Human and bovine respiratory syncytial virus vaccine research and development

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Abstract

Human (HRSV) and bovine (BRSV) respiratory syncytial viruses (RSV) are two closely related viruses, which are the most important causative agents of respiratory tract infections of young children and calves, respectively. BRSV vaccines have been available for nearly 2 decades. They probably have reduced the prevalence of RSV infection but their efficacy needs improvement. In contrast, despite decades of research, there is no currently licensed vaccine for the prevention of HRSV disease. Development of a HRSV vaccine for infants has been hindered by the lack of a relevant animal model that develops disease, the need to immunize immunologically immature young infants, the difficulty for live vaccines to find the right balance between attenuation and immunogenicity, and the risk of vaccine-associated disease. During the past 15 years, intensive research into a HRSV vaccine has yielded vaccine candidates, which have been evaluated in animal models and, for some of them, in clinical trials in humans. Recent formulations have focused on subunit vaccines with specific CD4+ Th-1 immune response-activating adjuvants and on genetically engineered live attenuated vaccines. It is likely that different HRSV vaccines and/or combinations of vaccines used sequentially will be needed for the various populations at risk. This review discusses the recent advances in RSV vaccine development.

Keywords: Vaccine; Respiratory syncytial virus; RSV

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Résumé

Les virus respiratoires syncytial humain (HRSV) et bovin (BRSV) sont des agents pathogènes étroitement apparentés, respectivement responsables d'affections de l'appareil respiratoire chez les jeunes enfants et chez les veaux. Des vaccins contre le BRSV sont commercialisés depuis deux décennies et ont probablement permis de limiter la prévalence de l'infection. Leur efficacité doit cependant être améliorée. La situation est différente pour le HRSV puisqu' aucun vaccin n'est actuellement disponible sur le marché, malgré de nombreuses recherches. La vaccination contre le HRSV se heurte à plusieurs difficultés, à savoir l'absence de modèle animal d'infection qui reproduise la pathologie chez l'enfant, la nécessité de vacciner de jeunes enfants dont le système immunitaire n'est pas totalement fonctionnel, la difficulté d'obtenir des vaccins vivants présentant un compromis efficace entre atténuation et immunogénicité et les défauts d'innocuité de certains vaccins. Pendant ces 15 dernières années, des efforts intensifs de recherche ont permis de développer de nouveaux candidats qui ont été évalués dans des modèles animaux et, pour certains, chez l'homme. Les recherches se sont concentrées sur la production de vaccins sous-unitaires avec des adjuvants orientant la réponse cellulaire T CD4+ vers une réponse de type Th-1 et sur des vaccins vivants atténués construits par génie génétique. Les stratégies vaccinales visent à l'utilisation prochaine de différents vaccins en fonction de la population à risque et/ou des combinaisons séquentielles de vaccins de différents types. Cette revue fait la synthèse des avancées récentes en terme de nouveaux vaccins contre le BRSV et le HRSV.

Mots clés: Vaccins; Virus respiratoire syncitial; VRS

1. Introduction

Human (HRSV) and bovine (BRSV) respiratory syncytial viruses (RSV) are two closely related, highly infectious, worldwide prevalent viruses that are the leading cause of serious respiratory tract disease in children and calves, respectively. RSV infection is extremely common in the first year of life. By 24 months of age, virtually all children have been infected by HRSV at least once, and about half have experienced two infections [1]. In the United States, about 70% of bronchiolitis hospitalizations are associated with HRSV infection [2]. HRSV has been estimated to account for 85,000 to 144,000 hospitalizations annually in infants of less than 1 year of age with an associated cost of $300,000,000 per year [3]. In Europe, RSV accounts for 42–45% of hospital admissions with low respiratory tract infection in children younger than 2 years of age [4]. HRSV mortality has been estimated as 0.005–0.02% in developed countries, due to the efficacy of symptomatic ventilation treatment. However, severe HRSV infection in the first 6 months of life is often followed by recurrent childhood wheezing, [5,6] or asthma [7]. There also are variable contradictory reports of an association with atopic disease [8,9]. HRSV reinfection is also gaining recognition as an important cause of disease in adults, especially the elderly [10], in patients with underlying cardiopulmonary illnesses [11], and in immunosuppressed patients, particularly bone marrow transplant patients [12,13].

Similarly, the frequency and seriousness of BRSV diseases are regarded as the principal health problem in calf rearing world-wide, as intensification of farming industry is developing. The frequency of BRSV infections is very high in cattle less than 1-year-old [14–17] and the virus might be responsible for more than 60% of the epizootic respiratory diseases observed in dairy herds [14,18,19] and up to 70% in beef herds [20,21]. Mortality of BRSV ranged generally between 2% and 3% but can reach up to 20% in some outbreaks.

The fight against HRSV is considered by the WHO as a priority and prevention of BRSV is a major concern in farming industry. Several BRSV vaccines have been commercialized for many years but there is a need to develop second generation vaccines with a greater efficacy in the presence of maternal antibodies (Abs) and capable of inducing a more durable protection. In contrast to BRSV, no vaccines are yet available against HRSV. Human vaccine development has been primarily hampered by disease potentiation observed after vaccination with an experimental RSV inactivated vaccine but other obstacles to vaccination exist, such as the lack of a relevant animal model that mimicks the natural disease in humans and the need to immunize immunologically immature young infants. This review discusses recent advances that have been obtained these last years to develop a new generation of safe and effective HRSV and BRSV vaccines.

2. Acquired immunity to RSV infection

BRSV and HRSV are members of the genus *Pneumovirus* in the family *Paramyxoviridae*. The virion is wrapped into a lipid envelope derived from the host plasma membrane that contains three viral transmembrane surface glycoproteins organized separately into spikes on the surface of the virion (Fig. 1). These glycoproteins are the attachment glycoprotein (G), the fusion protein (F) and the small hydrophobic protein (SH) [22]. The F and G glycoproteins are major antigens and were consequently extensively used in vaccine development. The envelope encloses a helical nucleocapsid, which consists of the nucleoprotein (N), the phosphoprotein (P), the viral RNA-dependent polymerase protein (L) and the genomic RNA. In addition, there is a matrix M protein that is thought to form a layer on the inner face of the envelope and a transcriptional anti-termination factor M2-1. The genome also encodes an RNA regulatory protein M2-2 and two non-structural proteins, NS1 and NS2 (Fig. 1). The viral genome, which consists of a negative-sense non-segmented single-stranded RNA genome of approximately 15,200 nucleotides, is first transcribed into 10 mRNAs in a sequential fashion from the 3′ to the 5′ end according to a polar transcription gradient [22]. The 10 mRNAs are then translated into 11 viral proteins, the M2 gene coding for two proteins M2-1 and M2-2. The properties of RSV proteins are shown in Table 1.

