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Interaction of Eosinophil Granule Major Basic Protein with Synthetic Lipid Bilayers: A Mechanism for Toxicity

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Summary. Eosinophil granule major basic protein (MBP) is a potent toxin for mammalian cells and helminths, but the mechanism of its toxicity is not known. Here we tested whether MBP toxicity is exerted through its effect on the lipid bilayer of its targets. Liposomes prepared from synthetic phospholipids were used as targets for MBP and their properties examined by fluorescence and circular dichroism (CD) spectroscopy. MBP caused a change in the temperature transition profiles of acidic liposomes (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl serine or an equimolar mixture of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid) and induced their aggregation as shown by fluorescence resonance energy transfer experiments. The CD spectra and fluorescence characteristics of MBP itself were altered by its interaction with acidic lipids. Blue shifts in the emission maxima of the Trp, and of the dimethylaminonaphthyl moiety in acrylodan-labeled MBP, and a reduction in the effectiveness of quenching of Trp fluorescence by acrylamide were observed in the presence of acidic lipids. None of these effects were noted with zwitterionic lipids. This MBP : lipid bilayer interaction resulted in fusion and lysis of liposomes as indicated by the fluorescent indicator calcein. The results demonstrate that MBP associates with acidic lipids and that it disrupts, aggregates, fuses, and lyses liposomes prepared from such lipids. Such interaction might account for its wide range of toxicity.

Key Words eosinophil major basic protein · liposomes · quenching · fluorescence polarization · fluorescence resonance energy transfer · lysis

Introduction

Eosinophils are important participants in the pathophysiology of a number of different diseases. They contain within their granules a number of cationic proteins, including the major basic protein (MBP)[1], that are involved in mediating their effector function (Gleich & Adolphson, 1986). Although MBP is highly toxic in vitro the mechanism of its toxicity is yet to be defined. MBP is deposited in damaged tissues in diseases associated with eosinophilia such as bronchial tissues of patients with asthma (Frigas & Gleich, 1986) and the myocardium of patients with endomyocardial fibrosis (Tai et al., 1987). MBP deposition occurs alongside infiltrating and degranulating eosinophils at sites of tissue damage. Deposition of MBP is also detected in vivo on the surface of damaged invading parasites (Kephart et al., 1984; Ackerman et al., 1990).

The in vitro effects of MBP are either toxic or stimulatory. The toxicity of MBP is directed towards a number of mammalian cells, parasites, and bacteria. MBP causes loss of ciliary motility (Hastie et al., 1987) and damage to the cilia of tracheal rings,

[1] *Abbreviations used:* Acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; CD, circular dichroism; DMPA, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; HEPES, N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid; K_{sv}, Stern-Volmer constant; k_q, bimolecular quenching coefficient; λ_{em}, emission wavelength; λ_{ex}, excitation wavelength; MBP, major basic protein; MOPC, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; NBD-PE, N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; nMBP, native major basic protein; PBS, phosphate-buffered saline; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl serine; raMBP, reduced and alkylated major basic protein; RHO-PE, rhodamine-phosphatidylethanolamine; Tes, N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid; and Tris, tris[hydroxymethyl]-aminomethane.

and at higher concentrations death and sloughing of the epithelial cells occur (Motojima et al., 1989). It is also helminthotoxic in vitro (Butterworth et al., 1979*a,b*) and possesses bactericidal activity (Lehrer et al., 1989). MBP stimulates smooth muscle contraction (Brofman et al., 1989), induces degranulation of neutrophils (Moy, Gleich & Thomas, 1990) and platelets (Rohrbach et al., 1990), causes histamine release from basophils and mast cells (O'Donnell et al., 1983), and induces bronchoconstriction and bronchial hyper-reactivity in primates (Gundel, Letts & Gleich, 1991).

MBP is localized to the crystalloid core of the specific eosinophil granule (Lewis et al., 1978; Peters, Rodriguez & Gleich, 1986). The molecular mass of MBP is 14 kDa (Barker, Gleich & Pease, 1988; Wasmoen et al., 1988). It is a strongly cationic protein with a P_I of > 10.9 as judged from the amino acid sequence; at pH 7.0 the net charge is +16.3 (Barker et al., 1988). The amino acid sequence shows alternating hydrophobic and hydrophilic segments (Barker et al., 1988; Wasmoen et al., 1988) and the protein contains 17 Arg, 4 His, 7 Trp, 6 Phe, 9 Cys, and 6 Tyr residues.

The *pleiotropic nature* of MBP's actions, its manifest nonselectivity with respect to the cells it affects, its ability to damage or "activate," and its frequent localization to cell surfaces, point strongly to the plasma membrane as the target of MBP action. Here we tested the hypothesis that the toxicity of MBP is due to its ability to perturb the membranes of its targets.

Materials and Methods

Materials

The materials used were as follows: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid (DMPA), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl serine (POPS), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MOPC), N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) amine labeled, egg lissamine rhodamine-phosphatidylethanolamine (RHO-PE) amine labeled (Avanti Polar Lipids, Alabaster, AL), 6-acryloyl-2-dimethylaminonaphthalene [(acrylodan), (Molecular Probes, Eugene, OR)], 1,6-diphenyl-1,3,5-hexatriene [(DPH), (Aldrich, Milwaukee, WI)], chloroform distilled in glass (Burdick & Jackson Laboratories, Muskegon, MI), N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid [(HEPES), (Research Organics, Cleveland, OH)], KCl, NaCl, KH_2PO_4, Na_2HPO_4, and sodium azide (Curtin Matheson Scientific, Houston, TX), dithiothreitol [(DTT), (Boehringher Mannheim, Indianapolis, IN)], iodoacetamide (Aldrich), tris[hydroxymethyl]-amino-methane [(Trizma Base), (Sigma Chemical, St. Louis, MO)], N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid [(Tes), (Sigma)], calcein (Molecular Probes), $CoCl_2$ (grade 1, Puratronic, Johnson Mathey Chemicals, Royston, England), citric acid anhydrous (Aldrich), morpholinopropane sulfonic acid [(Mops), (Serva, Heidelberg, NY), Bio-Gel A-5m agarose (Bio-Rad Laboratories, Richmond, CA), EDTA (E.M. Industries, Cherry Hill, NJ), and deoxycholate (Sigma).

