

SPECIFIC PRECIPITATION AND MOUSE PROTECTION IN TYPE I ANTIPNEUMOCOCCUS SERA*

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(Received for publication, June 17, 1930)

Specific precipitation has long been known to occur in antipneumococcus sera showing marked protective power in mice (1), but it has been possible only recently to obtain indications of a parallel variation in these two properties. Thus Friedlander, Sobotka and Banzhaf (2) showed that the number of mouse protection units in certain Type I and Type II antipneumococcus sera varied in the same sense as the "precipitin index." In the preceding communication Zozaya, Boyer, and Clark (3) have shown that it is possible by means of the precipitin test to obtain within 2 hours numbers which agree excellently with the mouse protection units found in fifteen sera.

The great advantages of a rapid *in vitro* test for the potency of antipneumococcus sera over the tedious, expensive, and often uncertain mouse protection test had led the writers to search for a possible relation between specific precipitation and mouse protection in Type I antipneumococcus sera on the basis of their recent quantitative study of the reaction between the specific polysaccharide of Type III pneumococcus and its homologous purified antibody (4) as well as on the basis of almost completed data on the corresponding Type I reaction. While this work has not resulted in so simple and rapid a routine test as that of Zozaya, Boyer, and Clark, the writers feel that it establishes on an experimentally verified and theoretically reasonable basis a definite parallel between maximum specific precipitation in Type I

* The work described in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital, New York City.

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antipneumococcus sera and mouse protection. Furthermore, this parallel can be drawn with sufficient exactness to warrant the statement that in the group of widely varying sera studied under the definite empirical conditions described below, sera containing 7 mg. or more of specifically precipitable protein per cubic centimeter showed a mouse protection value of 1000 units and over. It is suggested that should these, or similar values be found by other workers to hold for sera at their disposal, a quantitative grading of Type I antipneumococcus sera on the basis of their content of specifically precipitable protein would not only be feasible, but would also be far quicker, cheaper, and more susceptible of duplication in other laboratories than the present generally accepted mouse protection values.

EXPERIMENTAL

1. *Type I Pneumococcus Specific Polysaccharide.*—The Type I “soluble specific substance” used was prepared as in a preceding paper (5). Since an ash-free preparation of highest purity is not essential, an abbreviated method is here given.

20 liters of 5-day Type I pneumococcus culture in meat infusion phosphate broth containing 0.3 per cent of glucose are autoclaved and concentrated on the water bath to a volume of 1 liter. The concentrate is precipitated with 1.4 liters of alcohol and put through the initial 3-layer separation (6). The middle layer is treated with 200 to 250 cc. of hot water until all lumps are dissolved, and the mixture is centrifuged and the precipitate washed twice with small amounts of water. The clear solution and washings are chilled and acidified strongly to Congo red with 1:1 hydrochloric acid, centrifuging off in the cold and washing with a little 0.01 normal hydrochloric acid any precipitate formed at this point. The solution, the volume of which should not exceed 500 cc., is stirred vigorously, precipitated with 1.5 liters of chilled alcohol, and allowed to stand in the cold over night. The precipitate, after centrifuging, is taken up in water, with the addition of enough sodium hydroxide to maintain alkalinity. Water is added until all of the specific substance is in solution, and the remaining trace of insoluble material is centrifuged off. The supernatant, at a volume of 100 to 125 cc., is treated with 10 gm. of sodium acetate, and when this has dissolved, is chilled, precipitated with 70 cc. of cold alcohol, allowed to stand in the cold over night, and centrifuged in the cold. The precipitate is redissolved in 200 cc. of water, the solution is centrifuged, and the supernatant acidified with glacial acetic acid to pH 3.4, at which point the specific polysaccharide flocculates readily and is centrifuged off.

