rapid communication

Production of rabbit polyclonal antibody against apobec-1 by genetic immunization

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Abstract Circulating apolipoprotein B (apoB) exists in two forms; apoB-100 and apoB-48. ApoB-48 is a truncated form of apoB resulting from RNA editing. The editing enzyme, called apobec-1, converts a cytidine (C) at nucleotide 6666 in apoB-100 mRNA to a uridine (U) and changes a CAA codon to an in-frame stop codon, UAA. We have produced a specific rabbit polyclonal antiserum against apobec-1 by genetic immunization. The cDNA of mouse apobec-1 was inserted downstream and in-frame at the BamH I site in the last exon of human growth hormone cDNA driven by a cytomegalovirus promoter. This plasmid was injected together with another plasmid expressing granulocyte macrophage colony-stimulating factor into the thigh muscles of a rabbit. The resulting antiserum demonstrated high specificity on Western blots, and inhibited the apoB mRNA editing activity of mouse liver extract in a dose-dependent manner. This report demonstrates that DNA immunization is a powerful technique that can be readily applied to other sparse or difficult-to-purify proteins in lipid metabolism.—**Yeung, S-C. J., J. Anderson, K. Kobayashi, K. Oka, and L. Chan.** Production of rabbit polyclonal antibody against apobec-1 by genetic immunization. *J. Lipid Res.* 1997. **38:** 2627–2632.

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Apolipoprotein B (apoB) exists in the circulation in two forms: apoB-100 and apoB-48. ApoB-48 is a truncated form of apoB. The truncation is the result of RNA editing (1, 2), a novel genetic mechanism whereby the cytidine (C) at nucleotide 6666 in apoB-100 mRNA is converted to a uridine (U), changing a CAA codon for Gln-2153 to an in-frame stop codon, UAA (3, 4). The catalytic subunit of the enzyme complex is called apobec-1 (5, 6). It is a 27-kDa protein that is very sparse in quantity, and attempts to purify it have been unsuccessful. In order to produce a good antibody to the protein, most laboratories have resorted to using synthetic oligopeptides as an immunogen with problems (low specificity, low affinity) inherent to such an approach. Others have used fusion proteins (e.g., fusion with glutathione-S-transferase) produced in bacteria, which involves multiple production and purification steps. Here we describe a simple effective method to produce a polyclonal antiserum to apobec-1 by genetic immunization.

Genetic immunization refers to the generation of antibody or cell-mediated immunity using genetic materials encoding the antigen (7–9). Although direct mRNA injection can be used (10), it is generally unstable and plasmid DNA is preferred. Plasmid DNA encoding the antigen is delivered by ballistic microparticles (11–15), pneumatic (jet) injection (16, 17), or intramuscular injection (18–21). In this communication, we report the production of a rabbit polyclonal antiserum against mouse apobec-1 by intramuscular injection of DNA. This method can be used to produce antisera to any protein when the cDNA sequence is known.

MATERIALS AND METHODS

Genetic immunization

Plasmids pCMVI-GMCSF and pCMV-GH-F2 were generous gifts from Dr. M. Barry (22). Plasmid pCMVI-GMCSF encoded mouse granulocyte macrophage-colony stimulating factor (GMCSF) under the control of a cytomegalovirus (CMV) promotor (**Fig. 1**). Plasmid pCMV-GH-F2 was for fusion of the cDNA of a desired antigen to human growth hormone. Plasmid pCMV-GH-F2 was digested with BamH I and Sal I. A BamH I restriction site was introduced in-frame just 5′ to the first codon of mouse apobec-1 (23) by poly-

Abbreviations: apo, apolipoprotein; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; FCS, fetal calf serum; PS, penicillin–streptomycin; SDS, sodium dodecyl sulfate.

