

Original Article

Attempt to Curtail the Observation Period of Mice in the Tetanus Vaccine Potency Tests

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SUMMARY: Curtailing the observation of mice challenged with tetanus toxin in potency test of tetanus vaccine would reduce the agony of mice from spastic paralysis. From the viewpoint of animal welfare, we investigated the feasibility of this measure. The potencies of 85 lots of vaccine obtained on the 4th day after challenge were compared with those obtained on the 7th day. No significant difference was found ($P = 0.05$), indicating that the observation period could be curtailed from 7 days to 4 days without impairing the assessment of the vaccine's potency.

INTRODUCTION

Tetanus vaccines are used in many countries, each employing their own domestic standard to ensure the efficacy of the vaccine. The toxin challenge is the most convenient method for estimation of tetanus vaccine potency. In this method, mice immunized with the vaccine are challenged with tetanus toxin. The mice that have produced sufficient amount of anti-tetanus antibody by vaccination will not develop spastic paralysis, whereas those that have not been endowed with sufficient antibody will develop paralysis and die.

There is increasing controversy over the issue of animal welfare, in light of which it seems desirable to avoid any unnecessary agony of mice. Ideally, a tetanus vaccine potency testing method that would not use experimental animals will eventually be developed. At present, methods based on immunological assay are being considered as a possible alternative for the tetanus toxin challenge method (1-6). These methods are not based on the tetanus toxin-neutralizing ability but on its binding ability. In Japan, such alternative methods have not yet been accepted for use in a routine tetanus antibody measuring system.

Under these circumstances, curtailing the observation period would be one of the possible options to reduce the suffering period of mice. This report describes the results of our investigations in this area, which demonstrated that the observation period can be curtailed from 7 to 4 days without affecting the potencies.

MATERIALS AND METHODS

Tetanus vaccines: Forty-six lots of adsorbed diphtheria, tetanus, and acellular pertussis combined vaccine (DTaP), 14 lots of adsorbed diphtheria and tetanus combined toxoid vaccine (DT), and 25 lots of adsorbed tetanus toxoid vaccine (T) were used in this study.

Reference preparations: To measure the potency of DTaP,

a preparation of the Japanese reference tetanus vaccine (lot 2, 40 U/vial) was used. It was dissolved and diluted in sterile distilled water.

To measure the potency of DT and T, the Japanese standard tetanus toxoid preparation (Lot 3, 65 IU/vial) was used. It was dissolved and diluted in sterile saline.

Potency test: The potency tests were performed according to the method described in the Japanese Minimum Requirements for Biological Products (7). Several vaccine sample lots can be titrated simultaneously in one assay. Successive twofold dilutions were used to immunize mice; three doses for the reference preparations and two for the sample lots. Appropriate dilutions were employed for each sample lot and the reference preparation in order to obtain adequate dose-responses. Mice (Slc:ddY, SPF, female, body weight 22-24 g, 5 weeks old, 10 mice/group) were immunized subcutaneously, in the lumbar region, with 0.5 ml of each dilution.

At 32 days after immunization, the mice were subcutaneously challenged in the left lumbar region with 0.5 ml of a tetanus toxin solution containing approximately 200 mouse LD₅₀/ml. The time of death and the symptoms of the challenged mice were observed daily over 7 days, and this data was converted to scores according to the method of Murata et al. (8), with some modifications (Table 1). In 1952, the score method was established by Ipsen (9). The scores

Table 1. The scores assigned to the time of death and the symptoms of the challenged mice

| Symptoms | Score |
|------------------------------|-------|
| Death on 1st day | 0 |
| Death on 2nd day | 1 |
| Death on 3rd day | 2 |
| Death on 4th-7th day | 3 |
| Severe tetanus ¹⁾ | 3 |
| Mild tetanus ²⁾ | 4 |
| No tetanus ³⁾ | 8 |

¹⁾: Difficulty of moving, tonic spasm or respiratory distress.

