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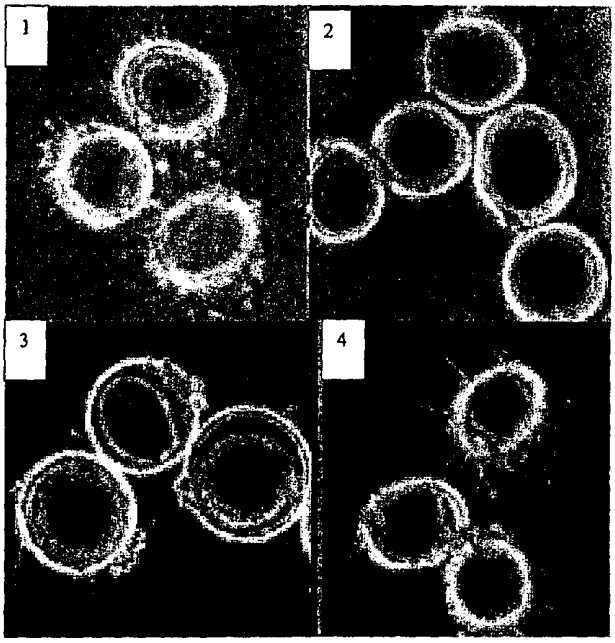
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(54) Title: VACCINE

Sperm–egg binding patterns for 1- red/red; 2 - red/grey; 3 – grey/red; 4 – grey/grey
Note sperm tails on zona of 1 and 4.



(57) Abstract: This invention relates to contraceptive vaccines for the control of pest species, and methods of preparing the same. The vaccines of the invention comprise a peptide that induces an antibody response in the pest species and antibody produced binds to a germ cell polypeptide, thereby reducing fecundity.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

VACCINE

This invention relates to contraceptive vaccines for the control of pest species.

There is an increasing demand for humane and effective control of pest species. For example, New Zealand has experienced an explosion in its possum population. The animals have become a formidable pest, chewing their way through thousands of tonnes of foliage a night, gorging themselves on birds' eggs and spreading bovine tuberculosis. The cost of controlling New Zealand's possum population, which currently stands at 60 million, is an estimated 100 million dollars per annum.

The UK is home to approximately three and a half million grey squirrels. A predominantly grey squirrel population poses a severe nuisance to conservation of the rapidly diminishing red squirrel population which is now largely restricted to remote areas of Cumbria and Scotland. Considerable concern has also arisen in relation to the severe forest damage caused by high numbers of grey squirrels.

Other examples of pest species in the UK include rabbits, rats, mice, badgers, foxes and all vermin injurious to livestock, game, crops or human health. In Canada, the control of seal populations poses a problem in the light of severely devastated fish populations.

Current methods of control include encouraging the target organism to ingest highly toxic substances or poisons. These may be delivered in a bait that when absorbed causes death or injury to the ingesting organism. The traditional way of controlling the possum population in New Zealand has been to scatter carrots laced with poison. Typically released into the environment at large, these substances are disadvantageously ingested by non-target organisms since current poison technology uses a non-specific agent. Considerable concern has also arisen concerning the build up of resistance to these toxic substances which become concentrated at the various

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levels of the food chain with huge potential for devastating and detrimental effects to higher life forms such as man.

In an attempt to bring possums under control, scientists at the Marsupial Cooperative Research Centre (MCRC) based in Sydney have created a genetically modified carrot which is designed to sterilise females. The carrots have been engineered to contain a protein that sabotages fertilisation by binding to a key protein on possums' eggs. An alternative approach has been taken by the Vertebrate Biocontrol Centre in Canberra to control rabbit or fox populations where the main strategy has been to use a recombinant viral vector (mixoma virus, cytomegalovirus) expressing a common egg protein (ZPC) as a vaccine.

The Genetically Modified Organism (GMO) approach is however questionable as there is limited control after release of the virus into the environment. These techniques also represent little improvement over more conventional control methods since they are all hampered by lack of species-specificity and have the potential to sterilise non-target animals.

Techniques for the inhibition of fertility through immunological interference of mammalian reproductive processes are well known in the art. Any approach must reduce the fecundity of the target population. It should involve an effective delivery system and be humane, cost effective and environmentally benign. There is a real need for target-specific action. Due to the non species-specificity of the egg antigen, any antigen should be based on sperm proteins that are species-specific.

Proteins and peptides isolated from tissues, or produced synthetically for use as contraceptive vaccines, are often only weakly antigenic, especially if autologous, and require immunostimulants to elicit an effective response. The mode of delivery and presentation of an antigen is crucial for its effectiveness. Oral vaccination has an advantage of preferentially inducing an immune response at mucosal surfaces such as the reproductive tract and is the most practical route of administration for wild

animals. However, oral vaccinations may produce weak immunity which may not be effective.

The aim of an immunocontraceptive vaccine is to induce a strong local mucosal immune response in the reproductive tracts. The vaccine must evoke an appropriate immune response that blocks one of the steps in the reproduction process. Antibodies generated from the immunisation should bind to the appropriate reproductive proteins preventing essential biological interactions and hence fertility. Ideally, the vaccine should be able to induce a strong mucosal immune response that is sustained over a period of time. A number of delivery systems have been reported to induce both a systemic and mucosal responses in mammals

The epithelium overlying the intestinal mucosa acts as a protective barrier against penetration of potentially harmful pathogens. It is now accepted however that most microorganisms must cross epithelial barriers to induce a mucosal immune response. Mucosa-associated lymphoid tissue (MALT) is present throughout the intestine either as isolated lymphoid follicles or as lymphoid follicle aggregates such as Peyer's patches in the small intestine (Bienenstock and Befus, 1984). The lymphoid tissue is separated from the intestinal milieu by the Follicle-associated epithelium (FAE) which contains highly specialised microfold cells or M cells. M cells facilitate the trans-epithelial transport of an antigen. By providing a gateway through which antigens and micro-organisms are rapidly delivered to the lymphoid tissue, M cells play a pivotal role in mucosal immunity.

It has been suggested that the FAE represents a highly dynamic tissue, which is able to modify its epithelial and lymphoid cells in response to antigenic stimuli. A previous study (Borghesi *et al.*, 1996) demonstrated that one hour exposure to living pneumococci induces dramatic alterations of the architecture and structure of the FAE. Transport of antigens through the follicle-associated epithelium (FAE) of Peyer's patch (PP) is the critical first step in the induction of mucosal immune responses.

Cross-species gamete binding experiments have been carried out to confirm the specificity of fertilisation mechanisms in the grey and red squirrel. Compared with homologous sperm-egg binding there was little binding of grey squirrel sperm to red squirrel oocytes or visa versa. The results indicate that there is a strong species-specific difference in the sperm/egg binding receptors of these two species. Cross-species fertilisation experiments have also been carried out using rabbit sperm and hare ova (or vice versa) and similar results were obtained.

The need for a safe and effective delivery system which can both be administered orally and produce an effective immune response remains paramount.

STATEMENTS OF INVENTION

In its broadest aspect there is provided a vaccine comprising a polypeptide wherein the polypeptide is a germ cell polypeptide.

