TETANUS ANTITOXIN TITRATION BY HAEMAGGLUTINATION AT A LOW LEVEL OF TEST

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(With 2 Figures in the Text)

It has been shown in an earlier paper (Fulthorpe, 1957) that tetanus antitoxins can be titrated fairly accurately by a haemagglutination inhibition method at the L+ level of test; results at the 0·01 L+ level were, however, rather variable. Antitoxin values calculated from direct agglutinin titre tended to be widely discrepant from the *in vivo* value, particularly those of sera from horses, hyperimmunized over a long period.

Landy, Trapani, Formal & Klugler (1955) suggested that diphtheria antitoxin titrations could be carried out satisfactorily by the direct method. An examination of their figures shows, however, that although there is a satisfactory general agreement between the haemagglutination titres and the *in vivo* antitoxin titres, the individual discrepancies are in some cases large. The haemagglutination titres of sera with the same *in vivo* antitoxin values may differ by a factor of as much as 30, and sera with the same haemagglutination titre may have *in vivo* antitoxin values which differ by a factor of 100. Similar results were obtained by Scheibel (1956) with diphtheria antitoxins of different avidity.

The present paper deals mainly with the problem of testing small quantities of low-value tetanus antitoxin by haemagglutination methods in the light of these observations. The technique of testing by haemagglutination inhibition at a low level of test was first investigated with horse sera before undertaking the examination of human sera from individuals immunized with ordinary tetanus formol-toxoid.

MATERIALS AND METHODS

Erythrocytes

Sheep cells were used exclusively. Washed cells were sensitized by Stavitsky's method (1954) and preserved with 20 % buffered formalin (Fulthorpe, 1957). Cell suspensions used for inhibition tests at the L+ or L_A level of test, or for direct agglutination tests were stabilized with 1 % normal horse serum, but those cells used for low-level inhibition tests ($L_A/100$) were stabilized with 2 % nutrient broth.

Controls

All sera were tested for anti-sheep haemagglutinins and anti-horse precipitins to exclude the possibility of non-specific effects. The usual diluent controls were also included in the test.

Antigen diluents

In low-level test ($L_A/100$) it was necessary to add a stabilizer to the tetanus toxin to prevent deterioration. Physiological saline containing only 5 % of nutrient broth (papain-digest of horse muscle, pH 7·0–7·2) was found satisfactory for this.

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Performance of direct agglutination test

Sera were diluted in 0·05 M sodium borate succinic acid buffered saline at pH 7·2; no stabilizer was necessary in the diluent as formalin-preserved cells were used. Dilutions of 1/2, 1/5, 1/10, 1/20 and so on, were dispensed in $\frac{3}{8} \times 3$ in. round-bottomed tubes in 1·0 ml. amounts and 0·1 ml. of 1·25 % suspension of cells added to each tube. The tubes were inverted to mix the contents and left overnight at room temperature.

Performance of haemagglutination inhibition tests

A test dose of toxin equivalent to one unit of standard antitoxin by haemagglutination (L_A dose) or to 0·01 unit ($L_A/100$) was dispensed in a number of tubes, and volumes of serum differing from each other by 20 % (L_A level of test) or 100 % ($L_A/100$ level of test), over a convenient range added. The tubes were inverted to mix, allowed to stand for 1 hr. at room temperature and the sensitized cells added. The arithmetic mean of the values obtained in tests on one serum was taken as the antitoxin value of the serum.

Reading haemagglutination tests

The tests were read by observing the pattern of cells on the bottom of the tubes, a smooth carpet of cells with a faint ring of unagglutinated cells at the periphery (+ + ±) was found to be the most consistent end-point. Lower degrees of agglutination, + + to ±, were found to be less reproducible from day to day.

Performance of in vivo *tests*

In vivo tests at the L+ and one-hundredth L+ level of test were carried out by the usual mouse subcutaneous method.

