A Rapid and Simple Approach to Preparation of Monoclonal Antibody Based on DNA Immunization

Yi Ni[1], Ke Ma[1], Jing Ni[1], Xiujuan Zheng[1], Ying Wang[1] and Sidong Xiong[1, 2]

Inoculation with purified specific protein is usually the first step for preparation of monoclonal antibody (mAb). But it is quite difficult to obtain pure proteins especially with natural structures. Here we attempt to replace the protein inoculation with DNA immunization in the preparation of mAb. The eukaryotic expression vectors pcDNA3-PreS2/S and pVAX-PreS2/S encoding the HBV M protein were constructed and prepared for DNA immunization. Female BALB/c mice developed a well antibody response to the target antigen after muscle injection with corresponding plasmids. The mice with effective antibodies induced were used for preparation of mAb. We found the mice immunized with three administrations of pcDNA3-PreS2/S and boosted by intrasplenic injection with the same plasmid could be exploited for preparation of mAb. And positive hybridoma cell 2D3 that can secrete specific mAb was cloned and analyzed. Our studies demonstrate that gene immunization may provide a convenient and efficient way to prepare mAbs. *Cellular & Molecular Immunology*. 2004;1(4):295-299.

Key Words: gene immunization, HBV, monoclonal antibody, intrasplenic immunization

Introduction

Gene immunization, by delivering the gene encoding the protein as a simple eukaryotic expression vector (naked DNA) rather than the protein itself, has been proved to be a good method for inducing humoral and cellular immunity (1, 2). In recent years, there are many reports about producing polyclonal antibodies against special proteins by DNA immunization, especially the proteins which are difficult to purify (3, 4, 5). But few researches were reported about production of mAbs to molecules of interest by this relatively simple strategy (6, 7). Theoretically, the advantages of DNA immunization over conventional protein immunization include: 1) efficient induction of antibody immune response without purified protein (3); 2) the ability to express proteins *in vivo* similar to their natural structures (4); 3) the relative ease of producing mAbs (8). Therefore, DNA immunization may provide a better way to produce specific monoclonal or polyclonal antibodies to proteins that are difficult to purify or whose structures are easy to be destroyed during purification or in the body of the immunized animals. In this study, HBV PreS2/S gene, which encodes HBV middle envelope protein (HBV M protien), was chosen as a model to investigate the possibility of mAbs production by DNA immunization. The regime of DNA immunization was explored and the positive hybridoma cells were cloned and analyzed.

Materials and Methods

Mice

Female BALB/c mice were obtained at 6-8 weeks of age from the department of laboratorial animals, Fudan University (Shanghai, China).

Cell line, media and cell culture

RPMI 1640 was supplemented with 2 mM L-glutamine, 20 mM HEPES and 100 U/mL ampenicilllin and streptomin. HAT medium was RPMI 1640 supplemented with 1 mM sodium pyruvate, 100 mM hypoxanthine, 400 nM aminopterin, 16 mM thymidine and 10% v/v FCS. HT medium was prepared as the HAT but the aminopterin was omitted. Sp2/0 cells (American Type Culture Collection CRL 1581) were routinely cultured in RPMI 1640 supplemented with 10% v/v FCS. They were subcultured at a split ratio of 1:4 at approximately 70% confluency. All cell cultures were maintained in a 5% CO_2 atmosphere at 37˚C.

Cloning of the gene encoding HBV M protein into vector

The middle envelope genes of HBV were amplified by PCR from plasmid pEHH (constructed by our lab), which contains the full-length HBV genome twice, and inserted into EcoR I sites in the polylinker region of plasmid pVAX to construct plasmid pVAX-PreS2/S. Then the pVAX-PreS2/S was digested with EcoR I. The fragment (approximately 800 bp) was inserted to pcDNA3 plasmid (Invitrogen). Miniprep plasmid DNA was purified from overnight

[1]Shanghai Medical College, Fudan University, Shanghai 200032, China.

[2]Corresponding to: Dr. Sidong Xiong, Department of Immunology, Shanghai Medical College of Fudan University, 130 DongAn Road, Shanghai 200032, China. Fax: +86-21-542-37749, E-mail: sdxiong@shmu.edu.cn.

