Molecular Interaction and Lateral Domain Formation in Monolayers Containing Cholesterol and Phosphatidylcholines with Acyl- or Alkyl-Linked C16 Chains

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The interactions of cholesterol with phosphatidylcholines having acyl- or alkyl-linked C16 chains have been determined in mixed monolayers using fluorescence microscopy to visualize lateral domain formation and cholesterol oxidase to probe for the relative strength of sterol−phospholipid interaction. The phosphatidylcholines of this study included 1,2-dipalmitoyl−*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (PHPC), 1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (HPPC), and 1,2-*O*-dihexadecyl-*sn*-glycero-3-phosphocholine (DHPC). As the two-dimensional crystallization of the liquid-condensed phase was visualized with fluorescence microscopy (using 0.5 mol % NBD-cholesterol as a probe), both DPPC and HPPC displayed a similar nucleation and growth of the liquid-condensed phase at the onset pressure of the liquid-expanded to liquid-condensed phase transition (although the size and shapes of the condensed domains differed). However, with both PHPC and DHPC, laterally condensed phases were evident well before the onset of the phase transition as determined fro the force−area isotherms of these lipids. Therefore, the pattern of formation of condensed phases was different, depending on the position of the alkyl function. When cholesterol was mixed with these phosphatidylcholines (at 20, 25, or 33 mol %), cholesterol-rich condensed domains were formed. The domain morphology was similar in cholesterol−DPPC and cholesterol−PHPC mixed monolayers, whereas cholesterol−HPPC and cholesterol−DHPC mixed monolayers had partly different domain morphologies, with more extensive fusion of the cholesterol-rich domains. When cholesterol oxidase was used to probe for the relative strength of intermolecular association between cholesterol and each of the phosphatidylcholines, it was observed that the interaction was loosest with DPPC (highest rate of cholesterol oxidation catalyzed by cholesterol oxidase), whereas the association was somewhat stronger in PHPC (lower rate of cholesterol oxidation) and much stronger in HPPC and DHPC mixed monolayers (no detectable oxidation). In conclusion, the presence of an alkyl function at position 1 or 2 (or both) of a phosphatidylcholine molecule markedly changed its properties in monolayer membranes.

Introduction

Ether-linked glycerophospholipids are natural components of biological membranes. For example, diether phospholipids are major components of the polar lipid fraction of extremely halophilic bacteria.[1] In addition, alkyl and alk-1-enyl ether-linked phospholipids occur in mammalian membranes of apparently normal cells[2,3] and in cancer cells.[4,5] A recent excellent review on ether lipids in biomembranes may be of interest to the reader.[6] Ether-linked phospholipids have been used to study the role of the C═O group of acyl-PCs in determining packing arrangements in bilayers. Physciochemical characterizations of ether−lipids have revealed that dialkyl phosphatidylcholines have a slightly higher gel-to-liquid crystalline phase transition temperature as compared with their diacyl counterparts, whereas phosphatidylcholines bearing both ether- and ester-linked chains have slightly lower transition temperatures than the corresponding diester phosphatidylcholines.[7−10] These transition temperature differences have been interpreted to suggest that dialkyl phosphatidylcholines display larger intermolecular attraction as compared to diester compounds.[9] A stronger intermolecular attraction should also lead to a denser molecular packing in monolayer membranes at a given surface pressure. It has indeed been reported that dialkyl phosphatidylcholines have a reduced mean molecular area requirement as compared to a comparable diacyl phosphatidylcholine.[10,11]

Based on permeability studies in liposomal systems, the interaction of cholesterol with acyl or alkyl phosphatidylcholines was reported to depend on the C═O group of diester-PC.[12,13] In another similar study with cholesterol, no difference in solute permeability was observed when the phospholipid was either diester or diether phosphatidylcholine,[14] suggesting that the carbonyl ester function was not important for cholesterol/phospholipid interaction. In addition, the segmental motion of phos-

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(1) Kates, M. In *Ether Lipids: Chemistry and Biology*; Snyder, F., Ed.; Academic Press: New York, 1972; p 351.
(2) Horrocks, L. A. In *Ether Lipids: Chemistry and Biology*; Snyder, F., Ed.; Academic Press: New York, 1972; p 177.
(3) Pugh, E. L.; Kates, M.; Hanahan, D. J. *J. Lipid Res.* **1977**, *18*, 710.
(4) Snyder, F. In *Ether Lipids: Chemistry and Biology*; Snyder, F., Ed.; Academic Press: New York, 1972; p 273.
(5) Friedberg, S. J.; Halpert, M. *J. Lipid Res.* **1978**, *19*, 57.
(6) Paltauf, F. *Chem. Phys. Lipids* **1994**, *74*, 101.
(7) Chupin, V. V.; Vasilenko, I. A.; Merkushkin, G. I.; Serebrennikova, G. A.; Evstigneeva, R. P. *Bioorg. Khim.* **1979**, *5*, 1515.
(8) Lee, T.-E.; Fitzgerald, V. *Biochim. Biophys. Acta* **1980**, *598*, 189.
(9) Bittman, R.; Clejan, S.; Jain, M. K.; Derro, P. W.; Rosenthal, A. F. *Biochemistry* **1981**, *20*, 2790.
(10) Hermetter, A.; Paltauf, F. *Chem. Phys. Lipids* **1981**, *29*, 225.
(11) Smaby, J. M.; Hermetter, A.; Schmid, P. C.; Paltauf, F.; Brockman, H. L. *Biochemistry* **1983**, *22*, 5808.
(12) Schwarz, F. T.; Paltauf, F. *Biochemistry* **1977**, *16*, 4335.
(13) Tirri, L. J.; Schmidt, P. C.; Pullarkat, R. K.; Brockerhoff, H. *Lipids* **1977**, *12*, 863.
(14) Clejan, S.; Bittman, R.; Deroo, P. W.; Isaacson, Y. A.; Rosenthal, A. F. *Biochemistry* **1979**, *18*, 2118.

