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Characterization of Epitope-Specific Anti-Respiratory Syncytial Virus (Anti-RSV) Antibody Responses after Natural Infection and after Vaccination with Formalin-Inactivated RSV

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ABSTRACT

Antibodies against the fusion (F) protein of respiratory syncytial virus (RSV) play an important role in the protective immune response to this important respiratory virus. Little is known, however, about antibody levels against multiple F-specific epitopes induced by infection or after vaccination against RSV, while this is important to guide the evaluation of (novel) vaccines. In this study, we analyzed antibody levels against RSV proteins and F-specific epitopes in human sera and in sera of vaccinated and experimentally infected cotton rats and the correlation thereof with virus neutralization. Analysis of human sera revealed substantial diversity in antibody levels against F-, G (attachment)-, and F-specific epitopes between individuals. The highest correlation with virus neutralization was observed for antibodies recognizing prefusion-specific antigenic site Ø. Nevertheless, our results indicate that high levels of antibodies targeting other parts of the F protein can also mediate a potent antiviral antibody response. In agreement, sera of experimentally infected cotton rats contained high neutralizing activity despite lacking antigenic site Ø-specific antibodies. Strikingly, vaccination with formalin-inactivated RSV (FI-RSV) exclusively resulted in the induction of poorly neutralizing antibodies against postfusion-specific antigenic site I, although antigenic sites I, II, and IV were efficiently displayed in FI-RSV. The apparent immunodominance of antigenic site I in FI-RSV likely explains the low levels of neutralizing antibodies upon vaccination and challenge and may play a role in the vaccination-induced enhancement of disease observed with such preparations.

IMPORTANCE

RSV is an importance cause of hospitalization of infants. The development of a vaccine against RSV has been hampered by the disastrous results obtained with FI-RSV vaccine preparations in the 1960s that resulted in vaccination-induced enhancement of disease. To get a better understanding of the antibody repertoire induced after infection or after vaccination against RSV, we investigated antibody levels against fusion (F) protein, attachment (G) protein, and F-specific epitopes in human and animal sera. The results indicate the importance of prefusion-specific antigenic site Ø antibodies as well as of antibodies targeting other epitopes in virus neutralization. However, vaccination of cotton rats with FI-RSV specifically resulted in the induction of weakly neutralizing, antigenic site I-specific antibodies, which may play a role in the enhancement of disease observed after vaccination with such preparations.

Human respiratory syncytial virus (RSV) is the leading cause of respiratory tract infection in children. Primary infection usually occurs during infancy, and essentially all children have been infected by 2 years of age. RSV infection is an important cause of bronchiolitis, severe cases of which may require hospitalization. Consecutive RSV infections in early life also increase the risk of developing asthma later in life (1, 2). In addition, RSV is recognized as a significant problem in adults and the elderly, causing morbidity and mortality similar to those seen with influenza virus (3). To date, there is still no effective antiviral or vaccine available for the protection of the general population (4).

The development of a vaccine against RSV has been hampered by the disastrous results obtained with formalin-inactivated RSV (FI-RSV) vaccine preparations in the 1960s. Vaccination with FI-RSV was shown to be poorly protective against natural RSV infection. Moreover, vaccinated children experienced immune-mediated enhancement of disease upon RSV infection. The vaccinees displayed low levels of RSV-neutralizing antibodies (Abs) (5, 6) and an exaggerated $CD4^+$ T lymphocyte response (7). This poorly neutralizing response is still not well understood but has been ascribed to denaturation of neutralization epitopes (5) as well as to deficient antibody affinity maturation (8).

RSV particles contain two major surface glycoproteins: attachment protein G and fusion protein F (9). Several current RSV vaccine approaches particularly focus on the induction of anti-F neutralizing antibodies (10). The RSV F protein forms metastable homotrimers (prefusion F) that can be triggered to undergo dra-

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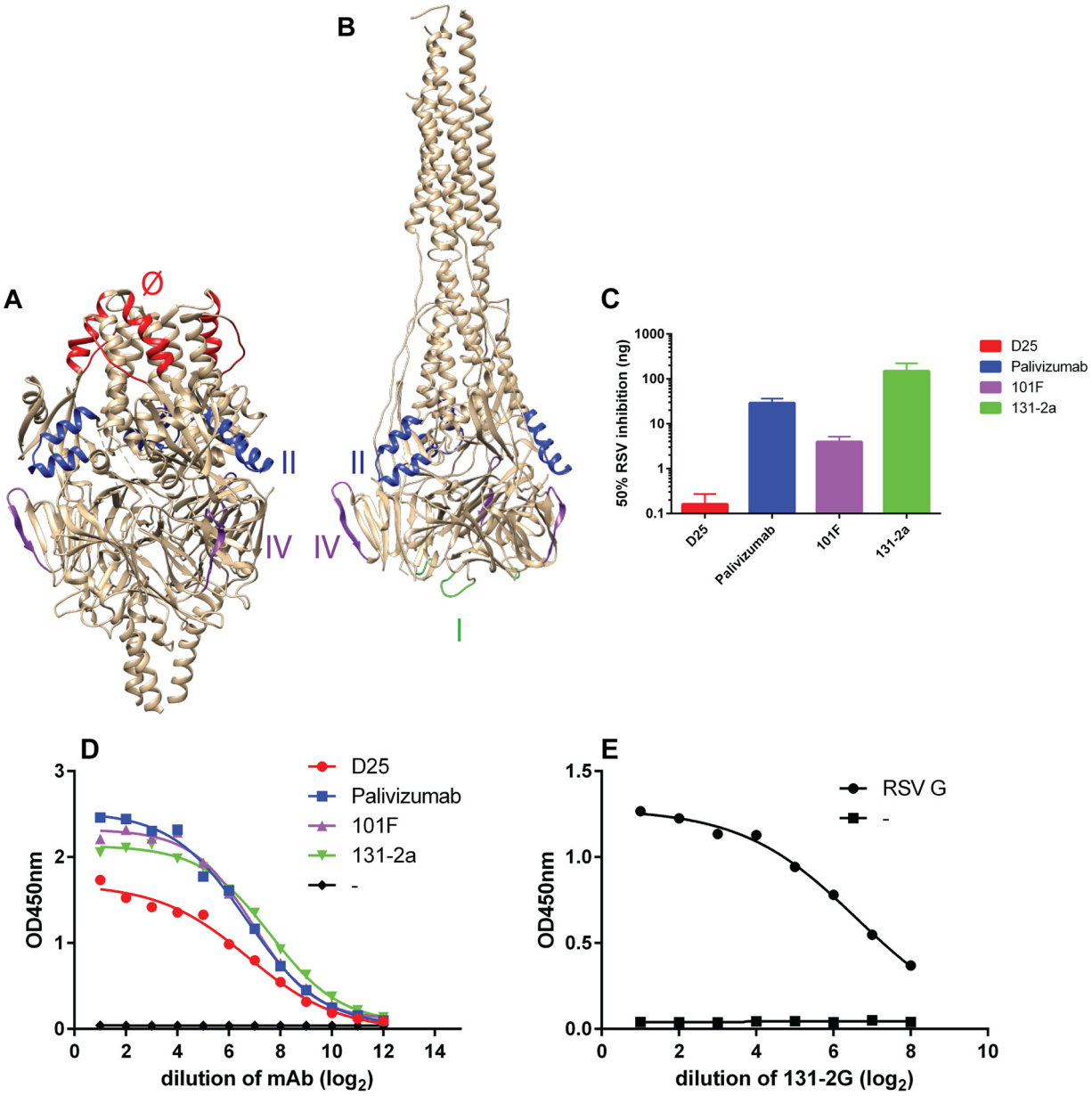

