

STUDIES ON THE ANTIGENICITY OF DIPHTHERIA TOXOID PART I. TITRATION OF IMMUNIZING POTENCY

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INTRODUCTION

Ramon's opinion to say that an Lf value represents the immunizing potency of toxoid quantitatively, has not been generally accepted yet. On the other hand, existence of some discrepancy has been known between the Lf value and the immunizing potency. But few reports have been published relative to the causes of such discrepancy. The followings are the factors which could be the causes responsible for such discrepancy.

1. The substance specifically combining with the antitoxin in flocculation is consisted of two or more different kinds of specific substances in respect to their immunizing potency.

2. A certain substance other than toxin, toxoid or antitoxin participates in specific flocculation.

3. Aside from specific substances to give antitoxic immunity, there is a certain unspecific substance which does not participate in flocculation but affects the antitoxic immunization (S. Schmidt^{26), 28}).

4. Or there is some question in the method of immunizing potency titration in animals causing the problem in direct comparison of the antigenicity titrated in vitro to those of in vivo.

Among above mentioned factors, there is no proved explanation to support the second, except unspecific flocculation. For the first and the third, though some authors are supporting them (Glenny², S. Schmidt^{26), 28}), Kolle et al⁹), Levine et al¹¹) no evidence proving precisely the entity concerned had yet been reported, except alum, calcium, lanolin or tapioca etc. The fourth cause has been ignored except by a few reports made by Kolle and Prigge etc.^{9), 10), 21), 22}), Greenberg et al³) and Jerne et al⁷).

As this problem is concerned so largely with the production of highly potent toxoid and also with the method of purification that it should be regarded as one of the most important problems.

Introduction of a method for precise quantitative titration is of the

prime importance for deeper understanding of the mechanism in question, nevertheless, in practice the studies concerned with such problems are in performance without making proper evaluation of the titration methods employed. Especially those studies pertaining to the first and the third causes can only be made by having a precise method of titration and proper interpretation of the results obtained.

Various methods of titration which have hitherto been in use were scrutinized in our laboratory as to their nature, their mutual relationship and their titrable limits. Not all of these methods were found to be based upon precise criteria, further, at least a portion of the discrepancy existing between the antigenicity titers measured *in vitro* and those measured *in vivo* was explained.

Several methods for titration have been devised which suit for a routine use in laboratories because of their comparative accuracy and small expense. The problems pertaining to the first and the third causes mentioned in the preceding are under study at present using the newly devised titration methods. Reports thereof will be made shortly.

METHODS

Direct challenge test; Usually, 10 to 12 guinea pigs weighing from 300 to 350 grams were challenged with a definite amount of toxin at the end of the 4th week after the injection of toxoid. For the challenge, the toxin PSSC18 prepared on Pope's medium²⁰) (MLD=0.004 ml, L₊=0.1 ml, L_f=20) was used throughout the whole experiments.

Schick test: The test was made 2 days in advance to the challenge. The toxin used was of some years old after its preparation and the MLD at the time of the experiments was 0.018 ml. For dilution 0.02% gelatine borate buffer (Moloney and Taylor¹⁶) was used. Reactions were read two days after the injection of the test toxin.

Measurement of blood antitoxin titer: Guinea pigs were bled 4 weeks after the toxoid injection unless otherwise described, and the sera obtained were examined of their antitoxin titers by using the intradermal method (Jensen⁶). The antitoxin titers shown in the tables were the figures adjusted with the finding in the controls tested simultaneously on the same rabbit.

"Quantitative Schick test" (Q.S.T.) (Glenny²): Descriptions will be made in the later chapter.

EXPERIMENT

1. Test for tolerance (or "toxin" method⁷)

It is common to give a subcutaneous injection of a definite amount

of toxoid on guinea pigs and to challenge with a definite amount of toxin after certain period of time, then to make comparison of the survival rate among toxoids. But there are some other methods, for instance, to give injections of graded doses of toxoid while using a fixed dose of challenge toxin or to give graded dose of challenge toxin while using a fixed dose of toxoid. These various methods were scrutinized.