Preferably the polypeptide is a cell surface polypeptide. More preferably the polypeptide is a receptor polypeptide. Preferably the peptide corresponds to at least part of the extracellular domain of a germ cell polypeptide. Most preferably the polypeptide is a sperm cell or sperm progenitor cell surface polypeptide

In one class of vaccines, the receptor is a 95-kDa phosphoprotein (95PP) located on the plasma membrane of the anterior acrosome in spermatozoa.

Other classes of vaccines use different sperm antigens as vaccine antigen candidates. By way of example and by no means of limitation, these proteins include Acrosin, FA-1 (Naz et al., 184), DE protein (Cuasnicu et al., 1884; Biol. Reprod. 55, 200 206), Fertilin (Blobel et al, 1990), PH-20 (Primakoff et al, 1988), SP17 (Richardson et al., 1994), LDH-C4 (Goldberg) and Sp56 (Bleil et al, 1990).

Antigen presenting cells are responsible for antigen processing and their subsequent presentation to lymphocytes. The antigens must be presented in such a way that the lymphocytes recognise and react to the antigen. Efficient antigen presentation is largely dependent upon the size of the antigen and peptides of 9 to 18 amino acids in length are most commonly used in the art.

Typically the peptide of the invention is at least nine amino acids in length and preferably from 9 to 18 amino acids in length. More preferably the peptide is 9, 12 or 13 amino acids in length.

Most preferably the peptide comprises the amino acid sequence AVTLGGVGFSDPVC (pep 1), or variant thereof. The variant may bind to antibodies to the cell surface polypeptide with increased affinity and may be characterised by conservative or other amino acid substitutions at any position in the sequence of pep 1. Although a variant may be characterised by amino acid substitution, deletion, insertion, etc., at any position in the peptide, it will be understood that the nature of the amino acids at the third and ninth position (i.e., the anchoring amino acids) will be particularly important.

Preferably the peptide is species specific. This feature is preferable because it eliminates possible interference with the fertility of non-target and especially endangered species. Various conserved and varying domains exist within 95PP from different species. Because the invention relies on the production of antibodies to that region of 95PP which is specific to a particular target species, non target organisms remain unaffected.

In one class of embodiments the peptide is conjugated, associated or cross-linked to a suitable adjuvant and/or carrier protein. Preferably the cross-link is a covalent linkage.

The terms adjuvant and carrier are construed in the following manner. Some polypeptide or peptide antigens contain B-cell epitopes but no T cell epitopes. Immune responses can be greatly enhanced by the inclusion of a T cell epitope in the polypeptide/peptide or by the conjugation of the polypeptide/peptide to an immunogenic carrier protein such as key hole limpet haemocyanin or tetanus toxoid which contain multiple T cell epitopes. The conjugate is taken up by antigen presenting cells, processed and presented by human leukocyte antigens (HLA's) class II molecules. This allows T cell help to be given by T cell's specific for carrier derived epitopes to the B cell which is specific for the original antigenic polypeptide/peptide. This can lead to increase in antibody production, secretion and isotype switching.

By way of example only and without limitation, carrier proteins include Keyhole Limpet Haemocyanin (KLH), ovalbumin, Bovine Serum Albumin and cholera toxin B.

An adjuvant is a substance or procedure which augments specific immune responses to antigens by modulating the activity of immune cells. Examples of adjuvants include, by example only, agonsitic antibodies to co-stimulatory molecules, Freunds adjuvant, muramyl dipeptides, liposomes. An adjuvant is therefore an immunomodulator. A carrier is an immunogenic molecule which, when bound to a second molecule augments immune responses to the latter.

In preferred embodiments, the peptide is conjugated, associated or cross-linked to cholera toxin B, preferably using a cross-linker compound. Chemical cross-linkers are well known in the art and are commercially available from Sigma Co. or Pierce and Warner. Preferably the cross-linker compound is succinimidyl 3-(2-pyridyldithio) propionate (SPDP). By way of example and by no means of limitation, other possible cross-linker compounds include AMAS (N-[α-Maleimidoacetoxy]succinimide ester), BMPS (N-β-Maleimidoproploxy]succinimide ester) and EMCS (N-ε-Maleimidocaproyloxy]succinimide ester).

It will be apparent to the skilled artisan that other methods besides chemical methods may also be used to achieve a cross-link. These include, by way of example, UV linkage.

In additional or alternative embodiments of the invention, the peptide is conjugated, associated or cross-linked with an adjuvant such as Freund's adjuvant, alum (aluminium hydroxide) or Immune stimulatory complexes (ISCOMS). An adjuvant is a reagent that augments the natural immune response, therefore acting as an immunopotentiator. Adjuvants can also act as immunomodulators. An adjuvant may stimulate the proliferation of antibody secreting B-cells, isotype switching and/or secretion of antibodies to increase serum antibody titre.

It will be appreciated that although an adjuvant does not typically require the formation of a stable linkage with the immunogen, the possibility that a linkage may be formed should not be excluded.

In one aspect of the invention the peptide is incorporated within a suitable delivery system.

Suitable delivery systems include, by way of example only, liposomes, Immune Stimulatory complexes (ISCOMS) and microparticles.

Liposomes are lipid based vesicles which encapsulate a selected therapeutic agent which is then introduced into an organism. The liposome is manufactured either from pure phospholipid or a mixture of phospholipid and phosphoglyceride.

Typically liposomes can be manufactured with diameters of less than 200nm. This enables their intravenous injection and ability to pass through the pulmonary capillary bed. They are permeable across blood vessel membranes and can gain access to selected tissues.

ISCOMS are colloidal spherical particles consisting of saponin Quil A, cholesterol and phosholipids such as phosphatidylcholine or phosphatidylethanolamine. They have a negative charge and a cage-like structure, typically an icosahedral structure with a 40 nm diameter in which antigen can be entrapped. ISCOMs are extremely stable and remain intact after lyophilisation. Once antigen is added, the ISCOM complex can be stored at sub zero temperatures, (e.g., -70 °C), depending on the stability of the added antigen.

Microparticles composed of, by way of example only, poly (D,L –lactic-co-glycolide) can be also be used as an effective vaccine delivery system (see Challacombe, 1995). The technique for the preparation of the biodegradable microparticles is known, (Rafati, *et al.* 1997). Recent developments in microparticle technology have shown that using the polymer Eudragit (R) L100-55 as stabiliser gives improved protection against gastric digestion of antigens as compared with the conventional polyvinyl alcohol. This method may increase the effective concentration of the antigen and may help to further retain immunogenicity of the antigen complex.

In one embodiment the peptide, preferably conjugated, associated or crosslinked with a carrier protein, is incorporated within a biodegradable microparticle. By way of example, suitable materials which might be used in the formation of a microparticle include Poly-lactiyl-glycolide (PLG), Dichloromethane and Polyvinyl alcohol.

In a particularly preferred embodiment of the invention the microparticle is comprised of poly(D,L-lactic-co-glycolide), or variant thereof.

Preferably the antigen concentration varies from 0.5 to 1.5 mg/dose. Preferably the antigen concentration is 1 mg/dose. Typically the size of a microparticle and antigen complex varies from 0.2 to 5.0 μm. Preferably the size of a microparticle and antigen complex is 1 μm.

