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A Replication-Defective Human Adenovirus Recombinant Serves as a Highly Efficacious Vaccine Carrier

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In this manuscript, an E1 and E3 deleted adenoviral recombinant expressing the rabies virus glycoprotein (G protein) under the control of the cytomegalovirus early promoter was tested for induction of a rabies virus-specific immune response in mice. The construct was found to induce neutralizing antibodies and cytolytic T cells to rabies virus. Mice vaccinated with the adenoviral construct either by the systemic route or by application into the airways were protected against a subsequent infection with a virulent strain of rabies virus. The efficacy of the replication-defective construct was far superior to that of a well-characterized vaccinia rabies glycoprotein recombinant.

INTRODUCTION

Recombinant replication-defective adenoviruses are being developed for use in human gene therapy to replace missing or faulty genes (Kozarsky and Wilson, 1993). These adenoviruses based on human serotypes 2 or 5 are rendered replication-defective by the deletion of essential genes from the E1 locus, required for initiation of viral replication (Ginsberg *et al.,* 1989). Furthermore, these constructs lack E3 expression which is not essential for viral replication but which encodes a protein that by down-regulating expression of major histocompatibility complex (MHC) antigens protects infected cells from T-cell-mediated destruction (Ginsberg *et al.,* 1989). Recombinant replication-defective adenoviruses are considered attractive candidates for human gene therapy; they can be readily grown and purified in large quantities and they have the ability to express high levels of the transantigen in most cells, including those that are nondividing. The replication-defective adenoviral constructs lack many of the risks inherent to multiplying agents and are thus well tolerated even at high doses. Experiments in animals as well as in humans using recombinant adenoviruses expressing a recombinant gene initially yielded promising results by showing high expression of the adenovirus-encoded protein either in hepatocytes after intravenous application or in cells of the airway mucosa after inhalation (Jaffe *et al.,* 1992; Kozarsky *et al.,* 1993; Rosenfeld *et al.,* 1992). But, expression was transient in immunocompetent individuals, causing a mononuclear inflammation concomitant with the loss of virally infected cells (Yang *et al.,* 1994). Further studies in inbred mice revealed that the adenoviral constructs induced a potent immune response including $CD8^+$ cytolytic T cells which destroyed the adenoviral antigen-expressing cells within 3 to 4 weeks (Yang *et al.,* 1994). On the other hand, immunodeficient SCID or RAG-2^- mice or mice lacking functional $CD8^+$ T cells showed persistent expression of the adenovirus-encoded protein (Yang *et al.,* 1994), confirming, in addition, that the construct per se had no cytopathic effects on infected cells.

The strong immune response induced by the replication-defective adenoviral recombinants led us to test this construct for its potential as a vaccine carrier using the well-defined murine rabies virus model. In this manuscript we describe the immune response to a rabies virus glycoprotein (G protein) expressing recombinant based on the replication-defective human adenovirus type 5 (Ad5). The replication-defective construct was shown to be highly efficacious in inducing protective immunity to rabies virus.

MATERIALS AND METHODS

Animals

Female C3H/He and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Outbred ICR mice were purchased from Harlan Sprague–Dawley (Indianapolis, IN). Mice were maintained at the Animal Facility of The Wistar Institute and used between 8 and 12 weeks of age.

Cells

Baby hamster kidney (BHK)-21 cells, L929 mouse fibroblasts, and HeLa cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), HEPES buffer, and antibiotics in a 10% CO_2 incubator. 293 cells (Graham *et al.,*

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1977) were grown in DMEM with 10% FBS without HEPES buffer in a 5% CO_2 incubator. A rabies virus-specific T helper cell clone derived from spleens of C3H/He mice immunized with the vaccinia rabies glycoprotein (VRG) recombinant virus was cultured in weekly intervals on irradiated syngeneic splenocytes pretreated with inactivated rabies virus in DMEM supplemented with 2% FBS, 10^{-6} *M* 2-mercaptoethanol, and 10% rat Concanavalin A supernatant as a lymphokine source as described previously (Otvos *et al.,* 1994). The B cell hybridoma cells 509-6, 1112-1, and 523-11 secreting antibodies to different antigenic sites of the rabies virus G protein (509-6 to site I, 1112-1 to site II, and 523-11 to site III; Wiktor and Koprowski, 1978) were grown in DMEM supplemented with 10% FBS. Ascitic fluid was prepared in BALB/c mice. L929 cells stably transfected with pSG5rab.gp[1] vector, expressing the rabies virus G protein as well as L929 cells transfected with pSV2neo, were maintained in 10% DMEM supplemented with 10% FBS. These cell lines used as target cells for cell-mediated cytolysis assays have been described in detail previously (Xiang and Ertl, 1994).