Preparation of MBP

MBP was purified from human peripheral blood eosinophils as described (Ackerman et al., 1983). Because native MBP (nMBP) precipitates at physiologic pH, the reduced and alkylated form of MBP denoted here as raMBP was used in all the experiments unless otherwise indicated. It is important to note that MBP treated as such retains its effectiveness as a toxin towards most of the targets affected by the native molecule (Hamann et al., 1990). In order to verify that nMBP interacts with lipid bilayers the effects of raMBP on liposomes were compared to nMBP in some experiments. MBP was reduced with DTT and alkylated with iodoacetamide as described (Ackerman et al., 1983). The reduced and alkylated MBP was dialyzed against phosphate-buffered saline [(PBS), (2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, and 0.02% sodium azide, pH 7.2)]. The level of MBP was measured by specific double antibody polyclonal (Ackerman et al., 1983) and two-site monoclonal immunoradiometric assay (J. M. Wagner, K. Bartemes, J. Checkel, S. Dunnette, K. Offord, K. Vernof, and G.J. Gleich, *unpublished work*).

Preparation of Liposomes

Lipids dissolved in chloroform (500 μl) at room temperature, or in the case of DMPC : DMPA dissolved in chloroform, water and methanol at approximately 3 : 1 : 1 (vol : vol : vol) ratios at 50°C, were dried to a film in a vessel under nitrogen flow at the concentrations indicated in each experiment. The appropriate buffered solution [20 mM HEPES and 125 mM KCl, pH 7.4, unless otherwise indicated] was added, and the mixture was tip sonicated at 4°C (while bubbling nitrogen in the sample) using a titanium probe in a Braunsonic 1510 sonicator (B. Braun Instruments, San Francisco, CA) for 10 min. The liposomes were annealed (10°C above the transition temperature of the lipids used) for 35 min and centrifuged at room temperature using a Dynac II centrifuge (with an angle rotor, tip radius 6.37 inches) at 2750 rpm to remove any contaminating titanium particles (Lawaczeck, Kainosho & Chan, 1976).

Fluorescence Measurements of DPH

DPH (2-μM final concentration), added to the lipids prior to drying, was used as an indicator of the fluidity of the lipids. Changes in its fluorescence polarization at different temperatures in the presence of raMBP, nMBP, and appropriate controls were monitored. The effect of different concentrations of raMBP on the fluorescence polarization of DPH in liposomes was studied. A steady-state fluorometer (SLM 4800, SLM Instruments, Champaign, IL) was employed for measurements of fluorescence polarization. Fluorescence polarization data were calculated and analyzed using the "Clinfo-2" program on the Mayo Clinic

Main Research Computer Facility. Results are presented as means ± SD, and the Student's t test was used for statistical analysis.

Study of Liposome Aggregation

The size of liposomes with and without MBP was measured by quasielastic laser light scattering (Malvern 4700 System, Malvern Instruments, Malvern, UK). MBP's ability to aggregate vesicles was also investigated by use of fluorescence resonance energy transfer (FRET) experiments. A mixture of two separate preparations of DMPC : DMPA liposomes or of POPS liposomes, one preparation containing NBD-PE at 2% molar concentration, the other containing RHO-PE at 2% molar concentration, was used as described by Düzgünes et al. (1987) and Meers et al. (1988). The measurements of FRET from NBD to RHO were conducted using a Perkin Elmer Fluorescence Spectrophotometer MPF-66.

Labeling of MBP with Acrylodan

MBP was alkylated with acrylodan at 4°C by a modification of earlier procedures (Prendergast et al., 1983; Clark & Burtnick, 1988). In brief, EDTA at 2 mM final concentration was added to nMBP (at 1 mg/ml) dissolved in acetate buffer (25 mM acetate and 150 mM NaCl, pH 3.0). DTT dissolved in trizma-buffered solution (1 M trizma base, pH 8) was added to MBP at a concentration of 1.6 mg DTT for each 1 mg MBP (volume of added DTT is one-tenth the volume of the MBP sample) and mixed by inverting for 20 min. The mixture was dialyzed against HEPES-buffered solution; after 10 min 100 μl acrylodan (in acetonitrile) was added to the dialysis bag to give a final 10-fold M excess to the MBP concentration. After three changes of the dialysis buffer (four liters each) over 48 hr an additional 100 μl of acrylodan was added and the dialysis continued for 6 additional hr. The dialyzed sample was centrifuged, and the supernatant containing acrylodan-labeled MBP collected and used. Absorbance measurements were made on a Cary 2200 spectrophotometer (Varian Instrument Group, Palo Alto, CA).

Fluorescence Characteristics of MBP

To determine if the interaction of MBP with liposomes affects intrinsic Trp fluorescence (MBP has seven Trp), Trp fluorescence spectra and fluorescence polarization measurements of both nMBP and raMBP were made in the presence and absence of liposomes. A steady-state fluorometer (SLM 4800, SLM Instruments, Champaign, IL) was employed for measurements of fluorescence polarization.

Acrylamide Quenching Experiments

Acrylamide was added to MBP in the presence and absence of liposomes at different concentrations, and fluorescence emission profiles of MBP Trp were collected. The average fluorescence lifetime $\langle\tau_0\rangle$ of MBP Trp fluorescence was measured by use of time-correlated, single-photon counting. A Nd : YAG laser (Coherent, Palo Alto, CA) was used as the excitation source (excitation wavelength (λ_{ex}): 295 nm); the excitation pulse was less than 5 psec in duration, and excitation was conducted under magic angle conditions. Fluorescence emission was selected with a double monochromator (American Holographics DB-10, Littleton, MA) and detected with a microchannel plate (Hamamatsu, Hamamatsu-City, Japan) as described by Hedstrom, Sedarous and Prendergast (1988). $\langle\tau_0\rangle$ is needed for calculation of the bimolecular quenching constant (k_q) from the Stern-Volmer relation by

