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Experiments designed to determine the mechanism of the adjuvant activity of Gram-negative organisms upon antibody production

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INTRODUCTION

The adjuvant action of Gram-negative bacteria upon antibody production has been known for some time (Ramon & Zoeller, 1926; Ramon, 1931; Maclean & Holt, 1940). Greenberg & Fleming (1947, 1948) observed the adjuvant effect of *Bordetella pertussis* vaccine on the response to diphtheria toxoid in guinea-pigs and Fleming, Greenberg & Beith (1948) obtained similar results in children. Since then plain diphtheria toxoid mixed with pertussis vaccine has been used as a combined prophylactic.

Farthing (1961) showed that the maximal effect in guinea-pigs was given by 500–1,000 million *B. pertussis* organisms per 5 Lf diphtheria toxoid and the response remained at the same level for higher doses of organisms. Lipopolysaccharide from these Gram-negative bacteria was found to be reponsible for this adjuvant effect and of this the lipid A component was the active part.

This investigation deals with some of the manifestations of the adjuvant action of *B. pertussis* and its lipopolysaccharide on antibody production to diphtheria toxoid and attempts to explain the possible mechanism of this enhancement.

MATERIALS AND METHODS

Diphtheria toxoid, Bordetella pertussis *vaccine*, Escherichia coli *vaccine*, *lipopolysaccharide and lipid A* were prepared, together with the reasons for doses used, as described in a previous paper (Farthing, 1961).

P.T.A.P. (Purified Toxoid precipitated by Aluminium Phosphate). Prepared as in W.H.O. monograph (1953).

Guinea-pigs. Porton strain. Albinos weighing 300–350 g. The animals were individually identified using a colour code which enabled accurate statistical analysis to be made of the serum antitoxin levels.

Mice. L.A.B. grey strain. Bred in this Institute. Approximately 20 g.

Rabbits. Weighing approximately 3 kg.

Injection procedure. In all the experiments on antibody response guinea-pigs were injected subcutaneously into the ventral wall of the abdomen (1 ml.), mice intraperitoneally (0·5 ml.), and rabbits intracutaneously in the left ankle (0·2 ml.).

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Sera. Guinea-pigs were bled by cardiac puncture, rabbits from the ear, and mice were bled from the heart under anaesthesia. The sera obtained from the samples of blood obtained from those bleedings were stored at $-20°$ C.

LD 50. Mice (L.A.B. grey) weighing 18–20 g. were used. LD 50 estimates were calculated using the method of Reed & Muench (1938).

Estimation of diphtheria antitoxin in serum. Individual serum diphtheria antitoxin titres were estimated by a micro-method based on the technique of Romer & Sames (1909) and the geometric means were calculated for each group. Mathematical analysis of results described in Farthing (1961). Unless otherwise stated, all values of antitoxin *titre* of groups of animals signify *geometric mean* titre in the group.

RESULTS

(1) *Earlier production of antibody*

One group of 18 guinea-pigs received 5 Lf plain diphtheria toxoid mixed with $1{,}000 \times 10^6$ *B. pertussis* organisms and a similar control group received 5 Lf diphtheria toxoid alone. They were bled 7, 14, 21 and 28 days after the primary inoculation.

Animals receiving toxoid alone did not begin to show titratable levels of antitoxin ($>$ 0·0002 units/ml.) until the 21st day, whereas those receiving toxoid and vaccine did as early as the 7th day (Table 1). There was, therefore, substantial speeding-up of the antibody response under the influence of the vaccine.

The 43-fold adjuvance manifested after 28 days by the presence of *B. pertussis* vaccine was in the range of enhancement usually found.

(2) *No sustained response*

Serum antitoxin titres of groups of guinea-pigs given 5 Lf of toxoid and increasing concentrations of *B. pertussis* vaccine from 125×10^6 to $1{,}000 \times 10^6$ organisms were observed over a period of 8 months. Fig. 1 shows the peak of each response to be at the same time following injection. Although there is a 40-fold adjuvant effect after 1 month, from the 2nd month onwards the adjuvance ratio between the optimal dose and the control is constant between four- and fivefold. Fig. 2 shows the responses from a similar experiment using increasing concentrations of *E. coli* lipopolysaccharide. The results are similar to those seen in Fig. 1. *B. pertussis* lipopolysaccharide was also tested but the animals had to be destroyed after 3 months because of infection, although again the results were similar to that of complete vaccine.

These experiments indicate that this initial adjuvant effect is not followed by a prolonged stimulation of antibody, although an enhancement of antibody titre is still shown after 8 months.

(3) *Effect only on primary response*

Four groups of guinea pigs were used: two groups received 5 Lf plain diphtheria toxoid subcutaneously in the primary inoculation and the other two groups 5 Lf toxoid $+ 1000 \times 10^6$ *B. pertussis* organisms. These animals were bled by cardiac

puncture after 28 days and reinjected. In each case one of the 2 groups received 5 Lf diphtheria toxoid and the other 5 Lf toxoid + 1000×10^6 organisms *B. pertussis*. The guinea-pigs were bled 5, 9, 12, 16, 19, 26, 36, 49 and 84 days after the secondary inoculation.

