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Adjuvant effect of aluminium monostearate paraffin gels on antitoxin response

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Clostridium welchii Type D toxoid has been dispersed in aluminium monostearate-hydrocarbon gels. When these are injected subcutaneously in laboratory animals the antitoxin response is influenced by the method of preparation of the gels. The level and duration of the antitoxin titre in the blood is related directly to the viscosity of the vaccine preparations. Six months after injection, the residual antitoxin titre produced by the most successful treatments was still as good as the peak titre obtained from simple aluminium hydroxide adsorbed vaccine.

THE blood level of antitoxin produced by a single dose of toxoid is frequently inadequate to develop satisfactory immunity so that a second or "booster" dose is required. When aluminium hydroxide gel is used as an adjuvant for toxoid vaccines, it significantly increases the response to the antigen, but it may also produce reactions at the site of injection. Moreover, the blood antitoxin levels fall fairly rapidly, even after a "booster" dose.

Improvement of antitoxin levels by dispersing antigen in pharmaceutical vehicles immiscible with serum has been demonstrated on several occasions, and the mechanisms of action of such systems has been reviewed (McKinney & Davenport, 1961; Davenport, 1961). Oil: aluminium monostearate gels have recently been shown to enhance antitoxin blood levels (Woodhour, Metzgar, Stim, Tytell & Hilleman, 1964; Stokes, Weibel, Drake, Woodhour & Hilleman, 1964).

It is well known that the use of such gels lowers the rate of release of procaine penicillin or the active principle in pollen extract from the site of injection (Bristol, 1952, 1954).

Our attention was drawn to the enhanced blood antitoxin levels obtained from injections of *Clostridium welchii* Type D toxoid when presented in liquid paraffin containing aluminium stearate (Dr. C. Moller, personal communication). The present communication describes an examination of liquid paraffin aluminium stearate suspensions and gels in this connection.

Experimental

MATERIALS

The paraffin oil used was a mixture of 75 parts (by volume) liquid paraffin B.P. and 25 parts light liquid paraffin B.P. 1958. It was sterilised by heating to 150° for 1 hr. Aluminium monostearate* had the following characteristics: Al_2O_2 (ash) 16·6%; free fatty acid (as stearic acid) 6·7%; water soluble salts 0·5%. It was sterilised by exposure

From Glaxo Laboratories Ltd., Greenford, Middlesex.

* Mallinkrodt special M grade.

to formalin vapour (24 hr), excess formalin vapour was then removed under reduced pressure.

The antigen used was a freeze-dried *Clostridium welchii* Type D purified formol toxoid comminuted by successive passage through 60 mesh and 100 mesh sieves. It had a potency of approximately 40 Lf units/mg.

PREPARATION OF PARAFFIN GELS

Two general methods were used.

Method A. Aluminium monostearate was dispersed in sufficient paraffin oil to constitute 20% by volume of the final vaccine. The suspension was then maintained at the required temperature for a fixed period (72° or 100° for 10 min, or 150° for 1 hr). The products were cooled to room temperature without agitation. The antigen was dispersed in the remainder of the cool sterilised oil, and the paraffin gel was then dispersed in this suspension; the mixture was finally passed once through an Ormerod homogeniser Type QR at 1,000 p.s.i. At 72° only partial gelation occurs; the product corresponds to that described by Moller.

Method B. Aluminium monostearate was dispersed in sufficient paraffin oil to produce 80% of the final vaccine volume. This suspension was heated to either 100° or 150° and stirred for 1 hr at this temperature; it was then cooled with continuous agitation. The antigen was dispersed in the remainder of the cool sterilised oil and blended with the paraffin gel; the vaccine was passed once through an Ormerod homogeniser, as above.

Suspensions of aluminium stearate (2% w/v) in paraffin oil decreased in viscosity as they were heated slowly to 80°, owing to a reduction in the viscosity of the oil. Microscopical examination of the suspensions indicated that the soap particles began to swell at 80 to 89°. At 95° complete solution of the soap yielded a visco-elastic gel. A further rise in temperature, to 135°, was accompanied by a sharp drop in the viscosity; this may be attributed to dissociation of the soap molecules by thermal motion.