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cultures of ten colonies and the orientation of the insert was checked by PCR. In order to ensure that the plasmid construct was intact and functional, the plasmid was sequenced across the gene insert. Kozak sequence (Kozak, 1987) was included in both plasmids.

Preparation of plasmid DNA

For large-scale preparation, the plasmid were amplified in *E.coli* DH5α and isolated using 'mega prep' plasmid isolation columns (Qiagen, UK). Purity and concentration of DNA were determined by spectrophotometer at 260/280 nm. The plasmid (pVAX/pcDNA3-PreS2/S) were resuspended in sterile saline solution and stored at -20˚C until use.

Dot-enzyme immunosorbent assay (Dot-EIA)

Supernatants of transfected cells were harvested at 24, 48, 72-hour after transfection, pumped into Cellulose Nitrate membrane by negative pressure equipment. The membrane was blocked by 5% defatted milk, and then incubated with anti-sera and goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (HRP, SABC) in turn. Immunoblots were developed with DAB reagents (Sigma).

Immunohistochemistry

Quadriceps muscles of mice injected with plasmid DNA 24 hours before were taken out quickly before making cryosections. Following treated with acetone and normal serum, sections were incubated with anti-serum (1:500) for 1 h at 37˚C and overnight at 4˚C. Following washing three times in PBS, filters were incubated for 1 h with biotinylated goat anti-rat IgG (1:200) at 37˚C. After washed three times, filters were incubated for half an hour with streptavidin-biotin-peroxidase complex (Vector, USA). Staining was controlled under the microscope.

DNA immunization

Six-week female BALB/c mice were divided into two groups, and each group contained five. They were anesthetized and injected with bupivacaine intramuscularly into quadriceps muscles. 24 h later, mice were injected in the same site with 100 mg plasmid. Blood samples were collected from the ophthalmic venous plexus of immunized mice for ELISA. Sera were separated and stored at -20˚C.

Enzyme-linked immunosorbent assay (ELISA)

The titers of antibody against the HBV M protein in the sera of mice or supernatants of hybridoma cultures were determined by indirect ELISA. Polystyrene plates were coated with 0.5 g HBV S antigen (purified from baculoviral extracts) overnight at 4°C in 100 μl PBS (89 mM Boric Acid, 90 mM NaCl, pH 8.3) per well. And wells were blocked with 10% bovine serum and 5% goat serum in PBS for 1 h at 37˚C. Then the plates were washed with PBS containing 0.1% Tween 20 and 100 μl samples were added to the wells and incubated for 1 h at room temperature. After washing, 100 μl goat anti-mouse immunoglobulin horseradish peroxidase conjugate (SABC, diluted 1:5000) were added to each well and incubated for 1 h at room temperature. The reaction was developed with a solution containing 2 mg/ml phenylenediamine, 0.03% H_2O_2 in 0.1 M citrate–phosphate buffer (pH 5.0); the reaction was stopped with 50 μl of 4 N H_2SO_4. The result was read at 492 nm in an ELISA Microplate Reader S960 (Metertech).

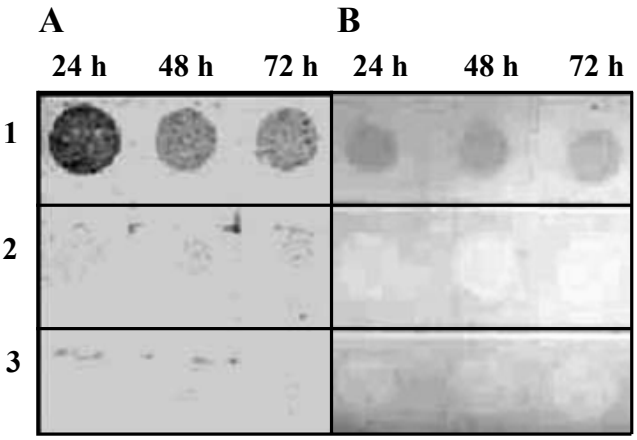

Figure 1. Expression of M protein in C2C12 cells transfected with pVAX-PreS2/S (A) and pcDNA3-PreS2/S (B). Supernatants of transfected cells were harvested at 24, 48, 72-hour after transfection, then M protein was detected by Dot-EIA.
1. Supernatant of pVAX-PreS2/S or pcDNA3-PreS2/S: 24-72 hour;
2. Supernatant of pVAX or pcDNA3: 24-72 hour;
3. Untransfected supernatant: 24-72 hour.

