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Señores
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

**Ref: Expediente No. 05.098.906**
**Solicitud de patente de invención titulada:**
**"EMULSIONES DE ACEITE EN AGUA MICROFLUIDIZADAS Y COMPOSICIONES DE VACUNAS".**
**Solicitante: PFIZER PRODUCTS INC.**

**SOLICITUD DE EXAMEN DE PATENTABILIDAD SEGÚN EL ARTÍCULO 44 DE LA DECISION 486, GACETA No. 562**

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, D.C., identificado tal como aparece al pie de mi firma, en mi condición de apoderado del solicitante, respetuosamente me permito solicitar, que se examine si la invención reúne los requisitos de patentabilidad previstos en la misma. Dando cumplimiento a lo establecido en el articulo 44 de la Decisión 486 de la comisión de la Comunidad Andina de Naciones. Adjunto recibo por el monto de $194.000 pesos para cubrir la tasa correspondiente, según la Resolución 35582 del año 2005.

Atentamente,

CARLOS R. OLARTE
C.C. 79.782.747 de Bogotá
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Anexo: Recibo por $194.000 pesos

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***** C O N C E P T O *****

| CANT. | RENTISTICO | CONCEPTO | TOTAL CONCEPTO |
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**DIVISION DE NUEVAS CREACIONES**

RADICACIÓN EXPEDIENTE No 05-98906

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **562** DE

FECHA **31** DE **MARZO DE 2006** EL TERMINO HABIL SEÑALADO POR LA

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NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____ NO__ __ **X** __ ____

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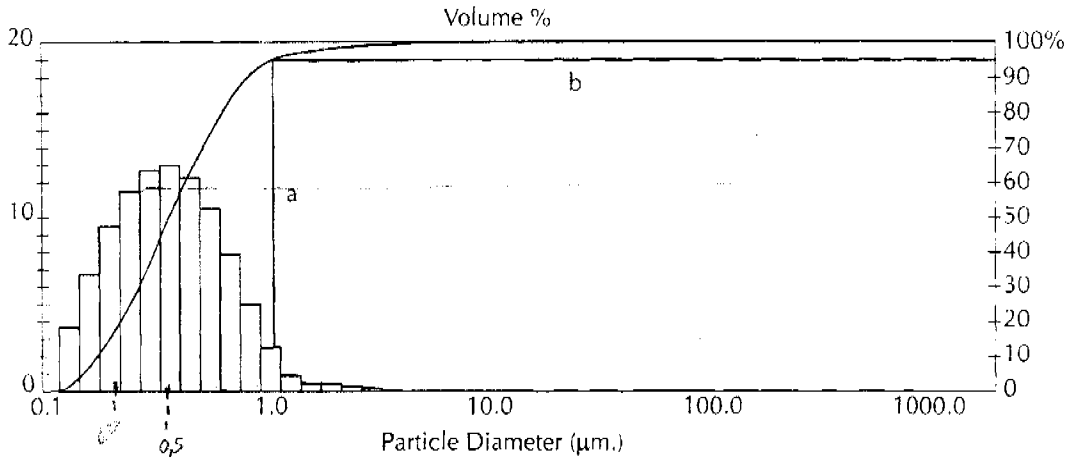
# (54) Adjuvants for use in vaccines

(57) The invention relates to adjuvants that contain a lecithin, an oil and an amphiphilic surfactant and that are capable of forming a stable oil-in-water emulsion vaccine so as to minimize local reactions to the vaccine in the injected animal.

FIG. 1

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## Description

### Field of the Invention

**[0001]** The invention relates to immunological adjuvants. In particular, the invention relates to adjuvants which comprise an oil-in-water emulsion and a surfactant. Adjuvants of the invention are useful in a variety of vaccine formulations, including vaccines comprising bacterial or viral components.

### Background of the Invention

**[0002]** The generation of immunity to infectious organisms is a powerful tool in disease control. Those antigens that induce immunity to infection are known as immunogens. The protective antibody they induce may collaborate with other natural defenses to inhibit the infective process, or they may neutralize harmful products of the infective organism such as toxins.

**[0003]** An effective means of enhancing the antibody response is the use of an adjuvant. Thus, an adjuvant is included in a vaccine as an additive or vehicle to enhance the response to the antigen. An adjuvant may function by different mechanisms, including (1 ) trapping the antigen in the body to cause a slow release, (2) attracting cells of the immune system to the injection site, (3) stimulating cells of the immune system to proliferate and to become activated, and (4) improving antigen dispersion in the recipient's body.

**[0004]** A number of agents with diverse chemical properties have been used as adjuvants, including water-in-oil and oil-in-water emulsions, mineral salts, polynucleotides and natural substances. One adjuvant, known under the trademark AMPHIGEN™, is described in U.S. Patent No. 5,084,269. AMPHIGEN™ adjuvant consists of de-oiled lecithin dissolved in an oil, usually light liquid paraffin. In vaccine preparations AMPHIGEN™ is dispersed in an aqueous solution or suspension of the immunizing antigen as an oil-in-water emulsion.

**[0005]** Problems were observed when using an AMPHIGEN™ adjuvant according to U.S. Patent No. 5,084,269, above. For example, the lecithin in the AMPHIGEN™ does not suffice to produce a stable emulsion of the oil, thus leading to a pool or depot of oil in the injected tissues. Mineral oil can not be metabolized or removed by the animal. As a result, the oil becomes a source of severe chronic inflammation and scarring. Emulsifying the AMPHIGEN™ directly in the antigenic preparation carries the risk of damaging the antigen. Also, if the desired emulsion fails to form, the valuable antigen must be discarded.

**[0006]** An adjuvant useful in vaccines for animals, including humans, that is effective and solves the above problems would therefore be highly desirable.

### Summary of the Invention

**[0007]** The invention relates to an adjuvant useful for the enhancement of the immune response of an animal to an antigen. In particular, the invention relates to an adjuvant that is capable of forming an oil-in-water emulsion in a vaccine composition. The invention also relates to an adjuvant that, when used in a vaccine formulation, causes minimal inflammation and scarring at the vaccination site. The invention further relates to a vaccine formulation that contains an adjuvant of the invention. Finally, the invention relates to a method of using an adjuvant of the invention in a vaccination.

