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GnRH-LEUKOTOXIN CHIMERASDescription10 Technical Field

The present invention relates generally to immunological carrier systems. More particularly, the invention pertains to leukotoxin-GnRH chimeras including more than one copy of a GnRH polypeptide.

15 The chimeras demonstrate enhanced immunogenicity as compared to the immunogenicity of GnRH polypeptides alone.

Background of the Invention

20 In vertebrates, synthesis and release of the two gonadotrophic hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH), are regulated by a polypeptide referred to as Gonadotropin releasing hormone (GnRH) (formerly designated LHRH).

25 Accordingly, one approach to fertility control in an animal population is to reduce the levels of GnRH, such as by immunization against GnRH, which effects a reduction in the levels of LH and FSH and the concomitant disruption of estrous cycles and

30 spermatogenesis. See e.g., Adams et al., *J. Anim. Sci.* (1990) 68:2793-2802.

Early studies of the GnRH molecule have shown that it is possible to raise antisera in response to repeated injections of synthetic GnRH

35 peptides (Arimura et al., *Endocrinology* (1973) 93(5):1092-1103). Further, antibodies to GnRH have

been raised in a number of species by chemical conjugation of GnRH to a suitable carrier and administration of the conjugate in an appropriate adjuvant (Carelli et al., *Proc. Natl. Acad. Sci.* 5 (1982) 79:5392-5395). Recombinant fusion proteins comprising GnRH or GnRH-analogues have also been described for use in peptide vaccines for the immunological castration or inhibition of reproductive function of various domesticated and farm animals 10 (Meloan et al., *Vaccine* (1994) 12(8):741-746; Hoskinson et al., *Aust. J. Biotechnol.* (1990) 4:166-170; and International Publication Nos. WO 92/19746, published 12 November 1992; WO 91/02799, published 7 March 1991; WO 90/11298, published 4 October 1990 and 15 WO 86/07383, published 18 December 1986).

However, attempts have fallen short of providing adequate immunological sterilization products due to the poor immunogenicity of GnRH peptides and due to the fact that chemical conjugation 20 protocols are difficult to control, rendering substantially heterogeneous and poorly-defined GnRH conjugates. Further, peptide vaccines based on GnRH have met with limited success in providing uniform effects on individual animal subjects even after 25 repeated vaccination. In this regard, prior GnRH constructs have failed to provide a uniformly successful immunological sterilization vaccine product due to the fact that GnRH is a small, "self" molecule that is not normally recognized by a subject's immune 30 system, rendering the molecule poorly immunogenic and inherently unable to induce a significant immune response against endogenous GnRH.

It is generally recognized that the immunogenicity of viral antigens, small proteins or 35 endogenous substances may be significantly increased by producing immunogenic forms of those molecules

comprising multiple copies of selected epitopes. In this regard, constructs based on two or four repeats of peptides 9-21 of herpes simplex virus type 1 glycoprotein D (Ploeg et al., *J. Immuno. Methods* 5 (1989) 124:211-217), two to six repeats of the antigenic circumsporozoite tetrapeptide NPNA of *Plasmodium falciparum* (Lowell et al., *Science* (1988) 240:800-802), two or four copies of the major immunogenic site of VP1 of foot-and-mouth disease 10 virus (Broekhuijsen et al., *J. gen. Virol.* (1987) 68:3137-3143) and tandem repeats of a GnRH-like polypeptide (Meloan et al., *Vaccine* (1994) 12(8):741-746), have been shown to be effective in increasing the immunogenicity of those molecules.

15 Small proteins or endogenous substances may also be conjugated to a suitable carrier in order to elicit a significant immune response in a challenged host. Suitable carriers are generally polypeptides which include antigenic regions of a protein derived 20 from an infectious material such as a viral surface protein, or a carrier peptide sequence. These carriers serve to non-specifically stimulate T helper cell activity and to help direct antigen to antigen presenting cells for processing and presentation of 25 the peptide at the cell surface in association with molecules of the major histocompatibility complex (MHC).

Several carrier systems have been developed for this purpose. For example, small peptide antigens 30 are often coupled to protein carriers such as keyhole limpet haemocyanin (Bittle et al., *Nature* (1982) 298:30-33), tetanus toxoid (Muller et al., *Proc. Natl. Acad. Sci. U.S.A.* (1982) 79:569-573), ovalbumin, and sperm whale myoglobin, to produce an immune response. 35 These coupling reactions typically result in the incorporation of several moles of peptide antigen per

mole of carrier protein. Although presentation of the peptide antigen in multiple copies generally enhances immunogenicity, carriers may elicit strong immunity not relevant to the peptide antigen and this may
5 inhibit the immune response to the peptide vaccine on secondary immunization (Schutze et al, *J. Immun.* (1985) 135:2319-2322).

Antigen delivery systems have also been based on particulate carriers. For example, preformed
10 particles have been used as platforms onto which antigens can be coupled and incorporated. Systems based on proteosomes (Lowell et al., *Science* (1988) 240:800-802), immune stimulatory complexes (Morein et al., *Nature* (1984) 308:457-460), and viral particles
15 such as HBsAg (Neurath et al., *Mol. Immunol.* (1989) 26:53-62) and rotavirus inner capsid protein (Redmond et al., *Mol. Immunol.* (1991) 28:269-278) have been developed.

Carrier systems have also been devised using
20 recombinantly produced chimeric proteins that self assemble into particles. For example, the yeast retrotransposon, Ty, encodes a series of proteins that assemble into virus like particles (Ty-VLPs; Kingsman, S. M., and A. J. Kingsman *Vacc.* (1988) 6:304-306).
25 Foreign genes have been inserted into the TyA gene and expressed in yeast as a fusion protein. The fusion protein retains the capacity to self assemble into particles of uniform size.

Other chimeric protein particles have been
30 examined such as HBsAg, (Valenzuela et al., *Bio/Technol.* (1985) 3:323-326; U.S. Patent No. 4,722,840; Delpeyroux et al., *Science* (1986) 233:472-475), Hepatitis B core antigen (Clarke et al., *Vaccines 88* (Ed. H. Ginsberg, et al., 1988) pp. 127-
35 131), Poliovirus (Burke et al., *Nature* (1988) 332:81-82), and Tobacco Mosaic Virus (Haynes et al.,

Bio/Technol. (1986) 4:637-641). However, these carriers are restricted in their usefulness by virtue of the limited size of the active agent which may be inserted into the structural protein without interfering with particle assembly.

Finally, chimeric systems have been devised using a *Pasteurella haemolytica* leukotoxin (LKT) polypeptide fused to a selected antigen. See, e.g., International Publication Nos. WO 93/08290, published 29 April 1993 and WO 92/03558, published 5 March 1992, as well as U.S. Patent Nos. 5,238,823 and 5,273,889. Inclusion of a LKT carrier portion in a peptide antigen chimera supplies enhanced immunogenicity to the chimera by providing T-cell epitopes having broad species reactivity, thereby eliciting a T-cell dependent immune response in immunized subjects. In this regard, inducement of adequate T-cell help is essential in the generation of an immune response to the peptide antigen portion of the chimera, particularly where the antigen is an endogenous molecule. However, the use of a leukotoxin polypeptide carrier in combination with multiple epitopes of the GnRH peptide has not heretofore been described.

Disclosure of the Invention

The present invention is based on the construction of novel gene fusions between the *P. haemolytica* leukotoxin gene, variants thereof, and one or more nucleotide sequences encoding multiple GnRH polypeptides. These constructs produce chimeric proteins that display surprisingly enhanced immunogenicity when compared to the immunologic reaction elicited by administration of GnRH alone.

Thus in one embodiment, the present invention is directed to a chimeric protein comprising

a leukotoxin polypeptide fused to one or more multimers wherein each multimer comprises more than one selected GnRH polypeptide. The leukotoxin portion of the chimera acts to increase the immunogenicity of the GnRH polypeptides. More particularly, the GnRH multimers used herein may correspond to more than one copy of a selected GnRH polypeptide or epitope, or multiple tandem repeats of a selected GnRH polypeptide or epitope. Further, GnRH multimers may be located at the carboxyl and/or amino terminal of the leukotoxin polypeptide, at sites internal to the leukotoxin polypeptide, or any combination of such sites. Each GnRH multimer may also correspond to a molecule of the general formula GnRH-X-GnRH, wherein X is selected from the group consisting of a peptide linkage, an amino acid spacer group and $[\text{GnRH}]_n$, where n is greater than or equal to 1, and further wherein "GnRH" may comprise any GnRH polypeptide. In one particular embodiment, a chimeric protein comprising a leukotoxin polypeptide fused to two GnRH multimers is provided. In this molecule, the C-terminus of one of the GnRH multimers is fused to the N-terminus of the leukotoxin polypeptide, and the N-terminus of the leukotoxin polypeptide is fused to the N-terminus of the other GnRH multimer.

Also disclosed are vaccine compositions comprising the chimeric proteins with a pharmaceutically acceptable vehicle, as well as methods for presenting one or more selected GnRH multimers to a host subject by the administration of an effective amount of the subject vaccine compositions.

In another embodiment, the invention is directed to DNA constructs encoding the chimeric proteins. The DNA constructs comprise a first nucleotide sequence encoding a leukotoxin polypeptide

operably linked to one or more selected nucleotide sequences, each selected nucleotide sequence encoding more than one copy of a GnRH polypeptide or epitope.

In yet another embodiment, the invention is directed to expression cassettes comprised of the above-described DNA constructs operably linked to control sequences that direct the transcription thereof, whereby the constructs can be transcribed and translated in a host cell.

In another embodiment, the invention is directed to host cells transformed with these expression cassettes.

Another embodiment of the invention provides a method of producing a recombinant polypeptide. The method comprises (a) providing a population of host cells described above and (b) culturing the population of cells under conditions whereby the chimeric polypeptide encoded by the expression cassette is expressed.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figures 1A and 1B show the nucleotide sequences and amino acid sequences of the GnRH constructs used in the chimeric leukotoxin-GnRH polypeptide gene fusions: Figure 1A depicts GnRH-1 which includes a single copy of a GnRH decapeptide; Figure 1B depicts GnRH-2 which includes four copies of a GnRH decapeptide when $n=1$, and eight copies of GnRH when $n=2$, etc.

Figure 2 depicts the structure of Plasmid pAA352 wherein *tac* is the hybrid *trp::lac* promoter from *E. coli*; *bla* represents the β -lactamase gene (ampicillin resistance); *ori* is the ColE1-based

plasmid origin of replication; lktA is the *P. haemolytica* leukotoxin structural gene; and lacI is the *E. coli* lac operon repressor. The direction of transcription/translation of the leukotoxin gene is indicated by the arrow. The size of each component is not drawn to scale.

Figures 3-1 through 3-9 show the nucleotide sequence and predicted amino acid sequence of leukotoxin 352 (LKT 352). Both the structural gene for LKT 352 and the sequences of the flanking vector regions are shown.

Figure 4 shows the structure of Plasmid pCB113 carrying a leukotoxin-GnRH (LKT-GnRH) gene fusion.

Figures 5-1 through 5-8 show the nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB113. The nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB112 are identical to the sequences of the chimeric protein derived from pCB113 except that the sequence for multiple copy GnRH was inserted twice as described above in regard to Figure 4.

Figure 6 shows the structure of Plasmid pCB111 carrying a leukotoxin-GnRH (LKT-GnRH) gene fusion.

Figures 7-1 through 7-5 show the nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB111. The nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB114 are identical to the sequences of the chimeric protein derived from pCB111 except that the sequence for multiple copy GnRH was inserted twice as described above in regard to Figure 6.

Figures 8-1 through 8-2 show the nucleotide sequence and predicted amino acid sequence of the blunt end fusion point of the truncated leukotoxin gene of plasmid pCB111 (Figure 8-2), where an internal DNA fragment (of approximately 1300 bp in length) was removed from LKT 352 by digestion with the restriction enzymes *Bst*B1 and *Nae*I (Figure 8-1).

Figures 9-1 through 9-6 show the nucleotide sequence and predicted amino acid sequence of the LKT-GnRH chimeric protein from pCB122.

Figure 10 shows the structure of Plasmid pAA101 carrying the LKT 101 leukotoxin polypeptide which lacks cytotoxic activity.

Figure 11 depicts the predicted amino acid sequence of the LKT 101 leukotoxin polypeptide.

Figure 12 shows a comparison of average serum anti-GnRH antibody titres in barrows, untreated boars, and immunocastrated boars (vaccinated with leukotoxin-GnRH fusion proteins) as described in Example 10.

Figure 13 shows a comparison of average serum testosterone levels in barrows, untreated boars, and immunocastrated boars (vaccinated with leukotoxin-GnRH fusion proteins) as described in Example 10.

