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RATE OF DISAPPEARANCE OF DIPHTHERIA TOXOID INJECTED INTO RABBITS AND GUINEA-PIGS: TOXOID PRECIPITATED WITH ALUM.

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IT has been shown by Glenny and Pope (1925) that diphtheria toxoid, when injected subcutaneously, will immunise rabbits but may fail when given intravenously. If however the toxoid be partially neutralised by antitoxin, rabbits can be immunised by the intravenous route. We have recently found that when toxoid is given intravenously to rabbits which have circulating antitoxin as a result of a primary stimulus there is a marked increase in the antitoxin titre in about seven days, whereas there is no such response after injection in rabbits which have no circulating antitoxin, although they may be already potentially immune, their tissues having been so stimulated that a rapid production of antitoxin would follow a subcutaneous injection of the antigen. This experiment confirms the suggestion of Glenny and Pope (1925), that "the intravenous injection of unneutralised toxoid may fail to act as a powerful antigen because of too rapid elimination, and that the presence of antitoxin in the mixture injected may delay absorption or elimination." Later in the same year Ramon and Descombey (1925) suggested that the absence of local reaction after subcutaneous injection favours the elimination and loss of a large proportion of the antigen, which occurs to an even greater extent after intravenous injection.

Glenny (1925), commenting on the use of purified toxoid, stated that the improvement in antigenic efficiency of this material has been but slight in spite of great concentration of antigen. He suggested that the immunity produced by the injection of an antigen into a non-immunised animal does not depend only upon the total amount of antigen injected; the time spacing of the stimulus or the continuance of the stimulus may be of great importance. If the mixture is too rapidly absorbed or eliminated, only slight stimulation may occur, however large the dose injected. The efficiency of toxin-antitoxin mixtures depends upon the slow dissociation of the toxin from the antitoxin, so that the animal injected is constantly stimulated. He concluded that "one of the aims of our work must be to determine the best method in which to present such a potent antigen as concentrated toxoid in such a way that the maximum stimulation occurs." Toxoid precipitated by alum offers such an improved method of presentation.

Glenny Pope Waddington and Wallace (1926 [1]) first demonstrated the high antigenic efficiency of diphtheria toxoid to which potash alum had been added and stated (1926 [2]) that such a suspension constituted one of the best antigens so far used by them. These authors further showed that a suspension of an alum toxoid precipitate after boiling for an hour was as efficient in antigenic properties as the average toxin-antitoxin mixtures for human immunisation against diphtheria. The increased antigenic value of toxoid on the addition of alum

has been further reported by Glenny and Waddington (1928) who compared the antigenic power of toxoid and alum-toxoid by means of the immunity index method. O'Brien and Glenny (1929) also stated that a single injection of formalised tetanus toxin containing 2 per cent. alum produced material active immunity in horses. Further details have recently been communicated by Glenny (1930) who showed the advantage derived from the addition of alum to both tetanus and diphtheria toxoids when used for the immunisation of horses in the production of therapeutic sera.

Prior to the use of alum, Ramon (1925) showed that the addition of tapioca to "anatoxine" (toxoid) used in the immunisation of horses against diphtheria and tetanus increases their antitoxic titre, and he suggested that the improved results were due to the ensuing local reaction causing a delay in absorption and elimination of the anatoxine. Ramon and Descombey (1930) have reported convincing results of the immunisation of horses with tetanus "anatoxine au tapioca." The titre of antitoxin produced by injections of this material corresponds closely to that obtained by ourselves and other workers (private communications) in England and America by the use of alum-precipitated toxoid. It is of interest to note that Ramon has found additions of tapioca of greater advantage in tetanus than in diphtheria immunisation, a difference that we ourselves also found with the use of alum-precipitated toxoid. Although the final result may be the same whether tapioca or alum be added to tetanus toxin used in the hyper-immunisation of horses, greater protection is afforded to horses given one or two prophylactic injections of tetanus toxoid with alum. The use of alum has several advantages over tapioca; it has already been used to a considerable extent for human immunisation against diphtheria, the mode of action is more easily explicable than that of tapioca and the strength of the toxoid after precipitation can still be titrated after redissolving the precipitate in Rochelle salt or other suitable solvent.

