

Quantitative Measurement of Precipitating Antibodies in Streptococcal Grouping Antisera by the Single Radial Immunodiffusion Technique

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A comparative study was made of the single radial immunodiffusion test and the classical quantitative precipitin test for determining the amount of precipitable antibodies present in streptococcal groups A and C antisera. The potency of 21 group A and 54 group C antisera was determined by both methods; purified group-specific carbohydrates were used as antigens. The coefficient of correlation between the results from the two methods was 0.976 for group A antisera and 0.946 for group C antisera. When the concentration of antigen, the volume of antiserum used, and the depth of the antigen-agar mixture are kept constant, the diameter of the precipitin disc is directly related to the concentration of precipitable antibodies present in the antiserum. The use of the radial immunodiffusion test for evaluating and standardizing streptococcal grouping antisera is discussed as well as the advantages and disadvantages of using a concentrated vaccine for producing these antisera.

The classification of streptococci into serological groups for clinical and epidemiological purposes depends upon the availability of reliable antisera. Most laboratories that prepare grouping antisera determine the potency by observing the amount of precipitate obtained and the time required for the precipitate to form in capillary tubes when homologous antigens are used. This qualitative method gives, at best, only a rough estimate of the actual potency of the antiserum. For example, one may have two antisera that give strong precipitin reactions in the capillary tube test, yet one may contain several times more precipitating antibody than the other. From the standpoint of stability during storage and shipment and the ability to react with acid extracts of cultures that may contain relatively low concentrations of grouping antigen, the stronger antisera are obviously preferred.

The single radial immunodiffusion test is uniquely suited for the quantitative measurement of antigen or precipitating antibodies when a purified antigen preparation is available for use in the test. Stiehm (8) and Vaerman et al. (9, 10) have shown that the "re-

verse" procedure in which antibody diffuses into antigen-containing gels operates in the same manner as the "direct" procedure originally described by Mancini (6, 7). In both tests the final area of precipitate is directly proportional to the mass of material placed in the well and inversely proportional to the concentration of reactant in the agar. The relative ease of obtaining purified preparations of streptococcal group A and C carbohydrate antigens suggested this procedure as a method for measuring the amount of precipitable antibody in antisera prepared against these antigens.

This report describes the use of the single radial immunodiffusion test for determining the amount of precipitating antibody present in streptococcal group A and C antisera and the correlation of this method with the classical quantitative precipitation test.

MATERIALS AND METHODS

Vaccine strains. Group A strain 23/14/0 and group C strain C74 were obtained from R.C. Lancefield, Rockefeller University. Stock cultures were maintained in sterile defibrinated rabbit blood at

-60 C.

Preparation of vaccines. The two cultures used for preparing vaccines were grown overnight at 37 C in Todd-Hewitt broth (Difco). The bacterial cells were collected by centrifugation, suspended in sterile 0.85% NaCl, and the cell suspension was adjusted to pH 2 with 1 N HCl. Sterile pepsin was added to the cell suspension to a final concentration of 1 mg/ml, and the mixture was incubated at 37 C for 2 hr. The bacterial cells were washed three times with 0.85% NaCl, adjusted to pH 7 to 7.2, and finally resuspended in 0.85% NaCl that contained 0.4% Formalin. The densities of both vaccines were adjusted so that a 1:20 dilution had an optical density of 0.4 at 660 nm when 1-cm cuvettes were used.

Antisera production. Antisera were prepared in New Zealand white rabbits by the method of Greenblatt et al. (3), except that a second series of inoculations was not given. The method consisted of three intravenous inoculations per week for 4 weeks. Maximum antibody response was obtained in most of the rabbits after they had received nine inoculations. To obtain antisera with low, medium, and high levels of precipitating antibodies, the rabbits were bled weekly, starting the second week after the first inoculation. From 30 to 40 ml of blood could be taken from the ear arteries without endangering the life of the rabbit. We used this procedure to collect 21 group A antisera and 54 group C antisera for study.

Group-specific carbohydrate antigens. Purified group A carbohydrate was kindly supplied by Leo Pine, Center for Disease Control, and purified group C carbohydrate by R. M. Krause, Rockefeller University. Both preparations were made by the method of Krause and McCarty (4). The rhamnose content, determined by the method of Dische and Shettles (2), was used as a quantitative measure of these antigens because it is an index of the group-specific carbohydrate (3).

