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Genetic delivery of an anti-RSV antibody to protect against pulmonary infection with RSV

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ABSTRACT

Respiratory syncytial virus (RSV) is a common cause of severe lower respiratory tract infections. Protection against infection with RSV can be achieved by monthly administration of the humanized monoclonal antibody palivizumab. The present study analyzes if genetic delivery of a murine version of palivizumab by single administration would achieve high-level and sustained antibody expression to protect mice against pulmonary infection with RSV. A murine version of the palivizumab antibody was constructed by replacing the human sequences with sequences from the constant region of a murine IgG1 antibody, while preserving the complementarity-determining region. As a proof-of-principle to test the validity of the strategy, the coding sequence for the heavy and light chains were cloned into a replication-defective serotype 5 human adenovirus vector (Ad α RSV). Antibody expression and specificity for RSV was confirmed by Western analysis. To determine if Ad α RSV would mediate production of anti-RSV antibodies *in vivo*, 5×10^{10} particle units of Ad α RSV or a control vector without transgene (AdNull), were administered intravenously to BALB/c mice. RSV neutralizing antibodies were detected in the serum after 4 days in mice receiving Ad α RSV but not in AdNull-infected or naive mice ($p < 0.05$). The mice that had received Ad α RSV had at least 5.4-fold lower RSV titers in the lung 4 days following intranasal challenge with RSV compared to the AdNull or naive group ($p < 0.01$). To evaluate long-term protection, the antibody construct was expressed in a non-human primate serotype rh.10 adeno-associated virus vector (AAVrh.10 α RSV). RSV neutralizing antibodies were detected in serum and bronchoalveolar lavage fluid for up to 21 wk following intrapleural administration of AAVrh.10 α RSV, but not with a control AAV vector expressing an unrelated transgene (AAVrh.10 α 1AT). Following challenge with RSV at 7 or 21 wk, 14.3-fold and 10.6-fold lower RSV titers were observed after 4 days in the lungs of mice that had received AAVrh.10 α RSV compared to AAVrh.10 α 1AT ($p < 0.05$). Together these data demonstrate that a gene transfer strategy for delivery of an anti-RSV antibody can generate protective immunity in mice against RSV infection in the respiratory tract and may provide an alternative to the administration of the antibody itself.

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Introduction

Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract infections in high-risk populations, such as infants, young children, the elderly and patients with chronic lung and heart disease or immunodeficiency (Hall, 2001; Smyth and Openshaw, 2006). Early severe infections with RSV have been associated with the development of recurrent wheezing or asthma (Perez-Yarza et al., 2007; Smyth and Openshaw, 2006). Treatment for RSV infection is limited to supportive care, often requiring prolonged mechanical ventilation (Hall, 2001; Smyth and Openshaw, 2006). As a licensed active vaccine for RSV is not available (Bennett et al., 2007; Polack and

Karron, 2004), the only preventive strategy to date is passive immunization with a monoclonal antibody or RSV immunoglobulin (Groothuis and Nishida, 2002; Mejias et al., 2005; The IMPact-RSV Study Group, 1998; Wu et al., 2008). Intramuscular administration of palivizumab, a recombinant humanized murine IgG1 antibody against a neutralizing epitope in the F-glycoprotein of RSV, to high-risk populations during the RSV peak season in the fall and winter has decreased RSV-related hospitalizations and morbidity (The IMPact-RSV Study Group, 1998). The antibody needs to be administered monthly and, due to its cost, is only available to high-risk populations (Groothuis et al., 1993; Nuijten et al., 2007).

Genetic delivery of antibodies using gene transfer vectors is a novel, attractive experimental strategy to achieve prolonged expression of a therapeutic antibody. A variety of viral vector systems have been used to express antibody molecules, including two of the most commonly used gene transfer vector systems, adenovirus (Ad) and adeno-associated virus (AAV) (BenAmmar-Cecoli et al., 2001; Fang et al., 2005, 2007;