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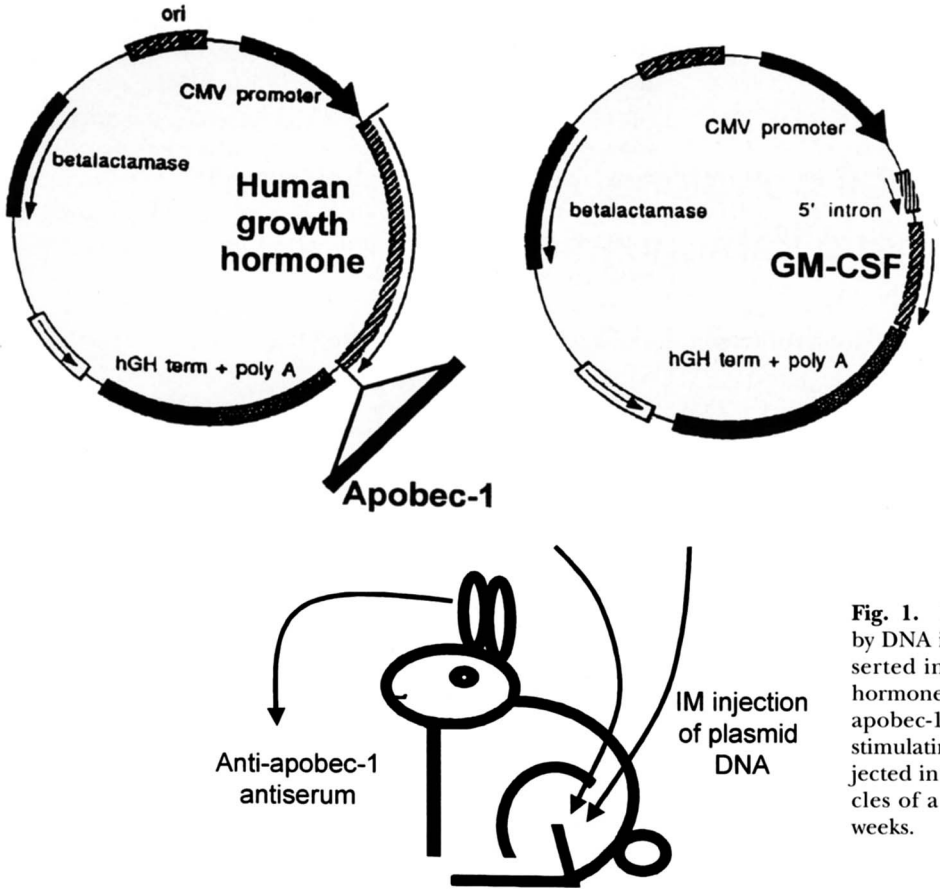

Fig. 1. Production of anti-apobec-1 antiserum by DNA immunization. Apobec-1 cDNA was inserted in-frame immediately after the growth hormone sequence. The growth hormone (GH)-apobec-1 and granulocyte macrophage colony stimulating factor (GMCSF) plasmids were co-injected intramuscularly (IM) into the thigh muscles of a rabbit. Antiserum was collected in 3 weeks.

merase chain reaction (PCR) with the following primers: (forward primer) 5′-GTC GGA TCC ATG AGT TCC GAG ACA GGC CCT GTA-3′; (reverse primer) 5′-GAC GCA TGC TCA TTT CAA CCC TGT AGC CCA AAG-3′. This PCR product was then digested with BamH I and Sma I. The 3′ portion of apobec-1 was obtained by digesting a plasmid containing the mouse apobec-1 cDNA sequence with Sma I and Sal I. By ligating these three fragments together, the full length cDNA of mouse apobec-1 was inserted downstream and in-frame at the BamH I site in the last exon of human growth hormone cDNA (i.e., removing the C-terminal 54 amino acids of growth hormone) driven by a CMV promoter (pCMV-GH-mAPOBEC-1). Errors in the reading frame and errors in PCR were excluded by DNA sequencing. *E. coli* (XL1-Blue, MRF strain, Stratagene) was transformed by pCMVI-GMCSF or pCMV-GH-mAPOBEC-1 and cultured in LB medium. Plasmid DNA was harvested from saturated cultures using alkaline lysis and affinity chromatography with P-2500 columns (Mega Plasmid Prep Kit from Qiagen) (24). DNA precipitated by isopropanol was resuspended in phosphate–buffered saline (PBS), precipitated again by adding 2 volumes of absolute ethanol, and then washed with 70% ethanol. This final DNA pellet was dissolved in PBS.

Male New Zealand white rabbits were immunized by bilateral intramuscular injection of 250 μg of pCMV-GH-mAPOBEC-1 and 250 μg of pCMVI-GMCSF into each quadracep muscle. Expression of GMCSF was shown to enhance the immune response (25,26). The first bleed was obtained 3 weeks after injection. Similar results were obtained with subsequent bleeds at the fourth and fifth week. Immunoglobulin was purified by affinity chromatography with a protein G-Sepharose column (Pharmacia).

