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COVALENT LINKAGE OF A SYNTHETIC PEPTIDE TO A FLUORESCENT PHOSPHOLIPID AND ITS INCORPORATION INTO SUPPORTED PHOSPHOLIPID MONOLAYERS

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A number of fluorescent peptide-lipid conjugates have been synthesized. Peptides with ten or eleven amino acids are linked through a single lysine residue to the headgroup of phosphatidylethanolamine, fluorescently labelled on one acyl chain, using homobifunctional disuccinimidyl crosslinking reagents. Peptide-lipids can be further derivatized with the hapten dinitrophenyl. Purified peptide-lipids have been incorporated into dimyristoylphosphatidylcholine monolayers at the interface of air and phosphate-buffered saline, at concentrations of up to 11 mol%. For equal average molecular areas, monolayers containing peptide-lipids have higher surface pressures than pure lipid monolayers; for equal surface pressures, peptide-lipid monolayers have higher average molecular areas than pure lipid monolayers. When the peptide-lipid monolayers are transferred to hydrophobic glass slides, the fluorescence appears uniformly distributed. Fluorescence recovery after photobleaching measurements indicate that peptide-lipids diffuse in the monolayer with coefficient $1.5 \cdot 10^{-9}$ cm^2/s, which is much smaller than that of typical lipids in fluid membranes. In addition, the diffusion coefficient of peptide-lipids decreases with increasing peptide-lipid concentration. We conclude that the peptide portion of the peptide-lipid associates with the lipid monolayer and/or that peptide-lipids oligomerize.

Introduction

The process of recognition among cells of the immune system provides one of the most interesting molecular problems in membrane biology today. It has already been shown that one class of cell-cell recognition processes can be mimicked by replacing one of the interacting cells with reconstituted membranes containing 'nonself' proteins, either foreign histocompatibility antigens [1–3] or viral proteins along with 'self' histocompatibility antigens [4,5]. It has also been shown that fluorescently labelled membrane components can be used to provide significant information on the physical state of such reconstituted membranes [4].

Recent work on a number of different systems shows that protein fragments often serve as well as proteins in evoking certain immune responses. Synthetic peptides representing segments of larger proteins, such as virus coat proteins, elicit antibodies that bind to the whole protein [6–8]. Peptides derived from larger, naturally occurring proteins have been used to study immune response specificities [9] and antigen presentation [10]. There is recent evidence that some immune responses, such as generation of helper T-lymphocytes, involve degradation of protein antigens to peptides, followed by the display of these peptides on the

Abbreviations: NBD-C_{12}, 12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminododecanoic acid; NBD-C_{12}-PE, 1-acyl-2-(12-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminododecanoyl)-phosphatidylethanolamine; DSS, disuccinimidyl suberate; EGS, ethylene glycol bis(succinimidyl succinate).

surfaces of antigen-presenting cells in proximity to histocompatibility antigens [11].

In the course of a biophysical study of antigen presentation on cell surfaces, we have devised a procedure for synthesizing a fluorescent peptide-lipid conjugate; this procedure is described here for the coupling of a peptide to the headgroup of phosphatidylethanolamine fluorescently labelled on one acyl chain, using disuccinimidyl cross-linking reagents [12–14]. The products can be purified and then incorporated into phospholipid monolayers at an air/water interface and transferred to hydrophobic glass, in a manner previously described for pure lipid monolayers [15–20] and monolayers containing membrane proteins [21]. We have also labelled cell membranes with peptide-lipid.

Synthetic procedures similar to the one described in this paper have been used previously for conjugating fatty acids or phospholipids to larger proteins. Protein-coated liposomes have been prepared with lipid-protein conjugates through a variety of methods [22–33]. In addition, one account of the construction of multibilayers containing fatty acid-antibody conjugates has been reported [34]. We believe that peptide-lipid conjugates may prove to be widely useful in studies involving membrane-membrane or molecule-membrane interactions.

