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Vaccine 38 (2020) 6088–6095

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

First-in-human administration of a live-attenuated RSV vaccine lacking the G-protein assessing safety, tolerability, shedding and immunogenicity: a randomized controlled trial

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ARTICLE INFO

Article history:
Received 7 April 2020
Received in revised form 17 June 2020
Accepted 14 July 2020
Available online 25 July 2020

Keywords:
Respiratory Syncytial Virus
Live-attenuated vaccine
phase I trial
Safety
Immunogenicity
Intranasal

ABSTRACT

Background: Human respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infections in early infancy and in elderly. A pediatric vaccine against RSV would not only prevent morbidity and mortality amongst infants and young children but could also reduce transmission to elderly. The RSVΔG vaccine consists of a live-attenuated RSV that lacks the G attachment protein. RSVΔG is severely impaired in binding to host cells and exhibits reduced infectivity in preclinical studies. Intranasal immunization of cotton rats with RSVΔG vaccine protected against replication of wildtype RSV, without inducing enhanced disease.

Methods: We performed a first-in-human trial with primary objective to evaluate safety and shedding of RSVΔG (6.5 $\log_{10}$ $CCID_{50}$) after intranasal administration. Healthy adults aged between 18 and 50, with RSV neutralizing serum titers below 9.6 $\log_2$, received a single dose of either vaccine or placebo (n = 48, ratio 3:1). In addition to safety and tolerability, nasal viral load, and systemic and humoral immune responses were assessed at selected time points until 4 weeks after immunization.

Results: Intranasal administration of RSVΔG was well tolerated with no findings of clinical concern. No infectious virus was detected in nasal wash samples. Similar to other live-attenuated RSV vaccines, neutralizing antibody response following inoculation was limited in seropositive adults.

Conclusions: A single dose of 6.5 $\log_{10}$ $CCID_{50}$ of RSVΔG was safe and well-tolerated in seropositive healthy adults. RSVΔG was sufficiently attenuated but there were no signs of induction of antibodies. Safety and immunogenicity can now be explored in children and eventually in seronegative infants.

Clinical trial register: NTR7173/EudraCT number 2016-002437-30.

1. Introduction

Respiratory syncytial viruses (RSV) are negative-sense, single-stranded, enveloped RNA viruses of the species *Human orthopneumovirus* [1]. RSV can cause acute respiratory tract infections in persons of all ages [2]. RSV-related acute lower respiratory tract infection accounts for approximately 3.2 million hospital admissions per year worldwide and is a major cause of mortality in children younger than 5 years [3,4]. Globally, RSV is estimated to be second to malaria as a cause of death in infants aged between 1 and 12 months due to a single pathogen [5]. By the age of two years almost all infants have been exposed to RSV [6]. However, immunity against RSV is incomplete and re-infections are common throughout life [7].

Currently there is no effective licensed treatment for ongoing RSV infections. Passive immunization with humanized F-specific monoclonal antibodies (palivizumab) is limited to high-risk infants

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https://doi.org/10.1016/j.vaccine.2020.07.029
0264-410X/

only and its application is primarily reserved to high-income countries due to its high cost. Despite the clear unmet medical need for a safe vaccine and ongoing vaccine development since the 1960s, there is still no effective vaccine available. This is partly due to a failed clinical trial in which a formalin-inactivated RSV vaccine caused enhanced disease following subsequent exposure to natural RSV infection, resulting in hospitalization of vaccine recipients and two fatalities [8,9]. However, multiple novel vaccine strategies against RSV are currently in development. The resurgence of RSV vaccine development is driven by innovations in biotechnology, such as reverse genetics.

With reverse genetic techniques recombinant RSV can be developed for use as a live-attenuated vaccine (LAV). Development of a LAV candidate against RSV has several advantages. Previous studies did not show enhanced RSV-related disease following LAV administration [10]. Live attenuated vaccines can be administered intranasally, thus mimicking the natural route of infection and thereby priming both local mucosal-and systemic immunity. In addition, intranasal inoculation is non-invasive and easy to administer.

Respiratory syncytial virus has two major surface glycoproteins, the attachment (G)- and fusion (F) protein. Both G- and F proteins contain neutralizing antibody binding sites [11]. Unlike the F protein, the presence of the surface protein G is not required for viral replication. Previous research showed that replication competence is reduced in absence of the G-protein [12]. A RSV lacking the G-protein is expected to be attenuated but still capable of inducing an effective immune response due to presence of the surface protein F as the major antigen site and the remaining infectivity. Using reverse genetics Intravacc (the Netherlands) constructed a LAV against RSV from which the coding sequence for the attachment (G) protein was deleted from the RSV genome (RSVΔG) [13]. Preclinical studies confirmed that recombinant RSV lacking the G protein was highly attenuated when administered intra-nasally and single dose administration conferred long lasting protection against wild-type (wt) RSV challenge in a cotton rat model [13]. Here we present the first-in-human (FIH) study aimed to assess the safety, tolerability, viral shedding and immunogenicity of RSVΔG in healthy adult volunteers.