Viruses

Rabies virus of the Evelyn Rokitniki Abelseth (ERA) strain was propagated on BHK-21 cells, purified, and inactivated with betapropionolactone (BPL, inactivated ERA: ERA-BPL) as described previously (Wiktor, 1973). The challenge virus standard (CVS)-24 strain of rabies virus, which is antigenically closely related to the ERA strain but shows higher virulence in mice, was derived from brain suspensions of infected newborn ICR mice (Wiktor *et al.,* 1977). The VRG virus was propagated on HeLa cells as described (Wiktor *et al.,* 1984).

The recombinant adenovirus expressing the rabies virus G protein of the ERA strain was constructed as follows: the pAdCMVlacZ vector (Wilson *et al.,* 1994), which contains the *lacZ* gene under the control of the cytomegalovirus (CMV) promoter and a segment encoding the 5′ portion of Ad5 upstream of the CMV promoter and flanking E2 sequences (downstream of the CMV promoter), was completely digested with *Not*I to remove the *lacZ* gene. The rabies virus G cDNA was purified from the pSG5rab.gp vector (Burger *et al.,* 1991) upon digestion with *Bgl* II. Following blunt-ending (Klenow) the G gene was cloned into the adenoviral vector. The appropriate orientation of the insert was confirmed by restriction enzyme mapping. The linearized plasmid was cotransfected into 293 packaging cells with an E3 deleted Ad5 DNA that had been digested with CLaI to remove the left end of the adenovirus, rendering the DNA noninfectious. In consequence, only products of homologous recombination could produce infectious virus in 293 cells (which had previously been stably transfected with the E1 gene of human Ad5 to allow replication of the adenoviral construct; Graham *et al.,* 1977). Several recombinant viral plaques were harvested and tested for expression of the rabies virus G protein as described below. One clone termed Adrab.gp was purified by two rounds of plaque purification and used for further studies.

The adenoviral recombinants, Adrab.gp, the previously described H5.010CMVlacZ expressing *Escherichia coli* lacZ (Wilson *et al.,* 1994), and a replication-competent adenovirus with E3 deleted [Ad5d17001] were grown on 293 cells for 72 hr. Virus was recovered on the third round of freeze–thawing. Cell-free supernatants either were used directly or were further purified by CsCl density centrifugation. Viral stocks were titrated on 293 cells using a plaque assay.

Indirect immunofluorescence

Cells adherent to glass coverslips were infected for 48 hr with 1 plaque-forming unit (PFU) per cell of the adenovirus recombinant. Cells were washed and treated on ice for 60 min with a monoclonal antibody (mAb) to rabies virus g protein. Cells were rinsed with phosphate-buffered saline and then incubated for 60 min on ice with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin (Ig) antibody. Cells were rinsed again and analyzed by confocal microscopy.

Immunization and infection of mice

Groups of ICR or C3H/He mice were injected subcutaneously (s.c.), orally, intranasally (i.n.), or upon anesthesia and surgical exposure of the trachea intratracheally (i.t.) with the adenoviral constructs diluted in 100 to 150 μl of saline. VRG virus was given s.c. For protection studies, groups contained five to eight mice; for most immunological studies, three to four mice were used per group. All data give the results for sera or spleens pooled from the different groups. Mice were bled by retro-orbital puncture in regular intervals after immunization to assess serum antibody titers. Mice were challenged with 10 mean lethal doses (LD_{50}) of CVS-24 virus given intramuscularly (i.m.) into the masseter muscle; they were observed for the following 3 weeks for symptoms indicative of a rabies virus infection. Mice that developed complete bilateral hindleg paralysis (proceeding death by 24 to 48 hr) were euthanized for humanitarian reasons.

Neutralization assay

Virus neutralizing antibody (VNA) titers were determined on BHK-21 cells using infectious ERA virus at 1 PFU per cell (Dietzschold *et al.,* 1987). Data are expressed as neutralization titers which are the reciprocal of the serum dilution resulting in a 50% reduction in the number of infected cells. Samples were assayed in duplicate in serial threefold dilutions starting with a dilution of 1:5. Standard deviations were within 10% for any given experiment.

