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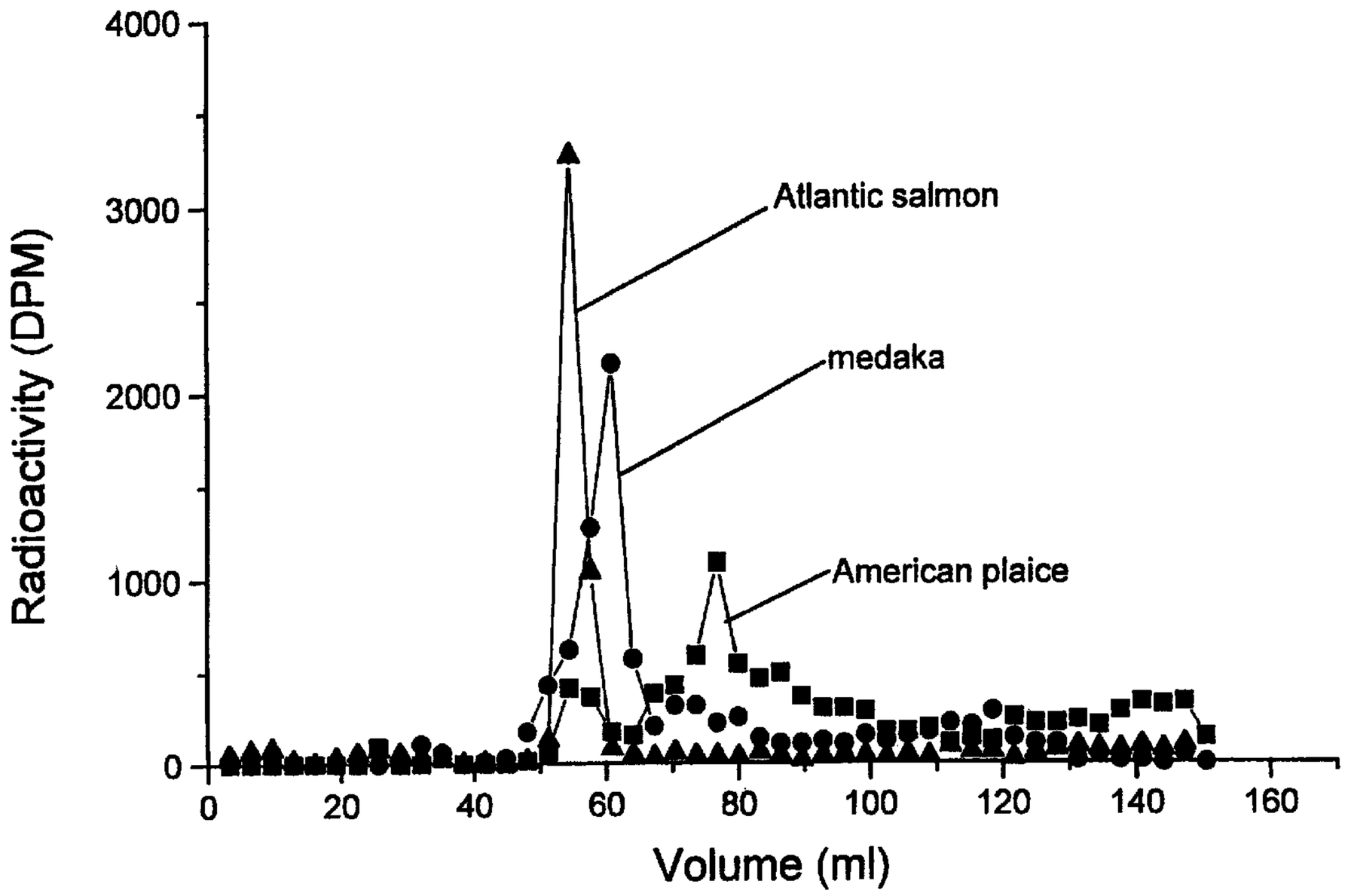

(57) Abrégé/Abstract:
Disclosed is an immunocontraceptive vaccine composition comprising a teleost homolog of zona pellucida (TH-ZP), together with a pharmaceutically acceptable diluent or carrier, for reducing or preventing fertilization in a fish, and a method for its use. Also disclosed is an immunocontraceptive vaccine composition comprising an antigen from an inner perivitelline layer (IPVL), together with a pharmaceutically acceptable diluent or carrier, for reducing or preventing fertilization in a bird, and a method for its use.

