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Study on the antifertility effects of the plasmid DNA vaccine expressing partial brLDH-C4′

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Abstract

Partial cDNA sequence coding for *Microtus brandti radde* (Brandt's vole) testes-specific lactate dehydrogenase (brLDH-C4) was amplified by reverse transcription-polymerase chain reaction (RT-PCR). By inserting the product into the eukaryotic expression vector pCR3.1, pCR3.1-brLDH-C4′ was obtained as the prototype of contraceptive DNA vaccine. Immunization with pCR3.1-brLDH-C4′ in BALB/c mice generated antibodies specific to purified brLDH-C4′ and native mouse LDH-C4 protein. The birth rate of the pCR3.1-brLDH-C4′ immunized mice was found to be decreased significantly (80% lower than that of those immunized with pCR3.1). Functions of the elicited antibodies in sera from pCR3.1-brLDH-C4′ inoculated mice were further explored. The results indicated that the antibodies from the mice injected with pCR3.1-brLDH-C4′ could cause the agglutination of normal sperm suspension, while the ovarian structure and the development of ovarian follicles of these mice were not impaired, which gives a possible explanation for the immunocontraceptive effects of the pCR3.1-brLDH-C4′ DNA vaccine.

Reproduction (2006) **131** 183–192

Introduction

Development of a vaccine based on sperm antigens is a promising approach to contraception (Naz & Zhu 1998). Sperm-specific isozyme of lactate dehydrogenase (LDH-C4) does not appear until puberty and then is isolated from the immune system by the blood-testis barrier (Wheat & Goldberg 1983) and catalyses the conversion of lactate to pyruvate (Goldberg 1985), has been selected as a vaccine target due to its quality of sperm surface localization (Burgos *et al.* 1995), antibody inhibition of sperm function (Beyler *et al.* 1985) and its unique occurrence in the testis (germ cell)/spermatozoa (Blanco & Zinkham 1963).

LDH-C4 was highly immunogenic in the production of humoral antibody in female mice (Gupta *et al.* 1996). Stimulation of a local mucosal immune response to LDH-C4 in the reproductive tract would guarantee the presence of antibodies at the site of fertilization, which should suppress fertility. After intrauterine immunization with LDH-C4, SJL/J female mice secrete antibodies specific for LDH-C4, into their uterine fluids inducing of a local immune response. This an effective alternative to systemic immunization for administering a contraceptive vaccine (Shelton & Goldberg 1986). The immunosuppressive determinant of LDH-C4 is cell-specific and dose selective (Gupta & Chaturvedi 2000).

LDH-C4 is currently the most characterized sperm antigen (Herr 1996), its potential as candidate vaccine immunocontraception has been under extensive investigation. Mice (Kille *et al.* 1978, Mahi-Brown *et al.* 1990), rabbits (Goldberg 1973) and baboons (Goldberg *et al.* 1981) actively immunized with purified LDH-C4 showed reduced fertility which was also induced by chemically modified LDH-C4 in mice (Gupta & Syal 1997). Small synthetic peptides bearing antigenic determinants of LDH-C4, while conjugated to diphtheria toxoid (Wheat *et al.* 1985, Hogrefe *et al.* 1989, O'Hern *et al.* 1995) or tetanus toxoid (O'Hern *et al.* 1997) T-cell epitope, could elicit an immune response to the native protein, which significantly reduces the fertility of treated animals. Athough immune responses could be elicited in foxes to oral doses of recombinant *Salmonella typhimurium* expressing fox LDH-C4 (Bird *et al.* 1998), and in mice to vaginal LDH-C4 DNA immunization (Shen *et al.* 2003), there is no literature reporting the contraceptive effects of a LDH-C4 DNA. In this study, partial cDNA of *Microtus brandti radde* (Brandt's vole) LDH-C4, which includes the coding sequence for amino acid 5–20, a B-cell epitope, was cloned and inserted into the eukaryotic expression vector pCR3.1. We then use the recombinant plasmid

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/rep.1.00262
Online version via www.reproduction-online.org

pCR3.1-brLDH-C4′ as the prototype of a LDH-C4 DNA vaccine to immunize BALB/c mice and examine its effects on immuno-antifertility. It was found that immune responses to LDH-C4 were induced in the female BALB/c mice dosed with the DNA vaccine and the suppressed fertility of the immunized mice were also observed.

Materials and Methods

Animals

The BALB/c mice were purchased from the Institute of Genetics of the Chinese Academy of Sciences, housed in a 12 h light: 12 h darkness and given food and water *ad libitum*. All experiments were conducted according to the guidelines of the Chinese Animal Care for Laboratory Animals, and the protocols were approved by the Animal Care and Use Committee at the Institute of Zoology, Chinese Academy of Sciences.

cDNA amplification

Based on the mouse and fox cDNA sequences in GenBank, the partial encoding sequence of the LDH-C4 protein was selected as the target gene, in which the region coding for the immunogenic epitope 5–20 was included. The primers for reverse transcription- polymerase chain reaction (RT-PCR) were as follows; upstream primer A: 5′-AACATGGCCACCGTCAAGGAGC-3′, downstream primer B: 5′-ACCCAGCTTCTCCCCAATCAGTTAACG-3′, primer A contains a start codon, while primer B contains a stop codon.

Total RNA was extracted from mature testes of *M. brandti radde* using Trizol reagent (Invitrogen, Carlsad, CA, USA) following the manufacturer's protocol. The RNA pellets were gently resuspended in 100 μl nuclease-free water. All RNA samples were stored at −20 ° C until use.

The amplification of cDNA fragment was performed by RT-PCR according to the manufacturer's instruction (Promega, Madison, WI, USA). The reverse transcription was allowed to proceed at 48 ° C for 50 min then followed by inactivation at 95 ° C for 2 min. The PCR cycling parameters were 94 ° C for 1 min, 65 ° C for 30 sec and 72 ° C for 2 min, cycle 40 times; 72 ° C for 10 min. The PCR product was examined by electrophoresis in 1.5% agarose gel (Promega).