Enzyme-linked immunosorbent assay (ELISA)

The ELISAs were conducted in 96-well microtiter plates coated with 2 μg per well of ERA-BPL virus or with purified adenovirus using an alkaline phosphatase-conjugated goat anti-mouse Ig as second antibody as described in detail (Xiang and Ertl, 1992).

T cell proliferation assay

The T cell clone was cultured (2×10^4 cells/well) in 96-well round-bottom microtiter plate wells with 5×10^5 irradiated C3H/He splenocytes pretreated with different antigen preparations in DMEM supplemented with 2% FBS and 10^{-6} *M* 2-mercaptoethanol. Proliferation of the cloned T cells was assessed 48 hr later by a 6-hr pulse with 0.5 μCi of [^{3}H]thymidine (Ertl *et al.,* 1991).

Cell-mediated cytolysis

Splenocytes were harvested from immunized C3H/He mice. Single cells were prepared and incubated at 6×10^6 cells per well with 1 PFU per cell of the Adrab.gp recombinant virus in 1.6 ml of DMEM supplemented with 10^{-6} *M* 2-mercaptoethanol and 2% FBS for 5 days in a humidified 10% CO_2 incubator. The effector cells were then cocultured with ^{51}Cr-labeled L929 cells expressing the rabies virus G protein upon stable transfection with the pSG5rab.gp vector at varied effector-to-target cell ratios. To assess spontaneous release, ^{51}Cr-labeled target cells were incubated with medium; to determine maximal release, target cells were cocultured with 10% sodium dodecyl sulfate. Cell-free supernatants were harvested 4 hr later and radioactivity was measured. Percentage of specific lysis was calculated by using the following formula (Yang *et al.,* 1994):

$$\frac{\text{Release in presence of effectors} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100.$$

RESULTS

Antibodies and T cells to the rabies virus G protein recognize cells infected with the Adrab.gp construct

To confirm that the Adrab.gp recombinant virus expresses the rabies virus G protein on infected cells in a form recognized by antibodies as well as T cell to rabies virus, a series of *in vitro* experiments were performed initially. To assess the conformation of the G protein as expressed by the Adrab.gp virus, HeLa cells were infected for 48 hr with 1 PFU of Adrab.gp virus per cell or as a control with a similar adenoviral construct expressing lacZ. Cells were then stained by an indirect immunofluorescence assay using mAbs (523-11, 509-6, and 1112-1) to different conformation-dependent binding sites of the rabies virus G protein. As shown for one of the antibodies (509-6) in Fig. 1, Adrab.gp virus-infected cells exhibited surface staining with the antibody, while cells infected with the control construct expressing lacZ were negative.

Further *in vitro* studies showed that the Adrab.gp construct induced proliferation of a rabies virus G protein specific T helper cell clone in the presence of syngeneic, γ-irradiated splenocytes (Fig. 2). In a separate experiment, this T cell clone was shown to not proliferate to the H5.010 CMVlacZ construct (data not shown). Furthermore, mouse fibroblasts infected with the Adrab.gp recombinant were rendered susceptible to lysis by rabies virus G protein induced H-2 compatible cytolytic T cells (Xiang *et al.,* 1995). Together these *in vitro* experiments demonstrated that the Adrab.gp construct causes expression of the rabies virus G protein in a form that is readily recognized by both rabies virus-specific antibodies and T cells of the helper and the cytolytic subset.

The Adrab.gp construct induces in mice a potent immune response including antibodies and cytolytic T cells to rabies virus

In the next set of experiments mice were immunized with the Adrab.gp recombinant virus at several doses using different routes of immunization. In some experiments an adenoviral construct expressing the *lacZ* gene was used as a negative control, and the VRG recombinant was used as a positive control. Mice were bled 10 to 14 days after a single immunization with the vaccine and VNA titers were determined from the sera (Table 1). Adenovirus given s.c., i.t., or i.n. induced a potent neutralizing antibody response if given at 10^6 PFU. Oral immunization with Adrab.gp or systemic immunization with H5.010CMVlacZ failed to induce a measurable antibody response to rabies virus. The antibody responses to different doses of the replication-defective Adrab.gp construct were clearly superior to the response induced by the VRG recombinant. For example, the antibody titers of mice inoculated with as little as 2×10^4 PFU of Adrab.gp were more than 10 times higher than those of mice infected with 2×10^6 PFU of VRG (Table 1).

