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ACCELERATED COMMUNICATION

Cross-Reactivity of Antibodies Raised Against Acetaldehyde Adducts of Protein with Acetaldehyde Adducts of Phosphatidylethanolamine: Possible role in Alcoholic Cirrhosis

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SUMMARY

This study measured the possible cross-reactivity of hapten-specific IgG antibodies purified from the sera of rabbits sensitized to an albumin-acetaldehyde conjugate [*N*-ethyl-rabbit serum albumin (*N*-ethyl-RSA)] with acetaldehyde-phosphatidylethanolamine adducts. The *N*-ethyl-RSA was coupled to an Affigel-10 column to affinity purify the IgG (anti-*N*-ethyl-RSA IgG). Dioleoylphosphatidylethanolamine (DOPE) was reacted with acetaldehyde to form a Schiff base, which was reduced to *N*-ethyl-DOPE, purified by high pressure liquid chromatography, and analyzed with direct chemical ionization mass spectrometry. Lamellar liposomes containing either 5% by weight *N*-ethyl-DOPE and 95% egg phosphatidylcholine or a mixture of 5% *N*-ethyl-DOPE, 71% DOPE, and 24% dioleoylphosphatidylcholine, as well as hexagonal phase micelles containing 5% *N*-ethyl-DOPE and 95% DOPE, were prepared by sonication. Anti-*N*-ethyl-RSA IgG was then incubated with each of these lipid mixtures for 30 min, a fluorescein-conjugated goat anti-rabbit IgG was added for an additional 30 min, and then binding of anti-*N*-ethyl-RSA IgG to *N*-ethyl-DOPE in the liposomes or micelles was measured by flow cytometry. Anti-*N*-ethyl-RSA IgG bound to *N*-ethyl-DOPE in both vesicles and hexagonal phase micelles, but the affinity was 16 times greater for the hapten in the hexagonal phase. This result demonstrates that physical presentation of the hapten can affect antibody recognition and that antibodies raised against *N*-ethyl-RSA can cross-react with acetaldehyde-phospholipid adducts.

Metabolism of ethanol by both alcohol dehydrogenase and cytochrome P450 produces acetaldehyde that permeates the liver and circulates in the blood (1, 2). *In vivo*, acetaldehyde forms stable *N*-ethyl-lysine adducts (3, 4) with intracellular proteins such as hemoglobin (5), cytochrome P450IIE1 (6), and a M_r 37,000 liver protein (4). Specific antibodies appear in the serum following ethanol ingestion and the titer of these antibodies correlates with the severity of hepatic damage (1). Synthetic adducts of acetaldehyde with proteins such as hemocyanin or albumin also elicit production of polyclonal antibodies in rabbits and monoclonal antibodies in mice that demonstrate a high affinity for these adducts (5).

The role of these antiacetaldehyde antibodies in alcoholic cirrhosis is a subject of much debate. It seems clear that they can serve as markers of alcohol consumption and as a warning of extensive liver damage (1). However, it also has been suggested that these antibodies may initiate antibody-mediated hepatotoxicity by binding to the surface of hepatocytes (7–10). This mechanism would suggest that, when antibodies have bound to the surface of hepatocytes, neutrophils or complement would recognize the antibodies and lyse the cells. The relevance of the latter step in this mechanism is strengthened by our recent demonstration that activated neutrophils can lyse hepatocytes (11). However, a difficulty with the suggestion of immune-mediated cytotoxicity is that proteins of the cytosol and endoplasmic reticulum that form adducts with acetaldehyde are unlikely to appear on the outer surface of the hepatocyte to act as antigens (12).

There is evidence that *N*-ethyl-phosphatidylethanolamines could appear on the surface of hepatocytes, where they could be recognized by circulating antibodies. Kenney (13, 14) has shown that, in an *in vitro* incubation with rat liver microsomes, acetaldehyde forms a Schiff base with phosphatidylethanolamine and this adduct is reduced to the corresponding *N*-ethyl-phosphatidylethanolamine. We have shown that similarly modified phospholipids rapidly equilibrate with the surface of other biological membranes during *in vitro* incubations (15, 16).

In these experiments we tested whether adducts of acetaldehyde with phospholipids could serve as haptenic binding sites for the antibodies that were elicited by acetaldehyde-protein

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ABBREVIATIONS: *N*-ethyl-DOPE, *N*-ethyldioleoylphosphatidylethanolamine; RSA, rabbit serum albumin; DOPE, dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; *N*-ethyl-RSA, adduct of acetaldehyde with RSA after reduction; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; DCI-MS, direct chemical ionization mass spectrometry; PBS, phosphate-buffered saline.

adducts. We investigated antibody binding to phospholipid adducts contained in two host environments: 1) lamellar phase liposomes, because most studies of antiphospholipid antibodies have been performed on them (17–20), and 2) hexagonal phase micelles, because recent studies have demonstrated that the antigenicity of phospholipids in hexagonal phase micelles is greatly enhanced (21–23). For example, lupus anticoagulants, a subclass of antiphospholipid antibodies, distinguish between lamellar and hexagonal phase phospholipids and bind only to the latter phase.