It has been shown by Glenny Pope Waddington and Wallace (1926[2]), and by Glenny and Waddington (1928) that the addition of turpentine or toluol to toxoid causes a similar increase in antigenic efficiency to that produced by the use of tapioca. Glenny Pope Waddington and Wallace (1925) also stated that the addition of sub-lethal doses of diphtheria toxin to fully modified toxin considerably increases the antigenic value as measured by the immunity index. There is some evidence to show that other toxic substances may act in the same way; in a single experiment it was found that the antigenic value of diphtheria toxoid was improved by the addition of *B. welchii* toxin.

Local damage follows the injection with toxoid of such substances as turpentine, tapioca, toluol, specific or non-specific toxin and it is conceivable that this may prevent rapid absorption. The increase in response on adding these substances appears therefore to be due to their irritant action, but adsorption by tapioca may play an additional part (Grasset 1926). The improvement following the addition of alum to toxoid may be partly attributed to local damage, but would appear mainly due to the relative insolubility of the precipitate.

In the experiments to be described we have endeavoured to show (1) that toxoid alone injected into an animal is so rapidly eliminated that very little is available to act as a continuous stimulus; (2) that the improved response due to the addition of alum to toxoid can be attributed to the fact that the antigen is in the form of a relatively insoluble precipitate which remains in the body sufficiently long to continue its action after the tissues have acquired the power of rapid response, *i.e.* have become potentially immune.

According to the principles of primary and secondary stimulation demonstrated by Glenny and Südmersen (1921), tissues increase greatly in their power of response some weeks after the primary injection. The effects of any injection into a normal animal may be regarded as a continual stimulation produced by lessening quantities of antigen acting on tissues whose power of response is rapidly increasing. The importance of the retention of antigen in the system is thus evident.

The rapid elimination of toxoid such as we demonstrate later, and which both Ramon and ourselves have previously suggested, is at variance with Ramon's (1929) statement that toxoid can be demonstrated in horses for many days after injection. Ramon has given no experimental data of his experiments on horses beyond stating that normal horses can be rendered immune by transfusion from another horse several days after its injection with anatoxine; it is possible that the horse in which anatoxine appeared to persist was already naturally immune to a slight degree, and that the presence of traces of antitoxin delayed elimination, as previously suggested by Glenny and Pope. It is difficult to reconcile the rapid excretion of toxoid with Ramon's (1929) contention that antigen enters into the composition of antibody, but at the same time it by no means disproves this early theory of antitoxin formation; a more potent argument against this hypothesis is that a single injection of toxoid may result in the production of enough antibody to neutralise many thousand times the amount of antigen injected. Further, horses hyperimmunised to produce therapeutic sera may yield enough antitoxin to neutralise 3000 or more times the total toxin or toxoid injected. Two examples may be given:—from one horse 22·4 million units of diphtheria antitoxin were produced within 37 days of the commencement of immunisation, during which time a total of 7236 Lf doses of toxin were injected; another horse received 42,726 Lf doses and yielded in 64 days 108·0 million units of antitoxin.

The first experiment concerning the rate of elimination of toxoid was originally undertaken with the object of determining the length of time that an antigen must remain in the animal body in order that immunity should result. A number of guinea-pigs were injected subcutaneously with 1 c.c. of diphtheria toxoid containing 26 Lf doses. Certain groups were injected intravenously after definite intervals with 26 units of diphtheria antitoxin. All the guinea-pigs were bled after 4 to 6 weeks, and the serum titrated for antitoxic value. The results are recorded in table I. The control guinea-pigs, which had received no antitoxin, all showed good immunity, averaging 0·1 unit per c.c. while all but one of the animals which had received antitoxin within 3 days produced less than 0·001 unit per c.c.

When the interval was increased to 7 days, 3 out of 13 guinea-pigs had produced over 0·01 unit of antitoxin 6 weeks after the injection

of toxoid. Actively produced circulating antitoxin has never been demonstrated in guinea-pigs 7 days after a primary stimulus, and no passively conferred antitoxin has been found in the serum as late as 5 weeks after the injection of antitoxic horse serum. We must therefore conclude that immunity was produced actively after the injection of antitoxin; either some of the antigen remains inaccessible to the antitoxin and continues to act as a stimulus, or 7 days' contact between the toxoid and the tissues was sufficient to cause the subsequent production of active immunity. As the interval between the injection of toxoid and antitoxin is increased to 14 and 21 days the number of guinea-pigs failing to produce antitoxin decreases.