2. Material and methods

2.1. Study design

This was a double-blinded, randomized, placebo-controlled, parallel-group, single-dose study in 48 healthy adult volunteers. The primary objective of this study was to assess safety and tolerability of the vaccine candidate RSVΔG. Secondary endpoints were related to the viral load and shedding of RSVΔG, as well as the immunogenicity. The trial was conducted at the Centre for Human Drug Research (CHDR) in Leiden, the Netherlands. The clinical trial was performed outside the Dutch RSV season to prevent concurrent wt-RSV infection during the trial [14]. Participants were randomized in blocks of four, one placebo and three RSVΔG treatment. Randomization codes were generated in SAS 9.4 for Windows (SAS Institute, Cary, NC, USA) by a study-independent statistician at the start of study. Participants were sequentially assigned to the intervention. Investigators, study staff and subjects were blinded to the allocated treatment.

Subjects were inoculated with a single intra-nasal dose of 0.2 mL (0.1 mL per nostril) of either RSVΔG (dose: 6.5 ± 0.5 $\log_{10}$ $CCID_{50}$) or placebo. Subjects completed follow-up visits on 4, 7, 14 and 28 days after inoculation and received a follow-up phone call after six months. Blood and nasal wash samples were collected on follow-up visits indicated in Fig. 1. Nasal washes were collected using the Naclerio method [15]. Into each nostril 4 mL of 0.9% NaCl was instilled. The solution was kept in the nostril for at least 20 s to allow sufficient dwelling time. The study was approved by the Central Committee on Research Involving Human Subjects (CCMO; The Hague, The Netherlands) and was registered in the European Clinical Trials Database (EudraCT number: 2016-002437-30) and the Dutch trial register (NTR: NTR7173). All subjects provided written informed consent prior to participation in the study. All study related procedures were performed in accordance with the Declaration of Helsinki and the Dutch Act regarding Medical Research Involving Human Subjects. As RSVΔG is a genetically modified organism, the environmental permit on 'deliberate release into the environment' (according to the directive 2001/18/EC of the European parliament and of the council) had been granted before the start of the study.

2.2. Participants

Eligible participants were non-smoking healthy volunteers, aged 18 to 50 years inclusive, with a body mass index between 18 and 30 kg/m^2. Subjects were invited for a full medical screening if they had relatively low levels of pre-existing RSV-specific neutralizing antibodies (nAbs) ($\leq$9.6 $\log_2$) [16]. Eligibility was further assessed on subject's medical history, physical examination (including anterior rhinoscopy, blood- and urine laboratory analyses including pregnancy test for women of childbearing potential), vital signs and electrocardiogram. Exclusion criteria included close contact with infants (<2 years of age) and immunocompromised individuals for 14 days following vaccine administration, any immune deficiency or use of immunomodulatory drugs, airway infection in the period of 14 days before vaccine administration, (active) allergic rhinitis or other allergies involving the airway, chronic airway diseases or history of frequent epistaxis. Participants received oral and written instructions on hygiene rules to prevent transmission of RSVΔG in the case viral shedding would occur.

2.3. Vaccine and intranasal administration

The investigational vaccine was a non-sterile live-attenuated recombinant RSV (RSVΔG, Intravacc, Bilthoven, The Netherlands, batch number 100046). Details on the construction of the RSVΔG vaccine candidate have been described previously [13]. A total of 0.2 mL (0.1 mL per nostril) was administered intranasally using a spraying device (Teleflex VaxiNator™). The inoculated dose consisted of a virus titer of 6.5 ± 0.5 $\log_{10}$ $CCID_{50}$. This dose provided 100% protection against wt-RSV in a cotton rat challenge model without inducing enhanced respiratory disease and was safe in a repeated dose toxicity and local tolerance study in Wistar rats. Placebo treatment consisted of the formulation buffer only and was indistinguishable from the active treatment.

2.4. Safety and tolerability assessments

Vital signs (blood pressure and pulse rate) were measured during every visit. Anterior rhinoscopy was performed by a physician prior to dosing and during every visit to examine the nasal mucosa. If symptoms were present during the visit, a symptom limited physical examination was performed. Blood chemistry and hematology tests were performed prior to inoculation and on day 7 and 14 post-inoculation at the Central Laboratories of Leiden University Medical Center (Leiden, the Netherlands). Tolerability was assessed by asking subjects to rate naso-oropharyngeal pain on a visual analogue scale (VAS), range: 0–100 mm, immediately after intra-nasal vaccine administration and approximately 5 min after administration. Subjects reported solicited adverse events

