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THE EFFECT OF THE AMOUNT OF ANTIGEN ON ANTITOXIN-FORMATION DURING THE PRIMARY AND SECONDARY IMMUNIZATIONS

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In a previous study (1) on antitoxin-formation after intravenous or subcutaneous injections of plain or alum diphtheric toxoid we found that more antitoxin is formed after repeated intravenous injections of 25 Lf than of 5 Lf alum toxoid. The purpose of the present experiments was to obtain more complete information on the effect of injections of increasing amounts of the antigen on antitoxin formation. The studies of Eaton (2) and Pappenheimer (3) showed that diphtheric toxin is a protein 0.003 mg of which constitutes approximately one Lf. Since in our previous experiments only 5 and 25 Lf, i.e., 0.015 and 0.075 mg toxoid protein antigen were injected per dose it seemed desirable to increase the amount of antigen considerably. In the present study we compared the effect of doses ranging between 25 and 10,000 Lf which would correspond to 3.0 mg diphtheric toxoid.

MATERIAL AND METHODS

Male rabbits of various breeds weighing from six to ten pounds were used. The alum precipitated diphtheric toxoid was made by the addition of alum to a formalized filtrate of the diphtheria bacillus grown on infusion broth. This preparation was used during the first course of immunization in experiment 2 and 3 and also during the second course of immunization in experiment 2 group A. A concentrated preparation was injected during the first course in Experiment 1 and during the second course of immunization in all experiments (except experiment 2 group A). The method of concentration was as follows. One part of chilled toxoid was mixed with two parts of chilled acetone in large centrifuge cups and the mixture kept for half an hour in the refrigerator. After centrifugalization 0.9 per cent salt solution was added to the sediment to bring it to one-tenth of the original volume of the toxoid. The suspension was made slightly acid with a small amount of normal hydrochloric acid. To the resulting clear solution dibasic sodium phosphate solution was added to contain 0.4 per cent phosphate. Then a 10 per cent alum solution was added in the proportion of 3 parts to 7 of toxoid. The mixture was kept at room temperature for 2 or 3 hours and in a refrigerator over night. An aliquot of the alum and toxoid mixture was centrifugalized and the sediment dissolved in sodium citrate. This solution was titrated with the aid of the flocculation reaction to determine the antigen content of the precipitate. The main bulk of the alum and toxoid mixture was centrifuged and the alum precipitate suspended in salt solution to

contain the desired amount of Lf per ml. The antitoxin titrations were made by the method of Fraser (4).

EXPERIMENTAL

In experiment 1 seven rabbits received intravenous injections of alum toxoid on three consecutive days and after a rest of four days they were given three similar injections. The doses were: 25, 50, 75, 100, 125, 150 Lf. After a rest of one month the rabbits were given weekly injections of 200 Lf for five weeks. The first injection during secondary stimulation was a single injection of 200 Lf but because some of the rabbits had convulsions the subsequent injections were given in divided doses, i.e., 100 Lf followed by another 100 Lf about three hours later. These injections were given in 20 ml volume. Graph 1 shows

TABLE 1

Antitoxin units per ml of sera of rabbits immunized with alum precipitated diphtheric toxoid

Experiment 1

RABBIT	DAYS AFTER THE 1ST COURSE		DURING THE 2ND COURSE OF WEEKLY INJECTIONS; 5 DAYS AFTER					DAYS AFTER THE 2ND COURSE
	15	30	1	2	3	4	5	21
1	2.5	2.5	40	40	40	40	70	30
2	2.5	1.0	20	20	20	40	40	15
3	1.0	1.0	20	30	30	20	30	15
4	1.0	1.0	30	40	30	30	20	10
5	1.0	1.0	30	60	60	40	30	
6	1.0	1.0	20	60	70	40	40	15
7	0.5	0.5	20	50	40	20	20	20
Average....	1.3	1.1	25.7	42.8	41.4	32.8	35.7	16.6
Median....	1.0	1.0	20.0	40.0	40.0	40.0	30.0	15.0

During the first course the doses were from 25 to 150; during the second course 200 Lf alum toxoid; the last four doses were given in divided form. All injections were made intravenously.

that 15 days after the last injection of the primary series the average titer was 1.3 and 15 days later 1.1 antitoxin units per ml. Titrations 5 days after each injection of the secondary immunization revealed a striking rise in circulating antitoxin after first and second injections. This rise was followed by a slight drop. Twenty-one days after the last injection the titer was low.