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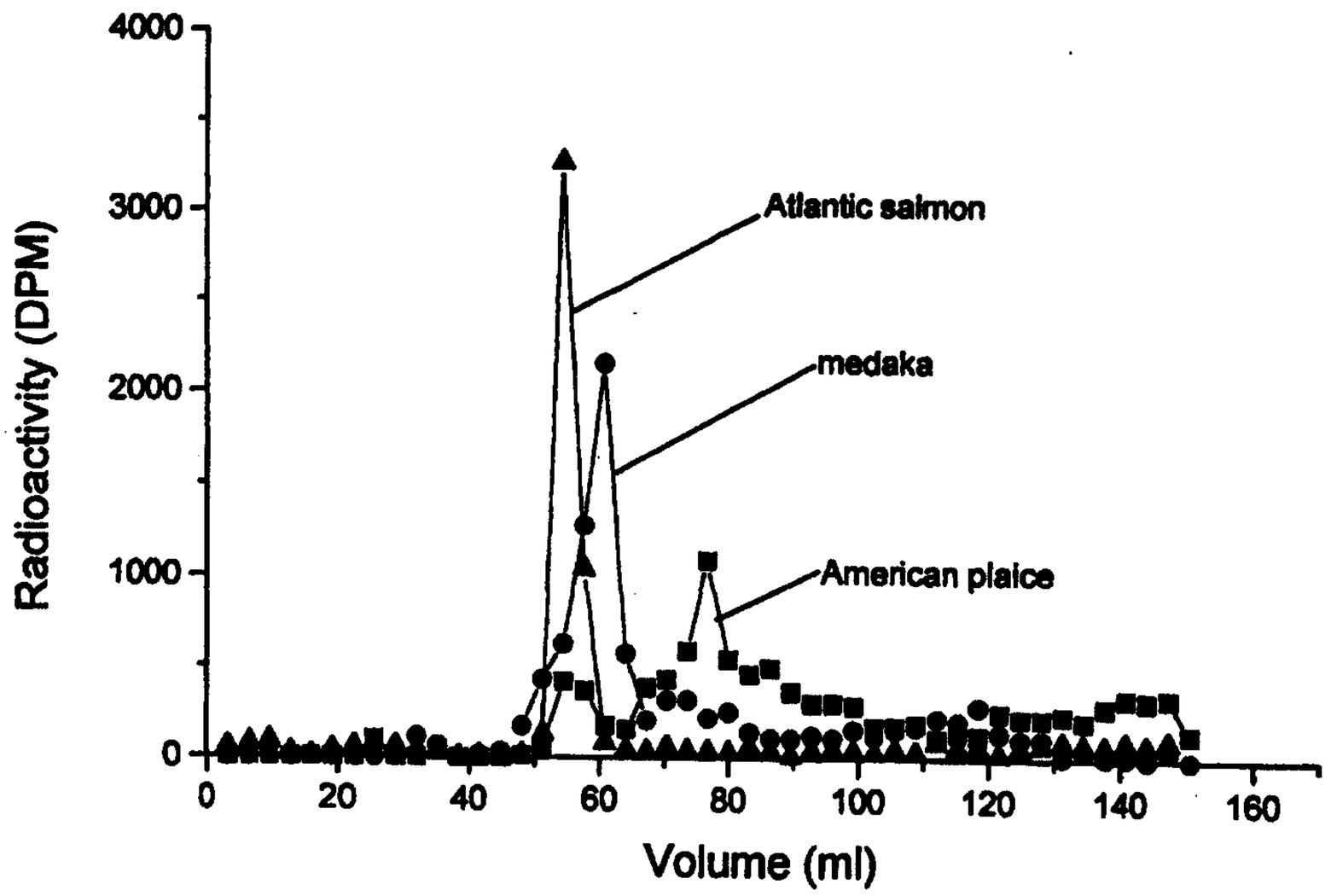

(57) Abstract

Disclosed is an immunocontraceptive vaccine composition comprising a teleost homolog of zona pellucida (TH–ZP), together with a pharmaceutically acceptable diluent or carrier, for reducing or preventing fertilization in a fish, and a method for its use. Also disclosed is an immunocontraceptive vaccine composition comprising an antigen from an inner perivitelline layer (IPVL), together with a pharmaceutically acceptable diluent or carrier, for reducing or preventing fertilization in a bird, and a method for its use.

