Genetic immunization for antibody generation in research animals by intravenous delivery of plasmid DNA

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Genetic immunization is an attractive approach to generate antibodies because native proteins are expressed in vivo with normal posttranscriptional modifications, avoiding time-consuming and costly antigen isolation or synthesis. Hydrodynamic tail or limb vein delivery of naked plasmid DNA expression vectors was used to induce antigen-specific antibodies in mice, rats, and rabbits. Both methods allowed the efficient generation of high-titer, antigen-specific antibodies with an overall success rate of Western detectable antibodies of 78% and 92%, respectively. High-titer antibodies were typically present after 3 hydrodynamic tail vein plasmid DNA deliveries, 5 weeks after the initial injection (i.e., prime). For hydrodynamic limb vein plasmid DNA delivery, two deliveries were sufficient to induce high-titer antibody levels. Tail vein delivery was less successful at generating antibodies directed against secreted proteins as compared with limb vein delivery. Material for screening was generated by transfection of the immunization vector into mammalian cell lines. The cell line (COS-7) that produced the highest level of antigen expression performed best in Western blot analysis screens. In summary, intravenous delivery of antigen-expressing plasmid DNA vectors is an effective genetic immunization method for the induction of antigen-specific antibodies in small and large research animals.

INTRODUCTION

Antibodies remain a vital tool for the detection of proteins in basic research and diagnostics. The sequencing of ever more genomes continually increases the number of proteins that could be detected by specific antibodies. To generate such antibodies, conventional immunization methods require the isolation and purification of the protein of interest, an often difficult and time-consuming task that is distracting from the real research objective. Frequently, proteins cannot be isolated with sufficient purity or the antigenic determinant (epitope) is altered or lost during the isolation procedure. Alternatively, peptides can be synthesized to substitute for the protein, provided the immunogenic epitope is linear and not dependent on the tertiary structure of the intact protein. While useful in many instances, not all peptides can be synthesized cost-effectively and they lack posttranscriptional modifications, which are sometimes part of the epitope of interest. Genetic immunization strategies promise a general solution to these issues (1). Instead of delivering a protein or peptide to the host, an expression vector encoding the protein (or protein fragment) is introduced. The vector subsequently produces the protein in vivo. This avoids the requirement to isolate a protein, or synthesize a peptide, and produces a native protein with appropriate posttranscriptional modifications. This approach has been used extensively to invoke protective or therapeutic immune responses (“genetic vaccination”), using a variety of gene delivery methods, including direct injection of naked plasmid DNA into skeletal muscle, lymph nodes, or the dermis, electroporation, ballistic (gene gun) delivery, and viral vector delivery (2–7). An added advantage of plasmid DNA delivery is that bacterially methylated DNA can serve as its own adjuvant (8,9).

The successful use of genetic immunization (i.e., for the specific purpose of inducing an antibody response) has been previously described (10). These authors used a gene gun to deliver plasmid DNA expression vectors into mice and reported successful antibody production for 84% of tested antigens (detection of 50 ng of antigen using 1:5000 diluted immune serum by Western blot analysis). Of the existing gene delivery methods, direct injection of naked plasmid DNA is most attractive for genetic immunization purposes: excellent expression vectors are readily available, plasmid DNA production is easy and affordable (in contrast to viral vectors), and delivery is simple and does not require any equipment (in contrast to ballistic
and electroporation methods). The disadvantage of direct intramuscular injection of naked plasmid DNA is the low transfection efficiency, especially in larger research animals (11). In recent years, two methods for the intravenous delivery of naked plasmid DNA have been described. Both rapidly deliver a large volume of plasmid DNA solution into the venous system, resulting in extravasation of the solution into the surrounding tissues and uptake and expression of the plasmid DNA. The first method delivers the plasmid DNA solution into the tail vein and transfects predominantly liver hepatocytes. This procedure is typically referred to as hydrodynamic tail vein (HTV) delivery (12,13). The second method delivers the plasmid DNA hydrodynamically into a limb vein (HLV delivery, trademarked as Pathway IV™; Mirus Bio, Madison, WI, USA) and appears to transfect myofibers exclusively (14). In the present study, we evaluated the suitability of these intravenous plasmid DNA delivery methods for the induction of an antibody response by genetic immunization. Intravenous plasmid DNA delivery provides an advantage over alternative nonviral methods because it has a very high transfection efficiency and does not require specialized equipment.