The results observed during the primary stimulation were unexpected. They indicate that the increase of dosage during the primary immunization did not stimulate more antitoxin formation than we had found in our previous experiment (1) in which each dose in the series was 25 Lf. During the secondary immunization, however, the increased dose of antigen resulted in high titers. With 25 Lf per dose during the secondary immunization we had observed an average maximum of 16 units per ml while in the present experiment with 200 Lf per dose the average titer reached was 43 units per ml.

TABLE 2

Antitoxin units per ml of sera of rabbits immunized with alum precipitated diphtheric toxoid

Experiment 2

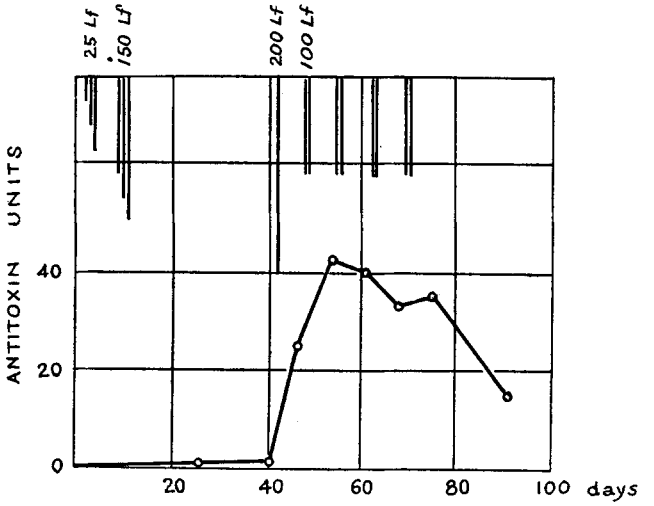
RABBIT	DAYS AFTER THE 1ST COURSE		DURING THE 2ND COURSE OF WEEKLY INJECTIONS, 5 DAYS AFTER							
	24	31	1	2	3	4	5	6	7	8
Group A										
1		2.5	12.5	20.0	20.0	20.0	17.5	15.0	10.0	
2		1.0	5.0	12.5	12.5	10.0	10.0	10.0	5.0	
3		1.0	20.0	12.5	7.5	5.0	7.5	7.5	7.5	
4		0.5	2.5	5.0	5.0	5.0	2.5	5.0	2.5	
5		0.1	2.5	2.5	5.0					
Average.....	0.6*	1.0	8.5	10.5	10.0	10.0	9.5	9.3	6.2	7.5*
Median.....		1.0	5.0	12.5	7.5	7.5	8.8	8.8	6.3	
Group B										
1		1.0	15.0	17.5	10.0	10.0	12.5	12.5	7.5	
2		1.0	10.0	25.0	35.0	35.0	20.0	25.0	12.5	
3		0.5	7.5	15.0	20.0	25.0	20.0	17.5	10.0	
4		0.1	5.0	7.5	15.0	20.0	12.5	10.0	7.5	
5		0.1	7.5	12.5	12.5	10.0	7.5	10.0	7.5	
6		0.05	1.0	7.5	25.0	30.0	30.0	35.0	17.5	
Average.....	0.6*	0.5	7.6	14.1	19.5	21.6	17.1	18.3	10.4	15.0*
Median.....		0.3	7.5	13.8	17.5	22.5	16.3	15.0	8.8	
Group C										
1		1.0	25.0	35.0	45.0	80.0	65.0	55.0	45.0	
2		1.0	60.0	35.0	40.0	50.0	35.0	40.0	20.0	
3		1.0	25.0	35.0	30.0	45.0	25.0			
4		0.5	15.0	17.5	15.0	20.0	20.0	17.5	10.0	
5		0.5	40.0	70.0	75.0	85.0	75.0	75.0	55.0	
6		0.5	12.5	7.5	7.5	7.5	5.0	5.0	2.5	
7		0.5	15.0	25.0	95.0	90.0	150.0	175.0	70.0	60.0
8		0.1	12.5	17.5	20.0	25.0	15.0	12.5	7.5	
Average.....	0.6*	0.6	25.6	30.3	40.9	50.3	48.7	54.2	30.0	30.0*
Median.....		0.5	20.0	30.0	35.0	47.5	30.0	40.0	20.0	