²⁾: Local spasm of the abdominal muscle in opposite side to the site of injection, or mouse's body bends to the injected side.

³⁾: No specific symptoms.

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of this method were prescribed so that mean scores of groups of mice would have approximately linear regression when plotted versus a log dose of toxoid. The potency of the tetanus vaccine sample lot was calculated by statistical analysis of scores by using the parallel line assay method (10,11). Pathological examination, not described in the Japanese Minimum Requirements (7), was not undertaken in this study.

For confirmation of the toxicity of the challenge toxin solution used for the assay, four groups of unvaccinated mice were challenged with 0.5 ml of 1:1 (undiluted), 1:50, 1:100, and 1:200 dilutions of the challenge toxin solution (i.e., 100, 2, 1, and 0.5 LD₅₀/mice, respectively). These mice, along with the vaccinated groups, were observed daily for 7 days.

RESULTS

Comparison of scores of challenged mice on the 4th and 7th days: The potencies of 85 tetanus vaccine sample lots were determined in 3,269 mice in 28 tests. A total of 1,175 mice (35.9 %) died by the 4th day after tetanus toxin challenge. Among the mice surviving on the 4th day after the

toxin challenge, those having the same scores on the 4th and the 7th days accounted for 94.1 % (1,971/2,094 mice). In these mice, there was no statistically significant difference in the score on the 4th and that on the 7th day ($r = 0.919$, $n = 2094$) (Figure 1).

Comparison of the potencies estimated from the 4th- and 7th-day scores: The regression of 4th and 7th day potencies were $y = 0.903x + 10.257$ in the DTaP group ($n = 46$), $y = 0.960x + 5.845$ in the DT group ($n = 14$), and $y = 0.871x + 12.980$ in the T group ($n = 25$) (Figure 2). The correlation coefficients were $r = 0.964$ in the DTaP group ($n = 46$), $r = 0.975$ in the DT group ($n = 14$), $r = 0.983$ in the T group ($n = 25$), and $r = 0.973$ in the whole group ($n = 85$). No significant difference ($P = 0.05$) was observed between the 4th and 7th day among groups.

Confirmation of toxicity of the challenge toxin solution: Based on the results of the observation of the groups for confirmation of the toxicity of the challenge toxin solution, the challenge toxin solution was confirmed to contain 68.0 LD₅₀/mouse on the 4th day and 103.4 LD₅₀/mouse on the 7th day. The toxicity of the toxin solution satisfied the minimum requirement.

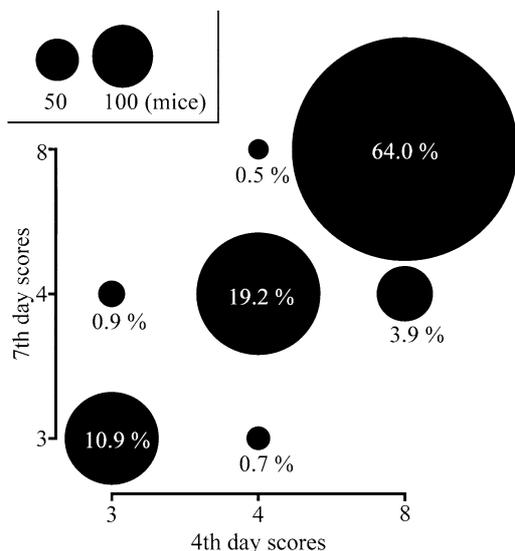


Fig. 1. Comparison of scores of mice immunized with tetanus toxoid vaccine at 4 and 7 days after toxin challenge. The square measure of a closed circle (●) indicates the number of mice with corresponding scores (see inset; the largest circle in the figure corresponds to 1,341 mice). The values in or below each circle indicate the % ratio of the number to the number of surviving mice at 4 days (2,094), rounded off to the first decimal place. Correlation coefficient: 0.919 ($n = 2094$).