The typical objective of genetic immunization is the isolation of polyclonal antibody containing sera or antibody-producing B cells, which can be used to generate monoclonal antibody (MAb)-producing cell lines (either by immortalization or by fusion with myeloma cells to form hybridomas). To evaluate whether an individual host has been effectively immunized, one typically uses the cognate immunization protein or peptide to screen sera for the presence of antigen-specific antibodies. The genetic immunization approach requires the generation of material for screening and confirmation purposes. Chambers and Johnston (10) introduced each gene of interest into two plasmid vectors: one placing the gene under transcriptional control of a mammalian promoter for genetic immunization in mice; another placing the gene under control of a bacterial promoter for the generation of protein in bacteria to be used for screening purposes. Not all proteins could be produced in bacteria (similar to the original problem of generating proteins for immunization purposes), and the benefit of mammalian posttranscriptional modification was lost. A more elegant solution to this problem is to use the same vector for both genetic immunization and antigen production. In these studies, we produced antigen in several mammalian cell lines transfected in vitro with the genetic immunization expression vector.

MATERIALS AND METHODS

Expression Vectors

Antigens were expressed under the control of the human cytomegalovirus (CMV) promoter in the pCI vector (Promega, Madison, WI, USA); pCI-cDys encodes canine dystrophin (predicted mass of the expressed protein approximately 425 kDa), pCI-hCD4 encodes a truncated human CD4 (approximately 46 kDa), pCI-EBVc encodes the hepatitis B virus pre-C antigen [predicted size 24 kDa, posttranslationally processed to the hepatitis B virus e (HBVe) antigen, approximately 16 kDa], and pCI-Ki67 encodes residues 1547–1742 of the human antigen recognized by MAb Ki-67 (approximately 23 kDa). The luciferase reporter gene (Luc+ from pSP-Luc+, predicted size approximately 61 kDa; Promega) and rat erythropoietin (rEPO, approximately 34 kDa) were expressed in the same expression cassette as in the pCI vector, but with a different plasmid backbone carrying a kanamycin resistance gene. Sustained expression of luciferase in the liver was accomplished by placing Luc+ under transcriptional control of the human ubiquitin C promoter and human ApoE hepatic control region (15). Endotoxin-free production of plasmids was by Aldevron (Fargo, ND, USA) or by EndoFree Plasmid Maxi Kits (Qiagen, Valencia, CA, USA).

In Vitro Transfections

All cell lines were obtained from ATCC (Rockville, MD, USA). Culture media were Cellgro® from Mediatech (Herndon, VA, USA) and supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Fetal bovine serum (FBS) and horse serum were from Hyclone (Logan, UT, USA). COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% FBS; HEK 293 (human embryonic kidney) cells were cultured in MEM plus 10% horse serum plus MEM nonessential amino acids (Invitrogen); Hepa-1c1c7 cells were cultured in minimal essential medium alpha (MEMalpha) plus 10% FBS; and HELa cells were cultured in MEM plus 10% FBS and MEM nonessential amino acids. Cells were transfected with TransIT®-LT1 Transfection Reagent (Mirus Bio). Forty-eight hours after transfection, the cells were harvested, counted, and lysed in a sample buffer containing 0.5% sodium dodecyl sulfate (SDS), 0.14 M Tris base, 0.1 M Tris-HCl, 0.5 mM EDTA, 0.425% bromophenol blue, and 0.375% β-mercaptoethanol at a cell density of 10⁷ cells/mL. For secreted proteins, the serum-containing medium was removed 16 h posttransfection, the cells were rinsed in phosphate-buffered saline (PBS), and nonserum-containing Opti-MEM® was added (Cellgro). After 48 h, the medium was collected and concentrated 5× using Slide-A-Lyzer® cassettes and concentrating solution (Pierce Biotechnology, Rockford, IL, USA). Luciferase activity was measured as previously described (16).

Genetic Immunization

All animals were obtained from Harlan (Indianapolis, IN, USA). For immunizations, 6-week-old ICR mice (approximately 20 g), 6- to 7-week-old (approximately 125 g) Sprague-Dawley rats, and 2-month-old (approximately 2.2 kg) New Zealand white rabbits were used. All procedures were approved by the Mirus Bio Institutional Animal Care and Use Committee (IACUC) and complied with all relevant laws, guidelines, and policies. Hydrodynamic delivery to mouse tail vein has been previously described (12,13). Briefly, plasmid DNA was diluted in Ringers’ solution, and a volume equal to 10% of the body weight was injected into the lateral tail vein in 6 to 7 s with
the maximum volume to be delivered capped at 3.0 mL (mice ≥ 30 g body weight). A similar procedure is used for hydrodynamic delivery to rats, with a delivery time of 18–22 s and a maximum volume of 20 mL (rats ≥ 200 g). HLIV gene delivery procedures were performed on isoflurane anesthetized animals essentially as previously described (14). A latex tourniquet was wrapped around the upper hind limb to block blood flow and tightened into place with a hemostat. A small incision was made to expose the distal saphenous vein, and a catheter was inserted into the vein. A syringe pump (Harvard Apparatus, Holliston, MA, USA) was used to deliver the plasmid DNA containing saline solution (0.05 mL/g in mice; 0.08 mL/g in rats; 33 mL/kg in rabbits). The needle was retracted and the tourniquet released 2 min after plasmid DNA delivery. Bleeding was controlled with pressure and a hemostatic sponge. The incisions were closed with 4-0 VICRYL® sutures (Ethicon, Cornelia, GA, USA). Analgesics were provided the first 48 h following HLIV. Direct intramuscular injections in mice were performed as described. For these experiments, 50 µg plasmid DNA were injected in 100 µL saline solution into the quadriceps, using a 30 gauge needle for 2 to 3 s. Blood samples were taken via retro-orbital bleed from rodents; rabbits were bled via the marginal ear vein.