TABLE I.

Showing the extent to which the immunity response of guinea-pigs to a subcutaneous injection of diphtheria toxoid is modified by the subsequent intravenous injection of sufficient antitoxin to neutralise all the toxoid injected.

Times elapsing between injections of toxoid and antitoxin.	Number of guinea-pigs with antitoxic values 4–6 weeks later of—			
	Under 1/1000. units per c.c.	1/1000–1/100.	1/100–1/10.	1/10–1.
Controls—no antitoxin . . .	0	1	10	13
No interval—antitoxin mixed .	6	1	0	0
No interval—antitoxin separate .	6	0	0	0
4 hours	7	1	0	0
24 ,, ...	8	0	0	0
3 days	8	0	0	0
7 ,, ...	9	1	3	0
14 ,, ...	5	4	2	0
21 ,, ...	2	0	7	4

In order to obviate the possibility of titrating passively administered antitoxin, those animals receiving their injection at intervals of 7 days or more from the time of injection of toxoid were bled 6 weeks after this date. The last three lines therefore refer to results of titrations of bleedings 6 weeks after the injection of toxoid, the remainder 4 weeks.

Guinea-pigs obviously vary considerably in what may be termed their "latent period of effective stimulation," 7 days being sufficient for 4 guinea-pigs out of 13, and 21 days insufficient for 2 out of 13. In order to determine whether any immunity had developed, the guinea-pigs which had not produced antitoxin were then Schick-tested at weekly intervals, and it was found that the animals receiving antitoxin 4 or 24 hours after the toxoid were definitely more immune than those receiving antitoxin at the end of 3 days. These results suggested that the failure of the guinea-pigs receiving antitoxin after 3 days was due to the fact that the toxoid remaining in these animals was more over-neutralised than that present in the guinea-pigs which had received antitoxin after a shorter interval. We therefore repeated

the experiment, using smaller doses of antitoxin. The results are given in table II, and show that sufficient toxoid remains in the body after 4 hours for the production of active immunity to be practically uninfluenced by the injection of 20 per cent. of the original quantity of antitoxin (see controls in table I).

TABLE II.

Showing the extent to which the immunity response of guinea-pigs to a subcutaneous injection of diphtheria toxoid is modified by the injection of sufficient antitoxin to neutralise only a fraction of the toxoid injected.

Times elapsing between injection of toxoid and of antitoxin.	Amount of antitoxin injected in terms of percentage of the amount required to neutralise all the toxoid injected.	Number of guinea-pigs with antitoxin values 4–6 weeks later of—			
		Under 1/1000. units per c.c.	1/1000–1/100.	1/100–1/10.	1/10–1.
4 hours . .	20	0	2	3	3
	10	2	2	6	2
24 „ . .	10	12	2	1	0
	2	0	2	1	2
3 days . .	10	11	0	0	0
	2	4	1	0	1
	1	3	1	3	1
7 „ . .	1	3	2	3	1
14 „ . .	1	1	4	7	3

If the antitoxin is not given until 24 hours after the injection of toxoid, one-tenth of the original amount is sufficient to interfere with immunisation, while 2 per cent. is insufficient. On increasing the interval to 3 days some interference occurs when the amount of antitoxin injected is as small as 1 per cent., and very little immunity results if the dose given is sufficient to neutralise 2 per cent. of the toxoid originally injected. These experiments suggest that most of the toxoid injected is eliminated within the first 24 hours, and at the end of three days only a trace remains; this, however, is of considerable importance in the development of immunity because of the rapidly increasing power of response by the tissues. Subsequent experiments were devised to investigate how much toxoid remains in the animal body, and how much is eliminated by the kidneys.

Four rabbits were injected intravenously with 5 or 10 c.c. of diphtheria toxoid (26 Lf doses per c.c.) and were then bled after intervals of 5 minutes, 6, 24 and 48 hours. The urine was collected by catheter during the first 6 hours and again during the next 18 hours. Both serum and urine samples were treated in the following way for the determination of the amount of toxoid present. Known amounts of toxin were added to the sample, and the combining power of the mixture determined by titration against antitoxin by means of

the intracutaneous method of testing. Control dilutions containing known amounts of toxoid were treated in the same way, and by comparison with these results an approximate estimation was made of the toxoid content of the sera and urine. The results of these experiments are recorded in table III, from which it will be seen that only 50 per cent. of the toxoid remained in the blood after 6 hours and that this figure was reduced to 15 to 25 per cent. after 24 hours. Since only a small percentage of the toxoid lost is detectable in the urine, it must be supposed that the rest is either destroyed in the animal or absorbed by certain tissues.