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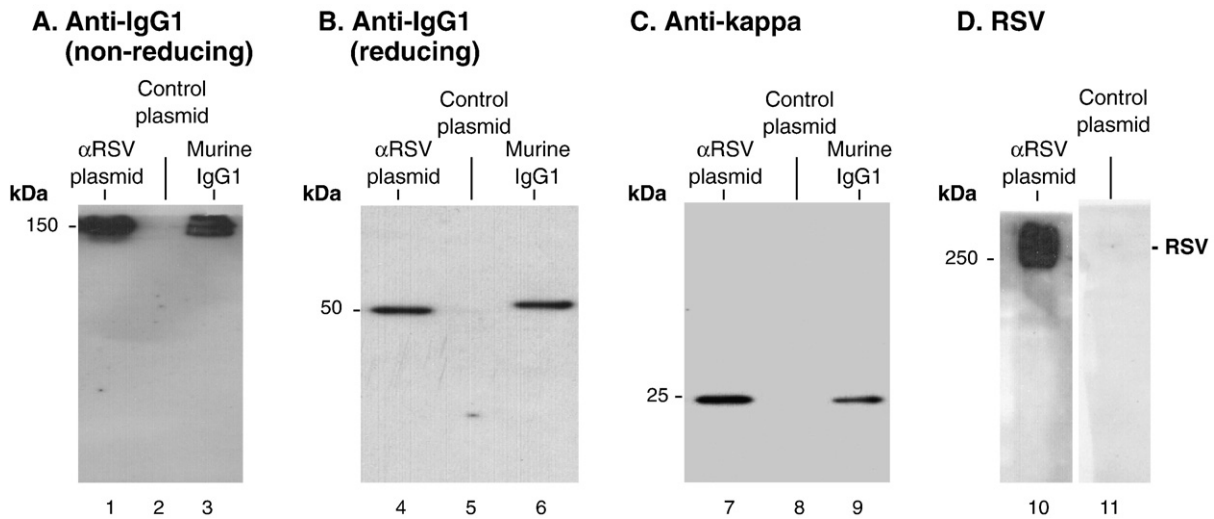


Fig. 1. Expression of a full length anti-RSV mouse monoclonal antibody *in vitro*. Supernatant of 293 cells transfected with the pShuttleCMV α RSV or a pShuttleCMV control plasmid were collected at 48 h and analyzed by Western analysis. A. Expression of the IgG1 heavy chain using an anti-mouse IgG1 antibody under non-denaturing conditions in cell supernatants transfected with pShuttleCMV α RSV (lane 1) or pShuttleCMV control plasmid (lane 2). Murine IgG1 control (lane 3). B. Expression of the IgG1 heavy chain using an anti-mouse IgG1 antibody under denaturing conditions in cell supernatants transfected with pShuttleCMV α RSV (lane 4) or pShuttleCMV control plasmid (lane 5). Murine IgG1 control (lane 6). C. Expression of the light chain using an anti-kappa antibody under denaturing conditions in cell supernatants transfected with pShuttleCMV α RSV (lane 7) or pShuttleCMV control plasmid (lane 8). Murine IgG1 control (lane 9). D. Binding to RSV. Purified RSV was resolved in a 4 to 12% native polyacrylamide gel and transferred onto a PVDF membrane. The blot was developed with the supernatant of cells transfected with pShuttleCMV α RSV (lane 10), pShuttleCMV control plasmid (lane 11) followed by anti-mouse IgG-HRP.

Koch et al., 2003; Lewis et al., 2002; Liang et al., 1997; Morimoto et al., 2001; Noel et al., 2002; Prosnjak et al., 2003; Jiang et al., 2006). Ad vectors generally achieve high levels of transgene expression with limited duration due to the clearance by the immune system and AAV vectors generally provide long-term expression with less immunological responses (Hackett et al., 2000). For the present study, we hypothesized that genetic delivery of a murine equivalent of palivizumab would result in sufficient expression of the anti-RSV antibodies to protect against pulmonary RSV infection in a murine model. Ad group C serotype 5 (Ad α RSV) and AAV rhesus macaque serotype rh.10 (AAVrh.10 α RSV) were designed to deliver the coding sequences for the heavy and light chains of the murine equivalent of palivizumab.

The data demonstrates that systemic administration of Ad α RSV results in efficient short-term expression of anti-RSV neutralizing antibody activity to protect mice against an intranasal challenge with RSV, and that intrapleural administration of AAVrh.10 α RSV results in protective anti-RSV neutralizing antibody activity in serum and lung epithelial fluid for up to 23 wk. These observations suggest that genetic delivery of an anti-RSV antibody can result in long-term anti-RSV protective immunity and that this strategy might be useful to achieve long-term passive immunization against other pulmonary pathogens.

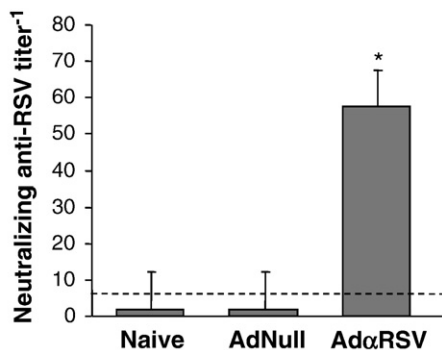


Fig. 2. RSV antibodies in serum following intravenous administration of Ad α RSV. Ad α RSV or AdNull (2×10^{10} pu) were administered intravenously to BALB/c mice ($n=5$). Serum was collected after 3 days and analyzed for anti-RSV neutralizing antibodies. Data is presented as mean \pm SEM. * denotes $p < 0.05$ compared to naive and AdNull group. The dashed line indicates the level of detection.

