8-10]. When used as a vector vaccine, MVA can provide similar levels of recombinant gene expression as compared to the fully replication competent strains of vaccinia virus and induces equal or better humoral and cellular immune responses [11-13]. The replication-deficient MVA vectors can be used under conditions of bio safety level 1 and several MVA recombinant vaccines have already entered clinical evaluation [14-17].

Vaccination of mice with recombinant MVA (rMVA) expressing the hRSV transmembrane glycoproteins F, G or both F and G, resulted in strong specific antibody responses and protection from challenge [18]. Vaccination of young macaques with recombinant MVA expressing the hRSV transmembrane glycoproteins F and G (rMVA-F and rMVA-G), did not predispose them for immunopathology, but also failed to confer protection. However, but the rMVA viruses used were not very immunogenic [19].

In the present study we have vaccinated seronegative calves with MVA expressing the bRSV F and G proteins (rMVA-F, and with rMVA-F plus rMVA-G), using calves vaccinated with formalin inactivated (FI-) bRSV and mock-immunized calves as controls.

Materials & methods

Construction of MVA recombinant viruses

MVA vectors were generated using a well established protocol based on transient K1L-based host range selection as described previously [20-23]. Briefly, for construction of recombinant viruses the bRSV-F gene sequence was excised from plasmid peVNeo 14 (kindly provided by Dr J.P.M. Langedijk) and the bRSV-G ORF was obtained from plasmid P-bRSV-Gsyn (kindly provided by Dr G.M. Keil, [24]) and inserted into the MVA transfer vector plasmids pIII-dHR-P7.5 and pVI-dHR-PH5, respectively. Upon transfection of these plasmids in MVA-infected CEF (chicken embryo fibroblasts) cells the bRSV-F gene was inserted into the site of deletion III of the MVA genome being placed under the transcriptional control of the natural vaccinia virus early-late promoter P7.5. The bRSV-G gene sequence was inserted into the site of deletion VI and expressed using modified vaccinia virus early-late promoter PH5 [25]. Clonal recombinant viruses were obtained during plaque passages on rabbit kidney (RK-13) and CEF cells. Final vector viruses were characterized for satisfying in vitro growth capacity (one-step and multi-step growth analysis) and quality controlled for genetic stability and recombinant gene expression using PCR analysis of genomic viral DNA and immune detection of bRSV G or F proteins produced upon infection (data not shown).

Vaccine preparation

Formalin inactivated (FI-) vaccine was prepared as described earlier[1]. Briefly; bRSV was cultured on embryonic bovine trachea (EBTr) cells. After clarification, supernatant was formalin treated (37% formaldehyde solution, 1:4,000 at 37°C for 72 hours), followed by ultracentrifugation. A vaccine dose consisted of 1ml sedimented virus material (at a protein concentration of 0.75mg per ml), mixed with 1ml home-made 2% $Al(OH)_3$. For the second (booster) vaccination, this vaccine batch was supplemented with a second vaccine batch, which was produced slightly different from the first batch. Virus material was formalin treated after ultracentrifugation. For the second vaccination: 2.85mg (in 1ml) from the first vaccine-virus batch was mixed with 2.17mg (in 0.61ml) vaccine-virus produced in the second batch. The mixed inactivated vaccine-virus materials were further diluted in PBS to a final protein-concentration of 0.75 mg/ml and mixed (1:1) with 2% $Al(OH)_3$ as described above.

Vaccine preparations of recombinant MVA were obtained as described previously [21,23]. Briefly, viruses were grown on large scale CEF cell cultures, purified by ultracentrifugation through a 36% sucrose cushion and a 20 to 60% sucrose gradient. Purified vector viruses were resuspended in 1mM Tris-HCl (pH 9.0), titrated, reconstituted in saline buffer (10 mM Tris pH 7.4, 140 mM NaCl) suitable for immunization and stored at -70°C.

Study design

Twenty-four caesarean-section-derived and colostrum-deprived (CD-CD) specific-pathogen-free (SPF) calves were reared and housed in separated isolation rooms. The calves were free of BVDV, as shown by BVDV antigen enzyme-linked immunosorbent assay (ELISA) (SERELISA BVD/MD/DB Ag; Synbiotics Europe). The calves were housed in isolation units holding three animals each. Starting at an age of 4 weeks, four groups of six calves were immunized twice at a three-week interval intramuscularly with either formalin-inactivated (FI-) bRSV (strain Lelystad) absorbed to $Al(OH)_3$, with 10^8 pfu (plaque forming units) of rMVA-F or with 10^8 pfu rMVA-F plus rMVA-G, or with phosphate buffered saline (PBS) as control. We choose to omit the empty-MVA control group, based on budgetary constraint and because the objectives of the study were to assess the induction of humoral and cellular immune responses by vaccination as well as the efficacy in reducing viral excretion and clinical disease after challenge infection. We placed a, with special emphasis on vaccine induced immunopathology.

Calves were challenged with bRSV (2 ml, at $10^{3.9}$ $TCID_{50}$/ml) four months after the second immunization. The animals were inoculated, as described previously [1], with bRSV strain Odijk, which had been isolated from a calf in a herd with severe respiratory tract disease. bRSV strain Odijk was in vivo passaged several times, as described elsewhere[26], and tested for absence of major bovine pathogens. All animal experiments were conducted in accordance with the Act on the use of Experimental Animals of The Netherlands supervised by the Ethical Review committee of the Animal Sciences Group.

Immunogenicity

To assess immunogenicity of the different vaccine preparations, we measured MVA and bRSV-specific humoral and cellular immune responses after vaccination. Calves were bled from the jugular vein once-a-week for serology, and sera collected on days -2, 19, 40, 61, 82, 103, 124, 131 and 141 were tested in commercially available double-antibody-sandwich ELISA (Cedi Diagnostics, Lelystad, The Netherlands), which detects antibodies directed against bRSV, a blocking G-peptide ELISA as described by Langedijk et al. [27], and a virus neutralizing antibody assay, carried out as described by Langedijk et al. [27]. CD8 T cell responses were evaluated approx three months (day 96) after the first vaccination. This time point was chosen to obtain an estimate of long-term vaccine-induced T cell immunity. To measure CD8 T cell responses, peripheral blood mononuclear cells (PBMC) from all calves were stimulated with MVA-F, MVA-G, or with wild-type MVA [28]. Un-stimulated cells were used as a negative control. PBMC were stimulated for 24 hours. Brefeldin A was added during the last 6 hours of stimulation, after which responding CD8 T cells were visualized by intracellular IFN-γ staining [29]. After surface and intracellular staining, cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry.

Parameters for bRSV vaccine efficacy and safety

In compliance with the European Pharmacopoeia (2005), a bRSV vaccine is determined as efficient if a significant decrease in bRSV secretion (in terms of amount of virus, and or duration of bRSV shedding) or a significant decrease of clinical symptoms is shown. For the clinical assessment, calves were examined once a day before challenge (from day -3 to day 0, the day of challenge) and twice a day thereafter (from day 0 until day 10 post-infection).

Calves were examined for signs of an upper respiratory tract disease; ocular discharge (runny eyes), nasal discharge, coughing, trachea sensitivity and respiratory noise (stridor), and signs of a lower respiratory tract disease; including respiratory rates, respiratory distress (dyspnoea, exaggerated intercostals and/or abdominal breathing, and irregular breathing) and lung auscultation. The severity of the clinical disease was expressed by allocating weighing factors to each observation as previously described [1]. Virological examinations were only performed on bronchoalveolar lung fluid (BALF) samples, as described previously [1,26]. BALF samples were collected on study days 128 (three days prior to challenge virus inoculation), 132, 135, 138 and 141 (1, 4, 7 and 10 days post challenge virus inoculation respectively). For the pathological assessment, blood samples were taken to determine the haptoglobin concentration (tested in serum) and lactate concentrations to classify the level of severity of disease using a portable blood lactate analyzer [30,31]. Post mortem examination included evaluation of the extent of macroscopic lesions which were scored according to Viuff et al. [32]. Necropsy samples from ten predetermined sites were collected and samples of three different lung sections (right cranial lobe, right caudal lobe and left cranial lobe) were used for histological examination. The severity of the microscopic changes was expressed by allocating weighing factors, varying from 1 (for minimal) to 5 (very severe), for ten observations per sample site. Since protection is associated with (local) memory responses, we took nasal fluid (NF) and bronchoalveolar lung fluid (BALF) samples, to detect local anti-bRSV specific IgA antibodies after challenge infection [33]. The vaccine‟s safety judgement was based on the absence of clinical signs after the challenge infection indicating an enhanced disease, absence of eosinophilic granulocytes in BALF samples, absence of an abundance of eosinophilic granulocytes in necropsy samples, and absence of specific IgE antibodies, which were analyzed using (i) an isotype-specific antibody capture assay and (ii) a standard bRSV ELISA which was precede by IgE affinity purification [34].

