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Immunization Against Neonatal Tetanus in New Guinea

4. Comparison of Tetanus Antitoxin Titres Obtained by Haemagglutination and Toxin Neutralization in Mice

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Haemagglutination (HA) has been used frequently for the titration of tetanus antitoxin but published results have varied in relation to the "unitage" determined by the mouse toxin-neutralization test (TN). This report gives the results of the titration of a group of sera by haemagglutination and compares them with the results obtained by toxin neutralization. Although there was marked variation between HA and TN titres of individual sera, results indicated that haemagglutination is a useful procedure for the over-all evaluation of the antitoxin responses to tetanus toxoids in field studies.

Passive haemagglutination (HA) has been used by several investigators for the titration of tetanus antitoxin since Stavitsky (1954a) applied Boyden's HA method (1951) for the titration of antitoxins. However, published results have varied in the correlation of the tetanus antitoxin titres measured by HA and toxin neutralization using mice (Marshall et al., 1957; Fulthorpe, 1957, 1958; Schubert & Cornell, 1958; Tasman et al., 1960; Surjan & Nyerges, 1962; Benenson et al., 1963; Levine & Wyman, 1964). Since the antitoxin titres of a large number of sera were to be determined in a study on the prevention of neonatal tetanus in New Guinea, it seemed worth while to compare the two methods of titration. The results of the toxin-neutralization titrations (TN) of the sera have been reported (MacLennan et al., 1965). The present report gives the results of the HA titrations and compares them with the TN results.

MATERIALS AND METHODS

Sera

The sera were from pregnant women immunized with either toxoid A_1 (mineral oil emulsified), toxoid B_1 (7-*n*-hexyloctadecane emulsified), toxoid C_1 ($AlPO_4$-adsorbed), toxoid D_1 (plain), or toxoid E (plain). Details of the toxoid preparations and the immunization and bleeding schedules were given in the previous report (MacLennan et al., 1965). The sera were stored at −20°C. There was one thawing prior to the HA test to remove a sample for TN and then the sera were refrozen until used for HA titration. The sera were inactivated at 56°C for 30 min and absorbed with an equal volume of packed fresh sheep erythrocytes (RBC). Haemagglutination Method I was used to test each of 430 sera from 117 pregnant New Guinea women and Method II was used to test 51 selected sera. Each of the selected sera had been shown to have detectable amounts of antitoxin by both TN and HA by Method I.

The US Standard Tetanus Antitoxin (Lot 96, 5 units/ml) was used as the standard of reference.

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Antitoxin titrations

Toxin-neutralization test (TN). The procedure for titration of antitoxin in mice is presented elsewhere (Barile et al., 1970). Undiluted and 10-fold dilutions of the sera were tested. The test system was designed to detect the presence of 0.001 unit/ml or more of antitoxin.

Haemagglutination (HA). Method I. This procedure was developed by Dr Lajos Csizmas while he was at the Division of Biologics Standards. It utilizes formalinized, tanned sheep RBC (Csizmas, 1960) in a microtitre system (Takatsy, 1951; Sever, 1962).

Toxoid. Tetanus toxoid, dried, Lot 208, Massachusetts Public Health Laboratories, was reconstituted in saline to contain 100 Lf/ml.

Preparation of toxoid-sensitized cells. A 50% (v/v) stock suspension of the formalinized sheep RBC was prepared and the same stock was used throughout the study. Sensitized cells were prepared daily for each test as follows: the stock cells were washed in 2 volumes of saline, centrifuged for 10 min at 650 *g* and reconstituted in saline to a 5% cell suspension. One volume of the 5% suspension of cells in physiological saline was added to an equal volume of 1 : 20 000 tannic acid, and the mixture was gently agitated for 30 min on a rotary shaker. The cells were washed 3 times in saline; after centrifugation they were resuspended in saline to a 5% cell concentration. A mixture of 1 volume of the 5% suspension of tanned cells, 1 volume of tetanus toxoid (100 Lf/ml), 2.5 volumes of saline and 2.5 volumes of phosphate buffer, pH 6.5, was shaken for 90 minutes. Then the cells were washed 3 times in 1% normal rabbit serum in saline (NRS), centrifuged, and diluted to a 1% suspension in the NRS. The rabbit serum had been inactivated at 56°C for 30 min before diluting. A non-sensitized cell control was prepared in the same manner except that 1 volume of saline replaced the toxoid.

