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(54) Titre : COMPOSITION D'ADJUVANT CONTENANT DU LIPOPOLYCACCHARIDE HYDROPHOBIQUE  
SYNTHETIQUE  
(54) Title: ADJUVANT COMPOSITION CONTAINING SYNTHETIC HYDROPHOBIC LIPOPOLYSACCHARIDE

(57) **Abrégé/Abstract:**

According to the present invention the adjuvants comprise an emulsion containing at least one synthetic hydrophobic lipopolysaccharide, which can either or not be provided with anionic groups, while maintaining the overall hydrophobicity, an interface-forming constituent (such as an oil) and optionally an aqueous solute. In particular the adjuvant and vaccines prepared with it contain a lipopolysaccharide which had an HLB value of less than 9, more favorably less than 4.

## ABSTRACT

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According to the present invention the adjuvants comprise an emulsion containing at least one synthetic hydrophobic lipopolysaccharide, which can either or not be provided with anionic groups, while maintaining the overall hydrophobicity, an interface-forming constituent (such as an oil) and optionally an aqueous solute. In particular the adjuvant and vaccines prepared with it contain a lipopolysaccharide which had an HLB value of less than 9, more favorably less than 4.

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## ADJUVANT COMPOSITION CONTAINING SYNTHETIC

## HYDROPHOBIC LIPOPOLYSACCHARIDE

The present invention is concerned with adjuvants and with vaccines containing these adjuvants.

5 According to the present invention the adjuvants comprise an emulsion containing at least one synthetic hydrophobic lipopolysaccharide, which can either or not be provided with anionic groups, while maintaining the overall hydrophobicity, an interface-forming constituent and  
10 optionally an aqueous solute.

In one aspect, the invention provides an adjuvant composition comprising at least one synthetic hydrophobic lipopolysaccharide and an interface-forming constituent, wherein the lipopolysaccharide has a  
15 hydrophilic-lipophilic-balance (HLB) value with the range of 4 to less than 9.

These adjuvants can be used in the preparation of veterinary or human vaccines.

The polysaccharides forming part of said synthetic  
20 hydrophobic lipopolysaccharides are esterified with straight or branched lipid groups of 8 to 40 carbon atoms; these lipid groups may be aliphatic or unsaturated, and optionally may contain an aromatic group. The mean ratio of lipid groups to sugar groups of the polysaccharides preferably is  
25 between 0.2 and 4, and more in particular can be about 1.

Optionally the lipopolysaccharide can be equipped with anionic groups, such as phosphate, sulfate, nitrate or carboxyl groups, preferably sulfate groups, while maintaining the overall hydrophobicity. Preferably, the

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mean ratio of anionic groups to sugar groups is between 0.1 and 2.

Hydrophobicity of SLPs is defined by the hydrophilic-lipophilic-balance (HLB)-value determined according to the method of Griffin (W.C. Griffin, J. Soc. Cosmet. Chem., 1, 311, 1949) and cited by Porter (M.R. Porter, Handbook of surfactants, Blackie, Glasgow and London, 1991, page 42) which is based on the appearance of a surfactant in water. HLB-values of the hydrophobic SLPs are below 9 (i.e. insoluble, poor and unstable dispersion or stable opaque dispersion), and in particular it can be below 4 (i.e. insoluble).

Examples of interface-forming constituents are:

- a) liquids immiscible with an aqueous phase e.g. mineral, animal, vegetable or synthetic oil or other organic liquid,
- b) insoluble salts e.g.  $\text{Al}(\text{OH})_3$ ,  $\text{AlPO}_4$ , calcium-oxalate, vermiculite, bentonite, silica,
- c) microparticles/microspheres of one or multiple polymers or copolymers e.g.

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polyacrylate, poly(methyl-methacrylate), polycyanoacrylate, polysaccharides, polypeptides, poly(ethylene-vinyl acetate), poly(lactic acid), poly(glycolic acid), poly(lactic/glycolic acid), polyoxyethylene-polyoxypropylene, polyethyleneimine, polyamidoamine.

- 5 d. lipid bilayers of lipophilic agents e.g. phospholipids, quaternary amines.
- e. micelles of one or more of the following surface-active agents:
- anionic (e.g. carboxylates, polyalkoxycarboxylates, N-acylsarcosinates, acylated protein hydrolysates, sulfonates, alkylbenzenesulfonates, sulfonates with ester linkages, sulfonates with amide linkages, sulfonates with ether linkages, alcohol sulfates, phosphate esters),
  - 10 - nonionic (e.g. ethoxylates, alcoholethoxylates, carboxylic acid esters, glycerolesters, polyoxyethylene esters, carboxylic amides, polyoxyethylene fatty acid amides, polyalkylene oxide block copolymers),
  - cationic (e.g. amines, oxygen containing amines, 2-alkyl-1-(2-hydroxyethyl)-2-imidazolines, quaternary amines), and
  - 15 - amphoteric (e.g. imidazolinium derivatives) surface-active agents.

From the partial point of view, interface-forming substances which give in combination with the SLP stable formulations and which are well tolerated by the recipients (either animals or humans) are preferred. In this respect, oils of animal and vegetable origin, insoluble salts such as  $Al(OH)_3$  and  $AlPO_4$ , and lipid bilayers of phospholipids of animal or vegetable origin are particularly suitable.

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In the examples described below, mineral oil or squalane is used as an interface-forming constituent. Squalane is preferred as this is an oil of animal origin and therefore biocompatibility and biodegradability are expected.