Construction of pCR3.1-brLDH-C4′

The purified PCR product was inserted into pCR3.1 (Invitrogen) according to the manufacturer's instruction. Successful ligations were confirmed by restriction mapping and sequencing.

Expression in cultured HeLa cells in vitro

pCR3.1-brLDH-C4′ and pCR3.1 were respectively transfected into cultured HeLa cells by use of LipofectAMINE (Gibco BRL, Rockville, MD, USA). Thirty-six h later, the transfected cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature. The slides were then rinsed three times in PBS; next, the cells were permeabilized with 0.1% Triton X-100 plus 0.1% sodium citrate in PBS for 2 min, washed with PBS, blocked with 0.5% BSA in PBS (pH 7.4) for 30 min at room temperature, and then incubated with the immunized sera (diluted 1:50 with PBS (pH 7.4) containing 0.5% BSA) at 4 °C overnight. These immunized sera were obtained from the mice at 6 weeks after being injected with pCR3.1-brLDH-C4′ or pCR3.1. After the cells were washed thoroughly, the secondary antibodies (goat anti-mouse IgG conjugated with FITC, diluted 1:100 with PBS (pH 7.4); Sigma) were added and incubated at 37 °C for 1 h. After washing the slides, the cells were counterstained with propidium iodine (PI, Sigma) for visualizing the nuclei and were then analyzed for gene expression using confocal microscopy (Leica, Solms, Germany).

Expression at mRNA level in vivo

Experimental BALB/c mice received 100 μl 0.25% bupivacaine-HCl i.m. by multi-spot injections in the leg muscles. Twenty-four h later, two mice were inoculated with pCR3.1-brLDH-C4′ (at 20 μg/mouse) in the same fashion and two other mice treated with pCR3.1 were controls. Total RNA was isolated from muscle of the injection sites on Week 1 post-inoculation and analyzed for mRNA expression *in vivo* by RT-PCR.

Immunization

The BALB mice were immunized with plasmid DNAs purified by using Qiagen Endofree Mega (Qiagen, Valencia, CA, USA). Female BALB/c mice housed as previously described, received 100 μl 0.25% bupivacaine-HCl by multi-spot injections in the leg muscles. Twenty-four h later (Wang *et al.* 1995, Xiang *et al.* 2003), each group of 20 female BALB/c mice were immunized i.m. with 10 μg (Group 1), 20 μg (Group 2) and 50 μg (Group 3) of recombinant pCR3.1-brLDH-C4′ respectively. Group 4 consisting of 20 female BALB/c mice were injected at the same schedule and in the same fashion as pCR3.1 as control. The mice were subsequently given booster doses twice by the same method at 2-week intervals.

Retro-orbital bleeds of immunized mice were collected at 2-week intervals, three times after priming. Serum was separated and stored individually at −20 °C. Preimmune sera were also collected for use from the mice in a similar manner as negative controls (Chen *et al.* 2002, Xiang *et al.* 2003).

Recombinant protein expressed in E. coli

M. brandti radde LDH-C4 partial cDNA sequence was inserted into pET28a (BamHI/EcoRI) to set up bacterial expression of the construct pET28a-brLDH-C4′. BL21

(DE3) was transformed with pET28a-brLDH-C4′ or pET28a was cultured in LB medium till OD = 0.5. Colonies containing brLDHC4′ partial sequence were confirmed by restriction enzyme digestion and sequencing. Recombinant protein was induced by 0.1, 0.5 and 1 mM IPTG (isopropyl thiogalactoside; Promega) respectively at 37 °C for 4 h, meanwhile the total protein of pET28a was induced with 0.5 mM IPTG, and was the control. Protein purification was performed with HiTrap chelating HP columns (Amersham Biosciences). The total protein including the specific recombinant protein was separated by 15% SDS–PAGE.

Western blot analysis

The total proteins of BALB/c mice testes and muscle were extracted with TRIzol Reagent (Invitrogen). After induction by IPTG, BL21 bacteria transformed with pET28a-brLDH-C4′ or pET28a were boiled, and the crude protein was diluted in the loading buffer. The two samples respectively contain specific LDH-C4 and purified recombinant brLDH-C4′ protein were separated by 15% SDS–PAGE with equal amount per lane, then electro-blotted onto a nitrocellulose membrane. After blocking overnight in TBST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% non-fat dry milk at 4 °C, the membrane was incubated with 1:100 immunized or preimmunized with sera in TBST at 37 °C for 2 h. Washed three times in TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (diluted 1:10 000 in TBST) for 1 h at room temperature. Following washing three times, the membrane was then processed using the enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA)

Antibody detection by ELISA

Recombinant LDHC-4′ protein and testes total protein (prepared as above) were selected as coated antigen. Ninety-six-well microtiter plates were coated with 100 μl (400 ng/ per well) of recombinant LDH-C4′ protein or testes total protein (2 μg per well) in bicarbonate buffer (pH 9.6) (Sigma) and incubated at 4 °C overnight. Following blocking 100 μl of 5% of non-fat dry milk was added to each well and then incubated at 37 °C for 1 h, 50 μl of sera of immunized mice (pCR3.1-brLDH-C4′) or sera of immunized mice (pCR3.1) at serial dilutions from 1:100 to 1:6000 was added to each well. Normal sera at 1:100 dilution was the control. After incubation at 37 °C for 2 h, 100 μl of secondary anti-mouse IgG conjugated with HRP (Promega) at dilution of 1:2000 was incubated at 37 °C for 2 h. The color development kit (R&D Systems Inc, USA) was used in order to detect the staining. The reaction was stopped by 1 M H_2SO_4, and the plate was measured with a plate reader (Bio-Rad) at 450 nm. Titers were defined as the final dilution giving an optical density of at least 0.1 unit above the optical density of the 1:50 dilution of the pre-immune serum. The pre-immune sera normally had an optical density of <0.1 units.