Methods of Statistics

The GraphPad Prism® software was used for analysis of the data. The repeated measures ANOVA and non parametric tests were used for comparison of the different groups. Level of significance was set at $p<0.05$.

Results

Immunization with rMVA-F and rMVA-F/G

Calves (n=6 per group) were immunized twice with 10^8 pfu of either rMVA-F or a cocktail of rMVA-F and rMVA-G at a three-week interval. Control animals were immunized with PBS. As a control for vaccine safety, a fourth group was immunized with FI-bRSV.

Vaccination with rMVA resulted in significant levels of vaccinia virus-specific antibodies in all MVA vaccinated animals (data not shown). The immunogenicity of the candidate bRSV vaccines was assessed by detecting total bRSV-specific and bRSV G specific antibody responses, and by measuring virus neutralizing antibody titers. BRSV-specific antibodies were first detected approximately three weeks (day 19) after the first vaccination (Figure 1). As expected, none of the rMVA-F vaccinated calves had G specific antibodies before challenge virus inoculation (day 131), whereas all rMVA-F/G vaccinated calves seroconverted after vaccination as determined in the G-peptide ELISA (Figure 1). All vaccinated calves had virus neutralizing antibodies, detected from day 40 until challenge infection at day 131.

As shown in Figure 2, MVA immunization induced substantial levels of MVA-specific memory CD8 T cells (0.53% of CD8 T cells, SEM = 0.23) in PBMC. To assess F-specific responses, we compared the CD8 T cell responses, based on intracellular IFN-γ staining, from immunized animals after in vitro stimulation with either MVA-F or wt MVA. The difference between these two should provide an estimate for the F-specific response. We observed that in vitro stimulation with MVA-F resulted in significantly ($P < 0.05$ by Students‟ t test) higher frequencies of IFN-γ-producing CD8 T cells (1.27% (SEM = 0.5~2-fold higher) than stimulation with either wt MVA (0.53%), yielding a difference of 0.73%, which should represent the or MVA-G in the MVA-F-specific response. immunized group ($P<0.05$ by Student‟s t test), but not in the other groups. This result indicates that priming with MVA-F results in the establishment of not only MVA-specific memory CD8 T cells, but also bRSV F-specific CD8 T cells. In present study we did not include assays aiming at detection of specific CD4+ T cell responses.

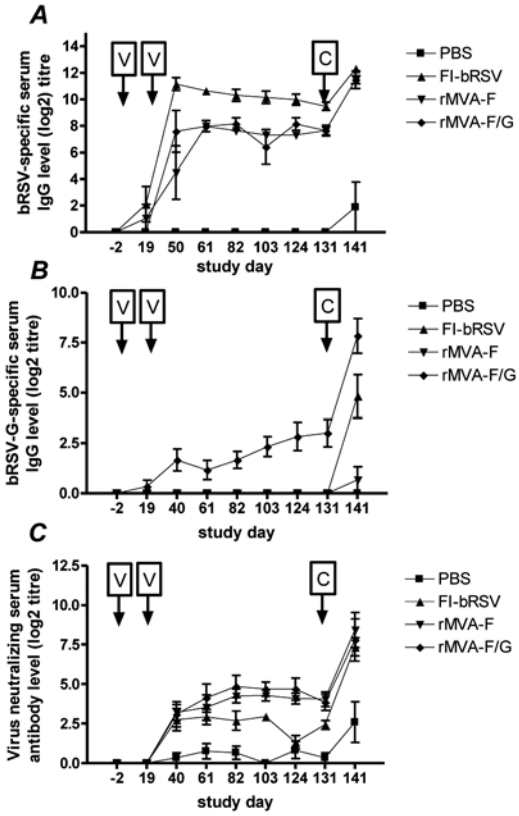

Figure 1 Average bRSV antibody titres with error bars (SEM), determined in blood samples collected at three-week intervals (days -2, 19, 40, 61, 82, 103, and 124) around vaccination (day 0 and 21), before challenge (day 131) and at the end of the in life phase (day 141). Six calves mock immunized (PBS), six control calves vaccinated with a formalin inactivated bRSV vaccine (FI-bRSV), and twice 6 calves vaccinated with a candidate recombinant vaccine (either rMVA-F or rMVA-F/G). The immunogenicity of the candidate bRSV vaccines was assessed by detecting total bRSV-specific (panel A) and bRSV G specific antibody responses (panel B), and by measuring virus neutralizing antibody titres (panel C). BRSV-specific antibodies were first detected approximately three weeks (day 19) after the first vaccination. As expected, none of the rMVA-F vaccinated calves had G specific antibodies before challenge virus inoculation (day 131), whereas all rMVA-F/G vaccinated calves seroconverted after vaccination as determined in the G-peptide ELISA (panel B). All vaccinated calves had virus neutralizing antibodies, detected from day 40 until challenge infection at day 131.

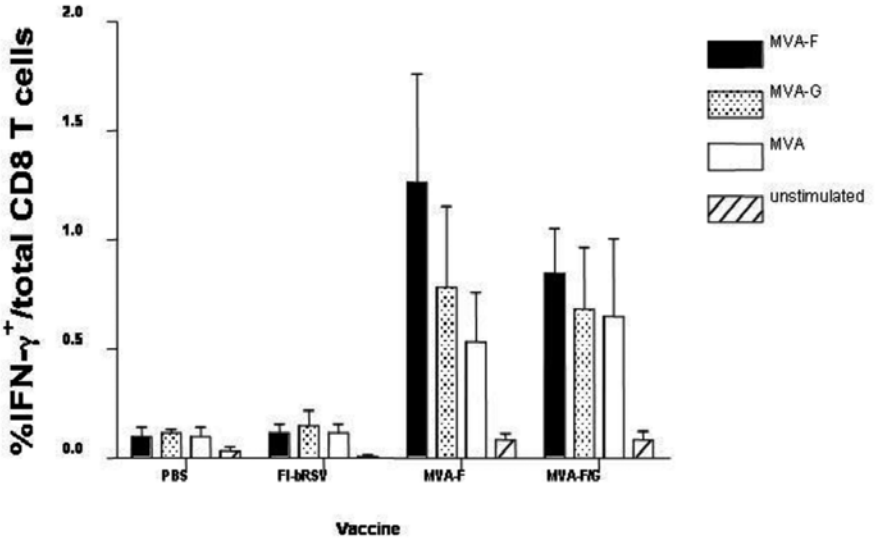

Figure 2 T cell responses post vaccination. CD8 T cell responses measured in blood samples collected at day 96. Peripheral blood mononuclear cells (PBMC) from all calves were stimulated with MVA-F, MVA-G or with wild-type MVA. Indicated are responses from the four experimental groups (PBS, FI-bRSV, MVA-F, MVA-F/G) after stimulation with MVA-F, MVA-G, MVA and PBS (un-stimulated). Different stimulations are indicated by different bars. Un-stimulated cells were use as negative control. MVA-immunized calves had relatively high levels of MVA-specific memory CD8+ T cells (0.53% of CD8+ T cells, n=12). Significant higher F-specific responses were measured in rMVA-F immunized calves.

BRSV challenge infection.

To assess the protective efficacy of the rMVA vaccines, all animals were challenged with bRSV four months after the last vaccination. Clinical assessment was done on the basis of the following five parameters: (i) general clinical symptoms, (ii) upper respiratory tract disease, (iii) lower respiratory tract disease, (iv) rectal temperature, and (v) breathing frequency. Clinical signs were observed from day 3, and were most severe in FI-bRSV immunized calves (Figure 3). The greatest increase of clinical signs was observed in the FI-bRSV immunized calves. The most obvious benefit of vaccination with rMVA-F or rMVA-F + rMVA-G was observed in the alleviation of lower respiratory tract disease (LRTD) symptoms (Figure 3). Thus, rMVA-F and rMVA-F+MVA-G significantly reduced LRTD symptoms compared to mock immunized animals ($P<0.01$ for rMVA-F, $P<0.005$ for rMVA-F/G). The duration of the LRTD was reduced in the rMVA-F/G immunized calves with a trend towards significance ($P=0.0693$).

