SOME FACTORS INFLUENCING THE RESPONSE TO IMMUNISATION WITH SINGLE AND COMBINED PROPHYLACTICS.

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EARLIER work (Barr and Llewellyn-Jones, 1953*a*, *b*) has shown that interference with the development of immunity to tetanus may occur in animals immunised with two injections of a combined prophylactic containing another antigen to which they already have some immunity. Similar interference occurred when the first injection was of the combined prophylactic and the second injection of tetanus toxoid alone, but not when the order of the injections was reversed. These results were obtained in diphtheria-immune guinea-pigs subsequently immunised with D.T.P. (diphtheria-tetanus prophylactic) and in T.A.B.-immune guinea-pigs immunised with T.A.B.T. (typhoid-paratyphoid A and B vaccine + tetanus toxoid). The interference with the response to tetanus toxoid, observed in groups receiving a combined prophylactic for the first dose, must have been due to an effect of the secondary response to the other component of the prophylactic.

Further experiments have now been done in an attempt to determine the circumstances in which interference occurs.

EXPERIMENTAL.

The Effect of Varying the Interval Between Injections of Combined Prophylactic.

Normal guinea-pigs were injected with a single dose (0·3 Lf) of a diphtheria prophylactic consisting of purified diphtheria toxoid adsorbed on aluminium hydroxide. In this and all other experiments, materials were suitably diluted in normal saline and injected subcutaneously in a dose of 1 ml. The animals were rested for three months, at the end of which time they were re-weighed. Normal guinea-pigs of comparable weight were selected as controls, and all received a dose of 0·005 ml. of diphtheria-tetanus prophylactic (D.T.P.) containing, per ml., 60 Lf of purified diphtheria toxoid and 7 Lf of purified tetanus toxoid adsorbed on aluminium hydroxide. The guinea-pigs were further subdivided and received a second dose of D.T.P. after different intervals of time, as shown in Table I, which gives the tetanus antitoxin titres of sera taken from the animals 10 days after the second injection. The sera were titrated by the mouse method described by Glenny and Stevens (1938). The numbers were small, but the figures in Table I suggest that both the diphtheria-immune and the normal control animals profited by delaying the second dose from 4 to 8 weeks, but that both groups gave a poorer response when the interval between injections was increased to 12 weeks. This result suggests that the tetanus toxoid injected was insufficient for the establishment of adequate basal immunity for longer than about 8 weeks after the first dose. The work was therefore repeated using a different prophylactic which

contained the same amount of diphtheria toxoid but approximately double the amount of tetanus toxoid. Table II shows the results of this experiment, in which the range of interval between injections was extended, and the results for each group except for 24-week ones consist of the sum of the figures obtained from two or more groups of 10–20 animals injected on different occasions.

It is clear from Table II that there was no significant difference between the tetanus antitoxin responses of the various groups of normal controls 10 days after the second injection of D.T.P. Some interference with the tetanus response occurred in all groups which had received an earlier injection of diphtheria prophylactic, but this was minimal in the animals which received the two doses of D.T.P. separated by an interval of 12 weeks. The distribution of antitoxin titres in these groups is interesting. In the 12-week group it approximated to the normal distribution, but the peak and the geometric mean (9·92) were lower than in the corresponding controls. This suggests that the pre-existing diphtheria immunity caused interference with the tetanus response of the animals in the group as a whole, but was insufficient to produce a sharp division between good and poor responders. In all other groups the peak of the main distribution was at a lower titre than that of the corresponding controls, but there was, in addition, a break in the distribution, so that the very poor responders of the groups became separated from the remainder. It may appear, on inspection of the figures in Table II, that the tetanus antitoxin titres of diphtheria-immune animals receiving the two injections of D.T.P. separated by an interval of 24 weeks were higher than in those receiving them separated by an interval of 16 weeks. It will, however, be noted that the number of animals in the latter group was greater, and that the peak of the distribution occurred at a higher antitoxin level than that for the guinea-pigs which received the two injections separated by an interval of 24 weeks.

