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CONSISTENCY IN POTENCY ASSAY OF TETANUS TOXOID IN MICE

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SYNOPSIS

The use of mice for the assay of tetanus toxoids would offer considerable advantages over the use of guinea-pigs, but mice cannot readily be immunized with the fluid tetanus toxoid at present designated as the International Standard. This study shows, however, that the mouse is a very suitable laboratory animal for the comparison of adsorbed tetanus toxoids, and that an $AlPO_4$-adsorbed vaccine, which is stable at 4°C, is a satisfactory reference preparation. The log-dose-response lines of toxoids adsorbed on different quantities of $AlPO_4$ and on various quantities of another adsorbent ran parallel to those of the reference vaccine. The 95% confidence limits for the potencies of tetanus vaccines, diphtheria-tetanus vaccines, and diphtheria-pertussis-tetanus vaccines, determined by assay against the reference vaccine in mice, showed a high degree of reproducibility of the results.

The Netherlands National Institute of Public Health regularly produces purified tetanus toxoid adsorbed on aluminium phosphate, often in combination with diphtheria toxoid or with diphtheria toxoid and pertussis vaccine.

It was obviously desirable to test the potency of these preparations in comparison with the International Standard for Tetanus Toxoid adopted by the WHO Expert Committee on Biological Standardization in 1952. This standard is a purified, freeze-dried, non-adsorbed tetanus toxoid, 0.03 mg of which was defined as the International Unit. In guinea-pig experiments, this quantity had the same potency as one *Schutzeinheit* of the German standard toxoid Tf 107, dissolved in peptone, developed by Istrati et al. (1940). As such experiments had also shown that the log-dose-response

lines of non-absorbed tetanus toxoids and of adsorbed ones run parallel (Prigge, 1940)—which is not the case with diphtheria toxoid—it was not thought necessary to introduce a separate standard for *adsorbed* tetanus toxoid, as has been done for adsorbed diphtheria toxoid.

Later investigations (Greenberg, 1953; Barr et al., 1957) showed that the mouse is not easily immunized with fluid tetanus toxoid. Moreover, Greenberg's work has shown that the results obtained with non-adsorbed preparations in mouse-experiments often do not fit the mathematical model. On the other hand, the mouse is a very cheap laboratory animal, requires little room, and pure strains can be bred in large numbers. Moreover, Ipsen et al. had found in 1953 that mice *can* be properly immunized with adsorbed tetanus toxoids. We therefore decided to examine the usefulness of this animal more closely.

In our experiments, the fluid standard was found unsuitable for this purpose, because of its poor immunizing effect in mice. We therefore tried a toxoid adsorbed on aluminium phosphate ($AlPO_4$) to see if this would be suitable as a laboratory reference preparation. It proved possible to define the reproducibility of the immunizing potency of our preparations (i.e., their " consistency ") by means of this preparation, using the mouse as laboratory animal.

Material and Methods

Laboratory animals

All the experiments were carried out using pure-bred Swiss mice, weighing 9-13 g, which had been bred in our Institute. The animals were segregated according to sex and divided into groups of 5 animals, each group being placed in a separate jar.

Reference preparations

Our reference toxoid was prepared in the following manner: A toxin obtained in the medium described by Mueller & Miller (1954), using Harvard strain No. 49205 of *Clostridium tetani*, which Mueller had received from the New York State Department of Health, was used in our laboratory by J. L. Sirks for the preparation of a crude toxoid. After having been purified by ultrafiltration and fractional salting out with ammonium sulphate (Tasman & Ramshorst, 1952), this toxoid (1580 Lf per mg N) was adsorbed on $AlPO_4$ (10 Lf toxoid and 3 mg $AlPO_4$ per ml), which had been prepared from alum and trisodium phosphate.

Challenge toxin

This was a tetanus toxin obtained in the Tarozzi medium (strain Wellcome CN 655, 9 days growth). After filtration it was saturated with am-

monium sulphate and the precipitated toxin protein dried, first on paper and then in a desiccator over phosphorus pentoxide. The dried toxin was thoroughly ground with dry, chemically pure sodium chloride (500 mg toxin+24.5 g NaCl). The LD_{50} of this mixture for the mice used was estimated to be 0.0147 mg. The standard deviation of this value was not calculated, as it was found to be within the limits of dilution errors.

Other preparations

The adsorbed purified tetanus toxoids were prepared in the same way as the reference toxoid.

The diphtheria toxoid used in the combined vaccines had also been purified by ultrafiltration and fractional salting out (Ramshorst, 1951), the crude toxoid having been prepared in a caseine-hydrolysate medium. The vaccines contained 30 Lf of diphtheria toxoid per ml. In addition to this, the pertussis-diphtheria-tetanus vaccines contained 40 000 million germs per ml of pertussis vaccine, obtained by means of a modified Bordet-Gengou medium (for formula see Cohen and Leppink, 1956).

