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Use of γ-inulin/liposomes/Vitamin E adjuvant combination in contraceptive vaccines

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Abstract

The adjuvanticity of two gamma inulin/liposomes/Vitamin E combinations was evaluated in the mouse, in contraceptive vaccines with sperm protein extracts or a synthetic HE2 peptide ("Human Epididymis gene product"; residues 15–28) as antigen. The HE2 peptide was not conjugated to a protein carrier, to ensure that the antibodies elicited were specific against the HE2 peptide. The adjuvant combinations were designed to increase adjuvanticity, as their components have complementary mechanisms, and their performance was compared to Freund's adjuvant. Antibody production against native sperm structures was determined in sera by ELISA immunoassay and immunohistology. Toxicity of adjuvants was determined by histopathological study and treated mice were monitored for signs of pain or distress. Our results show that the gamma inulin (1–2 μm particle size)/liposomes/Vitamin E combination, with sperm protein extracts, is better than Freund's adjuvant because it elicits good antibody titres without any toxicity. When the synthetic HE2 peptide is used as antigen, the gamma inulin (1–2 μm particle size)/liposomes/Vitamin E combination is less effective than Freund's adjuvant; nevertheless, the anti-HE2 antibodies elicited are highly specific and recognize native structures in sperm.

Keywords: HE2 sperm antigen; Contraception; Gamma inulin; Liposomes; Vitamin E; Freund's adjuvant

1. Introduction

Immunological adjuvants are of major importance as agents to potentiate the immune response for clinical and laboratory use. Freund's adjuvant is an extremely potent formulation that elicits high antibody production but it is too toxic for clinical use (Chapel and August, 1976). Aluminium-containing compounds such as hydroxides and phosphates (commonly referred to as "alum") are used in vaccines in humans, because of their low toxicity, although their response is usually less intense and persistent. It is of great interest to find alternative adjuvants that give good immune responses without inducing deleterious side effects.

Safe and effective contraceptive vaccines for use in humans or livestock particularly need such adjuvants.

Abbreviations: ASA, anti-sperm antibodies; FA, Freund's adjuvant; γ-IN, gamma inulin adjuvant; PBS, phosphate buffered saline; PO, horseradish peroxidase; DMAB, 3-dimethylaminobenzoic acid; MBTH, 3-methyl-2-benzothiazoline hydrazone hydrochloride; A_{620}, optical density at 620 nm

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Many fertility antigens have been studied for immunological contraception (Herr, 1996; Diekman and Herr, 1997); some of them have been sequenced and small peptides that are highly tissue-specific have been selected for immunization studies, but they are usually less immunogenic than the whole protein. One of such peptides is the amino acid sequence 15–28 of "Human Epididymis gene product" (HE2) sperm antigen (GELRERAPGQGTNGC; referred to as "HE2 peptide" in the text). This HE2 peptide is of great interest because it contains a functional adhesion motif (Leu-Arg-Glu: LRE) that may be evolutionarily conserved and it is highly probable that it is exposed on the surface of the molecule, accessible to oocyte receptors (Hunter et al., 1989). Therefore, the HE2 peptide is a promising antigen to be used in the development of contraceptive vaccines.

To be safe and effective, contraceptive vaccines with sperm antigens should elicit high antibody titres that are highly specific against native sperm structures, and do not cross-react with other tissues. This is difficult to achieve, specially with low molecular weight antigens, such as the HE2 peptide (15 amino acid residues). In laboratory use, small peptides are usually conjugated to protein carriers prior to immunization, to increase humoral immune response against the peptide. However, this also leads to immune response against the protein carrier, which in some cases (e.g. clinical use) may be undesirable or even toxic (Naylor et al., 1991; Kahn et al., 1992; Cooper, 1995). An alternative is the use of potent adjuvants to maintain a high and prolonged immune response.

The aim of the present study was to find an adjuvant that was highly effective and with low toxicity, which could be used safely in contraceptive vaccines. To this effect, we combined three non-toxic adjuvants: gamma inulin, liposomes and Vitamin E (α-tocopherol), approved for veterinary and clinical use (Lasic, 1993; Cooper, 1995). We selected these adjuvants because they have complementary mechanisms of adjuvant activity. We evaluated the performance of these adjuvants (given alone) and adjuvant combinations in a mouse model, with sperm antigens that show different levels of immunogenicity: mouse whole sperm (high immunogenicity), mouse sperm protein extracts (good immunogenicity) and the synthetic HE2 peptide (low immunogenicity, if uncoupled to protein carriers). The effectivity of the adjuvants and adjuvant combinations inducing humoral immune response to native sperm structures was compared to the use of Freund's adjuvant.