RSV replicates primarily in the superficial layer of the respiratory ciliated epithelium and to a lesser extent in type II pneumocytes [23,24]. No cytopathology is observed in infected human as well as bovine airway epithelial cells, suggesting that the host response to virus infection plays a major role in RSV pathogenesis [25]. Host

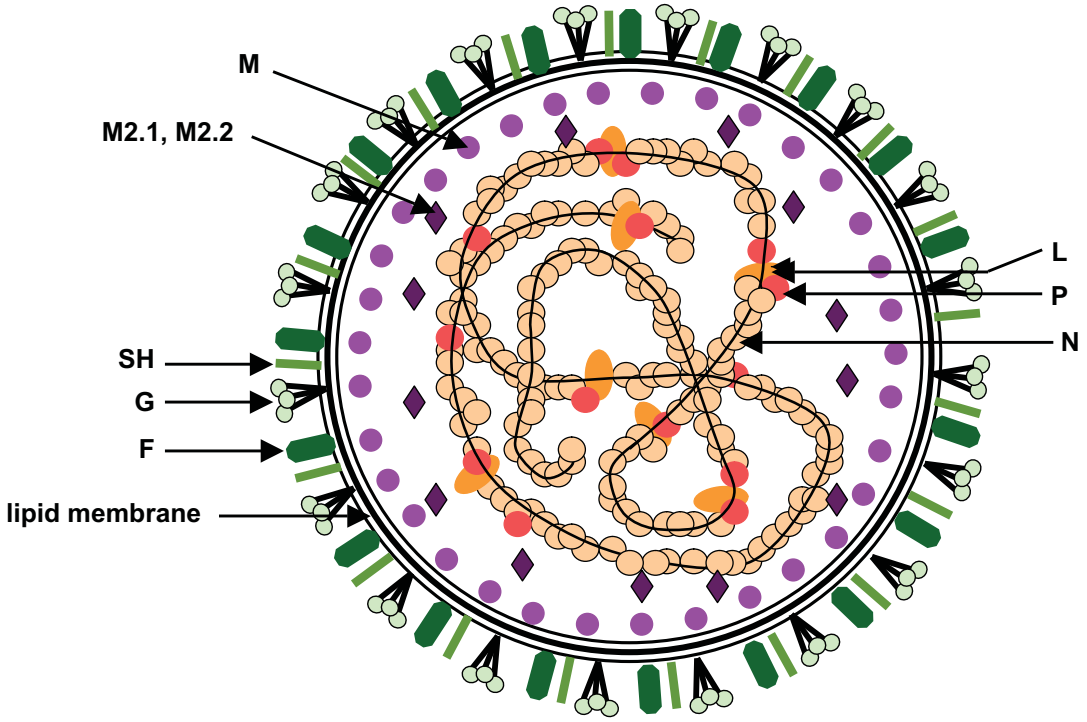

Fig. 1. Diagram of respiratory syncytial virus.

Table 1
RSV protein functions

Protein	Mr (kD)	Functions
NS1: Non-structural protein 1	13.8	Inhibition of and resistance to IFN-α/β Host tropism
NS2: Non-structural protein 2	14.5	Inhibition of and resistance to IFN-α/β Host tropism
G: G protein	70	Attachment Host tropism Escape immunity by binding to neutralizing Abs (G secreted form)?
F: Fusion protein	90	Attachment and membrane fusion Host tropism
SH: Small hydrophobic protein	7.5–60	Unknown function
M: Matrix protein	25	Virion assembly
M2-1: Matrix protein 2-1	22	Transcription anti-termination factor
M2-2: Matrix protein 2-2	11	RNA regulatory protein
N: Nucleoprotein	45	Protection of genomic and antigenomic RNA Transition from transcription to replication
P: Phosphoprotein	33	Polymerase co-factor Host tropism (HRSV)
L: Polymerase protein	250	RNA dependant RNA polymerase

defence mechanisms against RSV infection involve overlapping aspects of innate and acquired immunity where the magnitude and quality of the acquired immunity response is dependent on signals derived from the innate response. Indeed, the pattern of cytokine and chemokine expression induced during the innate response activates immune cell trafficking (macrophages, eosinophils, basophils, neutrophils and NK cells) to sites of infection, regulates B and T cell survival, and may enhance virus clearance or exacerbate disease. Features of the innate response were extensively studied these last few years and recently reviewed for HRSV [26–29] and BRSV [30]. Consequently, despite their importance, they will not be discussed in this paper.

Acquired immunity is mediated by B and T cell responses that endow diversity of antigen recognition, memory and immune self-tolerance. Established RSV infection is controlled primarily by the T cell response, which protects the host from severe RSV disease and limits virus shedding [31]. Both Th1 and Th2 T cell subsets are important for virus clearance but both also are connected with pathology and illness [32,33]. HLA class I or class II-restricted T cell HRSV epitopes have been described in the N, M, NS2, M2-1, F and G proteins [34–44]. HLA class II-restricted T cell responses to the F protein were studied in more detail by using a set of overlapping peptides, which resulted in the detection of multiple antigenic regions spanning the F protein [45]. Several studies have underlined the role of CD4+ T cells in RSV immunity and disease pathogenesis. CD4+ T cells express a spectrum of cytokines required for cell-mediated immune response, including IL-2 and IFN-γ. In BALB/C mice, primary infection induces a mixed Th-1/Th-2-type cytokine response with limited disease and it appears that early INF-γ expression is important in controlling the Th-1/Th-2 cytokine balance [27,46,47]. Absence of INF-γ early after primary infection or during subsequent RSV infections results in a predominant Th-2-type cytokine response and increased disease severity [46,48,49]. Thus, the response of CD4+ T cells and the pattern of cytokines they produce affect the outcome of immunity and disease pathogenesis.

The class I restricted CD8+ cytotoxic T lymphocyte (CTL) response is targeted against several proteins [50,51], the M2, F and N proteins being the major targets [44,52,53]. In mice, M2 primes strong CTL responses but also severe disease enhancement after RSV challenge [32]. In BALB/c mice the G protein is generally considered to not induce CTL [51,54,55] despite recent contradictory results [56]. Studies in humans have also been unable to demonstrate G-specific CTLs (4), whereas G-specific CD4+ T cells are readily detectable [57]. In contrast, in calves, it has been shown that G-specific CD8+ T cells can be detected after BRSV challenge [58]. CTLs have a major role in the clearance of virus infection and there is a robust expansion of RSV-specific CD8+ T lymphocytes in the lung. However, despite a rapid response, RSV-specific pulmonary CTLs with impaired cytokine secretion and cytotoxicity have been described [59].

If cell-mediated immunity is essential for virus clearance following acute infection, the antibody (Ab) response generally protects against reinfection. This is a major goal when developing vaccines against RSV. The humoral response is essentially directed against the F and G proteins, whose various neutralizing epitopes have been

mapped and characterized among RSV subtypes [60–67]. Selection of RSV escape mutants was also shown to be associated with amino acid substitution in these antigenic domains [60,61,68,69]. Nasal Ab levels are probably essential for protection against infection of the upper airway. Resistance to infection in the upper airways is mediated by transitory and local expression of secretory IgA, but more durable resistance is probably associated with IgM, and especially neutralizing IgG Abs [70]. Serum Abs were shown to play a significant role against lower airway infection [71].

The inability of the host to mount an effective immune memory response allows the occurrence of successive reinfections and constitutes a major obstacle to the efficacy of vaccination. The mechanisms involved in virus escape from the immune response are not well understood and could include an ineffective B cell response to RSV in the nasal cavity, probably due to a poor IFN-$\alpha\beta$ induction by the virus and/or a defective T cell memory compartment. Contact-dependent mechanisms, IL-1 receptor antagonist, IFN-α, IFN-β and IFN-γ together have each been implicated [28,72–75]. Alteration of dendritic cells (DCs) by RSV infection has also been proposed to explain why protection after natural RSV infection is incomplete and of short duration. However, infection of human monocyte-derived DCs (10–30%) by RSV was shown not to be effective and not to alter cell viability, suggesting that the virus does not significantly interfere with the function of these cells [76].