DISCUSSION

To reduce the period of experimental animals' suffering, we examined the possibility of curtailing the conventional observation period used in the tetanus toxin challenge method from 7 to 4 days. Based on previous findings that the symptoms of challenged mice progress until the 6th day after the tetanus toxin challenge (12), the Japanese official procedure currently requires an observation period of 7 days, which is notably longer than the 5 days required in the WHO method (13). However, from the results described herein, symptoms on the 4th and 7th days showed no significant difference when converted to scores. The parallel line assay method (10,11) is in current use to calculate vaccine potencies. The application of this method to statistical analysis of assigned scores requires satisfying the following three conditions: (i) similar variances of average scores of mice immunized with each dose of tetanus vaccine sample lots and that of the reference preparation; (ii) linearity of dose-response curves of each sample lot and the reference preparation; and (iii) parallelism between the dose-response curves of each sample lot and the reference preparation. In this study, all three conditions were satisfied. There was no significant difference in the variances of average scores of mice immunized with each tetanus vaccine sample lot and those immunized with the reference

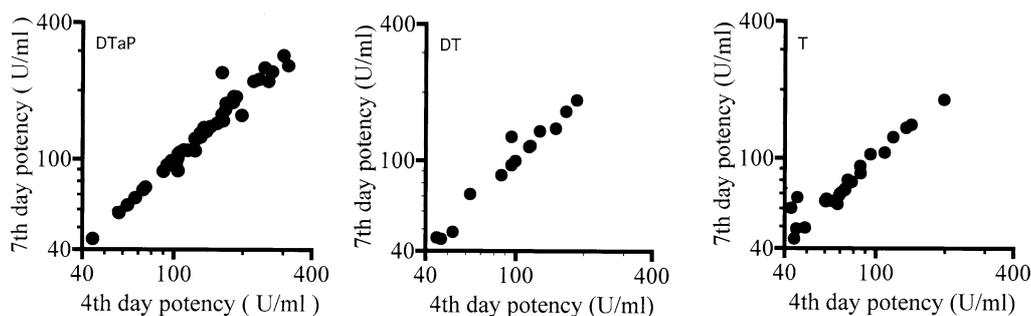


Fig. 2. Correlation of the potencies obtained from the scores on the 4th and 7th days. DTaP, DT, and T, respectively show adsorbed diphtheria, tetanus, and acellular pertussis combined vaccine, adsorbed diphtheria and tetanus combined toxoid vaccine, and adsorbed tetanus toxoid vaccine. Correlation coefficient (r) for DTaP: 0.964 ($n = 46$). r for DT: 0.975 ($n = 14$). r for T: 0.983 ($n = 25$). r for all the vaccines: 0.973 ($n = 85$).

Table 2. Common variance and coefficient of regression for reference and sample vaccines at 4 and 7 days

| Preparation | Common variance | | Regression coefficient | |
|----------------|-----------------|---------|------------------------|---------|
| | 4th day | 7th day | 4th day | 7th day |
| DTaP reference | 6.208 | 6.170 | 7.135 | 7.358 |
| DTaP vaccine | 7.591 | 7.380 | 6.631 | 6.718 |
| DT standard | 6.470 | 6.335 | 8.491 | 8.531 |
| DT vaccine | 7.116 | 7.079 | 9.257 | 9.300 |
| T standard | 6.659 | 6.282 | 7.941 | 8.005 |
| T vaccine | 8.244 | 8.208 | 9.899 | 9.546 |

DTaP, adsorbed diphtheria, tetanus, and acellular pertussis combined vaccine; DT, adsorbed diphtheria and tetanus combined toxoid vaccine; T, adsorbed tetanus toxoid vaccine; reference, Japanese national reference tetanus toxoid preparation (Lot 2, 40 U/vial); standard, Japanese national standard tetanus toxoid preparation (Lot 3, 65 IU/vial).

preparation. Based on the finding that the dose-response of each sample lot and that of the reference preparation showed no significant deviation from parallelism or linearity, it can be assumed that the assay results with a 4-day observation period are reliable. A comparison of the common variances and regression coefficients of potencies on the 4th and the 7th day indicated no statistically significant difference between the results, suggesting that the results can be considered interchangeable; the statistical parameters were essentially the same on both days (Table 2). In addition, no significant difference ($P = 0.05$) was observed between the potencies on the 4th and the 7th day for any of the three types of vaccine products (i.e., DTaP, DT, and T).

