Bull. World Health Organ. } 1974, **51**, 245-248
Bull. Organ. mond. Santé }

Quantitative measurement of precipitating antibodies and standardization of streptococcus group B antisera by the reverse radial immunodiffusion technique*

J. R. GEORGE[1] & W. K. HARRELL[2]

A comparative study was made of the reverse radial immunodiffusion (RRID) technique and the quantitative precipitin test for determining the amount of precipitable antibodies present in streptococcus group B antisera. The coefficient of correlation between the 2 tests was 0.963 when a purified carbohydrate antigen was used. The results of this study show that: (1) a group B antiserum should contain at least 3 mg of precipitable antibodies per ml to ensure that it will react with acid extracts of group B diagnostic cultures; and (2) the RRID technique is an accurate and simple procedure for determining the amount of precipitable antibody present in an antiserum, and it could be used for the standardization of these diagnostic reagents. A method for preparing the group B carbohydrate antigen used in the RRID and quantitative precipitin tests is described.

A simple method of quantitatively measuring the concentration of antibodies in *in vitro* diagnostic antisera would materially aid in the standardization of these reagents produced in different laboratories. This is particularly true for precipitating antisera, such as streptococcus grouping antisera, where the potency is usually determined by observing the amount of precipitate and the time required for the reaction to occur in capillary tubes when homologous antigens are used. The latter method is only roughly quantitative because 2 antisera may appear to give identical capillary precipitin reactions yet one may contain several times more precipitating antibodies than the other.

We have previously shown (1) that the reverse radial immunodiffusion (RRID) technique of Stiehm (2) and Vaerman et al. (3) is a simple, accurate, and reproducible procedure for quantitatively measuring the amount of precipitating antibodies in streptococcus groups A and C antisera when a purified carbohydrate antigen is used. In this report we describe the use of the same technique for quantitating the amount of precipitating antibodies in streptococcus group B antisera and the correlation between this technique and the classical quantitative precipitin test. The method used for preparing purified group B carbohydrate used in the test is also described.

MATERIALS AND METHODS

Vaccine strains

Streptococcus group B type Ia, strain 090R, was obtained from R. C. Lancefield (Rockefeller University).

Preparation of vaccines

Cultures were grown overnight in Todd-Hewitt broth (Difco) at 37°C. The bacteria were killed by heating at 56°C for 1 hour, collected by centrifugation, washed 3 times with 0.85% NaCl, and resuspended in 0.85% NaCl. The density of the vaccine was adjusted so that a 1 : 20 dilution had an internal transmission density of 0.4 at 660 nm when a 1-cm cuvette was used.

Antisera

Antiserum was prepared in New Zealand white rabbits, which were inoculated intravenously 5 times a week. The first 3 inoculations were of 0.5 ml and the rest were of 1.0 ml. The animals were bled from

* From the Center for Disease Control, Public Health Service, US Department of Health, Education, and Welfare, Atlanta, Ga., USA. Use of trade names is for identification only and does not constitute endorsement by the US Public Health Service or by the US Department of Health, Education, and Welfare.

[1] Microbiologist, Bacterial and Fungal Products Branch, Center for Disease Control, Atlanta, Ga., USA.

[2] Chief, Bacterial and Fungal Products Branch, Center for Disease Control, Atlanta, Ga., USA.

the centre ear artery after each week of inoculations to obtain antisera with high and low levels of antibodies. Maximum antibody response varied with individual rabbits and was obtained after 10–15 inoculations. Seventeen group B antisera were selected for this study.

Preparation of group-specific carbohydrate antigen

The group B antigen was extracted with formamide (4) from cell wall preparations (5) of heat-killed cells of strain 090R. The extract was adjusted to pH 7.0 and dialysed against several changes of 0.015 M phosphate buffer, pH 7.1; the precipitate that formed was removed by centrifugation and discarded. The supernatant was concentrated approximately 6 times with carboxymethyl cellulose and dialysed against pH 8.5 borax–boric acid buffer (0.05 M for borate). A Cellex D anion exchange column (Bio-Rad Laboratories, Richmond, Calif., USA) equilibrated with the borax–boric acid buffer was used to separate the grouping and typing carbohydrates. The 2 antigens were eluted from the column with the equilibrating buffer containing stepwise increasing concentrations of NaCl (0.05 M to 0.2 M). Each 8.0-ml fraction was tested for the presence of grouping or typing antigen by the capillary precipitin ring test using antiserum specific for each antigen. Tubes that were positive for the grouping antigen were examined chemically for the presence of rhamnose, the group-specific carbohydrate (6), and for galactose, the type-specific carbohydrate (7). Rhamnose was measured by the method of Dische & Shettles (8) and was used as a quantitative measure of the amount of antigen used in the precipitin test. Galactose was determined with the Worthington Galactostat and protein content by the method of Lowry et al. (9). The rhamnose concentration was adjusted to 100 μg/ml and stored at −70°C for use as a stock antigen solution.

