Critical Role of Lipid Composition in Membrane Permeabilization by Rabbit Neutrophil Defensins

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We have examined the interactions of the six known rabbit neutrophil defensin antimicrobial peptides with large unilamellar vesicles (LUV) made from various lipid mixtures based on the lipid composition of Escherichia coli membranes. We find that the permeabilization of LUV made from E. coli whole lipid extracts differs dramatically from that of single-component LUV made from palmitoyl-oleoyl-phosphatidylglycerol (POPG). Specifically, defensins NP-1, NP-2, NP-3A, NP-3B, and a natural mixture of the six defensins cause fast nonpreferential leakage of high molecular weight dextrans as well as the low molecular weight fluorophore/ quencher pair 8-aminonaphthalene-1,3,6 trisulfonic acid (ANTS)/p-xylene-bis-pyridinium bromide (DPX) from E. coli whole lipid LUV through large, transient membrane lesions. In contrast, release of ANTS/DPX from POPG LUV induced by the defensins is slow and graded with preference for DPX (Hristova, K., Selsted, M. E., and White, S. H. (1996) Biochemistry 35, 11888–11894). Interestingly, defensins NP-4 and NP-5 alone do not induce leakage from E. coli whole lipid LUV, whereas only NP-4 is ineffective with POPG LUV. Examination of the sequences of the six defensins suggests that the inactivity of NP-4 and NP-5 may be due to their lower net positive charge and/or the substitution of a Thr for the Arg or Lys that follows the fourth Cys residue. We found the presence of three major lipid components of E. coli whole lipid to be essential for creation of the large lesions observed in LUV: phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. Cardiolipin appears to play a key role because no leakage can be induced when only phosphatidylglycerol and phosphatidylethanolamine are present. These results indicate the importance of membrane lipid composition in the permeabilization of cell membranes by rabbit defensins.

Neutrophil defensins have been isolated from several species, including humans (1, 2) and rabbits (3). They are small (Mr ~ 4000) cationic antimicrobial peptides that act primarily by permeabilizing the cell membranes of a wide variety of microbes (4–6). Neutrophil defensins have 29–34 amino acids, are rich in arginine (4–10 per molecule), and have three disulfide bonds that stabilize a rigid β-sheet structure (see Ref. 7 for a review). The amino acid sequences of the six rabbit and four human neutrophil defensins are presented in Fig. 1 (residues are numbered according to the NP-1 sequence). Although neutrophil defensins kill bacteria, fungi, and enveloped viruses effectively, they are tolerated at high concentrations inside membrane-bound neutrophil granules. The suggestion has thus been made that antimicrobial peptide selectivity is determined by differences in the lipid compositions of the target and host membranes (8).

Available evidence supports the hypothesis that the principal mode of action of neutrophil defensins involves physical perturbation and permeabilization of the membranes of the target organisms (6, 9, 10). Therefore, studies of defensin-induced permeabilization of model membranes provide valuable insight to their permeabilization of biological membranes (11, 12). An important observation is that neutrophil defensins isolated from different species vary in their mode of action. Human neutrophil defensin HNP-2 forms large multimeric pores in large unilamellar vesicles (LUV)1 formed from POPG, causing the vesicle contents to be released in an all-or-none manner (11). Rabbit neutrophil defensins cause graded leakage (12). Despite high sequence identity and three-dimensional structural similarity of monomers, HNP-2 exists as dimers in aqueous solution, whereas rabbit defensins are always monomeric. This suggests that the lack of aqueous dimerization by rabbit neutrophil defensins leads to a fundamental difference in defensin assembly on the membrane surface and thus in the mechanism of permeabilization (11, 12). Another important observation is that the lipid composition of the LUV is important; small additions of neutral lipids (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)) to POPG LUV eliminate leakage even though defensins can bind to the mixed bilayers (12). This indicates that lipid composition can strongly influence membrane permeabilization by neutrophil defensins. A third observation of importance is that small differences in monomer sequence can have large effects on membrane permeabilization (12).

In the present study, our goal was to examine in greater detail the lipid requirements for rabbit neutrophil defensin action using defensin concentrations that are well within the normal physiological range (13). We began with an examination of the interactions of the six known rabbit neutrophil defensins, individually and as the “natural” mixture, with LUV

1 The abbreviations used are: LUV, extruded unilamellar vesicles of 100-nm diameter; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoyl phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; diPG, di-phosphatidylglycerol (cardiolipin); E. coli whole lipid, chloroform-methanol extract of lyophilized E. coli cells; ANTS, 8-aminonaphthalene-1,3,6 trisulfonic acid, Mr = 427.33; DPX, p-xylene-bis-pyridinium bromide, Mr = 422.16; HNP, human neutrophil peptide (human defensin) NP, rabbit neutrophil peptide (rabbit defensin); FD-4, fluorescein isothiocyanate-labeled dextran, Mr, 4,400; FD-90, fluorescein isothiocyanate-labeled dextran, Mr, 18,900; FD-70, fluorescein isothiocyanate-labeled dextran, Mr, 50,700; NBD-PE, 7-nitro-benzoxadizole phosphatidylethanolamine; HPLC, high pressure liquid chromatography.
made from whole lipid extracts of *Escherichia coli* membranes. The lipid content varies among strains but typically is about 20–50% charged lipid (PG and diphosphatidylglycerol (cardiolipin, diPG) and about 50–80% neutral lipid (PE) (14). We found that defensins NP-1, NP-2, NP-3A, NP-3B, and the natural mixture NP-1–5 induced membrane permeabilization, probably through large scale transient lesions. NP-4 and NP-5 alone had no effect on membrane permeability but did have synergistic effects when used in combination with other defensins. Under the same experimental conditions, bilayer permeabilization of POPG vesicles was fundamentally different as indicated by leakage that had a preference to DPX (12) and was slower. We then examined the permeabilization by NP-2 and NP-5 of LUV composed of various combinations of the three major *E. coli* lipid components, POPE, POPG, and cardiolipin, and confirmed the strong influence of lipid composition on the activity of rabbit neutrophil defensins. Cardiolipin was found to be especially important, although all three lipids must be present to mimic *E. coli* whole lipid. Further, we identified amino acid residues in the sequence of the six rabbit defensins that may be crucial for the observed activity. Our results indicate that permeabilization of microbial membranes is likely to be extremely sensitive to lipid composition, as well as to the composition of the defensin mixture.