**[0008]** In one embodiment, the adjuvant of the invention comprises a lecithin, an oil and an amphiphilic surfactant capable of emulsifying the adjuvant, for example, a Tween or a Span surfactant. In another preferred aspect, the surfactant is Tween 80, Tween 85, Span 80 or Span 85.

**[0009]** In another embodiment, the adjuvant of the invention comprises a lecithin, an oil and two amphiphilic surfactants capable of emulsifying the adjuvant or a vaccine composition that contains the adjuvant. In a preferred aspect, one of the two surfactants is predominantly found in the aqueous phase, for example, Tween 80, and the other surfactant is predominantly found in the oil phase, for example, Span 80.

**[0010]** A lecithin is a phosphatide. Crude preparations of lecithin may include triglycerides. For purposes of the present invention, "lecithin" encompasses both purified and crude preparations. In a preferred aspect, the lecithin is de-oiled.

**[0011]** Suitable oils include a mineral oil, for example, DRAKEOL™ light mineral oil.

**[0012]** In a further embodiment, the adjuvant of the invention contains an aqueous carrier solution, for example, a physiologically acceptable buffer, water or a saline solution.

**[0013]** In a preferred embodiment, the adjuvant of the invention contains a lecithin, a mineral oil, two amphiphilic surfactants and an aqueous carrier solution (*e.g.*, saline).

**[0014]** In another embodiment of the invention, a method to inactivate a culture of *Bordetella bronchiseptica ("B. bronchiseptica")* using formalin and glutaraldehyde is described. In another aspect, a culture of *B. bronchiseptica* is provided that was inactivated using formalin and glutaraldehyde. In yet another aspect, an antigen composition from

a *B. bronchiseptica* culture is provided that was inactivated using formalin and glutaraldehyde. In yet another aspect, a vaccine composition is provided that contains an antigen composition from a *B. bronchiseptica* culture that was inactivated using formalin and glutaraldehyde.

## Brief Description of the Drawings

**[0015]** Figure 1 presents a graph depicting the distribution of droplet sizes of an emulsion prepared as described below. Lines (a) and (b) depict that about 94% of the droplets have a diameter of 1 μm or less.

## Detailed Description of the Invention

**[0016]** The invention relates to an adjuvant useful for the enhancement of the immune response to an antigen. In particular, the invention relates to an oily adjuvant that is capable of emulsifying a vaccine formulation. Further, the invention relates to an adjuvant that, when used in a vaccine formulation, is capable of substantially avoiding the inflammation or scarring at the injection site, typical of vaccines containing mineral oil. Adjuvants of the invention comprise a lecithin, an oil and an amphiphilic surfactant capable of emulsifying the adjuvant or a vaccine composition that contains the adjuvant.

**[0017]** The invention is based, in part, on the discovery that adding from about 1.5% v/v (*i.e.*, 1.5% volume per volume concentration obtained by, *e.g.*, mixing 98.5 volumes of the vaccine comprising the adjuvant with 1.5 volumes of the amphiphilic surfactant) to 3.5% v/v of an amphiphilic surfactant to a vaccine containing an adjuvant as described in U. S. Patent No. 5,084,269 is effective to sufficiently emulsify a vaccine composition formulated with such an adjuvant and to minimize irritation in the injection site of the vaccinated animal.

**[0018]** In one embodiment, the adjuvant of the invention contains a lecithin and an oil and an amphiphilic surfactant. In one embodiment, the adjuvant of the invention contains a lecithin and an oil and an amphiphilic surfactant capable of emulsifying a vaccine composition formulated with an adjuvant of the invention. In another preferred embodiment, two amphiphilic surfactants are used in an adjuvant of the invention, for example a Tween and a Span surfactant.

**[0019]** A preferred adjuvant, herein referred to as No.1 Adjuvant", comprises about 2% v/v lecithin, about 18% v/v mineral oil, and about 8% v/v surfactant (e.g., about 5.6% v/v Tween 80 and about 2.4% v/v Span 80), with the remaining volume being a saline solution. In a preferred aspect, a vaccine composition is formulated comprising an antigen at a concentration of about 75% v/v and an adjuvant, preferably No. 1 Adjuvant, at a concentration of about 25% v/v of the vaccine composition. All concentrations provided herein in percentage are indicated in volume per volume unless the context indicates otherwise.

## Surfactants Useful in the Adjuvant of the Invention

**[0020]** Surfactants useful for the adjuvant of the invention are amphiphilic and acceptable for veterinary or medical use. Whether or not a particular surfactant is acceptable for medical or veterinary use can be determined by those of ordinary skill in the art. A surfactant is amphiphilic if a part of the surfactant molecule is hydrophobic and a part is hydrophilic. See U.S. Patent Nos. 5,690,942; 5,376,369; 4,933,179 and 4,606,918, which describe surfactants than can be used in the adjuvant of the invention. Examples of surfactants useful in the adjuvant of the invention include, but are not limited to, a Tween surfactant and a Span surfactant. Tween and Span surfactants include, but are not limited to, monolaureate (Tween 20, Tween 21, Span 20), monopalmitate (Tween 40, Span 40), monostearate (Tween 60, Tween 61, Span 60), tristearate (Tween 65, Span 65), monooleate (Tween 80, Tween 81, Span 80) and trioleate (Tween 85, Span 85). In a preferred embodiment, Tween 80, Tween 85, Span 80 or Span 85 is used.

**[0021]** It is preferred that a surfactant useful in the adjuvant of the invention is amphiphilic and has a hydrophilic-lipophilic balance ("HLB") value that is preferably at least about half the sum of the HLB values of all other components of the adjuvant. More preferably, the surfactant has an HLB value that is from about half to about twice the sum of the HLB values of all other components of the adjuvant. More preferably, the surfactant has an HLB value that is about the same as the HLB value of all other components of the adjuvant. HLB values are readily available for surfactants, lecithins, oils and carrier solutions or, if necessary, can be determined through routine experimentation. For example, see U.S. Patent Nos. 4,504,275 and 4,261,925 and references provided therein.

**[0022]** Amphiphilic surfactants useful in the adjuvant of the invention have HLB values from about 2 to about 20, preferably from about 3 to about 17. Methods for determinig the HLB value of particular surfactants are known in the art. See for example U.S. Patents Nos. 5,603,951; 4,933,179 and 4,606,918, which describe surfactants having particular HLB values.