Table 1. Correlation of amount of diphtheria toxoid inoculated in terms of Lf and number of animals died in each lot.

Number of animals died in each lot	Lf doses inoculated						Number of lots	
	18	25	38	50	75	100	tested	calculated*
0	2 lots	8 lots	13 lots	46 lots	27 lots	6 lots	102 lots	99 lots
1	1 "	1 "	2 "	9 "	6 "	3 "	22 "	22.7 "
2	1 "	2 "	1 "	1 "	1 "	0 "	6 "	
3	0 "	0 "	0 "	1 "	0 "	0 "	1 "	4.4 "
Total	4 "	11 "	16 "	57 "	34 "	9 "	131 "	

Materials were obtained from the protocols of Diphtheria Toxoid Assay Section, N.I.H. of Japan, performed during the first quarter of 1950, excepting the lots including uncertain death.

Challenge dose=10 MLD

* Calculated under the hypothesis that this distribution of number of animals died is in accord with Poisson distribution.

$$X^2 = 2.79, \quad n=2, \quad P=0.3-0.2.$$

The results of antigenicity test made on various kinds of toxoid manufactured by different plants in this country were summarized in Table 1. In these tests, injecting an amount of toxoid containing an Lf value from 10-15 to 100, the challenge was made with 10 MLD toxin 4 weeks later. The results showed that all survival rates were more than 80% with one exception which was less than 80% in the first and was more than 80% in the second test. In other words, no correlation was observed between the Lf values within a certain limit and its respective survival rate. Levine et al¹¹⁾ have reported that a number of highly purified toxoids of an Lf 13-46 show no difference in their survival rates against the challenge with 10 MLD, but when the Lf drops down below 10, there is a possibility of the survival rate becoming less than 80%. S. Schmidt²⁶⁾ has stated that antigenicity goes suddenly up at an Lf 1-3 and no marked difference of the antigenicity thereafter with the increase of the Lf value.

The results shown in Table 1 demonstrate practically no difference between the actual figures observed and the figures calculated on the basis

that the deaths caused by the challenge with 10 MLD among the groups consisted of 10-12 guinea pigs inoculated with toxoids show the Poisson distribution. Namely, when some percent of a certain guinea pig population is incapable of producing sufficient antitoxin to tolerate the challenge with 10 MLD after the inoculation of toxoid of within a certain Lf value, and if the population is divided into groups of 10-12 animals, the distribution of guinea pigs very poor in the ability of antitoxin production will occur merely by chance. When survival rates are in such a high grade, therefore, as are shown in Table 1, the survival rates have nothing to do any more with the grade of antigenicity, and it can be said that they depend entirely on chance.

Table 2. Antigenicity test of highly purified calcium ppt. toxoid.

Material: Ca-Td-I.

Lf=30, N/Lf=0.00069, Purity=65%.*

Dose inoculated is 1 ml.

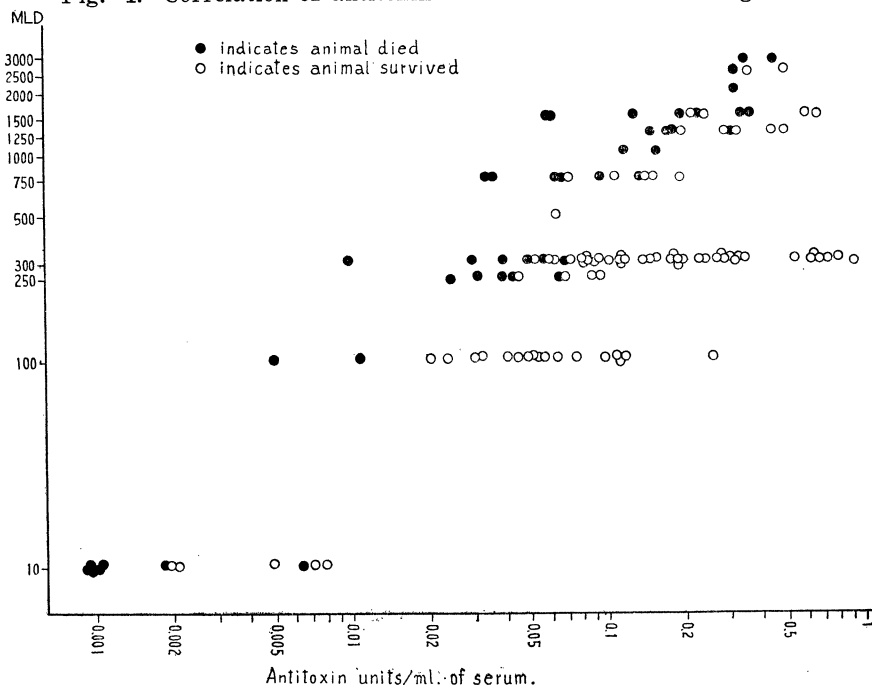
Antitoxin titers were determined 28 days after the inoculation of toxoid.