In this study, the vaccine potencies on the 3rd day were not examined. The challenge toxin solution used in this study was suitable for a 7-day observation period. The toxin solution did not have sufficiently high toxicity to show 50-200 LD₅₀/mouse on the 3rd day; the toxin solution did not satisfy the Japanese minimum requirement. The potencies on the 3rd day and the 7th day were not compared.

In conclusion, a reduction of the observation period in the tetanus toxin challenge method from 7 to 4 days is acceptable from the statistical point of view. This means that the results obtained on the 4th day can be considered to be a reliable indicator of a tetanus vaccine's potency. If widely adopted, this method will reduce animal suffering and provide significant cost benefits and labor-saving. Thus, curtailing the observation period from 7 to 4 days would be fully applicable to the toxin challenge method and adoptable in the forthcoming version of the Minimum Requirements for Biological Products of Japan.

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REFERENCES

1. World Health Organization (1997): ELISA methods for

the determination of diphtheria and tetanus antibodies. Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization. p. 135-141. WHO/BLG/95-1. World Health Organization, Geneva.

2. World Health Organization (1997): Toxin binding inhibition test as secondary method for testing the potency of vaccines containing tetanus and diphtheria toxoids. Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization. p. 142-150. WHO/BLG/95-1. World Health Organization, Geneva.

3. World Health Organization (1997): Direct ELISA as a secondary test for assessing the potency of vaccines containing tetanus toxoid. Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization. p. 151-156. WHO/BLG/95-1. World Health Organization, Geneva.

4. Nyerges, G., Virag, E. and Lutter, J. (1983): The potency testing of diphtheria and tetanus toxoids as determined by the induction of antibody in mice. J. Biol. Stand., 11, 99-103.

5. Hendriksen, C. F., van der Gun, J. W., Marsman, F. R. and Kreeftenberg, J. G. (1991): The use of the in vitro toxin binding inhibition (ToBI) test for the estimation of the potency of tetanus toxoid. Biologicals, 19, 23-29.

6. Winsnes, R., Hendriksen, C., Sesardic, D., Akkermans, A. and Daas, A. (1999): Serological assays as alternative to the Ph Eur challenge test for batch release of tetanus vaccines for human use. Dev. Biol. Stand., 101, 277-288.

7. Association of Biologicals Manufacture of Japan (1993): Minimum Requirement for Biological Products. p. 185-189. Association of Biologicals Manufacture of Japan, Tokyo.

8. Murata, R., Wada, E., Yamamoto, A. and Kubota, K. (1961): Studies on the standardation of tetanus toxoid. Jpn. J. Med. Sci. Biol., 14, 121-129.

9. Ipsen, J. (1952): The effect of environment temperature on the immune response of mice to tetanus toxoid. J. Immunol., 6, 273-283.

10. Finney, D. J. (1964): Statistical Methods in Biological Assay. 2nd ed. Charles Griffin Co., London.

11. World Health Organization (1997): Parallel line analysis. Manual of laboratory methods. Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization. p. 191-195. WHO/BLG/95-1. World Health Organization, Geneva.

12. Matuhasi, T. and Iwase, I. (1986): Report of a study group on simplification of potency test method of biological products (submitted to the Ministry of Health and Welfare on March 1986) (in Japanese).

13. World Health Organization (1997): Traditional method for testing the potency of vaccines containing adsorbed diphtheria and tetanus toxoids. Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization. p. 110-112. WHO/BLG/95-1. World Health Organization, Geneva.