2. Materials and methods

2.1. Animals

The immunization experiments used 6–8-week old B6CF1 female mice (C57BL/6 × BALB/c) with five to ten mice per experimental group. Fertite B6CBAF1 (C57BL/6 × CBA) were used as a source of gametes. Mice were purchased from IFFA-CREDO (Les Oncins, France) and housed under standard conditions at the vivarium of the Universidad Autónoma de Barcelona, with a diet of commercial food pellets and water ad libitum.

2.2. Synthetic peptides

The synthetic peptide segment corresponding to residue 15–28 of the deduced sequence of Human Epididymal gene product 2 (amino acid sequence: GELRERAPGQGTNGC) was chemisynthetically prepared as described in Osterhoff et al. (1994).

2.3. Obtention of mouse whole sperm

Sperm were obtained by dissection of cauda epididymis from B6CBAF1 males. Epididymis were placed in 60 mm Petri dishes in saline (150 mM NaCl) and were minced with a surgical blade. The supernatant was collected in sterile conical tubes and settled for 5–10 min to remove tissue debris. The supernatant containing sperm was washed twice at $300 \times g$ for 10 min in saline, then a sample was taken to assess sperm count.

2.4. Obtention of mouse seminal vesicle proteins

Seminal vesicles from B6CBAF1 males were dissected under sterile conditions and placed in 60 mm Petri dishes in chilled sterile bidestilled water. The seminal vesicle content was extruded with the aid of surgical pincers, was collected in sterile conical tubes and then was lyophilized. Seminal vesicle proteins were reconstituted in saline, then protein concentration

was determined by the Bradford's method (Bradford, 1976).

2.5. Mouse sperm protein extracts

Cauda epididymis sperm were centrifuged at 500 × *g* for 10 min, the pellet was resuspended in lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM PMSF pH 7.5; 50 × 10^6 sperm/ml lysis buffer) and incubated at 37 °C for 1 h. The solubilized samples were centrifuged at 500 × *g* for 10 min then the supernatant (sperm protein extracts) was collected in sterile conical tubes and the pellet (detergent-treated sperm) was resuspended in PBS and was analysed by indirect immunofluorescence assay (see below). Sperm protein extracts were centrifuged at 16,000 × *g* for 20 min and the supernatant was precipitated with 10 volumes of cold acetone (−20 °C) for 30 min to remove the detergent. After centrifugation at 16,000 × *g* for 20 min, the supernatant was removed and the pellet (sperm membrane proteins) was lyophilized. Sperm proteins were resuspended in saline buffer (50 mM Tris, 150 mM NaCl, 1 mM PMSF, pH 8.0) and then protein concentration was determined by the Bradford's method (Bradford, 1976).

2.5.1. Characterization of mouse sperm membrane proteins

The sperm membrane proteins were analysed by 12% PAGE (SDS-polyacrylamide slab gel electrophoresis; Laemli, 1970), 25 μg protein/lane, followed by Western Blot (Towbin et al., 1979); seminal vesicle proteins, which did not participate in fertilization processes, were run as a control, 25 μg protein/lane. Sperm surface antigens in the protein samples were detected using a mouse purified polyclonal antibody (PAbs+) raised against whole mouse cauda epididymis sperm, which recognizes native structures on the whole sperm surface and blocks mouse fertility in vitro and in vivo (80% sperm motility inhibition in vitro; 36% viable preimplantational embryos and 23% non-fertilized eggs observed after passive immunization in vivo; Fuentes and Martínez, 2000). PAbs+ antibody was also used to visualize antigens on detergent-treated cauda epididymis sperm, by indirect immunofluorescence assay (100 μg antibody/5 × 10^6 cells; see "Fluorescence microscopy". The immunofluorescence pattern obtained was compared to that of cauda epididymis sperm (without detergent treatment). Loss of immunofluorescence staining in detergent-treated sperm was interpreted as a result of successful solubilization of membrane proteins.

