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Abstract: Immunocontraceptive technology appears to be a viable approach for population control of nuisance species of wildlife. The administration of immunocontraceptive vaccines is presently performed by syringe injection or by remote delivery via darts or biobullets. In order for immunocontraception to be successful in wide application to free-roaming animals, the vaccine must be delivered in an oral form. Recent advances in molecular biology, immunology, and pathology of mucosal infections give us tools to develop effective oral vaccines. Oral vaccines encapsulated in either biodegradable microspheres, synthetic adhesive liposomes, or nonvirulent live vectors hold promise as a practical approach for immunocontraception of free-roaming wildlife. Issues of safety, species specificity, and field application of the vaccine will need to be addressed.

Keywords: Wildlife vaccines, immunocontraception, vaccine vectors, oral vaccine delivery

A growing need for nonlethal methodology for population control of nuisance or damaging species of wildlife has fostered research in immunocontraceptive vaccine technology. Kirkpatrick et al. (1990) demonstrated that reproductive rates of feral horses can be reduced by vaccinating these animals with native porcine zona pellucida (PZP). Turner et al. (1992) demonstrated that PZP was effective as an immunocontraceptive in the white-tailed deer *(Odocoileus virginianus).* Recent advancements in immunology and molecular biology have made it possible to produce and administer genetically engineered contraceptive vaccines, thus making reproductive control a very promising alternative in wildlife management.

In a previous study by Turner and Kirkpatrick (1991), the vaccine was delivered by darting or biobullet. This remote delivery is valuable for special applications. However, in order for this technology to have wide application, one must have a mode of application that can disseminate the vaccine to a large segment of a wildlife population at a reasonable cost (Garrott et al. 1992).

The most logical means of vaccine application to free-roaming animals is by oral delivery. Oral vaccination, however, is not without its problems (Bloom 1989). Because vaccines are proteins, they need a protective mechanism to prevent digestion in the gastrointestinal tract. Baiting with vaccines should be as species specific as possible and yet be designed to reach a large proportion of the selected wildlife population. Oral delivery of immunocontraceptive vaccines is an untested area of technology that will need several years of developmental research before the first vaccines are available for entry into the registration process.

The purpose of this paper is to review the immunological concepts of vaccination and how they may apply to immunocontraception, review the current technology of oral immunization, and propose some applications for oral immunocontraception in free-roaming pest vertebrates.

Reproduction and Immunology

Mammalian and avian reproduction involves interaction of spermatozoa and oocytes contributed by the male and female, respectively. Both these gametes have unique surface glycoprotein receptors against which an immune response can be elicited. The development of these gametes and corresponding hormones is under the control of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), gonadotropins secreted from the pituitary and flowing through the bloodstream to the gonads. Secretion of the gonadotropins is in turn regulated by gonadotropin-releasing hormone (GnRH), which also has a role in sexual receptivity that is in addition to its regulation of FSH and LH release and the stimulation of ovulation. Immunocontraception involves producing antibodies against these reproductive hormones and gamete proteins that will interfere with their biological activity.

The power and efficiency of vaccines in combating infectious diseases is well recognized and accepted.