Immunohistochemistry

Direct and indirect immunohistochemistry were conducted to detect the presence of specific antibodies in vaccinated serum binding to the LDH-C4 *in situ.* Antibodies associated with testes LDH-C4 *in situ* were analyzed by the direct immunohistochemistry staining. Briefly, for direct immunohistochemistry, frozen sections were prepared from testes of BALB/c mice ($n = 20$) immunized with pCR3.1-brLDH-C4′ or pCR3.1 as a mock, 'mock' vectors were used in addition to control vectors as shams to exclude effects of pCR3.1 on results. The sections were blocked with 3–6% non-fat dry milk, then blocked with normal goat serum for 20 min. The slides were washed 3 times in PBS and directly incubated with the secondary antibodies (goat anti-mouse IgG conjugated with HRP) at 37 °C for 30 min. After washing with PBS, the samples were developed in addition of DAB (diamino benzidine) and nuclei were stained by haematoxylin.

For indirect immunohistochemistry studies, frozen sections were prepared from testes of normal BALB/c mice ($n = 20$) as above and were incubated with antisera from the animals immunized with either pCR3.1-brLDH-C4′ vaccine or pCR3.1 6 weeks post-immunization, because the antisera titer would reach the highest level after 6 weeks post-immunization of DNA in most studies (Koide *et al.* 2000). The sections were blocked with 3–6% non-fat dry milk for 20 min, and then blocked with normal goat serum 20 min. Sections of testes reacted with the first antibodies, which was either the sera of immunized mice (pCR3.1-brLDH-C4′) or sera of immunized mice (pCR3.1) overnight at 4 °C. The sections then were washed three times in PBS and incubated with secondary antibodies (goat anti-mouse conjugated with HRP) at 37 °C for 30 min. Slides were washed again in PBS and the antibody-stains were developed in addition of DAB and nuclei were stained by hematoxylin (Xiang *et al.* 2003)

Sperm preparation

Caudal epididymal sperm were collected by placing two minced caudal epididymides into 5 ml of PBS at 37 °C Sperm were allowed to swim out for 1 h, and were then centrifuged at 500 ***g*** for 10 min. After two washes, the pellet was suspended with PBS (Yakirevich & Naot 2000). Sperm suspension was dropped onto poly-L-lysine-coated cover slips, smeared, air-dried and then fixed with 4% paraformaldehyde for indirect immunofluorescence as described above.

Immunocontraceptive test

One week after the last booster, treated and control mice were placed by pairing each immunized female mouse with one normal male for 2 weeks. They were checked

daily for mating as evidenced by a vaginal plug. Number and weight of each offspring born were recorded.

Statistic analysis

Values of the number and weight of offsprings were reported as the mean ± S.E.M. Statistical analysis of the birth rate was done by one-way ANOVA. When significant effects of treatments were indicated, the Student–Newman–Keuls multi-range test was employed among the groups.

Sperm agglutination assay

Sperm were collected from BALB/c mice as described above. The sperm suspension (diluted to 20×10^6 cell/ml) and sera were mixed in the proportion of 3:1(v/v) in a microcentrifuge tube. After incubation at 37 °C for 1 h, 50 μl of the mixture was dropped onto glass slides, and sperm agglutination and motility were observed using inverted microscope.

Histology analysis

Six weeks after the last booster, the ovaries of treated and control mice were fixed in 10% neutral-buffered formalin, embedded in paraffin and subsequently sectioned at a thickness of 8 μm. The slides were stained with hematoxylin and eosin (H & E).

Results

Construction of pCR3.1-brLDH-C4′

We selected the partial fragment of LDH-C4 gene as the target, which contains the sequence coding for the specific epitope of LDH-C4 antigen. When RT-PCR was performed with our designed primers, a brLDH-C4′ cDNA product of expected size (540 bp) was obtained as shown in Fig.1. The PCR product was sequenced, the sequence was submitted to GenBank (registration no. AY866433). We compared it with mouse, human and fox and found that they share 83%, 80%, 79% identity, respectively. Fig. 2 showed the comparison analysis of gene sequences between the PCR product and mouse. The product was then inserted into the eukaryotic expression vector pCR3.1 to construct pCR3.1-brLDH-C4′ as the prototype gene vaccine. The resulting construction was confirmed by restriction mapping (Fig. 3) and sequencing. Sequence analysis indicated that the nucleotide sequence of brLDH-C4′ was 83% homologous with the gene fragment of mouse LDH-C4 (Chang *et al.* 2003).

Expression of pCR3.1-brLDH-C4′ in vitro *and* in vivo

In order to determine whether brLDH-C4′ can be expressed in eukaryotic cells, pCR3.1-brLDH-C4′ plasmid was transfected into HeLa cells (human origin). pCR3.1 was used as negtive control, epifluorescence microscopy showed that sera from the mice immunized with pCR3.1-brLDH-C4′ could bind to the HeLa cells transfected with pCR3.1-brLDH-C4′, while in other HeLa cells transfected with pCR3.1 or pCR3.1-brLDH-C4′, reacted with sera from the pCR3.1 immunized mice, no similar signals were observed (Fig. 4). This indicates that brLDH-C4′ could be successfully expressed in HeLa cells *in vitro*, and sera from the mice injected with pCR3.1-brLDH-C4′ contained the antibodies specific to the expressed brLDH-C4′ protein.