No. of Guinea pig	Antitoxin units/ml of serum			
	of each guinea pig	arithmetic mean	geometric mean	pooled
264-1	2— 3	3	2.6	3—4
264-2	1— 3			
264-3	5			
265-1	5—10			
265-2	0.001			
265-3	1— 3			
265-4	1— 3			

*The % of purity was calculated assuming that the 100% purity is 0.00045 mgN/Lf.

The following is an example relating to the above problem. The results obtained from the antigenicity test performed on highly purified calcium precipitated toxoid prepared in our laboratory are shown in Table 2. While the antitoxin titers were from 1-3 to 5-10 u/ml in the majority of the animals employed, one animal showed an antitoxin titer less than 1/1000 u/ml. This particular case was rejectable with the level of risk lower than 1% as it was an exceptional titer. Similar instances could often be observed in other experiments too (e.g. Table 3, B group).

Fig. 1. Correlation of antitoxin titer of serum and challenge dose.



Antitoxin titer of the majority of the guinea pigs inoculated with various kinds of toxoid of within a certain Lf value were found to range from 1/20-1/50 to 1/2-1 u/ml; moreover, the logarithms of those antitoxin titers showed a normal distribution about which a detailed discussion will be made in the later chapter. Beside these which showed ordinary antitoxin titers, there were some animals which showed the antitoxin titers of far less than 1/100 u/ml. But the animals which showed intermediate titers were very scarce (Table 7, Fig. 1). It can be said, therefore, that some groups of guinea pigs can produce only but exceptionally low antitoxin titers.

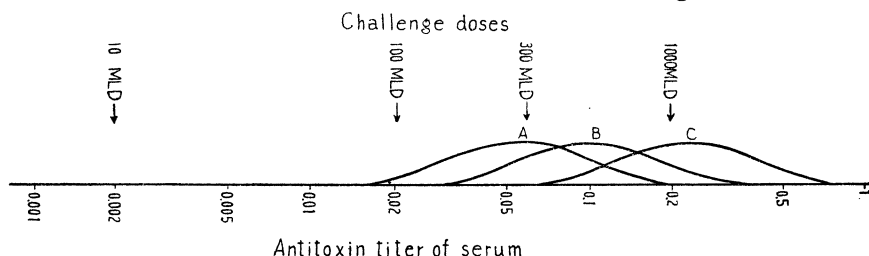
According to S. Schmidt et al²⁷⁾, the limit of the blood antitoxin of guinea pigs died of intoxication after the challenge with 10 MLD of his toxin was approximately below 0.0005-0.002 u/ml, and when the titers were higher than this limit, almost all of the animals tested did survive. Wadsworth et al³⁰⁾ have reported, though smaller in number tested, that the antitoxin level to tolerate the challenge with 100-175 MLD was about 1/100 to 1/50 u/ml which coincided with the results obtained by Kai⁸⁾. Glenn¹⁾ said that the antitoxin level of 0.14 u/ml 8 to 12 weeks after the inoculation of toxin-antitoxin mixture corresponds to a degree of tolerance supporting 400 MLD (6.25 Lo doses of the toxin used). Lately,

Jerne *et al*⁷⁾ have been claiming that this limit is about 0.01 u/ml against about 20 LD75.

