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Membrane Fluidity of Polymorphonuclear Leukocytes from Children with Primary Ciliary Dyskinesia

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ABSTRACT. Plasma membrane fluidity and heterogeneity of polymorphonuclear leukocytes (PMN) were investigated in seven children with primary ciliary dyskinesia (PCD) and 17 healthy controls. Membrane fluidity and heterogeneity were studied by measuring the steady state fluorescence anisotropy and fluorescence decay of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) incorporated into PMN plasma membrane. Our results show an increase in membrane fluidity at the surface level of PMN from patients with PCD. Distribution analysis of TMA-DPH lifetime values indicate an increase in membrane heterogeneity in subjects with PCD. The observed changes in the physicochemical properties of the membrane could lead to alterations in the function of PMN from children with PCD. (*Pediatr Res* 34: 725–728, 1993)

Abbreviations

PMN, polymorphonuclear leukocyte
PCD, primary ciliary dyskinesia
TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene
FMLP, N-formyl-methionyl-leucyl-phenylalanine
r_s, steady state fluorescence anisotropy
PMA, phorbol myristate acetate

PMN from subjects with PCD can have an abnormal locomotory system. Defects in orientation (1, 2), migration (1–3) and chemotaxis (3–5) have been reported. All these activities are the result of complex events mediated by the plasma membrane and cytoskeleton of PMN. Specific and dynamic interactions of plasma membrane components are essential for an adequate membrane function (6). Membrane fluidity has an important role in modulating cell functions, affecting the conformation of membrane proteins and the exposure and diffusion of membrane components. Fluidity is a complex physicochemical feature that depends upon mobility and order of membrane constituents (6, 7). Changes in composition and molecular organization are the principal determinants of alterations of membrane fluidity observed in many human diseases (8, 9).

In this study, we have evaluated plasma membrane fluidity of PMN obtained from children with PCD by measuring r_s and fluorescence decay of a probe incorporated into the plasma membranes. As a fluorescent probe, we have used TMA-DPH, which is incorporated at the lipid-water interface region bilayer.

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MATERIALS AND METHODS

Subjects. Seven children with PCD, five females and two males, with an age range between 1 mo and 9.4 y (mean 6 ± 3.2 y), were included in this study after appropriate informed consent had been obtained. Virtually all these children had a clinical history of upper and lower respiratory tract infections with onset in early childhood characterized by productive cough, rhinorrhea, severe otitis, chronic sinusitis, and recurrent bronchitis or pneumonia. Kartagener's syndrome (situs inversus, chronic sinusitis, and bronchiectasis) was observed in one female patient. Chest radiograph abnormalities present at the time of diagnosis included hyperinflation (six patients), situs inversus (two patients), and/or segmental bronchiectasis (three patients). In all subjects, the diagnosis of PCD was established by studying ciliary motion and ultrastructure. Nasal and bronchial brushings were obtained from each subject and handled for ciliary motion analysis and ultrastructural studies as previously described (10, 11). Analysis of ciliary motion examined by a light microscope equipped with Nomarsky differential interference contrast set as previously described (11) demonstrated a total absence of ciliary motion in three subjects and an uncoordinated and asyncrhonous motion with a reduced beat frequency of 2–6 Hz (normal range 12 ± 2.4 Hz) in four subjects. Ultrastructural studies, performed as previously described (10, 11), revealed microtubular transposition defects, radial spokes defects, and the total or partial lack of dynein arms (inner, outer, or both). Once diagnosis of PCD was confirmed, patients were treated with chest physiotherapy and intermittent courses of systemic antibiotic.

The control group consisted of 17 healthy children, 12 females and five males, between 6 mo and 10.2 y old (mean 6.4 ± 3.0 y). None of the children in the control group had a family or personal history of chronic respiratory disease or immunologic disorders.

All subjects were without signs of acute infection and had not suffered from acute infection in the previous 6 wk. In the case of the PCD group, patients had completed a course of systemic antibiotic therapy 6 wk earlier.

After overnight fasting, 13 mL of heparinized blood were obtained for isolation of PMN. Simultaneously, we measured erythrocyte sedimentation rate, differential white blood count in whole blood, IgM, IgA, IgG, complement factors C_3 and C_4, and plasma cholesterol levels as previously described (12).

Isolation of PMN. PMN were isolated using a Mono-Poly Resolving Medium (ICN Biomedicals, Milan, Italy) as previously described (13). Cells were resuspended in Krebs-Ringer phosphate solution supplemented with 5 mM glucose.

