Lecithin-Agar Assay for Lecithinase Antibodies in Serum

KYLE H. SIBINOVIC,1 FREDDIE A. BROWN, KAREN D. PETTIGREW,² AND ROBERT L. VOUGHT
Metabolic Diseases Epidemiology Unit, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland 20014

Received for publication 22 May 1970

A technique for assay of lecithinase antibodies in serum was developed in this laboratory by using a lecithin-agar plate diffusion procedure based on a combination of described plate assays. Egg yolk lipoprotein composed primarily of lecithin was used as a substrate for reaction with free or non-neutralized lecithinase C after incubation of known amounts of lecithinase C with various dilutions of control and test sera. It was found that the size of the reaction zone was a function of enzyme concentration and inversely proportional to the antibody concentration. Accuracy and precision of the assay were determined. In addition, lecithinase antibody levels in sera from experimentally inoculated rats and rabbits and sera from randomly selected human patients were studied.

The absence of a satisfactory assay method for detection of lecithinase antibodies in microquantities of serum led to the development of a lecithin-agar plate diffusion test. This test was based on a combination of the agar diffusion plate method for assay of lecithinase from Clostridium perfringens of Sheldon et al. (7) and the plate method for antibiotic assay of clinical specimens of Bennett et al. (2).

Accurate determination of antibody titers in such a system requires application of the principle that a linear relationship occurs between the square of zone diameters and antigen concentration (1). However, to facilitate calculation, the linear portion of the standard curve may be utilized for calculation of test results directly.

MATERIALS AND METHODS

Diluent. Borate-buffered saline (pH 7.3) was prepared by addition of 10.94 g of boric acid, 1.9 g of sodium borate, 8.5 g of sodium chloride, and 1.25 g of calcium chloride per liter of distilled water. This solution was used in the preparation of lecithin-agar plates and as a diluent for serum.

Preparation of lecithinase C. Lecithinase C [a gift from Ira Pastan, prepared by precipitation of C. perfringens culture filtrate to 50% saturation with (NH₄)₂SO₄, dialysis of the precipitate against water, and subsequent lyophilization of the dialysate, having a specific activity of 2 units/mg as determined by Pastan], prepared as described by Macchia et al. (6)

1 Present address: Bionetics Research Laboratories, Kensington, Md. 20795.
² Biometry Branch, National Institute of Mental Health, Bethesda, Md. 20014.
diameters of flocculation zones were measured, means were determined, and the average zone diameter was plotted against lecithinase C concentration. Optimal concentration was the lowest concentration to give a maximal zone.

Preparation of standard lecithinase C antibody curve. Standard lecithinase C antibody curves were prepared as follows. (i) A stock solution of type A antiserum containing 10 units of α-antitoxin per ml was prepared from Burroughs Welcome C. perfringens (type A) antiserum. The lot of antiserum used contained approximately 285 α-antitoxin units per ml. (We are indebted to Irene Batty, Welcome Reagents Ltd., Wellcome Research Laboratories, Beckenham BR3 3Bs, Kent, for furnishing information as to antibody concentrations in the horse sera used as standards.)

(ii) The standards were prepared as shown in Table 1.

Test preparations. Standard curves were run by using 500 μg of lecithinase C per ml as determined (see above). An equal volume of lecithinase C was added to each tube containing 1 ml of the diluted antibody solution used in the standard curve. After 15 min of incubation, 0.04 ml of each mixture was added to each of five replication wells on the lecinthinagar plate (see Fig. 1).

Calculation of the standard curve and unknowns. The diameter of the cloudy zone (y) is inversely proportional to the concentration of antibody (x); specifically, the more antibody, the smaller the zone formed by free or non-neutralized lecithinase. The relationship has been consistently linear within the range of antibody concentration specified in this report 0.4 to 1.2 units/ml in five 0.2-unit intervals. Hence the equation for the standard curve is of the form \( y = a + bx \). Since the concentration levels are equally spaced and since there are an equal number of replications at each level, a least-squares fit is easily obtained as follows. (A worksheet was developed to minimize arithmetic errors and copies are available.)

