A novel non-mineral oil-based adjuvant. I. Efficacy of a synthetic sulfolipopolysaccharide in a squalane-in-water emulsion in laboratory animals

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Sulfolipopolysaccharides (SLPs) were synthesized by reaction of the synthetic polysucrose polymer Ficoll-400 with chlorosulfonic acid and lauroyl chloride in anhydrous medium Hydrophobic derivatives were obtained by addition of a small number of sulfate and a large number of lipid groups Gel-permeation high-performance liquid chromatography (g p -h p l c) exhibited a wide range in molecular weight of both Ficoll-400 and SLP polymers The calculated weight-average molecular weight (M_w) of Ficoll-400 and SLP using polystyrene polymers as references was 187 000 and 380 000 respectively, exhibiting a twofold increase in molecular weight upon derivatization Adjuvanticity of hydrophobic SLPs with 0 2 sulfate and 1 5 lipid groups per sucrose monomer, a squalane-in-water emulsion (S/W), SLP incorporated into S/W (SLP/S/W), and a mineral oil-based emulsion (O/W) was investigated in combination with different antigens in mice and guinea-pigs Antibody responses in serum against ovalbumin (OVA), dinitrophenylated bovine serum albumin (DNP-BSA), inactivated influenza virus strain MRC-11 (MRC-11), a mixture of three influenza virus strains (iFlu3) and inactivated pseudorabies virus (iPRV) were measured by either haemagglutination (HA), haemagglutination inhibition (HI) or serum neutralization (SN) Vaccines were prepared by simply mixing one volume of antigen with one volume of adjuvant solution Antibody titres after one or two injections with these antigens were enhanced significantly by SLP/S/W, SLP, S/W and O/W and in most studies, SLP/S/W was demonstrated to be more effective than either the two constituent components or the O/W adjuvant A hydrophilic SLP-derivative with a high sulfate/lipid ratio incorporated into S/W appeared to be ineffective, which indicates the importance of a hydrophobic character of SLP Applicability of SLP/S/W as a novel non-mineral oil-based adjuvant is discussed

Keywords Adjuvant, sulfolipopolysaccharide, non-mineral oil, squalane-in-water emulsion, synthetic polymer, immunostimulation

It is well recognized that a novel generation of vaccines based on well defined pure antigens (polypeptides, proteins, poly/oligosaccharides etc) greatly depends on progress in the field of antigen presentation and immune stimulation In this respect, adjuvants play an important role as they are capable of stimulating different types of immune responses and, within limits, of directing the type of response Many compounds with adjuvant activity are at present known (for review see Refs 1 to 4) but only a few are applied routinely in human and veterinary vaccines Aluminium and calcium salts are the only adjuvants licensed for human use and are also applied in vaccines for companion animals Emulsions of mineral oil are often the adjuvant of choice for farm and laboratory animals since they evoke high immune responses These adjuvants, especially those of the water-in-oil type, can however provoke undesirable side-effects and therefore numerous attempts to develop strong and safe adjuvants have been undertaken The application of the novel adjuvants known at present might be limited for several reasons, such as disappointing

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efficacy in the target animal species, insufficient safety, problems with large-scale preparation, limited stability of the final formulation etc

Several years ago, we started a research programme on adjuvants without mineral oil for veterinary purposes After primary screening of experimental adjuvants with distinct antigens in laboratory animals, promising formulations were tested with appropriate antigens in target animal species The adjuvanticity of one of the most interesting preparations is presented here This novel formulation comprises a derivatized synthetic polysucrose which has been demonstrated to contain considerable adjuvanticity towards a particular antigen and a protein in mice[5] Activity of these so-called sulfolipopolysaccharides (SLPs) towards viral antigens was disappointing and therefore we attempted to improve efficacy by the use of physical vehicles SLPs were incorporated into different types of vehicles and adjuvanticity of a hydrophobic SLP in an emulsion of squalane-in-water was examined in laboratory animals

MATERIALS AND METHODS

Animals

Random outbred Swiss 10–12-week-old female mice (Harlan, Zeist, The Netherlands) with a minimum weight of 15 g and female 10–12-week-old guinea-pigs (Harlan) with a minimum weight of 200 g were used

Antigens

Dinitrophenylated bovine serum albumin (DNP-BSA, 22 DNP groups per BSA molecule) was prepared as described previously[6] Influenza virus strains MRC-11 (MRC-11), A/Swine (A/Swine) and X-79 (X-79) were grown in embryonated eggs, purified by centrifugation on a sucrose gradient and inactivated by incubation with 0 05% β-propiolactone plus 0 01% thimerosal for 4 days at 4°C and subsequently for 3 days at room temperature Pseudorabies virus (iPRV, Bartha strain) was grown on PD_5-cell cultures and inactivated by adding glutaraldehyde at a final concentration of 0 2% and subsequent incubation for 2 h at 37°C The following doses of antigen were injected in animals 10 μg ovalbumin (OVA), 10 μg DNP-BSA, 1 μg MRC-11, a mixture of three influenza virus strains (iFlu3) comprising 0 44 μg A/Swine + 0 40 μg MRC-11 + 0 2 μg X-79, and 10^4 $TCID_{50}$ iPRV

