Protective Immunity against Respiratory Tract Challenge with *Yersinia pestis* in Mice Immunized with an Adenovirus-Based Vaccine Vector Expressing V Antigen

Maria J. Chiuchiolo,[1,a] Julie L. Boyer,[1,a] Anja Krause,[1] Svetlana Senina,[3] Neil R. Hackett,[1,2] and Ronald G. Crystal[1,2]

[1]Department of Genetic Medicine and [2]Belfer Gene Therapy Core Facility, Weill Medical College of Cornell University, New York, New York; [3]Public Health Research Institute at the International Center for Public Health, Newark, New Jersey

The aerosol form of the bacterium *Yersinia pestis* causes the pneumonic plague, a rapidly fatal disease. At present, no plague vaccines are available for use in the United States. One candidate for the development of a subunit vaccine is the *Y. pestis* virulence (V) antigen, a protein that mediates the function of the *Yersinia* outer protein virulence factors and suppresses inflammato y responses in the host. On the basis of the knowledge that adenovirus (Ad) gene-transfer vectors act as adjuvants in eliciting host immunity against the transgene they carry, we tested the hypothesis that a single administration of a replication-defective Ad gene-transfer vector encoding the *Y. pestis* V antigen (AdsecV) could stimulate strong protective immune responses without a requirement for repeat administration. AdsecV elicited specifi T cell responses and high IgG titers in serum within 2 weeks after a single intramuscular immunization. Importantly, the mice were protected from a lethal intranasal challenge of *Y. pestis* CO92 from 4 weeks up to 6 months after immunization with a single intramuscular dose of AdsecV. These observations suggest that an Ad gene-transfer vector expressing V antigen is a candidate for development of an effective anti-plague vaccine.

The gram-negative bacterium *Yersinia pestis* is the etiological agent of plague and is classifie as a category A pathogen that is a potential agent of bioterrorism [1, 2]. There are 3 forms of the human disease: bubonic, septicemic, and pneumonic [2, 3]. Of these, the pneumonic plague is of most concern as a biological threat, because of the rapid onset, high mortality, and rapid spread. Although antibiotics can successfully treat plague, the fatality rate is high when treatment is delayed >24 h after the onset of symptoms [2–4].

Received 3 August 2005; accepted 14 May 2006; electronically published 25 September 2006.

Potential conflicts of interest: none reported.

Financial support: National Institutes of Health (grants U54 AI057158 and R01 AI 55844); Will Rogers Memorial Fund, Los Angeles, California; Robert A. Belfer (gift to support the development of an antibioterrorism vaccine).

[a] M.J.C. and J.L.B. contributed equally to this study.

Reprints or correspondence: Dr. R. G. Crystal, Dept. of Genetic Medicine, Weill Medical College of Cornell University, 515 E. 71st St., S-1000, New York, NY 10021 (geneticmedicine@med.cornell.edu).

The Journal of Infectious Diseases 2006; 194:1249–57

0022-1899/2006/19409-0010$15.00

At present, no plague vaccines are available in the United States. Several vaccines have been developed, including killed whole-cell formulations and the live attenuated EV76 vaccine [5–8]. Although these vaccines have been used in humans, they offer low levels of protection, have numerous adverse side effects, and require frequent immunizations with consequent prolonged time to develop immunity [5–8]. A promising subunit vaccine is based on the virulence (V) antigen (also referred to as "LcrV") [9–11]. V antigen is a 37-kDa multifunctional protein of *Yersinia* species encoded by the 70-kb low calcium response *(lcr)* plasmid. V antigen participates in the type III secretion system in *Y. pestis* regulating the production and facilitating the translocation of *Yersinia* outer proteins (Yops) with anti-host activity into the host cell [12, 13]. Active immunization with purifie V antigen or passive immunization with antiserum against V antigen provides protection against plague in mice [9, 10, 14, 15]. V antigen–based DNA vaccines are also being developed [16, 17]. These vaccines elicit low antibody titers, and

protection against a *Y. pestis* challenge is reached only after several immunizations [18].

The present study is focused on using replication-deficien adenovirus (Ad) gene-transfer vectors encoding V antigen to elicit protective immune responses against *Y. pestis*. Ad vectors are excellent candidates for vaccine platforms as they transfer genes effectively to antigen-presenting cells (APCs) in vivo, with consequent activation of APCs, thus conveying immune adjuvant properties and inducing strong, rapid humoral and cellular immune responses against the transgene product [19–26]. On the basis of these considerations, we constructed an $E1^{-}E3^{-}$ Ad-based vaccine vector that encodes a secreted human codon–optimized V antigen (AdsecV). The data demonstrate that AdsecV induces high IgG titers within 2 weeks after a single intramuscular immunization in mice. Importantly, mice immunized with a single intramuscular dose of AdsecV are protected from a lethal intranasal challenge of *Y. pestis*.