Results

Expression of anti-RSV antibody *in vitro*

To evaluate the expression of the anti-RSV antibody, 293 cells were transfected with the pShuttleCMV α RSV or a pShuttleCMV control plasmid. Cell supernatants were collected after 72 h and the expression of the antibody was assessed by Western analysis (Fig. 1). Under non-reducing conditions, a complex of 150 kDa corresponding to the size of the completely assembled antibody was detected in the cells transfected with pShuttleCMV α RSV (Fig. 1A, lane 1) but not with the control plasmid (lane 2). Under reducing conditions, individual heavy (50 kDa; Fig. 1B) and light (25 kDa, Fig. 1C) chains were detected in the cells transfected with the pShuttleCMV α RSV (lane 4, Fig. 1B and lane 7, Fig. 1C) but not with the control plasmid (lane 5, Fig. 1B and lane 8, Fig. 1C). Binding to RSV was detected with supernatants from cells transfected with pShuttleCMV α RSV (lane 10, Fig. 1D) but not with the control plasmid (lane 11).

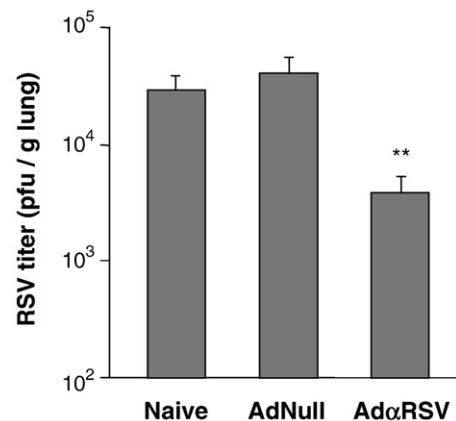


Fig. 3. Protection against RSV lung infection following Ad α RSV. Ad α RSV or AdNull (2×10^{10} pu) were administered intravenously to BALB/c mice ($n=5$). Seven days later, mice were challenged with RSV (10^6 pfu) by intranasal administration. Lungs were homogenized 4 days following challenge and the number of RSV PFU counts determined by lung plaque assay. Data is presented as mean \pm SEM of PFU counts per gram of lung. **denotes $p < 0.001$ compared to naive and AdNull groups.

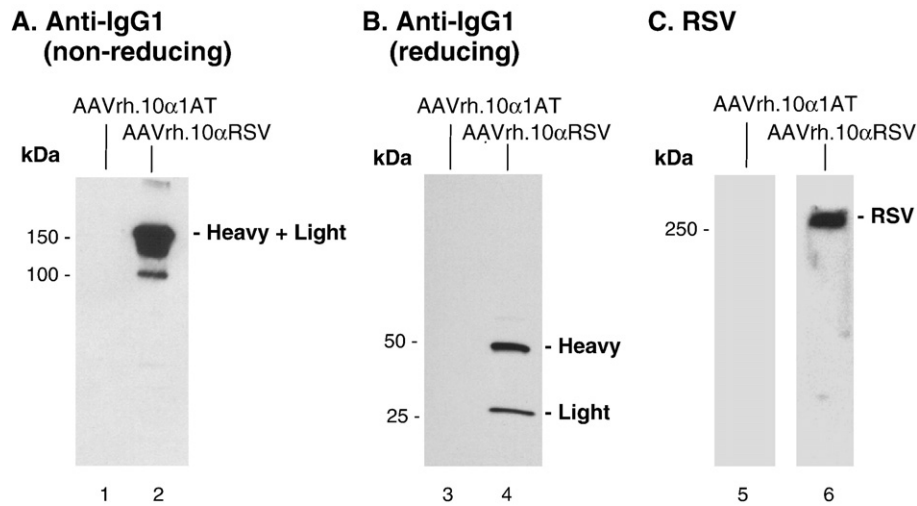


Fig. 4. Expression of a full length anti-RSV mouse monoclonal antibody in cells infected with AAVrh.10 α RSV. 293orf6 cells were infected with 10^4 genome copies per cell of AAVrh.10 α RSV or AAVrh.10 α 1AT as a negative control. Anti-RSV antibody expression was analyzed 3 days following infection in the cell supernatants by Western analysis. A. Expression of the IgG1 antibody using an anti-mouse IgG1 antibody under non-denaturing conditions in cell supernatants infected with AAVrh.10 α RSV (lane 1) or AAVrh.10 α 1AT (lane 2). B. Expression of the IgG1 antibody using an anti-mouse IgG1 antibody under denaturing conditions in supernatants of cells infected with AAVrh.10 α RSV (lane 3) or AAVrh.10 α 1AT (lane 4). C. Binding to RSV. Purified RSV was resolved in a 4–12% native polyacrylamide gel and transferred onto a PVDF membrane. The blot was developed with supernatant from cells infected with AAVrh.10 α RSV (lane 5) or AAVrh.10 α 1AT (lane 6) followed by anti-mouse IgG-HRP.

