Homogeneous Liposome Lysis Assay for Determination of Anti-Streptolysin O Antibody Titer in Serum

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Received 14 September 1987/Accepted 16 January 1988

We developed a liposome lysis assay for determining anti-streptolysin O antibodies (ASO) in human sera involving the use of carboxyfluorescein-entrapped multilamellar liposomes. This assay system was based on the inhibition of streptolysin O-induced lysis of rabbit erythrocytes by ASO. Briefly, after incubation of a given amount of streptolysin O with ASO for 30 min at 37°C, carboxyfluorescein-entrapped liposomes composed of egg yolk phosphatidylcholine and cholesterol in a molar ratio of 1:1 were added to the mixture to determine the residual streptolysin O activity. Liposome lysis, detected as carboxyfluorescein release from the liposomes, was inversely proportional to the ASO titer. The results of within-run and between-run precision studies indicated that the lysis assay is accurate and gives reproducible data. Bilirubin, hemoglobin, and triglycerides did not interfere with the lysis assay. The ASO titers of 100 patient sera, evaluated by our new method and the Rantz-Randall method, showed good correlation.

Streptolysin O (SLO) is a toxic immunogen produced and secreted by most Streptococcus pyogenes group A strains. It is cytolytic or cytotoxic toward erythrocytes and other types of eucaryotic cells (3). Clinically, elevated levels of anti-SLO antibodies (ASO) in sera have been shown to be associated with streptococcal diseases such as glomerulonephritis and rheumatic fever (6, 12).

The ASO titer in serum has previously been determined to be the serum dilution that inhibits 50% of the SLO-induced hemolysis of rabbit erythrocytes (7, 8). However, some problems are encountered during practical use of this method, the Rantz-Randall method; i.e., rabbit erythrocytes are unstable on storage, and erythrocytes from different rabbits vary in susceptibility to SLO. To overcome these disadvantages, we attempted to use carboxyfluorescein (CF); a fluorescent dye-entrapped multilamellar liposomes instead of rabbit erythrocytes, because such liposomes are stable on storage and the quality of the liposomes can be easily controlled.

Duncan and Schlegel studied the interaction between SLO and multilamellar liposomes composed of phospholipid and cholesterol (4). They reported that ring- or arc-shaped structures were observed electron microscopically on liposomes exposed to SLO, but no release of an internal marker (K2CrO4 or glucose) from the liposomes occurred. However, the results of our recent experiments indicated that multilamellar liposomes composed of phosphatidylcholine and cholesterol released the entrapped CF upon exposure to SLO (details to be published elsewhere). Based on these results, we have developed a liposome lysis assay (LLA) for determination of ASO titers in sera that uses CF-entrapped multilamellar liposomes. The LLA is spectrophotometric, simple, and accurate, and results are reproducible.

MATERIALS AND METHODS

Lipids and other chemicals. Egg yolk phosphatidylcholine was obtained from Nippon Fine Chemical Co. Ltd., Osaka, Japan, and cholesterol was from Sigma Chemical Co., St. Louis, Mo. These lipids were dissolved in chloroform and stored at −20°C under nitrogen gas. CF was purchased from Eastman Kodak Co., Rochester, N. Y., and purified as described by Weinstein et al. (9).

SLO. SLO was purified from the culture supernatant of S. pyogenes Richards by the method described by Bhakdi et al. (2). The purified SLO was lyophilized and then stored at −20°C after determination of the protein concentration (3) and hemolytic activity (in hemolytic units [HU] per milliliter) (5).

ASO standard. ASO was raised in rabbits by immunization with SLO (6,400 HU/ml) adsorbed on latex particles (0.22 μm in diameter; Japan Synthetic Rubber Co. Ltd., Tokyo, Japan). Rabbit antisera to SLO was assayed by the Rantz-Randall method (7), the titer being 833 Todd units (TU). To prepare a standard curve, the antisera was appropriately diluted with Veronal (Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.)-buffered saline (0.58 g of 5,5-diethylbarbituric acid, 0.38 g of sodium 5,5-diethylbarbiturate, 8.5 g of NaCl per liter, pH 7.4, 25°C) containing 0.1% gelatin (GVB). The standard value was expressed in arbitrary units, defined as 833 TU/dilution of antisera; e.g., 1.3 U = 833/640.

Preparation of CF-entrapped liposomes. Multilamellar liposomes were prepared from a lipid solution containing egg yolk phosphatidylcholine (0.5 μmol) and cholesterol (0.5 μmol) as described previously (11). Briefly, the lipid solution was evaporated to dryness in a rotary evaporator at 35°C and then further dried for 1 h under vacuum. CF-entrapped liposomes were obtained by mechanical stirring of the lipid film in 0.1 ml of a 0.2 M CF solution. Nonentrapped CF was removed by repeated centrifugation (20,000 × g for 20 min at 4°C). After the final centrifugation, the pellet was suspended in 1 ml of GVB containing 0.1% NaNO3 and then stored at 6 to 10°C. The stock liposome suspension (ca. 1 mmol of total lipids per liter) was diluted 1,000-fold before use.

