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The influence of mineral carriers on the simultaneous active and passive immunization of guinea-pigs against tetanus

By A. J. FULTHORPE*
Wellcome Research Laboratories, Biological Division, Beckenham, Kent

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INTRODUCTION

There is considerable interest in the use of adsorbed tetanus toxoid given concurrently or at close intervals with prophylactic horse tetanus antitoxin as a means of conferring immediate protection against tetanus, whilst promoting potential active immunity at the same time.

Simultaneous administration of antitoxin with fluid tetanus toxoid was found to inhibit the normal response to toxoid in humans (Barr & Sachs, 1955). This was also shown in rabbits by Suri & Rubbo (1961) and in guinea-pigs by Smith (1964) who showed that in similar tests with adsorbed preparations interference was much less.

Adsorbed preparations of tetanus toxoid in simultaneous active and passive immunization against tetanus have been used with satisfactory results by several workers in the field (Gold & Bachers, 1943; Ericcson, 1948; Tasman & Huygen, 1962; Eckmann, 1959; Smith *et al.* 1963).

The experiments recorded in this paper were carried out to investigate further the differences betweeen adsorbed and fluid preparations of tetanus toxoid in simultaneous immunization procedure.

MATERIALS AND METHODS

Tetanus antitoxin (horse)

A single preparation of pepsin refined horse globulin (Harms, 1948) was used in all experiments. The high-potency material was suitably diluted to contain 150 units of antitoxin in 1·0 ml.

Tetanus toxoid

Two preparations of concentrated tetanus toxoid were used to provide adsorbed toxoids of suitable strength. The first preparation was used in those experiments recorded in Tables 1–7 and the second preparation for those in Tables 8–14. No comparison has been drawn between these two series of results. The adsorbed materials were prepared by adding calculated amounts of concentrated toxoid (*ca.* 1000 Lf/ml.) to suitably diluted and buffered adsorbent. The material was left for 7 days at room temperature with occasional shaking for adsorption to take place.

* Dr Fulthorpe died while this paper was in the press. Requests for reprints should be addressed to The Secretary, Department of Immunology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent.

Toxoid estimation by flocculation

Constant volumes of a suitable dilution of toxoid were pipetted into a series of $\frac{3}{8} \times 3$ in. glass tubes. A working standard antitoxin (23 units/ml.) was added in volumes differing by 10%. The tubes were incubated at 50° C., with one-third length of the tubes immersed to allow for convection, and inspected at intervals. Estimation of the toxoid content of the preparation was based on the principle that the mixture which flocculated most rapidly contained an equal number of Lf doses of toxoid and provisional *in vitro* units of antibody.

Toxoid estimation by the in vivo *total combining power test (TCP)*

Volumes of toxoid, suitably diluted to contain about 1 unit equivalent of toxoid and differing by 20%, were measured into a series of tubes and a constant volume of antitoxin containing 2 units was added, the mixtures were gently shaken and allowed to stand for 1 hr. Constant volumes of a test toxin equivalent to 1 unit of standard antitoxin were then added and mixed and allowed to stand for $\frac{1}{2}$ hr. The whole mixture (restricted to a reasonable total volume) was then injected into mice. The mice were observed twice daily and the end-point of the *in vivo* test was based on the deaths of the test animal on the fourth day or definite symptoms of tetanus on the third day. If an animal was dead on the second or third day and the next animal in the series was alive, the end point was arrived at by interpolation. Several estimates were made on each preparation and the value allotted was the arithmetic mean of individual results. It has been observed that with a satisfactory toxoid the dose of toxoid equivalent to 1 unit, as determined by this method, was considerably lower than the Lf dose (Fulthorpe & Thomson, 1960).

Antitoxin titrations

The titrations of antitoxin in guinea-pig sera were carried out in mice by the technique of Glenny & Stevens (1938).

RESULTS

Simultaneous immunization with fluid toxoid

Guinea-pigs given two doses each of 2·25 Lf of fluid tetanus toxoid, at 28 days interval, were bled 10 days after the second dose of toxoid, and the antitoxin titres of individual animals estimated by *in vivo* titration in mice. The group was found to have responded well, with a geometric mean titre of 28·2 units/ml. (Table 1). When similar groups were also given 150 units of horse tetanus antitoxin (pepsin refined globulin) by the intraperitoneal route at different periods of time in relation to the first dose of toxoid, the antigenic responses were grossly impaired and the difference between these results and that with toxoid alone were significant ($P = < 0{\cdot}05$). There was no significant difference however, between results when antitoxin was given from 4 hr. before to 4 days after the toxoid ($P = > 0{\cdot}05$); in a considerable proportion of the guinea-pigs in these groups no detectable secondary response was observed. When doses of 20–50 Lf of toxoid were given concurrently with antitoxin very little improvement in response occurred.

Table 1. *The effect of horse tetanus antitoxin (pepsin refined globulin) 150 units, on the antigenic responses of guinea-pigs to two doses of 2·25 Lf of* fluid *tetanus toxoid given subcutaneously at 28 days interval*

(The horse antitoxin given intraperitoneally at different times in relation to the first dose of toxoid. Guinea-pigs bled 10 days after second dose of toxoid.)

