Novel Liposome Composition for a Rapid Colorimetric Test for Systemic Lupus Erythematosus

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This test for systemic lupus erythematosus utilizes a novel liposome composition entrapping the cation-responsive red dye Arsenazo III. In dilutions of normal sera the liposome membranes undergo a rearrangement when divalent cations are added, resulting in the release of the encapsulated dye and the rapid formation of a stable blue cation–dye complex. Microliter amounts of sera from patients with active lupus stabilize the liposome preparation such that the vesicles remain intact in the presence of the added divalent cations and thus maintain their red color for extended periods. The assay requires 1-min incubations in sera at room temperature and can be performed with standard microtiter plates, allowing the screening of large numbers of serum samples in a short time. Moreover, the unique absorption spectra of the complexed and uncomplexed dye allow for quantification of results.

Additional Keyphrases: screening • liposome-entrapped dye marker

Systemic lupus erythematosus (SLE) is a serious autoimmune condition in which abnormal humoral and cellular immune responses occur. This immune complex disorder manifests symptoms such as erosive inflammation of skin (producing a characteristic rash), blood vessel lesions, pleurisy, psychiatric disturbances, convulsions, and inflammation of the kidney glomeruli (leading to renal dysfunction or failure). Although various factors have been attributed to the onset of SLE, its precise etiology is not known. Significant concentrations of both gamma-globulin and complement are present in the tissues, and such immune complexes are considered the causative agents for the systemic cellular damage that is observed. A wide range of autoantibodies have been detected in SLE patients, including circulating antibodies to lymphocytes, erythrocytes, platelets, and neutrophils. All four subclasses of immunoglobulin recognize cellular components such as nuclei, ribosomes, mitochondria, and lysosomes.

At present, detection of serum antinuclear antibody (ANA) by indirect immunofluorescence against nucleus-rich substrate represents the accepted laboratory screening procedure for the diagnosis of SLE (1). Although this is a sensitive test, registering "abnormal" in >95% of patients with SLE (2), a positive result has virtually no specificity, serum ANA frequently being present in many chronic connective-tissue diseases; in the elderly, in psychiatric inpatients, and in relatives of SLE patients; in response to various drug therapies; and in a substantial number of healthy controls (1). Standard practice in most centers is to follow up positive tests for ANA with assays of high specificity for SLE, most notable of which are the various tests for antibody to double-stranded DNA (ds DNA) (3), a test of particular value because patients with these antibodies are more likely to manifest clinically active SLE or renal involvement (4).

Although anti-ds DNA is the hallmark antibody of SLE, the site of antigenicity remains unclear. Double-stranded DNA is a weak immunogen in animal models (5). Recently, monoclonal antibodies to DNA have been shown to react with a wide range of both natural and synthetic polynucleotides (6). Additionally, phospholipids (including cardiolipin, phosphatidylglycerol, and phosphatic acid) have been demonstrated to bind competitively to monoclonal anti-DNA antibodies. Further, the antinuclear reaction of a single monoclonal lupus autoantibody is specifically inhibited by cardiolipin (7). The implication of this is that a common phosphate-ester epitope represents the principal site of antigenic recognition of anti-ds DNA (8). By exploiting the fact that cardiolipin serves as an antigen for circulating SLE autoantibodies, one can design a liposome-based assay system, containing cardiolipin as a major constituent, for the detection of such antibodies. The system presented here takes advantage of the fact that cardiolipin can be induced to rearrange from a bilayer to a nonbilayer form in the presence of divalent cations, thereby destabilizing the liposome structure (9). By encapsulating a cation-responsive dye inside the liposome, the binding of antibodies and subsequent stabilization of the liposomal structure to the perturbative effects of added divalent cations can be detected by an inhibition of the color change that results from the formation of a cation–dye complex upon breakdown of the liposomal barrier.

Materials and Methods

Lipids
Palmitoyloleoylphosphatidylcholine (POPC), beef-heart cardiolipin, and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids, Birmingham, AL 35216. Cholesterol was purchased from Supelco, Inc., Bellefonte, PA 16823, and a-tocopherol from Sigma Chemical, St. Louis, MO 63178. All lipids were chromatographically pure.