3. Challenges and difficulties of RSV vaccine research

Despite the importance of HRSV infection, there are currently no licensed vaccines for prevention of this disease. From 1960 to 1980, trials of several candidate HRSV vaccines failed to attain the desired safety or to induce protection against natural infection, either by insufficient immunogenicity or by inducing exaggerated disease on natural exposure to the virus. Since the 1980s, there has been an intensive effort to develop an effective and safe RSV vaccine based on the molecular virology of RSV. Difficulties of vaccine development stem from the virus but also from the host. These difficulties include the lack of a relevant animal model, the need to immunize immunologically immature young infants with maternal RSV antibodies, the impact of RSV variability on vaccination and the risk of vaccine-associated disease enhancement. These problems also partially exist for BRSV vaccines. Since acceptation of the risk is different for livestock, several BRSV vaccines have nevertheless been commercialized.

3.1. Animal models

An accurate evaluation of the safety and efficacy of a RSV vaccine can only be made in clinical studies involving the target population, but pre-clinical studies need to be performed in animal models. Advantages and disadvantages of RSV animal models have recently been discussed [27].

For HRSV, mice and cotton rats, which are the models used currently, are semipermissive for viral replication and do not exhibit authentic RSV disease. This complicates the interpretation of clinical protection afforded by vaccines. Chimpanzees are the only experimental animals that approach humans in permissiveness to HRSV replication and disease [77–79] but their availability is limited. As HRSV and BRSV show similarity in epidemiological and clinical aspects of infection, BRSV infection in calves is also a good comparative model for the study of HRSV.

For BRSV studies, calves are the natural hosts and are used for vaccine evaluation. However, many models of infection using various concentrations of cell-culture-passaged virus by different routes failed to reproduce severe clinical signs or lesions as observed in natural conditions [80,81]. Consequently, clinical differences between vaccinated and non-vaccinated groups after challenge are frequently non-significant and protection is often demonstrated on reduction of virus titres in vaccinated calves. Recently, an improved clinical model of infection has been developed by using as inoculum a preparation of BRSV previously replicated in new-born calves [82–84]. Despite this progress, clinical variability between calves remains high and hampers statistical interpretation of the results. Finally, due to the high prevalence of infection and considering the age of the animals to vaccine, experimentations also ought to be done in calves possessing maternally derived Abs.

3.2. Age of hosts

An effective RSV vaccine has to stimulate an effective immune response during the first months of life when the peak incidence of severe disease occurs. However, immune response to infection or immunization exhibited by neonates is reduced and probably stems from multiple factors, including immunological immaturity [85,86] with limited B cell repertoire, inefficient mechanisms of antigen (Ag) presentation and T cell help, and inhibition by passively acquired maternal Abs [87]. The principal method of transfer of antibodies (Abs) from human mothers to infants is via the transplacental route, starting at 28 weeks of gestation and increasing until the time of birth. In ruminants, there is no transplacental transfer of Abs and the neonate lacks significant levels of serum Abs until it absorbs them from colostrum or breast milk via enteric Ab receptors. In the two cases, the IgG1 subclass of Abs is preferentially transferred, with little IgM, IgA, or IgE transferred. The role of maternal Abs during RSV infection or immunization is not clearly defined. Data suggest that passive Abs inhibit both the quantitative level and functional quality of mucosal and systemic Ab responses after natural infection or immunization in both mice, humans and calves [87,88]. Passively trasferred Abs are particularly suppressive of responses to RSV subunit vaccine candidates [89] but also to live vaccinia virus vectors that express the RSV surface glycoproteins [90,91]. Despite this inhibitory effect, protective efficacy against *wt* HRSV challenge was induced in mice or chimpanzees [87] by live attenuated HRSV infection in the presence of RSV Abs. In mice, CD4+ and CD8+ T cells are required for this protection [87]. Therefore, priming of the B cell repertoire for secondary Ab responses may occur during RSV passive/active

immunization experiments, even when primary Ab responses are suppressed at levels that are lower than detectable limits. This was also partially described for BRSV by Kimman et al. [88] who showed, in addition, that both the incidence and severity of disease were inversely related to the maternal Ab level, suggesting clinical protection by these Abs during natural infection [17].

Since maternal Ab inhibitory effect predominates in the lower respiratory tract [92], the most commonly admitted strategy today to overcome Ab-mediated immunosuppression is mucosal immunization, especially with live attenuated viruses. For BRSV, protection was afforded by intra nasal immunization with live virus, both in calves with and without maternal Abs [93]. This protection was associated with a strong and rapid mucosal Ab memory response. In contrast, intramuscular immunization with killed or live virus vaccines in the presence of maternal Abs proved to be little effective in inducing memory and protection against virus shedding [93]. Maternal immunization could be an alternate strategy to protect newborn calves from infection. One assay of vaccination of pregnant cows with BRSV live vaccines showed a boost of the systemic humoral and cellular responses to BRSV in cows and of the level of BRSV IgG1 in the colostrum [94]. A similar strategy for HRSV vaccination of pregnant women has been discussed but the fear was raised that some antigens might cross the placental barrier and predispose the infant to allergic responses because the foetal cytokine milieu exhibits a predominant Th-2-like bias [87].

3.3. RSV variability

The variability of RSV has a major impact on vaccine development, especially when addressing the development of sub-unit vaccines, which contain only one or a few viral epitopes. The antigenic and genetic variability among independent isolates of HRSV from different infected hosts or geographic regions has been extensively documented, and the existence of two major groups (A and B), as well as additional variability within each group, have been clearly established [95,96]. The most extensive variability was found in the attachment glycoprotein G, which differs by up to 45% in its aminoacid (aa) sequence between the two groups. Studies in animals and in humans have demonstrated that this difference should be taken into account in the development of vaccines. Thus, immunization with individual F or G proteins in animals has shown that the F protein is broadly cross-protective, whereas the G protein mostly provides group-specific protection [97–99]. A subunit vaccine based on G protein will therefore need G proteins from both antigenic groups to provide full protection.

The extent of antigenic variation observed among BRSV isolates is considerably less than that observed with HRSV isolates. Four antigenic subgroups (A, B, AB, untyped) have been identified in BRSV but they may only represent variants of a single major antigenic group [66,100–103]. Thus, the variability of the nucleotide sequence of the highly variable G gene does not exceed 15% between independent BRSV isolates [81,104]. The existence of six BRSV genetic subgroups based on the sequence of G and of five subgroups based on that of F or N has nevertheless been

established [104]. These classifications show a spatial clustering and a continuous evolution of BRSV isolates, probably driven by selective pressure as a result of the immune response induced by vaccination [104]. The biological significance of these subgroups remains unclear. Polyclonal sera obtained from calves vaccinated with the BRSV G protein from a subgroup A virus recognized a different subgroup A isolate but failed to recognize a subgroup B or an untyped isolate [105]. Moreover, recognition of a subgroup AB isolate was weaker than that of the subgroup A isolate. In contrast, vaccination with a strain isolated in 1969 and classified in genetic group II protected calves against challenge with a BRSV strain isolated in 2003 and belonging to the distant genetic group V (unpublished observation).