Assay principle. The LLA for the determination of ASO in serum is based on inhibition of SLO-induced liposome lysis by ASO. The liposomes are composed of egg yolk phosphatidylcholine and cholesterol in a molar ratio of 1:1. CF being encapsulated in interlamellar structures. CF is a self-quenching fluorophore when entrapped in liposomes at high concentrations, but it is highly fluorescent when released.
from the liposomes (10). CF release is measured as the fluorescence, with excitation at 490 nm and emission at 530 nm.

ASO-containing serum is incubated with a constant amount of SLO for 30 min at 37°C, and then the liposomes are added to the mixture. After incubation for an additional 30 min at 37°C, the residual SLO activity is measured and is inversely proportional to the ASO titer in serum.

**Standard assay system.** All dilutions were performed with GVB containing 1 mM mercaptoethanol. A 50-μl portion of diluted ASO-containing human serum and 25 μl of SLO (20 HU/ml) were added to each well of a microplate (Nunc, Roskilde, Denmark), which was then allowed to stand for 30 min at 37°C. A 25-μl amount of the 1,000-fold-diluted liposome suspension was added to the mixture, followed by incubation for another 30 min at 37°C.

A microplate fluorometer, model MTP-12F (excitation, 490 nm; emission, 530 nm; Corona Electric Co., Katsuta, Japan), was used for measuring CF release. Specific marker release (percent) was calculated by the following equation: specific marker release (％) = [(experimental release - spontaneous release)/(total release - spontaneous release)] × 100, where spontaneous release is releasable fluorescence obtained on adding 75 μl of GVB instead of human serum and SLO, and total release is that obtained on adding 50 μl of GVB instead of human serum. Specific marker release (percent) versus log SLO standards (units) was plotted on semilog paper to prepare a standard curve. ASO titers of serum samples were determined from the standard curve.

**RESULTS AND DISCUSSION**

**Liposome lysis by SLO.** Since multilamellar liposomes composed of equimolar amounts of phosphatidylcholine and cholesterol were found to be susceptible to SLO and to release the internal marker, we used such liposomes as an indicator throughout this study.

To determine the optimal conditions for measurement of SLO-induced liposome lysis, we examined the effects of the concentrations of liposomes and SLO on CF release from the liposomes. SLO (3,200 HU/ml) was diluted 10-fold and then serially diluted 4-fold for five dilutions. The stock liposome suspension was diluted 100- to 1,500-fold with GVB containing 1 mM mercaptoethanol. A 25-μl portion of the diluted SLO solution and 25 μl of the liposome suspension were added to each well of a microplate, in duplicate, and then 50 μl of GVB containing 1 mM mercaptoethanol was added to the mixture in each well. After the mixture was stirred and incubated for 30 min at 37°C, the fluorescence intensity was measured with a microplate fluorometer. Percent CF release was calculated as described in Materials and Methods.

Figure 1 shows representative CF release curves obtained with various liposome dilutions. The results indicate that SLO induced CF release from the liposomes in a dose-dependent manner and that there were no significant differences between the CF release curves when the liposome dilutions were in the range of 700- to 1,500-fold. With such liposome dilutions, 100% lysis was obtained with the SLO solutions that had been diluted <160-fold. Based on these results, we used the combination of 1,000-fold-diluted liposomes and 160-fold-diluted SLO (20 HU/ml) for the following assays.

Under the conditions described above, we followed the time course of CF release for 3 h. The results indicated that the CF release reached a plateau before 30 min of incubation (data not shown). Therefore, an incubation time of 30 min was used for the following assays.

**Standard curve for determination of ASO titer.** Figure 2 shows a representative standard curve obtained with a series of diluted ASO standards (rabbit serum). The assay was performed as described in Materials and Methods. CF release (percent) was found to be inversely proportional to the authentic ASO titers in the range of 0.5 to 2.5 U. The ASO titer of the test sample was calculated as follows: ASO titer = (sample dilution) × (value read from the standard curve), e.g., ASO titer (300 U) = 200 × 1.5 U.
TABLE 1. Precision of LLA for measurement of ASO titers in human sera

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Titer (U)</th>
<th>Mean</th>
<th>SD</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within run</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (12)</td>
<td>23</td>
<td>2.31</td>
<td></td>
<td>4.6</td>
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<tr>
<td>2 (50)</td>
<td>52</td>
<td>1.26</td>
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<td>2.4</td>
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<tr>
<td>3 (166)</td>
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<td>4.62</td>
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<td>2.7</td>
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<tr>
<td>4 (250)</td>
<td>200</td>
<td>6.02</td>
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<td>3.6</td>
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<tr>
<td>5 (833)</td>
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<td>46.1</td>
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<td>5.4</td>
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<td>6 (1,250)</td>
<td>983</td>
<td>45.2</td>
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<td><strong>Between run</strong></td>
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<td></td>
</tr>
<tr>
<td>1 (12)</td>
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<td>2.12</td>
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<td>2.7</td>
</tr>
<tr>
<td>2 (50)</td>
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<td>44.5</td>
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<td>3.3</td>
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</table>

* Numbers in parentheses are ASO titers determined by the Rantz-Randall method.
* Six individual sera were assayed, in 20 replicates (on 7 consecutive days for between-run samples).

