Influence of Mouse Strain on the Assayed Potency (Unitage) of Tetanus Toxoid

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The assayed potency of an adsorbed tetanus toxoid C2, relative to the international standard for tetanus toxoid (adsorbed), varied significantly with the use of different strains of mice. The unitage was highest with NIH mice, and it was not significantly different from that with CFW mice. With CDF, and BALB/c mice, however, the assayed potency was significantly lower than with NIH mice. C3H mice failed to respond to the dose range of the toxoids employed with the other four strains. The significance of the influence of the mouse strain on the designation of a prescribed unit requirement for tetanus toxoid, adsorbed, relative to human efficacy is discussed.

The designation of an International Standard for Tetanus Toxoid (Adsorbed) (38) has provided a basis by which comparisons of the quantitatively assayed potency of tetanus toxoid within and between laboratories may be made, as well as comparisons of the human responses to toxoids of titrated unitage. The use of a quantitative test for tetanus toxoids was proposed more than 20 years ago (12, 21, 23), and such an assay has been used by a number of laboratories (3, 9, 13, 14, 15, 18, 29, 33, 34). The United States has continued to prescribe a qualitative test (U.S. Department of Health, Education and Welfare, Minimum Requirements: Tetanus Toxoids, December 15, 1952). The U.S. toxoids have provided good protection in man (24). Nevertheless, only with quantitatively assayed toxoids would it be possible to compare the immunogenicity of different preparations and their unit relationship to primary and booster response and to duration of protective antitoxin levels in man. Recently we reported that the duration of protective antitoxin levels following primary immunization with plain and adsorbed toxoids was related directly to the unitage of the toxoids (32). This observation needs to be examined with the use of other toxoid preparations.

The guinea pig has been the most widely used animal for potency assay of tetanus toxoid (9, 17, 34); however, mice also have been used with apparent success (3, 12, 18, 19), and the WHO Expert Committee on Biological Standardization indicated that both guinea pigs and mice were suitable for use with the international standard (adsorbed) (38). Koerber and Mook (23) and Ipsen (18) found a wide variation in immunizability among different strains of mice, but they considered that the inclusion of a reference toxoid would control the variability. Wada et al. found different relative potencies in different strains of mice (37) immunized with plain toxoids, and Csizmas [cited by Pittman (31)] also found a variable response of mouse strains to tetanus toxoid. With pertussis and typhoid vaccines it has been shown that mouse strains not only vary in immunizability, but that the potency obtained by titration in such strains differs significantly (5, 31). This paper shows that the potency of a tetanus toxoid, adsorbed, assayed with the use of different strains of mice, varied significantly in international units (IU) per milliliter.

MATERIALS AND METHODS

Mice. Mice of the strains N: NIH (SW) formerly identified as NIH BX5, N:CFW, C3H/HeN, BALB/c AnN, and CDF, (BALB/c AnN × DBA/2N g) weighing 13 to 15 g were obtained from the Rabbit and Rodent Production Section, Division of Research Services, National Institutes of Health (NIH). For brevity, the strains are designated in this paper as NIH, CFW, C3H, BALB/c, and CDF1, respectively. Only female mice were used, except in one experiment with the C3H mice equal numbers of male and female mice were used. The mice were randomly distributed in test groups of 16 per cage, except the male and female C3H mice were caged separately in groups of 8, and in one assay with CDF1 mice only 10 were available for each group.
Toxoids. The international standard tetanus toxoid (adsorbed) was dried and contained 0.3 mg of AlCl₃ [Al(OH)₃] per 10 Lf (limit of flocculation) of toxoid (32) and 0.6667 mg was the equivalent of 1 IU (39). Tetanus toxoid C₂ which was used in the New Guinea study on the prevention of neonatal tetanus (27) had been held at 4 °C in the liquid form. It contained 1.1 mg of AlCl₃ (5.0 mg of AlPO₄) and 10 Lf/ml. Potency titrated in 1965 with the use of NIH mice was 251 IU/ml (32). Each toxoid was diluted in 0.85% NaCl solution just before injection of the mice.