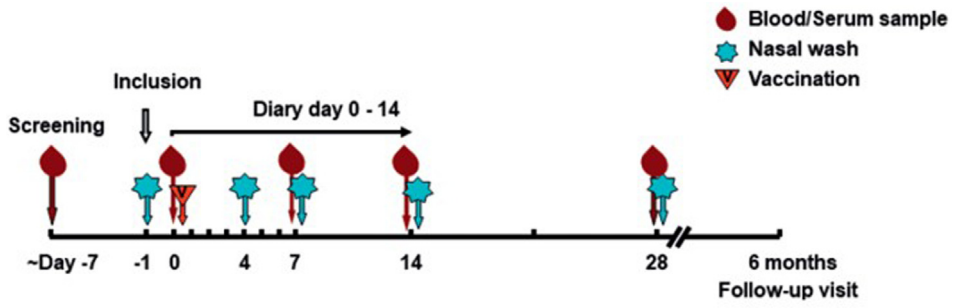

Fig. 1. Study Design.

by completing a daily questionnaire for 14 days following inoculation on a custom designed mobile application (E-diary) [17]. Solicited adverse events consisted of cold-like symptoms and/or reaction to the vaccine such as: sore throat, epistaxis, nasal congestion, rhinorrhea, sneezing, dyspnea, coughing, malaise, myalgia or arthralgia, headache, earache, eye irritation/complaints. Severity of symptoms were scored by the participant on an ordinal scale: 0 = not present, 1 = mild (easily tolerated, light complaints), 2 = moderate (bothersome but tolerable, able to perform daily activities), 3 = severe (difficult to tolerate, withholding daily activities). Symptom severity scores (range: 0–36) were calculated by summing up the scores (0–3) for each question in the E-diary per day. Participants recorded their oral temperature twice daily in the E-diary to assess the development of febrile temperature during the 14 days post-inoculation.

Non-solicited adverse events were assessed by the study physician throughout the study (until day 28). Follow-up phone calls were conducted six months after inoculation to assess late non-solicited adverse events, SAEs and concomitant medication use. For each non-solicited adverse event the relationship to inoculation was judged by the study physician as probable, possible, unlikely or unrelated. In addition, a diagnosis of upper respiratory tract infection (URTI) was given if several solicited (cold-like) adverse events coexisted at the same time and respiratory infection was clinically apparent in the opinion of the study physician. Cold-like symptoms (identical to the solicited adverse events) that were reported after 14 days were recorded in the same manner as non-solicited adverse events.

2.5. Viral shedding

Viral replication was assessed by quantitative culture (qCulture) and quantitative PCR (qPCR) in nasal wash samples on day −1, 4, 7, 14 and 28 after inoculation. All samples were analyzed by Viroclinics B.V. (Rotterdam, The Netherlands). After addition of Phocine distemper virus type 1 (PDV) as a universal internal control, nucleic acid was isolated from nasal wash samples using the MagNA Pure 96 instrument and MagNA Pure 96 kits (Roche Applied Science) [18]. A quantitative RT-PCR was performed for RSV-A on the purified nucleic acid using a Fast Virus Master Mix (Applied Biosystems, 4444436) on a 7500 Real Time PCR machine (Applied biosystems).The PCR target sequence was within the N gene.

Quantitative virus culture was performed by making serial dilutions of the nasal wash material and using these dilutions to infect Vero cells (ATCC® CCL-81™) with four replicates in a 96-well plate format. After 6 days of culture, the cells were fixed and immunostained with a murine monoclonal antibody directed against RSV F protein (Millipore, MAB858), followed by horseradish peroxidase conjugated goat-anti-mouse antibody (Life technologies, A16072) and TrueBlue (KPL, 50–78-02) to detect virus positive wells. The virus titer was calculated according to the Reed and Muench method for $TCID_{50}$ [19].

2.6. Immunogenicity measurement

Immunogenicity was assessed in blood and nasal washes on day −1, day 7 and 28 after inoculation. Virus neutralization assays for serum and mucosal RSV-specific nAbs were performed as previously described [16]. For the palivizumab competing antibody (PCA) assay serum samples were mixed with biotin-labelled palivizumab [20]. Competitive binding was performed in 96-well microtiter plates pre-coated with purified RSV-F. Serial 2-fold dilutions of serum samples were spiked with biotinylated palivizumab and added to RSV-F-coated plates. Unbound material was washed from the wells, and a peroxidase-conjugated streptavidin was added to the plates to determine antigen bound biotinylated palivizumab [20]. Competitive binding titers were expressed as the 50% inhibition titers and were calculated as described by Zielinska et al. [21]. Titers were reported as the reciprocal value of the serum dilution that resulted in 50% inhibition of biotinylated palivizumab binding. For the determination of IgA antibodies against RSV in nasal washes, a commercial ELISA kit was used (IBL International, RE56871) according to the manufacturer's instructions. The IgA concentrations were calculated by linear regression of the OD 450 nM values using the kit internal controls as reference. RSV F-specific antibodies in serum were determined similarly as described previously [22]. In short, ELISA plates (Nunc MaxiSorp; Thermo Scientific) were coated with 25 ng of RSV F protein and incubated with 5-fold serial dilutions of serum samples [23]. After extensive washing, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Pierce) diluted 1:1000. Detection of HRP reactivity was performed using tetramethylbenzidine substrate (Sigma) and an ELISA plate reader (EL-808 [from Biotek]). The IgG titer for RSV F protein was determined by calculating the end-point dilution with Gen5 software.

