A novel non-mineral oil-based adjuvant. II. Efficacy of a synthetic sulfolipopolysaccharide in a squalane-in-water emulsion in pigs

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The adjuvanticity of a sulfolipopolysaccharide (SLP) incorporated into a squalane-in-water emulsion (SLP/S/W) was compared with that of a mineral oil-in-water (O/W) adjuvant currently used in commercial porcine vaccines Groups of pigs were immunized twice with vaccines comprising either inactivated influenza virus (iFlu3 containing strains A/Swine, MRC-11 and X-79), inactivated pseudorabies virus (iPRV), live pseudorabies virus (PRV) or inactivated porcine parvovirus (iPPV) as antigen and SLP/S/W or O/W as adjuvant Antibody titres in serum 2 or 3 weeks after the second immunization were measured by haemagglutination inhibition (HI) or serum neutralization (SN) assays Both adjuvants significantly augmented the antibody responses against the antigens tested Mean factors of increase obtained by SLP/S/W and O/W were 315 and 91, respectively, for A/Swine, 478 and 137 for MRC-11, 362 and 128 for X-79, 69 and 49 for iPRV, and 23 and 7 for live PRV Increased humoral immunity against live PRV was affirmed by reduced levels and duration of virus excreted by pigs after challenge with virulent PRV Immunization of pigs with iPPV plus adjuvant SLP/S/W gave 36-fold higher titres than with O/W It was concluded that SLP/S/W is more effective than O/W in stimulating humoral immunity against the viral antigens examined and that the two constituents SLP and S/W interact synergistically Advantages of SLP/S/W over O/W include stronger adjuvanticity, better biocompatibility and lower doses of active substances

Keywords Adjuvant, sulfolipopolysaccharide, efficacy, pigs, synthetic polymer, squalane-in-water emulsion, immunostimulation, viral vaccines

The most common types of adjuvants used in vaccines for domestic food animals are still emulsions of either the oil-in-water or water-in-oil type, based on oil of mineral origin In general, these adjuvants exhibit strong activity with a wide range of antigens but, possibly owing to limited biodegradability and biocompatibility, their application is often accompanied with certain side-effects and risks Parenteral administration of mineral oil emulsions into animals frequently provokes reactions at the site of injection of which the severity and duration depend on the nature and concentration of the oil and physicochemical characteristics of the emulsions[1] Studies on the kinetics of mineral oil emulsions *in vivo* demonstrated that considerable quantities of oil remained at the site of injection[2,3] and in other anatomic compartments[4] for a long period of time As a consequence of this persistence, it cannot be excluded that consumers of food of animal origin are exposed to oil residues and although detrimental effects of such residues are not exhaustively documented, they might introduce certain risks to human health In addition, there exist also risks to veterinary surgeons or animal handlers of accidental autoinjection For these reasons, replacement of the mineral oil components while retaining adjuvanticity but reducing risks is very desirable Several attempts have been described and a few veterinary vaccines are at present on the market supplemented with novel adjuvants e g vitamin E, polyacrylate resins (Carbopol, of B F Goodrich), acetylated polymannose (Acemannan, Carrington Labs) and avridine Emulsions of oils of vegetable origin such as peanut, olive, sesame oil etc [5–8] or of animal origin, e g squalane and squalene[9], have also been investigated but activity was almost always insufficient compared to mineral oil

In an accompanying paper[10] we have described the adjuvanticity of a synthetic, high-molecular-weight polysucrose derivatized with fatty acid esters and sulfate

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groups and incorporated in a squalane-in-water emulsion towards various proteinic and viral antigens in laboratory animals Effects of this adjuvant formulation on immune responses against a number of porcine viral antigens in the target animal species are reported here

MATERIALS AND METHODS

Animals

Pigs of 8–10 weeks of age were screened for the presence of antibodies against the viral antigens in question and animals with detectable antibody titres were excluded