Generation of mAbs

After first DNA immunization, mice were boosted at 2th and 4th week. In the 6th week, intrasplenic immunization was done. For intrasplenic immunization, mice were anesthetized by intraperitoneal injection of Nembutal. The skin and peritoneum on the left side of the body were open to expose the spleen. A needle fitted to a 1 ml syringe with plasmid was deeply inserted into the spleen. After injection, the spleen was carefully pushed back into the peritoneal cavity. The peritoneal walls and skin were sutured with thread. Five days after the intrasplenic injection, splenocytes from BALB/c mouse immunized with pcDNA3-PreS2/S were fused with myeloma cells Sp2/0 by standard hybridoma technique using 50% polyethylene glycol (Sigma, USA). After HAT medium selection, culture supernatants were first analyzed by ELISA. Selected hybridomas were cloned by limiting dilution. Cloned hybridomas were grown as ascites in BALB/c mice.

Determination of the subclass of Ig in ascite

Conditions were the same as ELISA for subclass determination of the antibodies, except that HRP-anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Sigma, 1:5000).

Results

Construction of plasmids

To test the possibility for obtaining mAb by DNA vaccine, we constructed two plasmids DNA encoding HBV middle envelop protein (HBV M protein). The genes encoding HBV M protein (PreS2 plus S) were inserted into the eukaryotic expression vectors pVAX and pcDNA3 to construct plasmid pVAX-PreS2/S and pcDNA3-PreS2/S,

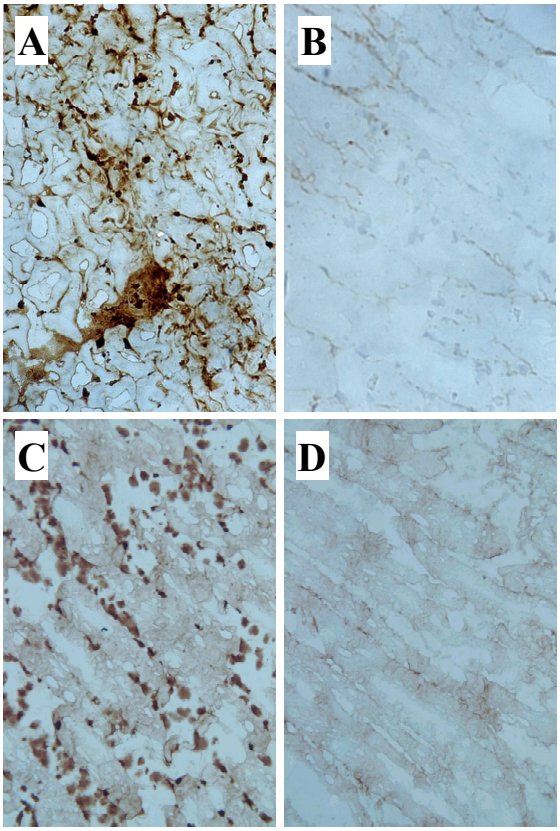

Figure 2. Expression of M protein in local quadriceps muscles in mice injected with pVAX-PreS2/S (A), pVAX (B), pcDNA3-PreS2/S (C) and pcDNA3 (D) (400×). The plasmids were injected in the quadriceps muscles of BALB/c mice. After 24 h, quadriceps muscles were taken out and made cryosections for immunohistochemistry.