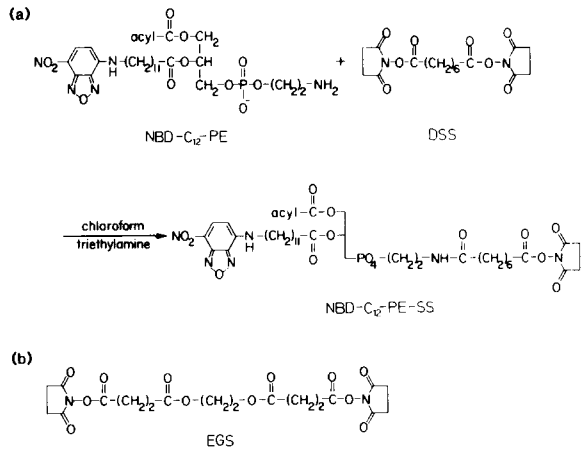
Materials and Methods

Reagents. The sequences and molecular weights of the peptides α-mating factor (Peninsula Laboratories, Inc., San Carlos, CA), renin inhibitor (Peninsula), and lysine vasopressin (Sigma Chemical Co., St. Louis, MO), all purchased lyophilized, are shown in Table I. We have also used the following reagents: L-α-dimyristoylphosphatidylcholine (DMPC), Calbiochem-Behring Corp., La Jolla, CA; disuccinimidyl suberate (DSS) and ethylene glycol bis(succinimidyl succinate) (EGS), Pierce Chem. Co., Rockford, IL; octadecyltrichlorosilane, Aldrich Chem. Co., Milwaukee, WI; 12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminododecanoic acid (NBD-C_{12}), Molecular Probes, Junction City, OR; 1-acyl-2-(12-[*N*-(-7-nitrobenz-2-oxa-1,3-diazol-4-yl)]aminododecanoyl)-phosphatidylethanolamine (NBD-C_{12}-PE), Avanti Polar Lipids, Birmingham, AL; Rabbit IgG (lyophilized), Miles Laboratories, Inc., Elkhart, IN; and Lympholyte M, Accurate Chemical and Scientific, Westbury, NY. The integrity of the DMPC was routinely checked by its ability to run as a single spot on TLC. Chloroform and acetone were spectroscopic grade.

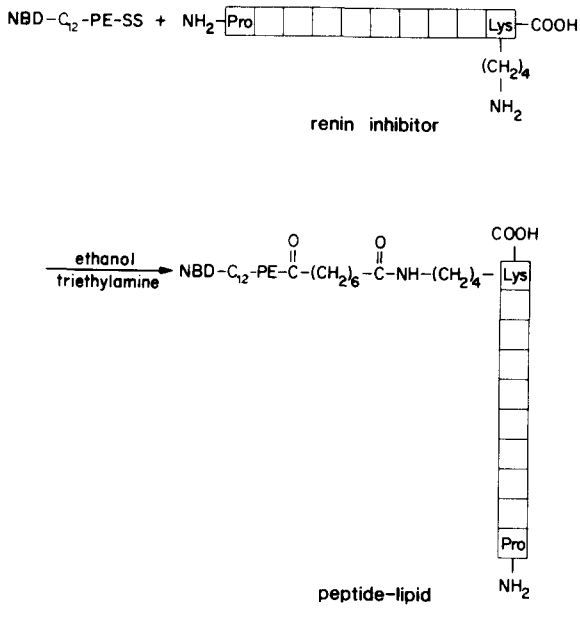
Thin-layer chromatography. Analytical and preparative thin-layer chromatography (TLC) was performed on small (10 cm × 1 inch, 250 μm) or large (20 cm^2, 1000 μm or 2000 μm) Silica gel G or GF (analytical only) plates. NBD-C_{12}-PE and derivatives were visualized either by direct observation of orange color or by observation of fluorescence excited by long wavelength ultraviolet illumination. The succinimide group was visualized by spraying first with a solution of 8.5 ml 14% sodium hydroxide mixed with 20 ml 14% hydroxylamine, and then, after 2 min, spraying with 5% ferric chloride in 1.2 M hydrochloric acid [39]. Primary amines were visualized by spraying with 0.2% ninhydrin [40] in ethanol and heating. Dinitrophenyl derivatives appeared as bright yellow spots; unreacted dinitrofluorobenzene appeared as a white spot, or could be visualized by its quenching of fluorescence on GF plates.

Absorption measurements. NBD-C_{12}-PE peak extinction coefficients, measured from known concentrations, and assuming a molecular weight of 850, were 24200 $M^{-1} \cdot cm^{-1}$ at 464 nm in ethanol and 27900 $M^{-1} \cdot cm^{-1}$ at 466 nm in methanol/ethanol (5 : 4, v/v).