	No. of guinea-pigs with antitoxin titres (units/ml.)														
Horse tetanus antitoxin	< 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	5·0–10·0	10–20	20–50	50–100	> 100	Total	Geometric means
Not given	—	—	—	—	—	—	—	—	—	1	1	6	1	1	10	28·2
150 units 4 hr. before toxoid	2	5	1	—	2	—	—	—	—	—	—	—	—	—	10	< 0·020
150 units concurrently with toxoid	4	3	1	—	1	—	—	—	—	—	—	—	—	—	9	< 0·016
150 units 4 hr. after toxoid	5	2	2	—	1	—	—	—	—	—	—	—	—	—	10	< 0·015
150 units 24 hr. after toxoid	7	—	—	1	2	—	—	—	—	—	—	—	—	—	10	< 0·020
150 units 4 days after toxoid	2	2	2	1	1	—	1	—	—	—	—	—	—	—	9	< 0·037

Simultaneous immunization with adsorbed toxoid

A similar experiment was carried out using 2·25 Lf of tetanus toxoid adsorbed on aluminium hydroxide (Table 2). The antigenic response to the toxoid alone (G.M. 38·7 units/ml.) was of the same order as that obtained with fluid toxoid. When passively administered antitoxin was given as before there was a reduction in the response to the toxoid. The differences in response were, however, not

Table 2. *The effect of horse tetanus antitoxin (pepsin refined globulin) 150 units, on the antigenic responses of guinea-pigs to two doses of* 2·25 Lf *of* adsorbed *tetanus toxoid given subcutaneously at 28 days interval*

(The horse antitoxin given intraperitoneally at different times in relation to the first dose of toxoid. Guinea-pigs bled 10 days after second dose of toxoid.)

Horse tetanus antitoxin	No. of guinea-pigs with antitoxin titres (units/ml.) 1·0–2·0	2·0–5·0	5·0–10·0	10–20	20–50	50–100	>100	Total	Geometric means
Not given		2			8	6	1	17	38·7
150 units 4 hr. before toxoid		2	1	3	1	3		10	15·3
150 units concurrently with toxoid		1	1	6	2	5		15	21·2
150 units 4 hr. after toxoid	3	1	3	4	2	1	1	15	9·9
150 units 24 hr. after toxoid		1	1	5	7	1		15	16·3
150 units 4 days after toxoid			1	2	5	5	1	14	33·1

significant between those results where the antitoxin was given 4 hr. before, concurrently with, 24 hr. or 4 days after the first dose of toxoid when compared with the response to toxoid alone. The response observed when the antitoxin was given 4 hr. after the first dose of toxoid was, however, significantly different from that obtained with toxoid alone ($P = < 0{\cdot}05$). There is a suggestion in these results that there is an optimum time for interference by passive antitoxin in the simultaneous immunization method. The immunizing efficiency of adsorbed toxoid in the presence of passive antitoxin was thus found to be satisfactory and remarkably different from that observed when fluid toxoid was used.

When the dose of antitoxin given concurrently with 2·25 Lf of aluminium hydroxide adsorbed tetanus toxoid was increased progressively (Table 3), it was found that there was no statistically significant reduction in response with 300, 600 or 1200 units of antitoxin but that a significant reduction in response occurred when 2400 units was given ($P = < 0{\cdot}05$).

The route of injection of the concurrently administered antitoxin did not significantly affect the responses to toxoid except where the intravenous route was used (Table 4). It must be assumed that the greater effectiveness of antitoxin administered in this way was due to rapid transfer to the site of toxoid injection.

Table 3. *The effect of increasing doses of horse tetanus antitoxin (pepsin refined globulin) on the antigenic responses of guinea-pigs to two doses of* 2·25 Lf *of* adsorbed *tetanus toxoid given subcutaneously at* 28 *days interval*

(The horse antitoxin given intraperitoneally at the same time as the first dose of toxoid. Guinea-pigs bled 10 days after the second dose of toxoid.)

	No. of guinea pigs with antitoxin titres (units ml.)								
Horse tetanus	1·0–2·0	2·0–5·0	5·0–10	10–20	20–50	50–100	> 100	Total	Geometric means
Not given	—	2	—	—	8	6	1	17	38·7
150 units	—	1	1	6	2	5	—	15	21·2
300 units	—	1	2	1	4	2	—	10	19·5
600 units	—	—	1	3	4	2	—	10	23·2
1200 units	—	1	1	3	3	1	1	10	18·4
2400 units	3	—	3	1	2	1	—	10	7·7

Table 4. *The effect of giving* 150 *units of horse tetanus antitoxin (pepsin refined globulin) on the antigenic responses of guinea-pigs to two doses of* 2·25 Lf *of* adsorbed *tetanus toxoid given subcutaneously at* 28 *days interval.*

(The horse antitoxin given by different routes at the same time as the first dose of toxoid. Guinea-pigs bled 10 days after the second dose of toxoid.)

	No. of guinea-pigs with antitoxin titres (units/ml.)										
150 units of antitoxin given	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	Geometric means
Intraperitoneal	—	—	—	—	1	1	6	2	5	15	21·2
Subcutaneous	—	—	—	—	2	1	4	1	2	10	13·6
Intramuscular	—	—	—	1	—	2	3	3	1	10	13·5
Intravenous	1	—	—	3	—	1	2	2	1	10	5·1

Elimination of passive antitoxin

In order to investigate the rise of actively produced antitoxin in actively and passively immunized animals it was first necessary to investigate the rate of turn-out of passively administered horse tetanus antitoxin in normal and serum-sensitive guinea-pigs. Groups of animals (250–350 g.) were injected with 150 units of antitoxin by the intraperitoneal route and separate groups were bled at intervals and the antitoxin titres estimated (Table 5). After 24 hr. the antitoxin titres ranged from 3·3 to 4·5 units/ml., after 7 days approximately one-tenth of this (0·42–0·47) and after 14 days most animals in the group had 0·02–0·05 units/ml. At 21 days there was no measurable antitoxin (< 0·01 units/ml.). In serum-sensitive guinea-pigs, i.e. animals which had received 150 units of antitoxin 3 months previously, the rate of loss of a further dose of antitoxin was much accelerated; at 7 days several members of the group had no measurable antitoxin and the titres in the remainder varied from 0·02 to 0·5 units/ml., and at 14 days there was no measureable antitoxin in that group. These observations indicate the scavenging effect of anti-horse antibodies and the considerable degree of variation between different individuals in the effectiveness of this process.

