Complement proteins C5b-9 induce transbilayer migration of membrane phospholipids

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ABSTRACT Transbilayer migration of membrane phospholipid arising from membrane insertion of the terminal human complement proteins has been investigated. Asymmetric vesicles containing pyrene-labeled phosphatidylcholine (pyrenePC) concentrated in the inner monolayer were prepared by outer monolayer exchange between pyrenePC-containing large unilamellar vesicles and excess (unlabeled) small unilamellar vesicles, using bovine liver phosphatidylcholine-specific exchange protein. After depletion of pyrenePC from the outer monolayer, the asymmetric large unilamellar vesicles were isolated by gel filtration and exposed to the purified C5b-9 proteins at 37°C. Transbilayer exchange of phospholipid between inner and outer monolayers during C5b-9 assembly was monitored by changes in pyrene excimer and monomer fluorescence. Membrane deposition of the C5b67 complex (by incubation with C5b6 + C7) caused no change in pyrenePC fluorescence. Addition of C8 to the C5b67 vesicles resulted in a dose-dependent decrease in the excimer/monomer ratio. This change was observed both in the presence and absence of complement C9. No change in fluorescence was observed for control vesicles exposed to C8 (in the absence of membrane C5b67), or upon C5b-9 addition to vesicles containing pyrenePC symmetrically distributed between inner and outer monolayers. These data suggest that a transbilayer exchange of phospholipid between inner and outer monolayers is initiated upon C8 binding to C5b67. The fluorescence data were analyzed according to a "random walk" model for excimer formation developed for the case where pyrenePC is asymmetrically distributed between lipid bilayers. Based on this analysis, we estimate that a net transbilayer migration of ~1% of total membrane phospholipid is initiated upon C8 binding to C5b67. The potential significance of this transbilayer exchange of membrane phospholipid to the biological activity of the terminal complement proteins is considered.

INTRODUCTION

The membranolytic activity of the complement system resides in a macromolecular complex composed of serum proteins C5b6, C7, C8, and C9, bound to membrane surfaces (reviewed in reference 1). This complex not only mediates immune cytolysis, but can also elicit nonlytic stimulatory responses resulting in cell activation (2–6). For example, human blood platelets exposed to the C5b-9 proteins are stimulated to secrete from intracellular storage granules and to express de novo binding sites for coagulation factor Va, initiating assembly of the prothrombinase enzyme complex of factors VaXa (7–9). This results in prothrombin conversion to thrombin and accelerated plasma clot formation. These observations suggest that platelet activation by the C5b-9 proteins results in increased exposure of acidic phospholipids on the platelet surface, providing negatively charged sites for membrane binding of factor Va (9–12). Transbilayer migration of acidic phospholipid from inner to outer monolayer of the plasma membrane has been demonstrated after platelet activation by calcium ionophore and collagen (10–13), raising the possibility that increased factor Va binding to C5b-9 platelets arises from cell activation mediated through increased cytosolic calcium. Alternatively, the C5b-9 proteins potentially affect the transmembrane distribution of phospholipid by directly altering lipid bilayer organization, for example, by creating local domains of nonbilayer lipid (14).

To determine whether the membrane inserted C5b-9 proteins directly catalyze transbilayer migration of phospholipids, we have examined the interaction of these proteins with unilamellar lipid vesicles under conditions permitting real-time determination of the transbilayer distribution of membrane phospholipid. Because the membrane permeability changes that are known to arise upon assembly of the C5b-9 complex precluded use of aqueous labeling reagents to assess phospholipid asymmetry, we have developed a method for directly assessing transbilayer distribution of a fluorescently labeled phos-

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pholipid, using a modification of the method of Everett et al. (15). In these experiments, changes in pyrene excimer and monomer fluorescence during assembly of the C5b-9 complex are employed to monitor the transbilayer migration of a pyrene-labeled phosphatidylcholine initially concentrated in the inner monolayer.

MATERIALS AND METHODS

Materials

3-Palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (pyrenePC)[1] was obtained from Molecular Probes Inc., Eugene, OR; L-α-lecithin and phosphatidylserine (disodium salt) from bovine brain were obtained from Avanti Polar Lipids, Inc., Birmingham, AL. All other chemicals were of reagent or analytical grade.

Solution

Na-TES: 10 mM 2-((tris-(hydroxymethyl)-methyl)-amino)ethane sulfonic acid, 50 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 0.02% (wt/vol) NaN_3, pH 7.4.

Exchange protein

Phosphatidylcholine-specific phospholipid exchange protein (PCEP) was isolated according to published methods (16) and assayed according to the methods of Brown et al. (17). The protein was stored at −20°C in 50% (vol/vol) glycerin, and dialyzed into Na-TES immediately before use.

Preparation of vesicles

Small unilamellar vesicles (SUV) were prepared according to Barenholz et al. (18). Large unilamellar vesicles (LUV) were prepared by detergent dialysis (19).

Preparation of asymmetric vesicles

Large unilamellar vesicles containing pyrenePC asymmetrically concentrated in the inner monolayer (ALUV) were prepared using a modification of the method introduced by Everett et al. (15). LUV containing 20 mol % pyrenePC were incubated (37°C, 24 h) with a 37-fold molar excess of SUV (5% phosphatidylserine, 95% phosphatidylcholine) in the presence of 122 μg/ml PCEP. Total lipid concentration was 29.4 mM (28.6 mM in small vesicles, 0.8 mM in large vesicles) in a total volume of 1 ml. After exchange, the large vesicles were separated from protein and small vesicles by gel filtration on Sepharose CL-2B (0.9 × 27 cm). Eluted fractions (1.16 ml) were assayed for total phospholipid (20) and pyrenePC (see below). The peak fraction of lipid eluting in the void volume was analyzed and used immediately. Contamination of large vesicles by aggregated small vesicles eluting in the void volume of the column was estimated to be <3% by phospholipid (data not shown).

Determination of pyrenePC concentration

PyrenePC concentrations of labeled vesicles were measured by a modification of the method of Van Den Zegel et al. (21). Samples were dissolved in 1% sodium dodecyl sulfate, incubated 1 h at 37°C, and transferred to a cuvette for measurement of fluorescence intensity (at 378 nm). Comparison was made to the measured fluorescence emission of standards of pyrenePC (0–5 μM) hydrated in 1% sodium dodecyl sulfate. Concentrations of the pyrenePC standards was based on phosphorus determination (20). The purity of the pyrenePC was confirmed by thin-layer chromatography on silica gel plates in the solvent system chloroform/methanol/water (65:25:4).