Succinimide esters of NBD-C_{12}-PE. 1 mg DSS in 1 ml chloroform or 10 mg EGS in 1 ml acetone were added to 1 mg NBD-C_{12}-PE in 1 ml chloroform so that the molar ratio of DSS or EGS to NBD-C_{12}-PE was approx. 25. 1 μl of triethylamine was then added with quick mixing, and reaction was allowed to proceed for 30 min at room temperature in the dark. The reaction mixture was analyzed and the desired product, hereafter referred to as NBD-C_{12}-PE-SS or NBD-C_{12}-PE-EGS (see Scheme I), was purified by TLC in acetone/water (95 : 5, v/v) and then in chloroform/methanol/water (95 : 25 : 3, v/v) (see Table II). The main band was scraped from each plate, extracted from the silica with 100 ml chloroform/methanol (2 : 1, v/v), and dried on a rotary evaporator. The dried film was redissolved in



Scheme I.



Scheme II.

several milliliters chloroform/methanol (2 : 1, v/v) after the first scraping (for application to the second plate), and in several milliliters ethanol after the second scraping. Occasionally some insoluble material remained. Product was stored dessicated at −5°C for not more than 48 h before use.

Reaction of succinimidyl NBD-C_{12}-PE with IgG. A volume of NBD-C_{12}-PE-SS or NBD-C_{12}-PE-EGS in ethanol containing 50 μg was dried under N_2. To the dried film was added 0.5 ml of 3 mg/ml IgG in 0.05 M $NaHCO_3$, 0.5% deoxycholic acid, pH 8.3, and reaction was allowed to proceed for 1–2 h at room temperature in the dark. Control samples were prepared identically with NBD-C_{12}-PE substituted for NBD-C_{12}-PE-SS or NBD-C_{12}-PE-EGS. The reaction mixtures were analyzed on a 2.3 cm diameter, 30 cm height, Sephadex G-75 column in the deoxycholate buffer (Fig. 1).

Reaction of NBD-C_{12}-PE-SS with peptide. The NBD-C_{12}-PE-SS resulting from the activation of 1 mg of NBD-C_{12}-PE was added in ethanol to 1 mg dry peptide along with 1 μl of triethylamine. The reaction was allowed to proceed for 1 h at room temperature in the dark, and was analyzed by TLC in chloroform/methanol/water (65 : 25 : 4, v/v) (see Table II). The covalent conjugate of NBD-C_{12}-PE-SS and renin inhibitor (Scheme II) was purified by extracting with 65 : 25 : 4 solvent and drying on a rotary evaporator. The product, hereafter referred to as 'peptide-lipid', was redissolved in several milliliters methanol/ethanol (5 : 4, v/v) and stored dessicated at −5°C.

The NBD concentration of a peptide-lipid solution was determined by absorbance at 466 nm. Peptide concentration was determined by the method of Lowry et al. [41], after first evaporating the alcohol and replacing it with water. Absorbance in the Lowry by NBD-C_{12}-PE was accounted for by testing known amounts of unreacted NBD-C_{12}-PE-SS. (Although this compound contains an extra succinimide group which is not present in peptide-lipid, we have determined that *N*-hydroxysuccinimide in such concentrations does not yield Lowry absorbance.) NBD-C_{12}-PE-SS yields Lowry absorbance equal to 40 ± 10% of that of an equimolar concentration of peptide-lipid.

Reaction of dinitrofluorobenzene with peptide-lipid. To 50–100 μg of peptide-lipid in several milliliters methanol/ethanol (5 : 4, v/v) was added 10 μl dinitrofluorobenzene and 2 μl triethylamine. The reaction was gently shaken for 30 min at room temperature in the dark. The reaction mixture was analyzed and the desired product purified by TLC in chloroform/methanol/water (65 : 25 : 4, v/v) (see Table II). The main band was extracted from the plate with 65 : 25 : 4 solvent, dried on a rotary evaporator, and redissolved in several milliliters methanol/

ethanol (5 : 4, v/v). Concentration was determined from NBD absorbance.

Peptide sequencing. Peptides were sequenced in the laboratories of Beckman Instruments, Inc., Palo Alto, CA, with a Beckman Automatic Sequencer.

Cell labelling. RDM-4 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Before labelling, cells were layered over Ficoll-sodium diatrizoate (Lympholyte-M), and then centrifuged to remove dead cells. The live cells were collected from the interface, washed twice with Hanks' balanced-salt solution (HBSS), and resuspended in HBSS with 1% fetal calf serum. After a 2 h incubation at 37°C, the cells were washed twice with HBSS and resuspended in HBSS at $4 \cdot 10^6$ cells/ml.