Systemic administration of Ad α RSV

To determine if Ad α RSV would direct expression of anti-RSV antibodies *in vivo*, 5×10^{10} particle units of Ad α RSV or the control AdNull were administered intravenously to BALB/c mice. Neutralizing RSV titers were elevated in the Ad α RSV infected but not in AdNull-infected or naive mice (Fig. 2; $p < 0.05$, both comparisons).

To assess protection against lung infection with RSV by Ad α RSV, Ad α RSV or AdNull were administered intravenously and the mice were challenged with RSV 7 days later by intranasal administration of RSV. The mice that received Ad α RSV showed more than 5.4-fold decreased RSV titers in the lung compared to the AdNull or naive group ($p < 0.01$, both comparisons; Fig. 3). Together, this indicates that systemic administration of Ad α RSV generates sufficient RSV-neutralizing antibodies after 7 days to protect the mice against a pulmonary infection with RSV.

Expression of RSV antibody from AAVrh.10 α RSV *in vitro*

To achieve long-term expression, the antibody construct was expressed from a serotype rh.10 AAV vector. To evaluate expression

of the anti-RSV antibody by the AAVrh.10 α RSV vector *in vitro*, 293 cells were infected with either AAVrh.10 α RSV or a control vector AAVrh.10 α 1AT, and binding of the anti-RSV antibody in the supernatant was evaluated by Western analysis (Fig. 4). Under non-reducing conditions, a complex of 150 kDa corresponding to the size of the completely assembled antibody was detected in AAVrh.10 α RSV-infected cells (lane 2, Fig. 4A) but not in AAVrh.10 α 1AT-infected cells (lane 1). Under non-reducing conditions, heavy (50 kDa) and light (25 kDa) chains were detected in AAVrh.10 α RSV-infected cells (lane 4, Fig. 4B) but not in AAVrh.10 α 1AT-infected cells (lane 3). Binding to RSV was detected with supernatants from AAVrh.10 α RSV-infected cells (lane 6, Fig. 4C) but not with supernatants from AAVrh.10 α 1AT-infected cells (lane 5).

Intraleural administration of AAVrh.10 α RSV

To determine if AAVrh.10 α RSV would mediate production of anti-RSV antibodies *in vivo*, 10^{11} genome copies of AAVrh.10 α RSV or the control vector AAVrh.10 α 1AT were administered intrapleurally, a route that had previously resulted in high levels of systemic transgene expression with an AAVrh.10 vector (De et al., 2004). Mice that had