* Pool of sera.

During the first course 25 Lf alum toxoid were injected intravenously four times three days apart; during the second course weekly injections were given; Group A: 25 Lf unconcentrated, Group B: 25 Lf concentrated and Group C: 200 Lf concentrated alum toxoid per dose.

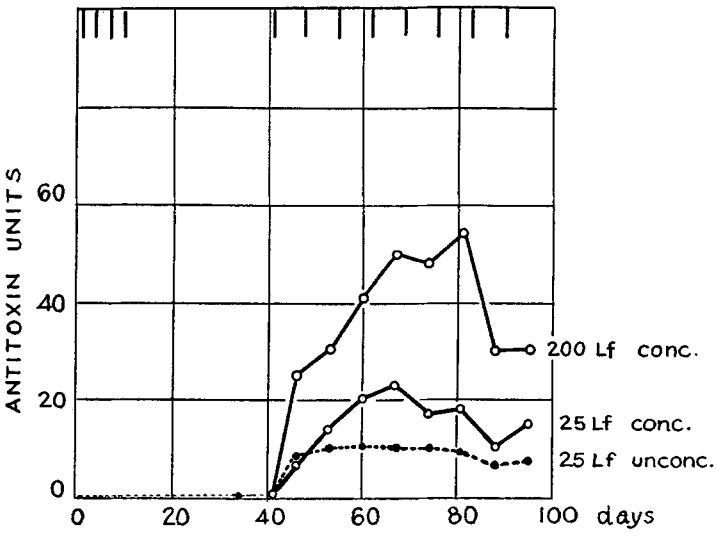
Since a comparison of our previous data (1) with Experment 1 indicates that the dosage influences antitoxin formation more during the secondary than during the primary immunization and since it is undesirable to subject rabbits to

repeated intravenous injections of large amounts of particulate matter, in experiment 2 all of the 19 rabbits received only 25 Lf per dose during the primary



Vertical lines = Dose of Antigen

GRAPH 1



Vertical lines = Day of Injection

GRAPH 2

immunization. This dose was given four times three days apart. One month after the last injection one group was given 25 Lf unconcentrated, one group 25 Lf concentrated and the third group 200 Lf concentrated alum toxoid per in-

jection, one injection per week for eight weeks. Titrations were made 24 and 31 days after the last injection during the first course of immunization. The

TABLE 3

Antitoxin units per ml of sera of rabbits immunized with alum precipitated diphtheric toxoid

Experiment 3

RABBIT	DAYS AFTER THE 1ST COURSE				DURING THE SECOND COURSE OF WEEKLY INJECTIONS; 7 DAYS AFTER				DAYS AFTER THE 2ND COURSE		
	15	30	60	90	1	2	3	4	30	60	90
Group A											
1					125	125	100	100	30	15	
2					125	125	50	70	15	5	
3					125	70	50	30	10	5	
4					125	60	50	30	5		
5					50	50	40	40	10	5	
Average.....	1.0	0.6	0.3*		110	86	58	48	14	7.5	2.5*
Median.....					125	70	50	40	10	5	
Group B											
1					175	175	100	90	30	30	
2					125	125	125	70	15	5	
3					100	100	100	90	30	15	
4					100	70	50	30	10	5	
5					5	150	90	90	30	15	
Average.....	1.0	0.6	0.3*		101	124	93	74	23	14	5.0*
Median.....					100	125	100	90	30	15	