Western Blots

Samples were prepared for loading by adding the appropriate amount of NuPAGE® LDS Sample Buffer (Invitrogen) plus 2% β-mercaptoethanol and heating to 70°C for 10 min. Lysate from 5 to 6 × 10^4 cells (membrane or intracellular proteins) or 12–15 µL of concentrated culture medium (secreted proteins) were electrophoresed into a 10% bis-Tris polyacrylamide gel with MOPS SDS running buffer or a 7% Tris-acetate polyacrylamide gel with Tris-acetate buffer (both from Invitrogen). TriChromRanger™ Prestained Molecular Weight Markers (Pierce Biotechnology) were included in each gel. Proteins were electro-transferred to a Hybond™-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked in PBS/5% milk/0.1% Tween® 20. Antisera and secondary antibodies were diluted in PBS/0.1% Tween 200/1.0% bovine serum albumin (BSA) and incubated with the blots, rocking, for 1 h at room temperature. Primary mouse, rat, or rabbit antisera were diluted as indicated in the individual experiments. Secondary antibodies were peroxidase-conjugated immunoglobulin (Sigma, St. Louis, MO, USA) and diluted 1:2000–1:5000. Results were detected using the ECL™ Western Blotting System (Amersham Biosciences) and BioMax™ X-ray film (Eastman Kodak, Rochester, NY, USA). Goat-anti-mouse erythropoietin (EPO) MAb (R&D Systems, Minneapolis, MN, USA) was used at a 1:100 dilution.

ELISA Tests

A sandwich enzyme-linked immuno-sorbent assay (ELISA) was used to measure anti-luciferase antibody levels in animal sera. MaxiSorp™ immunoplates (96-well; Nalge Nunc, Rochester, NY, USA) were coated with 200 µg/mL recombinant luciferase protein (Promega) in 0.1 M carbonate buffer, pH 9.6, and incubated overnight at 4°C (100 µL/well). The plates were washed three times with PBS/0.05% Tween 20 and blocked in PBS/1% milk for 1.5 h at room temperature. Primary antisera were diluted: 1:100–1:1,000,000 in PBS/1% milk and incubated in the plates for 2 h at room temperature (duplicate samples). The plates were washed as above, and a secondary peroxidase-conjugated antibody (Sigma) was added. The plates were then washed five times and detected with the TMB (3,3′,5,5′-tetramethyl-benzidine) Liquid Substrate System for ELISA (Sigma). The reaction was stopped by the addition of H_2SO_4 (0.5 M final concentration). Standards were generated using species-specific anti-luciferase antibodies (Sigma) with the highest concentration at 100 ng/mL and seven serial 1:2 dilutions. Plates were read at 450 nm using a SPECTRAMax® PLUS 384 Microplate Spectrophotometer and SOFTmax® PRO Software System (Molecular Devices, Sunnyvale, CA, USA).

MAb Generation

Splenocytes from candidate mice were harvested 4 days after a final plasmid DNA boost. Immunized mice were exsanguinated under anesthesia, the spleens removed and placed in 37°C serum-free DMEM, and the parenchymal cells were separated. The cells were cultured in DMEM plus 10% FBS for 1 h at 37°C, 5% CO_2, to allow fibroblasts to adhere. The nonadherent splenocytes were removed by shaking, counted, and frozen at 10^8 cells/mL in DMEM plus 10% FBS and 10% dimethyl sulfoxide (DMSO). Frozen splenocytes were shipped to Covance (Denver, PA, USA) or Rockland Immunocchemicals (Gilbertsville, PA, USA) for hybridoma fusion. Hybridoma clones were screened for specific antibodies production using purified protein (if available), native cells, or transfected cell lysates.