METHODS

Ad vectors. The *Y. pestis* V antigen gene (NCBI accession no. B33601) with mammalian-preferred codons was synthesized by overlap polymerase chain reaction and fused to the human Igκ signal sequence for extracellular secretion. The V antigen gene was cloned into a recombinant Ad5–based vector (E1a, partial E1b, and partial E3 deletion), to generate AdsecV. AdNull was used as a control vector with identical backbone but no transgene [27]. The vectors were produced in 293 cells and were purifie by double CsCl gradient centrifugation [28]. Dosing was based on particle units (pu), the physical number of Ad particles as measured by spectrophotometry [29].

Purificatio of recombinant V antigen. Recombinant V antigen was produced as a reagent for assessment of antibodies against V antigen. The V antigen gene was cloned into the pRSET expression plasmid (Invitrogen), and V antigen was purifie as a histidine-tag fusion by use of a Ni-NTA Superflo Column (Qiagen) under native conditions.

Expression of V antigen by AdsecV in vitro. The A549 lung epithelial cell line (CCL185; American Type Culture Collection) was maintained in complete Dulbecco's modifie essential medium. Cells were infected with AdsecV or AdNull (500 pu/cell) in low-serum medium. Twenty-four hours after infection, cells and medium were collected, and proteins were separated by SDS-PAGE (Invitrogen) and transferred to a polyvinylidene fluorid membrane (BioRad Laboratories). For Western blot analysis, the membrane was probed with a 1:1000 dilution of a rabbit anti–V antigen antibody (provided by S. Bavari, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD). A peroxidase-conjugated anti-rabbit antibody (Santa Cruz Biotechnology) and a chemiluminescent peroxidase substrate (ECL+ reagent; Amersham Biosciences) were used for detection.

To assess V antigen expression by immunofluo escence, 24 h after infection, cells were fixe with 4% paraformaldehyde and blocked with 5% goat serum (Jackson ImmunoLabs), 1% bovine serum albumin in PBS with 0.05% saponin (Sigma-Aldrich), and 0.1% Triton X-100 (Sigma-Aldrich), followed by incubation with the rabbit anti–V antigen antibody diluted 1:1000. A goat anti-rabbit secondary antibody conjugated to Alexa 488 fluo ophore (Jackson ImmunoLabs) was used at a fina concentration of 10 μg/mL. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes). The samples were observed by fluo escence microscopy with an Olympus IX70 inverted microscope (New York/New Jersey Scientific equipped with a $\times$60 PlanApo NA 1.4 objective, and digital image analysis was performed with Metamorph imaging software (version 4.6r9; Universal Imaging).

Mice. Female BALB/c mice were obtained from Taconic. Mice were housed under specific-pathogen-f ee conditions and were used at 7 weeks of age. Mice were immunized in a single vaccination by 2 intramuscular injections, with 50 μL of the vaccine preparation divided evenly between the quadriceps on each side. Ad vectors were diluted with saline to the specifie dose.

Transgene-specifi humoral responses. Serum samples from immunized mice were obtained from the tail vein, and anti–V antigen serum antibody titers were determined by ELISA. For vector dose response, serum samples were obtained from mice 4 weeks after immunization with AdsecV at doses ranging from 10^8 to 10^{11} pu. For time-dependent anti–V antigen antibody titers, mice were immunized with a single administration of 10^9 pu of AdsecV, and serum samples were obtained before and at 1, 2, 4, 6, and 8 weeks after vaccination. For IgG subtypes, mice were vaccinated with 10^9 pu of AdsecV, and serum samples were obtained 5 weeks after immunization.

For ELISAs, microtiter plates (Corning) were coated with 0.5 μg of recombinant V antigen per well. After blocking, serum samples were added in sequential 2-fold dilutions starting at 1:20 and were incubated for 1 h at 23°C. An anti–mouse IgG–horseradish peroxidase conjugate (Sigma-Aldrich) was used at 1:10,000 dilution. Detection was accomplished using a peroxidase substrate (BioRad Laboratories). Absorbance at 415 nm was read using a microplate reader (BioRad Laboratories). Class-specifi anti-IgG antibodies (IgG1, IgG2a, IgG2b, and IgG3) were determined using the Mouse Typer isotyping panel (Bio-Rad Laboratories). Antibody titers were calculated on the basis of a log(optical density) $-$ log(dilution) interpolation model and a cutoff value equal to 2-fold the absorbance of the background [30, 31].