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phatidylcholine molecules in liquid-crystalline bilayers of DPPC or DHPC has been reported to be unaffected by the presence of moderate amounts of cholesterol (10 mol %, ref 15). Furthermore, the anisotropic motion of cholesterol molecules was found to be similar in bilayer membranes prepared from either DPPC or DHPC, although the motion of cholesterol was slightly more restricted when the phosphatidylcholine molecule contained an O-alkyl rather than an acyl linkage at the *sn*-2 position.[15] The rates of passive diffusion of carboxyfluorescein across bilayers of DPPC and DHPC have been reported to be somewhat higher than those from bilayers of HPPC and PHPC.[15]

Pure bilayer membranes of DHPC and HPPC have been shown to include interdigitated phases in their gel phase state, whereas pure DPPC bilayers apparently do not.[16−18] In a recent synchrotron X-ray diffraction study on monolayer membranes containing diester or diether phosphatidylcholines, it was observed that the replacement of ester by ether linkages caused a marked reduction of the tilt angle of the molecules,[19] suggesting that the ester and ether linkages directly affected the lateral packing properties of these lipids. Finally, although the interfacial region of ether phospholipids is expected to be less polar than that of ester phospholipids, a study using polarity-sensitive fluorescence probes indicated that apoprotein A-I partitions into the interface of di-C14 ether phosphatidylcholine and dimyristoyl phosphatidylcholine in a similar manner.[20] This finding suggests that protein penetration into membranes is not directly affected by ether or ester linkages of phosphatidylcholines.

In this study we have made an additional effort to examine the interaction between cholesterol and phosphatidylcholines having either dialkyl or diacyl (or mixed alkyl−acyl) functions. We have both visualized the formation of lateral domains using monolayer fluorescence microscopy[21] and determined the relative strength of cholesterol−phosphatidylcholine interaction using cholesterol oxidase as a probe.[22]

Experimental Procedures

Materials. DPPC and cholesterol were obtained from Sigma Chemicals (St. Louis, MO) and DHPC from Alexis Biochemicals (Läufelfingen, Switzerland). PHPC and HPPC were synthesized as described before.[23,24] Stock solutions of all phospholipids were prepared in hexane/2-propanol (3/2, v/v), stored in the dark at −25 °C, and warmed to ambient temperature before use. The concentration of the various phospholipid stock solutions was determined by the method of Bartlett.[25] Cholesterol oxidase *(Streptomyces sp.)* was purchased from Calbiochem (San Diego, CA). The water used as subphase was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system, to yield a product with a resistivity of 18.2 MΩ/cm.

Force−Area Isotherms. Pure monolayers with each of the phospholipids were compressed on water at 20.5 ± 0.02 °C with a KSV surface barostat (KSV Instruments Ltd., Helsinki, Finland). The barrier speed did not exceed 3.4 Å²/molecule, min during compression. Data were collected using proprietary KSV software.

Oxidation of Monolayer Cholesterol. Monolayers of cholesterol and one of the phospholipids were prepared to have different initial molar ratios of sterol to phospholipid, and the monolayers were exposed to 56 mU/mL of cholesterol oxidase in the subphase. The subphase buffer consisted of 50 mM Tris-HCl containing 140 mM NaCl (pH 7.4). After preparation, the buffer was filtered through a nylon membrane filter (Gelman Science, MI) with a pore size of 0.45 μm. The temperature was kept at 30 ± 0.5 °C and the monolayer was held at constant surface pressure (20 mN/m) during the course of the oxidation. The average oxidation rate and the stoichiometry at which free cholesterol clusters disappeared was calculated as described previously.[22]

Monolayer Fluorescence Microscopy. The two-dimensional crystallization of phospholipids in monolayers, and the formation of lateral cholesterol-rich domains in mixed cholesterol−phospholipid monolayers was determined with monolayer fluorescence microscopy, using NBD-cholesterol as a fluorescent reporter molecule (at 0.5 mol %).[26] Micrographs were obtained using a sensitive video camera attached to a DT3851 digitizing board in a personal computer.

Results

Monolayers of Pure Phosphatidylcholines. The force−area isotherms of all the phospholipids of this study showed a liquid-expanded (LE) to liquid-condensed (LC) phase transition at room temperature (isotherms were run at 20.5 ± 0.2 °C). The transition region was most clearly defined with DPPC and less clearly defined with the alkyl phosphatidylcholines (Figure 1). The onset of the transition occurred slightly below a lateral surface pressure of 4 mN/m for DPPC and DHPC, and slightly above this pressure for HPPC and PHPC (Figure 1, indicated with an arrow). Similar isotherms of DPPC and DHPC have previously been reported in the literature.[27,28] Next, we examined the two-dimensional crystallization of the phosphatidylcholines using monolayer fluorescence microscopy with NBD-cholesterol as a fluorescent probe. Pure phosphatidylcholine monolayers containing 0.5 mol % NBD- cholesterol were compressed to a lateral surface pressure that was slightly below the onset pressure for the LE−LC transition. At this degree of compression, the monolayer should be in a LE state and should not display laterally condensed domains.[29,30] The results presented in Figure 2 show that DPPC (panel A, 3 mN/m) and HPPC (panel E, 4 mN/m) did not contain condensed domains before the onset of the phase transition. However, both PHPC (panel C, 3.8 mN/m) and DHPC (panel G, 3 mN/m) displayed condensed domains before the onset of the phase transition. When the monolayer surface pressure was increased beyond the onset pressure of the phase transition (as determined from the force−area isotherms in Figure 1), all four phosphatidylcholine monolayers showed condensed domains against a more expanded phase (Figure 2). The DPPC crystals showed a chirality-dependent long-range order (panel B of Figure 2), whereas the alkyl phosphatidylcholine crystals were more undefined in structure. The condensed HPPC domains were smaller in size than those of the DPPC monolayer and appeared to be uniformly distributed in the plane of the monolayer (panel F of Figure 2). With