Ideally the microparticle includes a stabiliser to increase the effective concentration of the antigen and help to retain immunogenicity of the antigen complex. A stabiliser may take the form of an enteric coating which resists degradation in the stomach of the target organism. This is important in oral administration of the vaccine. In a particularly preferred embodiment the stabiliser of use is Eudragit (R) L100-55. Other possible stabilisers which may be used include, by no means of limitation, carboxymethylcellulose.

In one class of embodiments, molecules actively released or induced by certain bacteria are used as antigen delivery proteins whereby they increase transport capability and enhance absorption of the orally delivered biologically active compound. Preferably the antigen delivery protein is Mucosal Antigen Delivery Protein (MADP).

In one embodiment of the invention, the antigen delivery protein is incorporated separately into the delivery system either as crude supernatant or purified material.

In yet an alternative embodiment of the invention, antigen delivery protein is conjugated directly to the peptide antigen in addition to the carrier protein. In yet a further embodiment, antigen delivery protein is not incorporated within the delivery complex and is administered as a separate entity. Recombinant antigen delivery protein may also be used.

Preferably the vaccine is formulated for oral delivery. From a practical standpoint, oral baiting is a feasible route for administration of the vaccine to wild animals. A problem associated with oral vaccinations however is that they have been found in some cases to produce only weak immunity and this may not be effective. The need for a safe and effective delivery system which can both be administered orally and produce an effective immune response is apparent.

We have found that four out of eight female test rabbits immunised via oral delivery of pep(1) showed significant titres of IgA antibodies in vaginal secretion at 6 and 12 weeks. Serum IgG responses were significantly elevated at 6 and 12 weeks in 3 of these females. Antibodies (IgG + A) were detected on rabbit sperm over the anterior acrosome in five out of eight treated females at 6 and 12 weeks after immunisation.

These data show that the animals raise antibodies to the antigen and this may correlate with a corresponding reduction in fecundity.

In an alternative embodiment of the invention there is provided a composition suitable for use as a contraceptive vaccine comprising a peptide corresponding to at least part of the extracellular domain of a sperm surface polypeptide wherein said peptide is formed synthetically using, by way of example only, an oligosynthesiser.

In an alternative embodiment of the invention the peptide is formed using recombinant techniques. A significant amount of published literature exists with respect to expression vector construction and recombinant DNA techniques in general: Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical Approach Vol III IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc.(1994).

The invention therefore provides for a method of manufacture of a peptide corresponding to at least part of the extracellular domain of a sperm surface polypeptide wherein said method includes peptide synthesis and/or recombinant techniques.

According to an alternative aspect of the invention, there is provided a method of preparing a composition for use as a contraceptive vaccine.

Preferably the method comprises:

(1) providing a composition comprising a polypeptide or peptide with a cross-linker, carrier protein and/or adjuvant and/ or an antigen delivery protein;

(2) conjugating, associating or cross-linking the polypeptide or peptide to a carrier protein or adjuvant and/or an antigen delivery protein to form a peptide-complex.

The invention includes a method in which the peptide (e.g., in the form of a peptide-complex) is incorporated within a suitable delivery system.

In a particularly preferred embodiment of the invention the method includes the step of incorporating the peptide or the peptide-complex into a microparticle, wherein said microparticle optionally comprises a stabiliser.

In yet a further preferred embodiment of the invention the method comprises one or a combination of any of the following steps;

(1)freeze-drying the prepared delivery system;

(2)preparing an oral bait comprising the prepared delivery system; or

(3)preparing an inoculum comprising the prepared delivery system for intramuscular, intradermal, intravenous or rectal administration.

Accordingly the invention provides for use of the vaccine of the present invention as a contraceptive.

The invention provides for a method of contraception comprising administration of the vaccine of the invention in an amount effective to reduce the fecundity of a mammalian species.

The invention also provides an oral bait or inoculum comprising the vaccine of the present invention.

In an alternative aspect of the invention, a vaccine according to the present invention is used as an immunogen.

In a further aspect of the invention there is provided an antibody which is produced by the immune response that is induced by the vaccine of the invention. Preferably the antibody is a monoclonal antibody.

It will be understood that specificity of the vaccine of the present invention may be such that it is not specific to only one particular pest species. Cell proteins of certain taxonomic groupings are characterised by homologous regions which are exclusive to that grouping.

Accordingly the invention provides for a method of testing the species specificity of a vaccine comprising:

(1) obtaining a sperm sample from a pest species of interest;

(2) incubating the sperm sample with at least one antibody;

(3) testing for the presence of any cross-reaction.

Method and Apparatus

Phase I fertility trial

Groups of grey squirrels were established in captivity for immunisation trials. Their reproductive status was assessed and pre-immune blood samples obtained.

Techniques were determined for the collection of viable spermatozoa and mature secondary oocytes from squirrels.

The effects of synthetic peptide (sperm peptide 1 (AVTLGGVGFSDPVC)) to the conserved region of the ZRK sperm receptor Burks D. *et al* (1995) on squirrel sperm function were investigated by sperm-egg binding *in vitro*. Significant inhibition of sperm binding was observed, corroborating the results of previous investigations that the antigen was a suitable candidate for vaccine development.

Synthetic peptides were successfully conjugated to carrier protein and encapsulated in microparticles. The microparticles consisted of lactide-glycolide polymer in the ratio 75: 25 and were prepared by high speed emulsification to produce particles of 0.5 – 2.0 μm as determined by scanning electron microscopy and fluorescent sorting techniques Rafati H *et al.* (1997).

Preparation of microparticles

The following procedures were strictly adhered to in order to reduce variation in microparticle size.

Reagents

All solvents were of HPLC grade.

- Poly-lactil-glycolide (PLG), 503 [50%:50%] cat no. 640661, or 755 [75%:25%] cat no. 640672, Boehringer Ingeheim
- Dichloromethane (DCM), Aldrich
- Poly vinyl alcohol (PVA), Alrish 13-23 000 MW, 87-89% hydrolysed, cat no. 36317-0

Equipment

- Silverson homogeniser SL2 (with a 1/5" microtubular).
- Stirrer
- Master sizer, or Fluorescence Activated Cell Sorter, or Scanning Electron Microscope
- Zeta sizer (measure the surface charge of microparticles)

Experimental protocol

1. 2 ml of 30 mg/ml of protein was made up in DW.

2. Protein from (1) was emulsified with 10 ml of 6% solution of PLG in DCM using a silverson SL2 for 2 minutes at full speed (11600 rev).

NB. The speed of homogenisation is important, therefore the speed of the homogenisation was switched to maximum quickly. The position of the blade in the emulsion was also standardised..

3. While still homogenising, 40 .ml of 10% of PVA was added and homogenisation was continued for a further 4 min. (PVA takes a couple of hours to dissolve).

4. The solvent was evaporated overnight at ambient temperature, with stirring, in fume hood.

5. The microparticles were centrifuged at 16 000 (31 000 x g) for 20 min at 4°C (Beckman, JA20 rotar).

Laboratory mice immunised subcutaneously with vaccine formulation generated antibodies to the anterior acrosome of grey squirrel spermatozoa. In a phase I trial, singly housed grey squirrels were immunised with microparticle vaccine (at two dose levels) or microparticles alone either subcutaneously or orally.