Figure 14 shows a comparison of feed conversion efficiency (expressed as the ratio of Kg feed:Kg weight gain) in barrows, untreated boars, and immunocastrated boars (vaccinated with leukotoxin-GnRH fusion proteins) as described in Example 10.

Figure 15 shows a comparison of average serum anti-GnRH antibody titres in animals injected with a vaccine composition containing a LKT::8 copy GnRH fusion protein, or a vaccine composition containing an 8 copy GnRH::LKT::8 copy GnRH fusion protein as described in Example 11.

Figure 16 shows a comparison of average ovarian weight (mg), average uterine weight (mg), and average serum estradiol (pg/mL), in control animals (solid bars) and animals treated with a vaccine composition containing an 8 copy GnRH::LKT::8 copy GnRH fusion protein as described in Example 13 (cross-hatched bars).

Figure 17 depicts a comparison in fat androstenone levels in barrows, boars, late castrated animals, and immunocastrated animals (vaccinated with leukotoxin-GnRH fusion proteins) as described in Example 14.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual; DNA Cloning, Vols. I and II (D.N. Glover ed.) ; Oligonucleotide Synthesis (M.J. Gait ed.); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds.); Animal Cell Culture (R.K. Freshney ed.); Immobilized Cells and Enzymes (IRL press); B. Perbal, A Practical Guide to Molecular Cloning; the series, Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., Blackwell Scientific Publications).

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

- 5 The term "Gonadotropin releasing hormone" or "GnRH" refers to a decapeptide secreted by the hypothalamus which controls release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH) in vertebrates (Fink, G., *British*
- 10 *Medical Bulletin* (1979) 35:155-160). The amino acid sequence of GnRH is highly conserved among vertebrates, and especially in mammals. In this regard, GnRH derived from most mammals including human, bovine, porcine and ovine GnRH (formerly
- 15 designated LHRH) has the amino acid sequence pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂ (Murad et al., *Hormones and Hormone Antagonists*, in The Pharmacological Basis of Therapeutics, Sixth Edition (1980) and Seeburg et al., *Nature* (1984) 311:666-668).
- 20 As used herein a "GnRH polypeptide" includes a molecule derived from a native GnRH sequence, as well as recombinantly produced or chemically synthesized GnRH polypeptides having amino acid sequences which are substantially homologous to native
- 25 GnRH and which remain immunogenic, as described below. Thus, the term encompasses derivatives and analogues of GnRH including any single or multiple amino acid additions, substitutions and/or deletions occurring internally or at the amino or carboxy terminuses of
- 30 the peptide. Accordingly, under the invention, a "GnRH polypeptide" includes molecules having the native sequence, molecules such as that depicted in Figure 1A (having an N-terminal Gln residue rather than a pyroGlu residue), and molecules with other amino acid
- 35 additions, substitutions and/or deletions which retain the ability to elicit formation of antibodies that

cross react with naturally occurring GnRH.

Particularly contemplated herein are repeated sequences of GnRH polypeptides such as in the oligomer depicted in Figure 1B (wherein each of the selected
5 GnRH polypeptides comprises a N-terminal Gln substitution, and further wherein every other GnRH polypeptide comprises an Asp residue substitution at position 2). Epitopes of GnRH are also captured by the definition.

10 The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. Since GnRH is a very small molecule, the identification of epitopes thereof which are able to elicit an antibody response is readily accomplished
15 using techniques well known in the art. See, e.g., Geysen et al. *Proc. Natl. Acad. Sci. USA* (1984) 81:3998-4002 (general method of rapidly synthesizing peptides to determine the location of immunogenic epitopes in a given antigen); U.S. Patent No.
20 4,708,871 (procedures for identifying and chemically synthesizing epitopes of antigens); and Geysen et al., *Molecular Immunology* (1986) 23:709-715 (technique for identifying peptides with high affinity for a given antibody).

25 As used herein the term "T-cell epitope" refers to a feature of a peptide structure which is capable of inducing T-cell immunity towards the peptide structure or an associated hapten. In this regard, it is accepted in the art that T-cell epitopes
30 comprise linear peptide determinants that assume extended conformations within the peptide-binding cleft of MHC molecules, (Unanue et al., *Science* (1987) 236:551-557). Conversion of polypeptides to MHC class II-associated linear peptide determinants (generally
35 between 5 - 14 amino acids in length) is termed "antigen processing" which is carried out by antigen

presenting cells (APCs). More particularly, a T-cell epitope is defined by local features of a short peptide structure, such as primary amino acid sequence properties involving charge and hydrophobicity, and
5 certain types of secondary structure, such as helicity, that do not depend on the folding of the entire polypeptide. Further, it is believed that short peptides capable of recognition by helper T-cells are generally amphipathic structures comprising
10 a hydrophobic side (for interaction with the MHC molecule) and a hydrophilic side (for interacting with the T-cell receptor), (Margalit et al., Computer Prediction of T-cell Epitopes, New Generation Vaccines Marcel-Dekker, Inc, ed. G.C. Woodrow et al.,
15 (1990) pp. 109-116) and further that the amphipathic structures have an α -helical configuration (see, e.g., Spouge et al., *J. Immunol.* (1987) 138:204-212; Berkower et al., *J. Immunol.* (1986) 136:2498-2503).

Hence, segments of proteins which include T-cell epitopes can be readily predicted using numerous
20 computer programs. (See e.g., Margalit et al., Computer Prediction of T-cell Epitopes, New Generation Vaccines Marcel-Dekker, Inc, ed. G.C. Woodrow et al., (1990) pp. 109-116). Such programs generally compare
25 the amino acid sequence of a peptide to sequences known to induce a T-cell response, and search for patterns of amino acids which are believed to be required for a T-cell epitope.

An "immunogenic protein" or "immunogenic amino acid sequence" is a protein or amino acid
30 sequence, respectively, which elicits an immunological response in a subject to which it is administered. Under the invention, a "GnRH immunogen" refers to a GnRH molecule which, when introduced into a host
35 subject, stimulates an immune response. In this regard, a GnRH immunogen includes a multimer

corresponding to more than one selected GnRH polypeptide; and, more particularly, to a multimer having either multiple or tandem repeats of selected GnRH polypeptide sequences, multiple or tandem repeats
5 of selected GnRH epitopes, or any conceivable combination thereof.

An "immunological response" to an antigen or vaccine is the development in the host of a cellular and/ or antibody-mediated immune response to the
10 composition or vaccine of interest. Usually, such a response includes but is not limited to one or more of the following effects; the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ T cells, directed
15 specifically to an antigen or antigens included in the composition or vaccine of interest. An immunological response can be detected using any of several immunoassays well known in the art.

The term "leukotoxin polypeptide" or "LKT polypeptide" intends a polypeptide which includes at
20 least one T-cell epitope and is derived from a protein belonging to the family of molecules characterized by the carboxy-terminus consensus amino acid sequence Gly-Gly-X-Gly-X-Asp (Highlander et al., *DNA* (1989)
25 8:15-28), where X is Lys, Asp, Val or Asn. Such proteins include, among others, leukotoxins derived from *P. haemolytica* and *Actinobacillus pleuropneumoniae*, as well as *E. coli* alpha hemolysin (Strathdee et al., *Infect. Immun.* (1987) 55:3233-3236;
30 Lo, *Can. J. Vet. Res.* (1990) 54:S33-S35; Welch, *Mol. Microbiol.* (1991) 5:521-528). This family of toxins is known as the "RTX" family of toxins (Lo, *Can. J. Vet. Res.* (1990) 54:S33-S35). In addition, the term
35 "leukotoxin-polypeptide" refers to a leukotoxin polypeptide which is chemically synthesized, isolated from an organism expressing the same, or recombinantly

produced. Furthermore, the term intends an immunogenic protein having an amino acid sequence substantially homologous to a contiguous amino acid sequence found in the particular native leukotoxin molecule. Thus, the term includes both full-length and partial sequences, as well as analogues. Although native full-length leukotoxins display cytotoxic activity, the term "leukotoxin" also intends molecules which remain immunogenic yet lack the cytotoxic character of native leukotoxins. The nucleotide sequences and corresponding amino acid sequences for several leukotoxins are known. See, e.g., U.S. Patent Nos. 4,957,739 and 5,055,400; Lo et al., *Infect. Immun.* (1985) 50:667-67; Lo et al., *Infect. Immun.* (1987) 55:1987-1996; Strathdee et al., *Infect. Immun.* (1987) 55:3233-3236; Highlander et al., *DNA* (1989) 8:15-28; Welch, *Mol. Microbiol.* (1991) 5:521-528. In the chimeras produced according to the present invention, a selected leukotoxin polypeptide sequence imparts enhanced immunogenicity to one or more fused GnRH multimers by providing, among other things, T-cell epitopes comprising small peptide segments in the range of five to fourteen amino acids in length which are capable of complexing with MHC class II molecules for presentation to, and activation of, T-helper cells. As discussed further below, these T-cell epitopes occur throughout the leukotoxin molecule and are thought to be concentrated in the N-terminus portions of leukotoxin, i.e., between amino acid residues 1 to 199.

As used herein, a leukotoxin polypeptide "which lacks cytotoxic activity" refers to a leukotoxin polypeptide as described above which lacks significant cytotoxicity as compared to a native, full-length leukotoxin (such as the full-length *P. haemolytica* leukotoxin described in U.S. Patent Nos.

5,055,400 and 4,957,739) yet still retains immunogenicity and at least one T-cell epitope. Leukotoxin polypeptides can be tested for cytotoxic activity using any of several known assays such as the
5 lactate dehydrogenase release assay, described by Korzeniewski et al., *Journal of Immunological Methods* 64:313-320, wherein cytotoxicity is measured by the release of lactate dehydrogenase from bovine neutrophils. A leukotoxin molecule is identified as
10 cytotoxic if it causes a statistically significant release of lactate dehydrogenase when compared to a control non-cytotoxic molecule.

The provision of LKT-GnRH chimeras comprising leukotoxin polypeptides which lack
15 cytotoxic activity provides several important benefits. Initially, a leukotoxin polypeptide which lacks cytotoxic activity is desirable since the injection of an active toxin into a subject can result in localized cell death (PMNs and macrophages) and, in
20 turn, cause a severe inflammatory response and abscess at the injection site. In this regard, cytotoxic activity resulting in the killing of macrophages may lead to reduced antigen presentation and hence a suboptimal immune response. The removal of the
25 cytotoxic portion as found in the non-cytotoxic LKT polypeptides used in producing the fusion proteins of the invention also results in a truncated LKT gene which is capable of being expressed at much higher levels than full-length LKT. Further, the use of non-
30 cytotoxic LKT polypeptides in the fusions constructed herein which retain sufficient T-cell antigenicity reduces the overall amount of leukotoxin-GnRH antigen which needs to be administered to a host subject to yield a sufficient B-cell response to the selected
35 GnRH polypeptides. Particular examples of immunogenic leukotoxin polypeptides which lack cytotoxic activity

include LKT 352, LKT 111, and LKT 101 which are described in greater detail below.

By "LKT 352" is meant a protein which is derived from the *lktA* gene present in plasmid pAA352 (Figure 2, ATCC Accession No. 68283). The nucleotide sequence and corresponding amino acid sequence of this gene are described in International Publication No. WO91/15237 and are shown in Figure 3. The gene encodes a truncated leukotoxin, having 914 amino acids and an estimated molecular weight of around 99 kDa, which lacks the cytotoxic portion of the molecule. The truncated gene thus produced is expressed at much higher levels than the full-length molecule (more than 40% of total cell protein versus less than 1% of total cell protein for the full-length form) and is more easily purified. The derived LKT 352 is not necessarily physically derived from the sequence present in plasmid pAA352. Rather, it may be generated in any manner, including for example, by chemical synthesis or recombinant production. In addition, the amino acid sequence of the protein need only be substantially homologous to the depicted sequence. Thus, sequence variations may be present so long as the LKT polypeptide functions to enhance the immunogenicity of antigen with which it is associated yet also lacks cytotoxic activity.

By "LKT 111" is meant a leukotoxin polypeptide which is derived from the *lktA* gene present in plasmid pCB111 (Figure 6, ATCC Accession No. 69748). The nucleotide sequence of this gene and the corresponding amino acid sequence are shown in Figure 7. The gene encodes a shortened version of leukotoxin which was developed from the recombinant leukotoxin gene present in plasmid pAA352 (Figure 2, ATCC Accession No. 68283) by removal of an internal DNA fragment of approximately 1300 bp in length. The

LKT 111 polypeptide has an estimated molecular weight of 52 kDa (as compared to the 99 kDa LKT 352 polypeptide), but retains portions of the LKT 352 N-terminus containing T-cell epitopes which are
5 necessary for sufficient T-cell immunogenicity, and portions of the LKT 352 C-terminus containing convenient restriction sites for use in producing the fusion proteins of the present invention. Under the invention, the LKT 111 leukotoxin peptide is not
10 necessarily physically derived from the sequence present in plasmid pCB111. Rather, it may be generated in any manner, including for example, by chemical synthesis or recombinant production. In addition, the amino acid sequence of the protein need
15 only be substantially homologous to the depicted sequence. Thus, sequence variations may be present so long as the protein functions to enhance the immunogenicity of antigen with which it is associated and lacks cytotoxicity.