Adjuvants

SLPs were synthesized as described previously[5] Briefly, 80 g polysucrose, M_w 400 000, (Ficoll-400, Pharmacia, Uppsala, Sweden) was dissolved in a mixture of 50 ml anhydrous pyridine and 300 ml dimethylformamide and this solution was then dried by molecular sieves (0 2 nm, Merck, Darmstadt, Germany) To this mixture, 125 ml lauroyl chloride (Merck) was added and the mixture was incubated for 6 h at 70°C and subsequently for 18 h at room temperature Then, 5 ml anhydrous chlorosulfonic acid (Merck) in 20 ml anhydrous dimethylformamide (DMF) was added and the mixture was incubated again for 6 h at 70°C and 18 h at room temperature Solvents were evaporated and the syrup was neutralized with 1 M NaOH solution, dialysed extensively against water and finally lyophilized Dried SLP was dissolved in ethanol and mixed with 10 ml squalane (Merck) per gram SLP The ethanol was evaporated thoroughly at low pressure and elevated temperature The SLP/squalane mixture was added to phosphate-buffered saline containing 2% Tween 80 (PBS/Tw80) at final concentrations of 1% w/v SLP and 10% v/v squalane This mixture was emulsified by microfluidization (Microfluidizer model M 110 Y, Microfluidics Corp, Newton, MA, USA) at a working pressure of about 10 000 psi (pounds per square inch) or by ultrasonic vibration (Branson Sonifier Power Company, Danbury, UK) The emulsification procedure was continued until no droplets with a diameter of >2–3 μm were visible under the microscope at 1000-fold magnification Squalane-in-water without SLP (S/W) was prepared as described for SLP/S/W SLP adjuvant solution was prepared by suspending SLP in PBS/Tw80 by microfluidizing or ultrasonic vibration The mineral oil-in-water emulsion (O/W) tested is the adjuvant applied in various commercial vaccines of Solvay Duphar BV (Weesp, The Netherlands) and comprised 50% mineral oil, 5% of a hydrophilic (Tween 80) and 5% of lipophilic detergent (Arlacel A) to stabilize the emulsion

Chemical analysis of SLP

Chemical composition of SLP was determined by gross chemical analysis as described previously[5] Briefly, sugar content was measured by the method of Dubois *et al* [7] SLP solubilized in DMF was hydrolysed by adding HCl at a final concentration of 4 M and incubating for 6 h at 100°C Sulfate and lipid contents in the mixture were determined according to the procedure described by Dodgson and Price[8] and Duncombe[9], respectively The amount of free lipid was determined before hydrolysis and the total amount of lipid was determined after hydrolysis The quantity of bound lipid was calculated by subtraction of the free lipid content from the total lipid content

Determination of the molecular weight of Ficoll-400 and SLP

The molecular weight of Ficoll-400 and SLP was determined by gel-permeation high-performance liquid chromatography (g p -h p l c) using a TSKgel G5000 H_{XL} column (TosoHaas, Montgomeryville, PA), and DMF with 0 05 M LiBr as eluent Samples of polystyrene with weight-average molecular weight (M_w) between 10 000 and 1 000 000 (TosoHaas) were used as standards Samples of SLP or Ficoll-400 in DMF were applied to the column and the retention time of the compounds was determined by refractive index detection M_w values of SLP and Ficoll-400 were calculated using the polystyrene standards

Characterization of the SLP/S/W emulsions

The emulsion of SLP/S/W was characterized by determination of the particle-size distribution of the oil droplets by laser diffraction using a microparticle sizer (Malvern Mastersizer Model S2 02, Malvern Instruments Ltd, Worcs, UK) and PBS/Tw80 as diluent

Vaccines and vaccination

Vaccines were prepared by mixing one volume of antigen solution with one volume of adjuvant solution

Groups of five animals were injected subcutaneously (s c) with 0 2 ml vaccine per animal, unless stated otherwise Mice were injected once at week 0 and blood was collected at week 3 Guinea-pigs were immunized twice, at week 0 and week 3, and blood samples were collected 3 weeks after the second injection (week 6)

Antibody titres against DNP and OVA

Anti-DNP and anti-OVA antibody titres were measured by a haemagglutination (HA) reaction using DNP- or OVA-derivatized sheep red blood cells (DNP-SRBC and OVA-SRBC, respectively) as indicator cells[10] Briefly, serum samples were serially diluted twofold in saline containing 1% normal rabbit serum in round-bottomed 96-well plates, suspensions of 0 25% DNP-SRBC or OVA-SRBC in saline were added and plates were incubated at room temperature The reciprocal serum dilution which just gave agglutination was considered to be the titre

