Contraception in Domestic and Wild Animal Populations Using Zona Pellucida Immunogens

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Introduction

The human population presently exceeds 6 billion and is continuing to expand at a startling rate. This population increase has resulted in the depletion of Earth's resources, which are essential for human survival. An unfortunate consequence of this expansion has been the destruction of wildlife habitats. As these habitats have diminished, numerous problems have arisen, including conflicts between wildlife and human populations. The threat of extinction of many plant and animal species has already become a reality; other wildlife populations have increased due to reductions in predator populations. While the increase in the human population must ultimately be checked, there is a need for effective and humane methods to regulate certain animal populations as well. Another factor relevant to animal overpopulation is regional distribution. Widespread overpopulation of such animals as white-tailed deer in North America and rabbits in Australia has caused environmental as well as health problems for humans. In Asia and Africa, the populations of some wildlife species, such as elephants, have been dramatically reduced. Often these animals have frequently been relegated to small areas of land that do not have sufficient resources to sustain them. There is a vicinal distribution of elephants in Asia and Africa whose localized high populations threaten the destruction of their own restricted habitats.

The rising domestic pet population also continues to be a problem, as more than 27 million dogs and cats are impounded annually in the United States, with more than 17 million of them being euthanized (Carter 1990). The extent of domestic pet overpopulation poses ethical as well as health-related dilemmas (Flowers 1979, Carter 1990). Countless dollars are spent each year to house and dispose of impounded animals and to combat diseases transmitted by the fleas and ticks that use the dogs and cats as hosts. As these problems continue to intensify, it is apparent that a serious need exists for effective and inexpensive methods of population control.

Conventional Methods of Animal Population Control

Despite the need for effective animal population control, few methods are available which are practical or cost effective for large-scale administration. The major methods of current methods are summarized below.

Surgical Sterilization

Of the surgical sterilization methods recommended for dogs and cats, the surgical removal of the uterus and cervix (where possible) as well as the ovary (ovariohysterectomy) has several advantages over partial removal of reproductive organs.

While neutering of male animals by castration is also common, this procedure may not ultimately have a dramatic impact on population reduction in many animal populations where one virile male can mate with numerous females. It is also apparent that surgical procedures are not practical for large-scale sterilization of major populations of mammalian wildlife.

Endocrine Regulation of Fertility

Numerous steroid treatments have been tested for their ability to regulate fertility in animals, including treatment with progestogens or androgens. In female dogs, these hormones have been shown to suppress normal ovarian cyclicity. Many of the treatment regimens that include progestins have been found to promote the development of cystic endometrial hyperplasia and subsequent uterine infection, mammary development, and posttherapy lactation, while androgens may induce external masculinization. Alternatively, endocrine administration for the inhibition of implantation is possible. Because these methods have adverse effects in some animal species and because they are expensive and require regular administration, these methods are not generally considered to be practical for regulation of large populations of wildlife; for these reasons, they will not be dealt with in more detail.

Contraceptive Vaccines for Animal Fertility Regulation

The theory that immunization could be used as a method of contraception has led to numerous investigations into the development of safe and effective contraceptive vaccines. These vaccines have proposed the use of a variety of antigens as targets that would be specific for hormones, gametes, or other reproductive tissues. These vaccines use a variety of antigens as targets which would be specific for hormones, gametes, or other reproductive tissues (see reviews by Alexander et al. [1990] and Dunbar and O'Rand [1992]). This brief overview is not intended to provide a detailed outline of all research in this area.

The development of contraceptive vaccines is distinct from that of vaccines to control infectious diseases. Therefore, many of the approaches used in this area are unique. First, the contraceptive vaccines will be administered to normal, healthy individuals and not to infected or ailing individuals. Second, most traditional vaccines are directed against foreign organisms such as viruses or bacteria; contraceptive vaccines must elicit an immune response against "self" molecules that would not normally be recognized as foreign. This means that the "self" molecule to be used in the vaccine must be presented to the body in a "foreign" or "nonself" form in order for the immune system to respond effectively. These unique features have provided a significant challenge to investigators in the field as described in more detail below.

