Adenovirus-Mediated Delivery of an Anti-V Antigen Monoclonal Antibody Protects Mice against a Lethal Yersinia pestis Challenge

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Pneumonic plague, caused by inhalation of Yersinia pestis, represents a major bioterrorism threat for which no vaccine is available. Based on the knowledge that genetic delivery of monoclonal antibodies (MAbs) with adenovirus (Ad) gene transfer vectors results in rapid, high-level antibody expression, we evaluated the hypothesis that Ad-mediated delivery of a neutralizing antibody directed against the Y. pestis V antigen would protect mice against a Y. pestis challenge. MAbs specific for the Y. pestis V antigen were generated, and the most effective in protecting mice against a lethal intranasal Y. pestis challenge was chosen for further study. The coding sequences for the heavy and light chains were isolated from the corresponding hybridoma and inserted into a replication-defective serotype 5 human Ad gene transfer vector (AdoV). Western analysis of AdoV-infected cell supernatants demonstrated completely assembled antibodies reactive with V antigen. Following AdoV administration to mice, high levels of anti-V antigen antibody titers were detectable as early as 1 day postadministration, peaked by day 3, and remained detectable through a 12-week time course. When animals that received AdoV were challenged with Y. pestis at day 4 post-AdoV administration, 80% of the animals were protected, while 0% of control animals survived (P < 0.01). Ad-mediated delivery of a V antigen-neutralizing antibody is an effective therapy against plague in experimental animals and could be developed as a rapidly acting antiplague therapeutic.

Yersinia pestis, the etiologic agent of plague, has been responsible for high mortality in epidemics throughout human history and remains a current threat as a potential biological warfare agent (35, 39). Y. pestis infection can result in bubonic, septicemic, or pneumonic plague, with the last being the most likely following a deliberate Y. pestis release (23, 35, 39, 47, 57) (http://www.bt.cdc.gov/agent/agentlist-category.asp). Pneumonic plague is highly contagious and is easily transmitted person to person through airborne droplets, resulting in a rapid onset of disease and a mortality rate of almost 100% if treatment is delayed more than 24 h postexposure (23, 39, 46, 57).

There are currently no plague vaccines available in the United States. Although several active vaccine candidates are being developed, most require multiple administrations to achieve protective immunity (1, 2, 4, 6, 15, 18, 27, 43, 46, 48, 49, 51, 53–55). In the context that it is improbable that nonmilitary populations will be prophylactically immunized against plague, vaccines requiring multiple administrations over weeks to months are not likely to be useful in response to a bioterror attack. However, several studies in experimental animal models have demonstrated the efficacy of passive antibody administration against plague (3, 16, 20–22, 33, 37). In combination with the capacity to effectively target antibiotic-resistant Y. pestis strains, the ability of passive immunotherapy to provide an immediate state of protection has increased interest in developing antibody-based therapeutics for plague.

The Y. pestis virulence (V) antigen has been identified as a potent protective antigen (PA) against plague and has consequently been evaluated as a subunit vaccine candidate and as a target for passive immunotherapy (2, 16, 20–22, 27, 33, 52, 54). V antigen has multiple roles during the course of Y. pestis infection. It is required for translocation of bacterial effector proteins into host cells via a type III secretion system and additionally is associated with increased interleukin 10 levels and decreased tumor necrosis factor alpha levels through an unknown mechanism (5, 36, 38, 41, 44). Transfer of immune sera from animals immunized with V antigen to naive animals confers immediate protection against Y. pestis challenge (16, 33). Additionally, passive transfer of an anti-V antigen monoclonal antibody (MAb) protects mice against a lethal challenge with Y. pestis (20–22).