Determination of titre. Serial 2-fold dilutions of the women's sera, and the standard, were made in NRS in the microtitre plates with use of calibrated droppers and loops. Undiluted sera were not tested. To each well containing 0.025 ml diluted serum was added, with a calibrated dropper, 0.025 ml of the suspension of toxoid-sensitized or non-sensitized cells. Controls included sensitized and non-sensitized cells in diluent and in dilutions of the standard antitoxin. The plates were incubated at room temperature (22°C) overnight. The highest serum dilution that showed a carpet of cells with end-folding was recorded as the end-point. The antitoxin " unitage " of each serum was determined by multiplying the unit concentration in the end-point dilution of the standard by the end-point dilution of the unknown serum. For example, if the end-point dilution of the standard (5 unit/ml) was 1 : 1024 which would contain 0.0049 unit/ml and the end-point dilution of the unknown was 1 : 2, the " unitage " would be 0.0049×2 or 0.0098 unit/ml. A negative reaction in the 1 : 2 dilution of the unknown (the highest concentration tested) was recorded as " no detectable antitoxin " (ND). If the non-sensitized cells were agglutinated in the same or higher dilution as were the sensitized cells, the reaction was recorded as " non-valid " (NV).

Method II. After Levine & Wyman (1964) reported good correlation between TN and HA titres obtained by the previously described method of Levine et al. (1960), 51 selected sera were retitrated by this procedure. Fresh sheep cells were sensitized with tetanus toxoid, Lot LP 250, kindly supplied by Mr Leo Levine of the Massachusetts Public Health Laboratories. The titrations were performed in 10 mm $\times$ 75-mm test tubes. The microtitre system was not applied.

Statistical analyses

Correlation coefficients were calculated between the TN and HA titres of the same sera (Snedecor, 1967). The structural relationship of the TN and HA (Method I) titres was determined by calculation of the orthogonal regression line (Diem, 1962), by fitting a regression line using the method of least squares (Snedecor, 1967) and by the Bartlett modification (1949) of the Wald procedure of ranking (1940).

RESULTS

Haemagglutination

Method I. The minimum detectable antitoxin levels of the standard in all of the HA tests ranged from 0.0006 unit/ml to 0.156 unit/ml. However, in the tests of 291 of 400 (73%) sera, the end-point was 0.0098 unit/ml $\pm$ one 2-fold dilution. No titrations were excluded because of a low level of antitoxin detection (low degree of cell sensitivity). Table 1 shows the results of the HA and TN titrations of the 430 sera from 117 women injected with 1 of the 5 toxoids. Thirty sera could not be titrated for HA because of non-specific agglutination and

TABLE 1

TETANUS ANTITOXIN UNITS DETERMINED BY HAEMAGGLUTINATION (METHOD I) AND TOXIN NEUTRALIZATION AFTER IMMUNIZATION WITH DIFFERENT TOXOIDS

Toxin neutralization (units/ml)	Haemagglutination (units/ml)														
	NV [a]	ND [b]	0.0006	0.0012	0.0024	0.0049	0.0098	0.019	0.039	0.078	0.156	0.312	0.625	1.25	2.5
Toxoid A_1															
<0.001	4	37				1		2	1						
>0.001–<0.01	1	16		1		1	2	1							
>0.01 –<0.1	1	8	1			6	3	3	3	1	1	1			
>0.1 –<1		1					2	6	3	8	1	2	1	1	
>1 –<10									1	1	1		2		1
Toxoid B_1															
<0.001	4	28					2		1		1				
>0.001–<0.01	1	10			3	3		1			1				
>0.01 –<0.1	1	7			2	2	6	4	3	3	1	2	1		
>0.1 –<1	1					2	1	4	4	6	1	3	2		1
>1 –<10													2	1	
>10 –<100														2	
Toxoid C_1															
<0.001	1	21													
>0.001–<0.01		1						1							
>0.01 –<0.1	1	7			3		3	4	2	2	1				
>0.1 –<1	1	2	2		1	1	1	3	3	3	1	1			
>1 –<10												1			
Toxoid D_1															
<0.001		20					1				1				
>0.001–<0.01	1	9													
>0.01 –<0.1	1	11			1	1		2	1	2	2				
>0.1 –<1	1				1		1	2		2		2			
Toxoid E															
<0.001	3	22													
>0.001–<0.01	3	3					1	1							
>0.01 –<0.1	2	6				1		2		2	1				
>0.1 –<1	3							2		1	2	3			