Studies to explain the molecular bases of RNA virus genetic variability showed that these viruses evolve as dynamic distributions of closely related mutant genomes that exist in equilibrium around a theoretical consensus sequence. Such *quasispecies structure* was observed *in vitro* and *in vivo* for BRSV with mutation frequencies ranging from 6.8×10^{-4} to 10.1×10^{-4} substitutions per nucleotide [106]. This genetic heterogeneity is shaped by the selective pressures of the environment and provides great adaptability among virus populations. For example, the emergence of HRSV escape mutants after passive administration of an anti-F monoclonal neutralizing Ab (palivizumab) has been observed both *in vivo* and *in vitro* [107,108]. It was also shown that only two aa changes (Arg 188 and Lys 192) in a dominant T cell epitope of the HRSV G protein were able to abolish the protection induced by this protein in a mouse challenge model [109]. The consequence of the existence of such a quasispecies on RSV vaccination is not clear to date but we can presume that an infecting virus will more readily escape the immune response directed against only one epitope than against several epitopes. In addition, a live attenuated vaccine strain with only one attenuation mutation will be more highly prone to reversion to virulence than a strain with several attenuation mutations.

3.4. Vaccine-associated disease enhancement

In fact, human vaccine development has essentially been hampered by HRSV vaccine-associated disease. In the 1960s, a formalin-inactivated RSV (FI-RSV) vaccine was evaluated in infants and children. It was found to be poorly protective and, unexpectedly, was associated with an increased frequency and severity of RSV disease upon subsequent natural infection [27,110,111]. Early studies suggested that FI-RSV induced high titres of RSV serum Abs as measured by ELISA but relatively low levels of RSV-neutralizing activity, suggesting that formalin inactivation selectively altered the protective epitopes located within the F and G surface glycoproteins [111]. In addition, lymphocytes from FI-RSV vaccinees showed a greater proliferative response to RSV antigens than did those obtained from children naturally infected with *wt* RSV, and a peripheral eosinophilia was observed in some FI-RSV vaccinees [111]. Mechanisms responsible for the FI-RSV vaccine-associated disease enhancement have been extensively studied and discussed [27,28,111] but they still are not completely understood. In mice, disease potentiation by FI-RSV was shown to depend on an increased stimulation of Th-2 CD4+ T lymphocytes

[111–113], while RSV-specific Abs were not sufficient to cause disease enhancement [114]. Further studies revealed a marked increase in the expression of Th-2-type cytokines (IL-5, IL-13, and IL-10) and a reduced expression of IL-12 in FI-RSV-immunized mice, indicating a Th-2 bias and an increase in proinflammatory cytokines [115]. In addition, the mouse model has provided evidence for down-regulation of CD8+ and CD4+ T cell responses [48,115,116]. Based upon these data, it was postulated that FI-RSV vaccinees remained susceptible to infection with *wt* RSV because vaccination produced inadequate levels of neutralizing Abs in serum and did not induce local immunity. Once the vaccinees were infected with *wt* RSV, the virus was not readily cleared because FI-RSV had not primed for CTL responses. In addition, immunization with FI-RSV primed for a Th-2-like response, an influx of lymphocytes and eosinophils, and the possible release of additional mediators resulting in local inflammation and bronchoconstriction.

By using recombinant vaccinia viruses (VV) expressing individual RSV proteins, it was shown that VV expressing the G glycoprotein primed a Th-2 cell response and led to secondary RSV disease [117], while VV expressing the F glycoprotein primed a Th-1 response including CTLs [118]. In addition, VV-M2 primed for a secondary RSV disease characterized by a strong CTL response [27]. However, immunization with VV recombinants expressing individual RSV proteins did not lead to enhanced disease of the same severity as that seen after FI-RSV vaccination. Also, immunization with the G protein was shown not to be necessary for FI-RSV-enhanced disease [119,120]. In non-human primates, patterns of FI-RSV-induced disease augmentation were generally similar to those seen in other animal species [121]. However, facilitation of RSV infection in cell culture by serum Ab from animals immunized with FI-RSV suggests that Ab might play a role in FI-RSV enhancement of disease [122].

The immunopathological FI-RSV model was also described for BRSV in calves, the natural host. Results showed that immunization with FI-BRSV generally resulted in an adequate neutralizing Ab response [123] and mainly primed inflammatory responses, which were associated with an eosinophilic influx into the bronchial alveolar lung fluid and lung tissues and high levels of immunoglobulin E serum Abs [82,124,125]. In addition, the FI-BRSV vaccine was not able to efficiently prime long-term T cell memory and several response patterns (Th-l/Th-2) seem to co-exist during BRSV infection. [58]. It has been suggested that the immunopathogenic immune response to BRSV may be mediated by the deposition of immune complexes and by complement activation in the lungs [126] and/or by the induction of a strong Th-2 biased immune response [112,114,115,119]. Immunopathological vaccine-associated disease was also demonstrated with commercialized inactivated BRSV vaccines after experimental [127] or natural [128,129] BRSV infections.

Finally, the clinical experience with FI-RSV and the information gleaned from animal models of disease enhancement suggest that a good vaccine should induce protective levels of neutralizing Abs as well as a strong CD8+ RSV-specific cytotoxic T cell response and elicit a pattern of CD4+ T cell response similar to that elicited by *wt* RSV.

4. Human and bovine respiratory syncytial virus vaccine development

4.1. Inactivated whole virus vaccines

In humans, the risk of disease enhancement has made vaccination of RSV-naïve infants with whole inactivated virus unacceptable to many registration authorities. However, the fact that disease enhancement is not observed if FI-RSV is administered as a boost to already infected animals suggests that an RSV subunit vaccine would be suitable to boost immunity in previously infected individuals including the elderly and individuals at high risk for RSV disease [130]. In addition, several sub-unit vaccines were shown not to be associated with disease enhancement. It is also possible that novel immunization strategies that combine non-replicating subunit vaccines with cytokines or new adjuvants will permit to drive the immune response towards a Th-1 pathway [131].

The situation is quite different in veterinary medicine. Despite a few observations of respiratory disease enhancement after infection in cattle previously vaccinated with inactivated virus [124,125,128,129], whole inactivated BRSV vaccines have been available for nearly 2 decades. Experimental studies have documented the efficacy of the new, recently licensed inactivated BRSV vaccines in significantly reducing the prevalence and severity of respiratory disease in cattle that were challenged with a virulent field isolate, and no disease enhancement was observed [82,127]. In addition, although millions of doses of vaccines have been used in the field, no immune-mediated enhancement of disease has been reported to date by pharmacovigilance authorities. One explanation could be that the current inactivated vaccines are adjuvanted with *Quillaja* saponin, which was shown to stimulate CD8+ and Th-1 CD4+ effector T cells. The dose of BRSV antigen, differences in formulation and inactivation process as well as differences in adjuvants may be responsible for the induction of different immune responses to BRSV vaccines [123,132]. It is therefore difficult to draw a general conclusion about the protective or disease-enhancing properties of whole inactivated BRSV vaccines.