Expression of epitope-tagged apobec-1 by adenoviral gene transfer

The specificity of the antiserum generated by DNA immunization was tested against mouse apobec-1 tagged with the HA-11 epitope in the C-terminal portion (27,28). The presence of the HA-11 epitope tag will allow confirmation of the identity of the protein by immunoreactivity to anti-HA-11 antibody (Rabbit polyclonal antiserum, Berkeley Antibody Co.). Hepa (mouse hepatoma cells) were cultured in DMEM with penicillin–streptomycin (PS) and 10% fetal calf serum

(FCS) to 80–90% confluence. They were washed with PBS twice at 37°C. An adenovirus containing an epitope-tagged mouse apobec-1 or luciferase cDNA (28) (1.8×10^9 pfu in 5 ml IMEM with PS and 2% FCS) was added to 75 cm^2 of cultured cells and incubated for 2 h at 37°C (29). The infection medium was replaced by DMEM with PS and 10% FCS, and the cells were incubated for another 24 h.

Protein electrophoresis and Western blotting

Hepa cell S-100 extract was prepared as previously described (28, 29). SDS-polyacrylamide gel electrophoresis was performed at room temperature at a current of 20mA/minigel (0.75 mm thick 4–15% polyacrylamide gradient mini-gel, Bio-Rad). Pre-stained protein markers (Rainbow Markers, Amersham) were used for molecular weight calibration. Two-dimensional gel electrophoresis was performed as adapted from O'Farrell's method (30, 31). Protein samples were isoelectric focused in 2-mm diameter tube gels on a pH gradient from 3–10 for 18 h at 300 V after a 30-min pre-run at 300 V. The second dimension gel was a 10% SDS-polyacrylamide gel.

The proteins separated on one- or two-dimensional polyacrylamide gels were electroblotted onto polyvinylidene difluoride membrane in 20mM Tris, 150mM glycine, 20% methanol, pH 8.0, at 25 V at 4°C overnight. After blotting, antibody incubation and visualization were performed with an enhanced chemiluminescence kit (Amersham Life Science). For reprobing the same membrane with a different antibody, it was incubated at 50°C for 30 min in a stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS, 50mM Tris, pH 6.8, and then washed with 0.1% Tween-20 in PBS. The stripped membrane was reblocked and then incubated with anti-HA-11 antibody.

Primer extension assay for apoB mRNA editing

Two μl (approximately 20 μg total protein) of wild-type mouse liver S100 extract was incubated with various amounts (8 μl, 4 μl, and 0 μl) of either antiserum or purified immunoglobulin at 4°C for 30 min. In order to balance the amount of protein, preimmune serum or buffer was added to bring the total volume of the antibody-S100 incubation to 10 μl. Each S100-antibody mixture was then incubated with 1 μl of chicken intestine S100 extract, 20 units of RNase inhibitor, and 2 ng of template cRNA (a 283 bp fragment containing the apoB mRNA editing site and recognition sequences) for 3 h at 30°C. RNA was then purified by proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation. The degree of editing was assessed by primer extension assay (32). A ^{32}P-labeled 35 bp DNA primer was used in a reverse transcriptase reaction in the presence of dCTP, dATP, dTTP, and dideoxy-GTP. This terminated transcription at the canonical C-6666 in unedited RNA and 5 bases further upstream in the edited RNA. The resulting products were separated on an 8% polyacrylamide gel and visualized by autoradiography. The ratio of the primer extension products of edited to unedited mRNA was quantified by phosphor-imaging equipment and software from Molecular Dynamics.

RESULTS

Production of polyclonal anti-apobec-1 antiserum by genetic immunization

We produced a rabbit polyclonal antiserum against mouse apobec-1 by genetic immunization. The procedure is described in detail in Materials and Methods. We tested the specificity of the antiserum and its capacity to inhibit apoB mRNA editing in vitro.

Specificity of anti-apobec-1 antiserum

We tested the specificity of the rabbit antiserum by Western blot analysis. In agreement with previous studies (e.g., reference 29), Western blotting of liver S-100 extract from a normal adult mouse using the antiserum failed to detect apobec-1, indicating that the protein is present at an extremely low concentration. However, the anti-apobec-1 antiserum recognized a 27-kDa protein band that was present only in mouse hepatoma (Hepa) cells infected with an adenovirus expressing the tagged-apobec-1 but not cells infected with a virus expressing luciferase (**Fig. 2, top**). Reprobing the same membrane with rabbit polyclonal anti-HA-11 antiserum showed that the same band contained the HA-11 epitope (data not shown).

To further define the specificity of the antiserum, we took advantage of the high resolving power of 2-dimensional gel electrophoresis. Hepa cell S100 proteins were separated on a 2-dimensional gel. Western blotting using the anti-apobec-1 antiserum revealed an immunoreactive spot at 27 kDa (Fig. 2, bottom).