We incorporated synthetic *N*-ethyl-DOPE into lamellar phase liposomes formed from either egg phosphatidylcholine or a mixture of DOPC and DOPE (24) or hexagonal phase micelles formed from DOPE (24, 25). Two kinds of lamellar liposomes were prepared because egg phosphatidylcholine liposomes are the most well characterized, whereas the mixture of DOPE and DOPC forms lamellar phases that have fatty acid chains with length and unsaturation identical to those in the DOPE micelles. The binding of affinity-purified IgG antibodies raised against an adduct of acetaldehyde with RSA (*N*-ethyl-RSA) to these liposomes or micelles was quantified by addition of a fluorescein-conjugated second antibody and subsequent measurement of fluorescence with flow cytometry.

Experimental Procedures

Materials. RSA, ovalbumin, gold-conjugated goat antibody raised against rabbit IgG, and trinitrobenzenesulfonic acid were purchased from Sigma (St. Louis, MO); fluorescein-conjugated goat antibody raised against rabbit IgG was purchased from Caltag (South San Francisco, CA); and Texas red-conjugated phosphatidylethanolamine was purchased from Molecular Probes (Eugene, OR). All phospholipids were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). Pyridine and sodium cyanoborohydride were purchased from Aldrich Chemicals (Milwaukee, WI), Affi-Gel 10 affinity resins were purchased from Bio-Rad (Richmond, CA), and all other reagents were purchased from J. T. Baker (Phillipsburg, NJ).

Synthesis of *N*-ethyl-RSA adducts. The synthesis of *N*-ethyl-RSA was performed by a modification of the techniques used by Means and Feeney (26) and Kenney (13, 14). RSA was dissolved at a concentration of 10 mg/ml in a 10 mM solution of sodium cyanoborohydride in PBS. Redistilled acetaldehyde was then added to the mixture (final concentration, 100 mM) and incubated at 37° for 18 hr. The samples were then removed from the incubator and dialyzed against 3 × 2 liters of Tris·HCl buffer, pH 8.3. The degree of modification of the RSA was determined to be 78 ± 2% by measurement of the content of free amino groups with trinitrobenzenesulfonic acid (27).

Production and purification of hapten-specific IgG. Six New Zealand White rabbits were injected with 100 μg each of the *N*-ethyl-RSA emulsified in Freund's complete adjuvant. Rabbits were boosted every 4 weeks with 100 μg of *N*-ethyl-RSA emulsified in Freund's incomplete adjuvant, and serum was collected on a weekly basis from each rabbit, starting 1 week after the first boost. On those weeks in which the rabbits were boosted, serum was not collected. Blood was collected from an ear vein and allowed to clot overnight at 4°. The IgG fraction was purified from the serum according to the procedures described by McKinney and Parkinson (28). Briefly, albumin and other proteins were first precipitated with caprylic acid and the immunoglobulins were then precipitated with ammonium sulfate. The hapten-specific IgG was then purified by affinity chromatography as follows. A preparation of *N*-ethyl-RSA was reacted with Affi-Gel 10 according to the procedures supplied with the preactivated resin. The affinity column was then charged with the purified IgG and washed with 100 volumes of PBS. The *N*-ethyl-RSA-specific IgG was eluted with 4 M KSCN and immediately diluted with 9 volumes of PBS. The preparation was then concentrated to a volume of 5 ml with a Millipore CX-30 vacuum concentrator and dialyzed against 3 × 2 liters of PBS at 4°. The affinity-purified IgG was aliquoted into 0.5-ml volumes and stored at −20° until used. Purified samples of IgG from preimmune rabbits (preimmune IgG) were also prepared, to be used as controls.

Synthesis of phosphatidylethanolamine-acetaldehyde conjugates. Synthesis of *N*-ethyl-DOPE was carried out essentially as described by Kenney (13, 14). Twenty milligrams of DOPE were dissolved in 250 μl of Ch_2Cl_2, in a 3-ml Reacti-vial that was flushed with N_2 and then placed in an aluminum block that was chilled by ice. Then, 20 μl of pyridine were added through a Teflon-sealed septum and 10 mg of freshly redistilled acetaldehyde dissolved in 200 μl of CH_2Cl_2 were chilled and then added to the phospholipid with a gas-tight syringe. The formation of the Schiff bases continued at 0° for 30 min and then a solution of 50 mg of $NaCNBH_3$ dissolved in 250 μl of methanol was added through the septum. The mixture was allowed to come to room temperature for 10 min, and then 500 μl of water, 200 μl of methanol, and 400 μl of $CHCl_3$ were added. The mixture was centrifuged and the lower phase was removed and blown dry with N_2.