COMPOSITIONS AND METHODS FOR REDUCING OR PREVENTING FERTILIZATION IN FISH AND BIRDS

FIELD OF THE INVENTION

The present invention relates to a vaccine composition for the immunocontraception of fish. The present invention also relates to a vaccine composition for the immunocontraception of birds.

BACKGROUND OF THE INVENTION

Among vertebrates, mating strategies involve behaviour, gamete structure and the specificity of recognition of sperm and egg. Mammalian oocytes are surrounded by an envelope called the zona pellucida that is composed of three glycoproteins in a ratio of 1:2:2 denoted by ZPA, ZPB, and ZPC (Harris,J.D., Hibler,D.W., Fontenot,G.K., Hsu,K.T., Yurewicz,E.C. and Sacco,A.G.(1994) "Cloning and characterization of zona pellucida genes and cDNA's from a variety of mammalian species : the ZPA, ZPB and ZPC gene families DNA sequence." J. Sequencing and Mapping 4:361-393). The zona pellucida contains species-specific sperm receptors composed mainly of O-terminal oligosaccharides. Fish eggs have a teleost equivalent of mammalian zona pellucida wherein the carbohydrate moiety has some structural similarity to the carbohydrate moiety of mammalian zona pellucida (Taguchi,T., Seko,A., Kitajima,K., Muko,Y., Inoue,S., Knoo,K-H., Morris,H.R., Dell,A. and Inoue,Y.(1994) "Structural studies of a novel type of pentaantennary large glycan unit in the fertilization-associated carbohydrate-rich glycopeptide isolated from the fertilized eggs of *Oryzias latipes*." J.Biol.Chem 269:8762-8771).

Mouse ZP2 (ZPA) contains a 241-amino acid domain at the C-terminus with 28% identity with a white flounder teleost egg protein (Lyons,C.E., Payette,K.L., Price,J.L. and

Huang,R.C.C.(1993) "Expression and structural analysis of a teleost homolog of a mammalian zona pellucida gene." J.Biol.Chem.268:21351-21358). A 348-amino acid sequence of mouse ZP1 (ZPB) is 47% similar (32% identical) to that of mouse ZP2 (ZPA) suggesting that this protein domain has been duplicated in mammals (Epifano,O., Liang,L-F. and Dean,J. (1996) "Mouse ZP1 encodes a zona pellucida protein homologous to egg envelope proteins in mammals and fish." J.Biol.Chem.270: 27254-27258). A smaller region of this sequence (275 amino acids) is 52% similar (36% identical) with a white flounder egg envelope protein that contains 509 amino acids.

Immunization of grey seals with a single administration vaccine containing soluble zona pellucida antigens encapsulated in liposomes has been shown to reduce female fertility by at least 90% for up to at least six years (Brown,R.G., Kimmins,W.C., Mezei,M., Parsons,J.L., Pohajdak,B. and Bowen,W.D. (1996) "Birth control for grey seals." Nature 379:30-31; Brown,R.G., Bowen,W.D., Eddington,J.D., Kimmins,W.C., Mezei,M., Parsons,J.L., and Pohajdak,B.(1997) "Evidence for a long-lasting single administration contraceptive vaccine in wild grey seals." J.Reproduct.Immunol. 35:43-51; and US Patent No. 5,736,141). The same vaccine prevented pregnancy in four rabbits (proven breeders) following 8 matings (unpublished observations).

An example of the use of liposome encapsulation of denatured recombinantly produced protein to raise antibodies against a native protein was shown with *Neisseria meningitidis* outer membrane protein P1. (Muttilainen,S., Idanpaan-Heikkila,I., Wahlstrom,E., Nurminen,M., Makela,P.H. and Sarvas,M. (1995) "The *Neisseria meningitidis* outer membrane protein P1 produced in *Bacillus subtilis* and reconstituted into phospholipid vesicles elicits antibodies to native P1 epitopes." Microb.Pathog.18:423-436).