Heating the gels to 100° followed by rapid cooling without agitation yielded a thick visco-elastic gel (Method A), whereas cooling associated with vigorous agitation yielded a thinner though still viscous gel (Method B). Rapid cooling of the gel from 150° (Method A) produced a solid grease which if dispersed in paraffin oil, yielded a product of viscosity similar to that cooled from 100°. If this was again heated to 150° (Method B) and stirred continuously while cooling, the least viscous gel of all was produced; it was much thinner than that produced at 100°.

An attempt was made to measure the viscosity of the gel systems with a rotary viscometer but no single parameter was found to express adequately the different characters of the gels. For this reason only visual observations of viscosity have been reported.

Antitoxin was induced in the serum of rabbits by subcutaneous injection of 2 ml (equivalent to 130 Lf units of toxoid) of each of these vaccines. Four weeks later a similar booster dose was given. The antitoxin levels

obtained are shown in Fig. 1. The titres presented as control were obtained by adsorbing antigen, from the same batch, on aluminium hydroxide gel.* Antigens before and after freeze drying were used for this purpose.

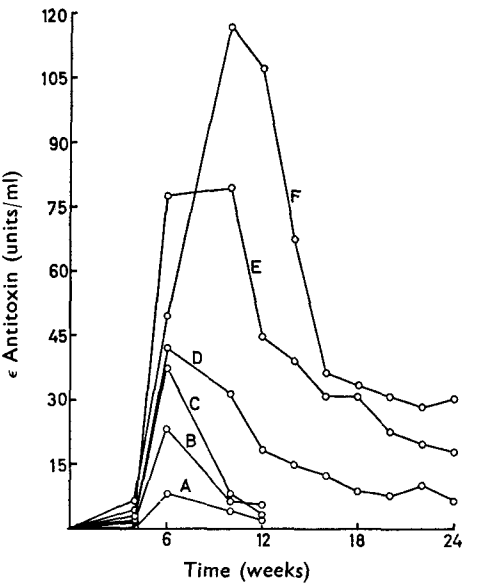

FIG. 1. Epsilon antitoxin titres produced in rabbits by a 2 ml subcutaneous injection of *Clostridium welchii* type D vaccine, followed by a booster dose at four weeks, A. Ungelled aluminium monostearate suspension. B. Reconstituted freeze-dried toxoid, aluminium hydroxide adsorbed. C. Aluminium hydroxide adsorbed toxoid. D. Aluminium monostearate gel prepared at 72°C. E. Aluminium monostearate gel prepared at 150°C. F. Aluminium monostearate gel prepared at 100°C.

A second series of vaccines was prepared both by methods A and B at the temperatures that had given most enhancement in the first experiments. The amount of aluminium stearate varied, 0·5, 1·0 and 2·0% being used. Antitoxin levels in guinea-pigs (after single 1 ml subcutaneous injection of vaccine) are shown in Table 1.

The addition of traces of many compounds, particularly those with pronounced co-ordinating properties, reduces the viscosity of gels (Gray & Alexander, 1949; Alexander & Gray, 1950). Non-ionic surface-active agents have this effect. A series of gels with a range of aluminium monostearate concentrations and incorporating 0·5% polysorbate 60† were prepared by Method B at 150°.

Guinea-pig and rabbit sera were titrated for their content of *Clostridium welchii* ε antitoxin. Approximately twofold dilutions of sera were

* Alhydrogel—Danish Sulphuric Acid and Superphosphate Works Ltd., Denmark.

† Tween 60—Honeywill-Atlas Ltd.

titrated in mice against standard *Clostridium welchii* ϵ toxin by the method of Batty & Glenny (1947).