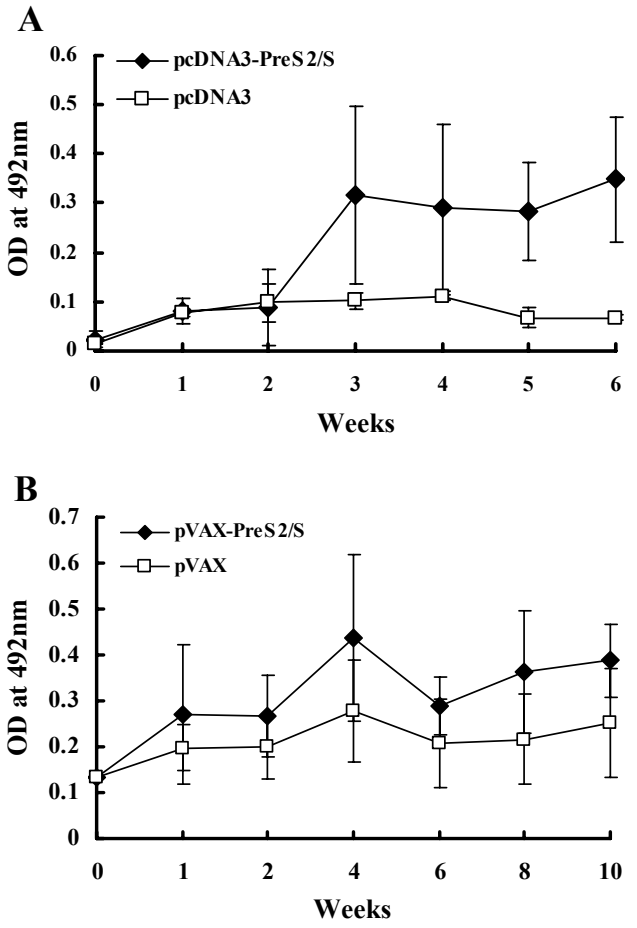

Figure 3. Antibody responses induced by DNA immunization determined by ELISA assay of anti-HBsAg specific IgG level. (A) DNA immunizion by pcDNA3-PreS2/S; (B) DNA immunizion by pVAX-PreS2/S. Mice were immunized intramuscularly with pVAX/pcDNA3-PreS2/S or pVAX/pcDNA3 with three administrations carried out at biweekly intervals. Sera were collected at appropriate time from each mouse and screened for the presence of anti-HBV M protein antibodies by ELISA.

respectively.

In vitro expression

The isolated plasmids were validated for expressing the corresponding proteins by analyzing the supernatant of transfected C2C12 cells with specific antibodies. As shown in Figure 1, anti-serum could specially react with the supernatants of pVAX/pcDNA3-PreS2/S transfected C2C12 cells. In contrast, the anti-serum could not react with the supernatants of pVAX/pcDNA3-transfected cells. It demonstrated that PreS2/S cloned into the two vectors could be expressed and secreted *in vitro*.

In vivo expression

As shown in the Figure 2, the expression of PreS2/S protein *in vivo* was observed by immunohistochemistry staining in the sections of tibilis anterior of BALB/c mice injected with pVAX/pcDNA3-hECD, not in the sections of muscular tissues injected with the control plasmid.

Evaluation of Ab response by ELISA

In order to produce antibodies against HBV M protein by injection of antigen-encoding plasmid DNA, intramusular immunization and direct immunization into spleen were used as inoculation routes. In this study, plasmid DNA encoding HBV M protein, termed pVAX/pcDNA3-PreS2/S was prepared. Mice were immunized intramuscularly with pVAX/pcDNA3-PreS2/S or pVAX/pcDNA3 with three administrations carried out at biweekly intervals. Sera were collected at 2-week intervals from each mouse and screened for the presence of anti-HBV M protein antibodies by ELISA. Four weeks after the first injection, all mice immunized with pVAX/pcDNA3-PreS2/S had high titers of IgG Abs, which could specially recognize the HBV M protein (Figure 3).

Production and characterization of mAbs

In our attempt to obtain hybridoma cells secreting the specific mAbs, we tried several immunization schemes. We found that mice immunized with either pVAX-PreS2/S or pcDNA3-PreS2/S generated specific antibodies after DNA inoculations, but no mAbs were obtained from mice that accepted a single dose or three doses intramuscular immunization. Eventually, we tried to improve the inoculating procedure by intrasplenic DNA immunization.

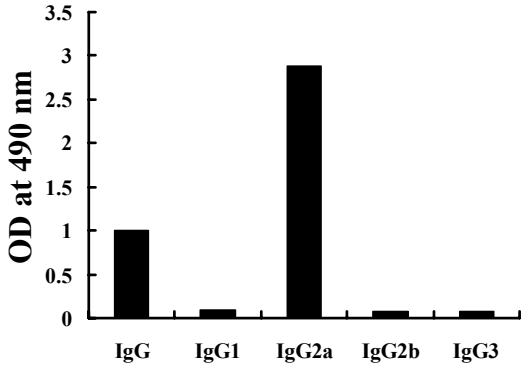

Figure 4. IgG subclass in ascites of mice inoculated by 2D3 hybridoma cells.

