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Water-in-oil solubilized vaccine adjuvants

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The solubilization of water and toxoid solutions in oils by non-ionic surface-active agents has been examined. Water-in-oil solubilized adjuvant formulations of vaccines containing *Clostridium welchii* type D toxoid as antigen have been tested in laboratory animals. The antitoxin titres in rabbit serum induced by the most successful formulation were as high 14 weeks after a single dose, as the peak titres after two doses of a simple aluminium hydroxide adsorbed vaccine. The vaccines are clear and of low viscosity which facilitates accurate measurement and handling by syringe.

APART from the early observations by Rabinowitsh (1897), the experimental results of Freund & Bonato (1944) first demonstrated the elevation and prolongation of antitoxin levels obtained by dispersing diphtheria toxoid in oily vehicles before injection. Water-in-oil emulsions have been used as adjuvants for a number of antigens, and the literature has been reviewed by Hilleman (1966). The adjuvant action of oil-in-water emulsions (Brit. Pat., 1963), multiple emulsions (Herbert, 1965, 1967), water emulsified in vegetable oils gelled with aluminium monostearate (Stokes, Weibel & others, 1964) and dried antigens dispersed in paraffin oil gelled with aluminium monostearate (Coles, Heath & others, 1965) has also been described. Although elevated and persistent antibody levels have been reported for all these adjuvants, they suffer from the practical disadvantages of high viscosity which limits their use in a syringe. They also require critical control of processing to ensure their physical stability and to allow their immunological advantages to be realized.

The solubilization of water in oil has been reported in the literature (Winsor, 1954; McBain & Hutchinson, 1955) and Higuchi & Misra (1962) reported studies on the solubilization of water in paraffin by cationic surfactants. Applications for such systems include dry cleaning (Fulton, Alexander & others, 1953), preparation of amphiphilic salts (Brit. Pat., 1941) and preparation of clear solutions of hydrated ephedrine alkaloid in paraffin (Bellafiore, 1965).

This paper describes water-in-oil solubilized adjuvant systems, which are effective, easily prepared, suitable for use by syringe and physically stable under practical storage conditions.

Experimental and results

Mixtures of commercial non-ionic surfactants and light mineral oil were examined, and formulae identified which permitted solubilization of water, or *Clostridium welchii* type D toxoid solution: clarity was used as the criterion of solubilization. Water contents above and below those which yielded clear products produced slightly hazy, birefringent gels which could be changed to thin clear solutions by gentle agitation and for this reason the use of the "Staggered Walk" technique (Boffey,

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Collison & Lawrence, 1959) for identification of phase boundaries was rejected.

To identify phase boundaries, accurately measured aliquots of solutions of surfactant in oil were distributed into neutral glass ampoules, and accurately measured incremental volumes of water were added. The ampoules were sealed, packed into cylinders and rolled at approximately 30 rev/min for 16 hr. The ampoules were then allowed to stand for 2 hr before examination.

A surfactant hydrophile : lipophile balance (HLB) of 10 (Griffin, 1949) is the near optimum value for the solubilization of water in paraffin oil. In general the use of mixtures of surfactants permitted the highest concentrations of water to be solubilized. The addition of a small proportion of a predominantly lipophilic surfactant to a system containing a single surfactant of HLB 10 enabled a markedly increased quantity of water to be solubilized. Fig. 1 shows the effect of the addition of Arlacel 80

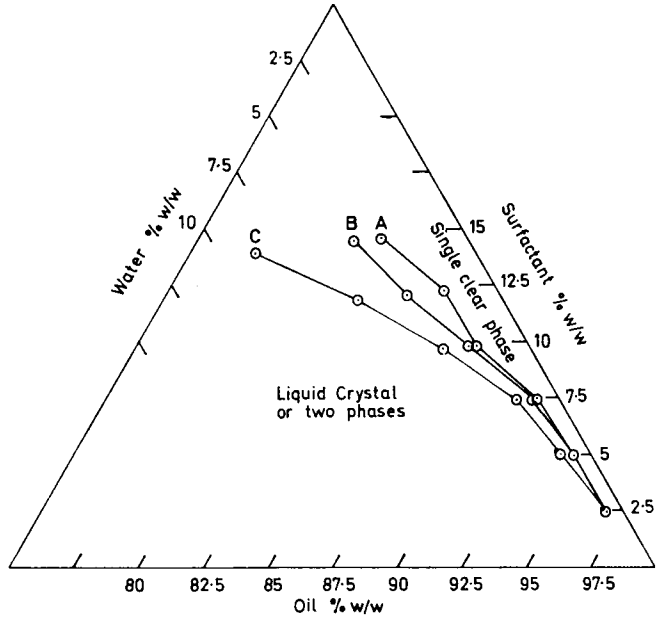

FIG. 1. Phase boundaries in the system light paraffin oil, surfactant, water. Surfactants: A. Tween 81. B. 5% Arlacel 80 in Tween 81. C. 10% Arlacel 80 in Tween 81.