It can be seen from Fig. 3 that *B. pertussis* vaccine has no adjuvant effect on diphtheria toxoid in the secondary response if the guinea-pigs have received vaccine with the primary inoculation.

B. pertussis vaccine had an adjuvant effect in the secondary response only when it was not present in the primary. The enhancement is only 4·3-fold ($0{\cdot}05 < P < 0{\cdot}10$) at the peak of the secondary and is reduced to 1·3 after 3 months. Although adjuvance seems to have taken place, the results are not statistically significant.

Table 1. *Relative rates of antitoxin production in groups of guinea-pigs receiving diphtheria toxoid* (*a*) *alone, and* (*b*) *mixed with* B. pertussis *vaccine*

		Days after inoculation			
		7	14	21	28
Inoculum	No./group	No./group each having titres > 0·0002 u./ml. serum			
5 Lf plain formol toxoid	18	0	0	2	18 (titre 0·003 u./ml.)
5 Lf toxoid + 10^9 pertussis organisms	18	1	10	18 (titre 0·02 u./ml.)	18 (titre 0·11 u./ml.)

(4) *No adsorption or admixture necessary but action exerted early*

An experiment was designed to demonstrate whether any adsorption takes place between diphtheria toxoid and *B. pertussis* vaccine in mixture with saline as diluent and in the presence of normal guinea-pig serum. *In vitro* tests on Lf values of supernatants and *in vivo* tests on antitoxin titres produced in guinea-pigs by the various fractions (complete mixture, supernatant and residue) were measured. The experiment was in 5 parts:

(*a*) Effect immediately on mixing with saline (usual diluent for dispensing purposes).

(*b*) Left overnight at room temperature with saline (18 hr.).

(*c*) Left for 36 hr. at room temperature with saline.

(*d*) After 15 min. at 37° C. with normal guinea-pig serum.

(*e*) Left overnight at room temperature with normal guinea-pig serum (18 hr.).

The mixtures used in the 5 parts each contained a final concentration of 50 Lf diphtheria toxoid and $10{,}000 \times 10^6$ *B. pertussis* organisms.

In vitro *test.* Lf values of the supernatants were found as shown in Table 2. The results are the average of 3 mixtures for each part of the experiment. Within the accuracy of the flocculation test no adsorption could be demonstrated.

In vivo *test.* In these tests with guinea-pigs the above fractions were diluted 1/10 to give 5 Lf diphtheria toxoid and 1000×10^6 *B. pertussis* organisms per ml.

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since this optimum value was that used in previous experiments. 1 ml. of each fraction was injected subcutaneously into each of 9 animals which were bled after 28 days and the individual antitoxin titres measured. The results are shown in Table 3. The complete mixtures of diphtheria toxoid and vaccine from all 5 parts of the experiment showed typical responses from 5 Lf toxoid and 1000×10^6 *B. pertussis* organisms (cf. Figs. 1, 3). No response was obtained from the washed bacteria alone after contact with the concentrated toxoid.

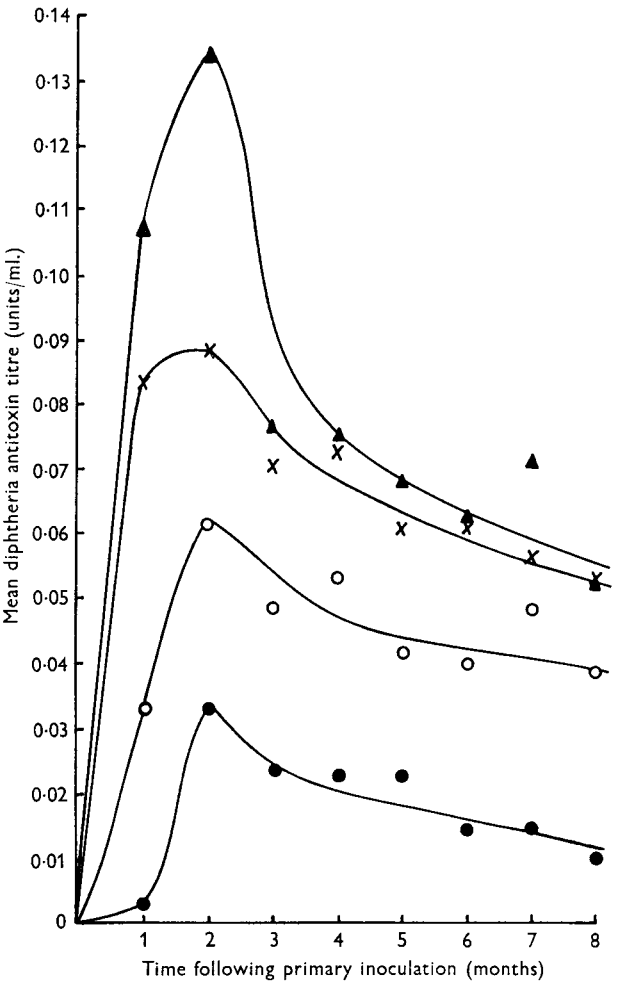

Fig. 1. Relation between diphtheria antitoxin response and various doses of *B. pertussis* vaccine over a prolonged period. ●—●, 5 Lf diphtheria toxoid; ○—○, 5 Lf diphtheria toxoid + 125 M *B. pertussis* organisms; ×—×, 5 Lf diphtheria toxoid + 375 M *B. pertussis* organisms; ▲—▲, 5 Lf diphtheria toxoid + 1000 M *B. pertussis* organisms.