2.7. Statistical analyses

SAS 9.4 for Windows (SAS Institute, Cary, NC, USA) was used to perform the statistical analysis. Safety measures were analyzed using descriptive statistics. Prior to analysis serum RSV-specific nAbs and F-specific antibodies were transformed to $\log_2$ and $\log_{10}$ titers, respectively. Mean and standard deviation (SD) were determined for RSV-specific nAbs at baseline (day −1) day 7 and 28 and for F-specific antibodies at baseline (day −1) and day 28. RSV-specific nAbs were further analyzed with a mixed model analysis of variance (ANCOVA) with treatment, day, and treatment by day as fixed factors and subject as random factor and the baseline measurement (at day −1) as covariate. The Kenward-Roger approximation was used to estimate denominator degrees of freedom and model parameters were estimated using the restricted

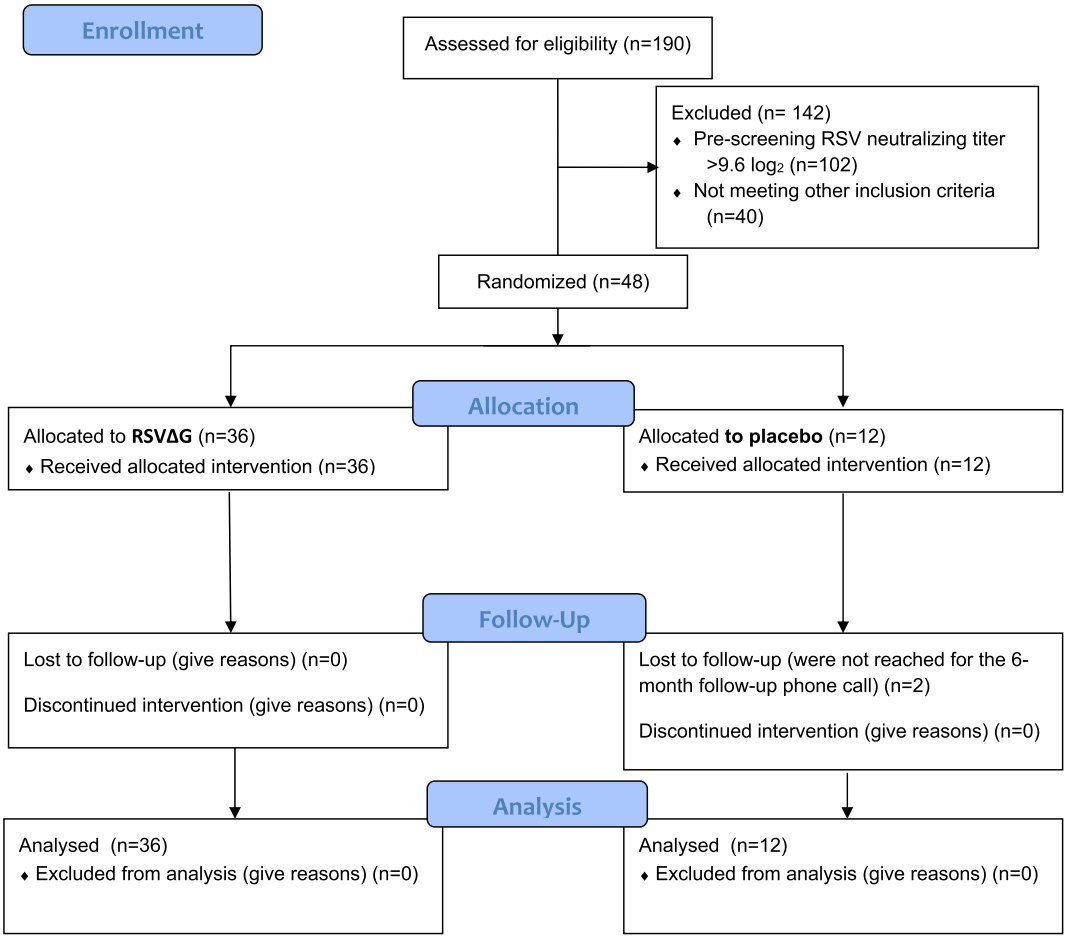

Fig. 2. CONSORT subject flow diagram.

maximum likelihood method. Contrasts were calculated within the model for RSVΔG versus placebo (overall [day 7, 28] and on day 28 separately). F-specific antibodies were analyzed with a general linear model of covariance with fixed factor treatment and baseline F-specific antibodies as covariate and same contrast as mentioned previously. The general treatment effect and specific contrasts were calculated.