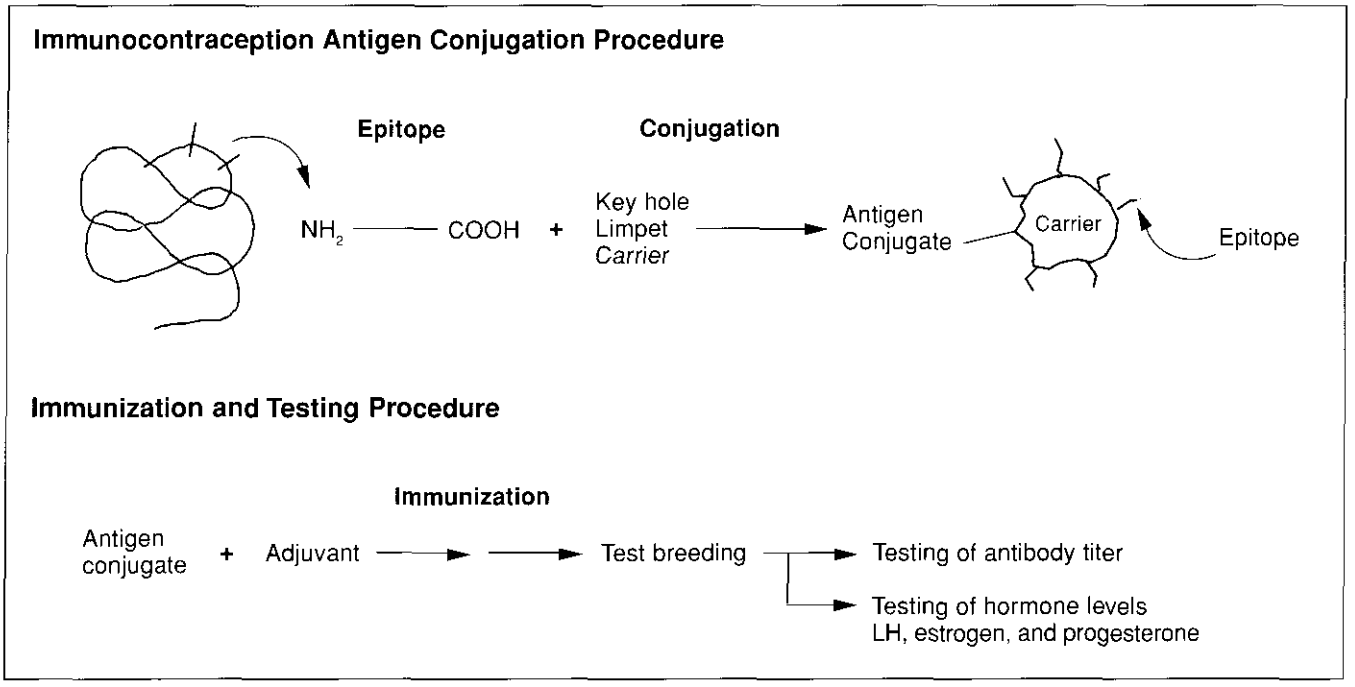

Figure 1. Reproductive "self" antigens are made "foreign" by coupling them (conjugation) to a protein foreign to the animal. Injecting the conjugate into the animal produces antibodies to the self antigen as well as the foreign protein.

The vast majority of vaccine research is concerned with the development of new and improved vaccines against viral and bacterial diseases. Antidisease vaccines are based on using immunologically foreign antigens, such as surface glycoproteins of viruses and bacteria, to stimulate the immune system to form antibodies that attack live viruses and bacteria just as they would the glycoproteins.

In order to understand the concepts of immunocontraception, one must understand how the immune system defends itself against outside organisms (Silverstein 1989). Development of infections and resulting immune responses are constantly in process because people live in a world filled with micro-organisms. Every facet of our existence brings us into contact with bacteria, fungi, viruses and a diversity of parasitic or potentially parasitic life forms. Yet we possess a rich, harmless, natural microflora on all body surfaces, within all body orifices, and throughout most of the gastrointestinal tract. Even vital digestive functions are mediated partly by the gastrointestinal flora. The body is able to differentiate normal flora and self-proteins from pathogens through a process called immune tolerance.

Antifertility vaccines are directed against self-reproductive antigens, either hormones or proteins, to which the recipient is normally immunologically tolerant (Jones 1983). These antigens are made "foreign" by coupling them to a protein foreign to the animal. The resultant vaccine induces immunity which interferes with the biological activity of that particular antigen. The result can be infertility (fig. 1).

An immunological approach to contraception is attractive because it requires only periodic vaccination. The approach is physiologically sound in the sense that antibodies induced in the target animal interfere with reproduction without the constant medication.