5 days after intrasplenic DNA immunization, mice were sacrificed for mAb production. One mAb was isolated after screening by ELISA. The mAb, which is derived from the group immunized with pcDNA3-PreS2/S, was named as 2D3 and demonstrated as IgG2a isotype (Figure 4).

Discussion

In most cases of DNA intramuscular immunization, high titers of antibodies have been found against the targeted protein. Although some researches demonstrated that a single intramuscular immunization of plasmid DNA induced antibody responses (9-11), boosters were required in several reports (12-14). Our experiences also suggested that repetitious immunization had better antibody responses than single dose inoculation. In contrast to intramuscular immunization, a single DNA intrasplenic injection could induce serum antibodies. These results indicate the possibility of using intrasplenic immunization for the induction of antibody responses by DNA immunization. In our results, specific antibody immune responses could be induced by DNA vectors encoding HBV M protein through intramuscular immunization, either by single dose or three doses administration. However, we did not get mAbs from these mice after several-time repetition. Therefore, we improved our experiments by another intrasplenic immunization after three-time intramuscular immunization. In fact, we did the intrasplenic immunization 5 days before the fusion of splenocytes and myeloma cells, and got a mAb 2D3. Intrasplenic immunization was also attempted by Kasinrerk (15) and his colleagues. During immunization with plasmid encoding several CD antigens, they compared different methods, and found that specific antibodies could by induced by single intrasplenic immunization and repetitious intramuscular immunization, but not by single intraperitoneal, single intravenous and single intramuscular immunization. It is also demonstrated in Xiong's research (16, 17) that strong immune responses could be induced by intrasplenic immunization.

Michel et al., (18) had constructed four recombinant vectors encoding different parts of HBV surface protein under different promoters/enhancers. They then compared the specific antibodies in mice inoculated with these plasmids. Titers of antibody were the highest in mice vaccinated with plasmid encoding PreS2/S under CMV promoter. In this study, we constructed plasmid expression vectors coding for HBV middle envelope protein PreS2/S and they all showed the ability of inducing antibody response against HBsAg in vaccined mice. However, even by three-time intramuscular immunization and intrasplenic immunization improvement, we obtained a mAb 2D3 from mice immunized with pcDNA3-PreS2/S, but failed to get mAbs from mice immunized with pVAX-PreS2/S. In fact, although pVAX is the only vector approved by FDA for administrator of human, most research on DNA immunization especially for preparing polyclonal and monoclonel antibody, the pcDNA3 was more widely used. Neverthness, it is hasty to get a conclusion that pcDNA3 was more suitable as a vecotor for mAb production than pVAX, and more experiments are needed to demonstrate this conception.

Theoretically, besides convenient immunization, other advantages of DNA immunization such as long-lasting expression of specific antigen *in vitro* and relatively natural construction of the antigen are promising for us to use this method in mAbs production. In our study, HBV M protein gene was transcripted and translated in eukaryotic cells, which may be similar to the nature HBV infection. After induction of humoral immunity in mice, we got a hibridoma cell, 2D3, which could secrete specific monoclonal antibody to HBsAg. But we need further experiments to determine the combine site of this mAb，the targeted epitope of HBsAg and the specificity of this mAb.

On the one hand, genetic immunization looks very promising as a new way to administer vaccines. Yet little is known about the biology underlying this technology. However, with the development of gene immunization in recent years, people realized that several factors had deeply close relation with the effect of immunization, such as the efficiency of the carriers, the expression level *in vivo*, immunogenicity of the target protein, the location of the protein which might be related to different method of gene inoculation, and the host animals. On the other hand, success of a mAb production could also be influenced by several aspects, such as proficiency of the operators, the fusion efficiency of cells, and the individual differences of animals. The both sides make it difficult to quantitatively determine the relation between gene immunization and mAb production. In our experiments, we demonstrated that mAb could be successfully produced by gene immunization. This is the first step that encourages the future experiments focused on how to boost immune responses, to improve the efficiency of selection, and to acquire high titer mAb.

In conclusion, the data presented here demonstrated that a mAb was produced through combined intramuscular and intrasplenic DNA immunization. These findings illuminate the way of mAb preparation.

Acknowledgements

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