To 125 μl of ice-cold cell suspension were added 125 μl ice-cold 0.01 M sodium phosphate/0.14 M NaCl, pH 7.4 and then peptide-lipid at either of two concentrations. For 'low' or 'high' concentration labelling, we added 2.5 μl of $2.4 \cdot 10^{-5}$ M or 12.5 μl of $4.8 \cdot 10^{-8}$ M peptide-lipid in 0.01 M Tris/0.14 M NaCl, pH 8.0 containing 1.0% or 0.2% deoxycholic acid, respectively. Thus, the final peptide-lipid per cell ratios were $7.2 \cdot 10^7$ or $7.2 \cdot 10^8$ molecules/cell. The final detergent concentration was 0.01%. Following this treatment, approx. 90% of the cells continued to exclude Trypan blue.

After a 30 min ice-cold incubation with peptide-lipid, cells were washed in HBSS with 1% fetal calf serum and transferred to the fluorescence microscope (see below) having a stage temperature of 5°C. The fluorescence of single cells was measured as a function of time from the beginning of 488 nm illumination; fluorescence values were measured by extrapolating the decaying fluorescence (due to photobleaching) to its time zero value. With our illumination intensities, the half-time of NBD photobleaching was several seconds, so that accurate time zero values could be obtained.

Supported phospholipid monolayers. Glass coverslips (No. 2, 18 mm square) were cleaned and alkylated by a slight modification of the methods of Von Tscharner and McConnell [16]. Coverslips were boiled for 10 min in Linbro 7X detergent/water (1 : 3, v/v), bath sonicated (while still warm) for 30 min, rinsed extensively with deionized water, dried at 150°C for 1 h, and placed in a plasma cleaner (Harrick PDC-3XG) under argon gas on the 'high' setting for 5 min. The coverslips were then dipped into hexadecane/carbon tetrachloride/ chloroform/ octadecyltrichlorosilane (80 : 12 : 8 : 0.1, v/v) for several seconds until they rapidly shed the solution upon being raised from it. The coverslips were then washed well in chloroform and baked for 1 h at 150°C.

Lipid monolayers were spread at the interface of air and 0.01 M sodium phosphate, 0.14 M NaCl, pH 7.4 (phosphate-buffered saline, PBS). A total of $5.25 \cdot 10^{-8}$ moles of lipids, consisting of DMPC and up to 11 mol% of NBD-C_{12}-PE, peptide-lipid, or DNP-peptide-lipid was dissolved in 50–200 μl chloroform/ methanol/ water (65 : 25 : 4, v/v) and then spread at the air/PBS interface. The monolayers were compressed and coated onto hydrophobic coverslips according to the methods of Von Tscharner and McConnell [15,16]. Surface pressure was measured before and after compression with a Wilhemy plate balance (Biolar Corp., North Grafton, MA). Values quoted as 'area/molecule at 39.2 dyn/cm' were measured after compressing to this pressure, waiting for approx. 30 min, and readjusting the average molecular area until the pressure was 39.2 dyn/cm. All monolayers were compressed to this pressure before coating onto the coverslips, regardless of the initial spreading pressure.

Fluorescence microscopy and photobleaching. Lipid monolayers on glass coverslips were mounted under PBS on microscope slides with a spacer, and then washed with several milliliters PBS. Monolayers were epi-illuminated on a Zeiss Photomicroscope III with the 488 nm line of an argon ion laser (Spectra Physics 265). NBD fluorescence was detected either by eye or by photomultiplier (RCA 34103A) at −30°C interfaced to an Archives microcomputer.

The technique of fluorescence recovery after photobleaching [42] using a Ronchi ruling pattern [43] was used to measure the lateral diffusion coefficient of NBD-PE and derivatives in DMPC monolayers. Photobleaching parameters were: objective, 40 × 0.65 N.A. Zeiss; ruling periodicity in the objective-focused beam, 8.5 μm or 22.5 μm;

observation and bleaching laser powers, 0.25 mW and 100 mW; bleach duration and recovery $1/e$-times, 200–500 ms and 5–100 s; depth of bleaching, 30–70%. No change in $1/e$-times was observed with a double in bleach duration and no significant recovery occurred when the Ronchi ruling was removed. The microscope stage temperature was 24–28°C.

In some experiments, 0.05 mg/ml free renin inhibitor peptide in phosphate-buffered saline was added to NBD-C_{12}-PE monolayers (see Results). In these experiments, lateral diffusion was measured after a 10 min incubation and without washing away free peptide.