20 By "LKT 101" is meant a leukotoxin polypeptide which is derived from the *lktA* gene present in plasmid pAA101 (Figure 10, ATCC Accession No. 67883). The predicted amino acid sequence of the *P. haemolytica* leukotoxin produced from the pAA101
25 construct is depicted in Figure 11. The LKT 101 polypeptide is expressed from a truncated form of the *lktA* gene which contains the 5' end of the gene up to the unique *Pst*I restriction endonuclease site. The truncated gene was fused to the β -galactosidase gene
30 (*lacZ*) to facilitate purification of the LKT 101 polypeptide. Under the invention, the LKT 101 leukotoxin peptide is not necessarily physically derived from the sequence present in plasmid pAA101. Rather, it may be generated in any manner, including
35 for example, by chemical synthesis or recombinant production. In addition, the amino acid sequence of

the protein need only be substantially homologous to the depicted sequence. Thus, sequence variations may be present so long as the protein functions to enhance the immunogenicity of antigen with which it is
5 associated and lacks cytotoxicity.

A leukotoxin-GnRH polypeptide chimera displays "increased immunogenicity" when it possesses a greater capacity to elicit an immune response than the corresponding one or more GnRH multimers alone.
10 Such increased immunogenicity can be determined by administering the particular leukotoxin-GnRH polypeptide and GnRH multimer controls to animals, and comparing anti-GnRH antibody titres thus obtained using standard assays such as radioimmunoassays and
15 ELISAs, well known in the art.

"Recombinant" proteins or polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired
20 polypeptide. "Synthetic" proteins or polypeptides are those prepared by chemical synthesis.

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a
25 polypeptide in vivo or in vitro when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus.
30 A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be
35 located 3' to the coding sequence.

DNA "control sequences" refer collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into mRNA, which is then translated into a chimeric polypeptide encoded by the two coding sequences. The coding sequences need not be contiguous to one another so long as the transcribed sequence is ultimately processed to produce the desired chimeric protein. A control sequence is "operably linked to" a coding sequence when it controls the transcription of the coding sequence.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become

integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

Two DNA or polypeptide sequences are "substantially homologous" when at least about 80% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., supra; DNA Cloning, vols I & II, supra; Nucleic Acid Hybridization, supra.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

By "vertebrate subject" is meant any member of the subphylum chordata, including, without limitation, mammals such as rodents, cattle, pigs, sheep, goats, horses and man; domestic animals such as

dogs and cats; birds, including domestic, wild and game birds such as cocks and hens including chickens, turkeys and other gallinaceous birds. The term does not denote a particular age. Thus, both adult and
5 newborn animals are intended to be covered.

B. General Methods

Central to the instant invention is the discovery that leukotoxin polypeptides, when coupled
10 to selected GnRH polypeptide repeats (or multimers), are able to confer superior immunogenicity to the associated GnRH moieties. In this regard, leukotoxin polypeptides act as carrier proteins which present selected GnRH multimers to a subject's immune system
15 in a highly immunogenic form. Thus, chimeric proteins constructed under the invention may be formulated into vaccine compositions which provide enhanced immunogenicity to GnRH polypeptides presented therewith. Fusion of the leukotoxin gene to selected
20 GnRH polypeptides also facilitates purification of the chimeric protein from cells expressing the same.

Accordingly, exemplified herein are leukotoxin chimeras which include leukotoxin fused to more than one GnRH polypeptide. Particular
25 embodiments of the present invention include chimeras comprising a leukotoxin polypeptide fused to one or more GnRH multimers, wherein said multimers have at least one repeating GnRH decapeptide sequence, or at least one repeating unit of a sequence corresponding
30 to at least one epitope of a selected GnRH molecule. Further, the selected GnRH peptide sequences may all be the same, or may correspond to different derivatives, analogues, variants or epitopes of GnRH so long as they retain the ability to elicit an immune
35 response. A representative nucleotide sequence of a GnRH decapeptide is depicted in Figure 1A. The

subject GnRH sequence is modified by the substitution of a glutamine residue at the N-terminal in place of pyroglutamic acid which is found in the native sequence. This particular substitution renders a molecule that retains the native glutamic acid structure but also preserves the uncharged structure of pyroglutamate. Accordingly, the resulting peptide does not require cyclization of the glutamic acid residue and may be produced in the absence of conditions necessary to effect cyclization.

Because the GnRH sequence is relatively short, it can easily be generated using synthetic techniques, as described in detail below. Under the invention, a leukotoxin polypeptide sequence is used to confer immunogenicity upon associated GnRH polypeptides (as a carrier protein) in order to help elicit an adequate immune response toward endogenous GnRH in a vertebrate subject. In this manner, immunization with GnRH can regulate fertility in a vaccinated subject by disruption of estrous cycles or spermatogenesis. A detailed discussion of GnRH can be found in U.S. Patent No. 4,975,420.

It is a particular object of the invention to provide a reliable and effective alternative to invasive sterilization procedures currently practiced in domestic and farm animal husbandry, such as surgical castration, surgical ovariectomy and the like. Immunosuppression of reproductive activity in vertebrate subjects using leukotoxin-GnRH chimeras constructed according to the present invention provides an effective alternative in that the constructs effect uniform inactivation of reproductive activity in immunized animals. In this regard, a suitable sterilization vaccine product must serve to uniformly inactivate reproductive capabilities in individual animals in response to a minimum of

vaccinations in order to provide a successful alternative to surgical procedures. This feature is particularly important for immunosterilization of herd animals, and particularly where it is desired to
5 immunocastrate male piglets to prevent "boar taint" which is produced by the synthesis of sex steroids in normally functioning testicles of male piglets. See e.g. Meloen et al., *Vaccine* (1994) 12(8):741-746. Prior attempts at developing such a product have not
10 produced uniform results due to the insufficient immunogenicity of GnRH peptides and/or related carrier systems, and the resultant inability of various prior GnRH-based vaccines to induce sufficient immune responses toward endogenous GnRH.

15 It is also a particular object of the invention to provide a method for reducing the incidence of mammary tumors in mammalian subjects using the leukotoxin-GnRH fusion molecules produced herein in a vaccine to block GnRH-regulated ovarian
20 functions such as the production of the ovarian hormones estrogen and progesterone in vaccinated subjects. The role of estrogen and progesterone in the etiology of mammary tumors is well established. These ovarian steroids are important in the early
25 stages of the cancer, but once the mammary tumors become established, some tumors become steroid independent. See e.g., the *Textbook of Endocrinology*, 7th Edition, Wilson et al. (eds), (1985) pp 68-69. Estrogen and progesterone are also known to be
30 carcinogenic and primarily responsible for mammary tumors in dogs.

Accordingly, leukotoxin-GnRH polypeptide chimeras contemplated herein comprise one or more GnRH
portions having a plurality of selected GnRH
35 polypeptide sequences in order to render a more immunogenic GnRH peptide antigen. This feature is

based on the recognition that endogenous proteins in general may be rendered effective autoantigens by multimerization of their epitopes as described in detail above. More particularly, the GnRH portions of the present leukotoxin-GnRH chimeras may comprise either multiple or tandem repeats of selected GnRH sequences, multiple or tandem repeats of selected GnRH epitopes, or any conceivable combination thereof. GnRH epitopes may be identified using techniques as described in detail above, or fragments of GnRH proteins may be tested for immunogenicity and active fragments used in compositions in lieu of the entire polypeptide. When more than one GnRH multimers are included in the chimeric molecules, each GnRH portion can be the same or different from other included GnRH portions in the molecule.

The sequence of one particular GnRH portion used herein is depicted in Figure 1B wherein four GnRH sequences, indicated at (1), (2), (3) and (4) respectively, are separated by triplet amino acid spacer sequences comprising various combinations of serine and glycine residues. In the subject oligomer, every other GnRH sequence (those indicated at (2) and (4), respectively) contains a non-conservative amino acid substitution at the second position of the GnRH decapeptide comprising an Asp residue in place of the His residue found in the native GnRH sequence. The alternating GnRH multimeric sequence thus produced renders a highly immunogenic GnRH antigen peptide for use in the fusion proteins of the invention. Other GnRH analogues corresponding to any single or multiple amino acid additions, substitutions and/or deletions are also particularly contemplated herein for use in either repetitive or alternating multimeric sequences. In one particular leukotoxin-GnRH fusion, four copies of the GnRH portion depicted in Figure 1B are fused to

a leukotoxin molecule such that the leukotoxin molecule is flanked on its N- and C- terminus with two copies of the subject GnRH multimer.

Furthermore, the particular GnRH portion
5 depicted in Figure 1B contains spacer sequences between the GnRH moieties. The strategic use of various spacer sequences between selected GnRH polypeptides is used herein to confer increased immunogenicity on the subject constructs.
10 Accordingly, under the invention, a selected spacer sequence may encode a wide variety of moieties of one or more amino acids in length. Selected spacer groups may preferably provide enzyme cleavage sites so that the expressed chimera can be processed by proteolytic
15 enzymes *in vivo* (by APC's or the like) to yield a number of peptides, each of which contain at least one T-cell epitope derived from the carrier portion (leukotoxin portion), and which are preferably fused to a substantially complete GnRH polypeptide sequence.
20 The spacer groups may be constructed so that the junction region between selected GnRH moieties comprises a clearly foreign sequence to the immunized subject, thereby conferring enhanced immunogenicity upon the associated GnRH peptides. Additionally,
25 spacer sequences may be constructed so as to provide T-cell antigenicity, such as those sequences which encode amphipathic and/or α -helical peptide sequences which are generally recognized in the art as providing immunogenic helper T-cell epitopes. The choice of
30 particular T-cell epitopes to be provided by such spacer sequences may vary depending on the particular vertebrate species to be vaccinated. Although particular GnRH portions are exemplified which include spacer sequences, it is also an object of the
35 invention to provide one or more GnRH multimers

comprising directly adjacent GnRH sequences (without intervening spacer sequences).

The leukotoxin-GnRH polypeptide complex can be conveniently produced recombinantly as a chimeric protein. The GnRH portions of the chimera can be fused 5' and/or 3' to the leukotoxin portion of the molecule, one or more GnRH portions may be located at sites internal to the leukotoxin molecule, or the chimera can comprise any combination of GnRH portions at such sites. The nucleotide sequence coding for full-length *P. haemolytica* A1 leukotoxin has been determined. See, e.g., Lo, *Infect. Immun.* (1987) 55:1987-1996; U.S. Patent No. 5,055,400. Additionally, several variant leukotoxin gene sequences are disclosed herein.

Similarly, the coding sequences for porcine, bovine and ovine GnRH have been determined, (Murad et al., *Hormones and Hormone Antagonists*, in The Pharmacological Basis of Therapeutics, Sixth Edition (1980)), and the cDNA for human GnRH has been cloned so that its sequence has been well established (Seeburg et al., *Nature* (1984) 311:666-668). Additional GnRH polypeptides of known sequences have been disclosed, such as the GnRH molecule occurring in salmon and chickens (International Publication No. WO 86/07383, published 18 December 1986). The GnRH coding sequence is highly conserved in vertebrates, particularly in mammals; and porcine, bovine, ovine and human GnRH sequences are identical to one another. The desired leukotoxin and GnRH genes can be cloned, isolated and ligated together using recombinant techniques generally known in the art. See, e.g., Sambrook et al., *supra*.

Alternatively, DNA sequences encoding the chimeric proteins can be prepared synthetically rather than cloned. The DNA sequence can be designed with

the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is
5 assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature* (1981) 292:756; Nambair et al. *Science* (1984) 223:1299; Jay et al. *J. Biol. Chem.* (1984) 259:6311.

10 Once coding sequences for the chimeric proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning
15 vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage lambda (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative
20 bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine
25 papilloma virus (mammalian cells). See, generally, DNA Cloning: Vols. I & II, supra; T. Maniatis et al., supra; B. Perbal, supra.

 The fusion gene can be placed under the control of a promoter, ribosome binding site (for
30 bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the chimeric protein is transcribed into RNA in the host cell transformed by a vector containing this
35 expression construction. The coding sequence may or may not contain a signal peptide or leader sequence.

