Novel homogeneous liposomal immunoassay for colorimetric estimation of serum IgG antcardiolipin antibodies

Stephen J. Frost, 1,2* Jessie Chakraborty, 2 and Gary B. Firth 1

Liposomes entrapping the dye sulforhodamine B were used to develop an assay for antcardiolipin antibodies (ACAs). In the presence of magnesium ions, IgG ACAs induced liposomal lysis and the resulting absorbance changes were dependent on the amount of ACAs present. The liposomal assay showed similar intra assay imprecision and detection limit to an ELISA for IgG ACAs, with a correlation coefficient of 0.90 ($y = 1.05x - 1.61$). No correlation with ELISA IgM ACA measurements was observed. Although ELISA remains the method of choice, particularly in examining different ACA classes, the liposomal assay offers advantages of speed and potential for processing large numbers of samples for IgG ACAs. This may facilitate study of the significance of increased IgG ACAs in the groups of conditions with which they are associated, and perhaps enable more laboratories to perform the test.

INDEXING TERMS: liposomes • sulforhodamine B • spectrophotometry

Liposomes are synthetic lipid vesicles. On their formation, measurable substances can be encapsulated within their aqueous interior. In addition, substances such as antigens or antibodies can be attached covalently to their surface. The liposomes can then be used as analytical reagents, most frequently in homogeneous immunoassays [1–6]. A common approach is to measure release of an entrapped substance after immune reaction at the liposome surface. Release of liposomal contents can be brought about by animal complement [1–4], cytolysins [5, 6], or (in cardiolipin-containing liposomes) divalent cations [7]. Liposomal immunoassays provide an alternative to other recent types of immunoassay. Each liposome can entrap many molecules of measured substance, giving a potential amplification of signal in an immunoassay. If the entrapped material is a sensitive marker (e.g., fluorophore), liposomal immunoassays have potential for increased sensitivity. The use of an intense chromogen (e.g., dye), on the other hand, can produce a simple and rapid assay by direct spectrophotometric measurement, provided a color change occurs when the chromogen is released.

Cardiolipin-containing liposomes have long been shown to be destabilized in the presence of divalent cations [8]. Janoff et al., in 1983, reported a change of color when liposomes containing the dye arsenazo III were lysed by magnesium ions [7]. The liposomal lysis and release of dye was found to be inhibited by systemic lupus erythematosus (SLE) serum. The procedure was essentially qualitative although the amount of color change could be measured by a spectrophotometer. The nature of the antibodies causing this inhibition was not established in the report; in 1983, the range of antibodies found in SLE sera had not been clearly elucidated. After the development of specific antcardiolipin antibody (ACA) assays involving RIA and ELISA [9, 10], ACAs are now known to exist as a discrete population of antibodies (although there appears to be overlap in reactivity in some cases with other SLE antibodies such as anti-double-stranded DNA). Distinct prognostic roles of ACA antibodies in SLE have also been established. IgG ACAs are a class of antiphospholipid antibodies (APAs) found in a subgroup of patients at risk of thrombosis, recurrent fetal loss, and thrombocytopenia [11–13]. Patients, with or without the autoimmune disease SLE, who have APAs and associated clinical conditions are included under the term "antiphospholipid antibody syndrome" (APS). When thrombotic features are found together with APAs but without the other clinical features of SLE, the condition is referred to as the primary APS. IgM ACAs tend to

1 Department of Clinical Biochemistry, The Princess Royal Hospital, Lewes Rd., Haywards Heath, West Sussex, RH16 4EX, UK.
2 School of Biological Sciences, University of Surrey, Guildford, Surrey, GU2 5XH, UK.
*Author for correspondence. Fax Int 44 1444 459635.
Preliminary findings have been reported in poster abstract: Frost Sj, Firth GB, Chakraborty J. Proceedings of the Association of Clinical Biochemists National Meeting 1994:147.
Received October 3, 1995; accepted February 9, 1996.

Nonstandard abbreviations: SLE, systemic lupus erythematosus; ACA, anticardiolipin antibody; APA, antiphospholipid antibody; APS, antiphospholipid antibody syndrome; PC, dipalmitoyl phosphatidyl choline; PTA, dipalmitoyl phosphatidic acid; and $\beta_2$-GPI, $\beta_2$-glycoprotein I.
be associated with a range of clinical features different from IgG ACAs, including livedo reticularis, chorea, and hemolytic anemia.

