DNA immunization protects nonhuman primates against rabies virus

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More than 40,000 people die annually from rabies worldwide[1]. Most of these fatalities occur in developing countries, where rabies is endemic, public health resources are inadequate and there is limited access to preventive treatment[2]. Because of the high cost of vaccines derived from cell culture, many countries still use vaccines produced in sheep, goat or suckling mouse brain[3]. The stability and low cost for mass production of DNA vaccines would make them ideal for use in developing countries[4]. To investigate the potential of DNA vaccines for rabies immunization in humans, we vaccinated *Macaca fascicularis* (*Cynomolgus*) monkeys with DNA encoding the glycoprotein of the challenge virus standard rabies virus, or with a human diploid cell vaccine (HDCV). The monkeys then were challenged with a non-passaged rabies virus. DNA or HDCV vaccination elicited comparable primary and anamnestic neutralizing antibody responses. All ten vaccinated monkeys (DNA or HDCV) survived a rabies virus challenge, whereas monkeys vaccinated with only the DNA vector developed rabies. Furthermore, serum samples from DNA- or HDCV-vaccinated monkeys neutralized a global spectrum of rabies virus variants *in vitro*. This study shows that DNA immunization elicits protective immunity in nonhuman primates against lethal challenge with a human viral pathogen of the central nervous system. Our findings indicate that DNA vaccines may have a promising future in human rabies immunization.

Rabies is an almost invariably fatal encephalomyelitis[5]. The World Health Organization estimates that 10–12 million people in developing countries receive one or more doses of rabies vaccine after exposure to the virus. An estimated 5 million people in China and over 1 million people in India begin treatment[1]. Many developing countries continue to use vaccines produced in sheep, goat or suckling mouse brain, with subsequent inactivation of the virus with ultraviolet light or phenol[6,7]. The World Health Organization recommends that vaccines derived from brain tissue be discontinued and replaced with vaccines produced in tissue culture[8]. Although vaccines derived from cultured cells, such as the human diploid cell vaccine (HDCV), are very effective and well tolerated, they are expensive to produce[3].

DNA vaccines offer a new and powerful approach for the generation of needed vaccines[4]. Advantages of DNA vaccines that distinguish them from conventional vaccines are the ease of their construction, their ability to induce a full spectrum of long-lasting humoral and cellular immune responses, and their high temperature stability, which would be a particular advantage for use in tropical areas, where refrigeration is difficult to maintain. Moreover, the low cost for their mass production makes DNA vaccines ideally suited for developing countries. Two important preliminary steps before human clinical investigation begins are to determine the utility of the DNA vaccine in eliciting protective immunity in nonhuman primates and to compare the effectiveness of DNA immunization with that of vaccination with a standard such as the HDCV. Thus, nonhuman primates (*Macaca fascicularis* (*Cynomolgus*) monkeys) were immunized with DNA encoding a rabies virus glycoprotein or with HDCV, and then challenged with a lethal dose of rabies virus.

Thirty days after immunization, a peak antibody titer was detected in the two monkeys that had been vaccinated with HDCV. The antibody titer of the four monkeys immunized with DNA by gene gun was much lower (Figs. 1 and 2). After the peak antibody response was attained at 30 days, the neutralizing antibody titer of the HDCV-immunized monkeys gradually decreased, whereas the antibody titer of the monkeys vaccinated by gene gun increased approximately fourfold by day 60, and then remained fairly constant for 180 days. Rabies neutralizing antibody was not detected in the four monkeys that had been vaccinated intradermally by needle injection with DNA. All monkeys were administered booster vaccinations 190 days after primary immunization (Figs. 1 and 2). Ten days after the booster, the geometric mean neutralizing antibody titer of the HDCV-vaccinated monkeys had increased more than 18-fold, whereas the geometric mean antibody response of the monkeys that had been vaccinated with DNA by gene gun had an even more dramatic increase of 176-fold. For the next 220 days, antibody titers of the monkeys that had been vaccinated with HDCV or DNA using the gene gun gradually decreased to similar levels. In distinct contrast to the anamnestic neutralizing antibody responses of the monkeys vaccinated by gene gun and HDCV, rabies neutralizing antibody remained undetectable in monkeys that had received primary and booster intradermal needle injection vaccinations of DNA. Consequently, at 60 days after booster immunization, the non-responsive monkeys vaccinated by intradermal injection were re-vaccinated, but with 100 μg of DNA delivered intramuscularly. Subsequently, a minimal antibody titer was detected in each of the four monkeys at day 10, increasing to a peak geometric mean titer by day 30 (Figs.1 and 2).