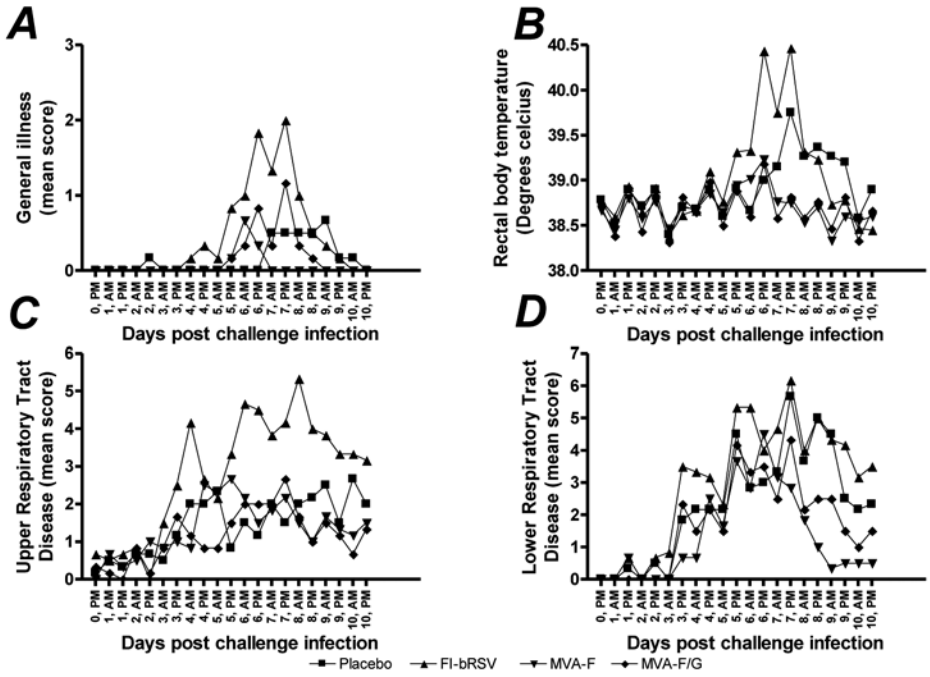

Figure 3 Clinical evaluations. Average clinical scores per group determined twice daily (AM and PM) from three days prior to challenge until the end of the study. Evaluation of the general illness (panel A) showed that the mean score of FI-bRSV vaccinated calves was significantly higher compared to mock-immunized calves (P<0.01), rMVA-F vaccinated calves (P<0.001) and rMVA-F/G (P<0.05). Evaluation of the rectal temperatures (panel B) showed that mean rectal temperatures of rMVA-F and rMVA-F/G immunized calves were significantly reduced compared to the mock immunized group (P<0.05 and P<0.005, respectively). Evaluation of the clinical signs of an upper respiratory tract disease (panel C) showed that the mean score of FI-bRSV vaccinated calves was significantly (P< 0.001) higher compared to mock-immunized calves, rMVA-F vaccinated and rMVA-F/G calves. Evaluation of the clinical signs of a lower respiratory tract disease (panel D) showed that the mean score of FI-bRSV vaccinated calves was significantly higher compared to mock-immunized calves (P<0.05), rMVA-F vaccinated calves (P<0.001) and rMVA-F/G vaccinated calves (P<0.001). Mean clinical scores of the rMVA-F vaccinated calves were significantly lower compared to the mock vaccinated calves (P<0.001).

Evaluation of the rectal temperatures showed that mean rectal temperatures of rMVA-F and rMVA-F/G immunized calves were significantly reduced compared to the mock immunized group (P<0.05 and P<0.005, respectively). All calves were found to have an increased respiratory rate (RR>40) varying from 1 to 9 days. The respiratory rates were significantly lower in the rMVA-F/G immunized group compared to the mock immunized animals (P<0.05). No significant improvement was observed for the rMVA-F vaccinated group. In summary, vaccination with rMVA-F/G led to improvements in three of the five parameters for clinical symptoms after bRSV challenge compared to control animals, whereas vaccination with rMVA-F resulted in alleviated LRTD symptoms (P<0.001) and reduced rectal temperatures. However, when compared to FI-bRSV immunized animals,

vaccination with either vaccine preparation resulted in significant improvements in all five clinical parameters (P<0.05). Indeed, FI-bRSV vaccinated calves developed an enhanced clinical response FI-after bRSV challenge infection (Figure 3). This shows that the enhanced disease phenomenon described earlier [1] was reproducible and that this group provided a suitable control for the evaluation of vaccine safety. Mean total clinical score was significant higher compared to all other groups (P<0.05). Furthermore, FI-bRSV animals experienced significantly stronger upper and lower respiratory tract disease (P<0.001 and P<0.05, respectively) compared to all other groups and had significantly higher mean daily respiratory rates compared to both rMVA vaccinated groups (P<0.01).

The kinetics of viral replication was analyzed by virus isolation from the bronchoalveolar lung lavage fluid samples collected 1, 4, 7 and 10 days post challenge virus inoculation (dpci) (Figure 4). BRSV was detected by virus isolation in four (out of six) mock-immunized calves (one calf on dpci 4 and four calves on dpci 7). Virus was detected in two (out of six) FI-bRSV immunized calf on dpci 7 and in one (out of six) rMVA-F immunized calf on dpci 10. No virus was isolated in any of the collected BALF samples of rMVA-F/G immunized calves.

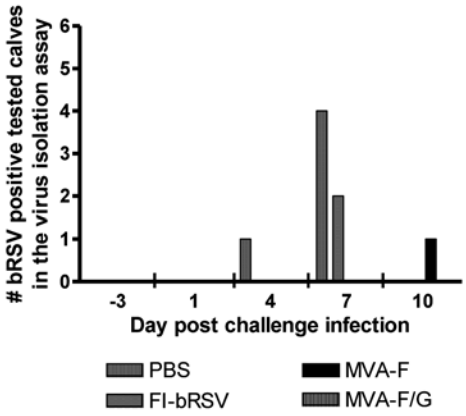

Figure 4 Total number of bRSV positive tested calves per group in the bRSV virus isolation assay. Bronchoalveolar lung lavage fluid samples, collected 1, 4, 7 and 10 days post challenge virus inoculation (dpci), were analyzed in a virus isolation assay. BRSV was detected in four (out of six) mock-immunized calves (one calf on dpci 4 and four calves on dpci 7). Virus was detected in two (out of six) FI-bRSV immunized calf on dpci 7 and in one (out of six) rMVA-F immunized calf on dpci 10. No virus was isolated in any of the collected BALF samples of rMVA-F/G immunized calves.

Acute phase responses and plasma lactate concentrations

The severity of respiratory disease was further assessed by analysing the acute phase responses (haptoglobin) and plasma lactate concentrations (PLC) (Figure 5). The magnitude of the haptoglobin response and PLC response did not correlate with the severity of disease in the control group (mock-immunized calves). However, aptoglobin concentrations were higher post challenge infection in the FI-bRSV vaccinated group ($P<0.01$) compared to the mock-immunized group from day 6 to 9. Compared to FI-bRSV immunized calves, haptoglobin responses were significantly lower ($P<0.01$) after vaccination with rMVA-F/G, confirming that rMVA vaccination did not predispose animals for enhanced disease. PLC levels were significantly lower ($P<0.01$) in rMVA-F treated calves post challenge infection compared to mock (PBS) immunized calves.

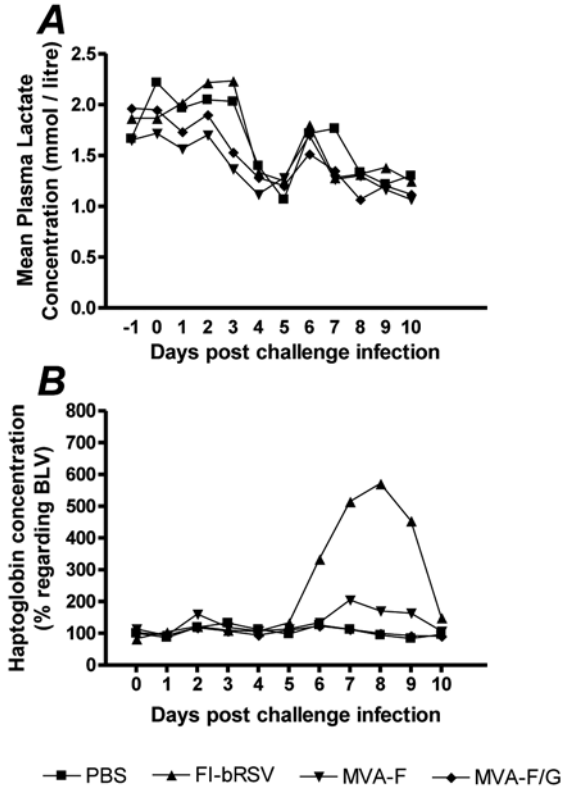

Figure 5 Haptoglobin and plasma lactate analysis. Plasma lactate levels post challenge infection in general were significantly lower in rMVA-F treated calves compared to mock-immunized calves and FI-bRSV vaccinated calves ($P<0.01$). Mean haptoglobin concentrations were significantly higher in FI-bRSV vaccinated ($P<0.01$) calves compared to the mock immunized calves and rMVA-F/G ($P<0.001$) vaccinated calves. It is interesting to note that haptoglobin levels seem to differentiate symptomatic from non-symptomatic animals, suggesting that haptoglobin could be used as a rapid monitor for disease.