4.2. Subunit and synthetic vaccines

4.2.1. Antigens

Research on HRSV vaccines has essentially focused on subunit vaccines including viral proteins or peptides. Two approaches have been developed to produce subunit vaccines. The first approach was to use chimeric FG glycoprotein or full-length F protein (PFP-1, PFP-2 and PFP-3) purified from infected cells. These vaccines were first tested in a variety of rodent and primate models [89,114,133–137]. In rodents, when administered parenterally, they were shown to protect the lower respiratory tract but not the upper respiratory tract against RSV challenge [114,133,135,136]. Intranasal immunization with PFP-1 protected the upper respiratory tract against challenge [138], while the combination of intranasal and subsequent parenteral immunization provided protection of both the upper and lower respiratory tracts as observed after wild-type virus infection [138]. However, enhanced pulmonary

pathologic changes were observed with the FG and PFP vaccine candidates in cotton rats, with high ratios of F-binding Abs to RSV-neutralizing Abs [114,139]. In African green monkeys, the FG candidate vaccine delivered intramuscularly was found to be safe but unfortunately induced only low levels of serum RSV-neutralizing Abs and afforded minimal protection of the lower respiratory tract to challenge with *wt* virus [114]. These candidates were finally tested in human clinical trials involving elderly volunteers [140,141], pregnant women [142] and children older than 1 year [143–147]. The PFP vaccines were shown to be safe and moderately immunogenic in pregnant women [142], children with chronic lung disease [146,147] and the elderly [140,141,146,147]. A meta-analysis was performed on PFP vaccine safety and efficacy studies that suggested that RSV subunit vaccines reduced the overall incidence of RSV infections, a conclusion which however needs to be confirmed by large field trials because of doubts about the appropriateness of pooling samples from different studies, the risk of publication biases and the fact that the clinically important RSV lower respiratory tract infection was not reduced in the vaccinees [4].

The second approach followed for the development of a subunit HRSV vaccine was that of BBG2Na, a recombinant fusion protein produced in bacteria that consisted of the central antigenic domain (amino acids 130–230) of the HRSV G protein of subgroup A fused to the C-terminal end of the albumin-binding domain of the streptococcal G protein [148]. BBG2Na was proved to be safe and moderately immunogenic in adult mice and in neonatal mice with or without maternal Abs [148–153]. Protective immune responses were demonstrated against both HRSV subgroups A and B but the duration of protective immunity against group B was insufficient to prevent infection for the duration of the epidemic season [154]. Priming of neonatal mice with BBG2Na formulated in either Al(OH)(3) or TiterMax (a Th-1-driving adjuvant) resulted in predominant Th-2- and Th-1-like responses, respectively. Despite these differences, no lung immunopathology was observed after challenge of the animals vaccinated with either vaccine candidate [150]. It has been suggested that the absence of disease enhancement may have been related to the fact that the G moiety in BBG2Na was not glycosylated, as it was produced in bacteria. Finally, unconclusive results about immunopathological safety of alum-adsorbed BBG2Na were obtained in infant macaques, leading to a request for more extensive studies before proceeding to clinical trials in seronegative infants [155]. BBG2Na was nevertheless investigated in a phase III clinical trial in adult volunteers, but the trial had to be stopped due to the occurrence of unexpected adverse events.

Many other studies on the development of RSV subunit vaccines have been published but these newer vaccine candidates are less far along in development. They include the RSV N protein (S. Riffault and J.-F. Ellouët, pers. comm.), a baculovirus-expressed chimeric protein created by fusing the RSV F and G ORFs [156], as well as synthetic peptides. Recently, peptide G20, a 69 amino acids peptide derived from the G protein deleted of the T helper cell immunopathogenic epitope, generated a highly protective Ab response against HRSV challenge in Balb/c mice [157]. A new recombinant protein was also obtained by fusing the N-terminus of the chimeric CTL epitope F/M2(81-95) from HRSV to the disulphide bond isomerase

used as a carrier. Administered by intraperitoneal injections into mice, the fusion protein elicited effective virus-specific CTL responses as well as protective immunity without association with enhanced disease [158]. The same M2 epitope was also fused to HRSV G protein fragment 125–225: the resultant fusion protein was shown to induce not only humoral but also cellular immunity and a balanced IgG1/IgG2a response [159].

4.2.2. Adjuvants

Whatever the purified recombinant protein or peptide used, it usually requires the co-administration of an adjuvant when injected alone, in order to elicit optimal immune responses. For RSV vaccines, new adjuvants are specifically tested to activate neutralizing Ab, CD8+ CTL and Th-1 CD4+ T cell responses, as well as mucosal immune responses.

Experimentally, several adjuvants were tested with some success: cholera toxin (CT), caprylic/capric glycerides (CCG) [160] and polyoxyethylene-20-sorbitan monolaurate (PS) [161] were tested with FG or PFP proteins, bacterial outer membrane vesicles (OMV) with inactivated RSV [162] and dimethyl dioctadecylammonium bromide (DDA) with BBG2Na [163]. A major concern for the use of enterotoxins is related to their toxicity especially for the central nervous system [164]. *Quillaja* saponi is the only adjuvant used in current commercialized inactivated BRSV vaccines. These vaccines were shown to reduce clinical disease [165] and virus shedding following experimental BRSV challenge, even in calves with high levels of maternal Abs at the time of vaccination [132,166,167]. The purified fraction 21 of Quillaja saponaria (QS-21) was also tested with the F protein of HRSV in Balb/c mice, in comparison with aluminium hydroxide (F/AlOH) adjuvant and wild-type infection [137]. Results showed that F/QS-21 induced a greater titre of seric virus-neutralizing activity than F/AlOH and local pulmonary immune responses similar to those obtained after experimental infection. It was also shown that QS-21 synergized with recombinant interleukin-12 to create a potent adjuvant formulation for the F protein of HRSV [168].

The immunostimulating complexes (ISCOMs) also are strong adjuvant and delivery systems for parenteral as well as mucosal immunization. The ISCOM technology relies on the multimeric presentation of an Ag with adjuvant molecules in a symmetrical particle composed of *Quillaja* saponins, cholesterol, phospholipids, and protein. ISCOMs were shown to have strong mucosal adjuvant activity when used by the nasal route. A panel of ISCOMs including the F and G HRSV glycoproteins and containing different Quillaja saponin fractions were tested for their capacity to induce innate and acquired immune responses [169]. All combinations induced various degrees of Th-1 biased responses in mice, with prominent production of IFN-γ and strong induction of IgG2a Abs. For BRSV, the protection induced by ISCOMs was evaluated and compared with that of a commercial inactivated vaccine in calves with BRSV-specific maternal Abs. The results showed that, in contrast to the inactivated vaccine, ISCOMs could overcome the suppressive effect of maternal Abs and induce a strong clinical and virological protection against a BRSV challenge [170]. HRSV ISCOM vaccines also induced

protection in the presence of passive Abs in animal models but the feasibility of this approach in human neonates is unclear at this time.

Another category of compounds with significant promise as adjuvants for subunit vaccine antigens is synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides. These compounds were reported to have adjuvant activity in mice and to drive the immune response towards a Th-1 phenotype [171,172]. The feasibility of using ODN–CpG motifs for subunit vaccines against HRSV or BRSV was tested in different models. In BALB/c mice, subunit HRSV F and FI-BRSV vaccines were compared with control vaccines [173]. Co-administration of these two vaccines with CpG–ODN resulted in statistically significant increases in serum neutralization titres, an enhanced generation of splenic antigen-dependent killer cell precursors, an accelerated clearance of infectious virus from lungs and, finally, an enhanced ability to elicit Th-1 immune responses. Cotton rats immunized with HRSV F protein and CpG–ODN as a nasal adjuvant showed enhanced pulmonary pathology after a live-virus challenge despite an increased humoral neutralizing-Ab response [174]. This observation has not yet been confirmed in non-human primates.