Precipitin tests

The quantitative precipitin test, the capillary precipitin ring test, and the RRID test were performed as previously described (1). The antigen–agar mixture for the RRID test contained 1.25 μg of the purified carbohydrate antigen per ml of agar.

RESULTS

Preparation of group B carbohydrate

The carbohydrate preparation was checked by the capillary precipitin ring test, double diffusion precipitin test, and immunoelectrophoresis and found to contain only 1 reactive antigen against group B specific antiserum. It was nonreactive against group B type Ia specific antiserum by these methods. When the antigen was examined with the Worthington Galactostat, no galactose was found. Since the type Ia antigen was reported to be 67% galactose by Wilkinson & Eagon (10), this further confirmed that no type Ia specific antigen was present.

Reverse radial immunodiffusion

The antibody concentration of the 17 antisera used in this study was determined by the quantitative precipitin test. The concentrations ranged from 0.76 mg to 10.73 mg of precipitable antibody per ml. When the antisera were tested by the RRID technique, in which 1.25 μg of antigen was used per ml of agar, the mean diameter of the precipitin disk varied from 4.05 mm for the weakest antiserum to 9.46 mm for the strongest. During the early phase of this work, it was determined experimentally that 1.25 μg of antigen per ml of agar was the optimum antigen concentration to use with group B antisera containing 1–11 mg of precipitable antibody per ml.

The values obtained from both the quantitative precipitin test and the RRID test are presented graphically in Fig. 1. The results from the RRID test were plotted against the quantitative test and a least squares line was fitted. The coefficient of linear correlation was 0.963, and the coefficient of determination was 0.927. Thus 92.7% of the variability among the mean diameter values for the 17 antisera was attributable to a linear association with the quantitative precipitin test, and 7.3% to other factors such as varying experimental conditions and accuracy of measurement.

The RRID data were examined by the analysis of variance technique. This analysis showed no measurable differences when the same antiserum was run on different days. When the antiserum to be quantitated was placed into 4 wells on the same slide and the results were reported as the mean diameter of the 4 precipitin disks, the estimate of reproducibility for the RRID technique was 0.24 mm. This estimate reflects the variation among slides ($\hat{\sigma^2}$ among slides = 0.056 mm^2) and experimental error ($\hat{\sigma^2}$ among wells within slides = 0.016 mm^2).

DISCUSSION

There is a definite need for a quantitative procedure to determine the potency of streptococcus

grouping antisera. This is particularly true for group B antisera because difficulties are encountered in producing an antiserum that reacts well with all strains of group B streptococci encountered in the diagnostic and research laboratories. Part of this difficulty may be due to the range of antigen levels found in different strains. In this laboratory 17 strains are used to evaluate group B antisera. When the rhamnose content of these strains was measured, it was found to vary from 310 μg to 750 μg per ml. Antisera that react well with antigens towards the middle of the range of concentrations sometimes produce prozones when antigen excess is encountered in strains producing large amounts of group B carbohydrate.

In our experience, group B antisera that contain 3 mg or more of precipitable antibody per ml give a strong capillary precipitin reaction with all the acid extracts of group B cultures tested. For this reason, we recommend that 3 mg/ml be the minimum concentration of antibodies in group B antisera.

The capillary precipitin ring test used in most laboratories offers little if any quantitation. The classical quantitative precipitin test has been used for many years in research laboratories, but is not practical for most laboratories to perform. The RRID technique, on the other hand, is simple, accurate, and requires a minimum of equipment. The reproducibility of the RRID test depends upon the volume of antisera placed in each well, the depth of the antigen–agar mixture on the slide, and the concentration of the antigen in the agar. The size of the precipitin disc is inversely proportional to the concentration of the antigen in the agar and no one concentration of antigen is ideal for all strengths of antisera encountered. The concentration of 1.25 μg/ml was selected in the knowledge that an antiserum containing less than 1 mg or more than 10 mg of precipitable antibodies per ml cannot be accurately measured by the present method. This point is illustrated by the curvilinear appearance of the data in Fig. 1.