Immunocontraception occurs when fertility is reduced by means of antibodies attaching to and interfering with the biological activity of hormones or reproductive tract proteins. Immunization against most reproductive antigens generally gives rise to a

reversible response. Antibodies decline in the course of time, and animals regain fertility (Dunbar and Schwoebel 1988).

Systemic Vaccination

Dose amount, frequency and timing, immunogenicity of the antigen vaccine preparation, and mode of immunization all influence the immune response. The nature of the immune response required for an anti-fertility vaccine is equivalent to the response obtained by immunization. Rendering reproductive self-antigens immunogenic involves conjugating these self-molecules with foreign substances in order to break the state of tolerance associated with these molecules. These vaccines must be designed to react with macrophages (the antigen presenting cells) as well as with the two immune-processing cells (T and B). For example, the T cells receive the antigen from the macrophage and present the foreign material to the B cells. Enhancement of B-cell activity is essential to the production of high levels of antibodies as well as creation of B memory cells to that specific antigen.

Traditional immunization has always been associated with adjuvants (nonspecific immune stimulants). The most common adjuvant is Freund's complete adjuvant (FCA). This substance is a mixture of mineral oil and killed bacteria cells. Booster injection is performed with Freund's incomplete adjuvant (FIA) (minus the killed bacteria) to prevent abscesses at the injection site. The protein to be injected is dissolved in water and mixed with the oily adjuvant to form a water-in-oil emulsion. This emulsion provides a depot at the injection site allowing a slow release of the immunogen to the immune system. The optimal length of antigen presence for maximum antibody production is unknown; however, if antigen presence is too short, the antibody quantity is suboptimal. Chronic presence of antigen leads to antigen tolerance and a lack of antibody production response.

The immune system, both systemic and mucosal, seems to respond best by giving a priming dose followed in several weeks by a booster dose. A single dose produces a short-lived antibody response and does not result in a long-lasting memory response. Many times, the best response is observed when the animal is boosted several months after the original antigen exposure. Continued presence of the antigen for several weeks is important for a long-lasting immune response. Slow-release vehicles such as microspheres or liposomes can provide this effect.

The standard form of vaccination involves 50–100 μg of antigen for small animals (mice to rabbits) and 200–400 μg for larger animals. The antigen is mixed with FCA to produce a thick water-in-oil emulsion. This emulsion is injected into the animal using multiple subcutaneous, intradermal, or intramuscular sites. Booster doses use the same or slightly less antigen in FIA. When incomplete Freund's is used for boosters, abscesses at the site of injection generally do not form. Highly immunogenic antigens can produce sufficient antibody with doses of 5 to 10 μg.

Scientists at the Denver Wildlife Research Center (DWRC) have demonstrated that the hypothalamic hormone GnRH, made foreign by coupling to keyhole limpet hemocyanin (KLH), can sterilize both sexes of wild Norway rats *(Rattus norvegicus)* for up to a year. White-tailed deer immunized with a porcine glycoprotein (PZP), the zona pellucida that surrounds all mammalian oocytes, remained sterile for at least two breeding seasons.

Oral Vaccination

Mucosal Immune System

The pharyngeal and intestinal mucosae represent a major interface with the external environment and come in contact with food and products of food digestion, ingested micro-organisms, drugs, and the vast quantity of resident flora that populate the distal small intestine and colon (Mestecky 1987).

The intestine is the body's largest immunologic organ. It comprises 70–80 percent of all of the body's immunoglobulin (Ig) (antibody)-producing cells and produces more secretory Ig (SIgA) than the total production of serum Ig in the body. The primacy of the intestine in making Ig is not surprising because the

majority of infectious disease organisms are first encountered through the intestinal mucosal membranes. The main antibody produced by the mucosal immune system is SIgA. Intestinal SIgA response is of relatively short duration, lasting from 2 to 4 weeks. The SIgA system exhibits potent immunologic memory and can be repeatedly stimulated by renewed contact with antigen. Systemic IgG production may also be stimulated by oral vaccination, and the presence of IgG as a result of vaccination may be detected in serum years later. It is the serum IgG that provides the long-term interference with the biological activity of reproductive hormones and proteins.