Table 5. *Antitoxin titres (units/ml.) of separate groups of guinea-pigs* (250–350 g.) *at different times following a single intraperitoneal injection of* 150 *units of horse tetanus antitoxin (pepsin refined globulin)*

(A) In normal guinea pigs.
(B) In serum sensitised guinea-pigs (450–650 g.).

(A)

Guinea-pig no.	Group 1 24 hr. later	Group 2 7 days later	Group 3 14 days later	Group 4 21 days later
1	4·0	0·42	0·01–0·02	< 0·01
2	4·0	0·45	0·02–0·05	< 0·01
3	3·6	0·45	0·02–0·05	< 0·01
4	4·5	0·42	0·02–0·05	< 0·01
5	4·0	0·45	0·02–0·05	< 0·01
6	4·0	0·45	0·02–0·05	< 0·01
7	4·5	0·45	0·02–0·05	< 0·01
8	4·0	0·45	0·05	< 0·01
9	3·3	0·47	0·01	< 0·01
10	4·0	0·42	0·01	< 0·01

(B)

	Group 5 7 days later	Group 6 14 days later
1	< 0·01	< 0·01
2	0·1–0·2	< 0·01
3	0·2–0·5	< 0·01
4	< 0·01	< 0·01
5	0·1–0·2	< 0·01
6	0·2–0·5	< 0·01
7	0·02–0·05	< 0·01
8	< 0·01	< 0·01
9	< 0·01	< 0·01

Rate of development of actively produced antitoxin

Two further groups of guinea-pigs (Table 6A and B) were immunized with two doses of 2·25 Lf of fluid toxoid at 42 days interval. One of these groups was in addition given 150 units of antitoxin intraperitoneally at the same time as the first dose of toxoid. All animals were bled at 14, 21, 28 and 42 days and the antitoxin titres following the primary response were estimated. A further titration was made at 52 days to estimate the value for the secondary response. At 14 days it was found that in the group given toxoid only, eight out of ten animals had measurable antitoxin and that active production of antitoxin had begun. In the group given antitoxin concurrently with the first dose of toxoid the values obtained were of the same order as those found when antitoxin alone was given (Table 5A).

Table 6. *Antitoxin titres (units/ml.) of two groups of guinea-pigs*

((A) Given 2·25 Lf of *fluid* tetanus toxoid subcutaneously, and (B) given 2·25 Lf of *fluid* tetanus toxoid subcutaneously and 150 units of tetanus antitoxin (pepsin refined globulin) intraperitoneally at the same time. All animals bled 14, 21, 28 and 42 days later, a further 2·25 Lf of toxoid given on the 42nd day and the animals again bled 10 days later.)

Guinea-pig no.	Day 14	Day 21	Day 28	Day 42	Secondary response Day 52
			(A)		
1	< 0·01	0·01–0·02	0·01–0·02	0·2–0·5	5–10
2	0·02–0·05	0·1	0·5–1·0	1–2	50–100
3	0·01	0·02–0·05	0·5–1·0	1·0	50–100
4	0·01–0·02	0·02–0·05	0·5–1·0	0·5–1·0	20–50
5	0·01–0·02	†	†	†	†
6	< 0·01	< 0·01	< 0·01	0·01–0·02	5–10
7	0·01–0·02	0·02	0·01–0·02	0·1	10
8	0·02–0·05	0·02–0·05	0·02	0·02–0·05	20–50
9	0·02–0·05	0·02–0·05	0·5–1·0	1–2	50–100
10	0·02–0·05	0·02	0·5	1–2	50–100
G.M.	< 0·016	< 0·026	< 0·15	0·31	28·6
			(B)		
1	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
2	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
3	0·02–0·05	< 0·01	< 0·01	< 0·01	0·5
4	0·02	< 0·01	< 0·01	< 0·01	0·01
5	0·02–0·05	†	†	†	†
6	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
7	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
8	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
9	0·02–0·05	< 0·01	< 0·01	< 0·01	1–2
10	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
11	0·05	< 0·01	< 0·01	< 0·01	< 0·01
12	0·01–0·02	< 0·01	< 0·01	< 0·01	0·01–0·02
13	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
14	0·05	< 0·01	< 0·01	< 0·01	< 0·01
15	0·02–0·05	< 0·01	< 0·01	< 0·01	< 0·01
G.M.	0·03	< 0·01	< 0·01	< 0·01	< 0·02

† Animal died.

At 21, 28 and 42 days the group given fluid toxoid alone showed a slow rise in titre with some animals responding much better than others. The final titres for

this group after a second dose of toxoid varied from 5 to 100 units/ml., the highest values being given by the animals in which antitoxin appeared at an early stage and increased most rapidly in titre after the first injection of toxoid. Where antitoxin was given concurrently with the first dose of toxoid, passive antitoxin was, as expected, not present at 21 days and no circulating antitoxin was found and, after the second dose of toxoid, only four animals of a total of fourteen gave a measurable secondary response.

Further groups of guinea-pigs were treated with aluminium hydroxide adsorbed tetanus toxoid (2·25 Lf) with and without concurrently administered antitoxin, and combined immunization was carried out in a similar fashion with serum sensi-

Table 7. *Antitoxin titres (units/ml.) of three groups of guinea-pigs*

((A) Given 2·25 Lf of tetanus toxoid *adsorbed* on aluminium hydroxide, subcutaneously, (B) 2·25 Lf of *adsorbed* toxoid subcutaneously and 150 units of tetanus antitoxin (pepsin refined horse globulin) intraperitoneally at the same time, and (C) horse serum sensitized guinea-pigs treated with both toxoid and antitoxin. All animals bled 14, 21, 28 and 42 days later, a further 2·25 Lf of *adsorbed* toxoid given on the 42nd day and the animals bled 10 days later.)

