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ABSTRACT  Respiratory syncytial virus (RSV) infections remain a major cause of respiratory disease and hospitalizations among infants. Infection recurs frequently and establishes a weak and short-lived immunity. To date, RSV immunoprophylaxis and vaccine research is mainly focused on the RSV fusion (F) protein, but a vaccine remains elusive. The RSV F protein is a highly conserved surface glycoprotein and is the main target of neutralizing antibodies induced by natural infection. Here, we analyzed an internalization process of antigen-antibody complexes after binding of RSV-specific antibodies to RSV antigens expressed on the surface of infected cells. The RSV F protein and attachment (G) protein were found to be internalized in both infected and transfected cells after the addition of either RSV-specific polyclonal antibodies (PAbs) or RSV glycoprotein-specific monoclonal antibodies (MAbs), as determined by indirect immunofluorescence staining and flow-cytometric analysis. Internalization experiments with different cell lines, well-differentiated primary bronchial epithelial cells (WD-PBECs), and RSV isolates suggest that antibody internalization can be considered a general feature of RSV. More specifically for RSV F, the mechanism of internalization was shown to be clathrin dependent. All RSV F-targeted MAbs tested, regardless of their epitopes, induced internalization of RSV F. No differences could be observed between the different MAbs, indicating that RSV F internalization was epitope independent. Since this process can be either antiviral, by affecting virus assembly and production, or beneficial for the virus, by limiting the efficacy of antibodies and effector mechanism, further research is required to determine the extent to which this occurs in vivo and how this might impact RSV replication.

IMPORTANCE  Current research into the development of new immunoprophylaxis and vaccines is mainly focused on the RSV F protein since, among others, RSV F-specific antibodies are able to protect infants from severe disease, if administered prophylactically. However, antibody responses established after natural RSV infections are poorly protective against reinfection, and high levels of antibodies do not always correlate with protection. Therefore, RSV might be capable of interfering, at least partially, with antibody-induced neutralization. In this study, a process through which surface-expressed RSV F proteins are internalized after interaction with RSV-specific antibodies is described. One the one hand, this antigen-antibody complex internalization could result in an antiviral effect, since it may interfere with virus par-
ticle formation and virus production. On the other hand, this mechanism may also reduce the efficacy of antibody-mediated effector mechanisms toward infected cells.

**KEYWORDS** antibodies, fusion protein, internalization, respiratory syncytial virus

Human respiratory syncytial virus (RSV) is a leading cause of severe lower respiratory tract disease in young children and a major cause in the elderly and immunocompromised patients worldwide (1, 2). Nearly all children are exposed to RSV by 2 years of age, and prematurity, bronchopulmonary dysplasia, and congenital heart disease are risk factors for developing severe RSV disease, including bronchiolitis and pneumonia (1). RSV may also cause significant disease in adults, and reinfection can occur throughout life (2). Despite the discovery of the virus in 1956, no safe and effective vaccine is currently available to control RSV infections (3). Treatment of severe infections is primarily supportive by maintenance of hydration and oxygenation. Palivizumab, a humanized monoclonal antibody (MAb), targets a conserved epitope of the RSV fusion (F) protein and is administered prophylactically to high-risk patients (4). Severe RSV disease appears to be linked to an unbalanced and incomplete immune response. Several factors that allow RSV to evade host defense have already been described (2, 5, 6).