[a] Not valid—non-specific agglutination. [b] Antitoxin not detected—no haemagglutination.

TABLE 2

COMPARISON OF HAEMAGGLUTINATION UNITAGE (METHOD I) WITH TOXIN NEUTRALIZATION UNITS

Toxin neutralization (TN)		Haemagglutination (no. of sera)				
Titre (units/ml)	No. of sera	NV [a]	ND [b]	Relative to TN		
				<	=	>
<0.001	150	12	128			10
>0.001-<0.01	61	6	39		11	5
>0.01 -<0.1	118	6	39	29	34	10
>0.1 -<1.0	89	6	3	59	19	2
>1.0 -<10.0	10			8	2	
>10.0 -<100.0	2			2		
Total	430	30	209	98	66	27
Percentage [c]				54	36	9

[a] Not valid—non-specific agglutinations.
[b] Antitoxin not detected—no haemagglutination.
[c] Percentage of the sera detectable by both TN and HA.

TABLE 3

CORRELATION COEFFICIENTS OF ANTITOXIN UNITAGE DETERMINED BY HAEMAGGLUTINATION (METHOD I) AND TOXIN NEUTRALIZATION

Sera from immunized women		Correlation coefficient
Toxoid	No. of sera	
A	54	0.62 [a]
B	61	0.62 [a]
C	33	0.24 [b]
D	17	0.05 [b]
E	16	0.61 [a]
Total	181	0.54 [a]

[a] Significant at 1 % level.
[b] Not significant.

209 others had no detectable antitoxin. Table 2 compares the units obtained by the HA and TN methods for all the sera. Of the 150 that had no detectable antitoxin (<0.001 unit/ml) by the mouse test, 140 showed either no HA (128) or non-specific agglutination (12). Thus, agreement between lack of detection of antitoxin by TN and HA was 93%. The remaining 10 sera (7%) showed a "false positive" HA; 6 of these false positive titres were equal to or greater than 0.01 unit/ml.

Of the 280 sera with TN antitoxin levels greater than 0.001 unit/ml there were 99 (35%) with NV or ND haemagglutination reactions. Of the remaining 181 sera, HA "unitage" relative to TN unitage was lower with 98 (54%), equal to with 66 (36%) and higher with 17 (9%).

The correlation coefficients (r) of the HA and TN titrations of the 181 sera with antitoxin detected by both procedures are shown in Table 3. The correlation coefficient for the combined sera was 0.54 ($P = 0.01$). A lack of significant correlation with sera from women given toxoids C_1 or D_1 was noted.

The accompanying figure shows the log units/ml of the 181 sera obtained by TN plotted against the log units/ml obtained by HA, the fitted regression line and the 95% tolerance limits. It was concluded from the analyses by the orthogonal regression, the method of least squares and by the Wald–Bartlett procedure that when the HA and TN "unitages" are determined in the reported manner (2-fold dilutions of sera for HA and 10-fold dilutions for TN), they may be used interchangeably as the estimate of the antitoxin level of a group of sera.