The chimeric proteins of the present invention can be expressed using, for example, native *P. haemolytica* promoter, the *E. coli* tac promoter or the protein A gene (*spa*) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular fusion coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular chimeric protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above.

Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

5 In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. It may also be desirable to produce mutants or analogues of the chimeric proteins
10 of interest. Mutants or analogues may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide
15 sequences, such as site-directed mutagenesis, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; Nucleic Acid Hybridization, supra.

A number of procaryotic expression vectors
20 are known in the art. See, e.g., U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent
25 Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491.

Depending on the expression system and host
30 selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The chimeric protein is then isolated from the host cells
35 and purified. If the expression system secretes the protein into growth media, the protein can be purified

directly from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

5 The chimeric proteins of the present invention may also be produced by chemical synthesis, such as by solid phase peptide synthesis, based on the determined amino acid sequences. Such methods are known to those skilled in the art. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide*
10 *Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New
15 York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology, supra*, Vol. 1, for
20 classical solution synthesis.

 Subjects can be immunized against endogenous GnRH by administration of vaccine compositions which include the present chimeric leukotoxin-GnRH proteins. Prior to immunization, it may be desirable to further
25 increase the immunogenicity of a particular chimeric protein. This can be accomplished in any one of several ways known to those of skill in the art. For example, the leukotoxin-GnRH polypeptide fusion
30 protein may be administered linked to a secondary carrier. For example, a fragment may be conjugated with a macromolecular carrier. Suitable carriers are typically large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like;
35 polymeric amino acids such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and

inactive virus particles. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art.

The protein substrates may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or selected GnRH polypeptides) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl) propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

Other suitable carriers for the chimeric proteins of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent No. 5,071,651. Also useful is a fusion product of a viral protein and a leukotoxin-GnRH immunogen, where that fusion product is made by methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the fusion proteins of the present invention may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

The chimeric proteins of the instant invention can also be administered via a carrier virus which expresses the same. Carrier viruses which will

find use herein include, but are not limited to, the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the novel chimeric proteins can be
5 constructed as follows. The DNA encoding the particular leukotoxin-GnRH chimeric protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine
10 kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant chimeric protein into the viral genome. The resulting
15 TK-recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

It is also possible to immunize a subject with the present chimeric proteins, either
20 administered alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Typically, vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior
25 to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is often mixed with vehicles containing excipients which are pharmaceutically
30 acceptable and compatible with the active ingredient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary
35 substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the ef-

fectiveness of the vaccine. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, oils, saponins and other substances known in the art. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990. The composition or formulation to be administered will, in any event, contain a quantity of the protein adequate to achieve the desired immunized state in the subject being treated.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal, oral formulations, and sustained release formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 1% to about 30% of the active ingredient, preferably about 2% to about 20%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention.

The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Controlled or sustained release formulations are made by incorporating the chimeric proteins into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures. The chimeric proteins can also be presented using implanted mini-pumps, well known in the art.

Furthermore, the chimeric proteins (or complexes thereof) may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

To immunize a subject, a selected GnRH-leukotoxin chimera is administered parenterally, usually by intramuscular injection in an appropriate vehicle. Other modes of administration, however, such as subcutaneous, intravenous injection and intranasal delivery, are also acceptable. Injectable vaccine

formulations will contain an effective amount of the active ingredient in a vehicle, the exact amount being readily determined by one skilled in the art. The active ingredient may typically range from about 1% to
5 about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired.

10 With the present vaccine formulations, approximately 1 μ g to 1 mg, more generally 5 μ g to 200 μ g of GnRH polypeptide per mL of injected solution, should be adequate to raise an immunological response when administered. In this regard, the ratio of GnRH
15 to leukotoxin in the Leukotoxin-GnRH antigens of the subject vaccine formulations will vary based on the particular leukotoxin and GnRH polypeptide moieties selected to construct those molecules. More particularly, in the leukotoxin-GnRH polypeptides used
20 in producing the vaccine formulations under the invention, there will be about 1 to 40% GnRH, preferably about 3 to 30% and most preferably about 7 to 27% GnRH polypeptide per fusion molecule. Increases in the percentage of GnRH present in the
25 LKT-GnRH antigens reduces the amount of total antigen which must be administered to a subject in order to elicit an effective B-cell response to GnRH. Effective dosages can be readily established by one of ordinary skill in the art through routine trials
30 establishing dose response curves. The subject is immunized by administration of the particular leukotoxin-GnRH polypeptide in at least one dose, and preferably two doses. Moreover, the animal may be administered as many doses as is required to maintain
35 a state of immunity.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

C. Experimental

Materials and Methods

Enzymes were purchased from commercial sources, and used according to the manufacturers' directions. Radionucleotides and nitrocellulose filters were also purchased from commercial sources.

In the cloning of DNA fragments, except where noted, all DNA manipulations were done according to standard procedures. See Sambrook et al., supra. Restriction enzymes, T₄ DNA ligase, *E. coli*, DNA polymerase I, Klenow fragment, and other biological reagents were purchased from commercial suppliers and used according to the manufacturers' directions. Double-stranded DNA fragments were separated on agarose gels.

cDNA and genomic libraries were prepared by standard techniques in pUC13 and the bacteriophage lambda gt11, respectively. See DNA CLONING: Vols I and II, supra.

P. haemolytica biotype A, serotype 1 ("A1") strain B122 was isolated from the lung of a calf which died of pneumonic pasteurellosis and was stored at -70°C in defibrinated blood. Routine propagation was carried out on blood agar plates or in brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 5% (v/v) horse serum (Gibco Canada Ltd., Burlington, Canada). All cultures were incubated at 37°C.

Example 1

Isolation of P. haemolytica Leukotoxin Gene

To isolate the leukotoxin gene, gene libraries

5 of *P. haemolytica* A1 (strain B122) were constructed using standard techniques. See, Lo et al., *Infect. Immun.*, supra; DNA CLONING: Vols. I and II, supra; and Sambrook et al., supra. A genomic library was constructed in the plasmid vector pUC13 and a DNA
10 library constructed in the bacteriophage lambda gt11. The resulting clones were used to transform *E. coli* and individual colonies were pooled and screened for reaction with serum from a calf which had survived a *P. haemolytica* infection and that had been boosted
15 with a concentrated culture supernatant of *P. haemolytica* to increase anti-leukotoxin antibody levels. Positive colonies were screened for their ability to produce leukotoxin by incubating cell lysates with bovine neutrophils and subsequently
20 measuring release of lactate dehydrogenase from the latter.

Several positive colonies were identified and these recombinants were analyzed by restriction endonuclease mapping. One clone appeared to be
25 identical to a leukotoxin gene cloned previously. See, Lo et al., *Infect. Immun.*, supra. To confirm this, smaller fragments were re-cloned and the restriction maps compared. It was determined that approximately 4 kilobase pairs of DNA had been cloned.
30 Progressively larger clones were isolated by carrying out a chromosome walk (5' to 3' direction) in order to isolate full-length recombinants which were approximately 8 kb in length. The final construct was termed pAA114. This construct contained the entire
35 leukotoxin gene sequence.

lktA, a MaeI restriction endonuclease fragment from pAA114 which contained the entire leukotoxin gene, was treated with the Klenow fragment of DNA polymerase I plus nucleotide triphosphates and
5 ligated into the SmaI site of the cloning vector pUC13. This plasmid was named pAA179. From this, two expression constructs were made in the ptac-based vector pGH432:lacI digested with SmaI. One, pAA342, consisted of the 5'-AhaIII fragment of the lktA gene
10 while the other, pAA345, contained the entire MaeI fragment described above. The clone pAA342 expressed a truncated leukotoxin peptide at high levels while pAA345 expressed full length leukotoxin at very low levels. Therefore, the 3' end of the lktA gene (StyI
15 BamHI fragment from pAA345) was ligated to StyI BamHI-digested pAA342, yielding the plasmid pAA352. The structure of pAA352 is shown in Figure 2 and the nucleotide sequence and predicted amino acid sequence of *P. haemolytica* leukotoxin produced from the pAA352
20 construct (hereinafter LKT 352) is shown in Figure 3.

Several truncated versions of the leukotoxin gene were expressed from pAA114. These truncated forms were fusions with the B-galactosidase (lacZ) gene. Two fragments, LTX1.1 and LTX3.2, from an EcoRV
25 PstI double digest, were isolated from pAA114 as purified restriction fragments (1.0 kb and 2.1 kb, respectively). These fragments were cloned into the cloning vector pTZ18R that had been digested with HincII and PstI. The resulting vector, termed
30 pLTX3P.1, was used to transform *E. coli* strain JM105. Transformed cells were identified by plating on media containing ampicillin plus Xgal and IPTG. Blue colonies indicated the presence of a functional lacZ gene. DNA from the transformed cells was analyzed by
35 restriction endonuclease digestion and found to

contain the 5' end of the leukotoxin gene (lktC and lktA).

A leukotoxin EcoRV/PstI 5'-fragment (from pLTX3P.1) was subcloned into the cloning vector pBR325 that had been digested with EcoRI and PstI. The pBR325 plasmid also contained the native leukotoxin promoter (obtained from pLTX3P.1) and a promoterless, full length lacZ gene. The resulting construct was used to transform *E. coli* JM105 and blue colonies were isolated from Xgal agar. The new construct was termed pAA101 (ATCC No. 67883) and is depicted in Figure 10. The predicted amino acid sequence of the *P. haemolytica* leukotoxin produced from the pAA101 construct (hereinafter LKT 101) is depicted in Figure 11.

Example 2

Construction of LKT-GnRH Fusions

Representative LKT-GnRH fusions were constructed as follows. Oligonucleotides containing sequences corresponding to single copy GnRH and GnRH as four multiple repeats were constructed on a Pharmacia Gene Assembler using standard phosphoramidite chemistry. The sequences of these oligonucleotides are shown in Figures 1A and 1B. The subject oligonucleotides were annealed and ligated into the vector pAA352 (ATCC No. 68283, and described above), which had been digested with the restriction endonuclease *Bam*H1. This vector contains the *P. haemolytica* leukotoxin gene. The ligated DNA was used to transform *E. coli* strain MH3000. Transformants containing the oligonucleotide inserts were identified by restriction endonuclease mapping.

An eight copy GnRH tandem repeat sequence was prepared by annealing the four copy GnRH oligonucleotides and ligating them into a vector which

had been digested with the restriction endonuclease BamH1. The oligomers were designed to disable the upstream BamH1 site when inserted and to ensure that the insertion of additional copies of the oligomer would be oriented in the proper reading frame. The sequence of the subject oligonucleotide is shown in Figure 1B. Plasmid DNA from the *E. coli* MH3000 strain was then isolated and used to transform the strain JM105. The recombinant plasmids were designated pCB113 (LKT 352:4 copy GnRH, ATCC Accession No. 69749) and pCB112 (LKT 352:8 copy GnRH). Recombinant plasmid pCB113 is shown in Figure 4, plasmid pCB112 is identical to pCB113 except that the multiple copy GnRH sequence (corresponding to the oligomer of Figure 1B) was inserted twice as described above. The nucleotide sequence of the recombinant LKT-GnRH fusion of pCB113 is shown in Figure 5. The nucleotide sequence of the recombinant LKT-GnRH fusion pCB112 is identical except that the multiple copy GnRH sequence was inserted twice.

Example 3

Construction of Shortened LKT Carrier Peptide

A shortened version of the recombinant leukotoxin peptide was constructed from the recombinant gene present on the plasmid pAA352 (as described above). The shortened LKT gene was produced by deleting an internal DNA fragment of approximately 1300 bp in length from the recombinant LKT gene as follows.

The plasmid pCB113, (ATCC Accession No. 69749) which includes the LKT 352 polypeptide fused to four copies of the GnRH polypeptide, was digested with the restriction enzyme BstB1 (New England Biolabs). The resultant linearized plasmid was then digested with mung-bean nuclease (Pharmacia) to remove the

single stranded protruding termini produced by the *Bst*B1 digestion. The blunted DNA was then digested with the restriction enzyme *Nae*I (New England Biolabs), and the digested DNA was loaded onto a 1% agarose gel where the DNA fragments were separated by electrophoresis. A large DNA fragment of approximately 6190 bp was isolated and purified from the agarose gel using a Gene Clean kit (Bio 101), and the purified fragment was allowed to ligate to itself using bacteriophage T4 DNA ligase (Pharmacia). The resulting ligation mix was used to transform competent *E. coli* JM105 cells, and positive clones were identified by their ability to produce an aggregate protein having a molecular weight of approximately 57 KDa. The recombinant plasmid thus formed was designated pCB111, (ATCC Accession No. 69748), and produces a shortened leukotoxin polypeptide (hereinafter referred to as LKT 111) fused to four copies of GnRH polypeptide. The structure of pCB111 is shown in Figure 6. Plasmid pCB114 is identical to pCB111 except that the multiple copy GnRH sequence (corresponding to the oligomer of Figure 1B) was inserted twice. The nucleotide sequence of the recombinant LKT-GnRH fusion of pCB111 is shown in Figure 7, the nucleotide sequence of the recombinant LKT-GnRH fusion of pCB114 is identical except that the multiple copy GnRH sequence was inserted twice.