FIG 1 RSV F and G ELISA. (A and B) Prefusion (A) (14) and postfusion (B) (13) structures of RSV F. Antigenic sites recognized by antibodies used in this study are indicated (according to reference 14) as follows: prefusion-specific site Ø (recognized by MAbs D25 and AM22), postfusion-specific site I (recognized by MAb 131-2a), site II (recognized by MAb palivizumab), and site IV (recognized by MAb 101F). (C) RSV neutralization by F-specific MAbs. The amount of each MAb needed to achieve 50% neutralization of RSV A2 infectivity is graphed. The error bars indicate the standard deviations. (D) Reactivity of different biotinylated monoclonal antibodies (MAbs) with RSV F (FlysGCN [21]) was tested by the addition of 2-fold serial dilutions of biotinylated D25, palivizumab, 101F, and 131-2a, starting with 10 μg/ml. Binding of MAbs was detected using HRP-conjugated streptavidin. The optical density at 450 nm (OD450nm) corresponds to binding of MAbs to F. (E) Reactivity of G-specific MAb 131-2G with recombinant G protein was assayed by the addition of 2-fold serial dilutions of this MAb. The OD_{450} value corresponds to binding of 131-2G to G.

matic conformational changes that ultimately result in the formation of the postfusion conformation. Both pre- and postfusion conformation can be found on the virion surface, suggesting that there is a conversion that occurs at an as-yet-undetermined rate (11, 12). The structures of these two F protein conformations have been solved (13–15). While some epitopes are found on both structures (antigenic sites II and IV), others appear to be specific for the prefusion form (antigenic site Ø) or the postfusion form (antigenic site I) of F (14, 16) (Fig. 1A and B). Monoclonal antibodies (MAbs) against the different antigenic sites differ in their neutralizing capacities, with pre- and postfusion-specific antibodies displaying the highest and lowest neutralizing capacities, respectively (16). In agreement, vaccination with F proteins stabilized in a prefusion-like conformation, which presumably results in the induction of highly neutralizing prefusion-specific antibodies, appeared to be more effective than vaccination with postfusion F proteins (17, 18).

Previous analyses of human sera showed that the majority of

the neutralizing antibodies are directed against prefusion F (19, 20) and revealed the importance of prefusion antigenic site Ø-specific antibodies (20), but detailed analysis of multiple epitope-specific F protein antibodies in human or animal sera and their contribution to virus neutralization has been lacking so far. In this study, we developed enzyme-linked immunosorbent assays (ELISAs) to determine RSV protein- and F epitope-specific antibody profiles in human sera and in sera of vaccinated and experimentally infected cotton rats. Analysis of human sera revealed substantial diversity in virus neutralization results and in F and G protein antibody titers as well as in F epitope-specific antibody levels. Our results not only indicate the importance of prefusion-specific antibodies binding antigenic site Ø but also show that low levels of these antibodies may be compensated by antibodies targeting other parts of the F protein. Vaccination with FI-RSV was shown to specifically induce antibodies recognizing antigenic site I but not antibodies recognizing other antigenic sites that were present in the preparations. We therefore conclude that antigenic site I is immunodominant in FI-RSV. The strong induction of poorly neutralizing antibodies targeting antigenic site I and the concomitant absence of more efficiently neutralizing antibodies may contribute to the enhancement of disease observed after vaccination with such preparations.

MATERIALS AND METHODS

Recombinant proteins. The expression constructs encoding the Fwt and Flys-GCN proteins (16, 21) and the heavy and light chains of palivizumab (22) and D25 and AM22 (23) antibody have been described previously. The expression constructs encoding the 101F antibody heavy and light chains were constructed on the basis of the published sequence (46) as described before (16, 21). The cDNA clones encoding a subtype A RSV G ectodomain (residues 64 to 298; GenBank accession number P03423.1) or the prefusion F protein DSCav1-T4fd (17) were synthesized by GenScript USA, Inc., using human-preferred codons and cloned in the appropriate expression vectors. The G protein-encoding cDNA was preceded by sequences coding for a CD5 signal peptide, One Strep tag, and GCN4 tetramerization motif (24–27). The DSCav1-T4fd protein-encoding cDNA was cloned into the pCD5 expression vector in frame with the CD5 signal peptide coding sequence.

Expression and purification of recombinant proteins. Recombinant proteins were expressed using HEK293T cells and purified as described previously (21). The Flys-GCN protein was purified using palivizumab (MedImmune) coupled to Hitrap *N*-hydroxy-succinimide (NHS)-activated HP Sepharose beads (GE Life Sciences) according to the manufacturer's instructions. The protein was eluted using 0.1 M glycine–HCl buffer (pH 3.0) and was immediately neutralized using 1 M Tris-HCl (pH 9.0).

Viruses. RSV-A expressing *Renilla* luciferase (RLuc-RSV) was kindly provided by Martin Moore (Emory University School of Medicine) and propagated as described previously (16). RSV-X (GenBank FJ948820), its derivative recombinant RSV-X (rRSV), and RSV-A2 were propagated and vaccine batches prepared as described previously (28). Formalin-inactivated RSV (FI-RSV) was prepared from RSV-A2 as described previously (29). Vaccination with similar preparations was previously shown to elicit enhanced disease phenotypes in cotton rats (data not shown). The F protein ectodomains of RSV-X and RSV-A2 are more than 98% identical.

Immunization and challenge of cotton rats. Two-month-old cotton rats (*Sigmodon hispidus*) 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). For intranasal immunization, 10 μl containing 1×10^4 or 1×10^5 50% tissue culture infective doses ($TCID_{50}$) of rRSV or phosphate-buffered saline (PBS) (mock control) was applied under narcosis conditions to 6 animals per group. A dose of 1×10^4 $TCID_{50}$ in 50 μl of FI-RSV was given intramuscularly. Cotton rats were challenged intranasally with 3×10^5 $TCID_{50}$ of RSV-X in 100 μl at day 28 after immunization. Plasma samples were collected at day 28 (prechallenge) and 5 days later (postchallenge). Animal studies were approved by the Animal Ethical Committee of the National Institute for Public Health and the Environment (RIVM), The Netherlands. Animal handling was carried out in accordance with Dutch national legislation.

Virus neutralization assay. Diluted, heat-inactivated human serum samples (obtained from 15 healthy volunteers with their consent) were analyzed for the presence of virus-neutralizing antibodies by using RLuc-RSV (1,000 $TCID_{50}$) as described previously (16). The neutralizing titers were determined at the 50% reduction point compared to mock-treated viruses using 4-parameter fit curve analysis (GraphPad). The neutralizing capacity of the cotton rat plasma samples was analyzed by fluorescence-based plaque reduction neutralization assay (30). Plaque reduction neutralization titers (PRNT) were reported as the $\log_2$ dilution that provided a 60% plaque reduction by regression analysis.

RSV F and G ELISA. ELISA plates (Nunc MaxiSorp; Thermo Scientific) were coated with 25 ng of RSV F or RSV G and incubated with 2-fold serial dilutions of MAbs or serum samples. After extensive washing, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (ITK Southern Biotech) or rabbit anti-mouse IgG (Dako) diluted 1:1,000, chicken anti-cotton rat IgG (Gallus Immunotech Inc.) diluted 1:5,000, or HRP-conjugated streptavidin (Thermo Scientific) (1 μg/ml). Detection of HRP reactivity was performed using tetramethylbenzidine substrate (BioFX) and an ELISA plate reader (EL-808 [from Biotek]). The IgG titer for RSV F or G was determined by calculating the corresponding dilution for an optical density (OD) value from the linear part of the curve (OD = 1 for RSV F and OD = 0.5 for RSV G).