(i) Five replications are made for each concentration (0.4, 0.6, 0.8, 1.0, 1.2 units/ml). That is, 25 wells are filled for the standard curve and, after 21 hr of incubation at 37°C, zone diameters are measured and the sum of the zone diameter is computed for each concentration [i.e., \( \sum (0.4) \), \( \sum (0.6) \), \( \sum (0.8) \), \( \sum (1.0) \), \( \sum (1.2) \) mm]. Also, the grand total for all 25 zones is computed; this is \( \sum (T) \) mm. The constants are estimated as follows.

The slope of the line is
\[
b = -0.2 \left( \sum (0.4) - \sum (1.2) \right)
- 0.1 \left( \sum (0.6) - \sum (1.0) \right).
\]
(Note: \( b \) must be negative since zone decreases as antibody increases.)

The \( y \) intercept is \( a = \sum (T)/25 - 0.8 b \).

Confidence limits may be calculated for predicted concentrations corresponding to observed mean zone diameters of unknown sera (4). In a typical assay, 95% confidence limits were calculated for the predicted concentrations corresponding to the given observed mean zone diameters where each mean is based on three observations.

In this assay, the precision was shown in Table 2.

**Estimation of accuracy of the assay.** To determine the accuracy of the test, antisera containing known amounts of α-antitoxin activity (C. perfringens type A, C, and D antisera) were diluted to contain 1.0, 0.8, and 0.6 units of activity per ml and tested in the plate assay. The zones of flocculation were measured and the antilecithinase activity was calculated as for unknown sera.

Determination of assay reliability. Two lecithinagar plates were prepared from the same preparation of diluent, agar, and egg on the same day. Dilutions of antibody for the standard curve were made by two of the co-authors. Using the independently prepared dilutions, one investigator prepared five standard curves and the other four, each operator using one of the two plates. Plates were incubated under identical conditions and were read after 21 hr of incubation. This procedure permitted us to make statistical comparisons of differences between the standard curves of the same operator as well as between operators.

**Development of antilecithinase activity in rats and rabbits injected with lecithinase C.** Norwegian hooded rats and New Zealand white rabbits were injected with increasing amounts of lecithinase C. Rats were injected intraperitoneally 11 times with the material in increasing amounts from 40 to 1,500 μg, and rabbits were injected subcutaneously 6 times with the material in increasing amounts from 40 to 1,500 μg and with two intraperitoneal injections of 1,500 μg each. Animals were bled and their sera were tested weekly.

**Determination of lecithinase antibody levels in 231 human sera.** Lecithinase antibody determinations were made in 231 sera obtained from two commercial

---

**Table 1. Standards for lecithinase C antibody curve**

| Stock type A antiserum (ml) | Diluent (ml) | Final vol. (ml) | Standard final concn (units/ml) |
|----------------------------|--------------|----------------|---------------------------------|
| 1.2                        | 8.8          | 10.0           | 1.2                             |
| 1.0                        | 9.0          | 10.0           | 1.0                             |
| 0.8                        | 9.2          | 10.0           | 0.8                             |
| 0.6                        | 9.4          | 10.0           | 0.6                             |
| 0.4                        | 9.6          | 10.0           | 0.4                             |

**Table 2. Calculated confidence limits and concentrations for observed mean zone diameters**

| Mean zone diameter (mm) | Predicted concn (units/ml) | Lower limit (unit/ml) | Upper limit (unit/ml) |
|-------------------------|-----------------------------|-----------------------|-----------------------|
| 14                      | 1.19                        | 1.13                  | 1.25                  |
| 15                      | 0.98                        | 0.93                  | 1.04                  |
| 16                      | 0.78                        | 0.73                  | 0.83                  |
| 17                      | 0.57                        | 0.52                  | 0.63                  |
laboratories. Laboratory A furnished 183 sera which were sent in for routine insurance physical examination profiles and laboratory B, which specializes in tests of thyroid function, supplied 48 sera. Clinical histories were not available. Three replications of 1:10 dilutions of sera in diluent were employed with lecithinase C (500 μg/ml) as antigen. Dilution of serum is necessary because it contains lecithin, or it may contain lecithinase(s). When undiluted serum is used, a reaction occurs between lecithinase C and serum lecithin within the well, resulting in small, cloudy, yellow zones which are not measurable. This effect was not seen at 1:10 or higher dilutions.