The supernatant should contain very little specific substance, but if it does, the pH should be adjusted in the direction of further flocculation and the precipitates combined. The specific substance is taken up in 100 cc. of chilled 0.05 normal acetic acid, centrifuged off again, and washed in the centrifuge bottle, first with redistilled alcohol and finally with redistilled acetone. It is then filtered on a Buchner funnel, washed with redistilled acetone, and dried *in vacuo* at room temperature. The yield in a typical instance was 0.8 gm.,* containing 0.5 per cent ash. Other analytical data, calculated to the ash-free basis, were: $[\alpha]_D + 299^\circ$, N, 5.0 per cent, values practically identical with those of the best preparations previously reported (5). If larger amounts of broth are worked up it is best to repeat each precipitation.

2. *Titration of Sera by the Precipitin Method.*—If, on addition of a 1:10,000 saline solution of the specific polysaccharide to a portion of the serum, precipitation is rapid and heavy, 0.5 cc. of the serum should be used for the quantitative determination. This will suffice for all except very low grade sera, of which it is better to use 1 cc. The sera should be measured in duplicate with accurately calibrated pipettes into wide agglutination tubes (dimensions of 10 x 75 mm. have been found suitable). If 0.5 cc. serum has been used, 1 cc. saline is then added to each tube, followed by 0.5 cc. of a saline solution containing 1 mg. of the specific polysaccharide per cubic centimeter, to make a total volume of 2 cc. Ordinary uncalibrated pipettes are adequate for the saline and polysaccharide. The tubes are plugged and the contents carefully and thoroughly mixed by a rotary motion imparted by drawing the finger tips rapidly and repeatedly diagonally down the side of the tube. After the tubes have been allowed to stand 2 hours in the water bath at 37° and over night in the icebox,† the plugs are removed and the tubes centrifuged for 10 minutes at 1000 revolutions per minute, either in a refrigerating centrifuge‡ or immersed in ice-water. The supernatant liquid is then carefully drained off by inverting the tube and wiping the mouth of the tube with filter paper after a few minutes. The tubes are then placed in ice-water and the precipitates are each washed with 2 cc. of an ice-cold 1:20,000 saline solution of the specific polysaccharide, mixing the contents as before. It appears to make little difference whether or not the disc of precipitate is loosened from the bottom of the tube. After $\frac{1}{2}$ hour in the cold the tubes are again centrifuged and drained

* Since the isoelectric substance is insoluble, solutions must be made up with the aid of alkali.

† Sterile technique should be employed up to this point. In the case of low grade sera identical values are obtained if the tubes are allowed to stand in the water bath only $\frac{1}{2}$ hour, followed by $\frac{1}{2}$ hour in ice-water before proceeding. In more potent sera results a few tenths of a milligram per cubic centimeter too low are obtained by shortening the process in this way.

‡ Supplied by the International Equipment Co., Boston, Massachusetts.

as before. About 0.5 cc. of water is then added to each tube, shaking as before until the precipitate is loosened, after which the disc is dissolved by the addition of 2 drops of normal sodium hydroxide solution with rotation of the tube until the precipitate has disappeared. If the disc is allowed to stick to the glass, solution is much slower. The solution is then rinsed quantitatively into a micro-Kjeldahl flask or tube and the nitrogen determined by any standard procedure. The Pregl method, slightly modified, was used in the present work. Nitrogen found $\times 6.25$ = specifically precipitated protein.

3. *Mouse Protection Tests.*—For the biological estimation of the amount of protective antibodies contained in various samples of Type I antipneumococcus sera, the method described by Felton (7) was employed. According to this method, a *unit* (of antipneumococcus protective antibody) is defined as “that fraction of a cc. of serum which will protect against one million fatal doses of an 18-hour serum broth culture. The culture used must be of such virulence that 3 to 10 organisms when injected intraperitoneally into a mouse kill the mouse in from 36 to 48 hours.”

Briefly, the method consists of the intraperitoneal injection into white mice of 0.5 cc. of a 1:200 dilution* of an 18-hour serum broth culture of the Type I pneumococcus mixed with an equal amount of the dilution of the Type I antipneumococcus serum to be tested. The immune serum dilutions were made in sterile normal saline and the dilutions used were 1:25, 1:50, 1:100, 1:200, 1:400, 1:600, 1:800, 1:1000, 1:1200, 1:1600, no attempts being made to titrate more closely by the use of intermediate dilutions. The organism and the immune serum dilutions were taken up into a syringe and injected as soon as possible intraperitoneally into mice. For each serum dilution tested, mice were injected in triplicate, and observed for 96 hours. The highest immune serum dilution protecting two out of the three mice for a period of 96 hours was taken as the protective unitage of the serum.