TABLE III.

Showing the rate of disappearance of toxoid from the serum of rabbits after intravenous injection.

Rabbit number.	Weight (kilo.)	Toxoid injected Lf doses.	Toxoid concentration (Lf doses per c.c.) at intervals after injection of—				Toxoid disappearing from serum. Percentage of total.		Toxoid detected in urine. Percentage of total.	
			5 mins.	6 hrs.	24 hrs.	48 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.
1	1·6	130	1·0	0·5	0·125	...	50	85	5	10
2	3·6	260	2	1	0·5	0·125	50	75	4	6
3	2·0	130	1·5	0·5	...	...	67	...	4	...
4	2·3	130	1	0·5	...	...	50	...	2	...

To determine the total amount of toxoid present in the body a further experiment was made in a different manner. A number of rabbits were injected intravenously with 5 c.c. of diphtheria toxoid containing 130 Lf doses; groups of the animals were then given, intravenously, an equivalent amount of antitoxin after various intervals. As controls, 2 rabbits received an injection of 130 units of antitoxin, no toxoid having been previously administered. Samples of serum were taken at definite intervals and titrated for antitoxic content. From the results of titration of the samples taken 5 minutes after the injection of antitoxin, it was possible to determine how much of the latter had combined with toxoid in the circulation. The values of the samples taken at longer intervals after the injection of antitoxin give an indication of the total amount of toxoid present in those parts of the body accessible to the antitoxin.

The results of the various titrations are given in table IV, from which it will be seen that when the antitoxin is injected 5 minutes after the toxoid, a portion of it is still detectable in the blood, although equivalent quantities of toxoid and antitoxin were injected. This is due to the relative looseness of the combination of toxoid with antitoxin; when toxin is added to the rabbit serum for the purpose of testing for excess of antitoxin, a small portion of the toxoid contained in the serum is displaced from combination with the antitoxin.

Though obviously the results of titration of antitoxin cannot be regarded as correct measures of the proportion remaining uncombined with toxoid, they may be accepted as fair approximations.

TABLE IV.

Showing the amount of antitoxin remaining in the circulation of rabbits after the intravenous injection of 130 *units of antitoxic horse serum given at definite intervals after a similar injection of an equivalent amount of toxoid.*

Group.	Rabbit.	Weight (kilo.)	Interval between injection of toxoid and antitoxin.	Antitoxic value of serum (units per c.c.) at intervals after injection of—			
				5 minutes.	4 hours.	24 hours.	8 days.
1	5	1·8	No toxoid	2·5	1·5	1·0	0·6
	6	2·1	,,	2·0	1·0	0·75	0·4
2	7	1·7	5 minutes	0·125	0·1	0·066	0·02
	8	1·9	5 ,,	0·33	0·2	0·125	0·04
3	9	1·8	4 hours	0·33	0·125	0·1	0·05
	10	...	4 ,,	0·75	0·2	...	...
	11	1·8	4 ,,	1·0	0·5	0·33	0·125
4	12	1·8	24 hours	1·5	1·0	0·6	0·33
	13	1·8	24 ,,	1·25	0·6	0·33	0·2
	14	2·0	24 ,,	2·0	1·0	0·5	0·4

The amounts of toxoid present 4 and 24 hours after injection, recorded in table V, have been calculated in the following manner. The difference between the average antitoxin values of the sera of rabbits in groups 1 and 2 shows the reduction in antitoxic titre caused

TABLE V.

Showing the percentage amount of toxoid remaining 4 *and* 24 *hours after intravenous injection, calculated from results given in table* IV.