Immunization and challenge methods

Both the reference toxoid and the vaccines to be examined were injected subcutaneously into groups of 20 mice in four dilutions. These dilutions were logarithmic, the dilution factor being 2. Thus, in one experiment, the dilutions used were 1:15, 1: 30, 1: 60 and 1: 120. The quantity of each dilution injected was 0.5 ml. Three weeks after immunization the animals were given a subcutaneous injection of 50 LD_{50} of the challenge toxin, the quantity injected being 1.0 ml. After 5 days (120 hours), the percentage of survivors in each group was calculated and from these figures we computed by probit analysis what dilution of the vaccine would have protected exactly 50% of the animals. This quantity of the vaccine is the ED_{50}.

Immunization period and challenge dose had already been determined in a series of preliminary experiments (Ramshorst & Cohen, 1957). These had shown that after the first two weeks there was an effective immunity, which increased very little during the next fortnight. The percentage of survivors was found to be almost constant, whether the challenge dose injected was 25, 50, or 100 LD_{50}. A dose of 50 LD_{50} was chosen.

Analysis of results

Probit analysis was applied. The determination of the confidence limits of the immunizing potency of our vaccines was carried out by G. J. Leppink, Statistics Department, Toegapast Natuurwetenschappelijk Onderzoek, The Hague, Netherlands.

Experiments

Choice of reference vaccine

Table 1 is a summary of two experiments which were made for the purpose of determining the potency of the fluid International Standard in experiments with mice.

TABLE 1. ED_{50} VALUES, b VALUES, AND χ^2 VALUES OF THE INTERNATIONAL STANDARD FOR TETANUS TOXOID IN TWO CHALLENGE TESTS IN MICE

Test number	ED_{50} values		b value	χ^2
	in ml *	in I.U.		
24	1/10	41	0.9	3.93
31	1/3	138	0.8	0.26

* 25 mg of the International Standard in 2 ml of 0.3M solution

It will be seen that very large quantities (>1 mg) of the standard vaccine are required to obtain reasonable protection in mice. Moreover, the slope of the log-dose-response line is very shallow on both experiments, so that the ED_{50} values found are very inaccurate. If these results are compared with those obtained with the adsorbed vaccine (vaccine No. 25), which are shown in Table 2, it will be seen how much better the mouse is immunized by adsorbed vaccines than by " fluid " ones.

In 28 experiments the ED_{50} values of the adsorbed toxoid ranged from 1/80 to 1/153 ml, and the b values (slope of the probit lines) from 2.4 to 4.8. In four cases the χ^2 value exceeded the 95% confidence interval ($\chi^2 = 5.99$ for 2 degrees of freedom). As this would not have been expected to occur more than once or twice, it may be an indication that the mathematical model is not quite satisfactory.

These experiments clearly show that a comparison between the two vaccines with regard to potency is fundamentally impossible because there is a considerable difference between the slopes of the probit lines. As it was clear that the fluid International Standard vaccine could not stand comparison with the adsorbed vaccine, we decided to undertake further experiments to find out whether our adsorbed vaccine No. 25 was a suitable laboratory reference preparation for use in mice.

Usefulness of vaccine No. 25 as a reference preparation

Stability

The adsorbed toxoid was available in a fluid, non-lyophilized form. It was decided to test the stability of this vaccine, expressed as the time

TABLE 2. ED_{50} VALUES, b VALUES, AND χ^2 VALUES OF ADSORBED TETANUS TOXOID IN MICE

Expt. No.	ED_{50} in ml	b value	χ^2
01	1/128	3.3	0.23
03	1/101	3.7	8.15*
04	1/143	4.5	0.03
05	1/98	4.6	0.41
06	1/120	3.7	0.51
07	1/96	4.3	0.28
08	1/89	3.8	0.78
09	1/153	4.0	1.23
10	1/97	3.9	0.91
11	1/90	3.7	0.50
12	1/80	3.6	1.10
13	1/111	3.3	10.86*
14	1/46	3.4	4.46
15	1/145	2.9	5.28
17	1/117	4.8	1.30
18	1/111	3.6	5.70
19	1/122	2.7	8.90*
20	1/107	3.4	2.54
22	1/85	3.3	1.50
26	1/96	3.5	3.96
27	1/140	3.2	7.06*
28	1/109	2.7	0.14
30	1/127	2.4	1.18
32	1/133	2.8	2.24
33	1/110	3.2	1.77
35	1/128	4.1	0.96
36	1/81	2.7	3.81
37	1/118	3.1	0.21