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Examples (with various trade names) of surface-active agents with appropriate hydrophilic/lipophilic balance (HLB) value to improve the stability of oil-in-water or water-in-oil emulsions are:

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- a. anionic surfactants e.g. Sandopan<sup>TM</sup> KST;
- b. nonionic surfactants e.g. Brij type numbers 30, 35, 58, 98, 721, Triton<sup>TM</sup> type numbers N-57, X-100, X-102, Span type numbers 20, 40, 60, 80, 85, Tween<sup>TM</sup> type numbers 20, 21, 40, 60, 80, 85, Pluronic<sup>TM</sup> type numbers PE 10100, PE 10500, RPE 2510, RPE 2520,
- 35 c. cationic surfactants e.g. Arquat<sup>TM</sup> 2HT-75, Arosurf<sup>TM</sup> TA100, bis(hydrogenated tallow alkyl) dimethylammonium chloride,

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d. amphotheric surfactants e.g. Lonzaine<sup>TM</sup> type numbers 10S, 12C, 16S,  
Scherotaine<sup>TM</sup> types CAB, IAB, MAB, Amphosol<sup>TM</sup>.

In the experiments described below a nonionic surfactant and especially Tween 80  
5 was used to stabilize the emulsion of SLP and oil.

Examples of animal oils are squalane and squalene. Suitable plant oils can be  
obtained from soya, peanut, sesame seeds, palm, etcetera. Examples of mineral oils  
10 are Markol<sup>TM</sup> 52, Krakel<sup>TM</sup>, Kremol<sup>TM</sup>, etc.

Aqueous solutes for use in the adjuvant according to the present invention are e.g.  
saline, phosphate buffered saline or pure water.

The adjuvant according to the invention preferably is prepared either by first mixing  
15 the synthetic hydrophobic lipopolysaccharide and the oil, and further dispersing the  
oily mass in water or by first mixing the lipopolysaccharide with a surface-active  
agent, adding water, and dispersing the oil in the water phase obtained.

The adjuvant is intended for use in vaccines for humans and animals. The vaccine  
20 can contain antigenic material characteristic for viruses, bacteria, mycoplasma or  
parasites, or for any other entity against which an immune response is intended to  
be raised. This antigenic material can for example consist of or contain live  
organisms, inactivated organisms, or so-called subunits (the latter e.g. prepared  
25 synthetically, or by recombinant DNA methods, or isolated from the intact  
organisms). For the preparation of the vaccines the antigenic material is mixed either  
well before or just prior to use.

The invention is further illustrated by the following experiments.

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#### EXAMPLE 1:

##### Use of SLP-dispersions as adjuvant

35 In the experiments described below, a lipophilic sulfolipopolysaccharide (SLP)-  
derivative was used, namely a SLP-derivative with a mean  
sulfate:lipid:monosaccharide-ratio of approximately 0.1:0.8:1.0, unless stated

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otherwise.

Vaccines used in the different experiments were prepared in one of the following manners:

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I. SLP was solubilized in the oil phase (squalane) at an appropriate concentration. Solubilization of the SLP could be improved by slightly heating the squalane (70 °C). Subsequently, the oil phase was added to an aqueous phase (phosphate buffered saline, containing 2% Tween 80) prewarmed at 70 °C, and vigorously stirred. The pre-emulsion obtained was emulsified further by either ultrasonic disrupture or microfluidizing. The emulsion was considered to be ready-for-use if the oil droplets were smaller than 1 micrometer (estimated by phase contrast microscopy at 1000-fold magnitude). The vaccine was prepared by mixing one volume of adjuvant solution (of about 4°C) with one volume of antigen solution.

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The quantity of SLP indicated is expressed as the sugar content using Ficoll<sup>TM</sup> as the reference (dry material is about two fold higher).

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II. SLP was solubilized in an organic solvent (e.g. ethanol, chloroform or dichloromethane) and mixed with the oil. The solvent was eliminated by extensive evaporation at 60 or 70 °C and low pressure. Subsequently, the oil phase was added to an aqueous phase (phosphate buffered saline, containing 2% Tween 80) prewarmed at 70 °C under vigorous stirring. The pre-emulsion obtained was emulsified further by either ultrasonic disrupture or microfluidizing. The emulsion was considered to be ready-for-use if the oil droplets were smaller than 1 micrometer (estimated by phase contrast microscopy at 1000-fold magnitude). The vaccine was prepared by mixing one volume of adjuvant solution (of about 4°C) with one volume of antigen solution.

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III. SLP was added to the adjuvant solution (e.g. mineral oil-in-water emulsion) and the SLP was dispersed by ultrasonic disrupture. The adjuvant solution was considered to be ready-for-use if no precipitate was visible by macroscopy (40-fold magnitude) and microscopy (400- and 1000-fold magnitude). One volume of the adjuvant solution was mixed with one volume of antigen solution.

35

IV. SLP, oil and aqueous phase with 2% Tween 80 were put in a vessel, mixed vigorously and subsequently emulsified by ultrasonic disrupture or microfluidizing

5 while maintaining the temperature at about 70 °C. The emulsion was considered to be ready-for-use if the oil droplets were smaller than 1 micrometer (estimated by phase contrast microscopy at 1000-fold magnitude). The vaccine was prepared by mixing one volume of adjuvant solution (of about 4°C) with one volume of antigen solution.