To ensure that the antibody response was caused by infection recombinant virus rather than by G protein fragments contaminating the virus-containing tissue culture supernatant used for immunization, mice were vaccinated with an equal dose of PFUs of unpurified and gradient purified recombinant adenovirus. Both groups of mice developed identical virus neutralizing antibody titers.

In addition to neutralizing antibodies, mice inoculated s.c. with Adrab.gp virus developed rabies virus G protein-specific cytolytic T cells (Fig. 3) able to kill H-2 compatible L929 target cells stably transfected with a plasmid vector expressing the rabies virus G protein under the control of the SV40 early promoter (Xiang and Ertl, 1994).

The Adrab.gp construct induces protection against challenge with a lethal dose of rabies virus

Mice immunized with the Adrab.gp construct or the VRG virus were challenged 2 to 5 weeks after immuniza-

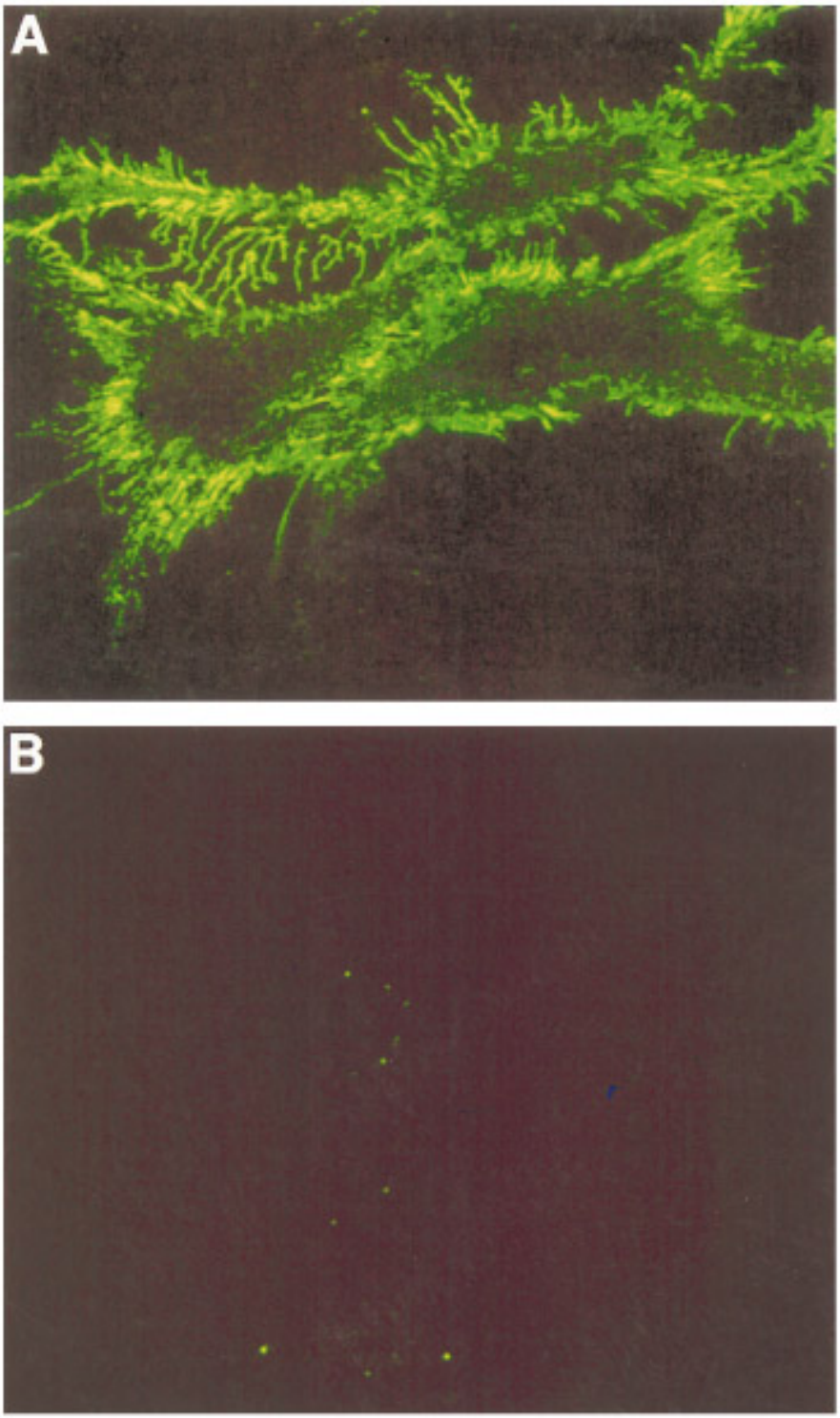

FIG. 1. The Adrab.gp construct causes expression of the rabies virus G protein on the surface of infected cells. L929 fibroblasts, plated on glass coverslips, were infected with 1 PFU of Adrab.gp (A) or of H5.010CMVlacZ (B). The following day cells were incubated for 60 min on ice with a 1:1000 dilution of ascitic fluid containing antibody 509-6. Cells were rinsed with phosphate-buffered saline and treated for 60 min with an FITC-labeled goat anti-mouse Ig preparation. Staining was assessed under a confocal microscope.

tion with 10 LD_{50} of the virulent CVS-24 strain of rabies virus given i.m. into the masseter muscle. As shown in Table 2, mice immunized with Adrab.gp s.c., i.t., or i.n. using doses ranging from 10^4 to 2×10^6 PFU were fully protected against infection, 87% of mice inoculated with 10^3 PFU