Results

We have synthesized and purified reaction products of NBD-C_{12}-PE with the disuccinimidyl compounds DSS and EGS. The R_f values of reactants and products are shown in Table II. The products have absorption spectra characteristic of NBD-C_{12}-PE; based on the peak absorption at 464 nm in ethanol, the yield is 38 ± 5% using DSS and approximately the same using EGS.

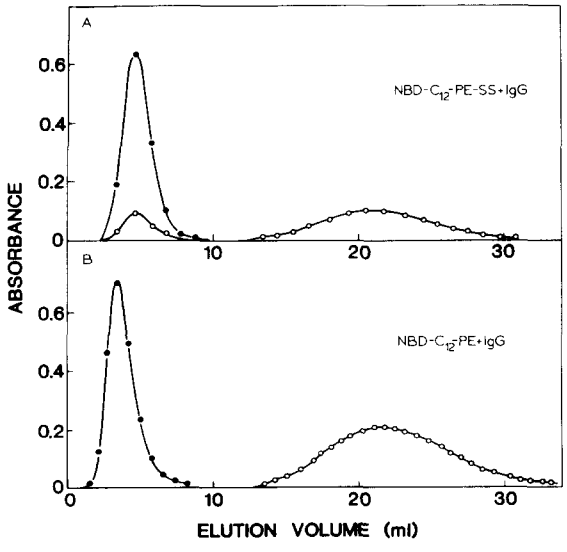


Fig. 1. (A) Purification of the NBD-C_{12}-PE-SS derivative of IgG on Sephadex G-75 in 0.05 M $NaHCO_3$/0.5% deoxycholic acid, pH 8.3. Solid circles (●) indicate absorbance at 278 nm; open circles (○) indicate absorbance at 476 nm; no circle indicates a measured absorbance ≤ 0.02. (B) NBD-C_{12}-PE + IgG.

To confirm reactivity of the succinimidyl NBD-C_{12}-PE compounds for amines, we reacted them with IgG. Purification of the NBD-C_{12}-PE-SS derivative of IgG on Sephadex G-75 in deoxycholate buffer is shown in Fig. 1A. As shown, the early eluting fractions contain material with tryptophan absorbance and NBD absorbance, and elute well ahead of another NBD peak. For NBD-C_{12}-PE which has not been derivatized with DSS, no NBD absorbance elutes with the IgG (Fig. 1B). Based on absorbances at 278 nm and 476 nm, the molar ratio of NBD to IgG in the conjugated fraction was 0.8 for a starting molar ratio of 4.3. Results are similar for NBD-C_{12}-PE-EGS.

Synthesis of reaction products of NBD-C_{12}-PE-SS with the three synthetic peptides (Table I) is demonstrated by the appearance of new fluorescent spots on analytical TLC (see Table II). The renin inhibitor-lipid product has been purified. NBD to peptide molar ratio is determined by comparing optical absorbance and the Lowry peptide assay, and equals 1.3 ± 0.4 averaged over ten independent preparations. The percent of starting NBD which is conjugated is 17 ± 2; the percent of peptide conjugated is 8 ± 1.

Peptide-lipid can be further derivatized with 2,4-dinitrofluorobenzene, as demonstrated by TLC (see Table II). Purified DNP-peptide-lipid has increased absorbance in the 250–450 nm range, which is characteristic of dinitrophenol derivatives. The yield of DNP-peptide-lipid, based on NBD absorbance, is 31 ± 11.

Sequencing analysis was performed on equimolar samples of unreacted renin inhibitor peptide, peptide-lipid, and DNP-peptide lipid; controls indicated that neither DNP-proline, ε-DNP-lysine, NBD-C_{12}-PE-SS or *N*-hydroxysuccinimide produced a peak in a typical sequencing cycle. Peptide-lipid sequenced identically to unreacted peptide, except that the lysine residue (C-terminal, see Table I) did not elute. The average ratio of amino acid content (excepting lysine) of equimolar amounts of peptide and peptide-lipid (based on NBD-absorbance) was 1.1 ± 0.1. Specifically, the observed ratio of proline in peptide to proline in peptide-lipid was 0.62. For the two histidines, the two ratios were 0.42 (the lowest of all ratios) and 1.1. These results indicate that most

TABLE I

PEPTIDE SEQUENCES AND MOLECULAR WEIGHTS

Peptide	Molecular weight	Sequence	References
Renin inhibitor	1319	Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-Lys	35, 36
α-Mating factor	1684	Trp-His-Trp-Leu-Glu-Leu-Lys-Pro-Gly-Gln-Pro-Met-Try	37, 38
Lysine vasopressin	1056	Cys-Try-Phe-Glu-Asn-Cys-Pro-Lys-Gly-NH_2	

TABLE II(a)

MEASURED R_f VALUES

Solvents are: A, acetone/water (95:5, v/v); B, chloroform/methanol/water (95:25:3, v/v); C, chloroform/methanol/water (65:25:4, v/v).