10 V. SLP was solubilized in an appropriate volume of liquid surface-active agent (e.g. Tween 80) and the aqueous phase was added under vigorous mixing, resulting in a clear or opalescent suspension. Squalane was added to this suspension and the mixture was emulsified by microfluidizing.

15 VI. As V except that the SLP in an organic solute was mixed with a liquid surface-active agent (e.g. Tween 80) and the organic phase was evaporated before or after addition of an aqueous phase.

Although no significant differences were seen in adjuvant activity, preparation methods I, II, V and VI were preferred as these are relatively easy procedures and easily applicable for large scale production.

20 A typical SLP/S/W (sulfolipopopolysaccharide/squalane/water) adjuvanted vaccine comprises per ml the following components: 2.5 mg SLP, 0.05 ml (40 mg) squalane, 0.01 ml (10 mg) Tween 80. The SLP-derivative used had a mean lipid/sulfate content (number of groups per monosaccharide units of the polysaccharide) of 0.8/0.1.

## 25 EXAMPLE 2

### SLP-H2/squalane/water as adjuvant for various antigens in mice and guinea pigs

#### 30 MATERIALS AND METHODS

##### Animals

35 Random Swiss, female mice and guinea pigs were obtained from Harlan, Zeist, The Netherlands. At the age of about 10 weeks, groups of five animals were immunized subcutaneously (sc), intraperitoneally (ip), or intramuscularly (im) with 0.2 ml vaccine at Week 0 and 3 and blood was collected at Week 3 and 6, unless stated otherwise.



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SLP-H2/squalane/w adjuvant

SLP-H2 in an organic solvent (either dichloromethane or chloroform) was mixed with squalane (Sigma Chem. Comp. St. Louis, USA) and the organic solvent was removed by evaporation in a Rotavapor (Büchi Lab.-Technik AG, Switzerland) at low pressure and 60 °C during at least 6 h. The squalane containing the SLP was mixed with phosphate buffered saline containing 2% Tween 80 (PBS/2%Tween80) and the mixture was emulsified in two steps. A preemulsion was made by vigorous shaking on a vortex. Subsequently, the preemulsion was microfluidized (Microfluidics Corp. Newton, USA) in two or three cycli with increasing pressure (200 to 600 atmospheres). The emulsion obtained was analyzed by assessment of the size of the oil droplets under the microscope (1000x magnification) and by a microparticle-sizer (Malvern Instruments). The emulsification procedure was repeated until most of the droplets had a diameter of less than 1  $\mu\text{m}$  and no particles with a diameter of more than 10  $\mu\text{m}$  were present. Merthiolate was added to the SLP-H2/s/w emulsion at a final concentration of 0.01% and the adjuvant was stored at 4 C. The adjuvant solution used comprised per ml 5 mg SLP, 0.1 ml (80 mg) squalane and 0.02 ml (20 mg) Tween 80, unless stated otherwise.

Vaccines and vaccinations.

The vaccines tested were prepared by mixing one volume of antigen solution with one volume of the adjuvant solution at least one day before injection. Mice received 0.2 ml of one of the following vaccins containing or not an adjuvant:

Vaccine I contained 10  $\mu\text{g}$  dinitrophenylated bovine serum albumin (DNP-BSA) per dose. (Experiment I),

Vaccine II contained 10  $\mu\text{g}$  DNP-BSA, 1  $\mu\text{g}$  MRC-11 and 10  $\mu\text{g}$  ovalbumin (OVA) per dose (Experiment II),

Vaccine III contained 1  $\mu\text{g}$  MRC-11 and 10  $\mu\text{g}$  OVA per dose (Experiments III, IV and V), and

Vaccine IV comprised 5log10 TCID50 inactivated pseudorabies virus particles (iPRV), 0.44  $\mu\text{g}$  A/Swine, 0.4  $\mu\text{g}$  MRC-11 and 0.2  $\mu\text{g}$  X-79. (Experiment VI). Guinea pigs were injected with vaccine IV with or without adjuvant. The antigenic composition of Vaccine IV is comparable with that of porcine Suvaxyn iAuj/Flu3 vaccine and one tenth of the dose for pigs is injected into mice or guinea pigs.

The following adjuvants were tested in mice:

1. An emulsion of mineral oil(Markol 52)/phosphate buffered saline plus 1 % Tween 80 (O/W) containing 40 mg of oil per dose (Group 182),

2. An emulsion of SLP/O/W containing per dose 0.5 mg of SLP and 40 mg of mineral oil (Markol 52; Group 188),
3. An emulsion of squalane/W (S/W) containing 8 mg of squalane per dose (Group 951, 1063, 1083, 1688, and 2322),
- 5 4. An emulsion of SLP/S/W containing 0.5 mg of SLP and 8 mg of squalane per dose (Group 952, 953, 1062, 1082, 1691, 1966, and 2334),
5. An emulsion of SLP/S/W containing 1.0 mg of SLP and 8 mg of squalane per dose (Group 1692),
- 10 6. An emulsion of SLP/S/W containing 2.5 mg of SLP and 8 mg of squalane per dose (Group 1693),
7. An emulsion of S/W containing 2 mg of squalane per dose (Group 1683),
8. An emulsion of SLP/S/W containing 0.5 mg of SLP and 2 mg of squalane per dose (Group 1685),
- 15 9. An emulsion of SLP/S/W containing 1.0 mg of SLP and 2 mg of squalane per dose (Group 1686),
- 10 An emulsion of SLP/S/W containing 2.5 mg of SLP and 2 mg of squalane per dose (Group 1687),
- 20 11 A suspension of SLP in phosphate buffered saline containing Tween 80 (PBS/T) containing per dose of 0.5 mg of SLP (Group 973, 974, 1061, 1081, 1964, and 2327),
- 12 A suspension of SLP in phosphate buffered saline containing Tween 80 (PBS/T) containing per dose of 0.2 mg of SLP (Group 1962),
- 25 13 An emulsion of S/W plus Ficoll containing 8 mg of squalane per dose and 0.5 mg of Ficoll per dose (Group 1998).