*** $\chi^2 > 5.99$, hence these values exceed the 95% confidence limits (2 degrees of freedom)**

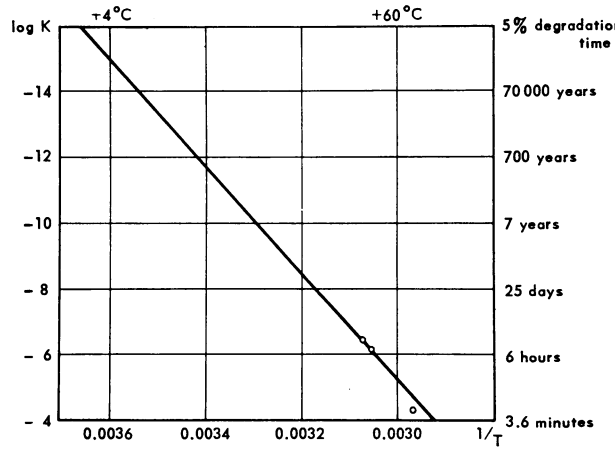
TABLE 3. RESULTS OBTAINED WITH THE REFERENCE VACCINE IN ACCELERATED DEGRADATION TESTS EXPRESSED AS THE PERCENTAGE POTENCY REMAINING AFTER VARIOUS PERIODS OF HEATING AT DIFFERENT TEMPERATURES AND AS THE CORRESPONDING LOG K VALUE

Temperature (°C)	Time of exposure (hours)	Potency after heating (%)	log K	Mean log K value
53	96	83	−6.6	−6.5
	192	53	−6.4	
55	32	97	−6.0	−6.1
	72	52	−6.0	
	144	44	−6.2	
	288	35	−6.4	
65	3	20	−4.2	−4.2

taken for the immunizing potency to decrease by 5% at 4°C (determined by means of the " accelerated degradation " test of Jerne & Perry (1956)). The results are shown in Table 3 and Fig. 1.

If the log K values are plotted against 1/T (the reciprocal of the absolute temperature at which K was determined) [1] the points found may be expected to lie roughly on a straight line. By extrapolation (Fig. 1) the time taken

FIG. 1. RELATIONSHIP BETWEEN LOG K AND THE RECIPROCAL OF THE CORRESPONDING ABSOLUTE TEMPERATURE



[1] K is computed by means of the formula $K = \frac{1}{t} \log \frac{a}{a-x}$, where t is the time of exposure in seconds and a - x is the potency remaining after heating a vaccine of initial potency a for t seconds.

for the vaccine to lose 5% of its potency can be estimated to correspond to a log K value between −14 and −15, i.e., more than 70000 years. Stored at 4°C, therefore, the vaccine is undoubtedly very stable.

Suitability for assaying toxoids adsorbed on other adsorbents or on different quantities of the same adsorbent

Although the same quantity of $AlPO_4$ is always used as an adsorbent in our Institute, we thought it advisable to test the usefulness of vaccine No. 25 as a reference preparation when other adsorbents or other quantities of adsorbent are used. Its value may be considered established if the slopes of the probit lines found for these preparations are the same, i.e., if one preparation behaves as a dilution of the other (Maaløe & Jerne, 1952).

Now it may be expected *a priori* that this will be the case with widely varying quantities of adsorbent, for the non-adsorbed toxoid immunizes the mouse so poorly that the presence of any non-adsorbed toxoid will have no perceptible effect on the immunization. Consequently, when only a small amount of toxoid can be adsorbed, the potency of the vaccines will decrease, but the log-dose-response lines will remain parallel to the probit line of the standard vaccine; it is only the ED_{50} value that will change. This was actually found to be the case for different quantities of $AlPO_4$ and $Al(OH)_3$, and the probit lines for these adsorbents also clearly run parallel (Table 4).

TABLE 4. SLOPE OF THE LOG-DOSE-RESPONSE LINES AND RELATIVE IMMUNIZING POTENCY OF TETANUS TOXOID ADSORBED ON INCREASING QUANTITIES OF $AlPO_4$ AND $Al(OH)_3$.

Adsorbent	Quantity of Al (mg/ml)	b value	Relative potency
$AlPO_4$	0.33	3.1	0.48
	0.66	3.2	1.35
	1.1	2.5	1.59
	Reference	2.4	—
$Al(OH)_3$	0.33	3.4	0.95
	0.66	3.1	1.64
	Reference	3.5	—

The relative immunizing potency varies, however, according to the quantity and the nature of the adsorbent.

Reproducibility of the mouse-assay

It was very important to know how far the results of the tests are reproducible if they are repeated with the same vaccine at different times. Table 5 shows the relative immunizing potencies of a number of tetanus vaccines and of combined vaccines containing tetanus toxoid, expressed in terms of ED_{50} reference/ED_{50} vaccine. A striking feature is that the values found when the experiment is repeated with the same vaccine differ very little, in other words the reproducibility of the animal test is very good.