Method II. Toxoid Lot LP 250, used to sensitize fresh RBC for Method II, did not differ significantly in sensitizing activity from Lot 208 used for the formalinized cells, i.e., fresh sheep RBC sensitized by the two toxoids gave approximately the same end-points in the standard antitoxin dilutions in concurrent tests. The results of the titrations of the 51 selected sera by Method II and TN are shown in Table 4. Table 5 compares the HA "unitages" of the sera obtained by Method I and Method II relative to TN "unitage". Of the HA titres determined by Method II, 31 (60.8%) were higher than the TN titres. In contrast, only 17.6% of the titres of the selected 51 sera determined by Method I were higher than the TN titres. However, the calculated correlation coefficient for the HA and TN titres of the 51 sera was similar for each HA method; with the fresh cells it was 0.75 and with the formalinized cells it was 0.74.

Method II appeared to be more sensitive than Method I in detecting antitoxin. For titration of 100% of the sera by Method II the detectable antitoxin of the standard was 0.00098 unit/ml ± 1 two-fold dilution, whereas for the titration of 73% (291 of 400) of the sera by Method I, the

TETANUS ANTITOXIN DETERMINED BY TOXIN NEUTRALIZATION PLOTTED AGAINST TETANUS ANTITOXIN DETERMINED BY HAEMAGGLUTINATION WITH THE FITTED REGRESSION LINE AND 95% TOLERANCE LIMITS

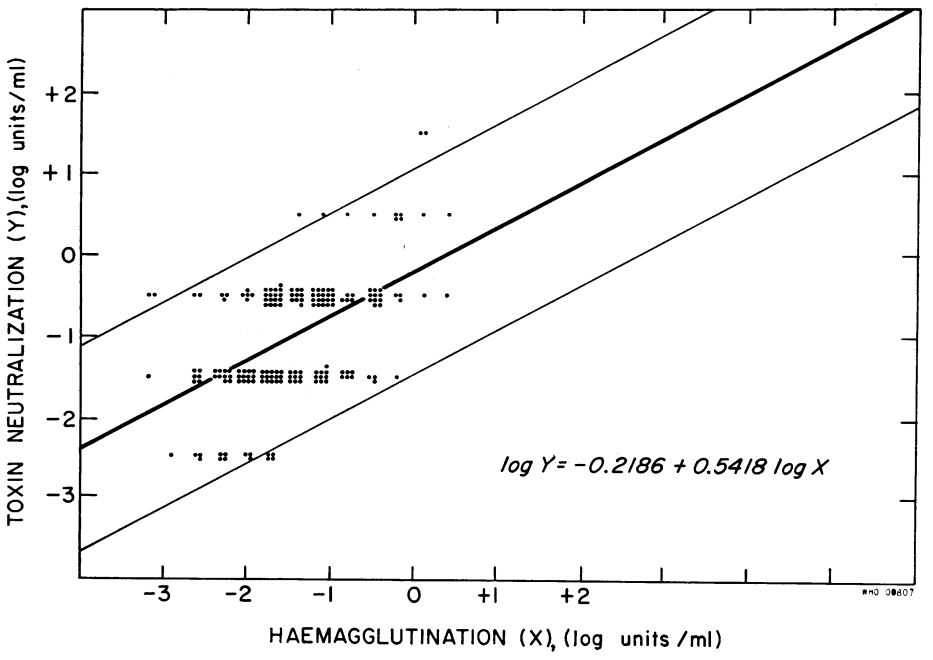

TABLE 4

TETANUS ANTITOXIN UNITS DETERMINED BY HAEMAGGLUTINATION (METHOD II) AND TOXIN NEUTRALIZATION

Toxin neutralization (units/ml)	No. of sera	Haemagglutination (units/ml)			
		(>0.001–<0.01) 0.007	(>0.01–<0.1) 0.015 0.03 0.06	(>0.1–<1.0) 0.125 0.25 0.50	(>1.0–<10.0) 1.0 4.0 7.9
>0.001–<0.01	9	2	1 2 2	1 1	
>0.01 –<0.1	27		2 6	5 5 7	2
>0.1 –<1.0	12		1	1 5	4 1
>1.0 –<10.0	2				1 1
>10.0 –<100.0	1				1
Total	51	2	14	25	10

detectable antitoxin of the standard was 0.0098 unit/ml ±1 two-fold dilution.