Guinea-pig no.	Day 14	Day 21	Day 28	Day 42	Secondary response Day 52	
			(A)			
1	0·01	1·0	2–5	2–5	100	
2	0·01	0·1	0·2	0·2–0·5	20–50	
3	0·02–0·05	2·0	5·0	5–10	100–200	
4	0·01	1–2	1–2	2–5	50–100	
5	0·02–0·05	1·0	2–5	5–10	10–20	
6	0·01	0·2	1·0	1·0	10–20	
7	0·01–0·02	2·0	2·0	5–10	20–50	
9	0·01–0·02	1·0	1·0	†	†	
10	0·01–0·02	1·0	2·0	2–5	100–200	
G.M.	0·014	0·78	1·6	2·8	47·9	
			(B)			
13	0·02	< 0·01	< 0·01	< 0·01	10–20	
16	0·02–0·05	< 0·01	< 0·01	< 0·01	5–10	
8	0·02–0·05	< 0·01	< 0·01	0·01–0·02	10–20	G.M.
4	0·02–0·05	< 0·01	< 0·01	0·02–0·05	10–20	11·9
1	0·02	< 0·01	< 0·01	0·05–0·1	10	
12	0·02–0·05	< 0·01	< 0·01	0·01	10–20	
19	0·02	< 0·01	0·02	0·1–0·2	20–50	
18	0·01–0·02	< 0·01	0·05–0·1	0·2	20–50	
2	0·02–0·05	0·01–0·02	0·5–1·0	0·5–1·0	10–20	G.M.
9	0·01	0·01–0·02	0·02–0·05	0·02–0·05	10–20	27·5
7	0·05	0·05	0·2–0·5	0·2–0·5	10–20	
10	0·02–0·05	0·02	0·5–1·0	0·5–1·0	20–50	
3	0·01–0·02	0·05–0·1	0·2–0·5	1–2	50–100	
6	0·02	0·05	0·2–0·5	1–2	20–50	
14	0·01–0·02	0·1–0·2	0·5	1–2	50	G.M.
20	0·01–0·02	1·0	1·0	2·0	10–20	28·1
17	0·05	1–2	2·0	2·0	20–50	
15	0·05	2·0	2–5	2–5	50	
G.M.	0·025	< 0·038	< 0·11	< 0·23	20·9	

† Animal died.

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Table 7 (*cont.*)

(C)

1	0·01–0·02	0·2–0·5	1–2	2–5	20–50
2	< 0·01	0·01–0·02	0·1	1–2	10
3	0·01–0·02	0·1–0·2	1–2	2–5	20–50
4	< 0·01	0·1–0·2	0·5	†	†
5	0·01–0·02	0·2	1·0	2–5	20–50
6	< 0·01	0·1–0·2	0·2–0·5	0·5–1·0	2–5
7	0·02–0·05	0·02	0·1–0·2	1·0	20
8	< 0·01	0·2–0·5	1–2	†	†
9	< 0·01	0·1–0·2	1·0	2–5	20
10	< 0·01	0·05	1·0	†	†
11	0·01	0·01	0·2–0·5	1·0	2–5
12	0·01	0·1	0·5	2·0	5–10
13	< 0·01	0·02	0·5	1–2	20–50
14	0·01–0·02	2–5	10	10–20	50–100
15	< 0·01	0·02	1–2	2–5	20–50
16	0·02–0·05	0·01–0·02	0·01	0·2–0·5	1–2
G.M.	< 0·011	0·078	0·55	1·9	14·1

tive animals. These groups were bled at intervals as before (Table 7A, B and C). The group given adsorbed toxoid only showed active antitoxin production at 14 days of a similar order to that found in Table 6A with fluid toxoid alone. Thereafter the rise in titre with adsorbed toxoid was more rapid and although the final titres after the second dose of toxoid were not proportionately as great, this could well be due to a flattening of the dose response curve when approaching the optimum response for this dose and time interval. When 150 units of antitoxin was given with the first dose of adsorbed toxoid (Table 7B), the antitoxin titres at 14 days were of the same order as those found with antitoxin alone. At 21 days there was evidence of active antitoxin production in ten out of eighteen animals; the titres at this time were, however, considerably lower than those found where no horse tetanus antitoxin had been given, and the subsequent rise in titre was much slower. Even at 42 days two animals had failed to produce measurable antitoxin, while others had responded well, and the picture was one of considerable individual variation between animals. All members of this group responded well to a second dose of toxoid, and arrangement of the results according to the primary response indicates that interference with the primary response did in fact have a slightly depressing effect on the secondary response.

Serum-sensitive guinea-pigs were immunized with adsorbed toxoid and given 150 units of antitoxin at the same time as the first dose of toxoid. At 14 days it was found that eight out of sixteen animals had no detectable antitoxin presumably as a result of rapid removal of the passively acquired antitoxin. The remainder of animals in the group had a low level of circulating antitoxin. It was hardly possible that this was horse globulin since when antitoxin alone was given to serum-sensitive animals (Table 5B) it was found that after 14 days no detectable antitoxin remained. *It must therefore be assumed that active antibody production had begun* in these animals, in spite of the concurrent administration of heterologous antitoxin. Moreover, when the results in Table 7B and C are compared it can be seen

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Table 8. *Antitoxin titres (units/ml.) of groups of guinea-pigs given two doses of 2·25 Lf of tetanus toxoid, with different aluminium hydroxide content, subcutaneously at 42 days interval and 150 units of tetanus antitoxin (pepsin refined horse globulin) intraperitoneally at the time of the first dose of toxoid. All animals bled 10 days after the second dose of toxoid*

Aluminium hydroxide (μg./Lf)	No. of guinea-pigs with 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	Geometric means
71·7 (M/27)	—	—	—	—	—	—	—	—	1	2	3	5	4	15	22·4
33·0	—	—	—	—	1	—	—	1	1	1	2	7	—	13	10·2
16·5	—	—	1	—	1	—	—	1	1	2	3	—	3	12	5·1
8·2	5	3	1	2	—	—	1	1	—	—	1	—	1	15	< 0·078
3·8	11	1	—	—	—	1	—	1	—	—	—	—	—	14	< 0·018
1·6	11	1	—	—	—	1	—	—	—	—	—	1	—	14	< 0·022
Nil	11	1	—	2	—	—	—	1	—	—	—	—	—	15	< 0·013