Pathology

At necropsy, the percentage of abnormal lung surface area ranged from 0 to 11.7% within the entire group of 24 calves. Macroscopically no consolidated lung areas (CLA) were observed in any of the rMVA-F or rMVA-F/G immunized calves. Evaluation of the CLA showed mean consolidated lung areas of the mock-immunized calves of 5.5% (±4.2%). CLA was significantly reduced in the rMVA-F and rMVA-F/G immunized groups compared to the control group. When consolidated lung tissue areas were scored as described by Viuff et al. [32], three (out of six) mock-immunized calves were scored 2 (5 to 15% CLA) and three were scored 1 (1-5% CLA).

Mock-immunized calves developed bronchitis, often with peribronchial interstitial infiltrates, interstitial hypercellularity accompanied with pneumonia. Significantly less ($p=0.002$) abnormalities were scored in rMVA-F and in rMVA-F/G treated groups. Two rMVA-F immunized calves, but none of the rMVA-F/G immunized calves, developed bronchitis after challenge infection. Pneumonia was not detected in any of the rMVA immunized calves.

Ten days post challenge infection, IgA antibodies were detected in bronchoalveolar lung fluids of none of the mock immunized calves, in all six FI-bRSV immunized calves, in one rMVA-F immunized calf and in two rMVA-F/G immunized calves (data not shown).

Evaluating vaccine safety: post-challenge eosinophilia and IgE responses

Dysregulated Th2-type responses are a hallmark of FI-vaccine mediated enhanced disease [1,34-36]. Therefore, we analyzed the presence of eosinophilic granulocytes in the bronchoalveolar lung lavage samples and in the lung tissue and IgE responses after vaccination and after challenge infection to assess the safety of the rMVA vaccines.

Analysis of the cell populations in the BALF and PBMC samples revealed a strong eosinophilic granulocyte infiltration in FI-bRSV immunized calves (Figure 6). Mean percentages eosinophils in FI-bRSV immunized calves were 27.5% (±17.76%) on day 7 post challenge infection and 17.5% (±13.6%) on day 10 post challenge infection. The number of eosinophils observed on these days was significantly higher in the FI-bRSV immunized group compared to the mock-immunized group. In the rMVA-F and rMVA-F/G immunized groups we observed significantly less eosinophilic infiltration: 0% and 0.33% (±0.47%) for days 7 and 10, respectively, in the rMVA-F immunized group, and 1.33% (±1.37%) and 3.0% (±3.74%) for days 7 and 10, respectively, in the rMVA-F/G immunized group. Unbound IgE antibodies were detected in serum samples of 5 (out of 6) FI-bRSV immunized calves two weeks after the first vaccination (data not shown), after challenge infection in two of the FI-bRSV immunized calves an IgE booster response was observed. In none of the rMVA-F and rMVA-F/G immunized calves an IgE response was detected after vaccination. Weak IgE responses were detected at ten days post challenge

infection, with low percentage positivity values, varying from 5.3 to 8.1. These values were significantly lower compared with the FI-bRSV vaccinated group (Figure 6).

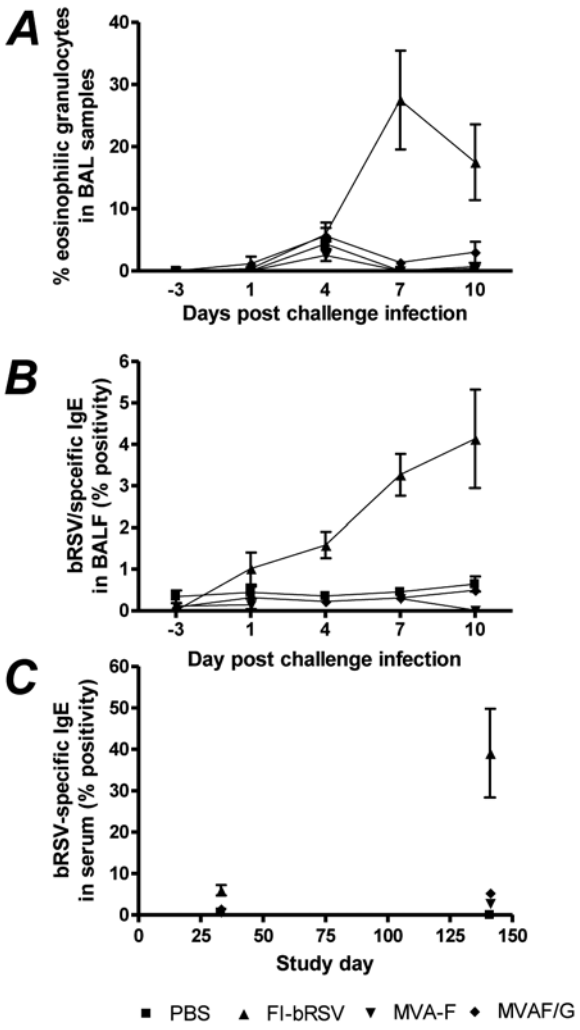

Figure 6 Markers of allergy. Mean percentages with error bars (SEM). Analysis of the cell populations in the BALF samples revealed a strong eosinophilic granulocyte infiltration in FI-bRSV immunized calves (panel A). The number of eosinophils observed on these days was significantly higher in the FI-bRSV immunized group compared to the mock-immunized group. In the rMVA-F and rMVA-F/G immunized groups we observed significantly less eosinophilic infiltration. BRSV-specific IgE antibodies were detected after vaccination, and after challenge infection in FI-bRSV immunized calves an IgE booster response was observed (panel C). Weak IgE responses were detected in rMVA-F and rMVA-F/G immunized calves at ten days post challenge infection, with low percentage positivity values, varying from 5.3 to 8.1. These values were significantly lower compared with the FI-bRSV vaccinated group (panel C).

Discussion

In the present study, we demonstrated the immunological safety and efficacy of two recombinant vaccinia virus based candidate vaccines; one vaccine consisting of modified vaccinia-virus Ankara (MVA) expressing the transmembrane bRSV protein F (rMVA-F), whereas the second comprised rMVA-F plus a construct expressing the bRSV glycoprotein G (rMVA-G), referred to as (rMVA-F/G). Both vaccines were evaluated in seronegative calves in our bRSV/cattle model, and were compared with mock-immunized calves to determine vaccine efficacy, and with FI-bRSV to evaluate their safety.

Vaccination with both rMVA preparations resulted in significant levels of vaccinia-virus specific antibodies. Furthermore, rMVA-F vaccination resulted in bRSV specific antibodies (ELISA and VNA), but no bRSV-G specific antibodies. Vaccination with rMVA-F/G also induced antibodies against the bRSV- glycoprotein G. F-specific CD8 T cell memory was measured after vaccination with rMVA-F, but surprisingly, was below detection limit after vaccination with rMVA-F/G. A possible explanation for this puzzling result may be the inherent immunodominance of endogenous MVA epitopes [37,38]. This immunodominance may be even more pronounced in the setting of a mixed infection, i.e., rMVA-F/G, in which a significant proportion of viruses does not contain F (i.e., MVA-G). It seems likely that the presence of F-negative viruses results in a more dominant MVA-specific response.

Both rMVA-F and rMVA-F/G provided protection against bRSV challenge infection, most strongly so with respect to symptoms of LRTD and fever (rectal temperatures). Moreover respiratory rates were lower in rMVA-F/G immunized calves. Thus, both rMVA vaccines clearly alleviated clinical symptoms. Although rMVA-F/G vaccination resulted in significant improvements in three of the five clinical criteria, compared to two for rMVA-F, the latter was associated with a lower overall clinical score and LRTD.

Our current data extend findings reported by Taylor et al.[39], that recombinant vaccinia viruses expressing F, G or N (used at the same dose as our MVA recombinants) induced bRSV-specific antibody responses, T cell responses and provided protection against challenge with the virulent Snook strain of bRSV. These data imply that neutralizing antibodies (which were only induced by vv-F immunization) are not per se required for protection, stressing the potential importance of anti-bRSV T cell responses. The results reported by Taylor and co-workers also imply that MVA-based vaccines provide a promising alternative to vaccinia virus based vaccines.