**EXPERIMENTAL PROCEDURES**

**Materials—** *E. coli* whole lipid extracts as PE from *E. coli* −50%, lot 41H8362, and fluorescein-labeled dextran FD-4, FD-20, and FD-70 were purchased from Sigma. The lipid composition of the extract was analyzed by Avanti Polar Lipids using normal phase HPLC with evaporative light scattering detection. Consistent with other studies (14–17), the three major components of the extract are PE (78%), diPG (14%), and PG (4.7%). The preparation also contained minor components identified as phosphatidylcholine (1.2%), phosphatidylethanolamine (0.8%), and lyso-PE (0.9%).

NBD- and rhodamine-labeled lipids were obtained from Avanti Polar Lipids (Birmingham, AL). ANTS and DPX were obtained from Molecular Probes (Eugene, OR). Water was glass distilled. The buffer composition was 10 mM HEPES, 50 mM KCl, 1 mM EDTA, 3 mM NaN₃, pH 7.0.

**Vesicle Preparation—** About 70 mg of lipid was combined in chloroform that was subsequently removed under a stream of argon. About 1 ml of buffer containing ANTS/dextran or dextran was added to the dry lipids, and the suspension was frozen and thawed 10 times to assure maximum entrapment prior to extrusion (11). The final lipid concentration was typically 100 mM. A stock solution of LUV of approximately 0.1 μm in diameter was formed by extrusion under N₂ pressure through Nucleapore polycarbonate membranes (11, 18). The concentrations of solutes used were 9 mM ANTS, 25 mM DPX, 8 mg/ml FD-4, 20 mg/ml FD-20, and 0.8 mg/ml FD-70. Assumption was that all the vesicles equaled the initial concentration, we estimated entrapment of about 200–300 dextran vesicles/entrapment of ANTS/dextran should be about an order of magnitude higher. The total KC1 concentration in ANTS/DPX-containing vesicles was adjusted so that the entrapped solutions had the same osmolarity as the external 50 mM KC1 buffer.

Unencapsulated ANTS and DPX were separated from encapsulated material using Sephadex G-100 packed in a 2.5-ml Pasteur pipette. Untrapped FD-4, FD-20, and FD-70 were removed on a 45 × 1.3-cm Sephadex G-100 column run at approximately 1 ml/min. Typically, about 50 μl of the vesicle stock solution (above) was applied to the column and eluted with several milliliters of buffer. The fraction of the effluent selected (about 90%) contained a lipid concentration of about 14 mM. After separation, nonfluorescent dextran of approximately the same molecular weight was added externally to the FD-4, FD-20, and FD-70 liposomal suspensions of *E. coli* lipids to eliminate any osmotic stress on the vesicles. No detectable leakage of any solutes in the absence of defensin was observed during periods of time corresponding to the duration of the experiments.

Fluorescence Spectroscopy—Fluorescence spectroscopy was performed using either a SPEX Fluorolog fluorimeter that was upgraded and interfaced to a computer by OLIS, Inc. (Jefferson, GA) or a SLM AMINCO 8100 spectrophotometer (Rochester, NY). Excitation and emission wavelengths were as follows: ANTS (SPEX): excitation, 380 nm (slit, 20 nm), and emission, 515 nm (slit, 50 nm); NBD-PE (SPEX): excitation, 465 nm (slit, 8 nm), and emission, 550 nm (slit, 8 nm); FD-20 (SPEX): excitation, 495 (slit, 8 nm), and emission, 525 (slit, 8 nm); and FD-4 and FD-70 (SLM AMINCO): excitation, 495 (slit, 1 nm), and emission, 525 (slit, 4 nm). Right angle geometry and no reference signal were used. Emission spectra covering the range of the fluorescence spectra of the fluorophores were collected from samples with and without vesicles to assure that light scattering made no contribution to the fluorescence signals of the fluorophores. We found in all cases that the scattering peak made no significant contribution to the fluorescence signals at the emission wavelengths used.

