Oral feeding and nasal instillation immunization with *Microtus brandti* lactate dehydrogenase c epitope DNA vaccine reduces fertility in mice via specific antibody responses

The immune responses induced by *Microtus brandti* lactate dehydrogenase C4 DNA vaccine via oral feeding and nasal instillation in mouse were investigated. The number of newborns of the vaccinated mice was statistically significantly reduced compared with the control mice, but the vaccine failed to affect the birthrate, as all vaccinated mice gave birth. (Fertil Steril® 2005;84:781–4. ©2005 by American Society for Reproductive Medicine.)

The human world population is growing at a tremendous rate despite the availability of various contraceptive modalities. Additional safe, convenient, and less-expensive means of contraception are urgently needed. The induction of immune responses against gamete-specific antigens represents one approach to developing contraceptive vaccines. Gamete-specific antigens to be used must be the functional molecules involved in the fertilization process, or in gamete development and transportation. A number of specific antigens that are involved in sperm–egg interaction have been identified (1). One such antigen that has been extensively studied is the sperm-specific antigen lactate dehydrogenase C_4 ($LDHC_4$).

$LDHC_4$ functions in lactate metabolism and glycolysis of sperm. Previous studies show that synthesis of $LDHC_4$ occurs only in the testis, and it is the predominant form of lactate dehydrogenase in mature spermatozoa. As Jethanandani and Goldberg (2) demonstrated, the expression of mouse $LDHC_4$ gene in non–germ cell nuclei is repressed by NF-I binding. Recently, it has been reported that $LDHC_4$ also is expressed in a wide range of cancer cells (3). Amino acid residues 1–20 of $LDHC_4$ have been shown to be particularly immunogenic in baboon and rabbit, and vaccinations of baboons with synthetic peptides representing this 20–amino acid region could reduce fertility by as much as 75% (4).

Inoculation by DNA vaccine coding an antigen directly into mammalian cells causes constitutive high-level expression of the antigen. Expression of this target antigen, in turn, can induce a full range of immunologic responses, including the production of antigen-specific antibody, cell-mediated killing, and cell-mediated cytokine release in various animals and in humans (5, 6). However, it is documented that tolerance has been developed by experimental animals immunized with self-antigen (7). As Wei et al. (8) described in 2001, immunogene therapy with a vaccine based on xenogeneic homologous molecules as the model antigen could overcome immune tolerance. Additionally, conventional methods of immunization with protein as the antigen could induce poor antigen-specific response in mucosal secretions yet produce adequate sera response.

The purpose of this study was to investigate the antifertility feasibility of a DNA vaccine based on xenogeneic LDHC, which was expected to break the immune tolerance and induce humoral and mucosal responses in the mouse model.

Female Balb/c mice were purchased from the Institute of Genetics, Chinese Academy of Sciences. All experiments were conducted according to the guidelines of the Beijing 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.

The pCR3.1 (Invitrogen, Groningen, The Netherlands) mock vector and pCR3.1–br$LDHC_4'$ (constructed and conserved by our laboratory) were purified with an endotoxin-free plasmid mega kit (Qiagen, Venlo, The Netherlands). The recombinant was confirmed by DNA sequencing.

Two experimental groups of mice (n = 10) received 20 μg of $LDHC_4'$ vaccine dissolved in 30 μL of saline via oral feeding and nasal instillation. Control groups were immunized with 20 μg of pCR3.1 in 30 μL of saline and only 30 μL of saline with oral feeding plus nasal instillation. We selected 20 μg as the vaccination dose based on a previous experiment that demonstrated that injection of 20

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μg of pCR3.1–brLDHC$_4$′ into the mouse can induce an antifertility response (Chang JJ, unpublished observations). Mice were given one booster in the same way at a week interval using the same amount of recombinant or mock DNA. One week after the second immunization, the sera and the vaginal fluids were collected.