As expected with subcutaneous injection, a high serum but low mucosal response was elicited. On oral immunisation (table 1), an antibody response was detected in 60% of animals displaying a level of immunity within mucosa and serum samples in keeping with levels in the rabbit that inhibit fertility. The duration of detectable antibody response by oral vaccination was 12-16 weeks.

Table 1

Summary of Phase I trial results for complete contraceptive vaccine (microparticles pep1+KLH) given orally and subcutaneously. Microparticles alone gave no response.

Treatment	Animals	Immune response	Duration of response
subcutaneous	5	100%, strong humoral; weak mucosal	More than 16 weeks
orally	5	60% medium humoral; medium mucosal	12-16 weeks

Phase II fertility trial

The fertility of caged animals cannot be assessed readily as breeding pairs will not reproduce in small laboratory cages. Grey squirrels require relatively large enclosures to promote appropriate reproductive and mating behaviour in the breeding seasons. Optimal conditions for captive breeding had been established previously. A phase II trial was therefore undertaken with vaccinations timed to produce a maximal response for the second breeding period (April – June). One large enclosure, C (0.5 ha), and two smaller enclosures (0.25ha), A and B were used.

20 adult females and 8 adult males were selected for the trial and housed singly in the animal house. Injecting an AVID LT310 transponder microchip into the base of the tail uniquely identified each squirrel. All animals were checked for health and condition. Pre-treatment blood samples (2ml) were taken and the weight of each squirrel recorded. Each female had a vaginal smear taken to determine reproductive condition at this time. Two squirrels exhibited an oestrus state as determined by vaginal epithelial morphology.

Vaccines were prepared using sperm peptide 1 and the carrier protein keyhole limpet haemocyanin or cholera toxin B. The latter is effective in enhancing oral immunity in several species, Jackson *et al.* (1993) and had been used successfully in rabbit vaccination studies.

The following schedule was used:

Day 1	Female squirrels given an initial oral vaccination.
Day 19 - 21	Animals weighed and examined for oestrus condition. Blood samples and vaginal swabs taken.
Day 22	Oral booster vaccination given 21 days after first vaccination.
Day 12 - 27	Enclosures cleared of any squirrels that may have entered (by trapping). Enclosures prepared for trial.
Day 28	All animals weighed. Females examined for oestrus with vaginal smear. Animals released into enclosures (table 3).
Day 76 - 92	Females re-captured for weighing, test bleeds, vaginal swabs. Vaccination (treatment and controls) Males re-captured for weighing and assessment of sexual condition/activity.
Day 126 - 139	Female re-captured for weighing, test bleeds and vaginal swabs Males re-captured for weighing and assessment of sexual condition/activity.
Day 174 - 188	Female re-captured for weighing, test bleeds and vaginal swabs. Final vaccination boost. Males re-captured for weighing and assessment of sexual condition/activity.
Day 240 – 246	Final re-capture and termination of trial.

Table 2

Vaccinations and animal groups for phase II trial

Substance		Amount	No. females
microparticles	(control)	2ml	4
microparticles + KLH + peptide1	(pep1+KLH)	2ml	8
microparticles + Cholera toxin-B + peptide1	(pep1+CTB)	2ml	8

Table 3

Distribution of animals in the various enclosures. Based on findings from previous studies, the density of animals in each enclosure was considered optimal for breeding.

Treatments	Control	pep1+KLH	pep1+CTB	Males	Total
Enclosure C	2	5	5	4	16
Enclosure A	1	1	2	2	6
Enclosure B	1	2	1	2	6

Pregnancy testing

Blood samples were centrifuged and plasma recovered for storage at -20 C. Progesterone concentration was determined by ELISA assay (OVUCHECK plasma EIA kit). Samples from treated and control animals were compared with known control samples; i.e. samples from known non-pregnant females and samples from two stages of pregnancy taken from a captive squirrel.

Results

(a) Management of trial

As expected it was not possible to capture every squirrel at each stage of the trial. There was a compromise between sample and data collection and stress to the animals.

In the first re-capture period, 17 out of the 20 treated females were re-captured. One female (treatment pep1+CTB) was dead in a nestbox on 16/3/99. Post mortem examination by the veterinarian revealed a rectal prolapse thought to be unrelated to the experimental procedure. All 8 males were re-captured. 5 unmarked adult squirrels (3 females; 2 males) were captured from the enclosure due to intrusion from outside. These were transported to the animal house and housed in individual cages.

In the second re-capture period, 16 females were re-captured. One female (pep1+CTB) was found dead having been caught between the two lifting trap doors of one of the open traps (19/5/99) and had probably died from dehydration. This was one of the two females that was not re-captured during the first re-trapping period. Two females were not recovered during this period. A female re-captured during this period that had been recovered during the first re-capture was vaccinated at this stage. All 8 males were re-captured. 11 unmarked males and one unmarked female were captured from the enclosures during the trapping period. A female squirrel (pep1+CTB) was found dead in nestbox on 21/6/99. The veterinarian was unable to determine the cause of death.

On the third re-capture period 16 out of the remaining 17 females were caught. One of these females captured (treatment pep1+KLH) had not been caught during the last trapping period and was re-vaccinated with twice the normal dose.

(b) Fertility of females

Fertility data (to 7/9/99) is shown in table 4.

Table 4 Number of females for each treatment that reached parturition

Treatment	Initial no of females	No died during trial	No Completing trial	No pregnant (%)	No reaching parturition
pep1+KLH	8		8	1 (12.5)	Not confirmed
pep1+CTB	8	3	5	1 (20.0)	1
Control	4		4	2 (50.0) **	2
Total	20	3	17	4	3
Un-marked*				1	3

*Un-marked squirrels located (pregnant or with litters)

** significantly different from combined treatment groups ($P \leq 0.05$)

Nestboxes were checked twice weekly for squirrels. The identity and condition of any squirrels found and which nestboxes they occupy were recorded. Of the trial animals, 2 control and 2 treated females had high levels of blood progesterone. Two controls and 1 treated female were located and had given birth. The second treated squirrel was not located and was thought to be nesting in a drey. No other pregnancies or births from trial animals were identified.

During the third re-capture period, the un-marked females (4) that were caught were marked, bled and returned. Three females had litters. The fourth squirrel was not pregnant.

(c) Breeding condition of male squirrels

Males in the trial exhibited large turgid testes and stained skin near the scent glands (androgen-dependent), features that are indicative of breeding condition. Five stock male grey squirrels were euthanised at approximately weekly intervals during

April/May. All males had large quantities of motile spermatozoa in their vas deferens and cauda epididymides.

(d) Immune Responses

The serum and immune responses were analysed in a single batch at the termination of the trial. Preliminary assays indicated immune responses in keeping with the oral vaccination data for the phase I trial. The original assays used cross-reacting rabbit anti-rat IgG to measure serum immune responses in squirrel. This was not ideal and rabbit anti-squirrel IgG serum has been produced which are effective in assays. Rabbit anti-squirrel IgA serum is now being generated for more accurate mucosal immune assessment using milk samples from lactating female squirrels.