RESULTS

Determination of test doses at different levels of test

The volume of toxin or toxoid required to inhibit multiples or fractions of one unit of the working standard antitoxin was found to be disproportionate to that required to inhibit one unit of antitoxin (Table 1) in these haemagglutination

Table 1. *The relative increase in test dose of agglutinin inhibiting toxins or toxoids as the level of test is reduced in haemagglutination inhibition tests*

Quantity of standard antitoxin used (units)	Volume of inhibiting toxin or toxoid required to produce standard end-point (ml.)			
	Toxoids		Toxin	
	TD 341 C	MW 1470	AW 2302	Level of test
100	—	1·07	0·8	100 L_A
10	0·135	0·10	0·08	10 L_A
1	0·015	0·012	0·009	L_A
0·1	0·00165	0·00165	0·0015	$L_A/10$
0·01	0·00025	0·00032	0·0003	$L_A/100$
0·001	0·00007	0·00011	0·00006	$L_A/1000$

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inhibition tests. As the concentration of antitoxin was reduced the quantity of inhibiting agent had to be relatively increased in order to obtain the accepted end-point. The relative increase in test dose was greatest at the lowest levels of test ($L_A/10$–$L_A/1000$).

Assay of horse sera for tetanus antitoxin

A number of sera were tested at the L_A and $L_A/100$ levels of test, and also *in vivo* at the L+ and 0·01 L+ levels of test. They were tested at the 0·01 L+ (i.e. one-hundred of the dose ≡ 1 unit) rather than the L+/100 level of test (i.e. dose ≡ 0·01 unit) in order to estimate their avidity (Fulthorpe, 1957). The mean direct haemagglutination titres were also found and antitoxin values from these results calculated by reference to the *in vivo* values of the working standard.

Table 2. *Assay of tetanus antitoxin in horse sera by haemagglutination inhibition at different levels of tests compared with the direct agglutination test and the* in vivo *values*

		Antitoxin values by haemagglutination inhibition (units/ml.) (level of test)		Calculated antitoxin values from direct agglutinin titres (units/ml.)	Antitoxin values by *in vivo* titration (units/ml.) (level of test)	
	Sera	L_A	$L_A/100$		L+	L+0·01
A	8480*	1700	1700	1700	1700	1800†
	9018	360	450	400	360	345
	8348	400	400	400	380	400
	9096	220	250	220	220	240
	65	1240	1250	1275	1200	1100
B	7897	1020	900	100	1350	1350
	7897/B	437	250	50	425	475
	8740	1320	1000	300	1600	1500
	7602	1200	600	128	1100	1100
	8737	167	125	64	180	200
	LX381/A	48	40	15	46	40
C	300	120	113	128	80	42
	302	165	104	34	100	42
	312	97	50	26	42	18

* Working standard.

† The *in vivo* values of these sera were tested at the 0·01 L+ level of test rather than the L+/100 in order to calculate the dilution ratios (avidity).

It can be seen from Table 2 that the sera in group A, which includes the working standard, have antitoxin values in good agreement by all methods. The sera in group B give antitoxin values by calculation from the direct haemagglutination titres which are in general too low, occasionally unrealistically low. Some of the values estimated at the $L_A/100$ level of test are also low, but do not show such gross discrepancies as those in the direct test. It has been pointed out before (Fulthorpe, 1957) that these discrepancies in the direct haemagglutination procedure are unlikely to be due to the avidity of the antitoxins; further evidence of this will be seen from the last two columns in Table 2. The three sera in group C were non-avid, but the haemagglutination titres at the L_A and $L_A/100$ levels of

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test differ less than do the *in vivo* values at the L+ and 0·01 L+ levels: this suggests that poor avidity does not have much effect on the antitoxin values at different levels of test by haemagglutination inhibition.

The observations on horse sera suggested that the inhibition test at the $L_A/100$ level might be more suitable than the direct agglutination test for testing human sera.