Leakage of Solutes—Vesicles containing ANTS/DPX, FD-4, FD-20, and FD-70 at about 400 μM were placed into 1 × 0.2-mm quartz cuvette (volume, 0.5 ml), and the fluorescence increase due to leakage and subsequent dilution of quenched dye was measured after the addition of defensin. Data are presented in terms of fractional fluorescence $F_\text{in}$ = ($F - F_\text{max}$)/($F_\text{in}$ − $F_\text{max}$), where $F$ is the measured fluorescence, $F_\text{max}$ is the initial quenched fluorescence, and $F_\text{in}$ is the fluorescence corresponding to 100% leakage as established by the addition of 0.4% Triton X-100. In the absence of a leakage, a fluorescence $F_\text{initial} = Q_\text{max} F_\text{in}$ is observed, where $Q$ is the quenching factor (all-or-none). If *E. coli* whole lipid/ANTS/DPX vesicles, 0.2 for *E. coli* whole lipid/FD-4, 0.5 for *E. coli* whole lipid/DF-20, and 0.6 for *E. coli* whole lipid/DF-70 vesicles. Note that $Q = 0$ corresponds to complete quenching, whereas $Q = 1$ corresponds to no quenching.

Mechanism of Leakage—There are two general mechanisms of leakage. It can be a graded process in which all vesicles release portions of their contents, or it can be an all-or-none process in which some vesicles lose all of their contents while others lose none. One can distinguish between these two possibilities using the so-called fluorescence re-quenching method, which is based on the idea that the ANTS molecules inside and outside the vesicles show different susceptibility to quenching with externally added DPX (11, 12, 19, 20). In brief, one measures the dependence of ANTS quenching inside the vesicles on the fraction of ANTS that has leaked out of the vesicles. If $Q_\text{a}$ is independent of the fraction of ANTS that has leaked out, then the leakage is all-or-none. If $Q_\text{a}$ increases with $f_\text{out}$ then the leakage is graded. For graded release, $Q_\text{a}$ depends on $f_\text{out}$ as follows (19),

$$Q_\text{a} = (1 + K_a [\text{DPX}_\text{a}][1 - f_\text{out}])^{-1} = \frac{1 + K_a [\text{DPX}_\text{a}][1 - f_\text{out}]}{1 + [\text{DPX}_\text{a}]}$$

(1) where $[\text{DPX}_\text{a}]$ is the initial DPX concentration inside the vesicles, and $a$ is the selectivity defined as the ratio of the rates of release of ANTS and DPX. The constant $K_a$ is the dynamic quenching constant, and $K_a$ is the association constant for the ANTS/DPX nonfluorescent complex. They were determined previously to be 50 M⁻¹ and 490 M⁻¹, respectively (19). The selectivity $a$ is determined by fitting this equation to the experimental data using standard nonlinear least squares methods (19).

Aggregation—The extent of aggregation of 400 μM vesicles of *E. coli* whole lipid by rabbit defensins was assayed by measuring the total concentration of defensin and lipid and the concentrations remaining in solution after centrifugation of the lipid/defensin mixture in a table top centrifuge (1000 × g) for 10 min. The pellet was resuspended in buffer and also assayed. The lipid concentration was determined by fluorescence measurements using the concentration inside the vesicles equal the initial concentration, we estimated entrapment of about 200–300 dextran vesicles/entrapment of ANTS/DPX should be about an order of magnitude higher. The total KC1 concentration in ANTS/DPX-containing vesicles was adjusted so that the entrapped solutions had the same osmolarity as the external 50 mM KC1 buffer.

Unencapsulated ANTS and DPX were separated from encapsulated
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Leakage of Low Molecular Weight Markers from E. coli Whole Lipid Vesicles—The leakage of low molecular weight markers from E. coli whole lipid LUV was examined using the dye/quencher pair ANTS/DPX as described under “Experimental Procedures.” Shown in Fig. 2 are the fractional fluorescence changes that occur following the addition of 30 μg/ml of NP-2, NP-5, or the natural mixture NP-1–5 corresponding to the naturally occurring ratio of the six rabbit defensins (26% NP-1, 11, 12, and 19). Briefly, when fluorescence reaches a plateau in detail elsewhere (see “Experimental Procedures” and Refs. 11, 12, and 19). Briefly, when fluorescence reaches a plateau after the addition of a particular amount of defensin, DPX is added externally to quench the released ANTS. The remaining unquenchable fluorescence is then due to the ANTS retained in the vesicles (if any). Such measurements allow one to determine the residual internal quenching of ANTS as a function of the fraction of ANTS leakage released in a series of release experiments. As discussed under “Experimental Procedures,” if Q_{in} is independent of f_{out}, then release is all-or-none, whereas if Q_{in} increases with f_{out}, then release is graded. One can further establish in the latter case whether the release is preferential for ANTS or DPX by means of the selectivity parameter α, which is the ratio of DPX to ANTS that leaked out of the vesicles. Fig. 5A shows the results of quenching experiments for release induced by NP-2 from E. coli whole lipid vesicles. The internal quenching, Q_{in}, of ANTS by DPX increases with

RESULTS

Leakage of Low Molecular Weight Markers from E. coli Whole Lipid Vesicles—The leakage of low molecular weight markers from E. coli whole lipid LUV was examined using the dye/quencher pair ANTS/DPX as described under “Experimental Procedures.” Shown in Fig. 2 are the fractional fluorescence changes that occur following the addition of 30 μg/ml of NP-2, NP-5, or the natural mixture NP-1–5 corresponding to the naturally occurring ratio of the six rabbit defensins (26% NP-1, 17% NP-2, 6% NP-3A, 6% NP-3B, 15% NP-4, and 30% NP-5). The zero level in Fig. 2 corresponds to zero leakage, and the maximum level corresponds to complete release of ANTS/DPX. The results show that both NP-2 and NP-1–5 induce leakage of contents of E. coli whole lipid vesicles and that NP-5 does not. NP-1, NP-3A, and NP-3B behaved similarly to NP-2, whereas NP-4, like NP-5, caused no leakage (data not shown). For both NP-2 and NP-1–5, a fast initial fluorescence signal increase followed by a slower one is observed. Although particular fluorescence levels are the same as for POPG LUV (12), the kinetics of the increase are much faster (5–10 min versus several hours for POPG). However, we observed subtle differences in the kinetics of release. For instance, as seen in Fig. 2, NP-2 causes a faster fluorescence increase toward a plateau level than NP-1–5.

