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NOTES

Genetic Immunization with Adeno-Associated Virus Vectors Expressing Herpes Simplex Virus Type 2 Glycoproteins B and D

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Intramuscular injection of mice with an adeno-associated virus (AAV) vector expressing herpes simplex virus type 2 glycoprotein B led to the generation of both gB-specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes and anti-gB antibody. AAV-mediated immunization was more potent than plasmid DNA or protein in generating antibody responses.

Adeno-associated virus (AAV), a single-stranded DNA virus, has been studied as a vector for gene therapy (7, 15). Many of the features that make AAV an attractive vector for gene therapy applications also make it an ideal candidate as a vector for genetic immunization. In sharp contrast to other viral vectors that have been used in vaccination such as vaccinia virus or adenovirus, AAV vectors do not express any viral genes. The only viral DNAs that must be included in an AAV vector are the 145-bp inverted terminal repeats (ITRs) (6, 9). As in immunization with naked DNA, the only gene expressed is the antigen itself. Genetic immunization with naked DNA has been done primarily by intramuscular injection (12). The mechanism of muscle cell uptake of naked DNA is poorly understood, and expression is only transient (3). Studies by several groups have shown that skeletal muscle is very efficiently transduced by recombinant AAV (rAAV) vectors and that expression is long-lasting (2, 4, 14). For vaccination then, AAV vectors represent a combination of the best properties of both viral and nonviral vectors. AAV vectors also have physical properties which make them ideal candidates as vaccine vectors. AAV vectors are small (25 nm), nonenveloped viruses with resistance to temperature, organic solvents, and both high and low pH. These characteristics may make AAV vectors ideal for oral and intranasal delivery, routes of administration important in the generation of potent mucosal immunity.

To examine the utility of AAV vectors for genetic immunization, we constructed vectors that expressed either herpes simplex virus type 2 (HSV-2) glycoprotein B (gB) or glycoprotein D (gD). To construct the gB vector, a gB mutant with the transmembrane region deleted (amino acids 703 to 776 deleted; kindly provided by Rae Lyn Burke, Chiron Corporation) was cloned into the polylinker of the expression vector pCI (Promega, Madison, Wis.). The fragment from this plasmid containing the cytomegalovirus promoter/intron, gB, and simian virus 40 poly(A) was cloned into pKm201, a modified AAV vector plasmid derived from pEMBL-AAV-ITR (11) in which the ampicillin resistance gene has been replaced with the gene for kanamycin resistance. The HSV-2 gD vector was constructed in a similar manner and contained a truncated mutant of gD (amino acids 1 to 317, with a terminal arginine added at position 318; kindly provided by Rae Lyn Burke). rAAV vector was packaged by cotransfection of 293 cells with the vector plasmid and an AAV helper plasmid pKSrep/cap, followed by infection with adenovirus *dl*312 as previously described (16). pKSrep/cap was constructed by cloning the AAV-2 genome, without the ITRs (AAV-2 nucleotides 192 to 4493 [10]), into pBluescript II KS+ (Stratagene, La Jolla, Calif.). Crude rAAV preparations were purified by two rounds of cesium chloride gradient centrifugation as previously described (13), and titers were determined by DNA dot blot analysis (11). No replication-competent adenovirus was detected in the purified rAAV preparations based on a cytopathic effect bioassay in 293 cells with a sensitivity of detection of 100 PFU per ml.

Intramuscular injection of rAAV-gB leads to the generation of gB-specific CTL and antibody. To investigate the ability of rAAV-gB to prime gB-specific, class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL), C57BL/6 mice were injected with 5×10^{10} particles of rAAV-gB (50 μl in 0.9% saline) into the tibialis anterior muscle. Assays for gB-specific, MHC class I-restricted CTL in C57BL/6 mice were performed as previously described (1). As shown in Fig. 1, intramuscular injection of rAAV-gB induced a vigorous gB-specific, MHC class I-restricted CTL response at both 4 and 11 weeks postimmunization. These data indicated that intramuscular injection of rAAV was an effective way to prime a CTL response in mice. At the time of sacrifice, the mice were bled and serum immunoglobulin G antibody titers to gB were determined by enzyme-linked immunosorbent assay (ELISA). To detect gB-specific antibody, microtiter plates were coated overnight at 4°C with 2.5 μg of HSV-2 gB protein (kindly provided by Rae Lyn Burke) per ml in phosphate-buffered saline (PBS). Plates were then blocked for 30 min at 37°C with PBS containing 1% goat serum and 3% Tween 20. Serially diluted samples were incubated on the plate for 1 h at 37°C. Microtiter plates were then washed and incubated at 37°C for 30 min with goat anti-mouse immunoglobulin G-