Tetanus toxin. Tetanustoxoid T₁, dried, was reconstituted and diluted in 0.067 M phosphate-buffered saline, at pH 7.4, containing 0.2% gelatin (PBGs) to provide 24 μg in 0.5 ml for the challenge dose of approximately 40 minimum lethal doses (MLD). The MLD was determined in preliminary titrations using the different mouse strains. A control titration was included in each potency assay. The method was as previously described (1). Two to four mice were injected per dose and observed for 120 hr.

Potency assay. The method was the same as we used in the collaborative assay of the tetanus toxoid preparation which later was designated as the international standard for tetanus toxoid (adsorbed) (38). This method was similar to the assays described by Cohen et al. (3) and Ikić (15). The mice were injected subcutaneously in the nape of the neck with a single dose of 0.5 ml of one of four or five twofold dilutions of the toxoids. After an interval of 3 weeks, they were inoculated in the right inguinal fold with the challenge dose of toxin, and survivals were recorded at 120 hr. Three experiments were performed. The NIH mice were included in each experiment, and mice of each of the other strains were included in two of the three experiments.

Statistical analyses. The amount of toxoid which was effective in protecting 50% of mice (ED₅₀), the 95% confidence limits of the ED₅₀, and the slope of the dose response curve were calculated by use of the probit method of analysis (7). These values were determined for each individual assay and for the combined assays of each toxoid for each mouse strain. The potency (IU) of toxoid C₂ as assayed with each mouse strain was estimated from the ratio of the ED₅₀ of the standard to the ED₅₀ of C₂ for the particular strain of mouse. For the NIH mice, combined test values were calculated for experiments 1 and 2 and experiments 2 and 3 to provide values for comparison with the combined test values for the different strains of mice.

RESULTS

Titration of MLD of challenge toxin for the different strains of mice. Two preliminary titrations using each strain of mouse, except BALB/c, indicated that the MLD was 0.6 ± 0.1 μg for each strain. Based on these results, 24 μg of toxin was selected to provide a challenge dose of approximately 40 MLD. The estimate of 0.6 μg per MLD was confirmed in the individual potency assays as follows: in five of seven assays with NIH mice, in three of five assays with CFW mice, in three of four assays with CDF₁ mice, and in two of four assays with C3H mice. With BALB/c mice the MLD was 0.7 μg in the two assays. Table 1 gives the combined results of all titrations and shows that there was no significant difference in the susceptibility of the five strains of mice to the challenge toxin T₁.

Unitage of tetanus toxoid C₂ assayed with the different strains of mice. (i) NIH mice. Table 2 gives the results of the three potency assays and the calculated values of the individual and the combined assays of experiments 1 and 2 and experiments 2 and 3. The two estimates of potency of C₂ were 231 and 190 IU/ml as determined by the respective combined tests. The latter estimate was influenced by a value of 158 IU obtained in experiment 3, which was the lowest unitage we have obtained in seven other assays (unpublished data).

(ii) CFW mice. The potency of toxoid C₂ assayed with use of CFW mice was 185 IU/ml (Table 3). It was not significantly different from the estimates obtained with NIH mice.

(iii) CDF, mice. The potency of toxoid C₂ assayed with CDF₁ mice was 142 IU/ml (Table 4). This value was significantly lower than the 231 IU/ml obtained with NIH mice tested concurrently (experiments 1 and 2, Table 2; p = 0.05).

(iv) BALB/c mice. The potency of C₂ assayed with BALB/c mice was only 105 IU/ml (Table 5). This value was significantly lower than the value obtained with NIH mice which were tested concurrently (experiments 2 and 3, Table 2; p = 0.01). Furthermore, the potency

| Table 1. Minimum lethal dose of the challenge toxin for five strains of mice |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Toxin T₁ (μg)  | NIH             | CFW             | CDF₁            | C3H             | BALB/c          |
|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| 0.85           | 27/28           | 20/20           | 14/14           | 13/14           | 8/8             |
| 0.80           | 26/28           | 20/20           | 14/14           | 14/14           | 7/8             |
| 0.75           | 28/28           | 18/20           | 14/14           | 14/14           | 8/8             |
| 0.70           | 28/28           | 18/20           | 14/14           | 14/14           | 8/8             |
| 0.60           | 28/28           | 16/20           | 14/14           | 14/14           | 6/8             |
| 0.50           | 10/24           | 4/16            | 8/10            | 7/10            | 6/8             |
| 0.40           | 0/24            | 1/16            | 4/10            | 1/10            | 2/8             |
| 0.30           | 0/24            | 0/16            | 0/10            | 0/10            | 0/8             |