The decrease in the degree of interference, brought about by lengthening the interval between the two injections of D.T.P. to 12 weeks, suggests that a prolonged but decreasing crowding-out effect was produced by the secondary response to diphtheria toxoid in the *first* dose of D.T.P. on the secondary response to tetanus toxoid in the *second* dose of D.T.P. The duration of the secondary diphtheria response effect cannot, however, completely account for the interference with the response to the course of immunisation with the tetanus toxoid in the combined prophylactic. If this were the sole cause, the effect of further increase, beyond 12 weeks, of the interval between injections would be finally to abolish any interference. It therefore seems probable that, in addition, the speed of the secondary response to the diphtheria toxoid in the first dose of D.T.P. brought about a crowding-out of the development of basal immunity to the tetanus toxoid present in the first dose. It is even possible that this latter phenomenon was the sole cause of the interference, but it will be shown later in this paper that antibody production following secondary stimulation may continue for at least 28 days.

An exactly similar experiment was carried out in which T.A.B.-immune and normal control guinea-pigs were immunised with two doses, each of 0·05 ml. of T.A.B.T. injected at varying intervals of time. The tetanus antitoxin titres, measured 10 days after the second injection, are shown in Table III. The results are substantially the same as those shown in Table II for diphtheria-immune animals immunised with D.T.P. On increasing the interval between injections, there was first an improvement and then a relapse in the responses of the T.A.B.-immune guinea-pigs. In this experiment, the optimal interval was 8 weeks. The

TABLE I.—*The Tetanus Antitoxin Responses of Diphtheria-immune and Normal Guinea-pigs Immunised with Two Doses of Diphtheria-tetanus Prophylactic (D.T.P.) Separated by Varying Intervals of Time.*

Interval between injections of D.T.P.	Group.	Number of guinea-pigs with tetanus antitoxin titres (units/ml.).													
		>/< 0·01	0·01 0·02	0·02 0·05	0·05 0·1	0·1 0·2	0·2 0·5	0·5 1·0	1·0 2·0	2·0 5·0	5·0 10	10 20	20 50	50 100	Total.
4 weeks	Diph.-immune	1	0	0	1	0	0	1	1	6	2	1	—	—	13
	Normal	—	—	—	—	—	—	—	—	1	3	4	3	—	11
8 „	Diph.-immune	—	—	—	—	—	—	—	5	2	5	2	—	—	14
	Normal	—	—	—	—	—	—	—	—	—	1	5	4	1	11
12 „	Diph.-immune	1	0	0	0	0	2	0	1	5	3	0	1	—	13
	Normal	—	—	—	—	—	—	—	—	2	4	7	1	—	14

The prophylactic contained, per ml., 60 Lf of purified diphtheria toxoid and 7 Lf of purified tetanus toxoid.

TABLE II.—*Tetanus Antitoxin Responses of Diphtheria-immune and Normal Guinea-pigs Immunised with Two Doses of Diphtheria-tetanus Prophylactic (D.T.P.) Separated by Varying Intervals of Time.*

Interval between injections of D.T.P.	Group.	Number of guinea-pigs with tetanus antitoxin titres (units/ml.).															Geometric mean.
		>/< 0·01	0·01 0·02	0·02 0·05	0·05 0 1	0·1 0·2	0·2 0·5	0·5 1·0	1·0 2·0	2·0 5·0	5·0 10	10 20	20 50	50 100	100 200	Total.	
4 weeks	Diph.-immune	—	—	—	1	0	2	0	2	6	7	5	1	—	—	24	3·96
	Normal	—	—	—	—	—	—	—	—	1	3	10	10	3	1	28	21·4
8 „	Diph.-immune	1	0	0	0	0	1	1	6	5	11	13	4	—	—	42	<5·5
	Normal	—	—	—	—	—	—	—	—	—	5	7	35	6	—	53	27·0
12 „	Diph.-immune	—	—	—	—	—	—	—	4	4	13	15	10	—	—	46	9·92
	Normal	—	—	—	—	—	—	—	—	—	3	13	24	3	—	43	23·6
16 „	Diph.-immune	—	1	0	1	0	0	1	1	4	4	12	7	—	—	31	7·27
	Normal	—	—	—	—	—	—	—	—	—	3	10	15	2	—	30	22·0
24 „	Diph.-immune	—	—	—	—	—	1	1	1	0	7	7	2	—	—	19	7·39
	Normal	—	—	—	—	—	—	—	—	—	2	7	6	—	—	15	17·8

The prophylactic contained, per ml., 60 Lf purified diphtheria toxoid and 13 Lf of tetanus toxoid.