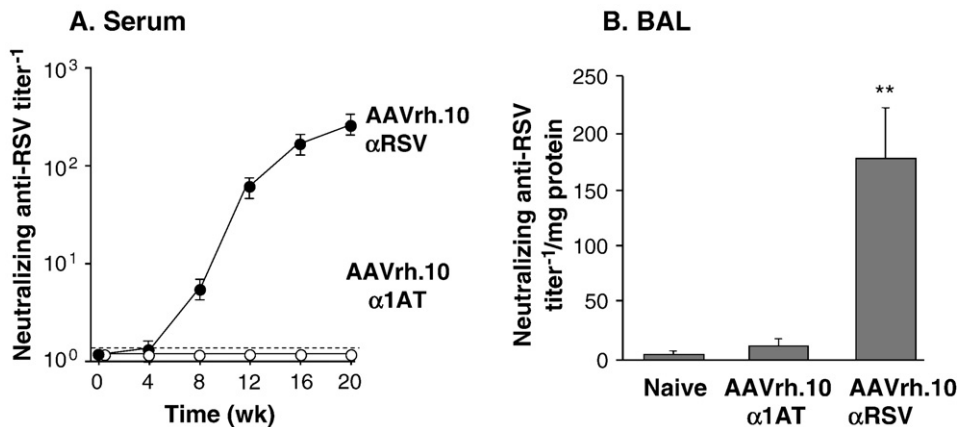


Fig. 5. Serum and lung RSV neutralizing antibodies following intrapleurally administration of AAVrh.10 α RSV. AAVrh.10 α RSV or AAVrh.10 α 1-AT (10^{11} gc) were administered intrapleurally to BALB/c mice ($n = 5$ /group). RSV neutralizing antibodies were determined in serum at 0, 4, 8, 12, 16 and 20 wk and in bronchoalveolar lavage fluid (BAL) at 23 wk following vector administration by neutralizing antibody assay. The sera or BAL of naive mice and mice immunized with AAVrh.10 α 1AT were used as negative controls. A. RSV neutralizing antibodies in serum. B. RSV neutralizing antibodies in BAL. Data is presented as mean \pm SEM. **denotes $p < 0.01$ compared to naive and AAVrh.10 α 1AT group.

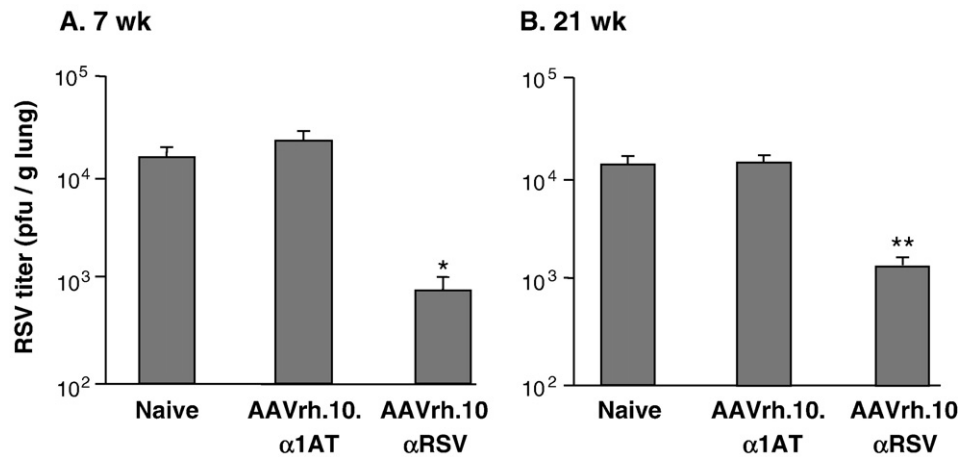


Fig. 6. Protection against RSV lung infection following AAVrh.10 α RSV administration. AAVrh.10 α RSV or AAVrh.10 α 1-AT (10^{11} gc) were administered intrapleurally to female BALB/c mice ($n=5$ /group). Seven and 21 wk later the mice were challenged with RSV (10^6 pfu) by intranasal administration. Lungs were homogenized 4 days following challenge and the number of RSV PFU counts determined by lung plaque assay. A. 7 wk. B. 21 wk. Shown is mean \pm SEM of PFU counts per gram of lung. *denotes $p < 0.05$ and **denotes $p < 0.001$ compared to naive and AAVrh.10 α 1AT group.

received AAVrh.10 α RSV had increased serum RSV neutralizing antibody titers as early as 8 wk post-administration for up to 20 wk (Fig. 5A). No anti-RSV antibodies were detected in the serum of mice that received the control vector or in the serum of naive mice. Interestingly, RSV neutralizing antibodies were elevated in BAL after 21 wk in the mice that had received AAVrh.10 α RSV compared AAVrh.10 α 1AT or naive mice ($p < 0.05$; Fig. 5B).

To assess protection against RSV by AAVrh.10 α RSV, AAVrh.10 α RSV or AAVrh.10 α 1AT were administered intrapleurally and the mice were challenged with RSV at 7 and 21 wk post-administration. Mice that had received AAVrh.10 α RSV showed 14.3-fold and 10.6-fold lower numbers of RSV pfu in the lung compared to the AAVrh.10 α 1AT and naive groups at 7 and 21 wk ($p < 0.05$, both comparisons; Fig. 6). These results indicate that protection against RSV can be sustained at least until 21 wk.

Discussion

Palivizumab is the only monoclonal antibody that has been licensed and widely used for an infectious disease (Casadevall et al., 2004). The present study investigates if genetic delivery of a murine form of palivizumab could be used as an alternative mode of delivery of a purified anti-RSV antibody preparation to provide protection against RSV. The data demonstrates that systemic administration of Ad α RSV results in efficient short-term expression of anti-RSV neutralizing antibody activity to protect mice against a pulmonary infection with RSV, and that intrapleural administration of AAVrh.10 α RSV results in protective anti-RSV neutralizing antibody activity in serum and bronchoalveolar lavage fluid for up to 21 wk. This suggests that genetic delivery of an anti-RSV antibody can result in long-term anti-RSV protective immunity and that this strategy might be useful to achieve long-term passive immunization against other pulmonary pathogens.