*Numbers of tests with mouse strains NIH, CFW, CDF₁, C3H, and BALB/c were 7, 5, 4, 4, and 2 respectively.
TABLE 2. Potency of toxoid C2: NIH mice

| Standard (IU) | Survivors/16 injected | C₄ (ml) | Survivors/16 injected |
|--------------|-----------------------|---------|-----------------------|
|              | Expt 1 | Expt 2 | Expt 3 | Expt 1 | Expt 2 | Expt 3 |
| 3.0          | 15     | 16     | 16     | 0.02   | 16     | 16     | 16     |
| 1.5          | 12     | 10     | 13     | 0.01   | 16     | 13     | 14     |
| 0.75         | 6      | 3      | 4      | 0.005  | 10     | 9      | 7      |
| 0.375        | 1      | 0      | 2      | 0.0025 | 2      | 1      | 0      |
| 0.187        | 0      | 0      | 0      | 0.00125| 0      | 0      |        |

| Determination | Assay values (IU) | Assay values (ml) |
|--------------|-------------------|-------------------|
|              | Expt 1 | Expt 2 | Expt 3 | Expt 1 | Expt 2 | Expt 3 |
| ED₅₀         | 0.9831 | 1.1989 | 0.9143 | 0.0042 | 0.0052 | 0.0058 |
| UCL          | 1.2680 | 1.4746 | 1.1580 | 0.0051 | 0.0067 | 0.0070 |
| LCL          | 0.7622 | 0.9748 | 0.7219 | 0.0034 | 0.0041 | 0.0047 |
| Slope*       | 3.4835 | 5.1700 | 3.9783 | 5.7385 | 4.0684 | 5.5400 |
| IU/ml        |        |        |        | 234.0  | 231.0  | 158.0  |

| Determination | Combined expt 1 and 2 | Combined expt 2 and 3 |
|--------------|-----------------------|-----------------------|
|              | Standard (IU) | C₄ (ml) | Standard (IU) | C₄ (ml) |
| ED₅₀         | 1.0841     | 0.0047  | 1.0443     | 0.0055  |
| UCL          | 1.2814     | 0.0055  | 1.2275     | 0.0064  |
| LCL          | 0.9172     | 0.0040  | 0.8884     | 0.0047  |
| Slope*       | 3.9997     | 4.5102  | 4.2523     | 4.6600  |
| IU/ml        | 231.0      | 190.0   |            |        |

* Abbreviations: ED₅₀, median effective dose; UCL, upper confidence limit; LCL, lower confidence limit; IU, international unit.

b Slope is a statistical term; therefore values are not given in units or milliliters.

TABLE 3. Potency of toxoid C2: CFW mice

| Standard (IU) | Survivors/16 injected | C₄ (ml) | Survivors/16 injected |
|--------------|-----------------------|---------|-----------------------|
|              | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| 3.0          | 16     | 16     | 0.02   | 16     | 16     |
| 1.5          | 15     | 14     | 0.01   | 16     | 15     |
| 0.75         | 8      | 2      | 0.005  | 10     | 6      |
| 0.375        | 1      | 0      | 0.0025 | 2      | 1      |
| 0.187        | 0      | 0      | 0.00125| 0      |        |

| Determination | Assay values (IU) | Assay values (ml) |
|--------------|-------------------|-------------------|
|              | Expt 1 | Expt 2 | Combined | Expt 1 | Expt 2 | Combined |
| ED₅₀         | 0.7500 | 1.0607 | 0.8859 | 0.0042 | 0.0054 | 0.0048 |
| UCL          | 0.9222 | 1.2570 | 1.0204 | 0.0051 | 0.0067 | 0.0055 |
| LCL          | 0.6100 | 0.8950 | 0.7692 | 0.0034 | 0.0041 | 0.0041 |
| Slope*       | 5.1764 | 7.6922 | 5.5331 | 5.7384 | 5.2064 | 5.1573 |
| IU/ml        |        |        |        | 179.0  | 196.0  | 185.0   |