Results obtained from the experiments performed on the antitoxin titers of sera and their tolerance to varying challenge doses in our laboratory are summarized in Fig. 1, which shows similar results to those mentioned above. Though the individual variation existing in guinea pigs and presumable experimental errors made it difficult to establish any relationship definitely, an almost rectilinear curve seemed to exist even against considerably large amount of toxin, in other words, the amount of challenge toxin to which guinea pigs could tolerate was roughly predictable, if informed of their antitoxin titers.

In view of the above findings, it can be said that the antitoxin titers of the guinea pigs unable to tolerate the challenge with 10 MLD of our toxin are far too lower than those of the average guinea pigs. Therefore, only those guinea pigs of exceptionally poor in the ability of antitoxin production are dying of intoxication by the challenge of such a small dose of toxin. This view agrees with the opinion mentioned in the first part of this report and even though the challenge dose is increased to 100 MLD or even 300 MLD, since the majority of guinea pigs are having sufficient amount of antitoxin to tolerate such challenge doses, there will be no significant changes (Fig. 2).

Fig. 2. Schematic diagram indicating the relation between the distribution of antitoxin titer of serum and the challenge doses.



If it is to draw a border line somewhere within the distribution from 1/50 to 1 u/ml by increasing the challenge dose still more, the comparison of survival rates will become significant. For instance, provided there are some products showing antitoxin titer distribution such as A, B, and C as are illustrated in Fig. 2, and if they are challenged with 300 MLD or 1000 MLD there will appear some difference in their survival rates. But even in this case, if it is expected to detect a minor difference in their survival rates, it will necessitate quite a number of animals to be used in such test. In fact, as is shown in Table 3, the difference of the survival

rate was significant at the 5% level of risk between C and D group both inoculated with the same amount of the same toxoid but challenged with different dose, the one with 300 MLD and the other with 1500 MLD.

If the intention of the test to know whether the antigenicity of a product is over certain level or not, in other words, if it is of somewhat a qualitative test, such as the potency test of the Minimum Requirements, sufficient indication can be obtained even with a small number of animals.

Table 3. Comparison of immunizing potency of the highly purified and original crude diphtheria toxoid, effect of diluent and dosis of toxoid inoculated.

Materials:

Crude toxoid (indicated as "C" in the table), from which the purified toxoid "A-V" was prepared.

Lf=60, Kf₆₀=48, N/Lf=0.33 mg.

Diluent: physiological saline.

Purified toxoid (indicated as "P" in the table): A-V.

Method of purification: Prec. with ZnCl₂, elut. with Na₂HPO₄, frac. with (NH₄)₂SO₄ and prec. with HCl.

Lf=650, Kf₆₅=35, N/Lf=0.00048 mg, purity=94%.

Diluent: 0.02% gelatine-borate buffer.

Groups	Kind of toxoid	Dosis inoculated		Schick test	Antitoxin units/ml		Challenge test	
		ml	Lf		AM**	GM (number of pigs)**	dose MLD	mortality rate
A	P (dilut. 1:52)	2.5	30	all (-)	0.12	0.10 (12)	300	2/12
B	C („ 1:5)	2.5	30	*	0.14	0.12 (10)	300	2/10
C	P („ 1:16.25)	2.5	100	all (-)	0.27	0.16 (12)	300	1/10
D	P („ 1:6.5)	1	100	all (-)	0.33	0.22 (12)	1500	5/9
E	C („ 1:1.5)	2.5	100	all (-)	0.44	0.32 (11)	300	0/7
F	P (undiluted)	2.5	650	all (-)	0.42	0.33 (11)	300	1/11

*One guinea pig is Schick positive (++) and its antitoxin titer of serum is far less than 1/100 unit/ml. All others (-).

**AM: arithmetic mean.

GM: geometric mean.

