Impaired Immune Response to Vaccination against Infection with Human Respiratory Syncytial Virus at Advanced Age

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ABSTRACT

Elderly humans are prone to severe infection with human respiratory syncytial virus (HRSV). The aging of today’s human population warrants the development of protective vaccination strategies aimed specifically at the elderly. This may require special approaches due to deteriorating immune function. To design and test vaccination strategies tailored to the elderly population, we need to understand the host response to HRSV vaccination and infection at old age. Moreover, the preclinical need for testing of candidate vaccines requires translational models resembling susceptibility to the (unadapted) human pathogen. Here, we explored the effects of aging on immunity and protection induced by a model HRSV vaccine candidate in a translational aging model in cotton rats (Sigmodon hispidus) and examined possibilities to optimize vaccination concepts for the elderly. We immunized young and aged cotton rats with a live-attenuated recombinant HRSV vaccine candidate and analyzed the induced immune response to and protection against challenge with HRSV. In old cotton rats, HRSV infection persisted longer, and vaccination induced less protection against infection. Aged animals developed lower levels of vaccine-induced IgG, virus-neutralizing serum antibodies, and IgA in lungs. Moreover, booster responses to HRSV challenge were impaired in animals vaccinated at an older age. However, increased dose and reduced attenuation of vaccine improved protection even in old animals. This study shows that cotton rats provide a model for studying the effects of aging on the immune response to the human respiratory pathogen HRSV and possibilities to optimize vaccine concepts for the elderly.

IMPORTANCE

HRSV infection poses a risk for severe disease in the elderly. The aging of the population warrants increased efforts to prevent disease at old age, whereas HRSV vaccines are only available in the developmental phase. The preclinical need for testing of candidate human vaccines requires translational models resembling susceptibility to the natural human virus. Moreover, we need to gain insight into waning immunity at old age, as this is a special concern in vaccine development. In this study, we explored the effect of age on protection and immunity against an experimental HRSV vaccine in aged cotton rats (Sigmodon hispidus), a rodent species that provides a model representing natural susceptibility to human viruses. Older animals generate fewer antibodies upon vaccination and require a higher vaccine dose for protection. Notably, during the early secondary immune response to subsequent HRSV infection, older animals showed less protection and a slower increase of the virus-neutralizing antibody titer.

At advanced age, approximately >65 years, people become more susceptible to severe infectious diseases caused by respiratory pathogens. This increased medical threat is caused by impaired functioning of the immune system (immunosenescence). Immunosenescence is currently best exemplified by limited immunity induced by vaccines to prevent infection with pneumococcal bacteria and influenza virus in the elderly (1–4). It is now becoming recognized that human respiratory syncytial virus (HRSV) contributes to an important part of severe respiratory disease and mortality in the elderly (1–3, 5, 6). This probably has been underestimated since HRSV is regarded as a common cause of lower respiratory tract infections primarily in infants, and disease caused by HRSV is potentially attributed to influenza virus due to its symptoms matching those of influenza-like illness (7, 8). Some studies suggest that the extent of HRSV-associated hospitalization in the elderly population is close to that for influenza (3, 7, 9).

As the proportion of the aged population is increasing, the need for prevention of age-related diseases is also increasing. Vaccines that elicit protection against respiratory viruses would significantly contribute to maintaining health and, hence, quality of life at old age. Despite intense efforts to design an effective HRSV vaccine, a licensed vaccine is not available. Most of these efforts are aimed at protecting children. To design vaccination strategies tailored to the elderly population, we need to understand how the immune system of this age group responds to vaccination. To enable testing of human candidate vaccines in the elderly, a model that resembles susceptibility to the natural human pathogen is required.

