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Birth Control Vaccine Targeting Leukemia Inhibitory Factor

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SUMMARY

The population explosion and unintended pregnancies resulting in elective abortions continue to impose major public health issues. This calls for a better method of contraception. Immunocontraception has been proposed as a valuable alternative that can fulfill most, if not all, of the properties of an ideal contraceptive. There are several targets that are being explored for contraceptive vaccine development. Leukemia inhibitory factor (LIF), a member of interleukin-6 family, is required for embryo development and successful blastocyst implantation in several mammalian species. The present study was conducted to examine if LIF can be a target for the development of a birth control vaccine. Three sequences from LIF and two sequences from LIF-receptor (LIF-R) that span the regions involved in ligand-receptor binding were delineated, and peptides were synthesized based upon these sequences. Antibodies raised against these five peptides reduced LIF bioactivity in the in vitro culture assay using BA/F3 mLIF-R-mpg130 cells. Vaccines were prepared by conjugating these peptides to various carrier proteins. Immunization of female mice with these peptide vaccines induced a long-lasting, circulating as well as local antibody response in various parts of the genital tract, and resulted in a significant ($p \leq 0.05$) inhibition in fertility in all the three trials; the LIF-R peptide vaccines proved to be a better vaccine target. The data indicate that LIF/LIF-R is an excellent target for the development of a birth control vaccine. This is the first study, to our knowledge, that examined LIF/LIF-R as a target for immunocontraception. The findings of this study can be easily translated to humans since LIF/LIF-R is also important for implantation and pregnancy in women.

Keywords

leukemia inhibitory factor; contraceptive vaccine; mucosal immunity; genital tract immunity; peptide vaccine; contraception; implantation; pregnancy

INTRODUCTION

Despite the availability of several modalities for contraception, the world population is increasing at a tremendous rate. It has exceeded 6.91 billion, and is increasing by one billion about every 11 years (World POP-Clock Projection, 2011). Also, unintended pregnancies resulting in elective abortions continue to impose a major public health burden: An estimated 80 million women have unintended/unwanted pregnancies worldwide annually, and 45 million of these end in abortion (WHO Press Release, 2006). Each year in the United States, half of all pregnancies are unintended, resulting in over 1 million elective abortions (Henshaw, 1998; Trussell, 2004). In over half of these unintended pregnancies, the women self-reported using some type of contraceptive. These data call for a better method of contraception that is acceptable, effective, and available both in developed and developing

nations. Such a contraception should be non-steroidal, non-barrier, non-surgical, intercourse independent, and reversible. Contraceptive vaccines have been proposed as valuable alternatives that can fulfill most, if not all, of the properties of an ideal contraceptive (Naz, 2011 a; b), and several targets that are being explored for their development (Naz et al., 2005). Due to their high target specificity, long-term action, low cost, and minimal side-effects, the development of contraceptive vaccines will indeed be an advancement in the field of contraception.

The recent database search indicated that there are at least 76 factors (cytokines/chemokines/growth factors/others) involved in various steps towards establishment of pregnancy (Lemons and Naz, 2011). Among these factors, two, namely chorionic gonadotropin (CG) and leukemia inhibitory factor (LIF), are unique and pregnancy-specific. Human chorionic gonadotropin (hCG) has been extensively explored as a target for immunocontraception. Vaccines based upon hCG have undergone phase-I and -II clinical trials in women, exhibiting a positive contraceptive effect (Talwar et al., 1994; Singh et al., 1998). Present studies are focused on increasing the immunogenicity and efficacy of the hCG vaccine for phase-III clinical trial (Purswani et al., 2011; Talwar et al., 2011).

Leukemia inhibitory factor (LIF), a member of the interleukin-6 family, is required for embryo development and successful blastocyst implantation (Robb et al., 2002). Mice expressing a mutated *LIF* gene that encodes a truncated, non-functional form of LIF, have a complete implantation failure (Stewart et al., 1992). Also, administration of a high-affinity LIF antagonist completely blocks implantation in mice (White et al., 2007). These findings provide a strong rationale for exploring LIF as a target for immunocontraception. It has not been investigated whether immunologic intervention via vaccination against LIF and/or its receptor (LIF-R) can prevent successful blastocyst implantation. We hypothesize that blocking the LIF/LIF-R interaction via immunologic intervention will cause a contraceptive effect.