Transgene-specifi cellular responses. Mice were immunized intramuscularly ($n = 5$) with either saline, AdNull (10^{11} pu), or AdsecV (10^{11} pu). The frequency of antigen-specifi T lymphocytes was determined using an interleukin (IL)–2–, in-

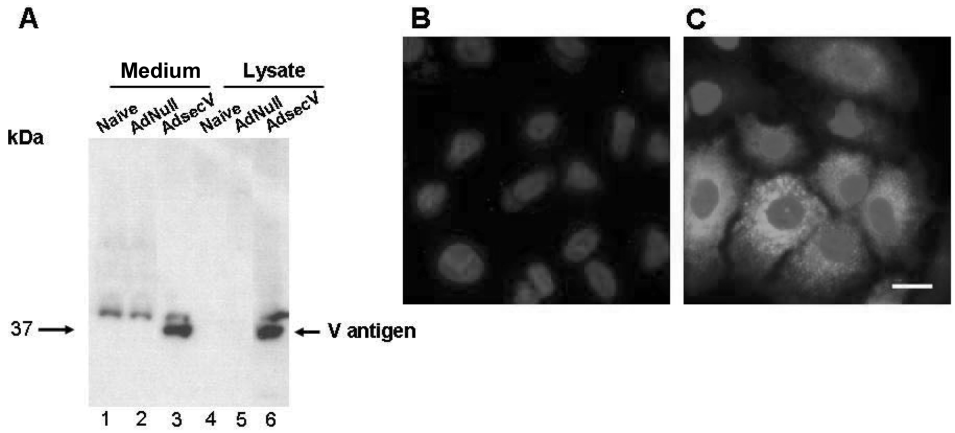

Figure 1. Expression of virulence (V) antigen in cells infected with AdsecV. A549 cells were assessed 24 h after infection with AdsecV or the control vector AdNull at 500 particle units/cell. Data are representative of results from 2 independent experiments. *A,* Western blot analysis of medium and cell lysate. V antigen was detected by use of anti–V antigen antibody. *Lane 1,* medium, naive cells; *lane 2,* medium, AdNull-infected cells; *lane 3,* medium, AdsecV-infected cells; *lane 4,* cell lysate, naive cells; *lane 5,* lysate, AdNull-infected cells; *lane 6,* lysate, AdsecV-infected cells. The extra band visible in the supernatant lanes is the result of the cross-reactivity of other antibodies in the polyclonal preparation with other proteins in the medium. *B* and *C,* Indirect immunofluorescence detection of V antigen. After 24 h, cells were fixed with 4% paraformaldehyde and were stained with anti–V antigen antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Panel B shows AdNull-infected cells, and panel C shows AdsecV-infected cells. The bar is 10 μm.

terferon (IFN)–γ–, and IL-4–specifi enzyme-linked immunospot (ELISPOT) assay (R&D Systems). Six days after administration of Ad, $CD4^+$ or $CD8^+$ T cells were purifie by negative depletion using SpinSep T cell subset purificatio kits (StemCell Technologies). Splenic dendritic cells (DCs) were purifie from naive mice by positive selection using CD11c MACS beads (Milentyi Biotec) and double purificatio over 2 MACS-LS columns (Milentyi Biotec). The purity of $CD4^+$ T cells, $CD8^+$ T cells, and DCs was assessed by staining with anti–CD4–phycoerythrin (PE), anti–CD8-PE, and anti–CD11c-PE antibodies (BD Biosciences), respectively. Cell purity evaluation and cell counts were performed using a FACScalibur flo cytometer running at a constant flo rate. For ELISPOT assays, 10^5 $CD4^+$ or $CD8^+$ T cells were incubated for 36 h with splenic DCs at a ratio of 4:1, with or without purifie V antigen. Spots were counted by computer-assisted ELISPOT image analysis (Zellnet Consulting).

Y. pestis *CO92 challenge.* The *Y. pestis* challenge studies were conducted at the Public Health Research Institute at the International Center for Public Health under biosafety level 3 conditions. Four weeks or 6 months after immunization, mice (10/group) were challenged intranasally with *Y. pestis* CO92. *Y. pestis* CO92 was grown aerobically in heart infusion broth (Difco) at 30°C and was diluted in saline solution at doses ranging from 10^3 to 10^6 cfu. Fifty microliters of bacterial suspension was used for intranasal infection of mice. Bacterial dose was controlled by plating on *Yersinia* selective agar (YSA; Oxoid). Survival was monitored daily for 15 days. From a subset of the mice that died after challenge, liver, spleen, and lungs were removed, homogenized in saline solution, and plated on YSA, to confi m that plague was the cause of death. A subset of the vaccinated mice that survived the challenge were killed 15 days after infection; liver, spleen, and lungs were removed, homogenized in saline solution, and plated on YSA to confi m that bacteria were not present in internal organs.