A novel chromogenic liposomal marker has recently been developed by using sulforhodamine B [4]. When this dye is entrapped at high concentration it forms dimers, which results in an altered absorption spectrum (major absorption peak at 530 nm) compared with dilute free dye (major absorption peak at 565 nm). When the liposomes release their contents, the dye is diluted into the surrounding aqueous medium and the absorption spectrum reverts to that of free dye. This results in an absorbance change that can be monitored at either 565 nm or 530 nm. This report utilizes this chromogenic marker, first to establish that the effects observed by Janoff et al. [7] on the divalent ion-dependent lysis of cardiolipin-containing liposomes are attributable to ACAs, and second to demonstrate that reasonable agreement can be achieved between a liposomal assay based on this phenomenon and a conventional ELISA technique.

**Materials and Methods**

**LIPOSOME PREPARATION**
The reverse evaporation technique of Szoka and Papahadjopoulos [14] was used, modified as described previously [4, 15]. The lipids dipalmitoyl phosphatidyl choline (PC), cholesterol, and cardiolipin of the highest available purity, and the dye sulforhodamine B were purchased from Sigma Chemical Co., Poole, UK. Other chemicals and Analar-grade reagents were supplied by Merck, Poole, UK. Cardiolipin was supplied as a sodium salt in ethanol solution, which first had to be removed to prevent possible liposome disruption. An appropriate volume (usually ~6 mL) of cardiolipin solution was dried in a rotary evaporator, redissolved in 6 mL of chloroform, and again evaporated to dryness. The cardiolipin was finally redissolved in 2 mL of chloroform. To this solution the other lipids, petroleum ether (2 mL), and aqueous sulforhodamine B (1 mL, 0.1 mol/L) were added. The reverse evaporation procedure was then carried out to synthesize liposomes. The lipid composition (substance fractions) used to prepare liposomes subsequently used in the immunoassay was PC, cholesterol, and cardiolipin 36, 24, and 40, respectively. Free dye was removed by four sequential dialyses into 40 volumes of phosphate buffer, 0.05 mol/L, pH 7.4, with $\frac{1}{2}$-inch dialysis tubing (Medicell International, London, UK). When the cardiolipin content was varied experimentally the molar ratio of PC to cholesterol was kept constant.

**ACA ASSAY PROCEDURE**
The release of dye from the liposomes could be monitored either with a spectrophotometer or an automated analyzer [4]. In this study a Cobas Bio Centrifugal Analyzer (Roche Diagnostic Systems, Welwyn Garden City, UK) was used. The settings used on the Cobas Bio are shown in Table 1 (modified as described to assess and optimize variables).

### Table 1. Reaction parameters used on Cobas Bio analyzer to assess MgCl$_2$ destabilization.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 (reagent 1): 5mL of phosphate buffer plus 40 µL of liposomes, 100 µL/cuvette</td>
<td>Sample: serum, 5 µL/cuvette</td>
</tr>
<tr>
<td>Dient (distilled water): 10 µL/cuvette</td>
<td>Diluent</td>
</tr>
<tr>
<td>R2 (reagent 2): 5 mol/L MgCl$_2$, 10 µL/cuvette</td>
<td>Temperature: 25 °C</td>
</tr>
<tr>
<td></td>
<td>Wavelength: 565 nm</td>
</tr>
<tr>
<td></td>
<td>Assay process: R1 and sample (+ diluent) dispensed and mixed; 3-min preincubation. R2 added and (after 3 s of mixing) change in absorbance in 120 s at 565 nm recorded.</td>
</tr>
</tbody>
</table>

**CALIBRATION OF ACA LIPOSOMAL ASSAY**
The assay was calibrated with an IgG ACA-positive serum (kindly donated by J. Faux, Churchill Hospital, Oxford, UK) with an antibody concentration of 1100 GPL units [GPL unit is the unit of activity (IgG; MPL for IgM) of a standardized affinity-purified serum from a patient [10]]. This was diluted in a serum pool to establish a calibration curve. To minimize the antibody concentration present in the pool, samples (n = 100) from patients without evidence of immunological disorders were collected. The 20 samples with the lowest lysis by MgCl$_2$, assessed on the Cobas Bio, were combined and used subsequently as a "negative pool" for dilution of the ACA-positive serum.