* For abbreviations, see Table 2.

b See footnote b, Table 2.
Table 4. Potency of toxoid C2: CDFS5

| Standard (IU) | Survivors/10 injected (Expt 1) | Survivors/16 injected (Expt 2) | C4 (ml) | Survivors/10 injected (Expt 1) | Survivors/16 injected (Expt 2) |
|---------------|-------------------------------|-------------------------------|---------|-------------------------------|-------------------------------|
| 3.0           | 10                            | 16                            | 0.02    | 10                            | 16                            |
| 1.5           | 10                            | 12                            | 0.01    | 7                             | 12                            |
| 0.75          | 4                             | 6                             | 0.005   | 3                             | 9                             |
| 0.375         | 2                             | 0                             | 0.0025  | 1                             | 0                             |
| 0.187         | 0                             | 0                             | 0.00125 | 0                             | 0                             |

**Assay values (IU)**

| Determination | Expt 1 | Expt 2 | Combined | Expt 1 | Expt 2 | Combined |
|---------------|--------|--------|----------|--------|--------|----------|
| ED50          | 0.6841 | 0.9786 | 0.8524   | 0.0065 | 0.0058 | 0.0060   |
| UCL           | 0.9027 | 1.2131 | 1.0170   | 0.0089 | 0.0073 | 0.0073   |
| LCL           | 0.5184 | 0.7894 | 0.7145   | 0.0048 | 0.0046 | 0.0050   |
| Slope*        | 4.5987 | 4.7905 | 4.3704   | 3.7342 | 3.4117 | 3.9147   |

**Assay values (ml)**

| Determination | Expt 1 | Expt 2 | Combined | Expt 1 | Expt 2 | Combined |
|---------------|--------|--------|----------|--------|--------|----------|
| ED50          | 105.0  | 169.0  | 142.0    | 0.0065 | 0.0069 | 0.0066   |

* For abbreviations, see Table 2.

Table 5. Potency of toxoid C2: BALB/c mice

| Standard (IU) | Survivors/16 injected | C4 (ml) | Survivors/16 injected |
|---------------|-----------------------|---------|-----------------------|
|               | Expt 2 | Expt 3 | Expt 2 | Expt 3 |
| 3.0           | 15     | 16     | 16     | 16     |
| 1.5           | 16     | 12     | 13     | 10     |
| 0.75          | 6      | 7      | 6      | 4      |
| 0.375         | 6      | 3      | 0      | 2      |
| 0.187         | 0      | 2      | 0      | 0      |

**Assay values (IU)**

| Determination | Expt 2 | Expt 3 | Combined | Expt 2 | Expt 3 | Combined |
|---------------|--------|--------|----------|--------|--------|----------|
| ED50          | 0.6698 | 0.7327 | 0.6938   | 0.0063 | 0.0069 | 0.0066   |
| UCL           | 1.0590 | 0.9943 | 0.8510   | 0.0077 | 0.0090 | 0.0077   |
| LCL           | 0.4110 | 0.5400 | 0.5656   | 0.0051 | 0.0054 | 0.0056   |
| Slope*        | 3.07   | 2.56   | 2.7914   | 5.17   | 3.46   | 4.04     |

**Assay values (ml)**

| Determination | Expt 2 | Expt 3 | Combined |
|---------------|--------|--------|----------|
| ED50          | 105.0  | 106.0  | 105.0    |

* For abbreviations, see Table 2.