RABBIT	DAYS AFTER THE 1ST COURSE				DURING THE 2ND COURSE, 7 DAYS AFTER INJECTION		DAYS AFTER THE 2ND COURSE			
	15	30	60	90	1	2	14	21	51	81
Group C										
1					100	50	60	50	15	
2					100	40	30	15	5	
3					90	30	30	30	15	
4					90	30				
Average.....	1.0	0.6	0.3	0.2*	95	37	40	31	11	2.5*
Median.....					95	35	30	30	15	

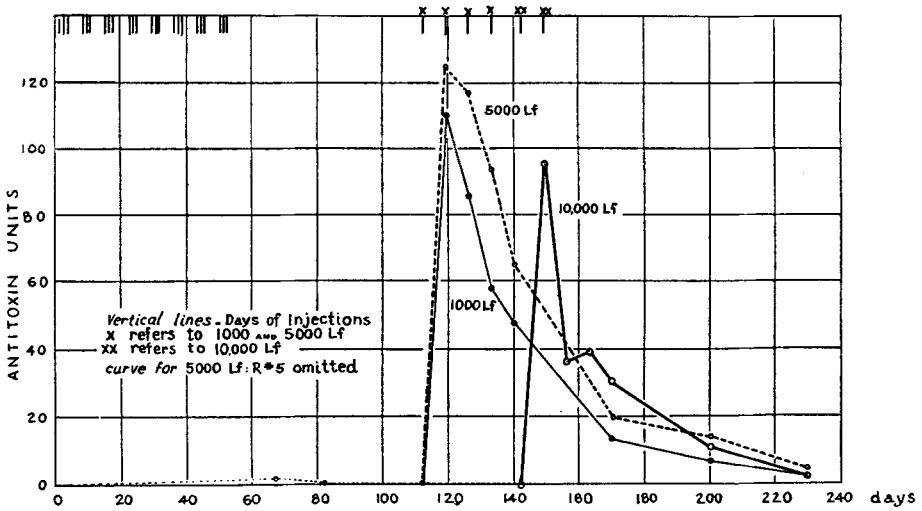
* Pool of Sera: Group A, 1000 Lf; Group B, 5000 Lf; Group C, 10,000 Lf.

All rabbits received 24 injections of 12.5 Lf alum toxoid intravenously on three consecutive days during eight weeks; Group A had 2 months rest and 1000 Lf intraabdominally four times one week apart; Group B the same treatment except 5000 Lf instead of 1000 Lf; Group C had 3 months rest and two intraabdominal injections of 10,000 Lf one week apart.

first two titrations showed 0.6 and 0.7 units per ml (average for all the rabbits). The first injection after rest had the greatest effect in all three groups. The

highest titers were reached in the rabbits that had received 200 Lf per injection; the group with 25 Lf concentrated toxoid had higher titers than the group with the same amount of unconcentrated toxoid. The titers in the group with 200 Lf of experiment 2 were similar to those of experiment 1.

In experiment 3 a group of 14 animals received intravenous injections of 12.5 Lf alum toxoid on three consecutive days per week for eight weeks. After a rest of two months five rabbits were given 1000 and five other rabbits 5000 Lf alum toxoid into peritoneal cavity once a week for four weeks. The remaining four animals were injected after 3 months rest because we wanted to await the outcome of the injections of 1000 and 5000 Lf of toxoid. These rabbits were finally given two intraperitoneal injections of 10,000 Lf toxoid one week apart. The injections were discontinued because one of the four rabbits died.