**[0023]** The concentration of a surfactant in a vaccine composition formulated with the adjuvant of the invention is from about 1.5% to 3.5% v/v, more preferably from about 1.5% to about 3% v/v, more preferably from about 1.5% to about 2.5%, and most preferably about 2% v/v. When more than one surfactant is used, the sum of the concentrations

**[0032]** The concentration of a lecithin in a vaccine composition formulated with the adjuvant of the invention is from about 0.25% to about 12.5% v/v, more preferably from about 0.5% to about 10% v/v, more preferably from about 0.5% to about 7.5%, more preferably from about 0.5% to about 5%, more preferably from about 0.5% to about 2.5%, and most preferably from about 0.5% to about 1.25% v/v.

**[0033]** The concentration of a lecithin in a 25% adjuvant is at least about 1 % v/v, preferably at least about 2% v/v. In another aspect, the lecithin concentration in a 25% adjuvant is from about 1% to about 50% v/v, more preferably from about 2% to about 40% v/v, more preferably from about 2% to about 30% v/v, more preferably from about 2% to about 20% v/v, more preferably from about 2% to about 10% v/v and most preferably from about 2% to about 5% v/v. The concentration of a lecithin in the adjuvant of the invention with a higher or lower concentration is determined as exemplified above.

**[0034]** The adjuvant of the invention contains an oil, for example an oil described in U.S. Patent Nos. 5,814,321; 5,084,269. In a preferred aspect, the adjuvant of the invention contains a mineral oil, for example DRAKEOL™. In another aspect, a mixture of oils is used. The oil may be provided for preparation of the adjuvant of the invention as pure oil or as a mixture that contains the oil and another component, for example the lecithin.

**[0035]** The concentration of an oil in a vaccine composition formulated with the adjuvant of the invention is from about 1% to about 23% v/v, more preferably from about 1.5% to about 20% v/v, more preferably from about 2.5% to about 15%, more preferably from about 3.5% to about 10%, more preferably from about 3.5% to about 7.5%, more preferably from about 4% to about 6% v/v, and most preferably about 4.5%.

**[0036]** The concentration of an oil in a 25% adjuvant is at least about 5% v/v, preferably at least about 8% v/v and more preferably at least about 12% v/v. In another aspect, the oil concentration in a 25% adjuvant is from about 4% to about 92% v/v, more preferably from about 6% to about 80% v/v, more preferably from about 10% to about 60% v/v, more preferably from about 14% to about 40% v/v, more preferably from about 14% to about 30% v/v, more preferably from about 16% to about 24% and most preferably about 18%. The concentration of an oil in the adjuvant of the invention with a higher or lower concentration is determined as exemplified above.

**[0037]** In another embodiment, an aqueous carrier is used in the adjuvant of the invention, for example saline (e.g., phosphate-buffered saline), tris-HCl, citrate-phosphate buffer, Hepes buffers, other pharmaceutically acceptable buffers known in the art or water. The pH of the carrier preferably is physiologically acceptable, for example between 6 and 8, most preferably around 7. The aqueous carrier used in the adjuvant of the invention preferably takes up the volume that is not needed for any of the other components.

**[0038]** The adjuvant of the invention is preferably provided at a concentration that is from about 2X to about 10X the concentration after formulation of the adjuvant in a vaccine composition, more preferably from about 2X to about 8X, more preferably from about 3X to about 6X and most preferably about 4X.

Uses of Adjuvants of the Invention

**[0039]** Adjuvants of the invention may be used to enhance the immune response to an antigen of a vaccine formulation. Adjuvants of the invention can be used with antigens derived from any bacteria or from any virus, provided the antigen does not get destroyed or denatured. Examples of antigens, and not by way of limitation, are *Erysipelothrix rhusiopathiae* antigens, *Bordetella bronchiseptica* antigens, antigens of toxigenic strains of *Pasteurella multocida*, antigens of *Eschericia coli* strains that cause neonatal diarrhea, *Actinobacillus pleuropneumoniae* antigens, *Pasteurella haemolytica* antigens, or any combination of the above. Adjuvants of the invention are also useful in vaccine compositions that contain an antigen described in U.S. Patent Nos. 5,616,328 and 5,084,269.

**[0040]** In a preferred embodiment, the adjuvant of the invention is used in a vaccine formulation containing an antigen obtained from the liquid phase of an *Erysipelothrix rhusiopathiae ("E. rhusiopathiae")* culture. In a preferred aspect, a culture of *E. rhusiopathiae* is inactivated by adding formalin (about 0.5% v/v final concentration) and, after incubation for 24 hours at 37° C, the cells were removed, for example by centrifugation or filtration. The culture supernatant, in a preferred embodiment, is concentrated about 10 fold and aluminum hydroxide gel (preferably REHYDRAGEL™) is added to the concentrated supernatant at a final concentration of about 30% v/v to stabilize the antigen. In another preferred embodiment, thimerosal (about 0.01% v/v final concentration) (Dimportex, Spain, imported through Flavine Inc., Klosters, New Jersey) with EDTA (about 0.07% v/v final concentration) are added to the antigens as preservatives. In another preferred embodiment, a vaccine composition is formulated comprising the antigen and the adjuvant of the invention (e.g. No. 1 Adjuvant) with the adjuvant comprising, for example, about 25% v/v of the vaccine composition. This preferred *E. rhusiopathiae* antigen is described in U.S. Patent Application Serial No. _____, filed January 29, 1999, entitled *"Erysipelothrix rhusiopathiae* Antigens and Vaccine Compositions", which is incorporated herein by reference.

**[0041]** In another preferred embodiment, the adjuvant of the invention is used in a vaccine composition containing antigens from a *B. bronchiseptica* culture that has been inactivated by adding formalin thereto in log phase, preferably late log phase, followed by the addition of glutaraldehyde. In addition to killing the bacterial cells, the purpose of this

**[0050]** All percentage concentrations herein are provided in volume per volume unless indicated otherwise. Percentage values, unless otherwise indicated, of an oil-lecithin adjuvant refer to the concentration of a mixture of lecithin (10% of the mixture) and a carrier oil (DRAKEOL™) (90% of the mixture) in an aqueous carrier (continuous phase). For example, a 20% oil-lecithin adjuvant contains 2% v/v lecithin (Central Soya, Fort Wayne, Indiana), 18% v/v DRAKEOL™ 5 (Penreco, Karns City, Pennsylvania) and 80% v/v saline solution (with the saline content being reduced if other components, for example surfactants, are added). The percentage values of an oil-lecithin adjuvant in a vaccine composition, i.e., following dilution of the adjuvant solution with the antigen solution, refer to the concentration of a mixture of lecithin (10% of mixture) and a carrier oil (DRAKEOL™) (90% of mixture) in the vaccine preparation which comprises the adjuvant and a solution containing an antigen, unless the context indicates otherwise. In all cases where a surfactant was added to an adjuvant composition, the percentage values for a surfactant concentration refer to the total concentration of all added surfactants in the adjuvant or the vaccine preparation, unless the context indicates otherwise.

