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Factors Influencing Haemagglutination Tests with Tetanus Antitoxin

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Summary. The agglutinin titres of tetanus antitoxins tested with preserved sheep cells sensitized with tetanus toxoid have been found to be inversely proportional to the cell concentration used.

The agglutinin titres per international unit of antitoxin were widely different among various electrophoretically separated globulin fractions of tetanus antitoxin. There were smaller differences between the agglutinating capacity of γ-globulin fractions of sera from different horses.

With haemagglutination inhibition tests there was always a relative increase in test dose between the L_A and $L_A/100$ level of test. This relative increase was greatest where the sensitivity of the cell suspensions was high.

Increased suspension sensitivity may be the result of treatment of cells with greater quantities of sensitizing antigen or of reduction in the concentration of sensitized cells in the suspension.

A greater volume of serum was required to give a standard end point, when mixed with a test dose (L_A) of toxin, if the mixtures were allowed to stand for increasing periods of time. These observations have been discussed.

INTRODUCTION

Work has already been carried out on the titration of tetanus antitoxin with sheep cells sensitized with tetanus toxoid and preserved with formalin (Fulthorpe, 1957, 1958). In the course of that work a number of observations were made which merited further examination.

The results of direct agglutination tests (agglutinin titre) were found to be only very roughly correlated with those of the antitoxin content of sera (international units/ml.) and there were some large discrepancies. In the case of hyperimmune horse sera they seemed to be related to the length and quality of immunization. Similar discrepancies, however, were found with human sera, where there was no correlation with immunization history.

In addition it has been shown that the sensitivity of a cell suspension could be influenced by the amount of sensitizing antigen used (Fulthorpe, 1957), and Scheibel (1956) demonstrated with diphtheria toxoid-sensitized cells that dilution of the sensitized suspension caused a progressive increase in direct agglutinin titre.

The sensitivity of a suspension has also been found to influence the end point in haemagglutination inhibition tests, with the result that agglutination could be produced by toxin-antitoxin mixtures of different degrees of neutralization as measured in mice.

These and several other points have been further investigated and are the subject of this paper.

MATERIALS AND METHODS

ERYTHROCYTES

Sheep cells were used exclusively. Washed cells were sensitized by Stavitsky's method (1954) and preserved with 20 per cent formalin (Fulthorpe 1957). Cell suspensions were stabilized either with 1 per cent normal horse serum or 2 per cent nutrient broth. Suspensions stabilized with nutrient broth were used for tests at low levels of test ($L_A/100$) where agglutination patterns tend to collapse if serum is used as a stabilizing agent.

It has been found advisable to standardize all suspensions by haemocytometer counts after preparation. Comparison of the concentration of suspensions by opacity methods (Grey Wedge photometer) and by direct estimations in a counting chamber showed considerable discrepancies. This may possibly be due to leakage of haemoglobulin from formalin preserved cells.

DILUENTS

Tests were set up in 0·05M sodium borate-succinic acid buffered saline at *p*H 7·2. Where toxin-antitoxin mixtures were used in high dilution, 5 per cent nutrient broth was used in the diluent to prevent deterioration of the diluted toxin.

DIRECT AGGLUTINATION TESTS

Sera were diluted 1/2, 1/5, 1/10, 1/20 and so on, and dispensed in $\frac{3}{8}\times 3$ in. round-bottomed tubes in 1·0 ml. amounts; 0·1 ml. of 1·25 per cent suspension of sensitized cells was added to each tube. The tubes were inverted to mix the contents and left overnight at room temperature.

HAEMAGGLUTINATION INHIBITION TESTS

In determination of test doses, convenient volumes of toxin or toxoid differing by 20 per cent were dispensed in a series of tubes and a fixed volume of serum containing 1·0, 0·1 or 0·01 unit of antitoxin (according to the level of test) added to each tube, the contents were mixed and 0·1 ml. sensitized cells added after one hour at room temperature.

In antitoxin titrations a test dose of toxin equivalent to one unit of standard antitoxin by haemagglutination (L_A dose) or to 0·1 or 0·01 unit ($L_A/10$ or $L_A/100$) was dispensed in a number of tubes, and volumes of serum added differing from each other by 20 per cent over a convenient range. The tubes were inverted to mix, allowed to stand 1 hour at room temperature, and 0·1 ml. of sensitized cells added, mixed and allowed to stand overnight.