RESULTS

A typical lecithin-agar assay plate after 20 hr incubation at 37 C is presented in Fig. 1. Table 3 summarizes the mean zone diameters of the standard curve derived from 10 assays in which different preparations of lecithin were used. Note that the range of mean values is not wide and that linearity is maintained in spite of small variations in zone diameters found in different assays. One unit of anti-lecithinase C antibody was required to neutralize completely 250 μg of lecithinase C per ml. The optimal concentrations of lecithinase C and commercial lecithinase C, i.e., the minimal concentrations yielding the maximal zone diameter of 22 mm, were 500 μg/ml and 600 μg/ml, respectively.

The 1:10 dilution of test sera from human patients prevented nonspecific reactions and insured that a large majority of the serum antibody levels could be determined in one assay. The optimal incubation time based on sharpness of zone margin and reproducibility of test results was 20 hr, although readings as late as 22 hr were satisfactory. After 22 hr, zones continued to increase.

![Photograph of typical lecithin-agar plate after 20 hr of incubation at 37 C. (A) Standard curve: five replications of standard antibody concentrations from highest (1.2 units/ml), upper left, to lowest (0.4 units/ml), fourth zone below upper left-hand corner zone. (B) Measured concentration of antibody in commercial antitoxin for a "recovery" experiment: three replications of each serum proceeding downward column by column. (C) Rabbit sera during course of immunization against lecithinase C: three replications of each serum. (D) Sera from patients. +, Spill due to overfilling the well or to an imperfectly cut well; zone not measured for calculation of antibody concentration. 0, Excess antibody present so that zone diameter is off the curve; must be repeated at lower concentration of antibody. (Note: The reproduction is a copy of a photographic negative so that the cloudy zones referred to in the text show in this reproduction as clear black zones.)

### Table 3. Mean zone diameters found in a series of 10 assays with different preparations of lecithin

| Date of assay | Mean zone diameters at various concn of antibody<sup>a</sup> |
|---------------|--------------------------------------------------------|
|               | 1.2 | 1.0 | 0.8 | 0.6 | 0.4 |
| 2/3/70        | 13.0 | 15.0 | 16.0 | 17.0 | 18.0 |
| 12/2/69       | 14.0 | 15.0 | 16.0 | 17.0 | 18.0 |
| 10/24/69      | 15.7 | 14.8 | 15.9 | 17.0 | 18.0 |
| 10/20/69      | 15.2 | 16.2 | 16.0 | 16.9 | 17.7 |
| 10/3/69       | 14.3 | 15.0 | 16.0 | 16.9 | 18.9 |
| 9/26/69       | 15.3 | 16.0 | 17.0 | 18.0 | 19.0 |
| 9/24/69       | 14.9 | 15.9 | 16.9 | 17.9 | 18.9 |
| 9/23/69       | 14.0 | 15.4 | 16.1 | 17.2 | 18.0 |
| 9/19/69       | 14.9 | 16.0 | 16.9 | 17.9 | 18.9 |
| 9/16/69       | 15.1 | 16.1 | 17.0 | 18.0 | 18.9 |
| Range, all assays | 13.0-15.3 | 14.8-16.2 | 15.9-17.1 | 16.9-18.1 | 17.7-19.1 |

<sup>a</sup> Diameters mean of five replications. Same lot of antitoxin was used in all assays.

<sup>b</sup> Units/ml of commercial α-antitoxin.
in diameter and to develop "fuzzy" margins that tended to merge with adjoining zones. Incubation time (approximately 20 hr) was chosen as a matter of convenience, and we made no attempt to measure the rate of increase in zone diameters. Antibody concentrations of 0 to 1.6 units/ml yielded a quadratic curve. Between 0.4 and 1.2 units/ml, the curve was substantially linear (Fig. 2).

The accuracy of the assay as determined by "recovery" experiments was satisfactory (Table 4). Recoveries ranged from 73 to 120%, with the majority of results falling between 90 and 110%. It should be noted that the poorest results occurred when type A antiserum, having the highest antibody concentration, was used. In this case, there was a large dilution factor of the order of 390:1 (diluent:antibody) so that minor errors in pipetting sera and diluent were magnified. The reliability of the assay based on a comparison of

---

**Fig. 2.** Graph showing the linear and quadratic nature of the standard curve when antibody concentrations are altered.