Immunocytochemistry

HeLa cells were grown on glass coverslips, fixed in 4% formaldehyde in PBS, permeibilized with 0.2% Triton® X-100 in PBS, and blocked with 2 mg/mL BSA in PBS. Culture supernatants from hybridoma clones were diluted 1:5, applied to the slides, and incubated 1 h at room temperature. The secondary antibody was Cy^3-labeled anti-mouse immunoglobulin G (IgG) (H+L) F(ab’)2 fragment (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), diluted 1:600 in 2 mg/mL BSA in PBS. The samples were counterstained with 13 nM TO-PRO®-3 DNA stain and 16.5 nM Alexa Fluor® 488-phalloidin actin stain (both from Invitrogen). The control antibody was a monoclonal anti-human Ki67 antibody from BD Transduction Laboratories (San Jose, CA, USA), diluted 1:1000. Fluorescent images were collected using an LSM 510 Confocal Laser-Scanning Microscope (Carl Zeiss, Thornwood, NY, USA).
RESULTS

Generation of Antigen in Mammalian Cell Lines by In Vitro Transfection

To determine the presence of antigen-specific antibodies in sera of genetically immunized hosts, we produced antigen in mammalian cell lines. This allowed us to use the same plasmid DNA antigen expression vector for genetic immunization and for the generation of antigen. In evaluating suitable cell lines for antigen production, two aspects were considered: (i) the high-level production of antigen and (ii) low background signal in Western blot analyses. Antigen production is a function of cell line growth characteristics and transfectability. Our previous work developing high-efficiency, low-toxicity transfection reagents had identified several cell lines that are consistently transfected with high efficiency (40%–70% of cells transfected) and generate high absolute levels of transgene expression. Several cell lines were evaluated for their suitability to produce antigen, including COS-7 (African green monkey kidney origin), HEK 293, and Hepa-1c1c7 (murine hepatoma). Many different assay types can be used for screening purposes, including immunocytochemistry, Western blot analysis, and ELISA. For these studies, we focused on Western immunoblots, in which transfected cell lysates were used as a source of antigen and detected with sera from immunized mice. Figure 1 shows an example of a Western blot analysis in which transfected cell lysates were blotted and probed with serum from a mouse genetically immunized with a luciferase reporter gene expression vector. The cell lines were transfected with either the same luciferase expression vector or a β-galactosidase reporter gene expression vector for control purposes. No signal was detected in any of the β-galactosidase transfected cell lysates, whereas a specific band at approximately 61 kDa was detected in all luciferase transfected cell lysates. Recombinant luciferase and β-galactosidase proteins were used as standards and demonstrated that this assay can detect as little as 5 ng protein. Under equal loading conditions, COS-7 cell lysates provided much stronger specific protein signals, reflecting the superior transfectability of these cells. Transfection of these cell lines with pCI-luc yielded luciferase expression levels of 357, 39, and 10 ng per plate for COS-7, HEK 293, and Hepa-1c1c7, respectively. In Western blots, the COS-7 cell lysates typically showed more cross-reactivity when probed with primary mouse sera compared with the other two cell lines (data not shown). However, the 10- to 35-fold higher amount of expressed antigen and accompanying stronger signal proved more important in screening assays. Thus, for further studies, we used transfected COS-7 cells as a source of antigen for Western blotting analyses because they provided the best signal-to-background ratio.

Genetic Immunization by HTV Plasmid DNA Delivery in Mice

HTV delivery results in high levels of transgene expression in the liver and lower levels in other organs, including heart, spleen, and kidneys (12,13). We tested if repeated HTV delivery of a luciferase expression vector would result in the development of an anti-luciferase antibody response. Mice were immunized 5 times with 50 μg pCI-Luc (delivered on days 0, 14, 21, 28, and 35), either by HTV or direct intramuscular (IM) injection. Sera were collected on days 35 and 42 for anti-luciferase antibody quantitation by ELISA. On day 35, the average level of anti-luciferase antibodies in HTV-immunized mice was 334.3 ± 122.2 μg/mL versus 6.6 ± 4.8 μg/mL for IM-immunized mice (n = 5 for each group, ±SEM). Antibody levels approximately doubled by day 42 (667.5 ± 182.8 vs. 14.3 ± 10.9 μg/mL). Using 100 μg pCI-Luc per immunization dose resulted in antibody levels only slightly higher than those obtained with 50 μg. Sera from mice injected with vehicle alone or plasmid DNA expressing other genes showed no response in the luciferase antibody ELISA (data not shown). Thus, under these rather maximal genetic immunization conditions (five gene deliveries), HTV gene delivery resulted in higher anti-luciferase antibody titers as compared with IM gene delivery.