Blocking ELISA. To measure antibody responses to antigenic sites Ø, I, II, and IV specifically, blocking ELISAs were performed. Briefly, ELISA plates were coated with 25 ng of Flys-GCN and incubated with 2-fold serial dilutions of serum samples or unlabeled MAbs (D25, palivizumab, 101F, and 131-2a [Millipore]) at a known concentration. After extensive washing, the plates were incubated with biotinylated D25, 101F, palivizumab, or 131-2a at 0.6 μg/ml, 0.4 μg/ml, 0.3 μg/ml, or 0.2 μg/ml, respectively. MAbs were biotinylated using an EZ-Link Sulfo-NHS-LC-Biotinylation kit (Thermo Scientific) according to the manufacturer's instructions. After washing, the plates were incubated with HRP-conjugated streptavidin (Thermo Scientific). Detection of HRP reactivity was performed as described above. Control wells were incubated with biotinylated MAbs in the absence of serum. Serial dilution of unlabeled MAbs was used as a standard curve in the competition ELISA. Percent inhibition was calculated for every dilution using the following equation: (OD of biotinylated MAb − OD of serum samples)/OD of biotinylated MAb × 100. Using 4-parameter fit curve analysis (GraphPad), the inhibition titer for each serum sample was determined as the serum dilution that resulted in 50% inhibition of palivizumab, 101F, or 131-2a binding. Similar results were obtained when 25% inhibition was used as the readout. For D25, the titer was determined at 25% inhibition, as several human sera contained lower D25-competing antibody levels. If the indicated level of inhibition could not be obtained, the lowest dilution was used as the inhibition titer. The concentration of F-epitope-specific antibody in sera was calculated based on linear regression of concentration-dependent MAb inhibition as described by Rao and Hsieh (31). All experiments were repeated three times.

Statistical analysis. Statistical analysis of the anti-F or -G antibody responses was performed using Student's *t* test or analysis of variance (ANOVA) (GraphPad). The correlation between the different serum responses was determined by linear regression and Pearson *r* analysis using the GraphPad software.

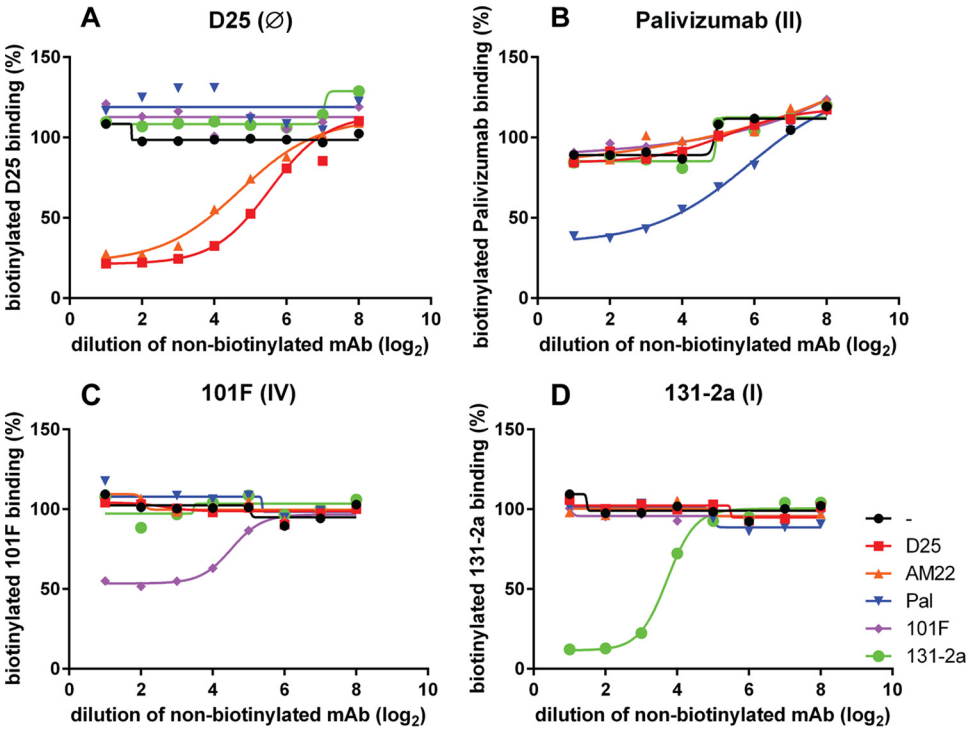

FIG 2 F epitope-specific blocking ELISA. Two-fold serial dilutions of nonbiotinylated MAbs were applied to RSV F protein-coated wells (Flys-GCN [21]), followed by addition of the indicated biotinylated MAbs at a fixed concentration. The *y* axes depict the percentage of biotinylated MAb binding in the presence of nonbiotinylated MAbs normalized to the binding in the absence of nonbiotinylated MAbs.

RESULTS

Development and validation of RSV protein- and F epitope-specific ELISAs. Different ELISAs were developed to analyze RSV protein- and epitope-specific antibody levels in human and animal sera. As an antigen to determine F protein-specific antibody levels, we used a recombinant soluble prefusion-like form of F consisting of the F ectodomain extended with a GCN4 trimerization motif and containing mutated furin cleavage sites (Flys-GCN) (21). Flys-GCN is recognized by prefusion-specific antibodies (D25; antigenic site Ø) and postfusion-specific antibodies (131-2a; antigenic site I) as well as by antibodies that recognize both conformations (palivizumab and 101F; antigenic sites II and IV, respectively) (Fig. 1A, B, and D). The neutralizing capacity of these antibodies is shown in Fig. 1C. To examine antibody responses to the RSV G protein, we produced and purified recombinant soluble G protein extended with a tetramerization domain. Blue native gel electrophoresis confirmed the oligomerization of the recombinant protein (data not shown). A monoclonal antibody (131-2G) against the G protein was shown to efficiently bind to the recombinant soluble G protein (Fig. 1E).

As Flys-GCN displays a large repertoire of F-specific epitopes, it is suitable for determination not of only the overall F protein reactivity but also of the different F epitope-specific antibody levels in sera. Flys-GCN was therefore used for the development of a blocking ELISA based on the competition between biotinylated monoclonal antibodies and serum antibodies for binding to different RSV F antigenic sites. After optimization of the antigen coating, concentration of the biotinylated MAbs, and different incubation times (data not shown), the specificity of the assays was verified by performing a blocking ELISA using labeled and nonlabeled MAbs. As shown in Fig. 2, binding of biotinylated MAb could be blocked only by the corresponding nonlabeled antibody, with the exception of biotinylated D25, which was blocked by D25 and AM22, both of which have been shown to bind antigenic site Ø (14, 23).

The different ELISAs (using F protein, G protein, and epitope-specific F protein) were validated using pooled human control sera that were obtained from beiRESOURCES and that have either a low or high RSV-neutralizing titer (referred to as serum "High" and serum "Low") (Fig. 3A). As expected, control serum High showed higher reactivity with the F and G proteins than control serum Low (Fig. 3B and C, line charts), although the differences were smaller for the G protein than for the F protein. Specific serum titer values corresponding to the OD at 450 nm (OD_{450}) within the linear part of the curve, which were determined in three independent experiments, significantly differed between the two control sera (Fig. 3B and C, bar charts).

Using our blocking ELISA, we checked whether the serum titer differences observed between the two control sera in the F protein ELISA were also observed for the different F epitopes. Indeed, control serum High showed stronger inhibition of binding for all antibodies (D25, palivizumab, 101F, and 131-2a) tested than control serum Low (Fig. 4, line charts). Serum titers corresponding to a specific level of inhibition of biotinylated antibody binding, determined in three independent experiments, were significantly different between the two control sera (Fig. 4, bar charts). These results show that, with our ELISAs, we can detect differences in F-, G-, and F-epitope-specific antibody levels in pooled control sera, in agreement with these sera differing in their virus-neutralizing titers.