Delivery of the coding sequences for MAbs with viral vectors has been effective against both infectious diseases and cancers and is an alternative platform to administration of purified antibodies (9, 10, 25, 26, 28, 45, 50). The rapid transgene expression kinetics from adenovirus (Ad) gene transfer vectors renders them applicable as antibody delivery vehicles for potential biowarfare. With this background, we generated an anti-V antigen MAb that neutralizes Y. pestis following passive transfer to experimental animals and constructed a replication-defective human Ad serotype 5 gene transfer vector expressing the coding sequences for this protective anti-V antigen MAb (AdoV). Following administration to mice, AdoV generates high-serum anti-V antigen antibody titers and, most importantly, protects mice against a lethal challenge with a fully virulent strain of Y. pestis.

Materials and Methods

Purification of recombinant V antigen. Recombinant V antigen from Yersinia pestis was produced as a reagent for screening reactivity of MAbs against V...
Novel V antigen-specific MAbs protect against Y. pestis challenge. Hybridoma supernatants from a panel of clones generated by immunizing mice with purified recombinant V antigen were screened for V antigen specificity by Western analysis. As examples, Western analysis of clones 2C12.4, 2G9.8, and 1A10.14 were assessed for reactivity against bovine serum albumin (BSA) (Fig. 1, lanes 1, 3, and 5) or purified recombinant

**RESULTS**

V antigen coding sequence was inserted into the T7 promoter-driven prokaryotic expression plasmid pRSET (Invitrogen, Carlsbad, CA) to generate the pRSET-V plasmid, expressing V as a histidine tag fusion protein. pRSET-V was transformed into the BL21(DE3)pLysS strain of Escherichia coli, and expression of the V antigen was induced with isopropyl-β-D-thiogalactopyranoside. The V antigen was affinity purified using a Ni-nitrilotriacetic acid (NTA) Superflow column (Qiagen, Valencia, CA) under native conditions. The purity of the protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NuPAGE system; Invitrogen), and its identity was confirmed by Western analysis with a rabbit anti-V antigen antibody (kindly provided by Sina Bavari, USAMRIID, Fort Detrick, MD).

**Adenovirus production.** expression and the specificity of the anti-v antigen antibody from AdoV following infection of cells in vitro were assessed by Western analysis. A549 cells were infected with AdoV (5,000 pfu/cell), and infected-cell supernatants were harvested at 72 h postinfection. Supernatants were concentrated using Ultracel YM-10 centrifugal filters (Millipore, Billerica, MA) and evaluated for the expression of anti-V antigen antibody by Western analysis under reducing and nonreducing conditions using a peroxidase-conjugated sheep anti-mouse IgG secondary antibody (Sigma, St. Louis, MO) and ECL reagent (Amersham). The specificity of the Ad-expressed anti-V antigen in infected-cell supernatants for the Y. pestis V antigen was determined by Western analysis with recombinant V as the target antigen. Negative controls included AdgPA-infected cell supernatants. Detection was with a peroxidase-conjugated sheep anti-mouse IgG secondary antibody and ECL reagent.

**Assessment of AdoV in vivo.** Female BALB/c and male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and Taconic (Germantown, NY), respectively. The animals were housed under specific-pathogen-free conditions and used at 6 to 8 weeks of age. Male C57BL/6 mice were administered AdoV (10^11 pu) via the intravenous route. Naive mice injected with AdNull or AdgPA were used as negative controls. Ad vectors were diluted with saline to the specified dose. To assess the ability of AdoV to generate anti-V antigen antibodies in vivo, at various times following vector administration, serum was collected via the tail vein, centrifuged at 8,000 × g for 20 min, and stored at −20°C. Anti-V antigen antibody levels in mouse serum were assessed by ELISA using flat-bottomed 96-well EIA/RIA plates (Corning, New York, NY) coated with 0.5 μg recombinant V antigen per well in a total volume of 100 liters of 0.05 M carbonate buffer, pH 7.4, overnight at 4°C. The plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween) and blocked with 5% dry milk in PBS for 1 h at 23°C. Serial serum dilutions were added to each well and incubated for 1 h at 23°C. The plates were washed four times with PBS-Tween, and 100 μl well of 1×10^3 diluted peroxidase-conjugated sheep anti-mouse IgG1 (Sigma) in PBS containing 1% dry milk was added and incubated for 1 h at 23°C. The plates were washed four times with PBS-Tween and once with PBS. Peroxidase substrate (100 μl; Bio-Rad, Hercules, CA) was added and incubated for 15 min at 23°C, and then a stop solution of 2% oxalic acid (100 μl/well) was added. Absorbance at 415 nm was read with a microplate reader (Bio-Rad). Antibody titers were calculated with a log/log plot and a cutoff value equal to twofold the absorbance of the background.