The expression of pCR3.1-brLDH-C4′ was further examined *in vivo*. Total RNAs were extracted from themuscle of BALB/c mice immunized with pCR3.1-brLDH-C4′ and pCR3.1. Expression of brLDH-C4′ was examined by RT-PCR. It was shown in Fig.5 that in either mouse

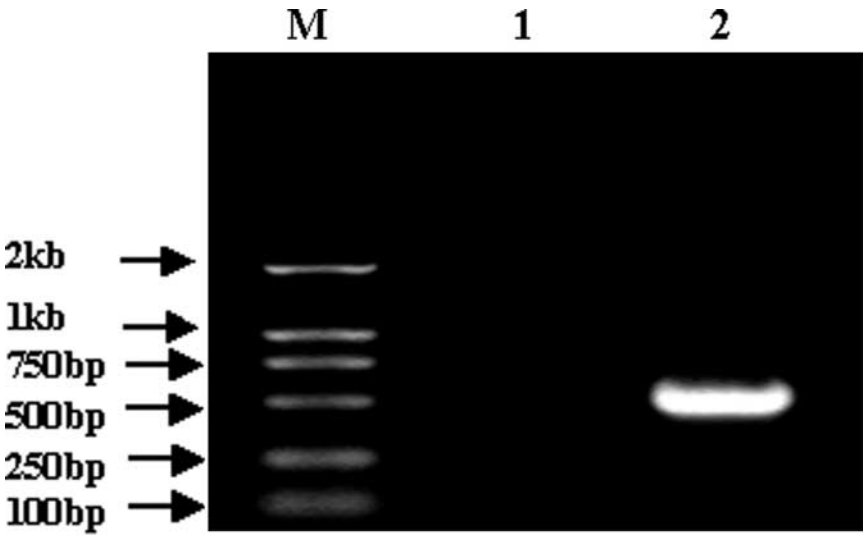

Figure 1 cDNA amplification of brLDH-C4′ by RT-PCR. Lane M shows the DNA marker (2 kb, 1 kb, 750 bp, 500 bp, 250 bp, 100 bp). Lane 1 shows the negative control. Lane 2 shows the cDNA of brLDH-C4′ amplified from *Microtus brandti radde*.

Figure 2 The comparison analysis of gene sequences between the PCR product and house mouse. Br, *Microtus brandti radde*; hm, house mouse.

inoculated with pCR3.1-brLDH-C4′, expression of brLDH-C4′ mRNA could be detected in muscle, while in each pCR3.1 immunized mouse, the expression of brLDH-C4′ was undetectable. The RT-PCR product was digested by *pvu*II and the two fragments correspond to the length of cloned brLDH-C4′ cDNA. The results of the expression of brLDH-C4′ both *in vitro* and *in vivo* suggest that pCR3.1-brLDH-C4′ as the prototype DNA vaccine is feasible.

Detection of specific antibodies to LDH-C4

To determine whether the expressed brLDH-C4′ in the mice inoculated with pCR3.1-brLDH-C4′ is immunogenic and whether the antibodies generated by such immunized mice can specifically bind to native mouse LDH-C4, sperm of normal mice were probed with immunized and preimmunized sera. It was observed that there was intense fluorescence along the principle pieces of the flagella and

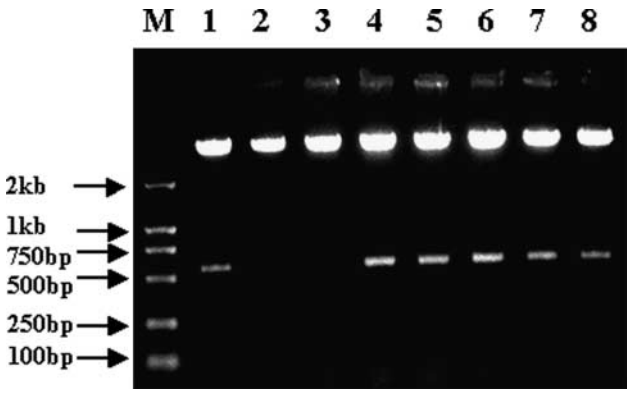

Figure 3 Identification of recombinant plasmid pCR3.1-brLDH-C4′ by restriction digestion. When digested with HindIII/HpaI, transformed clones with sense orientated brLDH-C4′ insertion produced fragments of about 600 bp (lane1 and lanes 4–8), while transformed clones with no brLDH-C4′ insertion did not produce the 600 bp fragments (lane 2 and lane 3). The 600 bp products were then analyzed by sequencing.

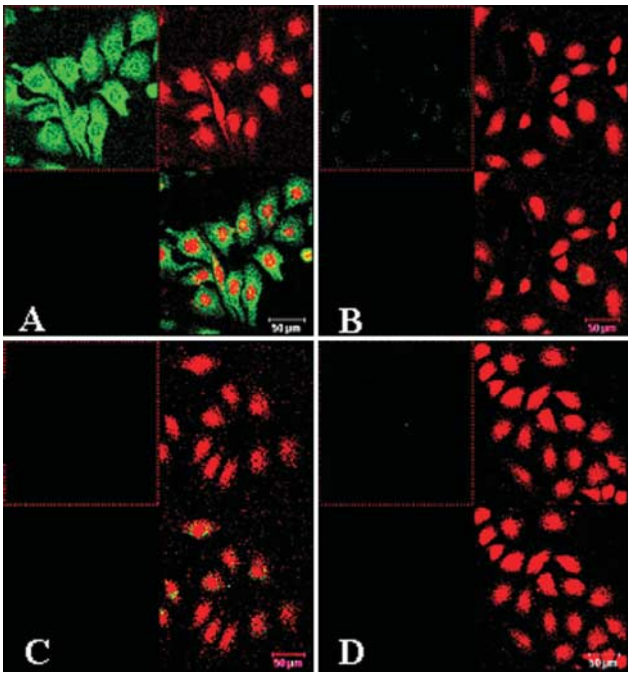

Figure 4 Analysis of expression of brLDH-C4′ protein in transfected HeLa cells. Transfected cells were reacted with sera as the primary antibody and secondary antibody conjugated with FITC as described in Materials and Methods. The cells were counterstained with propidium iodine for visualizing the nuclei of all cells. (A) HeLa cells transfected by pCR3.1-brLDH-C4′ were reacted with sera from the mice immunized with pCR3.1-brLDH-C4′. (B) HeLa cells transfected by pCR3.1-brLDH-C4′ were reacted with sera from the mice immunized with pCR3.1. (C) HeLa cells transfected by pCR3.1 were reacted with sera from the mice immunized with pCR3.1-brLDH-C4′. (D) HeLa cells transfected by pCR3.1 were reacted with sera from the mice immunized with pCR3.1. The green fluorescence emitted by activated FITC represents the expression of brLDH-C4′ protein and the red fluorescence from activated PI represents the nuclei of cells. Bar: 50 μm

