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Membrane Fluidity of Nonmuscle Cells in Duchenne Muscular Dystrophy: Effect on Lymphocyte Membranes of Incubation in Patient and Control Sera

CHRISTOPH HÜBNER, ALFRIED KOHLSCHÜTTER, AND JUTTA GÄRTNER

Department of Pediatrics, University of Hamburg, Hamburg, West Germany

ABSTRACT. The membrane fluidity of intact fibroblasts, erythrocyte ghosts, and intact lymphocytes from Duchenne muscular dystrophy (DMD) patients and controls was measured by steady state fluorescence polarization. The fluorescent probes used were diphenylhexatriene (DPH), trimethylammonium-DPH, and a set of n-(9-anthroyloxy) fatty acids. Fluorescence anisotropies in DMD fibroblasts and DMD erythrocyte ghosts were normal. In DMD lymphocytes (n = 10) fluorescence anisotropy of DPH was decreased *versus* controls (0.212 ± 0.028 *versus* 0.231 ± 0.012, $p < 0.05$). Linear regression analysis of creatine kinase activity in sera and DPH fluorescence anisotropy in lymphocytes from DMD patients showed a negative correlation ($r = -0.93$, $p < 0.001$). DMD lymphocytes and control lymphocytes were incubated for 4 h in sera from DMD patients and from controls. When incubated in DMD sera, DPH fluorescence anisotropy of DMD lymphocytes decreased from 0.211 ± 0.018 to 0.180 ± 0.028, and fluorescence anisotropy of control lymphocytes decreased from 0.239 ± 0.012 to 0.179 ± 0.025 reaching the same level as did DMD lymphocytes. When incubated in control sera, DPH fluorescence anisotropy of DMD lymphocytes increased to 0.224 ± 0.012, and fluorescence anisotropy of control lymphocytes decreased to 0.218 ± 0.017. The fluorescence anisotropy changes after incubation in DMD *versus* control sera were different ($p < 0.05$ for DMD lymphocytes and $p < 0.005$ for control cells). Our findings do not support the hypothesis of a general membrane defect but suggest a toxic serum factor in DMD which attacks lymphocyte membranes and possibly muscle membranes at the same time. (*Pediatr Res* 22: 488–492, 1987)

Abbreviations

16-AP, 16-(9-anthroyloxy)-palmitic acid
6-, 7-, 9-, and 12-AS, 6-, 7-, 9-, and 12-(9-anthroyloxy)-stearic acid
CK, creatine kinase (EC 2.7.3.2)
DMD, Duchenne muscular dystrophy
DPH, 1,6-diphenyl-1,3,5-hexatriene
GOT, glutamate oxaloacetate transaminase (EC 2.6.1.1)
GPT, glutamate pyruvate transaminase (EC 2.6.1.2)
LDH, lactate dehydrogenase (EC 1.1.1.27)
PBS, phosphate-buffered saline without Ca^{++} and Mg^{++} (pH 7.29)
TMA-DPH, 1-[4-(trimethylammonium)-phenyl]-6-phenyl-1,3,5-hexatriene

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Correspondence Dr. C. Hübner, Department of Pediatrics/Kinderklinik, University of Hamburg, Martinistr. 52, D-2000 Hamburg 20, West Germany.
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Membrane defects observed in nonmuscle cells in DMD patients led to the hypothesis that there could be a general membrane abnormality in DMD (1). In DMD neutrophils reduced spontaneous migration and chemotaxis suggest that there is a defect in the contractile system or cell membrane functions (2). In DMD fibroblasts capping capacity was reduced (3) and membrane fluidity was increased (4). Nuclear magnetic resonance studies revealed a decreased water permeability in DMD erythrocytes (5, 6). Some authors found different electron spin resonance spectra in DMD *versus* control erythrocytes (7, 8), other studies did not support these findings (9, 10). Diminished capping capacity in DMD lymphocytes was demonstrated in several studies (11–14), while others did not confirm this (15, 16).

