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Particle-mediated delivery of recombinant expression vectors to rabbit skin induces high-titered polyclonal antisera (and circumvents purification of a protein immunogen)

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ABSTRACT

Polyclonal antibodies were generated in rabbits by delivery to skin of gold particles coated with mammalian expression vectors encoding a cytoplasmic (β-galactosidase) or a nuclear (L1 capsid of cottontail rabbit papillomavirus) protein. One primary and one booster immunization of 30 μg DNA per rabbit yielded specific antisera with titers from 1:24 000 to 1:120 000 in each of eight rabbits, as detected by ELISA and Western blot analysis. Genetic immunization requires relatively small amounts of DNA, eliminates the need to purify the protein immunogen, and does not require irritating adjuvants.

Domestic rabbits (*Oryctolagus cuniculus*) have been the primary species for polyclonal antibody production for biomedical research for >30 years. Antibody production is often induced by subcutaneous inoculation of the protein of interest emulsified with an adjuvant, such as Freund's complete adjuvant, to enhance the immune response (1). Isolation and purification of the protein in sufficient quantity for optimal immunization is the most technically demanding and time-consuming step.

Previous studies in mice documented that long-lived humoral as well as cell mediated immune responses could be induced without preceding protein purification by delivering a mammalian expression vector for a protein directly to skin *in vivo* (2–6). Skin is an ideal target because it contains Langerhans and other dendritic cells that recognize foreign proteins and readily drain to lymph nodes where immune responses are generated/amplified (reviewed in 7). We report here that genetic immunization of skin is also an excellent method to induce high titered polyclonal antibodies in rabbits.

Two mammalian vectors were used. Plasmid pCMV-β (Clonetech, Palo Alto, CA) expressed the bacterial gene β-galactosidase (β-gal) under the transcriptional control of the cytomegalovirus (CMV) promoter. β-gal is a cytoplasmic protein. pCMV-CRL1 was derived from pCMV-β by replacing the *Not*I fragment containing the β-gal gene with a PCR-generated fragment containing the L1 gene of cottontail rabbit papillomavirus (CRPV) [nucleotides 5828–7345 (8) of the CRPV genome], flanked by *Not*I sites. Immunofluorescent studies of pCMV-CRL1-transfected CHO cells, performed with a CRPV L1 antibody (9), confirmed the ability of the vector to express the nuclear L1 protein (data not shown).

DNA-coated gold particles were prepared (D. McCabe, personal communication) by adding 50 μg of Qiagen-purified (Qiagen, Inc., Chatsworth, CA) supercoiled plasmid DNA to 25 mg of gold particles (1–3 μm average diameter) in 100 μl 0.1 M spermidine, followed by a 10 s sonication. Two hundred microlitres of 2.5 M $CaCl_2$ was added with continuous vortexing and the mixture was further incubated at 20°C for 10 min with intermittent vortexing. The DNA-coated particles were pelleted at 12 000 r.p.m. for 30 s, washed three times with 100% ethanol, and resuspended in 3 ml ethanol. A 21 inch length of Tefzel tubing (1/8″ outside diameter, 3/32″ internal diameter) (McMaster-Carr, Elmhurst, IL) was filled with the suspension and laid flat for 10 min to allow the particles to settle. The ethanol was then decanted, and the tubing was manually rotated to coat the inner walls with the particles. It was dried in a stream of nitrogen gas for 3–5 min. Individual 'shots' were generated by cutting the tubing into 1/2 inch lengths, each containing 1 μg DNA/0.50 mg gold, and loaded into 12-shot barrels of a helium-driven Accell™ Gene Delivery Device (Agracetus, Inc., Middleton, WI) (5,10).

Four to five pound Pasteurella-free female New Zealand white rabbits were anesthetized with ketamine (30 mg/kg) and xylazine (3 mg/kg). Hair was clipped from a 200 cm^2 area on the rabbits' backs, and residual hair and superficial keratin were treated with a commercial depilatory (Nair, Division of Carter-Wallace, Inc., New York, NY 10105). The Accell™ device was held against the skin, and each 'shot' was inoculated by the instantaneous release of 350 psi of helium pressure through the barrel. The ability of the pCMV-β vector to express β-galactosidase in rabbit skin 24 h after inoculation was confirmed in preliminary experiments by staining frozen sections of treated sites with X-gal and hematoxylin and eosin. Strongly staining cells were localized in the epidermis and probably included keratinocytes and Langerhans or other types of dendritic cells (Fig. 1).