Compound	Solvent		
	A	B	C
NBD-C_{12}-PE	0.04 ± 0.02	0.22 ± 0.02	0.41 ± 0.03
NBD-C_{12}	0.90 ± 0.02	0.65 ± 0.02	0.73 ± 0.03
DSS	0.95 ± 0.05	0.85 ± 0.03	0.88 ± 0.03
N-Hydroxysuccinimide	0.94 ± 0.06	0.53 ± 0.03	0.41 ± 0.03
All peptides	–	–	0.02 ± 0.02
Dinitrofluorobenzene	–	–	0.96 ± 0.04
Dinitrophenol	–	–	0.33 ± 0.03

TABLE II(b)

OBSERVED TLC R_f VALUES

Solvents are defined in Table II(a). Occasionally additional ninhydrin bands are visible which originated from triethylamine impurities.

	Purification of	R_f	Visualization	Compound
I.	NBD-C_{12}-PE-SS			
	Solvent A	0.88 ± 0.06	hydroxylamine/$FeCl_3$	DSS
		0.28 ± 0.12	orange	NBD-C_{12}-PE-SS
		0.02 ± 0.02	orange (faint)	NBD-C_{12}-PE
II.	NBD-C_{12}-PE-SS			
	Solvent B	0.58 ± 0.04	orange (faint)	unidentified
		0.34 ± 0.12	orange	NBD-C_{12}-PE-SS
III.	Peptide-lipid			
	Solvent C	0.47 ± 0.13	orange	unidentified
		0.17 ± 0.04	orange	peptide-lipid
		0.02 ± 0.02	ninhydrin	peptide
IV.	DNP-peptide-lipid			
	Solvent C	0.94 ± 0.06	yellow	dinitrofluorobenzene
		0.55 ± 0.10	orange	DNP-peptide-lipid
		0.36 ± 0.04	yellow	dinitrophenol

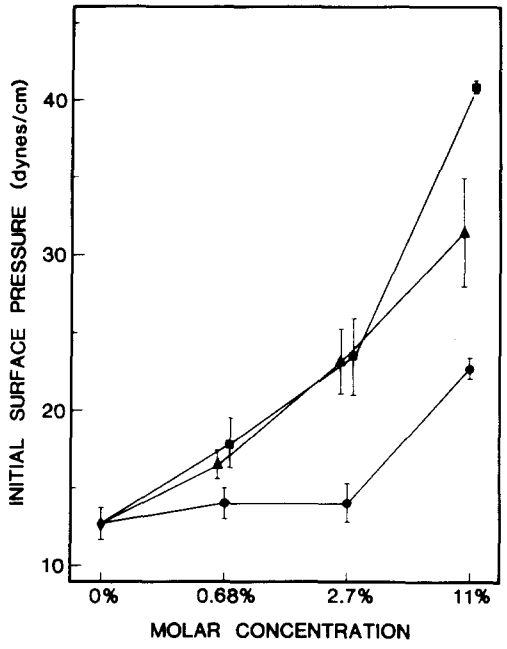


Fig. 2. Initial spreading pressure as a function of molecular composition. Shown is the surface pressure 30 min after spreading at 68.1 $Å^2$/molecule. The lipid composition is DMPC with: no fluorescent lipid (◆), NBD-C_{12}-PE (●), peptide-lipid (▲), or DNP-peptide-lipid (■).