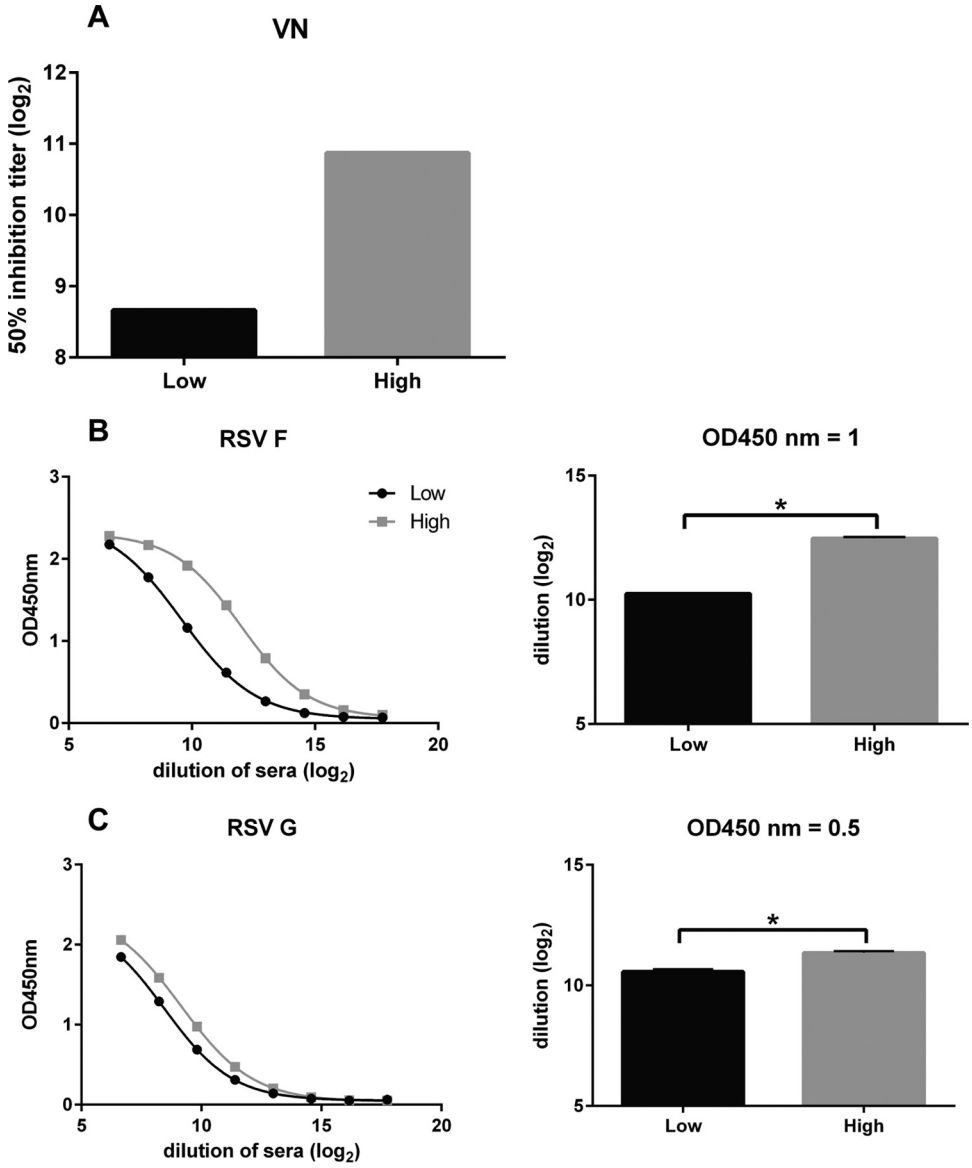

FIG 3 Reactivity of pooled control sera with F and G. (A) RSV A neutralization titer of pooled human control sera "Low" and "High" (beiRESOURCES NR-4023 and NR-4021, respectively). The dilutions (log_2) that gave 50% inhibition of RSV infection are graphed. (B and C) RSV F (FlysGCN) (B) and G (C) results of ELISA analyses of pooled control sera "Low" and "High." Two-fold serial dilutions of pooled control sera were applied to F or G protein-coated wells. The optical density at 450 nm (OD450nm) corresponding to binding of antibodies to F or G was graphed as a line chart. The bar graphs depict the serum dilutions (log_2) that correspond to $OD_{450} = 1$ for FlysGCN and $OD_{450} = 0.5$ for RSV G. The error bars indicate the standard deviations ($n = 3$). *, $P < 0.05$ (Student's t test).

RSV protein- and epitope-specific antibody levels in individual human sera after natural infection. After having analyzed the pooled human sera, we were interested in the RSV protein- and epitope-specific antibody responses in individual human sera and the correlation thereof with virus neutralization. Sera were collected from 15 healthy individuals and quantitatively analyzed for the presence of virus-neutralizing F protein- and G protein-specific antibodies (Fig. 5A to C) as described above. To determine the correlations among the different antibodies titers, linear regression analysis was performed and the Pearson correlation coefficients were determined. Analysis of the P values indicated a significant correlation between virus neutralization and F protein ELISA titers (Fig. 5E) but not between virus neutralization and G protein ELISA titers (Fig. 5F). F protein- and G protein-specific antibody titers also did not correlate significantly (data not shown).

Next, the human sera were subjected to the epitope-specific blocking ELISAs to determine the antibody levels against D25, palivizumab, 101F, and 131-2a epitopes for each serum sample (Fig. 6A to D). Subsequently, we analyzed the extent to which these values correlated with the F protein ELISA and RSV neutralization titers. The antibody levels against 131-2a, palivizumab, and 101F epitopes correlated significantly with the F protein ELISA titers, as indicated by the low P value ($P < 0.05$), but this was not the case for antibody against D25 epitope (Fig. 6E to H). Nevertheless, the highest positive correlation (indicated by the r