Vaccines

Antigens were prepared as described previously[10] The following doses of antigen (corresponding to 1 ml of antigen solution) were injected 4 4 μg influenza virus A/Swine + 4 0 μg MRC-11 + 2 0 μg X-79 (iFlu3), 10^8 $TCID_{50}$ inactivated pseudorabies virus (iPRV), 10^5 $TCID_{50}$ live pseudorabies virus (PRV), and 10^5 $TCID_{50}$ inactivated porcine parvovirus (iPPV) Adjuvants tested have been described elsewhere[10] Vaccines were obtained by either mixing 1 volume of antigen with 1 volume adjuvant solution or resuspending lyophilized virus (live PRV) in distilled water or adjuvant solution diluted with an equal volume of distilled water

Vaccination

Groups of at least five pigs were injected twice intramuscularly (i m) with 2 0 ml vaccine per animal at weeks 0 and 3 and blood was collected 2 or 3 weeks after the second immunization

Antibody titres against influenza and pseudorabies virus

Anti-influenza and anti-pseudorabies virus antibody titres were measured as described previously[10]

Antibody titres against PPV

Serum samples were inactivated by incubating for 30 min at 56°C and pretreated with 3 volumes of kaolin suspension (ICN/Flow Labs, Irvine, UK) and twice with 1 volume of a suspension of 50% guinea-pig red blood cells (GpRBC) in PBS Then, 50 μl of the serum samples were diluted in PBS in 96-well plates and 50 μl of a virus suspension containing 8 HA PPV were added to the serum dilutions After incubation for 45 min at room temperature, 50 μl of a 0 6% GpRBC suspension in PBS were added After 1–2 h, agglutination was detected and the reciprocal value of the highest serum dilution demonstrating HI was considered to be the titre

Virus excretion upon challenge with virulent PRV

Virus excreted after challenge with virulent PRV was determined by the method described by Vannier *et al*[11] Briefly, nasal swabs were taken daily from individual pigs from before challenge to 12 days postchallenge The swabs were weighed before and after sampling and soaked in 2 ml of culture medium and stored at −70°C for a maximum of 14 days Samples of 100 μl of these culture media were taken and the numbers of plaque-forming units were determined The means ($\pm$s e m) were calculated of the 10-log of $TCID_{50}$ on PD_5 cells per gram of mucus

Statistical analysis

Analysis of samples was performed by standardized tests and criteria for validity have been described before[10] Student's t test was carried out to analyse statistical significance of the results and $p > 0\,05$ was considered to be significant

RESULTS

Effect of SLP/S/W on the antibody response against iFlu3 in pigs

In five independent experiments, groups of pigs were immunized twice with a combination of three influenza virus strains plus different adjuvants, and blood samples were taken 3 weeks after the second vaccination Antibody titres achieved by either SLP/S/W, S/W or SLP were compared with those of antigen alone or antigen plus O/W (*Figure 1*)

In general, differences in titres against the three virus strains were seen between the individual experiments and responses against A/Swine were lower than those against the two other strains The mean factors of increase in responses to A/Swine, MRC-11 and X-79 observed varied