**[0051]** When an oil-lecithin adjuvant was used as an adjuvant in vaccine formulations, it was found that it does not emulsify aqueous preparations without the addition of extra surfactants as the lecithin in the oil-lecithin adjuvant did not suffice for emulsification. Therefore, vaccines made using inadequately dispersed oil-lecithin adjuvant formed a pool or depot of mostly mineral oil in the tissues at the injection site. This oil can not be metabolized or removed by the injected animal and so it remains as a source of severe chronic inflammation and scarring.

**[0052]** It was also determined that adding surfactants to a vaccine formulation comprising an oil-lecithin adjuvant and an antigen in order to emulsify the formulation was not an adequate solution. Problems encountered when adding oil and surfactants to the vaccine formulation before emulsifying were that the antigen could get damaged and, if a suitable emulsion was not achieved, that the formulation would have to be discarded including the valuable antigen.

**[0053]** Different adjuvant compositions were tested comprising an oil-lecithin adjuvant in combination with surfactants to emulsify the vaccine formulations.

Example 2. The Use of an Adjuvant Containing a Surfactant at a Low Concentration

**[0054]** The following example describes the use of an emulsion containing 40% oil-lecithin and 2% of synthetic surfactants, *i.e.*, Tween 80 and Span 80 (Van Water & Rogers, Omaha, Nebraska) in phosphate buffered saline. This adjuvant was prepared aseptically and separate from the antigen. The emulsion was added to the antigen preparation without further emulsification. The synthetic surfactants helped the oil-lecithin adjuvant to disperse as a coarse, relatively stable emulsion. The adjuvant emulsion was added to the aqueous antigenic preparation at the rate of one in eight, decreasing the oil-lecithin adjuvant content from 40% to 5%, and the surfactants from a combined 2% to 0.25%.

**[0055]** The adjuvant was used in several vaccines. It was found that because the emulsion is coarse and not very stable, the oil droplets tend to coalesce and to separate as a permanent, irritating depot of oil in the injected tissues. Another problem observed with this adjuvant was that it aggregates with Al gel. A number of vaccines contain Al gel for a number of purposes like, for example, as an adjuvant or to stabilize an antigen or to bind endotoxin. The oil-lecithin adjuvant carries a negative charge which causes it to bind to the positively charged Al gel to form coarse aggregates. These aggregates are unsightly, difficult to pass through a hypodermic needle, and very irritating to the injected tissues.

Example 3. The Use of an Adjuvant Containing a Surfactant at a High Concentration

**[0056]** An oil-lecithin adjuvant (5% v/v) was emulsified in the antigenic preparation with the help of Tween 80 and Span 80 surfactants, as above, but at a total surfactant concentration of 8% in the vaccine composition. The emulsion was very fine and stable. It had almost the clarity of a solution and it did not cream on standing. Under the microscope, with maximum magnification (resolution 0.2 micron), most droplets were too small to be visible. Thus, it was a microemulsion. This adjuvant, when used in a vaccine formulation, was found to be virtually free of injection-site reactivity and, when Al gel was added, there was no detectable aggregation of oil and gel. As a result of its high surfactant content, this adjuvant is easy to emulsify, attractive in appearance, stable, unreactive with Al gel, and virtually free of irritating effects at the site of vaccination. Despite these advantages, however, this emulsion had slightly lower adjuvant potency compared to the coarse version made with surfactants at a low concentration.

Example 4. The Use of an Adjuvant Containing a Surfactant at a Medium Concentration

**[0057]** An attempt was made to find an adjuvant emulsion that is acceptably smooth and fully potent as an adjuvant. A 20% oil-lecithin adjuvant was used in these experiments as it was found that a 20% oil-lecithin adjuvant emulsion is easier to make than a 40% oil-lecithin adjuvant emulsion. Its addition to vaccines at a rate of one in four, to make a final oil concentration of 5%, would leave 75% of the dose volume for antigens. Preliminary experiments showed that a smooth submicron emulsion (most droplets had a diameter of less than one micron, see Figure 1) could be prepared

US005961970A

# United States Patent [19]

Lowell et al.

[11] Patent Number: 5,961,970

[45] Date of Patent: Oct. 5, 1999

[54] SUBMICRON EMULSIONS AS VACCINE ADJUVANTS

[75] Inventors: **George H. Lowell**, Baltimore, Md.; **Shimon Amselem**, Rehovot, Israel; **Doron Friedman**, Carmei Yosef, Israel; **Haim Aviv**, Rehovot, Israel

[73] Assignees: **Pharmos Corporation**, New York, N.Y.; **The United States of America as represented by the Secretary of the Army**, Washington, D.C.

[21] Appl. No.: **08/637,756**

[22] PCT Filed: **Oct. 29, 1993**

[86] PCT No.: **PCT/US93/10402**

§ 371 Date: **Apr. 29, 1996**

§ 102(e) Date: **Apr. 29, 1996**

[87] PCT Pub. No.: **WO95/11700**

PCT Pub. Date: **May 4, 1995**

[51] **Int. Cl.**[6] ..... **A61K 39/00**; A61K 9/127; A61K 9/58; A01N 63/00

[52] **U.S. Cl.** ..... **424/93.1**; 424/184.1; 424/279.1; 424/283.1; 424/455; 424/450; 424/462; 424/278.1; 424/490; 424/452

[58] **Field of Search** ..... 424/279.1, 278.1, 424/283.1, 455, 452, 490, 450, 462, 184.1, 93.1

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*Primary Examiner*—Nita Minnifield

*Attorney, Agent, or Firm*—Pennie & Edmonds LLP

[57] **ABSTRACT**

A vaccine adjuvant composition of an oil-in-water submicron emulsion that has about 0.5 to 50% of a first component of an oil, about 0.1 to 10% of a second component of an emulsifier, about 0.05 to 5% of a nonionic surfactant, about 0.00001 to 1% of an immunogen, and an aqueous continuous phase. This submicron emulsion has a mean droplet size in the range of between about 0.03 and 0.5 $\mu$m, and preferably 0.05 and 0.2 $\mu$m.