Complement proteins

Human complement proteins C5b6, C7, C8, and C9 were purified and assayed with the following modification of methods previously described (22–24): C5b6 was subject to additional purification by ion exchange on Mono Q (Pharmacia Fine Chemicals, Piscataway, NJ), performed at pH 8 in the presence of 10% glycerol. C7 was purified using monoclonal immunoaffinity chromatography, followed by ion exchange chromatography on Mono Q. To exclude possible trace contaminating C9, purified C8 was absorbed with monospecific antibody (goat) to human C9, coupled to Sepharose CL-4B.

C5b67 vesicles

Membrane bound C5b67 complexes were prepared by mixing vesicles with C5b6 followed by the addition of C7 with rapid mixing. Final concentrations were 0.26 mM lipid, 0.25 mg/ml C5b6, and 0.05 mg/ml C7 except when specified otherwise. After 5 min incubation at room temperature the mixture was stored on ice and used within 60 min.

C5b-9 assembly

C5b-9 assembly was initiated by C8 and C9 addition to C5b67 vesicles suspended in a stirred fluorescence cuvette equilibrated at 37°C. C5b67 vesicles were diluted in Na-TES to yield a final lipid concentration of 1.9 μM. After 5 min equilibration, C8 (0–10 μg/ml) and/or C9 (0–16 μg/ml) were added as described in figure legends. Fluorescence was continuously monitored before and after protein additions (below).

Fluorescence spectroscopy

All fluorescence measurements were obtained with a SLM8000C photon-counting Spectrofluorometer equipped for sample stirring and temperature control. Excitation was provided by a xenon arc lamp and emission detected by model R928P photomultiplier tubes (Hamamatsu Corp., Middlesex, NJ). Wavelength selection was provided by a double grating excitation monochromator and single grating emission monochromators arranged in T-format. To reduce photobleaching, shutters were closed except during data acquisition; 1 cm pathlength quartz cuvettes containing 1.5–2.5 ml volume of sample were employed throughout. Unless otherwise indicated, spectra were recorded with an excitation bandwidth of 2 nm, emission bandwidth of 4 nm and the

[1] *Abbreviations used in this paper:* ALUV, asymmetric large unilamellar lipid vesicle; *E*, pyrene excimer fluorescence; LUV, large unilamellar lipid vesicle; *M*, pyrene monomer fluorescence; m_f, mole fraction of pyrenePC; PCEP, phosphatidylcholine-specific phospholipid exchange protein; pyrenePC, 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine.

excitation monochromator was set at 345 nm. All data were corrected for dilution due to additions to the cuvette, and for the wavelength-dependent response of the instrument, using correction factors supplied by the manufacturer.

DATA ANALYSIS

Dependence of pyrene excimer formation on probe concentration when pyrenePC is symmetrically distributed across bilayer

As discussed by Eisinger et al. (25), three models are available to describe the relationship of pyrene excimer (E) and monomer (M) intensities to the mole fraction of pyrenePC (m_f) incorporated into a lipid bilayer: a "diffusion" model (25), a "random walk" model (26, 27), and a "milling crowd" model (25). To determine the most appropriate model to account for the concentration-dependent change in E and M observed for pyrenePC under our experimental conditions, we first measured the dependence of E and M on the mole fraction of pyrenePC (m_f) when the probe lipid was symmetrically incorporated into large lipid vesicles, by hydration and detergent dialysis of premixed labeled and unlabeled lipids (see above). We found that the experimentally determined E/M vs. m_f deviated from linearity for $m_f > 0.1$, indicating that the diffusion model (25) cannot be applied for pyrenePC under our conditions (Fig. 1). Similar results for a pyrene-labeled glucocerebroside incorporated in lipid vesicles have been reported by Thompson and associates (28). By contrast, when evaluated according to a random walk model (Fig. 2), the data we obtained were in good agreement with the predicted relationship of E and

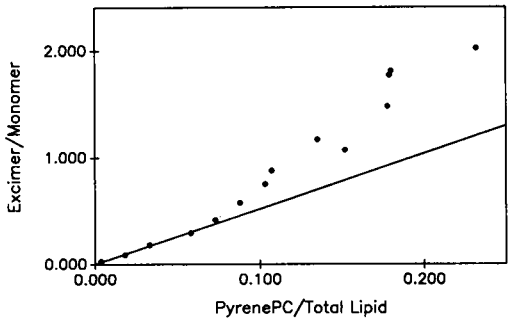

FIGURE 1 Dependence of E/M on mole fraction of pyrenePC. E/M was measured (at 37°C) for a series of vesicles containing varying amounts of pyrenePC (mole fraction pyrenePC indicated on abscissa), and analyzed according to the diffusion model (reference 25). Line represents a least squares fit through the origin according to diffusion model, for data with $m_f < 0.1$ (see Data Analysis in Materials and Methods).