In the field of BRSV, CpG–ODN appeared to be a suitable candidate adjuvant for inactivated vaccines. Used with FI-BRSV vaccine in calves, CpG–ODN increased the number of IFN-γ-secreting cells in the peripheral blood and bronchotracheal lymph nodes, enhanced the BRSV-specific serum IgG2 response and decreased the multiplication of BRSV in the lung tissue upon challenge of the vaccinated animals [175].

The capacity of dendritic cells (DCs) to present exogenous antigens into the MHC class I pathway and to induce CTL responses [176] could also represent a new and promising strategy for the development of new vaccines. Several molecules such as Fc receptors, C-type lectins, DC-SIGN mannose receptor and CD205, which have been shown to increase the capture and endocytosis of viral antigens by dendritic cells, have not yet been tested with RSV. Recently, nanoparticles of selected sizes (in the range of 20–120 nm) were combined with domains of RSV proteins G or M2.1 and assessed *in vivo* for uptake by DCs. Results showed that the balance between the Th-1 and Th-2 immune responses to vaccination was influenced by nanoparticle size, which could be useful for the development of effective vaccines against RSV [177].

4.3. Live virus vaccines

Since natural infection with RSV does not predispose to severe disease upon subsequent exposure to the virus, vaccine development has focused on live vaccines administered by a mucosal route, because they best mimic natural infection. Intranasal immunization with a live attenuated RSV vaccine should also induce both systemic and local immunity and therefore protect against upper as well as lower respiratory diseases. In addition, the mucosal route of immunization has the advantage of reducing the immunosuppressive effects of maternally derived Abs.

4.3.1. Classical live attenuated vaccines

Live attenuated vaccines against BRSV have been on the market since the 1970s. Attenuation was essentially obtained by serial passages of the virus in cell culture. Until 2006, these vaccines were administered by the parenteral route for fear of reversion to virulence, horizontal transmission, potential recombination between vaccine and wild-type field strains, and possible infection of humans. Live attenuated BRSV vaccines are currently used today and show the same efficacy as inactivated vaccines. In experimental studies in calves, these vaccines elicited a partially or completely protective immune response against BRSV challenge [178–181]. In-the-field studies have shown a variable effect of live attenuated BRSV vaccination on prevalence of clinical disease, needs for treatment or productivity of young calves, weaned animals and cows, perhaps related to variations in virus prevalence or prior exposure of the animals to the virus [182–185].

As recent studies suggested that HRSV and BRSV display a highly restricted host range *in vivo* [186], a new live attenuated BRSV vaccine administered by the intranasal route has been commercialized in 2006 [187]. Virus excretion was significantly reduced when vaccinated calves were challenged with a wild BRSV strain 10 days after vaccination [187]. However, transmission of the attenuated vaccine strain was observed from vaccinated to unvaccinated calves. Partial clinical protection was demonstrated in this study when vaccinated calves were challenged 21 days after immunization [187].

BRSV has also been considered as a possible vaccine against HRSV [186]. Wild-type BRSV was not directly tested as a vaccine in humans. However, a genetic recombinant BRSV vaccine was tested in chimpanzees and shown to be over-attenuated and not to protect the animals against challenge with HRSV, despite its high levels of replication in the upper respiratory tract [186]. Adaptation to the new host was improved by replacing the *G* and *F* genes of BRSV with their HRSV counterparts, but the resulting chimeric virus still remained overattenuated [186]. For this reason, BRSV is generally not considered as a suitable candidate vaccine for HRSV.

In humans, several strategies for the development of an attenuated HRSV vaccine were originally explored in the 1960s, including the creation of host range mutants, cold-passaged (*cp*) mutants, and temperature-sensitive (*ts*) mutants [110,188–190]. To summarize, these vaccine candidates were either underattenuated (*cp* and *ts*-1) or overattenuated (RSV *ts*-2), and reversion to the *wt* phenotype was observed in viral isolates obtained from children vaccinated with the RSV *ts*-1 mutant. Importantly, enhancement of disease was not observed when infants who received RSV *ts*-1 or *cp*-RSV were naturally infected with *wt* HRSV [191,192]. The most promising mutants (*cpts*) were obtained when wild-type HRSV strain A2 (subgroup A) was first subjected to extensive cold-passage *in vitro*, resulting in a moderately attenuated mutant called *cp*-RSV, which was then subjected to two rounds of chemical mutagenesis to yield temperature-sensitive (*ts*) derivatives. This strategy generated candidate vaccines with a range of shut-off temperatures (35–37 °C) that displayed a spectrum of attenuation in rodents and non-human primates [134,193–195]. The *cpts* 248/955 and 530/1009 vaccines retained the *ts* phenotype after prolonged replication

in RSV-seronegative children, indicating a stable *ts* phenotype [196]. These candidate vaccines were shown to protect chimpanzees against challenge with *wt* RSV [134,194,196] and to considerably restrict challenge virus replication in the upper and lower respiratory tract of seropositive animals [134], indicating that they could induce protective immune responses in the presence of passively acquired RSV Abs. Several promising *cpts* mutants were shown to induce a local and systemic protective immune response in seropositive as well as seronegative children [197,198]. However, to date, none of these vaccines, including the highly attenuated *cpts*-248/404, seems to be sufficiently attenuated to be used in 1–2-month-old RSV-naïve infants [198].

4.3.2. Live RSV vaccines derived via genetic engineering

The application of reverse genetics to RSV [199] has opened new ways to develop live attenuated viruses. First, it has provided significant information about the role of viral proteins in pathogenicity and the interplay of viral proteins with components of the host cell immune response. Second, it is a powerful tool to introduce combined attenuating mutations or deletions in the RSV genome to fine-tune the level of attenuation and to produce vaccines sufficiently infectious and immunogenic yet attenuated and genetically stable.

Reverse genetics was first used to evaluate the individual effects of the mutations observed in the *cpts* candidate vaccines, by introducing single attenuating mutations into RSV cDNA [200]. The combinations of several mutations and deletions were then tested *in vitro* and *in vivo*. The most promising candidate, designated rA2cp248/404/1030/_*SH*, is a recombinant version of *cpts*-248/404 that has been further attenuated by the inclusion of an additional *ts* mutation, called 1030, from *cpts*-530/1030, as well as by the deletion of its *SH* gene. This strain was more thermosensitive and attenuated and resulted in a lower level of virus shedding in seronegative children than *cpts*-248/404. This is the first RSV vaccine candidate to be sufficiently attenuated to be tested in young infants, although protective immunity against wild-type RSV challenge was not evaluated in this study [201].

The deletion of non-essential genes represents another promising strategy for vaccine development, either on their own or in combination with point mutations. Five genes, namely *NS1*, *NS2*, *SH*, *G*, and *M2-2*, can be deleted or silenced without much effect on virus yields *in vitro* [199,202–207]. The range of attenuation of HRSV deletion mutants was compared in chimpanzees and mutants could be ranked in order of increasing attenuation as follows: Δ*SH*-Δ*NS2*-*cpts*-248/404-Δ*NS1*-Δ*M2-2* [205,208].