GRAPH 3

Antitoxin titrations were made several times during the rest period, (seven days) following the injections of the second course and also at later occasions. The antitoxin titers before the secondary immunization were one unit or less per ml. The first injection of 1000 Lf caused a great rise in antitoxin titers; the average was 110 units; the antitoxin titers fell after the subsequent injections. In the group receiving 5000 Lf the rise after the first injection was of similar magnitude; in one animal, rabbit # 5 whose response to the first injection was very poor there was a further rise after the second injection; then a considerable fall in all the rabbits after the third and fourth injections. The group with injections of 10,000 Lf responded to the first injection with a rise to 95 units per ml and to the second injection with sharp decline to 27 units per ml. The antitoxin titers were followed in the 13 rabbits of experiment 3 for several months. During the first month the titers fell sharply and then more gradually. The individual variation of rabbits in antitoxin production may justify the presentation of the tables in addition to the graphs.

DISCUSSION

A comparison of the antitoxin titers found after the first course of immunization in experiment 1, 2, and 3 and also in those presented in our previous paper show that during the primary stimulation the size of the dose has very little effect on the antitoxin titers. Experiment 2 group A indicates that the same dose is more effective during the secondary stimulation than during the first one. With regard to the second course of immunization the effect of the amount of antigen is very striking. The antitoxin titers increased with increasing doses (5, 25, 200, 1000, and 5000 Lf).

An increase of the dose beyond 5000 Lf per injection did not result in higher titers. It is possible that the large amounts of antigens and non-antigenic material associated with alum precipitated toxoid may have a harmful effect on the animal, directly or indirectly inhibiting antitoxin formation.

It may be mentioned that two groups of rabbits immunized with 25 Lf alum toxoid per dose in our previous study (page 442) received, after a period of rest, a third course of injections. This course raised the antitoxin level but not to the highest titer reached during the second course of injections.

It is noteworthy that during the secondary immunization the curve of antitoxin titers rose sharply and then declined in spite of continued injections of the antigen. The fall was particularly conspicuous with the largest doses. The peaks were reached after one injection in all animals that received 1000 and 10,000 Lf per dose and in 4 or 5 animals in the group with 5000 Lf per dose. The accumulation of smaller amounts of antigen during repeated injections (experiment 2) did not make up for the larger single doses, used in experiment 3. It seems, therefore, misleading to compare methods of immunization on the basis of accumulated doses.

Some of the outstanding aspects of these observations, namely, the difference of the response during the primary and secondary stimulus and the effect of the amount of antigen per dose injected have been previously described (5). Glenny and Südmersen (6) studied diphtheric antitoxin formation under the primary and secondary stimuli. As immunizing agents they used either toxin-antitoxin mixture or toxin. Several species of animals were employed in their studies. The two rabbits mentioned in their paper received one injection of toxin-antitoxin mixture and 13 weeks later one injection of toxin; therefore the antigenic stimuli were not identical. Morgan and Olitsky (7) vaccinated mice with active or formalin-inactivated equine encephalomelitis vaccine. They found that six months after a course of vaccination a single dose of either active or inactivated virus gave a greater antibody response than the initial course of vaccination. Burnet and his associates observed a difference between primary and secondary responses to staphylococcal toxoid, rickettsiae, herpes virus and bacteriophage. For these studies and a review of the recent literature the reader is referred to the admirable monograph by Burnet and his associates (8).*

* Recently Loveless found a difference between the primary and secondary immune response in man immunized with pollen extract (Loveless, M. H., 1941. Immunological studies of pollinosis. Jour. Immun., **43**, 1–8.)

It may be mentioned that the antitoxin titers obtained in the rabbits which were given large amounts of antigen during the second course of immunization are unusually high for this species of animal. Pappenheimer (9) immunized rabbits by injections of highly purified alum toxoid. The highest titer he observed was 32 units per ml.

SUMMARY

Groups of rabbits were immunized by two courses of intravenous injections of alum precipitated diphtheric toxoid; the two courses were one or more months apart. One injection after the rest period gave far greater antibody response than could be obtained by the first course of immunization.

During the first course the response of rabbits to different doses of antigens was almost the same but during the second course more antitoxin was formed after the injection of larger amounts of antigens up to 5000 Lf per injection. The rise and fall of antitoxin titers were the sharpest with the largest doses of antigen. The accumulation of stimuli in the group with small doses did not produce as high titers as one single injection of 1000 Lf.

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