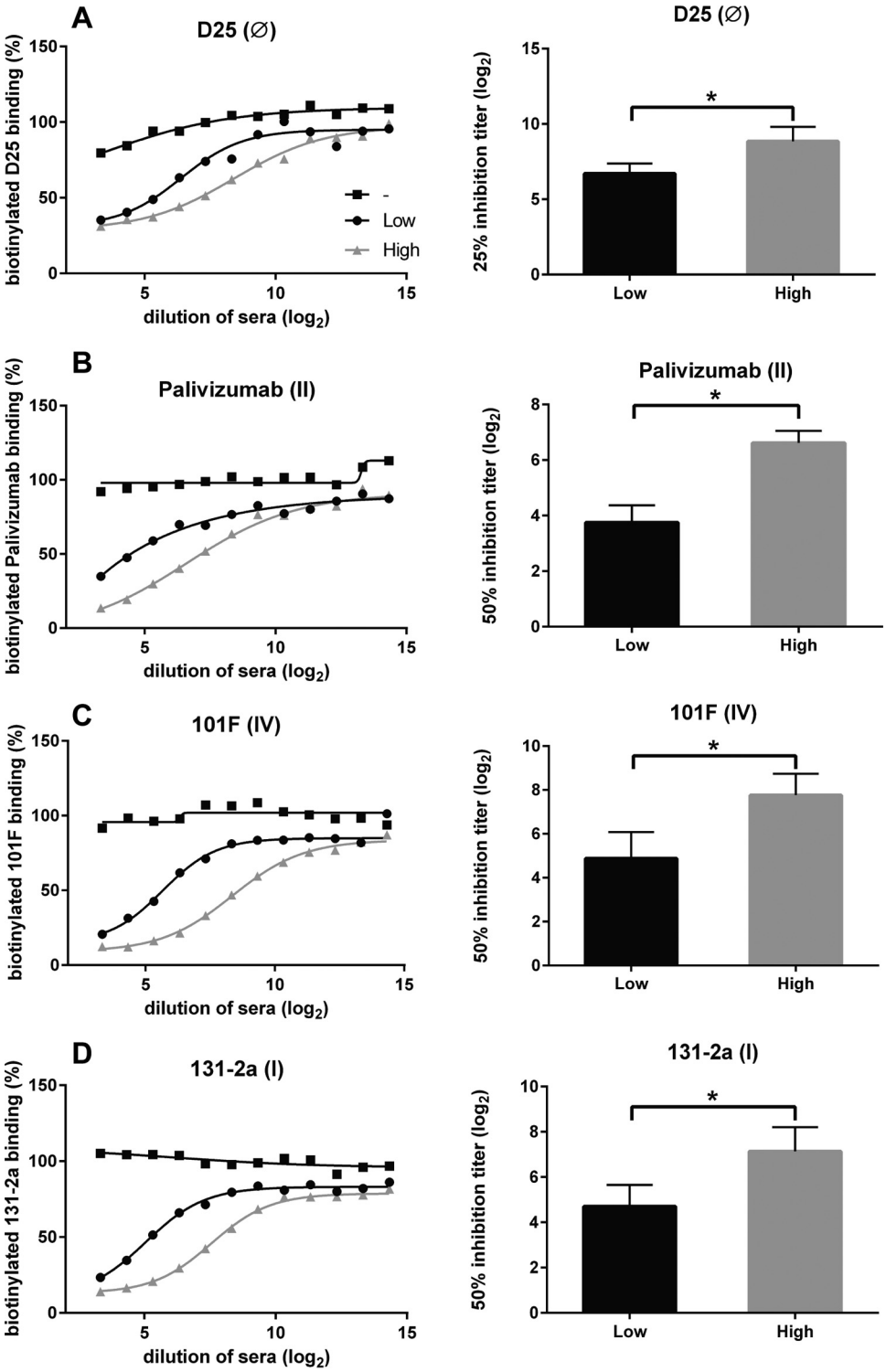

FIG 4 F epitope-specific reactivity of pooled control sera. Blocking ELISA analyses were performed to check for the presence of D25 (A)-, palivizumab (B)-, 101F (C)-, and 131-2a (D)-competing antibodies in the pooled human control sera "Low" and "High" (beiRESOURCES NR-4023 and NR-4021, respectively). Two-fold serial dilutions of sera were applied to RSV F (FlysGCN)-coated wells, followed by addition of the indicated biotinylated MAbs. The percentages of biotinylated MAb binding in the presence of sera normalized to the binding in the absence of sera were graphed as a line chart. The bar graphs depict the dilution ($\log_2$) that corresponds to 25% (D25) or 50% (palivizumab, 101F, and 131-2a) blocking of binding of biotinylated MAbs. The error bars indicate the standard deviations ($n = 3$). *, $P < 0.05$ (Student's t test).

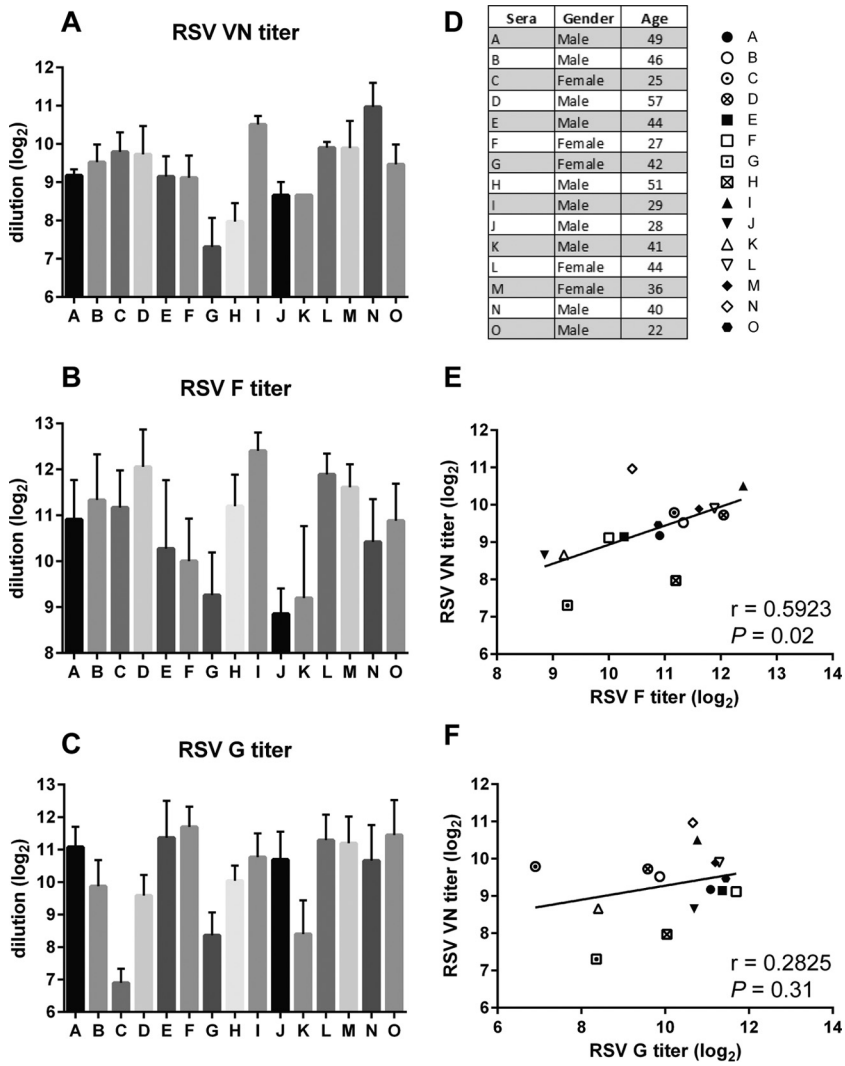

FIG 5 Characterization of RSV neutralization and RSV protein antibody responses in human sera and the correlation with RSV neutralization. Sera from 15 healthy individuals (indicated by capitals) were checked for the presence of RSV neutralization titer as described in Materials and Methods and anti-RSV antibodies as described in the Fig. 3 legend. (A to C) The bar graphs depict the dilution (log_2) that corresponds to 50% inhibition of RSV infection (RSV VN titer) (A), OD450 nm = 1 for RSV F (FlysGCN [21]) (B), or OD450 nm = 0.5 for RSV G (C). The error bars indicate the standard deviations ($n = 3$). (D) The age (in years) and gender of the 15 healthy volunteers from whom the sera were derived are indicated together with symbols that represent each individual. Linear regression analysis and calculation of the Pearson correlation coefficient were performed using the titer of RSV neutralization and RSV protein ELISA. (E) Correlation between overall F protein reactivity (depicted on the *x* axis) and virus neutralization (depicted on the *y* axis). (F) Correlation between G protein reactivity (depicted on the *x* axis) and virus neutralization (depicted on the *y* axis).

value) between the epitope-specific antibody and virus neutralization titers was observed for antibody against D25 epitope (Fig. 6I). Levels of antibodies against palivizumab and 131-2a but not 101F epitopes also showed a significant (but lower) positive correlation with virus neutralization titers (Fig. 6J to L).

To allow a better comparison of the relative amounts of the different antibodies, we also determined the fold difference between the antibody titers of each individual serum and the mean titer of the whole panel of human sera for each assay performed (Fig. 7). The antibody response to antigenic site Ø (antibody D25) displayed the largest variation among the antigenic sites. It furthermore appears that each individual displays a unique antibody profile. Some individuals contained either low or high antibody levels against all epitopes, resulting in low and high RSV-neutralizing antibody titers, respectively (represented by the red and green lines in Fig. 7). However, another individual had a low D25-like antibody titer but high antibody titers for other epitopes, concomitant with a relatively high virus neutralization titer (represented by the purple line in Fig. 7). In contrast, yet another individual displayed a high D25-like antibody titer while having a low antibody titer for the other epitopes and a relatively low virus neutralization titer (represented by the orange line in Fig. 7). We conclude from these results that, although antigenic site Ø-specific antibody levels in human sera show the highest correlation with virus neutralization titers of all the antigenic sites tested, antibodies targeting parts of the F protein other than antigenic site Ø can significantly contribute to the antiviral antibody response elicited by natural infection.