$$F_0/F = 1 + K_{sv} \cdot F = 1 + k_q \cdot \tau_0 \cdot F \quad (1)$$

where F_0 is the area under the emission curve in the absence of

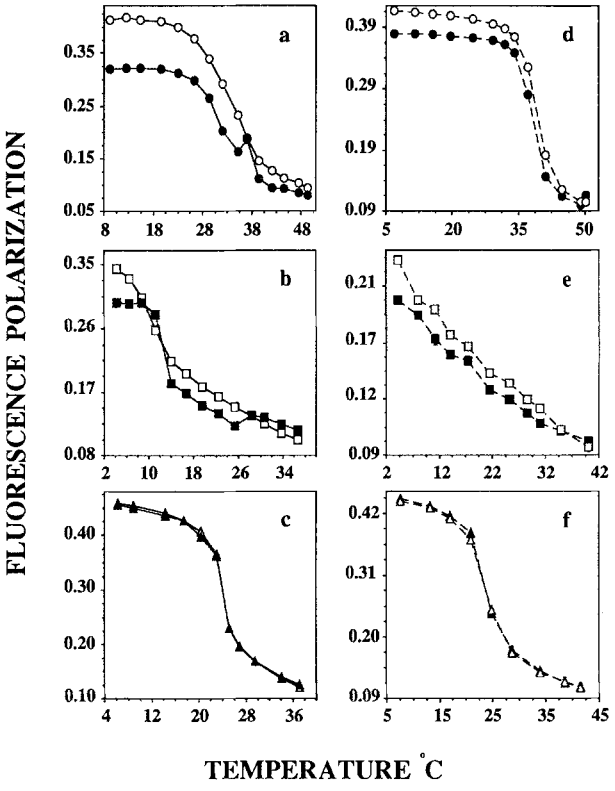

Fig. 1. The temperature transition profiles of liposomes (2 mM) made from different lipids in the presence of MBP (closed symbols) or an equivalent volume of buffer (open symbols). (*a*, *b*, and *c*) The effect of raMBP compared to PBS controls. (*d*, *e*, and *f*) The effect of nMBP compared to acetate buffer controls. Standard deviations are within the symbols. (*a*) DMPC : DMPA liposomes (circles) with raMBP (10 μM) or PBS. This is representative of four experiments. The difference upon addition of raMBP is significant ($P \leq 0.0001$) below 35°C. (*b*) POPS liposomes (squares) with raMBP (10 μM) or PBS. This is representative of nine experiments. The difference upon addition of raMBP is significant ($P \leq 0.0001$) below 10°C. (*c*) DMPC liposomes (triangles, 2 mM) with raMBP (5 μM) or PBS. This is representative of five experiments. (*d*) DMPC : DMPA liposomes (circles, dashed line) with nMBP (5 μM) or acetate buffer. This is representative of two experiments. The difference upon addition of nMBP is significant ($P \leq 0.0001$) below 48°C. (*e*) POPS liposomes (squares, dashed line) with nMBP (10 μM) or acetate buffer. The difference upon addition of nMBP is significant ($P \leq 0.0002$) below 31°C. (*f*) DMPC liposomes (triangle, dashed line) with nMBP (5 μM) or acetate buffer. λ_{ex}, 360 nm; emission cutoff filter, KV 450. Each reading is an instrument average of 10 and was repeated at least 7 times.

acrylamide, F is the area under the emission curve in the presence of each of the different concentrations of acrylamide, and K_{sv} is the Stern-Volmer constant (Eftink, 1991).

Circular Dichroism (CD) Measurements

CD spectra were collected for 5 μM MBP in Tes-buffered solution (5 mM Tes, pH 7.0) and in the presence of liposomes (200-μM lipid concentration). CD spectra for each of the liposome preparations in the absence of protein were also collected and used as background. A JASCO J-500 automatic recording spectropolarimeter (Japan Spectroscopic Company, Easton, MD) was employed for these measurements.

Study of Liposome Fusion and Lysis

The ability of nMBP and raMBP to induce lysis of liposomes was studied by use of the fluorescent probe calcein by a slight modification of the method originally described by Allen (1981). Briefly, DMPC : DMPA liposomes containing 80 mM calcein in 5 mM HEPES (osmolarity = 105 mOsm/kg as measured by an Advanced Digimatic Osmometer, Model 3D, Advanced Instruments, Needham Heights, MA) were prepared by bath sonication. Free calcein was removed by gel chromatography using a Bio-Gel A-5m agarose on a 1.4 × 23-cm column. The elution buffer was 5 mM HEPES and 50 mM KCl (100 mOsm/kg). To study liposome fusion and lysis the assay described by Kendall and MacDonald (1982) was used. Briefly, calcein (0.8 mM calcein, 50 mM Mops, and 1 mM $CoCl_2$, pH 7.4) or EDTA (1 mM EDTA and 50 mM Mops, pH 7.4) was added to lipids dried on the bottom of a vessel; the mixture was vortexed for 1 min then sonicated in a bath sonicator at 4°C for 30 min. Free calcein and free EDTA were removed by gel chromatography using Bio-Gel A-5m agarose on a 1.4 × 23-cm column; the elution buffer was 50 mM Mops and 100 mM KCl, pH 7.4. One-ml fractions were collected and monitored for scattering at 600 nm : 600 nm [λ_{ex}: emission wavelength (λ_{em})] and fluorescence intensity in the case of calcein at 490 nm : 520 nm ($\lambda_{ex} : \lambda_{em}$).

To study specific fusion, citrate : $CoCl_2$ was added to liposome mixtures (to give a final concentration of 0.4 mM citrate, 0.4 mM $CoCl_2$, and 50 mM Mops, pH 7.4). *Specific fusion* equals the change of calcein fluorescence in the presence of MBP and citrate : $CoCl_2$ in the medium minus the change of calcein fluorescence in the presence of buffer at an equal volume to MBP under identical conditions in the same experiment in the presence of citrate : $CoCl_2$ in the medium as indicated above. *Specific fusion and lysis* is identical to the definition of specific fusion but conducted in the absence of citrate : $CoCl_2$ in the medium. Additional controls included testing another of the eosinophil granule cationic proteins, the eosinophil-derived neurotoxin, at equimolar concentrations to MBP under identical conditions (R.I. Abu-Ghazaleh, *unpublished work*). Deoxycholate (0.1%) was used as a positive control for calcein release. The measurements were conducted using a Perkin Elmer Fluorescence Spectrophotometer MPF-66.