Precipitin tests. The quantitative precipitin test used in these studies was a Braun and Krause (1) modification of the McCarty and Lancefield (5) procedure. Purified group A and group C carbohydrates were used as antigens. The amount of precipitable antibody per milliliter of serum was calculated by using $E_{cm}^{1\%} = 15$ at 280 nm (1). The capillary precipitin "ring" test was used in all of these studies. In this test, an acid extract of whole cells is layered over the antiserum in capillary tubes (1.2 by 100 mm). The precipitin reaction occurs primarily at the interface of the two reagents.

Single radial immunodiffusion. The immunodiffusion slides were prepared by mixing the desired concentration of antigen with a 1% solution of Ionagar no. 2 (Colab Laboratories, Inc., Chicago Heights, Ill.) prepared in 0.02 M phosphate-buffered saline (PBS), pH 7.2, and adding 2.5 ml of this mixture to a microscope slide (1 by 3 inches). The slide was pretreated by allowing 0.5 ml of a 0.2% agar solution to dry on its surface. The group A antigen was used at a concentration of 2.5 μ g of rhamnose per ml of agar and the group C antigen at a concentration of 1.25 μ g of rhamnose per ml of agar. Four wells, each 3.0 mm in diameter and 12 mm apart, were cut in

the agar slide with a gel cutter (Gelman Instrument Co., Ann Arbor, Mich.) just before use. Undiluted antiserum measured with a 5- μ liter Eppendorf pipette (Brinkmann Instruments, Inc., Westbury, N.Y.) was placed in each well, and the plates were incubated at 4 C for 48 hr in a moist chamber.

After the antigen-antibody reaction reached equilibrium, i.e., at 48 hr, the diameter of the precipitin disc was measured with a spectrum plate reader (Bausch & Lomb Inc., Rochester, N.Y.) that had a 20-mm scale graduated in 0.1-mm divisions. The measurements were made either from photographic slides taken with a Polaroid CU-5 close-up camera which gave a 1:1 reproduction of the slide or from a slide that had been dried and, after removal of unreacted proteins, stained with a 0.1% solution of Buffalo Black NBR (Allied Chemical, Morristown, N.J.). The Buffalo Black NBR was prepared in a solution of 450 ml of methyl alcohol, 100 ml of acetic acid, and 450 ml of water. The slides were stained for 10 min and then destained in the same diluent used to prepare the stain. One slide containing four wells was used for each antiserum tested, and the mean diameter of the precipitin disc around three or more wells was calculated. The diameter of the well (3 mm) was included in the total diameter of each precipitin disc. The correlation between the mean diameter per slide and the corresponding quantitative precipitin test result was determined statistically.

RESULTS

Group A antisera. The antibody concentration of the 21 group A antisera varied from 0.55 to 9.3 mg of precipitable antibody per ml as determined by the quantitative precipitin test. When these 21 antisera were tested in the single radial immunodiffusion test in which 2.5 μ g of antigen was used per ml of agar, the mean diameter of the precipitin disc varied from 3.92 mm for the weakest antiserum to 7.75 mm for the strongest antiserum. When the results of these two tests were plotted against each other and a least squares line calculated, there was a random scatter of points around this line (Fig. 1). The coefficient of correlation between the two methods was 0.976, and the coefficient of determination was 0.953. Thus, 95.3% of the variability among the mean diameter values of the 21 antisera was due to a linear association with the concentration of antibody, as determined by the quantitative precipitin test, and 4.7% of the variability was due to other factors such as varying experimental conditions, accuracy of measurement, etc.

Three of the 21 antisera tested contained less than 1 mg of precipitable antibody per ml. The precipitin disc in the immunodiffusion test of these three antisera formed so close to the well that it was difficult to measure the