In case of immunizing with graded amount of toxoid and then challenging with a fixed amount of toxin, and also if precise quantitative results are expected, sufficient considerations should be made to the amount of the immunizing toxoid, challenge dose and number of animals to be used (Greenberg et al³). For example, group A, C and F in Table 3 showed no marked difference in their survival rates, despite of the inoculation made with 3 to 20 folds different amount of toxoid, against a fixed challenge dose. Prigge et al²² performed an exclusive study on this method, but Jerne et al⁷ discussed this "toxin" method on the experimental and sta-

tistical basis, and decided to use "antitoxin titration method".

On the other hand, there is not any fundamental difference in a method to immunize with fixed amount of toxoid and then to challenge with graded amounts of toxin in order to obtain the maximum toxin dose to which the animals are tolerable. As is shown in Fig. 1, even among guinea pigs of the same antitoxin titer, there is a certain width in their maximum tolerable toxin dose. Therefore, in this case also, careful considerations should be made on the amount of immunizing toxoid, challenge doses and the number of animals to be used in order to obtain reliable results.

2. Test for antitoxin production

The tests in this category, which have generally been employed hitherto, can be classified into two main groups: (1) several animals are bled after the inoculation of toxoid and equal amount of each serum is pooled for the antitoxin titration (Ramon²³), Levine *et al*¹¹) etc.) and (2) individual serum is titrated separately for the calculation of a geometrical mean (S. Schmidt²⁸), Manako¹³) and Levine *et al*¹¹).

In case of (1), when the antitoxin titers of individual animal are comparatively uniform, there will be little danger to be led into a false conclusion, but if the distribution of the titers is spread over a wide range, the results are liable to be misled into a false conclusion by a small number of particularly high titers.

Further, with this method, no information is obtainable as to whether the titers of the individual animal are uniform or showing wide distribution. There is no basis to say, therefore, that the average figure thus obtained represents the parameter of the population to which the animals are belonging. For instance, in our experiments (B and F in Table 4), the antitoxin titer of the pooled serum for the group B was more than 3 times as high as that of the group F, while individual animals showed similar titers in both B and F except one animal in B which showed an exceptionally high titer. It is evident that the average titer of B was increased by this single animal. Schick test made on these two groups showed no difference between them and the survival rate of B was 3/6 and that of F was 5/6 against the challenge with 10 MLD.

Thus, it can be said that the difference in the method of titration and interpretation may induce an entirely reverse conclusion.

Table 4. Immunizing potency of various kinds of purified toxoid.

Groups	Toxoids	Method of purif.	Purity		Dose inoc. Lf	Schick test + -	Antitoxin units/ml				Survival rate
			mgN/Lf	%			pooled serum	AM	GM	each animal	
B	H-orig	crude	0.099	0.45	3.7	3 3	0.05	0.047	0.014	0.005> 0.005> 0.01-0.02 0.05 0.2	3/6
C	H-6B	ZnCl ₂ -prec. + AmS-* frac.	0.0064	7	43	1 4	0.12	0.17	0.062	0.01> 0.1 0.1 0.12 0.5<	5/6
F	H-12N	CaCl ₂ -prec. + AmS-* frac.	0.012	3.8	5	2 3	0.015	0.015	0.01	0.01> 0.01> 0.02 0.02 0.05	5/6
H	H-12Z	CaCl ₂ -prec. + ZnCl ₂ -prec.	0.004	11	5.7	3 3	0.031	0.043	0.01	0.01> 0.01> 0.01> 0.01 0.1 0.14	6/6

*AmS = (NH₄)₂SO₄.

In the calculation of AM (arithmetic mean) and GM (geometric mean), the antitoxin units of less than 1/100 unit/ml were assumed as 0.002 unit.

In order to avoid the effects on the results to be obtained by exceptionally high titers, a geometric mean has been adopted in the place of an arithmetic mean (S. Schmidt, Manako, and Levine et al). According to this method of calculation, the titers of the two groups B and F were approximately the same. This may be a step advanced method, but still it has no basis to support to say that an average thus obtained is representing the population. What we are inquiring after should be the information of the population to which the sample is belonging, but it should not be the average titer of the sample group. Thus, it is considered that the application of statistics is much more convenient for such purpose.

