STANDARDIZATION OF THE ANTIMENINGITIS SERUM.[1]

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The difficulties encountered in standardizing the bactericidal type of immune sera are great and have not yet been successfully mastered. They are especially great in the case of the antimeningitis serum in which bacteriolytic power cannot be utilized as a measure of strength and protective and curative effects, and the experimental Diplococcus intracellularis infections in lower animals are also unavailable. The diplococcus of meningitis is not brought to bacteriolysis by the immune serum through amboceptor and complement, and the high variability in pathogenic power of its cultures makes the results of experimental inoculations very untrustworthy. The fatal dose of cultures and of autolyzed toxines fluctuate within wide limits for animals (guinea-pigs) of the same age and weight. The attempt to estimate quantitatively the strength of the antiserum by means of complement deviation cannot be said to have been successful,[2] and in our hands has not yielded accurate or uniform results.

The study of the changes which take place, under the influence of the antiserum, in inoculated animals and in human beings suffering from epidemic cerebro-spinal meningitis indicates unmistakably that the antiserum possesses power to destroy directly the diplococcus, to neutralize a certain amount of the endotoxin yielded by the organism, and above all to bring about an increased phagocytic inclusion and digestion of the diplococcus. The first convincing proof of the manner of action of the antiserum was supplied by the studies of Flexner[3] carried out on monkeys in which an experi-

[1] Received for publication March 1, 1909.

[2] Kolle and Wassermann, *Deut. med. Woch.*, 1907, xxxii, 609.

[3] Flexner and Jobling, *Jour. of Exper. Med.*, 1908, x, 141.

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mental cerebro-spinal meningitis had been produced by direct inoculation of the spinal membranes with cultures of Diplococcus intracellularis. In brief, Flexner found that in monkeys receiving a lethal dose of the culture an inflammation quickly set in and the diplococci appeared partly free in the fluid exudate and partly within leucocytes quite as in the natural disease in man. In the animals not injected with the antiserum the conditions remained essentially unaltered until death, except that some multiplication of the diplococci sometimes seemed to take place. In the animals receiving the antiserum, the diplococci diminished in number, tended to be entirely taken up by leucocytes, and to suffer rapid degeneration within the latter. These findings led to the view that part of the beneficial action of the antiserum was due to the increased phagocytosis caused by it, a conclusion which has since been verified in the study of many cases of epidemic meningitis in human beings which were treated with the antiserum.[4]

It should be mentioned that Jochmann[5] and Kolle and Wassermann[6] reported uniform results of tests with the antiserum in protecting small animals—mice or guinea-pigs—from the living cultures and thought that use could be made of this method of standardization. But a critical examination of their figures will soon dispel all confidence in its usefulness although they indicate a greater power of the immune serum to protect animals than is possessed by normal serum. A similar conclusion was independently reached by Flexner.[7]

Jochmann,[8] on the other hand, pointed out first that the antiserum possessed greater power of stimulating phagocytosis *in vitro* than normal serum, thus bringing it into line with other immune sera in respect to opsonic properties, but he did not attempt to determine the limits of the opsonic power.

It is difficult to express an opinion on the highly divergent results of Ruppel[9] who claims to have secured a strain of the diplococcus

[4] Flexner and Jobling, *loc. cit.*
[5] Jochmann, *Deut. med. Woch.*, 1907, xxxii, 788.
[6] *Loc. cit.*
[7] Flexner, *Jour. of Exper. Med.*, 1907, ix, 105, 142.
[8] *Loc. cit.*
[9] Ruppel, *Deut. med. Woch.*, 1906, xxxii, 1366.

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that is uniformly fatal to mice in doses of 1 cubic centimeter of a 1 to 1,000,000 dilution, and employs this culture in standardizing an antiserum. I believe that in the present state of our knowledge of Diplococcus intracellularis, we are justified in viewing Ruppel's claims with suspicion.