For each primary and booster immunization, rabbits were inoculated at each of 30 sites with 1 μg of DNA. Three rabbits were immunized with pCMV-β DNA and five with pCMV-CRL1

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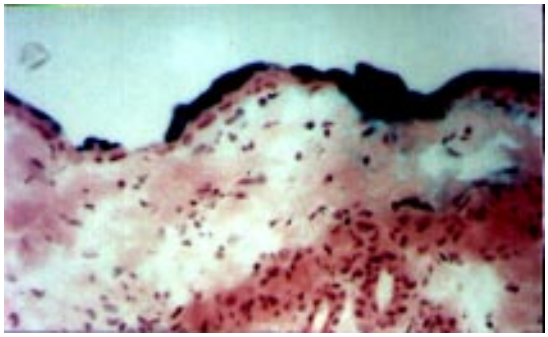

Figure 1. β-galactosidase expression in rabbit skin following particle-mediated delivery of pCMV-β DNA.

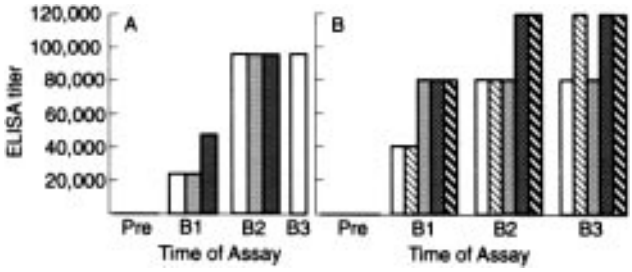

Figure 2. ELISA antibody titers of rabbits immunized by particle-mediated DNA delivery. Different bars represent different rabbits. (**A**) Rabbits immunized with pCMV-β. (**B**) Rabbits immunized with pCMV-CRL1. Time of assay: preimmune (Pre), or 10 days after boost 1 (B1), boost 2 (B2) or boost 3 (B3).

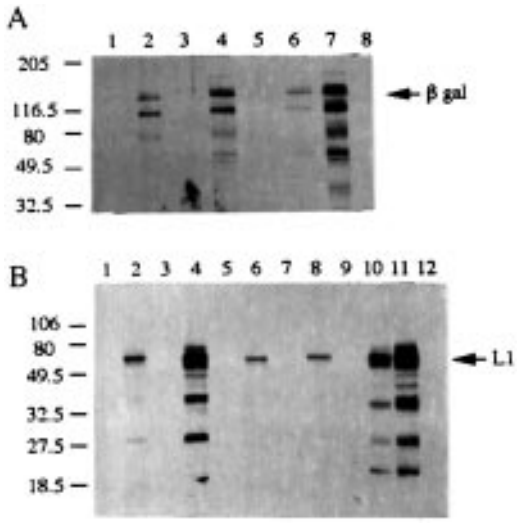

Figure 3. Antisera specifically recognize the protein encoded by the gene in the immunizing vector. (**A**) Western blots containing 1 μg of β-galactosidase were probed with preimmune (lanes 1, 3 and 5) or immune (lane 2, 4 and 6) sera from the three rabbits immunized with pCMV-β, immune serum from a rabbit immunized with pCMV-CRL1 (lane 8), and a control monoclonal antibody to β-gal (lane 7). Immune sera were from boost 3 (lanes 2 and 8) or boost 2 (lanes 4 and 6). Each rabbit serum was used at 1:50 000 dilution. The control mouse antibody was used at 1:5000. (**B**) Western blots containing 1 μg of CRPV virus-like particles were probed with preimmune (lanes 1, 3, 5, 7 and 9) or immune sera after boost 3 (lanes 2, 4, 6, 8 and 10) from the five rabbits immunized with pCMV-CRL1, a control antiserum to CRPV L1 (lane 11), and an immune serum from a rabbit immunized with pCMV-β (lane 12). All sera were used at a 1:20 000 dilution. Full length β-gal and L1 proteins are marked by arrows; the faster migrating bands are degradation products.