It is said that the statistical analysis of concentration or grade of dilution in serological studies, is generally better handled by employing their logarithms (Masuyama¹⁴). But in our knowledge, the report by Jerne et al⁷) is the only one based on the hypothesis that the logarithms of the antitoxin titers show a normal distribution.

The figures obtained on guinea pigs by S. Schmidt et al²⁷) and in our laboratory, when plotted on probability paper (Fig. 3), became practically a straight line, showing the existence of the relation of $(x-m)/d=t$. Namely, there is a possibility for the use of a precise testing method based on the normal distribution if logarithmic titers are used.

Table 5. Antigenicity test of highly purified toxoids;
Comparison of effect of diluents on antigenicity test.

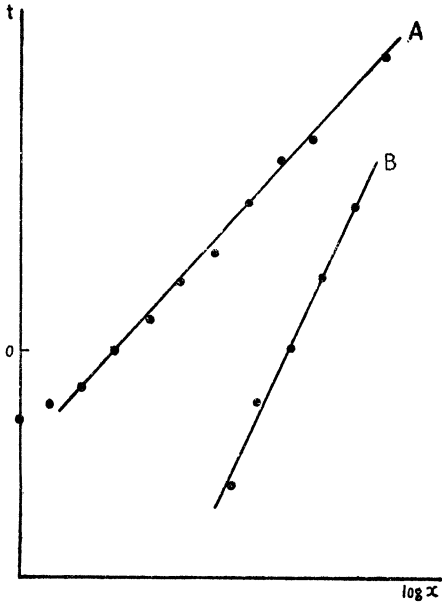
Materials used:

- MPII: Purified toxoid prepared from Martin broth.
- KID: " " " " infusion-free pepton broth.
- K-orig.: Crude toxoid, from which KID was prepared.
- Method of Purification of purified toxoid used:
ZnCl₂+(NH₄)₂SO₄+HCl, details of which are described in the text.
- Lf doses inoculated are 20 in all cases.
- Challenged dose is 10 MLD, and all animals survived in challenge test.

Toxoids	Kind of toxoid	Purity		Diluent	Schick test	Antitoxin units per 1 ml serum		
		mg.N/Lf	%			AM	G M	from each animal
MPII	P*	0.0028	16	S* *	+ 0 - 5	0.092	0.063	0.01-0.02, 0.02-0.05, 0.02-0.05, 0.05-0.1, 0.1-0.2
KID	P	0.00048	96	S	+ 3 - 1	0.01>		0.01>, 0.01>, 0.01>, 0.01-0.02
"	"	"	"	B* *	+ 0 - 4	0.137	0.137	0.05, 0.05-0.1, 0.1-0.2, 0.1-0.2, 0.2-0.5
K-orig.	C*	0.085		no	+ 0 - 5	0.292	0.274	0.1-0.2, 0.2-0.5, 0.2-0.5, 0.2-0.5

- * P: Purified toxoid.
- C: Crude toxoid.
- * * S: Saline.
- B: 0.02% gelatine-borate buffer.

Fig. 3



Curve A: Data from S.Schmidt (1933-1934)
Curve B: Authors' data..

Table 5 is the results of an experiment made on 2 kinds of purified toxoid and one crude toxoid. The antitoxin titers and the results of Schick test in this experiment showed that the titers of highly purified toxoid KID diluted with physiological saline were evident. While, the other three showed relatively similar results, still there was a difference, within 5% level of risk, between the geometric mean of the antitoxin titers of KID diluted with the buffer solution and that of the original crude toxoid. There was not any difference within 5% level of risk between the antitoxin titers of KID diluted with the buffer solution and that of MP11, a product of lower purity, diluted with physiological saline.