In vitro cross-fertilisation experiments to assess potential functional specificity of vaccine

In vitro fertilisation experiments have indicated the efficacy of candidate immunogens for the immunocontraceptive vaccine. To confirm the specificity, it was necessary to examine the potential for cross-reactivity with gametes (sperm/egg).

12 red squirrels (7 males; 5 females) were caught. Each squirrel was housed in an individual cage (approximately 1 m^3). The health and condition of each squirrel was checked. Nine red squirrels (5 males; 4 females) together with stock grey squirrels, were transported on three separate occasions to Sheffield University. The three remaining red squirrels (2 males; 1 female) were for use in the Thetford red squirrel breeding project.

Gametes and oocytes were collected and prepared. Experiments were carried out with a matrix design as shown in table 5. Typical binding patterns are shown in figure 1

Table 5 Inter-species binding of gametes

Gamete interaction (oocyte/sperm)	Number of oocytes	Number of sperm bound/oocyte	Proportion of acrosome reacted sperm bound to zona (%)
Red*/Red	22	90 ± 20	> 90
Red/Grey	26	2.4 ± 3.2**	5**
Grey*/Grey	16	75 ± 30	65
Grey/Red	25	6.2 ± 8.5**	22**

*gametes from Red squirrel or Grey squirrel

** significantly different from homologous gamete binding (P≤ 0.005)

Table 6 Laboratory rabbit (New Zealand White) oral contraceptive vaccine (sperm antigen) trials in 1999 and 2000 carried out at Sheffield.

Vaccine	Number of Females	Pregnancy Rate (%)	Fertility Rate* (%) (foetuses/copora lutea)
Trial 1999			
Complete	7	43	20 (15/74) [A]
CTB-MP[1]	6	83	83 (48/58)
MP	2	100	81 (17/21)
Trial 2000			
Complete	19	42	24 (50/209) [B]
CTB-MP	6	66	51 (26/51)
MP	3	66	58 (15/26)
Total			
Complete	26	42	23 (65/283)[C]
CTB-MP	12	75	68 (74/109)
MP	5	80	68 (32/47)
Complete	26	42	23 (65/283)[C]
Controls	17	76	68 (106/156)

* Fertility rate = Number of foetuses at 3 weeks /number of corpora lutea (= ovulation rate)

[1] Two rabbits may not have received final booster immunisation (record lost).

Compared with controls A = P<0.01; B = P<0.05; C=P<0.005

CTB = Cholera Toxin B

MP = Microparticles

Discussion and conclusions

The microparticle encapsulated vaccine inhibits fertility in treated squirrels compared with controls. A significant immune response was observed in some animals correlating with inhibition of fertility. Both KLH and CTB were effective apparently as carriers of the peptide vaccine. In the phase II trial there was a significant inhibition of fertility (pregnancy rate) in the combined treatment groups (15.4 %) compared with controls (50%).

Three females (all pep1 +CTB vaccinated) were found dead during the trial but these deaths were apparently unconnected and not obviously related to treatment. While caged before their release into enclosures, females showed no side effects after the initial and first booster vaccination at 21 days with any of the treatments. Cholera toxin B has been used successfully as a carrier protein in other studies in rodents without noticeable toxic effects, Jackson *et al.* (1993).

Vaccinations were timed to give an optimal immune response during a breeding season. As it was necessary to re-capture animals for blood samples further vaccinations were performed. Whether such repeated application of a vaccine is practical in the long-term is unclear. Because of the timing of the breeding season it is likely that only the first three vaccinations would have had an effect on fertility. Exactly when and how often an oral vaccine would have to be administered will require further investigation. The phase II trial covered the summer breeding period of the squirrels and additional trials should be performed that cover the winter breeding period.

The Cross-species *in vitro* fertilisation experiments between gametes of red and grey squirrels provide convincing evidence for differences in the binding affinities of spermatozoa with homologous and heterologous zona pellucida. These findings strongly support the contention of differences in the egg receptor on sperm from the

two species. The acrosome reaction involves vesiculation of the membranes over the anterior sperm head, which is essential for sperm penetration and fertilisation of the egg. The *in vitro* assay assessed secondary binding of spermatozoa to the zona pellucida, after the induction of the acrosome reaction, Brewis, I and Moore, H.D.M (1997). Where spermatozoa had bound to a heterologous zona, few had undergone the acrosome reaction. The antigen used in the contraceptive vaccine is to a region of the receptor involved in the induction of the acrosome reaction and one would therefore expect functional and structural differences in the receptor between the two species.

The results of the squirrel trial were in keeping with parallel studies on a contraceptive vaccine for the rabbit. Sperm peptide-based vaccine encapsulated in microparticles with CTB carrier gave an immune response which inhibited fertility in laboratory rabbits. Together the results in the squirrel and rabbit suggest that the overall approach is promising. Our detailed investigations (in the rabbit) indicate that the efficiency of microparticle uptake across the immune responsive tissue of the gastro-intestinal tract (Peyer's Patches) is probably the most important event in mounting an effective immune response against the sperm antigen for an oral vaccine.

Preliminary investigation of vaccine stability in bait

The animal trials detailed above involve administration of vaccine via dosing tube to the back of the mouth. This humane method delivers the vaccine with certainty to the gastro-intestinal (GI) tract ensuring that the efficacy of the vaccine in terms of the response to antigen can be determined. Clearly this technique gives no indication of the effectiveness of the vaccine under field conditions where bait delivery will be essential.

The incorporation of microparticles into commercial rodent chow and their subsequent stability was investigated. Vaccine was freeze-dried (previous studies

indicate that microparticles are stable for at least a year), rodent food pellets were ground up and vaccine was incorporated. The mixture was rehydrated, reconstituted into pellets by extrusion and redried. These pellets were examined under a variety of conditions (dry, wet, acid). Results indicate that microparticles remain stable and that there is no appreciable leakage of immunogen from their core. The palatability of the bait was also studied. Uptake of bait/vaccine by squirrels and their immune responses were monitored. It was important to ensure a sufficient vaccine dose was delivered over a brief period.

Squirrel-specific IgA assays

Anti-rat IgA antibody (which cross-reacts with squirrel IgA) was previously used in the assays to measure immunological response. This is not ideal and a homologous assay was devised. A squirrel IgA fraction was obtained from serum and milk by affinity chromatography. The purified fraction was used to immunise a rabbit and the antiserum tested for specificity and titre.

Problems were encountered in obtaining a reliable sample from the vagina of the squirrel to test IgA response in the genital tract. A plastic spatula is used to obtain 5 micro-litres for testing. Although this small sample is often sufficient for an assay, the position of the spatula near to the cervical secretion is important. A more detailed study of the local immune response may require immunolocalisation study on tracts removed from animals at the end of a fertility trial.

Relative merits of CTB and KLH carrier

The vaccine uses CTB carrier in preference to KLH. The rabbit studies indicate greater microparticle uptake with this molecule compared to KLH. There is no evidence to suggest CTB is toxic in the rabbit at least. The potential use of KLH should not however be excluded.

Costs of the vaccine was also considered. CTB is currently obtained in relatively small quantities and the costs are high. While there will be economy of scale when producing many doses of a vaccine, a cost-effective method of producing the immunogen will be required. In this regard there may be an opportunity to exploit existing techniques for producing a cholera vaccine for human use which is based on CTB subunit and which has been shown to be safe.