Results

Fluorescence Polarization Measurements of DPH

Both nMBP and raMBP caused a change in the ordered state of acidic liposomes only (DMPC : DMPA and POPS), as indicated by the temperature transition profiles of the lipids in the presence of protein or buffer controls (Fig. 1*a,b,d* and *e*). No change in the transition-temperature profiles was observed when either nMBP or raMBP were added to zwitterionic liposomes made of DMPC (Fig. 1*c* and *f*). raMBP did not interact with POPC bilayers (*data not shown*) or MOPC micelles (*data not shown*). The effect of raMBP on acidic liposomes at temperatures below the transition temperature of the lipid was dose dependent (Fig. 2*a* and *b*). The reduction in the fluorescence polarization of DPH in the presence of MBP with DMPC : DMPA and POPS liposomes shows that MBP causes increased disorder in the lipid. We could not interpret the data above the transition temperature because the sample precipitates.

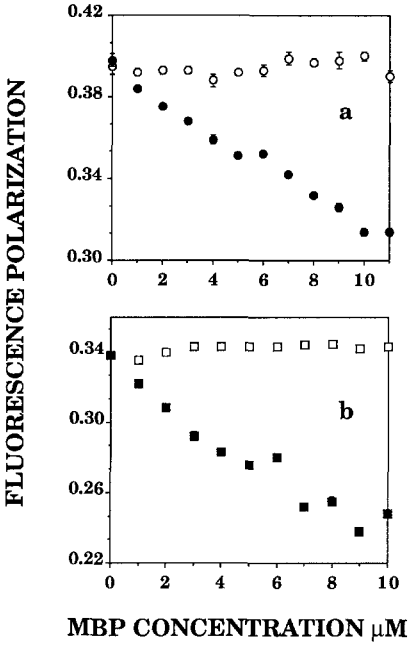

Fig. 2. The effect of adding increasing concentrations of raMBP (closed symbols) or PBS (open symbols) on the fluorescence polarization of DPH in acidic liposomes (2 mM). Data presented as means and standard deviations. (*a*) DMPC : DMPA liposomes (circles) at 20°C. Each point represents 10 different readings. The difference between sample and control is significant for all the points with MBP ($P \leq 0.0001$). (*b*) POPS liposomes (squares) at 4°C. Each point represents 10 different readings. The difference between sample and control is significant for all the points with MBP ($P \leq 0.0001$). The experimental parameters are as described in the legend to Fig. 1.

At temperatures above the transition temperature, the MBP : liposome mixture precipitated at a 1 : 200 MBP : lipid molar ratio. Precipitation was observed both visually and by a reduction of total emission intensities measured by

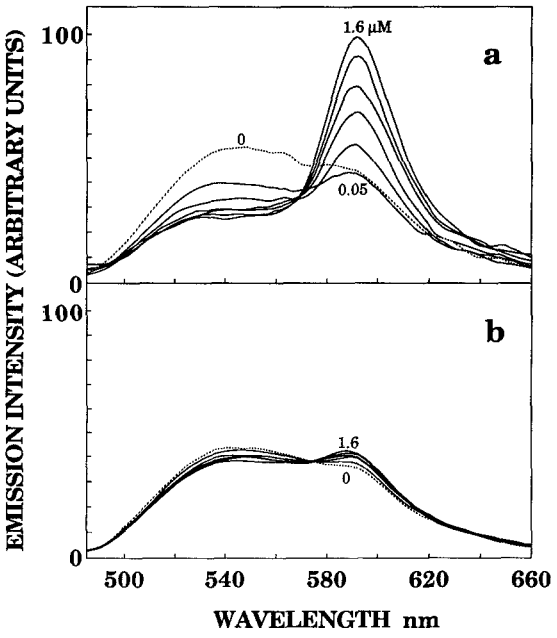

Fig. 3. The ability of raMBP to cause DMPC : DMPA liposome aggregation (20 μM) as measured by FRET from NBD-PE to RHO-PE at room temperature. (*a*) The addition of MBP at 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 μM show stepwise increase in the emission maximum of rhodamine (590 nm). (*b*) Control experiment adding equivalent volumes of PBS. This is representative of three equivalent experiments. λ_{ex}, 470 nm; excitation slit, 4; emission slit, 2; response time, 1; scan speed, 120 nm/min. Each scan is an average of five.

$$I_{||} + 2I_{\perp} \quad (2)$$

where $I_{||}$ and $I_{\perp}$ are the intensities of the emitted parallel and perpendicular light, respectively. The concentration of both MBP and lipid in some samples were measured to study the composition of the precipitate. MBP concentration was measured by radioimmunoassay. No MBP was detected in the supernatant of liposomes made from acidic lipids using either the immunoradiometric assay or the double antibody polyclonal radioimmunoassays. Almost all (99.7 ± 0.2%) the MBP added to the acidic liposomes (and not to the zwitterionic liposomes or micelles) was measured in the precipitate. DPH fluorescence intensities (emission maxima) in the supernatant and pellet (bath sonicated after reconstitution in an equal volume of buffered solution) were measured to calculate the total intensity. The percentage of the DPH intensity in the pellet, used as a rough indicator of lipid concentration, showed that the precipitate contained 74.5 ± 17.8% of POPS (n = 4) and 96.97 ± 3% of DMPC : DMPA (n = 3). These findings demonstrate that although MBP fully partitions into the acidic lipid compartment it does not interact with all the liposomes.

Aggregation of Liposomes with MBP

Quasielastic light-scattering data showed an increase in the size of measured particles upon the addition of MBP from a value of 400 nm (for DMPC : DMPA liposomes) to > 7000 nm. The curve, however, did not have a good fit, suggesting a wide variety of aggregate sizes. FRET experiments were conducted with two mixed DMPC : DMPA liposome preparations (Fig. 3) and with two mixed POPS liposome preparations (*data not shown*), one containing NBD-PE (energy donor) and the other RHO-PE (energy acceptor). In both sets of experiments, the ability of MBP (0.05 to 1.6 μM in twofold increments) to cause aggregation of those liposomes, in a dose-dependent manner and at lipid concentrations of 20 μM, was shown. At higher protein (up to 10 μM) and lipid (up to 2 mM) concentrations, a reduction of emission intensity of the NBD-PE : RHO-PE : acidic lipid : MBP mixture occurred. This phenomenon was also observed when MBP was added to acidic liposomes containing NBD-PE only (at 1% molar concentration). The reduction of fluorescence intensity could be either due to self-quenching of NBD fluorescence, precipitation of the formed aggregates, or a combination of both (*data not shown*).