In order to check the hypothesis of a generalized membrane defect in DMD we investigated different nonmuscle cell types from DMD patients by one method. We measured the membrane fluidity (17, 18) of intact fibroblasts, erythrocyte ghost preparations, and intact lymphocytes from DMD patients, using steady-state fluorescence polarization (19). We incubated lymphocytes from DMD patients and from control subjects in sera from DMD patients and from control subjects. The results of these incubation experiments led us to a new understanding of the pathophysiology of lymphocyte membrane defects in DMD.

MATERIALS AND METHODS

Patients and controls. For our fibroblast studies we examined the cultured skin fibroblasts of nine patients with DMD (age range 0.5–10 yr). The control group was comprised of 10 age-matched boys without infectious, metabolic, systemic, or malignant diseases.

The erythrocyte studies were performed on six patients and 17 control subjects. The lymphocyte study group (without incubation experiments in sera) consisted of 10 patients and 60 control subjects. Incubation experiments in sera were performed on the lymphocytes of four patients and of five control subjects. The age range of the DMD patients was 2–17 yr and that of the control subjects 1–46 yr. There were no correlations between age or sex and fluorescence anisotropy. For the lymphocyte studies a second control group consisted of three patients with congenital myopathy and one patient with necrotizing encephalitis and myoclonia (age range 1–5 yr).

Preparation of fibroblasts. Skin biopsies were taken in DMD patients during a muscular biopsy, usually from the thigh. In the

control boys, skin samples were taken during surgical interventions from the abdominal wall. Fibroblasts were grown in Falcon flasks according to Galjaard (20) and Wiesmann *et al.* (21) in Ham's medium (F-10 medium, Seromed, West Berlin) supplemented with fetal calf serum (13%), L-glutamin, and antibiotics. After reaching confluency in 75 cm^2 flasks (after 4 to 5 wk), the cells were frozen in liquid nitrogen. After further subcultures, the fibroblasts were trypsinated and washed twice with PBS. For all measurements, the passage numbers were between two and six. There were no differences in the fluorescence anisotropy of fibroblasts within the range of passage numbers 1 to 10.

Preparation of erythrocyte ghosts and lymphocytes. Erythrocyte membranes (ghosts) were kindly prepared according to Dzandu *et al.* (22) by Dr. Seitz in our department. Intact lymphocytes were isolated from EDTA anticoagulated blood within 1 h of sampling. Ficoll solution (Seromed) was used for gradient centrifugation according to Böyum (23). No precautions as to the state of fasting were taken.

Fluorescence polarization. The pellets of the cell preparations were suspended in PBS. The initial optical density of the cell suspensions was approximately 0.180 absorbance units at 365 nm (corresponding to 333 ± 93 fibroblasts/μl, 317 ± 30 ghosts/μl, 469 ± 57 lymphocytes/μl) before labeling (24). The fluorescent probes used were DPH (25, 26) (Sigma Chemical Co., St. Louis, MO), TMA-DPH (24, 25, 27, 28), and a set of five long-chain saturated fatty acids with the fluorophore 9-anthroyloxy attached to various positions of the fatty acid chain: 6-AS, 7-AS, 9-AS, 12-AS, and 16-AP (29, 30) (Molecular Probes, Inc., Junction City, OR). The stock solutions (1 mM) were diluted with PBS immediately before use and added to the cell suspension (24). The final concentrations of the fluorescent probes were: 0.4 μM of the anthroyloxy-fatty acids (fibroblasts, erythrocyte ghosts, lymphocytes), 0.4 μM of the DPH (fibroblasts, erythrocyte ghosts), 0.2 μM of the DPH (lymphocytes), 0.5 μM of the TMA-DPH (fibroblasts), and 0.1 μM of the TMA-DPH (erythrocyte ghosts, lymphocytes). Incubations and measurements of the fluorescence anisotropy were carried out at 26° C (fibroblasts), 37° C (erythrocyte ghosts), and 21° C (lymphocytes). Cell suspensions labeled with anthroyloxy probes were incubated for 50 min, those with DPH for 10 min (lymphocytes for 10 and 30 min), and those with TMA-DPH for 1 min. The final optical density ($\lambda = 365$ nm) of the labeled cell suspensions during measurement of the fluorescence anisotropy was about 0.160 ± 0.035 (fibroblasts), 0.160 ± 0.015 (erythrocyte ghosts), and 0.155 ± 0.019 (lymphocytes) absorbance units. Fluorescence anisotropy measurements $[r = I_{\parallel} - I_{\perp})/I_{\parallel} + 2I_{\perp})]$ (19) of the labeled cell suspensions were carried out in a "microviscosimeter MV-1a" [(Elscint Ltd., Haifa, Israel) excitation $\lambda = 365$ nm, emission $\lambda = 418$ nm]. The microviscosimeter was equipped with a separate excitation mercury lamp outside the measuring instrument and connected to the measuring unit by fiber optics. The fluorescence anisotropy, r, was corrected for scattered light (24). A decrease of fluorescence anisotropy indicates an increase in membrane fluidity.