The data obtained in these experiments were subjected to statistical analysis as described below.

TABLE 5. RELATIVE IMMUNIZING POTENCIES OF VARIOUS VACCINES IN ASSAYS REPEATED AT DIFFERENT TIMES

Vaccine	Series number	Relative potency $\frac{ED_{50}\text{ reference}}{ED_{50}\text{ vaccine}}$				
Tetanus	27	0.82	0.57	0.79		
,,	28	0.90	0.81			
,,	29	0.74	0.97	0.73	1.27	0.86
,,	30	0.90	0.83			
Diphtheria-tetanus	20	0.56	0.84	0.86	0.91	0.67
Diphtheria-pertussis-tetanus	27	1.40	1.47	1.36		
,,	31	1.68	2.07	1.75	2.37	1.63

Reproducibility of the results obtained in the production of vaccines

From Table 6 it will be seen that the values found for various *vaccines* are also remarkably consistent; in other words, the method adopted by our Institute for the production of tetanus vaccines yields reproducible results. Another striking feature is the strong adjuvant effect of the pertussis component.

Lastly, the data thus obtained made it possible to calculate by means of a statistical analysis the *confidence limits* within which the value found for any new vaccine series must lie for this series to be acceptable.

Statistical analysis

In view of the adjuvant effect of the pertussis component, a distinction was made between T and DT vaccines on the one hand and DPT vaccines

TABLE 6. RELATIVE IMMUNIZING POTENCIES OF A NUMBER OF VACCINES COMPARED TO THE REFERENCE VACCINE, EXPRESSED IN TERMS OF THE ED_{50}

Vaccine	Series number	Relative potency*
Tetanus	20	0.54
	27	0.73
	28	0.85
	29	0.91
	30	0.86
Diphtheria-tetanus	16	1.03
	20	0.66
	21	0.64
	22	0.74
	23	0.84
	24	0.86
Diphtheria-pertussis-tetanus	27	1.41
	28	0.92
	29	1.39
	30	1.32
	31	1.90
	32	2.10
	33	2.17
	34	1.40
	35	1.20
	36	1.28

* Whenever the potency was tested more than once (see Table 5) the average was taken.

on the other. The first 15 experiments were analysed by means of the probit method, the log ED_{50} values, slope b and χ^2 being calculated for each probit line.

When carrying out a series of experiments, wide variations will sometimes be found in the b or χ^2 values, or in both. There are two possible reasons for this: (1) some alteration may have occurred in the vaccine itself; (2) there may have been an inadvertent departure from the usual experimental procedure. In the former case, the same deviation will occur again when the experiment is repeated; in the latter case, the chance of such a recurrence is extremely small.

It follows, therefore, that if during the routine control of batches of vaccine one experiment is found to yield a different b or χ^2 value from the