It can be assumed that no adsorption takes place in the adjuvant action of *B. pertussis* vaccine on diphtheria toxoid, although the adjuvant appears to have become water-soluble

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In order to ascertain whether admixture of the toxoid and vaccine was essential for adjuvance to occur, the following procedures were adopted:

(*f*) 5 Lf plain diphtheria toxoid was administered subcutaneously in the ventral wall.

(*g*) 5 Lf diphtheria toxoid + 1000×10^6 *B. pertussis* organisms inoculated together subcutaneously in the ventral wall, having been mixed prior to injection.

(*h*) 5 Lf diphtheria toxoid and 1000×10^6 *B. pertussis* organisms both injected subcutaneously into the ventral wall at the same time but in separate sites 4 in. apart.

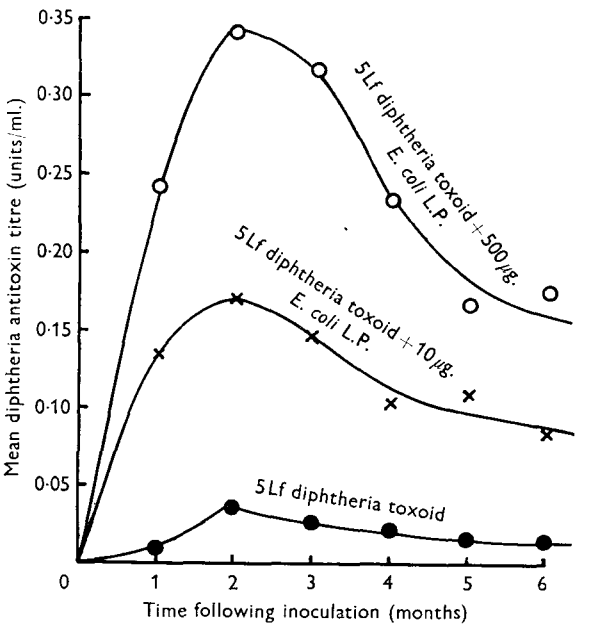

Fig. 2. Effect of *E. coli* lipopolysaccharide (LP) on diphtheria antitoxin production over a prolonged period.

Individual serum diphtheria antitoxin titres were determined 28 days after inoculation and are shown in Table 4. The responses from guinea-pigs receiving toxoid and vaccine in separate sites was slightly less than those receiving the admixture. But the responses from the former group greatly exceeded the antitoxin level attained by toxoid alone and was in the range of adjuvance normally found.

In another experiment *B. pertussis* vaccine was injected at various time intervals into groups of guinea-pigs, from 7 days before the antigen up to 7 days following it. Serum antitoxin levels were determined 1 month after injection of the toxoid and the results are shown in Table 5. All groups contained 9 animals except (*l*) which was part of another experiment included with the same batch of guinea-pigs. The time of injection of *B. pertussis* vaccine in relation to antigen administration is of critical importance in augmenting antibody levels. Vaccine given simultaneously or within 1 day following a single injection of 5 Lf diphtheria toxoid

enhanced the production of antibody in guinea-pigs. This did not occur when vaccine was given up to 24 hr. prior to the antigen. Furthermore, the stimulus appeared to be most effective when administered simultaneously with the antigen.

(6) *Effect of minimal stimulating dose and hyperplasia*

The minimal stimulating dose could be defined as the basic level of antigen that has to be acquired within a cell to activate its antibody-forming mechanism.

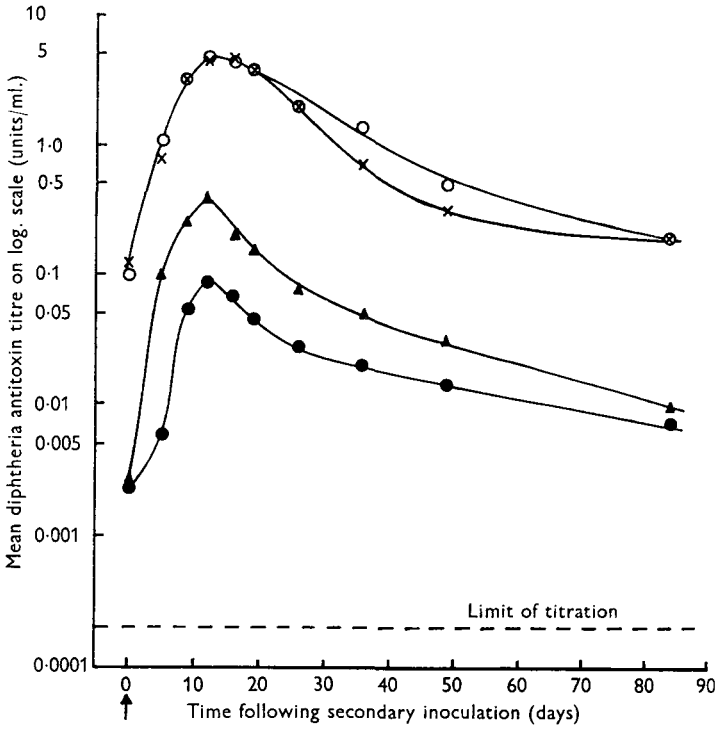

Fig. 3. Effect of *B. pertussis* vaccine on the secondary response to diphtheria toxoid.