2.6. Adjuvants

2.6.1. Gamma inulin

Gamma inulin standard formulation (γ-IN, a crystalline form of the carbohydrate inulin, particle size of 1–2 μm) and gamma inulin ultrafine formulation (γ-IN_{UF}, particle size of <1 μm) consisted of 50 mg γ-IN/ml in saline solution, and were prepared as described in Cooper and Carter (1986).

2.6.2. Liposomes

Liposomes were prepared from lyophilized hydrogenated phosphatidylcholine, from Lucas Meyer S.A., Barcelona, Spain. Hydrogenated phosphatidylcholine was resuspended by stirring in sterile deionized water at 70 °C for 30 min. The suspension was passed five times in a Microfluidizer 110S equipment (Microfluidics) at 70 °C and 4 Bar pressure, to reduce liposome size. Liposomes were cooled below 40 °C, were mixed with Vitamin E and with an equal volume of soluble antigen (sperm membrane proteins or synthetic HE2 peptide; whole sperm was not used as antigen because of its size), and were passed twice through a Microfluidizer at 4 Bar pressure. The final phospholipid and Vitamin E concentrations were 30 and 20 mg/ml, respectively. Liposomes with antigen were stored in sterile conditions at 4 °C until used.

2.6.3. Characterization of liposomes

Liposome structure was characterized by negative staining transmission electron microscopy (Johnson et al., 1971), and their mean diameter was measured by laser Doppler shift with a Micro-Track ultrafine particle analyzer 150 (Honeywell, USA) (Barnadas et al., 1997). The machine was operated in accordance with the manufacturers instructions.

2.6.4. Freund's adjuvant

Complete Freund's adjuvant (0.5 mg *Mycobacterium butyricum*/ml in a mixture of paraffin oil and mannide monooleate) and Incomplete Freund's adjuvant (without bacteria) were purchased from DIFCO laboratories, Detroit, MI.

2.7. Immunization schedules

2.7.1. Antigen dose

Mice immunized with sperm (Sp) received 2.5 million cells emulsified in adjuvant on days 1, 20, 40, and a final booster of 5 million cells in saline on day 60.

Mice immunized with sperm protein extracts (PE) received 12.5 μg antigen in adjuvant on days 1, 20, 40, and a final booster of 25 μg antigen in saline on day 60.

Mice immunized with HE2 peptide received 50 μg antigen in adjuvant on days 1, 20 and 40, with a final booster of 100 μg peptide in saline on day 60.

2.7.2. Inocula administration

Gamma inulin adjuvants (γ-IN and γ-IN_{UF}) were administered in saline, 72 h *before* injection of antigen, to a final dose of 500 μg adjuvant/100 μl.

Liposomes with antigen were prepared as described above (see "Adjuvants"), to administer the appropriate dose of antigen in 100 μl.

Freund's adjuvant (first injection in Complete Freund's adjuvant) was emulsified with an equal volume of antigen, prior to injection.

All immunizations were subcutaneous in 100 μl. This volume was divided in two aliquots of 50 μl administered at different injection sites (nape and base of tail). Mice were bled by retroorbital puncture on days 0, 80 and 90 of the experiment. Blood samples were kept at 4 °C for 1 h, centrifuged at 10,000 × *g* for 5 min and the clear supernatant was aliquoted and stored at −20 °C. Antisera were thawed and inactivated 30 min at 56 °C prior to use.

2.8. Experimental groups

NI ($n = 7$ mice)	non-immunized mice (control)
PBS ($n = 6$ mice)	injection of PBS (control)
FA + PBS ($n = 6$ mice)	injection of Freund's adjuvant emulsified with PBS (first injection with complete Freund's).
Sp ($n = 6$ mice)	injection of sperm in saline.
FA + Sp ($n = 6$ mice)	injection of sperm in Freund's adjuvant emulsion (first injection with complete Freund's)
γ-IN + Sp ($n = 6$ mice)	injection of standard gamma inulin adjuvant 72 h before injection of sperm in saline
γ-IN_{UF} + Sp ($n = 6$ mice)	injection of ultrafine gamma inulin adjuvant 72 h before injection of sperm in saline
PE ($n = 5$ mice)	injection of sperm proteins in saline.
FA + PE ($n = 5$ mice)	injection of sperm proteins in Freund's adjuvant emulsion (first injection with complete Freund's)
γ-IN + PE ($n = 5$ mice)	injection of standard gamma inulin adjuvant 72 h before injection of sperm proteins in saline
γ-IN_{UF} + PE ($n = 5$ mice)	injection of ultrafine gamma inulin adjuvant 72 h before injection of sperm proteins in saline
L_{VitE} + PE ($n = 5$ mice)	injection of sperm proteins in liposomes with Vitamin E
γ-IN/L_{VitE} + PE ($n = 5$ mice)	injection of standard gamma inulin adjuvant 72 h before injection of sperm proteins in liposomes with Vitamin E
γ-IN_{UF}/L_{VitE} + PE ($n = 5$ mice)	injection of ultrafine gamma inulin adjuvant 72 h before injection of sperm proteins in liposomes with Vitamin E
HE2 ($n = 6$ mice)	injection of HE2 peptide in saline
FA + HE2 ($n = 10$ mice)	injection of HE2 peptide in Freund's adjuvant emulsion (first injection with complete Freund's)