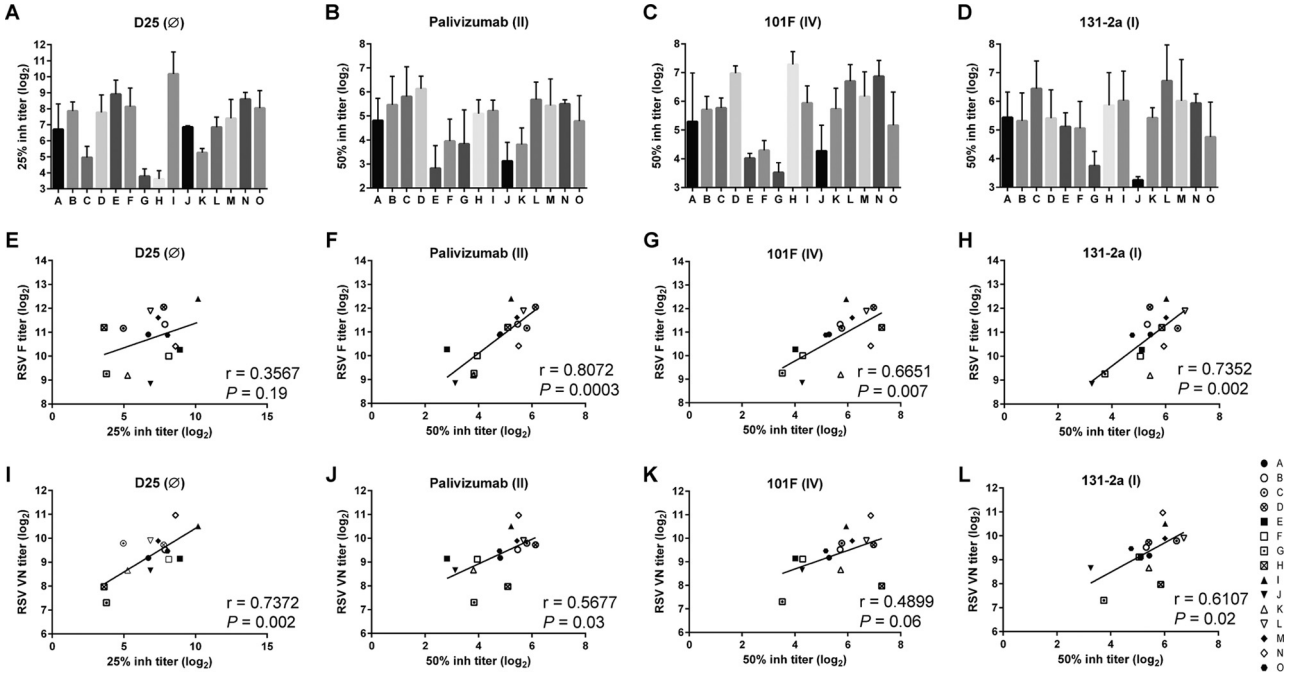

FIG 6 Characterization of RSV F epitope-specific antibody responses in human sera and their correlation with overall F protein reactivity or RSV neutralization. Sera from 15 healthy individuals (indicated by capitals) were checked for the presence of antibodies against F-specific epitopes as described in the Fig. 4 legend. The bar graphs depict the dilution ($\log_2$) that corresponds to 25% inhibition of binding of D25 (A) or 50% inhibition of other MAbs (palivizumab, 101F, and 131-2a [B, C, and D]). The error bars indicate the standard deviations ($n = 3$). Linear regression analysis and calculation of the Pearson correlation coefficient were performed using the titer of RSV F ELISA and RSV neutralization as described in the Fig. 5 legend and RSV epitope-specific ELISA. (E, F, G, and H) Correlation between the F epitope-specific reactivity (depicted on the *x* axis) and overall F protein reactivity (depicted on the *y* axis). (I, J, K, and L) Correlation between the F epitope-specific reactivity (depicted on the *x* axis) and RSV neutralization (depicted on the *y* axis). Different symbols are used for the different sera. The Pearson *r* correlation coefficient and *P* values are indicated.

Evaluation of antibody responses after vaccination with FI-RSV. Finally, we used our ELISAs to study RSV-specific antibody responses in the cotton rat model. Cotton rats were immunized either with FI-RSV or with live attenuated recombinant RSV X (rRSV), the latter at two different doses. As a control, mock-vaccinated cotton rats were included. At 28 days postvaccination, blood samples were taken (prechallenge samples) and the animals were challenged with RSV. At 5 days postinfection, the animals

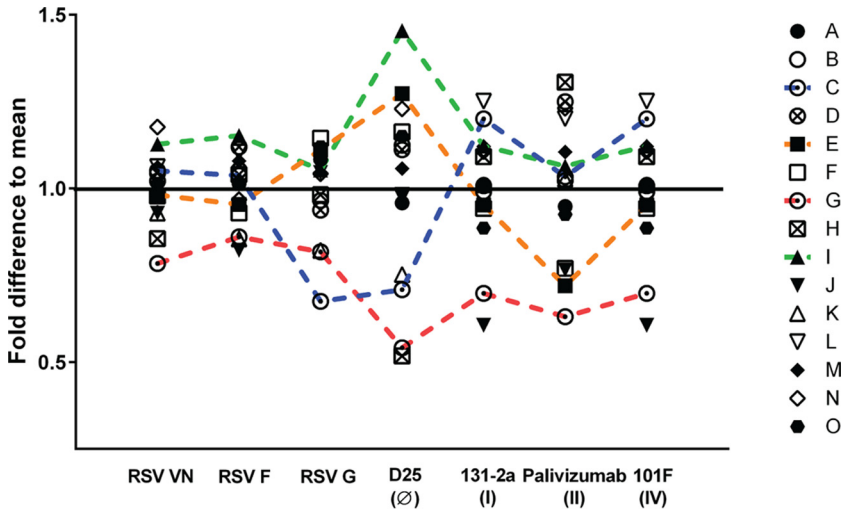

FIG 7 RSV-specific antibody profiles in human sera. The results corresponding to the titer of RSV neutralization and RSV protein/epitope-specific ELISA analysis of human sera described in the Fig. 5 and 6 legends are depicted as the fold difference between the value for each of the sera and the mean value for all sera tested. Each data point represents the mean of results from three independent experiments performed to determine the presence of virus-neutralizing (RSV VN), FlysGCN protein-specific (RSV F), G protein-specific (RSV G), and epitope Ø-, I-, II-, and IV-specific antibodies. Dashed lines with different colors that connect corresponding symbols represent different antibody profiles of 4 different individuals.

were euthanized, and lungs and blood samples were collected (postchallenge samples). Animals that had been immunized with rRSV or with FI-RSV displayed significantly decreased virus titers in the lungs compared to the mock-vaccinated animals, although FI-RSV-vaccinated animals displayed higher virus titers than rRSV-immunized animals (Fig. 8A). Pre- and postchallenge sera were analyzed for RSV neutralization, F protein ELISA, and F epitope-specific titers (Fig. 8B to F). On the day of the challenge, immunization with recombinant RSV had resulted in low but detectable virus neutralization and F protein ELISA titers. In contrast, vaccination with FI-RSV did not induce appreciable levels of either virus-neutralizing or F protein-specific antibody levels prior to the challenge (Fig. 8B and C). After the challenge, all animals except the mock-vaccinated ones showed an increase in their virus neutralization titers and in their response to RSV F (Fig. 8B and C), although these responses were somewhat lower for the animals vaccinated with FI-RSV. No detectable levels of antibody against D25 epitope were observed in any of the animals (data not shown). However, immunization with rRSV induced antibodies against palivizumab, 101F, and 131-2a epitopes, the levels of which increased after the challenge. In agreement with the F protein ELISA results (Fig. 8C), vaccination with FI-RSV did not induce detectable epitope-specific F protein responses prior to challenge (Fig. 8D to F). After challenge, appreciable competing antibody levels could be detected only for 131-2a and not for palivizumab or 101F. Similar results (Fig. 9) were obtained with pooled sera of cotton rats that had been vaccinated with FI-RSV and which displayed vaccination-enhanced disease upon challenge (21).