RSV belongs to the *Pneumoviridae*, genus *Orthopneumovirus*, which is comprised of enveloped viruses with a negative-stranded RNA genome. The 15.2-kb nonsegmented genome is comprised of 10 genes that encode 11 proteins. Among these are three surface glycoproteins, the G glycoprotein, the F protein, and the small hydrophobic (SH) protein (1). The G protein is responsible for attachment with host cells, which are predominantly ciliated airway epithelial cells (7, 8). Fusion of the viral and cellular membranes is facilitated by the RSV F protein, as is fusion between the membranes of infected cells with adjacent cells, which result in large, multinucleated syncytia. The smaller SH protein is considered to act like a viroporin and increases membrane permeability (5). Of these envelope glycoproteins, only the RSV F protein is indispensable for viral replication *in vitro* (9). It is the most conserved RSV glycoprotein and also the main target of neutralizing antibodies and vaccine development (10, 11). Initially, the RSV F protein assembles into a homotrimeric, metastable prefusion conformation that rearranges to a highly stable postfusion conformation during fusion of the viral and target cell membrane or spontaneously (12). Six major antigenic sites are currently identified that are located on the prefusion and/or postfusion trimer conformation of the RSV F protein (10, 13–15). Palivizumab, directed to antigenic site II, is the only approved immunoprophylaxis and provided a 55% reduction in RSV-associated hospitalizations in a phase III trial (16). At present, the use of potent neutralizing antibodies directed to other epitopes and/or targets is being extensively studied as an alternative approach for both therapy and prophylaxis. This research is mainly focused on highly potent antibodies that recognize the prefusion RSV F conformation. Three antibodies (5C4, AM22, and D25) were shown to bind the prefusion-specific antigenic site Ø, located at the apex of the prefusion trimer (14). Recently, two novel prefusion-specific antibodies, MPE8 and AM14, were characterized and shown to bind antigenic sites III and V, respectively (10, 15, 17). The epitope for MPE8 is located near the binding site of palivizumab in the groove between the helix-turn-helix and the ridge of antigenic site IV on the adjacent protomer. It partially competes with MAbs to sites II, IV, and V. This epitope is well conserved between other pneumoviruses of the *Paramyxoviridae* family (15). Antigenic site V, targeted by AM14, spans from the tip of the β3-β4 hairpin of one protomer to the distal end of antigenic site IV on the adjacent protomer (17).

Internalization of viral envelope proteins expressed on the surface of infected cells is a commonly seen characteristic of viruses, including paramyxoviruses (18–22). For most viruses, the relevance of this process is not yet fully understood. In the case of the Henipavirus fusion proteins, internalization from the surface is essential for proteolytic activation by cathepsin L (19). Also, virus assembly can be affected by the internalization of viral glycoproteins (23). Furthermore, internalization can be important for viral
pathogenesis by downregulation of viral antigen surface expression and reduced recognition of infected cells by the immune system (20, 24–26). Two different types of internalization have been described previously. Spontaneous endocytosis was observed for many herpesviruses, and human immunodeficiency virus (HIV) was observed among others. A second type of internalization is induced by the interaction of specific antibodies with viral proteins expressed on the surface of infected cells, followed by internalization of antibody-antigen complexes in the cell (25, 27, 28). Such viral protein internalization may result from cross-linking or depend on specific endocytic motifs in the cytoplasmic or transmembrane domains of glycoproteins, such as common tyrosine-based sorting motifs and dileucine motifs (20, 24, 29, 30).

Previous studies have shown that upon binding of goat anti-RSV polyclonal antibodies (PABs) to RSV antigens expressed on the surface of infected HEp-2 cells, internalization of these RSV antigen-antibody complexes may occur (31, 32). In this study, we examined this process in the context of a viral infection as well as at the level of individual RSV glycoproteins expressed at the surface of transfected cells. Both strategies demonstrated uptake of RSV antigen-antibody complexes in a time-dependent manner that resulted in a reduction of surface-expressed RSV antigens. This process modulates surface expression of RSV antigens and may affect induction of and recognition by RSV-specific antibodies. Since the RSV F protein elicits the most potent neutralizing antibodies and is currently the most interesting target for therapeutic and prophylactic purposes, this study aimed to characterize antibody-induced internalization of RSV antigens, in particular RSV F, in more detail.

RESULTS

Antibody-induced internalization of cell surface-expressed RSV antigens. To evaluate antibody-induced internalization of RSV antigens, RSV-infected HEp-2 and A549 cells were incubated with polyclonal goat anti-RSV IgG for 1 h and subsequently fixed and stained with AF488 donkey anti-goat IgG. At time point 0, membrane staining was observed for both cell types, and none of the infected cells showed vesicles in their cytoplasm (Fig. 1A). After 1 h of incubation at 37°C, multiple intracellular vesicles were present in the cytoplasm of the cells, and a reduction in surface expression was observed. No intracellular vesicles were observed in noninfected cells. Isotype control antibodies and secondary antibodies were used to confirm that the internalization process is specifically induced by the interaction of RSV-specific antibodies with RSV antigens (data not shown). In addition, the kinetics of antibody-induced internalization was analyzed. Figure 1B shows the kinetics of the percentage of cells positive for intracellular RSV antigen-antibody complexes, which reached a maximum at 60 min and 90 min for HEp-2 cells and A549 cells, respectively. Similar observations were made based on the number of internalized vesicles (Fig. 1C). By flow-cytometric analysis, the surface-bound antibodies were measured before and after induction of internalization to allow a more quantitative measurement of internalization (33, 34). For HEp-2 cells and A549 cells, the mean fluorescence intensity (MFI) of surface RSV proteins after the addition of antibodies was reduced to 44% and 40%, respectively, showing that internalization occurs but that not all RSV proteins are internalized (Fig. 1D). Taken together, antibody-induced internalization of RSV antigens is time dependent, but not all molecules are internalized upon binding of PABs. Addition of increasing concentrations of PABs resulted in rising levels of internalized vesicles between 0.001 and 0.01 mg/ml, followed by a slower increase between 0.01 and 0.3 mg/ml (Fig. 1E). At a concentration of 1 mg/ml, the internalization efficiency decreased.