**ELISAS**
ELISAs were performed for IgG ACAs and for IgM ACAs by using cardiolipin-coated microtiter plate assay kits of conventional design (SELISA; Cambridge Life Sciences, Ely, UK). Calibrators provided with the kits were used for the ELISAs. The manufacturer’s instructions were followed.

**SERUM SAMPLES**
Serum samples from patients on whom ACA analysis had been requested (n = 35) were obtained from the following Immunology Departments: Kings College Hospital, London, UK; Royal Hallamshire Hospital, Sheffield, UK; Royal Sussex County Hospital, Brighton, UK; and St. Helier Hospital, Carshalton, Surrey, UK. They were assayed on receipt by the liposomal immunoassay and by the ELISAs. In addition, to establish and compare reference ranges, plasma samples were collected from 20 healthy blood donors and also from 20 antenatal clinic attenders without evidence of immunological illness. Imprecision studies were conducted by using pools of samples with various ACA concentrations.

**Results**
Assessed by gel filtration, entrapment of dye (~21%) in the cardiolipin-containing liposomes was similar to that found previously in liposomes not incorporating cardiolipin but otherwise prepared under similar conditions [4]. The effect of addition of ACA on the Mg (II) ion-dependent absorption change is shown in Fig. 1. This also demonstrates that the dye release was dependent on the amount of cardiolipin used to form the
liposomes, and was virtually absent if cardiolipin was omitted. An additional observation was a “hook effect” at high ACA concentrations, but this was abolished up to 1000 GPL units when the cardiolipin content (substance fraction) of the liposomes was increased to 40. (Human serum rarely contains IgG ACAs at >100 GPL units, although occasionally concentrations up to 1000 GPL units can be found.)

When the reaction temperature was increased to either 30 °C or 37 °C, no appreciable improvement in sensitivity was observed. Instead, there was a significant increase in nonspecific dye release at higher temperatures in the absence of ACAs. The amount of liposomal dye release was also dependent on the concentration of MgCl₂ up to a reaction concentration of 0.4 mol/L. In its absence the rate was negligible. At physiological concentrations, magnesium ions and calcium ions did not cause detectable dye release.

The liposomal ACA assay was configured as a two-step assay. This provided a check of any liposomal lysis by human complement, which would have resulted in a high absorbance after the preincubation relative to the other cuvettes. No such anomalous absorbance changes were observed.

In preparing the diluent pool for calibrants in the liposomal assay, samples with low absorbance changes were selected to minimize ACAs. It is still likely that a small amount of antibodies was present, although the absorbance change due to the pool was only 0.018, which was low compared with that of added ACA (Fig. 1). The lowest absorbance change in any of these samples was 0.010. There was good agreement between the liposomal calibrator concentrations measured by the ELISA (which used its own calibrators) (y) and their target values assigned by diluting the reference serum (x) (r = 0.99). A regression slope of 0.98 and an intercept of 2 GPL was obtained. By using a calibration curve based on assays with cardiolipin-containing liposomes (Table 1), Fig. 2 shows the relation between the liposomal assay (y) and the IgG ELISA (x) when the results of 35 serum samples were compared. The linear regression equation obtained was: y = 1.05 (±0.18)x – 1.61 (±4.98), r = 0.90. Only four of the 35 samples analyzed by ELISA gave IgM ACA concentrations above the ELISA kit manufacturer’s reference interval (0.4–5 MPL units). There was no significant correlation between the dye release measured by absorbance and the ELISA IgM ACA concentrations [correlation coefficient (r) = 0.20, probability of null hypothesis (P) = 0.27].