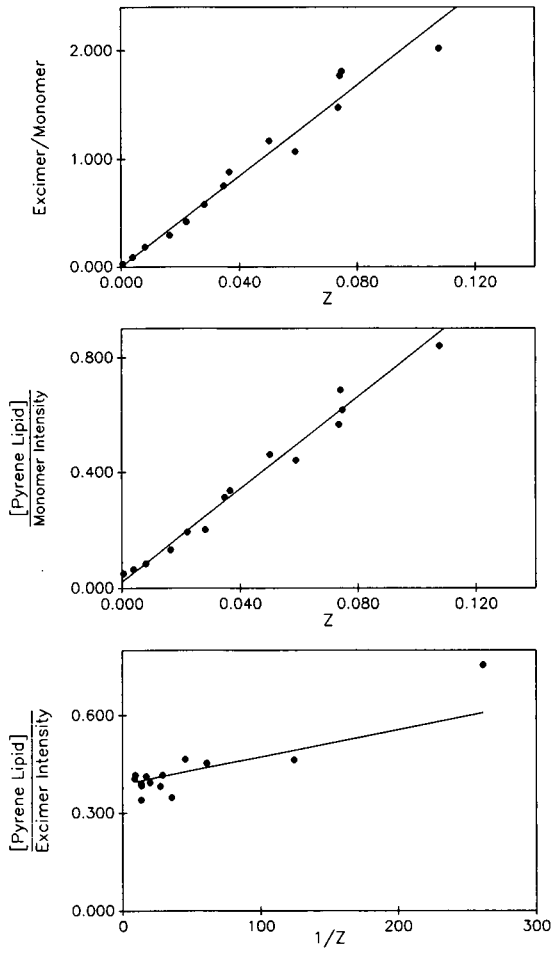

FIGURE 2 Fit of experimental data to random walk model. Data refer to the series of vesicles described in Fig. 1, analyzed according to Eqs. 1 and 2 (random walk model; see Data Analysis, Materials and Methods). (A) Measured E/M plotted as function of Z (from Eqs. 1 and 2). Line represents least-squares fit of the data through the origin ($r^2 = 0.98$). From the reciprocal of the slope of the regression line, $C/L = 0.046 \pm 0.006$. (B) Monomer fluorescence plotted as $(m_f * Q)/M$ vs. Z (see Eq. 1); $r^2 = 0.98$. Parameter V (defined in Data Analysis, Materials and Methods) derived from best fit of the data equals 0.0026 ± 0.0012. (C) Excimer fluorescence plotted as $(m_f * Q)/E$ vs. $1/Z$ (see Eq. 2); $r^2 = 0.87$. $V = 0.0022 \pm 0.0002$.

M to m_f, given by the following equations (adapted from reference 25):

$$M = m_f * Q * L * V/(V + Z) \quad (1)$$

$$E = m_f * Q * C * Z/(V + Z), \quad (2)$$

where Q is the total lipid concentration, L and C are proportionality constants, and Z is related to m_f by

$$Z = m_f/\ln(2/m_f). \quad (3)$$

V (in Eqs. 1 and 2) is that value of Z for which the

monomer and excimer yields are half-maximal. For these symmetrically distributed pyrenePC LUV (before PCEP-catalyzed exchange), we determined that $V = 0.0022 \pm 0.0002$ and $C/L = 0.046 \pm 0.006$, for m_f between 0 and 0.25. Because of the satisfactory fit of our data to the random walk model (Fig. 2), we have not further analyzed these data according to the generalized milling crowd model, developed by Eisinger et al. (25).

Dependence of pyrene excimer formation on probe concentration when pyrenePC is asymmetrically distributed across the bilayer

To deduce the quantitative change in the distribution of pyrenePC between inner and outer monolayers of the ALUV employed in our experiments, we considered the process of pyrene excimer formation for the case that pyrenePC is asymmetrically distributed across the bilayer (i.e., when the mole fraction of pyrenePC in the inner monolayer [$m_{f,in}$] is not equal to that in the outer monolayer [$m_{f,out}$]).

Model 1: pyrene excimer formation restricted to in-plane interactions within each monolayer

In this model we assume that pyrene excimers can only form between pyrenePC located in the same monolayer. According to this model, E and M are related to the mole fraction of pyrenePC by the following equations:

$$M = [m_{f,in} * Q_{in} * L * V/(V + Z_{in})] + [m_{f,out} * Q_{out} * L * V/(V + Z_{out})] \quad (4)$$

$$E = [m_{f,in} * Q_{in} * C * Z_{in}/(V + Z_{in})] + [m_{f,out} * Q_{out} * C * Z_{out}/(V + Z_{out})], \quad (5)$$

where L, V, and C are the constants introduced above, Q_{in} is the molar lipid concentration accounted for by inner monolayer lipid and Q_{out} is the molar lipid concentration accounted for by outer monolayer lipid. Z_{in} and Z_{out} are each related to the respective mole fraction of pyrenePC in the inner and outer monolayers by

$$Z_{in} = m_{f,in}/\ln[2/m_{f,in}] \quad (6)$$

$$Z_{out} = m_{f,out}/\ln[2/m_{f,out}]. \quad (7)$$

Combining Eqs. 4 and 5, E/M can be expressed as

$$E/M = (C/LV) * (N/D), \quad (8)$$

where

$$N = [(m_{f,in} * Z_{in})/(V + Z_{in})] + [(Q_{out}/Q_{in}) * (m_{f,out} * Z_{out})/(V + Z_{out})]$$

$$D = [m_{f,in}/(V + Z_{in})] + [Q_{out}/Q_{in}) * (m_{f,out}/(V + Z_{out})].$$

The ratio of phospholipid in outer and inner monolayers (Q_{out}/Q_{in}) of the ALUV employed in our experiments can be estimated from the change in total probe concentration when pyrenePC is removed from the outer monolayer (see Table 1 in Results). As these data indicate, the amount of pyrenePC in the vesicles decreases from 0.151 μmol (of a total of 0.75 μmol phospholipid before PCEP-catalyzed exchange) to 0.072 μmol (of a total of 0.74 μmol phospholipid after exchange and gel filtration). Assuming that pyrenePC is (*a*) distributed symmetrically across the monolayers of the initial vesicles formed during detergent dialysis and (*b*) removed only from the outer monolayer of the LUV during PCEP-catalyzed exchange with unlabeled SUV (described above), these data indicate that the ratio of total lipid in outer/inner monolayers of the large vesicles used in these experiments, $Q_{out}/Q_{in} = 1.1$. This result is in good agreement with the predicted mass distribution between monolayers, based on consideration of the radius of curvature of LUV formed by detergent dialysis (reviewed by reference 29). Using this value of Q_{out}/Q_{in}, Eq. 8 can be used to predict the relationship between E/M and $m_{f,out}$ as pyrenePC redistributes from the inner to outer monolayer of the ALUV formed under our experimental conditions (Fig. 3 *solid curve*).