Our key finding, i.e. that rMVA-based vaccines reduce the severity of post-challenge symptoms, differs from results published by the Waal et al. in the hRSV/macaque model [19]: these authors evaluated the efficacy of an rMVA-F/G vaccine in a hRSV model in infant macaques. In this study the recombinant MVA constructs used were substantially less immunogenic than in our study. A disadvantage of the macaque model is that these animals are not naturally susceptible for RSV and do not show URTD and LRTD. Protection in this model is

evaluated on the basis of pulmonary viral loads, and these were not reduced after vaccination with rMVA-F/G in this study. The authors proposed that the relative high challenge dose (10^6 $TCID_{50}$) or the relatively young age (31±18 weeks old, [40]) of the animals could be the underlying causes of the lack of efficacy. However, based on the assertion that RSV disease and vaccine induced enhancement is a particular problem during infancy, it would be better to reproduce this disease in a critical age range, and in a natural host model such as bRSV in cattle, as presented here. Both Wyatt et al. and Olszewska et al. studied the immunogenicity of the recombinant MVA based RSV vaccines in BALB/c mice, and got ambiguous results [18,41]. Wyatt et al. did show that recombinant MVA based RSV vaccines expressing the (human) RSV F or G protein were highly immunogenic in their mice, inducing higher RSV antibody titers than achieved by infection with RSV and greatly restricted the replication of RS challenge virus in both the upper and lower respiratory tracts. Also Olszewska et al. showed that the MVA vector vaccines reduced viral replication in the lungs and further showed that these MVA vector vaccines induced a balanced IgG1 and IgG2a response, higher levels of IL-12 in the lung, more IFNγ-producing cells and fewer cells making IL-4 and IL-5 compared to FI vaccines. But despite this favourable immune profile, vaccinated BALB-c mice did experience weight loss similar or greater that those vaccinated with FI-RSV.

In both humans and cattle, inactivated RSV whole-virion vaccines predispose to exaggerated responses after challenge infection. The enhanced responses are characterized by severe clinical symptoms, and increased pulmonary inflammation and eosinophilia [42-46]. Here, we show that vaccination with our candidate rMVA vaccines did not cause severe adverse reactions and did not predispose the calves for immunopathology: no IgE antibodies were detected post vaccination and no excessive eosinophilic infiltration into the lungs was observed post challenge infection. The most likely explanation for these findings is that recombinant MVA vaccines elicit Th1 based immune responses [41,47], and provide a more favourable context to bRSV antigens. In contrast, formaldehyde-treated RSV vaccine antigens amplify Th2-helper responses as shown by Moghaddam et al. [48]. Moreover, clinical symptoms and virus replication in the lungs post-challenge infections were reduced in the rMVA-F and rMVA-F/G immunized groups suggesting protective efficacy of vaccination.

An additional advantage of using recombinant MVA based bRSV vaccines is, that these vaccines have DIVA vaccine properties whilst using e.g. a RSV N-specific ELISA as developed by Samal et al. [49], which could be an important tool in assessing the effectiveness of a bRSV vaccination programme.

Overall, our data clearly indicate that further investigation of bRSV-specific MVA vector vaccines is warranted. Indeed, recombinant MVA could be a suitable tool to develop a candidate veterinary vaccine against infectious respiratory tract disease in calves and juvenile cattle. MVA can be readily produced at a commercial scale and vector technologies are sufficiently advanced to consider the development of multivalent vectors co-delivering antigens from different pathogens being of concern. A possible improvement would be the use of rMVA vaccine in a DNA/MVA

prime-boost vaccination schedule. Prime-boost vaccination has shown to augment specific immunity [47] and the combination of DNA priming with MVA boosting is a particularly effective one. Similar to MVA, clinical grade DNA plasmids can be readily produced on a commercial scale. On the basis of our work, the obvious choice is to use plasmids expressing the bRSV F and G proteins.

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Chapter 8

Summarising discussion

Summarising discussion

Human and bovine RSVs cause severe disease in humans and in cattle respectively. They have been recognised as important respiratory pathogens in the last five decades, and this has resulted in significant research activities on the pathogenesis and intervention strategies around the world. Physicians and veterinarians have been puzzled by conflicting results from field and experimental observations: for example which mechanism are involved in the development of disease signs and symptoms,; how to diagnose and treat the disease; what is the effect of vaccination with different vaccine candidates, and how to develop other intervention strategies for RSV infections?. The most intriguing observations occurred in the 1960s, when the first hRSV vaccines were evaluated in children under field conditions. The vaccine-enhanced RSV disease has triggered many studies about the possible mechanisms involved, however without delivering a final answer to this question so far. Despite many years of considerable research efforts there are still no effective hRSV vaccines available. For bRSV vaccines that are available on the market, there is still a need for more knowledge regarding efficacy and safety.

RSV associated disease and animal models

In view of vaccine-induced illness, evaluation of safety and efficacy of new or existing vaccines greatly depends on the availability of models of RSV infection that closely mimic the events in the natural host after natural infection in the field situation. This has been difficult to achieve, at least for hRSV infection.

Essentially two types of animal models can be distinguished: heterologous (so called surrogate) models in which non-natural hosts are used, and homologous (so called natural-host models) infection models. Surrogate RSV models, most commonly using mice and cotton rats, have extensively been used for hRSV research. Unfortunately these animals are semi-permissive for viral replication and do not exhibit hRSV disease as observed in naturally infected children. On the other hand, these laboratory animal models offer the advantage of working in a well defined genetic background, are easy to handle, relatively cheap, can be used more easily with statistically relevant numbers, and can allow all appropriate controls [1]. An other advantage of using mouse models is that many immunological reagents are available, in contrast to cotton rats, for which much less immunological reagents are available.

The laboratory animal models have provided useful tools to study specific aspects of both disease pathogenesis and host immune response. Among laboratory animal models, BALB/c mice have helped to delineate the mechanisms underlying vaccine-enhanced disease and cotton-rats have been used for pre-clinical safety and efficacy testing of vaccine candidates. However extrapolation of the data

obtained in this way to natural hRSV infections in humans remains controversial. No animal approaches the genetic relatedness that exists between chimpanzees and humans. However, practical and ethical considerations severely limit the use of chimpanzees in RSV research. These primates are available only through specific breeding programs, are very expensive to purchase and maintain, and pose ethical constraints. In Europe it is no longer possible to use chimpanzees as experimental animals for vaccine and pathogenesis studies. Furthermore, published studies have not reliably reproduced lower respiratory tract disease signs or symptoms during lower hRSV tract infection in chimpanzees. Enhanced disease after administration of formalin-inactivated RSV vaccine has not been described in chimpanzees, but has been reproduced and characterized in the African green monkey [2] and cynomolgus macaques [3]. For bRSV research, most commonly a natural-host model is used. This homologous natural-host RSV model in calves allows studying disease pathogenesis, host–pathogen interactions, and mechanisms of protection following vaccination, infection or treatment of disease in the natural host. In the natural-host model described in this thesis we were able to clearly demonstrate immune modulation by vaccination, when young calves were immunized using formalin-inactivated bRSV adjuvanted with Al(OH)3. These calves showed a significant increase of bRSV-specific IgE antibodies after being vaccinated and showed enhanced disease upon challenge inoculation [4]. An impressive IgE booster response upon challenge infection correlated with enhanced bRSV disease [5].

Apart from reproducing the disease after experimental infection, reproduction of bRSV immunopathology after vaccination is a valuable element of the infection model. Our group has developed a homologous challenge model in which we can reproduce both the classical bRSV infection and the vaccine-enhanced immunopathology with a formalin-inactivated (FI-) bRSV vaccine, which renders this model suitable for the evaluation of both vaccine efficacy and vaccine safety [5].

Reproducing bRSV immunopathology

In our studies, calves vaccinated with a FI-bRSV vaccine experienced a much more severe disease upon bRSV challenge infection, compared to the MLV vaccinated and non-vaccinated animals in a long-term (6 months) duration-of-immunity study. One of the most striking findings in our studies was the discrepancy between viral loads and humoral immune responses on the one hand and pathogenesis and clinical signs on the other hand. In mock-immunized and bRSV-infected animals, we observed viral replication in the lungs and concurrent pathological lesions. Clearance of the viral infection coincided with a rise in (neutralizing) IgG antibody titers. Vaccination with MLV-bRSV provided protective immunity: viral RNA was virtually undetectable in the lungs of vaccinated calves, only mild clinical signs were observed and (neutralizing) antibodies increased rapidly after challenge infection. Immunization with FI-bRSV resulted in a strong IgG antibody response against both F and G proteins. Neutralizing antibodies could also be detected, but titers were

lower than those of MLV-bRSV immunized calves, and did not prevent the early onset of severe clinical symptoms [5]. It was shown that immunization with FI-bRSV mainly primes for a Th2-like inflammatory response [4] that is characterized by a significant eosinophilic influx in the bronchial alveolar lung fluid and lung tissue as well as high levels of immunoglobulin E [4,5]. In all cases, bRSV infection induced potent pulmonary CD8 T cell responses. The kinetics of the post-challenge response in MLV-bRSV immunized animals was accelerated compared to the FI-bRSV and mock-immunized groups, suggesting that only the MLV-bRSV vaccine, and not the FI-bRSV vaccine, had primed for specific memory CD8 T cells. The existence of strong IFN-gamma+ CD8 responses in FI-bRSV vaccinated animals after challenge suggests (i) that these IFN-gamma+ responses in FI-bRSV immunized animals did not or not sufficiently protect against immunopathology, and (ii) that Th-2 biased responses upon bRSV challenge after vaccination with FI-bRSV have a limited impact on the CD8 responses in the bronchoalveolar lavage fluid. Thus, several response patterns (Th-l/Th-2) seem to co-exist during bRSV infection [6].