Immune follicles, including tonsils, are located in the pharyngeal area at the entrance to both the respiratory and digestive tracts. The pharyngeal area of the throat may be considered the first line of mucosal defense and immune response. As a second line of defense, thousands of lymphoid follicles are located in the distal portion of the small intestine. Aggregates of these follicles called Peyer's patches (PP) are also found throughout the small intestine. The lumenal surface of PP are covered by an epithelium which contains a unique cell type termed the M cell (Childers et al. 1990). Intact viruses and micro-organisms and particulate antigens up to 10 μm in size are taken up by M cells for antigen delivery to the underlying lymphoid cells. This uptake of micro-organisms enhances the ability of the host to respond immunologically to a microbial challenge and fight off an infection. These antigens activate T and B cells and, along with macrophages, soon migrate out of the PP to the mesenteric lymph nodes and into the bloodstream via the thoracic duct, thereby presenting the antigen to the systemic immune system (fig. 2).

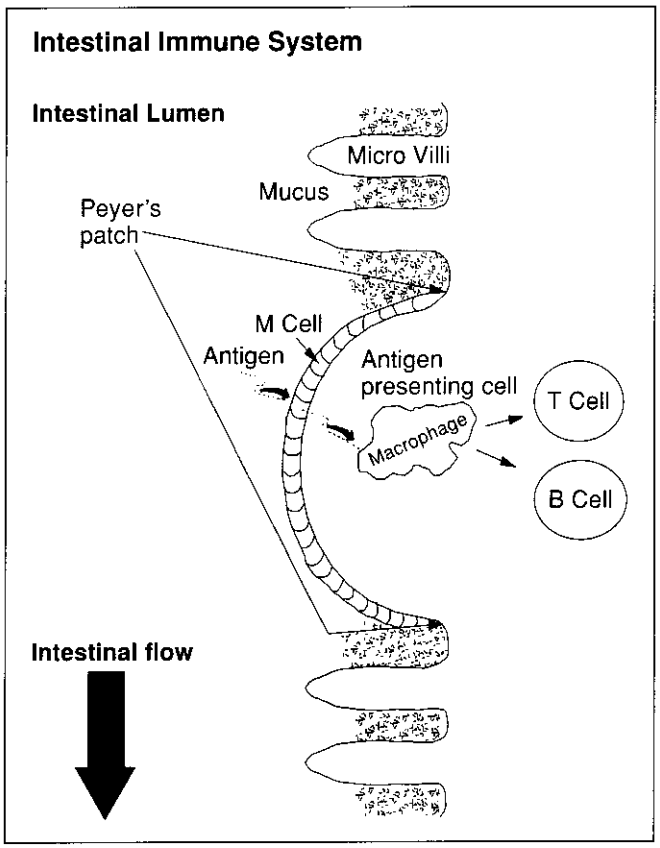

Figure 2. The small intestine contains thousands of immune follicles. Aggregates of these follicles are called Peyer's patches (PP). Their surface contains a unique cell type, the M cell, which takes up intact viruses, bacteria, and particulate antigens up to 10 μm in size. Once inside the PP, the antigens are processed by the macrophage and presented to the immune system.

Oral Delivery of Antigen to the Intestine

Many factors can influence the expression of mucosal immunity to a specific antigen. Most proteins are rather poor immunogens when given orally. This is the reason so few vaccines are currently administered by this route.