READING HAEMAGGLUTINATION TESTS

Tests were read by observing the pattern of cells on the bottom of the tubes. The most consistent end point was found to be a smooth carpet of cells with a faint ring at the periphery ($++\pm$).

IN VIVO TESTS

Antitoxin estimations and test dose estimations were made by the usual mouse subcutaneous method, at the L+ level of test.

RESULTS

FACTORS INFLUENCING DIRECT AGGLUTINATION TESTS

(a) Cell Concentration and Sensitivity

A series of direct agglutination tests were carried out with avid antitoxins using tetanus toxoid-sensitized cell suspensions in different concentrations. A comparison of the agglutinin titres obtained with the different concentrations of cells showed that as the cell concentration was reduced, the agglutinin titre increased. Fig. 1 shows that there was a linear log. relationship between cell concentration and agglutinin titre, and when the cell concentration was halved the agglutinin titre was doubled.

The sensitivity of indicator suspensions has been shown to vary if different quantities of sensitizing antigen are used (Fulthorpe 1957). Some degree of variation of sensitivity has been found when the same quantity of antigen (Lf/ml.) prepared by different methods was used. Moreover, unfortunately, variation in sensitivity has also been observed in batches of cells prepared in the same way at different times. The direct agglutinin titre was naturally affected by such variations.

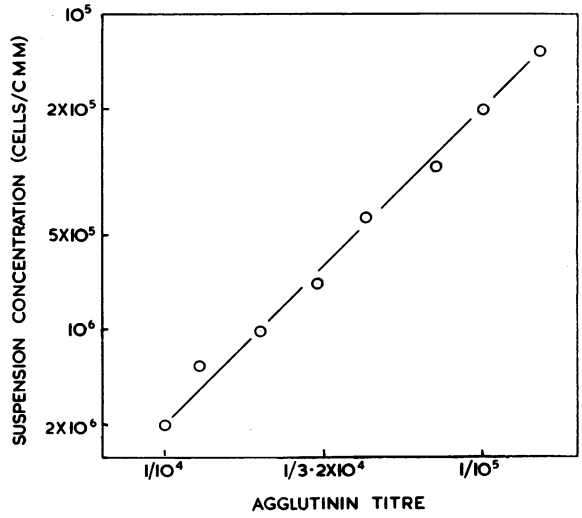

FIG. 1. Log relationship between the concentration of the agglutinable suspension and agglutinin titre.

(b) Qualitative Differences between Sera

A group of horse sera were fractionated by paper strip electrophoresis, the protein eluted and the antitoxin content of the fractions estimated by *in vivo* titration in mice. The direct agglutinin titres of the fractions were measured and the agglutinin titre per international unit of antitoxin calculated. It was found that the agglutinating capacity of the α-globulin fraction was very low, of the β-fraction intermediate and the γ-fraction much higher. Moreover the γ-antitoxin from horses after only one course of hyper-immunization had a greater agglutinating capacity per international unit than had the γ-fractions from horses after many courses. (Table 1.) This effect was only slightly noticeable with the β-fractions and there was little difference with the α-fractions.

The use of cells sensitized with toxoids of different purity in estimating the agglutinin

titre of different fractions did not significantly affect the relative values. It is therefore unlikely that the difference between fractions could be caused by a change in the distribution of non-specific antibodies.

TABLE 1
AGGLUTININ TITRE PER INTERNATIONAL UNIT OF TETANUS ANTITOXIN IN ELECTROPHORETICALLY SEPARATED GLOBULIN FRACTIONS

Sera	*Globulin fractions*			*Full courses of immunization*
	α	β	γ	
1333	1/300	1/2500	1/30,000	1
1291	1/1000	1/2000	1/25,000	1
929	—	1/2000	1/20,000	1
9350	1/200	1/650	1/3200	32
9154	1/300	1/1500	1/5000	36
8850	1/500	1/1400	1/4000	41
8657	1/300	1/1500	1/2500	55

FACTORS INFLUENCING HAEMAGGLUTINATION INHIBITION TESTS

(*a*) *Effect of Suspension Sensitivity on Test Dose Estimations*

When test dose estimations (L_A) were made with suspensions of widely different sensitivity, agglutination occurred with mixtures of toxins and antitoxin which were toxic to mice (Table 2). The degree of underneutralization of the mixtures capable of agglutination varied according to the sensitivity of the suspensions used.