Cardiolipin was converted to its cesium salt and DPPG to its potassium salt by titration with cesium hydroxide or potassium hydroxide, respectively, after incubation of the lipid with a cation-exchange resin (AG-50W, mesh 100-200;
Bio-Rad Laboratories, Richmond, CA 94804). Details of this procedure will be published elsewhere.

Procedures

Preparation of liposomes. We added cardiolipin, POPC, cholesterol, DPPG, and a-tocopherol, from stock solutions in chloroform, to a round-bottom flask in a 3:4/1.9/1.0/0.1 mol ratio. Routinely, we used 40 µmol of total lipid. After removing the chloroform by rotary evaporation, we resuspended the dried lipids in 10 mL of petroleum ether containing 0.1 mL of methanol. The ether–lipid mixture was briefly sonicated to facilitate solubilization and used as the solvent phase during liposome production.

Liposomes were prepared by the ether infusion method of Deamer and Bangham (10) as modified by Oestro et al. (11). We placed the ether–lipid solution in a 20-mL glass syringe mounted on a Sage infusion pump, then pipetted 2 mL of an aqueous phase (final concentration, per liter, 4.5 mmol of Arsenazo III (AIII) solubilized in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 5 mM/L, pH 7.4, containing 0.5 mM/L of EDTA and 145 mM/L of NaCl/KCl per liter) into a stopped Liebig condenser heated to 54 °C with a circulating water bath. The ether–lipid phase was infused into the aqueous phase at 0.36 mL/min through a 22-gauge needle inserted through the rubber stopper at the bottom of the condenser. During the infusion process, nitrogen was gently bubbled through the aqueous phase to promote mixing and reduce lipid oxidation. Solvent phase entering the heated aqueous phase rapidly evaporated, leaving behind a turbid solution of free and liposome-entrapped AIII. At the conclusion of the infusion, we removed the liposome suspension from the top of the condenser and evaporated the residual solvent under reduced pressure for 1 h. The entrapped AIII was separated from the free dye by gel filtration on a 25-mL Sepharose 4B column, pre-equilibrated with multilamellar liposomes composed of egg phosphatidylcholine and egg phosphatidic acid in a mole ratio of 8/2. The liposomes were eluted from the column in the void volume, clearly separated from the free dye.

General lupus test procedure. The liposome-based SLE assay procedure is as follows: Place 20 µL of serum into either small glass tubes or the wells of a microtiter plate. Add 150 µL of red liposomes containing uncoupled AIII at a previously calibrated dilution. Mix the serum–liposome combination and incubate at room temperature for 1 min. Then add 10 µL of a previously calibrated concentration of MgCl₂, mix, and let stand for 5 to 10 min. If the serum sample is obtained from a patient suffering with SLE, the assay mixture remains red. If non-SLE serum is used, the liposomes turn blue, because of the breakdown of the liposomal structure and the subsequent formation of the stable AIII–Mg²⁺ complex. In the presence of SLE serum the red color lasts for at least 24 h.

Calibration of lupus test. The ratio of liposome concentration to magnesium concentration is critical to the proper functioning of the assay. Optimization of the assay can be achieved by a ‘matrix’ dual-titration system. To accomplish this, we added 20 µL of SLE serum to each of 20 microtiter wells in a rectangular pattern, five wells across and four wells down, then repeated this process with normal serum in an additional 20 wells on the same microtiter plate. We assessed twofold serial dilutions of liposome concentration on the vertical axis and MgCl₂ on the horizontal axis. The normal range of dilution of the liposome material eluting in the void volume of a Sepharose 4B column was two-to-eightfold. The final concentration of magnesium ranged from 0.55 to 0.034 mM/L. Once the magnesium had been added to all 40 wells, we could determine which liposome and magnesium concentrations resulted in the largest difference between the red seen in the SLE wells and the blue seen in the normal wells.

Spectrophotometric methods. Spectral scans of AIII entrapped in liposomes in the presence and absence of magnesium and in the presence and absence of serum (SLE or normal) were generated with a dual-beam spectrophotometer blanked against AIII–liposomes. We quantified the color changes from red (lupus sera + Mg²⁺) to blue (normal sera + Mg²⁺) at 610 nm.