Hispid cotton rats (Sigmodon hispidus) provide a highly representative model for human infections since they are susceptible to a variety of human pathogens that do not require adaptation to
infect and replicate (10–14). HRSV is a good example, as infection, pathology, and protection induced by candidate HRSV vaccines in cotton rats have already been explored and reflect the human situation (11, 12, 15–17). In comparison to young cotton rats, aged cotton rats express a prolonged cytokine response and a high degree of pathology in the lungs in response to a primary HRSV infection (18, 19). However, age-related effects on the immune response and antiviral protection induced by HRSV vaccination remain to be clarified. To this end, we explored the effect of aging on vaccine-induced immunity and protection against HRSV infection in cotton rats in various age groups. We vaccinated young adult (2 months of age) and old (8 to 9 months of age) cotton rats with a live-attenuated HRSV vaccine candidate and explored the induction of HRSV-specific antibodies in serum and lung mucosa, the primary entry site of HRSV, and protection against challenge with wild-type virus.

**MATERIALS AND METHODS**

**Virus and vaccine preparation.** The propagation of virus and preparation of vaccine were described in detail previously (17). In brief, the HRSV that we used for infection and challenge experiments was wild-type HRSV-X (wtHRSV) (strain 98-25147-X). This clinically isolated HRSV serogroup A strain (17) also served as the basis for the recombinant HRSV (rHRSV) used as a vaccine in this study. Recombinant HRSV was recovered from a plasmid encoding cDNA containing directed mutations in the intergenic regions of the HRSV-X genome, as described previously (17, 20). This resulted in infectious rHRSV particles that express all the viral proteins but express an attenuated phenotype.

**Immunization and challenge of cotton rats.** Adult cotton rats (Sigmodon hispidus) of young (2 months of age), intermediate (6 months of age), and old (8 to 9 months of age) ages were obtained from a specific-pathogen-free breeding colony (Charles River Laboratories, The Netherlands) and held at the animal facilities of Intravacc (Bilthoven, The Netherlands). HRSV infection was done intranasally (i.n.) with 10^5 50% tissue culture infective doses (TCID50) of wild-type HRSV (17). Cotton rats were immunized i.n. with 10^6 TCID50 of live-attenuated rHRSV and challenged i.n. with 10^5 TCID50 of wild-type HRSV 28 days later (17). Animal studies were approved by the Animal Ethical Committee of RIVM. Animal handling was carried out in accordance with Dutch national legislation.

**Virus titration.** HRSV titers were analyzed by TCID50 determination (17). Lung titers were determined in homogenized right lungs. Nasal HRSV titers were determined from nasal wash specimens obtained by flushing the upper trachea with 2 ml phosphate-buffered saline (PBS) supplemented with 7.5% sucrose.

**Virus neutralization assay.** Neutralizing antibodies were analyzed in a fluorescence-based plaque reduction assay by testing 2-fold serial dilutions of cotton rat serum starting at a 1:10 dilution (17, 21). Diluted serum was mixed with an equal volume of HRSV containing an enhanced green fluorescent protein (EGFP) reporter gene and added to Vero cell monolayers. After centrifugation for 1 hour at 700 g and incubation for 1 hour at 37°C, the inoculum was removed, and cells were overlaid with 1% methyl cellulose and cultured for 2 days at 37°C. Plaques were detected with a fluorescence enzyme-linked immunosorbent spot (ELISpot) reader (Aidagnostika, Germany). Plaque reduction was calculated by regression analysis to provide a 60% plaque reduction titer.

**IgG and IgA analysis.** IgG was measured in blood serum, and IgA was measured in homogenized lung tissue. RSV-specific IgA and IgG antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) on polystyrene 96-well microtiter plates coated with Triton X-100-inactivated HRSV-X with horseradish peroxidase (HRP)-labeled goat anti-mouse IgA (AbD Serotec, Oxford, United Kingdom), cross-reactive to cotton rat IgA, and chicken anti-cotton rat IgG (ICL, Portland, OR), respectively.

**Statistics.** Multiple comparisons were analyzed by analysis of variance (ANOVA), including Tukey’s multiple-comparison test, to test for statistical significance of differences. Comparisons of two samples were analyzed by a t test. A P value of <0.05 was considered significant.