Selection of the promoter driving antigen expression may impact the immune response. CMV-driven expression reaches maximum levels between 10 and 20 h after HTV delivery but diminishes very rapidly afterwards (16). A single delivery of 10 μg pCI-Luc did not result in a very robust antibody response (0.3 μg/mL on day 48; high antibody titers were only obtained after three boosts (46.9 μg/mL on day 48). An expression vector with the ubiquitin promoter generated sustained expression in the liver and induced significant antibody titers after a single HTV delivery (14.5 μg/mL). Additional HTV deliveries of the ubiquitin-luciferase vector further increased titers (47.4 μg/mL).

We next tested antibody generation against a number of different mammalian proteins. Two examples are shown in Figure 2. In Figure 2A,
Mice were genetically demonstrated for any of the three HTV-antibody induction could not be antibody response. Interestingly, 272 of 276 mice yielded a measurable mice per antigen). For the 14 antigens (14 of 18 different antigens, at least 6 analyses with little to no background were easily detected in Western blot of the antigens yielded antibodies that combinations with nonsecreted antigens, 78% mice). Of all HTV genetic immuniza
tions with pCI-hCD4 for a total of 25 results are representative of multiple antigen with no specific signal detected with 50
or telophase and vice versa (data not shown). Immunization with bacterially
produced, nonphosphorylated recom-
binant proteins would not have yielded such an extensive palette of MAbs that enables the study of the dynamic changes of this protein during the cell cycle. The project was successful in numerical terms as well: of 960 wells of fused hybridomas, 164 passed the initial ELISA screen for reactivity with
the expressed Ki-67 fragment. In a more stringent screen based on immunocyto-
chemistry and Western blot analysis, 46 of these positive hybridoma lines gave characteristic Ki-67 staining patterns. None of these 46 MAbs cross-reacted with the mouse or hamster Ki-67 orthologs (44% identity to the human protein); only two cross-reacted with the rat ortholog (45% identity); while almost all could recognize the monkey ortholog (determined by immunohistochemistry). MAB-producing hybridoma cell lines were also generated for seven other HTV-delivered antigens.

Generation of MAB-Producing Hybridoma Cell Lines

We tested whether MAB-producing hybridoma cell lines could be generated using splenocytes from mice immunized via HTV. Results from a representative project using pCI-Ki67 (expressing part of the human antigen recognized by the Ki-67 MAB) are shown in Figure 3. Ki-67 is a nuclear protein expressed exclusively in proliferating cells. Its localization within the interphase nucleus and on condensed mitotic chromosomes during mitosis is regulated by cell cycle-specific kinases and phosphatases (17). Figure 3A shows staining of HeLa cells with a commercially available Ki-67 MAB. Figure 3, B–F shows results using five of the MAbs generated using the HTV genetic immunization method. The differences in the staining patterns are due to the antibodies reacting with specific phosphorylation-dependent Ki-67 epitopes present only at certain stages of the cell cycle. For example, the MAB shown in Figure 3D stains the perinucleolar region in interphase cells, but cannot recognize Ki-67 when it is hyperphosphorylated during mitosis (lack of staining on the condensed mitotic chromosomes). The MAB shown in Figure 3E reacts strongly with the mitotic form of the protein and stains the nucleolar region of interphase cells very weakly. In contrast, the MAB shown in Figure 3C yields very intense staining with all forms of Ki-67. There were several other MAbs that were able to distinguish even the various stages of mitosis, staining the chromosomes in pro- and metaphase, but not in anaphase or telophase and vice versa (data not shown). Immunization with bacterially produced, nonphosphorylated recombinant proteins would not have yielded such an extensive palette of MAbs that enables the study of the dynamic changes of this protein during the cell cycle. The project was successful in numerical terms as well: of 960 wells of fused hybridomas, 164 passed the initial ELISA screen for reactivity with

Hybridoma Cell Lines

Generation of MAb-Producing Hybridoma Cell Lines

We tested whether MAb-producing hybridoma cell lines could be generated using splenocytes from mice immunized by HTV delivery of pCI-hCD4 or pCI-cDys on days 0, 14, 21, 28, and 35. Sera were collected on day 42 and applied to blots of transfected COS-7 cell lysates (1:100 dilution). Control lanes contain lysates of COS-7 cells transfected with pCI-Luc (-; sample lanes (+) contain lysates of COS-7 cells transfected with pCI-hCD4 (panel A, predicted size 46 kDa) or pCI-cDys (panel B, predicted size 425 kDa). Each lane contains lysate from 5 × 10⁶ cells. Secondary HRP-conjugated anti-mouse polyvalent Ig was used at 1:2000. The blots were developed with HRP-conjugated anti-mouse polyvalent Ig was used at 1:2000. The blots were developed with horseradish peroxidase; Ig, immunoglobulin.