To test the efficacy of the DNA vaccine in comparison with HDCV, the twelve monkeys were challenged with a non-passaged rabies virus isolate from a salivary gland homogenate of a naturally infected rabid dog[9]. At least two human deaths in Texas have been associated with transmission of this virus since

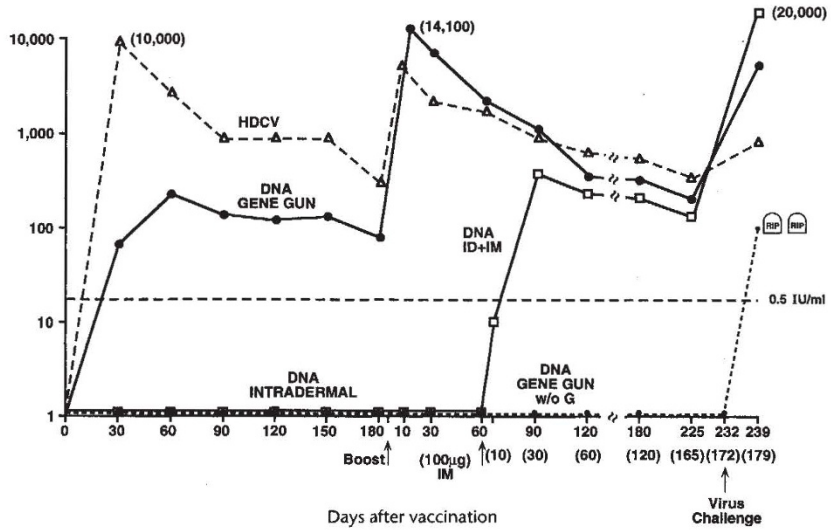

Fig. 1 Geometric mean anti-rabies-virus neutralizing antibody titers in serum after DNA or HDCV vaccination and subsequent rabies virus challenge. DNA encoding glycoprotein administered by intradermal and then intramuscular injection (□) or by gene gun (•—•): Four of four monkeys (100%) protected in each group. HDCV administered by intramuscular injection (△): two of two monkeys (100%) protected. DNA without glycoprotein (vector alone) administered by gene gun (•---•): Zero of two monkeys (0%) protected. Horizontal dashed line, 0.5 IU/ml, is the minimal acceptable human level of antibody titer. Numbers in parenthesis on horizontal axis indicate days after 're-boosting' of monkeys initially immunized intradermally (□). RIP: The two negative-control monkeys that were not protected (•---•) developed CNS signs of rabies and were sedated and killed.

1980. At the time of the challenge, the antibody titer of each vaccinated monkey exceeded the minimal acceptable human level[10] of 0.5 IU/ml (1:40 serum dilution) (Fig. 2). The titer of only one DNA-vaccinated monkey exceeded the titers of the monkeys that had received HDCV. One week after virus challenge, neutralizing antibody titers of the ten vaccinated monkeys had increased considerably. Furthermore, the individual titers of six of eight (75%) DNA-vaccinated monkeys exceeded the titers of the monkeys that had received HDCV (Figs. 1 and 2). At this time, neutralizing antibody was detected for the first time in the two negative control monkeys that had received only the DNA vector (Figs. 1 and 2). On days 10 and 11 after the challenge, the negative control monkeys displayed lethargy, anorexia, cranial nerve deficits, altered phonation and paresis, all abnormal CNS clinical signs associated with rabies. Consequently, they were sedated and killed. Brain impressions of both monkeys were positive for rabies virus antigen as detected by a direct fluorescent antibody test. Six months after the rabies virus challenge the ten vaccinated monkeys were killed. All brain impressions were negative for rabies virus antigen.

Table Worldwide isolates of rabies virus neutralized[a] with serum samples from monkeys vaccinated with DNA or HDCV

Reservoir	Country
Dog	Indonesia
Dog	Thailand
Dog[b]	Laos
Dog[b]	Philippines
Dog	China
Fox	USA-Alaska
Dog	India
Dog	USA-Texas
Dog[b]	Mexico
Laboratory strain (ERA)	USA-Alabama
Laboratory strain (CVS)	France-Pasteur Institute
Dog	Nigeria
Vampire bat	Latin America
Red bat	USA-Florida
Silver-haired bat	USA-Arkansas
Raccoon	USA-Massachusetts
Skunk	USA-Texas
Little brown bat	USA-Montana

[a]In each instance, 100% of the infectivity was neutralized. [b]Virus isolates from humans who had been bitten by rabid dogs and died from rabies after incubation periods of 1, 4 or 6 years. ERA, Evelyn-Rokitnicki-Abelseth; CVS, challenge virus standard.