Fig. 3 shows the fractional fluorescence increase f_{p} caused by leakage from E. coli whole lipid vesicles induced by NP-2 and NP-1–5 as a function of defensin concentration. The measurements were made in the plateau region (see Fig. 2) approximately 30 min after defensin addition. f_{p} is a sigmoidal function of defensin concentration, indicating defensin cooperativity. The results for NP-2 and NP-1–5 presented in Fig. 3 were obtained using a single LUV preparation; the exact shape varied somewhat between preparations. Also shown in Fig. 3 are measurements of increases in f_{p} caused by leakage from various mixed lipid vesicles induced by NP-2 (see below).

In our previous study of the interactions of rabbit neutrophil defensins with POPG LUV (12), no synergy was observed between the six peptides despite the occurrence of antimicrobial synergy in vivo (24). In the present case, however, there was significant synergy between NP-5 and a mixture of NP-1, NP-2, NP-3A, NP-3B, and NP-4 (NP-1–4). Fig. 4 shows the kinetics of ANTS/DPX leakage induced by 40 μg/ml NP-1–5 and by 30 μg/ml NP-1–4 mixture containing the same amounts of NP-1, NP-2, NP-3A, NP-3B, and NP-4. The data reveal that although NP-5 did not cause leakage by itself (Fig. 2), it enhanced the permeabilizing properties of NP-1–4. Similarly, NP-4 enhanced the permeabilizing properties of a mixture of NP-1, NP-2, NP-3A, NP-3B, and NP-5 (data not shown). The possibility of synergy among other defensin combinations remains to be examined.

The mechanism of ANTS/DPX leakage was established by the fluorescence requenching method that has been described in detail elsewhere (see “Experimental Procedures” and Refs. 11, 12, and 19). Briefly, when fluorescence reaches a plateau after the addition of a particular amount of defensin, DPX is added externally to quench the released ANTS. The remaining unquenchable fluorescence is then due to the ANTS retained in the vesicles (if any). Such measurements allow one to determine the residual internal quenching Q_{in}, as a function of the fraction f_{out} of ANTS released in a series of release experiments. As discussed under “Experimental Procedures,” if Q_{in} is independent of f_{out}, then release is all-or-none, whereas if Q_{in} increases with f_{out}, then release is graded. One can further establish in the latter case whether the release is preferential for ANTS or DPX by means of the selectivity parameter α, which is the ratio of DPX to ANTS that leaked out of the vesicles. Fig. 5A shows the results of quenching experiments for release induced by NP-2 from E. coli whole lipid vesicles. The internal quenching, Q_{in}, of ANTS by DPX increases with
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A. Ladokhin, personal communication.

2 A. Ladokhin, personal communication.

detergent, was assigned a value of 1. For two of the NP-1–5 concentrations, we performed several experiments with different FD-70 vesicle preparations to estimate experimental variability (error bars, Fig. 6B). Fig. 6 shows that NP-1–5 and NP-2 induce the leakage of all of the dextrans studied. Furthermore, the leakage increases with defensin concentration, up to about 20 μg/ml, and then levels off at about 40–50%, independent of dextran molecular weight.

The kinetics of the leakage of ANTS/DPX and FD-20 from E. coli whole lipid vesicles induced by NP-2 are compared in Fig. 7. The data show that NP-2-treated vesicles are leaky to both small molecules (ANTS/DPX) and large dextran molecules. Note, however, that although ANTS fluorescence quickly reaches its plateau level of almost 1, the fluorescence of FD-20 levels off at about 0.5 with a slower time course. The kinetics and extent of leakage of the release of FD-4 and FD-70 are very similar to those of FD-20 under similar conditions (data not shown).

The data presented in Figs. 6 and 7 reveal that dextran leakage is independent of molecular weight and that it is never complete (Fig. 6). We considered the possibility that the dextran-loaded E. coli whole lipid vesicles are incapable of releasing all of their contents regardless of the leakage mechanism.

FIG. 4. Demonstration of synergy between NP-5 and a mixture of NP-1, NP-2, NP-3A, NP-3B, and NP-4 (NP-1–4). Kinetics of ANTS/DPX leakage induced by 40 μg/ml NP-1–5 (●) and 30 μg/ml NP-1–4 (○). The amounts of NP-1, NP-2, NP-3A, NP-3B, and NP-4 are the same in both cases. The data reveal that although NP-5 does not cause leakage by itself (see Fig. 3), it enhances the lytic properties of NP-1–4.

f_{out} and is above the initial level of Q_{in} of 0.14. The dotted line in Fig. 5A shows the result expected had the leakage been all-or-none. The solid line in Fig. 5A is the best fit of the data to Equation 1, which yields a value for \( \alpha \) of 1.17 ± 0.24. Similar results were obtained for NP-1–5 as shown in Fig. 5B (\( \alpha = 1.13 \pm 0.26 \)) and for NP-1, NP-3A, and NP-3B (data not shown).