For asymmetric LUV prepared by PCEP-catalyzed depletion of the outer monolayer of 20 mol% (initial) pyrenePC vesicles (described above), the measured E/M (1.19) suggests a distribution of pyrenePC between LUV monolayers of $m_{f,out} = 0.003$, $m_{f,in} = 0.197$ (from Eq. 8

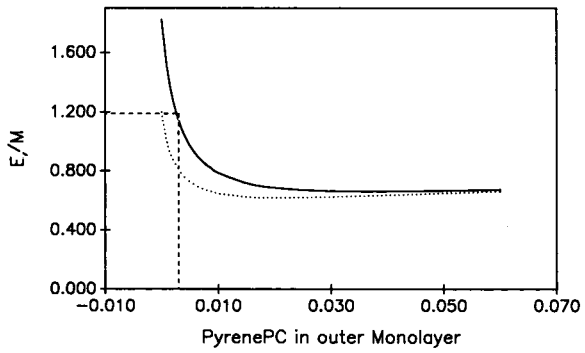

FIGURE 3 Dependence of pyrene excimer formation on the distribution of pyrenePC between monolayers. Solid curve depicts the theoretical relationship of E/M (ordinate) to $m_{f,out}$ (abscissa) based on Eq. 8, for $V = 0.0022$, $C/L = 0.046$, and $Q_{out}/Q_{in} = 1.1$ (model I; see Data Analysis in Materials and Methods). Curve is drawn for asymmetric large unilamellar vesicle containing 9 mol% pyrenePC (pyrenePC/total lipid). Dotted curve depicts the theoretical relationship of E/M to $m_{f,out}$ based on Eq. 11, for $C/LV = 13.8$, $C'/LV = 7.2$, $V = V' = 0.0022$, and $Q_{out}/Q_{in} = 1.1$ (model 2; see Data Analysis in Materials and Methods). Dashed lines denote experimental E/M measured for asymmetric LUV prepared by depletion of probe from the outer monolayer of an initial 20 mol% pyrenePC LUV (see text).

using coefficients $Q_{out}/Q_{in} = 1.1$; $V = 0.0022$; $C/L = 0.046$; see above). These values are depicted by dashed lines in Fig. 3. Note that this estimate is close to the predicted transbilayer distribution of pyrenePC at the end of PCEP-catalyzed exchange between 20 mol% pyrenePC (symmetric) LUV and a 37-fold molar excess of unlabeled SUV, as under our experimental conditions.

Model 2: pyrene excimer formation by both in-plane and transbilayer interactions of pyrenePC

As noted above, the formulation of model 1 assumes that pyrene excimers form only by in-plane interaction of pyrenePC distributed within the same monolayer, and that the observed E/M represents the contribution of excimers arising independently in each monolayer. According to this model, the discrepancy between the experimental E/M observed in asymmetric bilayers generated by depletion of pyrenePC from the outer monolayer ($E/M = 1.19$; fraction 6 of Fig. 4) from that of the initial symmetric pyrenePC containing LUV ($E/M = 1.85$ before PLEP-catalyzed exchange) reflects the fluorescence of a small amount of pyrenePC that remains in the outer monolayer of the newly formed asymmetric LUV. An alternative interpretation of these results is that excimer formation in each monolayer is not strictly independent, and that excimers can also arise by interaction between pyrenePC distributed in apposing monolayers. Because of the uncertainties inherent to preparing (and maintaining) asymmetric LUV completely devoid of pyrenePC in the outer monolayer, we were unable to unambiguously exclude this latter possibility. Nevertheless, an upper limit to the potential contribution of such a transbilayer excimer-forming process can be obtained by assuming that the observed discrepancy in the E/M of asymmetric vs. symmetric LUV is totally accounted for by the loss of transbilayer excimers when pyrenePC is removed from the outer monolayer. In this model, we assume that pyrene excimers can form in two ways, (*a*) between pyrenePC in the same monolayer (i.e., in-plane excimer formation) and (*b*) between pyrenePC in apposing monolayers (i.e., transbilayer excimer formation). In this model, E and M are given by:

$$M = [m_{f,in} * Q_{in} * L * V/(V + Z_{in})] + [m_{f,out} * Q_{out} * L * V/(V + Z_{out})] \quad (9)$$

$$E = [m_{f,in} * Q_{in} * C * Z_{in}/(V + Z_{in})] + [m_{f,out} * Q_{out} * C * Z_{out}/(V + Z_{out})] + [m_{f,in} * Q_{in} * C' * Z_{out}/(V' + Z_{out})] + [m_{f,out} * Q_{out} * C' * Z_{in}/(V' + Z_{in})], \quad (10)$$

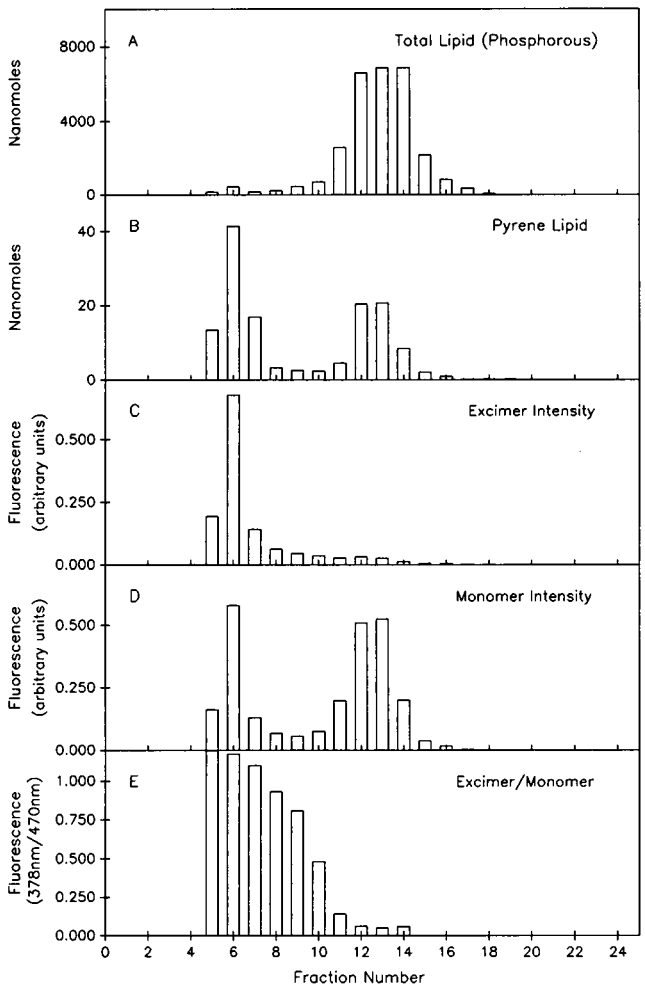

FIGURE 4 Gel permeation chromatography of asymmetric pyrenePC-containing LUV. Sepharose CL-2B elution profile of exchange mixture containing pyrene-PC LUV and unlabeled SUV after incubation with PCEP. (*A*) Total lipid phosphorus (nanomoles). (*B*) PyrenePC (nanomoles). (*C*) Relative fluorescence intensity of pyrene excimer, measured at 470 nm (arbitrary units). (*D*) Relative fluorescence intensity of pyrene monomer, measured at 378 nm (arbitrary units). (*E*) Ratio of excimer/monomer intensities. Fluorescence data were obtained at room temperature and corrected for temperature dependence and instrument response. See Materials and Methods.