A second major factor relating to the development of contraceptive vaccines is that the immune system interacts with the male gonads in a different way than it does with the female system. It is well established that there is a blood–testis barrier in the male that partially protects the developing sperm in the testis from the immune system. The developing spermatozoa are formed at a stage long after the immune system has developed the capability to distinguish self from nonself antigens. Because ovaries initiate oocytes before birth in the human and many other animal species, the same immunological barriers are not developed. These fundamental differences have required that vaccines targeted toward the male be developed in a different manner than those for the female.

Although the ideal vaccine has yet to be formulated, research has provided a great deal of information relating to the fundamental mechanisms of hormone action, gamete interaction, fertilization, and implantation. These detailed studies have been important not only in laying the foundation for future development of contraceptive methods but also in developing a better understanding of reproductive systems of many mammalian species.

While the development of a contraceptive vaccine for humans has the criteria that it must be safe, effective, and potentially reversible, the criteria for wildlife management are different. In some animal populations, it is desired (if not required) that animals be permanently sterilized. In other populations, such as some captive animals in zoo or farms, it is desirable that the vaccine be reversible. This is an essential requirement where it is important not to deplete a potential gene pool even though there is temporary abundance of a species or where there is a temporary lack of space for housing animals. It is likely, therefore, that several strategies will be necessary to develop optimal vaccines for different animal species.

Hormone-Based Contraceptive Vaccines

To date, many of the more applied studies have been carried out using peptide hormones as immunogens. These include the peptide hormones, human chorionic gonadotrophin (hCG), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and the gonadotropin-releasing hormones (see review by Stevens [1988] and articles by Thau et al. [1987] and Mougdal [1990]). Of these potential vaccines, the most extensive studies conducted to date have been on the hCG (see reviews by Gupta and Koothan [1990] and Griffin [1991a,b]). The hCG vaccine, which is most advanced in clinical studies for human contraception, is limited to efficacy in primates and is not applicable to the majority of wildlife species. Because it interrupts

pregnancy rather than preventing it, this vaccine may not be a universally accepted alternative to contraception. Although some of these vaccines have promise for human contraception, their modes of action make them less likely to be effective for large-scale animal populations.

Gamete-Based Contraceptive Vaccines

Sperm Antigens

The onset of spermatogenesis and the appearance of mature sperm in the male reproductive tract occur during puberty at a time well beyond the establishment of immunological competence and tolerance to autoantigens (see review by Simon and Alexander [1988]). The further development of the male gamete occurs elsewhere in that it must mature in the epididymis (Dacheux et al. 1990, Cameo et al. 1990) and pass through the male reproductive tract, during which time the sperm surface antigens are modified (Isojima and Koyama 1988). Finally, the sperm are further modified in the female reproductive tract, where sperm capacitation occurs (Yanagimachi 1989, Bedford 1991). It is therefore possible to interfere with sperm function at several points in the reproductive process.

Because antibodies are produced against spermatozoa, Rosenfeld (1926) suggested that women repeatedly injected with human semen would become infertile. Since these early investigations, numerous studies by many laboratories have concentrated on identifying sperm antigens that would be (1) specific for sperm and not cross-react with any other cell types so they would not elicit antibodies with deleterious side effects on other tissues, and (2) present on the sperm surface or involved in the fertilization process so that antibodies would be able to inhibit fertility. These studies have been reviewed in detail elsewhere (O'Rand and Fisher 1988, Simon and Alexander 1988). Again, although these vaccines hold great promise for human vaccine development, they may not be practical for large-scale animal populations unless temporary infertility is desired and continuous and long-term antibodies can be sustained.

Oocyte and Zona Pellucida (ZP) Antigens

Studies on the identification of oocyte-specific antigens have been less numerous than those of sperm antigens for the simple reason that large numbers of mammalian eggs are not readily available to carry out many of the biochemical studies necessary for such research. The development of procedures to isolate large numbers of oocytes with their surrounding egg coat, the zona pellucida (ZP), has made it possible to study the study the glycoprotein antigens of the ZP in great detail (see review by Timmons and Dunbar [1988]).

The antigens of the ZP have long been considered to be an attractive target for immunological contraceptive or sterilization vaccines for several reasons. For human contraceptive vaccines, antibodies directed against the ZP antigens expressed at later stages of oocyte development should inhibit fertilization (i.e., this would be a nonabortive method). Alternatively, if antibodies are directed against ZP antigens expressed early in oocyte growth and follicular development, permanent sterilization may result because all oocytes are ultimately destroyed and no steroids will be produced. This latter method would be preferable for use in animal sterilization.