HPLC of acetaldehyde-phosphatidylethanolamine adducts. HPLC was performed on a Beckman Altex system with a 25- × 1-cm Si 60-199 Brownlee cartridge column, at a flow rate of 2 ml/min. The products were detected with a Varian Varichrome multiwavelength detector set at 215 nm. A binary gradient system was formed from the following mixtures: hexane/isopropanol/0.1 M NH_4HCO_3, pH 7.0, at 40:58:2 (solvent A) and 40:50:10 (solvent B). The elution program was as follows: 100% A until 5 min, a linear gradient to 100% B from 5 to 35 min, and 100% B from 35 to 70 min. The actual shape of the gradient was monitored with a Waters refractive index detector. Two milligrams of the reaction mixture were dissolved in 100 μl of solvent A and were injected with a loop injector. Under these conditions, the *N*-ethyl-DOPE eluted at 38.2 min and the *N*,*N*-diethyl-DOPE adduct eluted at 16.5 min.

TLC of phosphatidylethanolamine adducts. The products that eluted at 38.2 and 16.5 min in HPLC were compared with the original reaction mixture by TLC on 5- × 20-cm Merck silica gel 60 F_{254} plates run in a solvent system of $CHCl_3$/methanol/concentrated NH_4OH (65:30:5). The spots were visualized by exposure to iodine vapor and molybdate spray reagent. Under these conditions, DOPE, *N*-ethyl-DOPE, and *N*,*N*-diethyl-DOPE had R_F values of 0.27, 0.42, and 0.57, respectively. Visual examination of the intensity of the resulting spots suggested that the *N*-ethyl and the *N*,*N*-diethyl adducts each were formed in approximately 30% yield.

Mass spectral analysis. Mass spectra were obtained by DCI-MS in the negative ion mode, using ammonia as a reagent gas, with a Hewlett Packard 5985A mass spectrometer modified as described by Jamieson and co-workers (29, 30). Phospholipids were dissolved at a concentration of 1 μg/μl in the HPLC solvent system, hexane/isopropanol/0.1 M NH_4HCO_3 (40:54:6). One microliter of the solution was applied to a polyimide-coated fused silica fiber used as an extended probe tip. The solvent was evaporated and the probe was inserted into the mass spectrometer without internal heating of the probe tip. The sample was vaporized by the ambient 350° source block temperature and was ionized in the ammonia gas plasma.

Preparation of liposomes and vesicles containing *N*-ethyl-DOPE. Liposomes containing either 5% (by weight) *N*-ethyl-DOPE and 95% egg phosphatidylcholine or a mixture of 5% *N*-ethyl-DOPE, 71% DOPE, and 24% DOPC, as well as hexagonal phase micelles containing 5% *N*-ethyl-DOPE and 95% DOPE, were prepared by mixing the lipids in $CHCl_3$ in 6-ml flat-bottomed Vari-Clean vials (Pierce Chemical, Rockford, IL), removing the solvent with a stream of nitrogen, and then removing traces of solvent in a vacuum desiccator. Before mixing, the concentrations of the various phospholipids were determined by phosphate analysis (31). The lipids were hydrated in PBS at a concentration of 200 μg/ml for 1 hr and then treated with sonication in a bath sonicator for 3 × 1 min at room temperature. The

flat-bottomed vials provided exceptional coupling, with the ultrasonic transducer, and rapidly produced clear suspensions of liposomes. In order to preserve homogeneity of size and concentration, sufficient liposomes and micelles for an entire experiment were prepared and then aliquoted.

Analysis of antibody affinity for *N*-ethyl-DOPE by flow cytometry. Mixtures containing a total of 100 μg of phospholipids in 0.5 ml of PBS were placed in 13- × 100-mm test tubes, and 5 μg of either anti-*N*-ethyl-RSA IgG or preimmune IgG were added. After a 30-min incubation at 22°, 5 μg of fluorescein-conjugated secondary antibody were added and the mixture was lightly agitated. After a 30-min incubation at 22°, the samples were analyzed by flow cytometry. All samples were prepared in triplicate. As a control for autofluorescence of the phospholipids or the anti-*N*-ethyl-RSA IgG, samples in which the fluorescein-conjugated secondary antibody was omitted were prepared. As a control for nonspecific binding of the fluorescein-conjugated IgG to liposomes or micelles, samples without anti-*N*-ethyl-RSA IgG were prepared. In preliminary experiments (data not shown), 100 μg of the total phospholipid mixtures were tested with a matrix of 1, 5, and 10 μg of the primary IgGs and 1, 5, and 10 μg of the fluorescein-conjugated secondary antibody. In addition, phospholipid mixtures containing 1, 5, and 10% *N*-ethyl-DOPE were probed with 5 μg of anti*N*-ethyl-RSA IgG followed by 5 μg of fluorescein-conjugated secondary antibody. The combination of 5% *N*-ethyl-DOPE with 5-μg amounts of both primary and secondary IgG was found to give an optimum compromise between fluorescence intensity and background.