In the present study, five sequences from LIF or LIF-R were delineated from the regions that are involved in ligand-receptor interaction, and peptides were synthesized based upon these sequences. To examine that they indeed inhibit LIF/LIF-R interaction, the antibodies were raised against these peptides in female rabbits and examined for their ability to neutralize the biological activity of LIF in vitro. Then, these synthetic peptides were conjugated to various carrier proteins as vaccines to examine if they can induce a long-lasting antibody response in circulation and genital tract that can cause a contraceptive effect in female mice in vivo.

RESULTS

Peptide antibodies and their ability to bionutralize LIF activity in in vitro cell assays

The bionutralizing ability of the five peptide antibodies raised against LIF or LIF-R was examined in an in vitro cell culture assay using BA/F3 mLIF-R-mgp130 cells, which grow only in the presence of LIF. These cells express murine LIF-R on the surface, and require LIF supplementation in the culture medium to proliferate; the cells grow at a low rate without LIF. The data from two representative experiments, with and without the presence of LIF, over a 4-day period is shown in Figure 1A. Addition of LIF caused a 2.5-fold increase in cell number in the presence of LIF after 72 hr, and 5-fold increase by 88-93 hr. Without LIF, cell proliferation was slow and was several-fold less than in the presence of LIF.

All the peptide antibodies significantly ($P<0.05$) reduced the bioactivity of exogenous LIF (Figure 1B). The LIF-R peptide antibodies showed better reduction than the LIF peptide antibodies, although the differences were not statistically significant ($P>0.05$). In these

assays, the pre-immune control sera did not reduce LIF activity, whereas the commercially available poly-LIF antibody reduced the LIF bioactivity. None of these antibodies were cytotoxic to the cells.

Immunogenicity and fertility trials

In the first trial (Trial 1), each mouse was immunized with all the five LIF and LIF-R peptide vaccines administered intranasally and intramuscularly, and the results of immunization on their fertility are summarized in Table 2. Overall, there was a significant ($P<0.05$) reduction (28.6%) in fertility in the vaccinated compared to control animals injected with phosphate-buffered saline (PBS)-adjuvant alone. Taking 3 standard-deviation (SD) units as a cut-off, 93% and 27% of the vaccinated mice were positive for peptide-specific IgG (Figure 2A) and IgA (Figure 2B) in the sera, and 53% for IgG (Figure 2C) and 67% for IgA (Figure 2D) in the vaginal washings, respectively. SD units in the antibody-positive animals were low, however, varying from 3-5 in sera and 3-11.6 in the vaginal washings. None of the animals in the control group demonstrated a positive antibody response to the peptides. The findings of the first trial indicated that the immunization with LIF and LIF-R peptide vaccines can induce a reduction in fertility, and formed the basis for carrying out the next trial, with an objective to increase the antibody response.

In the next trial (Trial 2), the animals were immunized via intramuscular and subcutaneous routes with: PBS-adjuvant alone (Group 1), three LIF peptide vaccines (Group 2), two LIF-R peptide vaccines (Group 3), or a combination of the five LIF + LIF-R peptide vaccines (Group 4). The results of immunization on their fertility are summarized in Table 3. There was an overall significant reduction in all three experimental groups compared to controls. Immunization with LIF peptide vaccines (Group 2) demonstrated a 26% reduction; with LIF-R peptide vaccines (Group 3) showed a 50.6% reduction; and with all the five LIF + LIF-R peptide vaccines (Group 4) demonstrated a 33.8% reduction in fertility. The antibody response of these groups in the sera (Panel A) and vaginal washings (Panel B) was analyzed using H- and L-chain specific secondary antibody, and is shown in Figure 3. All the animals (100%) developed peptide-specific antibodies in the sera to the LIF peptides (Group 2), and 83% of them also demonstrated antibodies in the vaginal washings. Eighty-three percent of the animals vaccinated with the LIF-R peptides (Group 3) developed peptide-specific antibodies in the sera and 8% in the vaginal washings. All animals (100%) immunized with both LIF and LIF-R peptide vaccines (Group 4) developed antibodies in the sera and vaginal washings. None of the animals injected with PBS-adjuvant alone (Group 1) demonstrated antibodies to the peptides.

Two to three weeks after delivery of the pups, some of the animals in Trial 2 were boosted with the respective peptide vaccine via intranasal and intramuscular routes. The sera and vaginal washings were examined for the antibody response 3-4 weeks after the booster, and the animals were mated again to examine the effect on fertility. The results of the fertility trial are summarized in Table 3. Animals immunized with LIF peptide vaccines (Group 2) demonstrated a 35.0% reduction, those immunized with LIF-R peptide vaccines (Group 3) showed a 51.3% reduction, and the one animal immunized with combined LIF and LIF-R peptide vaccines (Group 4) showed a 62.5% reduction in fertility. The differences between pre- and post-booster reduction in fertility in all the groups were not statistically significant ($p>0.05$). After the booster immunization, an antibody response was seen in both the sera and vaginal washings in the same range as seen before in the sera and vaginal washings of the pre-booster animals (data not shown).