The comparison of the potency values of toxoid C2 summarized in Table 7 emphasizes the significant influence the mouse strain may have on the quantitative titration of the potency of a toxoid relative to the international standard, and no doubt other reference standards that might be selected. The potency of toxoid C2 appears to have been stable during the 5-year interval between the two studies. The ED50 of toxoid C2 for NIH mice was almost identical in 1970-71 and in 1965, and the ED50 for CFW mice in 1970-71 was similar. The ED50 was greater for CDF1 and BALB/c mice. Conversely, the ED50 of the standard for (v) C3H mice. The immunizing doses used successfully with the other four mouse strains failed to induce sufficient protection against the challenge to permit calculation of a potency value for toxoid C2 (Table 6).
Table 6. Potency of toxoid C₄: C3H mice

| Survivors/8 injected | C₄ (ml) | Survivors/8 injected | C₄ (ml) |
|----------------------|---------|----------------------|---------|
| Standard (IU) | | | |
| 3.0 | 2 | 0.02 | 0 | 2 | 2 |
| 1.5 | 0 | 0.01 | 0 | 0 | 0 |
| 0.75 | 0 | 0.005 | 0 | 0 | 0 |
| 0.375 | 0 | 0.0025 | 0 | 0 | 0 |
| 0.187 | 0 | 0.00125 | 0 | 0 | 0 |

* For abbreviations, see Table 2. ED₅₀ values were: standard, >3.0 IU; experiment 1, q > 0.02 ml; experiment 2, q > 0.02 ml; experiment 3, > 0.02 ml. Unitage was not calculated because dose range used did not bracket the ED₅₀.

Table 7. Summary of the influence of the mouse strain on the assayed potency of toxoid C₄ and the ED₅₀ of the standard and of toxoid C₄

| Mouse strain | Year | No. of assays | IU/ml | ED₅₀ |
|--------------|------|--------------|-------|------|
| NIH | 1970–71 | 3 | 251 | 1.2312 | 0.0049 |
| NIH | 1965 (32) | 2 | 231 | 1.072a | 0.0050a |
| CFW | 1965 | 2 | 190 | 0.8959 | 0.0048 |
| CDF | 1965 | 2 | 142a | 0.8524 | 0.0060 |
| BALB/c | 1965 | 2 | 105a | 0.6938 | 0.0069 |
| C3H | 1965 | 2 | NC | NC | NC |

* Geometric mean of the ED₅₀ of the three experiments performed with NIH mice. See Table 2 for abbreviations and the ED₅₀ values calculated from combinations of experiments 1 and 2 and of experiments 2 and 3.

* Significantly different from potency values obtained with NIH mice (p = 0.05 and 0.01, respectively).

* Unitage was not calculated because dose range used did not bracket the ED₅₀.

NIH mice was less in 1970–71 than in 1965, but not sufficiently smaller to cause a significant difference in the assayed potency value. However, with CDF, and BALB/c mice the ED₅₀ values were lower than with NIH mice. The significant difference in the potency values obtained with these strains relative to NIH mice was due to the lower ED₅₀ values of the standard for CDF1 and BALB/c mice and the higher ED₅₀ values for NIH mice. The individual ED₅₀ values obtained with the four strains of mice ranged from 0.6598 to 1.1989 IU for the standard and 0.0042 to 0.0069 ml for toxoid C₄ (Table 2–6), a difference of 82 and 62%, respectively. The slopes did not test significantly different for the toxoids with any of the four mouse strains.

**DISCUSSION**

Although an international standard for tetanus toxoid (adsorbed) was not established until 1966 (38), several workers previously had used quantitative assays for adsorbed toxoids (3, 9, 13, 14, 17, 18, 33–35). Some had indicated that mice, as well as guinea pigs, were suitable for use in this type of assay (3, 13, 18). However, a wide variation in the immunizability of mouse populations to adsorbed toxoids was noted by Ipsen (18, 20). Similar observations were made by Koerber and Mook (23) and Wada et al. (37) in the mouse assay of fluid toxoids. The necessity of including a reference to control the variability of the mice was stressed by these workers.

Cohen et al. (3) described a method for the quantitative assay of the potency of adsorbed toxoids in mice which was essentially the method used to establish the International Standard Tetanus Toxoid (Adsorbed). A similar assay was used by Hegedus et al. (13). Both of these laboratories used only one strain of mice.