Inhibition of apoB mRNA editing by anti-apobec-1 antibody

To ascertain whether binding of anti-apobec-1 antiserum to mouse apobec-1 had any effect on the in vitro RNA editing activity, apoB mRNA editing activity of mouse liver S100 extract was assayed in the absence and presence of different amounts of anti-apobec-1 serum. The anti-apobec-1 antiserum inhibited the editing activity in a dose-dependent manner (**Fig. 3**). The ratios

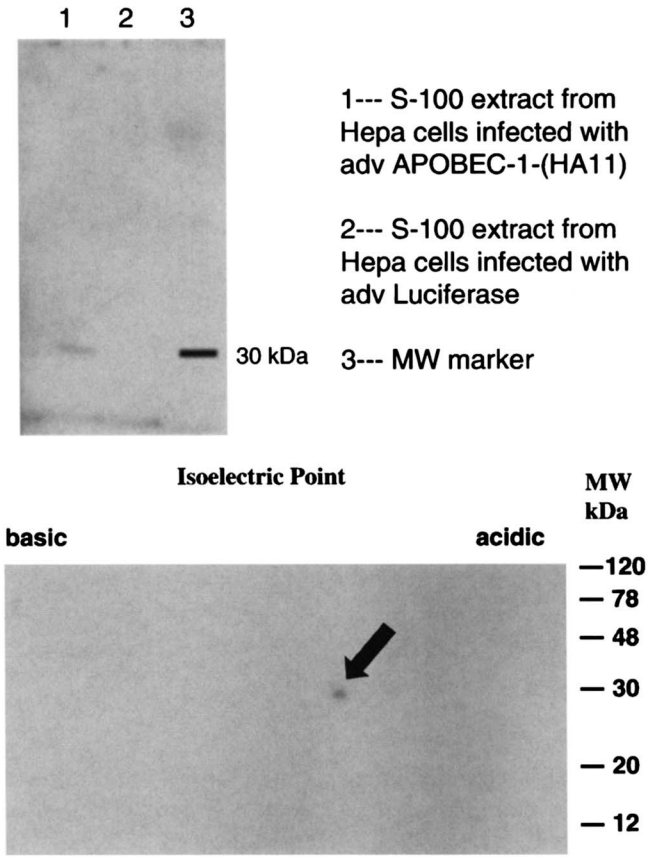

Fig. 2. (Top) Western blotting with anti-apobec-1 antiserum. SDS-polyacrylamide gel electrophoresis and Western blotting of Hepa cell S-100 extracts was performed as described in Materials and Methods. The chemiluminescence image recorded on film was shown. Lane 1 was from Hepa cells expressing HA-11 epitope-tagged apobec-1. Lane 2 was from Hepa cells expressing luciferase. Lane 3 prestained molecular weight markers and the position of marker closest to the molecular weight of apobec-1 was indicated. (Bottom) Western blotting of two-dimensional gel. Hepa cell S-100 with epitope-tagged apobec-1 was isoelectric focused on a pH 3–10 gradient and electrophoresed in the vertical dimension on a 10% SDS-polyacrylamide gel. The location of the protein detected by the anti-apobec-1 antiserum is indicated (arrow).