Binding of serum proteins to liposomes. Three liposome compositions were used to demonstrate the binding of normal and SLE serum to the membranes. Liposome preparation A contained POPC/cholesterol/DPPG/a-tocopherol, 7/1.9/1.0/0.1 (mol ratio). Preparation B contained the same lipid components as A but in a 3/1.9/5.0/0.1 mol ratio. Liposome preparation C was composed of POPC/cardiolipin/cholesterol/DPPG/a-tocopherol in a mol ratio of 3/4/1.9/1.0/0.1. For each formulation we used 40 µmol of total lipid in the ether infusion method described above. For liposome–serum incubations we placed three 20-µL aliquots each of normal and SLE serum in small glass tubes, plus 50 µL of liposome preparation A, B, or C. After 30 min at room temperature, the liposomes were centrifuged at 11 000 × g for 15 min. We collected the supernatant fluids, saving them for analysis by the liposome-based SLE test, and washed the pellets three times with phosphate-buffered saline, pH 7.4. The final pellets were mixed with an ethanol/chloroform solution (9/1 by vol) to precipitate bound proteins, then centrifuged at 13 750 × g for 15 min. The protein pellets were collected and prepared for electrophoresis by addition of 50 µL of urea (8 mol/L), 5 µL of sodium dodecyl sulfate (200 g/L), and 5 µL of undiluted 2-mercaptoethanol. After boiling for 5 min, samples were applied to a polyacrylamide slab gel. We similarly processed standards of known molecular masses.

Electrophoresis of liposome-bound protein. For sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis we followed the procedure of Laemmli (12), using a 120 g/L polyacrylamide separating gel containing 3.2 g of bisacrylamide per liter. Gels were formed in a 12 × 12 cm slab apparatus and electrophoresed for 5–6 h at a constant current of 25 mA. We stained the gels with Coomassie Brilliant Blue (2 g/L) and destained with a solution of acetic acid, methanol, and water.

Patients

We obtained single serum samples from 74 patients attending the outpatient Rheumatology Clinics of the Rutgers Medical School. These included 21 patients who met revised criteria for the classification of SLE (13), 13 with seropositive rheumatoid arthritis, 14 who met preliminary criteria for the classification of systemic sclerosis (scleroderma) (14), and two considered to have primary Sjogren’s syndrome. We also included 24 controls, seven in whom tests for serum ANA were ordered on clinical grounds (one with discoid lupus erythematosus and six with idiopathic Raynaud’s phenomenon) and 17 in whom results of ANA would not be considered clinically relevant but in whom we felt the possibility of false-positive results by liposomal colorimetric assay deserved consideration. This latter group consisted of five individuals with treated syphilis and positive tests for serum VDRL, eight with end-stage renal disease, and four normal individuals.

Serologic studies. Sera from blood allowed to clot at room temperature were stored at -70 °C and thawed just before the liposomal colorimetric assay. We used fresh samples for all serologic studies, which included tests by indirect immu-
of fluorescence for ANA (mouse liver substrate; Meloy Laboratories, Springfield, VA 22151) and anti-ds DNA (Crithidia luciliae substrate; Bio-Dx, Morristown, NJ 07960). Although the results of the liposomal colorimetric assays were read without knowledge of the sample type, sera were not randomly collected but rather were collected to reflect a desired balance of known serologic reactivities.

**Results and Discussion**

Liposomal Colorimetric Assay

Large unilamellar liposomes composed of POPC, cardiolipin, cholesterol, DPPG, and α-tocopherol (3/4/1.9/1.0/0.1 mol ratio) and containing the divalent cation-sensitive dye AII within their aqueous spaces appear red. Hereafter we refer to these as cardiolipin–liposomes. Addition of magnesium to these vesicles will break down the liposomal membrane, leading to the formation of a stable blue magnesium–AIII complex. This phenomenon is not blocked in the presence of normal serum, but addition of SLE serum before the addition of magnesium will inhibit the magnesium-dependent lysis and thus block the red-to-blue color change, presumably because the lupus autoantibodies bind to the cardiolipin. Figure 1 shows spectral curves we established for cardiolipin–liposomes in the presence of both SLE and normal serum 5 min after the addition of magnesium. The AII–magnesium absorbance maximum at 610 nm was significantly decreased in the presence of SLE serum as compared with the curve generated when normal serum was added (0.4 vs 1.6 A). The visual perception of this absorbance difference is shown in Figure 2.