Flow cytometry was performed on a Facstar II (Becton Dickinson). The analysis of particles was gated on forward scatter and 10,000 particles were counted from each sample. All samples were prepared and run in triplicate. The log scale intensities of fluorescein intensity, forward scatter, and obtuse scatter were recorded for each sample. In order to select for liposomes or micelles rather than small clumps of fluorescent antibodies, the fluorescence intensity of only those particles with forward scatter and obtuse scatter intensity greater than 40 (40 of 250 channels; see Fig. 2) was integrated (32).

In a control experiment to determine the homogeneity of the particles as well as to set the appropriate forward and obtuse scatter limits, 0.01 mol % of Texas red-conjugated phosphatidylethanolamine was mixed with DOPE in $CHCl_3$. The mixture was taken to dryness and then hydrated, and micelles were formed as described above for the other lipids. These micelles were studied by flow cytometry by collecting Texas red fluorescence, fluorescein fluorescence, forward scatter, and obtuse scatter (32).

Results

The structure of the synthetic *N*-ethyl-DOPE was confirmed by negative ion ammonium DCI-MS, as shown in Fig. 1. The base peak in the spectrum is the molecular ion at *m/z* 770 ($M - H^+$, as expected for negative ion mass spectrometry). A second major and diagnostic fragment is *m/z* 699 ($M - C_2H_4NHC_2H_5$, the *N*-ethyl-ethylamine headgroup of the phospholipid). The assignment of the *N,N*-diethyl-DOPE structure to the peak that eluted at 13 min from HPLC and had an R_F on TLC of 0.57 (14) was confirmed by a mass spectrum that had ions of *m/z* 798 ($M - H^+$, the base peak in the spectrum), 770, 752, 727, 719, and 699 (dioleoylglycerolphosphate). A control mass spectrum of DOPE had ions of *m/z* 742 ($M - H^+$, base peak in the spectrum), 724 (M − 18), and 699 (dioleoylglycerolphosphate). To our knowledge, this is the first reported DCI-MS mass spectrum of DOPE in which the molecular ion is the base peak.

The results of the flow cytometry analysis are shown in Fig. 2. In each case, the log forward scatter (a qualitative measure of the size and refractive index of a particle) is plotted versus the log fluorescein fluorescence intensity (32). In Fig. 2A it is seen that the *N*-ethyl-DOPE in hexagonal micelles strongly binds the anti-*N*-ethyl-RSA IgG. It is seen that the fluorescent intensity of the particles in Fig. 2A is much greater than in the controls in which preimmune IgG was substituted for the primary antibody (Fig. 2B) or in which the primary antibody was omitted (Fig. 2C). In contrast, it is seen that there is not a great change in the distribution of particle sizes as measured by forward scatter, indicating that binding of the antibodies did not cause aggregation or a change in lipid structure (32). In Fig. 2D it is seen that there is a small amount of binding of the primary anti-*N*-ethyl-RSA IgG to *N*-ethyl-DOPE incorporated in lamellar liposome structures composed of DOPC and DOPE, as compared with their respective controls in which preimmune IgG substituted for the primary antibody (Fig. 2E) and in which the primary antibody was omitted (Fig. 2F). However, the amount of antibody bound to the liposomal structure is much less than that bound to the hexagonal phase micelles seen in Fig. 2A.