Statistical analyses. Data are presented as mean ± SE values. For ELISPOT assays, statistical analyses were performed using 1-way analysis of variance followed by Fisher's protected least significan difference test. For survival comparison, Kaplan-Meier analysis was performed; reported *P* values are from Mantal-Cox analysis. Statistical significanc was determined at $P < .05$.

RESULTS

Expression of V antigen by AdsecV. V antigen expression by AdsecV was analyzed in vitro by Western blot analysis. Twenty-four hours after infection of A549 cells with AdsecV, a protein with the expected size for V antigen (37 kDa) was identifie in medium and cell lysates by use of an anti–V antigen antibody (figu e 1*A*). The protein was not detected in cells infected with AdNull (the control vector) or in uninfected cells. The localization of V antigen in the AdsecV-infected cells was evaluated by indirect immunofluo escence. At 24 h, V antigen (figu e 1*C*) exhibits a broad, diffuse staining pattern as well as a bright, punctate, perinuclear staining pattern consistent with localization of V antigen in the endoplasmic reticulum and Golgi apparatus of the secretory pathway. The protein was not present in AdNull-infected cells (figu e 1*B*).

Humoral immune responses to AdsecV. To evaluate hu-

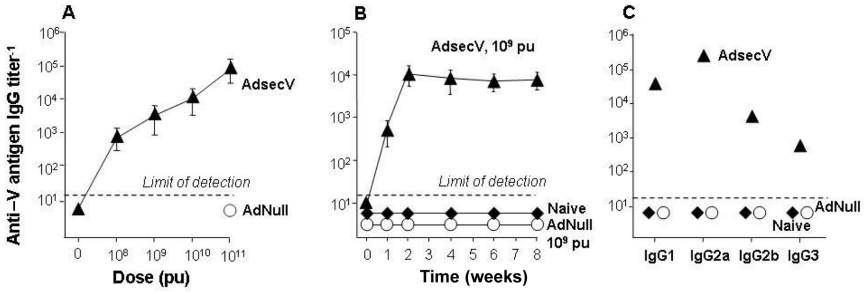

Figure 2. Anti–virulence (V) antigen antibodies in serum evoked by AdsecV after intramuscular immunization of mice. Antibody levels were quantified by ELISA. *A,* Dose-dependent induction of anti–V antigen IgG 4 weeks after immunization with AdsecV ($n = 10$ mice/group). *B,* Time course of induction of anti–V antigen IgG after immunization with AdsecV (10^9 particle units [pu]) or the control vector AdNull (10^9 pu). Serum samples were collected before and 1, 2, 4, 6, and 8 weeks after immunization ($n = 5$ mice/group). *C,* Anti–V antigen IgG subtypes at 5 weeks in pooled serum samples ($n = 10$ mice/group). Data are mean ± SE values except for those in panel C, in which data are from pooled serum samples. For all panels, black triangles indicate AdsecV-immunized mice, white circles indicate AdNull-immunized mice, and black diamonds indicate naive mice; dashed lines indicate the limit of detection of the assay. Data are representative of results from 2 independent experiments.

moral immune responses in AdsecV-immunized mice, anti–V antigen IgG titers were assessed in serum. Four weeks after a single intramuscular administration of 10^8–10^{11} pu of AdsecV, a dose response in the total anti–V antigen IgG titers was observed, with the total anti–V antigen IgG titers for immunized mice reaching a mean ± SE titer of 76,000 ± 16,000 for the mice vaccinated with 10^{11} pu of AdsecV (figu e 2*A*). No anti–V antigen IgG titers were detected in the naive mice (which received saline) or in the AdNull-immunized mice.

To evaluate the kinetics of the anti–V antibody response, total IgG titers were measured at different time points after a single vaccination. After administration of a 10^9-pu dose of AdsecV, anti–V antigen titers were detected in serum of immunized mice as early as 1 week (figu e 2*B*). The antibody titer reached a maximum level at 2 weeks and remained high through 8 weeks. Analysis of IgG subclasses at the 10^9-pu AdsecV dose showed a strong response for both IgG1 and IgG2a and a lesser response for IgG2b and IgG3 (figu e 2*C*). Similar antibody titers and subtypes were observed with an Ad vector expressing a nonsecreted form of V antigen (data not shown). Anti–V antigen antibody after immunization with 10^9 pu of the nonsecreted form could be detected in serum at 2 weeks, with a mean ± SE titer of 13,700 ± 2,900. Antibody levels remained high through week 8, as reported for AdsecV (figu e 2*B*). Because anti–V antigen antibodies in serum could already be detected 1 week after immunization with AdsecV and because titers were similar for both forms of the vaccine, we focused our study on the responses evoked by AdsecV.