**RESULTS**

**Older cotton rats clear HRSV infection slowly.** Cotton rats at the age of 2, 6, or 9 months were infected with wild-type HRSV-X (wtHRSV). This clinically isolated HRSV serogroup A strain (17) served as the basis for the recombinant HRSV virus used as a vaccine in this study. Naïve animals were infected intranasally and sacrificed at 4 to 10 days after inoculation to analyze virus titers in lung and nose. At 4 days after inoculation, the lungs (Fig. 1A) and nasal wash specimens (Fig. 1B) of all animals showed large amounts of virus but did not show different virus titers between the various age groups. However, at 6 days postinfection, the young animals (2 months of age) showed a significant drop in virus titer in the lungs compared to 6-month-old animals (Fig. 1C). This difference was pronounced compared to 9-month-old animals. In addition, virus titers in the nose of young adult cotton rats were slightly reduced compared to those of the older age groups of 6 and 9 months of age (Fig. 1D). At day 10 after challenge, virus could no longer be detected in nose and lungs of old animals (9 months of age) (data not shown), indicating that the virus was eventually cleared. Together, these data show that HRSV infection remains for a longer period in older cotton rats.

**Vaccination induces less protection against HRSV infection at older age in cotton rats.** To assess if age affects HRSV vaccination efficacy in cotton rats, we vaccinated cotton rats at the age of 2 months (young) or 8 to 9 months (old) with 10^4 TCID50 of live-attenuated rHRSV. Subsequently, we analyzed protection induced against challenge at 28 days postimmunization with wtHRSV (17). As measured at 5 days postchallenge, virus was not detectable in the lungs of young animals (Fig. 2A), whereas old immunized animals and mock-immunized controls showed detectable amounts of challenge virus. These data indicate that in cotton rats, vaccination efficacy is reduced at old age.

**Increasing the dose and reducing attenuation of virus improve vaccine efficacy in old cotton rats.** Since increasing the dose of a vaccine can improve its efficacy (22), we tested whether immunization with rHRSV using a dose of >10^5 TCID50 would induce protection against challenge virus in old cotton rats. The 10-fold-higher (10^4 TCID50) and 100-fold-higher (10^5 TCID50) doses of immunizing rHRSV resulted in lower titers of challenge virus in a dose-dependent fashion, as detected in the lungs 5 days after challenge (Table 1). Most (4 out of 5) animals immunized with the highest dose (10^5 TCID50) of rHRSV were free of detectable virus at this time point after challenge (Fig. 2B and Table 1). These data indicate that increasing the vaccine dose can restore protection in old cotton rats.

The rHRSV used in this study is attenuated by mutations made in intergenic regions of the viral genome (17). Attenuation of virus may influence its capacity to induce protection against infection (23). Hence, we analyzed protection against challenge virus induced by parental unattenuated wtHRSV at an immunizing dose at which the HRSV was not protective (10^4 TCID50) in old cotton rats. In contrast to the old cotton rats immunized with this dose of
attenuated rHRSV, all animals that had been immunized with $10^4$ TCID$_{50}$ of wtHRSV showed no detectable challenge virus by day 4 after challenge (Fig. 2B), indicating a stronger protective potency of nonattenuated virus in old cotton rats. Old cotton rats generate lower titers of IgG and virus-neutralizing antibody in response to primary immunization. The capacity to generate antibodies in response to vaccination and infection is reduced at old age (24–28). To assess whether this age effect is paralleled in the cotton rat model, we compared the production of antibodies in response to HRSV vaccine in young cotton rats to that in old cotton rats. Vaccination with rHRSV protected young adult cotton rats and partly old cotton rats. Therefore, we argued that rHRSV vaccination would allow an immunological window to analyze differences between young (2 months of age) and old (8 to 9 months of age) cotton rats. We set up an experiment to longitudinally analyze the response to immunization with rHRSV followed by a wtHRSV challenge given at day 28 postimmunization. An increase in HRSV-specific serum IgG titers in response to rHRSV was found starting 2 weeks after immunization. However, the antibody titer induced was significantly lower in old animals (Fig. 3).