Changes in the Fluorescence Characteristics of MBP

Fluorescence polarization measurements of MBP Trp fluorescence in the presence of DMPC : DMPA liposomes (Fig. 4*a*) were significantly higher than those in HEPES-buffered solution at temperatures below the transition temperature of the lipids ($P \leq 0.0001$). No such effects occurred when POPS or DMPC vesicles were employed (Fig. 4*b* and *c*). The emission spectrum of MBP showed a blue shift in wavelength maximum of approximately 8 nm for raMBP and 7 nm for nMBP in the presence of DMPC : DMPA liposomes (the Table, P = 0.0022 and 0.0023, respectively). This shift did not appear when raMBP was mixed with POPS or DMPC liposomes (the Table). A blue shift of 14 nm in the emission maximum of the dimethylaminonaphthyl moiety in acrylodan-labeled MBP was also seen in the presence of the DMPC : DMPA liposome preparations as compared with acrylodan-labeled MBP in simple solution. This shift was not observed in the presence of POPS or DMPC liposomes (the Table).

Acrylamide Quenching

The Stern-Volmer plots for acrylamide quenching of MBP intrinsic Trp fluorescence (Fig. 5) show a difference in the Stern-Volmer constants (K_{sv}) for

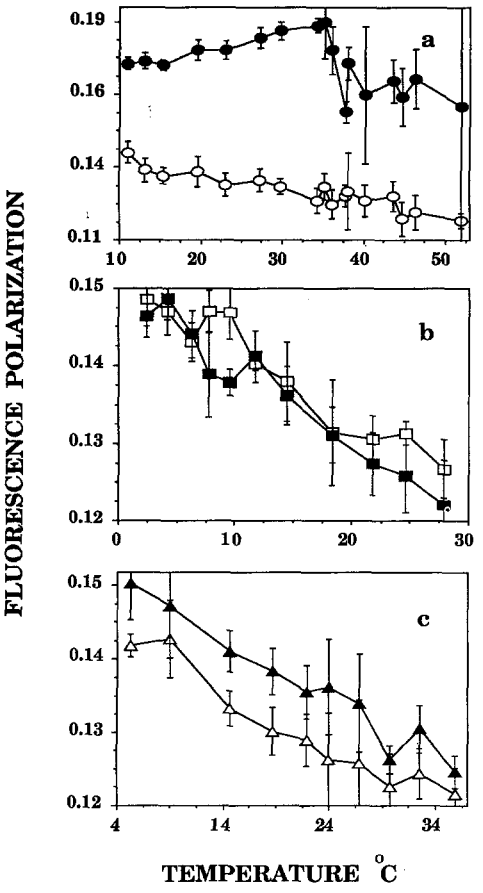

Fig. 4. Changes in the fluorescence polarization of MBP Trp fluorescence in buffered solution (open symbols) and MBP in the presence of liposomes (closed symbols). (*a*) The difference between MBP in HEPES-buffered solution (with 100 mM KCl) and MBP (10 μM) with DMPC : DMPA liposomes (circles, 200 μM) is significant below 52°C [below 35°C ($P \leq 0.0001$) and from 35 to 47°C ($P < 0.01$)]. The MBP : DMPC : DMPA mixture precipitates above 35°C, causing a reduction in the intensity of emitted light which required increasing the sensitivity of the instrument. (*b*) MBP in Tes-buffered solution or with POPS liposomes (squares, 200 μM), no difference observed. (*c*) MBP in Tes-buffered solution or with DMPC liposomes (triangles, 2 mM), no difference seen. λ_{ex}, 300 nm; emission cutoff filter, WG 345.

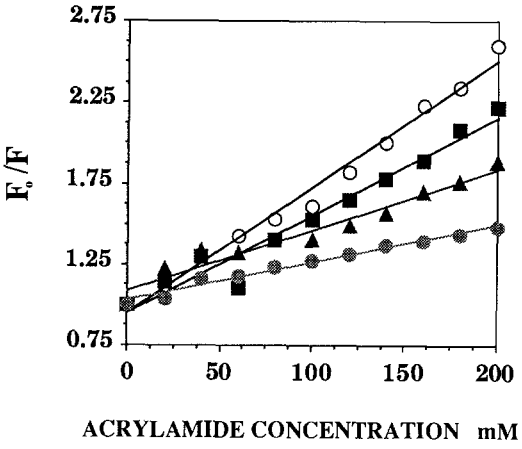

Fig. 5. Stern-Volmer plots of MBP (5 μM) in HEPES-buffered solution (open circles) and of MBP (5 μM) with liposomes at 2 mM [(DMPC : DMPA, closed circles), (POPS, squares), and (DMPC, triangles)]. The quencher used is acrylamide. F_0 is the area under the emission curve of MBP in the absence of quencher, F is the equivalent area in the presence of the indicated concentrations of quencher. λ_{ex}, 295 nm; excitation slit, 2; emission slit, 2; response time, 1; speed 60 nm/min. Each scan was an average of five. The experiments were conducted at 5°C. $\langle\tau_0\rangle$ of MBP in the presence and absence of each of the liposome preparations was also measured to calculate k_q. $\langle\tau_0\rangle$ of MBP changes in the presence of POPS and DMPC liposomes (1.79 and 1.73 nsec, respectively) compared with MBP in HEPES-buffered solution (3.02 nsec) and MBP with DMPC : DMPA liposomes (3.01 nsec).

MBP alone and for each of the MBP-lipid mixtures. This change suggested a reduction of the accessibility of the emitting Trp(s) to the quencher in the presence of liposomes. However, the bimolecular quenching constant ($k_q = K_{sv}/\langle\tau_0\rangle$) is a more accurate indicator of the accessibility of a fluorophore (Trp) to its quencher. In order to calculate k_q we measured $\langle\tau_0\rangle$ of MBP in the presence and absence of each of the liposome preparations. The maximum observed difference in k_q is between MBP alone (2.58×10^9 sec^{-1} M^{-1}) and MBP in the presence of DMPC : DMPA liposomes (0.78×10^9 sec^{-1} M^{-1}). In the presence of POPS and DMPC liposomes, the difference is less striking (3.36×10^9 sec^{-1} M^{-1}, and 2.16×10^9 sec^{-1} M^{-1}, respectively).