TABLE 7. ANALYSIS OF THE RESULTS OF 15 EXPERIMENTS ON THE RELATIVE IMMUNIZING POTENCIES OF VARIOUS VACCINES

Experiment No.	Vaccine	Probit analysis				Mean slope b = 3.49		
		b value	log ED_{50}	χ^2	Observations	log ED_{50}	(log ED_{50} vaccine) − (log ED_{50} reference)	Remarks
1	DPT 26	1.04	2.639	1.17	b too low			
	T 26	4.05	1.568	0.03		1.565	−0.238	
	DPT 27	1.22	1.973	3.01	b too low			
	reference	3.32	1.806	0.23		1.803		
2	T 29	3.18	1.752	1.10		1.747		
	T 27	2.70	1.847	2.65		1.823		
	DT 20	3.64	1.638	1.13		1.641		
3	T 3	3.17	1.702	4.82		1.700		
	T 1	3.58	1.655	1.48		1.654		
	reference	3.73	1.702	8.15	χ^2 too high			
4	DPT 28	2.93	1.818	1.01		1.824		
	DPT 27	5.14	1.983	0.35		1.994		
	reference	1.73	1.822	19.17	χ^2 too high			
5	DPT 29	2.84	1.832	2.46		1.837	+0.139	
	DT 21	2.74	1.432	5.95		1.187	−0.511	(log ED_{50} vaccine) − (log ED_{50} reference) too low
	reference	4.57	1.689	0.41		1.698		
6	reference	3.66	1.778	0.51		1.779		
7	DT 1	3.72	1.748	1.75		1.749	+0.068	
	DPT 30	3.82	1.799	0.92		1.806	+0.125	
	DT 22	3.48	1.550	0.61		1.536	−0.145	
	reference	4.34	1.679	0.28		1.681		
8	DT 3	2.74	1.578	3.60		1.578	−0.073	
	reference	3.82	1.648	0.78		1.651		
9	T 27	3.42	1.781	2.12		1.780	−0.121	
	T 29	5.96	1.735	0.64	b too high			
	DT 20	2.73	1.632	3.67		1.641	−0.260	
	reference	4.13	1.886	1.28		1.901		
10	T 30	3.39	1.640	3.17		1.618	−0.070	
	reference	3.91	1.686	0.91		1.688		
11	DPT 31	2.84	1.879	3.44		1.865	+0.216	
	DT 23	3.44	1.574	0.48		1.575	−0.074	
	T 30	3.97	1.572	1.71		1.573	−0.076	
	reference	3.72	1.651	0.50		1.649		
12	DPT 31	4.64	1.996	5.33		2.002	+0.319	
	T 29	4.51	1.668	1.48		1.620	−0.063	
	DT 20	3.62	1.605	1.10		1.583	−0.100	
	reference	3.84	1.680	0.72		1.683		
13	DPT 31	2.14	2.027	10.83	χ^2 too high			
	T 29	2.66	1.604	3.12		1.602		
	DT 20	3.02	1.677	2.31		1.673		
	reference	3.78	1.726	8.19	χ^2 too high			
14	DPT 31	2.58	2.006	2.62		1.993	+0.361	
	T 29	3.43	1.737	0.23		1.736	+0.104	
	DT 20	3.51	1.600	2.22		1.600	−0.032	
	reference	3.38	1.633	4.46		1.632		
15	DPT 31	3.68	2.074	0.56		2.075	+0.231	
	T 29	2.84	1.793	0.92		1.774	−0.070	
	DT 20	2.80	1.686	1.26		1.681	−0.163	
	reference	2.95	1.861	5.28		1.844		

others, this experiment will have to be repeated. (In most of the cases so far observed by us, the deviation was due to the fact that the series of dilutions which we had chosen for the vaccine was not very suitable.)

When, however, the object is to arrive at certain norms based on experiments of unquestionable accuracy, vaccines showing greatly deviating values of b and/or χ^2 cannot be used for the statistical analysis. In some of our experiments, the χ^2 value found was indeed too high and these were not included in the analysis. For the reference vaccine and for the different vaccines that we examined, the mean slope b and the standard deviation (" S between b's ") were calculated separately. The b values of a number of vaccines proved to be significantly larger or smaller than the others (according to the test for outlying observations proposed by Grubbs (1950)). For the reasons stated above, these vaccines were also excluded from further analysis.

Table 8 gives the mean slope and standard deviation found for the remaining vaccines. The value of " S between b's " is significantly smaller than the S_b estimated for each probit line, a result that can only be attributed to chance.

TABLE 8. MEAN SLOPE OF THE PROBIT LINES (b VALUES) AND STANDARD DEVIATIONS (" S BETWEEN b's ") FOR VARIOUS VACCINES

Vaccine	b value	" S between b's "	Number of tests
Reference	3.8	0.47	11
DT and T	3.3	0.94	8
DPT	3.6	0.49	23
All vaccines	3.5	0.8	42

An analysis of variance shows that there is no significant difference in slope between the probit lines for the three groups of vaccines shown in Table 8 ($P=0.1$). For the calculation of log ED_{50}, therefore, a mean slope $b=3.5$ may be used, with S equal to 0.8. The log ED_{50} values calculated using this slope—which differ very little from the values found from the slopes of the individual lines—are given in column 7 of Table 7.

In each of the 15 experiments except experiment No. 2, the various vaccines were examined side-by-side with the reference vaccine, and the results were corrected by deducting the corresponding log ED_{50} of the reference vaccine from the log ED_{50} of the vaccine under test. The calculation of such corrected values was possible in 10 experiments, yielding altogether 21 values. One of these (Table 7, experiment No. 5) diverged widely from the others and was omitted from the statistical evaluation. After making due allowance for repetition of vaccines, we could now estimate the

standard deviation of log ED_{50} " within the vaccines " and the standard deviation " between the vaccines " (S_t) (Table 9). At the same time, the standard deviation " within the vaccines " was calculated from the standard deviation found for each probit line. The latter value (S) worked out at 0.051. Therefore the standard deviation of the difference between two log ED_{50} values is $\sqrt{2S^2} = 0.072$.

As only three DPT vaccines were included in our investigation, the figures for all the vaccines collectively can only be considered as provisional (Table 9, column 4). If any given vaccine is compared with the reference vaccine in one single experiment, the standard deviation of (log ED_{50} vaccine)-(log ED_{50} reference) $= \sqrt{S_t^2 + S^2} = 0.088$.