γ-IN/L_{VitE} + HE2 ($n = 6$ mice)	injection of standard gamma inulin adjuvant 72 h before injection of HE2 peptide in liposomes with Vitamin E
γ-IN_{UF}/L_{VitE} + HE2 ($n = 6$ mice)	injection of ultrafine gamma inulin adjuvant 72 h before injection of HE2 peptide in liposomes with Vitamin E

2.9. ELISA immunoassay

Serum samples from day 0 (preimmune serum), 80 and 90 were assayed for differences in antibody production by ELISA immunoassay. Microtiter plates (96 wells, Dynatech M129B) were coated with HE2 peptide (5 μg/well) by incubation with 0.1 M bicarbonate buffer, pH 9.6 for 60 min (Lepp and Martínez, 1989) or with mouse cauda epididymis sperm (10^5 cells/well) by incubation with 0.25% glutaraldehyde (Benet-Rubinat et al., 1991).

Coated plates were washed and then incubated for 60 min with 250 μl/well of a blocking solution (3% w/v non-fat dry milk in PBS 0.05% v/v Tween 20 (PBS-T)), to eliminate non-specific background reactions. Washing steps involved three washes with PBS-T at 5 min intervals; all incubations took place in a moist chamber. Serum samples were two-fold diluted from 1/32 to 1/13,072 in PBS-T, then microtiter plates were incubated with 200 μl/well of each serum dilution, in duplicate, for 90 min at 37 °C; PBS-T was used as control. Goat anti-mouse IgG conjugated with horseradish peroxidase (BioRAD, USA), 200 μl/well of 1/3,000 dilution, were added to washed plates that were then incubated for 60 min at 37 °C. Plates were washed again and developed for 15 min with 200 μl/well of a chromogenic substrate (MBTH (0.8 mM), DMAB (40 mM), H_2O_2 (3 mM); Sigma, St. Louis, MO, USA). The reaction was stopped with 50 μl/well of 2 M sulfuric acid, and the absorbance at 620 nm (A_{620}) was automatically determined with a multiscan plate reader.

Anti-HE2 and anti-sperm antibody levels in serum were determined by a positive dilution threshold (PDT), defined as the lowest serum dilution with an absorbance at 620 nm greater than twice that of the control (PBS-T):

$$\text{PDT} = A_{620\,\text{serum}} \geq 2 \times (A_{620\,\text{PBS-T}})$$

2.10. Fluorescence microscopy

Mouse sperm were visualized by indirect immunofluorescence assays to localize the antigens recognized by immune sera from all experimental groups. Washed cauda epididymal sperm (1×10^6 sperm in 100 μl PBS) were incubated for 45 min at 37 °C with a 1/100 dilution of serum (100 μl/sample); PBS was used as control. Sperm suspensions were washed twice at $300 \times g$ for 10 min in PBS, and then were incubated for 30 min at 37 °C with a 1/100 dilution of Goat anti-mouse IgG conjugated with FITC (Jackson Immunoresearch Laboratories), 100 μl/sample. Sperm were washed thoroughly at $300 \times g$ for 10 min, three times, were mounted on a slide and observed under an epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with an FITC filter set (excitation wavelength = 490 nm).

2.11. Distress and pain evaluation

Mice were monitored periodically (twice on immunization days and at 3–5 days intervals) to evaluate if the animals suffered any pain or distress due to experimental procedures. The observed parameters were the general aspect (appearance, stance), behavioural responses to external stimuli (handling) and unprovoked behaviour (motility, vocalizing) of the animal, according to the guidelines from Morton and Griffiths (1985), with some modifications. Body weight was estimated every 3–7 days. Mice treated with PBS were used as control of normal appearance and behaviour.