The absence of palivizumab- and 101F-competing antibodies after vaccination with FI-RSV but not after vaccination with rRSV prompted us to analyze the preservation of the different epitopes in these preparations. As controls, postfusion F (Fwt [21]) and prefusion F (F DSCav1-T4fd [17]) were included. The results indicate that FI-RSV and rRSV display similar levels of reactivity for all epitopes tested, with both preparations having negligible binding of prefusion-specific MAb D25 (Fig. 10A and B). The reactivity observed with the different virus preparations was very similar to that of postfusion F (Fig. 10C) and clearly deviated from that of prefusion F (Fig. 10D). These results indicate that most F proteins in rRSV as well as FI-RSV are in the postfusion conformation. Furthermore, the lack of induction of palivizumab- or 101F-like antibodies was not due to the absence of their corresponding epitopes in FI-RSV preparations.

DISCUSSION

Results of recent studies performed using recombinant RSV F proteins and conformation-specific monoclonal antibodies indicate an important role for prefusion F-specific antibodies in protective immune responses (17–19). Despite these recent findings, detailed insight into multiple F epitope-specific serum responses after natural infection or after vaccination is lacking. This is important for the development and evaluation of novel vaccines, especially in the light of the FI-RSV vaccine failure that argues for a role of nonneutralizing antibodies as a factor that can prime for enhanced disease in seronegative individuals (32). Therefore, we developed novel assays to evaluate RSV protein- and F epitope-specific antibody profiles in human sera and in sera of vaccinated and experimentally infected cotton rats. We show that human sera contain various levels of antibodies with different specificities upon natural infection. The highest correlation with virus neutralization was observed for antibodies recognizing prefusion-specific antigenic site Ø. Our data also indicate that low levels of antigenic site Ø-specific antibody may be compensated by neutralizing antibodies targeting other parts of the F protein. Neutralizing sera of experimentally infected cotton rats were shown to contain high levels of antibodies recognizing antigenic sites I, II, and IV but not site Ø. Importantly, vaccination with FI-RSV failed to induce antibodies that recognize antigenic sites II and IV, as it specifically resulted in high (but poorly neutralizing) antibody levels against postfusion-specific antigenic site I either upon (Fig. 8) or prior to (Fig. 9) challenge infection with RSV.

Analysis of individual sera from healthy volunteers revealed substantial diversity in their virus neutralization and F and G protein ELISA titers (Fig. 7), consistent with previous studies (33, 34). Analysis of the F epitope-specific serum responses revealed similar differences between individuals, with the largest differences being observed for antibody levels against D25 epitope (Fig. 7). The relatively large differences in antibody levels against the D25 epitope might be related to antigenic site Ø being the least conserved region among the antigenic regions in protein F (14, 35), although this was not analyzed in the present study.

The variations among individuals allowed us to analyze the correlation between protein- and epitope-specific serum responses and the virus neutralization titers. Overall F protein reactivity in humans sera correlated well with levels of serum antibodies recognizing antigenic sites I, II, and IV but not antigenic site Ø. Similar observations were made when a previously published prefusion-stabilized F protein was used (F DSCav1-T4fd [17]; data not shown). Hence, reactivity in human sera with recombinant prefusion F is not predictive of antigenic site Ø-specific antibody levels. The low correlation between antigenic site Ø-specific antibody levels and overall F protein reactivity suggests that epitope Ø-specific antibodies are less abundant than antibodies recognizing other epitopes and therefore contribute less to the overall reactivity with F. Quantification of epitope-specific anti-F antibody levels based on monoclonal antibody standard curves indeed indicated higher levels of antibodies against palivizumab, 101F, and 131-2a than against D25 epitope (data not shown). The relatively low levels of antibody against D25 epitope may be related to the low stability of epitope Ø compared to other epitopes (12, 36, 37). Furthermore, the possibility of differences in levels of immunodominance between epitopes (with antigenic Ø possibly being subdominant) (38) or in levels of epitope-specific stability of antibodies cannot be excluded.

Strong correlation with virus neutralization was observed for RSV F-specific but not G-specific serum reactivity, in agreement with previous studies that showed that F is a more important target of neutralizing antibodies than G, at least in *in vitro* assays using immortalized cells (39–41). The abundance of antigenic site Ø-specific antibodies correlated better with virus neutralization than the abundance of antibodies binding other antigenic sites. These results suggest that antibodies against D25 epitope are responsible for a large proportion of the virus-neutralizing capacity of sera, in agreement with the known potency of prefusion-specific antibodies (14, 17) and with a recent study in which prefusion-specific antibody levels were correlated with virus neutralization (20). Nevertheless, our results indicate that antibodies targeting parts of the F protein other than antigenic site Ø can contribute significantly to the antiviral antibody response elicited

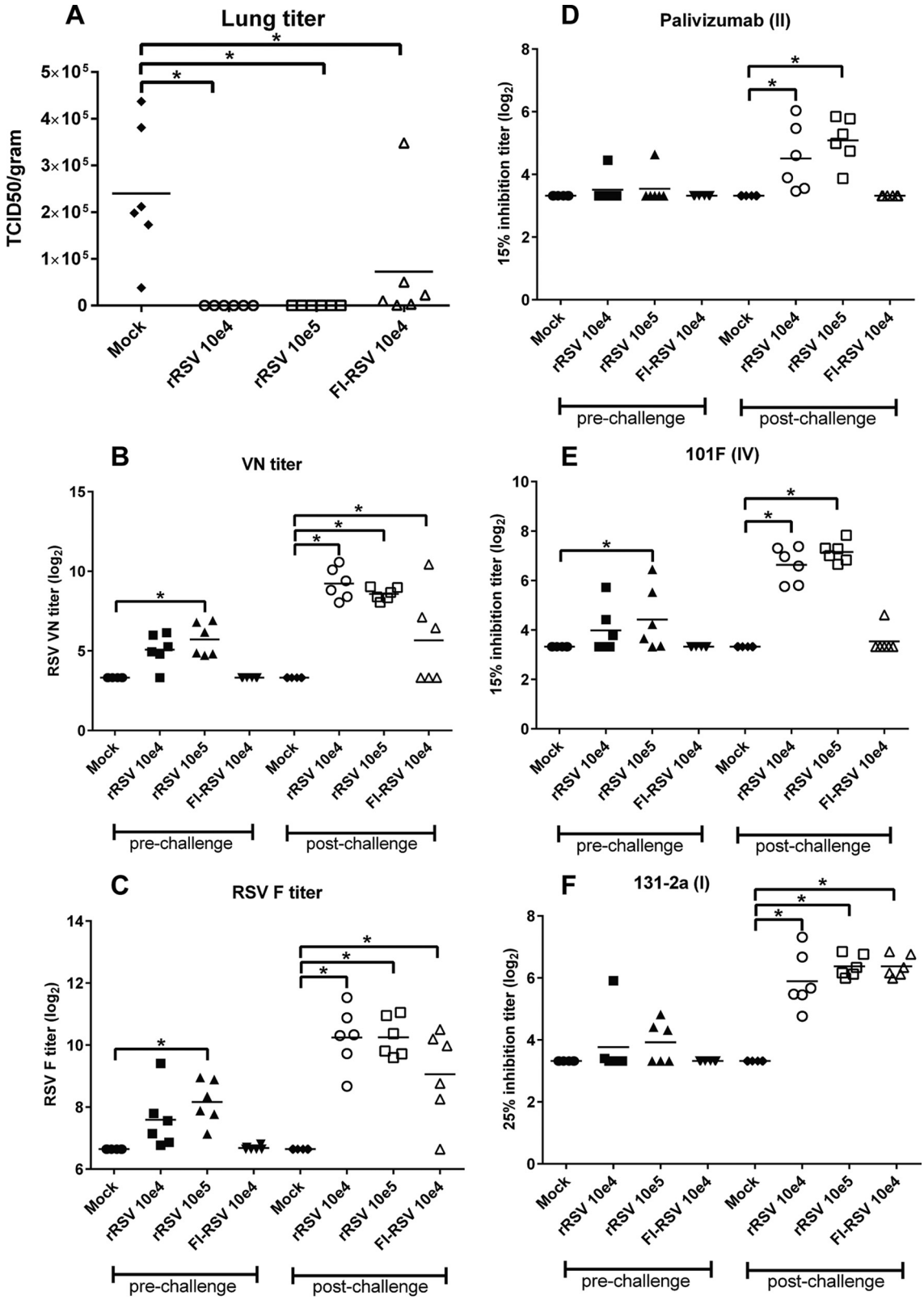

FIG 8 Characterization of cotton rat sera after vaccination with FI-RSV. Cotton rats were vaccinated intranasally with PBS (Mock) or rRSV (using two different doses, 10e4 and 10e5) or intramuscularly with FI-RSV followed by a challenge with RSV at 28 days after vaccination. Sera were collected on the day of challenge (pre-challenge) and 5 days after challenge (post-challenge). (A) Virus titer in the lungs at day 5 postchallenge ($TCID_{50}$/gram). (B) Pre- and postchallenge 60% RSV PRNT results. (C) RSV F (FlysGCN) ELISA titer pre- and postchallenge. (D, E, and F) FlysGCN epitope-specific ELISA titers pre- and postchallenge. *, $P < 0.05$ (using ANOVA).