To assess the ability of AdoV to protect mice against a lethal challenge with Y. pestis, male C57BL/6 mice (n = 5/group) were injected with AdoV. Mice injected with AdNull were negative controls. Four days after vector administration, each mouse was challenged intranasally with Y. pestis CO92. As a positive control, concentrated MAbs 2C12.4, hybridoma supernatant containing approximately 500 μg MAb was administered IP 2 h prior to intranasal challenge. A fully virulent strain of Y. pestis CO92 (described below) was infected supernatants from clone 2C12.4 provided the best protection against the lethal challenge, and this clone was chosen for further analysis.

**Ad vectors.** The coding sequences for the heavy and light chains of the protective anti-V antigen antibody 2C12.4 were obtained by RNA ligase-mediated rapid amplification of cDNA ends (GeneRacer kit; Invitrogen, Carlsbad, CA) using RNA extracted from the corresponding hybridoma cells. The isolated coding sequences were cloned into a replication-defective human Ad vector to generate AdoV. AdoV is a serotype 5, E1− E3− Ad gene transfer vector constructed to direct the expression of a full-size (heavy- and light-chain) murine MAb against the Yersinia pestis V antigen. The anti-V-light-chain and heavy-chain sequences were separated by a furin cleavage site and the self-cleaving 2A chain sequences were controlled by plating on hypoxanthine-, aminopterin-, and thymidine-containing media. As soon as the wells were 25 to 50% confluent, the fused cells were plated onto 96-well plates in hypoxanthine-, aminopterin-, and thymidine-containing medium. The medium from each of the wells was screened by ELISA using the same His-tagged V antigen, and ~480 wells were found to produce immunoglobulin G (IgG) antibodies that reacted with His-V antigen, using goat anti-mouse IgG isotype-specific antibody-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) as the secondary antibody. The positive wells were secondarily screened by ELISA for reactivity with His-tagged B7-H14-Ig (hB7) to identify any His binding antibodies. The medium from the 48 wells that were the most strongly positive for His-V antigen but negative for His-hB7 was screened by Western analysis for reactivity with recombinant V antigen, and the hybridoma cells from 20 wells were subcloned in soft agar (13). The supernatants of ~20 subclones from each well were checked for isotype and reactivity with V antigen. Totals of 12 IgG1, 4 IgG2a, and 4 IgG2b clones producing stable, cloned antibody from AdoV were used for initial screening of anti-V antigen antibodies in vivo, at various times following vector administration. The data are presented as means ± standard errors of the means. Statistical analyses were performed using the nonparametric two-tailed Student t test, assuming equal variance. Survival evaluation was carried out using Kaplan-Meier analysis. Statistical significance was determined at P < 0.05.