Scores of 0–3 were assigned to each of these variables in an animal, according to criteria listed below. If a score of 3 was recorded more than once, then all scores of 3 were given one extra mark. The sum of scores of variables from each experimental group was compared. Immunizations with a total score ≤4 were considered to be safe with minor side effects; immunizations with a total score between 5 and 8 were considered to be moderately toxic with some side effects; immunizations with a total score between 9 and 12 were considered to be toxic and to cause significant discomfort or distress to the animals; immunizations with a total score ≥13 were considered very toxic and cause significant pain to the animals.

Appearance

0 normal
1 mild swelling at injection site
2 moderate swelling at injection site; coat loses sheen
3 intense swelling and/or necrosis at the injection site; hair loss, coat unkempt or soiled

Unprovoked behaviour

0 normal behaviour pattern
1 normal posture and alertness, with occasional licking or scratching of the injection site
2 abnormal behaviour. Restlesness, with licking or scratching of the injection site
3 abnormal behaviour. Very restless or does not move at all (exaggerated signs of 2 above)

Behavioural responses to external stimuli (handling)

0 behavioural responses normal for the expected conditions (body tone, paw grip, vocalization, etc... on handling)
1 shows some minor depression or minor exaggeration of responses
2 moderate signs of abnormal responses; there may be a change of behaviour
3 animal reacts violently to stimuli or show very weak muscular responses

Bodyweight

0 normal bodyweight is maintained or increased
1 shows mild loss of body weight ($P > 0.05$), less than 5% of expected value
2 moderate loss of body weight ($P < 0.05$), 5–10% of expected value
3 severe loss of body weight ($P < 0.01$), more than 10% of expected value

2.12. Statistical analysis

The statistical significance of any differences found was evaluated by means of one-way Anova, with the StatView Program (Abacus Concepts, Inc.). A probability of 0.05 or less was considered significant; treatment mean differences were identified by Fisher's least significant difference test.

3. Results

3.1. Characterization of liposomes

Analysis by laser Doppler shift showed that liposomes had a mean size of 100 nm. Negative staining transmission electron microscopy revealed that liposomes had a oligolamellar structure.

3.2. Characterization of mouse sperm membrane proteins

The use of 1% Triton X-100 detergent proved to be effective to solubilize sperm membrane proteins

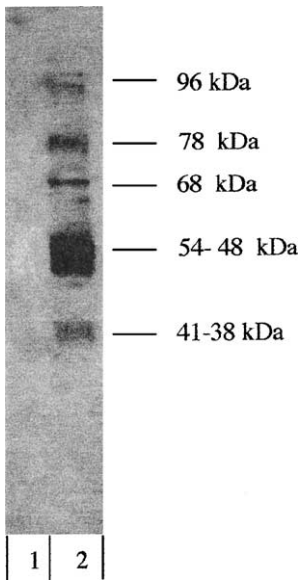

Fig. 1. Western blot analysis of mouse seminal vesicle proteins (lane 1) and mouse sperm membrane proteins (lane 2). PAbs+ polyclonal anti-sperm antibody reacted with several solubilized sperm membrane proteins but not with seminal vesicle proteins; estimated molecular weights of the proteins are indicated on the right.

in the mouse; the mean protein yield was of 7 μg protein/10^6 cauda epididymis sperm. PAGE and Western blot analysis, using PAbs+ mouse purified polyclonal anti-sperm antibody, detected several protein bands, with 96, 78, 68, 54–48, 48–38 kDa estimated molecular weights (Fig. 1) in the sperm membrane sample; no protein bands were detected in the seminal vesicle sample, under the same conditions. The extraction of sperm surface antigens was confirmed by indirect immunofluorescence assay using the same antibody (PAbs+): detergent-treated sperm showed less immunofluorescence staining than non-treated sperm, due to removal of antigens mainly localized on the acrosome and equatorial regions of sperm.

3.3. Antibody production

3.3.1. Immunization with mouse sperm

Mouse sperm is highly immunogenic on its own, and its injection elicited a good immune response (Table 1). The use of Freund's adjuvant increased up to 16-fold anti-sperm antibody production and proved more effective ($P < 0.01$) than gamma inulin (standard and ultrafine formulations), which increased 4-fold antibody production.