Antibody therapy against RSV

Passive antibody therapy has been successfully used against many microorganisms (Casadevall et al., 2004; Buchwald and Pirofski, 2003). Monoclonal antibodies have been developed for therapeutic use, but interestingly, RSV is the only infectious agent for which a monoclonal antibody has been licensed, now widely used for almost 10 yr (Groothuis et al., 1993; The Impact-RSV Study Group, 1998). RSV is the leading cause of severe lower respiratory tract infections in infants and children (Hall, 2001; Smyth and Openshaw, 2006). A licensed active vaccine is not available and efforts have been challenged by the failure more than 30 yr ago of a formalin-inactivated vaccine that was associated with exaggerated Th2 responses and respiratory disease on

subsequent infection with RSV following vaccination (van Drunen Littel- van den Hurk et al., 2007). There is no effective therapy and passive immunization with either RSV hyperimmune globulin or the monoclonal antibody palivizumab, and its newer version motavizumab (Wu et al., 2007), have been effective in diminishing disease severity in the high-risk populations (Mejias et al., 2005; Wu et al., 2007; Groothuis and Nishida, 2002; Groothuis et al., 1993; The Impact-RSV Study Group, 1998). Palivizumab is an IgG1 humanized monoclonal antibody against a neutralizing epitope in the A-antigenic site of the F-glycoprotein of RSV (Johnson et al., 1997). Due to its high cost, the antibody is only regularly provided to high-risk groups of premature infants and needs to be administered monthly (Groothuis and Nishida, 2002; Nuijten et al., 2007). An ideal passive immunization strategy would be a single administration with protective immunity lasting through the vulnerable period or at least throughout the RSV season. In the present study, we constructed a genetic murine equivalent of palivizumab by replacing the human sequences of the constant region with the sequence from the constant region of a murine IgG1 antibody while preserving the complementarity-determining region to generate an antibody that could be tested in a murine model without inducing immunity against the human portion of the antibody.

Genetic delivery of therapeutic antibodies

Genetic transfer of therapeutic antibodies is an attractive strategy to achieve long-term persistence of the antibody. Antibody molecules have been expressed using a variety of viral vector systems, including adenovirus, AAV, vaccinia virus, baculovirus and rhabdovirus (BenAmmar-Cecoli et al., 2001; Fang et al., 2005, 2007; Koch et al., 2003; Lewis et al., 2002; Liang et al., 1997; Morimoto et al., 2001; Noel et al., 2002; Prosnjak et al., 2003; Jiang et al., 2006). In the present study, a full length antibody that bound and neutralized RSV was expressed in two viral vector systems: an Ad vector, where the light and heavy chain genes were separated using a poliovirus IRES, and in an AAV serotype rh.10 vector, with the heavy and light chains separated by a furin cleavage site and the self-cleaving peptide from the foot-and-mouth disease virus. These platforms have been previously shown to efficiently express a full length antibody against protective antigen (PA) of *Bacillus anthracis* delivered by an Ad vector (De et al., 2007). In general, long-term persistence of antibodies expressed by Ad vectors is impaired by the immune response against the Ad vector, although expression for up to 4 wk has been demonstrated for an anti-HER-2 monoclonal antibody (Jiang et al., 2006) and 3 months following delivery of an anti-thyroglobulin antibody (Noel et al., 2002). Transgene expression following Ad5-mediated gene transfer to

BALB/c mice is usually limited to a few weeks. Sustained levels of antibodies following genetic delivery can be achieved using AAV vectors. For AAV vectors prolonged expression of an antibody for at least 6 months has been demonstrated with an AAVrh.10 serotype vector expressing an antibody against PA of *B. anthracis* (De et al., 2007), with an AAV2 vector for the HIV monoclonal antibody anti-gp120 (Lewis et al., 2002), and with an AAV8 vector for an anti-vascular endothelial growth factor receptor 2 antibody (Fang et al., 2005; Lewis et al., 2002). In the present study, sustained levels of the anti-RSV antibody in serum and bronchoalveolar lavage fluid were seen for at least 3 months with no difference in the protective effect against an RSV pulmonary infection between mice challenged after 7 or 21 wk. The reduction in RSV titers following challenge with RSV, although significant, was only moderate in magnitude. Future studies will need to assess antibody constructs with increased affinity such as motavizumab. In this study, to circumvent possible interference of the human parts of palivizumab, the human sequences were replaced by murine sequences. Future studies of genetic delivery of humanized antibodies should include an analysis of the immune recognition of the humanized antibody (which has been shown to work efficiently in mice) a theoretical concern if antibodies are being expressed over a prolonged period using AAV vector systems.