Antibody titres against influenza virus

Anti-influenza virus antibody titres were measured by a haemagglutination inhibition test (HI) Sera were inactivated at 56°C for 30 min and treated with 25% kaolin (Ref 11, ICN/Flow Laboratories, Irvine, UK) by mixing four volumes of kaolin with one volume of serum and subsequent incubation of the mixture for 30 min at room temperature The kaolin was spun down and supernatants were collected and serially diluted in veronal-buffered saline (pH 7 2, 0 025 M) containing 0 1% BSA (VBS/BSA) in round-bottomed 96-well plates Four HA units of the appropriate strain of influenza virus were added to each well and the plates were incubated for 1 h at room temperature Washed chicken red blood cells at a concentration of 0 25% in VBS/BSA were added and after a time period of 0 5–2 h depending on the virus strain tested, agglutination was detected The highest reciprocal serum dilution demonstrating HI was considered to be the titre

Antibody titres against PRV by serum neutralization (SN)

Serum samples of individual animals were inactivated by incubating for 30 min at 56°C Next, 50 μl of the serum samples were diluted twofold in culture medium in 96-well microtitre plates and subsequently 50 μl of a virus suspension containing 100 $TCID_{50}$ of PRV were added After incubation for 24 h at 37°C, 50 μl of a cell suspension containing 2×10^4 PD_5 cells were added to the wells Plates were incubated for 5 days at 37°C and the cytopathogenic effect in the different wells was scored The reciprocal value of the serum dilutions resulting in 50% neutralization of the virus was considered as antibody titre

Statistical analysis

Serum samples were always tested in duplicate and mean values were calculated provided that the difference between the two individual values was not more than one 2-log unit (one twofold dilution step) In each assay, negative and positive standard serum samples were included and the test was considered valid if the deviation of the mean value of these standards did not exceed one 2-log unit (one dilution step) Results are expressed as the arithmetic mean of n independent observations $\pm$ standard error of the mean (s e m) Factor of increase (FOI) was determined by calculation of the antilog value of the difference between geometric means of the experimental group immunized with antigen plus adjuvant and the control group immunized with antigen alone Student's t test was performed to analyse the statistical significance of the results Values of $p > 0\,05$ were considered not to be significant

RESULTS

Molecular weight of SLP

SLPs were synthesized using as backbone Ficoll-400, which is a synthetic copolymer of epichlorohydrin and sucrose[12] The precise epichlorohydrin/sucrose ratio is not known but the highly branched structure implicates a value $> 1\,0$ The molecular weights of Ficoll-400 and SLP were determined by g p -h p l c using polystyrene as reference DMF was employed as eluent as both SLP and Ficoll-400 are soluble in this solvent Analysis of polystyrene polymers of differing M_w revealed significant linearity in the range 10 000–1000 000 (*Figure1*) Ficoll-400 exhibited a wide range of polymers with molecular weights from < 10 000–1000 000 SLP gave a comparable pattern although a considerable polymer fraction displayed molecular weights > 1000 000 The calculated molecular weights of Ficoll-400 and SLP were 187 000 and 380 000, respectively

Chemical composition of SLP

Various laboratory batches of SLP with low sulfate and high lipid contents were synthesized and chemical composition of the SLPs was established by determination of sugar, sulfate and lipid contents before and after complete hydrolysis (*Table 1*) The sugar content of SLP was about 50% w/w Before hydrolysis, a significant amount of lauric acid but no sulfate was detected After hydrolysis with 4 M HCl, a considerable quantity of sulfate and a high quantity of lipid were detected From the difference in sulfate and lipid content before and after hydrolysis, the number of groups per sucrose monomer linked covalently was calculated, taking the molecular weight of the sucrose monomer of Ficoll-400 to be 400 The sulfate ratio was $0\,24 \pm 0\,08$ and the lipid

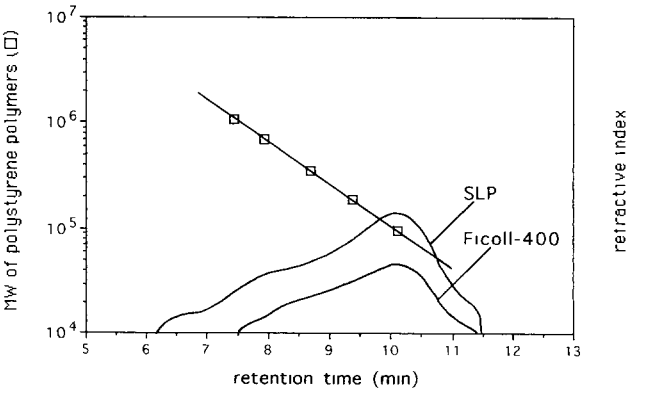


Figure 1 G p -h p l c patterns of Ficoll-400 and SLP analysed on a TSKgel G5000 H_{XL} h p l c column Polystyrene polymers with MWs ranging from 10 000 to 1000 000 (□) were used as reference