Incubation of lymphocytes with serum. Incubations of lymphocytes with sera of DMD-patients and controls were carried out according to Inbar *et al.* (31). Sera were prepared from fresh clotted blood and stored for up to 4 wk at −20° C. Eight ml of lymphocyte suspension in PBS (optical density 0.250 absorbance units at 365 nm) were mixed with 1840 μl serum and incubated for 2 or 4 h at 4° C. After incubation the lymphocyte suspensions were centrifuged (1400 × *g*, 20° C, 10 min) and the pellets washed once with 8 ml of PBS. In the incubation experiments DMD lymphocytes were incubated in control sera ($n = 4$) and in DMD sera ($n = 5$). The control lymphocytes were also incubated in control sera ($n = 22$) and in DMD sera ($n = 9$). There were no differences in fluorescence anisotropy measurements between the incubation experiments in autologous versus heterologous sera.

For incubation experiments with artificially CK-enriched human sera, we used CK from rabbit muscle (Serva, Heidelberg, West Germany). In all incubation experiments fluorescence anisotropy was measured with the fluorophore DPH (label incubation time 10 min).

Determination of enzyme activities in sera. CK, LDH, GOT, and GPT were determined in our department of laboratory medicine according to standard techniques.

Statistical analysis. The statistical analysis was carried out using the nonparametric Mann-Whitney test. The results are expressed as means ± SD. Correlations between CK and DPH fluorescence anisotropy were determined by least squares linear regression analysis. The significance test of the correlation coefficient r was carried out according to Fisher (32).

RESULTS

The fluorescence anisotropy measured revealed no differences in DMD fibroblasts (Table 1) and DMD erythrocyte ghosts (Table 2) *versus* controls.

In DMD lymphocytes fluorescence anisotropy measured with DPH showed a difference between DMD patients and controls, while measurements with the other labels showed no difference (Table 3). The standard deviation of the DPH anisotropy values was increased in DMD *versus* control values (Table 3). In order to find an explanation for this phenomenon, the CK activity in DMD serum was correlated with the DPH fluorescence anisotropy value in DMD lymphocytes isolated from the same blood sample. With increasing CK activity in sera the fluorescence anisotropy in lymphocytes decreased (Fig. 1). The correlation coefficient between CK activity and DPH fluorescence anisotropy in DMD was the same after 10 as it was after 30 min

Table 1. *Fluorescence anisotropy studies of DMD fibroblasts and control fibroblasts (26° C)*

Probe	Subject	Anisotropy	*n*
DPH	DMD	0.159 ± 0.014*	6
	Controls	0.164 ± 0.020*	7
TMA-DPH	DMD	0.272 ± 0.008*	9
	Controls	0.273 ± 0.006*	10
6-AS	DMD	0.142 ± 0.004*	9
	Controls	0.139 ± 0.007*	10
9-AS	DMD	0.119 ± 0.002*	6
	Controls	0.120 ± 0.004*	7
12-AS	DMD	0.085 ± 0.003*	9
	Controls	0.083 ± 0.003*	9
16-AP	DMD	0.047 ± 0.002*	6
	Controls	0.049 ± 0.005*	4

* NS.