TABLE 9. STANDARD DEVIATIONS OF (LOG ED_{50} VACCINE)-(LOG ED_{50} REFERENCE) BETWEEN AND WITHIN THE VACCINES EXAMINED

	DT and T vaccines	DPT vaccines	All vaccines
Standard deviation " between the vaccines " = S_t	0.036 (9)*	0.032 (3)*	0.050 (12)*
Standard deviation " within the vaccines " = S	0.089 (7)*	—	—
Standard deviation calculated for each probit line	—	—	0.072 (48)*

* The figures in brackets indicate the number of comparisons made.

The mean value of (log ED_{50} vaccine)-(log ED_{50} reference) for T and DT vaccines and for DPT vaccines is given in Table 10, together with the 95% confidence limits.

TABLE 10. MEAN VALUES OF (LOG ED_{50} VACCINE)-(LOG ED_{50} REFERENCE), ED_{50}VACCINE/ED_{50}REFERENCE, AND RELATIVE POTENCIES FOR T AND DT VACCINES AND FOR DPT VACCINES, WITH THE 95% CONFIDENCE LIMITS

	(log ED_{50} vaccine)-(log ED_{50} reference)	95% confidence limits	$\frac{ED_{50}\ vacc.}{ED_{50}\ ref.}$	95% confidence limits	Rel. potency $\frac{ED_{50}\ ref.}{ED_{50}\ vacc.}$	95% confidence limits
T and DT vaccines	0.0875	−0.0885 to 0.2635	1.22	0.81 to 1.83	0.82	0.55 to 1.24
DPT vaccines	−0.2319	−0.4079 to −0.0559	0.59	0.39 to 0.88	1.70	1.14 to 2.5

The relative potency in comparison with the reference vaccine is found by calculating the ratio ED_{50} reference/ED_{50} vaccine. The values of this ratio are also given in Table 10.

Assuming the experiment to have been successful (χ^2 value < 6 and slope of the probit line between 1.9 and 5.1) it may be expected that in 19 out of 20 cases the potency of T and DT vaccines, or of DPT vaccines, will be between the values given in Table 10. Deviations in either direction, especially if regular, are an indication that an undetected change has crept into the process of production—in other words, that the " consistency " has been disturbed.

Discussion

The potency of each fresh batch of a vaccine cannot be tested otherwise than in animals, as it is naturally impracticable to test it in man every time.

The validity of this procedure presupposes a correlation between the potency as determined in the animal test and that in man. In a purely qualitative sense, this is indeed generally the case. Such a correlation was shown to exist for pertussis vaccine in the second field trial carried out by the Medical Research Council of Great Britain (1956). In consequence of the results obtained, Armitage & Perry (1957) recently formulated minimum requirements to which this vaccine must conform in the mouse-protection test. Greenberg & Benoit (1956) showed that there is a correlation between the results obtained by the challenge method for diphtheria and tetanus vaccines in the guinea-pig and the potency in man.

In an extensive investigation, Ipsen (1953) showed that a correlation existed between the properties of four batches of $Al(OH)_3$-adsorbed tetanus toxoid, when tested on mice, guinea-pigs and man. Quantitatively, however, there was a statistically significant difference between the different species.

Thus, the use of different species for potency assays does not always yield comparable results. According to Prigge (1954) in Germany and Scheibel (1957) in Denmark, the standard tetanus toxoid adopted by the WHO Expert Committee on Biological Standardization in 1952 gives log-dose-response lines in the guinea-pig test which run parallel to those of adsorbed vaccines. It is only the ED_{50} value that changes as a result of the adjuvant effect of the adsorbent. Both investigators used pure-bred animals for their experiments.

Results in the mouse, a much cheaper laboratory animal, are far more doubtful. Greenberg (1953), when testing the International Standard for tetanus toxoid in mice, obtained irregular results which could not be made to fit the mathematical model. The non-adsorbed vaccine immunized poorly. More recently Barr et al. (1957) also pointed out that the mouse is comparatively insensitive to fluid tetanus toxoid as an immunizing agent. This is confirmed by our experiments (Table 1). Moreover, as the slope of the probit line for the International Standard vaccine in our experiments was found to be very shallow (< 1), this vaccine cannot be used as a means of estimating the potency of adsorbed vaccines in mice.