Table 1 Chemical composition of SLP

Component	Content before hydrolysis[a] (mg g^{-1})	Content after hydrolysis (mg g^{-1})	Difference between before and after hydrolysis	
			Content (mg g^{-1})	Ratio[b]
Sugar	510 (24)	NT	–	–
Sulfate	<5	43 (14)	43 (14)	0 24 (0 08)
Lipid	64 (39)	450 (140)	386 (121)	1 5 (0 6)

Values are given as mean (standard deviation)
[a]Sugar, sulfate and lipid contents of three laboratory batches of SLP were determined before and after treatment with 4 M HCl for 6 h at 100 C Ficoll-400, lauric acid and Na_2SO_4 were used as standards
[b]Ratio expressing the number of sulfate or lipid groups per sucrose unit was calculated assuming that one sucrose monomer of Ficoll-400 is 400

ratio was 1 5±0 6 On the basis of these results and data on chemical composition of Ficoll-400 described elsewhere, the structure of the SLP monomer was established and is represented in *Figure 2*

SLPs with low sulfate/lipid ratio were poorly soluble in water and readily soluble in organic solvents, e g ethanol, chloroform and dichloromethane

Microparticle-size distribution of SLP/S/W

The distribution of particle diameters of various SLP/S/W emulsions was determined and an example of the plot of frequency versus diameter is demonstrated (*Figure 3*) More than 98% of the particles were <1 μm and 99 9% <2 μm Different SLP/S/W emulsions can be compared by expression of the maximum diameter (MD) of the 10%, 50% and 90% portion of the smallest particles and for five different laboratory batches of SLP/S/W emulsions the following mean values (±s e m) were obtained MD(10%), 0 18±0 02, MD(50%), 0 34±0 08, and MD(90%), 0 91±0 60 μm

One batch of SLP/S/W was incubated for more than 7 months at 4°C and particle-size distribution was analysed Separation of phases could not be detected and MD(10%) remained 0 18 μm The values of MD(50%) and MD(90%), however, increased from 0 30 to 0 36 and from 0 59 to 1 97 μm, respectively The fraction of particles with a diameter of <1 0 μm decreased from 96 to 85%

Effect of SLP/S/W on humoral response to a protein and hapten carrier in mice

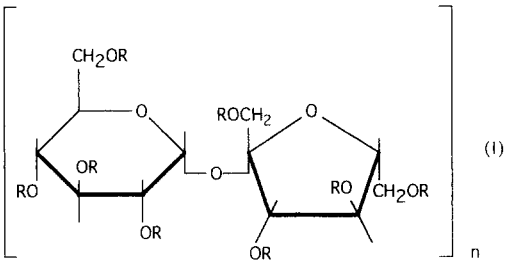
Groups of mice were injected once with OVA and antibody titres in serum were measured 3 weeks later OVA without adjuvant induced considerable levels of antibodies (*Table 2*) Neither SLP nor S/W enhanced the response whereas SLP/S/W provoked in both independent experiments an increase in antibody titres by a factor of 5 to 10

In two separate experiments, the effect of SLP/S/W, S/W, SLP and O/W on the antibody response to hapten conjugated to a protein was investigated in mice Low but detectable levels of antibody titres were observed after a single injection of antigen without adjuvant (*Table 2*) O/W and S/W emulsions did not enhance the responses whereas SLP alone and SLP/S/W induced significant increases of titres SLP alone gave a 16-fold and SLP/S/W 32- to 48-fold increase in antibody titre against the DNP hapten

Enhancement of antibody responses to inactivated viruses in mice

The effect of SLP/S/W, SLP and S/W on the humoral response against MRC-11 was examined in mice Three weeks after a single injection of antigen without adjuvant low titres of antibodies against MRC-11 were observed (*Table 3*) SLP, S/W and SLP/S/W induced significant increases in antibody titre and titres evoked by SLP/S/W were significantly higher than those obtained by the individual components

The influence of SLP/S/W on viral antigens was further investigated using an inactivated porcine herpes virus, iPRV Groups of mice were injected with iPRV with or without adjuvant and antibody titres in serum were measured by virus neutralization (*Table 3*) Addition of S/W did not result in augmented responses while application of either SLP or SLP/S/W gave significant six- to 14-fold increases