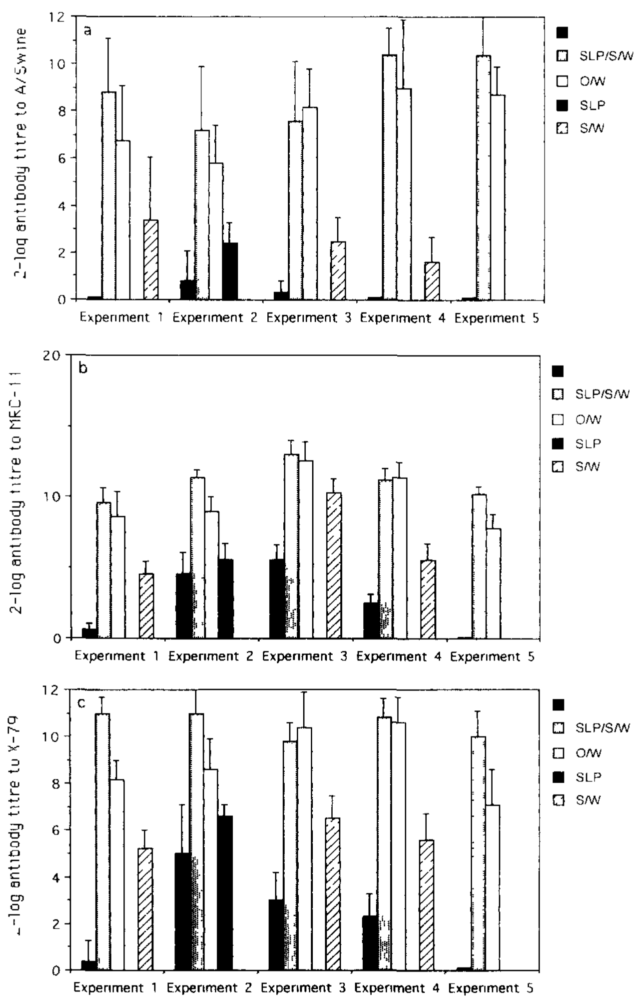

Figure 1 Effect of various adjuvants on the antibody response against influenza virus (a) A/Swine, (b) MRC-11 and (c) X-79 in pigs after two vaccinations Mean values for at least five animals are represented and vertical bars indicate s e m

Table 1 Comparison of the effect of various adjuvants on the antibody responses to iFlu3 in pigs

			2-log antibody titres at week 6 against											
			A/Swine				MRC-11				X-79			
Group	Adjuvant	*n*	Mean	s e m	FOI	S	Mean	s e m	FOI	S	Mean	s e m	FOI	S
1	–	25	0 8	0 6	–	*a*	2 7	0 8	–	*a*	2 1	1 1	–	*a*
2	O/W	30	7 3	1 8	91	*b*	9 8	1 2	137	*b*	9 1	0 9	128	*b*
3	SLP/S/W	35	9 1	1 6	315	*c*	11 6	1 1	478	*c*	10 6	0 8	362	*c*
4	S/W	15	2 5	2 1	3	*d*	6 8	0 9	17	*d*	5 8	0 9	13	*d*
5	SLP	5	2 4	0 9	3	*d*	5 6	1 1	7	*e*	6 6	0 5	23	*d*

Results of the five independent experiments of *Figure 1* were taken together Pigs were immunized intramuscularly with iFlu3 plus different adjuvants at weeks 0 and 3 and antibody titres were measured at week 6 by HI 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

Table 2 Comparison of the effect of various adjuvants on the antibody responses against iPRV and live PRV in pigs

		2-log antibody titres at week 6 against									
		iPRV					Live PRV				
Group	Adjuvant	*n*	Mean	s e m	FOI	S	*n*	Mean	s e m	FOI	S
1	–	25	1 3	0 7	–	*a*	20	7 5	1 1	–	*a*
2	O/W	30	6 9	1 5	49	*c*	20	10 3	1 4	7	*b*
3	SLP/S/W	35	7 4	2 0	69	*c*	20	12 0	1 4	23	*c*
4	S/W	15	3 2	1 3	4	*b*		NT			
5	SLP	5	4 0	1 7	4	*b*	5	9 0	1 6	3	*b*

NT, not tested
Results of the five independent experiments of *Figures 2* and *3* were taken together Pigs were immunized intramuscularly with iPRV or live PRV plus different adjuvants at weeks 0 and 3 and antibody titres were measured at week 6 by SN 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

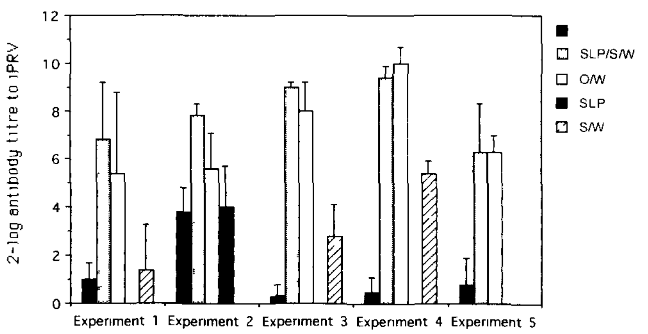

Figure 2 Effect of various adjuvants on the antibody response against iPRV in pigs after two vaccinations Mean values for at least five animals are represented and vertical bars indicate s e m