The results obtained are given in Table I.

DISCUSSION

In the course of a quantitative study of the precipitin reaction between Type I pneumococcus specific polysaccharide and purified Type I pneumococcus antibody, it was found that maximum precipitation occurred when the initial concentration of specific polysac-

* Calculated to contain 500,000 lethal doses.

TABLE I
Specific Precipitation and Mouse Protection in Type I Antipneumococcus Sera

Sample No.	Source	Agglutination titer	Units found in individual mouse protection tests	Individual determinations (in duplicate) of sp. pptble. protein	Average mouse protection units	Average specifically pptble. protein
				<i>mg. per cc.</i>		<i>mg. per cc.</i>
B108C	4*	1:10	50	0.7 0.6	50	0.7
101	1 (diagnostic)	1:60	100 100 100	1.7 1.7 1.7	100	1.7
110	5 (standard)**		200	1.9	200	1.9
108B	4*	1:40	200	2.0 2.0	200	2.0
105	3 (diagnostic)	1:60	800 400 800 400 600†	3.1 3.3 3.4	600	3.3
108A	4*	1:40	600	3.8 3.8	600	3.8
107	3 (diagnostic)		800	5.0 4.8	800	4.9
103	3 (therapeutic)	1:120	1200 1000 1000 800	6.9 6.5 6.7 6.6	1000	6.7
104	2 (diagnostic)	1:160	>1200‡ 1200 >1600‡ 800	7.0 6.9 7.4 7.1 6.9	1200	7.1
102	1 (diagnostic)	>1:120	>600‡ >800‡ 1200	7.4 7.9 7.6	1200	7.6
106	3 (therapeutic)	1:160	>1200‡ >1600‡	9.7	1600	9.7

* Number 8843 in the paper by Zozaya, Boyer and Clark.

** Number F 146 in the paper by Zozaya, Boyer and Clark.

† This dilution was not used until the first four results had been obtained.

‡ Highest dilution tested.

charide was 1:2,000. Since, however, all but a small fraction of the specifically precipitated protein is carried down at one-half this concentration, the latter strength is used in the test herein given, in the interest of economy of specific substance. Similarly, the precipitate is washed with a 1:20,000 solution of the polysaccharide as this concentration is adequate to prevent appreciable dissociation of the precipitate and the nitrogen content of a solution of this concentration is too small to interfere.

It can not be claimed that the nitrogen figures obtained are more than an approximate representation of the specifically precipitable protein in the sera tested, since a single washing of the compact disc-like precipitate would scarcely remove all entrained inert serum proteins. On the other hand, washing with 1:20,000 polysaccharide solution, as is done in the test, probably fails to prevent absolutely dissociation of the precipitate formed under optimum conditions, so that these two errors tend to balance each other. Since duplicates and repeated determinations usually check, at least consistent results are readily obtained. Moreover, any number of sera may be tested at a time.

From Table I it will be seen that the sera tested, when arranged in the order of increasing specifically precipitable protein per cubic centimeter, are also found to be in the order of increasing mouse protection units. By the methods employed, 7 mg. of specifically precipitable protein per cubic centimeter corresponded roughly with a mouse protection value of 1000 units, and it is felt that an easily reproducible and readily determinable standard system of grading could be worked out along these lines.

SUMMARY

1. A rapid and simple method is given for the approximate determination of the specifically precipitable protein in Type I antipneumococcus sera.
2. It is shown that a close parallel exists between the specifically precipitable protein and the number of mouse protection units in a wide variety of Type I antipneumococcus sera.
3. Owing to the consistent results obtained and the rapidity, simplicity, and economy of the method, its use is proposed instead of the

mouse protection test as a basis for the titration of standard sera and the comparison of others with a standard.

4. A method is given for conveniently preparing highly purified specific polysaccharide of Type I pneumococcus.

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