TABLE III.—*Tetanus Antitoxin Responses of T.A.B.-immune and Normal Guinea-pigs Immunised with Two Doses of T.A.B.T. Separated by Varying Intervals of Time.*

Interval between injections of T.A.B.T.	Group.	Number of guinea-pigs with tetanus antitoxin titres (units/ml.).														Total	Geometric mean.
		<0·01	0·01–0·02	0·02–0·05	0·05–0·1	0·1–0·2	0·2–0·5	0·5–1·0	1·0–2·0	2·0–5·0	5·0–10	10–20	20–50	50–100	100–200		
4 weeks	T.A.B.-immune	2	0	0	0	2	1	1	3	9	7	—	—	—	—	25	<1·56
	Normal	—	—	—	—	—	—	—	1	11	14	7	7	—	—	40	7·99
6 „	T.A.B.-immune	—	—	—	—	—	—	3	6	2	1	—	—	—	—	12	1·56
	Normal	—	—	—	—	—	—	—	—	4	7	11	1	1	—	24	9·95
8 „	T.A.B.-immune	—	—	—	—	—	—	—	—	2	5	4	—	—	—	11	7·86
	Normal	—	—	—	—	—	—	—	1	3	5	3	7	1	—	20	12·2
12 „	T.A.B.-immune	1	0	0	1	0	0	0	2	4	6	1	2	—	—	17	<3·2
	Normal	—	—	—	—	—	—	—	2	1	5	5	3	2	1	19	13·0
16 „	T.A.B.-immune	2	0	0	0	0	0	0	2	3	4	2	—	—	—	13	<2·8
	Normal	—	—	—	—	—	—	1	0	1	3	3	5	0	1	14	13·9

The prophylactic contained, per ml., 10^9 *S. typhi*, 5×10^8 each *S. paratyphi* A and B and 8 Lf of tetanus toxoid.

mean of the titres of the normal control animals showed a progressive but not significant increase as the interval between injections was increased up to 8 weeks. Thereafter there was no increase, but the responses of the T.A.B.-immune animals were poorer.

The least interference occurred when the interval between injections of the diphtheria-immune guinea-pigs immunised with D.T.P. was 12 weeks: for the T.A.B.-immune guinea-pigs immunised with T.A.B.T. interference was least when the interval was 8 weeks. This difference may be due to difference in dosage and composition of the prophylactics and the degree of potential immunity established by them, or to differences in the immunological effects of a secondary response to a toxoid and to a bacterial vaccine.

The Effect of Primary Stimulation with One Antigen on Responses to a Subsequent Course of Immunisation with Another Antigen.

In this experiment normal guinea-pigs were injected with a single dose of 0·05 ml. of T.A.B. vaccine containing per ml. 10^9 *Salmonella typhi* and 5×10^8 each *Salmonella paratyphi* A and B, at varying intervals of time before the first of two injections of tetanus toxoid separated by an interval of 2 months. The animals were bled 10 days after the second injection and the sera titrated for tetanus antitoxin content. The results are shown in Table IV.

TABLE IV.—*Effect on the Tetanus Antitoxin Response of a Single Dose of T.A.B. Vaccine given at Varying Times before the First of Two Injections of Tetanus Toxoid Separated by an Interval of 2 Months.*

Interval between T.A.B. injection and first dose of tetanus toxoid.	Number of guinea-pigs with tetanus antitoxin titres (units/ml.)													
	> <0·01	0·01 0·02	0·02 0·05	0·05 0·10	0·10 0·20	0·20 0·50	0·50 1·0	1·0 2·0	2·0 5·0	5·0 10	10 20	20 50	50 100	Total.
7 days	3	—	—	—	—	—	—	1	3	7	1	—	—	15
14 ,,	—	—	—	—	—	2	—	—	2	2	7	2	—	15
21 ,,	—	—	—	—	—	—	—	1	1	4	4	4	1	15
28 ,,	—	—	—	—	—	—	—	4	4	6	8	5	3	30
Control (no preliminary T.A.B.)	—	—	—	—	—	—	1	4	5	9	6	3	1	29

It will be seen that interference with the response to immunisation with tetanus toxoid occurred only in those groups of animals which had been primarily stimulated with T.A.B. vaccine 7 or 14 days previously. The primary stimulation had no effect on the results of tetanus toxoid immunisation if this were delayed for 21 days or longer after the dose of vaccine.