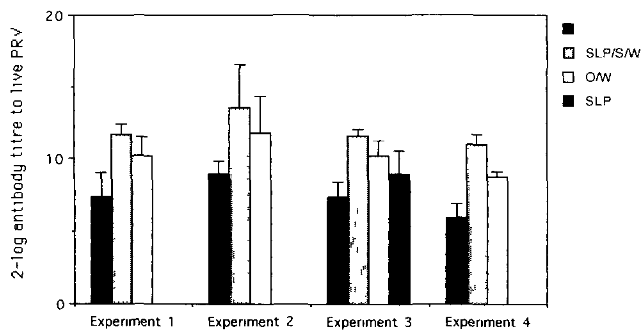

Figure 3 Effect of various adjuvants on the antibody response against live PRV in pigs after two vaccinations Mean values for five animals are represented and vertical bars indicate s e m

from 3 to 23 for SLP, 3 to 17 for S/W, 91 to 137 for O/W, and 315 to 478 for SLP/S/W (*Table 1*) SLP/S/W proved to be significantly more effective than either SLP, S/W or O/W

Stimulation of antibody responses to iPRV by SLP/S/W in pigs

In five separate experiments the effect of SLP/S/W on the antibody titre against inactivated PRV was compared with that of O/W, SLP or S/W, or with antigen alone (*Figure 2*) Both SLP and S/W induced mean fourfold increases whereas O/W and SLP/S/W evoked increases of 49- and 69-fold, respectively (*Table 2*) S/W and SLP were significantly less effective than SLP/S/W in stimulating antibody responses against iPRV

Effect of SLP/S/W on the antibody responses against live PRV in pigs

The effect of SLP/S/W, SLP and O/W on the antibody response against live PRV was studied in four separate experiments in pigs Animals were immunized twice with an interval of 3 weeks and antibody titres were measured 3 weeks after the second vaccination (*Figure 3*) SLP/S/W and O/W significantly augmented the humoral response against live PRV with a factor of 23 and 7, respectively (*Table 2*) SLP/S/W was significantly more effective than O/W SLP alone induced a slight, significant enhancement of antibody titres

Effect of SLP/S/W and O/W on virus excretion upon challenge

Groups of five pigs were vaccinated twice with a time interval of 3 weeks, 5 weeks after the second vaccination, they were challenged with virulent PRV Two out of five non-vaccinated control animals died shortly after challenge All animals that received live PRV vaccine with or without adjuvant survived the challenge

Virus titres in tonsillar swabs were monitored over 14 consecutive days (*Figure 4*) Geometric means of the number of virus particles ($TCID_{50}$) per gram sample were

Table 3 Effect of O/W and SLP/S/W on the antibody response against iPPV in pigs

			2-log antibody titres against PPV at								
			Week 0			Week 3			Week 6		
Group	Adjuvant	*n*	Mean	s e m	S	Mean	s e m	S	mean	s e m	S
1	O/W	5	<3 0	0 0	*a*	5 0	0 9	*a*	5 6	1 9	*a*
2	SLP/S/W	5	<3 0	0 0	*a*	6 7	1 5	*b*	10 8	0 5	*b*

Groups of five pigs were immunized intramuscularly with iPPV plus different adjuvants at weeks 0 and 3 Antibody titres were measured at week 0, 3 and 6 by serum neutralization Mean value, s e m and statistical significance (S) of the results are represented Groups which are not statistically different ($p>0\ 05$) are indicated by the same letter