The nucleotide sequence of the ligation fusion point of the subject clones has been confirmed by sequencing with a bacteriophage T7 polymerase sequencing kit (Pharmacia). The nucleotide sequences of these fusion points are shown in Figure 8.

Example 4

Construction of an LKT-GnRH Fusion Having 8 Copy
Amino Terminal and Carboxyl Terminal GnRH Multimers

A recombinant LKT-GnRH fusion molecule
5 having two 8 copy GnRH multimers, one arranged at the
N'-terminus of LKT 111 and the other arranged at the
C'-terminus of LKT 111, was constructed from the LKT-
GnRH fusion sequence obtained from the pCB114 plasmid
by ligating the multiple copy GnRH sequence
10 (corresponding to the oligomer of Figure 1B) twice at
the 5' end of the LKT 111 coding sequence. A
synthetic nucleic acid molecule having the following
nucleotide sequence: 5'-ATGGCTACTGTTATAGATCGATCT-3'
was ligated at the 5' end of the multiple copy GnRH
15 sequences. The synthetic nucleic acid molecule
encodes an eight amino acid sequence (Met-Ala-Thr-Val-
Ile-Asp-Arg-Ser). The resulting recombinant molecule
thus contains in the order given in the 5' to 3'
direction: the synthetic nucleic acid molecule; a
20 nucleotide sequence encoding a first 8 copy GnRH
multimer; a nucleotide sequence encoding the shortened
LKT peptide (LKT 111); and a nucleotide sequence
encoding a second 8 copy GnRH multimer.

The recombinant molecule was circularized,
25 and the resulting molecule was used to transform
competent *E. coli* JM105 cells. Positive clones were
identified by their ability to produce an aggregate
protein having a molecular weight of approximately 74
KDa. The recombinant plasmid thus formed was
30 designated pCB122 which produces the LKT 111
polypeptide fused to 16 copies of GnRH polypeptide.
The nucleotide sequence of the recombinant LKT-GnRH
fusion of pCB122 is shown in Figures 9-1 through 9-6.

Example 5

Purification of LKT-antigen Fusions

The recombinant LKT-GnRH fusions from Examples 2, 3 and 4 were purified using the following procedure. For each fusion, five to ten colonies of the transformed *E. coli* strains were inoculated into 10 mL of TB broth supplemented with 100 micrograms/mL of ampicillin and incubated at 37°C for 6 hours on a G10 shaker, 220 rpm. Four mL of this culture was diluted into each of two baffled Fernbach flasks containing 400 mL of TB broth + ampicillin and incubated overnight as described above. Cells were harvested by centrifugation for 10 minutes at 4,000 rpm in polypropylene bottles, 500 mL volume, using a Sorvall GS3 rotor. The pellet was resuspended in an equal volume of TB broth containing ampicillin which had been prewarmed to 37°C (i.e., 2 x 400 ml), and the cells were incubated for 2 hours as described above.

3.2 mL of isopropyl-B,D-thiogalactopyranoside (IPTG, Gibco/BRL), 500 mM in water (final concentration = 4 mM), was added to each culture in order to induce synthesis of the recombinant fusion proteins. Cultures were incubated for two hours. Cells were harvested by centrifugation as described above, resuspended in 30 mL of 50 mM Tris-hydrochloride, 25% (w/v) sucrose, pH 8.0, and frozen at -70°C. The frozen cells were thawed at room temperature after 60 minutes at -70°C, and 5 mL of lysozyme (Sigma, 20 mg/mL in 250 mM Tris-HCl, pH 8.0) was added. The mixture was vortexed at high speed for 10 seconds and then placed on ice for 15 minutes. The cells were then added to 500 mL of lysis buffer in a 1000 mL beaker and mixed by stirring with a 2 mL pipette. The beaker containing the lysed cell suspension was placed on ice and sonicated for a total of 2.5 minutes (5-30 second

bursts with 1 minute cooling between each) with a Braun sonicator, large probe, set at 100 watts power. Equal volumes of the solution were placed in Teflon SS34 centrifuge tubes and centrifuged for 20 minutes at 10,000 rpm in a Sorvall SS34 rotor. The pellets were resuspended in a total of 100 mL of sterile double distilled water by vortexing at high speed, and the centrifugation step repeated. Supernatants were discarded and the pellets combined in 20 mL of 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (Tris-buffered saline) and the suspension frozen overnight at -20°C.

The recombinant suspension was thawed at room temperature and added to 100 mL of 8 M Guanidine HCl (Sigma) in Tris-buffered saline and mixed vigorously. A magnetic stir bar was placed in the bottle and the solubilized sample was mixed at room temperature for 30 minutes. The solution was transferred to a 2000 mL Erlenmeyer flask and 1200 mL of Tris-buffered saline was added quickly. This mixture was stirred at room temperature for an additional 2 hours. 500 mL aliquots were placed in dialysis bags (Spectrum, 63.7 mm diameter, 6,000-8,000 MW cutoff, #132670, from Fisher scientific) and these were placed in 4,000 mL beakers containing 3,500 mL of Tris-buffered saline + 0.5 M Guanidine HCl. The beakers were placed in a 4°C room on a magnetic stirrer overnight after which dialysis buffer was replaced with Tris-buffered saline + 0.1 M Guanidine HCl and dialysis continued for 12 hours. The buffer was then replaced with Tris-buffered saline + 0.05 M Guanidine HCl and dialysis continued overnight. The buffer was replaced with Tris-buffered saline (no guanidine), and dialysis continued for 12 hours. This was repeated three more times. The final solution was poured into a 2000 mL plastic roller bottle (Corning) and 13 mL of 100 mM PMSF (in

ethanol) was added to inhibit protease activity. The solution was stored at -20°C in 100 mL aliquots.

To confirm that the fusion proteins had been isolated, aliquots of each preparation were diluted 20-fold in double distilled water, mixed with an equal volume of SDS-PAGE sample buffer, placed in a boiling water bath for five minutes and run through 12% polyacrylamide gels. Recombinant leukotoxin controls were also run.

All fusion proteins were expressed at high levels as inclusion bodies. The predicted molecular weights based on the DNA sequences of the fusion proteins were 104,869 (LKT 352::4 copy GnRH, from pCB113); 110,392 (LKT 352::8 copy GnRH, from pCB112); 57,542 (LKT 111::4 copy GnRH, from pCB111); 63,241 (LKT 111::8 copy GnRH from pCB114); and 73,886 (8 copy GnRH::LKT 111::8 copy GnRH from pCB122). The predicted molecular weight of the recombinant LKT 352 molecule was 99,338, and the predicted molecular weight of the recombinant LKT 111 molecule was 51,843.

Example 6

In Vivo Immunologic Activity of LKT-GnRH Fusions

To test for the ability of LKT-GnRH fusions to induce an anti-GnRH immunological response in vivo, and to compare this response to other GnRH carrier conjugates, the following vaccination trial was performed. Three groups of 8 male pigs, approximately 8 weeks of age (35-50 kg) were used which were Specific Pathogen Free. The animals were maintained in a minimal disease facility and were vaccinated on days 0 and 21 of the trial with the following formulations:

Group 1 -- placebo which consisted of saline formulated in Emulsigen Plus adjuvant containing 15 mg of dimethyldioctadecylammonium bromide (DDA) (2 ml);

Group 2 -- LKT 352-GnRH (250 μ g LKT, prepared as described in the previous examples) formulated in the same adjuvant (2 ml);

Group 3 -- VP6-GnRH, 0.5 μ g VP6 and 5 μ g GnRH, formulated in the same adjuvant (2 ml). The VP6 preparation was made as described in U.S. Patent No. 5,071,651, using the binding peptide described therein.

Blood samples were taken on days 0, 21 and 35, allowed to clot, centrifuged at 1500 g, and the serum removed. The serum antibody titres against GnRH were measured using the RIA procedure of Silversides et al., *J. Reprod. Immunol.* (1985) 7:171-184.

The results of this trial indicated that only those animals immunized with the LKT 352-GnRH formulation produced significant titres against GnRH (titres >1:70). Neither the placebo nor the VP6-GnRH groups produced anti-GnRH titres. Previously, multiple vaccinations with doses of GnRH of more than 100 μ g, conjugated to other carrier proteins, were required to induce anti-hormone titres. These results indicate that the LKT-GnRH carrier system provides a greatly improved immunogen over prior carrier systems.

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Example 7

In Vivo Immunologic Effect of Multiple Tandem GnRH Repeats Ligated to LKT

To test for the ability of recombinant LKT-GnRH fusion proteins containing multiple GnRH polypeptide repeats to induce an anti-GnRH immunological response in vivo, the following vaccination trial was performed. Cultures of *E. coli* containing plasmids pCB113 and pCB175 (having 4 and 8 copies of GnRH ligated to LKT 352, respectively) and a plasmid having 1 copy of GnRH ligated to LKT 352 were prepared as described above. Vaccines from each of

the above cultures were formulated to contain the equivalent of 5 μ g of GnRH in 0.2 mL of Emulsigen Plus. Three groups of 10 female mice were given two subcutaneous injections 23 days apart and blood samples were collected at days 23, 35 and 44 after the primary injection. Serum antibody titres against GnRH were measured at final dilutions of 1:100 and 1:1000 using a standard radioimmunoassay procedure. If less than 5% of the iodinated GnRH was bound, antibody was deemed to be undetectable. The antibody titres thus obtained are summarized in the Table 1.

The results of this study indicate that equal doses of GnRH presented as multiple tandem repeats (four or eight copy GnRH) gave a dramatic improvement in antibody production over single copy GnRH (as measured by binding to iodinated native GnRH). Further, the above results indicate that a fusion protein comprising a four copy GnRH tandem repeat ligated to LKT 352 represents an effective immunogenic GnRH antigen form, although immunogenicity may be influenced by dose or subject species.

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Sample Day	Group 1				Group 2				Group 3			
	LKT 352::1 Copy GnRH				LKT 352::4 Copy GnRH				LKT 352::8 Copy GnRH			
	No. responding		mean response (%)*		No. responding		mean response (%)*		No. responding		mean response (%)*	
	1:100	1:1000	1:100	1:1000	1:100	1:1000	1:100	1:1000	1:100	1:1000	1:100	1:1000
23	0	0	-	-	3	1	16	9	2	0	33	-
35	2	2	45	20	9	9	75	30	7	5	48	41
44	2	2	60	39	10	10	55	43	8	7	57	46

*mean response is the average binding of I¹²⁵-GnRH of only those animals with binding in excess of 5%.

Table 1

Example 8

In Vivo Immunologic Activity and Biologic Effect
of LKT 352::GnRH and LKT 111::GnRH Fusions

To test the ability of fusion proteins
5 comprising multiple tandem repeats of GnRH (ligated to
either LKT 352 or LKT 111) to elicit an anti-GnRH
immunological response in vivo and to manifest a
biologic effect in vivo, the following vaccination
trial was preformed. Cultures of *E. coli* containing
10 plasmids pCB113 and pCB111 (4 copy GnRH ligated to LKT
352 or LKT 111, respectively) were prepared as
described above. Vaccines from each of the above
cultures were formulated to contain the equivalent of
5 µg of GnRH in 0.2 mL of VSA-3 adjuvant, (a modified
15 Emulsigen Plus adjuvant), with a control vaccine
comprising 0.2 mL of the adjuvant also being prepared.
Three groups of 5 male Swiss mice were given two
subcutaneous injections 21 days apart, with the
initial injections (day 0) given at 5-6 weeks of age.
20 On day 49 the subjects were sacrificed.

Immunological activity of the subject GnRH-
LKT fusions was assayed by measuring anti-GnRH
antibody titres using a standard radioimmunoassay
procedure at a 1:1000 serum dilution. Biological
25 effect of the GnRH-LKT fusions was quantified by
standard radioimmunoassay of serum testosterone levels
with a sensitivity of 25 pg/ml, and testicular tissue
was weighed and histologically examined. The results
of this trial are summarized in Table 2.

30 In the trial, all animal subjects injected
with GnRH:LKT antigens had readily detectable antibody
levels; however, the LKT 111::GnRH fusion (from
plasmid pCB111) showed superior immunogenicity as
indicated by uniformity of response and titre. Serum
35 testosterone (produced by the testicular Leydig cells)
is secreted in a pulsatile manner, and accordingly,

low values and extreme variability of serum levels are expected in normal animal subjects. Under the trial, the control group (receiving the 0.2 mL adjuvant vaccine injections) had normal serum testosterone levels, while both groups of treated subjects had essentially undetectable serum testosterone.