	Primary	Secondary
●—●	5 Lf diphtheria toxoid	5 Lf diphtheria toxoid
▲—▲	5 Lf diphtheria toxoid	5 Lf diphtheria toxoid + vaccine
×—×	5 Lf diphtheria toxoid + vaccine	5 Lf diphtheria toxoid
○—○	5 Lf diphtheria toxoid + vaccine	5 Lf diphtheria toxoid + vaccine

Table 2. *Supernatant Lf values in various mixtures of diphtheria toxoid and* B. pertussis *vaccine*

Treatment		Lf/ml. added	Lf/ml. in supernatant
Original toxoid (no vaccine present)		50	50
Saline (vaccine present)	(*a*) immediate upon mixing	50	48
	(*b*) after 18 hr. at R.T.	50	50
	(*c*) after 36 hr. at R.T.	50	48
Normal guinea-pig serum (vaccine present)	(*d*) after 15 min. at 37° C.	50	50
	(*e*) after 18 hr. at R.T.	50	51

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Increasing doses of plain diphtheria toxoid (1, 2, 5, 50 and 200 Lf) were injected, subcutaneously in 1 ml., into 4 groups of 12 guinea-pigs. The responses are shown in Fig. 4 and the estimated slope of the regression line (x) was 0·6. Below 5 Lf the response fell away rapidly.

Table 3. *Antitoxin responses in groups of guinea-pigs from various mixtures of plain diphtheria toxoid and* B. pertussis *vaccine: measured 28 days after a single subcutaneous inoculation*

		Treatments diluted to 5 Lf toxoid + 1000 × 10^6 *B. pertussis* organisms				
Fractions		(*a*) immediate upon mixing	(*b*) after 18 hr. at R.T.	(*c*) after 36 hr. at R.T.	(*d*) after 15 min. at 37° C. in serum	(*e*) after 18 hr. at R.T. in serum
Complete mixture of toxoid + vaccine	No./group	9	9	9	9	9
	Titre	0·10	0·13	0·10	0·13	0·10
Supernatant of mixture	No./group	9	9	9	9	9
	Titre	0·11	0·11	0·16	0·13	0·14
Washed residue of mixture	No./group	9	9	9	9	9
	Titre	All < 0·0002 units/ml.				

Table 4. *Antitoxin responses in groups of guinea-pigs from toxoid and vaccine mixed or administered separately: measured 28 days after injection*

	(*f*) 5 Lf toxoid only	(*g*) 5 Lf toxoid + 1000 × 10^6 *B. pertussis* organisms injected together	(*h*) 5 Lf toxoid and 1000 × 10^6 *B. pertussis* organisms injected in different sites
No./group	12	12	12
Titre	0·003	0·098	0·046
Log σ	0·45	0·31	0·46
P (cf. (*f*))	—	< 0·001	< 0·001
P (cf. (*g*))	—	—	0·02 < P < 0·05

Table 5. *Antitoxin responses in groups of guinea-pigs to formol toxoid in relation to pertussis vaccine administered at various times before and after inoculation of the toxoid*

(Titres measured 28 days after injection of the toxoid.)

	Times of administration of vaccine before and after 5 Lf diphtheria toxoid						
	(*i*) 7 days before	(*j*) 3 days before	(*k*) 1 day before	(*l*) vaccine and toxoid together	(*m*) 1 day after	(*n*) 3 days after	(*o*) 7 days after
No./group	9	9	9	12	9	9	9
Titre	0·003	0·004	0·002	0·10	0·04	0·002	0·004
Log σ	—	—	—	0·31	0·77	—	—
P (cf. (R))	—	—	—	—	0·05 < P < 0·1	—	—

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If a decrease in the effective minimal stimulating dose were the only explanation of adjuvance then Fig. 5 shows what would be expected when *B. pertussis* vaccine was added to the doses of toxoid. This addition was done experimentally using the constant ratio of 200×10^6 *B. pertussis* organisms per Lf diphtheria toxoid at each of the 4 dosage levels used above. The doses of plain toxoid previously described and toxoid with vaccine were, in fact, all administered at the same time and in the same experiment. The responses are also shown in Fig. 4 with the estimated regression line (y). As well as showing a decrease in the effective