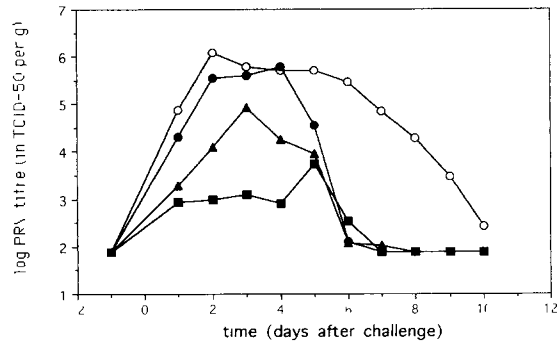

Figure 4 Virus excretion by vaccinated and non-vaccinated pigs at different time intervals after challenge with virulent PRV Mean titres of virus in nasal fluid of unvaccinated pigs (○), animals vaccinated twice with live PRV without adjuvant (●), live PRV+O/W (▲) or live PRV+SLP/S/W (■)

calculated The surviving unvaccinated animals excreted large amounts of virus during the time period from 1 to 10 days after challenge Titres higher than 10^5 virus particles per gram of sample were detected over a period of 6 days (days 1–7 after challenge) and virus excretion lasted for 10 days Animals that received antigen without adjuvant also excreted high amounts of PRV during the first 5 days but no significant excretion could be observed beyond day 7 postchallenge Immunization of animals with live PRV plus adjuvant significantly reduced virus excretion Animals immunized with PRV plus O/W excreted over 4 consecutive days 10^4–10^5 $TCID_{50}$ g^{-1} while those injected with SLP/S/W excreted about 10^4 $TCID_{50}$ of virus for only 1 day

Areas under the curves (AUCs) and above the background values (i e 10^2 $TCID_{50}$ g^{-1}) were calculated by integration and that of the group of control animals was considered to be 100% Vaccination of animals with antigen alone reduced the AUC to 57% Vaccination with O/W and SLP/S/W resulted in AUCs of 40 and 36%, respectively

Adjuvant activity of SLP/S/W and O/W for the antibody response against iPPV

Groups of pigs were immunized twice with iPPV plus either SLP/S/W or O/W as adjuvant and antibody titres were measured at different time intervals (*Table 3*) Before immunization, no antibodies were detected After the first injection, significant antibody titres in serum were observed and both adjuvants appeared to be equally effective Responses increased upon the second injection with vaccine and SLP/S/W evoked a significantly higher titre than O/W

DISCUSSION

In the literature, many adjuvants have been described but most data have been obtained from studies in laboratory animals Efficacy of these experimental adjuvants in target animals is often disappointing In this paper we described the adjuvant activity of a novel formulation in the target animal species, i e pigs It is the outcome of an extensive research programme on non-mineral oil adjuvants for veterinary purposes which included the screening of a large number of different compounds and formulations in mice or guinea-pigs and subsequent testing of promising substances in target animal species In laboratory animals, several experimental adjuvants displayed distinct activity with the different types of antigens tested and only those which exhibited strong overall activity were tested in target species Among several others, an experimental formulation comprising a synthetic polysucrose derivatized with sulfate and lipid groups and incorporated into a squalane-in-water emulsion (SLP/S/W) appeared to exert strong adjuvanticity against a protein, a hapten carrier, and two viral antigens[10] Subsequent testing in pigs revealed that this SLP/S/W was significantly more effective than several other experimental formulations (data not shown) As reported here, it enhanced antibody responses against three inactivated influenza viruses, inactivated and live PRV, and against inactivated PPV Relative to negative controls which received antigen without adjuvant, anti-influenza antibody titres were increased 315- to 478-fold Humoral responses against iPRV and live PRV were increased 69- and 23-fold, respectively As compared with the commercially applied O/W adjuvant, SLP/S/W was about three- to fourfold more effective in stimulating responses against the three influenza virus strains tested, live PRV and iPPV, and equally effective in enhancing responses against iPRV The two constituent substances SLP and S/W also augmented responses against the viral antigens but the combination thereof demonstrated synergistic activity Similar beneficial interaction has been observed in mice and guinea-pigs[10] In principle, adjuvants are used to compensate for lack of potency of inactivated antigens as compared with their live counterparts Experiments with inactivated and live PRV revealed that immune responses against both types of antigens can be enhanced upon addition of an adjuvant The stimulatory effect of adjuvants on the immunity induced by live antigens has been reported previously[11,12] and has resulted in improved vaccines against Aujeszky disease Considerable differences were seen in levels of anti-PRV antibody responses against either inactivated or live PRV Live PRV vaccine without adjuvant evoked antibody levels that were comparable with those obtained by iPRV plus adjuvant The possibility of increasing responses against live PRV by an adjuvant suggests that the immune system may not always react maximally to a live virus antigen