Optimization of sample dilution. When the ASO titers of sera are high, i.e., in the range of 12 to 2,500 TU, the samples should be diluted appropriately. To determine the optimal sample dilution, we assayed dilutions of three samples that had been previously measured by the Rantz-Randall method (50, 166, and 1,250 TU). The ASO titers of these samples could be determined within the measurable range of the standard curve when the samples containing 50, 166, and 1,250 TU were diluted in the ranges of 30 to 50, 30 to 200, and 500 to 1,400, respectively. On the basis of the results, the following dilutions were used for the assays: 40-fold dilution for samples containing 12 to 50 TU, 200-fold dilution for those containing 100 to 333 TU, and 1,000-fold dilution for those containing 500 to 2,500 TU. For screening sera, a 200-fold dilution would be optimal, because values of >333 TU are considered ASO positive.

Precision and reproducibility. The precision of the assay was examined with samples showing high, normal, and low ASO titers (Table 1). For within-run precision, determined by assaying 20 replicate samples with six different ASO titers, coefficients of variation in the range of about 2.5 to 5.5% were obtained. Between-run precision was determined by assaying samples in the same manner on 7 consecutive days, and the coefficients of variation were all <7%. The highly reproducible data of the between-run precision test were obtained in the following 6 months (data not shown). Furthermore, we determined the ASO titer of a sample containing 333 TU with three separately prepared batches of liposomes, and the coefficient of variation of the results obtained was ±6%. These results indicate that the LLA is accurate and the results are reproducible.

Interference studies. We studied the effects of bilirubin, hemoglobin, and triglycerides on ASO titer determination by using serum samples supplemented with bilirubin, hemoglobin, or triglycerides. The results obtained for 10 replicate samples with three different ASO titers showed that none of them appreciably affects the precision of the assay (Table 2).

Correlation studies. The ASO titers of 100 patient sera were determined by the two methods, i.e., the LLA (y) and the Rantz-Randall method (x). Comparison of the results by linear regression analysis showed good correlation between the results obtained by the two methods (y = 0.99x + 65; r = 0.94) (Fig. 3). Assuming that values of >333 U or 333 TU are ASO positive, 70 of the 100 sera were determined to be negative by the LLA method, whereas 76 were found to be negative by the Rantz-Randall method. The discrepancy seems to be due to the dilution scheme used for the Rantz-Randall method; i.e., sera categorized as 250 TU may have various ASO titers, ranging from 166 to 333 TU. In fact, only for sera containing 250 to 333 TU were discrepant results obtained with the two assays.

In this work, we have developed a simple, accurate, and reproducible LLA for determination of ASO titer in serum. It is based on inhibition of SLO-induced liposome lysis by ASO. As linear regression analysis (Fig. 3) of the data shows, there is good correlation between the results obtained by the LLA and the Rantz-Randall method. In addition, the present LLA has the following advantages over the Rantz-Randall method. (i) In contrast to the rabbit erythrocytes used for the Rantz-Randall method, the liposomes used for the LLA are stable on storage for at least 6 months at 6 to 10°C. (ii) There is no significant variation in susceptibility to SLO between separately prepared batches of

![FIG. 3. Correlation between results obtained with LLA and Rantz-Randall methods. The linear regression line was obtained by assaying the ASO titers of 100 patient sera by the Rantz-Randall method (X) and the LLA (Y), as described in Materials and Methods.](http://jcm.asm.org/Downloadedfrom)
liposomes. (iii) The Rantz-Randall method is a stepwise method involving the use of a series of serum dilutions, and the ASO titer of serum is determined as the serum dilution that inhibits nearly 50% of SLO-induced hemolysis. Therefore, the method gives stepwise ASO titers for various sera; e.g., sera categorized as 250 TU may contain different ASO titers ranging between 166 and 333 TU. By contrast, the present LLA is a linear analysis, in which ASO titer is directly calculated from percent inhibition of SLO-induced liposome lysis by the serum. (iv) Moreover, since the serial dilution of serum is not necessary and the assay procedure is homogeneous, the assay requires less time and labor. Thus, the LLA developed in our study may be suitable for diagnostic use.

LITERATURE CITED