To analyze the quality of antibodies at the day of challenge, we analyzed the virus-neutralizing (VN) titers in serum (Fig. 4A). Although all animals generated detectable VN antibodies in response to a dose of $10^5$ TCID$_{50}$ of rHRSV, the VN titer was significantly lower in old animals than in young animals that had received the same dose. A low response of old animals was also

FIG 1 Clearance of HRSV upon infection in cotton rats of different ages. At the age of 2, 6, or 9 months, cotton rats were infected intranasally with $3 \times 10^5$ TCID$_{50}$ of HRSV. Virus titers were analyzed in lungs and nose at 4 days postinfection (A and B) and at 6 days postinfection (C and D). At 10 days postinfection, virus was no longer detectable. *, $P < 0.05$, as determined by ANOVA.

FIG 2 Vaccine-induced protection against HRSV challenge in cotton rats at different ages. At the age of 2 or 9 months, cotton rats ($n = 5$ or 6 per group) were immunized with attenuated rHRSV (with doses indicated as TCID$_{50}$) or the mock control. One group of cotton rats at 9 months of age was immunized with wild-type HRSV (HRSV at $10^4$ TCID$_{50}$). Subsequently, all animals were challenged intranasally with $3 \times 10^5$ TCID$_{50}$ of wild-type HRSV at 28 days postimmunization. Virus titers were analyzed in lungs at 5 days postchallenge. Less protection induced by rHRSV in old cotton rats was found in two independent experiments; data from one of these experiments are shown. * indicates that none of the challenged animals were free of HRSV at 5 days postchallenge.
found after immunization with a dose of $10^4$ TCID$_{50}$. These data show that old cotton rats generate less IgG and VN antibodies in serum in response to rHRSV vaccine.

Old vaccinated cotton rats exhibit reduced VN antibody responses to challenge virus. At 5 days after challenge (day 33), a sharp increase in the IgG titer was observed in young and old animals that had been immunized with rHRSV (Fig. 3). This sharp increase is an early booster effect induced by challenge virus in previously immunized animals, since this quick and steep increase of antibody titers in immunized animals was found only upon challenge and not in nonimmunized cotton rats (data not shown). We questioned whether elicitation of this early recall response to challenge virus differed between the two age groups. To analyze this booster effect, we measured the increase in antibody levels from the day of challenge (day 28 postvaccination) to 5 days postchallenge (Fig. 5). All young and old animals that had been immunized with rHRSV, with either the higher ($10^5$ TCID$_{50}$) or the lower ($10^4$ TCID$_{50}$) dose, exhibited significant induction of IgA compared to nonimmunized controls. However, old animals that had been immunized with the lower rHRSV dose showed significantly lower IgA titers in lungs than those in young animals that had received the same vaccine dose. This indicates that when a low vaccine dose is used, old cotton rats generate a lower mucosal antibody response than young adult cotton rats do.

**DISCUSSION**

The elderly population is an important target group for vaccination, since this group is vulnerable to severe lung disease caused by respiratory infections with pneumococcal bacteria, influenza virus, and HRSV (1–3, 5, 29). Animal models allow controlled studies of age-related effects on vaccine-induced immune protection against infection. Cotton rats are permissive for infection with HRSV, develop human-like pathology upon infection with human virus, and allow analysis of the immune response to HRSV. Toward improving and testing vaccine-induced protection at old age, we analyzed the effects of aging on immunity and protection against virus challenge in this representative model. We showed that aged hispid cotton rats mount decayed protection induced by immunization against HRSV infection.