Among the four different test materials employed in this experiment, KID diluted with saline evidently showed the decrease of the Lf at the end of one week's incubation at 37°C, but no change of the Lf nor Kf was detectable with the other three exceeding the limit of experimental errors (Murata et al¹⁷). There was no difference in the survival rates among the 4 test materials. Namely, by using the statistical procedure in handling the antitoxin titers, conclusions were obtainable within 5% level of risk even by such a small number of animals as in the present experiments. The same conclusion to say that there is a slight difference between the immunizing potency of highly purified toxoid diluted with gelatine-buffer and that of the original crude toxoid, are obtainable from the results of the experiments of Table 3 and 6. It can be said, therefore, that the conclusion made in the above is reproducible.

Table 6. Correlation of antitoxin unit of serum and results of "Quantitative Schick test" (Glenny).

Materials:-

Purified toxoid: KV, Lf=275, Kf₅₅=60, N/Lf=0.00069 mg.

Crude toxoid from which KV was prepared: Lf=27.

Schick test toxin: PSSC-18, MLD=0.004, L+=0.1 ml.

Days after toxoid inocul.	Purified toxoid (Lf=27)		Crude toxoid (Lf=27)	
	Quantitative Schick test *	Antitoxin unit of serum	Quantitative Schick test *	Antitoxin unit of serum
26	5 MLD	0.5	10 MLD	0.36
	3	0.08	10	0.3
	1.5	0.076	3	0.15
	1.5	0.036	3	0.034
	1/10-1/20	0.015	1.5	0.07
	1/20-1/50	0.1		
33	5	0.16	25	0.3
	5	0.08	10	0.7
	5	0.08	10	0.35
	1	0.06	10	0.3
	1	0.035	10	0.15
	1/20	0.015	(2)?	0.16

46	2	0.16	2	0.18
	1	0.12	1	0.3
	1/2	0.15	1	0.16
	1/2	0.065	1/2	0.13
	1/5	0.03		
68	1/5	0.3	2	0.36
	1/5	0.26	1/2	0.34
	1/50	0.03	1/2	0.33
	1/100	0.02	1/5	0.27
			1/10	0.13

* Maximum toxin doses resulting negative reaction.

Within 5% level of risk, if the difference of the mean titers is about twice fold with variance of approximately 0.1 (in logarithm), satisfactory results are obtainable with groups each composed of about 12 animals. Even when 1% level of risk is taken, it will be sufficient if a group is consisted of 20 animals. When the difference of the mean titers is 4 times, animals from 6 to 8 in each group will be sufficient. When the difference is so small as 50%, more than 40 animals are necessary in one group even within 5% level of risk, indicating the difficulty of performing such experiment.

3. "Quantitative Schick test"

In order to establish a certain simple method for measuring immunizing potency of toxoid, the following method was examined as to its practicability. This method was accepted by Glenny *et al*²⁾ as an alternative tolerance test. A series of intracutaneous injections are to be made with graded doses of a certain toxin after bleeding for antitoxin titration, in order to obtain the minimum toxin dose which will produce the minimum toxin reaction or the maximum toxin dose which will not produce any positive reaction. According to Glenny, this method will be called as a "Quantitative Schick test" in this paper. The toxin used in the present test was PSSC18.

Table 7 shows the relation existing between the antitoxin titers of the blood samples from actively immunized guinea pigs and their tolerance against intracutaneous injection of toxin observed in several separate experiments. A correlation, though not fully satisfactory, is observable between them. This method, therefore, can be regarded as a relatively simple method for quantitative titration of the immunizing potency. For instance, the data of the 33rd day in Table 6, showed a difference between highly purified toxoid and crude toxoid, within 5% level of risk, when tested with "Q.S.T.", but it showed a difference, within 1% level, when examined on the basis of the blood antitoxin titers.

But as is evident in Table 6, the above mentioned relation will largely be affected by the interval spent between inoculation and titration, the detailed data of which will be reported in a separate paper. When "Q.S.T." is to be adopted, therefore, either the period of observation must be uniform or the test must be performed under the same conditions.