A chi-squared test showed that these values are not statistically different from 1.0 (reduced \( \chi^2 = 0.87 \)). Thus, the experiments did not distinguish preferential leakage for ANTS or DPX, suggesting that the release of solutes occurs in a nondiscriminative manner.

The nonpreferential leakage of ANTS and DPX suggested that the leakage path aperture might be much larger than the characteristic dimensions of ANTS and DPX. We therefore examined the effect of the rabbit defensins on the leakage of high molecular weight dextrans from E. coli whole lipid vesicles.

Leakage of High Molecular Weight Markers from E. coli Whole Lipid Vesicles—The leakage of high molecular weight compounds was examined using fluorescein-labeled dextrans (FD-4, \( M_r = 4,400 \); FD-20, \( M_r = 18,700 \); and FD-70, \( M_r = 50,700 \)). The possibility of these dextrans binding to E. coli whole lipid liposomes was examined as described elsewhere (25). No evidence of binding was found.2 We took advantage of the fact that the fluorescein-dextrans self-quench as indicated by the observation that the concentration dependence of dextran fluorescence in vesicle-free solutions deviates from linearity at high concentrations (data not shown). Self-quenching concentrations of dextrans were therefore entrapped, and defensin-induced leakage was detected by increases in fluorescence due to dextran dilution during leakage. The fractional fluorescence increases of \( f_p \) following additions of NP-2 and NP-1–5 to solutions of 400 μM E. coli whole lipid LUV with entrapped dextrans are plotted in Fig. 6 as a function of defensin concentration. All measurements were made in the plateau region of the release kinetics approximately 30–40 min after defensin addition. The zero level corresponds to vesicle fluorescence in the absence of defensins. The maximum level of fluorescence, determined by lysis of the vesicles with Triton X-100 detergent, was assigned a value of 1. For two of the NP-1–5 concentrations, we performed several experiments with different FD-70 vesicle preparations to estimate experimental variability (error bars, Fig. 6B). Fig. 6 shows that NP-1–5 and NP-2 induce the leakage of all of the dextrans studied. Furthermore, the leakage increases with defensin concentration, up to about 20 μg/ml, and then levels off at about 40–50%, independent of dextran molecular weight.

The kinetics of the leakage of ANTS/DPX and FD-20 from E. coli whole lipid vesicles induced by NP-2 are compared in Fig. 7. The data show that NP-2-treated vesicles are leaky to both small molecules (ANTS/DPX) and large dextran molecules. Note, however, that although ANTS fluorescence quickly reaches its plateau level of almost 1, the fluorescence of FD-20 levels off at about 0.5 with a slower time course. The kinetics and extent of leakage of the release of FD-4 and FD-70 are very similar to those of FD-20 under similar conditions (data not shown).

The data presented in Figs. 6 and 7 reveal that dextran leakage is independent of molecular weight and that it is never complete (Fig. 6). We considered the possibility that the dextran-loaded E. coli whole lipid vesicles are incapable of releasing all of their contents regardless of the leakage mechanism.
To examine this possibility, we took advantage of the fact that denatured defensins (reduced disulfide bonds) induce leakage by a different mechanism than native defensins and are far more effective in causing leakage of ANTS/DPX (11, 26). The time dependence of the fractional fluorescence increase accompanying FD-4 leakage induced by 15 μg/ml reduced NP-2 is shown in Fig. 8. Although the increase in $f_F$ does not go beyond about 50%, the fluorescence of FD-4 does not go beyond about 50% The error bars show the results of multiple measurements at the concentrations shown. These error estimates serve as a general estimate of the experimental variability observed for all of the results shown in the figure. Lipid concentration was 400 μM in all cases.

To examine this possibility, we took advantage of the fact that denatured defensins (reduced disulfide bonds) induce leakage by a different mechanism than native defensins and are far more effective in causing leakage of ANTS/DPX (11, 26). The time dependence of the fractional fluorescence increase $f_F$ accompanying FD-4 leakage induced by 15 μg/ml reduced NP-2 is shown in Fig. 8. Although the increase in $f_F$ is much slower, it nevertheless goes well above the value of 0.5 observed for native rabbit neutrophil defensins. We also examined the leakage induced by a very low concentration (0.001%) of Triton X-100 (27, 28) by a mechanism that is not well understood. The effects of reduced NP-2 and Triton X-100 are very similar. These results indicate that the partial release of dextrans is a characteristic of the native rabbit defensin release mechanism rather than a mechanism-independent characteristic of dextrans entrapped in E. coli whole lipid vesicles.

The dextran release experiments support the hypothesis that native rabbit defensins act by inducing large aperture lesions in the vesicle membranes. However, these lesions are likely to be transient because the leakage of the dextrans is never complete. Under “Discussion,” we present evidence that leakage is diffusion-limited, so that the time for diffusion of dextrans out of the vesicles is slow compared with the lifetime of the lesions.