DISCUSSION

Statistical analyses of the antitoxin titrations of 181 sera which had antitoxin detected by both HA and TN (Method I) confirmed the observation of other workers (Tasman et al., 1960; Surjan & Nyerges, 1962; Levine & Wyman, 1964) that there is a significant relationship between the titres obtained by the two procedures. However, the calculated *r* values with either the formalinized cells or the fresh cells

TABLE 5

COMPARISON OF TOXIN NEUTRALIZATION UNITS WITH HAEMAGGLUTINATION UNITS DETERMINED BY USE OF FORMALINIZED CELLS (METHOD I) AND FRESH CELLS (METHOD II)

Toxin neutralization (TN)		Haemagglutination (units/ml)					
		Method I			Method II		
Titre (units/ml)	No. of sera	Relative to TN			Relative to TN		
		<	=	>	<	=	>
>0.001–<0.01	9	0	6	3	0	2	7
>0.01 –<0.1	27	7	14	6	0	8	19
>0.1 –<1.0	12	4	8	0	1	6	5
>1.0 –<10.0	2	2	0	0	0	2	0
>10.0 –<100.0	1	1	0	0	1	0	0
Total	51	14	28	9	2	18	31
Percentage		27.5	54.9	17.6	3.9	35.3	60.8

were lower than those reported by Levine & Wyman (1964), Tasman et al. (1960) or Chatterjee (1964). Their calculated correlation coefficients ranged from 0.90 to 0.98. We also observed marked variations between HA and TN titres of individual sera as reported by others (Fulthorpe, 1957; Tasman et al., 1960; Chatterjee, 1964; Levine & Wyman, 1965).

Levine & Wyman (1964) found HA titres to be higher than TN titres, while Schubert & Cornell (1958) reported that in their series HA titres were higher than TN titres in 38% of sera, 49% were in the same intervals and 14% of HA titres were lower than TN titres. Olitzki (1963) found no significant difference between the two values of the sera of 15 subjects. In our study of the 181 TN and HA (Method I) positive sera, the HA "unitages" of 54% were lower, 36% were equal to, and 9% were higher than the TN "unitage". On the other hand, using the 51 selected sera the absolute relationship between HA and TN values was altered by the HA method of titration since Method II gave more titres (60.8%) greater than the TN titres, whereas only 17.6% of the titrations by Method I exceeded the TN titres. Although it is definite that HA does detect the presence of antitoxin, the degree of correlation of individual sera with TN "unitage" depends in part on the sensitivity of the HA method employed.

Had Method II been used for the entire study it seems likely that some of the sera with TN titres of <0.001 unit/ml would have had antitoxin detected by HA since the fresh-cell method was capable of detecting a lower titre of antitoxin. Daniel et al. (1963) observed that formalinized cells were sometimes less sensitive than fresh cells for certain antigens. However, since the methods of HA differ in factors other than the type of RBC (macro- as against microtechniques, amount of antigen used for sensitization, and other factors mentioned below), the cause of variable results cannot be attributed solely to the method of preparation of the RBC.

It has been reported that different lots of both fresh and formalinized cells have different capacities for sensitization (Daniel et al., 1963) and that HA titres differ as much as 30-fold when different batches of formalinized cells are used for HA (Hirata & Brandriss, 1968). Whether or not different *r* values would have been obtained by us using different batches of cells is not known.

Surjan & Nyerges (1962) have stated that the haemagglutination procedure alone cannot always be held responsible for the lack of a more exact correlation between HA and TN titres. Many variables influence the titration of antitoxin by either procedure (Petrie, 1943; Stavitsky, 1954a; Fulthorpe, 1957, 1958, 1959; Tasman et al., 1960; Surjan & Nyerges, 1964; Daniel et al., 1963). Some factors reported to influence titrations of antitoxins are the L+ level of the TN titration, the avidity of toxin or antibody, the nature of the antibody, and impurity of the immunizing or sensitizing antigen. Surjan & Nyerges (1962) found no greater than a 4–5-fold variation in HA and TN titres, but they reported a difference in correlation if the TN titrations were determined at the L+/10 level or at the L+/400 level. In contrast, Tasman et al. (1960) calculated similar correlation coefficients when either an L+/1000 (0.95) or an L+/10 000 (0.90) level was used for the TN. These workers as well as Fulthorpe (1957) and Levine et al. (1967) suggest that observed differences in HA and TN titres may be related to avidity.