Table 9. *Antitoxin titres (units/ml.) of groups of guinea-pigs given two doses of 2·25 Lf of tetanus toxoid with different aluminium phosphate content, subcutaneously at 42 days interval and 150 units of tetanus antitoxin (pepsin refined horse globulin) intraperitoneally at the same time as the first dose of toxoid. All animals bled 10 days after the second dose of toxoid*

Aluminium phosphate μg./Lf	No. of guinea-pigs with 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	0·2–1·0	1·0–2·0	2·0–5·0	5·0–10	10–20	20–50	50–100	Total	Geometric means
239 (M/10)	—	—	—	—	—	—	—	—	1	2	2	7	3	15	20·4
119	—	—	—	—	—	—	—	—	—	4	4	4	3	15	19·0
55·1	—	—	—	—	—	—	—	—	1	1	7	4	2	15	17·8
27·5	—	2	1	1	3	1	—	3	3	1	—	—	—	15	0·31
13·7	2	4	2	—	1	—	—	4	2	—	—	-	—	15	< 0·11
6·4	9	3	—	1	2	—	—	—	—	—	—	—	—	15	< 0·016
Nil	10	3	1	—	1	—	—	—	—	—	—	—	—	15	< 0·013

Table 10. *Antitoxin titres (units/ml.) of groups of guinea-pigs given two doses of 2·25 Lf of tetanus toxoid, with different calcium phosphate content, subcutaneously at 42 days interval with 150 units of tetanus antitoxin (pepsin refined horse globulin) intraperitoneally at the same time as the first dose of toxoid. All animals bled 10 days after the second dose of toxoid*

Calcium phosphate (μg./Lf)	No. of guinea-pigs with 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	Total	Geometric means
316 (M/20)	7	2	—	—	1	1	—	1	2	1	—	15	< 0·06
158	5	1	—	—	2	—	—	3	1	1	2	15	< 0·11
79	4	4	—	—	—	2	1	—	3	1	—	15	< 0·09
39·5	9	1	1	—	1	—	—	2	—	1	—	15	< 0·011
19·8	11	1	1	—	1	—	—	—	1	—	—	15	< 0·010
9·9	12	1	1	—	—	—	—	1	—	—	—	15	< 0·010
Nil	12	1	2	—	—	—	—	—	—	—	—	15	< 0·010

that there were no non-responders at 21 days in Table 7C, indicating that production of antitoxin had begun earlier. The titres attained at this time were not however so high as those found in Table 7A where no heterologous antitoxin had been given; the rate of increase of titre from 21 to 42 days was more rapid however, and at 42 days the titres were not significantly different from those found in Table 7A. The final titres in serum-sensitive guinea-pigs although not significantly different from those found with unsensitized animals were significantly lower than the results where heterologous antitoxin had not been given ($P = < 0{\cdot}05$).

Influence of concentration of adsorbents

The influence of different adsorbents at different concentrations on the antigenic efficiency of tetanus toxoids in the presence of heterologous antitoxin was investigated (Tables 8–10). Tetanus toxoid (approximately 50 Lf/ml.) was adsorbed into (*a*) aluminium hydroxide in concentrations from 71·6 μg./Lf (M/27) down to 1·6 μg./Lf; (*b*) aluminium phosphate 239 μg./Lf (M/10) down to 6·4 μg./Lf, and (*c*) calcium phosphate 316 μg./Lf (M/10) down to 9·9 μg./Lf. Groups of guinea-pigs were given two doses of 2·25 Lf of each of these materials at 42 days interval; 150 units of heterologous antitoxin was given intraperitoneally at the same time as the first dose of toxoid. Antitoxin titres were estimated from bleedings taken 10 days after the second dose of toxoid.

It was quite evident from the results obtained that the efficiency of the preparations depended on the concentration of the adsorbent. With the aluminium salts at concentrations customarily used in human prophylactics the antigenic response obtained was very satisfactory. Calcium phosphate, although an excellent adsorbent for proteins, was singularly ineffective. Although at the highest level of concentration there were a number of animals in the calcium phosphate group giving reasonable responses, there were many non-responders and the values were widely scattered. There was however a gradation of response which indicated that some effect had been produced. The aluminium salts produced results which demonstrated that the adsorbent content of the toxoid preparation was critical and that amounts below a certain level were quite inadequate. This critical concentration appeared to be slightly lower for the hydroxide than for the phosphate.

Adsorption characteristics of different adsorbents

The adsorption characteristics of different mineral carriers in relation to tetanus toxoid were investigated (Table 11). Tetanus toxoid of approximately 50 Lf/ml. was adsorbed into different concentrations of aluminium hydroxide, aluminium phosphate and calcium phosphate. The unadsorbed toxoid remaining in the supernatant fluid from such material was titrated by flocculation after 1 week and after 9 months. The concentrations of carrier used were of a similar order, the highest concentration of aluminium hydroxide being 71·7 μg./Lf (M/27) and subsequent reductions in concentration at about 50 % differences were made down to 1·6 μg./Lf. Aluminium phosphate was used at an initial concentration of 239 μg./Lf (M/10) and calcium phosphate at an initial concentration of 314 μg./Lf (M/20). After

adsorption for one week it was found that the most effective adsorbent on a basis of the molar concentration of the different preparations was calcium phosphate (complete adsorption at M/80): this material took up further toxoid on standing at 0–4° C. for 9 months. On this basis aluminium hydroxide was nearly as effective (complete adsorption at M/58), and there was only slight improvement in the position after 9 months. Aluminium phosphate was relatively poor as an adsorbent and complete adsorption was only effected at M/10, hardly any improvement taking place with this material after 9 months.