To evaluate the efficacy and safety of the pCR3.1–brLDHC$_4$′ vaccine, the mice were mated with normal adult males at 1 week after immunization, using the mating process described previously elsewhere (1).

The total RNA was isolated and purified by Trizol reagent (Invitrogen) from the uterus after immunization. The RNA was treated with DNase (Promega, Madison, WI), and reverse transcription polymerase chain reagent (RT-PCR) was used to detect the expression of brLDHC$_4$ in vivo (Promega):

Up primer: 5′-AACATGGCCACCGTCAAGGAGC-3′
Down primer: 5′-ACCCAG-CTTCTCCCCAATCAGTTA AGG-3′

The testes of the adult mice were decapsulated and homogenized in 50 mM Tris-HCl buffer, then 50 μg of the protein was separated on 5% to 15% SDS-PAGE gel and blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). After blocking, the proteins were incubated with the sera (1:50) or reproductive tract fluids (1:20) overnight at 4°C. Then the blot was washed and incubated with goat anti-mouse IgG, IgA conjugated with HRP. Proteins were detected using the chemiluminescent ECL system from Amersham Pharmacia Biotech.

HeLa cells transfected with 2 μg pCR3.1–brLDHC$_4$′ or mock DNA were cultured in Dulbecco's Modified Eagle's medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine sera (HyClone Laboratories, Logan, UT). After 48 hours, the cells were fixed and permeabilized, then the cells were incubated with sera (1:50) or vaginal washes (1:20) at 4°C overnight. After washing, the cells were stained with goat anti-mouse IgG, IgA conjugated FITC, then counterstained with PI (propidium iodine; Sigma Chemical, St. Louis, MO) for visualizing nuclei. The samples were observed with a confocal microscope (Leica TCS-NT; Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

Spermatozoa were collected as described by Burgos et al. (9), and the sperm were incubated with mock and recombinant DNA vaccinated sera (1:100) and vaginal fluids (1:100) at 37°C. After 3 hours, the samples were washed and incubated with horseradish peroxidase-labeled goat anti-mouse IgG or IgA. We added diaminobenzidine solution on sperm for color development. The samples were visualized at ×100 magnification using an Olympus microscope (Melville, NY).

To test the specificity of antibodies induced by the DNA vaccine, 10 μL of pCR3.1–brLDHC$_4$′ vaccinated sera and vaginal fluids were incubated with 10 μg of brLDHC$_4$′ antigen (Chang JJ, unpublished data) at 4°C overnight. Spermatozoa tests were conducted as previously described, and the sera (1:100) and vaginal washes (1:100) after neutralization were used as the first antibody.

The sperm agglutination assay was based on the agglutination test described by Yakirevich and Naot (10). Sperm suspension (15 μL) was mixed with 5 μL of inactivated sera or vaginal fluids from the mock or recombinant vaccinated mice. The mixture was incubated at 37°C for 1 hour and examined under an inverted microscope.

Values were reported as the mean ± standard error of mean (SEM). For comparison of individual points, one-way analysis of variance (ANOVA) was used. $P<.05$ was considered statistically significant.