(15) Bittman, R.; Clejan, S.; Lund-Katz, S.; Phillips, M. C. *Biochim. Biophys. Acta* **1984**, *772*, 117.
(16) Ruocco, M. J.; Siminovitch, D. J.; Griffin, R. G. *Biochemistry* **1985**, *24*, 2406.
(17) Lohner, K.; Schuster, A.; Deovics, G.; Muller, K.; Laggner, P. *Chem. Phys. Lipids* **1987**, *44*, 61.
(18) Haas, N. S.; Sripada, P. K.; Shipley, G. G. *Biophys. J.* **1990**, *57*, 117.
(19) Brezesinski, G.; Dietrich, A.; Struth, B.; Böhm, C.; Bouwman, W. G.; Kjaer, K.; Möhwald, H. *Chem. Phys. Lipids* **1995**, *76*, 145.
(20) Massey, J. B.; She, H. S.; Pownall, H. J. *Biochemistry* **1985**, *24*, 6973.
(21) Slotte, J. P. *Biochim. Biophys. Acta* **1995**, *1235*, 419.
(22) Slotte, J. P. *Biochemistry* **1992**, *31*, 5472.
(23) Ali, S.; Bittman, R. *Biochem. Cell Biol.* **1990**, *68*, 360.
(24) Guivisdalsky, P. N.; Bittman, R. *J. Org. Chem.* **1989**, *54*, 4643.
(25) Bartlett, G. R. *J. Biol. Chem.* **1951**, *234*, 466.
(26) Slotte, J. P.; Mattjus, P. *Biochim. Biophys. Acta* **1995**, *1254*, 22.
(27) Paltauf, F.; Hauser, H.; Phillips, M. C. *Biochim. Biophys. Acta* **1971**, *249*, 539.
(28) Albrecht, O.; Gruler, H.; Sackmann, E. *J. Colloid Interface Sci.* **1981**, *79*, 319.
(29) Subramaniam, S.; McConnell, H. M. *J. Phys. Chem.* **1987**, *91*, 1715.
(30) Rice, P. A.; McConnell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6554.

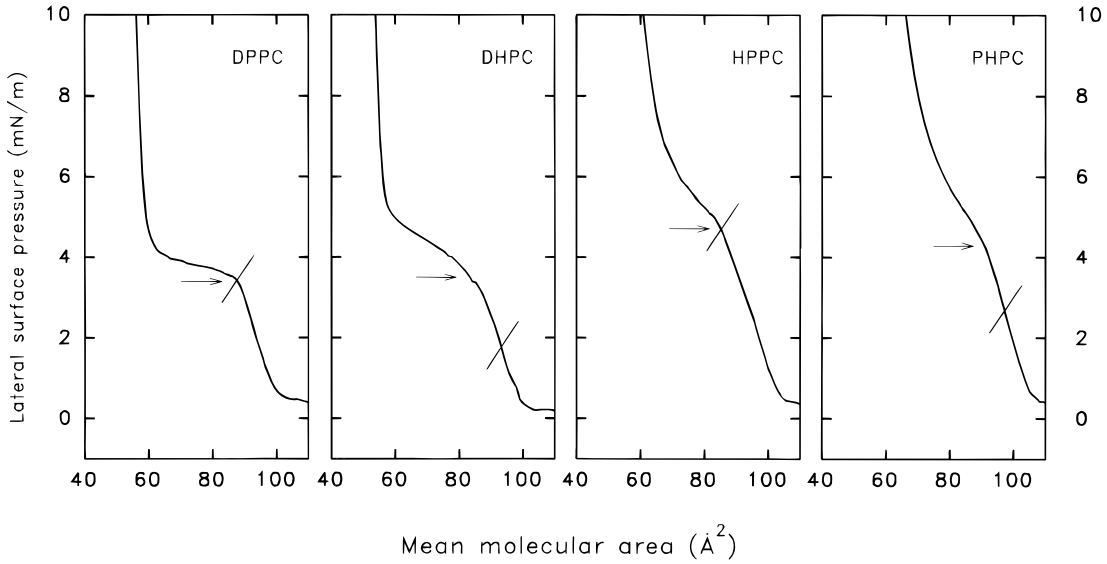

Figure 1. Transition region of the force−area isotherms of acyl and alkyl phosphatidylcholines. Force−area isotherms of the phospholipids were collected on highly purified water at 20.5 ± 0.2 °C. Only the phase transition region is shown. The arrow shows the onset of the liquid-expanded to liquid-condensed phase transition. The line marker shows at which pressure/area the liquid-condensed domains became visible in the fluorescence microscope (when the monolayer contained 0.5 mol % NBD-cholesterol as a fluorophore; cf. Figure 2). The monolayer isotherms shown in this figure did not include NBD-cholesterol.

