THE STANDARDIZATION OF ANTIPNEUMOCOCCIC SERUM TYPES I AND II

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The mouse protection test for establishing the value of antipneumococcic serum does not give consistent results unless a large number of mice are used. Friedlander, Sobotka and Banzhaf (1) used the specific polysaccharide of the pneumococcus as an antigen for precipitation tests, to determine the potency of serum. Their findings agreed fairly well with the mouse protection test of Felton (2).

In confirming the work of Heidelberger and Avery (3), on the pneumococcus polysaccharide, we were struck by the consistent results obtained in successive precipitation tests with any one serum. At the same time, we have long been aware of the difficulty in the interpretation of the mouse protection test in judging the potency of antipneumococcic serum. We, therefore, have undertaken parallel studies on a series of antipneumococcic serums, using both the specific polysaccharide precipitation and mouse protection tests. A close agreement between the results of the two methods was found to exist.

The difficulty in the preparation of polysaccharide of the high purity obtained by Avery and Heidelberger would be a barrier to the practical routine application of the polysaccharide precipitation test. We found, however, in comparing different lots of polysaccharides of varying purity, that results were comparable with each lot.

Crude polysaccharide prepared by the following technique was found very satisfactory for use as antigen in the precipitation test:

An 8 day broth culture of the type pneumococcus is evaporated to about one-fifteenth the original volume. The material is chilled and trichloracetic acid crystals added to make a 5 per cent solution. A precipitate is formed and removed by centrifugation as soon as possible. To the clear supernatant about one and one-half volumes of alcohol are added and centrifuged. The alcoholic pre-

cipitate contains the polysaccharide which is soluble in water and is purified by repeated re-precipitations with alcohol. The final precipitation is usually done with acetone. Further purification does not seem necessary.

Polysaccharide made by this method when dried and dissolved in saline 1 to 10,000 can be satisfactorily used for antigen in the precipitation test.

The technique of the precipitation test used by us is as follows: Equal volumes of serum dilutions and a constant polysaccharide dilution (1 to 10,000) are mixed and placed in a water bath at 37°C. The water level should be not more than one-half that of the test mixture. The test is read after 2 hours. Further precipitation in higher dilutions may occur after longer incubation at 37°C., or a subsequent period on ice, but the result is not as clear cut, variations being more frequent than when the 2 hour reading is used.

The interpretation of the test is based on a comparison of the titer of the complete precipitation of a standard serum, F-146 (supplied by Felton), as containing 200 protective units* per cubic centimeter for Pneumococcus Types I and II, with that of the unknown test serum.

In repeated tests, using a solution of Type I polysaccharide of 1 to 10,000, the standard serum gave complete precipitation in a dilution of 1 to 20. On this basis the number of units for Type I of the test serum would be expressed by the following equation.

$$\frac{\text{Dilution of test serum}}{20} \times 200 = \text{Number of units of the test serum.}$$

The results of comparative mouse protection and polysaccharide precipitation tests with a portion of the many polyvalent antipneumococcic Type I and II serums, tested, are shown in Table I. Only results of tests with Type I are given. Type II values can also be tested by the polysaccharide precipitation test, the equation of calculation, however, being different as the standard serum F-146 with a protective unit value of 200 units in Type II, results in a complete precipitation at a dilution of 1:35 with the specific Type II polysaccharide used by us.

* The standard value of this serum has recently been changed by Felton after testing with the different strains of pneumococcus used by the various laboratories, to 500 units per cubic centimeter Type I and Type II.

The interpretation of the mouse protection by direct calculation for unit value according to virulence is frequently difficult. The variations and irregularities in the time of the deaths frequently make necessary a repetition of the test before results are obtained which satisfactorily check with the established value of the standard serum.

TABLE I

Type I Antipneumococcus Protective Units Estimated by the Mouse Protection and Polysaccharide Precipitation Tests

Serum	Mouse test	SSS Ppt.
F-146	200	200
7892-N	100	120
7879-A	80	80
7895-G	200	220
7899-M	100	100
7900-H	50	80
7905-I	300	300
7908-I	300	300
8081-A	40	50
8082-A	50	50
MM8843-A	600	560
MM8843-B	100	90
MM8843-C	50	20
46130	50	50
48398	400	350

* "SSS Ppt." is the designation used by Heidelberger and Avery for the "soluble specific substance"—polysaccharide responsible for the precipitate.