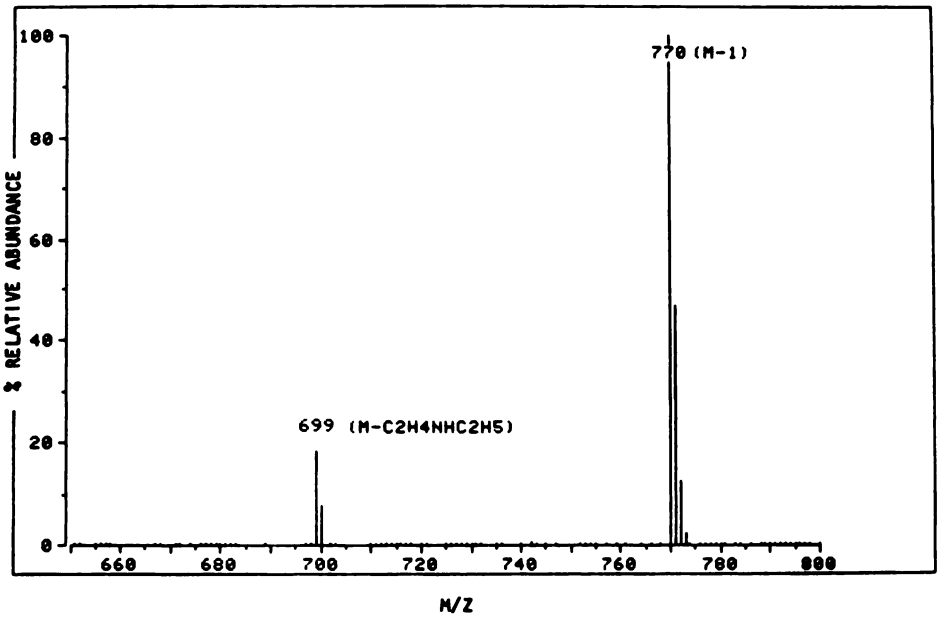

Fig. 1. Mass spectrum of *N*-ethyl-DOPE obtained by DCI-MS in the negative ion mode, using ammonia as a reagent gas, with a Hewlett Packard 5985A mass spectrometer modified as described by Jamieson and co-workers (29, 30). One microgram of N-ethyl-DOPE was applied to a polyimide-coated fused silica fiber used as an extended probe tip, from which the sample was vaporized by the ambient 350° source block temperature and then ionized in the ammonia gas plasma. The base peak in the spectrum is the molecular ion at *m/z* 770 ($M - H^+$, as expected for negative ion mass spectrometry). A second major and diagnostic fragment is *m/z* 699 ($M - C_2H_4NHC_2H_5$, loss of the *N*-ethyl-ethylamine headgroup from the phospholipid).