**Novel V antigen-specific MAbs protect against Y. pestis challenge.** Hybridoma supernatants from a panel of clones generated by immunizing mice with purified recombinant V antigen were screened for V antigen specificity by Western analysis. As examples, Western analysis of clones 2C12.4, 2G9.8, and 1A10.14 were assessed for reactivity against bovine serum albumin (BSA) (Fig. 1, lanes 1, 3, and 5) or purified recombinant protein. pRSET-V was transformed into the BL21(DE3)pLysS strain of Escherichia coli, and expression of the V antigen was induced with isopropyl-β-D-thiogalactopyranoside. The V antigen was affinity purified using a Ni-nitrilotriacetic acid (NTA) Superflow column (Qiagen, Valencia, CA) under native conditions. The purity of the protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NuPAGE system; Invitrogen), and its identity was confirmed by Western analysis with a rabbit anti-V antigen antibody (kindly provided by Sina Bavari, USAMRIID, Fort Detrick, MD).
V antigen (Fig. 1, lanes 2, 4, and 6). The isotypes of all MAbs were determined; clones 2C12.4, 2G9.8, and 2G9.10 are IgG2b, and the three showed strong reactivity with V antigen. Clones 1F1.3 and 1A10.14 are IgG1. Clone 1F1.3 was strongly reactive with V antigen, and clone 1A10.14 did not recognize V antigen.

Eleven MAbs screened by Western analysis were selected for studies of passive protection against a lethal Y. pestis challenge. Concentrated hybridoma supernatants (each containing approximately 500 µg antibody) were administered IP to mice 2 h prior to intranasal challenge with Y. pestis CO92, and survival was monitored for 14 days (Fig. 2A). Pooled V antigen-specific hyperimmune serum from mice immunized multiple times with AdsecV, an Ad gene transfer vector encoding V antigen, was used as a positive control (8). Negative controls for this experiment included PBS and clone 1A10.14, a MAb that did not react with V antigen. Shown in Fig. 2A is a representative experiment screening four V antigen-specific MAbs (1F3.3, 2G9.10, 2G9.8, and 2C12.4). In four independent replicate experiments, MAB 2C12.4 consistently mediated protection against Y. pestis. The dose-dependent efficacy of MAB 2C12.4 was also determined in passive protection experiments against Y. pestis challenge (Fig. 2B). Mice received 100, 250, or 500 µg of antibody IP and were challenged intranasally with Y. pestis at 2 h postadministration. The results of this single experiment indicate that MAb 2C12.4 protects animals in a dose-dependent manner, with all doses providing some degree of protection.

Characterization of AdoV-expressed full-length anti-V antibody. Based on the data demonstrating that antibody 2C12.4 was the most effective in protecting mice from a lethal challenge with Y. pestis, the coding sequences for the heavy and light chains of antibody 2C12.4 were isolated from the corresponding hybridoma line and cloned into a replication-defective serotype 5 human Ad gene transfer vector to generate AdoV. To examine expression of the anti-V antigen antibody by AdoV, A549 cells were infected with AdoV. AdoPA, an Ad vector expressing an unrelated antibody, was used as a positive control, and AdNull, an Ad vector with no transgene, was used as a negative control. At 72 h postinfection, cell supernatants were collected, and antibody expression was examined under nonreducing and reducing conditions to assess expression of the individual heavy and light chains (Fig. 3). Supernatants collected from AdoV- or AdoPA-infected cells demonstrated the presence of antibody heavy (50-kDa) and light (25-kDa) chains (Fig. 3A, lanes 2 and 4). No antibody was detected with uninfected or AdNull-infected cells (Fig. 3, lanes 1 and 3). When these supernatants were analyzed by Western blotting under native (nonreducing) conditions, samples from both AdoV- and AdoPA-infected cells contained a protein of the ex-
expected size for a completely assembled antibody (150 kDa; Fig. 3B, lanes 6 and 8). No antibody was detected with uninfected or AdNull-infected cells (Fig. 3B, lanes 5 and 7).