Kraus and Doerr[10] are the latest writers to recommend the use of soluble products of the diplococcus, prepared either by Flexner's toluol method[11] or by extraction with N/10 sodium hydrate, for standardization and determination of anti-endotoxic power of the antiserum. The figures given by them do not inspire confidence in the method.

My own experiments with extracts prepared by autolysis and by alkali extraction have been entirely disappointing. In the effort to secure more uniform results the proteids were precipitated from the extracts with alcohol, dried, redissolved in sodium chloride solution, reprecipitated and dried. The resulting powder readily dissolved in water and salt solution but acted irregularly whether administered by subcutaneous intraperitoneal or intracardiac injection in guinea-pigs weighing from 200 to 225 grams, which is the most uniformly susceptible size. The fatal dose varied from 0.001 to 0.01 gram. I am convinced that nothing is to be achieved by this method of standardization.

The methods which have been worked out recently for determining quantitatively the opsonins in serum offer more hope in securing a unit of value for standardization, especially since it has been established that the opsonic power of the antiserum is an important factor in respect to the therapeutic effects. Neufeld[12] is the first person to make quantitative estimations of the opsonic strength of antimeningitis sera and to publish his results. He tested several different samples of antimeningitis sera to determine the greatest dilutions at which they would still cause phagocytosis. The limits of variation were considerable: some samples were active in dilutions of 0.0002 to 0.0005 per cubic centimeter and others only on 0.01 per cubic centimeter.

[10] Kraus and Doerr, *Wien. klin. Woch.*, 1908, xxi, 12.
[11] Flexner, *Jour. of Exper. Med.*, 1907, ix, 105.
[12] Neufeld, *Med. Klin.*, 1908, iv, 1158.

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Neufeld is strongly of the opinion that the favorable action of the antimeningitis serum depends largely on its bacteriolytic effect and hence he believes that a measure of the opsonins would also be a measure of its activity and would suffice for standardization. He also admits other effects of the serum including an anti-endotoxic property but he regards them as subordinate to the opsonic property.

I used, at first, in the quantitative study of the opsonins of our antiserum the method recommended by Neufeld, but later I added to it the direct method of counting the included diplococci by a modified Leishman's method. I shall describe the two methods employed in brief and then state the results obtained.

Neufeld's Method.—The leucocytes are obtained from the peritoneal cavity of guinea-pigs by injecting aleuronat one day and collecting the exudate the next day. The cells are washed four times in 0.9 per cent. salt solution. After the last washing they are suspended in such a quantity of the saline solution as to equal in opacity a 0.3 per cent. lecithin emulsion in normal saline.

The emulsion of diplococcus is made by adding one cubic centimeter of an equal mixture of bouillon and salt solution to each slanted tube of twenty-four hours' growth of the diplococcus on beef-infusion-glucose agar. This emulsion is quite thick but it is desirable that it should be.

Not every culture of diplococcus intracellularis is suitable for the test. Neufeld laid great stress on the proper selection of the culture and my experience agrees with his. I examined and discarded numerous cultures as being unsuitable either for the reason that they were too readily digested by the leucocytes, or were not readily subject to phagocytic inclusion. Culture No. 720, which was the one selected, fulfilled the conditions very well. As illustrating the great variation in phagocytosis I might mention that certain antisera caused considerable phagocytosis of this culture in dilutions of 1 to 5,000, while with other cultures a corresponding amount of phagocytosis required a dilution of 1 to 500. Culture No. 720 had been under artificial cultivation for more than a year. But the factor of artificial cultivation is not highly important, as I found that certain recently isolated strains were more easily phagocyted than some strains grown in the laboratory for three years.