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**Fig. 6.** Fractional fluorescence increase caused by leakage of self-quenching fluorescein-labeled dextrans (FD-4, FD-20, and FD-70) that is induced by NP-2 (A) and NP-1-5 (B) as a function of defensin concentration. Measurements were made in the plateau region approximately 30 min after defensin addition. The zero level corresponds to vesicle fluorescence in the absence of defensins. The maximum level of fluorescence determined by lysis of the vesicles with Triton X-100 detergent is assigned a value of 1. The fractional fluorescence increases with defensin concentration and levels off at about 0.4–0.5. The fractional fluorescence increase does not depend on the molecular weight of the fluorescein-labeled dextran, indicating that leakage does not occur through a pore that can be sized. The error bars shown in B for FD-70 indicate the results of multiple measurements at the concentrations shown. These error estimates serve as a general estimate of the experimental variability observed for all of the results shown in the figure. Lipid concentration was 400 μM in all cases.

**Fig. 7.** Kinetics of ANTS/DPX and FD-20 leakage from E. coli lipid vesicles induced by NP-2. NP-2 at 40 μg/ml was added to 400 μM liposomal suspensions with encapsulated ANTS/DPX (●) and FD-20 (□) at time 0. The data show that NP-2 treated E. coli lipid vesicles are leaky to both small molecules (ANTS/DPX) and large dextran molecules. Although ANTS fluorescence quickly reaches its plateau level of almost 1, the fluorescence of FD-20 does not go beyond about 50%. The arrow indicates the addition of Triton X-100 to give a 0.4% solution. This concentration solubilizes the vesicles for the purpose of establishing the $f_F = 1.0$ reference level.

**Fig. 8.** Leakage of FD-4 from E. coli lipid liposomes induced by 0.001% Triton (●) and 15 μg/ml reduced NP-2 (▲). The arrow indicates the addition of Triton X-100 to give a 0.4% solution. This concentration solubilizes the vesicles for the purpose of establishing the $f_F = 1.0$ reference level. The increases in fractional fluorescence are well above the maximal level of 0.5 observed for the native rabbit neutrophil defensins.
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...fraction of ANTS released indicates graded nonpreferential leakage of ANTS/DPX. In Fig. 9A, the solid line represents the estimated 20% variability among different liposome preparations. The data also show that lipid charge alone is not responsible for the effect because the vesicle charge density is the same for 0% diPG points (PE:PG = 1:2) and the 50% diPG points (PE:diPG = 1:1). Requenching experiments with NP-2 demonstrated that increasing the diPG concentration from 33 to 67 mol% increased the release selectivity for ANTS/DPX from $\alpha = -1$ to 1.63 ± 0.13. This suggests that the exact molar...
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The results show that increasing the diPG concentration leads to a greater fluorescence increase, which proves the importance of cardiolipin in the induction of leakage. The vesicles with 0% diPG (PE:PG = 1:2) and those with 66% diPG (PE: diPG = 1:2) have the same surface charge density, meaning that the observed effect is not due to charge alone. The error bars of ± 20% are based upon observed variations among various vesicle preparations.

Together, these experiments show that 1) the activity of rabbit neutrophil defensins is very sensitive to lipid composition, 2) vesicles formed from PE, PG, and diPG generally mimic the naturally occurring mixture, and 3) no single lipid component can assure the exact mechanism of permeabilization observed for the E. coli whole lipid membranes. Overall, the synthetic systems are much less sensitive to defensins than the E. coli whole lipid system. This could be a result of either the uniform palmitoyl:oleoyl acyl chain composition of the synthetic systems or the absence of yet another type of lipid head-group present in the natural mixture.

Aggregation and Fusion of E. coli Whole Lipid Vesicles Induced by Rabbit Neutrophil Defensins—A final issue of concern to us was whether or not the leakage processes observed might be a side product of fusion events. Release of solutes from fusion intermediates is known to occur (29–31), and we therefore examined the aggregation and fusion of E. coli whole lipid liposomes induced by NP-2 and NP-1–5.

The turbidity of defensin solutions increased immediately upon the addition of the E. coli whole lipid LUV suspensions, suggesting aggregation of the LUV. The composition of the resulting aggregates was assayed using fluorescence measurements and HPLC (21), as described under “Experimental Procedures.” We found that for 400 μM E. coli lipid and 40 μg/ml NP-2, the pellet contained 27% of the lipid and about 75% of the peptide. For 60 μg/ml NP-2, these numbers increased to 41% lipid and about 90% peptide. For 60 μg/ml NP-1–5, as much as 92% of the lipid and no less than 90% of the defensins sedimented in the pellet, indicative of large peptide-lipid aggregates. Thus, aggregation is quite substantial, occurs immediately upon mixing of defensin and liposomes, and therefore precedes leakage. This is not surprising in the light of our previous findings that rabbit defensins bind strongly to negatively charged bilayers (12). For the analysis of leakage, aggregation is not problematic if the LUV remain intact. The crucial questions, then, are whether or not this aggregation leads to fusion intermediates and whether or not the fusion intermediates disrupt vesicle integrity.

We assayed fusion by means of the NBD-PE/Rhodamine-PE probe dilution assay as described under “Experimental Procedures” and in Struck et al. (22). Fig. 11 shows that fusion occurs because the NBD fluorescence increases following the addition of NP-2 and NP-1–5 at 60 μg/ml. However, leakage of vesicle contents occurs on a much faster time scale, as illustrated by the inclusion in Fig. 10 of the time course of the ANTS fluorescence increase. The ANTS release is virtually complete in 2 or 3 min, whereas the NBD fractional fluorescence is only about 15% complete after 2 or 3 min and is still increasing after about 20 min. We conclude that leakage of ANTS/DPX leakage is unlikely to be due to fusion events.