3.3.2. Immunization with mouse sperm proteins

Gamma inulin/liposomes/Vitamin E adjuvant combination proved to be very effective, and elicited 4–16-fold higher antibody levels than those obtained with liposomes with Vitamin E ($P < 0.01$) or gamma inulin ($P < 0.01$), given alone with the antigen (Table 1). When the gamma inulin/liposomes/Vitamin E adjuvant combination was prepared with standard gamma inulin (1–2 μm particle size; γ-IN/L_{VitE} + PE antibody levels were two-fold those obtained with ultrafine gamma inulin (<1 μm particle size; γ-IN_{UF}/L_{VitE} + PE). The highest humoral immune response was obtained with Freund's adjuvant, with antibody levels two- to four-fold higher than those obtained with the second most effective adjuvant (γ-IN/L_{VitE} + PE; $P < 0.01$).

Table 1
Antibody levels in sera of mice immunized with sperm antigens or HE2 peptide and adjuvants, determined by ELISA immunoassay

Immunizations		Anti-sperm antibodies (PDT)[a]		Anti-HE2 antibodies (PDT)[b]
NI	−	1/128 ($n = 7$)	−	>1/32 ($n = 7$)
PBS	−	1/128 ($n = 6$)	−	>1/32 ($n = 6$)
FA + PBS	−	1/128 ($n = 4$); 1/256 ($n = 2$)	−	>1/32 ($n = 6$)
Sp	+	1/4096 ($n = 6$)		n.a
FA + Sp	+++	1/65536 ($n = 3$); 1/131072 ($n = 3$)		n.a
γ-IN + Sp	++	1/8192 ($n = 1$); 1/16384 ($n = 5$)		n.a
γ-IN_{UF} + Sp	++	1/18192 ($n = 2$); 1/16384 ($n = 4$)		n a
PE	−/+	1/256 ($n = 2$); 1/512 ($n = 3$)		n.a
FA + PE	++	1/8192 ($n = 1$); 1/16384 ($n = 4$)		n.a
γ-IN + PE	−/+	1/256 ($n = 1$); 1/512 ($n = 4$)		n.a
γ-IN_{UF} + PE	−/+	1/256 ($n = 2$); 1/512 ($n = 3$)		n.a
L_{VitE} + PE	+	1/1024 ($n = 2$); 1/2048 ($n = 3$)		n.a
γ-IN/L_{VitE} + PE	++	1/4096 ($n = 3$); 1/8192 ($n = 2$)		n.a
γ-IN_{UF}/L_{VitE} + PE	+	1/4096 ($n = 5$)		n.a
HE2	−/+	1/256 ($n = 6$)	−/+	1/128 ($n = 2$); 1/256 ($n = 4$)
FA + HE2	+	1/4096 ($n = 10$)	++	1/8192 ($n = 3$); 1/16384 ($n = 7$)
γ-IN/L_{VitE} + HE2	+	1/512 ($n = 3$); 1/1024 ($n = 3$)	+	1/512 ($n = 3$); 1/1024 ($n = 3$)
γ-IN_{UF}/L_{VitE} + HE2	−/+	1/512 ($n = 6$)	−/+	1/512 ($n = 6$)

Serum samples were obtained on day 90 of the experiment. Sp: mouse sperm; PE: mouse sperm membrane proteins; HE2: HE2 peptide; FA: Freund's adjuvant; γ-IN: gamma inulin; UF: ultrafine particle size; L_{VitE}: liposomes with Vitamin E. Antibody levels: −/+, PDT ≤ 1/256; +, PDT ≤ 1/1024; ++, PDT ≤ 1/8192; +++, PDT ≤ 1/65536. Positive dilution threshold (PDT) being defined as the lowest serum dilution with an absorbance at 620 nm greater than twice that of the control (PBS-T), PDT = $A_{620\,serum} \geq 2(A_{620}$ control).

[a] ELISA plates coated with mouse sperm (10^5 cells/well).

[b] ELISA plates coated with HE2 peptide (5 μg/well); n.a.: not analysed.

3.3.3. Immunization with HE2 peptide and adjuvants

Mice immunized with HE2 peptide in gamma inulin/liposomes/Vitamin E adjuvant combinations γ-IN/L_{VitE} + HE2 or γ-IN_{UF}/L_{VitE} + HE2 raised anti-HE2 antibodies (Table 1), which recognized mouse cauda epididymis sperm, specifically ($P < 0.01$). However, the anti-HE2 antibody levels elicited were 16–32-fold lower than those obtained with Freund's adjuvant ($P < 0.01$).