3. Results

3.1. Study population

Subjects were recruited from April 2018 until September 2018. A total of 190 volunteers were screened for levels of RSV-specific nAbs. Of these volunteers 102 (53.7%) had pre-screening nAbs titers > 9.6 $\log_2$ and were excluded. Forty-eight subjects were found eligible to participate in the study based on in- and exclusion criteria. All 48 subjects completed the 28-day observation period and were analysed per protocol. See Fig. 2 for the CONSORT subject flow diagram. Subject characteristics were similar for vaccine and placebo recipients (Table 1).

Table 1
Subject characteristics.

Subject characteristics	Groups	
	RSVΔG (n = 36)	Placebo (n = 12)
Gender, *n* (%)		
Female	33 (91.7)	10 (83.3)
Male	5 (10.4)	2 (16.7)
Age, years, median (IQR)	23.5 (20.3, 26.0)	23.5 (21.0, 26.5)
BMI, kg/m^2, mean (SD)	22.5 (3.7)	22.8 (3.1)
Race (n, %)		
White	29 (80.6)	9 (75.0)
Black or African American	1 (4.2)	1 (8.3)
Mixed	5 (10.4)	0 (0)
Asian	1 (4.2)	1 (8.3)
Other	0 (0)	1 (8.3)

BMI = Body Mass Index; IQR: interquartile range.

3.2. Safety and tolerability evaluation.

Intranasal administration of RSVΔG was well tolerated. Naso-oropharyngeal pain VAS scores were similarly low in both the RSVΔG group (t = 0 min: mean = 1.4, SD = 6.1; t = 5 min: mean = 0.9, SD = 2.2) and placebo group (t = 0 min: mean = 0.6, SD = 1.7; t = 5 min: mean = 0.1, SD = 0.3). Examination by anterior rhinoscopy revealed no abnormalities related to vaccine administration. There were no findings of clinical concern in blood chemistry and hematology assessments and no clinically significant values or trends were observed in vital signs (data not shown). There was no apparent increase in body temperature following inoculation with RSVΔG compared to placebo. Two subjects reported a febrile temperature of 38.4 °C (RSVΔG) and 38.2 °C (placebo) on day 10 after inoculation that coincided with complaints of URTI.

In both the RSVΔG and placebo group the majority of subjects reported at least one solicited adverse event in the E-diary during the first 14 days after inoculation (Table 2). Sneezing and

Table 2
Solicited adverse events during first 14 days after inoculation.

	RSVΔG*N* = 36	Placebo*N* = 12
Symptoms	Number of subjects *(%)*	Number of subjects *(%)*
≥1 symptom	29 (80.6)	9 (75.0)
Nasal congestion	11 (30.6)	5 (41.7)
Sneezing	15 (41.7)	5 (41.7)
Rhinorrhea	16 (44.4)	4 (33.3)
Epistaxis	4 (11.1)	–
Coughing	11 (30.6)	2 (16.7)
Sore throat	11 (30.6)	7 (58.3)
Dyspnea	2 (5.6)	2 (16.7)
Eye irritation/complaints	4 (11.1)	–
Earache	2 (5.6)	1 (8.3)
Myalgia/arthralgia	12 (33.3)	4 (33.3)
Malaise	13 (36.1)	6 (50.0)
Fever	1 (2.7)	1 (8.3)

Table 3
Summary of possible or probable related non-solicited adverse events.

	RSVΔG (n = 36)	Placebo (n = 12)
Adverse events	Number of Subjects (%)	Number of Subjects (%)
Subjects with at least one adverse event	19 (52.8)	7 (58.3)
GENERAL DISORDERS		
Fatigue	1 (2.8)	–
Feeling hot	1 (2.8)	–
INFECTIONS AND INFESTATIONS		
Impetigo	1 (2.8)	–
NERVOUS SYSTEM		
Headache	1 (2.8)	–
RESPIRATORY TRACT		
Nasal congestion	3 (8.3)	1 (8.3)
Sneezing	1 (2.8)	–
Throat lesion	–	1 (8.3)
Upper respiratory tract infection	9 (25)	4 (33)
SKIN AND SUBCUTANEOUS TISSUE DISORDERS		
Dermatitis	–	1 (8.3)
Lip edema	1 (2.8)	–
Herpes simplex	1 (2.8)	–

rhinorrhea had the highest relative incidence in the RSVΔG group and sore throat and malaise had the highest relative incidence in the placebo group. Epistaxis and eye irritations/complaints were reported in the RSVΔG but not in the placebo group, however, few subjects reported these symptoms. Overall, the relative frequencies of solicited adverse events were similar in both groups. Severity of solicited adverse events was comparable in both groups (mild: 75% RSVΔG versus 78% placebo, moderate: 18% RSVΔG versus 20% placebo, severe 7.3% RSVΔG versus 2.2% placebo). Total symptom scores were also similar for both treatment groups (Fig. 3). Highest total symptom scores were observed on day 12 in the placebo group.