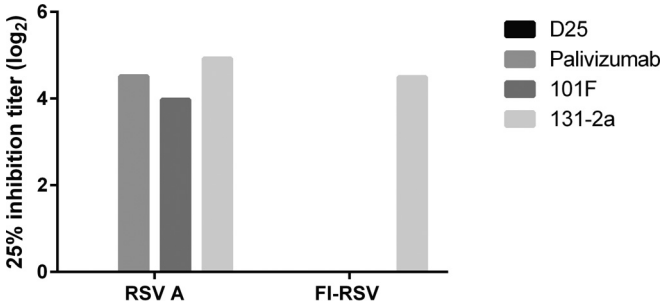

FIG 9 Epitope-specific antibody levels in pooled cotton rat sera. Pooled sera of cotton rats vaccinated with FI-RSV (21) were analyzed for the presence of D25-, palivizumab-, 101F-, and 131-2a-competing antibodies as described in the legend to Fig. 4. Sera collected 2 weeks after the last vaccination (at the day of challenge) were analyzed. Animals vaccinated with FI-RSV were shown to display vaccination-enhanced disease upon challenge (21). As a control, pooled sera from a parallel experiment in which cotton rats had been infected intranasally with 10^5 PFU of RSV/A/Long per animal were analyzed. Sera collected at day 42 postinfection (at the day of challenge) were analyzed. The latter animals did not display enhanced disease upon challenge. The animal experiment was performed by Sigmovir Biosystems, Inc. (Rockville, MD).

upon natural infection, as some individuals with low D25-competing antibody levels but with relatively high levels of antibodies against other neutralizing epitopes still display high virus neutralization activity (Fig. 7). We cannot exclude the possibility of a contributory role in this respect for antibodies recognizing other prefusion-specific epitopes such as AM14 (23, 42) and MPE8 (43) that were not analyzed in this study.

The protein- and epitope-specific ELISAs were also used to analyze sera from cotton rats that had been immunized with either live attenuated rRSV or FI-RSV followed by a challenge infection. In all cases, we could not detect the presence of antibodies against the D25 epitope in cotton rat sera or in cotton rats that had been infected with (live attenuated) RSV. This might be explained by low levels of virus replication (28), resulting in antibody responses being limited to those epitopes that were present in the virus preparation, which did not appreciably include antigenic site Ø (Fig. 10). Despite the absence of antigenic site Ø-specific antibodies, animals that had been immunized with live attenuated rRSV developed detectable titers of antigenic site I-, II-, and IV-specific antibodies. For the animals that had received FI-RSV, essentially only antigenic site I-specific antibodies were detected, in agreement with these sera displaying relatively low virus neutralization titers (Fig. 8B) and with the low neutralization capacity of 131-2a (Fig. 1C). Previous studies suggested the modification of F protein epitopes by formalin inactivation (5, 8). However, the failure of FI-RSV to induce antigenic site II- and IV-specific antibodies is not explained by the absence of the corresponding epitopes in the FI-RSV preparations, as we convincingly showed the reactivity of FI-RSV with palivizumab and 101F (Fig. 10). We therefore speculate that formalin inactivation specifically stimulates antibody responses to site I and/or downregulates site II and IV antibody responses, resulting in the presence of high levels of postfusion-specific, poorly neutralizing antibodies and the concomitant absence of neutralizing antibodies after vaccination with FI-RSV. The absence of neutralizing antibodies may lead to a high load of

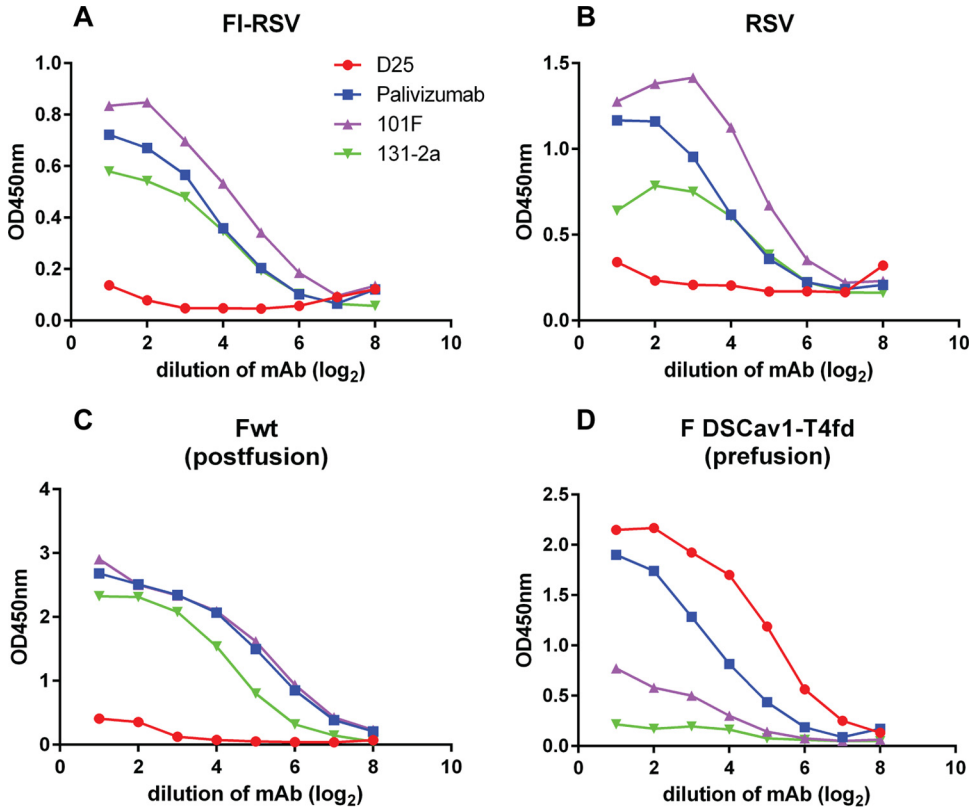

FIG 10 Reactivity of FI-RSV and rRSV with different MAbs. Reactivity of different MAbs with FI-RSV (A), rRSV (B), recombinant protein Fwt (C; postfusion F [21]), and recombinant protein F DSCav1-T4fd (D; prefusion F [17]) is shown.

RSV antigen that subsequently triggers an exacerbated detrimental immune response (44). Alternatively, opsonization of RSV with nonneutralizing antibodies may enhance disease by facilitating infection of macrophage-like cells (45).

Our results indicate that F epitope-blocking ELISAs provide additional insights into RSV-specific serum responses compared to conventional F protein ELISAs. F protein ELISAs cannot discriminate between pre- and postfusion F-specific responses, as many epitopes are shared by these two F protein conformations. Analysis of F epitope-specific antibody levels in cotton rat sera also revealed novel insights into the antibody responses induced by vaccination with FI-RSV. It remains to be determined to what extent the specific induction of poorly neutralizing antibodies against RSV F antigenic site I contributes to enhanced disease upon vaccination with FI-RSV. However, our data indicate that the antigenicity of a vaccine preparation is not predictive of the induced antibody responses.

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