Preimmune sera and serum from control mice immunized with Freund's adjuvant with PBS (FA + PBS) did not show anti-HE2 antibodies or specific anti-sperm antibodies.

3.4. Fluorescence microscopy

Antisera from mice immunized with cauda epididymis Sp recognized antigens on the whole surface of sperm. Antisera from mice immunized with PE recognized antigens on the acrosome and equatorial region of sperm; the staining pattern coincided with the origin of solubilized antigens (see "Characterization of sperm membrane proteins"). Antisera from mice immunized with HE2 peptide (HE2) showed higher specificity, and recognized antigens on the acrosome of mouse sperm. In all immunizations, the use of adjuvants gave a higher intensity of antigen recognition, that correlated with anti-sperm antibody levels determined by ELISA immunoassay. Preimmune serum and serum from mice injected with Freund's adjuvant in PBS (FA + PBS) did not show immunofluorescence staining of mouse cauda epididymis sperm.

3.5. Distress and pain evaluation

Mice immuniced with gamma inulin (standard or ultrafine formulation), liposomes with Vitamin E or gamma inulin/liposomes/Vitamin E combinations did not show signs of pain or distress during the immunization experiments (Table 2). On the contrary, mice given Freund's adjuvant showed moderate to severe behavioural and physical alterations, depending on the antigen used in the immunizations. The worse effects were observed with whole Sp as antigen (highly

Table 2
Physical and behavioural changes in mice immunized with sperm antigens or HE2 peptide and adjuvants

Immunizations	Aspect	UB	REE	Body weight	Total score	Toxicity level
NI	0	0	0	0	0	–
PBS	0	0	0	0	0	–
FA + PBS	2	1	2	1	6	+
Sp	1	0	1	1	3	–
FA + Sp	3	2	3	3	11 + 3 = 14	+++
γ-IN + Sp	1	0	1	1	3	–
γ-IN_{UF} + Sp	1	0	1	1	3	–
PE	1	0	0	1	2	–
FA + PE	3	2	2	2	9	++
γ-IN + PE	1	0	1	1	3	–
γ-IN_{UF} + PE	1	0	1	1	3	–
L_{VitE} + PE	1	0	1	1	3	–
γ-IN/L_{VitE} + PE	1	1	1	1	4	–
γ-IN_{UF}/L_{VitE} + PE	2	1	1	1	5	+
HE2	0	0	0	0	0	–
FA + HE2	2	1	2	1	6	+
γ-IN/L_{VitE} + HE2	1	1	1	1	4	–
γ-IN_{UF}/L_{VitE} + HE2	2	1	1	1	5	+

Sp: mouse sperm; PE: mouse sperm membrane proteins; HE2: HE2 peptide; FA: Freundt's adjuvant; γ-IN: gamma inulin; UF: ultrafine particle size; L_{VitE}: liposomes with Vitamin E; UB: unprovoked behaviour; REE: behavioural responses to external stimuli (handling). Scores: 0: normal; 1: mild changes; 2: moderate changes; 3: severe changes. Toxicity level: +: total score between 5 and 8, moderately toxic, with some discomfort to the animal; ++: total score between 9 and 12, toxic, with significant discomfort to the animal; +++: total score 13, very toxic, with significant pain or distress to the animal.

immunogenic): mice showed severe swellings at injection sites that caused pain and distress. The use of PE or HE2 as antigens, which are less immunogenic than whole Sp, caused less severe swellings at injection sites, which caused discomfort to the animals.

4. Discussion

The development of improved adjuvants that have the adjuvant properties of Freund's adjuvant but not the side effects is of great interest for human and veterinary use. Contraceptive vaccines for use in humans or livestock particularly need such adjuvants. Liposomes and gamma inulin have been proposed as alternatives to Freund's adjuvant, and will be briefly discussed here.

Entrapment of antigen in liposomes of $\leq$500 nm size (such as the ones employed in this work) enables antigen delivery into lymph nodes, where it is slowly released and taken up by antigen-presenting cells (Cullis et al., 1987). The addition of Vitamin E (anti-oxidant) to liposomes increase their stability and also may enhance their adjuvanticity. Vitamin E supplementation in mice has been associated with increased macrophage phagocytic activity, Th cell activity, antibody synthesis, lymphoblastic cell viability and neutrophil anti-microbial activity (Odeleye et al., 1992; Wang et al., 1995).