R =	ratio
R_1 = -CH_2-CHO(R_5)-CH_2-O(I)	> 2 0
R_2 = -SO_3H or -SO_3Na	0 24
R_3 = -CO-$(CH_2)_{10}$-CH_3	1 6
R_4 = -H	< 5 16
R_5 = R_2 or R_3 or R_4	

ratio is the number of groups per sucrose-monomer
n is about 500

Figure 2 Chemical structure of the sucrose monomer of SLP

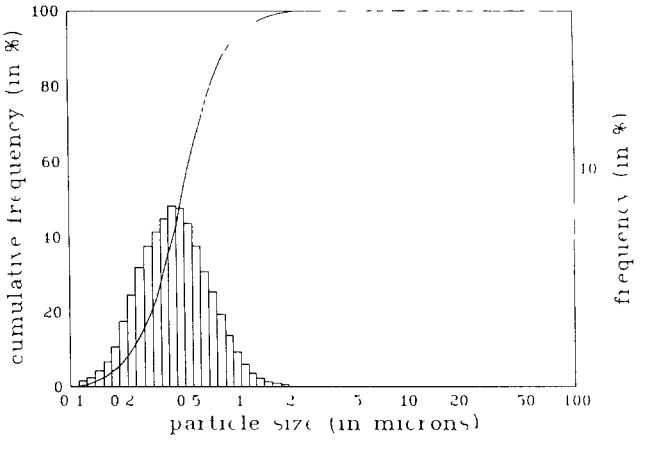


Figure 3 Distribution of the size of droplets of a SLP/S/W emulsion Bars indicate the relative frequency of particles with a given diameter and the line represents the cumulative values (%)

Table 2 Effect of SLP/S/W, S/W and SLP on antibody titres against two protein model antigens in mice

		2-log antibody titre 3 weeks after immunization							
		Experiment 1				Experiment 2			
Group	Adjuvant	Mean	s e m	FOI	S	Mean	s e m	FOI	S
Anti-OVA									
1	–	6 4	1 8	–	*a*	6 6	0 9	–	*a*
2	SLP/S/W	9 7	3 2	10	*b*	9 0	2 5	5	*b*
3	S/W	8 8	1 9	5	*a*	6 8	1 3	1	*a*
4	SLP	7 4	1 3	2	*a*	5 8	0 4	<1	*a*
Anti-DNP									
1	–	3 4	1 5	–	*a*	4 0	0 8	–	*a*
2	SLP/S/W	9 0	1 7	49	*b*	9 0	2 0	32	*b*
3	O/W	NT		–		4 8	1 8	2	*a*
4	S/W	2 2	1 6	<1	*a*	NT		–	
5	SLP	7 4	0 9	16	*b*	NT		–	

NT, not tested
Groups of five animals were immunized subcutaneously with either OVA or DNP-BSA and antibody titres against OVA and DNP were measured 3 weeks later by HA Mean value, s e m , factor of increase (FOI) and statistical significance (S) are represented Groups that are not statistically different ($p > 0\,05$) are indicated by the same letter

Table 3 Effect of SLP/S/W, S/W and SLP on antibody responses against two inactivated viruses in mice

		2-log antibody titre against									
		MRC-11					iPRV				
Group	Adjuvant	*n*	Mean	s e m	FOI	S	*n*	Mean	s e m	FOI	S
1	–	15	1 4	1 5	–	*a*	5	1 4	1 5	–	*a*
2	SLP/S/W	15	9 0	1 4	194	*b*	5	5 2	1 5	14	*b*
3	S/W	15	6 5	1 5	34	*c*	5	1 8	1 3	1	*a*
4	SLP	10	5 8	1 2	21	*c*	5	4 0	2 3	6	*b*

Groups of five mice were immunized with inactivated influenza virus MRC-11 or iPRV in combination with the adjuvants indicated Three weeks after immunization, antibody titres against MRC-11 or iPRV were measured by HI and SN, respectively Results of three independent experiments on MRC-11 were taken together Total number of animals tested (*n*), mean value, s e m , factor of increase (FOI) and statistical significance (S) are represented Groups that are not statistically different ($p > 0\,05$) are indicated by the same letter

Effect of SLP/S/W on antibody responses against influenza virus in guinea-pigs

Adjuvanticity of SLP/S/W was compared with that of O/W in guinea-pigs This animal species was selected as it is often used for potency tests of commercial products In five independent experiments, groups of five or ten animals were immunized twice with a mixture of three influenza virus strains (iFlu3) with or without adjuvant and antibody titres were measured 3 weeks after the second vaccination (*Figure 4*) Antigen without adjuvant evoked moderate titres against the three different viruses and both SLP/S/W and O/W increased the antibody levels Mean factors of increase (FOI) in immune responses against A/Swine, MRC-11 and X-79 were 26, 9 and 6 for SLP/S/W and 9, 6 and 3 for O/W, respectively (*Table 4*) If results of the five experiments were taken together, SLP/S/W was more effective than O/W in stimulating humoral responses against A/Swine and X-79 but not against MRC-11 strain

Augmentation of humoral immune responses against iPRV by SLP/S/W, S/W and O/W in guinea-pigs

Adjuvanticity of SLP/S/W for responses against iPRV was compared with that of either S/W and O/W (*Figure 5*) SLP/S/W and S/W but not O/W significantly enhanced the humoral response in guinea-pigs measured 3 weeks after the second vaccination S/W without SLP