TABLE 1. ANTITOXIN LEVELS (GUINEA-PIGS) FROM DIFFERENT ALUMINIUM STEARATE CONCENTRATIONS AND GELATION TEMPERATURES

Method of preparation	Temperature of gelation, °C	Aluminium monostearate %	Antitoxin units/ml				
			2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
A	100	0·5	0·06	0·28	0·4	0·4	0·14
		1·0	0·14	2·8	2·8	1·4	1·4
		2·0	0·14	5·6	4	5·6	5·6
A	150	0·5	0·14	0·8	1·4	1·4	0·56
		1·0	0·28	1·4	1·4	1·4	1·4
		2·0	2	14	14	14	14
B	100	0·5	0·56	0·8	2	2	2·8
		1·0	0·56	5·6	5·6	5·6	5·6
		2·0	1·4	28	28	14	14
B	150	0·5	0·28	0·04	0·28	0·14	0·08
		1·0	0·4	4	4	5·6	2·8
		2·0	0·28	2·8	2·8	5·6	4
Aluminium hydroxide adsorbate suspension			0·2	2·8	1·4	1·4	1·4

Results

The results in Fig. 1 show that a base prepared by the gelation of paraffins with aluminium monostearate increases and prolongs the titre of antitoxin in the blood. As the content of aluminium monostearate rises from 0·5 to 2%, the antitoxin level in guinea-pig serum rises irrespective of the method of preparation or the temperature of gelation (Table 1). Six months after injection the residual titre with the most successful vaccines was still as high as the peak titre obtained with a simple aluminium hydroxide adsorbed vaccine. The viscosity of the vaccines showed a positive correlation with the degree of enhancement and the prolongation of the antitoxin levels.

Products obtained by Method A confirm the effect of temperature on gelation although the results for vaccines prepared by Method B do not. The conclusion drawn from Fig. 1 is confirmed, namely that antitoxin titres and the viscosity of the vaccine are related.

TABLE 2. ANTITOXIN LEVELS IN GUINEA-PIGS FROM GELS WITH AND WITHOUT POLYSORBATE (P) 60

Aluminium monostearate %	Percentage P 60	Antitoxin units/ml				
		2 weeks	4 weeks	6 weeks	8 weeks	12 weeks
0·5	—	0·2	0·4	2·8	2·8	2
0·5	0·5	0·06	0·4	0·56	0·56	0·56
1·0	—	0·04	2·8	2·8	5·6	5·6
1·0	0·5	0·2	1·4	1·4	0·56	0·56
2·0	—	0·28	4	5·6	5·6	8
2·0	0·5	0·14	4	2·8	2·8	2·8

Table 2 confirms that antitoxin level is enhanced as the concentration of aluminium stearate increases. The addition of polysorbate 60 depresses antitoxin levels which moreover seem less prolonged. These effects are also related to the lower viscosity of these bases.

Acetoglycerides also reduce the viscosity of the gels, but they could not be evaluated in animals because gross reactions were produced by them at the site of injection.

Discussion

Hydrocarbon-aluminium stearate gels comprise a two-phase structure. One phase consists of a network of more or less solvated materials, whose interstices are filled with its saturated solution (Rideal, 1950). It is possible that particles of antigen are situated within the network of solvated material so that their diffusion from the vaccine is hindered. This hypothesis received some support from an experiment in which the freeze-dried toxoid was milled in oily suspension, to provide particulate toxoid: by microscopical appearance this exhibited a range of particle sizes. The samples of milled toxoid in paraffin-aluminium stearate gel however show negative correlation of particle size and antitoxin response.

The way in which oil adjuvant vaccines enhance and prolong antibody production is not clearly established. In simplified general terms two mechanisms have been suggested (Davenport, 1961): (*a*) local irritation at the site of injection attracts certain cells to the site so that an "antibody producing organelle" is formed and (*b*) that slow continuous release of antigen from the site of inoculation promotes more efficient utilisation of the antigen for its specific purpose. The local reactions from all the vaccine preparations (except those prepared with acetoglycerides) were macroscopically indistinguishable from each other and from those produced by the simple suspension of aluminium monostearate in paraffin oil.

These results suggest that, because the viscosities of the products described correlate directly with antitoxin response, the rate of release of the antigen from the injection site plays an important part in determining the adjuvant effect. In the absence of histological data the significance of other mechanisms which may operate at a cellular level cannot be assessed.

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