**25 Claims, 5 Drawing Sheets**

intrinsically and extrinsically as extensively detailed in the examples. In general, SME intrinsic formulations are prepared by emulsifying the antigen together with the SME components, while SME extrinsic formulations are prepared by adding externally the antigen to previously prepared plain SME.

Dehydrated SME Adjuvants

A further aspect of the invention provides dehydrated emulsions, made by dehydrating the submicron emulsion of the types described herein. Dehydrated submicron emulsions may be stored for prolonged periods with minimal degradation, then reconstituted with water shortly before use. Residual water content in the dehydrated emulsion is usually less than 5% (w/w), commonly less than 2%, and often less than 1%.

Dehydration may be performed by standard methods, such as drying under reduced pressure; when the emulsion is frozen prior to dehydration, this low pressure evaporation is known as lyophilization. Freezing may be performed conveniently in a dry ice-acetone or ethyl alcohol bath. The pressure reduction may be achieved conveniently with a mechanical vacuum pump, usually fitted with a liquid nitrogen cold trap to protect the pump from contamination. Pressures in the low millitorr range, e.g., 10–50 millitorr, are routinely achievable but higher or lower pressures are sufficient.

A cryoprotectant or anticoalescent compound may be added to the emulsion prior to dehydration to inhibit flocculation and coalescence upon rehydration. The cryoprotectant may be of any type known in the art, including sugars and polysaccharides such as sucrose or trehalose, and non-natural polymers such as polyvinylpyrrolidone. Cryoprotectants are usually present at less than 25%, commonly 10%, more commonly 5%, 4% (w/v) or less in the emulsion before lyophilization.

A preferred category of cryoprotectants is amino acids and oligopeptides. Preferred amino acids include valine, leucine, isoleucine, lysine, methionine, threonine, serine, arginine, alanine, glycine, histidine, proline, phenylalanine, taurine, and carnitine, although any of the other natural amino acids may also be present. Amino acids may be of either D or L configuration, or a mixture; the natural L form is preferred. Amino acids may be present as their salts or esters, and as mixtures of amino acids or as pure species.

A particularly preferred amino acid is glycine, which may be present either in pure form or as a component of a mixture, e.g. in an hydrolyzate of collagen or other glycine-rich protein.

Mixtures of oligopeptides, especially di- and tripeptides, are another preferred type of cryoprotectant. These may be prepared conveniently as partial protein hydrolysates or enzymatic digests.

The cryoprotective amino acids or oligopeptides are generally present in the emulsion at a concentration of about 0.25 to 25% (w/w), preferably about 0.5 to 12% (w/w), more preferably about 1 to 10% (w/w) and commonly 3–6% (w/w).

Cryoprotectants and methods of making lyophilized submicron emulsions are taught in more detail in Pharmos Corp. PCT International Application Publication WO 93/15736 entitled "Dry Compositions for Preparing Submicron Emulsions", the content of which is expressly incorporated herein by reference.

## EXAMPLES

This invention is illustrated by the following non-limiting examples:

### Example 1

Preparation of Intrinsic-gp160-SME vaccine

Antigen Description and Background

The urgency and high priority for developing an effective vaccine against the human immunodeficiency virus (HIV) are fully recognized. The reasons for using subunits of the virus as the basis of an HIV vaccine are the perceived overwhelming requirements for safety. Despite the high efficacy of many live attenuated viral vaccines, the requirement for product safety, especially in the case of retroviruses, has favored the subunit approach to the extent that all of the current candidate preparations in clinical prophylactic trials are of this type, being mainly gp160, the envelope protein of HIV, or part thereof. Studies have shown that gp160 attaches the virus to the cell and also facilitates the fusion of the cell and virus during the early stages of infection.

The gp160 antigen used in this example was supplied by MicroGeneSys Inc. This gp160 recombinant protein in alum-adjuvanted vaccine formulation is currently under evaluation in human clinical trials.

Preparation of Oil Phase

The oil phase was composed of MCT oil (2.0 g Mygliol 812, Hulls, Germany), lecithin (0.4 g, Lipoid E-80, Germany), and DL-α-tocopherol succinate (8.0 mg, Merck, Germany). The lipids and oil were weighed in a 250-ml beaker and mixed at room temperature using a magnetic stirrer during 2–4 hrs until a homogenous and almost clear solution was obtained.

Preparation of Water Phase

Polysorbate 80 (1% w/v, Montanox 80, DF, Seppic, France), Glycerol (2.2% w/v, Merck, Germany), EDTA (0.1% w/v, Merck, Germany), and purified water (to 100% w/v) were dissolved at room temperature in a 250-ml beaker by gentle shaking using a magnetic stirrer plate until a clear homogenous solution was obtained (about 15–20 min). A total volume of 40 ml of water phase was prepared. A vial containing 2.1 ml of gp160 recombinant protein (MicroGeneSys, Inc., CT, USA) at a concentration of 0.25 mg/ml in saline was added to the water phase and the mixture was gently shaken for 5 min.

Mixing of Oil and Water Phase

The oil phase was heated to 40° C. and added to the beaker containing the 40 ml of water phase. The mixture was gently stirred for 10–15 min at room temperature.

Preparation of Oil-in-Water Coarse Emulsion

An oil-in-water emulsion containing the antigen was prepared using the medium-sized disperser and homogenizing unit Polytron PT3000 (Kinematics, Switzerland) at 3,600 rpm for 30 sec. The resultant micronsize emulsion was cooled at room temperature.

Sizing of Emulsion to Submicron Range

The droplet size of the emulsion obtained after Polytron step was lowered to the submicron (nanosize) range by submitting the emulsion to high shear homogenization using the Gaulin Microlab 70 High Pressure Homogenizer (APV Gaulin, Germany) at 800 bar pressure. A total of 10 cycles were performed. The particle size distribution of the resultant formulation was determined using an N4MD Coulter Particle Size Analyzer (Coulter Electronics, England). The differential weight % mode of the instrument indicated the existence of homogeneous population of SME droplets with

D3 165

US006306405B1

(12) **United States Patent**
**O'Hagan et al.**

(10) **Patent No.: US 6,306,405 B1**
(45) **Date of Patent: Oct. 23, 2001**

(54) **USE OF MICROPARTICLES COMBINED WITH SUBMICRON OIL-IN-WATER EMULSIONS**

(75) Inventors: **Derek O'Hagan**, Berkeley; **Gary Van Nest**, El Sobrante; **Gary S. Ott**, Oakland; **Manmohan Singh**, Hercules, all of CA (US)

(73) Assignee: **Chiron Corporation**, Emeryville, CA (US)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/564,416**

(22) Filed: **May 2, 2000**

**Related U.S. Application Data**

(62) Division of application No. 09/015,736, filed on Jan. 29, 1998.