Table 11. *Degree of adsorption of tetanus toxoid* (*approximately* 50 Lf/ml.) *by different concentrations of* (a) *Aluminium hydroxide*, (b) *Aluminium phosphate*, (c) *Calcium phosphate*

Aluminium hydroxide (μg./Lf)	Toxoid content of supernatant fluid (Lf/ml.) After 1 week	After 9 months
71·7 (M/27)	< 2 (< 1)*	< 2
33·0	< 2 (< 1)	< 2
16·5	23 (25)	8·7
8·2	45 (67)	39
3·8	52·5 (91)	52·5
1·6	52·5 (91)	52·5
Nil	52·5 (91)	52·5
Aluminium phosphate (μg./Lf)		
239 (M/10)	< 2 (< 1)	< 2
119	10·8 (18)	6
55	25 (50)	26
27·5	39 (67)	41
13·7	47·5 (77)	47·5
6·4	49 (83)	49
Nil	49 (83)	49
Calcium phosphate (μg./Lf)		
314 (M/20)	< 2 (< 1)	< 2
157	< 2 (< 1)	< 2
78	< 2 (1·7)	< 2
39	10·3 (14)	< 2
19·5	32 (42)	25
9·7	46 (61)	41
Nil	51 (91)	51

* Figures in parentheses represent toxoid content of supernatant fluid estimated by total combining power method in mice (unit-equivalents/ml.).

It was demonstrated by Holt (1950) that elution of diphtheria toxoid from mineral carrier occurred in normal human serum. This characteristic was investigated (Table 12). Mineral precipitates from fully adsorbed toxoid were resuspended in an equivalent volume of normal horse serum and incubated at 37° C. for 2 and 6 hr. The supernatants from these materials were then tested by the

Table 12. *Elution of tetanus toxoid from totally adsorbed preparations by incubation of precipitates with an equivalent volume of normal horse serum at* 37° *C.*

Total combining power (TCP) estimation of eluates (unit-equivalents/ml.)

Time of elution	Aluminium hydroxide adsorbed	Aluminium phosphate adsorbed	Calcium phosphate adsorbed
2 hr.	5·0 (5·5 %)	27·7 (33·3 %)	16·7 (18·3 %)
6 hr.	4·5 (5·0 %)	26·3 (31·6 %)	12·5 (13·7 %)
TCP of unadsorbed control	91	83	91

Table 13. *Percentage reduction in antitoxin combining power (unit-equivalents/ml.) of tetanus toxoid adsorbed on to different quantities of three different adsorbents*

Aluminium hydroxide			Aluminium phosphate			Calcium phosphate		
Adsorbent content (μg./Lf)	Combining power unit-equivalents/ml.	Reductions (%)	Adsorbent content (μg./Lf)	Combining power unit-equivalents/ml.	Reductions (%)	Adsorbent content (μg./Lf.	Combining power unit-equivalents/ml.	Reductions (%)
71·7 (M/27)	50	45·1	239/(M/10)	83	Nil	314 (M/20)	59	35·2
33·0	53	42·2	119	83	Nil	157	59	35·2
16·5	56	37·7	55	80	3·6	78	59	35·2
8·2	77	14·4	27·5	80	3·6	39	59	35·2
3·8	83	7·7	13·7	83	Nil	19·5	67	26·4
1·6	91	Nil	6·4	83	Nil	9·7	83	8·8
Nil	91	—	Nil	83	—	Nil	91	—

total combining power (TCP) method for any toxoid which had been eluted. Only 5 % of the total toxoid adsorbed was eluted from aluminium hydroxide precipitates by this method, whereas 31–35 % of the toxoid was eluted from the aluminium phosphate carrier and 13–18 % was eluted from the calcium phosphate carrier.

The ability of antitoxin to combine with toxoid adsorbed on to mineral carriers was investigated by TCP tests with adsorbed materials (Table 13). Toxoid adsorbed on to aluminium hydroxide did not combine so well with antitoxin, and at the highest level of concentration of adsorbent, this resulted in a 45 % reduction in combining power. With aluminium phosphate-adsorbed toxoid, however, there was no significant reduction in combining power for antitoxin. With calcium phosphate-adsorbed toxoid there was some reduction in combining power which, as with the aluminium hydroxide-adsorbed material, reached an optimum point consistent with almost complete adsorption as judged by adsorption experiments shown in Table 11. In the circumstances the failure of aluminium phosphate to interfere with antitoxin combination was inexplicable.

Table 14. *Combining power of antitoxin in the presence of different mineral carriers at the calculated concentration used in the total combining power test for toxoid*

	Apparent antitoxin value (units/ml.) in the presence of:		
No carrier	Aluminium hydroxide	Aluminium phosphate	Calcium phosphate
42	42	42	45

The power of adsorbents to interfere with toxin–antitoxin titrations was investigated to ensure that the results obtained with toxoid were not due to adsorption of toxin in the second part of the total combining power test (Table 14). In this laboratory standard antitoxin was titrated in the presence of such quantities of adsorbent as would be used in the total combining power test, if adsorbed toxoid were under test. By this method there was no evidence of interference with the results using the aluminium compounds, but a slight increase of 7 % in the apparent antitoxin value where calcium phosphate adsorbent was added to the mixture. This would have the effect of lessening the percentage reduction in combining power with calcium phosphate at the highest level of adsorbent concentration in Table 13.

The observations on the characteristics of different adsorbents when taken in conjunction with the results of the antigenicity tests with adsorbed toxoids in the presence of heterologous antitoxin, indicated that the efficiency of toxoids adsorbed on aluminium compounds could hardly be due to the physical fact of adsorption alone. This suggests that the very different efficacy of the two adsorbents might be associated with some specific difference in action of calcium and aluminium ions on tissue cells.