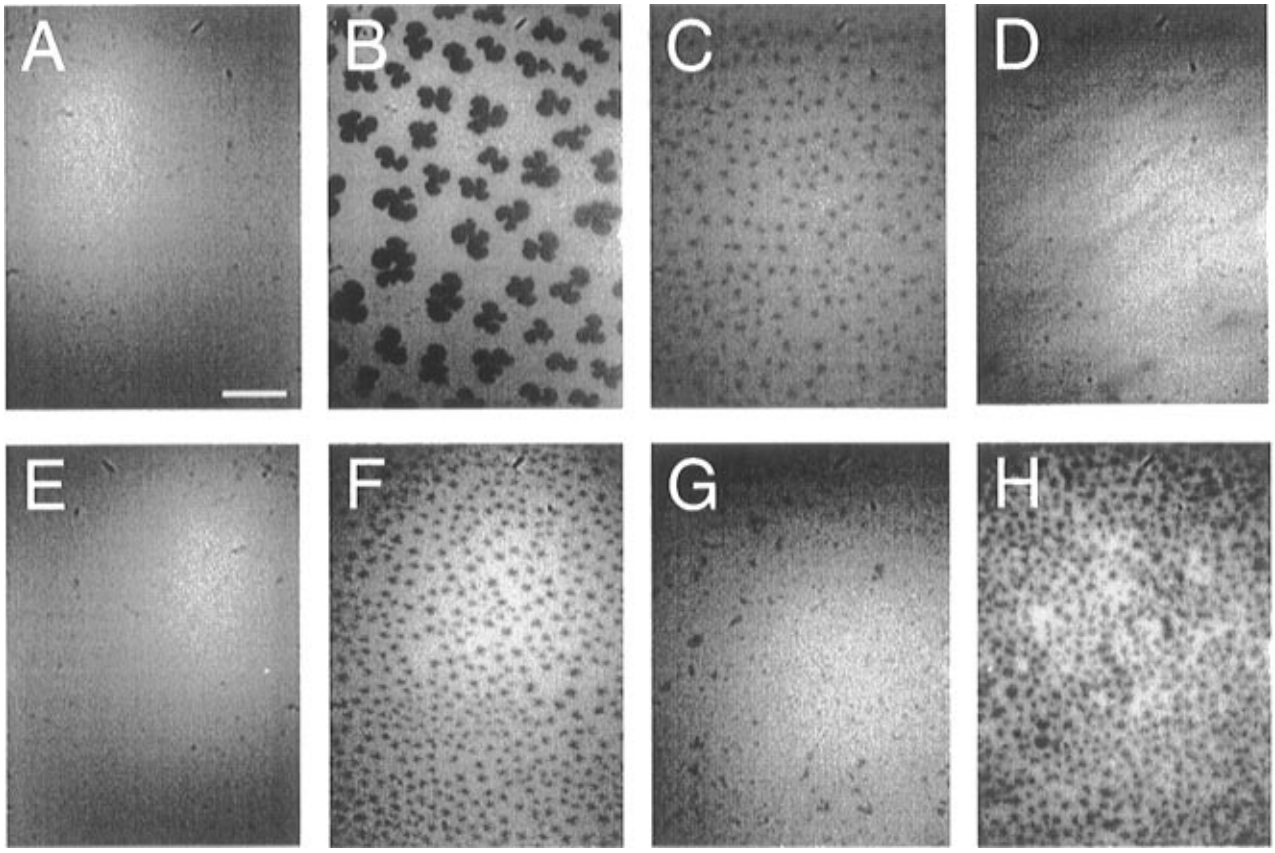

Figure 2. Visualization of the two-dimensional crystallization in the pure phosphatidylcholine monolayers. Monolayers of DPPC, PHPC, HPPC, or DHPC were prepared on pure water and compressed slowly (3.4 Å^2/molecule min) through the phase transition region (cf. Figure 1). The panels are as follows: DPPC at a surface pressure of 3 mN/m (A) and 4 mN/m (B), PHPC at a surface pressure of 3.8 mN/m (C) and 5.5 mN/m (D), HPPC at a surface pressure of 4 mN/m (E) and 5.5 mN/m (F), and DHPC at a surface pressure of 3 mN/m (G) and 4.7 mN/m (H). The scale bar represents 50 μm.

both PHPC and DHPC, the condensed domains became fuzzy in appearance and aggregated to some extent when the monolayer was compressed beyond the onset of the phase transition pressure.

Mixed Monolayers of Cholesterol and Phosphatidylcholines. The domain properties of cholesterol−DPPC mixed monolayers have recently been published,[21] and micrographs of this system are not included in the present study. Mixed monolayers of cholesterol and PHPC were observed to have domain morphologies similar to what was seen with the cholesterol−DPPC system (cf. ref 21, Figure 1). At 20 mol % cholesterol during initial compression, circular condensed domains were formed (with a fairly broad size distribution; Figure 3A was

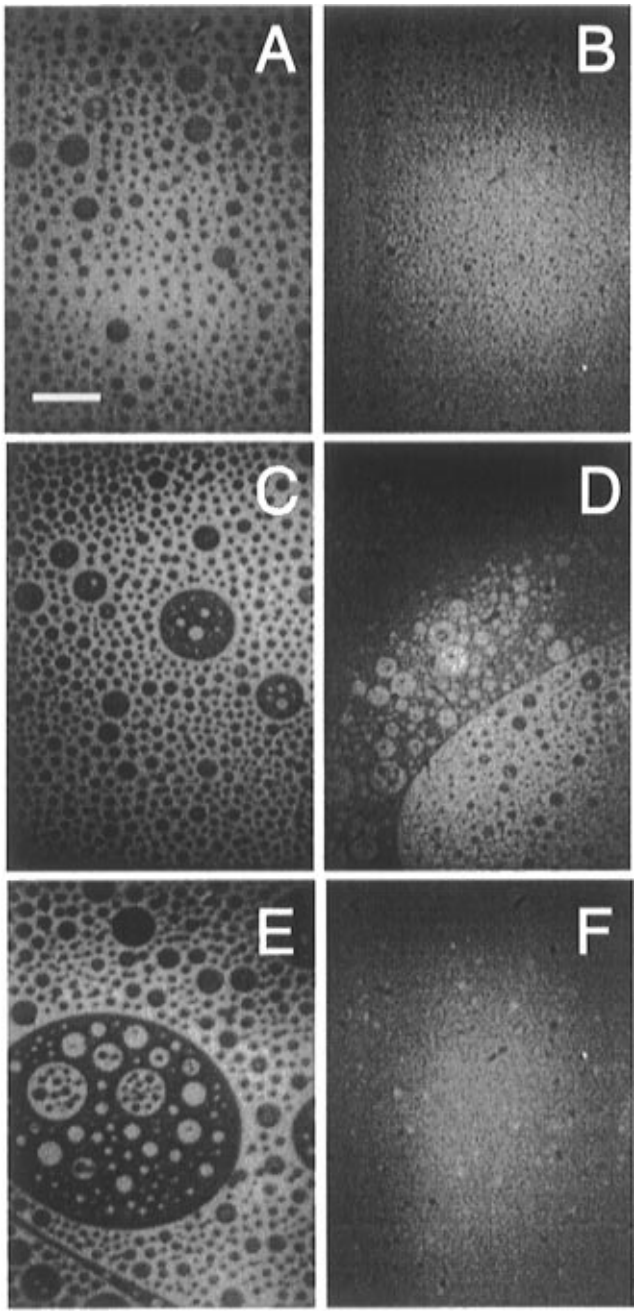

Figure 3. Lateral domains in a PHPC monolayer as a function of the cholesterol concentration. Monolayers of PHPC were prepared to include 20 (panels A and B), 25 (panels C and D), or 33 mol % cholesterol (panels E and F) together with 0.5 mol % NBD-cholesterol. The monolayers were compressed slowly (3.4 Å^2/molecule min), and the lateral domains were documented at 0.5 mN/m during initial compression (panels A, C, and E) and at 1 mN/m following a monolayer compression/expansion cycle (panels B, D, and F). The scale bar represents 50 μm.