Binding of Serum Proteins to Liposomes

To determine the extent of antibody binding to liposomes, we mixed liposomes with either normal sera or SLE sera as previously described and analyzed the absorbed proteins by gel electrophoresis (Figure 3). We used three liposome compositions to determine whether the negative charge of cardiolipin liposomes was in itself sufficient to stimulate the binding of appropriate serum factors. In lanes 1 and 4 we analyzed the proteins bound primarily to lecithin liposomes (POPC substituted for cardiolipin). Obviously, under these conditions, virtually no discrete protein could be detected. The substitution of DPPG for cardiolipin (lanes 2 and 5) results in a substantial amount of protein bound to the liposomes after incubation in both normal and SLE sera. In fact, we could discern little, if any, difference in the banding patterns in these two lanes. Lanes 3 and 6 represent the proteins bound by cardiolipin–liposomes with normal and SLE serum, respectively. Lane 6 (SLE serum) shows large increases in both the number and intensity of protein bands, with the most relevant being the massive accumulation of a 50 000-Da protein and the first presence of a noticeable band at 25 000 Da. In light of the denaturing conditions used on this gel, the 50 000- and 25 000-Da proteins bound to the cardiolipin liposomes from SLE serum probably represent the heavy and light chains of immunoglobulin. This conclusion is supported by the fact that, although the SLE serum incubated with both POPC- and DPPG-substituted liposomes retained its ability to inhibit the magnesium-induced color change when mixed with standard red cardiolipin liposomes, the SLE serum incubated with cardiolipin-containing liposomes did not (Figure 3).

Antibody Specificity

To determine the specificity of the antibody detected by the liposomal colorimetric assay, we added various amounts of ds DNA, single-stranded DNA (ss DNA), polyinosinic acid

![Graph](image-url)

**Figure 1.** Spectral scan of Alli–cardiolipin liposomes in the presence of either lupus or normal serum and in the presence and absence of Mg**2+**. 140 mmol/L.

All curves were generated after a 1-min incubation of liposomes and serum and a 5-min incubation after the addition of MgCl₂.

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**Figure 2.** Liposomal colorimetric assay: Alli–cardiolipin liposomes incubated with normal sera (left) and SLE sera (right). Incubation and Mg**2+** addition as in Fig. 1.

[an analog for polyphosphate (6)], or polyvinyl sulfate, which has a polymeric negative charge, to SLE serum before testing with the liposome assay system (Table 1). The chemical nature of the additive that best eliminated the ability of the SLE serum to inhibit the described color change was taken as evidence for the nature of the specific-

| Table 1. Inhibition of Liposomal Assay

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<th>Inhibitor</th>
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<tr>
<td></td>
<td>0.5</td>
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<td>dsDNA</td>
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<td>ssDNA</td>
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* + = total inhibition of assay; – = 0 inhibition of assay;± = marginal inhibition of assay.
Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of protein bound by liposomes from normal (NS) and lupus (LS) serum. PC, proteins bound to liposomes composed of POPC/cholesterol/DPPG/α-tocopherol in a 7/1.9/1.0/0.1 mol ratio. PG, proteins bound to liposomes composed of the same components in a 3/1.9/5.0/0.1 mol ratio. CL, proteins bound to liposomes composed of POPC/cardiolipin/cholesterol/DPPG/α-tocopherol in a 3/4/1.9/1.0/0.1 mol ratio. MWM, molecular weight marker. In samples marked (−) the supernatant remaining after the adsorption of serum proteins by liposomes had no ability to inhibit a red-to-blue color shift in the liposomal colorimetric assay system; samples marked (+) inhibited the color shift. Arrows on the electrophoretogram mark the migration of M, 50,000 (upper) and 25,000 (lower) proteins.

Sensitivity of the antibody detected by the liposomal colorimetric assay. Polyvinyl sulfate, ds DNA, and ss DNA failed to inhibit the assay unless present at 20 μg/test (100 μg/mL). Polyinosinic acid, however, totally inhibited the positive lupus response at 5 μg/test (25 μg/mL), indicating that the liposomal colorimetric assay is primarily detecting antiphosphatidylate antibody. This supports the contention of Rauch et al. (8) that DNA itself need not be the antigenic stimulus for autoantibody formation in SLE.