The incorporation of contraceptive vaccines (antigen conjugated to carrier protein) into several possible delivery systems was explored and the effectiveness of these vehicles in enhancing mucosal antibody responses was investigated by animal trials. Vaccine encapsulated in poly lactide-glycolide microparticles induced a significant increase in IgA in the reproductive tract and serum and reduction in fertility.

The ability of the rabbit alimentary canal to absorb and process microparticles was examined in detail by developing an *in vivo* mucosal assay using the isolated intestinal loop technique. The uptake of particles (latex microspheres) across specialised regions (follicle-associated epithelium (FAE) via specialised M cells) was demonstrated initially along with a dramatic increase in particle uptake following short-term exposure to the bacteria, *streptococcus pneumoniae.*

Subsequent experiments established that a sterile, cell-free extract from bacteria-stimulated intestine was capable of mimicking the effects of viable bacteria on FAE. This finding implied that molecule(s) actively released or induced by certain bacteria could be exploited to enhance delivery of oral vaccine. The active moiety responsible for enhancing M cell development and activity was designated "mucosal antigen delivery protein", MADP. A limited fertility trial in female rabbits using a uteroglobin peptide vaccine indicated that the addition of MADP to microparticles induced a reduction in the number of pregnancies as compared with controls (60 and 90% respectively).

Further investigations of other immunostimulatory factors were carried out. Sperm peptide antigen was conjugated to cholera toxin B (CTB), a non-toxic protein component of bacteria *Vibrio cholerae* which binds to the gut mucosal cells. This vaccine was encapsulated in microparticles and fed to female rabbits. A significant immune response (IgA) was induced leading to a marked reduction in fertility (83%) compared to control animals fed CTB microparticles alone. (See table 6)

These results indicate that an oral contraceptive vaccine approach is feasible for the control of rabbit fertility. A vaccine based on sperm peptide may be more effective than uteroglobin peptide.

A number of delivery systems that have been reported to induce both a systemic and mucosal responses in mammals were investigated for use with sperm-specific antigens.

Methods and Results:

***Liposomes*:** Liposomes with the composition of distearoylphosphatidylcholine (DSPC), phosphotidyl serine and cholesterol are stable in the gastrointestinal tract and taken up by Peyer's patches ([Fujii *et al.*, 1993). Studies have demonstrated that liposome vaccines can induce both mucosal and systemic immune response against, for example, hepatitis B ([Diminsky *et al.*, 1993); and influenza (Ben Ahemeida *et al.*, 1993).

The method of preparing liposomes was carried out according to (North, 1982), and the liposome composition was those prepared by (Fujii *et al.*, 1993). Phospholipids consisting of a 1:1:2 molar ratio of phosphatidylserine, distearoyl L-α-phosphatidylcholine (DSPC) and cholesterol in chloroform/methanol (9:1 v/v) were evaporated to dryness in a 250 ml round bottomed rotary evaporation flask. To this flask was added 5-7 ml of sperm antigen (pep1+KLH carrier) or KLH carrier antigen alone (control) containing 250 mg of n-Octyl-β-D-thioglucopyranoside. This was left

at 4°C for 48 hrs before a brief water sonication to resuspend the lipid from the walls of the flask. The preparation was then dialysed for 5 days with four daily changes of PBS (500ml).

Following dialysis the preparation was adjusted to 40% sucrose by addition of an equal volume of 80% sucrose. This was layered onto 1 ml of 75% sucrose in PBS in polycarbonate centrifuge tubes, followed by 3 ml of 30% and 3 ml of 15% sucrose. These tubes were balanced with PBS. The preparation was then centrifuged at 26 000 rpm for 24 hrs. The liposome bands were pooled and dialysed against 8 changes of 500 ml PBS for 48 hrs. It was estimated that the antigen concentration per immunisation was ~ 500 μg/ml.

New Zealand White adult female rabbits (8) were immunised (x2 at 2 week intervals) with 2 ml of liposomes carrying 500 μg/ml of antigen preparation (or control) in 1 M Na_2CO3 buffer pH8.0 and delivered to the back of the throat using a 5 ml syringe. Liposome preparations were examined by electron microscopy and liposome vesicles were identified.

Rabbits were assessed in serum and vaginal secretions for immune responses by enzyme-linked immunosorbent assay (ELISA) up to 20 weeks after immunisation. ELISA plates were coated with 5 μg of BSA-peptide. Rabbit sera IgGs were detected with anti-rabbit IgG alkaline phosphatase conjugate, while vaginal IgAs were detected with goat anti-rabbit IgA followed by mice anti-goat IgG alkaline phosphatase conjugate.

Immune stimulating complexes: ISCOMs carrying antigens were shown to stimulate both B and T cell response by intramuscular, intranasal, oral and parenteral routes (reviewed by McGhee, 1993).

The method of preparation of ISCOM was a modification of the one used by (de Vries, 1988). Sperm antigen (as above with controls) cholesterol,

phosphatidylcholine and Quil A were mixed in the following ratio (weight: weight): 0.4:1:1:4 and heated to 37° C. The mixture was sonicated three times for 3 min at amplitude of 18 μ and then placed in a rotary shaker for 60 min at 4°C. This was followed by extensive dialysis into1 1 of PBS at 4°C with three changes of PBS per day.

Antigen concentration was estimated at 0.5 – 1 mg /ml. Rabbits (8 +4 controls) were immunised and assessed as described above.

Microparticles: Biodegradable microparticles composed of poly (D,L –lactic-co-glycolide) can be used as a vaccine delivery system (see Challacombe, 1995). The technique for the preparation of the biodegradable microparticles was developed by Davis, SS., School of Pharmaceutical Sciences, University of Nottingham. (Rafati, *et al.* 1997) and is sensitive to minor alterations to the protocol.

Sperm antigen (as above) was emulsified into 10 ml of 6% poly lactide glycolide (75:25) in dichloromethane using a Silverson SL2 homogeniser at 11600 revs for 2 minutes. 40 ml of 10% poly vinyl alcohol were added to the emulsified polymer and homogenised for a further 4 minutes. The dichloromethane was evaporated from the emulsion overnight at ambient temperature.

Microparticles were recovered, washed twice with distilled water by centrifugation at 31 000 g for 20 minutes at 4°C and resuspended in 5 ml of distilled water. The microparticles were examined with a Fluorescence Activated Cell Sorter (FACS) and their mean size was determined to be 0.5 - 2.0 μm (figure 1). The particle size was confirmed by scanning electron microscopy (figure 2). The microparticles were then freeze-dried which increases their shelve life for up to 1 year. Antigen concentration was estimated at 0.5 – 1 mg /ml.

Rabbits (8 +4 controls) were immunised and assessed as described above. Compared with controls, 4/8 females immunised with sperm antigen had significant titres of IgA

antibodies in vaginal secretion (1/400 – 1/1500 titre) at 6 and 12 weeks after immunisation and 3/8 females at 20 weeks. Serum IgG responses were significantly elevated at 6 and 12 weeks in 3 of these females.