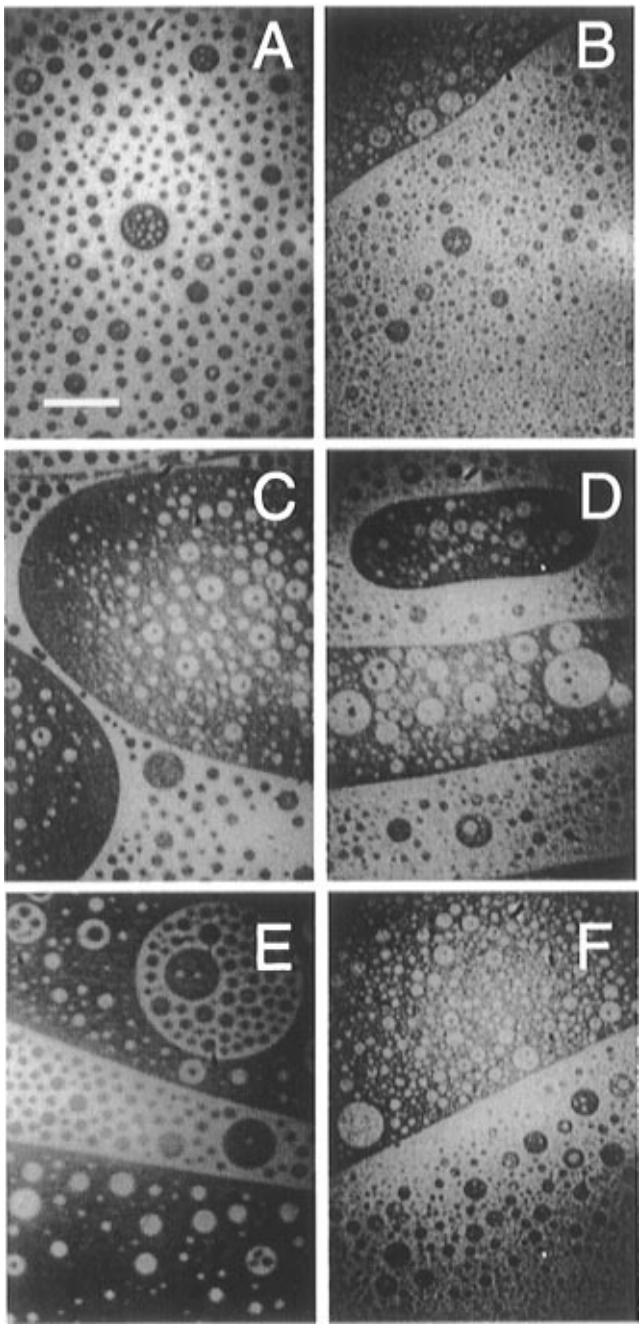

Figure 4. Lateral domains in a HPPC monolayer as a function of the cholesterol concentration. Monolayers of HPPC were prepared to include 20 (panels A and B), 25 (panels C and D), or 33 mol % cholesterol (panels E and F) together with 0.5 mol % NBD-cholesterol. The monolayers were compressed slowly (3.4 Å^2/molecule min), and the lateral domains were documented at 0.5 mN/m during initial compression (panels A, C, and E) and at 1 mN/m following a monolayer compression/expansion cycle (panels B, D, and F). The scale bar represents 50 μm.

obtained at 0.5 mN/m). After a compression/expansion cycle (the monolayer was compressed to 15 mN/m and then expanded back to about 1 mN/m) the condensed domains became much smaller in size and had a much narrower size distribution as well (Figure 3B). At higher cholesterol concentration (25 and 33 mol %), the fusion of condensed domains became fore pronounced, especially after the compression/expansion cycle (Figure 3C−F).

With cholesterol at 20 mol % in HPPC mixed monolayers, the condensed domains formed during initial compression were again circular in shape and variable in size. Some fusion of condensed domains was evident after a compression/expansion cycle (Figure 4A,B). At higher cholesterol concentrations (25 and 33 mol %), small circular condensed domains were seen together with larger fused condensed domains. This pattern was evident both before and after a compression/expansion cycle (Figure 4C−F). The domain morphology for cholesterol−HPPC at 33 mol % sterol differed clearly from that seen with cholesterol−PHPC (and cholesterol−DPPC, ref 21) in that the condensed domains were not completely fused in the HPPC monolayer after the compression/expansion cycle, as opposed to the situation with both PHPC and DPPC mixed monolayers.

The domain morphology during initial compression in cholesterol−DHPC mixed monolayers differed slightly