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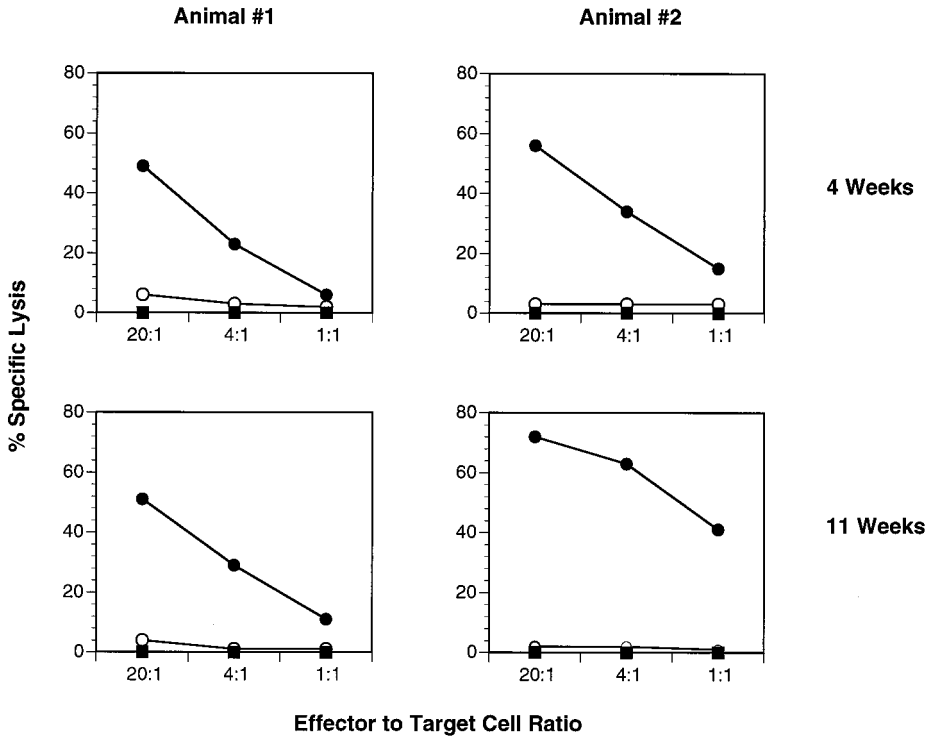

FIG. 1. CTL responses in rAAV-gB-immunized C57BL/6 (*H-2*^{b}) mice. CTL assays were performed at 4 and 11 weeks after intramuscular vaccination. CTL were tested against untreated (○) or gB peptide (amino acids 496 to 503)-sensitized (●) MC57 (*H-2*^{b}) targets or gB peptide-sensitized (■) SvBalb (*H-2*^{d}) targets. Two mice were tested at each time point. MC57 and SvBalb cells do not express class II antigens.

horseradish peroxidase (Boehringer Mannheim Corp., Indianapolis, Ind.) at 1:2,000. *O*-Phenylene diamine substrate was used to develop the plate. Plates were read at 492 nm and a cutoff of 0.5 optical density units was used to determine titer. As shown in Table 1, gB-specific antibodies were present at 4 and 11 weeks postvaccination.

Lymphocytes from rAAV-gB-vaccinated mice proliferate in response to gB. The ability of rAAV-gB vaccination to prime a helper T-cell response was assessed by using a lymphoproliferation assay. Briefly, splenocytes from the rAAV-gB-vaccinated mice were plated at 2×10^5 cells per well in a 96-well round-bottomed plate in the presence or absence of 10 μg of HSV-2 gB protein per ml. After 120 h, the plates were pulsed with 1 μCi of [^{3}H]thymidine per well for 6 to 8 h. As shown in Table 1, the mice demonstrated gB-specific lymphoproliferation at 4 and 11 weeks postvaccination. Splenocytes from mice similarly injected with rAAV-LacZ did not demonstrate gB-specific lymphoproliferation (data not shown).

Minimum dose of rAAV-gB needed to elicit immunity. To determine the minimum dose of rAAV-gB required to generate antibody, groups of four C57BL/6 mice were injected intramuscularly with doses of rAAV-gB ranging from 1×10^6 to

TABLE 1. gB-specific antibody and lymphoproliferation after immunization with rAAV-gB

Immunization (wk)[a] and animal no.	gB antibody titer[b]	LPA SI[c]
rAAV-gB (4)		
1	7,500	3.2
2	20,000	3.7
rAAV-gB (11)		
1	35,000	3.9
2	25,000	6.4

[a] C57BL/6 mice were injected intramuscularly with 5×10^{10} particles of rAAV-gB. Sera was collected at the time of sacrifice, either 4 or 11 weeks postinjection.

[b] Reciprocal ELISA titers, cutoff was 0.5 optical density units.