By indirect immunofluorescence, antibodies (IgG +A) were detected on rabbit sperm over the anterior acrosome in 5/8 treated females at 6 and 12 weeks after immunisation. Rabbits were subjected to mating at the end of the trial (figure 3). There was a trend but no significant difference in fertility between controls (5 mated, 100% pregnancy, mean litter size 6.5) and treated animals (7 mated, 57% pregnancy (4/7); mean litter size 5.8).

Conclusion: Microparticles induced the most significant immune response in a proportion of females (IgA and IgG). There was no significant inhibition in fertility at the end of the trial but the number of rabbits in the mating trial was low.

The results of a previous study (Borghesi *et al.*, 1996) prompted an investigation as to whether the pneumococci-induced modifications were accompanied by enhanced ability of the FAE to transport antigens.

The ability of the FAE to bind, internalise, and transport fluorescent latex microparticles, highly specific to rabbit M cells, after exposure to *S. pneumoniae* was evaluated. Quantitative studies revealed a marked increase in the number of microspheres bound to PP tissues exposed to S. pneumoniae compared to tissues exposed to either phosphate-buffered saline ($p<0.001$) or *Escherichia coli* DH5 alpha ($p<0.001$) as controls (figure 4). A comparable increase of internalised particles in FAE exposed to *S. pneumoniae* was also seen (figure 5).

No evidence of relevant paracellular transport of microparticles within the FAE or bacterially induced damage to the epithelial barrier was observed. Confocal microscopy analysis of the FAE surface showed that a significant increase in the number of cells that showed both morphological and functional features of M cells

took place within pneumococci-treated PP tissues. Thus it appeared that challenge with *S. pneumoniae* upregulated the ability of the FAE to bind and internalise microparticles.

The most striking difference was observed when the number of particles translocated to the subepithelial lymphoid tissue was examined (figure 6). In this case the number of particles increased 7.1 fold as compared to PP treated with PBS ($p<0.001$) and 6 fold compared to E. Coli treated PP ($p<0.001$). This result is noteworthy since the lymphoid tissue underlying the FAE of PP is the location of initiation of mucosal immune response.

Scanning electron microscopy indicated that *S.pneumoniae* R36a induced the conversion of enterocytes to cells showing morphological and functional features of intestinal M cells (figure 7). Further experiments carried out using both heat-killed and formalin –fixed bacteria showed that dead bacteria were not able to induce any changes of the FAE morphology and function. Overall, these data provide the first direct evidence that the FAE-specific antigen sampling function may be manipulated to improve antigen and drug delivery (Meynell *et al.*, 1999).

The above findings indicated that exposure of the isolated rabbit intestinal loop to *S. pneumoniae* R36a upregulated microsphere transport across follicle associated epithelium (FAE) of Peyer's patch (PP). Further studies were carried out therefore to isolate the factors responsible.

A series of experiments established that a sterile, cell-free extract from bacteria-stimulated intestine is capable of mimicking the effects of viable bacteria on the FAE. Contents of stimulated intestinal loops were partially purified by centrifugation and sterile filtration through 0.22 μm syringe filters. When this material was instilled into isolated intestinal loops of rabbits there was a significant improved ability to bind particles (latex microspheres) from the intestinal lumen and translocate them to the gut-associated lymphoid tissue (GALT) and intestinal lymphatic system. This

result implied that molecules actively released or induced by certain bacteria can be exploited to enhance absorption of orally delivered biologically active compounds.

In order to determine the physical characteristics of the active material, supernatant was fractionated according to molecular weight through a molecular weight filter. Extract supernatant (1 ml) was centrifuged for 1 hour through a Centricon-3 filter (Amicon, USA). The filtrate (<3kD) was removed and the > 3 kD fraction was washed by dilution and reconcentration. Biological activity was retained in the higher molecular weight (>3 kD) fraction (figure 8). The active moiety responsible for enhancing M cell development and activity was designated "mucosal antigen delivery protein" MADP.

The discovery of a soluble factor with the ability to up-regulate mucosal antigen uptake provides the first direct evidence that the immunologically relevant antigen sampling function of the FAE in the gut can be manipulated *in vivo*. Such factors can be used to improve antigen delivery to the intestinal immune system.

Since the intestinal loop experiments indicated that bacterial components may increase microparticle uptake, further studies were undertaken with the known bacterial immunostimulating protein, Cholera toxin (CT). enterotoxin produced and secreted by the bacterium *Vibrio cholerae* which infects the mucosal membranes of the gastrointestinal tracts and activates adenylate cyclase/cAMP signal transduction pathway of cells. Cholera toxin comprises of two subunits, A (CTA) and B (CTB). CTB is a pentameric protein and mediates the binding of the toxin to a glycosylated lipid (ganglioside G_{m1}) on the surface of mucosal cells, and is the non-toxic component of CT (Jackson, *et al.* 1993, Holmgren *et al.* 1994). The property of CTB is thought to play a useful role in the development of a oral vaccine as it can assist in the uptake of the vaccine and display to the GALT. Both CTA and CTB have been fully characterised and cloned.

CTB was conjugated to sperm peptide 1 (AVTLGGVGFSDPVC) using the cross-linker succinimidyl 3-(2-pyridyldithio)propionate (SPDP). 4 mg CTB was dialysed into 20 mM phosphate buffer pH 7.5 and mixed with 25 µl of 25 mM SPDP for 60 min at room temperature. Excess SPDP was removed using a G-10 column and the CTB-SPDP complex was mixed with peptide for 18 hrs. The CTB-peptide was dialysed in PBS and then encapsulated in microparticles as described previously.

Female New Zealand rabbits were orally immunised as described previously with complete vaccine (8 females, pep1–CTB microparticles , ~ 1 mg), with microparticles alone (2 females), or with microparticles with CTB (4 females). Booster immunisations were given at 4 and 8 weeks. Rabbits were monitored (test bled and vaginal swab) at 8 and 18 weeks and mated at 20 weeks (vaginal swab taken). One rabbit in the complete vaccine group was culled at 16 weeks due to infection after fighting.

The pregnancy rate of females immunised with complete vaccine (43 %) was less than in the combined controls (83%, $p<0.1$). Of more importance, the fertility rate as determined by the number of foetuses/number of corpora lutea (thus ovulations) was markedly reduced for females given complete vaccine (20.2%) compared to controls (80 %, $p<0.01$; figure 9). Most females (6/8) displayed a significant elevation in serum and/or vaginal IgA levels compared to controls but little or no change in serum IgG levels. Generally, there was a correlation between immune response and fertility which seemed to be related to elevated levels of serum IgA at the time of mating. Difficulties in obtaining sufficient vaginal secretion from swabs may have affected accurate detection of IgA in the vagina.

In order to assess the immunostimulatory of MADP, a vaccine was prepared that incorporated uteroglobulin peptide (T4) conjugated to Keyhole limpet haemocyanain (KLH) with biological solutions containing MADP activity. The results suggest that MADP induced a reduction in the number of pregnant rabbits as compared with control groups (figure 10).

Conclusion: Vaccines incorporating immunostimulatory bacterial protein (CTB or MADP) and specific reproductive antigen (sperm pep1 or uteroglobulin T4) caused a decrease in fertility. With sperm-based vaccine there was significant decrease in the fertility as measured by the total number of foetuses produced divided by the number of corpora lutea (indication of oocytes ovulated). This result was in keeping with the premise that this vaccine inhibits fertilisation rather than pregnancy. By contrast, the site of action of uteroglobulin vaccine is at implantation. The addition of MADP seemed to reduce pregnancy rate, but the number of rabbits in this preliminary trial was too small to make any definite conclusion.