**ESTABLISHMENT OF REFERENCE INTERVALS FOR DIGG ACAS**

The manufacturer of the ELISA kits recommended that each user should establish a reference range. A local reference range was established with 20 samples of citrated plasma from healthy blood donors and also 20 serum samples from antenatal clinic attenders without evidence of immunological illness. The samples were analyzed by using both IgG ACA ELISA and the liposomal method. The distribution of results was nongaussian (skewed towards increased concentrations); therefore logarithmic and square-root transformations of the data were compared. The square-root transformation proved a better means of
normalizing the results, and the normalized means and standard deviations from this transformation are shown in Table 2. The differences between the normalized mean values for the two sources of samples (i.e., blood donor and antenatal clinic attenders) were not significant by two-sample t-tests (Table 3), except for the difference between the ELISA results for the two sources of samples, which was weakly significant (P < 0.05). By using combined data for the two groups of samples, a square-root-transformed reference range of 1.1–19.9 GPL units was obtained for the liposomal assay and 1.9–20.5 GPL units for the ELISA. For comparison, the range for 5th to 95th percentiles was 2.8–16.5 GPL units for the liposomal assay and 4.2–18.5 GPL units for the ELISA.

**Comparison of Imprecisions**

Since in ELISA the tests are normally assayed in duplicate, the samples were assayed on the Cobas in the same way. The Cobas performs tests by using rotors with a maximum capacity of 24 tests per sample tray. The ELISA imprecision was calculated from results obtained from a single microtiter plate.

Samples could be analyzed on the Cobas either in a similar way to the ELISA method, i.e., with calibrators in the same rotor, or with a calibration curve in an earlier rotor, provided there was little drift in results from rotor to rotor. To assess both these options, imprecisions were calculated within a single rotor and also over three rotors. The results are shown in Fig. 3. The imprecisions were similar, except that over three rotors the Cobas performed considerably worse at low antibody concentrations.

**Limits of Detection**

The limits of detections were estimated as equal to 3 SD of a value close to zero, calculated from the imprecisions of the lowest pool (~5 GPL units). The limits of detections were 2 GPL units for ELISA and also 2 GPL units for the within-rotor liposomal assay, and 4 GPL units for the liposomal assay over three rotors.

**Discussion**

On the face of it, the augmenting of liposomal lysis by ACAs is contradictory to the report of Janoff et al. [7]. Their assay showed inhibition of liposomal lysis by SLE serum. However, both the preparation of liposomes and the reaction conditions used by Janoff et al. differed from this study, and some of these differences are compared in Table 4. As the procedures varied so markedly, it is perhaps not surprising that dissimilar effects were observed. Unfortunately it is not possible to estimate the relative amounts of liposomes used in the two techniques, since the liposome dilution of Janoff et al. was not indicated.

No antibody-dependent change in lysis rate was found in the absence of cardiolipin, even though PTA, which like cardiolipin is a negatively charged lipid, was present in the liposomes. This

---

**Table 2. Normalized values for mean and standard deviation.**

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>Liposomal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood donors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean*</td>
<td>10.2 (10.9)</td>
<td>8.4 (9.5)</td>
</tr>
<tr>
<td>SD of roots*</td>
<td>0.82 (5.9)</td>
<td>0.94 (5.7)</td>
</tr>
<tr>
<td><strong>Antenatal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean*</td>
<td>7.3 (7.8)</td>
<td>6.6 (7.0)</td>
</tr>
<tr>
<td>SD of roots*</td>
<td>0.68 (4.0)</td>
<td>0.72 (3.7)</td>
</tr>
<tr>
<td><strong>Overall</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean*</td>
<td>8.8 (9.4)</td>
<td>7.5 (8.5)</td>
</tr>
<tr>
<td>SD of roots*</td>
<td>0.78 (5.2)</td>
<td>0.85 (4.9)</td>
</tr>
</tbody>
</table>

* Mean is defined as the square of the mean of the square roots.

* SD of roots is the standard deviation of the square roots.

Numbers in parentheses are the untransformed arithmetic means and standard deviations, given for comparison.

---

**Table 3. Analysis of significance of differences in means (from Table 2).**

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA vs liposomal (overall)</td>
<td>0.26</td>
</tr>
<tr>
<td>ELISA donors vs liposomal donors</td>
<td>0.62</td>
</tr>
<tr>
<td>ELISA antenatal vs liposomal antenatal</td>
<td>0.51</td>
</tr>
<tr>
<td>ELISA donors vs ELISA antenatal</td>
<td>0.047</td>
</tr>
<tr>
<td>Liposomal donors vs liposomal antenatal</td>
<td>0.14</td>
</tr>
</tbody>
</table>
accords with the view that lysis is specific for cardiolipin. Significant liposomal lysis was observed in this study only in the presence of both ACAs and Mg (II) ions, suggesting that under these assay conditions, their combination is necessary for any degree of liposomal lysis.