where C of model 2 is now a constant proportional to the probability of pyrene excimer formation by in-plane (intramonolayer) interaction and C' is a constant proportional to the probability of transbilayer excimer formation. V of model 2 is now that value of Z_{in} at which the in-plane excimer formation is half-maximal, and V' is the equivalent parameter for the process of transbilayer excimer formation. It should be noted that the constants introduced in model 2 are not necessarily numerically equivalent to the analogous constants employed in model

1. Combining Eqs. 9 and 10,

$$E/M = [(C/LV) * (N) + (C'/LV) * (N')]/D, \quad (11)$$

where N and D are defined as in Eq. 8 (above) and $N' = [(m_{f.in} * Z_{out})/(V' + Z_{out})] + [(Q_{out}/Q_{in}) * (m_{f.out} * Z_{in})/(V' + Z_{in})]$. In the case of symmetrically distributed pyrenePC, $m_{f.in} = m_{f.out} = m_f$, $Z_{in} = Z_{out} = Z$, and Eq. 11 condenses to:

$$E/M = Z\,[C/LV + (C'/LV) * (V + Z)/(V' + Z)]. \quad (12)$$

When pyrenePC is symmetrically distributed across the bilayer, measured E/M is observed to be linearly related to Z (see Fig. 2 *A*), implying from Eq. 12 that $V \approx V'$. Consequently, in this case (where pyrenePC is symmetrically distributed across the bilayer), Eq. 12 simplifies to

$$E/M = Z * (C/LV + C'/LV). \quad (13)$$

Using the data of Fig. 2 *A* to solve for the coefficient of Eq. 13, we estimate $(C/LV + C'/LV) = 21$.

After total removal of pyrenePC from the outer monolayer ($Z_{out} = m_{f.out} = 0$), the possibility of transbilayer excimer formation is precluded, and the relationship between E/M and Z in the resulting asymmetric vesicle can be expressed by

$$E/M = Z_{in} * (C/LV), \quad (14)$$

where $Z = Z_{in}$ (before any redistribution of pyrenePC from inner to outer monolayers). For vesicles containing an initial 20 mol% pyrenePC (i.e., $Z = 0.087$; see Eq. 3), the measured E/M was observed to decrease from $E/M = 1.85$ (in the initial LUV formed by detergent dialysis) to $E/M = 1.19$ (after depletion of outer monolayer pyrenePC by PCEP-catalyzed exchange and gel filtration; data not shown). By substituting these values of E/M into Eqs. 13 and 14, respectively, and setting $(C/LV + C'/LV) = 21$ (see above), the coefficients (C/LV) and (C'/LV) can be directly solved: $C/LV = 13.8$ and $C'/LV = 7.2$. Based on the estimated mass relationship of outer and inner monolayers for these large vesicles ($Q_{out}/Q_{in} = 1.1$; see above), the numerical solutions to these coefficients can then be used to derive the relationship of E/M to $m_{f.out}$ predicted by Eq. 11 (dotted curve in Fig. 3).

Inspection of the two curves depicted in Fig. 3 reveals that when one allows for the possibility of a transbilayer pyrene excimer (i.e., dotted curve predicted by model 2), there is less than a twofold change in the initial slope of E/M vs. $m_{f.out}$ from that predicted when one assumes complete independence of excimer formation in each monolayer (solid curve predicted by model 1). These data therefore suggest that intramonolayer pyrenePC excimer formation is dominant to the transbilayer process $(C/C' = 2)$, the latter process presumably requiring interdigitation of the acyl chain–tethered pyrenes. Also to be noted, model 1 (which excludes transbilayer excimer formation) and model 2 (which assumes transbilayer excimer formation) refer to limiting cases, and the actual relationship of E/M to $m_{f.out}$ is likely to be intermediate to the two curves plotted in Fig. 3. According to both models, when $m_{f.out} < 0.01$, the measured E/M is predicted to be extremely sensitive to changes in $m_{f.out}$ arising from subsequent transbilayer phospholipid exchange (resulting in equilibration of pyrenePC from the inner to outer monolayers).

RESULTS

The distribution of total phospholipid and pyrenePC between LUV and SUV after incubation with PCEP and chromatography over Sepharose CL-2B are summarized in Table 1 and Fig. 4. LUV (eluting in the void volume) contained 99% of initial phospholipid (added as large vesicles to the exchange mixture) and 48% of their initial pyrenePC. Assuming a symmetric distribution of pyrenePC between inner and outer monolayers of LUV (before exchange), these data suggest near complete depletion of pyrenePC from the LUV outer monolayer (see Data Analysis, Materials and Methods). Consistent with this interpretation, the ratio of excimer/monomer intensities of the peak fraction of large vesicles eluting from the column ($E/M = 1.19$) indicates that the vesicles retain nearly 20 mol% pyrenePC in the inner monolayer, <0.3 mol% pyrenePC remaining in the outer monolayer (see Data Analysis and Fig. 3, Materials and Methods). When spontaneous transbilayer exchange was induced by heating (1 h, 85°C), E/M decreased to 0.83 (data not shown), consistent with a decrease in mole fraction of pyrenePC in the inner monolayer due to redistribution of the probe from inner to outer monolayers.

TABLE 1 Analysis of vesicles eluting from Sepharose CL-2B

	Before exchange	After exchange
	μmol	*μmol*
Phospholipid in large vesicles	0.75	0.740*
Phospholipid in small vesicles	27.9	28.0‡
PyrenePC in large vesicles	0.151	0.072*
PyrenePC in small vesicles	(0)	0.066‡

Phospholipid (measured as total phosphorous) and pyrenePC in large and small vesicle pools, before and after exchange catalyzed by PCEP. Exchange conditions were as described in Materials and Methods.
*Refers to ALUV eluting in void volume (pool of fractions 5–7 eluting from Sepharose CL-2B; Fig. 4).
‡Pool of fractions 8–19 eluting from Sepharose CL-2B; Fig. 4.