Cellular immune responses to AdsecV. The frequency of T cell responses to V antigen in vaccinated mice was analyzed by ELISPOT assay. Six days after mice were immunized with 10^{11} pu of AdsecV, purifie $CD4^+$ and $CD8^+$ T cells from the spleens of the vaccinated mice were stimulated with syngeneic DCs pulsed with V antigen, and cytokine production was assessed.

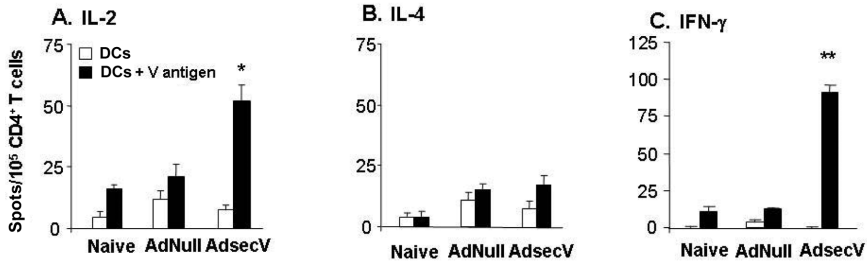

Figure 3. Virulence (V) antigen–stimulated cytokine production by $CD4^+$ T cells from mice immunized with AdsecV. Mice were immunized intramuscularly with either saline (naive mice), AdNull (10^{11} particle units [pu]), or AdsecV (10^{11} pu). Six days after immunization, $CD4^+$ T cells were isolated from spleens and were stimulated for 36 h with syngeneic dendritic cells (DCs) pulsed with 100 μg/mL purified V antigen. Cytokine expression was assessed by enzyme-linked immunospot assay. Panel A shows interleukin (IL)–2 expression, panel B shows IL-4 expression, and panel C shows interferon (IFN)–γ expression, both for stimulation with DCs alone and for stimulation with DCs plus V antigen. Data are mean ± SE values ($n =$ 5 mice/group) and are representative of results from 3 independent experiments. *$P < .005$ and **$P < .0001$, compared with the 2 control groups (analysis of variance followed by Fisher's protected least significant difference test).

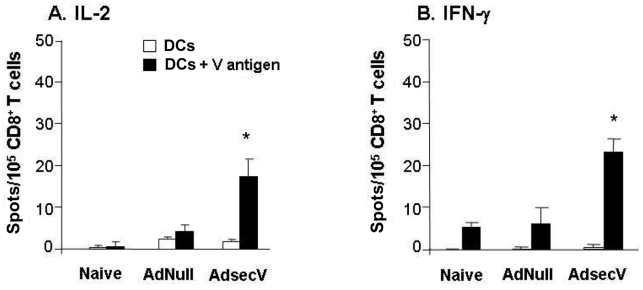

Figure 4. Virulence (V) antigen-stimulated cytokine production by $CD8^+$ T cells from mice immunized with AdsecV. Mice were immunized intramuscularly with either saline (naive mice), the control vector AdNull (10^{11} particle units [pu]), or AdsecV (10^{11} pu). Six days after immunization, $CD8^+$ T cells were isolated from spleens and stimulated for 36 h with syngeneic dendritic cells (DCs) pulsed with 100 μg/mL purified V antigen. Cytokine expression was assessed by enzyme-linked immunospot assay. Panel A shows interleukin (IL)–2 expression and panel B shows interferon (IFN)–γ expression, both for stimulation with DCs alone and for stimulation with DCs plus V antigen. Data are mean ± SE values ($n = 5$ mice/group) and are representative of results from 3 independent experiments. *$P < .005$, compared with the 2 control groups (analysis of variance followed by Fisher's protected least significant difference test).

V antigen–specifi IL-2 secretion (mean ± SE, 52 ± 6 spots/10^5 $CD4^+$ T cells) (figu e 3*A*) as well as IFN-γ secretion (mean ± SE, 91 ± 3 spots/10^5 $CD4^+$ T cells) (figu e 3*C*) was significantl higher ($P < .005$ and $P < .0001$, respectively) in $CD4^+$ T cells from the AdsecV-immunized mice than in the 2 control groups. In contrast, no significan differences were observed for V antigen–specifi IL-4 production (figu e 3*B*). $CD8^+$ T cell activation was evaluated by V antigen–specifi IL-2 and IFN-γ secretion. Both IL-2 (mean ± SE, 19 ± 4 spots/10^5 $CD8^+$ T cells) (figu e 4*A*) and IFN-γ (mean ± SE, 24 ± 3 spots/10^5 $CD8^+$ T cells) (figu e 4*B*) responses were higher in the AdsecV-vaccinated mice than in the 2 control groups ($P < .005$). The naive mice and the mice immunized with AdNull showed no significan signal of V antigen–induced cytokine production above background.