Two major antigenic subgroups (A and B) have been described for RSV. With regard to the RSV surface proteins F and G, an amino acid identity of 91% and 51%, respectively, exists between the subgroups (35). After infection with the RSV B1 reference strain, internalization of RSV-specific PABs in complex with RSV surface antigens was also observed (Fig. 2A). Additionally, a clinical strain (A1998/3-2) was evaluated and numerous intracellular vesicles were also observed, and surface expression of RSV proteins was reduced compared to the level at time point 0 (T0) (Fig. 2A).
These findings suggest that internalization of RSV surface antigens is a general feature of RSV. In addition to polyclonal goat anti-RSV antibodies obtained after immunization, a human anti-RSV reference serum (obtained after natural RSV infection) was also evaluated. After a 60-min incubation at 37°C, internalization and antigen-antibody complexes were detected in the cell cytoplasm, and some complexes remained at the surface of infected HEp-2 cells (Fig. 2B). Finally, the internalization process was also analyzed in RSV-infected well-differentiated primary bronchial epithelial cells (WD-PBECs). After 120 min of incubation with human RSV-specific antiserum, internalized RSV antigen-antibody complexes were observed in more than 80% of the RSV-infected cells (Fig. 3A and B).

**Determination of the cell surface-expressed RSV proteins involved in the internalization process.** Since RSV F and G proteins are the two major surface antigens and the only RSV proteins that induce neutralizing antibodies, the involvement of the respective RSV surface glycoproteins was identified. For this purpose, RSV glycoprotein-specific MAbs were used. For both surface proteins and in both cell types, internalization and intracellular vesicles were observed (Fig. 4A). Analysis of internalization in BSR T7/5 cells transfected with the individual RSV F or G proteins was performed to
investigate their respective roles in this process. Internalization of RSV antigens was also observed after RSV infection of BSR T7/5 cells and incubation with RSV-specific antibodies, showing that this process also occurs in these cells (data not shown). For both proteins, multiple internalized antigen-antibody complexes were present in vesicles in the cytoplasm of cells transfected with the RSV F or G protein and after incubation with either RSV-specific PAbs or RSV glycoprotein-specific MAbs (Fig. 4B). These observations show that internalization of both RSV surface proteins occurs independent of other viral proteins.

**Internalization of surface-expressed RSV F proteins is clathrin mediated and mainly triggered after antibody-induced cross-linking of the RSV F protein.** As mentioned earlier, RSV F is the major target of neutralizing antibodies and plays a central role in the development of new immunoprophylaxis and vaccine strategies. Therefore, the characteristics of RSV F internalization were studied in more detail. The endocytic route through which RSV F-antibody complexes are internalized was investigated by using inhibitors that block different mechanisms of endocytosis. Dynamin inhibitory peptide (DIP) is an inhibitor of the GTPase dynamin that blocks the binding of dynamin to amphiphysin (36). Addition of this peptide during internalization resulted in a reduction of approximately 50% of internalized vesicles (Fig. 5A). Inhibition by dynasore, an inhibitor that blocks dynamin by disturbing the plasma membrane cholesterol homeostasis (37), resulted in similar reductions (data not shown). In addition, BSR T7/5 cells expressing a recombinant RSV F protein were cotransfected with dominant-negative (DN) proteins to inhibit a specific internalization process. By using an enhanced green fluorescent protein (eGFP)-tagged DN mutant of dynamin 2(aa) in RSV F-transfected BSR T7/5 cells, a significant reduction of internalized vesicles (62.01%)
was observed in cells transfected with DN dynamin compared to wild-type (WT) dynamin-transfected cells (Fig. 5B and G). Both results show the involvement of a dynamin-dependent mechanism. Since GTPase dynamin is recruited to both clathrin-coated pits and caveolae, further distinction was made between these endocytic routes (38). Amantadine was used to test the dependence of clathrin and resulted in a dose-dependent reduction of intracellular vesicles with a maximum of 74.09% (Fig. 5C).