(sorbitan mono-oleate) to a system of Tween 81 [polyoxyethylene (5) sorbitan mono-oleate], light paraffin oil and water. When toxoid solution was substituted for water, the volume which could be solubilized was, in contrast, decreased in the presence of small proportions of lipophilic surfactant.

Water could be solubilized in paraffin oils and pure hydrocarbons, both straight chain and branched; in fatty alcohols and their fatty acid

esters in significantly lower concentrations, and in extremely low concentrations in vegetable oils, pure triglycerides and fatty alcohol ethers.

A series of vaccines was prepared by sterilization of solutions of the surfactants in oil by membrane filtration, followed by aseptic addition of antigen solution, with gentle agitation. The formulae of the vaccines thus prepared are shown in Table 1.

TABLE 1. FORMULAE OF VACCINES TESTED

No	Antigen (Lf/ml)	Surfactants*		Surfactant conc. % w/v	Oil
1	CWD 65	AA/T 20	65:35	8·75	Puremor**
2	CWD 65	A 80/T 20	60:40	8·25	Puremor
3	CWD 65	AA/T 80	55:45	9·50	Puremor
4	CWD 50	AA/T 20	60:40	8·25	Puremor
5	CWD 50	T 81		10·00	Puremor
6	CWD 50	T 81		10·00	Tridecyl myristate
7	CWD 50	A 80/T 80	10:90	10·00	Squalane
8	CWD 250	Tn x-100/Tn x-15	50:50	18·00	Puremor
9	CWD 50	T 81		10·00	Puremor
10	CWD 50	T 81		10·00	Puremor
11	CWD 50	T 81		10·00	Puremor

*Abbreviations:
T = Tween (Honeywill Atlas Ltd.)
A = Arlacel (Honeywill Atlas Ltd.)
Tn = Tritons (Charles Lennig & Co.)
** Puremor extra light white oil (Burmah Oil Co.).

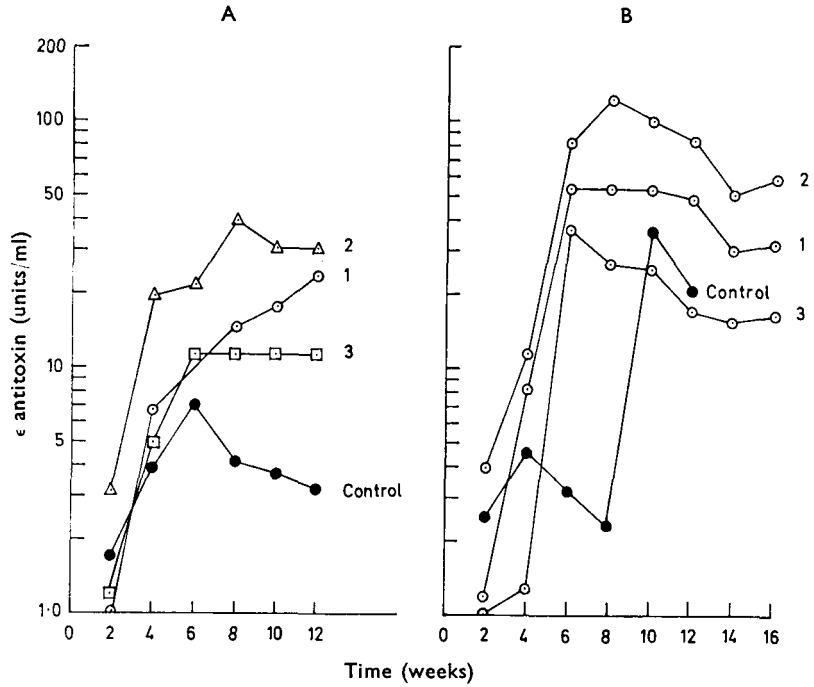

FIG. 2. ϵ-Antitoxin titres produced in rabbit serum by: (A), a 2 ml subcutaneous injection of *Clostridium welchii* type D vaccine, and (B), a second dose after 4 weeks. Formulae 1, 2 and 3. Control, 65 Lf/ml aluminium hydroxide adsorbed toxoid.

Vaccines were injected subcutaneously into laboratory animals. Groups of six rabbits or six guinea-pigs were used, and serum samples were titrated for their content of *Clostridium welchii* ϵ antitoxin. Results for rabbits are the mean titres from six animals; those for guinea-pigs are from pooled sera of six animals.

Vaccines 1–3, containing 65 Lf/ml of *Clostridium welchii* type D toxoid, were injected subcutaneously into rabbits and guinea-pigs. Dose schedules and serum antibody titres are shown in Fig. 2 and Table 2.

Vaccine 4 was prepared to the same formula as the most successful vaccine above (No. 2) but with the toxoid content reduced to 50 Lf/ml. Antibody levels measured in rabbits are shown in Fig. 3.