The specificity of the Ad-expressed anti-V antibody was assessed by Western analysis. This experiment tested antibody expression in vitro in AdV-infected cell supernatants and in vivo in serum from AdV-administered mice. AdPA was used as a negative control, and MAb 2C12.4 hybridoma supernatant was used as a positive control. When purified recombinant V antigen was probed with supernatants from AdV-infected cells, sera obtained from AdV-immunized mice, or the 2C12.4 hybridoma supernatant, a strong signal corresponding to the expected molecular weight of V antigen was detected (Fig. 4, lanes 3, 4, and 5). In contrast, supernatants from AdPA-infected cells or sera from AdPA-immunized mice did not recognize V antigen (Fig. 4, lanes 1 and 2). Thus, AdV functions to direct expression of a fully assembled MAb that has specificity for V antigen in vitro and in vivo.

**Time-dependent AdV-mediated in vivo expression of an anti-V antibody.** The time-dependent in vivo expression profile of the anti-V antibody was assessed with mice administered either AdV or AdPA. Serum anti-V antigen antibody levels were measured with a V antigen-specific ELISA over 12 weeks. Mice that received AdV had high serum anti-V antibody levels as early as 1 day postadministration (Fig. 5). These antibody titers peaked at day 3 postadministration with a titer of 10^5 and then gradually decreased over time, with a titer of 10^2 at the end of the 12-week experiment. The serum antibody concentration was determined to be 1.15 mg/ml on day 3 postadministration and 0.85 mg/ml on day 5 postadministration. Mice that received the AdPA or AdNull control vectors did not have any measurable anti-V antibody titers at any time point.

**Protection against intranasal challenge with Y. pestis.** To determine the efficacy of Ad-expressed anti-V antibodies against Y. pestis, mice were immunized with AdV or AdNull and were then challenged at 4 days postadministration (Fig. 6). Although both bubonic and pneumonic plague
models were considered for the challenge experiments, an intranasal challenge route was selected to most closely mimic pneumonic plague, the most likely form of plague to be encountered in an act of bioterrorism. Concentrated MAb 2C12.4 hybridoma supernatants containing approximately 500 μg MAb were used as the positive control and were administered 2 h prior to challenge. All animals were challenged at the same time. Administration of AdαV to mice resulted in substantial protection against *Y. pestis* challenge (93.3% survival), with the protective effect comparable to that observed for positive-control mice that received MAb 2C12.4. In contrast, animals immunized with AdNull were not protected against the challenge (*P* < 0.01, AdαV versus AdNull). The data presented in Fig. 6 show the averages from three independent experiments.

In a similarly designed experiment, mice were immunized with AdαV and were then challenged at 4 days postadministration with increasing doses of *Y. pestis*, ranging from 363 LD₅₀ to 9,090 LD₅₀ (Fig. 7). At each challenge dose, AdαV immunization conferred 100% protection.

The relative timing of protection against challenge was determined by administering AdαV at various time points, both pre- and post-363 LD₅₀ *Y. pestis* challenge (Fig. 8). In two independent experiments, mice were immunized with AdαV 4 days prior to challenge, at the same time as the challenge, 6 h after challenge, or 24 h after challenge. After a 1-month time period, animals that received AdαV at 24 h following the challenge were partially protected (12.5% protection). However, the animals immunized with AdαV 4 days prior to challenge, at the time of challenge, or 6 h after challenge...
challenge survived (93.7% protection) without any signs of adverse health effects, demonstrating the potential utility of AdoV as both a pre- and a postexposure therapy. Additionally, the survival rates for these experiments were unchanged from day 7 through day 30 postchallenge, demonstrating robust protection with no evidence of vaccine breakthrough. The data shown in Fig. 8 represent the averages from two independent experiments.

DISCUSSION

The ability to express full-size antibody molecules with Ad gene transfer vectors facilitates the use of this convenient platform as a therapy for potential agents of bioterrorism. We generated and characterized a protective MAb against the Y. pestis V antigen and demonstrated that a single administration of AdoV, an Ad gene transfer vector encoding the protective antibody, rapidly generates a high concentration of antibody in serum of immunized mice and most importantly, protects immunized mice against a lethal Y. pestis challenge as effectively as administration of a V antigen-specific antibody preparation. In the context that there is no vaccine available for treatment of Y. pestis, the strategy of genetic delivery of an anti-V antibody by AdoV as an antibody-based drug may be a useful strategy to develop for use in a Y. pestis bioterrorism attack.