By using a DN mutant of Eps 15 (DIII) and the control plasmid DIIIΔ2, the role of clathrin was further analyzed. DIII-transfected cells showed a significant reduction in internalization compared to DIIIΔ2-transfected cells (Fig. 5D and G). No difference in the amount of internalized vesicles was observed after treatment of the cells with nystatin, a sterol-binding agent which disrupts caveolae (Fig. 5E). These results were confirmed in RSV F-transfected cells cotransfected with a GFP-tagged DN mutant of caveolin-1, which showed no significant differences in internalization of RSV F compared to cells transfected with the control plasmid, eGFP-tagged WT caveolin-1 (Fig. 5F and G).

In addition, we investigated the extent to which spontaneous endocytosis occurs for RSV proteins by labeling the surface proteins of infected cells with a membrane-impermeable biotinylation reagent, followed by an internalization assay. Cell surface biotinylation was efficient (Fig. 6E), and the addition of cell-impermeable glutathione removed almost all biotin from the cell surface (Fig. 6D). Antibody-induced internalization of biotinylated surface proteins resulted in protection from glutathione-mediated biotin cleavage. A difference in the amount of intracellular (biotinylated) RSV F proteins could be observed between internalization induced by antibodies (Fig. 6C) and spontaneous endocytosis in the absence of antibodies (Fig. 6B), confirming that the majority of RSV F internalization is triggered by antibodies.

Intact monoclonal IgG antibodies can cross-link surface-expressed antigens and stimulate their internalization (39, 40). To determine whether cross-linking of RSV F proteins is required for internalization, monovalent Fab fragments of RSV F-specific
MAbs were compared with the intact MAbs (Fig. 7A). Internalization of RSV F proteins was significantly reduced when induced by the monovalent Fab fragments (Fig. 7B), indicating that cross-linking plays a role in efficient RSV F internalization. Internalization of surface RSV F proteins is triggered by binding of different neutralizing RSV F epitope-specific MAbs. Different neutralizing epitopes on the RSV F protein have already been identified, and MAbs directed against these epitopes are available (10, 13–15). Since MAbs recognizing different epitopes of the same protein could have different effects on internalization, the epitope dependence of RSV F internalization was analyzed (41). The amount of internalized RSV F-MAb complexes was determined by flow-cytometric analysis. After shifting RSV-infected HEp-2 cells in suspension to 37°C, anti-RSV F MAbs attached to surface RSV F proteins were internalized and the amount of internalization was quantified by a reduction of surface expression. A reduction in MFI was seen for all MAbs compared to the MFI of cells that were kept at 4°C and did not undergo internalization. The amount of internalization, as calculated by the reduction of surface fluorescence, ranged from 31 to 57% for MAb 131-2A and AM22, respectively. Overall, no major differences could be observed between prefusion RSV F-specific MAbs or MAbs that bound both pre- and postfusion RSV F (Table 1). The results also showed that not all RSV F molecules are internalized upon stimulation with the MAbs. These findings were confirmed by analysis with fluorescence microscopy, where surface expression of RSV F was still observed after induction of internalization (data not shown).

**DISCUSSION**

Our results show the potential of RSV-specific antibodies and human sera from RSV-infected patients to trigger internalization of RSV antigens expressed on the surface of infected cells. These findings were clearly documented by confocal micro-
FIG 5 Effect of inhibitors and DN proteins on antibody-induced RSV F internalization. RSV-infected HEp-2 cells were incubated with MAb 131-2A in the presence of different concentrations of the endocytic inhibitors DIP (A), amantadine (C), and nystatin (E). After 90 min of incubation, cells were fixed, permeabilized, and stained. The amount of intracellular vesicles was quantified and expressed as a percentage relative to the number of vesicles in the absence of the inhibitor. BSR T7/5 cells were cotransfected with both RSV F (red) and DN (green) proteins (Continued on next page)
scopic analysis and flow cytometry and observed in different epithelial cell lines (HEp-2, A549, and BEAS-2B cells) (44, 45). In addition, WD-PBEC cultures were used to confirm that this process also occurs in a more representative model of primary airway epithelial cells. The amount of intracellular vesicles increased with increasing time of incubation and reached a plateau after 60 to 90 min in monolayer cells. Furthermore, surface-expressed glycoproteins of different RSV strains, including both reference strains (A2 and B1) and a clinical isolate (A1998/3-2), were shown to be susceptible to antibody-induced internalization (46). Taken together, our findings suggest that the internalization process is a general feature of RSV-infected cells. Using inhibitors and plasmids encoding dominant-negative proteins of endocytic pathways, the internalization was shown to be clathrin dependent. However, since complete inhibition of RSV F internalization was not achieved, other mechanisms might be involved or could be induced upon blocking a specific pathway (47). To exclude the latter possibility, combinations of inhibitors were tested but did not show any difference compared to treatment with a single inhibitor (data not shown) (48).