Test dose estimations at different levels of test (L_A, $L_A/10$ and $L_A/100$) were made using suspensions of different sensitivity with the same cell concentration estimated by haemocytometer (Table 3). The relative increase in test dose from L_A to $L_A/100$ was greatest with suspensions of high sensitivity. The increase in test dose with increasing suspension sensitivity was greater at the $L_A/100$ than at the L_A or $L_A/10$ levels of test.

TABLE 2
TEST DOSE ESTIMATION (L+) BY IN VIVO TITRATIONS COMPARED WITH THE RESULTS OF TEST DOSE ESTIMATIONS BY HAEMAGGLUTINATION (L_A) USING TETANUS TOXOID SENSITISED SUSPENSIONS OF DIFFERENT SENSITIVITY

Volume (ml.) of toxin mixed with one unit of standard antitoxin	*In vivo mouse test**	*In vitro haemagglutination inhibition tests with different sensitized suspensions*			
		J2	191	168	155
0·008	—	+++			
0·010	—	+++	+++		
0·012	T	++±	+++	+++	
0·015	3	—	++±	+++	+++
0·018	2	—	—	++±	+++
0·022	1		—	—	+++
0·027	1			—	—
0·033					—
Sensitivity of suspensions, *i.e.* direct agglutinin titre with standard		1/10^6	1/3×10^6	1/5×10^6	1/15×10^6

* Results of mouse test. T—marked symptoms of tetanus on the fourth day. 1, 2, 3 represent day of death of animal with specific symptoms of tetanus.

(b) Effect of Different Cell Concentrations

A reduction in the concentration of sensitized cells also increased the sensitivity of agglutination inhibition tests.

The test doses of toxin necessary to inhibit agglutination were determined using sensitized cells in three different concentrations and at three levels of test. These determinations were made using a batch of toxin before and after treatment with formaldehyde for conversion into toxoid. Table 4 shows that the relative increase in test dose between the L_A and $L_A/100$ was greatest with the lowest cell concentration, and the increase in test dose with reduction in cell concentration was greatest at the $L_A/100$ level of test. At a low level of test with low cell concentration, inhibition of agglutination was not so effective with toxoid as it was with unmodified toxin.

TABLE 3

RELATIVE QUANTITIES OF TETANUS TOXIN REQUIRED TO INHIBIT AGGLUTINATION OF TETANUS TOXOID SENSITIZED SHEEP CELLS OF DIFFERENT SENSITIVITY BUT THE SAME CELL CONCENTRATION BY 1·0, 0·1 AND 0·01 UNITS OF STANDARD ANTITOXIN

Quantity of antitoxin used	*Relative volumes of antigen required*			*Level of test*
	A	B	C	
1·0 unit	1·00	1·37	1·83	L_A
0·1 unit	0·13	0·18	0·23	$L_A/10$
0·01 unit	0·015	0·028	0·045	$L_A/100$
Relative increase of test dose from L_A-$L_A/100$.	50%	100%	150%	
Cell sensitivity (agglutinin titres/international unit of three suspensions A.B.C.)	1/500	1/2500	1/10000	

For ease of comparison the volume of antigen required to give a standard end point with 1·0 unit of antitoxin with suspension A has been converted to 1·0 and all other inhibiting doses related to this.

TABLE 4

RELATIVE QUANTITIES OF (A) TETANUS TOXIN AND (B) TETANUS TOXOID REQUIRED TO INHIBIT AGGLUTINATION OF TETANUS TOXOID SENSITIZED SHEEP CELLS IN THREE DIFFERENT CONCENTRATIONS, BY 1·0, 0·1 AND 0·01 UNITS OF STANDARD ANTITOXIN

Quantity of antitoxin used		*Relative volumes of antigen required*			*Level of test*
		I[a]	*II*[a]	*III*[a]	
A	1·0 unit	1·00	1·17	1·25	L_A
	0·1 unit	0·14	0·15	0·17	$L_A/10$
	0·01 unit	0·018	0·028	0·030	$L_A/100$
B[b]	1·0 unit	1·00	1·12	1·25	L_A
	0·1 unit	0·14	0·15	0·26	$L_A/10$
	0·01 unit	0·026	0·035	0·045	$L_A/100$

[a] Sensitized cell concentrations I 560,000/c.mm.
II 168,000/c.mm.
III 56,000/c.mm.