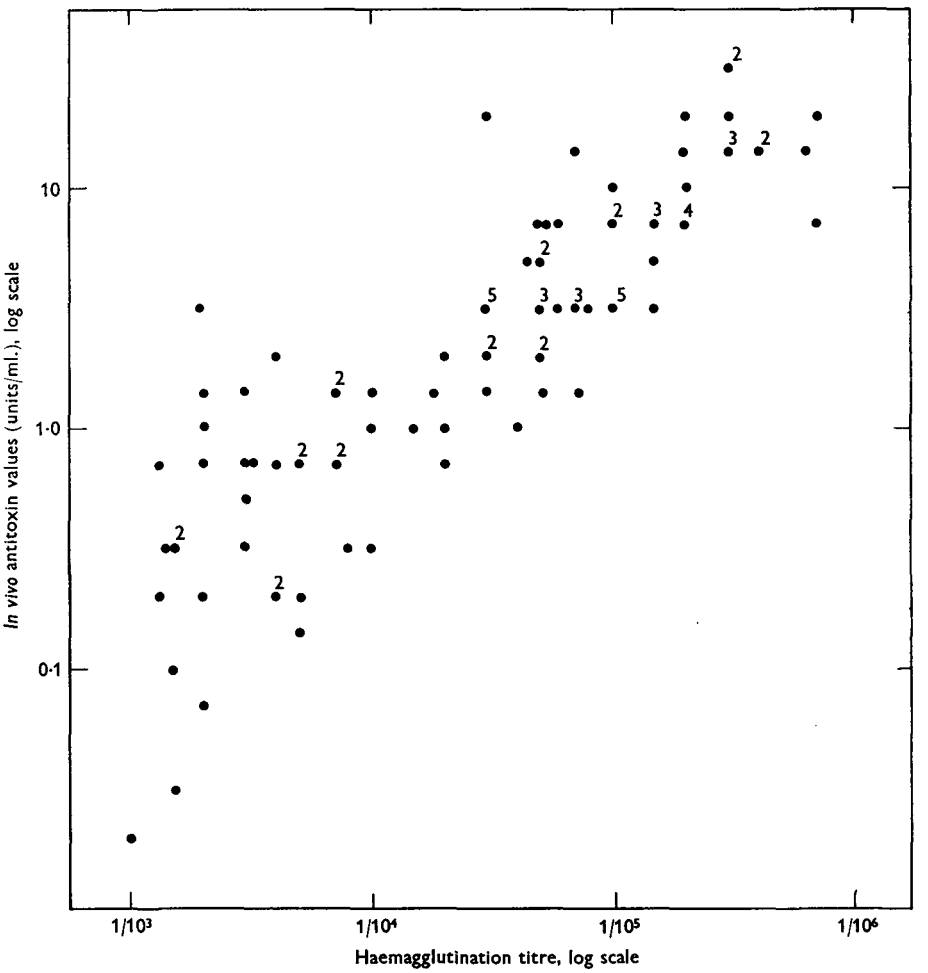

Fig. 1. Haemagglutination titres (direct agglutination test) of 100 human sera plotted against the *in vivo* antitoxin values.

Direct agglutination tests on human sera

One hundred sera were collected from members of the staff who had been immunized against tetanus. The immunization history of these people varied a great deal, some having had regular boosting doses of toxoid for 15 years or more, while others had only had two doses of toxoid at a 6 weeks interval. Sera from individuals who have received only two doses of toxoid may be non-avid.

The sera were tested first by direct agglutination and the mean haemagglutination titres plotted over a log scale against the *in vivo* antitoxin values (Fig. 1). The

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general correlation is undoubted but the individual discrepancies are very great. Many of the sera were tested in the same way using cells sensitized with toxoids of widely different purity (738 Lf/mg. P.N. to 2500 Lf/mg. P.N.), and it was quite evident that the differences in agglutinating capacity of sera with the same *in vivo* antitoxin content was not due to the purity of the cell-sensitizing toxoids. Since the poor agglutinating capacity of some horse sera appeared to be related to immunization history (Fulthorpe, 1957), this aspect of the human sera was investigated. No correlation was found, however, between agglutinating capacity per *in vivo* antitoxin unit and the immunization records.

A number of immune guinea-pig and mouse sera were also tested by direct agglutination; the immunization histories of the animals in these groups were the same. It was found that when the haemagglutination titres were plotted against the *in vivo* antitoxin values, similar discrepancies occurred as had been observed with human sera, so that it would appear that high and low agglutinating capacity per *in vivo* unit of antitoxin exists in sera from these animals also.