The following adjuvants were tested in quinea pigs:

1. An emulsion of O/W containing 40 mg of oil (Markol 52) per dose (Experiment IV, Group 1; Experiment V, group 2),
- 30 2. An emulsion of S/W containing 8 mg of squalane per dose (Experiment I; Group 2; Experiment II; Group 4; Experiment III; Group 9; Experiment IV; Group 6; Experiment V; Group 3),
3. An emulsion of SLP/S/W containing 0.5 mg of SLP and 8 mg of squalane per dose (Experiment I; Group 3; Experiment II; Group 5; Experiment III; Group 10; Experiment V; Group 8),
- 35 4. An emulsion of SLP/S/W containing 0.25 mg of SLP and 8 mg of squalane per dose (Experiment IV, Group 7),
5. An emulsion of SLP/S/W containing 1.25 mg of SLP and 8 mg of squalane per

dose (Experiment IV, Group 8).

The following adjuvants were tested in pigs:

1. An emulsion of O/W containing 400 mg of oil per dose,
2. An emulsion of S/W containing 80 mg of squalane per dose,
3. An emulsion of SLP/S/W containing 5 mg of SLP and 80 mg of squalane per dose, and
4. A suspension of SLP in PBS/T containing 5 mg of SLP per dose .

#### Determination of antibody titers in serum

Anti-DNP-BSA antibody responses were measured by a haemagglutination (HA) reaction using dinitrophenylated sheep red blood cells (DNP-SRBC) as indicator cells. Briefly, serum was serially diluted twofold in saline containing 1% normal rabbit serum (which has been preadsorbed with sheep red blood cells) in round-bottom 96-wells plates and DNP-SRBC suspension was added. The reciprocal serum dilution which just gave agglutination was considered to be the titer.

Anti-OVA antibody responses were measured by heamagglutination of SRBC conjugated with OVA.

Anti-iPRV antibody titers were measured by a serum neutralization test (SN). Briefly, serum was serially diluted twofold in 96-wells plates. The serum dilutions were combined with an Aujeszky virus suspension of 100 TCID<sub>50</sub>. After incubation for 24 h at 37 °C, 2.10e4 PD5-cells per well were added to the serum-virus mixture. After five days of incubation at 37 °C, virus plaques were enumerated and the reciprocal dilution of serum causing 50% neutralized of the virus was considered to be the antibody titer.

Anti-iFlu antibody titers were measured by a heamagglutination inhibition reaction (HI). Sera were pretreated with kaolin by adding one volume of serum to four volumes of kaolin (50  $\mu$ l plus 200  $\mu$ l, respectively) in a 96-well roundbottom plate. After incubation for 30 min at room temperature, the kaolin was spun down and the supernatant collected and serially diluted twofoldly in veronal buffered saline containing 0.1 % BSA in round-bottom 96-wells plates. Four HA units of influenza virus were added to the wells and plates were incubated for 1 h at room temperature. Chicken red blood cells were washed three times in veronal buffered saline with 0.1 % BSA and were added to the wells at a concentration of 0.25%. After 30 minutes and within 2 h, agglutination was detected and the highest reciprocal serum dilution demonstrating inhibition of heamagglutination was considered to be the titer.

RESULTSAdjuvanticity in mice.

5 Adjuvanticity of SLP/S/W for the antibody responses against various antigens was  
studied in mice in six separate experiments (Table 1). The effects of SLP/S/W were  
compared with either SLP, S/W, O/W, or antigen without adjuvant. Antibody  
responses against DNP-BSA were enhanced significantly by SLP/S/W but not by O/W  
10 (Exp. I). SLP alone was less effective than SLP/S/W and S/W was less effective than  
both SLP or SLP/S/W (Exp. III). Anti-MRC-11 antibody responses were stimulated  
significantly by different adjuvants tested. SLP/S/W was more effective than S/W  
alone (Exp. III to VI) except in Exp. II. SLP alone demonstrated considerable  
adjuvanticity which was comparable to (Exp. IIIa, IIIb, and VIb) or less than that of  
SLP/S/W (Exp. II, V, VIa). Anti-MRC-11 antibody response increased with increasing  
15 dose of SLP and increasing amount of squalane in SLP/S/W (Exp. V).  
Antibody responses against OVA were enhanced by SLP, S/W and SLP/S/W but  
SLP/S/W was significantly more effective than SLP or S/W alone (Exp. II, IIIa, IIIb,  
and IV). Adjuvanticity of SLP/S/W for anti-OVA responses did not increase with  
increasing dose of either SLP or squalane within the range tested.  
20 Humoral responses against iPRV were measured and SLP/S/W augmented  
significantly the response (Exp. VIa and VIb) and proved to be more effective than  
S/W. SLP alone was about as effective as SLP/S/W.  
The results are summarized in Tables 1A and 1B.