Table 2. *Fluorescence anisotropy studies of DMD erythrocyte ghosts and control erythrocyte ghosts (37° C)*

Probe	Subject	Anisotropy	*n*
DPH	DMD	0.215 ± 0.008*	6
	Controls	0.212 ± 0.011*	17
TMA-DPH	DMD	0.272 ± 0.002*	6
	Controls	0.269 ± 0.011*	17
6-AS	DMD	0.174 ± 0.005*	6
	Controls	0.172 ± 0.010*	17
7-AS	DMD	0.172 ± 0.005*	6
	Controls	0.166 ± 0.011*	17
9-AS	DMD	0.154 ± 0.004*	6
	Controls	0.152 ± 0.011*	17
12-AS	DMD	0.112 ± 0.003*	6
	Controls	0.109 ± 0.008*	17
16-AP	DMD	0.053 ± 0.010*	6
	Controls	0.052 ± 0.009*	16

* NS.

Table 3. *Fluorescence anisotropy studies of DMD lymphocytes and control lymphocytes (21° C)*

Probe	Subject	Anisotropy	*n*
DPH 10 min*	DMD	0.212 ± 0.028†	10
	Controls	0.231 ± 0.012†	60
DPH 30 min*	DMD	0.210 ± 0.023†	10
	Controls	0.223 ± 0.011†	60
TMA-DPH	DMD	0.304 ± 0.009‡	9
	Controls	0.309 ± 0.010‡	60
6-AS	DMD	0.172 ± 0.014‡	8
	Controls	0.174 ± 0.008‡	50
7-AS	DMD	0.167 ± 0.010‡	8
	Controls	0.167 ± 0.010‡	50
9-AS	DMD	0.142 ± 0.012‡	8
	Controls	0.148 ± 0.009‡	50
12-AS	DMD	0.106 ± 0.007‡	8
	Controls	0.111 ± 0.009‡	50
16-AP	DMD	0.104 ± 0.021‡	8
	Controls	0.099 ± 0.010‡	50

* Incubation time.
† $p < 0.05$.
‡ NS.

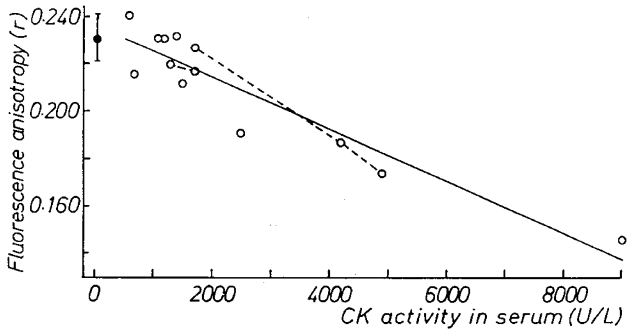

Fig. 1. CK activity in sera correlated with DPH fluorescence anisotropy (label incubation time 10 min) in lymphocytes from DMD patients (○, $n = 10$). In two DMD patients CK activity and fluorescence anisotropy were measured in several blood samples taken at various times from the same patient (○---○). The correlation coefficient of the linear regression analysis was $r = -0.93$ ($p < 0.001$). Controls (●, $n = 60$, mean ± SD).

incubation time of DPH ($r = -0.93$, $p < 0.001$, $n = 10$). Similar correlations between enzymes and fluorescence anisotropy were found with GOT ($r = -0.90$, $p < 0.001$), LDH ($r = -0.86$, $p < 0.001$), and GPT ($r = -0.76$, $p < 0.01$). There was no significant correlation between the age of the DMD patients and fluorescence anisotropy. In a second control group, the lymphocytes of patients with different disorders were measured. A patient with necrotizing encephalitis and myoclonia showed increased enzyme activities in serum (CK 2130 U/liter, LDH 1278 U/liter, GOT 140 U/liter, GPT 73 U/liter), DPH fluorescence anisotropy in lymphocytes was within the normal range (0.223, result of 10 min DPH incubation). Three patients with congenital myopathy showed normal DPH fluorescence anisotropy values in lymphocytes (range 0.229–0.238, results of 10 min DPH incubation), while one showed elevated enzyme activities in serum (CK 536 U/liter, LDH 414 U/liter, GOT 38 U/liter, GPT 24 U/liter). DMD lymphocytes and control lymphocytes were incubated in DMD sera and in control sera. Before incubation in sera, the initial values of DPH fluorescence anisotropy in the DMD lymphocyte membranes were again lower than in the controls (Fig. 2). After incubation in DMD sera, fluorescence anisotropy values of DMD lymphocytes and control lymphocytes decreased to the same level (Fig. 2). When incubated in control sera, fluorescence anisotropy values of DMD lymphocytes increased, and the values of control lymphocytes decreased (Fig. 2). Corresponding to the wide range of CK activity in DMD sera (mean = 1829 ± 1159, $n = 14$), the standard deviation of fluorescence anisotropy after incubation in DMD sera was greater than in the control sera (Fig. 2). CK activity in the control sera was 37 U/liter ± 21 ($n = 10$). Incubations of two control lymphocyte samples in human control sera enriched with rabbit muscle CK (CK levels between 1800 and 50000 U/liter) showed no effect on fluorescence anisotropy after 2 and 4 h incubation in these sera.