Group.	Rabbit.	Interval.	Percentage amount of toxoid remaining, calculated from results of titration at intervals after antitoxin injection of—			
			5 minutes.	4 hours.	24 hours	8 days.
3	9	4 hours	93	100	100	96
	10	4 ,,	72	98	...	...
	11	4 ,,	60	70	70	80
4	12	24 hours	36	20	35	36
	13	24 ,,	48	60	70	64
	14	24 ,,	12	23	48	21

by the full amount of toxoid present. A similar calculation from the results of groups 1 and 3 shows the reduction caused by the amount of the toxoid still remaining after 4 hours. The ratio of these differences indicates the proportion of toxoid remaining at the end of this time.

The figures given for the proportion of toxoid remaining after 24 hours were obtained by similar calculation using the results of tests on groups 1 and 4 in place of groups 1 and 3.

If, at the end of any given time, some of the toxoid is absorbed in the tissues and is still accessible to the antitoxin, the latter will disappear from the circulation at a greater rate than in normal rabbits. An inspection of the figures in table V shows that this does occur. The average amount of toxoid remaining after 4 hours is about 75 per cent. in the circulation and 85 per cent. in the whole body; after 24 hours the figures are roughly 25 per cent. and 40 per cent. respectively. The great variation occurring among individual rabbits does not permit any accurate conclusion to be drawn from an experiment involving the use of so few animals.

With one group of 4 rabbits an endeavour was made to correct to some extent errors due to individual variation. The diphtheria antitoxic horse serum, before injection into each rabbit, was mixed with the same number of units of tetanus antitoxic horse serum. The ratio between the two antitoxic values (diphtheria and tetanus) of any given sample of serum showed the reduction in diphtheria antitoxin due to the presence of toxoid. The result of this more accurate experiment showed that after 4 hours 36 per cent. of toxoid remained in the circulation and 70 per cent. in the whole body; after 24 hours the corresponding figures were 0 and 15 per cent. The extent of experimental error may be indicated by comparing the percentage variation in the ratio of tetanus to diphtheria titrations of the samples of serum taken at different intervals from rabbits in groups 1 and 2, which was 10 per cent. for group 1, and 14 per cent. for group 2.

There is some definite evidence to show that *alum toxoid* is not absorbed and eliminated at the same rate as toxoid alone. In a

TABLE VI.

Showing the extent to which the response of guinea-pigs to a subcutaneous injection of 1·0 *c.c. of diphtheria toxoid* (26 *Lf doses*) *is influenced by the addition of various precipitating agents.*

Addition per 100 c.c. toxoid.	Number of guinea-pigs with antitoxic values 8 weeks later of—				
	Under 1/1000. units per c.c.	1/1000–1/100.	1/100–1/10.	1/10–1.	Over 1.
Nil—controls	3	8	3	2	0
Alum 1 gr.	0	0	1	5	0
,, 2 gr.	0	0	2	8	3
Cerium nitrate 2 gr. . .	0	0	0	9	0
Zinc sulphate 1 gr. . .	0	0	0	6	0
Calcium chloride 1 gr. . .	0	0	1	5	0
,, ,, 10 gr. . .	0	1	1	3	0
Dialysed iron 2·4 gr. . .	0	0	3	7	0
Tungstic acid 1·7 gr. . .	0	0	2	7	0

preliminary experiment several guinea-pigs were injected intracutaneously with these two products. After 3 days a portion of skin containing the site of injection was excised and an emulsion injected into normal guinea-pigs. The animals receiving the emulsion of skin from the alum toxoid injected guinea-pigs were successfully immunised, but not the others. Both sets of donors became immune.

If the increased antigenic efficiency of alum toxoid is due to the decrease in the rate of elimination, then similar advantage should follow the addition of other salts causing precipitation of a similar nature. Groups of guinea-pigs were injected with diphtheria toxoid to which various precipitants had been added. After an interval of 3 weeks the animals were bled and their serum tested for antitoxoid. The results of these titrations, recorded in table VI, show that precipitated toxin has a higher antigenic efficiency than the original toxoid. It would appear from the figures recorded in table VI that more favourable results follow the use of alum, but it must be pointed out that the optimum amount of precipitant was not determined. This work is being continued in conjunction with Miss M. Llewellyn Smith who kindly provided the material for this experiment.

Summary.

(1) The rapid elimination of diphtheria toxoid after injection has been experimentally demonstrated.

(2) The increased antigenic efficiency of alum toxoid is due to the resulting slow absorption and elimination.

(3) Other precipitants act in a manner similar to alum.

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