Increased antibody titres against live PRV upon vaccination corresponded closely to decreased titres of virus in nasal fluid at different intervals after challenge with virulent PRV A comparable, inverse relationship between humoral response and protection against virus excretion has been described by other investigators[11,13] The role of circulating antibodies in protection against virus excretion can be deduced from investigation with inactivated antigen administered parenterally as this route of immunization is thought to be incapable of inducing significant levels of either cell-mediated or local immunity Intramuscular immunization of animals with purified protein gp50 of PRV evoked neutralizing antibodies in serum and reduced virus excretion upon challenge[13]

Adjuvanticity of mineral oil emulsions is believed to be at least partially related to the persistence of oil components in the host, as emulsions of oil of either vegetable or animal origin are considerably less effective Additional active substances such as microbial glycolipids[14], synthetic block polymers of polyoxyethylene and polyoxypropylene with or without microbial products[15], avridine[16] or SLP, can compensate for low activity of the biodegradable oil emulsions

The SLP/S/W adjuvant formulation was developed to replace mineral oil-based adjuvants, thereby reducing toxic side-effects of vaccination Vaccines at present used in pigs often contain 25–60% mineral oil Concentrations of SLP and squalane used in the experimental vaccines are considerably lower, namely 0 5 and 5% respectively Furthermore, low toxicity and high biocompatibility of squalane is expected since squalane is a normal constituent of animal tissue and as such is present in low concentrations in most animal species Next to squalene, it is the most common hydrocarbon in human sebum[17] As a consequence, plain emulsions of squalane are considered to be biodegradable and to be of low or no risk to consumers of food containing residues thereof Toxicological studies on squalane affirmed relative safety[17] Squalane has been used in cosmetics in high concentrations for more than 25 years and in pharmaceuticals as a carrier for lipid drugs[17] The SLP used in this novel adjuvant formulation is a new chemical entity and besides immunostimulatory activity, biological effects are not known As it is built up of naturally occurring compounds, i e sucrose, fatty acids and sulfate, low toxicity of SLP and degradation products might be expected The sugar backbone, Ficoll-400, is a relatively inert copolymer of sucrose and epichlorohydrin and degradation of SLP *in vivo* is considered to include cleavage of lipid and sulfate–ester bonds rather than breakdown of the polymer Such a degradation will yield fatty acids, sulfate and polysucrose Taking into account (1) low doses of active components, (2) biocompatibility of squalane, (3) chemical composition and the most likely route of degradation of SLP, and (4) very low absorption rate of a polysucrose analogue with a 25-fold lower molecular weight by the gastrointestinal tract[18,19], SLP/S/W is thought to be of low risk to the consumers of food made from animals treated with this adjuvant

In summary, the novel SLP/S/W is an effective adjuvant for humoral responses against influenza virus strains A/Swine, MRC-11 and X-79, for inactivated and live PRV and for iPPV, and the two active components (i e SLP and S/W) interact synergistically Despite lower doses of active substances and biocompatibility of the oil component, it is more effective overall than the O/W adjuvant currently used As far as we know, this is the first time that a non-mineral oil adjuvant has been proven to have such a high efficacy in pigs and thus it is a potential candidate for use in porcine vaccines

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