Changes in pyrenePC fluorescence due to C5b-9 assembly

To determine whether membrane insertion of the terminal complement proteins is accompanied by a redistribution of phospholipid between inner and outer monolayers, these asymmetric pyrenePC-containing LUV (ALUV) were exposed to the purified C5b-9 proteins and monitored for changes in pyrene excimer/monomer intensities (Table 2 and Figs. 5–8). No change in E/M from control values was observed upon deposition of the membrane C5b67 complex (by incubation of vesicles with C5b6 + C7 without added C8 or C9; Table 2). By contrast, formation of the C5b-9 complex (by addition of C8 + C9 to C5b67 ALUV) resulted in an approximate 8% decrease in E/M (Table 2). This change in E/M was also observed upon C8 addition to C5b67 ALUV (in the absence of C9), but was not observed upon C8 addition to ALUV lacking preformed C5b67 complexes.

The change in E/M upon assembly of the membrane attack complex was found to depend upon both the number of preformed C5b67 sites (as varied by changing the initial input of C5b6; Fig. 5) as well as upon the input of C8 (to ALUV expressing a constant number of preassembled C5b67 complexes; Fig. 6). Furthermore, no significant difference in the C8 dose-dependent change in E/M was observed when experiments were performed either in the presence (Fig. 6 *A*) or absence (Fig. 6 *B*) of saturating amounts of C9 (see also Table 2), suggesting that this change in E/M occurs when C8 binds to the membrane C5b67 complex (see Discussion). This change in E/M observed for C5b-8–treated ALUV was detected within seconds of C8 addition, and reached completion within 1 min (Fig. 7).

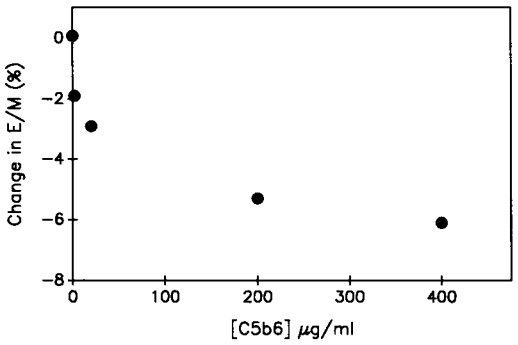

FIGURE 5 Loss of pyrenePC asymmetry upon C5b-9 assembly: relationship to input of C5b6. Asymmetric LUV (0.22 mM total lipid; mole fraction of pyrenePC in inner monolayer = 0.197) were exposed to C7 (40 μg/ml) plus amounts of C5b6 indicated (abscissa). After 5 min at 23°C, an aliquot was diluted and equilibrated at 37°C in a stirred cuvette for fluorescence measurements (final lipid concentration, 2.1 μM). Plotted is the change in excimer/monomer fluorescence measured upon addition of C8 (5 μg/ml). Data are representative of two similar experiments performed on separate days.

TABLE 2 Change in pyrenePC fluorescence due to membrane assembly of the terminal complement proteins

Vesicles*	Treatment‡	Change in E/M§
		%
ALUV	C5b6, C7, C8,‖ C9	−7.9 ± 0.8 (n = 3)¶
ALUV	C5b6, C7, C8‖	−8.4 ± 1.2 (n = 5)¶
ALUV	C5b6, C7, C8	−6.4 ± 0.8 (n = 14)¶
ALUV	C5b6, C8	0.07 ± 0.4 (n = 3)
ALUV	C7, C8	−0.03 ± 0.4 (n = 3)
Symmetric	C5b6, C7, C8, C9	−0.01 ± 0.01 (n = 3)
Symmetric	C5b6, C7, C8	0.5 ± 1.7 (n = 3)
Symmetric**	C5b6, C7, C8	−0.3 ± 1.9 (n = 3)

*Symmetric LUV (20% pyrenePC) and asymmetric ALUV (20% pyrenePC in inner monolayer only) were prepared as described in Materials and Methods and used at 0.26 mM (final concentration in cuvette).

‡Vesicles were incubated with C5b6 (0 or 0.25 mg/ml), C7 (0 or 0.05 mg/ml), C8 (0 or 5 μg/ml), and C9 (0 or 16 μg/ml) under conditions described in Materials and Methods.

§Mean ± SD. Change in E/M represents the response measured upon addition of C8, expressed as a percentage of the initial value. E/M measured for C5b67 ALUV (in absence of C8) did not significantly differ from that measured for controls (omitting C5b6 or C7; see Materials and Methods).

‖C8 was added at 10 μg/ml.

¶Significant vs. complement-free control, $P < 0.05$.

**ALUV were incubated for 1 h at 85°C to induce spontaneous transbilayer redistribution of pyrenePC before exposure to complement proteins.

Relationship of change in E/M to transbilayer exchange of pyrenePC

The change in E/M detected upon C8 binding to membrane C5b67 suggests a decrease in the concentration of pyrenePC in the inner monolayer arising from exchange with unlabeled lipid in the outer monolayer (see Data Analysis and Fig. 3; Materials and Methods). Alternatively, this spectral change could potentially reflect a change in either the local environment or the mobility, orientation, or lateral distribution of the chromophore arising upon C8 binding and insertion into the membrane (25–27). As illustrated in Table 2, no change in E/M was observed when C5b-8 (or C5b-9) was assembled on LUV containing pyrenePC symmetrically distributed between inner and outer monolayers. A similar result was obtained when ALUV were first allowed to spontaneously equilibrate pyrenePC between monolayers before exposure to the terminal complement proteins (Table 2). These data suggest that the decrease in E/M observed upon mem-