In all three fertility trials, there was a general trend between the antibody titers (SD units), especially in the vaginal washings, with a reduction in fertility in the sense that animals having no antibodies (<3 SD units) or low titers (3-5 SD units) showed the least inhibition in

fertility. Whether in sera or in vaginal washings, however, SD units did not show a statistically significant linear correlation with reduction in fertility in any of the trials. Also, there was no significant linear correlation between the antibody titers in the sera and vaginal washings.

To examine the reversibility of the immune response, some of the animals were kept up to one year. The residual low peptide-specific antibody titers (3-5 SD units), especially IgG, were still detected in the sera, but not in the vaginal washings of these mice. No peptide-specific IgA was detected in the sera or vaginal washings in any of the mice. We do not expect these low antibody titers (IgG) in sera to affect fertility since there are no detectable antibodies in the vagina.

The fourth trial was conducted to examine the development of antibodies to individual peptides in the sera, especially along various parts of the genital tract (vagina/uterus/oviduct) after vaccination. In this trial, CD-1, 6-8 week old, virgin female mice (n=11-12) were immunized intranasally/intramuscularly, exactly as described for the first trial except that they received the last booster injection intravaginally along with intranasal and intramuscular injections. Instead of performing a fertility trial, the mice were euthanized, and the sera and various parts of the genital tract were collected and flushed with PBS as described in the "Materials and Methods" section. The animals demonstrated antibody titers to all the individual peptides in various parts of the genital tract, as summarized in Figure 4. In the sera, all the animals (100%) developed antibodies to almost all peptides except LIF-Rp1, where only 64% of the animals showed a positive reaction (>3 SD units). In the vagina, 100% of the animals showed a positive reaction for LIFp1, LIFp3, and LIF-Rp2; 92% for LIFp2; and 73% for LIF-Rp1. In the uterus, 92% showed a positive reaction for LIFp1; 42% for LIFp2; 17% for LIFp3; 45% for LIF-Rp1; and 91% for LIF-Rp2. In the oviduct, 100% of animals were positive for all the peptides. Of all parts of the genital tract, the uterus had the least amount of antibodies to various peptides. None of the PBS-adjuvant injected control animals (0%) showed a positive reaction with any of the peptides.

DISCUSSION

LIF is required for embryo development and successful implantation. Mice expressing a mutated LIF gene have a complete implantation failure (Stewart et al., 1992; Robb et al., 2002), and administration of a high affinity LIF antagonist completely blocks implantation in mice (White et al., 2007). In the present investigation, we tested if blocking LIF activity via immunologic intervention can prevent pregnancy. We synthesized three LIF and two LIF-R peptides representing the regions that are involved/essential in LIF/LIF-R binding. The idea was that the antibodies developed against these peptides will block the LIF/LIF-R binding, resulting in implantation failure leading to block in pregnancy.

The first objective was to examine that indeed the antibodies against these peptide regions will neutralize the bioactivity of LIF. For this, antibodies were developed against these five peptides in rabbits. These high titer antibodies were tested in an in vitro culture assay using BA/F3 mLIF-R-mgp130 cells for their ability to neutralize the bioactivity of LIF. These are pro-B-cells that have been transfected with mLIF-R and mgp130 cDNA, and proliferate many-fold more in the presence of LIF than without LIF (Underhill-Day et al., 2003). Our data indicates that indeed the cells proliferate multi-fold more in the presence of LIF than in its absence. Pre-incubation of LIF with the antibody against each peptide significantly reduced the LIF bioactivity; the LIF-receptor peptides showing better bionutralization. Yet, none of the peptide antibodies, whether against LIF or LIF-receptor, totally neutralized LIF bioactivity; even combining all the antibodies against LIF or LIF-R did not completely neutralize the LIF activity. The incomplete bionutralization could be due to: a) the

antibodies, though have high titers, may have lower affinity than the LIF/LIF-R binding affinity, and/or b) the peptide sequences which we selected do not cover all the sites that are involved in ligand-receptor binding. We examined various concentrations of sera to neutralize the LIF activity, but none of the concentration of sera against any of the peptides was able to totally block the LIF bioactivity in this assay.