(60) Provisional application No. 60/069,724, filed on Dec. 16, 1997.

(51) **Int. Cl.**[7] ..... **A61K 45/00**; A61K 39/12; A61K 39/29

(52) **U.S. Cl.** ..... **424/278.1**; 424/204.1; 424/228.1; 424/283.1

(58) **Field of Search** ..... 424/283.1, 70.11, 424/70.19, 204.1, 228.1, 278.1, 280, 288.1

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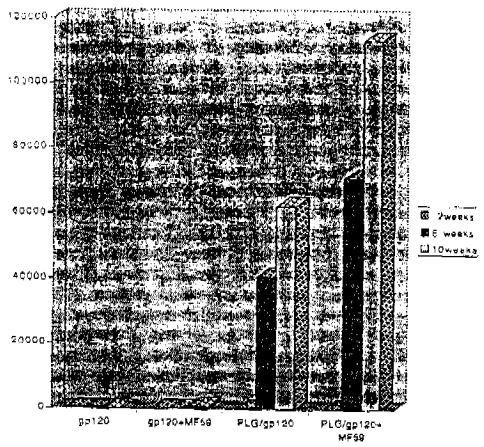
*Primary Examiner*—Hankyel T. Park
*Assistant Examiner*—Stacy S. Brown
(74) *Attorney, Agent, or Firm*—Roberta L. Robins; Alisa A. Harbin; Robert P. Blackburn

(57) **ABSTRACT**

Compositions are provided which include biodegradable microparticles with entrapped or adsorbed antigens, in combination with submicron oil-in-water emulsions. Also provided are methods of immunization which comprise administering to a vertebrate subject (a) a submicron oil-in-water emulsion, and (b) a therapeutically effective amount of a selected antigen entrapped in a microparticle.

**20 Claims, 1 Drawing Sheet**



while PLG particles biodegrade by random nonenzymatic hydrolysis of ester bonds to lactic and glycolic acids which are excreted along normal metabolic pathways.

Recent studies have shown that PLG microparticles with entrapped antigens are able to elicit cell-mediated immunity. For example, microencapsulated human immunodeficiency virus (HIV) gp120 has been shown to induce HIV-specific CD4+ and CD8+ T-cell responses in mice (Moore et al., *Vaccine* (1995) 13:1741–1749). Similarly, microparticle-encapsulated ovalbumin has been shown to be capable of priming cellular immune responses in vivo and can induce mucosal IgA responses when administered orally (O'Hagan et al., *Vaccine* (1993) 11:149–154). Additionally, both antibody and T-cell responses have been induced in mice vaccinated with a PLG-entrapped *Mycobacterium tuberculosis* antigen (Vordermeier et al., *Vaccine* (1995) 13:1576–1582). Antigen-specific CTL responses have also been induced in mice using a microencapsulated short synthetic peptide from the circumsporozoite protein of *Plasmodium berghei*.

However, the use of microparticles with entrapped or adsorbed antigen, in combination with submicron oil-in-water emulsions, has not heretofore been described.

## DISCLOSURE OF THE INVENTION

The present invention is based on the surprising and unexpected discovery that the use of biodegradable microparticles, such as those derived from a poly (α-hydroxy acid), and including entrapped or adsorbed antigen, in combination with submicron oil-in-water emulsions, serves to enhance the immunogenicity of the antigen. The use of such combinations provides a safe and effective approach for enhancing the immunogenicity of a wide variety of antigens.

Accordingly, in one embodiment, the invention is directed to a composition comprising a submicron oil-in-water emulsion, and a selected antigen entrapped in, or adsorbed to, a biodegradable microparticle.

In another embodiment, the invention is directed to a composition comprising (a) a submicron oil-in-water emulsion which comprises 4–5% w/v squalene, 0.25–0.5% w/v TWEEN 80®, and 0.5% w/v SPAN® 85, and optionally, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-huydroxyphosphoryloxy)-ethylamine, and (b) a selected antigen entrapped in, or adsorbed to, a biodegradable microparticle.

In yet another embodiment, the subject invention is directed to a method of immunization which comprises administering to a vertebrate subject (a) a submicron oil-in-water emulsion, and (b) a therapeutically effective amount of a selected antigen entrapped in, or adsorbed to, a biodegradable microparticle.

In still further embodiments, the invention is directed to a method of making a composition comprising combining a submicron oil-in-water emulsion with a selected antigen entrapped in, or adsorbed to, a biodegradable microparticle.

In particularly preferred embodiments, the microparticle is derived from a poly (α-hydroxy acid), preferably poly (L-lactide), poly (D,L-lactide) or poly (D,L-lactide-co-glycolide).

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows total IgG titers at 2, 6 and 10 weeks following initial vaccination in mice immunized with gp120; gp120+MF59; PLG with entrapped gp120; and PLG with entrapped gp120+MF59.

## DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., *Remington's Pharmaceutical Sciences*, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); *Methods In Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I–IV (D.M. Weir and C.C. Blackwell, eds., 1986, Blackwell Scientific Publications); and Sambrook, et al., *Molecular Cloning*: A Laboratory Manual (2nd Edition, 1989).

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

I. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The term "microparticle" as used herein, refers to a particle of about 100 nm to about 150 μm in diameter, more preferably about 200 nm to about 30 μm in diameter, and most preferably about 500 nm to about 10 μm in diameter. Preferably, the microparticle will be of a diameter that permits parenteral administration without occluding needles and capillaries. Microparticle size is readily determined by techniques well known in the art, such as photon correlation spectroscopy, laser diffractometry and/or scanning electron microscopy. Microparticles for use herein will be formed from materials that are sterilizable, non-toxic and biodegradable. Such materials include, without limitation, poly (α-hydroxy acid), polyhydroxybutyric acid, polycaprolactone, polyorthoester, polyanhydride. Preferably, microparticles for use with the present invention are derived from a poly(α-hydroxy acid), in particular, from a poly (lactide) ("PLA") or a copolymer of D,L-lactide and glycolide or glycolic acid, such as a poly (D,L-lactide-co-glycolide) ("PLG" or "PLGA"), or a copolymer of D,L-lactide and caprolactone. The microparticles may be derived from any of various polymeric starting materials which have a variety of molecular weights and, in the case of the copolymers such as PLG, a variety of lactide:glycolide ratios, the selection of which will be largely a matter of choice, depending in part on the coadministered antigen. These parameters are discussed more fully below.