The results with adsorbed toxoids proved to be much more favourable (mean value of the slope approximately 3.5). One of the series of $AlPO_4$-adsorbed tetanus toxoids (No. 25) was selected as a reference preparation. It was found that with this vaccine very constant ED_{50} values can be obtained in mice (Table 2). However, the χ^2 values of the calculated probit lines turned out to be too high more often than one would have expected (95% confidence limit for 2 degrees of freedom > 6). This may have been due to the set-up of our experiment, in which 4 doses of vaccine were used. Consequently extreme values occurred fairly often (0%-5% or 95%-100% survival) and because of this the results do not fit very well into the mathematical model. On the other hand, this discrepancy may be caused by a biological deviation from the model. Jerne & Maaløe (1949) have pointed out that theoretically with higher doses the dose-response curve will approach an asymptote. Should this be the case, a deviation from the model may be expected on biological grounds.

The reference vaccine is very stable at 4°C. An " accelerated degradation test " (Jerne & Perry, 1956) gave some indication of this. At 4°C the 5% degradation time, found by extrapolation, is more than 70 000 years (Table 3, Fig. 1). In our laboratory, the vaccines containing a tetanus component are all adsorbed on $AlPO_4$ (3 mg/ml, i.e., 0.66 mg Al/ml). This quantity of $AlPO_4$ is still not the most favourable. As shown in Table 4, a further increase in the quantity of $AlPO_4$ may result in even greater potency. We know from experience, however, that two injections of 0.5 ml of our vaccines, with an interval of one month between them, produce good serological immunity in man. The use of larger quantities of $AlPO_4$ increases the risk of local reactions and probably of general reactions as well.

If the $AlPO_4$-adsorbed toxoid is to be suitable for use as a reference vaccine it is important that the slopes of the log-dose-response lines should also run parallel to those obtained using other adsorbents, or other quantities of adsorbent. As Table 4 shows, this did, in fact, prove to be the case for different quantities of $AlPO_4$ and $Al(OH)_3$. The quantitative differences which we found between $AlPO_4$-adsorbed and $Al(OH)_3$-adsorbed toxoids, and which have also been described by Levine et al. (1955), will not be discussed here. We believe that the use of $AlPO_4$ has simplified our production and has thus resulted in greater reproducibility.

In view of the favourable results obtained with the reference vaccine, we decided to use the mouse as laboratory animal for the comparison of our adsorbed vaccines.

In the second part of the investigation, we tried to obtain an impression of the reproducibility of the immunizing potency (the " consistency ") of our vaccines, the 95% confidence limits around the mean potency—expressed in (log ED_{50} vaccine)-(log ED_{50} reference)—being taken as a measure of this. A distinction was made between DT-vaccines and T-vaccines on

the one hand and DPT-vaccines on the other, the pertussis component having a strong adjuvant effect, as described by Levine & Stone (1954) among others. This effect had to be taken into account, because a vaccine that does *not* show this effect may have a poor tetanus component. However, the mean effect of the DPT vaccines was calculated from only 3 series, so that the result (Table 10) cannot be regarded as definitive.

The 95% confidence limits around (log ED_{50} vaccine)-(log ED_{50} reference) are determined by two factors:

(*a*) fluctuations caused by differences in potency between vaccines;

(*b*) fluctuations caused by experimental irregularities.

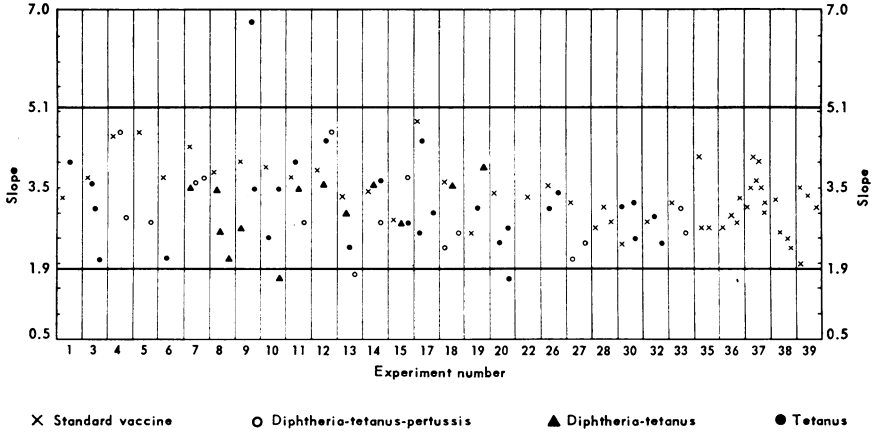
These limits of confidence are given in Table 10. For the present, we have decided to accept a vaccine that comes within the 95% confidence interval. If the potency falls between the 95% and the 99% confidence interval, the experiment is repeated, the number of mice being doubled. A vaccine the potency of which is found to be outside the 99% confidence interval is rejected if the potency is on the *low* side. If, however, the immunizing effect is too high, an inquiry will have to be made into the cause of this unexpected improvement in quality.