Murine LIF and LIF-R, and the peptides representing them, are “self” molecules in mice. To make them immunogenic, they were coupled to various T-cell carrier proteins. Conjugation of self molecules, such as hCG, to carrier proteins, namely tetanus toxoid/diphtheria toxoid, have been shown to make them immunogenic (Talwar et al., 1994; 2011). Indeed conjugation of these peptides to various carriers made them immunogenic, and immunization of mice with these vaccines via various routes induced peptide-specific antibodies both in the circulation and locally in the genital tract. In the first trial, the animals were immunized intranasally and intramuscularly with all the peptide vaccines together. Although there was a significant reduction in fertility (28.6%), the animals developed low titers of antibodies. In the second trial, the animals were immunized intramuscularly and subcutaneously, and they raised high antibody titers. The group immunized with the LIF-R peptide vaccines showed the highest reduction, more than the group immunized with LIF peptide vaccines in all the trials. These findings agree with the in vitro bionutralization data. The LIF-R peptide antibodies showed a better reduction of LIF bioactivity than LIF peptide antibodies. The different effects on fertility does not seem to be due to antibody titers since the animals immunized with LIF peptide vaccines developed antibody levels in a similar range as those immunized with LIF-R peptide vaccines. After booster immunization via intranasal and intramuscular routes, these animals again showed an anti-fertility effect in all three groups. In previous studies, we found that immunization with these carriers alone does not affect fertility in mice (Naz and Chauhan, 2002; Naz, 2008).

Overall, there was no significant linear, negative correlation between the antibody titers in sera/vaginal washings with the number of pups born. The antibody titers in sera/vaginal washings could not be used to predict whether there will be a total or a partial block in fertility of an animal. We hypothesized that it is possible that the presence of antibodies in the upper part of the genital tract, namely the uterus and fallopian tubes (ampulla-isthmus junction), may be more predictive of a contraceptive effect. LIF is expressed transiently in the murine glandular uterine epithelium on day 1 and then on day 4 of pregnancy, the day of implantation. This suggests that LIF is primarily essential for uterine function, although it may be trophic for the embryo (Kimber 2005; Hu et al., 2007). In the first three trials, we examined the presence of antibodies in the sera and vaginal tract. Since LIF is secreted into the uterus, the fourth trial was conducted to investigate if the antibodies after vaccination are present in various parts of the genital tract, especially the uterus. Intravaginal/intranasal booster vaccination after/along with systemic injection (intramuscular/subcutaneous) enhances both the mucosal and system immunity to an antigen (Naz, 2012). Although the antibodies were detected in all parts of the genital tract (vagina/uterus/oviduct), the uterus had the least amount of antibodies against all the peptides. These findings may indicate that, in addition to the factors discussed above, incomplete (~51%) reduction in fertility, rather than a complete block (100%), may be due to a low concentration of antibodies against LIF/LIF-R in the uterus.

Mice expressing a mutated *LIF* gene or administered with a high-affinity LIF antagonist have a complete (100%) implantation failure (Stewart et al., 1992; White et al., 2007). Also, administration of anti-LIF antibody has been shown to inhibit blastocyst implantation in mice (Terakawa et al., 2011), and in primates (Sengupta et al., 2006). Intraperitoneal injections of anti-LIF antibody between D3 and D4 (D1=day of vaginal plug detection) caused a complete block (100%) in C57BL/6J, and 73% reduction in embryo implantation

of ICR mice (Terakawa et al., 2011). In mice, LIF is essential for implantation; however, the total dependency of embryo implantation on LIF seems to differ among various murine strains. The LIF pathway appears to function in a strain-dependent manner in mice deficient in p53, which regulates expression of LIF (Hu et al., 2007). Implantation failure is more severe in C57BL/6J background pg53-deficient mice than in 129SV mice (Hu et al., 2007).

There are several lines of evidence indicating that LIF also has a role in human implantation and pregnancy. LIF mRNA and LIF protein levels in the endometrium peak at the time of implantation (Charnock-Jones et al., 1994; Vogiagis et al., 1996; Cullinan et al., 1996). LIF-R is expressed in endometrial tissue in both the proliferative and secretory phases of the menstrual cycle (Cullinan et al., 1996). LIF-R is expressed in human blastocysts in a stage-dependent manner (Sharkey, 1995). LIF concentrations are lower in cultures of endometrial explants from women with repeated implantation failure and from women with unexplained infertility (Delage et al., 1995; Chaouat et al., 1995). Also, women with unexplained infertility and endometriosis have *LIF* gene mutations that correlate with poor in vitro fertilization outcome (Novotny et al., 2009). These cumulative findings indicate the importance of LIF and LIF-R in human trophoblast differentiation, embryonic development, implantation and pregnancy.