were protected, while all mice immunized with only 10^2 PFU of the adenoviral construct or inoculated with the H5.010CMVlacZ control (2×10^6 PFU) construct or with Adrab.gp *per os* developed a fatal rabies virus encephalitis within 10 days after infection. Mice vacci-

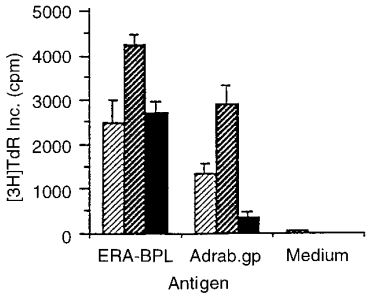

FIG. 2. The Adrab.gp construct induces in the presence of antigen presenting cell proliferation of a rabies virus G protein T helper cell clone. Irradiated C3H/He splenocytes plated at 5×10^5 cells per round-bottom microtiterplate well were incubated with 5 (▨), 1 (▨, or 0.2 (■) μg/ml of ERA-BPL virus or Adrab.gp at ~1 (▨), 0.1 (▨), and 0.01 (■) PFU per cell or as a negative control medium for 60 min at 37°. Cloned T cells (2×10^4/well) were added. Cells were pulsed for 6 hr with 0.5 μCi of [^{3}H]thymidine 48 hr later, harvested onto filtermats, and counted in a β-counter. Data present counts per minute (cpm) ± SD.

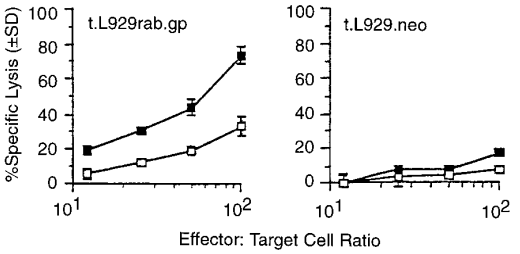

FIG. 3. The Adrab.gp construct induces cytolytic T cells to the rabies virus G protein. Groups of C3H/He mice were inoculated with 2×10^6 PFU of Adrab.gp (■) or H5.010CMVlacZ (□). Splenocytes were harvested 14 days later and cocultured for 5 days with 1 PFU of Adrab.gp virus per cells. Activated lymphocytes were then tested at different effector to target cell ratios on H-2 compatible L929 cells stably transfected with a rabies virus G protein-expressing vector (t.L929rab.gp, left) or as a control with a neomycin-expressing vector (t.L929.neo, right) in a 4-hr ^{51}Cr-release assay. Data are means of triplicates ± SD.

nated with VRG showed partial protection; the group receiving the highest dose, i.e., 2×10^6 PFU had a mortality rate above 50%, rising to ~90% in mice inoculated with 2×10^4 PFU of VRG.