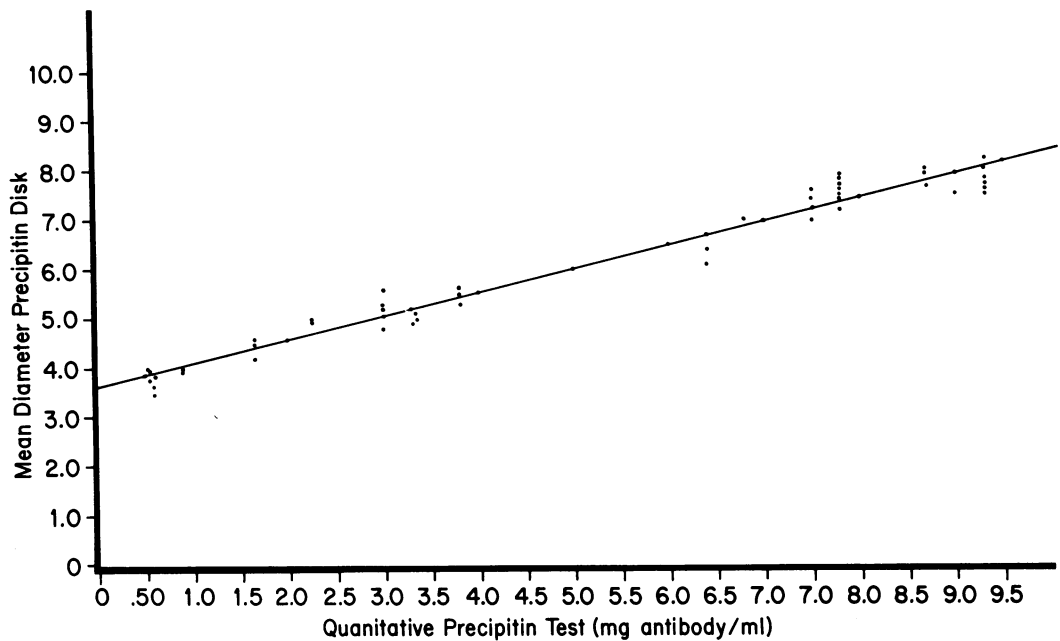


FIG. 1. Measurement of precipitating antibodies in 21 group A antisera by the single radial immunodiffusion test versus the quantitative precipitin test. Least squares line. Coefficient of correlation = 0.976.

reaction accurately. This could be overcome by decreasing the antigen concentration to 1.75 $\mu\text{g}/\text{ml}$ of agar. However, the latter concentration was not suitable for the stronger antisera because the outer boundary of the precipitin disc was fuzzy, making it difficult to measure. Because the three weaker antisera were borderline in acceptability for use in the capillary precipitin ring test, the higher antigen concentration was preferred. Each of the other 18 antisera tested contained 1.5 mg or more of precipitable antibody per ml. These antisera gave an immediate and strong precipitin reaction with routine acid extracts when the ring test was performed in capillary tubes.

Sera taken from six rabbits after nine inoculations were checked for cross-reactivity with acid extracts of groups A through O. Antisera from four of the rabbits gave weak to moderate precipitin reactions with extracts of groups B, C, E, F, G, L, and M; antisera from the other two rabbits reacted only with extracts of groups L and M. These reactions did not appear immediately, as the homologous reactions did, but required 5 to 20 min before they were evident. A test pool prepared from these bleedings could be made specific for group A extracts by two absorptions with group C cells when a ratio of one part of wet-packed cells to three parts of antiserum was used.

Group C antisera. The antibody concentra-

tion of the 54 group C antisera ranged from 0.58 to 9.62 mg of precipitable antibody per ml. When these 54 antisera were tested in the single radial immunodiffusion test, by using 1.25 μg of antigen per ml of agar, the mean diameter of the precipitin disc varied from 3.6 mm for the weakest antiserum to 8.85 mm for the strongest antiserum. When these results were plotted against each other and a least squares line calculated, there was a random scatter of points around the line (Fig. 2). The coefficient of correlation between the two methods was 0.946 and the coefficient of determination was 0.895.

Four of the group C antisera tested had less than 1 mg/ml of precipitable antibody. The precipitin disc formed by these antisera was similar to that formed by the three weak group A antisera, i.e., they were so close to the well that it was difficult to measure them accurately. Because these four antisera were of questionable value for use in the capillary precipitin ring test, no attempt was made to adjust the size of the precipitin disc by lowering the antigen concentration in the agar. The 50 other antisera tested contained 1.5 mg or more of precipitable antibody per ml; these gave an immediate and strong precipitin reaction with routine acid extracts when the ring test was performed in capillary tubes.