Older naive cotton rats cleared primary lung infection with wtHRSV more slowly than did young animals. This confirms previous studies that reported similar findings with the prototype Long strain of HRSV in older animals (18, 19). In old cotton rats, immunization with rHRSV vaccine induced less protection against HRSV challenge than in young adult animals. Reduced vaccine efficacy in old cotton rats was also found upon immunization with another live-attenuated HRSV (ΔG HRSV [30]) (data not shown) or with formalin-inactivated HRSV (data not shown). These results indicate a waning of the ability to mount a protective immune response to vaccination at an advanced age. However, increasing the dose improved vaccine efficacy in aged animals. In addition, wtHRSV conferred protection upon immu-

<table>
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<th>Titer (SD)</th>
<th>Nose virus titer % positive</th>
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$^a$ As a positive control, one group was immunized with $10^6$ TCID$_{50}$ of wtHRSV. Shown are the percentage of animals that showed HRSV 5 days after challenge and the mean titer detected in infected animals per group for lungs and nose. As a control for age effects in old cotton rats shown in this table, young animals were vaccinated at 2 months of age with mock vaccine or with HRSV at $10^5$ TCID$_{50}$ and challenged. Of these young mock-immunized animals, 100% of animals had HRSV titers in lungs upon challenge. Of the young animals immunized with rHRSV at $10^5$ TCID$_{50}$, 0% of animals had HRSV titers in lungs upon challenge.

**FIG 3** rHRSV vaccine-induced serum IgG. Cotton rats at the age of 2 months (white bars) or 9 months (black bars) ($n = 6/group$) were immunized with an rHRSV vaccine dose of $10^5$ TCID$_{50}$, and HRSV-specific IgG was analyzed in serum weekly. Animals were challenged with wild-type HRSV ($3 \times 10^5$ TCID$_{50}$) at day 28. This graph shows data from a single experiment with lower levels of rHRSV-induced antibodies in old cotton rats found in three independent experiments. *$P < 0.05$ for differences between the 2-month and 9-month age groups, as determined by a $t$ test. OD, optical density.
nization with a low dose at which attenuated rHRSV did not. This indicates that vaccine-induced protection against infection in the elderly may be achieved by increasing the dose or by reducing the level of attenuation of vaccine.

Reduced clearance at old age was found in the lungs and in the upper airways, although virus was retained in the nose for a longer period than in the lungs both after primary infection and upon challenge in vaccinated animals. This is a general finding in cotton rats (15,17) and has also been described in humans (31). Observed differences in viral loads between nose and lungs might partly be due to differences between sampling methods. However, cotton rat studies showed that virus-neutralizing antibodies protect the lower airways more effectively than the upper respiratory tract (15). This suggests that slower clearance from the upper respiratory tract reflects protection against infection being more effective in the lungs than in the upper respiratory tract in humans and in cotton rats.

Antibodies are important players in the protection induced by vaccination and natural infection. Previous cotton rat studies showed that HRSV-specific antibodies confer protection, and human studies suggest that a basic level of antibodies is needed for protection against deep lung infection (15, 32–36). Generally, the level of induced virus-neutralizing antibodies tends to wane in elderly people. However, the relationship between antibody levels induced by HRSV and age in humans is still subject to speculation. Observations on the effect of age on the immune response to vaccine may be blurred since social behavior, environment, frailty, and a varying history of natural infections can influence the outcome of human studies (25, 28, 32, 37–40). To clarify the effect of aging on the capacity to mount antibody production in response to HRSV vaccine, we analyzed the antibodies produced in response to vaccination with rHRSV, which induced suboptimal protection in old animals but fully protected young cotton rats. Old cotton rats showed significantly reduced vaccine-induced titers of IgG and neutralizing antibodies in serum at the time of challenge, 4 weeks after vaccination. Since antibodies contribute to protection, this finding suggests that immunosenescence of vaccine-induced immunity contributes to reduced clearance of virus. Moreover, old animals immunized with a higher dose of rHRSV showed more neutralizing antibodies and less replication of challenge virus, suggesting that more neutralizing antibodies induced by a higher antigen dose attribute to better protection in immunosenescent animals.

Also, immunization with nonattenuated wtHRSV induced a proper level of neutralizing antibodies (data not shown) and protection in old cotton rats. Together with a previous study by Curtis et al., who reported that wtHRSV did not induce significantly
fewer antibodies in older cotton rats (18), this finding may explain why wtHRSV is a more potent inducer of protection than attenuated rHRSV at old age.