Although, theoretically, an inferior vaccine might still come within the 95% interval and thus be wrongly accepted, the risk is considerably reduced by the fact that reliance may be placed on the "consistency". When a large number of lots of a vaccine with a certain average potency and standard deviation have been prepared, the chance of one lot suddenly turning out to be very bad is extremely slight, and it is highly improbable that, if this did occur, that particular lot would also happen to show a misleadingly good result. Reliance on " consistency " is therefore supported by experience in the production of the vaccine, which enables stricter standards to be applied than would have been possible in formulating rejection criteria in general. This reliance on " consistency " is not unusual in biological control methods. In the case of poliomyelitis vaccine, several successive lots of vaccine that do not contain any living virus must have been produced before permission is given to distribute it for use.

The results of our experiments prove that the mouse is a very suitable laboratory animal for the comparison of adsorbed tetanus toxoids. As the mouse is comparatively cheap and does not take up much room, its use could greatly simplify the potency assay of adsorbed tetanus toxoids in many laboratories. But first an adsorbed vaccine will have to be adopted as the International Standard.

We are fully aware that the confidence intervals found in our Institute are only valid for *our* vaccines and that they depend, moreover, on the conditions of our own experiments. For instance, the slope of the log-dose-response line seems to vary for different strains of mice (Ipsen et al., 1953). Besides, the validity of the minimum requirements which we have formulated

FIG. 2. "QUALITY CONTROL CHART" FOR b-VALUES



for our products will also have to be tested in man. The differences found between the various series in the mouse test will probably not lead to measurable differences in man, as the difference in potency between the series of the best and of the worst quality appears to be very slight. Furthermore, it is not impossible that an investigation of the effect on man may lead to a reduction in the quantity of tetanus toxoid in the DPT-vaccines, since the pertussis component also has an adjuvant effect in man.

Finally we should like to draw attention to the use of " quality control " charts. After a series of experiments, a small but recurring deviation may show a systematic " trend ", which, though still within the confidence interval, strikes the eye. In this way, one may be put on the track of small changes in the method of production or the method of assay. In Fig. 2, an example is given of such a "quality control" chart for the b-values (slope of the probit line). The figure 3.5, also given in Table 8, was taken as a mean value for the slope, with a 95% confidence interval ranging from 1.9 to 5.1. Although a value exceeding these limits was found only four times in 103 measurements (expected occurrence 5/103), one is struck by the fact that especially in the later tests too few higher values appear to have been found.

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RÉSUMÉ

Il existe actuellement un seul étalon international d'anatoxine tétanique — anatoxine simple — qui permet l'étalonnage des anatoxines simples et des anatoxines adsorbées, car les courbes log dose-réponse pour ces deux types d'anatoxines sont parallèles chez le cobaye, qui est l'animal utilisé pour l'essai biologique de ces substances.

La souris, qui serait préférable comme animal d'essai en raison de son moindre coût et de la facilité de son élevage, ne peut être employée, parce que l'anatoxine simple n'a guère de pouvoir immunisant pour cet animal et que les courbes dose-réponse sont très peu inclinées.

Les auteurs ont repris ce problème et cherché à établir le pouvoir immunisant pour la souris de préparations d'anatoxine adsorbée. Ils ont constaté que l'une de leurs préparations d'anatoxine adsorbée sur $AlPO_4$ donnait des résultats satisfaisants et uniformes, et qu'elle se montrait très stable à l'épreuve de dégradation accélérée par la température. De plus, les courbes obtenues pour l'anatoxine adsorbée sur une substance différente ($Al(OH)_3$) ou sur des quantités différentes d'adsorbants ($AlPO_4$ et $Al(OH)_3$) étaient parallèles à celle obtenue avec l'anatoxine adsorbée sur $AlPO_4$. Ils ont donc utilisé cette préparation comme référence de laboratoire et adopté la souris comme animal d'épreuve.

Dans la seconde partie de leur travail, les auteurs ont mis à l'épreuve sur la souris, par l'intermédiaire de cette préparation de référence, la « constance » et la régularité de l'activité de divers lots d'anatoxine tétanique, les uns en combinaison avec l'anatoxine diphtérique, les autres avec l'anatoxine diphtérique et le vaccin anticoquelucheux. Ils décrivent en détail les résultats d'essais comparatifs de ces vaccins avec leur préparation de référence et indiquent les limites de sécurité entre lesquelles un vaccin peut être accepté.

Les résultats de tous ces essais montrent que la souris peut convenir à la comparaison de l'activité des anatoxines tétaniques adsorbées, à condition que l'on dispose d'une préparation de référence d'anatoxine adsorbée.

Les recherches se poursuivent en vue de déterminer le rapport entre les résultats obtenus sur la souris et ceux auxquels on peut s'attendre chez l'homme.

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