The cross-reactivity of antisera taken from

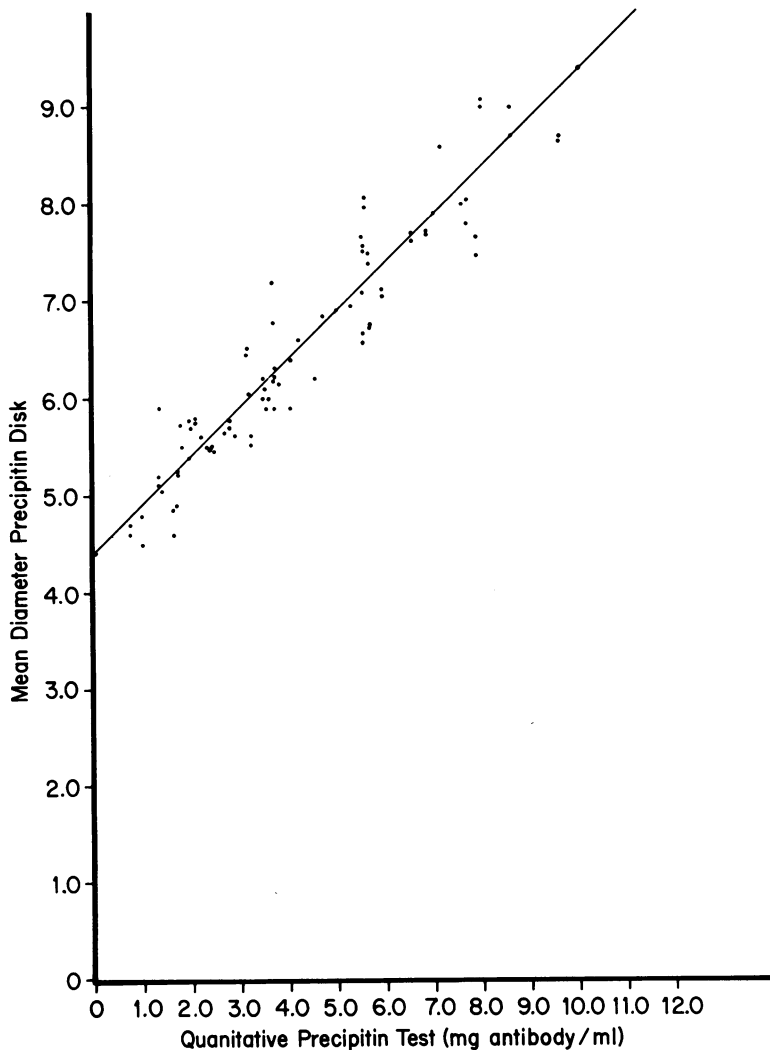


FIG. 2. Measurement of precipitating antibodies in 54 group C antisera by the single radial immunodiffusion test versus the quantitative precipitin test. Least squares line. Coefficient of correlation = 0.946.

five rabbits after nine inoculations was as follows. Antiserum from one rabbit reacted only with group O extracts; antisera from three rabbits reacted with extracts of groups A, B, D, E, F, G, K, and O; and antiserum from one rabbit reacted with all the extracts of groups A through O. These were weak to moderate reactions that appeared 5 to 10 min after the tests were set up. Antiserum from the rabbit that reacted with all the extracts could not be absorbed, whereas a pool of the antisera from the other rabbits could be made specific by two absorptions with group B cells at a ratio of one part of wet-packed streptococcus cells to three parts of antiserum.

From these results it is apparent that, when standard conditions are used, the single radial immunodiffusion test can be substituted for the quantitative precipitin test for measuring the concentration of precipitable antibody in groups A and C streptococcal antisera. The simplicity of the immunodiffusion test compared with the quantitative precipitin test makes the former a more practical procedure for routine use in most laboratories. For example, when standardized test conditions are used as described previously, group A antisera that give a precipitin disc 4.5 mm or greater in diameter and group C antisera that give a precipitin disc 5.0 mm or greater in diameter are

satisfactory for use in the precipitin ring test performed in capillary tubes. These antisera contain at least 1.5 mg of precipitable antibody per ml.