CD Spectra

The CD spectrum of MBP in Tes-buffered solution suggested that its structure was mostly random with an element of β-sheet (Fig. 6*a*). CD spectra of MBP in the presence of liposomes are reduced in intensity, in part due to scattering (Fig. 6*b–d*). The spectrum of MBP in the presence of POPS and DMPC liposomes did not change (Fig. 6*c* and *d*). In contrast, the spectrum of MBP in the presence of DMPC : DMPA liposomes (Fig. 6*b*) changed. This result could be due either to a change in the structure of MBP or to an undefined change in the CD signal due solely to interference from the liposomes. It should be noted, however, that MBP also induces the aggregation of POPS liposomes without an apparent change in the structure of MBP.

Lysis and Fusion of Liposomes

Preliminary experiments utilizing high concentrations of calcein within the aqueous interior of liposomes (self-quenched) showed the ability of both

Table 1. Changes in the fluorescence emission maxima of MBP

MBP (5 μM) in HEPES-buffered solution or with 2 mM liposomes made from:	Emission maxima (nm)		
	raMBP Trp[a]	nMBP Trp[a]	Dimethylaminonaphthyl moiety[b]
MBP in solution	343	341	522
DMPC : DMPA	335	334	508
POPS	343	ND[c]	521
DMPC	343	ND	523

[a] λ_{ex}, 295 nm.
[b] λ_{ex}, 390 nm.
[c] ND = not done.

nMBP and raMBP to increase the fluorescence of calcein once they are added to the liposomes, indicating their lysis (*data not shown*). In subsequent experiments a mixture of two liposome preparations, one containing calcein and its quencher $CoCl_2$ and the other containing EDTA, were used. Both raMBP (Fig. 7*a*) and nMBP (Fig. 7*b*) were found to induce fusion and lysis of the liposomes as indicated by an increase in the fluorescence of calcein. The ability of $CoCl_2$ and citrate outside the liposomes to quench some of the calcein fluorescence was used in parallel experiments to measure specific fusion. The relative fusion and lysis were calculated from corrected spectra (Fig. 7). The results demonstrated that the initial fusion event is followed by leakage of calcein outside the liposomes.

Discussion

MBP appears to be one of the major participants in the pathophysiology of diseases associated with eosinophilia. This work was aimed at an elucidation of the pathophysiology, i.e., mechanisms involved in those diseases. Because of the wide range of effector functions and toxicities of MBP and its interspecies actions, we hypothesized that MBP acts on the lipid bilayer of its targets. Synthetic lipid bilayers were used as models of the targets for MBP. The data showed that MBP interacts with and eventually disrupts the integrity of those lipid bilayers. In turn, interaction with the lipids is attended by changes in the protein itself.

The Effect of MBP on Liposomes

From a survey of different lipid types, it was found that MBP interacts most obviously with anionic lipid species. This interaction was evidenced by de-

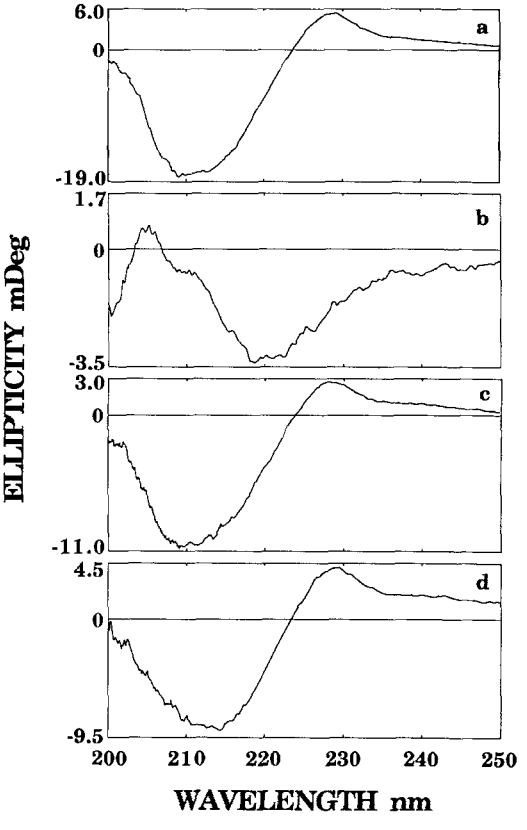

Fig. 6. CD spectra of MBP (5 μM) in the presence and absence of liposomes (200 μM). The spectra were corrected for the presence of liposomes or the buffered solution. (*a*) MBP in Tes-buffered solution. (*b*) MBP with DMPC : DMPA liposomes. (*c*) MBP with POPS liposomes. (*d*) MBP with DMPC liposomes. The experiments were conducted at room temperature using a cuvette with 0.5-cm light path. Step resolution, 0.04 nm; scan speed, 5 nm/min; number of scans, 10; time constant, 16 sec; sensitivity, 1 mdeg/full scale. The sample compartment was purged with nitrogen 30 min prior to the start of each measurement and continued throughout the experiment. All the spectra were reproducible.

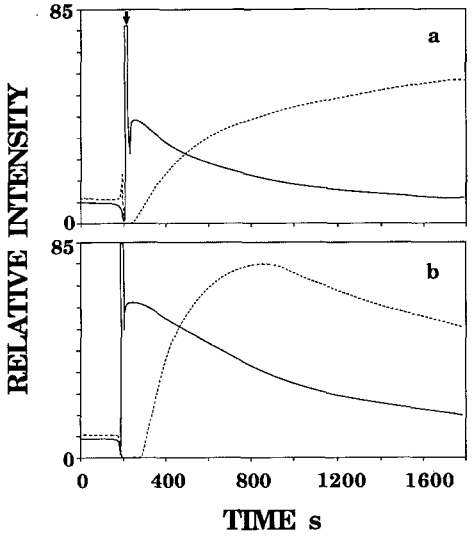

Fig. 7. The ability of MBP to induce lysis and fusion of calcein-containing liposomes. Deoxycholate (0.1%) was used as a positive control for lysis and fusion. The data shown are for 5 μM MBP added at the arrow to liposomes (30 μM) (DMPC : DMPA). The fusion curves (solid lines) were calculated by dividing the corrected time course of the increase in calcein fluorescence (in the presence of $CoCl_2$ and citrate in the buffer) by the time course of the total lysis. The lysis curves (dotted lines) were calculated by subtracting the relative protein-induced fusion from the relative protein-induced lysis and fusion. Each experiment was reproduced three times. (*a*) raMBP. (*b*) nMBP. λ_{ex}, 490 nm; λ_{em}, 520 nm; excitation slit, 5; emission slit, 5; response time 0.05 sec.

creases in the fluorescence polarization of DPH in DPH-labeled liposomes and alteration in the thermal transition profile of the lipids. The net inference is that the MBP : lipid complex was much more disordered than the original lipid complex (similar experiments employing lipids with zwitterionic headgroups showed no effects by MBP). These effects are not specifically influenced by the form of the protein used, i.e., the effects of raMBP on the fluidity of lipid bilayers were not different from those of nMBP.