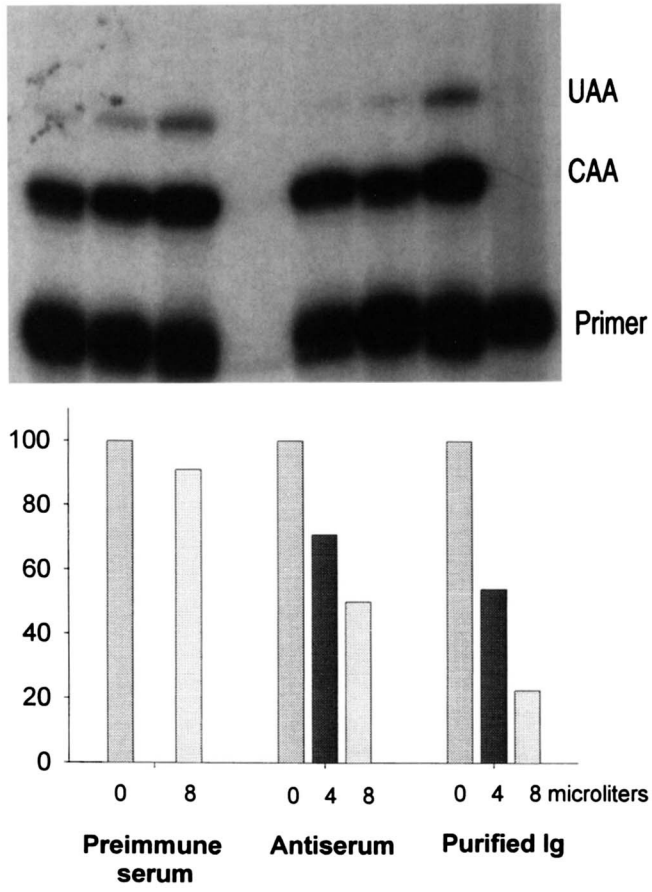

Fig. 3. Inhibition of apoB mRNA editing activity by anti-apobec-1 antibody. The autoradiograph shows the products of primer extension in the in vitro assay. The positions of unextended primer, extended primer on unedited apoB mRNA, and extended primer on edited apoB mRNA were labeled on the right as Primer, CAA, and UAA, respectively. The three lanes on the left were samples incubated with anti-apobec-1 antiserum of the amount indicated, and the three lanes on the right with anti-apobec-1 purified immunoglobulin. (Bottom) Quantitation of the degree of inhibition of apoB mRNA editing. The radiolabeled bands corresponding to the reaction products in A) were quantified by a phosphorimager. The percentage of edited over unedited apoB mRNA was 15.4% in the control reaction with no antiserum added and 16.9% in the control reaction with no purified immunoglobulin added. The effect of addition of antiserum or purified immunoglobulin was expressed as a percentage of the respective controls.

of edited to unedited RNA in the product were reduced by increasing amounts of antiserum from approximately 15% in wild type liver S100 with no antiserum to approximately 8% editing in the S100 reacted with 8 μl antiserum (Fig. 3). In contrast, preimmune serum had no effect on editing (Fig. 3, bottom). Immunoglobulin was purified from anti-apobec-1 antiserum by Protein G affinity chromatography. The purified immunoglobulin also inhibited RNA editing in a dose-dependent fashion (Fig. 3). In this experiment, the ratios of edited to unedited message were reduced by increasing amounts of purified immunoglobulin from approximately 17% in wild type liver S100 with no antibody down to approximately 4% editing in the S100 reacted with 8 μl immunoglobulin (Fig. 3, bottom). Therefore, the active component in the antiserum that inhibited apoB mRNA editing in vitro was the immunoglobulin fraction.

DISCUSSION

In the characterization of a gene product, specific antibodies to the protein are often invaluable. For most

cloned proteins, there are three conventional approaches to produce antibodies against the protein. The first approach is to purify the protein and use it as an immunogen. However, this approach is not feasible for minor proteins. The second approach is to express the cDNA in an expression vector with subsequent purification of the expression product. This approach has the disadvantages of being labor-intensive and of frequent contamination by expression of host proteins which may lead to low specificity of the antisera. The third approach is to synthesize selected oligopeptide sequences in the protein and then conjugate them to a carrier protein for immunization. This approach has the limitations that oligopeptides are often not highly immunogenic and specificity would be limited because the antisera would recognize only small portions of the protein molecule. The technique of DNA immunization presented in this communication circumvents all the problems associated with these conventional methods and allows for the production of highly specific antisera in a simple, direct, and expeditious manner.

DNA immunization has broad applications from production of antibodies for research to potential therapeutic benefits in a variety of diseases (7–9). The in vivo synthesis and secretion of the antigen by transfected cells in the host animal obviates the need for production and purification of the antigen. Furthermore, the antigen produced in situ is much more likely to be in the native state than proteins isolated in vitro. DNA immunization using intramuscular injection of DNA has been successfully used in mice (19, 21), piglets (20), and monkeys (33). However, this technique has not been used previously in rabbits.

The quality of DNA injected is important as impurities in the DNA may decrease transfection efficiency. When DNA is injected intramuscularly, the myocytes are the primary site of transgene expression. The antigen-presenting cells are the cells responsible for processing the antigen for class I MHC presentation to the lymphocytes (21). Co-injection of a plasmid expressing GMCSF enhances the immune response (25, 26). The quantity of DNA needed for injection of rabbits is small and well within the production capacity of most laboratories without the need for large-scale fermentors. Furthermore, by using the intramuscular route, one does not need sophisticated DNA "guns" that are used for intradermal immunization.

In conclusion, production of a polyclonal anti-apobec-1 antiserum from rabbit was achieved by simple intramuscular injection of plasmid DNA using ordinary needles and syringes. The antibody was specific and the immunoglobulin in the antiserum was the active component that inhibited apoB mRNA editing by mouse liver S100 extract in vitro. A similar approach can be applied to the production of antibodies to a variety of low abundance proteins related to lipid metabolism (e.g., most of the intracellular enzymes such as hormone-sensitive lipase and acyl-coenzyme A: cholesterol acyltransferase).

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