Additionally, it was shown that RSV F internalization is mainly triggered upon binding of antibodies and is not merely spontaneous internalization, a well-described feature of several paramyxoviruses (18, 19, 21, 23). The results of a biotin internalization assay showed internalization of RSV F proteins after Ab triggering. Only a weak signal was observed in the absence of antibodies to induce internalization, which might be a consequence of membrane turnover or residual noncleaved proteins. Interestingly, a

FIG 5 Legend (Continued)
(dynamin 2 [B], Eps15 [DIII] [D], and caveolin-1 [F]). For each DN protein, a control construct also was used (WT dynamin 2 [B], inactive Eps15 [D3Δ2] [D], and WT caveolin-1 [F]). After induction of internalization and staining of the cells, the amount of internalized vesicles was determined by fluorescence microscopy. Data represent the means (±SD) from three replicates. *, P < 0.05.
reduction in the molecular weight of the RSV F protein was observed upon internalization. A possible explanation is that upon internalization, an event occurs which cleaves the disulfide bridge between F1 and F2 subunits. This can be mediated by the presence of enzymes in the endolysosomal system that reduce disulfide bonds and would thus also cleave the F1-F2 bonds (49, 50). Since the MAb used in this experiment is specific for F1, this cleavage will result in detection of a lower-molecular-weight band on Western blotting, corresponding to the F1 subunit. In addition to binding, antibody-induced cross-linking is most likely needed, since we observed that RSV F protein-specific, monovalent Fab fragments were not efficient in inducing internalization. Furthermore, at the highest Ab concentration tested, internalization was induced less efficiently. This could indicate that with high antibody concentrations, cross-linking of RSVF proteins does not efficiently occur because every F protein is bound by a different antibody, and thereby cross-linking-induced internalization signals would be lost.

Previous work showed that internalization of surface-expressed viral glycoproteins can be influenced by interactions with other viral proteins expressed in infected cells. For the Suid herpesvirus I glycoproteins gB and gD, antibody-induced cross-linking was shown to be required for efficient internalization in infected monocytes (22). In contrast, viral core proteins of measles virus are known to regulate the expression of viral glycoproteins and may inhibit internalization during infection to promote virus assembly (18). In our work, internalization was observed in cells transfected with a single RSV

![FIG 7 Internalization of Fab fragments. RSV-infected HEp-2 cells were incubated with RSV F-specific MAbs D25, AM14, SC4, and MPE8 or corresponding monomeric Fab fragments at the same concentration for 90 min to induce internalization. Afterwards, the cells were fixed, permeabilized, and stained with AF488 human anti-goat IgG or AF488 chicken anti-mouse IgG (green). Nuclei were visualized with DAPI (blue). The amount of internalized vesicles was quantified in 50 positive cells. Results are shown as means (±SD) from three independent replicates. *, P < 0.05.](image)

| TABLE 1 Capacity of different RSV F-specific MAbs to induce internalization^a |
|---------------------------------|-------------------------------|------------------------------|
| RSV F-specific MAb             | % Reduction^b (±SD) | RSV F antigenic site | RSV F conformation^c | Reference |
| 131-2A (murine)                 | 31 ± 9                      | I                           | Post (∞ pre)        | 42        |
| 101F (murine)                   | 47 ± 15                     | IV                          | Pre and post        | 13        |
| Palivizumab                     | 40 ± 18                     | II                          | Pre and post        | 4         |
| Motavizumab                     | 34 ± 17                     | II                          | Pre and post        | 43        |
| AM22                            | 57 ± 2                      | Ø                           | Pre                | 14        |
| D25                             | 50 ± 16                     | Ø                           | Pre                | 14        |
| SC4 (murine)                    | 39 ± 14                     | Ø                           | Pre                | 14        |
| MPE8                            | 44 ± 9                      | III                         | Pre                | 15        |
| AM14                            | 42 ± 17                     | V                           | Pre                | 17        |

^aRSV-infected HEp-2 cells in suspension were incubated with the indicated anti-RSV F MAbs for 1 h at 4°C, followed by a shift to 37°C to induce internalization of the attached antibodies. After staining the cells with secondary antibodies, the mean fluorescence intensity was measured by flow cytometry.