Assay of human sera for tetanus antitoxin at the $L_A/100$ *level of test*

The human sera previously tested by direct haemagglutination (Fig. 1) were retested by haemagglutination inhibition at the $L_A/100$ level of test and the *in vivo* antitoxin values were plotted against the *in vitro* values by this method (Fig. 2).

The agreement between *in vivo* and *in vitro* values in this figure is quite good. The haemagglutination titres of sera of the same *in vivo* value in no case differ by more than a factor of 7, nor do the *in vivo* antitoxin titres of sera of the same haemagglutination titre.

Comparison of geometric means of groups of human sera tested by different methods

The hundred human sera tested previously were divided at random into six groups. The geometric means of the antitoxin values obtained by *in vivo* titration and by haemagglutination inhibition at the $L_A/100$ level of test were calculated, as was the geometric mean haemagglutination titre by the direct test, on each group.

In Table 3 a comparison of these mean values are shown. The figures in brackets are corrected results obtained by reference to the mean value found in the *in vivo* tests on Group I.

DISCUSSION

The direct haemagglutination procedure for assaying antitoxin is convenient and economical; it is therefore likely to be popular. But these advantages must be balanced against the limitations of the test. It has the advantage of sensitivity, and it can be seen from Table 3 that a little over 1·0 unit of antixon can be diluted 1/20,000 and still have detectable agglutinin. This is a far smaller quantity of tetanus antitoxin than can be detected by an *in vivo* test. Haemagglutination tests can be set up quickly and read the following day, whereas *in vivo* tests take 4 days to complete. The quantity of serum required for a test can be limited to exceedingly

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small amounts, and in consequence, many tests can be done on any one sample. When the method is used for the comparison of geometric means of groups of sera it is evident (Table 3) that the method can have a wide application. However, when the discrepancies seen in Fig. 1 are considered, in addition to evidence in papers by Scheibel (1956) and Landy *et al.* (1955) with diphtheria antitoxin systems, it would appear that great caution should be observed in assessing the antitoxin