Histological observations

Guinea-pigs were injected subcutaneously with 1·0 ml. amounts of suspensions of (*a*) aluminium phosphate, and (*b*) calcium phosphate. Small volumes (0·2 ml.)

of suspensions of approximately ten times the strength used in antigenicity tests at the highest level of concentration of mineral carrier, were used to exaggerate any effects produced. An equal number of animals was treated with the same materials to which 10 Lf of tetanus toxoid had been added. Direct comparison between the effects of the two minerals was made by injecting both mineral carriers into the same animal in each experiment. Different individual animals were killed after 1, 2, 3, 5 and 9 days, and the lesions excised and examined histologically.

Histological appearances showed both qualitative and quantitative differences in the tissue reactions between aluminium and calcium phosphate treated animals, both with and without toxoid, over the nine-day observation period. As the differences in reaction were more easily seen when the salts alone were injected, the features of these will be described first.

After one day both salts were detected in the lymphatics. Polymorphonuclear leukocytes were seen around the inoculum, but the degree of cellular response showed a marked difference between the two salts, that for calcium being profound whilst this was not so for aluminium. Macrophages were also present and both salts were detected in them.

After 3 days in the aluminium-treated animals, in addition to an increase in the cellular response, many cells of a different type first appeared in or near the lymphatics. These cells had a pale-staining cytoplasm with a well-defined, medium-sized nucleus; the chromatin was vesiculated. They were phagocytic as shown by the presence of aluminium in the cytoplasm. The blood vessels did not show any leukocytosis.

During the rest of the observation period, this special type of cell increased in numbers and appeared to be attracted to the aluminium, forming zones around it. These cells were not present except in aluminium treated animals.

The calcium salt appeared to be more noxious and induced a more severe cellular reaction, active phagocytosis was seen to be present, but the cell types involved did not include the particular type observed in aluminium treated animals. Giant cells were in evidence at 5 and 9 days and calcium was detected in them, but the majority of the mineral was extracellular. The development of fibrous tissue was considerable only in the calcium treated animals. At 9 days at the site of injection, the calcium salts were much less in evidence than the aluminium salts.

The addition of toxoid to both mineral carriers had the effect of increasing the polymorphonuclear response. The large pale-staining cells were still in evidence in those animals receiving aluminium salt, but the presence of an increased polymorphonuclear response confused the picture, making the differences between the effects of the different forms of treatment more difficult to distinguish.

DISCUSSION

It is well known that the presence of heterologous antibodies interferes with the primary response to fluid tetanus toxoid (Ramon & Laffaile, 1925; Cooke & Jones, 1943; Barr & Sachs, 1955). These observations were confirmed in animals by Suri & Rubbo (1961) who also observed that the degree of interference depended on the

ratio of toxoid to antitoxin, and that postponement of the toxoid injection for 7 days improved the response to it. The degree of interference with the response to fluid toxoid in guinea-pigs has been amply demonstrated in this paper in Table 1. It was, however, surprising to find that passive antitoxin was capable of severe *interference when given up to 4 days after the first dose of fluid toxoid. This* observation has been made previously by Uhr & Baumann (1961), with diphtheria toxoid and antitoxin.

It is reasonable to assume that when antitoxin was present at the time of injection of fluid toxoid, it caused interference by combining with and rendering unrecognizable the determinant antigenic groups. Antigen-antibody mixtures, especially when antibody is in excess, are poorly antigenic. However, it would be expected that when antitoxin was injected 4 days after the first dose of toxoid, antigen would have already been taken up by the receptor cells. It was therefore surprising that interference should still be evident at that stage.

It has been shown that many phagocytic cells such as the Kupffer cells of the liver which are not responsible for antibody production will take up antigens (White, 1963). Moreover, antigen taken up by many different cell types disappears in a few days (Coons, Leduc & Kaplan, 1951). The work of Gowans, McGregor, Cowan & Ford (1962) demonstrated that the primary antigenic response depends on the presence of small lymphocytes. This suggests either that antigen is passed from phagocytic cells to the small lymphocytes or that some processed material is handed on in this way, or that only antigen taken up by small lymphocytes is responsible for the initiation of the immune response and that phagocytosis serves some other purpose. There is a strong possibility that antibody-producing plasma cells are derived from small lymphocytes following stimulation by an antigen, and that this development process is essential to the production of specific antibody (McGregor & Gowans, 1963). In that case phagocytic cells may act merely as a holding depot, capable of transforming additional cells of the lymphocyte series. It is possible that antigen may be transferred directly from phagocytes to immunologically competent cells (White, 1963).

Buxton & Allen (1963) demonstrated in chickens that leucocytes sensitized *in vivo* with *Salmonella gallinarum* polysaccharide were sensitive to antisera to that organism. They attributed the cytotoxic effect to an anaphylactic type of reaction. It is possible that destruction of cells of the lymphatic series, from which antibody-producing cells are probably derived (Gowans *et al.* 1962) could be responsible for the failure to respond when toxoid was followed by antitoxin 4 days later.

When simultaneous active and passive immunization was carried out with aluminium hydroxide-adsorbed toxoid the antitoxin responses were of quite a different order. Although some interference with the responses was evident it was of a low degree where an adequate amount of mineral carrier was used. This difference did not seem to be a simple quantitative effect, for if the dose of fluid toxoid was increased to 20 or 50 Lf, with 150 units of antitoxin, no great improvement in active response was achieved.

It is well known that foreign particles such as insoluble aluminium salts are readily taken up by phagocytic cells, but it does not follow that soluble antigens

are so readily taken up, although Robineaux & Pinet (1960) demonstrated the uptake of fluorescein-labelled human serum protein by chicken macrophages. However, Glenny & Pope (1925) showed that, although fluid diphtheria toxoid injected intravenously into rabbits failed to immunize, if the toxoid was partly neutralized when antigen-antibody aggregates would be formed an immune response was obtained, and this applied to both heterologous and homologous antitoxins. Moreover, Glenny, Buttle & Stevens (1931) found that fluid toxoid was rapidly eliminated from the body, and they found some evidence that it was lost rapidly from the site of subcutaneous injection whereas adsorbed toxoid was not. These observations strongly suggest that mineral adsorbents produce an enhanced effect by promoting phagocytosis and acting as a long-term reservoir of antigen, protected from interference by antibody.