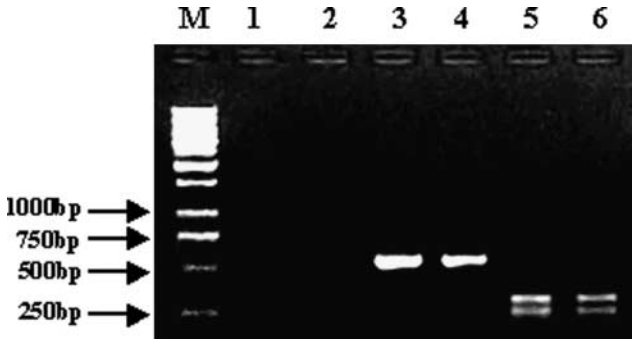

Figure 5 Determination of the expression of brLDH-C4′ at mRNA level by RT-PCR. Lane M shows the DNA marker. Lanes 1–2 show no expression of brLDH-C4′ at mRNA level detected in muscle of two individual mice immunized with pCR3.1. Lanes 3–4 show the expression of brLDH-C4′ at mRNA level detected in muscle of two individual mice immunized with pCR3.1-brLDH-C4′. Lanes 5–6 show the digestion of RT-PCR product by pvuII.

a slightly faint fluorescence in the heads and midpieces when the sperm were reacted with sera from the mice immunized with pCR3.1-brLDH-C4′; however, on the sperm incubated with sera from the mice injected with pCR3.1 or preimmunized mice, specific fluorescence could not be found (Fig. 6).

The specificity of antibody binding to brLDH-C4′ antigen was confirmed by Western blot. BrLDH-C4′ protein was expressed in the bacteria BL21, and after induction with IPTG, the volume of the desired protein was increased (Fig. 7A).

Purified recombinant brLDH-C4′ protein and total testes protein were tested with immunized or preimmunized sera, at the same time total muscle protein was used as a control by Western blot. Only purified recombinant brLDH-C4′ protein and total testes protein probed with immunized sera showed a specific protein band, approximately 20 kDa and 35 kDa, respectively, but no signal was found in total muscle protein probed with immunized sera. No signal appeared in purified recombinant brLDH-C4′ protein, total testes protein and total muscle protein probed with preimmunized sera (Fig. 7B).

ELISA

IgG specific to purified brLDH-C4′antigen and testes total protein in sera samples of the vaccinated mice at serial dilution were detected by standard ELISA. The results were shown in Fig. 8A, B, and indicated that brLDH-C4′-specific antibody elicited by pCR3.1-brLDH-C4′ was highly significant compared with that elicited by the pCR3.1 mock vector ($P < 0.01$).

Immunohistochemistry

In order to determine if the antibodies generated by immunized animals can specifically bind to LDH-C4 of testis *in situ* with immunohistochemical analysis, sections of testes from mice immunized pCR3.1-brLDH-C4′ or pCR3.1 vector were used. We have set up two immunohistochemical analyses.

Direct immunohistochemistry analysis

To examine the immunized animals that have develop anti-brLDH-C4 antibodies bound to the LDH-C4 antigen, frozen sections from immunized animals, and sections from the mock vector immunized animals were used reacted with goat anti-mouse IgG conjugated with HRP. After stain developed, we have observed that germ cells (spermatocyte, spermatid) from the mice that been immunized with pCR3.1-brLDH-C4′ were stained brown *in situ*, whereas no staining appeared from the animals vaccinated with the mock vector (Fig. 9 A1, A3). It suggests that anti-brLDH-C4′ antibodies were generated from the mice immunized with pCR3.1-brLDH-C4′ that were specifically bound to LDH-C4 antigen.

Indirect immunohistochemistry analysis

An indirect immunohistochemistry analysis was performed to examine whether sera from immunized animals can

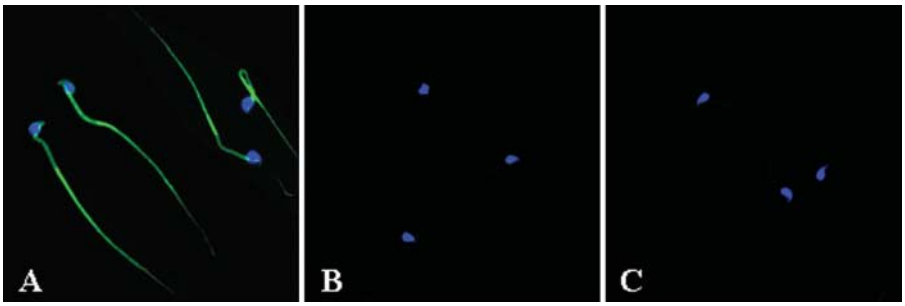

Figure 6 Antibody detection in sera by indirect immuno-fluorescence assay. The sperm from normal mice were reacted with antisera and stained with goat anti-mouse IgG conjugated with FITC. All the sperm cells were counterstained with DAPI (4′, 6-diamino-2-phenylidole) for visualization of the heads of the sperm. (A) Sperm were reacted with antisera from the mice inoculated with pCR3.1-brLDH-C4′, (B) Sperm were reacted with sera from the mice inoculated with pCR3.1. (C) Sperm were reacted with sera from the pre-immunized mice. The green fluorescence represents the binding of the antibodies elicited by brLDH-C4′ protein to native mouse LDH-C4, while the blue fluorescence represents the heads of all the sperm. The magnification is × 400.