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The tests are conducted in test-tubes of about twelve millimeters in diameter and five centimeters in length. To each tube are added two drops of the diluted antiserum, one drop of emulsion of diplococcus and two drops of suspension of leucocytes. The tubes are incubated at 37° C. for one and one-half hours. At the expiration of the incubation the leucocytes have settled to the bottom of the tubes so that by careful manipulation the supernatant fluid can be poured off, leaving the leucocytes adherent to the bottom of the tube. The spreads are made from the mass of leucocytes and diplococci by placing a platinum loopful on a slide and spreading it with the inclined edge of a second slide. After drying in the air, the spread is fixed for one minute in methyl alcohol and then stained in a 1 to 10 dilution of Manson's methylene blue solution. The control tube contains salt solution instead of antiserum. Where many tests are being carried on at one time a control tube is made for each six or eight of the series. The serum tested ranged in age from ten days to fifteen months. When the sera were less than fifteen days old they were heated at 60° C. for thirty minutes before testing.

The readings are based on the gross appearance of the spreads without counting the phagocyted diplococci. The spreads made from the tubes containing the higher concentrations of the antiserum showed that very marked phagocytosis had occurred. Many of the leucocytes were completely filled with diplococci; the control spreads would sometimes show leucocytes containing two or three diplococci. In dilutions of the serum at 1 to 1,000 and 1 to 2,000 no difficulty was experienced in interpreting the appearance since the degree of phagocytosis greatly exceeds the controls. When the dilutions exceeded 1 to 2,000 the decision was sometimes less readily made. Whenever doubt existed the tests were repeated several times so as to avoid the experimental error as far as possible.

Modified Leishman[13] *Method.*—Feeling that the figures obtained by the Neufeld method were subject to some difference in interpretation, all the antisera were tested as follows: the leucocytes were derived from the circulating blood of the dog, the emulsion of the diplococcus was prepared much thinner, and capillary tubes were used for the mixture to be incubated, after the manner recom-

[13] Leishman, *Brit. Med. Jour.*, 1902, i, 73.

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mended by Leishman. The included cocci were counted and the greatest dilution yielding a higher count than the saline control was taken as representing the ultimate strength of the antiserum.

The figures yielded by the two methods are in remarkable agreement so that it will be necessary to give only one table showing the general results obtained. In practice, the second or Leishman method is somewhat more convenient.[14]

The two methods were applied to the antisera obtained by mixing the sera from the three horses used for immunization as is done in preparing the antiserum for distribution. The table gives the numbers of the sera tested, the numbers of the horses and the age of the antisera at the time of the testing.

TABLE I.

Opsonic Strength of the Mixed Antisera.

Number of Serum.	Horses Used.	Age of Serum.	Greatest Dilution.
7	Nos. 1 and 2	15 months	1– 800
12	Nos. 1 and 2	13½ "	1– 2,000
17	Nos. 1 and 2	10 "	1– 2,000
19	Nos. 1 and 2	9 "	1– 5,000
20	Nos. 1, 2 and 3	8½ "	1– 2,000
21	Nos. 1, 2 and 3	8 "	1– 2,000
22	Nos. 1, 2 and 3	7½ "	1– 2,000
23	Nos. 1, 2 and 3	7⅛ "	1– 3,000
24	Nos. 2 and 3	7 "	1– 1,000
25	Nos. 2 and 3	6½ "	1– 1,000
26	Nos. 1 and 2	4 "	1– 1,000
27	Nos. 1, 2 and 3	3½ "	1– 800
28	Nos. 1, 2 and 3	3 "	1– 2,000
29	Nos. 1, 2 and 3	2⅛ "	1– 5,000
30	Nos. 1, 2 and 3	2 "	1– 5,000
31	Nos. 1, 2 and 3	1½ "	1– 8,000
32	Nos. 1, 2 and 3	15 days	1–10,000
Normal horse serum.		30 and 60 days	1– 80

[14] I am indebted to Dr. R. V. Lamar for a slight modification of the methods which has led to more accurate results. Owing to the readiness with which the diplococcus is dissolved in the leucocytes, it is important to leave them in contact for the briefest possible period. By incubating the serum dilution and diplococci at 37° C. for one hour, before adding the leucocytes, and the mixture of the three components for an additional thirty minutes, maximum phagocytosis is secured and the diplococci are not so acted upon as to lose their staining power.