Specificity of recognition of sperm and egg is

essential in any species. However, the mechanism of fertilization varies widely, both physiologically and biochemically, between species. Fertilization in fish differs from that in mammals in that most teleostean fish spermatozoa lack an acrosomal structure. Penetration by a spermatozoon of the fish egg envelope occurs via a discrete micropyle with closure of the micropyle after penetration of the first spermatozoon.

Sperm-egg interaction in birds is significantly different from that in mammals and different again from fish. In birds, sperm-egg recognition is initiated by the binding of spermatozoa to the inner perivitelline layer (IPVL), a proteinaceous structure surrounding the avian ovum (Bakst,M.R. and Howarth,B. (1977) "Hydrolysis of hens perivitelline layer by cock sperm *in vitro*." Biol. Reproduct. 17:370-379). There is no block to polyspermy in avian species but a further proteinaceous layer, the outer perivitelline layer (OPVL), is laid down about 15 minutes after the IPVL in chickens and appears to prevent further penetration of sperm. Therefore, if spermatozoa can be prevented from entering the avian egg between the laying down of the IPVL and OPVL, by antibodies directed against the IPVL, then immunocontraception would be realized.

There is some similarity between reproduction in mammals and fish but also many differences. Unlike the C-terminus, the N-terminus domain of white flounder egg protein is quite dissimilar to mouse ZP2 (ZPA) and a transmembrane domain characteristic of all mammalian zona pellucida proteins is not present in teleost egg protein indicating the divergence of these species 650 million years ago (Epifano,O., Liang,L-F. and Dean,J. (1996) "Mouse ZP1 encodes a zona pellucida protein homologous to egg envelope proteins in mammals and fish." J.Biol.Chem.270: 27254-27258).

The carbohydrate moiety of teleost egg glycoproteins

is also dissimilar, for example, rainbow trout egg envelope glycoprotein has a unique N-linked glycan containing KDN (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) in the second layer of the vitelline envelope (Tezuka,T., Taguchi,T., Kanamori,A., Muto,Y., Kitajima,K., Inoue,Y. and Inoue,S. (1994) "Identification and structural determination of the KDN-containing N-linked glycan chains consisting of bi- and triantennary complex-type units of KDN-glycoprotein previously isolated from rainbow trout vitelline envelopes." Biochem.33:6495-6502). This KDN-glycoprotein is exposed to the outer surface around the micropyle through which sperm enter the egg at fertilization. Most fish sperm lack an acrosome and penetrate the fish egg envelope via a discrete micropyle. The micropyle forms a guidance system in teleost fertilization that enhances sperm penetration (Amanze,D. and Iyengar,A. (1990) "The micropyle: a sperm guidance system in teleost fertilization." Development 109:495-500). A chemical attractant may also emanate from the micropyle to enhance the chance of fertilization.

In spite of the significant structural differences between fish egg envelope protein and mammalian zona pellucida, fish egg envelope proteins have been designated the teleost homolog of zona pellucida (TH-ZP for convenience of reference). In fish, TH-ZP3 is made in the liver and transported via the blood to the ovary, while TH-ZP2 is made in the ovary (Hamazaki,T.S., Nagahama,Y. and Yamagami,K. (1989) "A glycoprotein from liver constitutes the inner layer of the egg envelope (zona pellucida interna) of the fish, *Oryzias latipes*." Dev.Biol.133:101-110; Murata,K., Sasaki,T., Yasumasu,S., Iuchi,I., Enami,J., Yasumasu,I. and Yamagami,K. (1995) "Cloning of cDNAs for the precursor protein of a low-molecular weight subunit of the inner layer of the egg envelope (chorion) of the fish *Oryzias latipes*."; Chang,Y.S., Wang,S.C., Tsao,C.C. and Huang,F.L. (1996) "Molecular cloning,

structural analysis and expression of carp ZP3 gene." Mol.Reprod.Dev.44:295-304; Murata,K., Sugiyama,H., Yasumasu,S., Iuchi,I., Yasumasu,I. and Yamagami,K. (1997) "Cloning of cDNA and estrogen-induced hepatic gene expression for chorigenin H, a precursor protein of the fish egg envelope (chorion)." Proc.Natl.Acad.Sci. USA 94:2050-2055; Chang,Y.S., Hsu,C.C., Wang,S.C., Tsao,C.C. and Huang,F.L. (1997) "Molecular cloning, structural analysis and expression of carp ZP2 gene." Mol.Reprod.Dev.46:258-67).