Protection against intranasal challenge with* Y. pestis *CO92. The ability of AdsecV to confer protective immunity was evaluated by challenging immunized mice with the fully virulent *Y. pestis* strain CO92. The mice received a single intramuscular administration of AdsecV at doses ranging from 10^8 to 10^{11} pu. Four weeks after vaccination, the mice were infected intranasally with 3×10^3 cfu of *Y. pestis* strain CO92. All (10/10) of the mice in the group vaccinated with 10^{11} pu of AdsecV survived the *Y. pestis* challenge ($P < .005$) (figu e 5*A*). The survival of mice that were immunized with 10^{10} pu of AdsecV ranged from 40% (4/10; $P < .05$) (figu e 5*A*) to 60% (6/10) in a different experiment (data not shown). The 10^9- and 10^8-pu doses were not protective; the mice in those groups died according to the same time frame as did the mice in the control groups that received either saline or 10^{11} pu of AdNull. Assessment of the data at 15 days after challenge showed that the mortality of mice was dependent on the vaccine dose (figu e 5*B*).

To evaluate the protective capacity of the vaccine at different challenge doses, mice were infected intranasally with 10^3–10^6 cfu of *Y. pestis* CO92 4 weeks after a single administration of 10^{11} pu of AdsecV. Mice were protected at all doses (figu e 6). All AdsecV-immunized mice (10/10) survived the challenge with 10^3 ($P < .005$) and 10^4 ($P < .0001$) cfu, whereas the naive mice and the mice immunized with 10^{11} pu of AdNull died within 3–5 days. At higher challenge doses, 80% (8/10) and 90% (9/10) of the mice survived after intranasal infection with 10^5 and 10^6 cfu *Y. pestis* CO92, respectively ($P < .0001$).

The capacity of the vaccine to confer long-term protection was evaluated by challenge at 6 months after a single immunization with 10^{11} pu of AdsecV. Anti–V antigen total IgG titers in immunized mouse serum before challenge were a mean ± SE of 106,000 ± 16,500 ($n = 20$). Mice were infected intranasally with 10^4 or 10^6 cfu of *Y. pestis* CO92. All AdsecV-immunized mice (10/10) survived the challenge with 10^4 cfu and 90% (9/10) survived the challenge with 10^6 cfu ($P < .0001$, for both doses) (figu e 7), whereas none of the 10 naive mice survived the challenge with 10^4 cfu *Y. pestis* CO92.

DISCUSSION

In the present study, AdsecV, a replication-defective Ad vector expressing a secreted form of the *Y. pestis* V antigen, was evaluated as a vaccine against plague. The V antigen sequence in AdsecV was targeted for extracellular expression by an Igκ

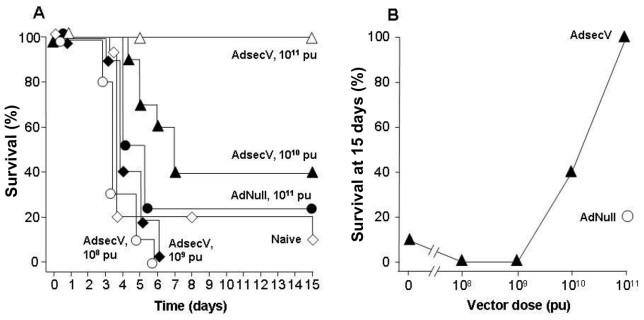

Figure 5. Survival of mice after intranasal challenge with *Yersinia pestis* CO92 after a single intramuscular administration of AdsecV. Mice were immunized intramuscularly with either saline (naive mice), the control vector AdNull (10^{11} particle units [pu]), or AdsecV (10^8, 10^9, 10^{10}, or 10^{11} pu) ($n = 10$ mice/group) and were challenged 4 weeks later by intranasal administration of 3×10^3 cfu of *Y. pestis* CO92. Data are representative of results from 2 independent experiments. *A,* Time course of survival after challenge. For the mice that received 10^{10} or 10^{11} pu of AdsecV ($n = 10$ mice), $P < .05$ and $P < .005$, respectively, compared with the 2 control groups. *B,* Dose-dependent survival of mice immunized with AdsecV at day 15 after challenge.