[b] Toxoid B prepared from toxin A by formalin treatment.

For ease of comparison the volume of antigen required to give a standard end point with 1·0 unit of antitoxin with suspension A has been converted to 1·0 and all other inhibiting doses related to this.

(c) Effect of Time of Combination of Mixtures

Toxin-antitoxin titrations at the L_A level of test were set up in multiplicate and sensitized cells were added after different periods of time from 1 hr. to 8 days (Table 5). These tests showed that there was a progressive increase in the volumes of serum required to give a standard end point when time of combination was increased. Fig. 2 compares these results with others obtained at the $L_A/100$ level and shows that the curve at the lower level of test was steeper in the early stage.

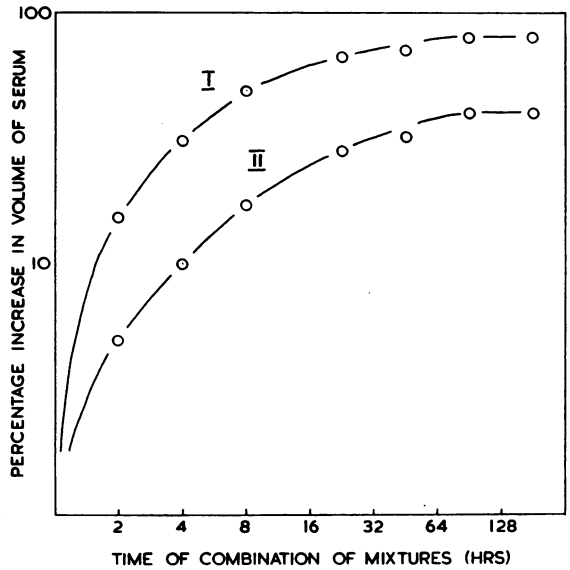

FIG. 2. Rate of increase in the volume of serum required to give a standard end point with (I) an $L_A/100$ dose and (II) with an L_A dose of toxin, when the mixtures are allowed increasing periods of time for combination before the addition of sensitized cells.

Several antitoxins were titrated with toxin at the L_A and $L_A/100$ levels of test. The mixtures were made up in duplicate; to one series, sensitized cells were added after 1 hr. and to the other after 24 hrs. (Table 6). In this experiment the L_A and $L_A/100$ test doses

TABLE 5

TITRATION OF TETANUS ANTITOXIN USING AN L_A DOSE OF TOXIN, THE MIXTURES ALLOWED TO STAND FOR DIFFERENT PERIODS BEFORE ADDING THE SENSITIZED CELLS

Volume of serum (ml.)	*Time of combination of toxin and antitoxin (hrs.)*							
	1	2	4	8	24	48	96	192
0·0013							+++	+++
0·0010					+++	+++	+++	+++
0·00083	+++	+++	+++	+++	+++	++±	−	−
0·00067	+++	+++	+++	+++	++±	−	−	−
0·00056	+++	++±	++	−	−	−	−	−
0·00045	+	−	−	−	−	−	−	−
0·00036	−	−	−	−	−	−	−	−

used were those determined previously for a combining time of 1 hr. It was found that with all sera allowed to stand for 24 hrs. there was a reduction in the apparent antitoxin value, of about 20 per cent at the L_A level of test, and of about 50–70 per cent at the $L_A/100$ level of test. Similar titrations *in vivo* did not produce this effect, showing that there had been no change in the degree of neutralization of the mixtures. The change in end point by haemagglutination cannot therefore be due to further simple combination of toxin and antitoxin.

TABLE 6

TITRATION OF A GROUP OF TETANUS ANTITOXINS BY HAEMAGGLUTINATION INHIBITION AT TWO LEVELS OF TEST (L_A AND $L_A/100$). THE MIXTURES ALLOWED TO STAND FOR (*a*) 1 HR. AND (*b*) 24 HRS. BEFORE THE ADDITION OF SENSITIZED CELLS

Apparent antitoxin values (units/ml.)