By "antigen" is meant a molecule which contains one or more epitopes that will stimulate a host's immune system to make a cellular antigen-specific immune response when the antigen is presented, or a humoral antibody response. Normally, an epitope will include between about 3–15, generally about 5–15, amino acids. For purposes of the present invention, antigens can be derived from any of several known viruses, bacteria, parasites and fungi. The term also intends any of the various tumor antigens. Furthermore, for purposes of the present invention, an "antigen" refers to a protein which includes modifications, such as deletions, additions and substitutions (generally conservative in nature), to the native sequence, so long as the

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO**
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2020
Bogotá, D.C.

Abogado
**CARLOS R.OLARTE**

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| ASUNTO | Radicación | 0598906 |
| | Trámite | 011 |
| | Evento | 379 |
| | Actuación | 411 |
| | Oficio | [illegible] 59 |
| | Folios | 015 |

Patente de Invención ■ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (fase nacional) ☐ Cap.I ☐ Cap. II ■

No. Sol. Internacional PCT/IB2004/000937

Fecha de Presentación Internal. Marzo 22 de 2004

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

**1. Documentos estudiados**

| | | | |
|---|---|---|---|
| 1.1. | Descripción: | Folios: 41 a 114 | como se radicó inicialmente |
| 1.2. | Reivindicaciones: | Folios: 115 a 119 | como se radicó inicialmente |
| 1.3. | Prioridad: | Abril/ 04/2003, US,60460301 | |

**2. Objeto de la invención**

Título de la invención: **EMULSIONES DE ACEITE EN AGUA MICROFLUIDIZADAS Y COMPOSICIONES DE VACUNAS.**

Problema Técnico Planteado: La necesidad de un adyuvante, con larga vida útil, para potenciar la capacidad inmunógena de los antígenos.

Solución: Proporciona formulaciones de emulsiones submicrométricas de aceite en agua como adyuvante de vacuna.

IPC: A61K39/39.

## 3. De la solicitud de Patente

3.1. Descripción *(artículo 28 D. 486)*

Se utiliza el término "aproximadamente" el cual no es conciso.

3.2. Reivindicaciones *(artículo 30 D 486)*

La reivindicación 3, utiliza el término "aproximadamente" el cual no es conciso.

## 4. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

| Nº | Documento | Título | Fecha de publicación* |
|---|---|---|---|
| **D1** | EP1023904 | Adjuvants for use in vaccines | febrero/08/2000 |
| **D2** | US5961970 | Submicron emulsions as vaccine adjuvants | Octubre/05/1999 |
| **D3** | US6306405 | Use of microparticles combined with submicron oil-water emulsions | Octubre/23/2001 |

D1 (folios 158-162); D2 (folios 163-164 ); D3 (folios 165-166)

## 5. Análisis de patentabilidad (artículo 14 D486)

5.1 Novedad (artículo 16 D486)

La reivindicación 1, reclama una emulsión submicrométrica de aceite en agua como adyuvante de vacuna, con un tamaño de gota inferior a 1µm, que comprende:

1-Un aceite no metabolizable ligero
2-Un tensioactivo
3-Componente acuoso

**D1**, divulga una emulsión submicrométrica de aceite en agua como adyuvante de vacuna, con tamaño de gota inferior a 1µm (folio 159, párrafo 07, líneas 36-37, párrafo 9, línea 44; párrafo 12, línea 53) y folio 162 , párrafo 57, líneas 57-58) que comprende:

1-Un aceite no metabolizable ligero 18% V/V (folio 159 párrafo 11, línea 51, folio 160 párrafo 19, línea 27)
2-Un tensioactivo 2% V/V (folio 160 párrafo 19, línea 27)
3-Componente acuoso

De la comparación anterior se concluye que las reivindicaciones 1 y 2, no son nuevas.

La reivindicación 6, reclama una composición que comprende una emulsión de aceite y agua (reivindicación 1) y un antígeno.

**D1**, divulga una emulsión submicrométrica de aceite en agua como adyuvante de vacuna, con tamaño de gota inferior a 1µm (folio 159, párrafo 07, líneas 36-37, párrafo 9, línea 44; párrafo 12, línea 53) y folio 162, párrafo 57, líneas 57-58) que comprende:

1-Un aceite no metabolizable ligero 18% V/V (folio 159 párrafo 11, línea 51, folio 160 párrafo 19, línea 27)
2-Un tensioactivo 2% V/V (folio 160 párrafo 19, línea 27)
3-Componente acuoso
4-un antígeno (vírico ó bacteriano, ej: E. coli) ( folio 161, párrafo 39, líneas 36-40).

De la comparación anterior se concluye que las reivindicaciones 6-9, no son nuevas.

5.2 Nivel inventivo *(artículo18 D486)*

La reivindicación 3, reclama una emulsión submicrométrica de aceite en agua como adyuvante de vacuna, con tamaño de gota de aceite entre 0.1 y 0.5µm, que comprende:

1-Un aceite no metabolizable ligero aproximadamente 40 % V/V
2-Tensioactivos aproximadamente 10% V/V de lecitina, aproximadamente 0.18% V/V de Tween-80 y 0.08% V/V de Span-80
3-Componente acuoso

El documento del Estado de la Técnica más próximo es **D1,** que divulga una emulsión submicrométrica de aceite en agua como adyuvante de vacuna, con tamaño de gota inferior a 1µm (folio 159, párrafo 07, líneas 36-37, párrafo 9, línea 44; párrafo 12, línea 53) y folio 162, párrafo 57, líneas 57-58) que comprende:

1-Un aceite no metabolizable ligero 18% V/V (folio 160, párrafo 19, línea 27)
2-Un tensioactivo 2% V/V (folio 160, párrafo 19, línea 27)
3-Componente acuoso

La diferencia entre la materia reclamada y **D1,** es que la solicitud menciona otras concentraciones de aceite metabolizable ligero: aproximadamente 40 % V/V y de tensioactivos: aproximadamente 10% V/V de lecitina, aproximadamente 0.18% V/V de Tween-80 y 0.08% V/V de Span-80.