In summary, genetic delivery of an anti-RSV antibody is feasible to achieve long-term protection against pulmonary infection with RSV and could provide an alternative passive immunization strategy for this and other pulmonary infectious diseases.

Methods

Murine anti-RSV antibody construct

A murine form of an anti-RSV antibody (α RSV) was constructed synthetically based on the sequence of palivizumab (Johnson et al., 1997). The complementarity-determining region was preserved from the palivizumab sequence (Beeler and van Wyke Coelingh, 1989) (US patent 5,824,307) and the human sequences of the remaining parts of the antibody were replaced with the sequence from the constant region of a murine IgG1. Overlapping oligonucleotides were chosen with the DNA Builder software (http://ecce.pnl.gov/help/child/dna_toolkit). The light chain design consisted of 14 seventy-mers, overlapping by ~20 bases each, assembled by 2 rounds of PCR amplification. The heavy chain design included 28 seventy-mers, overlapping by ~20 bases, assembled by 3 rounds of PCR amplification. The light and heavy chain genes were separately cloned into TOPO-expression vectors and sequenced. To facilitate bicistronic expression of heavy and light chains from a single vector, the light and heavy chain coding sequences were then both cloned into the pShuttleCMV plasmid, separated by the poliovirus internal ribosome entry site (IRES). Accurate orientation was confirmed by restriction analysis and sequencing.

Ad vectors

The anti-RSV antibody construct was cloned into a first generation E1-E3- Ad vector, encoding the anti-RSV light chain and heavy chain cDNAs separated by a poliovirus internal ribosome entry site (IRES). The expression cassette in the Ad α RSV vector contains (5' to 3') the cytomegalovirus promoter/enhancer followed by cDNAs encoding the anti-RSV light chain, the polio virus internal ribosomal entry site (IRES), the anti-RSV heavy chain, and the SV40 polyadenylation signal. AdNull, an Ad5 vector containing no transgene in the expression cassette, was used as a negative control (Hersh et al., 1995). The vectors were produced in 293 cells and purified by centrifugation twice through CsCl gradient as previously described (Rosenfeld et al., 1992), and the particle units (pu) determined spectrophotometrically (Mittereder et al., 1996).

AAV vectors

AAVrh.10 was chosen based on previous studies demonstrating that intrapleural administration of a vector with AAVrh.10 capsid and AAV2 internal terminal repeats (ITR) yielded the highest serum levels of α 1-antitrypsin (De et al., 2004) and anthrax anti-protective antigen antibodies (De et al., 2007). AAVrh.10 α RSV encodes the α RSV antibody heavy and light chains, separated by furin and self-cleaving 2A protease sites for expression of the CMV promoter. The AAVrh.10 α RSV expression cassette contains (5' to 3') the cytomegalovirus promoter/enhancer followed by cDNAs encoding the α RSV heavy chain, a 13 amino acid myc tag, a 4 amino acid furin cleavage site, the 24 amino acid self-cleaving 2A peptide from foot-and-mouth disease virus (De et al., 2006; Fang et al., 2005), the α RSV light chain and the human growth hormone polyadenylation signal. All recombinant AAVrh.10 vectors were produced using a three plasmid system: (1) an expression plasmid (pAAV α RSV for the vectors expressing α RSV antibody or pAAV α 1AT for vectors expressing α 1AT); (2) pAAV44.2, an AAVrh.10 packaging plasmid that provides the AAV Rep proteins (derived from AAV) needed for vector replication and the viral structural (*cap*) proteins VP1, 2, and 3 derived from AAVrh.10, which determines the serotype of the AAV vector; and (3) pAdDeltaF6, an Ad helper plasmid that provides the Ad helper functions of E2, E4 and VA RNA (Gao et al., 2004; Rabinowitz et al., 2002; Xiao et al., 1998). For AAVrh.10 vector production, the expression plasmid pAAV α RSV or the α 1AT expression plasmid pAAV α 1AT, the AAVrh.10 packaging plasmid pAAV44.2, and helper plasmid pAdDeltaF6 were cotransfected in 293 cells. The AAVrh.10-based vectors were purified, concentrated and the vector genome titers determined by Taq-Man real-time PCR as previously described (De et al., 2006).