Further under the trial, histological evaluation of testicular tissue revealed varying degrees of Leydig cell atrophy, reduced seminiferous tubule diameter and interruption of spermatogenesis in treated subjects; however, testicular weight remained close to normal in treated animals -even in the presence of high anti-GnRH antibody titres- although there was clear evidence of testicular regression in 2 of 5 subjects receiving the LKT 111::4 copy GnRH fusions.

Accordingly, these results show that multiple copies of GnRH ligated to either LKT 352 or LKT 111 comprise potent immunogens; and further, it is indicated that vaccination with the subject fusion proteins triggers production of antibodies which are able to neutralize endogenous GnRH in vivo, and that a concomitant in vivo biological effect is discernable in animal subjects receiving such vaccinations.

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Animal	Group 1			Group 2			Group 3		
	Control			5 µg LKT 352::4 Copy GnRH			5 µg LKT 111::4 Copy GnRH		
	Anti-body Titre*	Testic- ular Wt.(mg)	Serum Testos- terone†	Anti-body Titre*	Testic- ular Wt.(mg)	Serum Testos- terone†	Anti-body Titre*	Testic- ular Wt.(mg)	Serum Testos- terone†
1	7.0	252	.04	73.0	282	.13	75.0	163	.00
2	4.0	327	.18	14.0	334	.10	59.0	296	.07
3	0.0	276	2.73	18.0	254	.03	54.0	260	.24
4	0.0	220	.36	55.0	222	.05	66.0	265	.03
5	1.0	232	1.44	61.0	226	.19	64.0	50	.00
Mean	2.4	261	.95	44	263	.10	64	206	.07
Std Error	1.4	19	.51	12	21	.03	4	45	.04

* % Binding of I¹²⁵-GnRH at a 1:1000 serum dilution

† ng/ml

Table 2

PCT/CA97/00559

Example 9

In Vivo Immunologic Activity of
LKT::GnRH Fusions in Porcine Subjects

To test the ability of fusion proteins comprising multiple tandem repeats of GnRH (ligated to either LKT 352 or LKT 111) to elicit anti-GnRH immunological response in vivo in porcine subjects, the following vaccination trial was preformed. Cultures of *E. coli* containing plasmids pCB113, pCB111, pCB175 and pCB114 (LKT 352::4 copy GnRH, LKT 111::4 copy GnRH, LKT 352::8 copy GnRH, and LKT 111::8 copy GnRH, respectively) were prepared as described above. Vaccines from each of the above cultures were formulated to contain the equivalent of 50 µg GnRH and were administered in VSA-3 adjuvant in a 2.0 mL volume. Four groups of 5 male and 5 female weanling pigs, 35 days old (at day 0), were injected at day 0 and reinjected at day 21 of the trial. Blood samples were collected at days 0, 21 and 35, with anti-GnRH antibody titres being measured at a final dilution of 1:1000 using a standard radioimmunoassay procedure. The assay results are summarized in Table 3.

Under the trial, anti-GnRH antibodies could not be detected in any subjects prior to immunization, but were readily detected in most subjects by day 35 (one subject in treatment group 4 died due to an infection unrelated to treatment). The results in this trial indicate that fusion proteins comprising multiple GnRH repeats ligated to either a LKT 352 or LKT 111 carrier polypeptide form useful immunogens in porcine subjects. Based on the predicted molecular weights of the decapeptide GnRH (1,200), the LKT 111 polypeptide (52,000) and the LKT 352 polypeptide (100,000), the percentages of GnRH in the LKT-GnRH antigen fusions are as follows: 4.9% (LKT 352::4 copy GnRH); 8.5% (LKT 111::4 copy GnRH); 9.3% (LKT 352::8

copy GnRH) and 15.7% (LKT 111::8 copy GnRH).
Accordingly, the practical result thus obtained
indicates that by using LKT-GnRH fusions comprising
the LKT 111 polypeptide carrier, the overall amount of
5 antigen (LKT-GnRH) administered to the subject may be
halved (as compared to vaccination compositions using
the LKT 352 carrier polypeptide system) to obtain an
equivalent anti-GnRH response.

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35	30	25	20	15	10	5
Animal Number	Group 1	Group 2	Group 3	Group 4		
	LKT 352::4 copy GnRH 50 µg	LKT 111::4 copy GnRH 50 µg	LKT 352::8 copy GnRH 50 µg	LKT 111:: 8 copy GnRH 50 µg		
	day 35 1:1000 dilution	day 35 1:1000 dilution	day 35 1:1000 dilution	day 35 1:1000 dilution		
1	♂ 47.7	♀ 46.0	♂ 68.3	♂ 51.0		
2	♀ 50.3	♂ 71.6	♂ 65.1	♂ 31.7		
3	♀ 66.0	♀ 21.4	♀ 50.7	♀ 35.7		
4	♀ 70.2	♂ 46.2	♂ 4.7	♀ 65.9		
5	♂ 17.3	♀ 48.9	♀ 38.3	♀		
6	♂ 18.3	♂ 69.4	♀ 17.4	♂ 11.3		
7	♀ 14.7	♂ 47.9	♀ 51.4	♀ 28.3		
8	♂ 37.0	♀ 44.4	♂ 18.0	♂ 43.0		
9	♂ 26.0	♂ 70.8	♂ 83.5	♀ 78.7		
10	♀ 2.7	♀ 37.8	♀ 24.2	♂ 55.9		
Mean	35.0	50.4	42.2	44.6		
Standard Deviation	7.3	5.1	8.1	6.9		
Responders	9/10	10/10	9/10	9/9		

Table 3

Example 10

Evaluation of LKT 111::8 Copy GnRHImmunocastration Vaccine Efficiency

To evaluate the efficacy and commercial

5 usefulness of a vaccine formulation containing the LKT
111::8 copy GnRH fusion protein, the following
vaccination trial was carried out. A culture of *E.*
coli containing the plasmid pCB114 (LKT 111::8 copy
GnRH) was prepared as described above. A vaccine
10 formulation from the above culture was prepared which
contained the equivalent of 50 µg GnRH. The vaccine
formulation was administered in VSA-3 adjuvant at a
2.0 mL final volume. Three treatment groups, with 30
male pigs (boars) each, were established. The three
15 groups consisted of 30 barrows (boars surgically
castrated before sexual maturity), 30 control boars
and 30 immunocastrates (boars castrated by vaccination
with the GnRH immunogen). At weaning (day 21), the
barrow and control boar group animals were injected
20 with placebo (VSA-3 adjuvant alone), while the
immunocastrate group was injected with the above-
described vaccine formulation. When the animals
reached a predetermined weight about 3 weeks before
slaughter, the immunocastrate group was given a
25 booster dose of the vaccine, while the barrow and
control boar groups were again given placebo
injections. Measurements included serum antibody
titres to GnRH, blood testosterone levels, carcass
traits, animal behavior, feed efficiency, rate of
30 weight gain, and salivary gland and body fat
androstenone levels (as a measure of boar taint).

(a) Serum Anti-GnRH Antibody Titre:

Immunological activity of the 8 copy GnRH-
35 LKT fusion vaccine formulation was assayed by
measuring anti-GnRH antibody titres using a standard

radioimmunoassay procedure at a 1:5000 serum dilution. A comparison of serum antibody titres in the three experimental groups is provided in Figure 12. As can be seen, anti-GnRH antibody titres increased
5 dramatically in the immunocastrate (vaccinated) boars and remained at levels significantly in excess of the minimal amount required to produce a biological effect (approximately 10 to 20 % binding in Figure 12) for over 20 days post vaccination.

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(b) Biological Effect of the Immunocastrate Vaccine on Sexual Gland Size:

The biological effect of the 8 copy GnRH-LKT fusion vaccine formulation was determined by comparing
15 the weight and measurements of sexual glands from the control boars and the immunocastrate (vaccinated) boars, as well as by assaying and comparing serum testosterone levels in those two experimental groups. In particular, the bulbourethral glands and testes
20 from the animals were weighed and measured. The results are depicted below in Table 4. As can be seen, the average weight of the bulbourethral glands in the vaccinated animals was reduced approximately 32% relative to the control animals. In addition, the
25 average weight of the testes in the vaccinated animals was reduced approximately 25% relative to the control animals. These results are consistent with reduced testosterone production from the testes in the vaccinated animals.

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TABLE 4							
Treatment	No. of Animals	Bulbourethral Gland				Testes	
		Average Weight (gm)	% of Control	Average Length (cm)	% of Control	Average Weight (gm)	% of Control
Control Boars	22	60.5±3.5*		11.4±.21		263±10.9	
Immunocastrate Boars	27	41.3±5.2	68.3	9.5±.47	83.3	198±11.3	75.3

*means ± standard errors

The average serum testosterone levels in all three experimental groups was determined using a standard radioimmunoassay of serum testosterone levels with a sensitivity of 25 pg/mL. The assays were conducted on Day 0, Day 7, Day 14, and Day 21 after the booster immunizations (and placebo vaccinations in the control boar and barrow groups). The results of the assays are depicted in Figure 13. As can be seen, the serum testosterone levels in the vaccinated animals decreased after vaccination, while the levels in the control boars increased.

(c) Carcass Composition:

Commercial aspects of the carcass composition of animals from each experimental group were assessed after slaughter of the animals. In particular, average body weights and fat content were determined, average measurements of the loin eye were taken, and the average weight of trimmed hams and loin was determined. The results of the carcass assessments are reported in Table 5. As can be seen, the carcass data show that the control boars and immunocastrates (vaccinated animals) had very similar carcass compositions, whereas the barrows had appreciably more body fat, less body lean. In addition, the growth performance of the barrows reached a plateau over the last 24 days of life (results not shown). These carcass data are consistent with the objective of having the carcass compositions of the immunocastrated animals mimic that of the control boars for all but the final few days of their growing period.

TABLE 5			
Carcass Data			
	Borrows	Control Boars	Immunocastrates
Kill wt (kg)	110.5	115.2	115.4
Fat (mm)	19.1	15.7	15.3
Loin eye (cm ²)	41.5	44.5	44.2
Trim Primal (kg)	27.3	28.4	28.2
Trimmed ham (kg)	7.70	8.23	8.11
Trimmed loin (kg)	7.38	7.79	7.65

(d) Feed Conversion:

The feed conversion efficiency of animals from each of the experimental groups was measured over the period of weaning to slaughter. In particular, average feed conversion efficiency was expressed as the ratio of Kg feed:Kg weight gain in the animals. The results are depicted in Figure 14. As can be seen, feed conversion in the control boars and the immunocastrates (vaccinated animals) was about 10% more efficient than feed conversion in the barrows.

(e) Boar Taint Component Levels:

The ability of the 8 copy GnRH-LKT fusion vaccine formulation to reduce boar taint in vaccinated animals was assessed by assaying the androstenone levels (a boar taint component) in fat and salivary glands of animals from each of the experimental groups. Androstenone levels were quantified by a standard chemical method on fat and salivary gland specimens obtained from each group. The results are reported in Table 6. As can be seen, the control

boars had appreciably higher androstenone concentrations relative to the barrows and the immunocastrates (vaccinated animals).

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TABLE 6			
	Barrows	Control Boars	Immunocastrates
Fat Androstenone	0.14 $\mu\text{g/g}$	0.44 $\mu\text{g/g}$	0.26 $\mu\text{g/g}^*$
Salivary Androstenone	33.76 $\mu\text{g/g}$	40.46 $\mu\text{g/g}$	30.18 $\mu\text{g/g}$

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*p less than .01

All of the above results indicate that immunocastration vaccine formulations containing the short LKT::8 copy GnRH fusion molecules provide a commercially viable alternative to surgical castration methods.

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Example 11

Comparison of In Vivo Immunogenic Activity of Fusion Molecules Having One or Two GnRH Multimers

In order to compare the ability of LKT-GnRH fusion proteins comprising either a single GnRH multimer (containing 8 tandem repeats of GnRH), or two GnRH multimers (both containing 8 tandem repeats of GnRH), to elicit an anti-GnRH immunological response in vivo, several vaccination trials were carried out.