Because there are a limited number of glycoproteins associated with the ZP, the major proteins of several species have been studied in great detail. To date, three major glycoproteins have been identified in most species (see review by Timmons and Dunbar [1988]). Many studies have demonstrated that both the immunogenicity and antigenicity of the ZP glycoproteins is extremely complex (see discussion below on general aspects of immunogenicity and antigenicity). Antibodies can be identified which recognize amino acid and carbohydrate epitopes as well as conformational or structural epitopes of the ZP (Maresh and Dunbar 1987). Further, it is not possible to predict the immune response of a given animal when immunized with ZP antigens of a different species. Because isoimmunization with the ZP glycoproteins of the same species does not elicit a significant response, it has become apparent that immunogenicity of ZP is primarily

due to the foreign epitopes associated with the ZP of different species. These observations have further been demonstrated by the necessity to conjugate the A mouse peptide to the foreign molecule, keyhole limpet hemocyanin, to elicit an immune response. The distinct species differences in the antigens of ZP of different mammalian species and the need to define antigenic epitopes which will elicit antibodies that prevent fertilization but do not alter ovarian function foster the necessity to dissect the antigenic domains of the ZP glycoproteins.

Studies have also demonstrated that immunization with some ZP antigens can elicit an immune response which interferes with development of ovarian follicles (Skinner et al. 1984, Dunbar et al. 1989, Rhim et al. 1992, and Lee and Dunbar 1992). These effects vary among different mammals and may mimic such clinical conditions as premature ovarian failure, and polycystic ovarian disease. In the development of animal sterilization vaccines, it is preferable to eliminate growing oocytes along with the hormone-secreting granulosa and theca cells, which are responsible for estrous behavior. These side effects are not acceptable for the development of a human contraceptive vaccine.

In view of these observations and the need to develop different vaccines which may cause temporary or permanent infertility, it has been necessary to study the formation and development of the ZP during ovarian follicular development. These studies have demonstrated that the ZP proteins are produced in follicle stage-specific sequence (Wolgemuth et al. 1984, Lee and Dunbar 1992) and that the distinct ZP antigens are synthesized and secreted at different stages of development. It should therefore be possible to identify distinct antigenic epitopes that are associated with the ZP matrix following the differentiation of the steroid-producing granulosa cells.

Molecular biology techniques have allowed for the further study of ZP proteins' antigenic domains. Initial studies of molecular cloning and characterization of complimentary deoxyribonucleic acids (cDNA's) encoding the ZP glycoproteins of mouse and rabbit have been described (Ringuette et al. 1986, Liang et al. 1990, and Schwoebel et al. 1991). These studies have demonstrated the rabbit ZP 55 Kd antigen has a distinct amino acid sequence (Schwoebel et al. 1991) from the two sequences of mouse ZP proteins (Ringuette et al. 1986, Liang et al. 1990). Furthermore, the 55 Kd protein does not appear to be present in the mouse genome. A rabbit 75 Kd ZP protein has demonstrated significant similarity (70 percent) with the amino acid sequence of the mouse ZP2 protein, and cDNA isolated against the 55 Kd rabbit ZP protein has demonstrated that the pig has a homologue of this rabbit protein.

The complete amino acid sequences of ZP proteins for several species will ultimately be needed to determine their species similarities and determine vaccine efficacy. Results of these studies could explain why immunization of mice or rats with the ZP of pig ZP antigens does not cause infertility (Drell et al. 1984, Sacco et al. 1981), while immunization of other mammalian species including nonhuman primates with pig ZP proteins effectively reduces fertility (Drell et al. 1984, Skinner et al. 1984 and 1989, Dunbar et al. 1989).