Chemiluminescence measurements. Luminol-amplified chemiluminescence was measured in the Autolumat LB 953 (Berthold Co., Wilbad, Germany) and PMN were activated by addition of PMA 10^{-6} M (Sigma Chemical Co., St. Louis, MO).

Chemiluminescence measurements were followed for the next 60 min as previously described (14).

Chemotaxis assay. Chemotaxis was tested by a modified Boyden technique as described by Governa *et al.* (15) with the use of blind-well chambers and 5-μm pore cellulose acetate filters (Millipore Corp., Bedford, MA). Zymosan-activated serum, prepared as described by Metcalf *et al.* (16), was used as a chemoattractant. Zymosan was obtained from Sigma Chemical Co. Tissue culture medium 199 (ICN Biomedicals) was used to determine random motility. After incubation of cells for 3 h at 37°C, the filters were removed and stained. The number of cells that completely migrated through the filter was counted within 10 microscopic fields (400×) using a 5 × 5-mm photographic reticle, and the chemotactic index was calculated (15, 16). Samples were tested in duplicate.

Fluorescence measurements. The r_s measurements were performed at 37°C with a Perkin Elmer Spectrofluorimeter MPF-66, equipped with a Perkin Elmer 7300 Personal Computer for data acquisition and elaboration (Perkin Elmer Corp., Norwalk, CT), using TMA-DPH (Molecular Probes Inc., Eugene, OR) as a hydrophobic fluorescent probe at a final concentration of 10^{-6} M as previously described (17). The computer program calculated fluorescence anisotropy by using the expression $(I_{\parallel} - I_{\perp} \times g)/(I_{\parallel} + 2I_{\perp} \times g)$, where g is an instrumental correction factor and $I_{\parallel}$ and $I_{\perp}$ are the emission intensities with the polarizers parallel and perpendicular respectively to the direction of the polarized exciting light. The optimal cell concentration for fluorescence measurements has been determined as previously described (17). After incubation with TMA-DPH for 5 min, the basal r_s was measured for 5 min. PMN were later activated by the addition of FMLP at a final concentration of 10^{-6} M and r_s measurements were followed for the next 15 min as previously described (18).

Fluorescence lifetime measurements were performed as previously described (19) with a multifrequency phase fluorometer. The instrument was equipped with an ADC interface (ISS Inc., Champaign, IL) for data collection and analysis; the excitation wavelength was set at 325 nm (UV line of a helium/cadmium laser, model 42240 NB, LiCONix, Santa Clara, CA). The range of modulation frequencies used for TMA-DPH was 7–130 MHz. Data were accumulated at each modulation frequency until the SD of the phase and modulation values were below 0.1° and 0.002, respectively. The fluorescence was measured through a long-pass filter (type RG 370 from Janos Technology, Townshend, VT), which showed negligible luminescence. The experimental data were analyzed by a model that assumes a continuous distribution of lifetime values characterized by Lorentzian shape centered at a time C and having a width W as previously described (20, 21). For this analysis, the program minimizes the reduced χ^2 defined by an equation reported elsewhere (22). The temperature of the samples was maintained at 37°C with an external bath circulator.

Statistical method. The nonparametric Mann-Whitney U test was used to determine the significance of differences between the PCD group and the control group. To determine the significance of the data obtained in each group, we used the *t* test.

RESULTS

Erythrocyte sedimentation rate, white blood count, serum level of immunoglobulins, C_3, C_4, and plasma cholesterol values from the PCD and control groups were not significantly different ($p > 0.5$).

Luminol-amplified chemiluminescence has been used to verify that in isolated PMN the superoxide-generating oxidase system is dormant under basal conditions and can be activated by PMA. All samples used in this study demonstrated an activatable NADPH-oxidase system (data not shown). Peak activity was noted within 15–20 min of the addition of PMA and there was no significant difference in the peak value of PMA-stimulated chemiluminescence between the two groups ($p > 0.5$).

Chemotactic index of PMN from subjects with PCD ranged between 86 and 110 (mean 94.5 ± 8.6), whereas in the control group values obtained ranged between 108 and 152 (mean 122 ± 12.4).