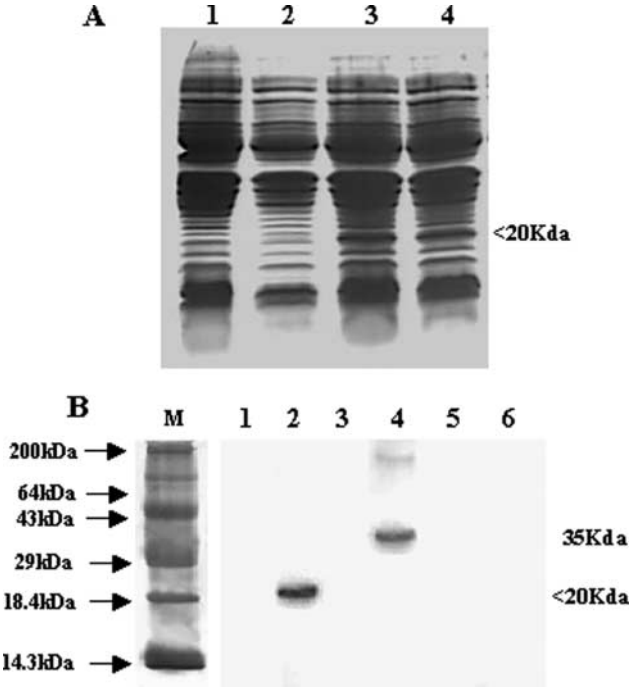

Figure 7 Detection of antibody in sera. (A) Recombinant protein expressed in *E.coli.* brLDH-C4′ partial cDNA segment was inserted into pET28a to construct bacterial expression vector-pET28a-brLDH-C4′. The total protein of BL21 was transformed with pET28a-brLDH-C4′ or pET28a and was separated by SDS–PAGE. Lane1: BL21 transformed with pET28a after 4 h induction of 0.1M IPTG; Lane 2: BL21 transformed with pET28a-brLDH-C4′ without IPTG induction; Lane 3: BL21 transformed with pET28a-brLDH-C4′ after 4 h induction of 0.1 M IPTG; Lane 4: BL21 transformed with pET28a-brLDH-C4′ after 4 h induction of 0.5 M IPTG. (B) Detection of specificity of the antibody to brLDH-C4′ from immunized sera. Lane M showed standard protein marker; Lane 1 showed that purified recombinant brLDH-C4′ protein was probed with preimmunized sera; Lane 2 showed that purified recombinant brLDH-C4′ protein was probed with immunized sera; Lane 3 showed that total testes protein was probed with preimmunized sera; Lane 4 showed that total testes protein was probed with immunized sera; Lane 5 showed that total muscle protein was probed with preimmunized sera; Lane 6 showed that total muscle protein was probed with immunized sera.

bind to LDH-C4 *in situ*. The frozen sections of testes from normal animals were reacted with anti-sera from the brLDH-C4′ DNA vaccinated mice and subsequently reacted with the goat anti-mouse IgG conjugated with HRP as the secondary antibodies. Sera from the unimmunized animals and mock vector immunized animals were used as the negative controls. After staining, we have observed that anti-sera from the animal immunized with pCR3.1-brLDH-C4′ was able to bind LDH-C4 of normal testis *in situ*, whereas no similar stains developed from the sera of animals immunized with the control vector and from the normal animals (Fig. 9 B1, B3). Both direct and indirect immunohistochemical analysis indicated that both mice immunized with pCR3.1-brLDH-C4′ generate specific anti-brLDH-C4′ antibodies, which specifically bind to the LDH-C4 of testis.

Immunocontraceptive effects of pCR3.1-brLDH-C4′ vaccine

An immunocontraception test was carried out to determine the effects of pCR3.1-brLDH-C4′ DNA vaccine. The results of this experiment clearly indicate that the pCR3.1-brLDH-C4′ vaccine has an effect on mice birth rate. This effect of birth rate is seen in immunized female mice, of which only 20% are able to give birth (Table 1). The data also showed there was no evident difference with the immunocontraceptive effects between 20 μg and 50 μg doses (Table 1)

Functional analysis of pCR3.1-brLDH-C4′ vaccine

The reduced fertility of the mice immunized with pCR3.1-brLDH-C4′ led us to investigate the inhibitory activity of pCR3.1-brLDH-C4′ vaccine on spermatozoa. Sperm were mixed with sera either from the pCR3.1-brLDH-C4′ immunized mice or from the mice injected with pCR3.1. Sera from the preimmunized mice were also used as control. Sperm agglutination was observed as the cross-linking or clumping together only appeared in the samples treated with sera from the pCR3.1-brLDH-C4′ inoculated mice (Fig.10). This indicates that the antibodies generated in the mice immunized with pCR3.1-brLDH-C4′ are

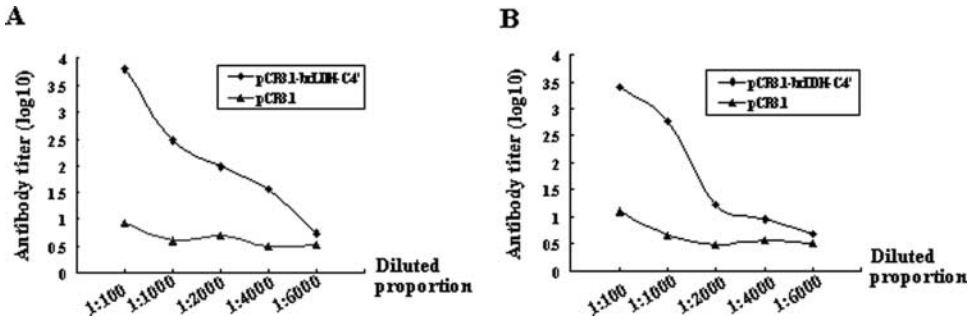

Figure 8 Detection of the antibody titers specific to LDHC4′ in sera by ELISA. (A) Recombinant LDH-C4′ protein was chosen as coated antigen. Sera samples (1:100 to 1:6000) from the mice immunized with pCR3.1-brLDH-C4′ or pCR3.1. B, Testes total protein was chosen as coated antigen, and the sera were serial diluted from 1:100 to 1:6000. The antibody titers were pooled and expressed as $\log_{10}$ titers.