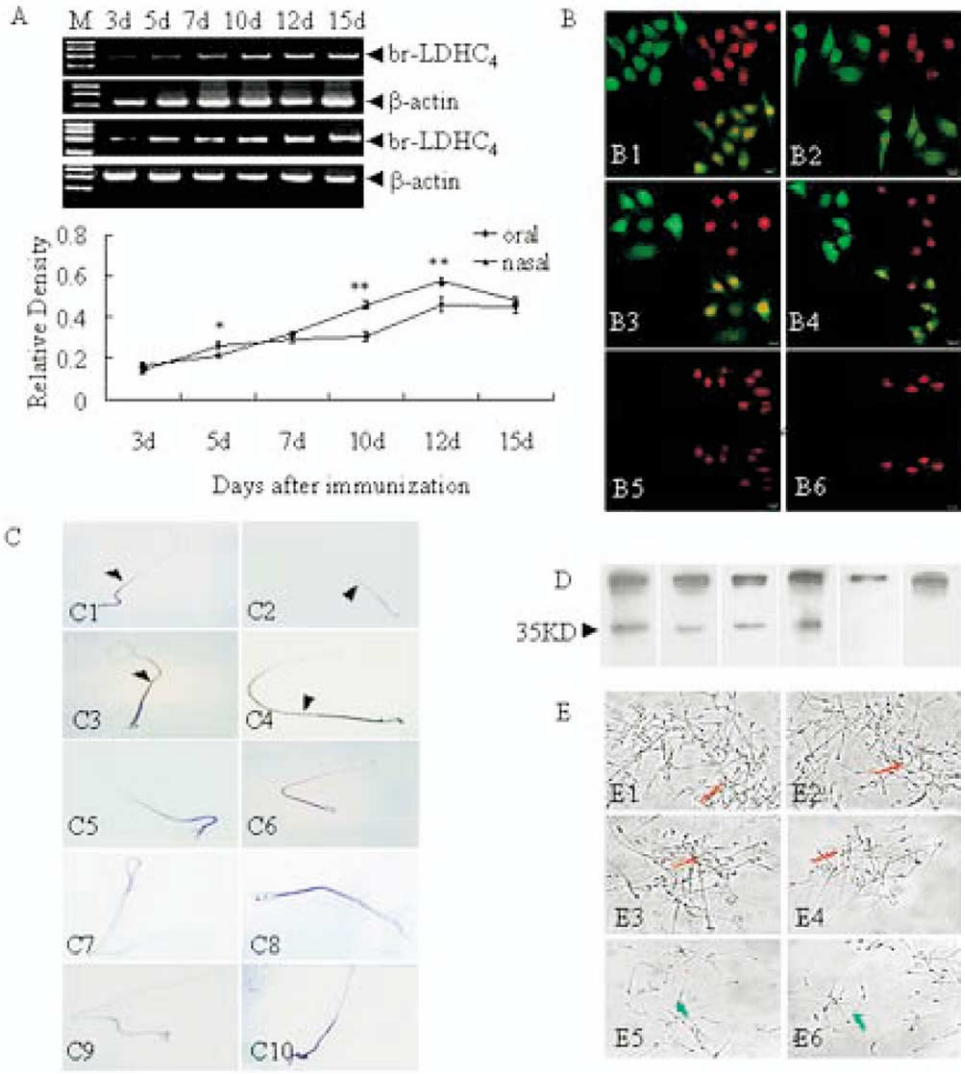
As seen in Figure 1A, after the mice were immunized with pCR3.1–brLDHC$_4$′ via oral feeding and nasal instillation, the LDHC$_4$′ mRNA were expressed in vivo, even in uterus, and the expression was time dependent during the detection period. The antigen expression of the nasal group was higher compared with that of the oral group at 10 and 12 days after DNA immunization ($P<.01$). Indirect immunofluorescence assay (see Fig. 1B) indicated that pCR3.1–brLDHC$_4$′ could be efficiently expressed in cultured cells, and the majority of the protein presented in cytoplasm. The IgG in sera and IgA in the genital tract induced by vaccine can bind the partial LDHC$_4$ protein. Further enzyme immunoassay (see Fig. 1C, 1–6) showed that the middle and principal pieces, especially middle pieces, of the sperm were stained after the motile mouse sperm were treated with the vaccinated sera and vaginal secretions, whereas no staining was observed in the control group. This result is in accordance with the study from Burgos et al. (9).