Deletion of the *G* gene was thought unlikely to be useful since G was one of the antigens that elicited the greatest and most frequent immune responses [204,205]. Recombinant BRSV lacking the G protein (Δ*G*) was nevertheless tested by the intranasal route in calves and appeared to be overattenuated. More interesting results were obtained with BRSV expressing only the membrane-anchored form of the G protein. This mutant efficiently replicated in the nasopharynx of calves but viral load was reduced in the lungs. Intranasal and intratracheal inoculation induced a complete protection against subsequent BRSV challenge [30]. However, this strain differs from wild-type BRSV by only one single-point mutation, facilitating potential

reversion. Protection and safety was also demonstrated in the mouse model with an HRSV mutant lacking the ability to secrete the G protein [209].

Several other deletion mutants have been tested. The *SH* gene is not essential for the replication of either HRSV or BRSV *in vitro*. *In vivo*, BRSV Δ*SH* replicated as efficiently as wild-type virus in the bovine nasopharynx but was attenuated in the lungs [206,210]. The immunogenic potential of the BRSV Δ*SH* strain has not yet been evaluated in calves. Immunization of chimpanzees by the intranasal and intratracheal routes with a HRSV Δ*SH* strain-induced seric titre of virus-neutralizing Abs comparable to those induced by wild-type HRSV [208]. The HRSV Δ*SH* strain was shown to be moderately attenuated.

HRSV mutants with deletions of the *NS2* (Δ*NS2*) or *NS1* (Δ*NS1*) genes induced serum-neutralizing Ab levels comparable to or slightly lower than those induced by wild-type HRSV when tested in chimpanzees but clinical protection was not tested in this study [205]. Experiments performed in seronegative calves with BRSV Δ*NS1* and Δ*NS2* showed that intranasal and intratracheal vaccination induced a specific Ab response, primed BRSV-specific CD4+ T cells and protected calves against a subsequent challenge with a virulent strain of BRSV [211]. The Δ*NS2* mutant seemed to induce a stronger immunity than Δ*NS1* in calves.

Silencing the M2-2 ORF resulted in a virus in which transcription and antigen expression was increased, whereas RNA replication and virus production were decreased. This regulation of RNA replication could provide a new phenotype that might increase vaccine immunogenicity. Deletion of M2-2 in HRSV indeed produced a virus that was attenuated and immunogenic in chimpanzees [212] and African green monkeys [213,214]. No such deleted mutants were tested for BRSV.

Two points must be emphasized regarding these studies. First, in the chimpanzee model of HRSV vaccination, attenuation was only estimated on the basis of reduction of virus excretion in the upper and lower respiratory tracts of vaccinated and infected animals. Actually, due to the limited availability of animals, challenges were performed with a non-virulent strain and clinical protection was therefore not fully investigated. In this context, the BRSV model of vaccination may provide a more accurate information. Second, deletion mutants have only been tested so far in seronegative animals: we do not know the impact of maternal Abs on their immunogenicity and, consequently, on their possible efficiency in the field.

Other genetic approaches have been derived from studies on RSV pathogenicity. The HRSV G protein was shown to be a structural and functional mimetic of fractalkine [215], a proinflammatory CX3C chemokine that mediates leucocyte migration and adhesion. This mimicry interferes with the host immune response [216,217] and is a factor in pathogenesis during HRSV infection [218–220]. Mutagenesis or deletion of the CX3C motif in G did not affect virus growth *in vitro* or infectivity in mice. A similar approach was performed with the F protein of BRSV. The cleavage of BRSV F0 protein during the virus replication cycle at two sites of the sequence results in the formation of the F1 and F2 subunits and in the release of a small 27-mer peptide, pep27 [221,222]. This peptide is further converted into a virokinine, which induces smooth muscle contraction *in vitro*, and may therefore contribute to bronchoconstriction *in vivo* [223]. Recombinant BRSV with

mutations that abolished cleavage at the second site (rFCS2) or from which pep27 was deleted (Δp27) were produced. Intranasal and intratracheal inoculation of these two recombinants into calves induced a priming of BRSV-specific T cells and a protective immune response similar to that induced by *wt* virus [210], despite a 10-fold lower BRSV-specific neutralizing Ab titre with FCS-2 than with Δp27 or wild-type. Reverse genetics was also used to increase the efficiency of protective antigen expression by moving the *G* and *F* genes from their natural positions in the genome as the seventh and eighth genes in the gene order to promoter–proximal positions, resulting in an increase in their expression [224]. It was also possible to further increase their levels of expression by codon optimization.

Finally, it is possible to insert a small gene in the RSV genome, like genes expressing immunomodulatory proteins, so as to increase the immune response to the virus. For example, insertion of the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene in the RSV genome resulted in a dramatic increase in the number of pulmonary DCs and macrophages in mice following intranasal inoculation of the recombinant [225].

4.4. Live recombinant vaccines

This approach was evaluated initially in BALB/c mice with recombinant vaccinia viruses (VV) expressing RSV proteins. VV-M2 conferred short-lived protection in BALB/c (*H-2d*) mice while VV-F and VV-G each induced a long-term protection against *wt* RSV challenge in BALB/c mice of three major histocompatibility complex haplotypes [226]. However, these recombinants were poorly immunogenic when administered parenterally to chimpanzees [227] and were considered to be not suitable for vaccination against HRSV. For BRSV, modified vaccinia virus Ankara (rMVA)-based vaccine candidates expressing the F protein, in combination or not with the G protein, were recently shown to induce BRSV-specific IgGs and CD8+ T cell responses as well as partial protection after challenge [228].

Regarding HRSV, the more advanced project concerns heterologous mononegaviruses as vectors for RSV antigens. One major advantage would be that the vector itself is a needed vaccine rather than simply a carrier. Since human parainfluenza virus type 3 (HPIV3) is second only to RSV in importance as a paediatric viral respiratory tract pathogen, a chimera was constructed between HPIV3 and its antigenically related bovine counterpart, BPIV3. BPIV3 is attenuated in primates due to a natural host range restriction and was found to be immunogenic, safe and genetically stable in seronegative infants and children [229]. First, BPIV3 was modified by reverse genetics so that its major protective antigen genes F and HN were replaced with the homologous genes from HPIV3. This chimeric virus, rB/HPIV, combines the major protective antigens of HPIV3 with the attenuated backbone of BPIV3 [230] and is a potential vaccine candidate for HPIV3. Next, the HRSV *G* and *F* glycoprotein genes were inserted singly or together into rB/HPIV3 at a promoter–proximal insertion site, so as to maximize their expression [231]. The resulting chimeric rB/HPIV3-RSV viruses replicated efficiently *in vitro* and expressed high levels of the RSV G and F glycoproteins [232]. When

inoculated into the respiratory tract of rhesus monkeys, they were somewhat more attenuated than their rB/HPIV3 parent but nonetheless proved to be highly immunogenic against both HRSV and HPIV3 [231]. Immunogenicity and protective effects of similar rB/HPIV3 constructions containing soluble or native HRSV F protein were also demonstrated in African green monkeys and hamsters [233,234]. Finally, mixing two rB/HPIV3-RSV recombinants with HRSV subgroups A and B specificity, respectively, provided a trivalent paediatric vaccine against RSV-A, RSV-B and PIV-3. Interestingly, the rB/HPIV3-RSV chimeric viruses were not neutralized by RSV-specific Abs, raising the possibility that a PIV-vectored RSV vaccine might be particularly useful as a booster immunization in infants previously immunized with an RSV vaccine.