Y. pestis therapeutics. Aerosol transmission of virulent Y. pestis results in pneumonic plague, a rapidly fatal disease that is a threat related to the potential use of the organism as a biological weapon (23, 35, 39, 46, 57; http://www.bt.cdc.gov /agent/agentlist-category.asp). The currently recommended plague therapy is antibiotic treatment, but multidrug-resistant Y. pestis isolates have been identified, thus limiting the general utility of antibiotics (10, 11, 14, 23, 30, 40).

Both live attenuated and killed whole-cell vaccines against plague have been used in humans for decades, but none are very effective, and none are available in the United States (43, 48, 49). The live attenuated vaccines are all based on mutants of fully virulent strains, but they are not licensed for use in the United States, relating to significant safety and efficacy concerns (21, 24, 43; http://cdc.gov/ncidod/dvbid/plague/prevent .htm). A formaldehyde-killed vaccine has been developed, but this vaccine is also no longer licensed or available for use in the United States, relating to adverse side effects and the inability to provide complete protection against pneumonic plague (21, 43). Current vaccine development efforts are focused largely on recombinant Y. pestis proteins, with V antigen and the capsular F1 protein as the primary targets. Although these vaccine candidates demonstrate efficacy, most require multiple administrations and time for the development of protective immunity (2, 27, 48, 49, 54).

MAbs have several advantages over antibodies and vaccines in the prevention of disease caused by many extracellular pathogens, including the ability to treat antibiotic-resistant pathogens and the immediate state of protection conferred upon administration (7, 56). There have been a variety of studies demonstrating the efficacy of polyclonal and MAbs as a treatment for Y. pestis infection (3, 16, 20–22, 33, 37). For example, a murine MAb raised against F1 protects mice in models of bubonic and pneumonic plague (3). Also, V antigen-specific MAbs protect mice in bubonic and pneumonic plague models when administered IP (20, 22). When anti-F1 and anti-V antigen MAbs are coadministered to mice, a synergistic effect is observed in response to Y. pestis challenge (20, 21).

Genetic delivery of MAbs. The limitations of MAb therapy include complex and costly production and purification methods as well as the relatively short half-life of some antibody molecules. Genetic transfer of therapeutic antibodies is an effective strategy for generating specific antibodies in vivo and a consequently attractive strategy for a variety of therapeutic applications, including infectious diseases. It has been possible to express antibody molecules with a variety of viral vectors either in vitro or in vivo, including baculovirus, rhabdovirus, vaccinia virus, Ad, and adeno-associated virus (AAV) (9–11, 17, 26, 28, 32, 34, 45, 50). The ability to directly administer viral vectors expressing MAbs to a host is an alternative technology to the standard practice of administering preparations of purified antibodies. With Ad or adeno-associated virus vectors, this technology has demonstrated efficacy against a variety of infectious diseases (9, 26, 28, 50). When administered to mice, an Ad vector encoding an antibody against the PA component of anthrax toxin is effective at protecting animals from an anthrax toxin challenge as early as 1 day postadministration (9). Similarly, an AAV vector encoding the same antibody protects immunized mice from anthrax toxin challenge throughout a 6-month time period (9). In other experiments, administration of an AAV vector encoding a human MAb that neutralizes human immunodeficiency virus to murine muscle results in significant serum levels of human immunodeficiency virus-neutralizing activity for over 6 months (28). Finally, we have demonstrated the efficacy of AAV-delivered anti-respiratory syncytial virus antibodies in reducing viral load in mice following challenge with wild-type respiratory syncytial virus (50). Collectively, these data indicate the convenience and efficacy of this strategy in producing both acute or durable protective immune responses and underscore the utility of genetic antibody delivery as an infectious disease therapeutic.

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