It is undesirable that transgenic fish escape from fish farms and mate with fish in the wild. This problem would be reduced if females were sterile. Such sterile fish could also redirect their food reserves to increase their body size rather than roe production. Triploid fish are sterile but triploid salmon grow poorly (MacKenzie, D. (1996) "Can we make supersalmon safe?" New Scientist pp 14-15). Triploidy can be induced in fish by a pulse of pressure that prevents embryos from ejecting one set of chromosomes.

With respect to birds, population control of certain species is of great environmental importance. For example, some Canada geese (*Branta canadensis*) populations in the USA, Canada and Europe have increased to a point that threatens other bird populations and are a nuisance to the enjoyment of parks, golf courses, etc. Burgeoning populations of snow geese (*Chen caerulescens*) are wreaking havoc on precious tundra habitat (Struzik,E. (1998) "The snow geese dilemma." Equinox 97:50-57) and have resulted in compensation claims in Quebec, Canada alone of $844,000 in 1996. Some tundra habitats have been described as 35% overgrazed, 35% damaged and 30% destroyed by snow geese. In addition, many populations of small birds such as pigeons (*Columba livia*) and starlings (*Sturnus vulgaris*) cause significant economic loss in many parts of the world. As a consequence, there is need for management of some bird populations.

SUMMARY OF THE INVENTION

The present invention provides a single administration immunocontraceptive for fish.

More specifically, the present invention provides an immunocontraceptive vaccine composition comprising a teleost homolog of zona pellucida (TH-ZP), together with a pharmaceutically acceptable diluent or carrier, for preventing fertilization in a fish.

In another aspect, the present invention provides a method for preventing fertilization in a fish comprising administering an effective amount of the composition of the invention, comprising a teleost homolog of zona pellucida (TH-ZP), to the fish.

It is preferred that an adjuvant, such as Freund's complete adjuvant (FCA) or another biologically acceptable adjuvant, be present to assist in stimulation of an immune response in fish. It is also preferred that the TH-ZP be encapsulated into a liposome for administration. Preferably the liposome is multilamellar and comprises L-α-lecithin (soybean) and cholesterol, since this will effect slow release of TH-ZP resulting in an extended period of antibody production and thereby an extended period of contraception in fish. In addition, antibodies raised by this immunological procedure will be directed to the native protein antigens.

The present invention also provides a single administration immunocontraceptive for birds.

Accordingly, in another aspect, the present invention provides an immunocontraceptive vaccine composition comprising an antigen from an inner perivitelline layer (IPVL) of a bird egg, together with a pharmaceutically acceptable diluent or carrier, for reducing or preventing fertilization in a bird.

In another aspect, the present invention provides a method for preventing fertilization in a bird comprising

administering an effective amount of the composition of the invention, comprising the antigen from an inner perivitelline layer (IPVL), to the bird.

It is preferred that an adjuvant, such as Freund's complete adjuvant (FCA) or another biologically acceptable adjuvant, be present to assist in stimulation of an immune response in birds.

It is preferred that the antigen from the IPVL, e.g. in an IPVL portion, be encapsulated into a liposome for administration. Preferably the liposome is multilamellar and comprises L-α-lecithin (soybean) and cholesterol, to effect slow release of antigen/IPVL and increase production of antibodies that bind to the target proteins. This will result in an extended period of antibody production and thereby an extended period of contraception in birds.

As well as FCA, other adjuvants that can be used in vaccine compositions of the present invention include non-ulcerative Freund's complete adjuvant, Freund's incomplete adjuvant, TITERMAX™, MF89, Gerbu, Bacillus Calmette-Guerin, RIBI (MPL+TDM+CWS), bacterial lipopolysaccharide, sodium phthalate derivative of bacterial lipopolysaccharide, sodium phthalate derivative of lipopolysaccharide plus alum, SUPERCARRIER™, ADJUPRIME™ and Alum.

In general, any suitable liposome can be used in the fish or bird vaccine compositions disclosed herein. Anionic and neutral liposomes are well-known in the art (see, *e.g.*, Liposomes: A Practical Approach, RPC New Ed, IRL press (1990), for a detailed description of methods for making liposomes) and are useful for delivering a large range of products.