In conclusion, our data indicates that the immunization with LIF and LIF-R peptide vaccines prevent pregnancy. In all three trials, fertility was inhibited after vaccination. The effect was long-lasting, with the antibody response declining with time. The peptide vaccination decreased fertility up to 51.3%. To increase the immunogenicity and efficacy of the vaccine and contraceptive effect, we plan to use the whole recombinant LIF/LIF-R molecule and DNA vaccination in the future studies. DNA vaccination has several distinct advantages, including easy manipulation, use of a generic technology, simplicity of manufacture, and chemical and biological stability (Whalen, 1996; Robinson 1999; Liu, 2003; Patil et al., 2005). Our data indicates that LIF/LIF-R is an excellent target for the development of a birth control vaccine. Vaccines targeting LIF-R provided better results. This is the first study to our knowledge that examined LIF/LIF-R as a target for immunocontraception. The findings of this study can be easily translated to humans since LIF/LIF-R is also important for implantation and pregnancy in women.

MATERIALS AND METHODS

Peptide Sequence Selection

The selected sequences from the published murine (m) LIF and mLIF-R proteins were previously shown to be important in their interaction (Gearing et al., 1987; Gearing et al., 1988; Hudson et al., 1996; Huyton et al., 2007). Three sequences (LIFp1, LIFp2, and LIFp3) were selected from LIF protein and two sequences (LIF-Rp1 and LIF-Rp2) were selected from LIF-R protein. Pro 51 and Phe 52 amino acids (aa) in the LIF sequence are essential for binding to the receptor, so the LIFp1 sequence was selected to span 40-54 aa to include these amino acids. The Ile-103, Asn-105 and Ser-107 residues are important in the second binding site, so the LIFp2 sequence was selected to span 100-115 aa to include these amino acids. The Phe-156 and Lys-159 residues are important in the third binding site, thus the LIFp3 sequence was selected to span 156-180 aa to also included nine C-terminal amino acids (166-188aa) that are required for LIF biological activity.

LIF has been shown to interact with LIF-R through the Ig-like domain (260-263aa), with Ser-262 providing a hydrogen bond with Lys-159 of LIF (Huyton et al. 2007). This was the rationale for selecting LIF-Rp1 sequence (250-263aa). Other amino acids that are important for the interaction include Val-314, Pro-316 and Glu-338, thus providing the basis for selecting LIF-Rp2 sequence (314-339aa). Their sequences are described in Table 1.

Peptide Synthesis and Developing Antibodies to these Peptides in Rabbits for in vitro Testing

These five sequences were analyzed using the Invitrogen Peptide Select™ Online program to examine their hydrophilicity and immunogenicity, and the peptides were synthesized using Fmoc chemistry at GenScript Corp. (Piscataway, NJ). Deprotection was achieved by 20% (w/v) piperidine in dimethylformamide, and the peptides were cleaved from the resin by 85% (v/v) trifluoroacetic acid (TFA). The peptides were then precipitated in methyl tert-butyl ether and purified by using reverse-phase high performance liquid chromatography. The fractions eluted with 0.5% (v/v) TFA in acetonitrile were dried in a speed vacuum, re-dissolved in water, and lyophilized. All the peptides were water-soluble and had >85% purity level. Glycine-cysteine (GC-) residues were added at the NH₂-terminal of each peptide for conjugation to the carrier protein if the peptide sequence did not have cysteine at the NH₂- or COOH-terminals.

Peptides were conjugated to keyhole limpet hemocyanin (KLH), and antibodies were raised in female rabbits at a commercial company (Antagene Inc., Sunnyvale, CA). The sera from these animals indicated high titer antibodies (>250,000 antibody titer) against each of these peptides. These sera were heat-inactivated (56°C, 30 min) and tested for their ability to neutralize the biological activity of LIF using BA/F3 mLIF-R-mgp130 cells in an in vitro cell culture assay, as described below.

Cell Culture Assay to Examine the LIF Bionutralization Ability of Anti-peptide Antibodies

BA/F3 mLIF-R-mgp130 cells, pro-B-cells transfected with mLIF-R and mgp130 cDNA (Underhill-Day et al., 2003), were cultured in RPMI 1640 (Invitrogen, Carlsbad, California) supplemented with 10% (v/v) FBS (Invitrogen), 50 U/ml penicillin-50 µg/ml streptomycin (Invitrogen) and 40 ng/ml recombinant mouse LIF (eBioscience, San Diego, CA). These cells have been shown to proliferate in the presence of LIF or other cytokines of IL-6 family. Anti-LIF antibody bionutralizes LIF activity in this assay, and the cells do not grow when the antibody-neutralized LIF, instead of LIF *per se*, is added to the culture (Hill and Vernallis, 2008).