The colorimetric assay of Janoff et al. [7] was based on the reaction of released dye with magnesium ions external to the liposome. A similar ion-dependent color change has been reported for the chromogen \(2-(2-\text{thiazolylazo})-4\)-methyl-5-(sulfo-methylamino)benzoic acid (TAMSMB), which reacts with chromium (III) ions [16]. As previously described [4], the use of sulforhodamine B as a marker is potentially simpler and more robust, since the color change is solely dependent on the dye's dilution and not on a second reactant (e.g., ion) concentration. Compared with ELISA, the sample dilution in the liposomal assay is much lower. The use of a greater amount of serum in the assay might have a beneficial effect compared with ELISA, since the concentration of \(\beta_2\)-glycoprotein I (\(\beta_2\)-GPI), a cofactor, is also much greater.

There has been considerable recent debate over the role of \(\beta_2\)-GPI since its involvement in binding ACAs was first suggested by Galli et al. [17]. The claim that \(\beta_2\)-GPI was the agent to which ACAs bind, rather than cardiolipin, sparked controversy. Their findings were apparently supported by the detection of APAs by using an ELISA with \(\beta_2\)-GPI as antigen [18], but may have been due to trace amounts of phospholipid contaminants in \(\beta_2\)-GPI preparations [19-21] or nonspecific binding to plates [22]. It now appears that \(\beta_2\)-GPI at least enhances ACA binding to cardiolipin bound to microtiter plates and the antigen seems to be a complex of cardiolipin and \(\beta_2\)-GPI. It is probable that adequate \(\beta_2\)-GPI was present in the liposomal immunoassay. The addition of animal serum is necessary in ELISAs, which involve large predilutions of sera.

There are several potential interferences, other than low \(\beta_2\)-GPI concentrations, that can affect ELISAs for ACAs, such as nonspecific binding to the plates [23] and possible heat-labile inhibitors of binding [24]. In addition, aspects of the liposomal technique differ markedly from ELISA. For example, the presentation of cardiolipin in the lipid membrane might have a different configuration and orientation from that of cardiolipin coated directly onto a microtiter plate. Despite these differences, a satisfactory agreement between IgG ELISA and the liposomal assay \(r = 0.90\) was obtained.

Although the liposomal immunoassay correlates with IgG ACAs measured by ELISA, there was no apparent correlation with IgM ELISA measurements. The main clinical applications of ACAs are related to their thrombotic effects, particularly in recurrent miscarriages and neurological and cardiological associations. These effects are reflected by IgG ACAs more than by IgM ACAs.

ELISA is likely to remain the main technique for APA measurement for some time. It is widely used, can be adapted to measure a range of antilipid antibodies, and extensive efforts have been invested to standardize the technique [25, 26]. Nevertheless, the liposomal assay has advantages because of its simplicity, greater assay speed (a few minutes compared with several hours), and ease of automation. A liposomal technique could be valuable in rapid preliminary tests for IgG ACAs, perhaps enabling wider study of the incidence of these antibodies. The assay may also facilitate preliminary investigations in smaller laboratories whose workload is too small to justify on-site testing by ELISA at present.

One potential clinical application that might benefit from a method for rapid screening of risk populations was suggested by the study of Hamsten et al. [27]. They found that raised IgG ACAs were associated with a high risk of recurrent cardiovascular events in postmyocardial infarction patients. ACAs were also shown in postmyocardial infarction patients in another small study [28]. However, no influence of raised ACAs soon after myocardial infarct on immediate patient outcome was reported in other studies [29, 30]. A liposomal assay may have a role in helping to reconcile these conflicting findings, which may be due to different selected populations, timing of samples relative to the myocardial event, and durations of study.

We are pleased to acknowledge the financial assistance of the South West Thames Locally Organized Research Scheme, The British Heart Foundation, and The Television South Trust.

### References

5. Freytag JW, Litchfield WJ. Liposome-mediated immunoassays for...