In the next set of experiments we tested the kinetic of the induction of protective immunity upon vaccination with the Adrab.gp virus. Vaccination to rabies virus is in general given postexposure, hence it is crucial for the vaccine to induce a rapid immune response before the rabies virus has reached the central nervous system. As shown in Fig. 4, mice vaccinated with Adrab.gp virus 10 days previously were completely protected while more than half of the animals were protected as early as 7 days after a single injection. Mice vaccinated 3 days before challenge succumbed to the infection.

The Adrab.gp construct induces a B and T cell response to rabies virus in spite of preexisting immunity to adenovirus

To test if preexisting immunity to adenoviral proteins interferes with stimulation of a rabies G protein-specific

TABLE 1

Adrab.gp Recombinant Induces Neutralizing Antibodies to Rabies Virus[a]

Vaccine	Dose	Route of immunization	Time after	VNA titer immunization
Adrab.gp	2×10^6	s.c.	Day 10	3645
Adrab.gp	2×10^5	s.c	Day 10	405
Adrab.gp	2×10^4	s.c.	Day 10	405
VRG	2×10^6	s.c.	Day 10	45
VRG	2×10^5	s.c.	Day 10	15
VRG	2×10^4	s.c.	Day 10	5
None	—	—	Day 10	<5
Adrab.gp	10^4	s.c.	Day 14	1215
Adrab.gp	10^3	s.c.	Day 14	405
Adrab.gp	10^2	s.c.	Day 14	<5
Adrab.gp	10^6	i.n.	Day 14	1215
Adrab.gp	10^6	i.t.	Day 14	3645
Adrab.gp	10^6	*Per os*	Day 14	<5
None	—	—		<5

[a] Groups of ICR mice were immunized in three separate experiments with the different vaccine constructs given at the indicated doses i.m., i.n., i.t., or *per os.* Mice inoculated into the trachea or i.n. were anesthetized prior to vaccination. Mice were bled 10 to 14 days later and serum antibody titers to rabies virus were tested by a neutralization assay. Data are expressed as neutralization titers, which are the reciprocal of the dilution resulting in a 50% reduction in the number of infected cells.

TABLE 2

Adrab.gp Recombinant Induces Protective Immunity to a Challenge with Rabies Virus[a]

Vaccine	Dose	Route of immunization	% Mortality
Adrab.gp	2×10^6	s.c.	0
H5.010CMVlacZ	2×10^6	s.c.	90
Adrab.gp	2×10^6	s.c.	0
Adrab.gp	2×10^5	s.c.	0
Adrab.gp	2×10^4	s.c.	0
VRG	2×10^6	s.c.	56
VRG	1×10^5	s.c.	71
VRG	2×10^4	s.c.	86
None	—	—	100
Adrab.gp	10^4	s.c.	0
Adrab.gp	10^3	s.c.	13
Adrab.gp	10^2	s.c.	100
None	—	—	100
Adrab.gp	10^6	i.n.	0
Adrab.gp	10^6	i.t.	0
Adrab.gp	10^6	*Per os*	100
None	—	—	100

[a] This table summarizes the results from four different experiments. Mice immunized as described in legend to Table 1 were challenged 21–28 days after immunization with 10 LD_{50} of rabies virus. Mice that subsequently developed complete bilateral hindleg paralysis indicative for a terminal rabies virus infection were euthanized for humanitarian reasons. Survivors were observed for a total of 21 days.

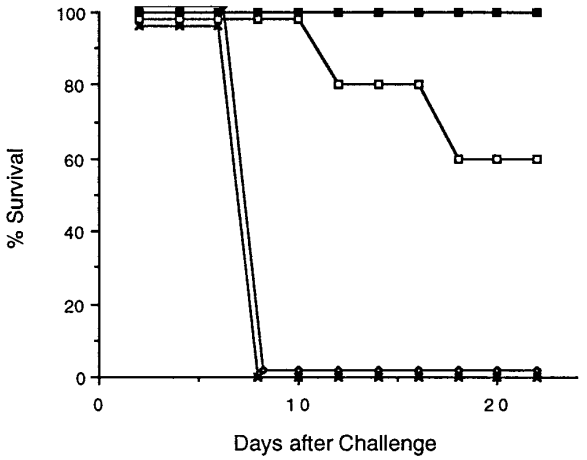

FIG. 4. Mice were immunized with 10^6 PFU of Adrab.gp s.c. Immunized mice were challenged 3 (◇), 7 (□), and 10 (■) days after vaccination with 10 LD_{50} of rabies virus given i.m. Naive mice (×) served as controls. Mice were observed for 4 weeks to record mortality.