Table 4 Comparison of the effects of SLP/S/W, S/W and O/W on antibody responses against inactivated influenza and pseudorabies virus in guinea-pigs

		2-log antibody titre at week 6				
Group	Adjuvant	*n*	Mean	s e m	FOI	S
Anti-A/Swine						
1	–	25	5 5	2 0	–	*a*
2	SLP/S/W	25	9 7	1 5	18	*b*
3	O/W	30	8 9	1 3	11	*c*
Anti-MRC-11						
1	–	25	6 7	1 0	–	*a*
2	SLP/S/W	25	9 7	0 9	8	*b*
3	O/W	30	9 5	1 4	7	*b*
Anti-X-79						
1	–	25	6 6	0 9	–	*a*
2	SLP/S/W	25	9 4	0 6	7	*b*
3	O/W	30	8 4	0 8	3	*c*
Anti-iPRV						
1	–	25	6 3	1 5	–	*a*
2	SLP/S/W	25	12 0	2 4	52	*b*
3	O/W	10	7 0	1 5	2	*a*
4	S/W	15	9 6	2 1	10	*c*

Results of the five independent experiments of *Figures 4* and *5* were taken together Guinea-pigs were immunized intramuscularly with either iFlu3 or iPRV plus different adjuvants at weeks 0 and 3 and antibody titres against iFlu3 and iPRV were measured at week 6 by HI and SN, respectively Total number of animals (*n*), mean value, s e m , factor of increase (FOI) and statistical significance (S) of the results are represented Groups which are not statistically different ($p > 0\,05$) are indicated by the same letter

was less effective than SLP/S/W and mean factors of increase were 10 and 52, respectively (*Table 4*)

Adjuvanticity of hydrophobic and hydrophilic SLP derivatives

The effects of a hydrophobic and two hydrophilic SLPs in combination with a squalane-in-water emulsion were tested with either OVA in mice or iPRV in guinea-pigs The sulfate/lipid ratio of the hydrophobic SLP was 0 1/0 8 while the hydrophilic SLP tested in mice and guinea-pigs gave values of 0 6/0 01 and 1 6/0 8, respectively

In mice, antibody responses against OVA were stimulated by the hydrophobic but not the hydrophilic SLP in squalane-in-water (*Table 5*) In guinea-pigs, the hydrophilic SLP formulated in S/W induced a slight, insignificant enhancement of antibody levels against iPRV while the hydrophobic derivative gave a high, 256-fold increase

DISCUSSION

The incidence, morbidity and mortality of a large number of infectious diseases in humans and animals have been diminished by vaccination Both live and inactivated antigens are used for this purpose Inactivated vaccines usually require supplementation with adjuvants for sufficient efficacy but addition of such compounds is often accompanied by increased toxicity The ideal adjuvant is considered to be a product which stimulates adequate immunity against the appropriate antigen(s) or epitope(s) of a pathogen without generating detrimental side-effects It exerts long-lasting stability, is easy to handle and is effective in combination with different pathogens and in different animal species It is well recognized that the adjuvants at present available do not meet all these criteria and compromises are made between efficacy, toxicity, performance etc In this paper, the adjuvanticity of a combination of SLP and squalane-in-water for