Sera	L_A *level of test*			$L_A/100$ *level of test*			*No. of immunization courses*
	Time of combination of mixtures 1 *hr.*	24 *hrs.*	*% reduction of titre at 24 hrs.*	*Time of combination of mixtures* 1 *hr.*	24 *hrs.*	*% reduction of titre at 24 hrs.*	
8480*	1700	1350	21	1700	525	69	7[c]
9018	380	300	21	380	150	61	3[c]
65	1200	1000	20	1100	330	70	1
8348	450	370	18	400	145	64	3[c]
990	1075	800	25	1150	300	74	1
7897	1100	900	20	800	425	47	20
7602	1150	900	22	500	230	54	55
8740	1450	1200	20	1000	525	47	6
8859	675	550	18	425	190	58	36

* The working standard antitoxin with an *in vivo* value of 1700 units/ml.

7[c] Seven full courses on tetanus antigens preceded by hyperimmunization with some other unrelated antigen.

DISCUSSION

The observations which have been made on haemagglutination tests with tetanus antitoxin demonstrate the necessity for careful standardization of the conditions of these tests. In direct haemagglutination tests comparison of the agglutinin titres of sera must take into account the variation produced by cell concentration and cell sensitivity. These must be kept constant by haemocytometer counts and reference to a standard antitoxin. The variable agglutinating capacity of the antitoxins in different sera presents at the moment an unavoidable difficulty in the use of the direct agglutination test for the comparison of the antitoxin content of the individual sera. This method has however been shown to be of use in comparison of groups of sera (Fulthorpe, 1958).

Haemagglutination inhibition tests must be carried out with agglutinable suspensions of constant sensitivity if reasonable comparisons are to be made. Table 2 shows that sensitized cells may be agglutinated by underneutralized mixtures of increasing toxicity when the degree of cell sensitivity is increased. Agglutination by such mixtures is probably carried out by toxin-antitoxin complexes in which the toxin is in excess. The existence of such toxic complexes was demonstrated by Eagle (1937).

The time allowed for the combination of mixtures before the addition of sensitized cells must be kept constant, since there is a progressive change in the volume of serum required to give a standard end point with an L_A dose of toxin with time (Table 5).

The effect is most noticeable at low levels of test ($L_A/100$). This change does not occur in *in vivo* titrations on sera of reasonably good avidity. It is possible that these changes are due to progressive aggregation of particles of the toxin antitoxin complex resulting in a reduction in the capacity of such aggregated large particles to cover the sensitized cell.

Table 6 shows that with different sera the fall in titre is fairly constant when mixtures are allowed to stand 24 hours before the addition of sensitized cells. There is a suggestion that qualitative difference between sera may show at the $L_A/100$ level of test, under these conditions, and that this difference may be related to immunization procedures. It is possible that aggregation of antigen antibody complexes may be slower when using antitoxins from horses after long periods of hyperimmunization. The flocculation time of sera from horses after prolonged hyperimmunization is usually long (Kekwick and Record, 1941).

The test doses required to inhibit agglutination by 0·1 and 0·01 unit of antitoxin are greater than $\frac{1}{10}$ and $\frac{1}{100}$ of the L_A dose. This relative increase is greatest with suspensions of high sensitivity, irrespective of the cause of the increased sensitivity. It is evident from these results that estimation of the antigen content of a preparation should be made at the same level of test and with cells of the same sensitivity and preferably with a reference toxin or toxoid as a control. It is possible that this relative increase in test dose at different levels of test is related to the speed of aggregation of the agglutinating antigen-antibody complex in mixtures of widely different concentration.

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REFERENCES

EAGLE, H. (1937). 'On the mutual multivalence of toxin and antitoxin.' *J. Immunol.*, **32**, 119–27.

FULTHORPE, A. J. (1957). 'Tetanus antitoxin titration by haemagglutination.' *J. Hyg. Camb.*, **55**, 382–401.

FULTHORPE, A. J. (1958). 'Tetanus antitoxin titration by haemagglutination at a low level of test.' *J. Hyg. Camb.*, **56**, 183–9.

KEKWICK, R. A. and RECORD, B. R. (1941). 'Some physical properties of diphtheria antitoxic horse sera.' *Brit. J. exp. Path.*, **22**, 29–49.

SCHEIBEL, INGA (1956). 'A comparative study on intracutaneous and haemagglutination procedures for assaying diphtheria antitoxin, with special reference to the avidity of the antitoxin.' *Act. path. microbiol. scand.*, **39**, 455–68.

STAVITSKY, A. B. (1954). 'Micro methods for the study of proteins and antibodies.' *J. Immunol.*, **72**, 360, 368–75.