Recently, studies have been carried out using the expression of ZP proteins produced using recombinant DNA techniques to begin to define specific epitopes that may be effectively used to develop a contraceptive vaccine. This vaccine would inhibit fertilization but not affect the early stages of ovarian follicular development (Schwoebel et al. 1991). These studies are the first to demonstrate that ZP proteins produced using bacterially expressed recombinant DNA techniques can elicit in monkeys antibodies that recognize the native ZP antigen. Furthermore, cynomolgus monkeys immunized with 55 Kd rabbit ZP protein show no alteration of ovarian follicular development, but antibodies to this protein inhibit monkey sperm from binding to monkey ZP. This is in contrast to monkeys immunized with a portion of the rabbit 75 Kd rabbit ZP protein, which does alter ovarian follicular development. Thus, antigenic domains of ZP proteins can also be dissected to define which ZP proteins will elicit specific antibodies that either (1) inhibit sperm binding to the ZP without affecting ovarian follicular development or (2) reduce the number of ovarian follicles and ovulations.

It is apparent that many studies need to be carried out to define specific ZP antigens which can effectively produce an immune response that results in reversible infertility or permanent sterilization. The advances in researchers' understanding of the development of the ovary, as well as the advances in molecular cloning techniques should allow investigators to identity specific antigens rapidly.

Trophoblast Antigens

The principal precursor of the fetal placenta is the trophoblast, which is composed of a group of cells that surround the developing embryo at the blastocyst stage. Research to identify and characterize specific trophoblast antigens has been important because they have provided the basis for studying reproductive failure. The research into the structure and function of these antigens is important in studies on the maternal–fetal interactions that can be used to devise strategies for improving reproductive success (see review by Faulk and Hunt [1990]). Although the antigens of the trophoblast could be used as targets for immunocontraception, the probability that many of antibodies directed against these antigens would result in abortive mechanism has made the development of this type of vaccine more controversial.

Major Goals and Hurdles in Contraceptive Vaccine Development

Large-Scale Production of Vaccinogens

To date, development of protein-based contraceptive vaccines, particularly those using gamete antigens, has been hampered by the inability to purify sufficient quantities of protein. However, rapid advances in recombinant DNA technology and genetic engineering have now made it possible to generate sufficient quantities of any target protein antigen (see review by Chin [1990]). The development of vectors designed for vaccine delivery systems using virus expression systems to express large proteins—such as the vaccinia virus that has been devised for rabies vaccination of wild animals—may also prove useful.

Optimizing Immune Enhancement for Development of Contraceptive Vaccines

The successful development of contraceptive vaccines, like other vaccines, will ultimately depend on the production of an immune response which is sufficient to elicit antibodies which will neutralize either the hormone or specific gamete antigens (see review by Woodrow and Levine [1990]). It is therefore necessary to understand the potential problems in generating a desired immune response using such vaccines. In general, *antigens* contain multiple regions (antigenic determinants) that are capable of being recognized by an antibody. These antigenic determinants may be associated with almost any molecular structure and may be associated with the peptide backbone of a protein or a carbohydrate of structure conformation. *Immunogens* have been defined as those chemical substances that are capable of inducing a specific immune response (see general discussions by Benaceraf and Unanue [1979] and Berzofsky and Berkower [1989]). Many molecules that are antigenic are not always immunogenic. In general, immunogenicity can often be achieved by covalently attaching defined molecules to a larger molecule called a carrier. Although many such studies have been carried out to study the immune response experimentally, fewer studies have been carried out to define specific molecules that can be effectively used in vaccines.

Another critical factor in the development of vaccines has been the identification of molecules that enhance the immune response. The use of *adjuvants,* which are agents that potentiate the immune response, has therefore become common. The term "adjuvant" has been defined as an agent that augments a specific immune response to antigens (Allison and Byars 1990). The adjuvant most commonly used is Freund's complete adjuvant, which contains a bacteria suspension in an oil vehicle. When it is inoculated with other specific antigens, this adjuvant induces a heightened immune response. Although Freund's is useful as an enhancing agent, it may also cause a variety of adverse reactions and is therefore not generally acceptable for use in clinical trials.

Recent studies using highly purified antigens alone have demonstrated the need for adequate adjuvants that can be used to enhance the immunogenicity molecules that are to be used in vaccines. In response, a variety of adjuvants have been introduced (see reviews by Allison and Byars [1990], Anderson and Capetola [1990], and Alam et al. [1991]), but their efficacy has yet to be proven in extensive clinical trials. It is apparent that, until such immune enhancement agents are readily available, many of the immunogens targeted for contraceptive vaccines cannot effectively be evaluated. The advances being made in other areas of vaccine development will therefore be critical for the future development of effective contraceptive vaccines.