For the antibody bionutralization assay, 2µl of each rabbit anti-LIF/LIF-R peptide serum or pre-immune serum (control) was incubated (2hr, 37°C) with 40 ng of recombinant mLIF. A commercially available LIF polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was also included as a positive control in these assays. Cells were added to a 24-well culture plate (500µl/well), and incubated with LIF or antibody-neutralized LIF. A positive control was cultured in the presence of LIF *per se*, and the negative controls were cultured without LIF or antibody alone. Cell proliferation (72 hr, 37°C, in 5% CO₂) was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen) (50µl of MTT/ 500 µl culture medium). Two hundred and fifty microliters of 10% (w/v) SDS/0.01 M HCl was then added, and the absorbance (A₅₇₀) was determined after overnight incubation at 37°C, as described previously (Karow et al., 1996; Hill and Vernallis, 2008). The assay was run in triplicate, and repeated several times (2-5) on various days.

Conjugation of Synthetic Peptides to Carrier Proteins for Vaccine Development for in vivo Testing

Four different carrier proteins, namely keyhole limpet hemocyanin (KLH) (Calbiochem, San Diego, CA), bovine serum albumin (BSA) fraction V (Sigma-Aldrich, St. Louis, MO), chicken γ-globulin (Calbiochem), and chicken ovalbumin (Calbiochem), were used for conjugation. The peptides were conjugated to the carrier proteins using heterobifunctional cross-linker N-hydroxysuccinimidyl ester of maleimido-alkyl-carboxylate (MBS) following the manufacturer's protocol (peptide:carrier ratio, wt/wt; 1:1), as described earlier (Naz,

2008). The coupling efficiency was generally >80%. Peptides were also conjugated to cholera toxin B subunit (CTB) (List Biological Labs, Campbell, CA) (peptide:CTB ratio, wt/wt; 2:1) using a two step glutaraldehyde (Sigma-Aldrich) reaction, as described previously (McKenzie and Halsey, 1984; Naz and Chauhan, 2002). The conjugates were dialyzed in phosphate-buffered saline (PBS) for 48 hr using the Slide-A-Lyzer dialysis cassette system before using them for immunization (Pierce Biotechnology, Rockford, IL).

Vaccination

Virgin BALB/c, 8-10 week old, female mice were immunized (designated at Day 0) with LIF/LIF-R peptide vaccines (as described below) while the controls were injected with PBS, intramuscularly (i.m.), subcutaneously (s.c.), and/or intranasally (i.n.). In Trial 1 (table 2), each mouse in the experimental group (n=11) was immunized with all the five LIF/LIF-R peptide vaccines i.n. and i.m. In the control group (n=10), each mouse was immunized similarly with equal amount of PBS with adjuvant. In Trial 2 (table 3), the animals were divided into four groups. Each mouse in Group 2 (n=12) was immunized i.m. and s.c. with all the three LIF peptide vaccines, in Group 3 (n=12) with the two LIF-R peptide vaccines, and in Group 4 (n=10) with all the five LIF/LIF-R peptide vaccines. Group 1 (n=12), control group, received equal amount of PBS with adjuvant. After the fertility trial, several animals in this group (table 3) were boosted i.n. and i.m. with respective LIF/LIF-R peptide vaccines with which they were immunized previously.

For i.m./s.c. immunizations, each animal received 50 μ l of total volume of the vaccine containing 50 μ g of each peptide conjugated to a carrier protein, emulsified with Freund's adjuvant (Sigma-Aldrich). The first injection was given with Freund's complete adjuvant, while the subsequent booster injections were given with Freund's incomplete adjuvant. For i.n. administration, each animal received 10-15 μ l volume of the vaccine per nostril containing a total of 50 μ g of each peptide conjugated to CTB. Each animal received a total of 3-4 booster injections. For i.m./s.c. injections, a vaccine formulation comprised of a different carrier protein was used for primary and subsequent booster immunizations to avoid carrier-induced immunosuppression. Two-three weeks after the last booster injection, sera and vaginal washings were collected. Blood was collected through the saphenous vein while the vaginal washings were collected by washing the vagina with 50 μ l of PBS. Animals were maintained at the West Virginia University Office of Laboratory and Animal House. All experimental protocols used in this study were approved by the West Virginia University Animal Care and Use Committee (WVU-ACUC).