Table 1A

Group adjuvant [mg per dose]	2log antibody titers against					
	DNP-BSA		MRC-11		OVA	
	mean	sd	mean	sd	mean	sd
Experiment I (ip)						
181 -	4.0	0.8				
182 O/W	4.8	1.8				
188 SLP/O/W	9.0	2.0				
Experiment II (ip)						
951 S/W [8.0]	2.2	1.6	7.8	1.3	5.3	2.1
952 SLP/S/W [8.0]	8.8	1.9	3.2	1.1	8.4	1.4
973 SLP	8.2	0.8	3.0	1.2	5.4	2.7
953 SLP/S/W [8.0]	9.0	1.7	4.2	1.8	4.0	1.6
974 SLP	7.4	0.9	1.4	0.5	4.0	1.9
978 -	3.4	1.5	1.4	0.9	0.8	0.8
Experiment IIIa (ip)						
1061 SLP			6.2	1.6	7.4	1.3
1062 SLP/S/W			5.7	0.6	9.7	3.2
1063 S/W			4.8	1.1	8.8	1.9
1068 -			3.2	1.3	6.4	1.8
Experiment IIIb (sc)						
1081 SLP			3.2	1.9	5.8	0.4
1082 SLP/S/W			3.6	2.2	9.0	2.5
1083 S/W			1.6	1.5	6.8	1.3
1088 -			2.6	1.7	6.6	0.9
Experiment IV (ip)						
1683 S/W [2.0]			3.0	2.4	1.8	2.5
1685 SLP/S/W [0.5/2.0]			4.4	2.6	2.2	1.9
1686 SLP/S/W [1.0/2.0]			5.4	2.1	3.8	1.9
1687 SLP/S/W [2.5/2.0]			6.0	0.7	2.0	1.9
1688 S/W [8.0]			8.6	0.5	1.8	1.9
1691 SLP/S/W [0.5/8.0]			7.2	1.3	4.6	3.5
1692 SLP/S/W [1.0/8.0]			9.2	1.1	2.6	2.2
1693 SLP/S/W [2.5/8.0]			10.6	1.1	2.2	2.3
1697 -			2.0	2.0	2.0	2.8

Table IB

Group adjuvant [mg per dose]	2log antibody titers against					
	MRC-11		OVA		iPRV	
	mean	sd	mean	sd	mean	sd
Experiment V (ip)						
1962 SLP [0.2]	6.4	1.8	5.6	3.0		
1964 SLP [0.5]	7.4	1.1	3.6	1.3		
1966 SLP/S/W	9.0	0.7	6.6	2.3		
1998 Ficoll + S/W	7.2	2.4	6.2	3.6		
Experiment VIa (ip; three weeks after the first immunization )						
2321 -	0.2	0.4			1.4	1.5
2322 S/W	3.8	1.5			1.8	1.3
2327 SLP	4.2	1.5			4.0	2.3
2334 SLP/S/W	7.2	1.3			5.2	1.5
Experiment VIb (ip; three weeks after the second immunization)						
2321 -	4.8	1.8			6.8	1.5
2322 S/W	6.6	0.8			7.2	1.9
2327 SLP	10.4	1.1			10.2	
2334 SLP/S/W	10.0	1.2			10.2	1.1

#### Adjuvanticity in guinea pigs

The effect of SLP/S/W on the antibody response to porcine iPRV + iFlu3 vaccine was compared with that of S/W and O/W in guinea pigs. This animal species has been used extensively as a model for porcine vaccines and antibody responses against both iPRV and iFlu components, and are considered to be indicative for efficacy of the vaccine in pigs.

Groups of five animals were immunized subcutaneously with 0.2 ml of the vaccine at week 0 and at week 3, and antibody titers were measured at week 6. The following vaccine compositions were tested:

- a. SLP/S/W for inactivated pseudorabies virus (Table 2; Experiment I-V),
- b. SLP/S/W for inactivated influenza virus strain A/Swine, MRC-11 and X-79 (Experiment IV and V), and

Experiments I-III indicate that antibody titers against iPRV were enhanced significantly by squalane-in-water (S/W) and SLP/S/W, and that SLP/S/W is more effective than S/W.

Table 2:

Group Adjuvant	[mg per dose]	2log antibody titers at week 6 against							
		iPRV		A/Swine		MRC-11		X-79	
		mean	sd	mean	sd	mean	sd	mean	sd
Experiment I (iPRV + Flu3)									
1 -		5.0							
2 S/W		6.4							
3 SLP/S/W		8.2							
Experiment II (iPRV)									
3 -		7.6							
4 S/W		12.3							
5 SLP/S/W		14.8							
Experiment III (iPRV + iFlu3)									
8 -		5.0							
9 S/W		6.5							
10 SLP/S/W		9.3							
Experiment IV (iPRV + iFlu3)									
1 O/W		12.3	3.4	11.3		13.3		12.1	
6 S/W		10.9	1.6	8.8		10.3		8.9	
7 SLP/S/W	[0.25]	10.5	0.8	12.3		13.5		12.7	
8 SLP/S/W	[1.25]	11.0	2.3	11.3		12.7		11.9	
Experiment V (iPRV + iFlu3)									
1 -		9.7		6.9		7.9		8.6	
2 O/W		9.0		10.1		9.9		8.0	
3 S/W		9.4		7.7		8.7		8.4	
8 SLP/S/W		9.8		10.6		10.5		> 10. 2	

In experiment IV and V little differences in anti-iPRV antibody titers were seen between the different groups. In these experiments, however, significant differences in anti-iFlu3 antibody titers were seen. SLP/S/W and O/W induced higher antibody titers than S/W or antigen alone. SLP/S/W was as effective as O/W.