The background fluorescence of PMN was checked before each measurement and was less than 0.1% of the fluorescence when TMA-DPH was added. In basal conditions, without stimulation, the r_s of TMA-DPH incorporated in plasma membrane of PMN from the PCD and control groups was stable and did not show significant changes ($p > 0.5$) for 30 min after addition of TMA-DPH. The TMA-DPH r_s values of unstimulated PMN from the PCD and control groups are reported in Table 1. The basal values for PMN from subjects with PCD (mean 0.270 ± 0.002) are significantly lower than the corresponding values for PMN from the control group (mean 0.288 ± 0.003).

When resting PMN from the PCD and control children were stimulated with FMLP, a statistically significant ($p < 0.05$) increase in r_s value was observed in both groups.

Figures 1 and 2 show TMA-DPH fluorescence lifetime distribution in PMN from the control and PCD groups. Data analysis revealed the presence of two distinct components of lifetime distribution in the samples. In the control group, a long component with a central lifetime value of 6.5 ns (fractional intensity of 0.8) and a short component with a central lifetime value of 0.8 ns (fractional intensity of 0.2) were observed. The width of the distribution was 0.30 ns for the long component and 0.6 ns for the short one. In PMN from the PCD group, the long component has a central lifetime value of 6.3 ns (fractional intensity of 0.8) and the short component has a central lifetime

Table 1. *Mean ± SD of r_s of TMA-DPH at 37°C in PMN from control and PCD groups in basal conditions and after stimulation with FMLP*

	r_s	
	Controls ($n = 17$)	PCD ($n = 7$)
Unstimulated PMN	0.288 ± 0.003	0.270 ± 0.002*
Stimulated PMN	0.360 ± 0.004*	0.300 ± 0.001†‡

* $p < 0.05$ compared with unstimulated PMN from the control group.
† $p < 0.05$ compared with unstimulated PMN from the PCD group.
‡ $p < 0.05$ compared with stimulated PMN from the control group.

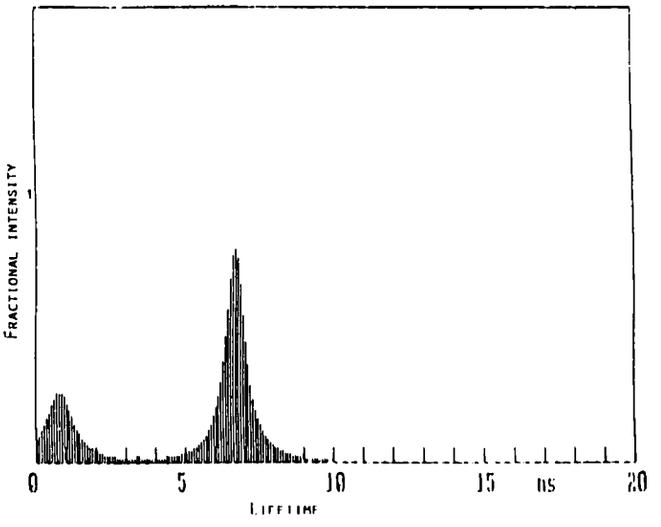

Fig. 1. TMA-DPH lifetime distribution in PMN from the control group. The distribution width of the long component has a value of 0.30 ns and is centered at 6.5 ns.

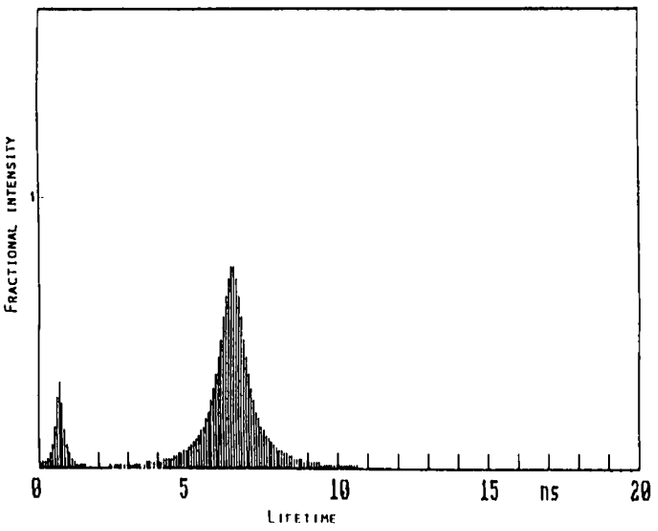

Fig. 2. TMA-DPH lifetime distribution in PMN from children with PCD. The distribution width of the long component has a value of 0.5 ns and is centered at 6.3 ns.

value of 0.8 ns (fractional intensity of 0.2). The distribution widths were 0.5 ns for the long component and 0.05 ns for the short component.