To examine the presence of antibodies in various parts of the genital tract, a group of animals were euthanized by CO₂ asphyxiation 2-3 weeks after the last booster, and blood and genital tract were collected from each animal. The genital tract was separated into the vagina, uterus and oviduct. Each vagina was rinsed with 50 μ l, each uterine horn with 200 μ l, and each oviduct was macerated in 200 μ l of PBS from every animal. The vaginal washings were sonicated at 4°C with 10-s bursts of 30% full power to solubilize cervical mucus, if any (Naz et al., 1995).

Analysis of Antibodies

The presence and titers of antibodies in sera and vaginal washings were analyzed using the enzyme-linked immunosorbent assay (ELISA), as described earlier (Naz and Chauhan, 2002; Naz, 2008). Each well was coated overnight (at 4°C) with peptide (individually or in combination) (4 μ g each peptide/well in 200 μ l) diluted in carbonate buffer (0.1M, pH 9.6). The wells were washed three times (for 5 min each) with PBS containing 0.05% (v/v) Tween-20 (PBS-T). To block non-specific binding sites, the wells were incubated with PBS-T containing 1% (w/v) BSA at 37°C for 45min and washed three times (5 min each) with

PBS-T. The wells were incubated (37°C, 3hr) with the serum (1:40 dilution) or the vaginal/uterine/oviduct washings (1:20 dilution) (200 µl/well), diluted in PBS-T containing 0.5% (w/v) BSA. The wells were washed (five times) with PBS-T, and then incubated (37°C, 1.5 hr) with alkaline phosphatase conjugated anti-mouse IgG (gamma-chain specific, or H- and L-chain specific) or anti-mouse IgA (α-chain specific) immunoglobins (Sigma-Aldrich) diluted 1:800 and 1:400, respectively, in PBS-T containing 0.5% (w/v) BSA (200 µl/well). The wells were washed as before, and then incubated (37°C, 30 min) with the substrate solution (1 mg/ml disodium p-nitrophenyl phosphate diluted in 0.05 M carbonate buffer, pH 9.8). The reaction product was read at 405 nm. The absorbance readings were converted to standard deviation (SD) units by using the following formula: SD units=absorbance(test)-mean absorbance(control group)/SD of control group, as described elsewhere (Naz et al., 1993; Naz and Chauhan, 2002; Naz, 2008). The antibody titers/reactivity was defined as SD units. The test samples with 3 or >3 SD units were considered as having a positive reaction with a peptide.

In the fourth trial, the presence and titers of antibodies were analyzed in various parts of the genital tract. In this trial, sufficient sera and washings from various parts of the tract were obtained, and thus we could test the presence and titers of antibodies against all of the five individual peptides (three LIF and two LIF-R).

Fertility Testing

Starting 3 weeks after the last injection, the animals were mated overnight with 11-13 week old male mice (two females with one male in each cage). The next morning, the mating was confirmed by the presence of a vaginal plug, and the mated animals were separated until they delivered pups. The number of pups delivered by each mated animal was counted, and the pups were killed by CO₂ asphyxiation. Fertility was defined as the mean number of pups born by the peptide(s)-vaccinated group divided by the mean number of pups born by the control group, multiplied by 100.

Reversibility of the Immune Response

Some of the animals were kept up to one year to examine the reversibility of the immune response. Sera and vaginal washings were collected every 2-3 week intervals, and analyzed for the presence of peptide-specific antibodies in ELISA.

Statistical Analysis

The significance of difference between the mean pups born in the vaccinated and control groups was analyzed by using an unpaired Student's *t*-test in Table 2. In Table 3, which is comprised of multiple groups, ANOVA was used to determine if differences exist among means of various groups. Since the differences were significant, the follow-up Fisher's Significant Difference (FSD) methods were used to examine the pair-wise differences. Correlation between the antibody titers (SD units) and fertility (number of pups born) was analyzed by linear regression. A *P* value of ≤ 0.05 was considered significant.