Effective mucosal immunogens appear to have certain characteristics: (1) They are not degraded in the mucosal environment (e.g., the intestine); (2) they can bind to and penetrate into the mucosal epithelium (thus allowing efficient uptake in the PP, as typified by cholera toxin (CT), one of the most effective mucosal immunogens known; and (3) they may also have adjuvant immunostimulating activity (Holmgren et al. 1992, McGhee and Kiyono 1994).

Live micro-organisms with mucosal adhesive properties are highly effective mucosal immunogens; killed and inert antigens without mucosal binding properties are poor mucosal immunogens. Most food antigens are poor mucosal immunogens because they are rapidly degraded into nonimmunogenic fragments in the mucosal environment. Food antigens generally do not bind to epithelial receptors.

As pointed out previously, immune lymphoid follicles are located in the pharyngeal area as well as distal portion of the small intestine. Most oral immunization studies use the gavage technique, which means the antigen was delivered into the stomach through a blunted needle. Lavage delivery, in which the antigen is delivered in the pharyngeal area, can stimulate the immune follicles in this area as well as in the small intestine. Delivery of unencapsulated protein antigens to the pharyngeal area may be an effective means of oral immunization since it precedes the stomach's digestive enzymes.

Enteric-coated capsules are commonly used for delivery of drugs to the small intestine. Enteric capsules are resistant to acid but are soluble in the alkaline solution of the small intestine. They provide only one-half of the formula of effective antigen delivery (i.e., protection from the stomach) because they generally cannot be made small enough to be taken up by the PP. Also, enteric-coated vaccines can get the protein past the stomach, dissolve, and release the antigen in the small intestine, but proteolytic enzymes in the small intestine may digest these proteins into nonimmunogenic peptides before they are absorbed by the immune cells. The safest way to deliver the antigen orally is to protect it until it is taken up by the PP and delivered to macrophages.

Combining two approaches—(1) enteric coating or using delivery vehicles that slow the intestinal degradation of the antigen and (2) targeting the vaccine design to attach to the immune follicles with M cell binding—could lead to an effective antigen uptake and potentiation of mucosal immune response.

The quantity of antigen used in oral immunization depends on how well the antigen is protected from degradation and how immunogenic it is. The antigen dose may vary from 12.5 µg to 1 g per dose, with larger animals receiving the larger quantities of antigen. Most studies indicate that two doses given 3–4 weeks apart are needed to produce a long-lasting immune response. Ahren et al. (1993) found that a third dose given within 3 weeks was counterproductive, probably because SIgA stimulated from the second dose interfered with the uptake of the antigen.

Two oral doses of a live salmonella vector produced IgG responses similar to the response of a systemic vaccination of the killed form of the same vaccine (Morona et al. 1994). Oral boosting after 42 days was needed for this response. A single dose or boosting after 14 days gave a much lower antibody titer.

Scientists at DWRC have demonstrated that white-tailed deer can be successfully vaccinated using a genetically engineered *Bacillus Calmette Guerin* (BCG). These bacteria were designed to deliver an outer surface protein A (Osp A) antigen onto the surface of the bacteria. A good IgG response to the Osp A antigen was demonstrated after two oral doses of bacteria. DWRC is also testing different oral immunocontraceptive vaccines in wild Norway rats.

Immune Tolerance

The constant systemic presence of antigen can induce a state of immune tolerance in which antibody production is reduced (Ernst et al. 1988). This process is probably a protective mechanism to prevent the animal from an excessive immune response. What is excessive depends on the antigen. However, gram quantities of antigen are generally considered excessive. The mucosal immune system seems to have a built-in limitation in terms of the magnitude of response to any single immunogen (oral immune tolerance). This limitation is in contrast to the systemic immune system, which responds vigorously to nonself-antigens. It would be impossible and perhaps even harmful for the intestine to mount a vigorous immune response to each of the thousand foreign antigens it encounters each day. The term "oral tolerance" is used when the animal's immune system ceases to respond to a given antigen. Oral tolerance is commonly found when a large dose of an antigen is given or when the antigen is highly immunogenic and therefore likely to cause the animal harm due to a severe immunologic reaction. An example of this second type of antigen is a bacterial surface lipoprotein, lipopolysaccharide (LPS).