**Table 4.** Recovery of lecithinase C antibody from commercial equine Clostridium perfringens antisera

| C. perfringens antisera type | Antibody* present (unit/ml) | Test-derived antibody (unit/ml) | Ranges of three recovery attempts (per cent recovered) |
|-----------------------------|-----------------------------|--------------------------------|--------------------------------------------------|
| A                           | 1.0                         | 0.91                           | 73-110                                           |
|                             | 0.8                         | 0.80                           | 89-114                                           |
|                             | 0.6                         | 0.64                           | 95-117                                           |
| C                           | 1.0                         | 1.03                           | 90-113                                           |
|                             | 0.8                         | 0.85                           | 87-116                                           |
|                             | 0.6                         | 0.64                           | 95-120                                           |
| D                           | 1.0                         | 0.98                           | 97-100                                           |
|                             | 0.8                         | 0.78                           | 96-100                                           |
|                             | 0.6                         | 0.59                           | 95-102                                           |
| Control                     | <0.4                        | <0.4                           |                                                   |

* Anti-α-toxin concentrations.

---

**Table 5.** Comparison of reliability of assay between curves and between operators

| Operator and curve no. | No. of wells | Standard curves |
|------------------------|--------------|-----------------|
|                        |              | Intercept (mm)  | Slope |
| KHS I                  | 24           | 21.00           | -5.00 |
| KHS II                 | 25           | 20.86           | -4.80 |
| KHS III                | 25           | 20.92           | -4.85 |
| KHS IV                 | 25           | 21.00           | -4.90 |
| KHS V                  | 25           | 20.94           | -4.90 |
| KHS overall            | 124          | 20.94           | -4.89 |
| FAB I                  | 25           | 20.88           | -4.85 |
| FAB II                 | 25           | 21.12           | -5.10 |
| FAB III                | 25           | 20.74           | -4.70 |
| FAB IV                 | 25           | 20.86           | -4.85 |
| FAB overall            | 100          | 20.90           | -4.88 |

---

**Fig. 3.** Development of lecithinase C antibody in rats and rabbits injected with increasing amounts of lecithinase C. Arrow indicates days on which lecithinase C was injected. Mean and ranges for each week's tests are plotted. (A) Concentration of antibody developed in eight rats after intraperitoneal injection of 40 to 1,500 μg. (Death of two rats occurred in the 4th week when animals with titers of less than 20 units/ml were injected with 400 μg of lecithinase C.) (B) Concentration of antibody developed in rabbits after subcutaneous or intraperitoneal injection of 1,500 μg. (Death of two rabbits occurred in the 5th week and one in the 6th week when animals with titers of less than 25 units/ml were injected with 1,000 μg of lecithinase C subcutaneously.)
TABLE 6. Determination of lecithinase antibody in the serum of patients

| Lecithinase antibody (unit/ml) | Laboratory A | Laboratory B | Total |
|-------------------------------|-------------|-------------|-------|
|                               | No. | Per cent | No. | Per cent | No. | Per cent |
| <4.0                          | 169 | 92.3     | 41  | 85.4     | 210 | 90.9     |
| 4.0-5.9                       | 12  | 6.6      | 7   | 14.6     | 19  | 8.2      |
| 6.0-7.9                       | 2   | 1.1      | 0   | 0        | 2   | 0.9      |
| 8 or more                     | 0   | 0        | 0   | 0        | 0   | 0        |
| Total                         | 183 | 100.0    | 48  | 100.0    | 231 | 100.0    |

Replicate standard curves (Table 5) was also satisfactory. There were no statistically significant differences between the standard curves of either operator individually, and none between the overall performance of the two operators.

Some minor variations occurred between different lots of antisera. However, careful testing of each lot of antiserum against a standard curve set up from the previous lot permitted rapid determination of the dilution of antisera needed to replicate the curves.

Eight rats and 7 rabbits immunized with increasing amounts of lecithinase C showed typical response curves (Fig. 3). All rabbits had titers of more than 15 units/ml by the 14th day, and all rats but one had titers of 5 units/ml or more. The potency of lecithinase C preparation was such that three rabbits with titers of less than 25 units/ml died when injected with 1,000 µg of lecithinase C subcutaneously. Similarly, two rats with antibody titers below 20 units/ml injected with 400 µg of lecithinase C intraperitoneally died.