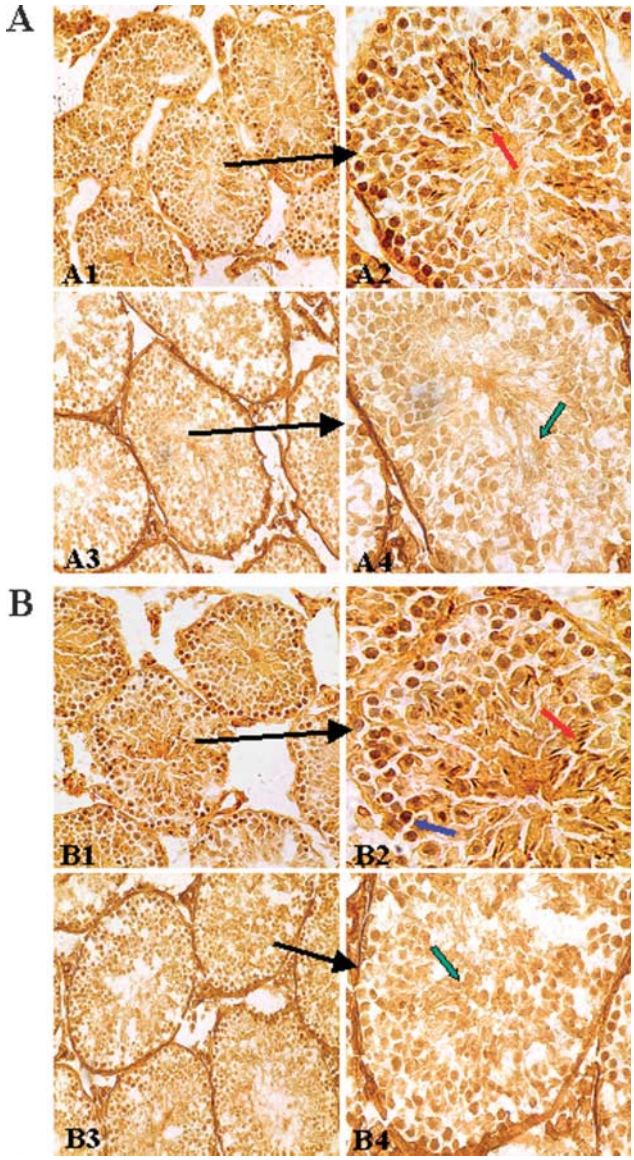

Figure 9 Analysis of direct or indirect immunohistochemstry. (A1) mice testes section from pCR3.1-brLDH-C4′ immunized group was fixed and stained with goat anti-mouse IgG conjugated with HRP (magnification: × 200). (A2) a higher magnification (× 400) of A1. (A3) sections from pCR3.1 vector immunized group were fixed and stained with goat anti-mouse IgG conjugated with HRP (× 200). (A4) a higher magnification (400 ×) of A3. (B1) testes sections of normal mice were reacted with the sera from pCR3.1-brLDH-C4′ immunized mice as the first antibodies and stained with goat anti-mouse IgG conjugated with HRP (× 200). (B2) a higher magnification (× 400) of B1. (B3) testes sections of normal mice were reacted with the sera from pCR3.1 vector immunized mice as the first antibodies and stained with goat anti-mouse IgG conjugated with HRP (× 200). (B4) a higher magnification (× 400) of B3. Green arrow, spermatid; Red arrow, spermatid with brown stains; Blue arrow, Primary spermatocyte with brown stains.

directed against native LDH-C4 on the sperm surface. Their interaction possibly restrained sperm motility and thus disturbed the normal sperm-egg interaction. We then studied the effects of pCR3.1-brLDH-C4′ vaccine on ovary morphological structure and follicle development. Ovary specimens from pCR3.1-brLDH-C4′ or pCR3.1 immunized mice were examined by the method of H & E staining. Histopathological slides of ovaries were evaluated by an independent observer. In all experimental mice including those immunized with pCR3.1-brLDH-C4′ and pCR3.1, there was no sign of abnormal development of ovarian follicles at multiple developmental stages, and ovarian structures were histologically normal in all experimental mice.

Discussion

As the world population is estimated to approach 10 billion by year 2050, there is increased need for simple, safe and reliable means of birth control (Bongaarts 1994). Immuno-contraception, the induction of antibody production to antigens associated with either gametes or reproductive hormones (Tollner *et al.* 2002), is a novel contraceptive strategy that has attracted much attention. Development of vaccines to sperm antigens is a promising way to carry out this scheme. Among the identified sperm antigens, LDH-C4 is perhaps the most characterized. Homotetrameric LDH-C4 functions in metabolic transition processes of pyruvate and lactate, and it uniquely exists in testes. Although most of the LDH-C4 is intracellular, a small proportion of LDH-C4 is located on the surface of sperm (Wheat & Goldberg 1977). It is also reported that antibodies to LDH-C4 do not cross-react with its somatic isozymes (Liang *et al.* 1986). With these traits, LDH-C4 is an excellent target for the development of a new kind of immunocontraceptive vaccine.

Peptide-based LDH-C4 vaccines have been reported to reduce the fertilities of mice, rabbits and baboons, yet there is still no literature regarding the contraceptive effects of a LDH-C4 DNA vaccine. In this current study, we have described for the first time, the construction of a pCR3.1-brLDH-C4′ DNA vaccine and its effects on fertility.