Recent studies have identified many rabies virus variants associated with different animal reservoirs and geographical areas[11–14]. To determine whether antibody produced in nonhuman primates immunized with a DNA vaccine would neutralize variant viruses that have been isolated worldwide, serum samples obtained before the virus challenge were tested in neutralization assays against 18 different viruses (Table). Serum samples from the DNA- or HDCV-vaccinated monkeys that had been adjusted to contain 0.5 IU/ml of antibody neutralized 100% of the infectivity of each worldwide isolate of virus, as well as the rabies virus laboratory strains, challenge virus standard and Evelyn-Rokitnicki-Abelseth (Table). In contrast, antisera generated by vaccination with the vector alone failed to neutralize any of the viruses (data not shown). These neutralization data, in combination with the protection data, indicate that a DNA vaccine encoding for the glycoprotein of challenge virus standard could protect against a global spectrum of rabies viruses.

The method of DNA vaccination proved to be important parameter in these studies. Rabies neutralizing antibody was not detected in the four monkeys that had been vaccinated intradermally by needle injection with DNA, whereas monkeys that received DNA by gene gun delivery or by a combination of intradermal and intramuscular needle injections produced appropriate antibody responses. The failure of the monkeys to develop appropriate antibody responses after intradermal needle injection vaccination alone was somewhat perplexing, as the same plasmid injected intradermally elicited high levels of neutralizing antibody in rodents[15]. The data demonstrate that immunization results in other species cannot necessarily be used to predict results extrapolated to nonhuman primates. The determination that DNA vaccination does protect nonhuman primates against a considerable rabies virus challenge permits the investigation of additional issues such as the requirement for a booster vaccination, the duration of protection and the possibility of prophylaxis after exposure.

This study indicates that DNA vaccines may have a promising future in human rabies immunization. The elevated neutralizing

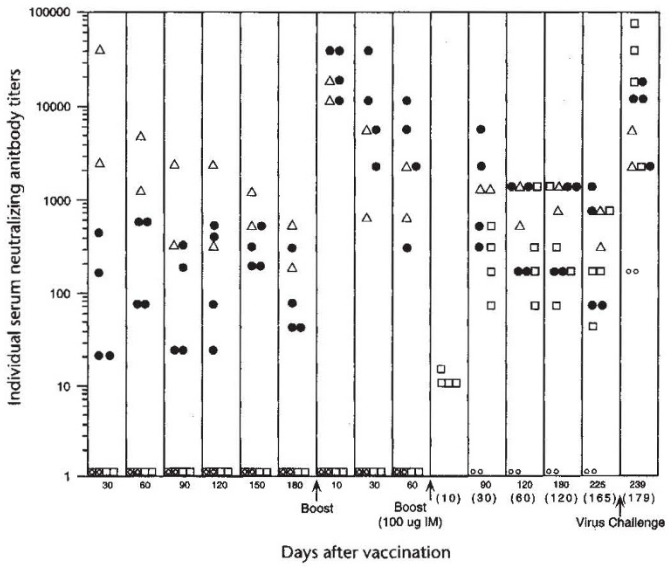

Fig. 2 Individual anti-rabies-virus neutralizing antibody titers in serum after DNA or HDCV vaccination and subsequent rabies virus challenge. HDCV administered by intramuscular injection (△) or DNA encoding glycoprotein administered by gene gun (●). DNA encoding glycoprotein administered intradermally and intramuscularly by needle injection (□). DNA without glycoprotein administered by gene gun (○). Numbers in parenthesis on horizontal axis indicate days after 're-boosting' of monkeys initially immunized intradermally (□).

antibody titers of the DNA-vaccinated monkeys after virus challenge are particularly noteworthy, as neutralizing antibody is the primary source of protection against rabies in the rodent system[16]. DNA vaccines could eliminate immunization with vaccines derived from tissues of the nervous system and could also provide an alternative to the costly HDCV and other cell-culture vaccines, especially for prophylactic purposes. Although nonhuman primates have been used in other DNA vaccination studies[17–20], this DNA vaccine study is the first, to our knowledge, to show complete protection of nonhuman primates against lethal challenge with a primary isolate of a human viral pathogen that invades the immune-privileged CNS.

Methods

Plasmid construction. Construction of the pCMV4 plasmid DNA vaccine encoding the glycoprotein of the challenge virus standard rabies virus has been described[15,21].

***Macaca fascicularis* (*Cynomolgus*)**. The *Macaca fascicularis* (*Cynomolgus*) colony at the Rocky Mountain Laboratories (RML) originated with captive monkeys from Mauritius Island. Ten of the experimental monkeys used in this study were wild-caught stock (3 to 8 years in the RML colony), while two were captive-born (from the RML colony). The monkeys in the RML colony are negative for retroviral and herpes B virus antibodies. The monkeys, randomly assigned to experimental groups, ranged in age from 3 to 10 years, with the six females ranging from 3.5 to 4.4 kg in weight and the males, from 4.1 to 9.9 kg in weight.