## EXAMPLE 3

SLP-H2/squalane/water as adjuvant for iPRV, iFlu,  
and live PRV in pigs

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MATERIALS AND METHODSPigs

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Groups of five pigs at an age of about 10 weeks, were used in the experiments. The animals were immunized intramuscularly with 2.0 ml vaccine at Week 0 and 3 and blood was collected at Week 6.

Vaccines and vaccinations.

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The vaccines tested contained one volume of antigen and one volume of adjuvant solution. The antigen solution comprised 6log<sub>10</sub> TCID<sub>50</sub> inactivated pseudorabies virus particles, 4.4 µg of influenza virus strain A/Swine, 4.0 µg of MRC-11 and 2.0 µg of X-79. The adjuvant solution used was either SLP/S/W (5 mg of SLP per ml adjuvant solution and 80 mg of squalane per ml adjuvant solution) as described earlier or a standard mineral oil-in-water emulsion (50% mineral oil).

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Freezedried live PRV was reconstituted in diluent or in adjuvant solution (previously diluted 1:1 with diluent) and injected within 10 min.

RESULTS

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Stimulation of antibody responses against iPRV and iFLU3.

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In five separate experiments, adjuvanticity of SLP/S/W was compared with that of standard O/W in pigs. Groups of at least five pigs were immunized with iPRV + iFLU3 combined with either SLP/S/W or standard O/W or with iPRV + iFLU3 without adjuvant (Table 3). Anti-iPRV antibody titer measured after immunization with SLP/S/W did not significantly differ from those elicited with O/W ( $p > 0.20$ ). In four out of five experiments, three- to four-fold higher titers against the different influenza virus strains were detected after injection with SLP/S/W in comparison to standard O/W. Combination of the results and statistical analysis proved a significant increase in the titers against A/Swine ( $p < 0.01$ ), MRC-11 ( $p < 0.01$ ) and X79 ( $p < 0.01$ ).

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Table 3

Group Adjuvant	n	2log antibody titers against:								
		Auj		A/Swine		MRC-11		X-79		
		mean	sd	mean	sd	mean	sd	mean	sd	
Experiment I										
1 SLP/S/W	5	6.8	4.6	8.8	2.3	9.6	0.6	11.0	0.7	
2 O/W	5	5.4	3.4	6.8	2.3	8.6	1.8	8.2	0.8	
Experiment II										
1 SLP/S/W	5	9.0	0.7	7.6	2.5	13.1	1.0	9.8	0.8	
2 O/W	5	8.0	1.2	8.2	1.6	12.6	1.6	10.4	0.8	
Experiment III										
1 SLP/S/W	5	9.4	0.5	10.4	1.1	11.2	0.8	10.8	0.8	
2 O/W	5	10.0	0.7	9.0	3.2	11.4	1.1	10.6	1.1	
Experiment IV										
1 SLP/S/W	15	6.3	2.0	10.0	1.1	12.1	1.0	10.2	0.6	
2 O/W	10	6.3	0.7	7.1	1.5	8.7	1.2	7.8	1.0	
Experiment V										
1 SLP/S/W #2L	5	7.8	0.5	7.2	2.7	11.4	0.5	11.0	1.4	
2 O/W	5	5.6	1.5	5.8	1.6	9.0	1.0	8.6	1.3	
Combined results of experiments I to V										
1 SLP/S/W	35	7.4	2.0	9.1	1.6	11.6	1.1	10.6	0.8	
2 O/W	30	6.9	1.5	7.3	1.8	9.8	1.2	9.1	0.9	
Factor of increase		1.4		3.5		3.5		2.8		
P value		>0.20		<0.01		<0.01		<0.01		
Significance		NS		S		S		S		

Groups of n pigs were immunized im with iPRV + iFlu3 plus adjuvant at Week 0 and 3.

Antibody titers were measured at Week 6. Factor of increase was calculated by the following formula:  $2e^{(\text{titer with SLP/S/W} - \text{titer with O/W})}$ . P values of the differences between SLP/S/W and O/W were calculated by the student's test. Values of  $> 0.05$  were considered to be not significant. NS: not significant; S: significant. Four out of the 15 animals of group 1 of experiment IV did not develop a significant antibody response against influenza strain MRC-11 (mean titer in these animals was  $< 5.6$ ) but normal responses against the other antigens were measured.

Table 4

		2log antibody titers against PRV at:				
		Week 3		Week 6		
Group adjuvant	n	mean	sd	mean	sd	
Experiment I						
1	-	5	.6	0.9	7.4	1.7
2	O/W	5	5.2	1.1	10.2	1.3
3	SLP/S/W	5	5.2	0.8	12.0	0.0
Experiment II						
1	O/W	5	6.8	1.5	11.8	2.6
2	SLP/S/W	5	5.5	1.8	13.6	3.0
5	-	5	3.5	1.0	9.0	0.9
Experiment III						
1	-	5	2.4	0.5	7.4	1.1
2	SLP	5	3.8	0.4	9.0	1.6
4	O/W	5	4.8	1.3	10.2	1.1
5	SLP/S/W	5	4.6	0.9	11.6	0.5
Experiment IV						
1	-	5			6.0	1.0
2	SLP/S/W	5			11.0	0.7
3	O/W	5			8.8	0.4
Combined results of Experiments I to IV						
1	-	20			7.5	1.1
2	O/W	20			10.3	1.4
3	SLP/S/W	20			12.0	1.4
			Factor of increase	P value	significant	
O/W versus '-'			7.0	<0.01	significant	
SLP/S/W versus O/W			3.4	<0.01	significant	
SLP/S/W versus '-'			23.0	<0.01	significant	

Stimulation of antibody responses against live PRV.