A similar experiment was carried out, in which groups of normal guinea-pigs received a moderately large dose (1 Lf) of diphtheria prophylactic at various intervals of time before the first of two injections of purified tetanus toxoid adsorbed on aluminium hydroxide. The diphtheria prophylactic used consisted also of purified toxoid adsorbed on aluminium hydroxide. The guinea-pigs were bled 10 days after the second injection of tetanus prophylactic and the antitoxin titres are shown in Table V.

TABLE V.—*Effect on the Tetanus Antitoxin Response of a Single Dose of Diphtheria Prophylactic, Given at Varying Times before the First of Two Injections of Tetanus Toxoid, Separated by an Interval of 28 Days.*

Interval between diphtheria injection and first dose of tetanus toxoid	Number of guinea-pigs with tetanus antitoxin titres (units/ml.)												
	> <0·01	0·01 0·02	0·02 0·05	0·05 0·10	0·10 0·20	0·20 0·50	0·50 1·0	1·0 2·0	2·0 5·0	5·0 10	10 20	20 50	Total.
3 days	6	0	0	0	1	2	2	4	7	3	0	1	26
7 ,,	8	1	1	1	0	2	1	6	3	2	1	1	27
14 ,,	3	0	0	1	0	0	0	6	7	6	6	1	29
21 ,,	4	0	0	0	0	2	0	8	7	7	1	—	29
28 ,,	—	—	—	—	—	—	—	5	6	7	9	—	27
Control (no preliminary diphtheria injection)	—	—	—	—	—	—	—	—	—	5	7	3	15

It is evident that very considerable interference occurrred with the response to tetanus immunisation, and that this interference was greatest in the group which received the first dose of prophylactic 7 days after the single dose of diphtheria prophylactic. Considerable interference occurred when the first dose was given as early as 3 days after the primary stimulation with diphtheria prophylactic, and as late as 28 days the responses, though much improved, were significantly lower than those of the control group ($P = \leqslant 0{\cdot}01$).

This interference cannot be associated with the time of appearance of diphtheria antitoxin in the blood. If this were the explanation, no interference would be expected to occur in guinea-pigs receiving the first dose of tetanus prophylactic 3, 7 and possibly also 14 days after the single dose of diphtheria prophylactic. The results of experiments of this kind might well be expected to depend on the type of prophylactic used for the " non-specific " primary stimulus (whether fluid or adsorbed), the dosage and the proximity of the sites of injection of the two antigens. In this work all injections were made subcutaneously through the abdominal wall.

The Effect of Secondary Stimulation with One Antigen on Responses to a Subsequent Course of Immunisation with Another Antigen.

Normal guinea-pigs received two doses of diphtheria prophylactic separated by an interval of 28 days. At varying times after the second dose, the first of two doses of tetanus toxoid was injected : the second dose was given 28 days after the first, and the guinea-pigs were bled 10 days later.

Table VI gives the tetanus antitoxin titres of the groups of guinea-pigs. Fifteen diphtheria-immune animals were injected in each experiment, but the control experiment was performed on two occasions, using a total of 29 animals.

Interference with the response to tetanus toxoid occurred in all groups, but was least in those which received the first dose 3 days after secondary stimulation with diphtheria toxoid. In this group all the animals produced detectable antitoxin, but the scatter of titres was large. When the interval between the second dose of diphtheria toxoid and the first dose of tetanus toxoid was increased, some animals failed to produce 0·01 unit of antitoxin (the lowest level tested) even when the interval was as long as 28 days. It would therefore appear that antibody-forming activity concerned in crowding-out responses is of longer duration after secondary stimulation than after primary stimulation, but is initiated slightly later.