The lower RSV-specific IgG level induced by immunization at older age in cotton rats reflects the general notion of immunosenescence in humans. In contrast, clinical studies indicate that old humans express similar or even higher titers of RSV-specific IgG antibodies (28, 31, 39, 41). However, several of those studies indicate that the functionality of these antibodies is inferior compared to that of the antibodies of younger people (28, 39). Of note, old humans have been exposed more often to RSV infection during their lifespan than young people. This may cause a different RSV-specific immune status that is not fully mimicked in animal models such as our cotton rat model and likely explains differences found between animal models and humans. Such differences in observations between models and clinical studies should be taken into account when interpreting data from models of immune responses at old age.

Naive cotton rats immunized in our study had not been exposed (as confirmed by serology) to HRSV infection before vaccination. Antibodies analyzed prior to wtHRSV challenge in our study therefore reflect a primary response to vaccine. This does not completely reflect the natural situation, since aged people are not naive. However, we also measured antibodies in rHRSV-immunized animals after subsequent challenge with wtHRSV. Since rHRSV is a live virus (17), rHRSV immunization can be regarded as a mild HRSV infection, and challenge with wtHRSV can be regarded as a boosting reinfection. A booster effect in previously immunized individuals becomes apparent by the quick and steep increase of the antibody titer during the recall immune response to infection within 5 to 7 days, as shown in our cotton rat studies (our unpublished data), lambs (42), young children (43), and aged humans (31). The early booster effect was significantly lower in old animals, and this was most pronounced for the increase in the level of neutralizing antibodies, rather than the boost of IgG, indicating that aged individuals are less capable of mounting recall responses of functional antibodies to HRSV. This may explain why aged people have lower antibody functionality rather than lower levels of total IgG against HRSV in serum (18, 38, 39).

It is tempting to speculate that the rate at which production of functional antibodies increases early after infection may determine the control of viral load during the first days of infection. Therefore, it may be interesting to further investigate the function of the rapid increase in antibody levels in a secondary immune response, the final maximum titer reached, and the role of the slower initial increase thereof at old age. Besides antibodies, T cells are important for an effective immune response required to clear the virus. Since functionality and numbers of RSV-specific CD4+ and CD8+ T cells drop with age (28, 44, 45), it would be useful to unravel how T-cell responses at old age are involved in the induction of protective immunity by RSV vaccines and how these responses can be improved to act in concert with antibodies to achieve protection at old age.

The lung mucosa is the primary entry site where protection against deep lung infection with HRSV by antibodies should be exerted. Moreover, mucosal antibodies have been reported to more closely resemble the HRSV-neutralizing function of the immune response than does serum IgG (35, 36). Since data on age-related effects on HRSV-induced mucosal IgA are relevant but scarce, we analyzed HRSV-specific IgA produced in the lungs of RHSV-immunized animals upon challenge. Prominently, in response to rHRSV vaccine, old cotton rats produced significantly less IgA in the lungs than did young animals.

Collectively, our data show an age-dependent reduction of the ability to mount protective immunity to human RSV in cotton rats, in both primary and secondary (booster) immune responses. Moreover, these data indicate that the cotton rat is a useful model to study human vaccines designed for the aged population as well as possibilities to promote vaccine efficacy in the elderly by increasing the vaccine dose or by reducing the level of attenuation.

ACKNOWLEDGMENTS

This work was supported by the Strategic Vaccine Research Program (grant S/000207) provided by the Dutch Ministry of Health, Welfare and Sport (VWS). We thank D. Elberts, P. van Schaijk, P. de With, C. Soputan, T. Schouten, and J. Rigters for excellent biotechnical support. We thank M. S. Boukhvalova and H. Kraan for advice on and help with setting up assays for the analysis of cotton rat IgA antibodies. We have no conflicts of interest to disclose.

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