In order to obtain an immunogenic peptide small enough to avoid cytotoxic responses (Xiang *et al.* 2003), partial cDNA of *M. brandti radde* LDH-C4 containing the sequence coding for residues 5–20, LDH-C4 B-cell epitope, was amplified and cloned into pCR3.1 to get the prototype DNA vaccine pCR3.1-brLDH-C4′. After the immunization of experimental mice with pCR3.1-brLDH-C4′, brLDH-C4′ protein was expressed as an immunogen, which induced immune responses to LDH-C4. The generated antibodies could not only identify the purified brLDH-C4′ protein, but could also recognize native mouse LDH-C4 either on the sperm surfaces or in testes total protein, which is likely to reduce the enzyme action of LDH-C4. The interaction between the antibodies elicited by expressed partial brLDH-C4 protein and native LDH-C4 indicates the antifertility potential of pCR3.1-brLDH-C4′ DNA vaccine. Further ELISA assays show that

Table 1 Fertility of mice immunized with pCR3. 1-brLDH-c4′ or pCR3.1 values are mean ± S.E.M.

Group	**Dose** (μg)	**Birth Rate** (%)	**Number** (newborn g)	**Weight** (newborn g)
pCR3.1-brLDH-C4′	10	40 (n=8)$^{+}$	6.0 ± 1.0 (n = 48)$^{+}$	1.33 ± 0.11
	20	20 (n = 4)$^{++}$	4.5 ± 0.7 (n = 18)$^{++}$	1.28 ± 0.06
	50	20 (n = 4)$^{++}$	5.0 ± 0.4 (n = 20)$^{++}$	1.30 ± 0.08
pCR3.1	10	100 (n = 20)	4.9 ± 0.3 (n = 98)	1.35 ± 0.10

+ , Number of newborns is significantly different from control group ($P < 0.05$).
++ , Number of newborns is significantly different from control group ($P < 0.01$).
n, total number of female mice who gave birth after mating; *n*, total number of newborn in each group.

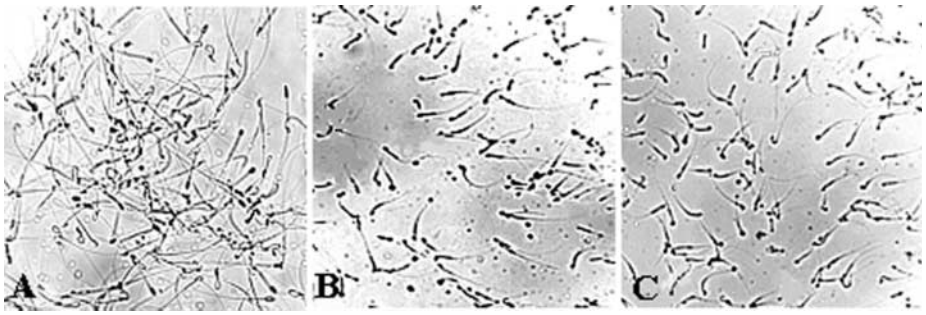

Figure 10 Sperm agglutination analysis. Agglutination in a tangled pattern was only observed in the sample treated with antisera from the mice immunized with pCR3.1-brLDH-C4′. (A) Sperm suspensions of normal mice were mixed with antisera from the mice inoculated with pCR3.1-brLDH-C4′; magnification: × 200. (B) Sperm suspensions of normal mice were mixed with antisera from the mice inoculated with pCR3.1; magnification: × 100. (C) Sperm suspensions of normal mice were mixed with antisera from the pre-immunized mice, magnification: × 100.

the DNA vaccine was able to induce the high antibody titre above 1:6000 in mice.

In this study, the fertility rate of pCR3.1-brLDH-C4′ immunized mice did decrease significantly, demonstrating the immunological and contraceptive functions of this DNA vaccine. However, there was almost no difference with the contraceptive efficacy between 20 μg and 50 μg (Table 1), despite that the efficacy of 10 μg was paralleled with 20 μg, suggesting that 20 μg of pCR3.1-brLDH-C4′ is perhaps enough to elicit strong immune responses. It has also been reported that serum antibody titers have no direct relationship with infertility rate (Goldberg *et al.* 1990, Herrera *et al.* 1992). The reason for no difference in infertility between 20 μg and 50 μg might involve cell-mediated immunity of DNA vaccine (Shelton & Goldberg 1990, Dufour 2001, Wang *et al.* 1995). We presume that T-cell immune responses possibly impact on one or various central stages of the antifertility process of the DNA vaccine.

Sera from pCR3.1-brLDH-C4′ immunized mice, when mixed with normal sperm suspension, caused the agglutination of sperm. This provides a possible explanation for the antifertility effects of pCR3.1-brLDH-C4′, i.e. the elicited antibodies impair the activity of LDH-C4, restrain sperm motility, prevent sperm from accessing the upper reproductive tract, and thereby inhibiting the binding of sperm to egg, reducing fertility. We also detected IgG antibodies to native LDH-C4 in vaginal fluids by Western blot assay.

Although the fertility of pCR3.1-brLDH-C4′ immunized mice was suppressed, their ovary structures and the development of ovarian follicles were unimpaired. This indicates that the antifertility effects of pCR3.1-brLDH-C4′ is working, the DNA vaccine didn't interfere normal ovarian functions. It seems that immunization with pCR3.1-brLDH-C4′ led to the reduction of fertility mainly by interfering with the function of sperm, but not the development of ovarian follicles.

The present study showed that immunocontraception could be achieved by immunization with a DNA vaccine directed to the sperm antigen LDH-C4. However, the contraceptive efficacy was far from satisfactory. In order to optimize the outcome, other trials, such as the use of DNA vectors encoding T helper 2 cytokines, are necessary for analyses. Alternatively, exploration of a contraceptive vaccine targeting multiple sperm antigens is also of significance. Our study, although preliminary, shows a new way for the development of simple, effective, safe and reliable forms of birth control.

Funding

This research was funded by grants from the National Natural Science Foundation of China (No. 30470204) and the Key Innovation Research Programs of Chinese Academy of Sciences (KSCX2-SW-201). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 2 April 2004
First decision 14 June 2004
Revised manuscript received 30 June 2005
Accepted 14 July 2005