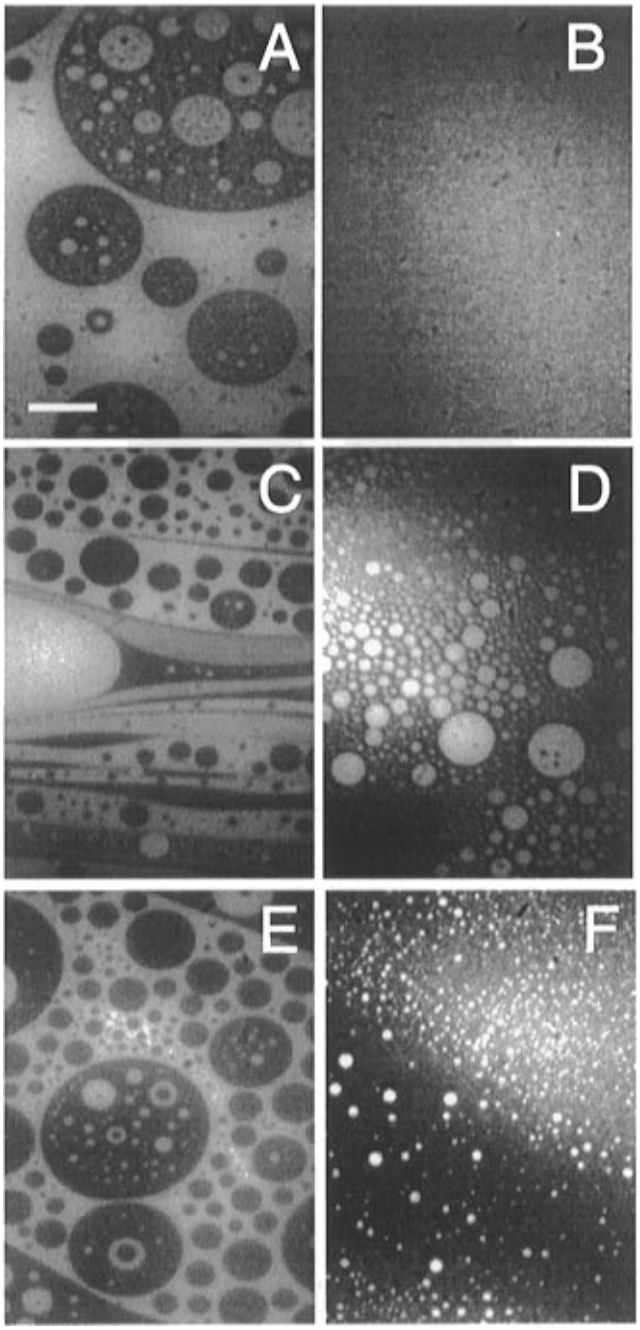

Figure 5. Lateral domains in a DHPC monolayer as a function of the cholesterol concentration. Monolayers of DHPC were prepared to include 20 (panels A and B), 25 (panels C and D), or 33 mol % cholesterol (panels E and F) together with 0.5 mol % NBD-cholesterol. The monolayers were compressed slowly (3.4 Å^2/molecule min), and the lateral domains were documented at 0.5 mN/m during initial compression (panels A, C, and E) and at 1 mN/m following a monolayer compression/expansion cycle (panels B, D, and F). The scale bar represents 50 μm.

from that seen with the other phosphatidylcholines. Extensive fusion of condensed domains was evident already at 20 mol % cholesterol (Figure 5A), whereas this pattern did not appear to change much with increasing cholesterol concentration (Figure 5C,E). The domain morphology in monolayers which had gone through a compression/expansion cycle was similar to those observed in cholesterol−PHPC mixed monolayers. At 20 mol % cholesterol, the condensed domains became very small and appeared uniform in size after the compression/expansion cycle (Figure 5B). At 25 and 33 mol % cholesterol, the condensed domains had coalesced as a result of the compression/expansion cycle (Figure 5D,F).

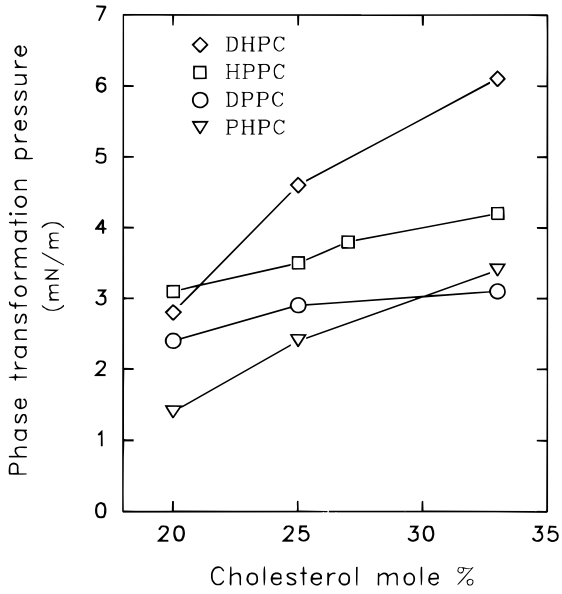

Figure 6. Phase transformation pressure versus cholesterol concentration. Monolayers of the phospholipids were prepared to contain 20, 25, or 33 mol % cholesterol (together with 0.5 mol % NBD-cholesterol). The monolayers were compressed, and the surface pressure at which the lateral domain boundary line dissipated was registered visually.

The phase transformation pressure, i.e., the surface pressure at which the line boundary between condensed and expanded domains dissipated, was determined by visual observation of the mixed monolayers at different cholesterol concentrations, although the phase transformation pressure can also be derived from an analysis of the force−area isotherm of the mixed monolayers.[21,31] The phase transformation pressure as a function of cholesterol concentration was recently reported for the cholesterol−DPPC system[21] and is again shown in Figure 6. The phase transformation pressure for DPPC was fairly similar with the cholesterol concentration ranging between 20 and 33 mol % (varies between 2.4 and 3 mN/m). With the mono- or dialkyl phosphatidylcholines, the phase transformation pressure increased more markedly as the cholesterol concentration increased from 20 to 33 mol % (Figure 6). Both HPPC and DHPC mixed monolayers gave higher phase transformation pressures as compared to DPPC mixed monolayers, whereas PHPC mixed monolayers had a slightly lower phase transformation pressure than the DPPC system.

Oxidation of Cholesterol in Mixed Monolayers. Since the mode of interaction between cholesterol and the different phosphatidylcholines appeared to differ, as based on their slightly differing domain forming properties, and their different phase transformation pressures, we also determined the relative strength of intermolecular association, using cholesterol oxidase as a probe.[22] It is known that at a given surface pressure and temperature, the stronger the sterol−phospholipid interaction is, the more resistant is cholesterol to oxidation by cholesterol oxidase.[22,32] When the oxidation susceptibility of cholesterol in a mixed phospholipid monolayer is determined as a function of the cholesterol-to-phospholipid (C/PL) mole ratio, one can determine the C/PL mole ratio at which readily oxidizable cholesterol domains (or "clusters") disappear [when the C/PL (cholesterol to phospholipid) molar ratio is successively decreased, ref 22. In this study, cholesterol in a DPPC monolayer became less susceptible

(31) Seul, M.; Sammon, M. J. *Phys. Rev. Lett.* **1990**, *64*, 1903.
(32) Mattjus, P.; Slotte, J. P. *Chem. Phys. Lipids* **1994**, *71*, 73.