In four separate experiments, the effects of SLP/S/W and standard O/W on the antibody responses against live PRV were studied. Pigs were vaccinated twice and anti-PRV antibody titers were measured three weeks after each vaccination. In all cases, low to moderate antibody titers were observed after the first injection and titers increased by the second immunization (Table 4). In comparison to live PRV in diluent, SLP/S/W and O/W enhanced slightly the antibody titers after the first

immunization. After the second vaccination antibody titers were increased significantly by SLP/S/W and O/W.

SLP/S/W induced a 23-fold increase while O/W evoked an seven-fold increase in anti-PRV antibody titers.

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Reduction of virus excretion after challenge by immunization with live PRV plus SLP/S/W as adjuvant.

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Efficacy of live PRV vaccines comprising either SLP/S/W or O/W was investigated by measurement of virus excretion after challenge. Animals were challenged intranasally five weeks after the second immunization and virus titers in tonsillar swabs were monitored during 14 subsequent days (Fig. 1). Titers are expressed as  $10\log\text{TCID}_{50}$ .

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Nonvaccinated animals excreted large quantities of PRV from 11 to 24 days after challenge. Two out of five animals of this control group died within the period of examination. Immunization of the animals without adjuvant reduced both the period and the titer of virus excreted.

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Vaccination with PRV reconstituted in either SLP/S/W or O/W caused further reduction of the period of excretion and the amount of virus excreted. With O/W, virus titers of at least  $4\log_{10}$  were excreted during four days while with SLP/S/W a maximal virus titer of  $3.5\log_{10}$  was measured at only a single day. Total virus excretion was determined by calculation of the area under the curve (AUC) of the log virus titer versus the time interval after challenge. The AUC of control animals was considered to be 100%. AUC of animals which received antigen without adjuvant was 57%. Vaccination with O/W and SLP/S/W resulted in AUCs of 39% and 26%, respectively.

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#### EXAMPLE 4

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##### Adjuvanticity in chicken

Groups of ten chickens were immunized with 0.5 ml vaccine at Week 0 and 3 and antibody titers were measured at Week 0, 3, and 6. SLP/S/W mixed with inactivated Newcastle disease (NCD) virus. The results are summarized in Table 5.

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Table 5

group	adjuvant	mean 2log antibody titers against NCD <sup>a</sup> at Week:		
		0	3	6
1	W/O	4.0	10.0	10.0
2	-	4.0	5.6	8.1
3	SLP/S/W	4.0	7.3	8.5

**EXAMPLE 5****Immunomodulatory activity versus immunoadjuvanticity**

Effects of a hydrophilic SLP-derivative with proven stimulatory activity on non-specific resistance (Patent publication EP 295,749) on specific immune responses were studied. In different animal models, the adjuvanticity of the hydrophobic SLP-derivative with a mean sulfate/lipid-ratio of 0.1/0.8 was compared with that of a hydrophilic SLP-derivative with a mean sulfate/lipid-ratio of 0.6/0.01 or 1.6/0.8. The different SLP/S/W emulsions were prepared by adding the SLP to an emulsion of S/W with oil droplets of less than 1 micrometer and subsequent ultrasonication of the emulsion. Groups of five mice or guinea pigs were immunized sc with 0.2 ml of a vaccine comprising per dose 0.5 mg of SLP-derivative and 8 of mg squalane. Pigs were immunized with 2.0 ml of vaccine comprising per dose 5 mg of SLP-derivative and 80 mg of squalane.

Results are shown in Table 6.

Table 6.

Group	sulfate/lipid ratio	mean ( $\pm$ SEM) 2log antibody titers	
		at week 6	against
Experiment I (mice)			
1	0.1/0.8	9.7 + 3.2	ovalbumin
2	0.6/0.01	6.0 + 1.0	ovalbumin
Experiment Ib (mice)			
1	0.1/0.8	9.0 + 2.5	ovalbumin
2	0.6/0.01	4.8 + 0.8	ovalbumin
Experiment II (guinea pigs)			
1	0.1/0.8	13.1 $\pm$ 2.5	PRV
2	1.6/0.8	7.7 $\pm$ 2.3	PRV
Experiment IIIa (pigs)			
1	0.1/0.8	4.3 $\pm$ 1.2	PRV
2	0.6/0.01	<0.5 $\pm$ 0.0	PRV
Experiment IIIb			
1	0.1/0.8	8.5 $\pm$ 1.0	A/Swine
2	0.6/0.01	<2.7 $\pm$ 0.9	A/Swine
Experiment IIIc			
1	0.1/0.8	>10.5 $\pm$ 0.5	X-79
2	0.6/0.01	6.5 $\pm$ 0.8	X-79
Experiment IIIId (pigs)			
1	0.1/0.8	>9.8 $\pm$ 1.0	MRC-11
2	0.6/0.01	6.3 $\pm$ 1.4	MRC-11

From the results of the experiments with different antigens and in different animal species it can be clearly observed that the hydrophobic SLP in S/W is a more effective adjuvant than the hydrophilic SLP in S/W.