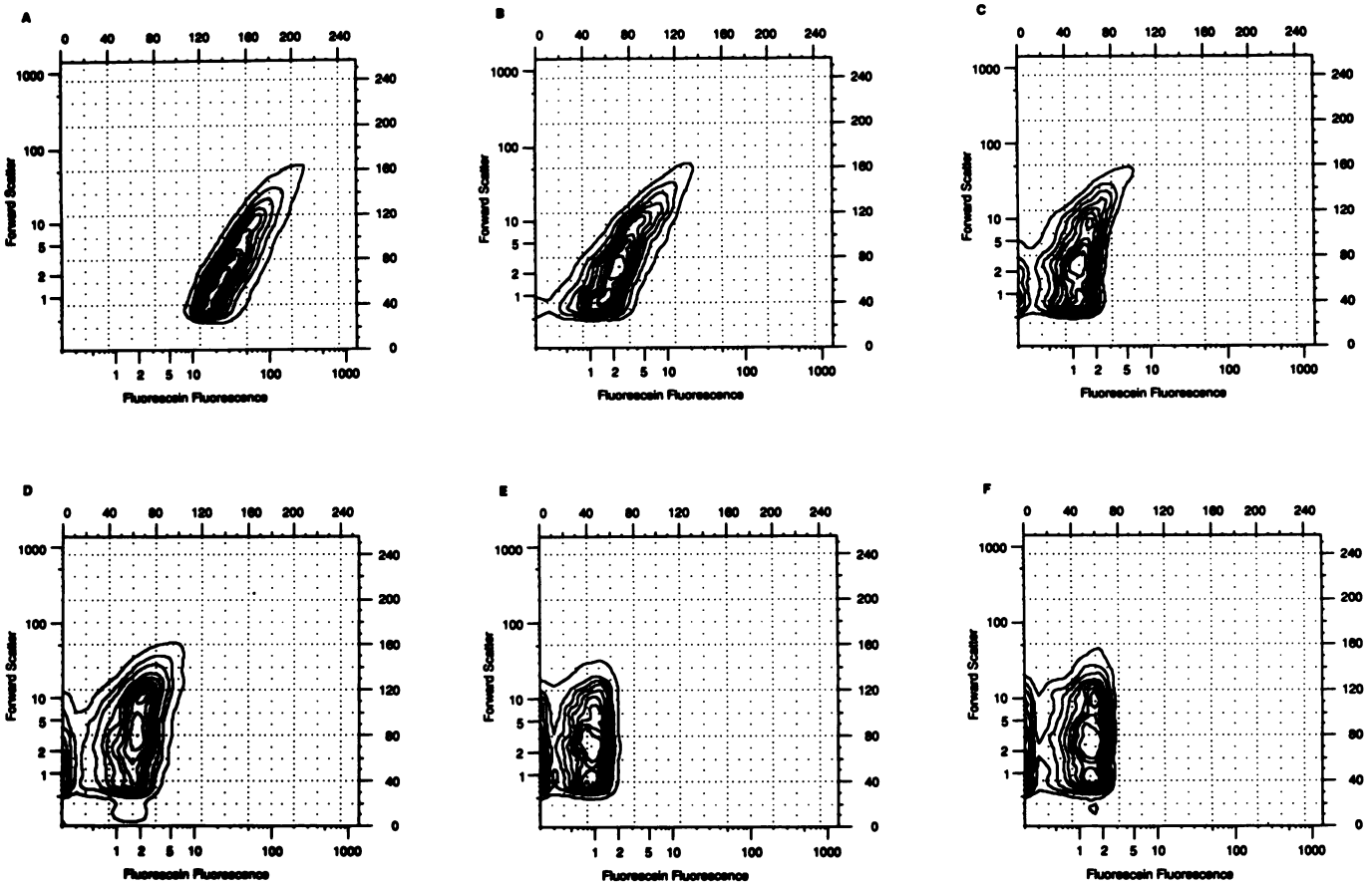

Fig. 2. Flow cytometry analysis of 100 μg of total lipid of hexagonal phase micelles (5% *N*-ethyl-DOPE and 95% DOPE) exposed to 5 μg of anti-*N*-ethyl-RSA IgG (A), 5 μg of preimmune IgG (B), or no primary IgG (C), or lamellar phase liposomes (5% *N*-ethyl-DOPE, 24% DOPC, and 71% DOPE) exposed to 5 μg of anti-*N*-ethyl-RSA IgG (D), 5 μg of preimmune IgG (E), or no primary IgG (F). After binding of the primary antibody, 5 μg of fluorescein-conjugated goat anti-rabbit IgG were added. In each case, log forward scatter (a qualitative measure of the size and refractive index of a particle) is plotted versus log fluorescein fluorescence intensity. The scales on the *top* and *right* of each figure are the 250 channels into which data are collected. The fluorescence in channels 40–250 was integrated for Table 1. The contour plot shows equal 10% intervals of probability; that is, an equal number of cell data points will fall between adjacent contours. Therefore, the number of contours drawn in a given area is related to the number of cells in that area.

TABLE 1

Integrated fluorescence intensities of micelles and lamellar liposomes

The fluorescence intensities of only those particles that had a size measured by obtuse scatter of greater than 40 of 250 channels and a fluorescence greater than 40 of 250 channels were integrated. The samples were either hexagonal phase micelles (5% *N*-ethyl-DOPE and 95% DOPE) or lamellar phase liposomes (5% *N*-ethyl-DOPE, 24% DOPC, and 71% DOPE) of 100 μg of total lipid that were exposed to either 5 μg of anti-*N*-ethyl-RSA IgG, 5 μg of preimmune IgG, or no primary IgG. All samples were then mixed with 5 μg of fluorescein-conjugated goat anti-rabbit IgG. The values are means ± standard errors (three experiments). In parentheses, the integrated intensities of the micelles and the liposomes are expressed as a ratio of their respective controls (no primary IgG).

	Anti-*N*-ethyl-RSA IgG	Preimmune IgG	No primary IgG
5% *N*-Ethyl-DOPE 95% DOPE	52.4 ± 3.8 (32.8)[a]	3.8 ± 0.2 (2.3)[b]	1.6 ± 0.1 (1)
5% *N*-Ethyl-DOPE 24% DOPC 71% DOPE	3.3 ± 1.0 (2.1)[b]	1.2 ± 0.1 (0.8)	1.6 ± 0.3 (1)

[a] $p < 0.001$ for analysis by two-tailed unpaired *t* test versus sample with no primary IgG.
[b] $p < 0.05$.

The integrated fluorescent intensities are shown in Table 1. In this analysis, an additional selection factor, obtuse scatter, was included to differentiate small clumps of antibodies from the desired liposomes and micelles. The fluorescence intensity of only those particles that had a size measured by obtuse scatter of greater than 40 of 250 channels and a fluorescence greater than 40 of 250 channels was integrated. In parentheses, the integrated intensities of the micelles and the liposomes are expressed as a ratio of their respective controls (no primary IgG). The fluorescence intensity of the IgG bound to *N*-ethyl-DOPE in the hexagonal phase micelles is 33 times greater than the control without primary IgG and 16 times greater than that bound to *N*-ethyl-DOPE in the lamellar phase liposomes.