The ability of antibodies to bind to sperm can be abrogated after treating the vaccinated sera and vaginal fluids with brLDHC$_4$′ antigen (see Fig. 1C, 7–10), which indicates that the antibodies induced by the DNA vaccine were specific to LDHC$_4$. The molecular weight of mouse LDHC$_4$ is about 35 kd; Western blotting indicates that the antibodies elicited by pCR3.1–brLDHC$_4$′ specially recognize the band of 35 kd on nitrocellulose membrane that contains the LDHC$_4$ from the mouse testes (see Fig. 1D). That means the antibodies induced by *Microtus brandti* (Brandt's vole) LDHC$_4$′ vaccine are able to cross-react with the mouse LDHC$_4$.

The results of the agglutination test demonstrate that the agglutination took place when the motile sperm were incubated with the vaccinated antibodies (see Fig. 1E). The vaccine-induced antibodies can generate sperm agglutination, but it is not clear whether the immune-reaction specific to LDHC$_4$ can affect the motility and number of sperm

FIGURE 1

(**A**) Representative expression of brLDHC′4 (520 bp) in the uterus of mice that received 20 μg of pCR3.1-brLDHC$_4$′ via oral feeding (up) and nasal instillation (down). The expression of β-actin served as an internal standard for mRNA (548bp). The volume is expressed as the mean ± SEM of three independent experiments (*$P<.05$; ** $P<.01$). (**B**) Transfected HeLa cells were reacted with sera (B1) and vaginal washes (B2) from the orally vaccinated mice, sera (B3) and vaginal washes (B4) from the nasally vaccinated mice, and sera (B5) and vaginal washes (B6) from the mice immunized with pCR3.1 mock DNA via oral plus nasal routes. *Green fluorescence* represents the specific antibody binding to brLDHC$_4$′ protein. *Red color* denotes the cell nuclei. (Bar = 20 μm). (**C**) Immunocytochemistry localization of antibody binding to mouse sperm. The sera and vaginal washes used in C1–C6 were the same order as B1–B6. D7 through D10 represent the immunocytochemistry experiments with sera and vaginal fluids (C1–C4) treated with brLDHC$_4$′ antigen. *Black arrow* shows the specific association between antibody and LDHC$_4$. Original magnification ×100. (**D**) Western blot analysis of LDHC$_4$′–specific antibodies. The first antibody used for incubation with the target band in lanes 1–6 was the same as the order of (*B*). (**E**) The sera or vaginal fluids used for sperm agglutination assay (E1–E6) were in accordance with B1–B6. *Red arrow* shows a specific agglutination response, and *green arrow* denotes a negative response.



Shi. LDHC$_4$ reduces fertility via antibodies. Fertil Steril 2005.

in the reproductive tract in vivo. It is also possible that cellular responses or other unknown mechanisms play roles in the antifertility efficacy generated by the brLDHC$_4$′ DNA vaccine.

Our data also show that immunization of female animals with the 20-μg DNA vaccine both orally and nasally significantly affected their fertility. The number of newborns of the vaccinated mice (oral: 5.0 ± 0.9, nasal: 4.0 ± 0.5) were statistically significantly reduced compared with the control mice (saline: 11.0 ± 0.9, mock: 10.6 ± 0.9; $P<.01$). Although the vaccine decreased the number of newborns, it failed to affect the birthrate, as all mice that had been immunized with the DNA vaccine gave birth.

Our experiments have shown that immunization with pCR3.1–brLDHC$_4$′ DNA vaccine both via oral feeding and nasal instillation can reduce fertility in female mice via humoral and mucosal responses. In addition, the efficacy of the orally and nasally delivered LDHC$_4$-based DNA vaccine has implications for further development of such convenient contraception methods. Because it has been reported that the female genital tract has been considered a component of the common mucosal immune responses, vaginal DNA immunization with LDHC$_4$ could overcome the barriers to DNA administration caused by the estrus cycle and physical environment of the vaginal tract, and could generate mucosal immune responses (11). Whether the immune responses triggered by brLDHC$_4$′ DNA immunization via vaginal tract also can reduce fertility remains to be investigated.

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