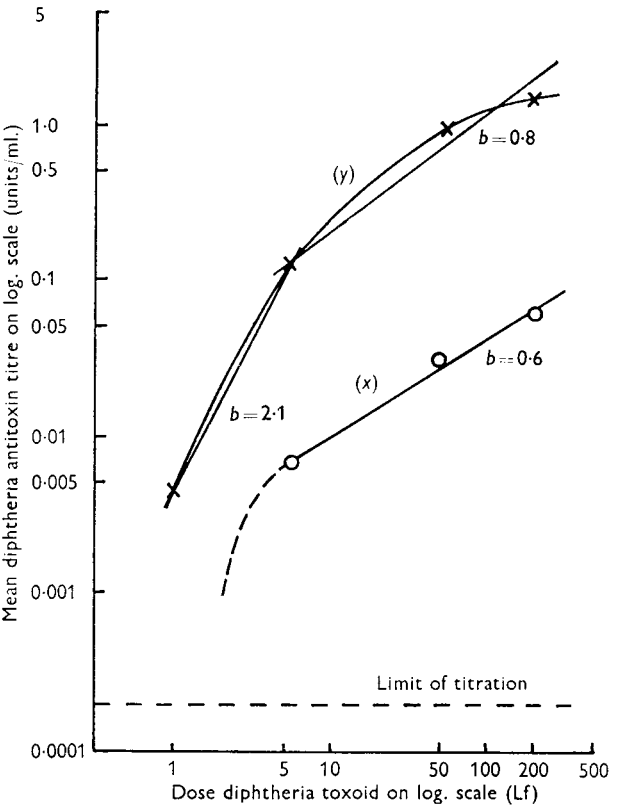

Fig. 4. Antibody response to various doses of toxoid alone and mixed with *B. pertussis* vaccine.

minimal stimulating dose the slope of the graph was also altered. The amount of adjuvance remains fairly constant and only varies between 19- and 31-fold. Holt, Barnes, Bousfield, Spiller & Croake (1959) found the slope of the regression line with toxoid/vaccine mixture was 2·2. In their experiment the line was straight but they only used small doses of toxoid (2·5, 1·25 and 0·625 Lf). As seen in Fig. 4 the curve tends to flatten and two straight lines can be drawn from (y). That in the lower dose range (1 and 5 Lf) has a slope of 2·1 whilst the higher range is 0·8. The steeper slope of the lower range has only two points on it, of which the upper one is twice the highest dose of Holt *et al.* (1959) but is consistent with their

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findings. These results give an indication that above 50 Lf toxoid and vaccine a possible maximum number of antibody-producing cells have been stimulated.

Since the slope is also altered with vaccine, and is greater than 1·0, it seems likely that hyperplasia of antibody-producing cells is taking place as well (Holt, 1951).

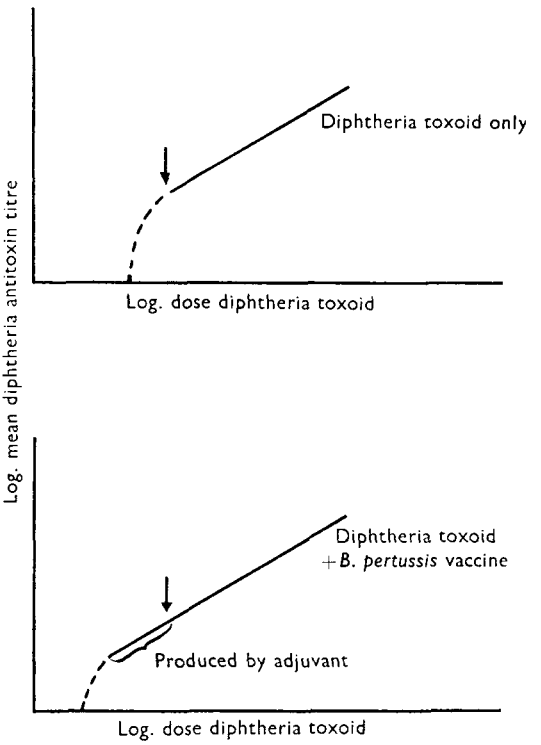

Fig. 5. Diagrammatical representation of the possible effect of a decrease in the effective minimal stimulating dose.

In an attempt to demonstrate a decrease in the effective minimal stimulating dose by *B. pertussis* vaccine, without hyperplasia, it was given with different dosage levels of P.T.A.P. The amount of antigen was reduced but the carrier concentration kept constant to ensure similar distribution. Fig. 6 from Holt (1950) diagrammatically illustrates the responses from guinea-pigs receiving increasing amounts of diphtheria toxoid while keeping the amount of $AlPO_4$ carrier constant. Using this graph as a guide three groups of 12 guinea-pigs were injected subcutaneously with the following mixtures:

(i) 6 Lf P.T.A.P. containing 1 mg. $AlPO_4$ at saturation point.
(ii) 0·03 Lf P.T.A.P. containing 1 mg. $AlPO_4$ at 1/5 critical ratio
(iii) 0·03 Lf P.T.A.P. containing 1 mg. $AlPO_4 + 1000 \times 10^6$ *B. pertussis* organisms.

All dilutions were made in the $AlPO_4$ suspension used in the routine production of P.T.A.P. to ensure that all three preparations were at a concentration of 1 ml. $AlPO_4$ per ml. Groups (ii) and (iii) contain a small amount of toxoid and all possible use is made of this by the mineral carrier, including hyperplasia. Since

the carrier is at its optimum level, if the diphtheria antitoxin levels of group (iii) are greater than (ii) then the vaccine presumably will have decreased the effective minimal stimulating dose. The animals were bled 28 days after inoculation and the results are shown in Table 6. There is a 13-fold difference in antitoxin levels between those animals receiving 1/5 critical ratio and those receiving this together with vaccine. This increase indicates that there is a decrease in the effective minimal stimulating dose when *B. pertussis* vaccine is added to diphtheria toxoid.