Clinical Trials
We compare the results of testing by the liposomal colorimetric assay with results of standard laboratory diagnostic tests for SLE (ANA, anti-ds DNA) in Table 2. Nineteen of 21 SLE patients were successfully identified by the liposomal colorimetric assay in a blinded analysis. Although the liposomal assay was less sensitive than ANA (90.5% vs 100%) with this sample of SLE sera, the liposomal assay was more sensitive than anti-ds DNA, as determined by *Crithidia luciliae* immunofluorescence (CLIF), 90.5% vs 61.9%. Three false-positive results were obtained in the liposomal test: two patients with rheumatoid arthritis (one of whom had a low-titer and anti-ds DNA, 1:20), and in one normal control. Table 2 also compares the specificity of the

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<th>Table 2. Sensitivity and Specificity of Liposomal Assay and Standard Serologic Assays for SLE</th>
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<td><strong>Assay</strong></td>
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<td>SLE</td>
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<td>Rheumatoid arthritis</td>
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<td>Sjogren’s syndrome</td>
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<td><strong>Specificity</strong></td>
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<td>Adjusted control population†</td>
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* Sensitivity = (no. of patients with positive tests/no. of patients tested) × 100.
* Nos. in parentheses indicate no. of patients giving positive test results (for sensitivity; or for specificity, no. of negative results) and total no. of patients in that group.
* Controls include tests done on serum from patients with syphilis, end-stage renal disease, and Raynaud’s disease. Four normals were also included.
* Specificity = (no. of patients with negative tests/no. of patients without SLE) × 100.
* Excluding patients with SLE.
| Total population minus patients in whom ANA testing would not be performed in routine clinical testing.
liposomal colorimetric assay with that of ANA and the CLIF test. Specificities were calculated both for the total group of sera tested and for an adjusted control population, eliminating those 17 patients in whom ANA testing would be unlikely in routine clinical practice (syphilis, uremia, and normals). As expected, positive results by serum ANA had low specificity in this sampling, whereas both the liposomal colorimetric assay and the assay of anti-ds DNA by CLIF had a comparably high specificity for the diagnosis of SLE. There was no apparent influence of age, sex, race, or ongoing medication on the results of the liposomal colorimetric assay.

**Perspectives**

Over a decade ago, Rand and Sengupta (15) showed that cardiolipin precipitated by either calcium or magnesium formed hexagonal structures, as determined by x-ray diffraction. More recently it has been demonstrated that addition of divalent cations to liposomes containing cardiolipin results in bilayer–hexagonal (HII) transitions as evidenced by 31P nuclear magnetic resonance and freeze-fracture electron microscopy (9). While the extent of hexagonal-phase lipid that forms when cardiolipin liposomes are challenged with divalent cations seems to be quenched by the presence of other lipids, clearly the rearrangement of cardiolipin results in a leak of the liposomal contents (16, 17). Further, the bilayer-to-hexagonal transition appears to be highly cooperative and thus should occur only when extended groups of molecules are capable of undergoing the same phase change.

Figure 4 depicts a schematic representation of how the cardiolipin-based assay described here probably functions. A detailed investigation of the biophysical events involved in the assay will appear elsewhere. Briefly, intact liposomes containing uncomplexed AIII ($A_{\text{max}} = 550 \text{ nm}$) are destabilized in the presence of added magnesium, the result of a magnesium-dependent lamellar-to-hexagonal rearrangement of the cardiolipin in the liposomal membrane. The released AIII binds to the added magnesium, forming a stable blue AIII–Mg$^{2+}$ complex ($A_{\text{max}} = 610 \text{ nm}$). In the presence of lupus autoantibodies, specific binding to cardiolipin occurs, thus blocking the cooperative lamellar-to-hexagonal rearrangement. In this case the liposomal membrane is stabilized and the AIII remains uncomplexed.

As formulated here, the cardiolipin-based liposomal assay system is unusual, in that binding of antibody stabilizes the membrane rather than destabilizes it through complement-mediated damage, as has been reported for other cardiolipin-based systems (18, 19). Such differences probably relate to differences in supramolecular structure.

In conclusion, this liposomal assay method may offer a substantial methodological advance over other assays currently available for the diagnosis of SLE. The liposomal system is more sensitive than the assays for ds-DNA, without losing specificity.

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**References**