A summary of all possible and probable related non-solicited adverse events is provided in Table 3. Adverse events related to the respiratory tract were most frequently reported. The diagnosis of upper respiratory tract infection was made in 9 subjects (25%) in the RSVΔG and in 4 subjects (33%) in the placebo group (Table 3). All non-solicited adverse events were mild except for three events that were of moderate severity. These three adverse events consisted of a urinary tract infection (placebo group) and two cases of URTI (RSVΔG group). All adverse events, solicited and non-solicited, resolved without sequelae before the last visit (approximately 28 days after inoculation). At the six month follow-up phone call no SAEs or non-solicited adverse events were reported by the subjects (n = 46). Two subjects (placebo group) could not be contacted for the six-month telephone follow-up interview. No serious adverse event (SAE) occurred during the study and no adverse events resulted in the withdrawal of subjects during this trial.

Concomitant medication to treat adverse events predominantly consisted of the use of paracetamol. Ibuprofen was used in one instance for complaints of URTI. One subject (placebo) was treated with nitrofurantoin to treat a urinary tract infection. One subject (RSVΔG) was treated with acyclovir and valacyclovir to treat a

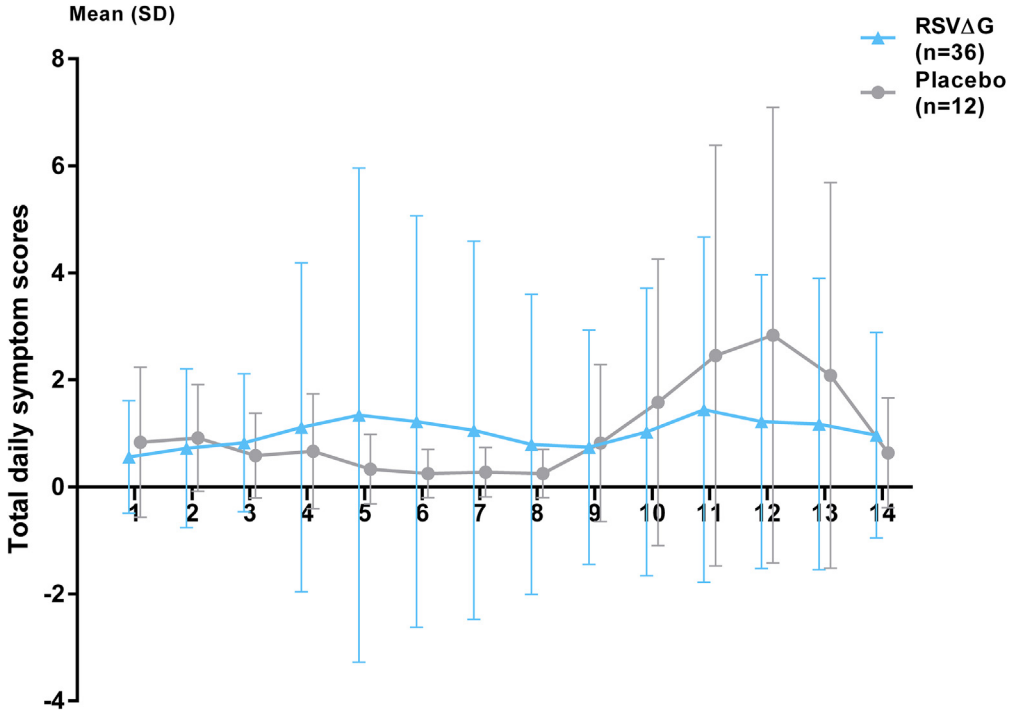

Fig. 3. Mean and SD of total symptom scores (range 0–32) during days 1–14 for RSVΔG and placebo treatment. SD = standard deviation.

Table 4
Mean (SD) titers of serum RSV-specific neutralizing antibodies and F-specific antibodies.

		RSV-specific neutralizing antibodies			F-specific antibodies		
Treatment	*n*	Baseline(Day −1)	Day 7	Day 28	*n*	Baseline(Day-1)	Day 28
RSVΔG	36	8.50 (0.87)	8.49 (0.96)	8.45 (1.07)	35	5.42 (0.31)	5.49 (0.36)
Placebo	12	8.28 (1.38)	8.22 (1.40)	8.22 (1.35)	12	5.36 (0.46)	5.49 (0.37)

Mean (SD) RSV-specific neutralizing antibodies are expressed as $\log_2$ titer, mean (SD) F-specific antibodies expressed as $\log_{10}$ titer. RSV = respiratory syncytial virus.

herpes simplex infection (the subject was familiar with herpes simplex re-activations) following the onset of a URTI. The same subject was later also treated with topical fucidic acid for impetigo and edema of the lip following the herpes simplex infection.

3.3. Viral load

In the RSVΔG group, 3 of 36 subjects (8.3%) had quantifiable qCulture results of nasal wash samples compared to 3 of 12 (25%) subjects in the placebo group. All positive qCulture results were observed on single time points only and in different subjects. Two positive qCulture results were found prior to inoculation (RSVΔG = 1, placebo = 1) and single positive results on day 4 (placebo), day 7 (RSVΔG), day 14 (RSVΔG) and day 28 (placebo). All qCulture results were equal to the lower limit of quantification (LLOQ) (0.75 $\log_{10}$ $TCID_{50}$/mL) except for the single day 14 sample (RSVΔG) with a titer of 1.0 $\log_{10}TCID_{50}$/mL.