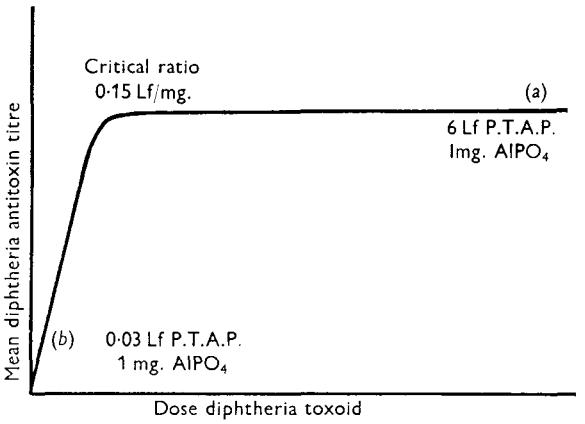

Fig. 6. Response to increasing amounts of diphtheria toxoid with a constant amount of aluminium phosphate.

Table 6. *Effect of* B. pertussis *on the antitoxin response of guinea-pigs to a suboptimal ratio of toxoid to $AlPO_4$ in P.T.A.P.*

	(i) 6 Lf P.T.A.P. containing 1 mg. $AlPO_4$ (normal ratio)	(ii) 0·03 Lf P.T.A.P. containing 1 mg. $AlPO_4$ (suboptimal ratio)	(iii) 0·03 Lf P.T.A.P. containing 1 mg. $AlPO_4$ + 1000×10^6 *B. pertussis* organisms
No./group	12	12	12
Titre	0·32	0·01	0·13
Log σ	0·17	0·78	0·28
P (cf. (*b*))	—	—	$< 0·001$

(7) *Ability of host to produce characteristic stress symptoms*

Landy & Johnson (1955) and Johnson, Gaines & Landy (1956) support the hypothesis that the ability of the host to respond physiologically with the usual lipopolysaccharide stress symptoms is necessary for antibody enhancement to occur. These workers failed to obtain any adjuvance using lipopolysaccharide and diphtheria toxoid in guinea-pigs. The present findings and those of Farthing (1961) are based mainly on the adjuvant effect of Gram-negative organisms and their lipopolysaccharides in the guinea-pig, and do not agree with the observations of Landy & Johnson and of Johnson *et al.* The pyrexia response in the guinea-pig

has been studied and the conclusion reached that these animals will give an endotoxin fever response, although there is a wide variation in doses required to produce this effect. This, combined with the great temperature fluctuation even in normal guinea-pigs (compared with the rabbit), makes quantitative work difficult. 0·05 μg. lipopolysaccharide was the dose always tested in rabbits. Guinea-pigs seldom gave a fever response at this level but generally did when the dose was increased to 0·5 μg.

Rabbits were also tested, since Johnson *et al.* did most of their work on this animal; their observations were confirmed. One group of 5 rabbits was given 20 Lf plain diphtheria toxoid while an identical group containing 7 animals was given 20 Lf diptheria toxoid + 15 μg. *E. coli* lipopolysaccharide. All injections were given subcutaneously into the left ankle in 0·2 ml. The rabbits were bled from the ear 3 weeks after the primary inoculation and individual serum diphtheria antitoxin titres determined. A 56-fold enhancement of antibody levels was shown ($P < 0{\cdot}001$) and when 0·05 μg. of this same lipopolysaccharide preparation was injected intravenously into the ear of each of 3 other rabbits they all gave a typical fever response.

Table 7. *Antibody responses in groups of* 12 *mice to diphtheria toxoid inoculation alone and with pertussis vaccine*

Toxoid dose (Lf)	Diphtheria antitoxin titres of pooled sera measured 28 days after inoculation (u./ml.)		Adjuvance shown
	Toxoid-only group	Toxoid + vaccine group	
5	0·002–0·005	0·02–0·05	10
50	0·005	0·05–0·1	15
100	0·02–0·05	0·1–0·2	4·3

The mouse apparently exhibits a state of relative resistance to endotoxin (Burrows, 1951). Landy & Johnson (1955) could not demonstrate a rise in temperature following injection of 10 μg. lipopolysaccharide into these animals but did not mention if any immunological response was measured. Not knowing whether mice respond to diphtheria toxoid, groups of 12 mice were given 3 different dosage levels of toxoid with and without *B. pertussis* vaccine. At each dose the optimum ratio in guinea-pigs of 200×10^6 *B. pertussis* organisms/Lf was maintained. The primary injection was made intraperitoneally in 0·5 ml. After 28 days the mice were killed, blood removed from the heart and diphtheria antitoxin titres of the pooled sera found for each group. The results are shown in Table 7. The mouse demonstrated clearly the adjuvant effect of lipopolysaccharide on diphtheria toxoid. The LD50 of lipopolysaccharide in mice depended on its origin. Lipopolysaccharide from the *E. coli* strain used was 50–100 μg. while that from the *B. pertussis* strain was 750 μg.–1 mg.