The monkeys were fed commercial high-protein monkey chow supplemented with fresh fruit and commercial monkey treats. Automatic watering systems provided water *ad libitum*. All monkeys, except the two colony-born males, had been used in previous experimental research involving a benign, self-limiting ocular infection with *Chlamydia trachomatis*. Inoculations and blood samples (collected from the cephalic vein using a 20-guage needle and a 5-ml syringe) were done while the monkeys were sedated with ketamine hyrochloride.

The animal facilities and animal care and use programs at the RML are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and function in accordance with all United States Department of Agriculture, Department of Health and Human Services and National Institutes of Health regulations and standards. The monkeys were housed indoors in artificial light (12 h/12 h dark/light cycle) and housed paired or singly in aluminum barred cages (15.1 square feet floor space × 63 inches high or 6.3 square feet floor space × 32 inches high, respectively). The colony rooms are maintained at 21 °C to 26 °C at 50% humidity. For virus challenge, the monkeys were shipped to the Centers for Disease Control and Prevention, Atlanta, Georgia by a domestic, commercial air freight provider, in full accordance with all United States Department of Agriculture, Department of Health and Human Services and National Institutes of Health regulations and standards. The husbandry of the Centers for Disease Control and Prevention animal facility is similar to that of the RML facility.

Immunizations. Primary immunizations with DNA were done either by the Dermal PowderJect XR gene gun delivery system (PowderJect Vaccines, Madison, Wisconsin) as described[21] or by intradermal needle injection. Four monkeys, each receiving 8 μg of DNA, were included in each group. The monkeys treated by gene gun were vaccinated above the axillary area of each upper arm and above the inguinal area of each upper thigh with 2 μg of DNA in each site. The DNA was coated onto 0.5 mg of gold beads 2.6 μm in diameter, according to the instructions provided by the gene gun manufacturers. The beads were administered with a helium pressure setting of 400 p.s.i. The monkeys vaccinated by intradermal injection were inoculated by needle in the same areas as the monkeys vaccinated by gene gun with an identical amount of DNA suspended in phosphate-buffered saline. Two negative-control monkeys were vaccinated by gene gun with a total of 8 μg of vector DNA only. Two positive-control monkeys received 0.5 ml of HDCV (Pasteur Merieux Serums and Vaccins S.A., Lyon, France) intramuscularly in each triceps muscle. A second vaccination identical to the first was given 7 days later. Booster immunizations identical to the primary vaccinations were given 190 days after primary immunization. The HDCV-vaccinated monkeys did not, however, receive a second booster 7 days after the initial 190-day booster. Sixty days after the 190-day booster immunization, the monkeys injected intradermally by needle were re-boosted, but with 100 μg of DNA delivered intramuscularly in the triceps muscle. Blood was collected at regular intervals and serum samples were tested for anti-rabies-virus neutralizing antibody.

Neutralizing antibody assay. Serum anti-rabies-virus neutralizing antibody titers were established by using the rapid fluorescent focus inhibition test[22] with chicken-embryo-related cells[23] and Evelyn-Rokitnicki-Abelseth virus at a multiplicity of infection of 1. The antibody titers in Fig. 1 are expressed as geometric means for each group of monkeys, whereas individual antibody titers are shown in Fig. 2. Antisera with known IU/ml of rabies virus neutralizing antibody, a rabies hyperimmune mouse serum and the U.S. Standard Human Rabies Immunoglobulin R2 were included as positive controls in all rapid fluorescent focus inhibition tests. An experimental titer of 1:40 was equivalent to 0.5 IU/ml. To test serum samples in neutralization assays against viruses that have been isolated worldwide, monkeys vaccinated with DNA or the HDCV were bled before the virus challenge. Serum samples were diluted to contain 0.5 IU/ml of neutralizing antibody, and then tested in a rapid fluorescent focus inhibition test against each virus that had been adjusted to a multiplicity of infection of 0.1:1.

Rabies virus challenge. The monkeys were challenged with rabies virus at the Centers for Disease Control and Prevention, Atlanta, Georgia. One week before the challenge (225 days after the initial booster; 422 days after primary vaccination), the monkeys were sedated with ketamine hydrochloride (10 mg/kg, injected intramuscularly), and blood samples were obtained to determine anti-rabies-virus neutralizing antibody titers. For the viral challenge, the monkeys were again sedated and then were inoculated in the right and left masseter muscles with 0.5 ml of a 1:5 dilution of a salivary gland homogenate obtained from a rabid dog naturally infected with a coyote rabies virus variant[9]. The viral titer of the stock salivary gland homogenate was $10^{6.5}$ mouse intracranial lethal $dose_{50}$/ 0.03 ml. After the challenge, the monkeys were observed several times daily for abnormal clinical signs associated with rabies. Animals showing abnormal CNS clinical signs associated with rabies were sedated and killed. At necropsy, the brains

were removed and brain impressions were made. The brain impressions were tested for rabies virus antigen by the direct fluorescent antibody test .

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