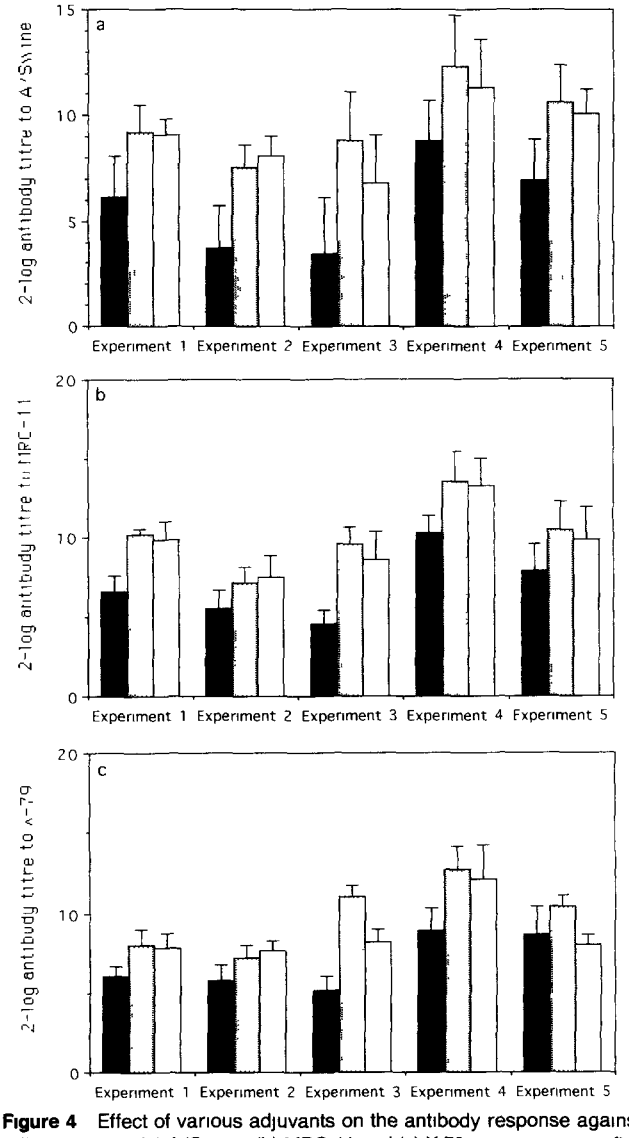


Figure 4 Effect of various adjuvants on the antibody response against influenza virus (a) A/Swine, (b) MRC-11 and (c) X-79 in guinea-pigs after two vaccinations Mean values of five animals are represented and vertical bars indicate s e m ■, no adjuvant, ⊡, SLP/S/W, □, O/W

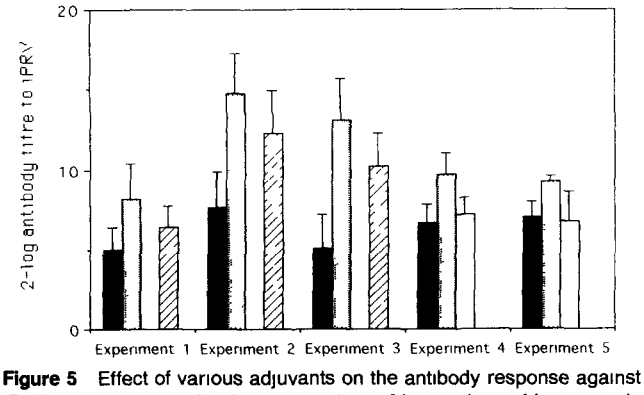


Figure 5 Effect of various adjuvants on the antibody response against iPRV in guinea-pigs after two vaccinations Mean values of five animals are represented and vertical bars indicate s e m ■, no adjuvant, ⊡ SLP/S/W, □, O/W, ▨, S/W

Table 5 Adjuvanticity of hydrophobic and hydrophilic SLPs incorporated in S/W in mice and guinea-pigs

			2-log antibody titre against									
			OVA in mice					iPRV in guinea-pigs				
Group	Adjuvant	S/L ratio	*n*	Mean	s e m	FOI	S	*n*	Mean	s e m	FOI	S
1	–		10	6 5	1 4	–	*a*	5	5 1	2 0	–	*a*
2	SLP/S/W	0 1/0 8	10	9 4	2 4	7	*b*	5	13 1	2 5	256	*b*
3	SLP/S/W	0 6/0 01	10	5 9	0 9	<1	*a*		NT		–	
4	SLP/S/W	1 6/0 8		NT				5	7 7	2 3	6	*a*

S/L ratio, sulfate/lipid ratio, NT, not tested
Groups of *n* animals were immunized with the antigen indicated plus different SLP/S/W formulations Antibody responses against OVA in mice were measured 3 weeks after the first injection by HA whereas antibody titres against iPRV in guinea-pigs were determined 3 weeks after the second injection by SN Mean values, s e m , factor of increase (FOI) and statistical significance (S) are represented Groups that are not statistically different ($p > 0\,05$) are indicated by the same letter

humoral response against various types of thymus-dependent antigens was investigated in two species of laboratory animals, mice and guinea-pigs

Originally, SLPs were synthesized to mimic the synergistic adjuvant activity of combinations of a sulfated polysaccharide, i e dextran sulfate, and surface-active agents, e g dimethyldioctadecylammonium bromide, non-ionic block polymers of polyoxyethylene plus polyoxypropylene[13,14] The polysucrose backbone of SLP is Ficoll-400, a synthetic copolymer of sucrose and epichlorohydrin with a highly branched, approximately spherical structure[12] Properties of the polymeric SLP were determined by chemical characterization of the monomer and establishment of the molecular weight distribution The chemical composition of the SLP derivatives investigated was determined by gross analysis which included the assessment of sugar, sulfate and lipid content before and after acid hydrolysis Differences between lipid content before and after this treatment revealed that $\geqslant 85\%$ of the lipid present was coupled covalently to the polysucrose backbone Sulfate could only be detected after hydrolysis, indicating that sulfate was completely linked covalently The mean numbers of sulfate and lipid groups per sucrose monomer of the hydrophobic SLP batches investigated were 0 24 and 1 5, respectively On the basis of these data plus information on Ficoll-400 obtained from the supplier[12], the chemical composition of the sucrose derivative as a monomer of SLP could be deduced (*Figure 2*) Additional analysis largely confirmed this chemical composition (Hilgers *et al*, manuscript in preparation)