An experiment was performed with guinea-pigs in which one group was rendered tolerant to lipopolysaccharide by repeated injection of this material. Tolerance was measured by failure to produce a thermal response. This group (*a*) was given

5 Lf diphtheria toxoid + 200 μg. *B. pertussis* lipopolysaccharide while (*b*) was given 5 Lf toxoid alone and another normal group (*c*) received toxoid and lipopolysaccharide. When antitoxin responses in these three groups were measured, 28 days after inoculation, the mean levels of (*b*) and (*c*) were those normally obtained, but that from (*a*), although significantly lower than (*c*), was just significantly higher than (*b*). This inconclusive result probably reflects the wide variation in the thermal response of guinea-pigs to lipopolysaccharide (see above).

Finally, it was found that lipid A gave rise to an enhancement of antibody response to diphtheria toxoid (Farthing, 1961). When quantities of *E. coli* lipid A, up to 10 μg, were injected intravenously into a group of 3 rabbits no fever response was observed: whereas 750 μg. of this lipid was not toxic for mice, the LD 50 of the parent lipopolysaccharide was found to be 70 μg.

DISCUSSION

The experiments described indicate some of the manifestations of the adjuvant activity of Gram-negative organisms and their lipopolysaccharides.

Antibody response was demonstrable much earlier in guinea-pigs that received *B. pertussis* vaccine with diphtheria toxoid than those receiving the toxoid alone. However, this earlier production of antibody was not followed by a sustained response, although the differential advantage was maintained. Increased tissue retention of the antigen does not seem to be characteristic of the lipopolysaccharide type of adjuvant (Condie, Zak & Good, 1955) compared with water-in-oil emulsions. With certain adjuvants, such as mineral carriers, one of the main explanations for their activity is the association between the antigen and adjuvant. If the organism or lipopolysaccharide combined with the antigen the physical state of the latter would be altered. The ability to stimulate antibody production could then be increased by directing the antigen to those cells actively concerned with antibody formation and causing less wastage of the antigen, as in the case of mineral carriers, or by delaying release of the antigen. Thus the question arises—'Is there close association, such as adsorption, between the toxoid and vaccine?' Barr (1956) using tetanus toxoid and T.A.B. vaccine could find no evidence that the vaccine acted as an adsorbent of toxoid. The supernatant of the mixture was measured over several days and there was no change within the error of the flocculation test. Faragó & Pusztai (1949) using rabbits believed the adjuvant effect of *B. pertussis* vaccine on diphtheria toxoid might be due to depot formation. Some lipids combine with protein molecules and Farthing (1961) found the active fraction of Gram-negative adjuvants was lipid A (12 % of the lipopolysaccharide in *E. coli* and 19 % in *B. pertussis*). From the results reported in this paper using *in vitro* and *in vivo* methods it seems certain that combination with toxoid is not one of the mechanisms behind the enhancing action of this type of adjuvant. Barr (1956) stated that T.A.B. vaccine may act as a local irritant and delay the absorption of tetanus toxoid. She had found that, in one experiment, the tetanus antitoxin response of guinea-pigs to two doses of toxoid previously mixed with

T.A.B. vaccine was significantly higher than the response of another group of animals to the same doses of these two materials injected into different sites, although no figures were given. However, our results showed that admixture of *B. pertussis* vaccine and diphtheria toxoid was not necessary in that when guinea-pigs were given one dose of the two at the same time but 4 in. apart the degree of adjuvance was only slightly less than when the two were mixed before injection. This agrees with the findings of Johnson *et al.* (1956) who gave typhoid lipopoly-saccharide and ovalbumin intravenously in different sites (opposite ears) separated by an interval of 15 min., and found an enhancement of antibody levels similar to that observed in animals receiving the two given as a mixture. All this confirms the indication that the adjuvance is mediated through the host and is not due to a direct effect on the antigen.

The adjuvant action of these substances shows a marked time-factor effect in relation to the injection of the antigen in that no effect was observed when the vaccine was given 24 hr. before the antigen, nor any time after 24 hr. following it. The stimulus was most effective when the vaccine was given simultaneously with the toxoid. Kind & Johnson (1959) observed that typhoid endotoxin did not produce adjuvance even if it was given as short a time as 6 hours before human serum albumin although it did when mixed with the antigen. These results appear to show that a fairly high concentration of antigen is required at the time of injection of the adjuvant for antibody enhancement to occur and that there is a crucial early step in antibody production, after which the adjuvant has no specific effect.

The adjuvant effect of the vaccine is effective for primary responses only and not secondary responses. *B. pertussis* vaccine will enhance the secondary response maximum titre when it is not present in the primary, but the adjuvance is only just significant at this peak of the secondary and is not after 3 months. In comparing two injections of toxoid with two of toxoid and vaccine, the increase in diphtheria antitoxin levels is 40-fold ($P < 0{\cdot}001$) at 28 days—a similar value to that found earlier; adjuvance is 54-fold ($P < 0{\cdot}001$) at the peak of the secondary response and 27-fold 3 months after the secondary injection. From the practical point of view it is seen to be most important that pertussis vaccine should be present in the primary inoculation and, although its presence makes no difference to the diphtherial antibody in the secondary, presumably it would add to the immunity of the child to pertussis.