The presence of RSV-specific RNA, determined by qPCR, was only detected on day 4 post-inoculation in nasal wash samples of three (8.3%) subjects in the RSVΔG group. All of these samples had qPCR titers below the LLOQ (2.23 $\log_{10}$ vp/mL) and did not coincide with quantifiable qCulture results.

3.4. Immunogenicity

3.4.1. RSV neutralizing antibody titers in serum

All subjects were seropositive for RSV neutralizing antibodies at baseline. Mean $\log_2$ titers of RSV-specific nAbs of RSVΔG and placebo group were similar prior to inoculation (Table 4). The overall fold change in nAbs titers following inoculation was < 2 (Fig. 4). The highest individual observed seroresponse was a 2-fold increase in nAbs titer on day 7 and day 28 in one subject after inoculation with RSVΔG. No treatment effects on RSV-specific nAbs were observed at day 28 and overall.

3.4.2. Palivizumab competing antibodies (PCA) in serum

At baseline, 23% (n = 11) of all subjects had positive serum samples for PCA. Subjects with positive samples on day 7 and 28 also had positive samples at baseline. No evident changes in PCA titers were observed following inoculation with RSVΔG. In the group vaccinated with RSVΔG, the number of PCA seropositive subjects declined from 9 (25%) on baseline, to 7 (19%) on day 7, and 5 (14%) on day 28. In the placebo group there were two (17%) subjects with PCA seropositive samples on baseline, these subjects remained seropositive throughout the follow-up visits.

3.4.3. RSV F-specific antibodies in serum

There were no evident increases in F-specific antibody titers following inoculation. Mean titers of F-specific antibodies of RSVΔG and placebo were similar on baseline (day −1) and day 28 (Table 4). No treatment effects on F-specific antibodies were observed.

3.4.4. RSV neutralizing antibodies and IgA in nasal wash

Titers of mucosal RSV neutralizing antibodies in nasal wash samples were all below the LLOQ (<8). One subject in the RSVΔG group (2.8%) had an IgA reciprocal titer of 31.8 on day 28 only. The increase in IgA titer did not coincide with an increase in other immunogenicity endpoints or with viral shedding. No IgA was detected in the placebo group.

4. Discussion

The results of this first-in-human study showed that a single dose of 6.5 ± 0.5 $\log_{10}$ $CCID_{50}$ RSVΔG is safe and well tolerated. Solicited and non-solicited adverse events were generally of mild to moderate severity, of short duration and resolved without sequelae. Symptom scores of the RSVΔG group were similar to those in the placebo group and showed no substantial rise in the first two weeks following inoculation, confirming the full attenuation phenotype of RSVΔG.

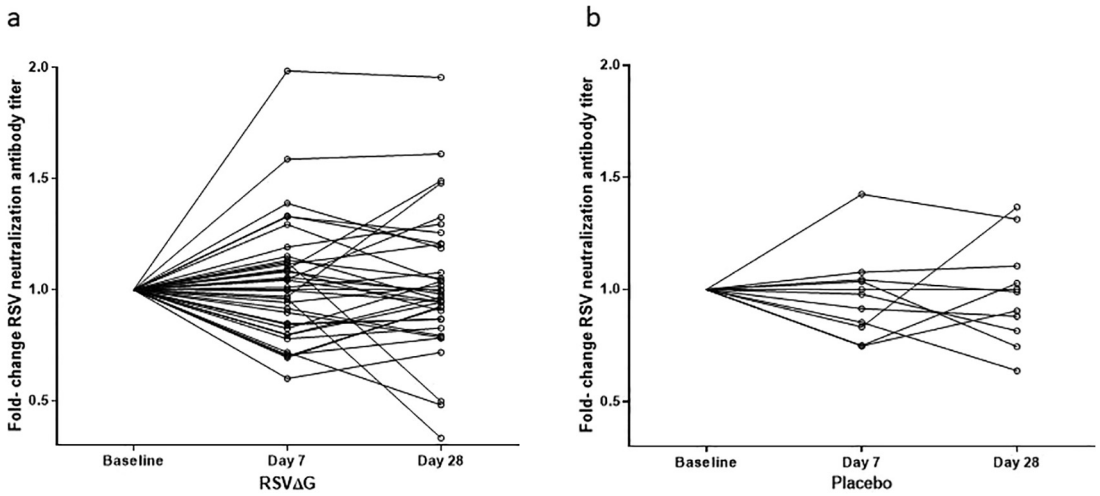

Fig. 4. Fold-change in RSV neutralization antibody titer, day 7 and day 28 post-inoculation versus baseline. (*a*) Fold- change in RSVΔG group (n = 36). (*b*) Fold- change in placebo group (n = 12). RSV = respiratory syncytial virus.