TABLE VI.—*Effect on the Tetanus Response of Secondary Stimulation with Diphtheria Prophylactic, at Varying Times before the First of Two Injections of Tetanus Prophylactic Separated by an Interval of* 28 *Days.*

Interval between diphtheria injection and first dose of tetanus toxoid.	Number of guinea-pigs with tetanus antitoxin titres (units/ml.).												
	> <0·01	0·01 0·02	0·02 0·05	0·05 0·10	0·10 0·20	0·20 0·50	0·50 1·0	1·0 2·0	2·0 5·0	5·0 10	10 20	20 50	Total.
3 days	—	—	—	—	—	1	2	1	2	6	1	2	15
7 „	2	—	—	—	—	—	—	2	5	3	3	—	15
10 „	3	—	—	—	—	—	—	1	4	3	4	—	15
14 „	3	—	—	1	—	—	—	2	4	2	3	—	15
21 „	4	—	1	—	—	—	1	1	4	2	2	—	15
28 „	1	1	—	—	—	—	1	5	3	2	1	—	14
Control (no preliminary diphtheria injection)	—	—	—	—	—	—	—	3	11	8	7	—	29

DISCUSSION.

These experiments confirm our earlier observation that there may be danger in using a combined prophylactic for the purpose of simultaneously boosting the titre of one antibody and producing another in primary immunisation. The mechanism of interference with the primary immunisation by the secondary response of the animal to the other antigen remains obscure. There is evidence from the data in Tables II and III that the speed of the secondary response to the antigen to which there was pre-existing immunity brought about crowding-out of the development of basal immunity to tetanus toxoid. In addition, results in Table VI show that prolonged antibody-forming activity follows secondary stimulation with toxoid ; this may have interfered with the response to the tetanus toxoid in the second injection of combined prophylactic (Tables II and III).

Other results (Tables IV and V) show that unsatisfactory responses may follow courses of immunisation in which the first dose of one course is given at too short an interval before the first dose of a second course. The primary response to the first may crowd out the development of basal immunity to the second. In our experiments interference with the response to immunisation with one antigen occurred when the first dose was given as early as 3 days after primary stimulation with another antigen. This is long before any antitoxin becomes detectable in the circulation, and indeed the interference had already diminished when the first dose of the course was delayed until 14–21 days after the non-specific primary stimulation ; it is at this time that antitoxin is demonstrable in the circulation of most animals.

It also appears dangerous to start a course of immunisation with one antigen until some weeks have elapsed since the completion of a course in which another antigen was used. The prolonged activity involved in the secondary response to the final injection of the first course may interfere with the development of basal immunity to the first dose of the second course. The results suggest that antigenic stimulation caused by subclinical or clinical infections might cause similar interference with the response to artificial immunisation with prophylactics, if this were started before the effects of the natural stimulation had died down.

If these results are directly applicable to human immunisation, injections of prophylactic should not be given without making sure that no other injection of antigen, whether a primary or secondary stimulus, has been given for at least a

month previously. The facts should also be borne in mind in devising programmes of immunisation of babies involving smallpox vaccination and separate courses of immunisation with diphtheria and tetanus prophylactics and *Haemophilus pertussis* vaccine.

The duration of antibody-forming activity following primary and secondary stimulation may well depend on the type of prophylactic and the dosage used. It is possible that the marked interference with the response to immunisation shown in Table V would have been less if the dose of diphtheria prophylactic given previously had been smaller, or if the dose had been administered as fluid toxoid with no mineral carrier. It is also possible that such interference might be reduced if the second antigen were injected into a different site from the first, and if animals with immunity to one component of a combined prophylactic were immunised, not with the combined prophylactic as such, but with the component antigens administered in widely separated sites. Much work therefore remains to be done. It is possible that in later work some light may be thrown on the more fundamental processes of interference with antibody production.

SUMMARY.

Interference with the response to two injections of tetanus toxoid occurred in guinea-pigs immunised with two injections, separated by different intervals of time, of a combined prophylactic containing another antigen to which they already had immunity. As the interval between injections was increased, the interference became less but eventually greater again. It is suggested that activity connected with the secondary response of the animals to the other antigen, present in the first dose of combined prophylactic, caused crowding-out of the response to the tetanus toxoid in the first and second doses.

A primary stimulus of diphtheria prophylactic suppressed the response to a course of two injections of tetanus toxoid when the first of these was given at times ranging from 3 to 21 days later; when the interval was 28 days interference, though significant, was reduced. The greatest interference occurred when the first dose of tetanus toxoid was given 7 days after the injection of diphtheria prophylactic. Less interference occurred in another group primarily stimulated with T.A.B. vaccine.

A secondary stimulus of diphtheria prophylactic suppressed the response to two injections of tetanus toxoid when the first of these was given at times ranging from 3 to 28 days.

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