COS-7 cell extracts transfected with pCI-hCD4 (expressing a truncated form of the human CD4 gene) were probed with serum from a mouse immunized with pCI-hCD4. In Figure 2B, COS-7 cell extracts transfected with pCI-cDys (expressing the canine dystrophin gene) were probed with serum from a mouse immunized with pCI-cDys. The mice were immunized five times with 50 μg of the respective plasmid DNA using the HTV procedure. In both cases, a strong, specific immune response was mounted against the antigen with no specific signal detected in the negative control lanes. These results are representative of multiple tests for each antigen (e.g., 4 experiments with pCI-hCD4 for a total of 25 mice). Of all HTV genetic immunizations with nonsecreted antigens, 78% of the antigens yielded antibodies that were easily detected in Western blot analyses with little to no background (14 of 18 different antigens, at least 6 mice per antigen). For the 14 antigens that resulted in antibodies, a total of 272 of 276 mice yielded a measurable antibody response. Interestingly, antibody induction could not be demonstrated for any of the three HTV-delivered plasmid DNAs expressing secreted proteins [vascular endothelial growth factor (VEGF), rEPO, or HBVe].

Genetic Immunization by HLV Plasmid DNA Delivery in Mice

HTV gene delivery is an efficient means of gene transfer in mice. However, other than in rats, it is not readily applied in other mammals that are frequently used for antibody generation (e.g., rabbit, goat, or sheep). While catheters can enable transcutaneous hydrodynamic delivery into the liver of larger animals (18–20), a better alternative is available. Intravenous plasmid DNA injection into veins of limbs temporarily isolated by a tourniquet results in very high gene transfer to skeletal muscle cells (14). This HLV procedure is readily applied in small rodents and larger mammals with similar transfection efficiencies. We tested whether HLV gene delivery could be used for genetic immunization to generate antigen-specific antibodies and determined how many HLV gene deliveries are required to induce a maximal humoral immune response. Mice were injected with 1, 2, 3, or 4 doses of 10 μg pCI-Luc. We also tested whether effective immunization required the transgene to be delivered in the same limb each time. Groups of mice received repeat doses of pCI-Luc in the same limb or in the alternate limb. ELISA results are presented in Figure 4 and show that very high levels of anti-luciferase antibodies were generated with as few as two HLV gene deliveries. Additional HLV gene deliveries did result in increasing titers. Repeat gene delivery to a single limb or to alternating limbs resulted in similar antibody levels (the apparent difference seen in this experiment for Figure 2. Antibody induction following genetic immunization by hydrodynamic tail vein delivery of plasmid DNA. Mice were genetically immunized by HTV delivery of pCI-hCD4 or pCI-cDys on days 0, 14, 21, 28, and 35. Sera were collected on day 42 and applied to blots of transfected COS-7 cell lysates (1:100 dilution). Control lanes contain lysates of COS-7 cells transfected with pCI-Luc (-; sample lanes (+) contain lysates of COS-7 cells transfected with pCI-hCD4 (panel A, predicted size 46 kDa) or pCI-cDys (panel B, predicted size 425 kDa). Each lane contains lysate from 5 × 10⁶ cells. Secondary HRP-conjugated anti-mouse polyvalent Ig was used at 1:2000. The blots were developed with HRP-conjugated anti-mouse polyvalent Ig was used at 1:2000. The blots were developed with horseradish peroxidase; Ig, immunoglobulin.
the triple immunization protocol was not observed in other studies; data not shown).

Animal-to-animal variation was consistently less for HLV procedures than for HTV procedures in mice. In a direct comparison, anti-luciferase antibody titers following HLV gene delivery were 571 ± 231 μg/mL and following HTV gene delivery, 609 ± 480 μg/mL (n = 5, average titer ± sd, day 35). Similar differences in variability were observed at other time points and in other experiments (data not shown). In larger animals, HLV gene delivery variability appeared somewhat larger, probably reflecting much larger differences in body weight and other features (e.g., vasculature) between individual animals.

We next tested whether HLV gene delivery would be successful in generating antibodies to secreted proteins in mice. Mice were injected via HLV four times (days 0, 14, 21, and 28) with 50 μg of plasmid DNA vectors expressing the HBVe or rEPO. Representative Western blot results are shown in Figure 5A for HBVe antigen, showing detection of the unmodified C antigen precursor (24 kDa) and the mature e antigen (16 kDa) in the pCI-HBVc-transfected COS-7 cell lysate (right lane). Figure 5B shows detection of rEPO, (predicted size 34 kDa) by pCI-rEPO-immunized polyclonal antiserum (left blot) or cross-reactive goat anti-mouse EPO MAb (right blot). Mice immunized via HLV generated specific, high-titer antibodies to expressed proteins in 83% of tested antigens (5/6; luciferase, human gp100, rEPO, HBVe, hVEGF165). Thus, HLV gene delivery is an effective method for generating antibodies to expressed proteins in mice, including secreted proteins.