Strategy for Development of Contraceptive Vaccines for Wildlife Populations

Based on observations that all nonrodent mammalian species tested to date have multiple ZP proteins which share antigenic determinants with other mammalian species, it is likely that the ZP proteins of elephant ZP will also contain similar antigenic determinants. It is possible to identify such antigens using as few as 20–30 zonae pellucidae using immunoblot analysis of ZP glycoproteins separated by one- and two-dimensional polyacrylamide gel electrophoresis. In this manner, it is possible to rapidly identify candidate ZP antigens. These procedures have been successful for identification of horse and deer antigens. Specific polyclonal as well as monoclonal antibodies are now available to ZP proteins of numerous mammalian species, and analyses can easily be used to define specific ZP antigens of the zonae pellucidae of different wildlife species. Furthermore, it has been possible to use cDNA's from the rabbit (Schwoebel et al. 1991) to isolate a cDNA from the pig's zonae pellucidae and to use mice ZP cDNA's to isolate human cDNA's (Chamberlain and Dean 1990, Liang and Dean 1993).

Despite the availability of cDNA sequences for ZP proteins of numerous species, it will ultimately be necessary to express sufficient quantities of proteins to use in fertility studies in target animal species. Initially, it will be essential to determine if immunization of individual species with ZP proteins will elicit a humoral immune response resulting in the production of antibodies to self ZP antigens. Secondly, it will be important to establish the stage of ovarian development during which the zonae pellucidae are formed, as well as the time of transition of oocyte recruitment from meiotic prophase to the time of ovulation. This timeframe is estimated to be 2 weeks in mice and up to 6 months in humans (Gougeon 1982). Because the time of exposure of oocytes in the developing follicle to antibodies is critical, it is important to understand the basic development of the ovary in each mammalian species for which contraceptive vaccines are being evaluated. The stage-specific expression of ZP proteins during ovarian development can easily be carried out using established immunocytochemistry methods or in situ hybridization methods. Once these studies are carried out for a target species, it should be possible to rapidly evaluate the potential for efficacy, safety, and reversibility of immunocontraception using these procedures. In some instances, small numbers of animals (e.g., elephants and some zoo animals) will need to be vaccinated for contraception or sterilization as compared to other large animal or human populations. It may be necessary, however, to identify the "species-specific" ZP antigenic domains for different target animals. Although this identification could pose a significant challenge, such species-specific vaccines will be essential if large-scale oral vaccine delivery systems are to be utilized.

Considerations for the Development of Vaccines for Wildlife Management

In order to design an effective contraceptive or sterilization vaccine for any wildlife species, it is necessary to take into consideration the long-term effects on the animal populations. For example, it may be desirable to develop methods for large-scale sterilization in animal species that are clearly not in danger of becoming extinct but whose population has become so excessive that starvation or other problems arise.

If extinction or reduction of the gene pool is, in fact, a consideration in an animal species, it will be important to consider contraception rather than sterilization. Because the zonae pellucidae are composed of distinct antigens that occur at different stages in oocyte growth and ovarian follicular development, they provide target antigens that can be developed to cause permanent sterilization instead of temporary contraception. However, the dissection of these antigenic domains can be carried out only by using advanced techniques such as recombinant DNA or peptide chemistry.

The efficacy of these methods is dependent upon the development of the ovary and the nature of the ovarian cycle, and it is important to develop a better understanding of the reproductive cycles of the females of any animal.

Summary

Development of most of the contraceptive vaccines, particularly those using gamete antigens, has been hampered by the inability to purify sufficient quantities of protein for development and use. The rapid advances in recombinant DNA technology and genetic engineering have now made it possible to generate sufficient quantities of any target protein antigen that has been identified. The use of these techniques for development of vaccines will ultimately depend on the production of an immune response sufficient to elicit antibodies that will neutralize either the hormones or the specific gamete antigens. This aspect of vaccine development is greatly dependent upon the production of more effective and safe adjuvant.

Although the "ideal" vaccine has yet to be formulated, research in this area has provided a great deal of information relating to the fundamental mechanisms of hormone action, gamete interaction, fertilization, and implantation. These detailed studies have been important not only in laying the foundation for the future development of contraceptive and sterilization methods but also in developing a better understanding of reproductive systems that may in turn shed light on reproductive dysfunction, disease, and infertility.

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