DNA, at the same time. The primary immunization was given on day 0, and booster immunizations on days 21 and 42. One rabbit immunized with pCMV-β and all five immunized with pCMV-CRL1 were boosted for a third time on day 63. Sera was obtained prior to primary immunization and 10 days after each booster immunization.

Serum antibodies were detected in enzyme-linked immunosorbent assays (ELISAs) on plates coated with 100 ng of purified β-galactosidase (Sigma Chemical Co., St. Louis, MO) or 100 ng of purified CRPV virus-like particles composed of CRPV L1 and L2 proteins (11). As positive controls, we used a commercial monoclonal antibody to β-gal (Sigma Chemical Co., St. Louis, MO) that reacted at 1:6400 in the β-gal ELISA, and a rabbit polyclonal antibody generated by protein immunization with a CRPV trpE-L1 fusion protein (9) that reacted at >1:120 000 in the CRPV ELISA. Primary immunization and one boost induced antibodies to β-gal in all three rabbits immunized with pCMV-β DNA and antibodies to CRPV L1 in all five rabbits immunized with pCMV-CRL1 DNA. Antibody titers to β-gal and to CRPV were already high after the first boost (1:24 000–1:120 000) and showed relatively minor increases (to 1:80 000–1:120 000) after the second and third boosts (Fig. 2).

The nature of the humoral immunity was further studied by Western blot analysis, performed as described (12). None of the preimmune sera reacted with β-gal or CRPV proteins (Fig. 3A and B). Antisera generated by pCMV-β-immunization reacted with purified β-gal and not with CRPV proteins at dilutions of 1:50 000 (Fig. 3A, lanes 2, 4 and 6; Fig. 3B, lane 12). The stronger β-gal staining by the control antibody (Fig. 3A, lane 7) can be attributed to its 10-fold lower dilution (1:5000). Antisera generated by pCMV-CRL1-immunization reacted with CRPV L1 protein and not with β-gal at dilutions of 1:20 000 (Fig. 3B, lanes 2, 4, 6, 8 and 10; Fig. 3A, lane 8). They reacted equally strongly (Fig. 3B, lane 4), nearly as strongly (lane 10), or less strongly (lanes 2, 6 and 8) than the control antiserum from a TrpE-L1 protein-immunized rabbit (9).

The specificity of the immune responses was demonstrated by three findings (Fig. 3). First, preimmune sera bound only background levels of antigen. Secondly, rabbits immunized with pCMV-β did not produce antibodies to CRPV, and rabbits immunized with pCMV-CRL1 did not produce antibodies to β-gal. Thirdly, immune sera generated by DNA immunization reacted specifically to the protein encoded by the immunizing gene.

Genetic immunization with DNA and biologically inert gold particles greatly simplifies the production of polyclonal rabbit antisera by eliminating the need to isolate and purify the protein immunogen. It offers the added benefit of reducing discomfort to the rabbits, as compared to protein immunization, because it does not require irritating adjuvants. Relatively small amounts of DNA are required. Two inoculations of 30 μg of DNA each per rabbit induced strong specific antibody responses in all rabbits treated with pCMV-CRL1 or pCMV-β. Each of the polyclonal rabbit sera to CRPV L1 had a titer that was approximately equal to the titer of the control rabbit antiserum generated by protein immunization (9). Rabbits immunized once with just 6 μg of pCMV-β DNA have also produced antibodies to β-gal with titers from 1:600 to 1:2400 by ELISA (data not shown). Lower DNA doses

may also be effective in rabbits, since as little as 16 ng of DNA can induce strong antibody responses in mice (10). The mechanism of immune recognition must involve intracellular processing of endogenously synthesized protein and presentation of peptides in the context of major histocompatibility antigens, since β-gal is cytoplasmic and CRPV L1 is nuclear. These results strongly imply that the method will be applicable to a broad array of genes.

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