**Genetic Immunization by HLV Plasmid DNA Delivery in Larger Animals**

Next, we investigated if the HLV genetic immunization method is effective in larger research animals. Rats were injected 4 times with 500 μg of the luciferase expression vector pCI-Luc (days 0, 14, 21, and 28). Sera were collected at several time points after immunization and analyzed for anti-luciferase antibodies by ELISA. The average anti-luciferase antibody level was 375.1 ± 246.9 μg/mL on day 35.
and 392.2 ± 296.5 μg/mL on day 42 (average ± SEM, n = 4). In contrast, HTV genetic immunization in rats resulted in an average antibody level of only 0.6 ± 0.4 μg/mL on day 35 and 0.5 ± 0.2 μg/mL on day 42 (average ± SEM, n = 4), which coincides with the generally lower HTV transfection efficiency in rats as compared with mice (data not shown).

Rabbits were injected with 1 mg/kg body weight pCI-Luc, either two (days 0 and 14) or three times (days 0, 14, and 21), via the hind limb saphenous vein (two rabbits per group). Individual rabbit anti-luciferase antibody levels were measured by ELISA on days 35 and 42. High levels of anti-luciferase antibodies were present in the sera on day 35 after only two gene deliveries (100.4 μg/mL and 585.0 μg/mL) or three gene deliveries (286.8 and 345.7 μg/mL). Titers were approximately double on day 42 (two deliveries: 534.1 and 681.4 μg/mL; three deliveries: 533.8 and 612.4 μg/mL). In rabbits, 100% of HLV gene delivery tests generated high-titer, specific antibodies (6/6).

**DISCUSSION**

In this study, we set out to determine the effectiveness of intravenous hydrodynamic plasmid DNA delivery methods to induce a humoral immune response, with the purpose of obtaining polyclonal antibody containing sera or B lymphocytes for the production of monoclonal antibodies. Intravenous hydrodynamic plasmid DNA delivery offers a simple, highly effective methodology that generates high titers of antibodies in a relatively short time frame. It only requires plasmid DNA and no specialized, costly equipment.

The success rate of antibody induction by HTV gene delivery is similar to that reported for gene gun delivery, 78% versus 84% (10), respectively. HLV gene delivery, in tests in three different animal species, showed an overall success rate of 92%. Failure to generate antibodies for certain antigens can be caused by poor immunogenicity of the antigen, inadequate immunization by the hydrodynamic genetic immunization technique, or inability to detect the antibody response (e.g., because of low production of the antigen by in vitro transfection). In the current study, antigens were expressed from a CMV promoter without any alterations to the antigen coding region. In the gene gun study, codon-optimized genes were expressed fused to an α1-antitrypsin secretion signal and a cartilage oligo-merization matrix protein in order to impart antigen secretion and improve solubility and immunogenicity. It is conceivable that such optimized expression cassettes, while slightly more complex to generate, may further increase the success rate for hydrodynamic genetic immunization. The addition of secretion signals on antigens has been reported to increase antibody induction success rates (10, 21, 22). However, HTV delivery of three different secreted protein expression vectors was not successful in generating antibodies, while high levels of the secreted proteins were measured in the serum 1 day after gene delivery (data not shown). The same secreted proteins did induce a strong antibody response following HLV gene delivery, thus these proteins are immunogenic in mice. It is unclear if the failure of antibody induction against secreted proteins by HTV genetic immunization is a function of the three antigens that were tested or whether this is a general phenomenon. Following HTV gene delivery, the majority of gene expression is found in hepatocytes (but not in Kupffer cells), with smaller amounts in the spleen, lung, and cardiac muscle (12). Thus, a variety of directly transfected cell types could provide antigen presentation by serving as facultative antigen presenting cells (APCs). Alternatively, antigen presentation could be provided by professional APCs following direct transfection and endogenous antigen expression or uptake and processing of exogenously produced antigen. HLV delivery results in seemingly exclusive gene expression in skeletal muscle. Classical immunological dogma dictates that for T cell-dependent antibody responses, activation of CD4 T-helper cells is requisite and requires processed antigen in the context of major histocompatibility complex (MHC) class II molecules. While under normal conditions muscle expresses...
minimal MHC class I or class II cell surface molecules, they can serve as facultative APCs to present antigen on MHC class I and/or class II under appropriate conditions such as inflammation (23). Such conditions may possibly be generated by HLV gene delivery. In addition, these inflammatory conditions may also trigger recruitment of professional APCs to muscle tissue, serving to stimulate CD8 and CD4 cellular response as well as facilitate humoral responses (24). Thus, the observed dichotomy regarding induction of humoral immunity between HTV and HLV genetic immunization may be a combination of numerous intrinsic characteristics of these two hydrodynamic gene delivery methods and target tissue environments. Manipulation of the antigens (e.g., adding secretion signals to intracellular proteins and membrane anchoring domains to secreted proteins) may provide more insight beyond the relatively small sample of antigens investigated in this study.