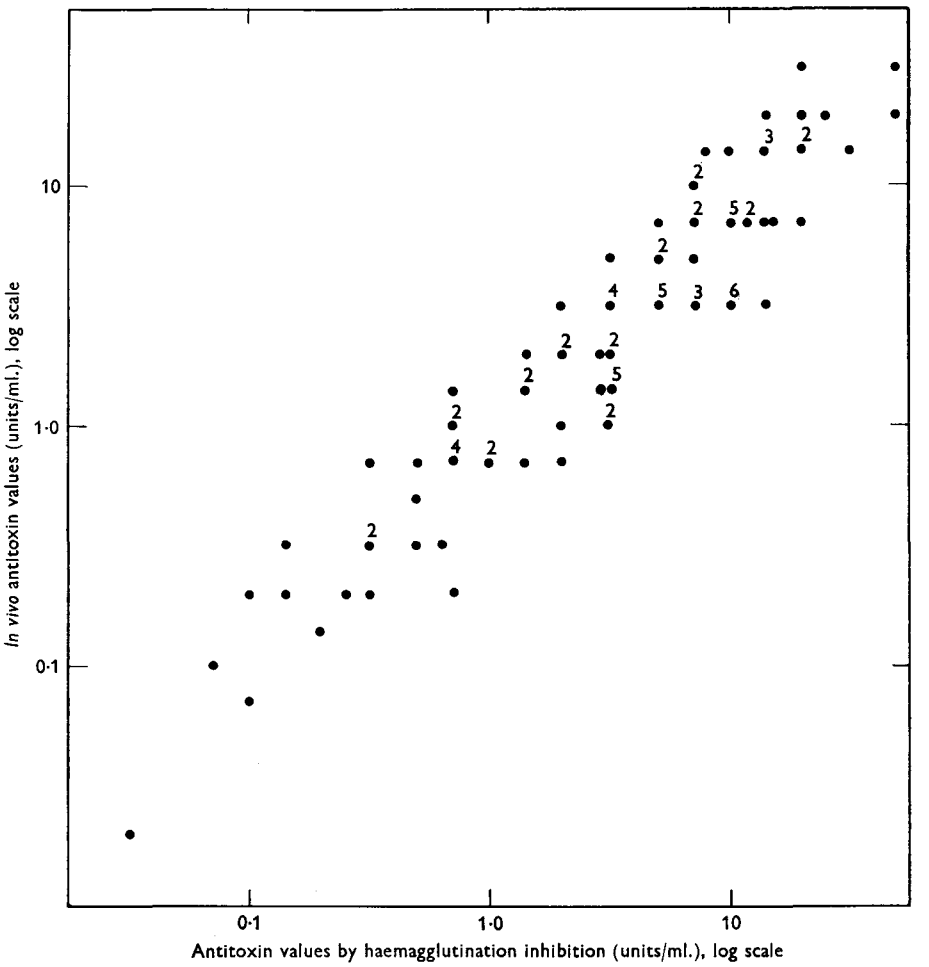

Fig. 2. The *in vivo* antitoxin values of ninety-eight of the sera shown in Fig. 1 plotted against the antitoxin values obtained by haemagglutination inhibition at the $L_A/100$ level of test.

values of *individual* sera by this method. Scheibel (1956) points out that the dose-response curves for direct haemagglutination tests with avid and non-avid antitoxin show a greater degree of parallelism than do similar curves for *in vivo* assay. This does not, however, dispose of the disadvantage of a method which gives such a wide variation in individual haemagglutination titres with sera of the same *in vivo* antitoxin content. These differences of agglutinating capacity have also been observed in direct agglutination tests on avid hyperimmune horse sera, and on

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Table 3. *Comparison of the geometric means of the titres of six groups of human-tetanus antitoxins estimated by different methods*

Groups of sera	Antitoxin values by *in vivo* titration (units/ml.)	Direct agglutination test: Haemagglutinin titres	Direct agglutination test: Calculated antitoxin values (units/ml.)	Haemagglutination inhibition test at $L_A/100$ (units/ml.)
I	1·3	1/20,000	(1·3)	1·7 (1·3)
II	1·3	1/28,000	(1·8)	1·7 (1·3)
III	1·4	1/23,000	(1·5)	1·7 (1·3)
IV	2·2	1/30,000	(2·0)	3·6 (2·4)
V	3·6	1/40,000	(2·6)	4·8 (3·1)
VI	4·7	1/63,000	(4·1)	7·6 (5·8)

The figures in brackets in column 4 represent the geometric means of the haemagglutinin titres converted to antitoxin units by reference to the *in vivo* mean value of Group I. The geometric mean values by haemagglutination inhibition in column 5 have been corrected in the same way. The mean values in column 5 are higher than the *in vivo* values because the test dose ($L_A/100$) was estimated against an avid antitoxin, and many of the human sera, being non-avid, would give higher values by this method. (See Table 2, cols. 3 and 6, group C.)

groups of sera from guinea-pigs which had the same immunization history, and it therefore seems improbable that the discrepancies are due to gross differences in avidity of the sera. It has been suggested that antitoxins of poor agglutinating capacity may consist of polymerized molecules (Fulthorpe, 1957). Scheibel (1956) has suggested that high avidity molecules have an agglutinin inhibiting effect.

The haemagglutination inhibition test at the $L_A/100$ level of test has the advantage of reducing the individual discrepancies of the direct test, and it would appear to be more suitable for testing individual sera. It is however less sensitive and requires a greater quantity of serum for testing.

SUMMARY

An investigation has been made of methods of estimating the antitoxin content of small samples of human sera by haemagglutination procedures.

The direct agglutination test appears to be satisfactory when dealing with groups of sera, but was unreliable for testing individual sera.

The discrepancies observed in the direct agglutination test were greatly reduced by adopting an agglutination inhibition test at the $L_A/100$ level of test.

The difference in agglutinating capacity of both avid and non-avid sera per *in vivo* unit of antitoxin did not appear to be related to avidity.

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