^bThe surface expression reduction is expressed as a percentage relative to the level at T0 (100%). Data represents the mean of 3 independent repeats.

^cPre, prefusion conformation; post, postfusion conformation; ∞ pre, antibody recognizes the epitope mainly on the postfusion conformation and to a limited extent on the prefusion conformation.
protein as well as in RSV-infected cells, indicating that RSV F internalization occurs independently and is not affected by the expression of other viral proteins. How exactly RSV F activates internalization upon antibody binding is not clear. For some viruses, it was shown that internalization can depend on specific endocytic motifs in cytoplasmic and/or transmembrane domains of the viral protein, such as common tyr-based motifs and di-leu motifs (18, 29, 30, 51). For other viruses, it is less clear that internalization depends on specific motifs, and internalization may result from cross-linking. Analysis of the cytoplasmic tail of the RSV F protein did not reveal any known amino acid motifs involved in internalization. Furthermore, analysis of antibody-induced internalization using a mutant RSV F protein lacking the cytoplasmic tail only resulted in approximately 30% reduction of internalization (data not shown). This suggests that for RSV, specific amino acid motifs are not involved, and that cross-linking is a major driver for internalization, consistent with our finding that monovalent Fab fragments cannot efficiently induce internalization.

Several neutralizing RSV F-specific MAbs are described here (Table 1). For some MAbs, it is shown that neutralization results from blocking virus fusion with host cell membranes by fixing RSV F proteins in their prefusion conformation (14, 52). Interestingly, their neutralizing activity may be directed against not only virions but also virus-infected cells, since cell-to-cell fusion can be inhibited by MAbs like palivizumab and motavizumab (52). In this study, flow-cytometric analysis showed that all RSV F-specific MAbs tested had the ability to decrease surface expression of RSV F proteins, which may impact cell-to-cell fusion. While this internalization may not affect cell-to-cell fusion for MAbs that directly block the RSV F fusion activity, it can still affect cell-to-cell fusion indirectly for MAbs that do not interfere with the RSV F fusion activity. As previously shown, different levels of epitope recognition by MAbs could change the internalization pattern of the target antigen (41). However, we observed no differences between the different MAbs, all specific for one of the six known RSV F epitopes. These findings suggest that the antibody-induced internalization process of RSV F is not epitope dependent.

Reinfection with RSV can occur throughout life, indicating that RSV antibody responses only partially provide protection and only for a limited period of time (2, 53). Prophylactic palivizumab is able to prevent severe RSV-induced respiratory tract disease in most, but not all, patients. The cause of the partial failure of this immunoprophylaxis remains unknown but could be attributed to variations in the dose of inoculum, the efficiency of Ab transfer to the airways, and the size of the airways. The process we observed may also affect the activity of anti-F antibodies if the internalization strongly decreases the amount of RSV-specific antibodies. Presumably, this decrease will not be sufficient to impair the neutralization of free virus particles. Furthermore, the process we observed may also result in an antiviral effect, since internalization of the F protein could interfere with the formation of virus particles, and thus spreading of the virus would be restricted already early in infection. On the contrary, interference in effector-mediated destruction of virus-infected cells by this process is more likely. In HIV-1- and simian immunodeficiency virus-infected cells, internalization of Env proteins provided protection from elimination by antibody-dependent cell-mediated cytotoxicity. Based on these findings, it was suggested that the efficacy of antibody-based therapeutics and HIV-1 vaccines could be improved by disturbing Env internalization (54). For herpesviruses, a similar observation was made (25). Surface-expressed virus proteins were internalized by addition of specific antibodies, and antibody-dependent complement-mediated cell lysis was reduced by approximately 50% in infected cells upon antibody-induced internalization (25). Our results showed a remarkable decrease of surface-expressed F proteins after internalization, yet a portion remained on the cell surface. In this regard, further research is needed to elucidate whether there is sufficient internalization to protect RSV-infected cells from antibody-mediated effector responses.

In conclusion, this study describes a mechanism by which the RSV proteins expressed on the surface of infected cells are removed from the cell surface, together with RSV-specific antibodies, by internalization. Whether this process affects the activity of
RSV F-specific antibodies and also has consequences for the in vivo replication and immune response remains to be elucidated.