RSV

RSV strain A2 (VR-1540, ATCC, Manassas, VA) was grown and quantified on Hep-2 cells and was prepared as described. Hep-2 cells were maintained in Eagle-modified minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Expression of anti-RSV antibodies *in vitro*

Expression of the anti-RSV antibody from Ad α RSV or AAVrh.10 α RSV following infection of cells *in vitro* was evaluated by Western analysis. A549 cells were transfected with the pShuttleCMV α RSV or a pShuttleCMV control plasmid and supernatants were harvested after 48 h. 293 orf6 cells were infected with AAVrh.10 α RSV and supernatants were harvested after 72 h. All supernatants were resolved by SDS-polyacrylamide gel electrophoresis under non-reducing or reducing conditions followed by Western analysis using a horseradish peroxidase conjugated goat anti-mouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA) for detection of full length antibody and the heavy chain or a goat anti-mouse kappa antibody (Santa Cruz Biotechnology) for the light chain followed by ECL reagent (Amersham, Piscataway, NJ). Binding of the antibodies to RSV was evaluated by separating RSV [1.2×10^7 plaque-forming units (PFU)] in a 4–12% native polyacrylamide gel and Western analysis. The analysis was carried out with antibody-containing cell supernatant followed by anti-mouse IgG-HRP and ECL reagent.

Administration of Ad and AAV vectors to mice

Ad α RSV or AdNull (2×10^{10} particle units) were administered via the tail vein to BALB/c mice. AAVrh.10 α RSV or AAVrh.10 α 1AT (10^{11} genome copies), were delivered into the pleural space of the left lung of 6 wk old female BALB/c mice. The AAV vectors were administered in 50 μ l following a thoracotomy in the third intercostal space through the exposed pleural space using a pipette (De et al., 2004). The dose of the AAV vector was chosen based on efficient gene transfer to the pleura of

α 1-antitrypsin (De et al., 2004) and an anthrax anti-protective antigen antibody (De et al., 2007).

Assessment of RSV neutralizing antibodies in vivo

To evaluate anti-RSV neutralizing antibody titers in serum and bronchoalveolar lavage following intravenous administration of Ad α RSV or AdNull (2×10^{10} particle units), or intrapleural administration of AAVrh.10 α RSV or AAVrh.10 α 1AT (10^{11} genome copies), the vectors were administered intravenously (Ad vectors) or to the left pleural cavity (AAV vectors) of 6 wk old female BALB/c mice. Naive mice that had received PBS either intravenously or intrapleurally served as additional controls. Sera from the Ad-immunized mice were collected at 3 days post-administration and sera from AAVrh.10-immunized mice were collected at 2, 4, 8, 12, 16 and 21 wk following vector administration. Bronchoalveolar lavage (BAL) was performed by three intratracheal instillations and removal of 1 ml PBS, pH 7.4 at 23 wk following vector administration. BAL fluid was centrifuged at 6000 rpm at 4 °C, 20 min. Serial dilutions of sera and BAL fluid in infection medium (MEM supplemented with 1% penicillin/streptomycin) were incubated with RSV (strain A2) for 1 h at 37 °C and then incubated on Hep2 cells for 90 min at 37 °C. The medium was then changed to 1% methylcellulose medium (with MEM, 5% fetal bovine serum, 1% penicillin/streptomycin). Palivizumab (Synagis, 1 μ g/ml; Medimmune, Gaithersburg, MD) was used as a positive control for the neutralizing antibody assay. After 4 days, the cells were fixed with 4% paraformaldehyde for 10 min, stained with 1% crystal violet and the plaques counted under a microscope.

Protection of mice from intranasal challenge with RSV

To evaluate protection against RSV infection of the respiratory tract following administration of Ad α RSV or AAVrh.10 α RSV, BALB/c mice received either Ad α RSV or AdNull (2×10^{10} pu) intravenously or AAVrh.10 α RSV or AAVrh.10 α 1AT (10^{11} gc) intrapleurally. The mice that had received the Ad vectors were challenged with RSV 7 days following vector administration; the mice that had received the AAVrh.10 vectors were challenged at 7 and 21 wk following vector administration. Following anaesthesia with isoflurane the mice were intranasally inoculated with RSV (10^6 pfu). Four days later the mice were sacrificed, the lungs homogenized in 1 ml MEM (supplemented with 1% penicillin/streptomycin) and the homogenate centrifuged at 1200 rpm at 4 °C for 10 min. Ten-fold serial dilutions of the lung homogenate supernatant were incubated in infection medium on Hep2 cells for 3 h at 37 °C. The medium was then replaced with 1% methylcellulose medium (with MEM, 5% fetal bovine serum, 1% penicillin/streptomycin) and incubated for 4 days. The cells were fixed and the number of PFU's quantified as outlined above.

Statistics

The data are presented as mean \pm standard error of the mean. Statistical analyses were performed using the non-paired two-tailed Student's t-test, assuming equal variance. Statistical significance was determined at $p < 0.05$.

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