Benenson et al. (1963) reported that dogs irradiated prior to tetanus immunization formed haemagglutinating antibody sooner than neutralizing antibody. They concluded that either (1) the antibody formed is less avid for the toxin and unable to neutralize it or (2) HA may detect an antibody against some constituent or fragment of the toxoid other than the physiologically active prosthetic group. Previously Boyden (1951) and Stavitsky (1954b) suggested that HA may measure reactions of impure antigens and antibodies. However, from the ana-

lyses of our data it cannot be ascertained that HA and TN are measuring different antigen-antibody reactions.

The toxoids themselves may have influenced our results as there were differences in the calculated *r* values for the sera from women given the different toxoids. The greatest differences were obtained with the $AlPO_4$-adsorbed and one plain toxoid. However, the number of sera in each toxoid group was too small to permit a complete evaluation of these various factors. Further, the time of serum collection may have affected the molecular size of the antibody and its avidity for sensitized RBC. The role of 19S or 7S antibody in these systems was not examined.

From this and other studies, we conclude that haemagglutination may be used for the titration of tetanus antitoxin. The toxin-neutralization test in mice does offer the advantage of being very specific in that symptoms due to tetanus are altered by the presence of antitoxin. However, the HA procedures are rapid and economical. Haemagglutination may be very useful in measuring the response to tetanus toxoids of a population but we would prefer to use the TN test to measure the antitoxin response of an individual.

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RÉSUMÉ

IMMUNISATION CONTRE LE TÉTANOS DU NOUVEAU-NÉ EN NOUVELLE-GUINÉE: 4. COMPARAISON DES TITRES D'ANTITOXINE DÉCELÉS PAR L'ÉPREUVE D'HÉMAGGLUTINATION ET L'ÉPREUVE DE NEUTRALISATION DE LA TOXINE CHEZ LA SOURIS

On a titré par l'épreuve d'hémagglutination passive (HA) l'antitoxine tétanique présente dans les sérums de femmes de Nouvelle-Guinée immunisées par diverses préparations d'anatoxine. La méthode basée sur l'emploi d'hématies de mouton traitées par le formol (méthode I) a été appliquée à 430 sérums et celle qui utilise des globules rouges frais de mouton (méthode II) a servi au titrage de 51 échantillons. On a comparé les titres HA ainsi obtenus aux titres décelés par l'épreuve de neutralisation de la toxine chez la souris (TN).

Sur 150 sérums ne renfermant en épreuve TN aucune antitoxine décelable (<0,001 u/ml), 140 ont été trouvés négatifs ou ont donné une agglutination non spécifique en épreuve HA (méthode I). La concordance entre les deux tests sous le rapport de la non-détection d'antitoxine a été de 93%. Dix sérums (7%) ont donné des réactions HA faussement positives. Dans chacun des 280 sérums restants, l'épreuve TN a décelé la présence d'antitoxine (>0,001 u/ml), alors qu'avec l'épreuve HA un résultat positif n'a été obtenu que dans 181 cas. On a noté de fortes variations entre les titres HA et les titres TN dans un certain nombre de sérums. Le coefficient de corrélation entre les titres HA et TN de ces sérums a été de 0,54.

La méthode II s'est révélée plus sensible que la méthode I et a permis de déceler des teneurs moins élevées en antitoxine. Les coefficients de corrélation entre les titres TN et les titres HA de 51 sérums ont été quasi identiques pour les deux méthodes (0,75 avec la méthode I; 0,74 avec la méthode II).

De ces résultats, et des constatations faites par d'autres auteurs, on conclut que l'épreuve d'hémagglutination, d'exécution rapide et peu coûteuse, peut rendre de grands services lorsqu'il s'agit d'évaluer globalement la réponse immunitaire dans une population immunisée par l'anatoxine tétanique.

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