In a similar way, a bovine herpesvirus-1 (BoHV-1) recombinant expressing the G protein of BRSV was constructed by insertion of a synthetic G gene behind the BoHV-1 gE promoter, to give a gE-negative, BoHV-1/G recombinant. This recombinant induced virological protection in calves against BRSV challenge but expression of the G protein of BRSV increased the virulence of BoHV-1 for calves after vaccination [235].

4.5. DNA vaccines

DNA vaccination is an efficient way of inducing CD8 + T-cell responses, although responses are generally weaker than those induced by live vectors. However, DNA vaccines offer several advantages. They are simple to store and administer and generate endogenous synthesis of antigen, allowing encoded proteins to enter the major histocompatibility complex (MHC) class I presentation pathway and securing efficient induction of CD8 + T cells [236]. Furthermore, there is evidence that DNA vaccines may circumvent the inhibitory effects of maternal Abs [237], although this has not been a consistent finding [238–240]. However, DNA vaccines do not appear to be significantly immunogenic in humans, so the utility of this strategy for immunizing human neonates is unclear at this time.

The ability of DNA expressing either the F or G protein to protect against infection has been successfully tested for HRSV in mice or cotton rats and for BRSV in calves [236,241,242]. In addition, DNA immunization with the *G* gene did not significantly enhance pulmonary pathology following virus challenge [243,244]. When tested in non-human primates, HRSV DNA vaccines were not highly immunogenic and required one or more booster immunizations that would be difficult to administer to infants in the short-time window between birth and the peak of serious RSV disease. Regarding BRSV, the protection of calves afforded by intramuscular or intradermal vaccination with BRSV DNA encoding the F glycoprotein was similarly not as great as that induced by prior BRSV infection [245]. Serum RSV Abs of calves vaccinated with DNA-F developed more slowly when compared with those previously infected with BRSV and only the intramuscular route primed for a rapid BRSV-specific IgA response after viral challenge [245]. Combination of genes and codon optimization may improve efficacy of vaccination as recently shown by Boxus et al. [84]. In this study, two

administrations of codon-optimized plasmids encoding the BRSV F and N proteins elicited a Th-1 biased immune response, but again protection of calves against a highly virulent challenge was not complete in spite of a drastic reduction of virus replication in the host [84].

In all these studies, the immunization regimens required large quantities of DNA. Therefore gene-gun immunization, which allows one to use much smaller quantities of DNA, was tested for HRSV with DNA encoding the F or G proteins or the Kd-restricted 282–90 epitope of M2-2. This technique was shown to be more immunogenic [246] and to protect mice against RSV infection [247–249]. However, gene-gun immunization with DNA-F or DNA-G was associated with an unwanted Th-2-biased response and cutaneous gene-gun immunization of BALB/c mice with a DNA vaccine encoding the Kd-restricted M2-2 epitope was followed by non-specific enhancement of RSV disease after challenge [250]. Gene-gun DNA vaccination, therefore, does not appear to be suitable for RSV vaccination.

4.6. Association of different vaccines

Until recently, vaccination was based on single or repeated administrations of the same vaccine preparation. A novel strategy involving priming and boosting with different immunogens has resulted in the generation of unprecedented levels of cell-mediated immunity. This could be advantageous for new vector delivery systems, such as plasmid DNA vector or modified Ankara virus (MVA) expressing RSV antigens since they induced correct cellular and humoral immune responses when administered individually but were insufficient to provide protection against challenge. The safety and immunogenicity of the HRSV live attenuated *cpts* 248/404 mutant and of the PFP-2 vaccine candidate were tested in a placebo-controlled trial in 60 healthy young adults and 60 healthy elderly subjects using simultaneous or sequential (*cpts* 248/404 followed by PFP-2) vaccination schedules. In both age groups, sequential immunization elicited higher RSV F IgG and IgA titre than simultaneous immunization [197]. Recently, immunization with BRSV plasmids encoding the F and N proteins was shown to elicit a Th-1 biased immune response and partial protection in calves against challenge with a highly virulent BRSV strain. When DNA vaccination was followed by a boost with a killed BRSV vaccine, protection of the animals was complete without disease enhancement [84].

5. Conclusions

The basic goal of HRSV and BRSV vaccination is not to prevent RSV infection but to prevent RSV-associated lower respiratory tract disease. The strategies of vaccination are however different for HRSV or BRSV. In humans, several factors complicate the development of an effective and safe vaccine and there still is no licensed vaccine for prevention of HRSV disease to date. Scientifically, these factors

also exist for BRSV but vaccines are available because minor risks are acceptable in livestock. Inactivated or attenuated BRSV vaccines have consequently been extensively used in Europe for the past 2 decades with no major associated-disease enhancement observed in the field since 2000. Nevertheless, as for HRSV, obstacles to BRSV vaccination remain, which include short induced protection, the need to immunize immunologically immature animals and insufficient efficacy of the vaccine in the presence of maternal Abs. Efforts are being made to use BRSV attenuated vaccines or subunit vaccines with mucosal adjuvants by the intranasal route. Licensure of a live attenuated intranasal vaccine in 2006 is too recent to make conclusions about the efficacy of the vaccine in cattle.

In this context, the ability to manipulate the HRSV and BRSV genomes and better understanding of the protective and putative disease-enhancing mechanisms associated with infection have permitted considerable progress in RSV vaccine development during the past 10 years. More complete understanding of RSV immunobiology is still however necessary to understand, in particular, why immunity to RSV is less complete and durable than, for example, that to influenza virus. In human vaccine research, recent advances have focused on different HRSV vaccines such as subunit vaccines with specific CD4+ Th-1 immune activating adjuvants and on genetically engineered live attenuated vaccines. This diversity must be considered as an advantage. Indeed, as a number of age-related factors may influence immunity and severity of disease, it is likely that success will require different vaccine strategies for infants and the elderly. A successful RSV vaccine for infants would have to induce a protective response in an immature immune system in the presence of maternal Abs. In this context, the most promising candidates for young infants are live engineered attenuated RSV strains and RSV antigens vectored from a live attenuated HPIV strain that can be used by the mucosal route. The actual difficulty for these live vaccines is to develop a correct balance between attenuation and immunogenicity in young infants. In addition, even if attenuating mutations can be fine-tuned in a reasonably systematic way by reverse genetics, the level of attenuation specified by a combination of attenuating mutations is not always the sum of individual mutations. Efficacy tests in animal models using highly virulent challenge strains are therefore required to test HRSV candidate vaccines. For the elderly, efficacious vaccines would have to overcome waning immune functions and the presence of neutralizing Abs. Safe subunits vaccines or sequential combinations of different vaccines are promising ways but need further investigation. Success of subunit vaccines will essentially depend on the development of strong adjuvants, which selectively activate neutralizing Abs, CTL and Th-1 CD4+ T cell responses, as well as mucosal immune responses.

Finally, production of safe and efficacious vaccine is a major but only one step of vaccination. Strategies of vaccination and the issue of vaccine storage and delivery will also have to be addressed. This is particularly true for live attenuated vaccines, which must currently be stored at $-70\,^{\circ}C$ and are administered as nasal drops. It is hoped that delivery and storage systems will be developed that will make RSV vaccines suitable worldwide.

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