[c] Results are presented as lymphoproliferation assay (LPA) stimulation index (SI), calculated as follows: SI = (mean experimental counts per minute)/(mean counts per minute in the absence of antigen). Each SI value represents the average of six wells.

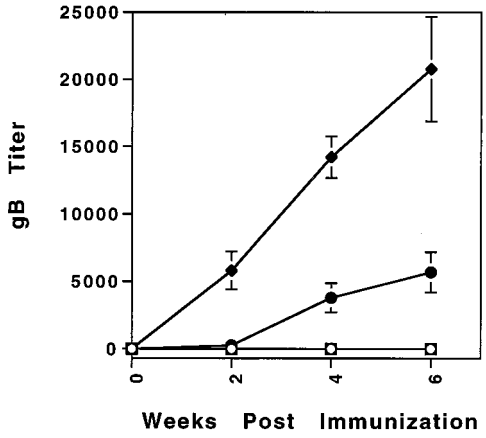

FIG. 2. Minimum dose of rAAV-gB required for the generation of anti-gB antibody. Groups of four C57BL/6 mice were immunized intramuscularly with 1×10^6 (○), 1×10^7 (▲), 1×10^8 (△), 1×10^9 (□), 1×10^{10} (●), or 5×10^{10} (◆) particles of rAAV-gB. Mice were bled at 0, 2, 4, and 6 weeks postimmunization. Values represent the mean ± standard error of the mean.

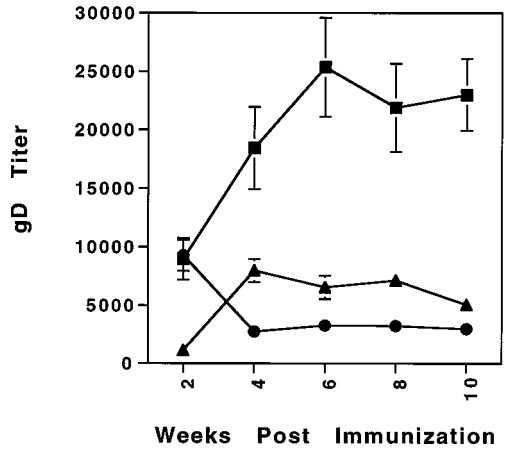

FIG. 3. Antibody response in mice immunized with either rAAV-gD, pAAV-gD plasmid DNA, or gD protein. Groups of three BALB/c mice were immunized intramuscularly with 10^{10} particles of rAAV-gD (■), 100 μg of pAAV-gD plasmid DNA (▲), or 10 μg of gD protein (●) in MF59. Values represent the mean ± standard error of the mean.

5×10^{10} particles. As shown in Fig. 2, no antibody to gB was seen in any of the animals injected with less than 10^{10} particles of rAAV-gB.

Immunization with one dose of rAAV-gD is more effective than plasmid DNA or recombinant protein in generating gD-specific antibody. To compare the efficacy of rAAV-mediated genetic vaccination to plasmid DNA or protein immunization, we injected groups of three BALB/c mice with either 10^{10} rAAV-gD particles, 100 μg of pAAV-gD plasmid DNA, or 10 μg of recombinant HSV-2 gD (kindly provided by Rae Lyn Burke) formulated with the adjuvant MF59 (8). The mice were bled every other week for 10 weeks, and antibody titers to gD were determined by ELISA (as described previously for gB). At week 6, the gD antibody titer was four- to fivefold higher in the rAAV-gD-immunized mice than in the mice immunized with either naked DNA or gD in MF59 (Fig. 3). We are continuing to monitor the antibody titers in these animals to determine whether the longevity of antibody titer is greater in the rAAV-immunized mice.

In summary, we have shown that a single intramuscular injection of rAAV vectors can prime specific CTL and antibody responses. This is in sharp contrast to the results in several recent reports which state that intramuscularly administered rAAV vectors are not immunogenic in mice (2, 14). Our results (Table 1, Fig. 2 and 3) show that antibody titers to gB or gD increased over time. AAV vectors may result in prolonged expression of antigen, especially secreted ones such as gB, providing internal boosting without readministration of vaccine. In addition, this prolonged expression of antigen could be important in the maintenance of memory CTL (5).

Our experiments suggest that the minimum dose of rAAV needed to elicit an immune response after intramuscular injection in the mouse is 10^{10} particles. This is in agreement with our results using rAAV-LacZ which suggest that a dose of approximately 10^{10} particles is required to see β-galactosidase expression in mouse muscle (6a).

It has been difficult to achieve strong and sustained immune responses by direct injection of naked DNA in the muscles of large animals. The ability of rAAV to efficiently transduce muscle and to persistently express antigen may thus prove to be useful for the genetic immunization of humans.

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