It can be seen in Tables 8 and 9 that the response to adsorbed tetanus toxoid by the simultaneous immunization procedure was directly related to the concentration of aluminium adsorbent and that on an equimolar basis the responses to the two preparations were very similar. However, in Table 10 the results when calcium phosphate was used as an adsorbent were very unsatisfactory, although there was some evidence of an increased effect at the highest levels of concentration of adsorbent.

Investigation of the adsorption characteristics of these three preparations gave no clue to the considerable differences in results. When the three different adsorbents were compared (Table 11) on a molecular basis, the calcium phosphate was the best and the aluminium phosphate preparation the least effective, and there was some slight improvement with time. The relative capacity of such adsorbed materials to cling to the adsorbent when inactivated in the presence of normal serum, which has been regarded as of significance in antigenicity (Holt, 1950), was investigated (Table 12). It was found that aluminium hydroxide-adsorbed toxoid was the most stable under these conditions and aluminium phosphate-adsorbed toxoid the least. There was then no correlation with immunizing capacity in the simultaneous immunization procedure.

Further, observations on the ability of the adsorbents to cover the antibody combining sites of the toxoid (Table 13) showed a similar lack of correlation with immunizing capacity.

White, Coons & Connolly (1955*a*) investigated the morphological changes in rabbits and guinea-pigs following the injection of alum precipitated diphtheria toxoid. They concluded that the improved antigenicity of alum precipitated toxoid was due to the development of an alum granuloma in which antigen was retained and released over a prolonged period, and that the development locally of antibody producing plasma cells contributed to the improved performance. Observations showed that the zone of macrophages built up around the alum deposit contained numerous alum particles. Further observations by White *et al.* (1955*b*) demonstrated a marked increase in the proliferation of macrophages both locally and in regional lymph nodes when the wax fraction of *Mycobacterium tuberculosis* was injected in a water-in-oil emulsion, and this was associated with plasma cell proliferation in the regional nodes; this material increased antibody titres to

antigens incorporated in the mixture. Humphrey (1963) has demonstrated that mycobacteria in oily emulsions cause a rise in γ-globulin in guinea-pigs without any increase in any known antibody. Moreover, the antitoxin response to diphtheria toxoid can be enhanced by a delayed hypersensitivity response to purified protein derivative at the site of injection of toxoid (Humphrey & Turk, 1963). These observations suggest that adjuvants have a double effect in that there is increased production of macrophages in which White *et al.* (1955*a*) found no evidence of antibody production, and proliferation of antibody producing plasma cells.

In the present experiments it was found that aluminium phosphate alone produced the typical macrophage reaction in tissue and calcium phosphate did not. The relative inefficiency of calcium phosphate-adsorbed toxoid as an antigen in the presence of passive antitoxin could be due simply to its poor qualities as an adjuvant, but very large doses of fluid toxoid do not produce satisfactory immunity when passive antitoxin is present. This suggests that some special condition may be operative. Since a feature of the alum granuloma is rapid phagocytosis it is possible that toxoid is protected from interference by antitoxin when it is incorporated within the intact cell. In addition localization of antigen at the site of injection rather than the rapid dispersion of a fluid antigen would expose the material to relatively less antitoxin.

SUMMARY

Guinea-pigs given two doses of 2·25 Lf of fluid tetanus toxoid at 28 days interval had very satisfactory antitoxin titres 10 days after the second dose of toxoid (G.M. 28·2 units/ml.). Similar groups of animals given 150 units of horse tetanus antitoxin simultaneously with the first dose of toxoid responded very badly (G.M. $< 0{\cdot}016$).

Interference by passive antitoxin occurred even when the antitoxin was given as late as 4 days after the first dose of toxoid.

Interference by passively administered antitoxin was minimal when aluminium hydroxide-adsorbed toxoid was used. It was necessary to increase the dose of antitoxin from 150 to 2400 units before significant interference occurred.

The route of administration of antitoxin did not significantly affect the results except when the antitoxin was given intravenously.

When guinea-pigs were immunized and bled at regular intervals it was found that with both fluid and aluminium hydroxide-adsorbed preparations, titratable antitoxin was present on the 14th day. The increase in titre thereafter was more rapid with the adsorbed preparation, but after a second dose of toxoid there was no significant difference in titre.

Passively administered antitoxin virtually abolished the active response to fluid toxoid, but with aluminium hydroxide-adsorbed preparations the primary response was not abolished but reduced and delayed and there was much individual variation.

Horse serum-sensitive guinea-pigs given adsorbed toxoid with simultaneous passive horse antitoxin gave a better primary response to the toxoid than did unsensitive animals.

The effectiveness of adsorbed tetanus toxoid in the simultaneous immunization procedure was directly related to the concentration of aluminium hydroxide or phosphate used: this concentration was critical and amounts below a certain level were ineffective. Calcium phosphate used as an adsorbent was unsatisfactory in this way, although it was an excellent adsorbent.

Investigation of the adsorbent characteristics of aluminium hydroxide and phosphate and of calcium phosphate, showed that the calcium salt on a molar basis was the most effective and that aluminium phosphate was the least effective.

Elution of toxoid from centrifuged precipitates of the three types of adsorbent showed that only 5% of toxoid was removed from the aluminium hydroxide, 13–18% from the calcium phosphate and 31–33% from the aluminium phosphate preparation when incubated with normal serum at 37° C.

Aluminium hydroxide adsorption *in vitro* interfered with the ability of antitoxin to combine with toxoid and to a lesser extent calcium phosphate had the same effect; aluminium phosphate, however, did not appear to interfere at all in this way

Histological observations on the tissue response to aluminium phosphate and calcium phosphate indicated that the typical alum granuloma produced by aluminium phosphate was not produced by the calcium salt.

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