Cationic lipids are also known in the art. Such lipids include Lipofectin™ also known as DOTMA (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethylammonio)propane), DDAB

(dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidologlycyl spermine) and cholesterol derivatives such as DC-Chol (3 beta-(N-(N',N'-dimethyl aminomethane)-carbamoyl) cholesterol). A description of these cationic lipids can be found in EP 187,702, WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5,527,928.

The route of administration of the vaccine compositions disclosed herein can be any route used typically used in the vaccine field. As general guidance, administration can be *via* a mucosal surface, *e.g.*, an ocular, intranasal, pulmonary, oral, intestinal, rectal, vaginal, and urinary tract surface; or *via* a parenteral route, *e.g.*, by an intravenous, subcutaneous, intraperitoneal, intradermal, intraepidermal, or intramuscular route. The choice of administration route depends on the formulation that is selected as well as on the animal to be vaccinated.

Administration is achieved in a single dose or repeated as necessary at intervals, as can be determined readily by one skilled in the art. An appropriate dose depends on various parameters including the recipient (*e.g.*, adult or infant), the particular vaccine antigen, the route and frequency of administration and the presence/absence or type of adjuvant as can be determined by one skilled in the art.

It should be noted that all of the antibody titers referred to in the specification are measured in comparison with the antibody titer in a reference serum. The titer in the reference serum was arbitrarily assigned a value of 100. That value has no relationship to the absolute titer required to produce an immunocontraceptive effect. In fact, titers of only a few percent of those found in the reference serum are sufficient to produce an immunocontraceptive effect in some cases. While the reference serum clearly contains sufficient antibody to effect immunocontraception, it does not represent

an indication of the minimum antibody titer needed for immunocontraception.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a gel chromatography profile of herring TH-ZP. Fractions 60-75 and 78-80 ml were pooled, dialyzed and freeze-dried.

Figure 2 shows an ion exchange chromatography profile of American plaice, Atlantic salmon and medaka TH-ZP. Each major peak (64-83 ml, American plaice; 54-70 ml Atlantic salmon; 54-64 ml medaka) was pooled, dialyzed and freeze dried.

Figure 3 shows the results of an isoelectric focussing purification of herring TH-ZP. Tubes 12-15 (inclusive) were pooled, dialyzed and freeze dried.

Figure 4 shows the production of anti-TH-ZP antibodies by rainbow trout immunized with Atlantic salmon TH-ZP, American plaice TH-ZP, tilapia TH-ZP, medaka TH-ZP and haddock TH-ZP.

DETAILED DESCRIPTION OF THE INVENTION

1. FISH

Preferred methods of purifying TH-ZP from the eggs of exemplified fish species are set out below. Rabbits were conveniently used for production of anti-TH-ZP sera for screening fractions obtained during purification of TH-ZP. Any species of fish can be immunized provided the TH-ZP used in the vaccine is different enough from the targeted fish species to provoke a good immune response but similar enough that the antibodies produced cross-react with the targeted species TH-ZP. In practice, species that are farmed commercially, including transgenic fish such as salmon, rainbow trout and tilapia, would be important targets.

Collection of fish eggs.

Atlantic salmon (*Salmo salar*), American plaice

(*Hippoglossoides platessoides*), herring (*Clupea harengus*) and haddock (*Melanogrammus aeglefinus*) eggs were obtained from local commercial suppliers. Tilapia eggs were obtained from a colony of hybrid tilapia (*Oreochronis mossambicus X hornorum*) maintained in the Aquatron, Dalhousie University. Medaka eggs were harvested daily from a colony of Indian medaka (*Oryzias latipes*) and stored at -20°C until extracted. Perch (*Perca flavescens*) and smelt (*Osmerus mordax*) eggs were obtained from fish caught in Lake Simcoe, Ontario and stored at -20°C until extracted.

Extraction of TH-ZP.

The method used to extract the teleost homolog of zona pellucida (TH-ZP) depended on the quantity of eggs available. Method 1 was used when the wet weight of eggs was under 100 g. Method 2 was used when the wet weight of eggs was over 100 g.

Extraction method 1.