Assays of 183 human sera from Laboratory A (Table 6) showed 92.3% with less than 4 units/ml of lecithinase antibody, whereas 85.4% of sera from Laboratory B had less than 4 units/ml. The difference is not statistically significant.

Discussion

The agar diffusion assay reported herein is an indirect but accurate and reproducible method for determination of antilecithinase activity in serum. Microquantities of sera can be assayed so that capillary blood specimens would be satisfactory. A 1:10 dilution will permit titration of most sera on a one-test basis. Dilution of sera also diminishes or eliminates possible errors due to endogenous lecithinase that may be present in some sera, e.g., in serum from patients on heparin therapy (3).

The linear form of the standard curve appears to depend on the narrow range of antibody concentration used—0.4 to 1.2 units/ml. If the range is increased to 0 to 1.6 units/ml, the curve becomes quadratic, flattening out between 0 and 0.4 units/ml and dropping sharply at concentrations higher than 1.2 units/ml (Fig. 2). This is apparently due to the kinetics of diffusion in agar and because of the cylindrical geometry of the agar wells and their surrounding cloudy zones. Becker (1) has studied this and has demonstrated a linear relationship between the square of zone diameters and antigen concentration. We agree with Becker's interpretation, but fitting the standard curve and calculation of precision is far simpler when using the narrow range of antibody concentrations. In the average laboratory, it may be more satisfactory to redilute sera that are out-of-range until zone diameters fall in the linear portion of the curve.

The test worked well in measuring increasing antibody levels in rats and rabbits (Fig. 3). The antibody ranges are wide as compared between animals, but we attribute this to differences in individual animal tolerance to highly toxic lecithinase C. The assay was used to determine antibody concentration in rat and rabbit sera prior to inoculation with additional lecithinase C. When animals were injected with amounts of lecithinase larger than the titers indicated they could tolerate, severe local reactions, bleeding, and death followed. We have found the assay useful in the measurement of rising antibody concentrations in rat serum during feeding of pure cultures of several strains of C. perfringens (to be published).

Slightly more than 9% of sera obtained from Laboratories A and B had more than 4 units/ml of lecithinase antibody. Since clinical histories were not obtained, we cannot report whether this reflects prior exposure to C. perfringens or its toxins. We conclude that 4 units/ml is the lower limit of sensitivity of the assay; the data (Table 6) also cautiously suggest that more than 8 units/ml may indicate (i) exposure either to C. perfringens or its toxins or (ii) the presence of antibody to other lecithinase(s) that cross-react with lecithinase C. Samples with 4.0 to 7.9 units/ml may represent individuals with previous exposure to toxin; however, until studies can be made on samples from patients with known clostridial infection exact limits cannot be established.

Literature Cited

1. Becker, W. 1969. Determination of antiserum titres using the single radial immunodiffusion method. Immunochemistry 6:539–546.
2. Bennett, J. V., J. L. Brodie, E. J. Benner, and W. M. M. Kirby. 1966. Simplified, accurate method for antibiotic assay of clinical specimens. Appl. Microbiol. 14:170–177.
3. Berlin, R., C. O. Oldfols, and O. Vikrost. 1969. Increased lecithinase activity after heparin administration. Acta Med. Scand. 185:433–437.
ASSAY FOR LECITHINASE ANTIBODIES

4. Brownlee, K. A. 1969. Statistical theory and methodology in science and engineering. John Wiley and Sons, Inc., New York.

5. Cassidy, J. T., G. W. Jourdain, and S. Roseman. 1965. The sialic acids. VI. Purification and properties of sialidase from Clostridium perfringens. J. Biol. Chem. 240:3501-3506.

6. Macchia, V., R. W. Bates, and I. Pastan. 1967. The purification and properties of a thyroid-stimulating factor isolated from Clostridium perfringens. J. Biol. Chem. 244:3726-3730.

7. Sheldon, D. R., M. Moskowitz, and M. W. Deverall. 1959. Agar diffusion procedures for the assay of lecithinase from Clostridium perfringens. J. Bacteriol. 77:375-382.