Studies on vaccine-exacerbated bRSV disease were also described by Gerswhin et al. [7]. Further studies using sera from our studies demonstrated a correlation between severity of clinical signs and serum bRSV-IgE levels [8]. Kalina et al. compared in two studies FI-bRSV vaccines which differed in viral protein dose. These authors showed that using a ten-fold less protein concentration, resulted in development of a much more severe vaccine-enhanced disease characterized by a strong Th2 immune response particularly to the N protein and to a lesser extent to the F1 and G proteins [8].

It may thus be concluded that animal models are an important tool to elucidate mechanisms of pathogenesis and immuno-pathogenesis as they have been used in experiments aiming at understanding the process of vaccine-enhanced RSV disease and the development of safe and effective vaccines. In addition to rodents (cotton rats, guinea pigs, hamsters, rats and mice) primates and cattle, RSV researchers have also used ferrets [9,10]. Advantages and disadvantages of the respective animal models have been discussed in many papers as reviewed by Domachowske et al. [1]. Although hRSV models have been useful in addressing specific questions, they all have one major disadvantage in common: the relatively low susceptibility of the experimental host to the -heterologous- virus.

Domachowske concluded that no animal model of hRSV infection replicates the complete spectrum of disease severity seen in humans. It was also concluded that the study of bRSV infection in the bRSV-calf model is more appealing. BRSV is a natural and ubiquitous pathogen of calves, and using calves in experimental studies provides a homologues, or natural-host model. We have described (chapter 2) a bRSV-challenge model in which both classical bRSV respiratory infection and vaccine-enhanced immunopathology have been reproduced [5,11]. This natural-host model prevents extrapolation problems encountered with heterologous animal models. As human and bovine RS virus infections share many features like viral properties, pathogenesis, immune-pathogenesis, clinical disease and pathology; it

may be concluded that the bRSV-calf model may be used to answer many research questions related to both bRSV and hRSV infections and the development of safe and effective intervention strategies.

There has been extensive speculation on the nature of enhanced RSV disease after vaccination with certain vaccines. So called “sub-neutralizing” antibodies were the first potential culprits to be identified [12]. We observed in our model that after subsequent challenge infection, viral loads in formalin-inactivated (FI)-bRSV-immunized calves were strongly reduced and that the FI-bRSV vaccine did induce virus-neutralizing antibodies. Thus our data are inconsistent with a model of disease enhancement that implies inadequate antibody function and increased viral load. In contrast, our data support the hypothesis that immunization with FI vaccines mainly primes Th2-mediated inflammatory responses [13]. A vaccine-induced Th2-biased memory response would then set stage for the expansion of Th2-polarized CD4+ T-cells and, indirectly, the enhanced pulmonary lesions found after a subsequent challenge infection. Interestingly, these Th2-like responses have been related to a hyperactive immunoglobulin (Ig) E response. We showed in our homologous calf model that immunization with FI-bRSV primes for a Th2-like inflammatory response that is characterized by a significant eosinophilic influx in the bronchial alveolar lung fluid and lung tissues and high levels of IgE serum antibodies.

We also compared antiviral CD8+ T cell responses in bRSV infected calves that had been immunized with either FI- or live-attenuated (L) bRSV, with evidence of immunopathology following challenge of calves vaccinated with FI-bRSV (chapter 3). In all cases, bRSV infection induced potent pulmonary CD8 T cell responses. The kinetics of the post-challenge response in L-bRSV immunized animals was accelerated compared to the FI-bRSV and PBS groups, suggesting that only the L-bRSV vaccine, and not the FI-bRSV vaccine, had primed memory CD8 T cells. The differences between primary and post-vaccination secondary infection were minor, in terms of the proliferation status of pulmonary CD8 T cells. Functional IFN-gamma+ CD8 responses were slightly higher in the FI-bRSV vaccinated animals. Furthermore, the existence of strong IFN-gamma+ CD8 responses in FI-bRSV vaccinated animals after challenge suggests (i) that these IFN-gamma+ responses in FI-bRSV immunized animals do not protect against enhanced disease, and (ii) that Th-2 biased responses during bRSV challenge after vaccination with FI-bRSV have a limited impact on the CD8 responses in the bronchoalveolar lavage fluid. Thus, several response patterns (Th-l/Th-2) seem to co-exist during and after bRSV infection. Severe RSV disease therefore seems to be associated with an imbalanced immune response rather than with massive viral replication [6].

To better study the hypothetical role of IgE in bRSV disease enhancement using our homologous challenge model in calves, we developed a bRSV-specific IgE capture ELISA (chapter 4). This test was used to examine whether calves vaccinated with FI-bRSV antigens responded with bRSV-specific IgE production. Vaccinated calves did indeed develop a bRSV-specific IgE immune response. Upon challenge these vaccinated calves developed an exacerbated airway

hypersensitivity response, and an augmented IgE memory response. We therefore concluded that the bRSV-specific IgE ELISA reported here can be used to evaluate immunopathologic responses and vaccine safety in a homologous experimental infection model of RSV disease in the natural host [4]. The conflicting results from previous studies might be related to difficulties in reproduction of severe clinical signs mimicking the natural infection.
Neutrophil migration into the airways and pulmonary tissue has been a common finding in bRSV infections. We studied rolling and firm adhesion of peripheral blood neutrophils isolated from healthy and bRSV-infected calves to tumour necrosis factor –alpha (TNF-α) activated pulmonary endothelium under flow conditions in vitro (chapter 5). We demonstrated an important function for alpha4- and beta2-integrins in rolling and firm adherence of bovine neutrophils, to TNF-alpha-activated endothelium and showed a dynamic use of these integrins for adhesion and migration by neutrophils in the course of BRSV infection [14].

The severity of respiratory syncytial virus (RSV) infections appears to differ between ages in both humans and bovines (chapter 6). A primary RSV infection in naïve infants and in young calves runs a more severe course when they are one-to-six months old compared to their first month of life. However, to our knowledge a controlled study with newborn calves has never been performed before with bRSV. We experimentally infected one-day-old colostrum-deprived calves (neonates) and six-week-old calves. Neonates did show more extensive virus replication and lung consolidation, but lower proinflammatory (in particular TNF-alpha) responses, specific humoral immune responses, lung neutrophilic infiltration, and clinical signs of disease compared to six-week-old calves. The lack of correlation between virus replication and clinical signs suggests a role of proinflammatory cytokines, in particular of tumour necrosis factor-alpha (TNF-α) in the disease. The capacity to produce proinflammatory TNF-α appears to increase with age, and might explain age-dependent differences in RSV pathogenesis [15]. We speculate that this age effect, in addition to the immunosuppressive effect of maternal antibodies, might affect vaccine efficacy in different age groups. It also requires more study on the use of animals of the appropriate age in bRSV vaccination experiments. Vaccine efficacy demonstrated in older animals might not be valid for extrapolation to young animals, whilst those are the key target population for vaccination. This might also apply for use of (older) cotton rats, macaques or other experimental animals used in hRSV vaccine development studies.

We have shown that our bRSV-calf model is useful for evaluating the safety and efficacy of novel RSV vaccine strategies. In our last experiment, we have evaluated the efficacy and safety of modified vaccinia virus Ankara (rMVA)-based vaccine candidates, expressing the bovine RSV-F protein, either or not in combination with the G protein (chapter 7). Vaccination induced bRSV-specific IgG and CD8 T cell responses. Importantly, no IgE responses were detected. After bRSV challenge, rMVA vaccinated calves experienced less severe symptoms of lower respiratory tract disease compared to the mock-immunized control group. Immunized animals showed reduced pulmonary virus loads, and no eosinophilic infiltration or enhanced respiratory distress. We concluded that these candidate rMVA/bRSV candidate

vaccines induced protective and safe immune responses in calves under experimental conditions.

Conclusion

Our homologous challenge model for bRSV in cattle, in which severe disease is observed upon aerosol infection, and vaccination-enhanced disease can be reproduced 4-6 months after vaccination with inactivated and adjuvanted vaccine by challenge-infection, appears instrumental in better understanding the mechanisms underlying disease augmentation. In particular, it has become clear that serological data alone are inadequate or at least insufficient to evaluate safety or potency of vaccines and the data presented in this thesis suggest that a combination of serological, cellular and immunological parameters must be measured in a suitable animal model and preferably in field studies. In this way the potential and limitations of bRSV vaccine candidates may be better assessed in the field. Our studies therefore have added major tools to the field of safety and efficacy evaluation of bRSV vaccines. The higher predictive value of a homologous animal model in natural host species may also provide important insights and tools that can be used in hRSV vaccine development.