The majority of adverse events were related to the respiratory tract, however, RSV-infection was not confirmed by qPCR or culture in subjects with upper respiratory tract symptoms. Because of the lack of confirmation of RSV infection and the fact that the frequency of these symptoms was equally distributed amongst inoculated and placebo volunteers, it is likely that they were caused by concurrent infections with other respiratory pathogens. The observed incidence of respiratory complaints is in line with subjects being biased to recall solicited adverse events (cold-like symptoms) more often, leading to higher reporting rates [24,25].

We did not observe clear evidence of viral shedding of RSVΔG based on qCulture and qPCR results in nasal wash samples. The presence of viral RNA determined by qPCR was only observed in 8.3% of the subjects (3/36) inoculated with RSVΔG, occurred on day 4 post inoculation and was below the LLOQ. These results further confirm that the RSVΔG vaccine candidate is sufficiently attenuated for testing in the pediatric population. However, the timing and frequency of sampling of nasal washes were tailored towards capturing the viral kinetics of wt-RSV and other RSV LAVs [26–28]. Although we expected RSVΔG to exhibit similar kinetics, we cannot fully rule out that transient shedding of the RSVΔG occurred in between the pre-determined sampling days. Alternatively, the low incidence of viral shedding could also be due to the presence of pre-existing neutralizing antibodies in healthy adults. Even though we selected adult volunteers with relatively low levels of RSV-specific nAbs, all subjects had pre-existing nAbs due to previous exposure to RSV. Finding low to absent levels of viral shedding after intranasal inoculation with a LAV have been described previously [29,30]. A study investigating a similar vaccine concept *cp-52* (a cold passaged RSV B1 LAV lacking a large part of the coding sequence for both SH and G surface proteins) showed that only 6% (1/17) of healthy adults and ultimately only 13% (2/16) of seronegative children shed virus in nasal washes [31]. The authors concluded that *cp-52* was restricted in replication and appeared to be overattenuated [31]. Many more live attenuated RSV concepts have been evaluated since and the general conclusion is that LAV face the challenge of achieving sufficient attenuation to be safe, while remaining immunogenic enough to induce a protective immune response [32,33]. Live-attenuated RSV vaccines that showed viral replication and immunogenicity in seronegative infants were overattenuated in seropositive children and adults [28,34,35]. Minimal or absent viral shedding in adults and RSV-non naïve children is a prerequisite to proceed to safe vaccine evaluation in RSV-naïve children [33]. To further assess the attenuation phenotype and replication-competence, RSVΔG should be evaluated through age de-escalation in the pediatric population.

Analysis of immunogenicity endpoints showed no apparent signs of induction of local or system immune responses in healthy adults following inoculation with RSVΔG. For many live-attenuated vaccines a minimal level of replication is needed to reach adequate immunogenicity. The poor immunogenicity of RSVΔG in this study may be related to the limited viral replication in healthy adults with pre-existing neutralizing antibodies. Absent and low immune responses in healthy adults volunteers have been described previously for other live-attenuated RSV vaccines candidates [31,36]. It is also possible that the dose of 6.5 $\log_{10}$ $CCID_{50}$ was insufficient to overcome natural immunity and induce an immune reaction in adults.

During this trial we applied the commonly used Naclerio method of nasal washing [15]. This method has proven to be effective for determining mucosal IgA after intranasal inoculation with a live-attenuated influenza vaccine [37]. For this reason, we also expected to detect induction of IgA antibodies following intranasal inoculation with RSVΔG (if it were to occur). However, some trials apply a more stringent method for nasal wash collection. In a study by Ascough et al. the nasal cavity was washed by alternatively withdrawing and advancing the plunger of the syringe 10 times. This study showed detectable levels of mucosal IgA prior to inoculation with a RSV subunit vaccine [38]. Although there are no studies comparing the different nasal wash techniques and the extraction of mucosal antibodies, the mucosal immune response in our trial could potentially be underestimated by our nasal wash approach.

In conclusion, a dose of 6.5 $\log_{10}$ $CCID_{50}$ of RSVΔG was safe and well-tolerated in healthy adults. In this first-in-human study, the live-attenuated genetically modified RSV variant RSVΔG did not shed following inoculation, confirming its attenuation in adults. However, with the tested dose there were no clear signs of induction of an immune response in seropositive adult subjects. Safety and immunogenicity of RSVΔG in a dose of 6.5 ± 0.5 $\log_{10}$ $CCID_{50}$ should be further explored in seropositive children and eventually in seronegative infants. In addition, dose-escalation studies may be performed in adults to test whether higher doses of RSVΔG would yield higher rates of immunogenicity, while still having a favourable safety profile.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the participants, staff and study teams at CHDR; the researchers, product development and the analytical team of Intravacc for development and manufacturing and quality control of the vaccine; and Gijsbert van Willigen, biosafety officer LUMC, for his contribution and advice regarding the application of the environmental permit for this trial.

Role of the funding source

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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