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Abbreviations

LIF	leukemia inhibitory factor
LIF-R	leukemia inhibitory factor receptor
PBS	phosphate-buffered saline
SD	standard deviation

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Figure 1. LIF bionutralizing ability of LIF and LIF-R peptide antibodies using BA/F3 mLIF-R-mgp130 cells in vitro. The cells ($0.4 \times 10^6/\text{ml}$) were cultured with/without LIF, and their proliferation was analyzed using an MTT assay over the course of 96 hr (A). The results are expressed as the absorbance at 570nm. Sera from rabbits immunized with LIF peptides (LIFp1, LIFp2, LIFp3, and all combined LIFp1-3), and LIF-R peptides (LIF-Rp1, LIF-Rp2, and both combined LIF-Rp1-2), and all (combined antibodies against all five peptides) were examined for their ability to neutralize the biological activity of recombinant mouse LIF (B). A commercially available polyclonal LIF antibody was used as a positive control (poly-LIF), and the pre-immune rabbit sera and the cell culture without LIF was used as negative

controls. The results are expressed as fold-increase compared to the negative control without LIF. In this figure, the results of two representative experiments are shown. The bar represents a mean $\pm$ SEM, and the star (*) indicates statistically significant ($P < 0.05$) difference.

Figure 2. Antibody response, IgG (A,C) and IgA (B,D) in mice immunized with combined LIF + LIF-R peptide vaccines via intranasal and intramuscular routes in sera (A,B) and vaginal washings (C,D). Each spot represents one animal. The horizontal dotted line (---) indicates 3 standard-deviation units, and the values above this line are considered positive.

Figure 3. Antibody response in mice immunized with LIF or LIF-R or LIF+LIF-R peptide vaccines via intramuscular and subcutaneous routes in sera (A) and vaginal washings (B). Each spot represents one animal. The horizontal dotted line (---) indicates 3 standard-deviation units, and the values above this line are considered positive.

Figure 4. Antibody response to individual five peptides in mice immunized with combined LIF + LIF-R peptide vaccines via intranasal and intramuscular routes in sera, and various parts of the genital tract (vagina/uterus/oviduct). Each spot represents one animal. The horizontal dotted line (---) indicates 3 standard-deviation units, and the values above this line are considered positive. PBS-adjuvant group was tested against all the five peptides coated together in each well of the ELISA plate.

Table 1
Murine LIF and LIF-R Peptide Sequences Used for Immunization

Peptide	Designation	Sequence
LIF (40-54)	LIFp1	GC-LFISYYTAQGEPFPN
LIF (100-115)	LIFp2	GC-DQKVLNPTAVSLQVK
LIF (156-180)	LIFp3	GC-FQRKKLGCQLLGRYKQVISVVVQAF
LIF-receptor (250-263)	LIF-Rp1	GC-AIHILNIPVSENSG
LIF-receptor (314-339)	LIF-Rp2	GC-GPRNTEYTLFESISGKSAVFHRIEG

Table 2
Contraceptive Effect of LIF/LIF-R Vaccines Injected Intranasally and Intramuscularly

Antigen	Animals (n)	Pups born (n)	Pups born/animal (mean $\pm$ SEM)	Fertility reduction (%)
PBS control	10	70	7.0 $\pm$ 0.86 [†]	---
LLIF+LIF-R peptides	11	55	5.0 $\pm$ 0.76 [*]	28.6%

[†] Values with different superscripts are statistically significant ($P < 0.05$).

^{*} Values with different superscripts are statistically significant ($P < 0.05$).

Table 3
Contraceptive Effect of LIF/LIF-R Vaccines Injected Systemically Followed by Booster Injection

Systemic Immunization (i.m./s.c.)					Booster (i.n./i.m.)			
Antigen	Animals (n)	Pups born (n)	Pups born/animal (mean $\pm$ SEM)	Fertility reduction (%)	Animals (n)	Pups born (n)	Pups born/animal (mean $\pm$ SEM)	Fertility reduction (%)
Group 1 (PBS control)	12	92	7.7 $\pm$ 0.48 [†]	---	10	80	8.0 $\pm$ 0.52 [†]	---
Group 2 (LIF peptides)	12	68	5.7 $\pm$ 1.25 [*]	26.00%	6	31	5.2 $\pm$ 1.51 ^{†*}	35.0%
Group 3 (LIF-R peptides)	12	45	3.8 $\pm$ 1.27 [*]	50.60%	10	39	3.9 $\pm$ 0.95 [*]	51.3%
Group 4 (LIF + LIF-R peptides)	10	51	5.1 $\pm$ 1.05 [*]	33.80%	1	3	3.0	62.5%

[†] Values with different superscripts are statistically significant ($P \leq 0.05$), and with the same superscript are statistically non-significant ($P > 0.05$).

^{*} Values with different superscripts are statistically significant ($P \leq 0.05$), and with the same superscript are statistically non-significant ($P > 0.05$).