In additional experiments, the effect of using 1, 5, or 10% *N*-ethyl-DOPE in hexagonal phase micelles containing a balance of DOPE was found to give a mean fluorescence intensity of 4.3 ± 0.7, 10.6 ± 1.8, and 31.2 ± 4.4 (mean ± SE). Each of these values is significantly different from the others by an unpaired *t* test ($p < 0.05$). The increase in affinity of the primary antibody as a function of increasing concentration of antigenic phospholipid has been previously shown (17–19). In an experiment to determine the effect of the concentration of the micelles themselves, the standard micelles formed from 5% *N*-

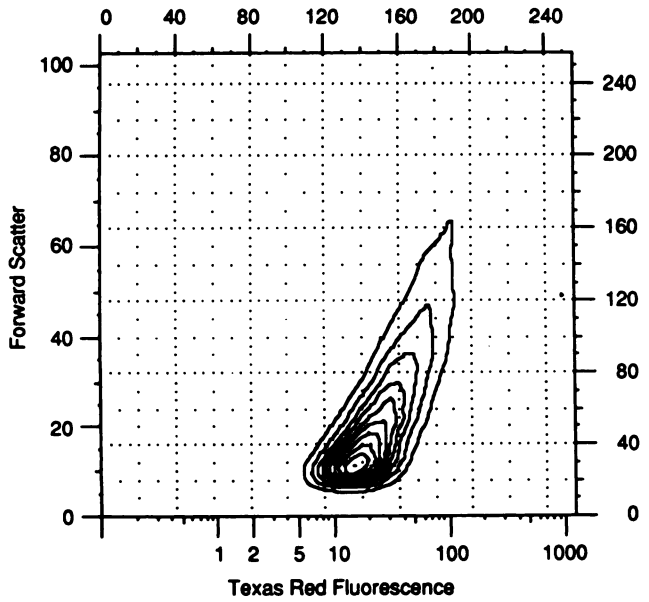

Fig. 3. Flow cytometry analysis of 100 μg of total lipid of hexagonal phase micelles (5% *N*-ethyl-DOPE and 95% DOPE) containing 0.01% Texas red-conjugated phosphatidylethanolamine. The forward scatter is plotted versus Texas red fluorescence. The experiment shows that the size distribution (forward scatter) of the pure micelles corresponds well to that of the micelles after binding of both the primary and secondary antibodies (Fig. 2A).

ethyl-DOPE and 95% DOPE were first diluted 1:10 or 1:100 in PBS before the usual amounts of anti-*N*-ethyl-RSA IgG and then fluorescein-conjugated secondary antibody were added. It was found that there was no significant difference between the integrated fluorescent intensity of the stock suspension and the two diluted suspensions.

Hexagonal phase micelles of DOPE were made fluorescent by the addition of 0.01% Texas red-conjugated phosphatidylethanolamine and then were analyzed by flow cytometry, as shown in Fig. 3, where the forward scatter is plotted against Texas red fluorescence on a linear scale. This experiment had several uses. The correct assignment of the forward scatter and obtuse scatter gating that was used in the experiments with fluorescein was determined. In addition, it was shown that there were neither very small nor very large particles that were not being properly labeled or quantified by the fluorescent secondary antibody technique. This experiment also demonstrated that the addition of either the primary or secondary antibody did not change the size or refractile nature of the particles of the micelles, as measured by forward and obtuse scatter, and that the DOPE micelles exhibited only low autofluorescence, which could be eliminated by setting channel 40 as a lower limit gate in the fluorescence channel.

Dot-blot experiments showed that anti-*N*-ethyl RSA IgG bound with high affinity to *N*-ethyl-RSA but not to any of the phospholipid mixtures spotted on nitrocellulose. The failure to detect binding in this experiment may reflect the low antigenicity of *N*-ethyl-DOPE in lamellar liposomes.

Discussion

Fig. 2 and Table 1 clearly show that anti-*N*-ethyl-RSA IgG cross-reacts with *N*-ethyl-DOPE with high affinity when the lipid is incorporated in a hexagonal phase micelle but with low affinity in a lamellar phase liposome. These host lipids were chosen for examples of lamellar and hexagonal phases because they are so well characterized (25) and because the addition of only a small amount (21%) of DOPE has been shown to cause the transition from hexagonal to lamellar phase, with a minimum of other dissimilarities in phospholipid chain length and head group environment (24). To further substantiate the effect of the phase of the phospholipid, we obtained results identical