Analysis of Ficoll-400 and SLP by g p -h p l c exhibited a wide range of molecular weights between $<10\,000$ and $>1\,000\,000$ This polydisperse profile of Ficoll-400 has been described previously by others using Sephadex G-200 for gel-permeation chromatography[15] The values for M_w of Ficoll-400 and SLP calculated using polystyrene standards were 187 000 and 380 000, respectively, which is in accordance with the increase expected on the basis of the number of sulfate and lipid groups added The M_w of Ficoll-400 found is less than the value of 400 000 indicated by the producer as measured by light scattering, which might be due to differences in the test system Analysis by classical gel-permeation column chromatography and sedimentation also presented significantly lower M_w values for Ficoll-400 than the 400 000 suggested[15] From the data presented, it was concluded that the polymeric structure of Ficoll-400 is preserved during the synthesis procedure of SLP, M_w increased twofold upon derivatization as expected, and SLP is a collection of polymers with a wide range of molecular weights and a M_w of about 380 000

Because of the presence of both hydrophobic and hydrophilic moieties, SLP is considered to be a surface-active agent The hydrophobic character of SLP is brought about by the aliphatic chains (lauroyl groups) while the sulfonate and hydroxyl groups establish the hydrophilic character The ratio between sulfate and lipid groups determines the hydrophilic–lipophilic balance (HLB), which is low for the adjuvant-active SLPs on the basis of solubility in aqueous and organic solvents Although many SLP derivatives displayed considerable adjuvanticity depending on sulfate and lipid content and length of the lipid chain, activity was insufficient for application in veterinary vaccines Several possibilities to improve activity was considered and examples of increasing adjuvanticity of surface-active agents by the use of a physical support have been described in the literature Non-ionic block polymers of polyoxyethylene and polyoxypropylene[16], and monophospholipid A-derivative plus trehalose dimycolate[17] incorporated into an emulsion of squalane-in-water demonstrated enhanced activity

Hydrophobic SLPs incorporated into a squalane-in-water emulsion (SLP/S/W) displayed significant adjuvanticity for the antibody responses to various types of antigens in both mice and guinea-pigs Within limits, the ratio of sulfate to lipids in SLP could vary without significant effect on adjuvanticity in combination with S/W Both constituent components S/W and SLP possessed adjuvanticity but S/W was ineffective in combination with OVA, DNP-BSA and tPRV while SLP failed to increase antibody titres against OVA The combination of these two compounds was effective towards all antigens tested and an overall higher activity was observed Both additional and synergistic interaction between the two components could be detected

Comparison of SLP/S/W with O/W revealed that the experimental adjuvant was more effective than the commercially supplied one with the exception of the response against the influenza virus strain MRC-11 in guinea-pigs The hydrophobic character of the SLP seemed to be important as hydrophilic derivatives (with relatively high sulfate/lipid ratios) in combination with S/W were not effective

Differences in antibody responses between independent experiments could be observed (*Figures 4* and *5*) Constant results with standard serum samples included in each test and simultaneous testing of serum samples from distinct experiments (data not shown) rule out the possibility that variation was introduced during serum analysis Differences in antibody responses to antigen without adjuvant, however, suggest that variation in either immunogenicity of antigen preparation or immuno-responsiveness of the animal rather than differences in SLP/S/W-batches contributed to interexperimental variation

In the literature, several other hydrophobic surfactants with apparent adjuvant activity have been described, e g lipophilic amines[18], polyoxyethylene– polyoxypropylene copolymers[19], lipid A derivatives[20], lipophilic muramyl-dipeptide[21], tripalmitoyl peptide[22] and trehalose di-mycolate[23] SLP distinguishes itself from these surface-active adjuvants as it is of much higher molecular weight (M_w of 380 000 versus $<10\,000$) In combination with an oil-in-water emulsion, these hydrophobic surfactants are assumed to reside on the interface between oil and water where they are capable of binding proteins or other substances[15 19 23] Adsorption of antigens or components of the host immune system to the surfactants on the oil surface has been considered to be important to the adjuvant activity Accumulation of SLP on the surface of squalane droplets and subsequent binding of antigen or components of the immune system have not been established but, together with other possible mode(s) of action, are the subject of further research

Stability of an emulsion is an important requirement for commercial exploitation In general, the size of the oil droplets in an oil-in-water emulsion is indicative of stability Emulsions with particles smaller than 1 μm are considered to be very stable The SLP/S/W prepared on a laboratory scale contained very small droplets and

more than 90% were smaller than 1 μm Analysis of a SLP/S/W batch after incubation at 4°C for more than 7 months demonstrated a very slight modification of the particle-size distribution but no separation of phases Another SLP/S/W batch incubated for more than 2 years at 4°C demonstrated a similar change in particle-size distribution (data not shown) Although these results suggest that these experimental adjuvant formulations are relatively stable emulsions, additional research is required to substantiate these data

In summary, SLP/S/W is a promising non-mineral oil-based adjuvant for different types of antigen and is at least as effective as the mineral oil adjuvant presently used in various veterinary vaccines

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