Table 1. Oxidation of Cholesterol in Phospholipid Mixed Monolayers[a]

monolayer type	average oxidation rate at 1:1 molar ratio (molecules/s)	stoichiometry[b]
cholesterol−DPPC	0.79×10^{13}	1:1
cholesterol−PHPC	0.24×10^{13}	2:1
cholesterol−HPPC	∼0	2:1
cholesterol−DHPC	∼0	2:1

[a] Monolayers were prepared to contain an equimolar ratio of cholesterol and either DPPC, PHPC, HPPC, or DHPC. The monolayers were held at a constant surface pressure of 20 mN/m at 30 °C and exposed to 56 mU/mL cholesterol oxidase. [b] The C/PL stoichiometry at which free cholesterol clusters disappear in the mixed monolayer (based on average oxidation rate data).

to oxidation when the C/PL mole ratio in the mixed monolayer decreased below 1 (Table 1). This finding is similar to our previously reported results concerning cholesterol−phosphatidylcholine mixed monolayers.[22,32] with alkylacyl, acylalkyl, or dialkyl phosphatidylcholines, the C/PL mole ratio below which cholesterol begun to show a decreased susceptibility to oxidation was 2:1, a limit which is similar to what has been reported for cholesterol−sphingomyelin mixed monolayers.[22,32] The average rate of cholesterol oxidation at 1:1 C/PL (20 mN/m, 30 °C) in a cholesterol−PHPC mixed monolayer was markedly lower (3.3-fold) as compared to the cholesterol−DPPC system, and the oxidation of cholesterol in HPPC or DHPC mixed monolayers was almost undetectable (Table 1). It thus appeared that the presence of one or two alkyl functions in the phosphatidylcholine molecule led to a stronger interaction with cholesterol. These results suggest a similarity between alkyl phosphatidylcholines and sphingomyelins in their interaction with cholesterol (at least as seen by cholesterol oxidase).

Discussion

In this study we have examined the importance of ether functions at positions *sn*-1 or *sn*-2 of palmityl phosphatidylcholines on their monolayer properties and on cholesterol/phospholipid interactions in mixed monolayers at the air/water interface. The extent of interaction was evaluated from the formation of lateral sterol-rich domains in mixed monolayers (using monolayer fluorescence microscopy, ref 21), and from the availability to oxidation by cholesterol oxidase of cholesterol.[22] As a reference phospholipid we used DPPC which contains two ester-linked palmitate chains.

Pure monolayers of all examined phosphatidylcholines displayed a liquid expanded to liquid condensed phase transition at ambient temperature (20.5 °C). This two-dimensional crystallization was obvious both from an analysis of the force−area isotherms and from the direct visualization of phosphatidylcholine crystallization using monolayer fluorescence microscopy. The sharpness of the on-set transition from an apparent homogenous liquid expanded phase to a coexistence of LE and LC phases was most pronounced in DPPC monolayers, whereas the onset of the LE → LC transition was less clear with the alkyl phosphatidylcholines. This transition is believed to have at least a limited cooperativity, which results from the formation of surface micelles with an aggregation number of 10−1000.[33] Consequently, the marked differences seen in the force−area isotherms (in the transition region) of acyl phosphatidylcholines and alkyl phosphatidylcholines apparently derived from differences in the nanoscale aggregation of surface micelles and nucleation sites. Clearly, the bonds which link the hydrocarbon chains to the glycerol backbone have marked effects on how the phospholipids interact with each other at the air/water interface.

Using monolayer fluorescence microscopy, it was possible to directly examine the macroscopic formation of nucleation centers and to follow the crystallization of pure phosphatidylcholines as a function of variable surface pressure. Phosphatidylcholines with an acyl function at the *sn*-2 position (i.e., DPPC and HPPC) showed the expected macroscopic condensed domains immediately at the onset of the LE → LC phase transition (cf. ref 34). However, both PHPC and DHPC (with an alkyl function at the *sn*-2 position) were observed to have condensed domains already before the onset of the LE → LC phase transition. This finding suggests that *sn*-2 alkyl phosphatidylcholines did crystallize at lower surface pressures and, consequently, displayed a higher degree of long-range order than their *sn*-1 acyl counterparts. The higher gel-to-liquid crystalline phase transition temperature reported for DHPC (as compared with DPPC) is in line with our monolayer results showing a more extensive crystallization of DHPC at lower surface pressures as compared to the DPPC system. These results together imply that the alkyl function, at least when placed at *sn*-2, can result in a more favorable intermolecular attraction between similar molecules. The observation that the 1-(alkyl/acyl)-2-alkyl phosphatidylcholines formed two-dimensional condensed phases as low surface pressures may relate to another observation with bipolar amphiphiles, which also were shown to form two-dimensional, densely packed clusters even at low surface densities.[35] It is possible that the condensed phases formed at low surface pressures with 2-alkyl phosphatidylcholines were not in equilibrium because the compression rate may have been too fast. Therefore, the presence of such two-dimensional clusters may become detectable from the analysis of force−area isotherms, provided that equilibrium conditions are achieved.

The molecular interactions between cholesterol and the phosphatidylcholines were examined both using monolayer fluorescence microscopy (for lateral domain formation) and cholesterol oxidase (to probe for the affinity of interaction). With monolayer fluorescence microscopy, macroscopic lateral domains (>10 μm) can be visualized in cholesterol/phosphatidylcholine mixed monolayers.[21] Such domains display a fairly condensed molecular packing density, since the domains exclude effectively fluorescent lipid reporter molecules. Despite their dense lateral packing, the domains still display liquid-like properties (e.g., circular shapes). The mixing of cholesterol into the different alkyl phosphatidylcholines led to the formation of laterally condensed, sterol-rich domains. These sterol-rich domains were liquid, similar to what was reported for a comparable cholesterol/phosphatidylcholine systems.[21,36] The stability of the domain boundaries between laterally expanded and condensed phases was almost similar with diacyl and acyl/alkyl phosphatidylcholines, in that the domain boundary dissipated above a surface pressure ranging from 2 to 6 mN/m (the value varied as a function of cholesterol concentration). The dissipation of the domain boundary at elevated surface pressures has been described to relate to dramatic change in the domain wall energy (which derives from attractive van der Waals forces between the molecules in the condensed domains), because at elevated surface pressures the repulsive electrostatic interactions between the polar groups of the molecules tend to destabilize the do-

(33) Israelachvili, J. *Langmuir* **1994**, *10*, 3774.
(34) Weis, R. M. *Chem. Phys. Lipids* **1991**, *57*, 227.
(35) Vogel, V.; Möbius, D. *Thin Solid Films* **1985**, *132*, 205.
(36) Slotte, J. P. *Biochim. Biophys. Acta* **1995**, *1238*, 118.

mains.[29,31,37] With DHPC, the sterol-rich domains had a slightly higher stability (at 33 mol % cholesterol), a finding which is comparable to the situation observed in cholesterol/*N*-palmitoyl sphingomyelin mixed monolayers.[21]

Even though the domain boundary dissipated when the monolayer was compressed beyond the phase transformation pressure, domains re-formed when the monolayer surface pressure was eventually lowered below the phase transformation pressure. Domain morphology of all cholesterol/phosphatidylcholine systems was different during initial compression and after a compression/expansion cycle. It is believed that domains which reform after a compression/expansion cycle are thermodynamically more stable than those which are formed during initial compression.[29,37,38] The sterol-rich domains in monolayers containing phosphatidylcholines with an alkyl function in the *sn*-2 position had a clear tendency to coalesce (at or above 25 mol % cholesterol) after a compression/expansion cycle, whereas extensive coalescence of sterol-rich domains were seen in cholesterol/DPPC monolayers only at 30 mol % cholesterol,[21] and with cholesterol/HPPC extensive fusion of condensed domains was not seen even at 35 mol % cholesterol. These findings could indicate that an acyl function at the *sn*-2 position hindered the coalescence of condensed domains much more than was the case with *sn*-2 alkyl phosphatidylcholines.