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Samenvatting

Respiratoir syncytieel virus (RSV) is een virus dat luchtweginfecties kan veroorzaken. Bij mensen en runderen komen verschillende RS virussen voor. De rundervariant wordt bovine RSV (bRSV) genoemd. De menselijke variant heet humaan RSV (hRSV). Beide virussen behoren tot het genus *Pneumovirus* van de familie van de *Paramyxovirussen*. Virusoverdracht geschiedt meestal via de lucht. De symptomen van een bRSV-infectie bij runderen lijken op die van een hRSV-infectie bij mensen. Het eerste contact tussen het lichaam en het geïnhaleerde virus vindt plaats op slijmvliescellen in de luchtwegen. Eenmaal binnengedrongen in deze cellen gaat het virus zich vermenigvuldigen. In het algemeen worden RSV-infecties gekenmerkt als een infectie van de bovenste (mensen) of voorste (runderen) luchtwegen. Symptomen van een RSV-infectie zijn in het algemeen mild. Een voorste luchtweginfectie kenmerkt zich doorgaans als een verkoudheid. Bij het klinische beeld staan neusuitvloeiing en hoesten op de voorgrond. RSV is echter ook één van de belangrijkste veroorzakers van een ernstige lagere (mensen) of achterste (koeien) luchtweginfectie. Aantasting van dit deel van de luchtwegen kan resulteren in veel ernstigere aandoeningen zoals bronchitis (ontsteking van de grotere luchtwegen gelegen tussen de luchtpijp en longblaasjes), bronchiolitis (ontsteking van de kleinste buisjes die naar de longblaasjes voeren) of zelfs een pneumonie (ontsteking van de longblaasjes en het omringende weefsel). Ernstige aantasting van de diepere luchtwegen leidt bij jongere kinderen vaak tot een ziekenhuisopname, en bij kalveren zal een dierenarts overgaan tot een meer of minder gerichte behandeling. Infecties met dodelijke afloop komen voor, vaak door bijkomende bacteriële infecties.

Ondanks dat hRSV al in 1956 werd ontdekt en geïsoleerd, is er nog steeds geen effectief en veilig vaccin beschikbaar. Vroege pogingen in de zestiger jaren van de vorige eeuw om een vaccin te maken en te testen verliepen desastreus. Deze vaccins bestonden destijds uit een, in het laboratorium gekweekt, humaan RSV, dat vervolgens geïnactiveerd was door middel van een behandeling met formaline. Dit geïnactiveerde virus werd in combinatie met aluminiumhydroxide als adjuvans (hulpstof die de afweerreactie tegen het geïnactiveerde virus moest versterken) aan kinderen toegediend. Deze procedure was in die periode reeds een aantal malen succesvol toegepast voor verschillende andere virusvaccins. De ervaringen met het formaline-geinactiveerde hRSV vaccin waren echter dramatisch. Meer dan 80% van de gevaccineerde kinderen moest tijdens de eerste winter na de vaccinatie opgenomen worden in een ziekenhuis omdat ze na natuurlijke hRSV infectie veel zieker werden dan niet-gevaccineerde kinderen, waarbij zelfs twee gevaccineerde kinderen zijn overleden.

Niet lang na de ontdekking van hRSV werd het nauw verwante bRSV aangetoond in kalveren. In de jaren die daarop volgden, werd bRSV in toenemende mate als verwekker van ernstige luchtwegproblemen bij kalveren aangetoond. BRSV is, net

als zijn humane equivalent, wereldwijd één van de belangrijkste veroorzakers luchtwegproblemen. Opvallend is dat ook de veterinaire wereld geconfronteerd werd met ernstige ziekteproblemen bij gevaccineerde kalveren na natuurlijke infectie.

De negatieve ervaringen met het hRSV-vaccin hebben geleid tot zeer vele pogingen om veilige en tegelijkertijd effectieve vaccins te ontwikkelen. Er is echter tot op heden geen hRSV-vaccin geregistreerd voor humaan gebruik. Voor runderen daarentegen zijn er sinds 1977 vele bRSV-vaccins op de markt verschenen. Informatie over de effectiviteit en de veiligheid van deze vaccins is over het algemeen schaars en registratie-eisen zijn minder streng dan bij humane vaccins.
Het onderzoek beschreven in dit proefschrift is verricht om het mechanisme van de vaccin-geïnduceerde overgevoeligheid, zoals die bij kinderen en kalveren was waargenomen, te onderzoeken. Daarvoor moest eerst een diermodel ontwikkeld worden, waarin klinische verschijnselen onder experimentele omstandigheden konden worden opgewekt en vaccin-geïnduceerde overgevoeligheid gereproduceerd kon worden. Dit model zou dan geschikt moeten zijn om de effectiviteit en de veiligheid van bRSV vaccins te kunnen evalueren.

In hoofdstuk 2 van dit proefschrift worden de resultaten van een dierexperiment in kalveren beschreven. In dit diermodel bleek het mogelijk onder experimentele omstandigheden klinische verschijnselen na toediening van bRSV op te wekken. Overeenkomstig met de wijze waarop in de zestiger jaren het hRSV vaccin werd geproduceerd is een bRSV vaccin gemaakt en gebruikt om jonge kalveren te vaccineren. Deze kalveren werden, onder experimentele omstandigheden, tweemaal gevaccineerd. Zes maanden na de tweede vaccinatie werd de afweerreactie van de gevaccineerde kalveren getest met een zogenaamde “challenge” infectie. De gevolgen van de bRSV-infectie werden geëvalueerd door dagelijks klinische observaties uit te voeren, het verloop van de lichaamstemperatuur te vervolgen en aan het einde van het dierexperiment de longen van de kalveren op de sectietafel macroscopisch en later microscopisch te beoordelen. In de loop van het dierexperiment werden bloedmonsters verzameld en longspoelingen uitgevoerd. Vervolgens werden in het laboratorium met behulp van een aantal specifieke diagnostische technieken de kwantitatieve en de kwalitatieve gevolgen van de virusinfectie en de respons van het afweersysteem op de bRSV infectie gemeten. De verkregen resultaten werden vergeleken met die van kalveren die niet of met een levend bRSV vaccin gevaccineerd waren. In het diermodel konden we de vaccin-geïnduceerde overgevoeligheidreactie, zoals ook humaan waargenomen, reproduceren onder goed gecontroleerde omstandigheden. We konden laten zien dat de afweerreactie door vaccinatie en het type vaccin een ongewenste kant kan worden opgestuurd. Ons diermodel blijkt een bruikbare methode om zowel de effectiviteit als de veiligheid van bRSV vaccins te beoordelen.

In hoofdstuk 3 worden de resultaten van een onderzoek naar de bijdrage van T lymfocyten in de specifieke immuunrespons tegen bRSV beschreven, na een experimentele bRSV infectie al dan niet voorafgegaan door vaccinatie met een

levend of formaline-geïnactiveerd bRSV vaccin. T lymfocyten zijn witte bloedcellen die een onderdeel vormen van de cellulaire component van het immuunsysteem. Een deel van deze T cellen (CD8+ T cellen) kan geïnfecteerde cellen doden. De kwantiteit en kwaliteit van de T cel responsen na bRSV infectie werden in de loop van de tijd vervolgd. In zowel de kwantitatieve als de kwalitatieve CD8+ T cel responsen worden geen grote verstoringen door vaccinatie met een formaline-geïnactiveerd bRSV vaccin aangetoond. De T cel respons was versneld in de kalveren gevaccineerd met een levend bRSV vaccin, vergeleken met de niet-gevaccineerde kalveren en kalveren gevaccineerd met een formaline-geïnactiveerd bRSV vaccin. Functionele IFN-□+ CD8 responsen waren hoger in de met formaline-geïnactiveerd bRSV gevaccineerde kalveren. Deze CD8+ T cellen kunnen de vaccin-geïnduceerde overgevoeligheid klaarblijkelijk niet voorkomen.

Hoofdstuk 4 behandelt een specifieke antistofrespons die optreedt na vaccinatie en experimentele infectie met bRSV. Antistoffen zijn eiwitten die geproduceerd worden door B lymfocyten, die net als de T lymfocyten behoren tot de witte bloedcellen. We laten zien dat vaccinatie met formaline-geïnactiveerd bRSV leidt tot een tegen bRSV gerichte productie van zogenaamde IgE antistoffen. Deze IgE antistoffen worden vooral gezien bij overgevoeligheidsreacties. De luchtwegovergevoeligheid die na de bRSV infectie wordt waargenomen blijkt gepaard te gaan met een versterkte IgE respons. Een bRSV-specifieke IgE ELISA lijkt daarom een welkome aanvulling op de reeks beschikbare tools om de veiligheid van bRSV vaccins te onderzoeken.