The unit values given in Table I are therefore calculated by direct comparison with the 200 unit standard serum F-146.

Table II shows the results obtained with the polysaccharide precipitation and mouse protection test, on using serum from consecutive bleedings from horses under immunization. The protection test used in this series was that endorsed by the Hygienic Laboratory and controlled by their standard serum. The serums are classified as *good, fair* or *poor*, according to the degree of protection, expressed in percentage, obtained in the group of mice placed on test. The ease with which one can be misled by the use of the mouse test alone is shown. For example in Serum 7892 the value of the bleeding of 2–15–30 and of

3–15–30 is more accurately determined by the polysaccharide precipitation test. The results of tests on bleedings which prove horses to be producing serum of a higher value—such as Serum 7905—are consistent with the mouse protection test. The exact status of serum from horses on the borderline of productivity is much more accurately

TABLE II

Antipneumococcic Type I

Protective Units in Consecutive Horse Bleedings

Date	SSS Ppt.	Mouse	Date	SSS Ppt.	Mouse
Serum 7879			*Serum 8083*		
1–28–30	90	Fair	12–26–29	180	Fair
2– 1–30	80	Good	1–26–30	100	Fair
2–15–30	50	Fair	2–10–30	150	Fair
3– 7–30	30	Poor	3– 7–30	80	Fair
3–31–30	30	Fair	3–15–30	100	Fair
			4– 1–30	80	Fair
Serum 7892			*Serum 8110*		
1–30–30	180	Good	12– 4–30	—	Poor
2–15–30	220	Fair	1–30–30	240	Good
3– 1–30	100	Fair	2–15–30	180	Good
3–15–30	90	Fair	3– 7–30	30	Fair
			3–22–30	50	Fair
Serum 7905			*Serum 8113*		
1–15–30	200	Good	9–25–29	—	Poor
1–30–30	300	Good	12– 4–29	—	Fair
2–15–30	280	Good	1–30–30	180	Good
3– 1–30	280	Good	2–15–30	250	Fair
			3– 1–30	280	Good

determined by the polysaccharide precipitin test, as shown in bleedings of Serums 8083, 8110 and 8113.

At present the immunization of all horses used in the production of antipneumococcic serum is followed by means of the precipitation test. Mixtures of bleedings for concentration or other purposes, are checked with the mouse protection test.

DISCUSSION

The standardization of antipneumococcus serum Types I and II has been a very difficult problem on account of the variable results obtained with the mouse protection test. The difference in the virulence of the cultures used, of Pneumococcus Types I and II makes comparison of the mouse tests of various laboratories impossible. Even within one laboratory, in which the same strain is always used, and with every care for absolute duplication of method and accuracy of technique, variations occur which often make the interpretation of the test difficult, and costly repetition necessary. This is due to the very nature of the test, susceptibility of the mouse to a virulent pneumococcus being so marked that the smallest deviation in the number of organisms injected into the test animal leads to most confusing irregularity in the time of death. Also variation in number of organisms is unavoidable by the method because of the procedure of making dilutions as a basis for dosage.

We have found that the precipitation test is practical and gives comparable results on the repetition of tests. Similar conclusions have been reached by Heidelberger, Sia, and Kendall (see the following paper (4)).

It seems highly probable that in the case of organisms elaborating specific polysaccharides this method of testing can be used for the standardization of the antiserums. We have succeeded with anti-anthrax serum, the standardization of which has been very unsatisfactory, and we are proceeding with tests of other antibacterial sera.

CONCLUSIONS

1. The mouse protection test used for the standardization of antipneumococcic serum Type I and II is very unreliable.
2. The specific polysaccharide precipitation test can be used to establish the comparative value of a test serum to a standardized serum.
3. The polysaccharide used for such tests does not have to be of the highest purity.

BIBLIOGRAPHY

1. Friedlander, M., Sobotka, H., and Banzhaf, E. J., *Jour. Exp. Med.*, 1928, **47**, 79.
2. Felton, L. D., *Jour. Inf. Dis.*, 1928, **43**, 540.
3. Heidelberger, M., and Avery, O. T., *Jour. Exp. Med.*, 1923, **38**, 73.
4. Heidelberger, M., Sia, R. H. P., and Kendall, F. E., *Jour. Exp. Med.*, 1930, **52**, 477.