The extent to which the contents of the vesicles were mixed during bilayer fusion, indicative of complete fusion, was assayed as described under “Experimental Procedures.” NP-2 at 60 μg/ml was added to a 400 μM solution of E. coli LUV containing either the fluorescent dye ANTS or quencher DPX. If the vesicles had fused fully, then we should have observed a decrease in ANTS fluorescence as DPX quenched the ANTS upon contents mixing (23). However, we could not detect any mixing of contents (results not shown). Similar effects were observed when POGP vesicles were added to human defensin HNP-2 solutions (11).
ecules in proximity to the membrane leak out slowly, as in the case of POPG LUV, it can be expected that leakage occurs because of small scale perturbations. The fast nonpreferential release of contents from *E. coli* whole lipid vesicles suggests the generation of lesions of much larger size, and this is confirmed by the observed leakage of high molecular weight dextrans.

The leakage of the dextran was unusual, however, because leakage was independent of dextran molecular weight and was never complete. If leakage were to occur through multimeric pores, they would have to have at least the apparent Stokes radius of FD-70, which is about 60 Å (32). We estimate that at least 50 defensins would be needed to form such a pore. Although we cannot completely rule out such a possibility, the existence of such large multimeric pores seems unlikely.

The failure to observe a size-selective pore induced by peptides is not without precedent. For example, Ostolaza *et al.* (33) found no substantial differences in the leakage of dextran of molecular weights 4400, 9400, and 17200 induced by α-hemolysin in LUV and therefore concluded that the leakage occurs through bilayer disruption. Our results are consistent with a major disruption of vesicle integrity, but the disruption cannot be a permanent one such as fragmentation because the release of the dextran is never complete. The most likely explanation is that leakage occurs through large scale but transient membrane lesions such as those induced in LUV by osmotic stress (34).

At defensin concentrations that induce almost full release of ANTS/DPX, dextran release never exceeded approximately 50%. If our hypothesis of short-lived large scale lesions is correct, then the amount of solute released will be limited not by the pore size but rather by the diffusion rate of the molecules. A very simple model for free diffusion of solutes out of the liposome volume supports this hypothesis and provides an estimate of the lifetime of the lesions. One can easily derive an expression for diffusion in three dimensions from the one-dimensional case (35),

$$c(x, y, z, t) = \frac{c_0}{8} \left[ \text{erf} \left( \frac{R + x}{2 \sqrt{Dt}} \right) + \text{erf} \left( \frac{R - x}{2 \sqrt{Dt}} \right) \right] \left[ \text{erf} \left( \frac{R + y}{2 \sqrt{Dt}} \right) + \text{erf} \left( \frac{R - y}{2 \sqrt{Dt}} \right) \right]$$

$$+ \text{erf} \left( \frac{R - z}{2 \sqrt{Dt}} \right) \left[ \text{erf} \left( \frac{R + z}{2 \sqrt{Dt}} \right) + \text{erf} \left( \frac{R - z}{2 \sqrt{Dt}} \right) \right]$$

(Eq. 2)

In this equation, \(t\) is time, \(D\) is the diffusion coefficient of the encapsulated molecules (which is inversely proportional to their size), \(c_0\) is the initial concentration of the molecules, \(R\) is the effective radius of the liposome (0.1 μm), and \(\text{erf}\) is the error function (35). We assume that the distribution of the molecules within the vesicles is uniform prior to leakage induction, that diffusion out of the vesicles is transient and occurs once per vesicle, and that diffusion is unrestricted while the membranous lesions are open. Given estimates of the diffusion coefficients of the entrapped solutes, one can calculate the time interval \(t\) that the lesions must be open to be consistent with the observed release of ANTS/DPX and the dextrans.

The solid line in Fig. 12 is the expected fraction of molecules released from the vesicles at \(t = 7 \times 10^{-6}\) seconds as a function of diffusion coefficient \(D\) predicted by the model. The data points (■) are the experimentally observed increases in the fractional fluorescence of the fluorescein-labeled dextran at the plateau region in Fig. 6B that are induced by NP-2. The error bars represent the estimated precisions of the measurements based upon several different liposome preparations. A data point corresponding to ANTS/DPX release is also shown.

Between the prediction of the simple model and the experimental results is quite good and supports the hypothesis that release is transient and diffusion-limited. The important feature of the model, which is completely consistent with observation, is that diffusion-limited release of dextran will not increase above a certain threshold (Fig. 6) of defensin concentration.

The *E. coli* lipid mixture generally contains about 20–50% charged lipids (PG and diPG) and 50–80% neutral lipids (mainly PE). Our measurements of the permeabilization of LUV made from different combinations of these components show that only membranes composed of all three major components generally mimic the *E. coli* whole lipid extract with respect to the mode of action of both NP-2 and NP-5, yet the model system is less sensitive to defensin activity. Of the major lipid components, diPG has the greatest influence on the permeabilization activity of rabbit defensins because 1) its absence eliminates leakage from PE/PG liposomes altogether and 2) leakage from PE/diPG vesicles increases with diPG concentration. However, no model whole lipid mixture comprised of PE, PG, and diPG can reproduce exactly the behavior of the *E. coli* whole lipid LUV. This suggests that defensin activity is strongly affected by general physical properties of the lipid.