In hoofdstuk 5 wordt beschreven dat een bRSV infectie de migratie van neutrofiele granulocyten van de bloedbaan naar het longweefsel beïnvloedt. Neutrofiele granulocyten in het bloed rollen en stuiteren langs de (endotheel)cellen van de bloedvatwand voordat ze zich vasthechten en tussen deze cellen door bewegen, een proces dat transendotheliale migratie wordt genoemd. Dit proces wordt gereguleerd door zogenaamde adhesie moleculen (integrinen). Transendotheliale migratie werd beïnvloedt door een bRSV infectie. Bij gezonde kalveren speelden met name β2- en α4 integrinen een essentiële rol. Bij met bRSV geïnfecteerde kalveren namen andere regulerende cytokinen het proces over.

Hoofdstuk 6 beschrijft leeftijdsafhankelijke verschillen in de gevolgen van een bRSV infectie. Data verkregen uit het veld suggereren dat grote hoeveelheden afweerstoffen verkregen via de biest (moedermelk) de reden zijn voor het beschermd zijn tegen bRSV-infectie van kalveren in de eerste levensmaand. Onder experimentele omstandigheden hebben wij in afwezigheid van maternale afweerstoffen gekeken naar het leeftijdsafhankelijk effect. Pasgeboren (neonatale) kalveren en zes-weken-oude kalveren werden op dezelfde dag geïnfecteerd met bRSV. Hoewel bij pasgeboren kalveren een grotere virusreplicatie en aantasting van de longen werd aangetoond, waren de klinische verschijnselen beduidend minder dan bij de zes-weken-oude kalveren. Een leeftijdsafhankelijk effect kan dus niet alleen verklaard worden door hoge, maternaal verkregen, antistoftiters. De data verkregen uit dit experiment suggereren een rol voor pro-inflammatoire

(ontstekings-)cytokines, die bij jongere kalveren minder sterk gevormd lijken te worden.

In hoofdstuk 7 worden de effectiviteit en de veiligheid van twee in ontwikkeling zijnde bRSV vaccins geëvalueerd. In een dierexperiment werden kalveren gevaccineerd met recombinant modified Vacciniavirus Ankara (MVA): vaccinvirussen die oppervlakte-eiwitten van bRSV aan cellen van het afweersysteem kunnen presenteren. MVA zelf vermenigvuldigt zich niet in zoogdierencellen, kan zich dus niet spreiden en is daarom veilig voor gebruik in zoogdieren. Eén kandidaat-vaccin bestond uit recombinant MVA virus dat het zogenaamde fusie(F)-eiwit van bRSV tot expressie brengt. Het andere kandidaat-vaccin bestond uit twee recombinant MVA-virussen: één dat het F-eiwit en één dat het zogenaamde aanhechtingseiwit (G-eiwit) van bRSV tot expressie brengt. Beide kandidaat-vaccins bleken een goede immuunrespons te induceren, die de kalveren beschermde tegen een experimentele (challenge) bRSV-infectie en vaccin-geïnduceerde overgevoeligheidsreacties werden niet waargenomen. Onze conclusie was dat beide kandidaat-vaccins veilig en effectief zijn.

In hoofdstuk 8 worden de bevindingen, die in dit proefschrift beschreven zijn, samengevat en bediscussieerd in de context van de reeds bestaande literatuur.

Dankwoord

De aanhouder wint
Wie opgeeft is laf
Wie wint krijgt de bloemen
Voor niets op zijn graf
Verpulverd tot as
Waait mee met de wind
De aanhouder wint
De aanhouder wint

De aanhouder wint

(Liesbeth List, tekst Boudewijn de Groot & Stef Bos)

De aanhouder wint
Mijn leven lijkt aan elkaar te hangen van bijzondere ontmoetingen met bijzondere mensen. Wanneer ik terug kijk naar en denk aan al die bijzondere, warme, inspirerende mensen, realiseer ik me dat ik alleen dankzij hen ben geworden wat en wie ik ben.

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De aanhouder wint
Piet Vellema heb ik in 1999 leren kennen als een bevlogen dierenarts, onderzoeker, leidinggevende, maar vooral ook mens. Na mijn vertrek bij de GD heb ik het genoegen gekend regelmatig contact met hem te mogen onderhouden. Eén van de eerste vragen die hij mij keer op keer stelde was, hoe staat het met je promotie, gevolgd door een bijna “vaderlijke” preek, dat het toch doodzonde zou zijn als ik het niet af zou ronden. Hij was één van de eerste personen die ik gebeld heb, toen de laatste pennestreek geveld was.

Wie opgeeft is laf
Met angst in zijn ogen, te laat voor de vlucht, geen weg meer terug. Steeds meer mensen om mijn heen vonden het nodig mij met enige regelmaat te attenderen op het onafgeronde “project”, dat steeds dieper in de la leek te verdwijnen. Wim van der Poel en Tjeerd Kimman hebben me door het laatste jaar gesleept. Ook zonder hen was er geen promotie.

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Wie wint krijgt de bloemen
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Waait mee met de wind
Voor wie mij niet, of niet zo goed ☺, kent, ik ben “geel”, enthousiast, een dromer, heb de neiging om te gaan zweven. Ik heb iemand nodig die mij af en toe terug haalt op aarde. Zo zei mijn zoon een jaar geleden, toen hij alle stapels literatuur op mijn bureau weer eens mocht aanschouwen: “Pap waarom maak jij nooit eens iets af” en hij had gelijk. Er is weer iets af. Ik wil in het bijzonder nog eens Christianne Bruschke, Paul Steverink, Godelieve Spaes, Wim Boersma en Rob Moormann bedanken. Zij waren belangrijk en vooral bijzonder voor mij.

De aanhouder wint
De man van het eerste uur, Robbert van der Most, altijd tijd, veel tijd, inspirerend, bevlogen een bijzonder mens, hij nam mij mee aan de hand. Het was goed dat je me los liet.

De aanhouder wint
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De aanhouder wint
Nu zijn er heel veel mensen heel belangrijk in mijn leven geweest en ik ben blij dat zij er waren. Zonder mijn ouders stond ik hier niet, zij waren er altijd, ze staan altijd voor me klaar! Ik ben dan ook blij dat hun bijdrage aan mijn “wetenschappelijke carriere” verzilverd wordt in dit boekwerk. Ik weet zeker dat het een mooie plek in hun boekenkast krijgt.
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Emiel, “de aanhouder wint”, papa is nu klaar.

Curriculum vitae

Adriaan Antonis werd op 26 okober 1969 geboren, 8 hoog in de Dodonaeusstraat te Zaandam. Al van kinds af aan was duidelijk dat hij dierenarts moest worden. Een goed besluit was de eerste stap. In 1990 haalde hij zijn VWO diploma aan het Thomas More College te Oudenbosch, lootte in voor de studie Diergeneeskunde en verruilde het brabantse land voor de stad *Utereg*. De weg naar succes was niet geplaveid met rozen, maar de wilskracht bleek opgepompt te kunnen worden. Tijdens zijn studie Diergeneeskunde werd een onderzoeksstage uitgevoerd bij prof. Dr. Frans van Knapen op het raakvlak van de veterinaire en humane gezondheid: een prevalentie onderzoek naar toxoplasmose bij Nederlandse melkgeiten, deels bij het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) en deels bij de Gezondheidsdienst voor Dieren. De contacten met Daan Dercksen in het kader van deze onderzoeksstage bleken bepalend voor het verdere verloop van zijn carriere. Door Daan Dercksen raakte hij nauw betrokken bij een veld- en experimenteel onderzoek naar caseous lymphadenitis (CL) bij schapen en geiten, veroorzaakt door Corynebacterium pseudotuberculosis. *In vivo* experimenten werden uitgevoerd bij het Centraal Diergeneeskunde Instituut (CDI) in samenwerking met Elbarte Kamp. Na zijn afstuderen in 1999 heeft Adriaan ruim een half jaar bij de Gezondheidsdienst voor Dieren bij de afdeling Kleine Herkauwers gewerkt. Begin 2000 werd hij aangenomen door Prof Dr Jan van Oirschot en Dr Christianne Bruschke, om binnen de afdeling Zoogdiervirologie te gaan werken met het Bovine Virale Diarree Virus (BVDV) in een aantal grote vaccinatie-challenge experimenten. Al snel werden er enkele uitstapjes gemaakt naar andere endemische virusen: o.a. Porcine Parvovirus (PVV), Bovine Herpes Virus type 1 (BHV-1) en uiteindelijk het Bovine Respiratoire Syncytieel Virus (BRSV). Niet lang daarna werd het doel, promoveren op bRSV, geformuleeerd. De jaren daarna waren nodig om het leven te kunnen organiseren naar het doel. De weg was mijn doel.
Inmiddels werkt Adriaan Antonis ruim tien jaar bij het onderzoeksinstituut in de Flevopolder. Met name klinisch virologische vraagstukken hebben zijn bijzondere interesse.

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