Fish eggs were placed in a Wheaton tissue homogenizer (30 ml) equipped with a Teflon plunger. The plunger was pushed to the bottom of the tube and up to the top until microscopical examination indicated that most eggs were broken. The egg ghosts were collected on a nylon screen (48 µm pore size) and washed with cold saline to remove cytoplasm. Egg ghosts were replaced in the tissue homogenizer and agitated with the plunger to wash any remaining cytoplasm out of the ghosts. Microscopical examination was used to judge when the egg ghosts were free of cytoplasm. The egg ghosts were suspended in Tris buffer (20 mM, pH 8.0) and incubated at 75°C in a water bath for 25 minutes. The suspension was vortexed and centrifuged (16,000 X g for 15 minutes). The supernatant fluid was dialyzed, freeze dried and stored at -20°C.

Extraction method 2.

Fish eggs were suspended in saline and the suspension placed in a Waring blender. The suspension was blended for 30

seconds and the egg ghosts collected on a nylon screen (pore size 500 µm). The ghosts were washed with liberal amounts of cold saline. The egg ghosts were resuspended in saline and replaced in the Waring blender for 30 seconds. The egg ghosts were collected on a nylon screen (pore size 209 µm) and washed with cold saline. The egg ghosts were extracted with Tris buffer as described in method 1.

Detection of TH-ZP.

Proteins in fish egg extracts were labelled with ^{14}C by reductive methylation (Jentoft, N. and Dearborn,D.G. (1979) "Labelling of proteins by reductive methylation using sodium cyanoborohydride." J.Biol.Chem.254:4359-4365) so that fractions obtained during purification procedures could be monitored by determination of radioactivity. Crude extracts (10 mg) were dissolved in Hepes buffer (20 ml, pH 7.5, 0.1 M) to which ^{14}C-formaldehyde (10 µCi, 37 mCi/mmol) was added. $NaCNBH_4$ was added in two equal portions, one at the beginning and one following 30 minutes incubation at 20°C, to give a final concentration of 20 mM. After 60 minutes incubation, the reaction mixture was acidified with acetic acid and dialyzed overnight. The labelled product was recovered by freeze drying.

To produce TH-ZP that was not radioactive, purification procedures were repeated with unlabelled egg extracts. In this case, fractions were monitored for protein with bicinchoninic acid (Sigma) using bovine serum albumin as a reference standard.

Fractions were also monitored by ELISA using rabbit anti-haddock TH-ZP serum during purification of herring, smelt and perch TH-ZP. Aliquots of fractions from gel chromatography, ion exchange chromatography and isoelectric focussing were diluted to contain protein in the range 10-100 µg/ml with sodium carbonate/bicarbonate buffer (Na_2CO_3 0.015 M; $NaHCO_3$,0.035 M; pH 9.6). The diluted fractions (100 µL) were

placed in wells of a microtiter plate and proteins allowed to absorb at 37°C for 1 hour. Material not absorbed was removed and the wells coated with gelatin (3 % in TBST buffer - Tris,0.01 M; NaCl,0.15 M; 0.05 % Tween 20; pH 8.0) for 10 minutes followed by washing 5 X's with TBST buffer. Rabbit anti-haddock TH-ZP serum (100 µL, diluted 1:100 with TBST buffer) was added to each well and the microtiter plate incubated at 37°C for 1 hour. Unbound antibody and other serum proteins were removed by washing with TBST buffer (5 X's). Bound antibody was measured with protein A/alkaline phosphatase using a Dynatech ELISA plate reader at 405 nm.

Chromatography.

Gel chromatography used TSK-gel (toyopearl HW-65F, 1.5 X 58 cm) eluted with Tris buffer (0.01 M, pH 7.5 containing 0.01 % NaN) at a flow rate of 15 ml/hr. Crude TH-ZP extracts were dissolved in Tris buffer (0.01 M, pH 7.5, 5 ml), centrifuged to remove any insoluble material and aliquots (2 ml) used for gel chromatography. Fractions (3 ml) were collected and aliquots from each fraction were analyzed for radioactivity, protein or ELISA using rabbit anti-haddock TH-ZP serum.

Ion exchange chromatography used Sephacel DEAE (1.5 X 22 cm) eluted with Tris buffer (0.01 M, pH 8) having a linear gradient from 0 to 0.3 M NaCl in a total volume of 150 ml at a flow rate of 6.4 ml/hr. Fractions were collected (3.1 or 6.2 ml) and aliquots of each fraction were analyzed for radioactivity, protein or by ELISA using rabbit anti-haddock TH-ZP serum.

Isoelectric focussing.