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CLAIMS:

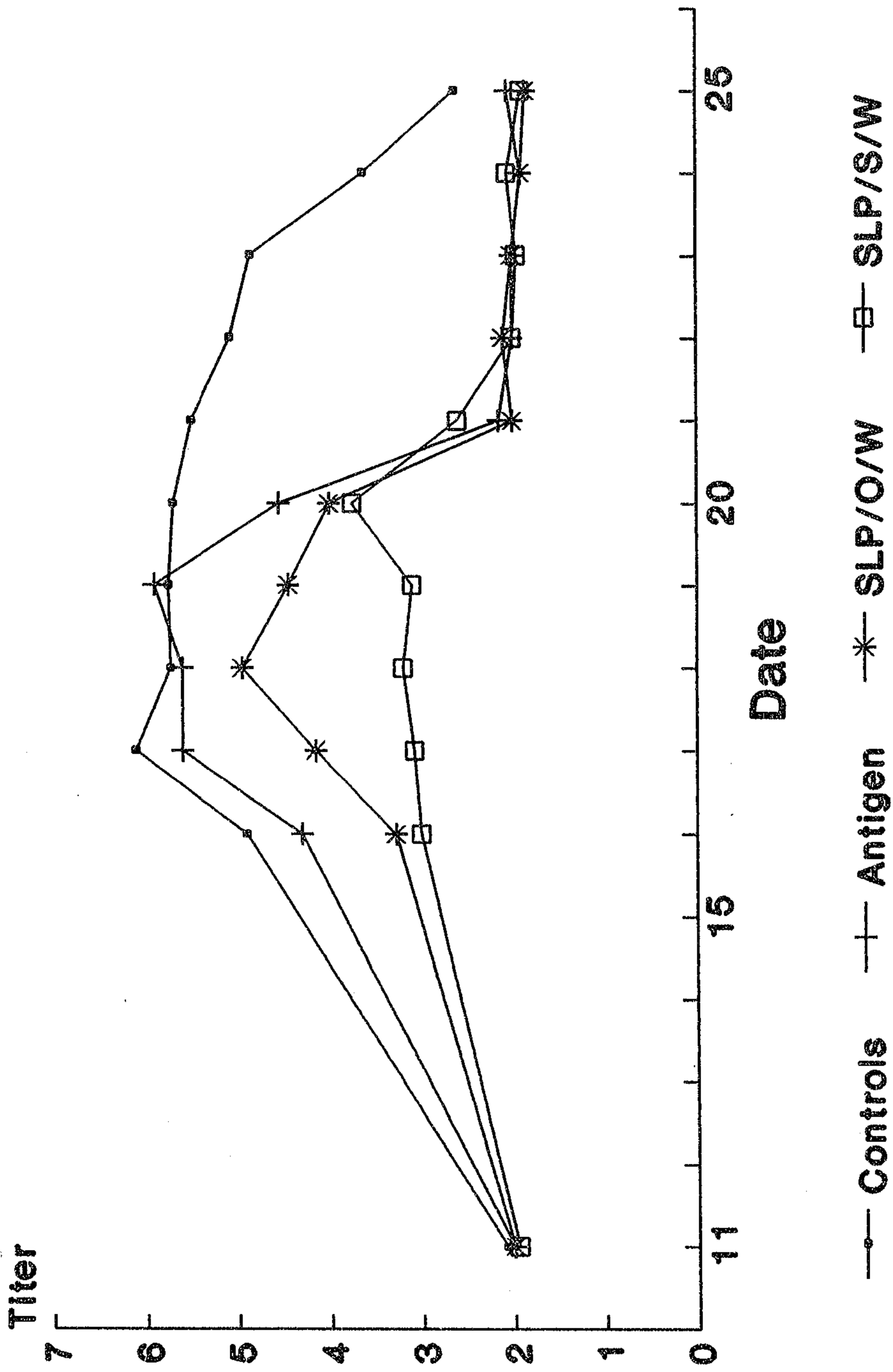
1. An adjuvant composition comprising at least one synthetic hydrophobic lipopolysaccharide and an interface-forming constituent, wherein the  
5 lipopolysaccharide has a hydrophilic-lipophilic-balance (HLB) value with the range of 4 to less than 9.
2. An adjuvant composition according to claim 1, further comprising an aqueous solute.
3. An adjuvant composition according to claim 1 or 2,  
10 wherein the lipopolysaccharide has a mean ratio of lipid groups to sugar groups of between 0.2 and 4.
4. An adjuvant composition according to claim 1, 2 or 3, wherein the lipopolysaccharide is provided with anionic groups while maintaining the overall hydrophobicity  
15 of the lipopolysaccharide.
5. An adjuvant composition according to any one of claims 1 to 4, wherein the interface-forming constituent is a liquid immiscible with an aqueous phase.
6. An adjuvant composition according to any one of  
20 claims 1 to 4, wherein the interface-forming constituent is a solid insoluble in an aqueous phase.
7. A vaccine comprising an adjuvant according to any one of claims 1 to 6, and an immunogen.

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PATENT AGENTS

FIGURE 1



Patent Agents  
Fetherstonhaugh & Co.