Efecto técnico: El hecho de utilizar otras concentraciones para los componentes que hacen parte de la emulsión submicrométrica, no muestra un efecto técnico inesperado.

El problema técnico objetivo en esta solicitud es: obtener una composición, alternativa, de emulsión submicrométrica de aceite en agua, como adyuvante de vacuna, con tamaño de gota inferior a 1µm.

Por esta razón se considera que modificar las concentraciones de los componentes de la emulsión no le confiere paso inventivo a la materia reclamada.

Como resultado del análisis anterior, se concluye que la reivindicación de 3, **no tiene Nivel Inventivo.**

La reivindicación 4, reclama un procedimiento para preparar la emulsión submicrométrica que comprende:

1-mezclar el aceite no metabolizable ligero, con un tensioactivo y el componente acuoso.
2-emulsificar la mezcla anterior
3-microfluidizar la emulsión de 2.

El documento del Estado de la Técnica más próximo es **D2,** que divulga un método de preparación de una emulsión submicrométrica, que consiste en:

1-mezclar la fase oleosa con la acuosa
2-homogenizar para obtener una emulsión.
3-La emulsión 2) se somete una etapa para obtener un tamaño de partícula submicrométrica. (folio 164, columna 10, líneas 28-59)

La diferencia entre el método reclamado y **D2,** es que la solicitud menciona un aceite no metabolizable ligero.

Efecto técnico: El hecho de utilizar un aceite no metabolizable ligero en el proceso de fabricación de la emulsión reclamada, no da evidencia de un efecto técnico inesperado.

El problema técnico objetivo en esta solicitud es: como obtener una composición, alternativa, de emulsión submicrométrica de aceite en agua, como adyuvante de vacuna, con tamaño de gota inferior a 1µm.

Por esta razón se considera que modificar el tipo de aceite en los componentes de la emulsión, ya mencionado en **D1** (aceite no metabolizable) no le confiere paso inventivo al proceso de fabricación reclamado.

La reivindicación 5, reclama un procedimiento de fabricación de acuerdo con la reivindicación 4, para obtener una emulsión con un rango de concentraciones de aceite y lecitina, lo cual no le da paso inventivo al procedimiento reclamado.

Como resultado del análisis anterior, se concluye que las reivindicaciones 4 y 5, **no tienen Nivel Inventivo.**

La reivindicación 10, reclama un procedimiento para preparar la emulsión submicrométrica que comprende:

1-mezclar el aceite no metabolizable ligero, con un tensioactivo y el componente acuoso.
2- añadir un antígeno a la mezcla 1).
3-emulsificar la mezcla anterior
4-microfluidizar la emulsión de 3)

El documento del Estado de la Técnica más próximo es **D2,** que divulga un método de preparación de una emulsión submicrométrica, que consiste en:

1-mezclar la fase oleosa con la acuosa
2-homogenizar para obtener una emulsión con antígeno (folio 164, columna 10, línea 52)
3-La emulsión 2) se somete una etapa para obtener un tamaño de partícula submicrométrica. ( folio 164, columna 10, líneas 28-59)

La diferencia entre el método reclamado y **D2,** es que la solicitud menciona un aceite no metabolizable ligero.

Efecto técnico: Por el hecho de utilizar un aceite no metabolizable ligero en el proceso de fabricación de la emulsión reclamada, no se evidencia un efecto técnico inesperado.

El problema técnico objetivo en esta solicitud es: como obtener una composición, alternativa, de emulsión submicrométrica de aceite en agua, como adyuvante de vacuna, con tamaño de gota inferior a 1µm.

Por esta razón se considera que modificar el tipo de aceite en los componentes de la emulsión, ya mencionado en **D1** (aceite no metabolizable ligero) no le confiere paso inventivo al método de fabricación reclamado.

Las reivindicación 11-13, reclaman un procedimiento de fabricación de acuerdo con la reivindicación 10, que no agregan características nuevas ni inventivas al proceso reclamado en la reivindicación 10, por lo tanto no le dan paso inventivo a dicho procedimiento.

Como resultado del análisis anterior, se concluye que las reivindicaciones 10-13, **no tienen Nivel Inventivo.**

La reivindicación 14, reclama una composición de vacuna que comprende:

1-un antígeno microencapsulado y una emulsión de aceite en agua (reivindicación 1).

El documento del Estado de la Técnica más próximo es **D3,** que divulga una composición, que comprende:

1- un antígeno microencapsulado en combinación con una emulsión submicrométrica de aceite en agua (folio 166, columna 3, líneas 25-31).

La diferencia entre la composición reclamada y **D3,** es que la solicitud menciona un aceite no metabolizable ligero.

Efecto técnico: Por el hecho de utilizar un aceite no metabolizable ligero, en la composición reclamada, no se muestra un efecto técnico inesperado.

El problema técnico objetivo en esta solicitud es: obtener una composición, alternativa, de vacuna con un antígeno microencapsulado y una emulsión submicrométrica de aceite en agua, como adyuvante de vacuna, con tamaño de gota inferior a 1µm.

Por esta razón se considera que modificar el tipo de aceite en los componentes de la emulsión, ya mencionado en **D1** (aceite no metabolizable ligero) no le confiere paso inventivo a la composición reclamada.

La reivindicación 15, reclama una composición de acuerdo con la reivindicación 14, con un rango de concentraciones de aceite y lecitina, lo cual no le da paso inventivo a la composición reclamada.

## 6. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

| N° | Documento N° | Reivindicaciones afectadas | Requisito que afecta |
|---|---|---|---|
| D1 | EP1023904 | 1-2, 6-9<br>3-5, y 10-15 | Novedad<br>Nivel Inventivo |
| D2 | US5961970 | 4-5, y 10-13 | Nivel Inventivo |
| D3 | US6306405 | 14-15 | Nivel Inventivo |

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.

**ALIX CARMENZA CÉSPEDES DE VERGEL**
Jefe de División de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de fecha 10 JUL. 2009

| **CONCEPTO TÉCNICO** No.1940 | **EXAMINADOR TÉCNICO**<br>Código No.202078 |
|---|---|