Preparative isoelectric focussing used a Rotofor (Biorad) at a constant power input of 12 W for 4 hr. The RotoLytes (Biorad) used were in the range pH 3-9 formed from combining RotoLytes in the range 2.9-4.1;4.5-6.1;6.4-7.5 and 7.8-8.9. Twenty fractions were collected and the pH of each

fraction was adjusted to pH 7-8 with acetic acid or solid $NaHCO_3$. Aliquots of each fraction were analyzed for TH-ZP by determination of radioactivity or ELISA using rabbit anti-haddock TH-ZP or rabbit anti-herring TH-ZP sera.

SDS-PAGE.

SDS-PAGE used gradient gels (Biorad) and kaleidoscope standards to determine molecular weights. Gels were stained with coomassie blue or used for Western blotting with rabbit anti-haddock TH-ZP.

Rabbit anti-TH-ZP sera.

Rabbit anti-TH-ZP sera were produced by immunizing one rabbit for each TH-ZP type with a preparation of haddock TH-ZP that produced a single band (44 kDa) following SDS-PAGE and coomassie blue staining or a preparation of herring TH-ZP obtained by gel chromatography and isoelectric focussing that Western blotting indicated contained a single band (44 kDa).

Immunization of rainbow trout.

Three rainbow trout for each TH-ZP preparation were immunized by a single intramuscular injection (18 gauge, 2.5 in. needle) with TH-ZP (50 µg) encapsulated in liposomes containing phospholipon 90G (Nattermann Phospholipid, Cologne, Germany, 0.04 g) and cholesterol (0.004 g) in saline (0.3 ml). A single dose of the vaccine contained liposomes (0.3 ml, 50 µg TH-ZP) emulsified in Freund's complete adjuvant (FCA, 0.3 ml). Mean body masses of rainbow trout at the time of vaccination were in the range 1.4-1.8 kg. Six rainbow trout were not immunized and served as controls.

Determination of rainbow trout anti-TH-ZP antibody titers.

Rainbow trout were anesthetized with MS 222 and blood samples taken from the caudal vein before immunization and 1,3,5,6 and 8 months post-immunization.

Anti-TH-ZP antibody titers were measured by ELISA using a 96-well microtiter plate. To each well, TH-ZP (1 µg) in sodium carbonate/bicarbonate buffer (100 µL) was allowed to

adsorb at 37°C for 1 hour. TH-ZP not adsorbed was removed. Plates were coated with gelatin as previously described. Rainbow trout serum samples were added in doubling dilutions using TBST from 1/25 to 1/3200 and incubated at 20°C for 1.5 hours. Unbound antibody and other serum proteins were removed by washing with TBST (5 X's). Mouse monoclonal IgM anti-chinook salmon antibody (100 µL, 1/100 dilution in TBST) was added to all wells. Although the mouse MAb was raised against chinook salmon antibody, the mouse MAb bound strongly to rainbow trout antibody reflecting the close phylogenetic relationship between the two salmonid species. The plate was incubated for 1.5 hours at 20°C. Unbound antibody was removed by washing with TBST (5 X's). Bound mouse monoclonal antibody was measured with goat anti-mouse IgM-alkaline phosphatase solution (100 µL, diluted 1:1000 with TBST from liquid stock, Sigma) using a Dynatech ELISA plate reader at 405 nm. One row in each plate did not receive serum (antibody) and served as a blank. Another row in each plate received doubling dilutions of a reference serum. The reference serum was anti-medaka TH-ZP serum that has a titer of 6,400. Production of antibodies by rainbow trout is expressed relative to this serum to avoid interassay variability.

Ova production.

Rainbow trout normally spawn in the spring, however, the rainbow trout used in this study were from St. Peter's fish hatchery, Nova Scotia, Canada and spawn in the autumn (Herbinger,C.M., Doyle,R.W., Pitman,E.R., Paquet,D., Mesa,K.A., Morris,D.B., Wright,J.M. and Cook,D. (1995) "DNA fingerprint based analysis of paternal and maternal effects on offspring growth and survival in communally reared rainbow trout. Aquaculture 137:245-256). To measure ova production, treated and control rainbow trout (three fish in each treatment group were weighed then the ova were removed and weighed. Rainbow trout immunized with smelt TH-ZP, herring TH-ZP and perch TH-ZP