Table 7. Correlation of antitoxin titers of serums and results of "Quantitative Schick test (Glenny)"
(Summarized table of several independent experiments)

MLD *	Antitoxin unit per ml. of serum														Total
	1-1/2	1.2-1.5	1.5-1.10	1/10-1/20	1/20-1.50	1.50-1.100	1/100-1/200	1/200-1.500	1.500-1.1000	1.1000-1/2000	1/2000-1/4000	1/4000	Total		
25	1	2											3		
10	1	5	4	2									12		
5	1	1	2	5									9		
2		8	9	2	4	1							24		
1		3	4	4	2	1							14		
1/2				3	3	1							7		
1/5						1							1		
1/10							1						1		
1/20								1					0		
1/50													0		
1/100								1	1	1			3		
1/200									1	1	2		3		
1/500									1	2	2		5		
1/1000											1	1	2		
1/2000												3	3		
1/4000												1	1		
Total	3	19	19	16	9	4	0	2	2	4	5	5	88		

* Maximum toxin dose resulting negative reaction.

The tests and titrations were performed from 2 to 4 weeks after the inoculation of toxoid.

Similar situation may be encountered in an ordinary tolerance test. Unless this possibility is denied definitely, it should be reasonable to say that only those data obtained from the experiments performed at the same interval after the injection of toxoid are comparable in ordinary tolerance tests.

SUMMARY

1. Method based on the survival rate of guinea pigs inoculated with an amount of toxoid and then challenged with a fixed amount of toxin after a certain period of time is a method which is only capable of indicating the grade of antigenicity of the toxoid tested. In other words, it is merely a method for qualitative measurement. But here is a possibility that even this method can serve as a method for quantitative measurement if a large number of animals are employed and if an adequate amount of toxoid for the first stimulus and an adequate amount of toxin for challenge are used.

Other method to use various doses of toxoid as well as various amounts of toxin are of not so much fundamental difference from the above mentioned method unless sufficient number of animals are used.

Studies of this point have almost been neglected except by Kolle and Prigge *et al*^{9), 10), 21), 22)} and Greenberg *et al*³⁾. It is considered, therefore, that a part of the discrepancy found in the titers of the antigenicity measured *in vitro* and *in vivo*, has been caused by the imperfect understanding of the nature of the method employed and improper interpretation of the results obtained thereof.

2. As to the methods for titration of antitoxin, the method to compare the mean averages of the groups employed is liable to lead into a false conclusion, especially when there exists a marked difference in the titers of individual guinea pig. It is considered, out of our experiments, that a method to apply statistical procedure to the antitoxin titers obtained from individual animal, is the method which enables relatively precise quantitative measurement with comparatively small expense. This view agrees with the opinion of Jerne and Maaløe⁷⁾.

3. The quantitative Schick test (Glenny) was also examined and found to be not sufficiently precise, but it may be of value for some other purposes.

4. Experiments presented in this reports showed that highly purified toxoids diluted with saline or gelatine-buffer could produce in animals the antitoxic immunity of evidently different in titers from the original crude toxoids of having the same Lf values.

The authors are intending to make reports as to whether this result

is dealing with the entity of toxoid or the diluent used, or else if there exist some toxoid of entirely different in its immunizing potency.

Hitherto, a number of reports have been made on the antigenicity of purified toxoid (Glenny et al²⁾, S.Schmidt²⁴⁾, ²⁵⁾, ²⁸⁾, Jensen⁵⁾, Theorell et al²⁹⁾, Jakowkiewicz et al⁴⁾, Mino¹⁵⁾, Manako¹²⁾, ¹³⁾, Ramon et al²³⁾, Levine et al¹¹⁾, Parfentiev et al¹⁹⁾ and Pappenheimer et al¹⁸⁾), but only 2 or 3 of them were using the toxoid products of the purity comparable to those used in our experiments (Jakowkiewicz et al, Pappenheimer et al, Levine et al, etc.) The conclusions made in these reports, further, are not necessarily acceptable, because of the reasons for which considerations have been made in this paper.

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