In the present report we have shown that NIH, CFW, CDF₁, and BALB/c mice, but not C3H mice, can be readily immunized with adsorbed toxoids. Although the standard toxoid was included in each assay, the assayed unitage of toxoid C₄ was dependent upon the strain of mouse used for assay. The effect of the changing relationship of the ED₅₀ of the standard and toxoid C₄ was responsible for this unitage difference. Why the ED₅₀ of one toxoid should increase while that of another decreases is not understood. The most significant difference in unitage was observed between NIH and BALB/c mice, excluding the "nonresponding" C3H mice. The dose-response slopes were determined not to be significantly different between the strains.

We have reported previously that the unitage calculated for toxoid C₄ assayed with use of NIH mice was essentially the same as that calculated for toxoid C₄ assayed with use of guinea pigs (32). The present data indicate that if mice are to be used for the assay of international unitage of tetanus toxoids, the strain of mouse selected for such assays may...
be an important factor. Ikić (17) and Scheibel (34) have suggested that the minimum unitage of a toxoid should be 100 IU total dose or 100 IU/ml. If the value obtained with BALB/c mice were used, toxoid C₂ would just meet this minimum unitage, whereas if the values obtained with NIH mice were used, the toxoid would exceed by about twofold this minimum value.

A variety of antigens have been shown to induce different responses in various strains of mice (2, 4, 5, 8, 28, 30, 31, 36). Most workers indicate that these varying responses are due to genetic factors (26). The observation that the unitage of toxoid C₂ in the F₁ hybrid of the BALB/c mice, CDF₁, was intermediate between that obtained in BALB/c and the other mice is compatible with this concept. Whether the variable responses of the mouse strains reported herein are dependent on the antigen per se, or an interaction of antigen and adjuvant, has not been determined. There could be some difference in the response of different strains to the toxin challenge even though the MLD was similar for all strains. The standard and toxoid C₂ do not contain the same adjuvant nor the same Al⁺⁺ equivalent. The adsortbent for the standard was Al(OH)₃ (0.31 mg of Al⁺⁺/10 Lf), whereas for toxoid C₂ was AlPO₄ (1.1 mg of Al⁺⁺/10 Lf) (32). Cohen et al. (3) and Levine et al. (25) have indicated that toxoids with equivalent quantities of aluminum give different relative potencies in mice when AlPO₄ or Al(OH)₃ is the adjuvant. Further, Cohen et al. (3) and van Ramshorst (35) have shown that the relative potency of toxoids is influenced by the amount of adsortbent present. The effect of various quantities of adsorbents and different types of adsorbents in different mouse strains needs further examination. In addition, the effect of other antigens such as pertussis vaccine, a known adjuvant (6, 11, 22), on the potency of tetanus toxoid titrated with use of different strains of mice should be examined. Prigge [cited by Istrati et al. (21)] suggested that similar strain differences in immunogenicity of toxoids might also be expected in guinea pigs. The influence of the different variables on the laboratory assay of tetanus toxoid emphasizes the need to standardize these factors relative to the antitoxin response of man.

A quantitative test for the control of tetanus toxoids offers many advantages. Standardization of toxoids by such assays allows a comparison of the immunogenicity of different toxoids prepared in the same or different laboratories, allows for a better comparison of the immunological response in man to different toxoids, and may allow for the establishment of a minimum quantity of toxoid to be given for primary immunization or a maximum quantity to be given for a booster. In addition, it would appear that the duration of protective antitoxin titers may be directly related to the unitage of a toxoid (10, 32). The validity of comparisons of human responses to toxoids assayed by the use of different strains of mice becomes questionable. The low incidence of tetanus in persons immunized with toxoids which have met the current potency requirements (Minimum Requirements: Tetanus Toxoid) (24) indicates that potency of products now in use has been adequate. Therefore, it appears that prior to prescribing a quantitative assay in the United States for adsorbed toxoids, the relationship between assayed unitage and the present requirements of the induction of 2 units of antitoxin/ml in guinea pigs should be determined.

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