γ-IN is able to enhance humoral and cellular immune responses with very different immunogens (KLH, influenza virus, sheep red blood cells; hepatitis B surface antigen; Taenia ovis recombinant antigen; malaria peptide/diphtheria toxoid conjugate protein) (Cooper and Steele, 1988; Cooper et al., 1991; Deol et al., 1995; Jones et al., 1996) by strong activation of the alternative-pathway complement system, by partial activation of macrophages and by deposition of C3-fragments on the surface of antigen-presenting cells (Kerekes et al., 2001); this effect of gamma inulin is very important, as it has been shown before that C3-fragments covalently bound on antigen-presenting cells interact with CR1/2 expressed on activated T cells and play an important role in the augmentation of the adaptive response (Kerekes et al., 2001). In addition to this, administration of gamma inulin up to 9 days before injection of antigen proved to enhance specific IgG responses to KLH, and antibody production was found to be optimal if pretreatment was at 2–3 days (Cooper, 1995); similar results were observed with sperm extracts as antigen (Fuentes, unpublished data). Pretreatment with gamma inulin before antigen injection may lead to the formation of immunostimulating factors at injection sites and efferent lymph nodes, and to improvement of specific immune response against the antigen.

In the present study, we evaluated the effectivity and toxicity of gamma inulin adjuvant and liposomes with Vitamin E, compared to Freund's adjuvant. Freund's was the most effective adjuvant tested, with mouse whole sperm, mouse sperm proteins or HE2 peptide used as antigen. Repeated subcutaneous injection did not cause severe histopathological lesions like those reported by others (Broderson, 1989) who administered higher volumes of adjuvant emulsion (>50 μl) per injection site but it caused significant pain and distress to the animals. The nature and dose of antigen contributed to the toxic effects of adjuvant emulsions, as mice injected with Freund's adjuvant and HE2 peptide showed milder swellings at injection sites and less signs of discomfort than mice injected with Freund's adjuvant and mouse sperm. These results are in concordance with the reports by others authors (Toth et al., 1989), and give support to the substitution of Freund's adjuvant by adjuvants with less side effects whenever possible.

In contrast, the use of γ-IN (standard or ultrafine particle size), liposomes with Vitamin E, and two gamma inulin/liposomes/Vitamin E combinations proved to be safer than the use of Freund's adjuvant. The combined use of gamma inulin and liposomes with Vitamin E improved adjuvanticity and enabled higher (4–16-fold) humoral immune responses against sperm proteins than those obtained with the same adjuvants, given alone ($P < 0.01$). This result can be explained because gamma inulin, liposomes and Vitamin E have complementary mechanisms of adjuvant activity, as has been discussed above. Another benefit of this adjuvant combination is that it is not toxic and does not cause discomfort, pain or distress to the animals. Pretreatment with standard gamma inulin (1–2 μm particle size) 3 days (72 h) before injection of antigen in liposomes with Vitamin E was slightly more effective than pretreatment with ultrafine gamma inulin (<1 μm particle size), with sperm proteins or with HE2 peptide as antigen.

If the synthetic HE2 peptide was used as antigen, antibody levels raised with gamma inulin/liposomes/ Vitamin E combinations were substantially lower (16–32-fold) than those obtained with Freund's adjuvant; nevertheless, the anti-HE2 antibodies elicited were highly specific and recognized native structures in sperm. It should be taken into consideration that the HE2 peptide has low molecular weight (15 amino acid residues), no neat electrical charge, and it was not coupled to a protein carrier prior to injection. We did not use protein carriers to ensure that the immune response was specific against the HE2 peptide sequence and to avoid cross-reactions or any side effects. However, considering the characteristics of the HE2 peptide, it is possible that entrapment in liposomes was not very effective. If this is the case, coupling the HE2 peptide to the liposomes surface may increase peptide stability in the liposomes and improve humoral immune response against the HE2 peptide.

We conclude that pretreatment with gamma inulin standard formulation (1–2 μm particle size) plus liposomes with Vitamin E is a good adjuvant, and may be a good alternative to Freund's with protein antigens. Moreover, gamma inulin, liposomes and Vitamin E are non-toxic and non-pyrogenic at the doses injected, and they are expected to be safe in human and livestock vaccines with a wide range of antigens, including fertility antigens.

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