immune response to the Adrab.gp construct, groups of mice were inoculated with an E3 deleted adenovirus of the human strain 5. Three weeks later mice were boosted with 10^6 PFU of Adrab.gp. Mice preimmunized with adenovirus developed VNA to rabies virus upon booster immunization with the Adrab.gp construct; titers were slightly lower when compared to those in control mice that had only received the Adrab.gp construct (Table 3), indicating that antibodies to adenoviruses marginally inhibit the B cell response to proteins expressed by adenovirus recombinants. When tested in comparison to a reference serum provided by the World Health Organization, sera from preimmune (both doses of adenovirus) or naive mice were shown to have titers of 40 IU to rabies virus. Protection to rabies virus is correlated to antibody titers and 2 IU are considered sufficient to protect against a severe challenge. Preimmunity to adenovirus does, thus, not impair the ability of the Adrab.gp vaccine to elicit protective immunity. Similar data were obtained for the stimulation of cytolytic T cells to rabies virus-infected cells, and preimmune animals showed somewhat lower

TABLE 3

The Effect of Preexisting Immunity to Adenovirus on the Rabies VNA Response to the Adrab.gp Vaccine[a]

Preimmunization	Immunization	VNA titer
None	10^6 PFU Adrab.gp	3.645
10^5 PFU Ad5d17001	10^6 PFU Adrab.gp	3.645
10^6 PFU Ad517001	10^6 PFU Adrab.gp	1.215
None	None	<5

[a] Groups of C3H/He mice were immunized with 10^5 or 10^6 pFU of a replication-competent adenovirus human serotype 5 that had the E3 gene deleted. Mice were injected 4 weeks later with 10^6 PFU of Adrab.gp. Control mice were only injected with Adrab.gp (10^6 PFU). Mice were bled 12 days later and neutralizing antibody titers were determined.

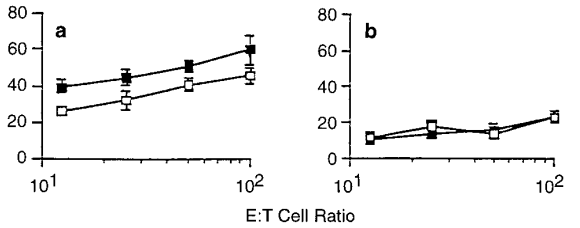

FIG. 5. The cytolytic T cell response to rabies virus G protein expressing target cells upon immunization with Adrab.gp is only slightly reduced in animals immune to adenovirus. C3H/He mice were inoculated with 10^6 PFU of replication-competent E3 deleted adenovirus. They were boosted 3 weeks later with 10^6 PFU of Adrab.gp (□). Control mice were inoculated with Adrab.gp only (■). Mice were sacrificed 4 weeks later and upon restimulation with 1 PFU of Adrab.gp per cell tested on a 4-hr ^{51}Cr-release assay on L929 cells stably transfected with pSG5rab.gp (a) or with pSV2neo (b). Data are expressed as the mean percentage specific lysis of triplicate results ± SD.

lysis compared to the control group. Nevertheless, adenovirus-immune mice still developed significant T cell responses to the rabies virus G protein upon immunization with Adrab.gp (Fig. 5).

DISCUSSION

The advance of molecular biology allowing manipulation of genetic material has benefited both the development of vaccines and more recently the generation of reagents suitable for gene therapy. In some instances, these two fields that fundamentally differ in their goals, one aiming at the induction of a protective immune response to pathogens, the other at the permanent replacement of faulty or missing genes, have been shown to overlap. For example, i.m. transfer of plasmid DNA was shown by gene therapists to result in long-term expression of vector-encoded sequences (Wolff *et al.,* 1990, 1992); immunologists subsequently modified the approach by using plasmids encoding foreign antigens such as viral proteins and demonstrated induction of protective immune responses, opening the new field of DNA vaccines (Ulmer *et al.,* 1993). Similarly, in this manuscript, a construct based on an E1 and E3 deleted Ad5 that was initially developed for gene therapy to cure diseases such as cystic fibrosis (Rosenfeld *et al.,* 1992) was found to be highly immunogenic and thus, in its current form, unsuitable for its intended purpose. The potent immune response elicited by the replication-defective adenovirus leading to $CD8^+$ T-cell-mediated elimination of infected cells within 3 to 4 weeks (Yang *et al.,* 1994) led us to test its potential use as a vaccine carrier. The recombinant adenovirus expressing the rabies virus G protein under the control of the CMV early promoter in a form that was readily recognized by antibodies to conformation-dependent epitopes as well as by activated rabies virus G protein-specific T cells induced by either live rabies virus or the VRG recombinant was found to be extraordinarily efficacious at inducing protective immunity to challenge with rabies virus; mice immunized