Experiments are described in which hyperplasia of cells, a decrease in the effective minimal stimulating dose, and these two factors operating concurrently, are deduced. Proliferation of plasma cells, believed to be the cell of origin of circulating antibody, was shown histologically following the administration of endotoxin containing organisms such as typhoid–paratyphoid vaccine (Good, 1955). Biozzi, Benacerraf & Halpern (1955) found an increase in size of the liver and spleen following intravenous or subcutaneous injection of typhoid endotoxin and suggested that this was due to an increase in the number of cells in these organs. Johnson *et al.* (1956) showed that typhoid lipopolysaccharide given *intravenously* with bovine serum albumin resulted in a faster clearance of the

latter from the circulation together with the usual increased production of antibody. However, when lipopolysaccharide was given 4 days after the antigen no such acceleration occurred. They postulated that since plasma-cell proliferation must have occurred, making more antibody cells available, this absence of accelerated antigen clearance from the circulation indicates that plasma-cell proliferation might not be the main factor responsible for adjuvance. Ward, Johnson & Abell (1959), studying the spleen, could not detect plasma cells or any new cellular types in rabbits following administration of *E. coli* endotoxin. High levels of antibody to bovine gamma globulin were produced by simultaneous *intravenous* injection of endotoxin but again no evidence was found of the appearance of plasma cells. On the other hand, a striking increase in the number of modified reticular cells was observed which correlated closely with antibody production. They did not infer that plasma cells are not associated with antibody production but suggested that possibly this type of cell arises only following secondary or intense antigenic stimulation. Condie *et al.* (1955) suggested that the adjuvant effect of endotoxin could be directed specifically at the mechanism of antibody synthesis at a cellular or subcellular level. They postulated that alterations in the distribution of the antigen based on changes in circulatory pattern in key organs or even alterations in vascular or cellular permeability or phagocytic function might be responsible for the enhancement of antibody production.

Landy & Johnson (1955) and Johnson *et al.* (1956) stated that the host must produce the typical lipopolysaccharide stress symptoms if it is to show adjuvance. One of their arguments is that their guinea-pigs were tolerant to lipopolysaccharide (no fever response from 10 μg.) and, since they could find no antibody enhancement with lipopolysaccharide in this animal, the two properties are connected. In demonstrating the latter part of their statement Johnson *et al.* (1956) using typhoid lipopolysaccharide failed to augment antibody responses in the guinea-pig to 5 Lf diphtheria toxoid, employing both the intracardial and subcutaneous routes of inoculation. Furthermore these workers report that doses of lipopolysaccharide from 10 to 200 μg. and a secondary injection of the toxoid and of endotoxin did not raise the antibody titres above the controls which received diphtheria toxoid only. The results presented in this paper do not confirm these observations on guinea-pigs. However, rabbits, when tested, upheld the hypothesis of a relation between physiological response and adjuvance. Mice demonstrated antibody enhancement although according to Landy & Johnson they failed to show any thermal response. Lipid A preparations produced an adjuvant effect without hyperthermia. Although the lipid is the active fraction of the lipopolysaccharide, even in excess, it did not produce the same degree of adjuvance as the parent material (Farthing, 1961). This may be related to the degree of dispersion of the lipid—being well dispersed in the lipopolysaccharide due to the combined polysaccharide—and may account for reports of pyrogenicity and toxicity associated with lipid A when combined with an inert carrier and made water-soluble (Westphal, Nowotny, Lüderitz, Hurni, Eichenberger & Schönholzer, 1958), or the relatively low degree of adjuvant action of lipid A may be due to the effect of acid hydrolysis (Ribi, Haskins, Landy & Milner, 1961). These present results indicate

that the stress symptoms of lipopolysaccharide are not directly related to their ability to enhance antibody formation. It is possible that the cells believed to be responsible for some of the physiological reactions to these endotoxins are not those concerned with antibody formation.

Lipids other than those extracted from Gram-negative bacteria have also been shown to have an adjuvant effect when administered together with antigen or separately using different routes of inoculation (Pound, 1958; Stanley, 1958; Dresser, 1961). It would be of interest to compare the chemical properties and histological effect upon the host of all lipids shown to possess this power to enhance antibody production.

SUMMARY

The characteristics of Gram-negative organisms and some of the underlying reasons for their adjuvant action with diphtheria toxoid are described.

The adjuvant effect was shown by an earlier production of antitoxin, with a maintained differential advantage over controls, but with the usual decline in titre with passage of time. The adjuvant effect only occurred with a primary stimulus. There was no adsorption between toxoid and vaccine and mixture of the two was not necessary, but the vaccine had to be given simultaneously with or within 24 hr. following injection of the toxoid. There was evidence for believing that these adjuvants decreased the minimal stimulating dose of antigen and caused hyperplasia of antibody-producing cells. No direct link could be found between the characteristic stress symptoms caused by lipopolysaccharides and their ability to enhance antibody formation.

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