DISCUSSION

Biologic membranes are a complex mixture of different types of lipid and protein molecules. Different modes of organization of the compositionally and functionally differentiated domains correspond to different functional states of the membrane (23). Chemical and physical events that take place within the membrane allow the cells to carry out their specific functions (23). Fluorescence spectroscopy is a useful tool in the study of the structure and function of biologic membranes because its high sensitivity, low degree of membrane perturbation, and favorable time scale allow one to observe a wide range of molecular processes (24). TMA-DPH has been used intensively for studying membrane structure and fluidity because of its advantageous structural and photophysical properties (25, 26). Because of its hydrophobic structure, TMA-DPH incorporates into the membrane but remains at the lipid-water interface region because of its cationic residue (26). TMA-DPH r_s reflects the packing of membrane lipid fatty acid chains and can be related to the order parameter S, if certain precautions are taken (27, 28). Lipid fluidity may be defined as the reciprocal of the lipid structural order parameter S (27), and thus a decrease of TMA-DPH r_s value corresponds to an increase in membrane fluidity. In our study, we have found that r_s of TMA-DPH in resting PMN from the PCD group is significantly lower than that in PMN from the control children. This indicates an increase in membrane fluidity that reflects a decrease in lipid ordering in the exterior part of PMN plasma membrane from patients with PCD. After stimulation of PMN from both groups with FMLP, significant differences in r_s values were observed between the two groups.

The TMA-DPH fluorescence lifetime value is dependent on the dielectric constant of the medium in which the probe is embedded (29). Therefore, the width of the lifetime distribution can be related to the different physicochemical properties of the environment surrounding the probe. The distribution analysis, although based on phenomenologic ground, offers a good description of membrane heterogeneity (20, 21, 30). In our distribution analysis, it was necessary to include a second component at a shorter lifetime with a very low fractional intensity. The origin of this second component is still debated; for diphenylhexatriene, this component has been referred to as a photochemical derivative of the probe (31) or, alternatively, it can represent a fraction of probe molecules localized in a very polar environment (32). In any case, the relative fractional intensity of the short component did not differ significantly between the two groups. Our data for TMA-DPH lifetime distribution show that PMN from children with PCD have a broad distribution width of the long component with respect to the control PMN. No significant differences were observed in the central lifetime value between the two groups. These results indicate an increase in membrane heterogeneity of PMN in children with PCD.

The plasma membrane is a complex structure exhibiting specific and dynamic interactions between cytoskeletal proteins and membrane lipids that determine the mobility and distribution of these constituents (33).

Functional studies of PMN motility implicating a requirement for an integrated cytoplasmic microtubule-membrane interaction have been recently reported (33–35). Decreased chemotaxis associated with alterations in membrane fluidity was reported in PMN treated with microtubule disrupting agents such as lead or 2,5-hexanedione (15, 36) in PMN from subjects with Chédiak-Higashi syndrome (34) or from healthy newborn infants (37, 38). Cytoskeletal architecture might directly affect the local composition of the plasma membrane (34, 35). Cytoskeleton-related changes in the lipid composition of the membrane or in membrane surface tension might afford regions of minimal free energy to certain classes of molecules such as chemotactic receptors (39).

The finding of an impaired chemotactic activity of PMN from the PCD group is consistent with previous studies (1–5) that have attributed the reduced motility to alterations in the cytoskeleton. Because the cytoskeleton can influence the distribution and dynamics of membrane components, we could speculate that alterations in the cytoskeleton may result in the above-described changes of membrane fluidity and heterogeneity in PMN from subjects with PCD.

Experimental evidence indicates that each integral membrane protein interacts with its neighboring "boundary" lipids in a specific manner (40). Alterations in the composition of this lipid boundary may lead to changes in enzyme activity as well as in ligand specificity and affinity (41).

Recently, Wolach *et al.* (38), using diphenylhexatriene as a fluorescent probe, have indicated that increased fluidity in neonatal PMN contribute to their deficiency in chemotactic ability, demonstrating an improvement of chemotactic capacity after membrane rigidification (38). At the moment, we cannot exclude the existence of a primary defect in membrane composition and organization. This hypothesis may be supported by the observation reported by Wolburg *et al.* (4) of a reduced number of intramembranous particles in PMN from PCD patients.

In conclusion, we believe that the observed alterations in the physicochemical structure of plasma membranes may contribute to the impairment of motility of PMN from children with PCD.

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