MATERIALS AND METHODS

Cells, virus, and antibodies. The human epidermoid carcinoma larynx cell line (HEp-2) and A549 cell line were obtained from the ATCC. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12K (Kainh's) medium, respectively, both supplemented with 10% inactivated fetal bovine serum (fBS) (Thermo Fisher Scientific). BSR T7/5 cells were a gift of K. K. Conzelmann (Max-von-Pettenhofer-Institut, Munich, Germany) and grown in Glasgow’s minimal essential medium (GMEM) supplemented with 10% fBS and 2% minimal essential amino acids (Thermo Fisher Scientific). Well-differentiated primary pediatric bronchial epithelial cell (WD-PBEC) culture was described previously (55). RSV reference strains A2 and B1 and clinical isolate A1998/3-2 were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI resources) and propagated in HEp-2 cells. Recombinant RSV encoding the far-red fluorescent protein monomeric Katushka-2 (mKate2) was recovered as described previously (56). Commercially available goat anti-RSV PAb (Virostat), mouse anti-RSV F IgG (clone 131-2A; Millipore), and mouse anti-RSV G IgG (clone 131-2G; Millipore) were used as reference antibodies. Human reference antiserum was obtained from BEI Resources (BEI NR-4020). A panel of RSV F-specific MAbs and their corresponding Fab fragments were provided by J. A. Melero, J. S. McLellan, and B. S. Graham. Secondary antibodies donkey anti-goat IgG, chicken anti-mouse IgG, and goat-anti human IgG, conjugated with Alexa Fluor (AF) 488 or 555 (obtained from Thermo Fisher Scientific), and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Dako) were used to visualize the antigens.

Construction and expression of recombinant RSV proteins. Synthesis of the RSV F and RSV G protein was performed by GenScript and delivered in pUC57, a commonly used plasmid for cloning. Restriction enzymes (New England BioLabs) were used to subclone the recombinant sequences in a mammalian expression vector, pBuDCE4.1 (Thermo Fisher Scientific). Transfection of the resulting plasmids was performed with Lipofectamine 2000 (Thermo Fisher Scientific) in Opti-MEM (Thermo Fisher Scientific) to obtain surface expression of recombinant RSV proteins. Briefly, BSR T7/5 cells were seeded on coverslips in 24-well plates to be confluent at the time of transfection. Plasmid DNA was mixed with Lipofectamine 2000 and incubated at room temperature for 20 min. The transfection complexes next were added to the cells and incubated for 2 h at room temperature. Further incubation at 37°C was performed after adding complete GMEM for 6 h. Finally, the complexes were removed, replaced by complete GMEM, and incubated overnight at 37°C.

Antibody-induced internalization assay. Cells were seeded on coverslips in 24-well plates to be subconfluent or confluent at the time of infection or transfection, respectively. RSV infection was performed by diluting the virus stock in basal growth medium and subsequently adding the virus suspension to the cells. After 2 h of incubation at 37°C, the inoculum was removed, replaced by complete growth medium, and further incubated at 37°C. Transfections were performed as described above. After 24 h of incubation, RSV-infected or transfected cells were incubated with antibodies against RSV for 1 h at 4°C to allow only attachment of the antibodies. To remove unbound antibody, cells were washed three times with growth medium, followed by incubation at 37°C to start the internalization process. After different time points, cells were fixed with 4% paraformaldehyde (Merck) and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich). To visualize antigen-antibody complexes, cells were stained with appropriate secondary antibodies conjugated with AF 488, AF 555, or FITC. As a control, cells were fixed after the 4°C incubation (T0).

Flow-cytometric analysis of RSV internalization. Surface expression after internalization was quantified by flow cytometry. HEp-2 and A549 cells were seeded in 6-well plates to be subconfluent after 24 h of incubation at 37°C or kept in suspension in precoated glass vials (Sigmacote) for 24 h. Infection of the cells was performed as described above. After 24 h, infected cells in 6-well plates were detached by incubation with 1 mM EDTA (Sigma) during 30 min at 4°C and pelleted by centrifugation (210 g, 10 min, 4°C). The pellet was resuspended in RPMI supplemented with 10% fBS and then incubated with primary antibodies for 1 h at 4°C and washed to remove unbound antibodies. Cells were kept at 4°C as time point 0. The other sample cells were then shifted to 37°C by the addition of warm RPMI medium and further incubated at 37°C for 90 min. To stop internalization, cells were shifted to 4°C for 15 min. The cells then were incubated with AF 488-conjugated secondary antibodies for 1 h at 4°C, washed with PBS, and analyzed by flow cytometry with a FACSCalibur. Dead cells were excluded by staining the cells with LIVE/DEAD fixable far-red dead cell stain (Thermo Fisher Scientific). Forward-scattered light (FSC), side-scattered light, and the AF 488 (FL-1) and far-red fluorescence (FL-4) signals were stored for further analysis. Mean fluorescence intensity (MFI) was calculated from three independent repeats. The reduction in surface expression was calculated with the following equation: 100 – (MFImin – MFIbackground)/ (MFI0 – MFIbackground) × 100, where MFIbackground is the mean fluorescence signal of stained noninfected cells.