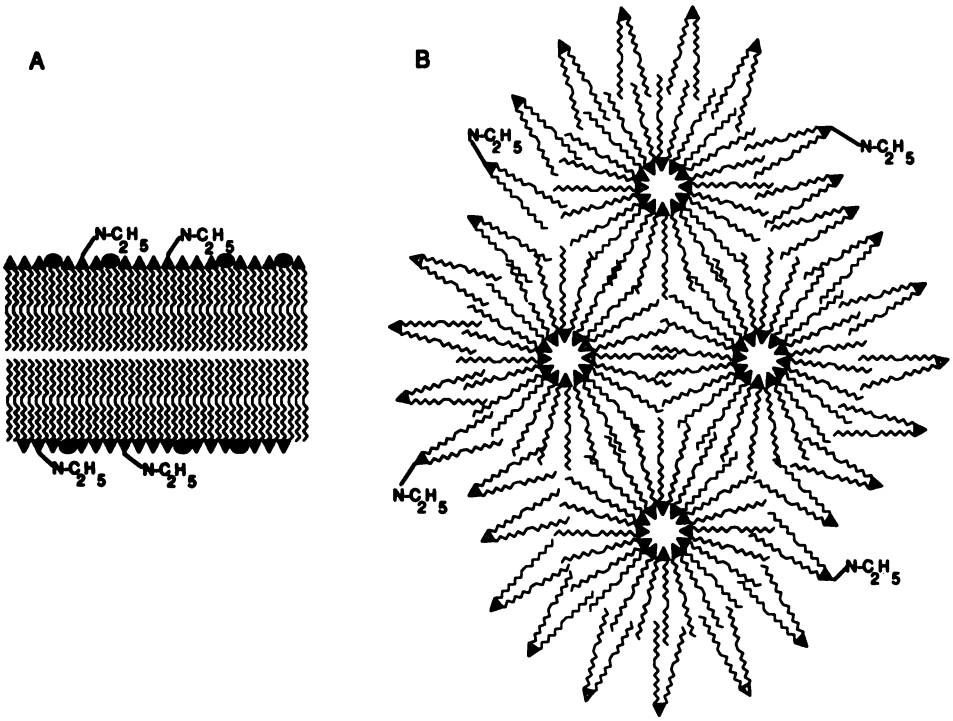

Fig. 4. Drawing of a bilayer composed of 5% *N*-ethyl-DOPE, 24% DOPC, and 71% DOPE (A) and hexagonal phase micelles composed of 5% *N*-ethyl-DOPE and 95% DOPE (B). *Circles*, DOPC headgroups; *triangles*, DOPE headgroups; *N-ethyl-triangles*, *N*-ethyl-DOPE headgroups.

to those in the lamellar phase DOPE/DOPC mixture with *N*-ethyl-DOPE in 95% egg phosphatidylcholine lamellar phase liposomes.

This strong preference for IgG binding to *N*-ethyl-DOPE in the hexagonal phase is consistent with previous studies on the preference of lupus antiphospholipid antibodies for hexagonal phase lipid (22, 23).

The difference in surface structures surrounding the antigenic phospholipids is illustrated in a drawing of a bilayer composed of 5% *N*-ethyl-DOPE, 24% DOPC, and 71% DOPE (Fig. 4A) and hexagonal phase micelles composed of 5% *N*-ethyl-DOPE and 95% DOPE (Fig. 4B). The lamellar structure of the lipid bilayer is well established and haptenic groups on phospholipids require a spacer length of several carbon atoms in order to be recognized by antibodies (19, 20). This may be a function of the tight packing of the phospholipids and the resulting high surface pressure in this phase (33).

The structure of a hexagonal phase micelle, in particular the outer monolayer that presumably encloses the hexagonal tubes (23, 34), is very controversial and the subject of considerable research (24, 25, 35–37). The property of phosphatidylethanolamines that causes them to be more stable as hexagonal tubes, the low cross-section of the head group with respect to the fatty acid chains makes it very difficult to form an outer monolayer with high surface density of head groups. In Fig. 4B this outer monolayer has been drawn as being partially interdigitated with the fatty acid chains of the inner hexagonal tubes (24, 34, 36). The result of the curvature and interdigitation is that the area per phospholipid headgroup is much greater than in a bilayer, resulting in greater exposure and accessibility of the *N*-ethyl-DOPE. It may be that this increased accessibility to the haptenic group confers the greater antigenicity of phospholipids in hexagonal phases.

It is interesting to note that recent studies demonstrate the existence within biological membranes of lipidic particles, domains with conformations and isotropic motions of phospholipids that are much like hexagonal phases (24, 25, 34, 35). These lipidic particles could provide domains on the hepatocyte surface in which *N*-ethyl-phosphatidylethanolamines are accessible to circulating antibodies. Clearly, many additional experiments will be required to test whether antibodies also bind to the surface of hepatocytes and whether this binding has a role in alcoholic cirrhosis.

Acknowledgments

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