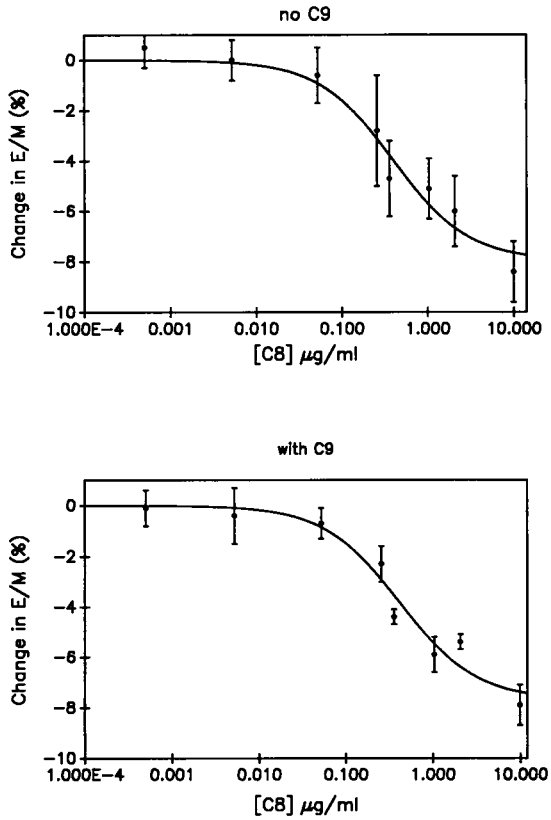

FIGURE 6 Loss of pyrenePC asymmetry upon C5b-9 assembly: dependence on C8. Asymmetric pyrenePC-containing LUV (0.22 mM phospholipid) were pretreated with C5b6 (0.25 mg/ml) plus C7 (40 μg/ml) before dilution and suspension at 37°C for fluorescence measurements. Plotted is the change in pyrene excimer/monomer intensity (percent change from initial value) measured upon addition of C8 at concentrations indicated (abscissa). (*A*) Data obtained in the absence of C9. (*B*) Data obtained in the presence of 5 μg/ml C9, added immediately before C8 additions. Data plotted as mean ± SD of three to nine determinations at each C8 concentration. Curves represent the least-squares fit of the data to the following equation: change in E/M (%) = $D \cdot [C8]/([C8] + K)$. For *A*, $D = 7.96$ and $K = 0.40$ μg/ml; For *B*, $D = 7.70$ and $K = 0.42$ μg/ml. Analysis by Students *t*-test indicates no significant difference between data of *A* vs. *B* at each concentration indicated ($P > 0.2$).

brane assembly of the C5b-8 complex is not due to changes in fluorescence secondary to perturbation of pyrenePC located in either inner or outer monolayers, but represents the change in pyrenePC concentration in each monolayer that is predicted when this lipid redistributes across the bilayer.

In addition to potential artifacts arising from changes in probe environment or orientation that might directly affect pyrene excimer and monomer intensities, we considered the possibility that the apparent decrease in the concentration of pyrenePC distributed in the inner monolayer of ALUV exposed to the complement proteins arose

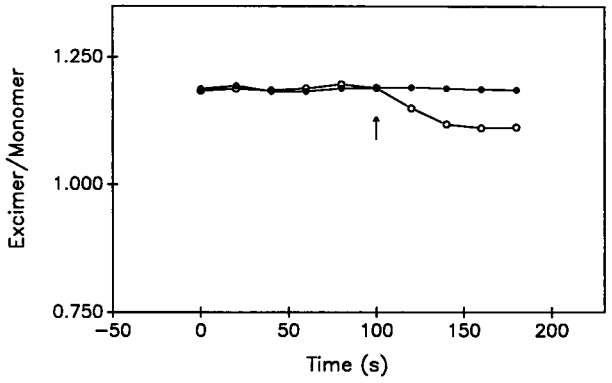

FIGURE 7 Rate of change in E/M upon C5b-8 assembly. The time-dependent change in the ratio of pyrene excimer/monomer fluorescence (E/M) is shown for C5b67 ALUV exposed to 5 μg/ml C8 (*open symbols*). C8 was added at time indicated by arrow. Change in E/M for controls (lacking C7) is also shown (*solid symbols*). C5b67 assembly was performed as described in Fig. 6. Data representative of three separate experiments performed under identical conditions.

from a complement-induced fusion of these vesicles. To address this possibility, LUV containing 20 mol% pyrenePC (symmetrically distributed) were mixed with an equal number of unlabeled LUV, and then C5b-9 assembly initiated. Under these conditions, no change in E/M was detected, whereas fusion and mixing of lipid (induced by light sonication) showed the expected decrease in E/M (data not shown).

Comparison of the emission spectra of C5b67 vs. C5b-8

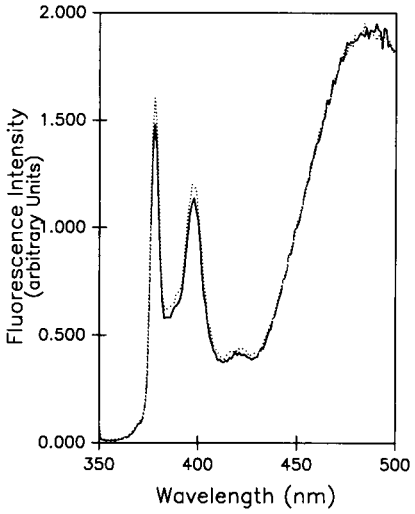

FIGURE 8 Fluorescence emission spectra of complement-treated asymmetric LUV. Fluorescence emission spectra measured before (*solid line*) and after (*dotted line*) addition of 10 μg/ml C8 to C5b67 ALUV. Excitation was at 345 nm. C5b67 assembly was performed as described for Fig. 6.

ALUV suggests that virtually all of the change in E/M upon C8 binding arises from an increase in monomer emission (at 378 nm), with virtually no change in excimer emission (at 470 nm) detected (Fig. 8). This result is in agreement with the expected change in excimer and monomer yields arising from a decrease in the concentration of pyrenePC in the inner monolayer (initially at ~20 mol%) through exchange with phospholipid in the outer monolayer (<0.3 mol% pyrenePC). According to Eqs. 4 and 5 (model 1) or Eqs. 9 and 10 (model 2; see Data Analysis in Materials and Methods), an 8% increase in monomer emission due to transbilayer exchange or pyrenePC under these conditions is predicted to decrease excimer emission by only 0.3% (cf. data of Fig. 8).

DISCUSSION

The results of these experiments suggest that membrane assembly of the terminal complement proteins is accompanied by a redistribution of lipid between inner and outer monolayers. Furthermore these data suggest that this transbilayer migration is initiated upon C8 binding to the membrane C5b67 complex, independent of subsequent incorporation of C9. These results therefore provide evidence that in addition to modifying membrane permeability to aqueous solute, insertion of these pore-forming serum proteins can directly affect the physical properties of the membrane surface, specifically through changes in the phospholipid composition of the outer monolayer.