Spontaneous endocytosis assay. Infection of subconfluent HEp-2 cell cultures was performed as described above. Surface proteins of the infected cells were biotinylated using EZ-link Sulfo-NHS-SS-biotin (Thermo Fisher Scientific) at 4°C. Cells then were incubated at 37°C to allow endocytosis to occur. As a control of antibody-induced internalization, one sample was incubated with RSV-specific antibodies. Biotin was removed from proteins by the addition of cleavage buffer (60 mM i-glutathione, 75 mM NaCl, 10 mM EDTA, pH 7.5) for 30 min at 4°C. One sample was neither incubated nor reduced with cleavage buffer to determine the total amount of biotinylated proteins. The efficiency of biotin removal by cleavage buffer was determined by cleavage of a nonincubated sample. After lysis of the cells with...
radioimmunoprecipitation assay (RIPA) lysis buffer (Millipore), biotinylated proteins were immunoprecipitated using streptavidin Mag Sepharose (Sigma) and then SDS-PAGE under nonreducing conditions. The presence of RSV F proteins was detected by transferring the gel to a polyvinylidene difluoride (PVDF) membrane and subsequent incubation with RSV F-specific MAb.

**Internalization inhibition assays.** To inhibit antibody-induced internalization, two strategies were carried out as previously reported (57). First, inhibitors were used in different concentrations before and during the internalization assay. Amantadine and nystatin, purchased from Sigma, and myristoylated dynamin inhibitory peptide (DIP), purchased from Tocris Bioscience, were diluted in their solvent prior to dilution in cell culture medium. After 1 h of pretreatment of the cells with the inhibitors at 37°C, fresh inhibitor was added to the cells together with antibodies to induce internalization. As a control for each drug, cells were incubated with the corresponding solvent of the inhibitor. Controls were applied to confirm the effectiveness of the drugs, including biotinylated transferrin for clathrin-mediated endocytosis and FITC-labeled bovine serum albumin (BSA) for caveola-mediated endocytosis. The biotinylated transferrin was visualized with streptavidin-FITC. Second, inhibition of internalization was performed using plasmids encoding dominant-negative (DN) proteins that inhibit clathrin-, caveola-, or dynamin-mediated endocytosis. These constructs and their WT counterparts were originally provided by A. Benmerah (58, 59), A. Helenius (60, 61), and M. McNiven (62, 63), respectively, and further modified as described before (64). Cotransfection of BSR T7/5 cells was performed by diluting equal amounts of DNA of the RSV F protein and the DN or WT protein. WT and DN dynamin 2(aa) with a C-terminal enhanced GFP (eGFP) tag were used to inhibit dynamin-dependent endocytosis. To inhibit clathrin-mediated endocytosis, the DN mutant of the protein Eps15 (DIII), essential for the docking of adaptor protein-2 during assembly of clathrin-coated pits, was used (64). This EGFPlabeled mutant has a deletion at the Eps15 homology and coiled-coil domains. A construct with a supplementary deletion of the AP-2-binding site served as a negative control (D3Δ2). WT and DN caveolin-1 constructs were used for caveola-mediated endocytosis.

**Microscopic analysis.** All high-resolution images of monolayer cultures were obtained using ApoTome 2 with an Axio Observer inverted microscope with an HXP 120C compact light source (Zeiss) and using a Nikon Eclipse Ti-E inverted microscope attached to a micromels-enhanced dual-spinning-disc confocal system (UltraVIEW VoX; PerkinElmer, Zaventem, Belgium) equipped with 405- and 488-nm diode lasers for excitation of blue and green fluorophores, respectively. The images were obtained with ZEN 2012 and Velocity 3D Image Analysis Software. WD-PBEC images were obtained with a Nikon eclipse 90i inverted microscope.

**Statistical analysis.** Data are presented as means (± standard deviations [SD]) from three independent repeats and were analyzed by a Student’s t test using GraphPad Prism 6. P values of <0.05 were considered statistically significant.

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