Stok et al. (1994) discovered that conjugating cholera toxin (CT) to ovalbumin and revaccinating with the conjugate orally could reverse an earlier ovalbumin-induced oral immune tolerance.

Oral Vaccine Delivery Vehicles

Synthesized Vectors

Microspheres—Biodegradable microspheres have been used as a slow-release antigen-delivery system. These spheres are copolymers of DL-lactide and glycolide that are synthesized to contain trapped antigen. When these spheres are injected into the host animal, they dissolve, slowly releasing the antigen. The microsphere can be designed to deliver the antigen for from 1 week to several months, depending on the size and the polymer ratio of lactide to glycolide. In most applications, microspheres have been given systemically; however, they can be given orally (Eldridge et al. 1989 and 1990). Microspheres of 1–10 μm are taken up by the PP; however, the efficiency of the uptake is only 1–2 percent. The remaining microspheres pass out of the intestine. The microspheres taken up by the PP dissolve, releasing the antigen directly to the immune system.

Liposomes—Liposomes are spherical, artificial biological membranes made up of phospholipids and cholesterol (Alving et al. 1991). Liposomes contain lipids, chosen for their stability in the gastrointestinal tract. These lipids can protect the antigen from gastrointestinal degradation. Cholesterol in the liposome stabilizes the membrane and makes it attractive to the macrophage because of its lipophilic nature. The phospholipids in liposomes are amphipathic, i.e., they possess a hydrophilic (polar) head and hydrophobic (hydrocarbon) tail. In an aqueous medium, phospholipids exist as micelles or bilayers; the polar heads are at the outer layer due to their affinity to water (fig. 3).

Because of the nature of the membrane, the liposome mimics the microbial cell when the liposome is presented to the immune system. During the synthesis of the liposome, antigen is trapped inside, providing a protective vehicle for delivery of the protein antigen. The liposome acts as an antigen microcarrier and an adjuvant, capable of targeting the antigen directly to the PP. Liposomes have been used to deliver the antigen systemically or orally. When given orally, liposomes with a diameter less than 10 μm are preferentially taken up by the PP and may persist there for up to several weeks (Alving et al. 1991). Liposomes, especially small ones (1–2 μm), can be expected to reach the blood circulation rapidly through the intestinal lymphatics.