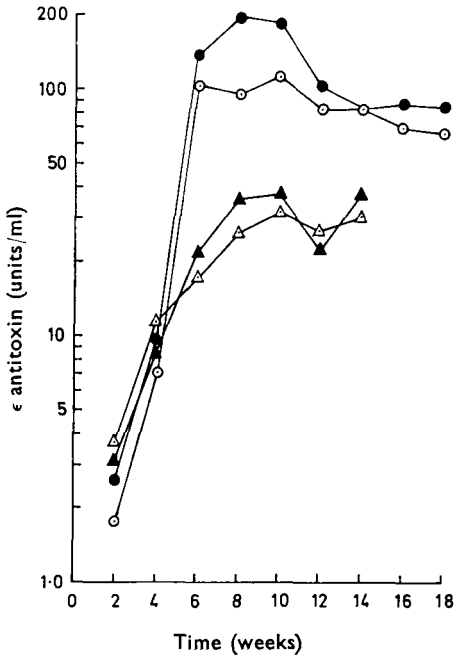

FIG. 3. ϵ-Antitoxin titres produced in rabbit serum after subcutaneous doses of vaccine 4. ● 1 × 2 ml followed by 1 × 2 ml after 4 weeks; ⊙ 1 × 1 ml followed by 1 × 1 ml after 4 weeks; ▲ 1 × 2 ml; △ 1 × 1 ml.

Vaccines 5–8 were prepared using a range of oils and types of surfactant. Antibody levels measured in guinea-pigs are shown in Fig. 4.

One vaccine, to the same formula as vaccine 5, was prepared by three different methods, namely, addition of antigen solution to a solution of surfactant in oil (vaccine 9), addition of surfactant to a crude emulsion of antigen solution in oil (vaccine 10) and addition of oil to a blend of antigen solution and surfactant (vaccine 11). All these products were stable and physically indistinguishable. Tests made in guinea-pigs showed negligible differences in antibody levels induced.

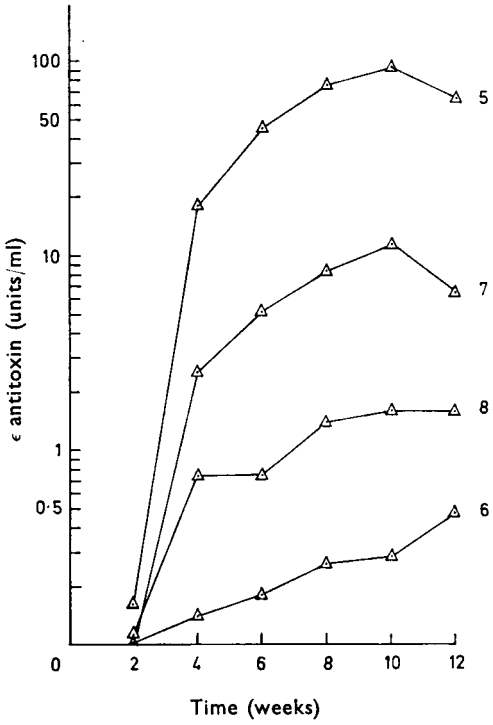

FIG. 4. ϵ-Antitoxin titres in guinea-pig serum after 1 ml subcutaneous doses of vaccines 5–7 and a 0·2 ml dose of vaccine 8.

Discussion

All but two formulations tested were good adjuvants. Levels of circulating antibodies in experimental animals were much elevated compared with those in control animals vaccinated with the same toxoid dose adsorbed on aluminium hydroxide gel before injection, and there is some evidence of prolongation of elevated levels (Figs 2 and 3 and Table 2). Neither reduction of the toxoid dose (Figs 3 and 4) nor

TABLE 2. ϵ-ANTITOXIN TITRES IN GUINEA-PIG SERUM AFTER 1 ML SUBCUTANEOUS INJECTION OF VACCINES 1–3
Controls 1, single 1 ml dose; 2, second 1 ml dose after 4 weeks of 65 Lf/ml aluminium hydroxide adsorbed toxoid

Time (weeks)	Formula			Control 1	Control 2
	1	2	3		
2	0·8	0·4	1·4	0·28	0·28
4	14	14	14	5·6	—
6	14	28	14	5·6	80
8	14	28	—	5·6	14
10	28	28	28	5·6	14
13	28	28	28	5·6	14

halving of the dose volume (Fig. 4) had significant effects on the responses.

Of the two vaccines that failed, that prepared in tridecyl myristate (No. 6) was physically unstable *in vitro* at 37°. The other vaccine that failed (No. 8) was in a dose volume of 0·2 ml, the phase equilibrium of the formulation requiring this vaccine to be prepared containing 250 Lf/ml. The small dose volume may have contributed to the poor result obtained.

All the vaccines reported contained *Clostridium welchii* type D toxoid as antigen, but *Cl. welchii* type B and C, *Cl. tetani*, *Cl. oedematiens* and *Cl. septicum* toxoids have also been examined; similar results were obtained.

In contrast to emulsified vaccines, the vaccines described are physically stable, easily prepared, and their clarity and low viscosity permit accurate measurement and convenient administration with a hypodermic syringe.

These vaccine formulations are the subject of patent applications (B.P. Application No. 25,595/66).

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