DISCUSSION

There is a definite need for quantitative procedures to determine the potency of diagnostic reagents used in clinical and research laboratories. The potency of most diagnostic antisera is reported as a titer, i.e., the reciprocal of the highest dilution that gives some observable reactions such as agglutination, lysis of red blood cells, or neutralization of toxins. Generally, the tests used to determine these titers are considered to have an accuracy of only ± 1 twofold dilution which means, in effect, that the titer of one particular serum may vary as much as fourfold when the test is performed at different times or in different laboratories. The potency of precipitating antisera used to identify the serological groups of streptococci is usually determined by the amount of precipitate that forms when the antisera are reacted with extracts of whole cells. In most laboratories this is recorded as a strong (4+), moderate (2+), or weak (1+) reaction. This, of course, is a very subjective method for determining potency and frequently leads to confusion in the interpretation of a test when different individuals read it. Although the classical quantitative precipitin test has been used for many years in research laboratories, it is not practical for most laboratories to perform. The single radial immunodiffusion test, on the other hand, is simple to perform and requires a minimum of equipment. From the data presented in this paper, it is apparent that there is an excellent correlation between the results obtained with the radial immunodiffusion test and that of the classical quantitative precipitin test when the former is performed as described. In recent years, procedures have been published for purification of the carbohydrate antigens of many of the serological groups of streptococci. Although we have presented data on only groups A and C streptococcal antisera, there is no reason to think that this test would not be applicable to the other serological groups. To date, preliminary studies in our laboratory indicate that the potency of group B streptococcal antiserum can also be quantitated with this test by using a purified preparation of group B carbohydrate antigen. It is not difficult to visualize the possibility of an international or national organization preparing and

supplying a standard purified carbohydrate antigen to be used in this test for determining the potency of streptococcal grouping antisera. Being carbohydrate in nature, the purified antigens are stable for long periods of time and can be quantitated rather easily with sensitive, well-established chemical tests. The availability of a standard purified antigen would make it possible to recommend a minimum concentration of antibodies that should be present for an antiserum to be considered satisfactory for the serological grouping of streptococci. This would be a major step towards standardizing and increasing the quality of diagnostic antisera.

The reproducibility of the radial immunodiffusion test depends on the volume of antiserum placed in each well, the depth of the antigen-agar mixture on the slide, and the concentration of the antigen in the agar. There are a number of devices available that will accurately dispense 5 μ liters of antiserum; the depth of the antigen-agar mixture can be controlled by dispensing a known volume (2.5 ml) onto a standard 1 by 3 inch microscope slide, and the concentration of the purified carbohydrate antigen can be determined chemically. A concentration of 2.5 μ g of group A and 1.25 μ g of group C purified carbohydrate per ml of agar gives a measurable precipitin disc with antisera containing 1 to 10 mg of precipitable antibody per ml. Antisera that are much stronger than this may require a higher concentration of antigen; otherwise the precipitin disc is very large and somewhat diffuse at the edges, making it difficult to measure accurately.

Greenblatt et al. (3) recently suggested that group A and group C antisera should contain a minimum of 5 mg and preferably 10 mg of precipitable antibodies per ml. This recommendation is not necessarily in conflict with our suggested lower limits of 1.5 mg of precipitable antibody per ml. Greenblatt et al. (3) used the capillary precipitin test in which the antiserum is placed on top of the acid extract; because the serum is heavier, it "falls" through the extract causing a rapid, heavy precipitate to form when a highly potent antiserum is used. They pointed out that a weaker antiserum does not react as well as a stronger antiserum in this precipitin test and, therefore, is less satisfactory. The precipitin test we use is a ring test in which the acid extract is layered over the antiserum in a capillary tube and the precipitin reaction occurs primarily at the interface of the two reactants. Antiserum con-

taining only 1 mg of precipitable antibody per ml will give a strong precipitin reaction within 1 min in the latter test, although the amount of precipitate is less than that given by a stronger antiserum. Although we have had only limited experience with the test recommended by Greenblatt et al., it appears the type of test may dictate to a certain extent the potency of antiserum that should be used.

The availability of streptococcal antisera containing 5 mg or more of precipitable antibody per ml would obviously be beneficial to the clinical diagnostic laboratory. Such antisera would give a stronger and a more rapid precipitin reaction with extracts of diagnostic cultures and presumably would be more stable and therefore have a longer shelf life than weaker antisera. Obtaining antisera of this potency necessitates the use of a rather concentrated vaccine which also stimulates cross-reacting antibodies. In our studies, this was particularly true for group A antisera which reacted with extracts of many of the heterologous groups. In the past, our group A antisera, containing only 1.5 to 2 mg of precipitable antibody per ml, was prepared with a weaker vaccine and rarely reacted with heterologous grouping extracts. However, the cross-reactivity of group C antisera prepared with a concentrated vaccine appeared to be about the same as that previously prepared with a weaker vaccine. These antisera can be made group specific by absorption and, in our opinion, this must be done before an antiserum can be considered acceptable. Otherwise, there would be a great deal of confusion in the interpretation of the precipitin test in laboratories that are not familiar with the reactivity of a particular antiserum.

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