Cultures of *E. coli* containing plasmids pCB114 (one 8 copy GnRH multimer, ligated to the C'-terminus of LKT 111), and pCB122 (two 8 copy GnRH multimers, one ligated to the N'-terminus of LKT 111 and the other ligated to the C'-terminus of LKT 111) were prepared as described above. Vaccines derived from cultures containing the pCB114 plasmid were formulated to contain 160 μg of the fusion molecules (25 μg total of GnRH) in a 2 mL final volume of VSA-3

adjuvant. Vaccines derived from cultures containing the pCB122 plasmid were formulated to contain 185 µg of the fusion molecules (50 µg total of GnRH) in a 2 mL final volume of VSA-3 adjuvant. In this manner, the amount of the LKT carrier molecule was kept constant (135 µg total of LKT per formulation) in both preparations. The vaccine formulations were used in the following vaccination trials.

10 (a) Anti-GnRH Antibody Titre and Functional Activity of the Anti-GnRH Antibody Molecules:

A comparison between anti-GnRH antibody titres elicited by the two experimental vaccine formulations was carried out, wherein the ability of the elicited antibodies to block the effect of endogenously produced GnRH was also assessed. In particular, three groups of male pigs were established as follows: 50 animals were injected with the single GnRH multimer vaccine composition (LKT 111::8 copy GnRH fusions obtained from pCB114), 10 animals were injected with the plural GnRH multimer vaccine composition (8 copy GnRH::LKT 111::8 copy GnRH fusions obtained from pCB122), and 10 control animals were injected with 2 mL of the VSA-3 adjuvant alone.

25 Vaccinations were carried out at weaning (21 days of age), and the animals were boosted 30 days later. Blood was collected 14 and 28 days after the booster immunization. Serum was obtained and assayed for anti-GnRH antibody titer and serum levels of Luteinizing Hormone (LH). Serum anti-GnRH antibody titres were determined at a final serum dilution of 1:5000 using iodinated GnRH in a standard radioimmunoassay. Serum levels of LH were assayed using porcine LH as a reference standard in a standard radioimmunoassay. The results of the assays, given as mean values ± standard errors, are reported in Table

7. As can be seen by the data depicted in Table 7, anti-GnRH antibody titres were higher in animals injected with the plural GnRH multimer vaccine composition (8 copy GnRH::LKT 111::8 copy GnRH) than seen with the animals receiving the single GnRH multimer vaccine (LKT 111::8 copy GnRH). Further, the animals receiving the plural GnRH multimer vaccine had lower serum LH levels. This reduction in serum LH reflects the ability of the anti-GnRH antibodies produced in the immunized animals to block the effect of endogenously produced GnRH. Finally, 100% of the animals receiving the plural GnRH multimer vaccine responded to the vaccine by producing anti-GnRH antibodies, whereas 90-92% of the animals receiving the single GnRH multimers responded.

TABLE 7			
	GnRH Antibodies at Day		Serum LH at Day
Day after the Booster	14	28	14
Treatments 1 (Control)	0.5 ± .3	0.5 ± .3	1.16 ± .22
Treatment 2 LKT III::8 copy GnRH 160 µg (25 µg GnRH)	44.6 ± 4.1	37.2 ± 4.1	0.13 ± .04
Treatment 3 8 copy GnRH::LKT III::8 copy GnRH 185 µg (50 µg GnRH)	60.5 ± 6.9	51.8 ± 7.5	.06 ± .02

(b) Comparison of Anti-GnRH Titres and
Assessment of the Effect of Increased Vaccine Dosages:

The immunogenicity of the two vaccine formulations (the 8 copy GnRH single multimer antigen and the 16 copy GnRH plural multimer antigen) was again assessed as follows. Two experimental groups of

20 male pigs each were established. Animals in the first group were vaccinated at weaning (Day 21 of age) with 160 μ g of the single multimer antigen preparation, and then boosted 33 days later with the same dosage. Animals in the second group were vaccinated at weaning (Day 21 of age) with 185 μ g of the plural multimer antigen preparation and also boosted 33 days later. Blood was collected at 8, 14, and 24 days after the booster injections, and serum was assayed for anti-GnRH antibody molecules at a final dilution of 1:5000 using standard radioimmunoassay as previously described. The results are depicted in Figure 15. As can be seen, the antibody response to the plural multimer vaccine (8 copy GnRH::LKT 111::8 copy GnRH) was higher ($P<.001$) than for the single multimer vaccine (LKT 111::8 copy GnRH). Referring still to Figure 15, the horizontal line at 20% on the Y axis represents an antibody titre which, in previous trials not reported herein, have been shown to suppress secretion of LH in vaccinated animals. Once again, 100% of the animals receiving the plural GnRH multimer vaccine responded (produced anti-GnRH antibodies), while approximately 90-92% of the animals receiving the single multimer vaccine responded.

In order to determine if the increased immunogenicity observed with the plural GnRH multimer vaccine is due to the increased dosage of the GnRH antigen (e.g., 50 μ g GnRH in the [8 copy GnRH::LKT 111::8 copy GnRH] vaccine, as compared to 25 μ g GnRH in the [LKT 111::8 copy GnRH] vaccine), the following study was carried out. Three groups of 20 pigs each were vaccinated at weaning (21 days of age) and boosted approximately 30 days later with the single GnRH multimer vaccine composition (LKT 111::8 copy GnRH fusions obtained from pCB114) at the following

dosages: 50 μ g, 150 μ g and 450 μ g of the fusion protein, respectively. Blood was collected at 14, 28 and 64 days after the booster injection. Serum was assayed for anti-GnRH antibodies at a final dilution of 1:5000 as described above. The results are reported in Table 8. As can be seen, no appreciable increase in anti-GnRH antibody titres were obtained in response to vaccination with increased dosages of the single GnRH multimer vaccine composition. This indicates that the increased immunogenicity observed with plural GnRH multimer vaccine (8 copy GnRH::LKT 111::8 copy GnRH fusions obtained from pCB122) is not due to increased GnRH antigen concentration; rather the increased immunogenicity is likely due to the three dimensional structure of the particular LKT-GnRH fusion molecule, or in the physical presentation of the GnRH antigen to antibody producing cells.

TABLE 8			
Dose (μ g) LKT III::8 copy GnRH	% Binding at 1:5000 Dilution at Day after Boost		
	Day 14	Day 28	Day 64
50 μ g	60.9 $\pm$ 4.8	50.7 $\pm$ 5.8	22.0 $\pm$ 4.7
150 μ g	59.0 $\pm$ 4.9	46.0 $\pm$ 4.9	16.8 $\pm$ 3.6
450 μ g	62.6 $\pm$ 4.0	56.5 $\pm$ 4.7	22.8 $\pm$ 4.8

Example 12

Dose Response Study With LKT-GnRH

Fusion Molecules Having Two GnRH Multimers

In order to determine optimal dosages of vaccine compositions formed from LKT-GnRH fusion proteins comprising two GnRH multimers (both containing 8 tandem repeats of GnRH), the following in vivo dose response study was carried out.

Cultures of *E. coli* containing plasmid pCB122 (two 8 copy GnRH multimers, one ligated to the N'-terminus of LKT 111 and the other ligated to the C'-terminus of LKT 111) were prepared as described above. Seven vaccines derived from cultures containing the pCB122 plasmid were formulated at the following dosages of total fusion protein: 0 μ g (control); 1 μ g; 5 μ g; 10 μ g; 20 μ g; 40 μ g; and 80 μ g, each in a 1 mL final volume of VSA-3 adjuvant.

Seven experimental groups of 20 animals each were assembled and vaccinated with the above-described vaccine formulations. A blood sample was taken at day 35 after the vaccination, and anti-GnRH antibody titres were measured at a final dilution of 1:100 in a standard radioimmunoassay as described above. The results of the assay are reported in Table 9. The titres are expressed as % binding as above. As can be seen, statistically 0 μ g of the fusion protein was different from all other values. The 1 μ g fusion protein dose was lower ($p < .009$) than all other values obtained from groups receiving the protein antigen. The 5 μ g dose was less than the 20 μ g dose ($p < .06$), however, all values for doses above 10 μ g total fusion protein were statistically similar. These data show that the optimal dosage of the vaccine derived from the fusion protein of plasmid pCB122 (8 copy GnRH::LKT 111::8 copy GnRH) is approximately 20 - 40 μ g of the fusion protein.

TABLE 9

	8 copy GnRH::LKT 111::8 copy GnRH Dose (μ g)						
	0	1	5	10	20	40	80
Titre $\bar{x}$	2.6	20.5	47.9	52.0	59.6	62.0	64.6
S $\bar{x}$	$\pm .6$	5.0	5.8	4.6	4.4	3.4	3.6

Example 13

GnRH Immunization of Female Rats

In order to assess biological effects of GnRH immunization of female subjects, the following study was carried out.

Cultures of *E. coli* containing the pCB122 plasmid (two 8 copy GnRH multimers, one ligated to the N'-terminus of LKT 111 and the other ligated to the C'-terminus of LKT 111) were prepared as described above. A vaccine derived from the cultures was formulated to contain 185 µg of the fusion molecules (50 µg total of GnRH) in a 1 mL final volume of VSA-3 adjuvant. The formulation was then used in the following vaccination trial to assess the effect of GnRH immunization on ovarian weight, uterine weight, and serum estrogen concentration in female subjects.

Two experimental groups of female Sprague Dawley rats, 10 animals per group, were assembled. A control group (Group 1) was given a placebo injection (VSA-3 adjuvant only) at day 0 of the trial. Animals in the second group received a single injection of the GnRH/LKT vaccine formulation. Anti-GnRH antibody titres were monitored after treatment, and animals in Group 2 showed a rise in titer that began 21 days after injection to reach maximum levels at approximately day 50 of the study, after which the levels declined gradually until the animals were sacrificed on day 224 of the study.

Ovarian weight, uterine weight, and serum estradiol levels were then determined and recorded. The results of these measurements are depicted in Figure 16. As can be seen, ovarian weights in the treated animals (immunized with the GnRH-LKT vaccine formulation) were reduced dramatically relative to the control animals. Histological examination of the tissue revealed no active follicles in the ovarian

tissue. Uterine weights were also dramatically reduced in the treated animals. Uterine weight provides a good reflection of serum estrogen concentrations, and is related to gonadal steroid secretion. Furthermore, serum estradiol levels were reduced in the treated animals to about 20 pg/mL, whereas serum estradiol was about 50 pg/mL in the control animals. Since estrogen is derived from the ovary, it was expected that the serum estradiol would be reduced in the treated animals. These results demonstrate that the GnRH/LKT immunizations of the present invention are effective in controlling ovarian function, indicating a viable alternative to procedures such as ovariectomy or treatment with GnRH antagonists.

Example 14

Immunocastration of Male Porcine Subjects

Using LKT-GnRH Fusion Molecules Having Two GnRH

Multimers

In order to determine the ability of vaccine compositions formed from LKT-GnRH fusion proteins having two GnRH multimers (both containing 8 tandem repeats of GnRH) to reduce androstenone in fat, the following study was carried out.

Cultures of *E. coli* containing plasmid pCB122 (two 8 copy GnRH multimers, one ligated to the N'-terminus of LKT 111 and the other ligated to the C'-terminus of LKT 111) were prepared as described above. Vaccine compositions derived from the cultures were prepared as also described above. Four experimental groups of male porcine subjects were formed as follows: Group 1, comprising 6 Barrows (male animals surgically castrated within a few days of birth); Group 2, comprising 7 Boars (intact males left intact throughout the study); Group 3, comprising 6

Late Castrates (intact males left intact until approximately 135 days old at which time the animals were anesthetized and castrated surgically); and Group 4, comprising 10 intact males which were immunized with the LKT-GnRH vaccine composition at weaning (21 days old) and at approximately 135 days old.

After 42 days, the study was completed, and the animals sacrificed. Fat androstenone levels (a boar taint component) in fat specimens from animals in each experimental group were quantified by standard chemical methodology. The results are depicted in Figure 17. As can be seen in the figure, fat androstenone was similar in the barrows (Group 1), late castrates (Group 3) and immunocastrates (Group 4, treated with the LKT-GnRH vaccine), and all three groups had lower fat androstenone levels relative to the boars of Group 2.

Various aspects of the carcass composition in the experimental animals was also determined. In particular, carcass weight, back fat measurements, testicular weight (where appropriate) and bulbourethral (BU) gland length were determined in each group, and the average measurements are depicted below in Table 10. The BU gland is dependent on testosterone for maintenance of size and function.

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Table 10				
Treatment Group	Carcass Weight (kg)	Back Fat (mm)	Testicular Weight (gm)	BU Length (cm)
LKT-GnRH (n=10)	90.4	24.5 (18-32)	261 (145-480)	9.6 (8.0-11.0)
Late Castrates (n=6)	88.8	24.3 (18-32)	---	10.1 (8.8-12.1)
Boars (n=7)	90.3	18.3 (15-26)	641 (458-800)	14.2 (11.9-16.5)
Barrows (n=6)	83.6	28.0 (22-36)	---	---

As can be seen in Table 10, both testicular weight and BU gland length was significantly reduced in the immunocastrated animals of Group 4 relative to the untreated boars of Group 2, indicating that the LKT-GnRH vaccine composition was effective in reducing the levels and/or effects of serum testosterone in the vaccinated animals.