Studies with the various formulations for vaccine delivery indicated that microparticle encapsulation of antigen was the most efficient method to evoke a significant immune response after oral administration in the rabbit. Whereas a parenteral (injection) approach leads primarily to a humoral IgG immune response, oral vaccination with microparticles induced an IgA response in both serum and vaginal secretion. This would be critical for the efficacy of an immunocontraceptive vaccine where immune inhibition of fertility in the reproductive tract is required.

Investigations with the intestinal loop procedure demonstrated clearly that microparticles adhere and are internalised by the follicle associated epithelium of Peyer's patches of the gastro-intestinal tract and that this route of presentation of antigen is a pivotal aspect of induction of a mucosal immune response. More significantly it was shown that *S. pneumoniae* bacteria (of non-intestinal origin) substantially increased the uptake of particles and therefore exposure to antigen. This finding was exploited in two ways. Further studies were conducted to examine the nature of immunostimulatory factors that may be present in the gut after exposure to *S. pneumoniae*. This approach lead to the discovery of " mucosal antigen delivery protein" (MADP) which may be extremely useful for increasing immunostimulation and is an important step in designing new strategies for oral vaccination. Initial vaccine trials indicated that MADP enhanced immune response.

Microparticle vaccines containing bacterial protein (cholera toxin B subunit protein) were constructed also to enhance immune response and inhibition of fertility. With sperm-based vaccines (pep1-CTB and microparticles) a significant decrease in fertility was observed in laboratory rabbits. This is an important finding because it indicates that a practical oral contraceptive vaccine approach is indeed feasible and is in keeping with experimental observation *in vitro*.

The research findings may be adapted for other oral immunisation procedures in any mammalian species.

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CLAIMS

1. A vaccine comprising a polypeptide wherein the polypeptide is a germ cell polypeptide.

2. A vaccine according to claim 1 wherein the polypeptide is a cell surface polypeptide.

3. A vaccine according to claim 1 or 2 wherein the polypeptide is a receptor polypeptide.

4. A vaccine according to claim 2 or 3 wherein the polypeptide corresponds to at least part of the extracellular domain of a germ cell polypeptide.

5. A vaccine according to any of claims 2 to 4 wherein the polypeptide is a sperm cell or sperm progenitor cell cell surface polypeptide.

6. A vaccine according to claim 5 wherein the polypeptide is a 95-kDa phosphoprotein (95PP).

7. A vaccine according to any of the preceding claims wherein the polypeptide is a peptide of at least 9 amino acids in length.

8. A vaccine according to claim 7 wherein the polypeptide is a peptide from 9 to 18 amino acids in length.

9. A vaccine according to claim 8 the polypeptide is a peptide of 9, 12 or 13 amino acids in length.

10. A vaccine according to any of the preceding claims wherein the polypeptide comprises the amino acid sequence AVTLGGVGFSDPVC, or is a variant thereof.

11. A vaccine according to any of the preceding claims wherein the peptide is species specific.

12. A vaccine according to any of the preceding claims wherein the vaccine is formulated for oral delivery.

14. A vaccine according to any of the preceding claims wherein the polypeptide is conjugated, associated or cross-linked to a suitable carrier protein.

15. A vaccine according to claim 14 wherein the carrier protein is selected from the group consisting of: keyhole limpet haemocyanin; ovalbumin; bovine serum albumin; or cholera toxin B.

16. A vaccine according to claim 15 wherein the polypeptide is conjugated, associated or crosslinked to cholera toxin B.

17. A vaccine according to any of the preceding claims wherein the polypeptide is conjugated, associated or cross-linked with an adjuvant.

18. A vaccine according to claim 17 wherein the adjuvant is selected from the group consisting of: Freunds adjuvant; muramyl dipeptides; or liposomes.

19. A vaccine according to any of claims 14 to 18 wherein the cross-linker compound is succinimidyl 3-(2-pyridyldithio) propionate (SPDP).

20. A vaccine according to any of the preceding claims wherein the polypeptide or peptide concentration is from about 0.5 to1.5 mg/dose.

21. A vaccine according to claim 20 wherein the polypeptide or peptide concentration is 1 mg/dose.

22. A vaccine according to any preceding claim wherein the vaccine is formulated into a delivery system selected from the following group: liposomes; immune stimulatory complexes; or microparticles.

23. A vaccine according to claim 22 wherein the delivery system comprises microparticles comprising of poly(D,L-lactic-co-glycolide), or any variant thereof.

24. A vaccine according to any of claims 22 to 23 wherein the size of a microparticle and peptide complex is from 0.2 to 5.0 μm.

25. A vaccine according to claim 24 wherein the size of a microparticle and peptide complex is about 1 μm.

26. A vaccine according to any of claims 22 to 25 wherein the microparticle includes a stabiliser.

27. A vaccine according to claim 26 wherein the stabiliser is Eudragit (R) L100-55.

28. A vaccine according to any of the preceding claims comprising an antigen delivery protein.

29. A vaccine according to claim 28 wherein the antigen delivery protein is Mucosal Antigen Delivery Protein (MADP).

30. A vaccine according to any of the preceding claims wherein the peptide is formed by a peptide synthesiser.

31. A method of preparing a vaccine of any of the preceding claims comprising:

(i) providing a composition comprising a polypeptide or peptide with a cross-linker, carrier protein and/or adjuvant and/ or an antigen delivery protein;
(ii) conjugating, associating or cross-linking the polypeptide or peptide to a carrier protein or adjuvant and/or an antigen delivery protein to form a peptide-complex.

32. A method of incorporating a peptide within a suitable delivery system comprising incorporating the peptide or the peptide-complex into a microparticle, wherein said microparticle optionally comprises a stabiliser.

33. A method of claim 32, further comprising one or a combination of any of the following steps;
(i)freeze-drying the prepared delivery system;
(ii)preparing an oral bait comprising the prepared delivery system; or
(iii)preparing an inoculum comprising the prepared delivery system for intramuscular, intradermal, intravenous or rectal administration.

34. A vaccine of any of claims 1 to 30 for use as a contraceptive.

35. A method of contraception comprising administering a vaccine of any of claims 1 to 30 in an amount effective to reduce the fecundity of a mammalian species.

36. An oral bait or inoculum comprising the vaccine of any of claims 1 to 30.

37. A vaccine of any of claims 1 to 30 for use as an immunogen.

38. An antibody produced by the immune response that is induced by the vaccine of any of claims 1 to 30.

39. An antibody according to claim 38 wherein the antibody is a monoclonal antibody.

40. A method for testing the species specificity of a vaccine of any of claims 1 to 30 comprising:

(i) obtaining a sperm sample from a pest species of interest;

(ii) incubating the sperm sample with at least one antibody of claim 39;

(iii) testing for the presence of any cross-reaction.

1/1

Figure 1 Sperm–egg binding patterns for 1- red/red; 2 - red/grey; 3 – grey/red; 4 – grey/grey

Note sperm tails on zona of 1 and 4.