The observation that cholesterol appeared to interact differently (or with different affinity) with diacyl, acyl/alkyl, and dialkyl phosphatidylcholines was further strengthened by our cholesterol oxidation results. We have previously demonstrated that cholesterol is more resistant to oxidation by cholesterol oxidase in tightly packed monolayers as compared to more loosely packed membranes.[21,32,39] In this study we could show that cholesterol oxidation was greatly reduced when the phosphatidylcholine was changed from DPPC to PHPC. The oxidation of cholesterol was almost undetectable in mixed monolayers having phosphatidylcholine with an alkyl function at the *sn*-1 position under the experimental conditions used. These results would suggest that the acyl and alkyl functions in phosphatidylcholines have marked effects on the strength of the cholesterol/phospholipid interaction. Previous studies, in which solute permeability have been examined in cholesterol/phospholipid liposomes, have failed to demonstrate a significantly different effect of cholesterol on solute permeability in acyl versus alkyl phospholipid systems.[9,10,14,15]

In addition to having effects on the rates of cholesterol oxidation (inhibitory) at a given C/PL mole ratio, the presence of alkyl functions in the phosphatidylcholine molecules also appeared to affect long-range order parameters in the monolayer. Whereas the oxidation susceptibility of cholesterol in a DPPC mixed monolayer started to decrease below an equimolar C/PL ratio, it begun to decrease already at 2:1 C/PL if the phosphatidylcholine component contained one or two alkyl chains. Assuming that no specific or long-lived molecular complexes exist in binary monolayers, this finding suggests that alkyl-containing phosphatidylcholines were able to cooperatively and over a longer range affect the oxidation susceptibility of cholesterol in a way not possible for DPPC. Taken together, all these results suggest that phosphatidylcholines containing one or two alkyl functions resemble sphingomyelins in their mode of interaction with cholesterol in monolayers. This point is of interest, since an electron diffraction analysis of the molecular packing of DHPC in minimally hydrated crystals revealed that the bilayer packing of DHPC resembled more the packing observed for sphingomyelin than for DPPC.[40]

Studies on both phosphatidylcholine crystals[41−43] and liquid-crystalline bilayers[44] show that the orientation of the glycerol backbone is almost parallel to the bilayer plane. The *sn*-1 chain is extended perpendicular to the membrane surface, while the *sn*-2 chain begins parallel to the membrane surface and is bent perpendicular to the membrane surface after the C-2′ segment.[45] It is plausible that the presence of one or two alkyl functions in a phosphatidylcholine molecule affects the orientation of both the backbone and the polar moiety at the interfacial region of the monolayer. Recent calorimetric and spectroscopic evidence, obtained from experiments with hydrated bilayers of diacyl or dialkyl phosphatidylcholines, indeed shows that the environment of the polar headgroup and the polar/apolar interface is different in diacyl and dialkyl phosphatidylcholine bilayers.[46] This difference was interpreted to arise from a difference in the conformation of the glycerol backbone of diacyl and dialkyl phosphatidylcholines. Changes in phospholipid conformation at the lipid/water interface are likely to also affect their interaction with cholesterol. The recently observed difference in tilt angle for DPPC and DHPC in monolayers also suggest that the orientation of the glycerol backbone is different in diacyl and dialkyl phosphatidylcholines.[19] Finally, the finding that the alkyl phosphatidylcholines resembled sphingomyelins in that they have an increased capacity to solubilize cholesterol as compared to their acyl counterparts may have physiological relevance in cancer cells, which are known to contain alkyl phospholipd[4,5] and which also are known to have an impaired regulation of endogenous cholesterol biosynthesis.[47] It appears possible that alkyl phospholipids (or at least alkyl phosphatidylcholines) function in cancer cells in part as a buffer that can solubilize excess cholesterol during conditions of deregulated cholesterol biosynthesis.[48]

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(37) Keller, D.; Korb, J. P.; McConnell, H. M. *J. Phys. Chem.* **1987**, *91*, 6417.
(38) Yu, H.; Hui, S. W. *Chem. Phys. Lipids* **1992**, *62*, 69.
(39) Mattjus, P.; Hedström, G.; Slotte, J. P. *Chem. Phys. Lipids* **1994**, *74*, 195.
(40) Dorset, D. *Biochim. Biophys. Acta* **1987**, *898*, 121.
(41) Hitchcock, P. B.; Mason, R.; Thomas, K. M.; Shipley, G. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3036.
(42) Pearson, R. H.; Pascher, I. *Nature* **1979**, *281*, 499.
(43) Pascher, I.; Lundmark, M.; Nyholm, P.-G.; Sundell, S. *Biochim. Biophys. Acta* **1992**, *1113*, 339.
(44) Eklund, K. K.; Virtanen, J. A.; Kinnunen, P. K. J.; Kasurinen, J.; Somerharju, P. J. *Biochemistry* **1992**, *31*, 8560.
(45) Seelig, J.; Browning, J. L. *FEBS Lett.* **1978**, *92*, 41.
(46) Lewis, R. N. A. H.; McElhaney, R. N. *Biophys. J.* **1995**, *68*, A430.
(47) Coleman, P. S.; Sepp-Lorenzino, L. In *Advances in Cholesterol Research*; Esfahani, M., Swaney, J. B., Eds.; The Telford Press Inc.: New Jersey, 1990; p 201.
(48) Yachnin, S.; Golomb, H. M.; West, E. J.; Saffold, C. *Blood* **1983**, *61*, 50.