Wiedmer et al. have previously shown that human platelets treated with the C5b-9 proteins express de novo binding sites for coagulation factor Va and exhibit increased prothrombinase activity which can accelerate plasma clotting (7, 8). Because surface-expressed acidic phospholipid is thought to provide the catalytic surface required for assembly of the platelet prothrombinase enzyme complex (VaXa), these results suggested that the C5b-9 proteins induce transbilayer exchange of phosphatidylserine from the inner to outer monolayer of the platelet plasma membrane, either through stimulatory mechanisms associated with platelet activation, or through direct effects of the complement proteins on membrane bilayer configuration (9). Evidence for transbilayer migration of phosphatidylserine from inner to outer monolayers due to cell activation by collagen and other platelet agonists has previously been described (10–12). The data of the present study suggest that for cells exposed to the activated complement proteins, increased factor Va binding may also arise due to the direct effects of the terminal complement proteins on transbilayer phospholipid asymmetry, irrespective of concomitant C5b-9-induced activation of the cell's stimulatory pathways.

Analysis of the data of Table 2 and Fig. 6 according to Eq. 8 (model 1) or Eq. 11 (model 2; see Data Analysis in Materials and Methods) suggests that under the conditions of our experiments, maximally 0.09% (model 2) to 0.14% (model 1) of total pyrenePC is translocated from inner to outer monolayers upon C8 binding to membrane C5b67. Because in our experiments pyrenePC initially constitutes 20% of the inner monolayer (i.e., 9% of total bilayer lipid), these results imply that 1–2% of total membrane lipid participates in this exchange. Implicit to this estimate are the assumptions that (*a*) translocation of lipid between monolayers occurs by one-for-one exchange, and (*b*) pyrenePC and unlabeled lipid are equivalent in the transbilayer exchange process (see Materials and Methods). This transbilayer exchange of phospholipid commences immediately upon C8 addition and is complete within 40–60 s at 37°C (see Fig. 7), approximating the kinetics of C8 binding and activation of the functional complement pore observed at this temperature (30, 31). Assuming a random distribution of preassembled C5b67 complexes among ALUV, these data suggest that transbilayer exchange of phospholipid is related to the incorporation of C8 into the membrane C5b-8 complex, and that this transbilayer migration of lipid is only transiently activated, resulting in net exchange of only a small fraction of total membrane phospholipid. This implies that transbilayer migration of lipid occurs only during insertion of C8 into the membrane, and is likely to be localized to the vicinity of the bound C5b-8 complex.

One surprising result of the present study was that we were unable to detect any difference in the amount of transbilayer migration of pyrenePC upon C8 vs. C8 + C9 interaction with membrane C5b67 (cf. Table 2 and Fig. 6). These results imply that most of the alteration in bilayer phospholipid asymmetry arising from assembly of these proteins occurs upon binding of C8, and not upon incorporation of C9 (and C9 polymer) into the membrane attack complex. Although we cannot exclude the possibility that the C5b6–C8 proteins we employed in this study were contaminated with trace quantities of C9 sufficient to induce the observed changes in phospholipid asymmetry, we consider this possibility unlikely based on the following considerations. First, we have been unable to detect functional C9 (by hemolytic assay; 22–24) in these proteins when prepared as described in Materials and Methods. Furthermore, we observe no change in the measured response to C8 (added to C5b67 ALUV) for protein repeatedly absorbed against monospecific antibody to C9 (data not shown). In this context it is of interest to note that (*a*) although acyl chain restricted photoaffinity probes show minimal labeling of protein in the membrane C5b67 complex, addition of C8 results in marked photolabeling of both the α- and γ-chains of this

protein, suggesting peptide insertion into the bilayer upon C8 binding (32–34); (*b*) measurement of phospholipid binding to the C5b-9 proteins suggest that the C5b-8 complex expresses 80% of the phospholipid binding sites exhibited by the C5b-9 complex (35); (*c*) analysis of electron spin resonance spectra obtained with oriented lipid multimers indicates that a large change in acyl chain organization occurs upon C8 binding to C5b67, accounting for ~50% of the spectral change observed for the fully assembled C5b-9 complex (14); and (*d*) the C5b-8 complex has been shown to lower the activation energy for membrane insertion and polymerization of C9 (36, 37). As has been suggested by Esser and associates (14, 38, 39), one explanation for this effect of the membrane-inserted C5b-8 complex on C9 activation may relate to the capacity of this complex to create a focal defect in lipid packing, promoting insertion of hydrophobic domains of C9 into the membrane.

It is of interest to note that in addition to these data for the C5b-9 proteins, there is evidence that other pore-forming molecules enhance the rate of transbilayer migration of phospholipid, potentially through the formation of local domains of nonbilayer lipid (40, 41). For example, Classen et al. (41) have recently shown that incorporation of gramicidin into the membrane of human erythrocytes enhances the rates of transbilayer migration and that this enhancement was accompanied by the formation of hexagonal H_{II} lipid structures. As suggested by Cullis and De Kruijff (42), reversible formation of nonbilayer structures in the membrane would provide a mechanism for translocation of lipid between monolayers. As indicated above, there is data suggesting that a local disruption of bilayer structure occurs upon C8 insertion into the membrane, which may account for the transbilayer exchange of phospholipid we observe upon C5b-8 assembly. The possibility that the increased membrane permeability to aqueous solute arising upon insertion of these proteins may also be related to formation of domains of nonbilayer structure has been discussed previously (14, 43).

Finally, it should be noted that the well-known capacity of the C5b-9 proteins to increase membrane permeability (and ultimately lyse cell membranes) complicates the interpretation of any results relating transbilayer asymmetry that might be obtained using techniques which rely upon exclusion of an aqueous component from the internal compartment. This includes methods that rely upon chemical labeling of phospholipid head groups (44, 45), phospholipase cleavages (46, 47), and the use of fluorescence quenching and nuclear magnetic resonance shift reagents (48, 49). By using a phospholipid probe which is itself asymmetrically distributed across the bilayer, the method we have employed circumvents potential artifacts arising from changes in membrane permeability. As shown above this technique is extremely sensitive to transbilayer migration of the labeled lipid, enabling ready detection of the translocation of <0.1% of pyrenePC. We suggest that future application of this method to analysis of membrane composition asymmetry in other circumstances of altered membrane permeability be considered.

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