The group B specific antigen used in this study is not considered to be pure in a chemical sense, but is purified to a level at which it does not react with any other antibody normally encountered in the production of the homologous antiserum. This antigen, like other streptococcus group-specific antigens, is

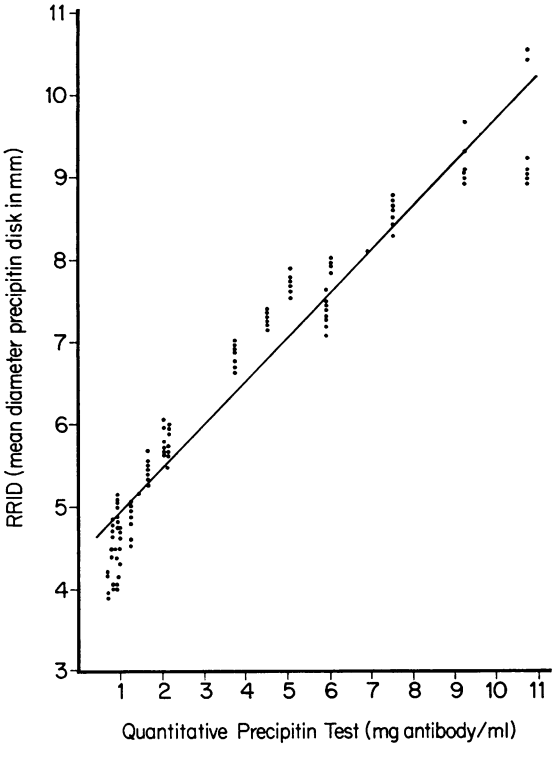

Fig. 1. Measurement of precipitating antibodies in 17 group B streptococcus antisera by the reverse radial immunodiffusion (RRID) test as compared to the quantitative precipitin test. A least squares line has been fitted. Coefficient of correlation = 0.963.

carbohydrate in nature, stable for long periods of time, and easily quantitated with established chemical tests. It is not difficult to visualize an international organization preparing and supplying a purified carbohydrate antigen for streptococcus groups A, B, and C to be used for the RRID test in determining the potency of grouping antisera. The availability of a standard antigen for these groups 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 these streptococci. This would be a major step toward standardizing and improving the quality of streptococcus diagnostic antisera produced in different laboratories in different parts of the world.

ACKNOWLEDGEMENT

We are indebted to Dr Ross Wood and his associates at the Bureau of Laboratories, Center for Disease Control, Atlanta, Ga., USA, for the statistical analysis of our data.

RÉSUMÉ

DOSAGE QUANTITATIF DES ANTICORPS PRÉCIPITANTS ET STANDARDISATION DES SÉRUMS ANTI-STREPTOCOQUES DU GROUPE B PAR LA TECHNIQUE DE L'IMMUNODIFFUSION RADIALE INVERSÉE

On a procédé à une étude comparative de l'épreuve d'immunodiffusion radiale inversée et de l'épreuve quantitative des précipitines pour déterminer la teneur en anticorps précipitants des sérums anti-streptocoques du groupe B. Les deux techniques ont été utilisées pour mesurer l'activité de 17 antisérums en présence d'un antigène hydrocarboné purifié. Le coefficient de corrélation entre les résultats des deux épreuves a été de 0,963.

L'étude a montré que les antisérums du groupe B doivent contenir au moins 3 mg d'anticorps précipitants par millilitre pour réagir avec les extraits acides des cultures à identifier. L'immunodiffusion radiale inversée est apparue comme une épreuve fiable et d'exécution simple qui permet de déterminer la teneur d'un antisérum en anticorps précipitants et qui peut servir à normaliser ce genre de réactif. Les auteurs exposent une méthode pour préparer l'antigène hydrocarboné du groupe B utilisé dans la présente étude.

REFERENCES

1. HARRELL, W. & GEORGE, J. Quantitative measurement of precipitating antibodies in streptococcus grouping antisera by the single radial immunodiffusion technique. *Appl. Microbiol.*, **23**: 1047-1052 (1972).
2. STIEHM, E. R. Radial diffusion technique for the quantitative estimation of precipitating antibody. *J. lab. clin. Med.*, **70**: 528-534 (1967).
3. VAERMAN, J. ET AL. Further studies on single radial immunodiffusion. 1. Direct proportionality between area of precipitate and reciprocal of antibody concentration. *Immunochemistry*, **6**: 279-285 (1969).
4. FULLER, A. T. Formamide method for extraction of polysaccharide from hemolytic streptococci. *Brit. J. exp. Path.*, **19**: 138 (1938).
5. BLEIWEIS, A. ET AL. Improved technique for the preparation of streptococcus cell walls. *J. Bact.*, **88**: 1198-1200 (1964).
6. CURTIS, S. & KRAUSE, R. Antigenic relationships between groups B and G streptoccocci. *J. exp. Med.*, **120**: 629-637 (1964).
7. WITTNER, M. & HAYASHI, J. Studies of streptococcus cell walls. VII. Carbohydrate composition of group B cell walls. *J. Bact.*, **89**: 398-402 (1965).
8. DISCHE, D. & SHETTLES, L. A specific color reaction of methylpentose and a spectrophotometric micromethod for their determination. *J. biol. Chem.*, **175**: 593-603 (1948).
9. LOWRY, O. ET AL. Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**: 265-275 (1951).
10. WILKINSON, H. & EAGON, R. Type-specific antigens of group B type Ic streptococci. *Infection & Immunity*, **4**: 596-604 (1971).