Example 15

Prediction of T-cell Epitopes in the Recombinant LKT 352 and LKT 111 Molecules

In order to predict potential T-cell epitopes in the leukotoxin polypeptide sequences employed in the LKT-GnRH chimeras of the present invention, the method proposed by Margalit and co-workers (Margalit et al., *J. Immunol* (1987) 138:2213) was performed on the amino acid sequence corresponding to numbers 1 through 199 of the LKT molecule as depicted in Table 11. Under the subject method, the amino acid sequence of the leukotoxin polypeptide sequence was compared to other sequences known to induce a T-cell response and to patterns of types of amino acids which are believed to be required for a T-

cell epitope. The results of the comparison are depicted in Table 11.

As can be seen by the predictive results thus obtained, there are several short sequences in the leukotoxin peptide which are identified as potential T-cell epitopes using the criteria suggested by Margalit et al (supra). More particularly, 9 sequences were identified as having a (Charged/Gly - Hydrophobic - Hydrophobic - Polar/Gly) sequence (indicated as pattern "1" in Table 11), and 3 sequences were identified as having a (Charged/Gly - Hydrophobic - Hydrophobic - Hydrophobic/Pro - Polar/Gly) sequence (indicated as pattern "2" in Table 11). By coupling these data with the *in vivo* anti-GnRH activity produced by both the LKT 352 and the LKT 111 carrier systems in Examples 7 and 8 above, it is indicated that critical T-cell epitopes are retained in the shortened LKT 111 molecule, and that those epitopes are likely contained within the N-terminal portion of the LKT 352 and LKT 111 molecules.

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Table 11
LKT Sequence Patterns Corresponding
To Potential T-cell Epitopes

5	<u>LKT Amino Acid Sequences Showing Pattern "1":</u>	
	GTID	(aa's 27-30)
	GITG	(aa's 66-69)
	GVIS	(aa's 69-72)
	HVAN	(aa's 85-88)
10	KIVE	(aa's 93-96)
	DLAG	(aa's 152-155)
	KVLS	(aa's 162-165)
	DAFE	(aa's 171-174)
	KLVL	(aa's 183-186)
15	GIID	(aa's 192-195)

LKT Amino Acid Sequence Showing Pattern "2":

	RYLAN	(aa's 114-118)
20	KFLLN	(aa's 124-128)
	KAYVD	(aa's 167-171)

Example 16

25 Prediction of the Physical Structure
of LKT-GnRH Fusion Proteins Obtained From pCB122

In order to predict the physical structure of the B-cell epitopes of the 8 copy GnRH::LKT 111::8 copy GnRH fusion molecules obtained from the pCB122 construct, the pCB122 amino acid sequence (depicted in Figures 9-1 through 9-6) was analyzed using previously described methods for determining physical protein structure. Rost et al. (1993) *J. Mol. Biol.* 232:584-599, Rost et al. (1994) *Proteins* 19:55-72, and Rost et al. (1994) *Proteins* 20:216-226. In particular, the prediction was performed by a system of neural

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networks where the input data consisted of a multiple sequence alignment. The network analysis was performed using the program MaxHom (Sander et al. (1991) *Proteins* 9:56-68, where training for the residue solvent accessibility was taken from Kabsch et al. (1983) *Biopolymers* 22:2577-2637. The neural network analysis assessed each amino acid in the pCB122 sequence, and predicted if the residue would be present as a loop, helix or exposed structure. In the prediction, the 8 copies of GnRH at the amino terminal of the pCB122 molecule were predicted to exist mainly as a loop structure, while the 8 copies of GnRH at the carboxyl terminal have a mixture of predicted structures (loop, helix and exposed residue).

These data suggest that the enhanced immunogenicity observed with the 8 copy GnRH::LKT 111::8 copy GnRH fusion molecules obtained from the pCB122 construct may be related to the different three-dimensional structures of the GnRH antigens in the molecule.

D. Industrial Applicability

The leukotoxin-GnRH chimeras of the present invention are of use in providing immunogens that, when administered to a vertebrate host, serve to immunize the host against endogenous GnRH, which in turn acts to inhibit the reproductive function or capability of the host.

Notwithstanding the specific uses exemplified in this specification, the novel chimeric molecules disclosed herein provide a means for obtaining fusion proteins comprising more than one GnRH polypeptide, occurring in either multiple or tandem repeats, which are fused to immunogenic epitopes supplied by the leukotoxin polypeptide portion of the molecule (and in some cases by spacer

peptide sequences occurring between selected GnRH sequences). The subject chimeric proteins constructed under the present invention provide enhanced immunogenicity to the fused GnRH peptide sequences, allowing an immunized vertebrate host to mount an effective immune response toward endogenous GnRH; effecting an interruption in the synthesis and release of the two gonadotropic hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) and rendering the host temporarily sterile. In this manner, the novel leukotoxin-GnRH constructs may be employed in immunosterilization vaccines to provide an alternative to invasive sterilization procedures currently practiced in domestic and farm animal husbandry.

The leukotoxin-GnRH fusion molecules can also be used to reduce the incidence of mammary tumors in mammalian subjects using vaccines comprising those molecules to block ovarian functions such as the production of the ovarian hormones estrogen and progesterone. In much the same manner, immunologically-sterilized canine and feline subjects will not develop pyometra (infection of the uterus), since the immunized animals will not produce progesterone which predisposes to that condition.

Other contemplated uses of the instant fusion molecules include population control, for example the interruption of reproduction capabilities in wild rodent populations. In this regard, the LKT-GnRH fusion molecules may be used as an alternative to population control measures currently practiced, such as poisoning and the like. The fusion products of the instant invention may also be administered in constructs having both slow and fast release components. In this manner, the need for multiple vaccinations may be avoided. Further, since the amino

acid sequence of GnRH is highly conserved among species, a single leukotoxin-GnRH fusion vaccine product may be produced which will exhibit broad cross species effectiveness.

5 Thus, various chimeric proteins comprising leukotoxin fused to selected GnRH polypeptides have been disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made
10 without departing from the spirit and the scope of the invention as defined by the appended claims.

Deposits of Strains Useful in Practicing the Invention

15 A deposit of biologically pure cultures of the following strains was made with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. The deposits were made
20 under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for a period of
25 thirty (30) years from the date of deposit and at least five (5) years after the most recent request for the furnishing of a sample of the deposit by the depository. The organisms will be made available by the ATCC under the terms of the Budapest Treaty, which
30 assures permanent and unrestricted availability of the cultures to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12). Upon the
35 granting of a patent, all restrictions on the

availability to the public of the deposited cultures will be irrevocably removed.

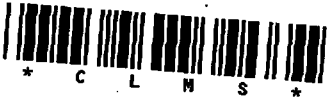
These deposits are provided merely as convenience to those of skill in the art, and are not an admission that a deposit is required under 35 USC §112. The nucleic acid sequences of these plasmids, as well as the amino acid sequences of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with the description herein. A license may be required to make, use, or sell the deposited materials, and no such license is hereby granted.

	<u>Strain No.</u>	<u>Deposit Date</u>	<u>ATCC No.</u>
15	<i>P. haemolytica</i>	February 1, 1989	53863
	serotype 1 B122		
	pAA101 in <i>E. coli</i>	February 1, 1989	67883
	JM105		
	pAA352 in <i>E. coli</i>	March 30, 1990	68283
20	W1485		
	pCB113 in <i>E. coli</i>	February 1, 1995	69749
	JM105		
	pCB111 in <i>E. coli</i>	February 1, 1995	69748
	JM105		

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Claims:

1. A chimeric protein comprising a leukotoxin polypeptide fused to first and second
5 multimers, wherein the C-terminus of the first multimer is fused to the N-terminus of the leukotoxin polypeptide and the N-terminus of the second multimer is fused to the C-terminus of the leukotoxin polypeptide, and further wherein each of said
10 multimers comprises more than one selected GnRH polypeptide.
2. The chimeric protein of claim 1 wherein the first and second GnRH multimers are different and
15 comprise molecules according to the general formula $[\text{GnRH-X-GnRH}]_n$, wherein:
GnRH comprises a GnRH polypeptide;
X is selected from the group consisting of a peptide linkage, an amino acid spacer group and a
20 leukotoxin polypeptide; and
n is an integer greater than or equal to 1.
3. The chimeric protein of claim 1 wherein the first and second GnRH multimers are the same and
25 comprise molecules according to the general formula $[\text{GnRH-X-GnRH}]_n$, wherein:
GnRH comprises a GnRH polypeptide;
X is selected from the group consisting of a peptide linkage, an amino acid spacer group and a
30 leukotoxin polypeptide; and
n is an integer greater than or equal to 1.
4. The chimeric protein of any of claims 2 or 3 wherein X is an amino acid spacer group having at
35 least one helper T-cell epitope.



5. The chimeric protein of any of claims 2 or 3 wherein n is 4.

6. The chimeric protein of claim 1 wherein the leukotoxin polypeptide lacks cytotoxic activity.

7. The chimeric protein of claim 6 wherein the leukotoxin polypeptide is LKT 352.

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8. The chimeric protein of any of claims 1-7 wherein the first multimer further comprises the amino acid sequence (Met-Ala-Thr-Val-Ile-Asp-Arg-Ser) fused to the N-terminus thereof.

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9. The chimeric protein of claim 1 comprising the amino acid sequence depicted in Figures 9-1 through 9-6, or an amino acid sequence substantially homologous and functionally equivalent thereto.

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10. A vaccine composition comprising the chimeric protein of any of claims 1-9 and a pharmaceutically acceptable vehicle.

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11. A method for presenting selected GnRH multimers to a subject comprising administering to said subject an effective amount of a vaccine composition according to claim 10.

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12. A DNA construct encoding a chimeric protein, wherein the chimeric protein comprises a leukotoxin polypeptide fused to first and second multimers wherein the C-terminus of the first multimer is fused to the N-terminus of the leukotoxin polypeptide and the N-terminus of the second multimer

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is fused to the C-terminus of the leukotoxin polypeptide, and further wherein each of said multimers comprises more than one selected GnRH polypeptide, said DNA construct comprising:

5 a first nucleotide sequence encoding the first GnRH multimer; and

 a second nucleotide sequence encoding the second GnRH multimer;

 wherein said first and second nucleotide
10 sequences are operably linked by a third nucleotide sequence encoding a leukotoxin polypeptide.

13. The DNA construct of claim 12 wherein the first and second GnRH multimers are different and
15 comprise molecules according to the general formula $[\text{GnRH-X-GnRH}]_n$, wherein:

 GnRH comprises a GnRH polypeptide;

 X is selected from the group consisting of a
peptide linkage, an amino acid spacer group and a
20 leukotoxin polypeptide; and

 n is an integer greater than or equal to 1.

14. The DNA construct of claim 12 wherein the first and second GnRH multimers are the same and
25 comprise molecules according to the general formula $[\text{GnRH-X-GnRH}]_n$, wherein:

 GnRH comprises a GnRH polypeptide;

 X is selected from the group consisting of a
peptide linkage, an amino acid spacer group and a
30 leukotoxin polypeptide; and

 n is an integer greater than or equal to 1.

15. The DNA construct of any of claims 13 or 14 wherein X is an amino acid spacer group having
35 at least one helper T-cell epitope.



16. The DNA construct of any of claims 13 or 14 wherein n is 4.

17. The DNA construct of claim 12 wherein the leukotoxin polypeptide lacks cytotoxic activity.

18. The DNA construct of claim 17 wherein the leukotoxin polypeptide is LKT 352.

19. The DNA construct of any of claims 12-18 wherein the first multimer further comprises the amino acid sequence (Met-Ala-Thr-Val-Ile-Asp-Arg-Ser) fused to the N-terminus thereof.

20. The DNA construct of claim 12 wherein the chimeric protein comprises the amino acid sequence depicted in Figures 9-1 through 9-6, or an amino acid sequence substantially homologous and functionally equivalent thereto.

21. An expression cassette comprised of:
(a) the DNA construct of any of claims 12-20; and

(b) control sequences that direct the transcription of said construct whereby said construct can be transcribed and translated in a host cell.

22. A host cell transformed with the expression cassette of claim 21.

23. A method of producing a recombinant polypeptide comprising:

(a) providing a population of host cells according to claim 22; and



(b) culturing said population of cells under conditions whereby the polypeptide encoded by said expression cassette is expressed.

5 24. A method for reducing the incidence of mammary tumors in a mammalian subject comprising administering a therapeutically effective amount of the vaccine composition of claim 10 to said subject.

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