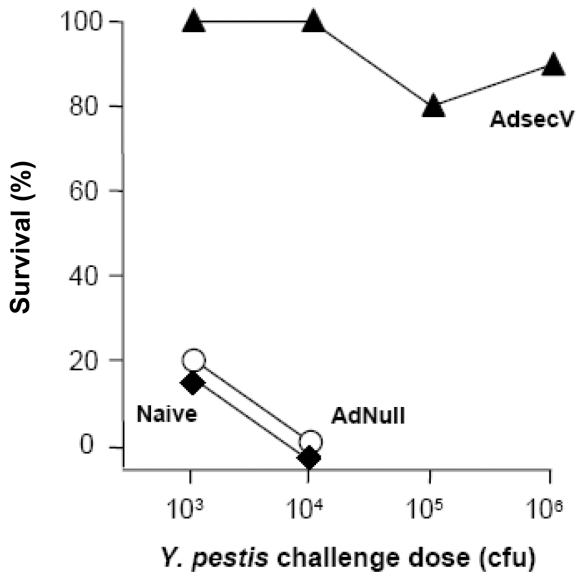

Figure 6. *Yersinia pestis* CO92 challenge dose response of AdsecV-immunized mice. Mice were immunized intramuscularly with either saline (naive mice), the control vector AdNull (10^{11} particle units [pu]), or AdsecV (10^{11} pu) and were challenged 4 weeks later by intranasal administration of 10^3–10^6 cfu of *Y. pestis* CO92. Data are from 1 experiment ($n = 10$ mice/group). Survival at 15 days is plotted against dose. Because all naive and AdNull-immunized mice died at a dose of 10^4 cfu, higher doses of *Y. pestis* were not assessed in the 2 control groups. For the mice that received 10^3 cfu and for the mice that received the higher challenge doses, $P < .005$ and $P < .0001$, respectively, compared with the 2 control groups.

secretion signal, and V antigen expression in infected cells was confi med by Western blot analysis and indirect immunoflu orescence. After a single intramuscular immunization with AdsecV, mice developed strong humoral responses within 2 weeks, with anti–V antigen IgG titers predominantly of the IgG2a and IgG1 subtypes, suggesting a strong Th1 and Th2 response. The cellular immune responses observed in splenic T cells from vaccinated mice were V antigen–specifi Th1 helper ($CD4^+$) and $CD8^+$ responses. Most importantly, immunized mice were protected from an intranasal challenge with a lethal dose of 10^6 cfu of *Y. pestis* CO92, from 4 weeks through 6 months after a single administration of the vaccine.

Y. pestis *vaccines.* Plague is one of the most devastating acute infectious diseases experienced by humankind [1–5]. Antibiotics are only marginally effective once symptoms of pneumonic plague develop; moreover, some antibiotic-resistant isolates have been identified Given these characteristics, there is concern that an aerosolized form of *Y. pestis* may be exploited as a bioweapon.

There is no licensed *Y. pestis* vaccine for use in the United States. Killed whole-cell vaccines have been used since the late 1890s [5–8]. Although these vaccines have been shown to protect against the bubonic form of the disease, they do not protect against pneumonic plague. These vaccines also have disadvantages, such as a significan incidence of transient local and systemic adverse side effects and the need for frequent boosting to maintain adequate immunity [5, 7, 8]. A live attenuated vaccine based on the pigmentation-negative *Y. pestis* strain EV76 has been available since 1908 [6]. This vaccine had questionable efficac in evoking effective immune responses in humans and presents the risk for reversion to virulence in vivo.

In recent years, the development of a safe and effective plague vaccine has been focused on using recombinant protein subunits of *Y. pestis* [5, 9–11]. Several virulence factors have been identifie as possible vaccine candidates, but the most promising are the V antigen and the F1 protein [7–11, 32–34]. Antibodies against V and F1 confer protection against both bubonic and pneumonic plague in mice, guinea pigs, and nonhuman primates [9–11, 14, 15, 33, 35]. Recent studies have shown that a single intramuscular immunization with both recombinant antigens delivered with adjuvants and combined in a molar ratio of 2:1 (F1:V antigen) protected mice against an aerosol challenge with *Y. pestis* [10, 11, 36–39]. Although this protection was correlated with high IgG levels [40], it has been suggested that nonhumoral immune responses also participate, because immunized IL-4–deficien mice, which do not mount effective humoral immune responses, have been shown to be protected against plague [41]. Also, immunization with the combined subunits failed to protect signal