Sustained antigen expression in the liver (ubiquitin promoter-driven) resulted in significant antibody titers after 7 weeks. In contrast, short-term (CMV-driven) antigen expression gave very low antibody titers over the same time frame. Both promoters enabled very high antibody titers when given repeatedly. Thus, the choice of expression vector can reduce the number of antigen immunizations, while still promoting the induction of high titers of antigen-specific antibodies. Weekly boosts with a strong promoter (e.g., CMV) antigen expression vector can shorten the time span required for high antibody titer development. The use of plasmid-based self-amplifying expression vectors (e.g., based on alphaviruses) (25) may be another option for improving genetic immunization success rates or shorten the time required for antibody induction. Most plasmid DNA used in our studies was prepared endotoxin free (<0.1 EU/endotoxin unit)/µg by an outside service provider using anion exchange column purification. Commercially available anion exchange plasmid DNA purification kits were also used and resulted in effective hydrodynamic gene transfer, expression, and antibody induction. We therefore do not anticipate any problems using plasmid DNA prepared by alternative methods for genetic immunization via hydrodynamic delivery.

Chambers and Johnston (10) determined that the codelivery of granulocyte/macrophage colony-stimulating factor (GM-CSF) and FMS-like tyrosine kinase 3 ligand (Flt3L) expression vectors improve the immune response about 9-fold. It is conceivable that inclusion of these factors in an HTV or HLV genetic immunization protocol would facilitate a similar improvement. In the present study, we purposefully maintained the simplest methods to achieve antibody induction. While hydrodynamic gene delivery requires more plasmid DNA per immunization than the gene gun, higher antibody levels were achieved in a shorter time frame: high antibody levels after 5 to 6 weeks compared with 12 weeks for four gene gun deliveries at 3-week intervals (10).

The same plasmid DNA construct was used for genetic immunization and for in vitro protein generation required for screening purposes, thus eliminating the additional step of preparing bacterial expression vectors. Importantly, this avoids problems with the generation of screening materials because expression vectors capable of generating sufficient antigen in vivo to induce an effective antibody response will usually generate ample antigen in vitro for screening purposes. Moreover, the in vitro produced antigen will typically have similar posttranscriptional modification and thus provide more suitable screening material than can be obtained from bacteria. Our assessment of various transfected cell lines for the generation of protein showed that a nonhuman primate cell line (COS-7) produced the best signal-to-background ratio. We had anticipated higher background from nonmurine lines for screening polyclonal mouse sera, but this was not an issue. It appears that the level of antigen produced in the cells is the critical factor, thus the cell lines that are transfected most effectively in vitro (i.e., COS-7, HEK 293) perform best in screening tests. We have typically used antigen-transfected cell lysates in Western blot assays for screening, yet other methods can also be used, such as immunocytochemistry, flow cytometry, and ELISA. If desired, hydrodynamic gene delivery could also be used to generate antigen for screening in vivo. For instance, the liver could be used as a source of antigen 1 day after HTV plasmid DNA delivery in mice, potentially reducing background. Indeed, luciferase antibody containing sera could identify a specific luciferase band in Western blots of HTV-transfected mouse livers (data not shown); however, in vivo antigen expression levels are less than those obtained by in vitro transfection.

In summary, our results show that hydrodynamic tail and limb intravenous gene delivery methods can be used to generate high-titer antibody responses to expressed proteins. No additional specialized equipment is necessary.
is required for immunization or screening. High-titer, antigen-specific antibodies may be generated in as few as 5–6 weeks in mice, rats, and rabbits. The HLV gene delivery procedure was especially successful in generating antibodies against a wide variety of expressed antigens. HLV should be readily adapted for use in larger animals such as goats and sheep to generate large amounts of polyclonal antibodies. Screening methods are simple and straightforward even in the absence of purified antigen, using the genetic immunization plasmid DNA to transfect cultured cells and evaluating using standard protein screening techniques.

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COMPETING INTERESTS STATEMENT

M.K.B, Z.C.N, M.G.S, and H.H. are employed by Mirus Bio, the developer of the technology described here and manufacturer of a reagent employed in this study. G.Z. and J.A.W. are consultants to Mirus Bio; J.A.W. holds an equity interest in the company.

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