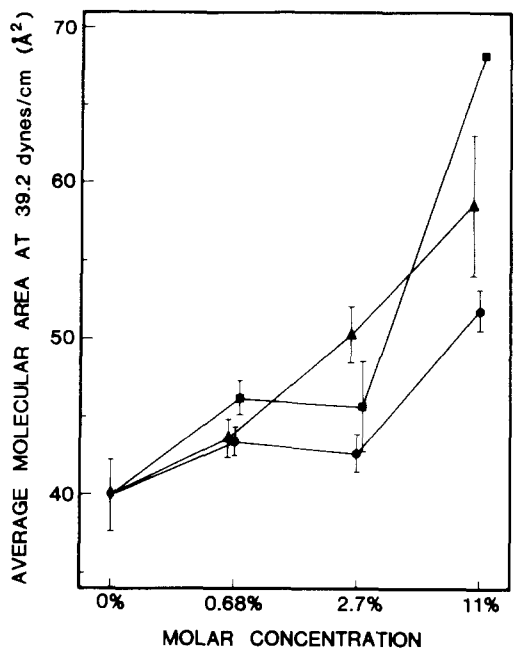


Fig. 3. Average area per molecule at 39.2 dyn/cm as a function of molecular composition. Shown is the average area/molecule when the monolayer has a surface pressure of 39.2 dyn/cm. DNP-peptide-lipid at 11% spreads with an initial pressure > 39.2 dyn/cm; its value is plotted as the initial molecular area. Symbols are defined in Fig. 2.

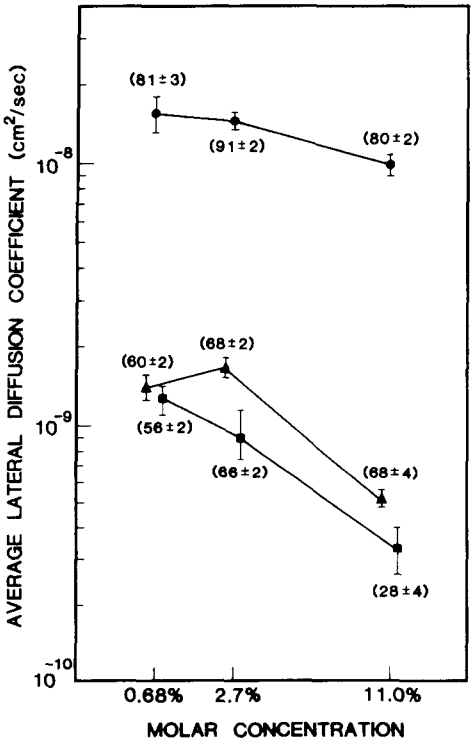


Fig. 4. Average lateral diffusion coefficient of NBD-C_{12}-PE and peptide-lipids as a function of molar concentration. Symbols are defined in Fig. 2. Above each point is the mobile ($D \geq 10^{-10}$ cm^2/s) fraction.

of the lipids are conjugated to the lysine amine and not the proline N-terminal or the histidines [44]. DNP-peptide-lipid could not be sequenced from the N-terminal, indicating that the N-terminal proline was modified by DNP.

Cells treated with peptide-lipid in detergent solution were more fluorescent than untreated cells. The ratios of the average fluorescence of treated cells (minus background) to the average fluorescence of untreated cells (minus background) were: low concentration labelling, 2.6 ± 0.5; high concentration labelling, 5.1 ± 2.3.

Peptide-lipid and DNP-peptide lipid could be incorporated into DMPC monolayers at an air/PBS interface, using chloroform/methanol/water (65 : 25 : 4, v/v) as the spreading solvent, with the same apparent efficiency as underivatized NBD-

C_{12}-PE. A plot of the surface pressure vs. the logarithm of the average area per molecule of a DMPC monolayer containing peptide-lipid has nearly the same shape as one containing only lipid (see, for example, Ref. 15). However, the initial spreading pressure and the average area/molecule at which the surface pressure is 39.2 dyn/cm is higher for peptide-lipid containing monolayers than for pure lipid monolayers (Figs. 2 and 3). Peptide-lipids were incorporated at molar concentrations of up to 11%.

The peptide-lipid containing monolayers could be transferred to alkylated glass coverslips, producing uniform (but slightly grainy) fluorescence over the whole coverslip. In addition, the peptide-lipids diffused laterally in the monolayer. The average lateral diffusion coefficient of peptide-lipids was 10^{-9} cm^2/s (at 2.7% molar concentration), a factor of approx. 10 smaller than that of NBD-C_{12}-PE in a DMPC monolayer. In addition, as shown in Fig. 4, the diffusion coefficient of peptide-lipids decreases slightly with increasing peptide-lipid concentration. On the average, monolayers containing peptide-lipids have a higher immobile fraction than those made with NBD-C_{12}-PE. Free renin inhibitor peptide at a concentration of 50 μg/ml reduced the diffusion coefficient of NBD-C_{12}-PE in DMPC from $(1.5 \pm 0.1) \cdot 10^{-8}$ cm^2/s to $(7.2 \pm 0.5) \cdot 10^{-9}$ cm^2/s.