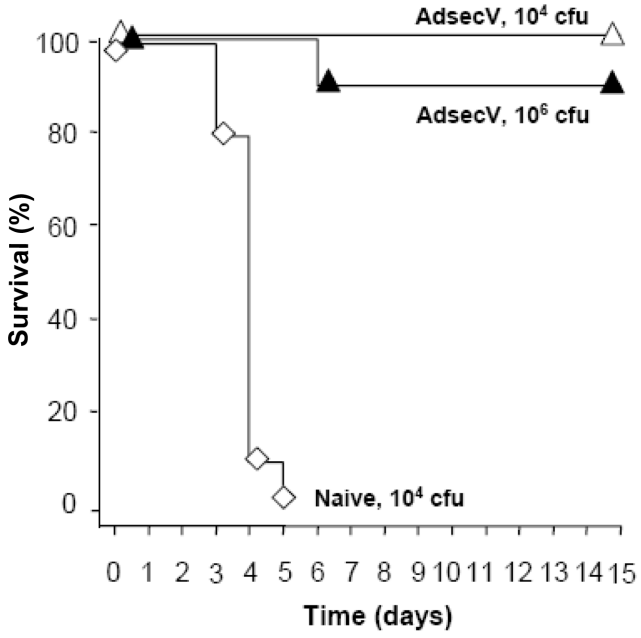

Figure 7. Long-term protection of AdsecV-immunized mice. Mice were immunized intramuscularly with either saline (naive mice) or AdsecV (10^{11} particle units [pu]) and were challenged 6 months later by intranasal administration of 10^4 or 10^6 cfu of *Yersinia pestis* CO92 ($P < .0001$, compared with the naive mice). Survival is plotted as a function of time. Data are from 1 experiment ($n = 10$ mice/group).

transducer and activator of transcription (Stat) $4^{-/-}$ mice against plague; Stat $4^{-/-}$ mice are diminished in their capacity to mount type 1 cytokine responses [42]. It was shown recently that cellular immunity in the absence of antibody can protect against pulmonary *Y. pestis* infection; transfer of *Y. pestis*–primed T cells to naive B cell–deficien μMT mice protected the mice against a *Y. pestis* challenge [43].

V antigen–based vaccines. V antigen is a good candidate for *Y. pestis* vaccine development because it can protect from infection with either $F1^+$ or $F1^-$ strains [44]. V antigen plays important roles in the virulence of plague. It participates in the regulation and translocation of the effector proteins Yops into host cells through a type III secretion system. Yops virulence factors produce cytoskeletal rearrangements and apoptosis in macrophage-like cells, allowing bacteria to escape phagocytosis and to proliferate extracellularly [12, 13, 45, 46]. In addition, purifie V antigen has been shown to suppress the normal inflammato y response in the host by down-regulating the expression of tumor necrosis factor (TNF)–α and IFN-γ, which promotes the production of IL-10 by macrophages, and by inhibiting the chemotaxis of neutrophils [47–50].

The basis for the protection conferred by the recombinant V antigen vaccine is not well known. The correlation with high IgG titers and protection of immunodeficien SCID/Beige mice against pneumonic plague by passive transfer of anti–V antigen–specifi immune serum [14, 15, 51] suggests that anti–V antigen antibodies play a significan role. It has been shown that anti–V antigen antibody administered during infections of mice with *Y. pestis* restore the production of TNF-α and IFN-γ [49], and in vitro assays have shown that anti-V antigen antibodies can partially block the delivery of Yops (and the consequent downstream effects of Yops) in infected macrophage-like cells [52]. Thus, the protection conferred by a V antigen vaccine might be achieved by opsonization through antibody association with surface V antigen on *Y. pestis,* by blocking the delivery of Yops to host cells, by preventing early bacterial growth in macrophages, and/or by neutralizing the immunomodulatory activity of V antigen, allowing the host to mount an inflammato y response.

Ad vectors as vaccine platforms. Recombinant Ad vectors are attractive for vaccine strategies against pathogens for many reasons. They are stable, easy to manipulate, can be produced inexpensively at high titer, and can be purifie by commonly available methods [53]. Ad vectors are capable of delivering genes to a broad variety of cell types, and relevant to their use as a vaccine is their ability to infect DCs and other APCs in vivo. The Ad vector itself may act as adjuvant by inducing a strong inflammato y response at the injection site and by promoting the differentiation of immature DCs into professional APCs [54–56]. Recombinant Ad vectors expressing a wide variety of pathogen-specifi genes have been used in vaccination studies in rodents, canines, and nonhuman primates [57–72]. Ad vectors induce protective adaptive immune responses against the transgene product very rapidly after a single application [19–26]. This feature is particularly useful for postexposure vaccination or to combat infectious agents that cause infrequent but rapidly spreading outbreaks associated with high mortality.

The present evaluation of the efficac of an Ad vaccine, AdsecV, expressing the *Y. pestis* V antigen demonstrated the induction of rapid protective humoral and cellular immune responses. AdsecV antibody responses were elicited rapidly (within 2 weeks after administration) and showed the characteristic Th1/Th2 responses elicited by Ad vectors. Most importantly, the immune responses evoked by AdsecV were sufficien to protect immunized mice against an intranasal challenge with a fully virulent strain of *Y. pestis*. Together, these data suggest that AdsecV is a promising vaccine candidate for protection against plague.

Acknowledgments

We thank T. Niven, for performing the antibody assays; Dr. D. Perlin and the staff of the Public Health Research Institute at the International Center for Public Health, for help with the biosafety level 3 studies; and N. Mohamed, for help in preparing the manuscript.

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