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**Membrane Lipid Composition and Action of Rabbit Defensins**

**Fig. 12. Predictions of a simple model of diffusion-limited leakage.** The solid line is the expected fraction of molecules released from the vesicles at \(t = 7 \times 10^{-6}\) seconds as a function of diffusion coefficient \(D\) predicted by the model. The data points (■) are the experimentally observed increases in the fractional fluorescence of the fluorescein-labeled dextran at the plateau region in Fig. 6B that are induced by NP-2. The error bars represent the estimated precisions of the measurements based upon several different liposome preparations. A data point corresponding to ANTS/DPX release is also shown.
matrix that can be affected by alkyl chain composition and minor amounts of other lipid headgroup types. Together, these findings are consistent with the hypothesis that defensin selectivity is determined by the differences in the lipid composition of cell membranes of bacteria, viruses, and host cells (8). Significantly, cardiolipin is a common constituent of bacterial and plant membranes but is confined largely to mitochondria in animal tissues (36).

Divalent cations are known to induce inverted hexagonal phase in cardiolipin model systems (37, 38). Furthermore, the positively charged bee venom toxin melittin destabilizes cardiolipin bilayers and promotes the formation of hexagonal phase (39). The tendency of cardiolipin to destabilize bilayers and to form hexagonal phases upon interaction with polycations may be the basis for its crucial role in our experiments. Despite its obvious importance, however, diPG appears to have primarily a modulating effect on the permeabilization of E. coli whole lipid vesicles by rabbit defensins, because the absence of PE changes the nature of the leakage of ANTS/DPX. PE, PG, and diPG are completely miscible with each other, whereas phosphatidylcholine is not fully miscible with PG and diPG on the microscopic level (40). This may explain why cardiolipin fails to change the permeabilization properties of the phosphatidylcholine bilayer.

In contrast to human neutrophil defensin HNP-2, which causes all-or-none release of POPG vesicles contents by means of multimeric pores (11), rabbit neutrophil defensins do not form multimeric pores in either POPG (12) or E. coli whole lipid vesicles. Only NP-4 fails to permeabilize POPG LUV, whereas both NP-4 and NP-5 fail to permeabilize E. coli whole lipid LUV. This is interesting because NP-5 was found to be more potent in causing leakage from POPG vesicles than NP-2 (12). NP-2 bears a higher charge than NP-5 (Fig. 1). Indeed, NP-5 bears the lowest positive charge (+4) of all the defensins tested. One can speculate that this is the factor that determines its highest activity in POPG LUV permeabilization. A peptide with a very high positive charge such as NP-2 may be immobilized at the boundary of the pure POPG surface and thus unable to penetrate to the hydrocarbon core of the bilayer (41). In the E. coli whole lipid mixture, the negatively charged lipid species comprise only about 45% of the total lipid (14) so that NP-2 can penetrate the surface charge layer and create the leakage pathway. However, this simple explanation fails to account for the fact that dilution of POPG by POPE or POPC completely inhibits rabbit neutrophil defensin activity. Clearly, subtle dependences on lipid composition exist that remain to be determined.

In a previous study, we observed no synergy in the permeabilization of POPG bilayers by rabbit neutrophil defensins (11). In the permeabilization of E. coli whole lipid bilayers, we found that NP-5 enhanced the activity of a mixture of NP-1, NP-2, NP-3A, NP-3B, and NP-4 and that NP-4 enhanced the activity of a mixture of NP-1, NP-2, NP-3A, NP-3B, and NP-5. These findings are consistent with previous observations of antimicrobial synergy in vivo (24).

Structural Features of Neutrophil Defensins That May Be Crucial for the Mode of LUV Permeabilization—Rabbit neutrophil defensins, although exhibiting high sequence and structural similarities, can be divided into two groups with respect to their activity in E. coli lipid LUV permeabilization: 1) NP-1, NP-2, NP-3A, and NP-3B, which are active, and 2) NP-4 and NP-5, which are not. This prompted us to search the sequences of the six defensins for residues that differ between the two groups but within a group are almost identical.

We were able to identify a residue in position 21 with such a property, shown in Fig. 1 (numbered according to NP-1). NP-1, NP-2, NP-3A, and NP-3B have a charged residue (Arg$^{21}$ or Lys$^{21}$), whereas NP-4 and NP-5 both have Thr$^{21}$. It is thus possible that an Arg or Lys in position 21 is essential for the permeabilizing activity that occurs. One can speculate that membrane permeabilization results from the binding of multiple charged residues in a specific three-dimensional structure with respect to the E. coli lipid or diPG in particular. NP-4 and NP-5 must modify this structure in a way that increases permeabilization because NP-4 or NP-5 enhance the activities of mixtures of the remaining five defensins while being ineffective alone.

Fig. 13 shows the three-dimensional backbone structures of rabbit neutrophil defensin monomers NP-2 and NP-5 determined by NMR methods (42–44) and of the human HNP-3 dimer, which is very similar to HNP-2, determined by crystallography (45). The residues in equivalent position 21 (Arg for NP-2, Thr for NP-5, and Ile for HNP-3) are indicated by means of space-filling representations. Notice that Ile$^{21}$ in HNP-3 is located at the monomer-monomer interface. We speculate that a nonpolar residue at position 21 may be important for dimerization and may affect the mode of membrane permeabilization by defensins.
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