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The variations shown in the table may at first sight appear contradictory, but on analysis they will prove not to be so. In the beginning of the work on the antimeningitis serum, only one horse was used in the preparation of the serum and it was not until several months later that a second horse was subjected to immunization. Sera Nos. 7, 12, 17 and 19 were prepared from a mixture of the sera of horses Nos. 1 and 2. The power rose in this mixed serum from 1 to 800 to 1 to 5,000 in about six months time. Somewhat later a third horse was added, and serum No. 20 is the first lot containing serum from horse No. 3.

The drop in opsonic activity has been attributed to the effect of serum from horse No. 3 that was of low strength. Gradually the strength of the mixture rose to 1 to 3,000 (sample No. 23). Shortly after the preparation of sample No. 23, the horse No. 1 developed fever which continued for several days so that the horse was not bled the next regular time. Hence serum (sample No. 24) was composed of sera from horses Nos. 2 and 3 which had been treated a shorter time than horse No. 1. The drop in opsonic activity is marked, as the limit of the next two samples was only 1 to 1,000. After serum No. 25 was prepared the horses were sent to the farm for the summer—from June until September, during which period they received no inoculation. Samples Nos. 26 and 27 show the falling off due to the summer's rest. Inoculations were resumed and a progressive increase in opsonic strength produced in the combined sera, the highest titre being reached in sample No. 32 in which the limit of dilution was 1 to 10,000. The opsonic value of normal horse serum is low and in the sample recorded was 1 to 80.

TABLE II.

Opsonic Strength of the Separate Antisera.

No. of Serum.	Horse No. 1	Horse No. 2.	Horse No. 3.
No. 26, Sept. 19, '08	1– 2,000	1–1,000	1– 200
No. 28, Oct. 16, '08	1– 8,000	1–2,000	1–1,000
No. 29, Oct. 30, '08	1–10,000	1–2,000	1–2,000
No. 32, Jan. 7, '09	1–20,000	1–5,000	1–2,000

Beginning September, 1908, the sera from the several horses were tested separately several times. Table II gives the results of four tests of each horse and exhibits clearly the effects of inoculations

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continued over a long period of time. In September, 1908, Horse No. 1 had been under treatment for two years; horse No. 2 for fourteen months; and horse No. 3 for eight months.

The serum sample of September 9 was drawn before inoculation was re-commenced after the summer's rest. Horse No. 3 has always given a smaller yield of serum for the amount of blood withdrawn than either horse No. 1 or No. 2. Horse No. 1 yields the greatest volume of serum of the three horses. Hence in the mixtures, the diluting effects of the sera of horses Nos. 2 and 3 are less than would have been the case had the yield of serum been approximately equal.

CONCLUSIONS.

The high variability in infectivity of Diplococcus intracellularis makes it impracticable to standardize the antimeningitis serum on the basis of the virulence of the diplococcus.

The irregularity of reaction of small animals to the poison or endotoxin of Diplococcus intracellularis makes it impracticable to standardize the antimeningitis serum on the basis of endotoxic value.

The want of uniformity in the complement-binding power of the antimeningitis serum, and the absence of established relation between complement-binding power and therapeutic activity make the employment of this method of standardization impracticable.

The part taken by specific opsonins in promoting recovery from infection with Diplococcus intracellularis suggests their employment as a measure of the therapeutic activity of the antiserum. Methods of quantitative estimation of opsonic content of the antimeningitis serum being available, it would seem advantageous to adopt for the present as a standard of value a definite and suitable strength in opsonins of the antimeningitis serum.

As a definite and suitable standard of strength a minimum dilution activity of a 1 to 5,000 dilution of the antiserum is proposed.

Since the immune opsonins of the antimeningitis serum appear to be highly durable under proper conditions of refrigeration of the antiserum, the test proposed will be applicable when the antimeningitis serum shall have become an article of commerce.

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