In the course of this investigation the MBP : acidic lipid dispersions were found to precipitate above the lipid transition temperature. We reasoned that this might be due either to the precipitation of the lipids or the protein or more likely to the ability of MBP to induce liposome aggregation and then precipitation of the complex. Fluorescence measurements with acidic liposomes (composed of DMPC : DMPA mixtures or composed of POPS) containing NBD-PE and RHO-PE showed that MBP was able to induce FRET from NBD to RHO and cause self-quenching of NBD. These results confirm that MBP causes aggregation of negatively charged liposomes.

In the experiments presented above, MBP was able to interact with liposomes at an ionic strength equivalent to 0.125 M. It should be noted here that at similar ionic strengths purely electrostatic interactions of proteins with liposomes are markedly diminished (Perides, Harter & Traub, 1987; Benfenati et al., 1989). At equivalent ionic strengths MBP not only interacts with the lipids mentioned above but also retains its bactericidal ability (Lehrer et al., 1989). In contrast, another of the eosinophil granule proteins, the eosinophil cationic protein, loses its bactercidal ability at 140 mM NaCl (Lehrer et al., 1989).

Perturbation of the Fluorescence Characteristics and Structure of MBP

Intuitively one assumes that lipid binding would perturb protein structure and dynamics. Evidence of this was adduced from the effects of DMPC : DMPA liposomes on the intrinsic (Trp) fluorescence polarization of MBP. *A priori,* it was evident that interpretation of those data in terms of detailed structural changes are not possible because of the presence of multiple Trp residues in MBP. The fluorescence polarization data, however, showed a significant increase in Trp polarization in the presence of DMPC : DMPA liposomes. This finding suggests a strong interaction between MBP and those liposomes. We cannot exclude the possibility, however, that this increase in fluorescence polarization is in part due to light scattering resulting from liposome aggregation. However, this effect is unlikely because the interaction of MBP with POPS induces the aggregation of those liposomes yet it does not affect the polarization of MBP Trp.

Furthermore, the fluorescence emission intensity profiles of MBP show blue shifts in the emission maxima of the intrinsic MBP Trp(s) and the fluorescence of an extrinsic, covalently bound fluorescent probe, the dimethylaminonaphthyl-moiety in acrylodan-labeled MBP, also shows a blue shift by about 14 nm in the presence of DMPC : DMPA liposomes. These data indicate a shift of both the Trp residue(s) and the naphthyl moieties to less polar microenvironments in the presence of the DMPC : DMPA liposomes.

The next question we then sought to answer is whether the Trp moieties of the protein are buried subsequent to interaction with the lipid bilayers. The ability of acrylamide to quench the fluorescence of the emitting Trp(s) was therefore studied. In the presence of DMPC : DMPA liposomes, the bimolecular quenching rate of acrylamide was about three times lower than that of MBP in solution. This

change indicates a reduction in the accessibility of acrylamide to the emitting MBP Trp(s), implying either that the Trp residues of the MBP have been more deeply buried in a lipid-induced refolded protein molecule or more deeply embedded in the lipid bilayer, or both.

The crystal structure of MBP has not yet been solved. It is known, however, that MBP possesses at least two free sulfhydryl groups (Gleich et al., 1974; Wasmoen et al., 1988); it is with at least one of these that acrylodan covalently binds. Computer analysis of the structure of MBP suggests the presence of some β-sheet structure (Wasmoen et al., 1988). Similarly our CD analyses have shown that in solution raMBP exists largely as a random coil with an element of β-sheet. Our results also show that when raMBP is mixed with DMPC : DMPA liposomes its structure changes such that an element of α-helix appears. Admittedly, we cannot exclude that this change in the spectrum is due to interference from the aggregated-liposomes but this is unlikely because the spectrum of MBP in the presence of the aggregated-POPS liposomes does not change. These data further support our earlier inference that the MBP : bilayer interaction results in the formation of a new structural form of the protein.

In the above described experiments it was also clear that the interaction of MBP with lipid bilayers composed of POPS is different from its interaction with bilayers composed of DMPC : DMPA. Although MBP causes a small change in the temperature transition profile of POPS and induces its aggregation, the fluorescence characteristics and the apparent structure of MBP do not change in the presence of POPS. In contrast, the interaction of MBP with DMPC : DMPA liposomes not only affects the lipid bilayers but also induces changes in the Trp fluorescence of MBP, the latter implying an alteration in MBP structure. It is unlikely that the differences in saturation or the length of the alkyl groups between the two lipids is the cause of the differential interaction with MBP. No change in the temperature transition profiles of DMPC or POPC is observed upon the addition of MBP. It seems plausible, therefore, that the difference in headgroups between DMPA and POPS dictates the nature of their interaction with MBP.

The Ability of MBP to Breach the Lipid Bilayer

Pore formation is one of the mechanisms by which some toxins such as complement (McCloskey, Dankert & Esser, 1989) mediate their toxicity. The ability of MBP to act as a lysin was therefore examined and the calcein entrapment method was employed (Kendall & MacDonald, 1982). The results showed that MBP first induced fusion of liposomes followed by lysis.