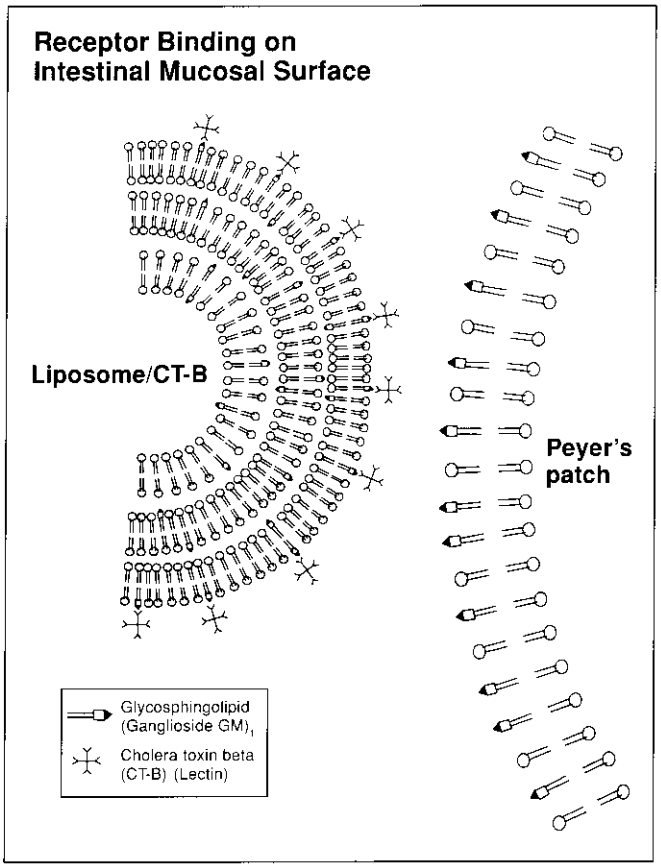

Figure 3. Liposomes are spherical artificial biological membranes. During the synthesis of the liposome, antigen is trapped inside the sphere. The result is a protective vehicle for oral delivery of the antigen. Cholera toxin B is attached to the outer surface of the liposome to provide intestinal adhesive properties. This adhesion to the intestine enhances immune response to the liposome and its antigen contents.

Liposomes Enhanced With Cholera Toxin B—Until recently, based on the relatively poor mucosal immunogenicity of soluble antigens, it was widely assumed that only live vaccines would effectively stimulate a mucosal immune response (Nedrud and Lamm 1991). Recent understanding of the mechanisms by which pathogenic viruses and bacteria colonize and infect the intestinal tract gives researchers new tools to develop successful oral vaccines. For example, a bacterium must survive the presence of the stomach's acid and proteolytic enzymes in order to infect the small intestine successfully. After surviving the stomach, the bacterium must have surface adhesive properties allowing it to adhere to and colonize the intestinal wall, resulting in an infection. Bacteria without these adhesive properties will be carried out of the gut with undigested food material.

Because of their lipophilic nature, liposomes are avidly taken up by the macrophages (Rooijen 1990). However, the liposome must bind to the mucosal surface of the intestine before it can be taken up. This mucosal adhesive property increases the mucosal uptake resulting in greater efficiency and allowing one to use a smaller oral vaccine dose. The most common liposome adhesive is the bacterial lectin CT, a member of a family of enterotoxins produced by several strains of enteropathogenic bacteria (Ahren et al. 1993, McGhee 1992, Mestecky and McGhee 1989). Lectins have multiple binding sites and can bind to receptors on the liposome as well as to intestinal receptors.

CT consists of two subunits—alpha (CTA), which has the toxic properties, and beta (CTB), which has the adhesive or mucosal binding properties. CTB bound to the liposome provides the adhesive properties without the toxicity associated with CT. The CTB bound to the ganglioside GM_1 receptor inserted in the liposome also binds to the ganglioside GM_1 receptors present on the surface of intestinal epithelial cells, thus providing the binding activity needed for mucosal antigens (fig. 3).

Heat-labile toxin (LTB) from pathogenic *Escherichia coli* bacteria represents another adhesive lectin that can be attached to liposomes to provide an intestinal mucosal binding.

Live Vectors

The common forms of existing vaccines are killed bacteria or modified live viruses that, when injected into the host animal, produce immunity by producing antibodies against surface proteins of these organisms. New techniques in molecular biology have introduced the concept of delivering the vaccine surface proteins in harmless live bacteria or viruses that act as a delivery system and therefore are called vectors. Vectors can be used to deliver the vaccine proteins systemically or orally. Vectors that are effective orally must have the ability to attach or adhere to mucosal surfaces. After attachment, these vectors are taken in by the mucosal immune system and thereby deliver the vaccine proteins directly to the immune system. Nonattaching vectors would be carried out of the intestine with the food bulk.

The ideal immunocontraceptive vaccine should be species specific; however, at the present time, species specificity is difficult to achieve. Live vectors can help provide species specificity by employing species-specific viruses or bacteria, such as swinepox, which was used to develop a vaccine carrier for the control of the feral hog.