Discussion

The purpose of this study was to synthesize and purify covalent conjugates of peptides and phospholipids to be used in studies of the interaction of immunological cells with model membranes. For this purpose, we chose synthetic peptides of 10 or 11 amino acid residues and fluorescent phosphatidylethanolamine. We found that purified products could be easily incorporated into cell membranes and supported phospholipid monolayers.

The presence of peptide-lipid in a DMPC monolayer at the air/PBS interface is immediately detected by increased surface pressure upon spreading. The increased spreading pressure indicates a higher degree of repulsive interactions between molecules (see, for example, Ref. 45). The increased pressure may arise from peptide-lipid interactions with other peptide-lipids or DMPC molecules, or it may simply arise from a large average molecular area of peptide-lipid.

The incorporated peptide-lipids undergo diffusion in a manner similar to fluorescent lipids in supported phospholipid monolayers. However, the average diffusion coefficient is $1.5 \cdot 10^{-9}$ cm^2/s, which is substantially smaller than that of many other molecules in reconstituted model membranes. We can consider the possibility of interactions of the lipid or the peptide portion of the peptide-lipids with their environments.

First, the NBD-C_{12}-PE from which the peptide-lipids are derived has one acyl chain fluorescently labelled with NBD and the other acyl chain of heterogeneous chain length and degree of saturation (derived from egg phosphatidylcholine). This NBD-C_{12}-PE in its unconjugated (to peptide) form diffuses with coefficient $1.5 \cdot 10^{-8}$ cm^2/s (Fig. 4) in DMPC monolayers, which is a factor of two slower than that of head-labelled NBD-C_{12}-PE [14]. Thus, a small part of the reduced mobility of peptide-lipid can be ascribed to the lipid portion of the molecule.

Second, we observe that the addition of free renin inhibitor peptide to a DMPC monolayer reduces the NBD-C_{12}-PE diffusion coefficient by a factor of two (see Results), which implies that renin inhibitor interacts in some manner with the phospholipid monolayer. We can compare these results with two studies of small hydrophobic peptides which directly incorporate into membranes. Wu et al. [46] found that NBD-labelled Gramicidin S (M_r 1141) in DMPC multibilayers at 4% and 20% molar concentration (and 25°C) diffused with coefficient 10^{-8} to $5 \cdot 10^{-9}$ cm^2/s. Smith et al. [47] measured the diffusion coefficient of fluorescein-labelled M-13 phage coat protein (M_r 5260) in 25°C DMPC multibilayers to be $3 \cdot 10^{-8}$ cm^2/s. These diffusion coefficients are considerably higher than that which we have measured for peptide-lipid. If the reduced mobility is to be ascribed to incorporation (or association) of the peptide portion of peptide-lipid with the monolayer, it would have to be of a nature different from the association of Gramicidin S and M-13 coat protein with DMPC membranes.

Peptide-lipid oligomerization within the monolayer might account for the low diffusion coeffi-

cient. At least one synthetic molecule is known to form stable hexamers in model membranes [48]. A concentration dependence of the diffusion coefficient might or might not be observed, depending on the equilibrium constant for oligomerization. We observe a small concentration dependence for both peptide-lipid and unconjugated lipid (Fig. 4). The diffusion of peptide-lipid is found to be 'more' dependent on concentration than that of unconjugated lipid; the ratios of diffusion coefficients at 0.68% to those at 11% molar concentrations are: NBD-C_{12}-PE, 1.5 ± 0.3; peptide-lipid, 2.7 ± 0.4; DNP-peptide-lipid, 3.8 ± 0.9.

The original purpose of this work was to synthesize a molecule which, when incorporated into a model membrane, could act as antigen to immunological cells. We believed that the synthesis, purification, and reconstitution of peptide conjugated with lipid at a single amino acid residue would allow a high degree of precision in the control of the physical and chemical state of an antigen-containing model membrane. The fact that the peptide-lipid diffuses when incorporated into a model membrane indicates that it behaves in a manner similar to other membrane components. However, the particular peptide chosen for this study may associate with the lipids of the monolayer, and/or may oligomerize. Model building (data not shown) is compatible with either possibility. The present work does indicate that the conformation of a membrane associated peptide may be a highly sensitive function of its molecular environment.

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