The Mechanism of the MBP : Bilayer Interaction

It is clear from the experimental findings presented above that the MBP-lipid interaction is at least in part electrostatic as has been described for other peptides and proteins (Niggli et al., 1986; Perides et al., 1987; Benfenati et al., 1989; Bazzi & Nelsestuen, 1991; Kim et al., 1991; Mosior & McLaughlin, 1991). However, cationic charge is not by itself sufficient to explain the functions of MBP; another of the eosinophil proteins which is also frankly cationic, the eosinophil-derived neurotoxin, does not affect the temperature transition profile of DMPC : DMPA liposomes, nor does it induce liposome fusion or lysis (R.I. Abu-Ghazaleh, *unpublished work*). Accordingly, a role for the hydrophobic residues of MBP in mediating or enhancing the interaction with lipid bilayers has to be posited and such an inference is buttressed by the relative lack of effect of ionic strength on the cytocidal activities of MBP (*see above*). In fact, the hydrophobicity of nMBP very likely plays a role in precipitation of the protein at physiologic pH.

Working Model for the Effector Function of MBP

Although a molecular interpretation of the mechanism of the MBP toxicity in biological systems is still lacking, the experimental data presented here clearly demonstrate the ability of MBP to perturb and breach lipid bilayers. Biological cell membranes are invariably negatively charged not only because of their content of acidic phospholipids but also because of the presence of glycolipids and glycoproteins which are also negatively charged (Honig, Hubbell & Flewelling, 1986; Tsong & Astumian, 1987; Cevc, 1990; Devaux, 1991).

The model we propose for the mechanism of action of MBP on lipid bilayers is therefore as follows: MBP binds to target membranes via electrostatic and hydrophobic interactions and induces clustering of the negatively charged components of those membranes (Tsong & Astumian, 1987), as well as aggregation of adjacent membranes. This destabilizes the membranes, induces fusion of adjacent bilayers, and results in leakage of cell contents and promotes osmotic imbalance (Fig. 8). Once such a

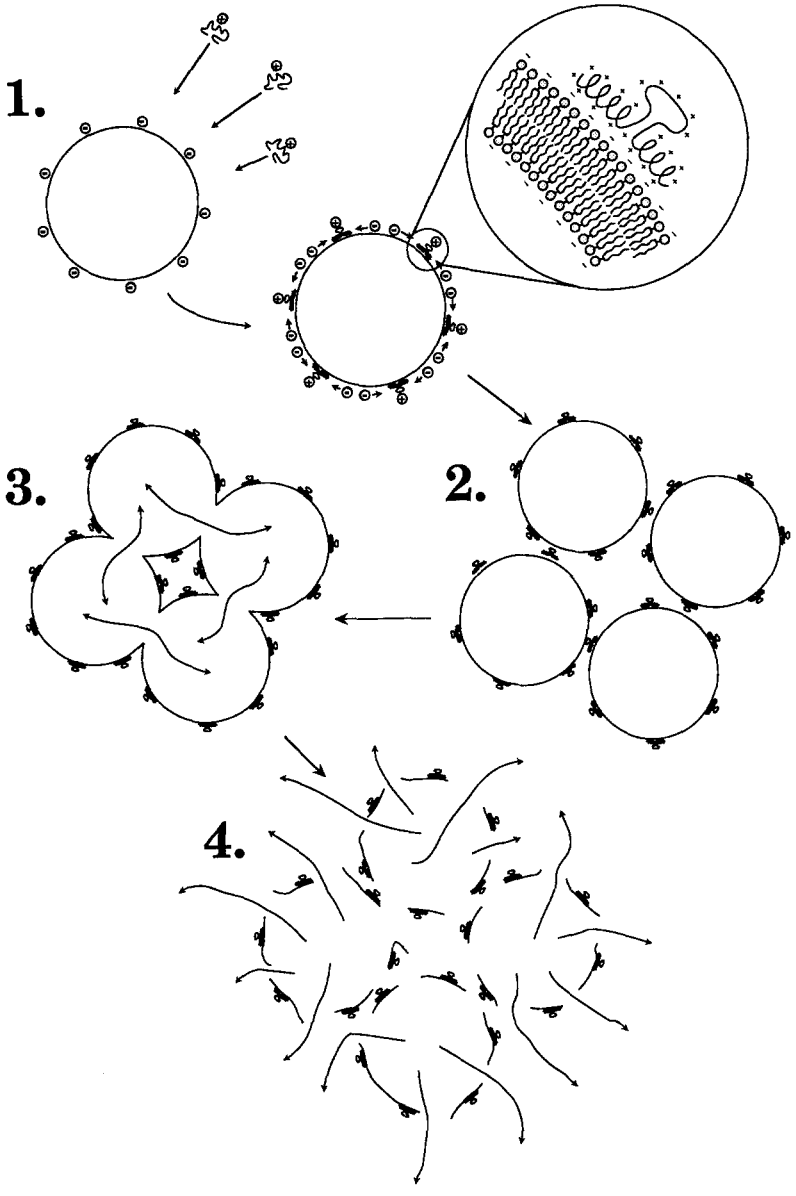

Fig. 8. Proposed model for the effect of MBP on synthetic lipid bilayers. An interaction mediated by electrostatic and hydrophobic interactions will cause (*1*) the binding of MBP to the surface of the bilayer and clustering of the surface charged molecules, (*2*) aggregation of liposomes and destabilization of the bilayer, inducing (*3*) fusion of the bilayer, and subsequently (*4*) lysis. Because we have no evidence supporting the actual penetration of the protein into the bilayer or formation of a channel we drew the protein on the surface of the membrane.

breach has been established, a cascade of events is likely, including osmotic swelling, loss of cellular contents, and intrusion of inimical agents such as Ca^{2+} into the cytoplasm. Any of these would be sufficient to cause cell death; all are possible.

In instances when MBP induces degranulation of its cellular targets, e.g., platelets (Rohrbach et al., 1990), neutrophils (Moy et al., 1990), or basophils and mast cells (O'Donnell et al., 1983), additional mechanism(s) might be at play. In such cases, an influx of cations caused by the breach of the membrane integrity, or the clustering of the acidic lipids, as suggested by Bazzi and Nelsestuen (1991), might provide the trigger for exocytosis. Alternatively, in these situations there may be a protein membrane receptor, the binding to which triggers degranulation. However, the ability of MBP to affect targets from a wide number of species (mammalian cells, parasites, and bacteria) makes the existence of a specific membrane receptor for MBP a less attractive hypothesis.

To further elucidate the mechanism(s) of toxicity of MBP, kinetic studies using stopped-flow analysis of the lytic and fusogenic capabilities of MBP (and fragments of it) in artificial and biological systems are underway. Also studies employing digital imaging microscopy are planned to evaluate directly the effects of MBP on target cells.

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