Viral Vectors—The DNA representing several vaccines has been inserted into harmless viruses. The inserted DNA synthesizes the vaccine protein as the virus multiplies in the host animal, thereby vaccinating the animal. The most noted viral vector has been the vaccinia virus a member of the poxvirus family (Moss 1991). This virus has been genetically engineered to deliver a rabies vaccine. Given orally, the harmless vaccinia virus multiplies in the body and synthesizes a surface rabies protein. Antibodies produced against this protein protect the animal against a future rabies virus infection. This viral construct has been used successfully in eliminating most of the rabies in foxes and raccoons in Europe (U.S. Department of Agriculture, Animal and Plant Health Inspection Service 1991). The viral vectors can also be designed to contain immunocontraceptive proteins (Morell 1993).

Bacterial Vectors—As in viral vectors, bacteria can be genetically rendered harmless (nonpathogenic) and have immunocontraceptive vaccine DNA inserted into

them. This recombinant bacteria can deliver a immunocontraceptive protein, coded by the inserted DNA, to an animal host. The two bacterial vectors in use today are an attenuated BCG and a double gene-deleted *Salmonella typhi* bacillus. Both bacteria vectors are considered safe and have been used in many vaccine delivery applications. *S. typhi* strains, with deletion of two genes, are avirulent in animals, birds, and humans. These strains retain the intestinal adherence property found in unmodified *Salmonella* spp. and are absorbed by the intestinal immune cells. It appears to be safe and effective as a live vector for oral delivery of immunocontraceptive vaccines.

Morona et al. (1994) found that two oral doses of a live *Salmonella* construct elicited serum IgG responses that were comparable to intramuscular vaccination with formalin-killed *Salmonella*. Therefore, it appears that—even with live vectors—one needs at least two presentations of the antigen.

Live bacterial and viral vectors would be more economical to produce than the synthetic vectors; however, the public acceptance and safety issues have to be addressed.

Field Applications of Oral Immunocontraceptive Vaccines

Oral immunocontraceptive protein vaccines are untested. The protein vaccine must be mixed into liquid or solid baits that require some protection from the environment for at least several weeks. The bait must be attractive to a large segment of the target animal population. Present vaccine designs would require baiting an animal population twice about one month apart. Vaccine application should start about 2 months before the start of the breeding season. If the vaccine itself is not species specific, the delivery system should be. Problems of multiple visits to the bait and repeat baiting of dominant animals need to be understood in the practical application of population control. With the exception of the vaccinia virus rabies vaccine, safe use of recombinant bacterial and viral vectors has yet to be proven in a field application.

Summary

Immunocontraceptive vaccines delivered by injection or by darting have been shown to be a viable technique in preventing conception when used in confined or limited field applications. However, in order for immunocontraception to have widespread success against free-roaming animal populations, the vaccine must be delivered in an oral form in a designed bait. Because oral vaccines are proteins, they are subject to digestion by stomach gastric contents; therefore, the oral vaccine must be protected by some form of encapsulation. Inconsistent antibody responses to multiple oral doses may be due to the presence of intestinal IgA antibodies, which may prevent uptake of the antigen by the intestinal immune system.

Recent understanding of the mechanisms by which pathogenic viruses and bacteria colonize and infect the intestinal tract give us new tools to develop successful oral vaccines. Synthesized vectors, such as biodegradable microspheres and liposomes, can protect the protein vaccines and deliver them to the mucosal immune cells. Liposomes can be designed to contain lectin receptors that mimic the adhesive properties of intestinal pathogens, thereby enhancing their mucosal uptake and immunogenic properties.

Understanding the molecular genetics of oral pathogenic bacteria and viruses allows one to attenuate these virulent strains and insert the DNA of the vaccine to be expressed. Because these vaccine proteins are "self," they need to be linked to the DNA of a more immunogenic protein and be expressed together by the live vector. The use of these attenuated live vectors to deliver immunocontraceptive vaccines can provide economical vaccines of a consistent nature. These new tools should provide the basis for successful oral immunocontraceptive vaccines of the future. Successful field application of these vaccines needs careful study and is yet to be attempted.

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