with as little as 10^4 PFU of Adrab.gp were completely protected against rabies. Partial protection was already seen 7 days after immunization. The efficacy of this replication-defective construct was thus far superior compared to that of the previously described VRG vaccine that is currently being used for oral wildlife immunization (Brochier *et al.,* 1994). The VRG virus has previously been shown to replicate for at least 48 hr in infected mice (United States Department of Agriculture, Animal and Plant Health Inspection Service, 1992), thereby increasing the antigenic load by several orders of magnitude. The finding that the replication-defective Adrab.gp construct was the more efficacious of the two vaccines was thus unexpected. Other parameters such as appropriate presentation of antigen by professional antigen presenting cells or cytokine release by infected cells might influence the magnitude of the immune response. Pox viruses are known to subvert the immune system by encoding a protein that mimics the IL-1 receptor and an additional protein that inhibits the enzyme that cleaves the IL-1β precursors protein (Marrack and Kappler, 1994). Wild-type adenoviruses evade immune surveillance by down-regulating MHC expression, a function of products of the E3 locus (Ginsberg *et al.,* 1989) deleted in all of the viruses used in this study. In addition to the antigenic load, the kinetics of antigen expression might affect the magnitude of the specific immune response. Short-lived antigens such as peptides that are rapidly degraded by ubiquitous peptidases are generally poorly immunogenic unless administered at high concentrations in adjuvant. Cells infected with the VRG recombinant rapidly express substantial amounts of the rabies virus G protein on the cell surface but then die shortly after infection. The Adrab.gp construct on the other hand results in slow accumulation of the rabies virus G protein on the surface of infected cells without causing visible cell damage (data not shown). As shown previously *in vivo* using an adenovirus recombinant carrying the *lacZ* reporter gene, expression of proteins encoded by the replication-defective adenoviral construct persists for at least 7 days in immunocompetent mice until $CD8^+$ cytolytic T cells eventually eliminate the infected cells (Yang *et al.,* 1994). The duration of antigen expression on individual cells is thus considerably longer upon expression of the rabies virus G protein by the adenoviral recombinant than upon infection with the VRG virus which in turn might result in a superior immune response. Adenoviral constructs deleted in E1 and E3 have previously been used as vaccines (Eloit *et al.,* 1990; Ragot *et al.,* 1993). In contrast to the adenoviral recombinant described in this manuscript, these constructs expressed the foreign viral gene under the control of an adenoviral promoter. Although they induced in animals an antibody response to the inserted viral protein, protection to viral challenge was poor, suggesting that differences in the level of expression caused by promoters has a significant impact on the efficacy of an adenoviral vaccine. Replication-competent, recombinant adenoviruses have also been previously used as vaccine carriers. One of these recombinants expressing the rabies virus G protein was shown to induce protective immunity in animals to challenge with rabies virus (Prevec *et al.,* 1990). The efficacy of this vaccine was comparable to that observed with a vaccinia recombinant; doses above 10^6 PFU of this replication-competent virus were required to induce complete protection to viral challenge.

The Adrab.gp vaccine was found to be efficacious upon systemic application or administration into the airways. Unlike the previously described replication-competent adenovirus recombinants immunity could not be elicited upon oral immunization (Prevec *et al.,* 1990).

Adenoviruses cause early childhood infections that are manifested by mild respiratory symptoms. Most children become seropositive within their first year of life, which might potentially interfere with active immunization using a vaccine based on human adenoviruses. Data presented here show only a small reduction of the immune response to the rabies virus G protein in mice that had recently been infected with a high dose of replication-competent adenovirus. Further studies are needed to establish the specificity and the kinetics of this inhibition.

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