DISCUSSION

When examining membrane fluidity in DMD nonmuscle cells by fluorescence anisotropy, we found no differences in intact DMD fibroblasts (Table 1). Shaw *et al.* (4) examined isolated fibroblast membranes and found an increased membrane fluidity, measured by fluorescence polarization of DPH and by electron spin resonance. Although we use different methods, our results do not support these findings.

In DMD erythrocyte ghosts membrane fluidity was also within the control range (Table 2). These findings are in accordance with Clark *et al.* (10) and Butterfield *et al.* (9) who studied red cell membrane fluidity by electron spin resonance and found no differences in lipid fluidity of DMD erythrocytes *versus* controls. However, other results do exist which indicate membrane fluidity changes in DMD erythrocytes (7, 8).

Our results in intact DMD lymphocytes are more complex. We found an increased membrane fluidity in DMD lymphocytes only when DPH was used as a probe (Table 3). TMA-DPH (24, 28) and several isomers of anthroyloxy fatty acids (29, 30) probing different depths of the bilayer did not reveal any differences between the levels of fluorescence anisotropy in DMD and control lymphocytes (Table 3). At first glance, there is a discrepancy between the data obtained with DPH and 12-AS as both labels are localized in the hydrophobic membrane interior. However, we have to take into account that DPH anisotropy rather reflects the static parameter of lipid motions (when the anisotropy exceeds 0.100) (26) whereas anthroyloxy fatty acids monitor to a large extent lipid motion itself (30). We therefore believe that DMD lymphocyte membranes exhibit a different degree of lipid order in the internal membrane region as compared to controls. An explanation for this difference in lipid order is beyond the scope of this study.

An alternative interpretation would be that the membrane permeability (18, 33) for DPH is increased in DMD compared

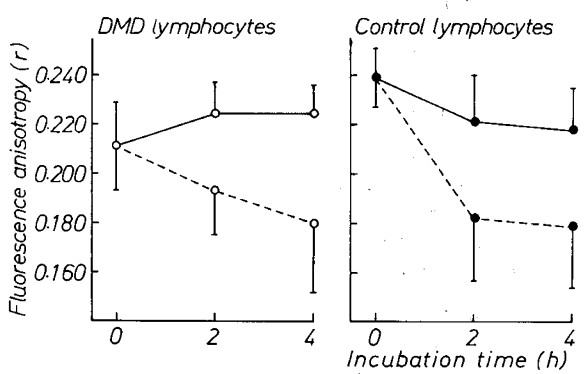

Fig. 2. DPH fluorescence anisotropy after incubation of DMD lymphocytes in control sera (○——○, $n = 4$) and in DMD sera (○---○, $n = 5$) and after incubation of control lymphocytes in control sera (●——●, $n = 22$) and in DMD sera (●---●, $n = 9$). The fluorescence anisotropy values after 4 h incubation in control *versus* DMD sera differed significantly ($p < 0.05$ for DMD lymphocytes, $p < 0.005$ for control lymphocytes).

with control lymphocytes. Our finding that fluorescence anisotropy in DMD lymphocytes did not change from 10 to 30 min DPH incubation time but did change in control lymphocytes (Table 3, 0.231 ± 0.012 to 0.223 ± 0.011, $p < 0.005$), is compatible with this view. Membrane fluidity strongly influences the passive bilayer permeability for nonelectrolytes, so that high fluidities are connected with enhanced diffusion rates of the solutes (18, 33).

Correlations between capping capacity and DPH fluorescence anisotropy values in lymphocytes are well documented. Hoover *et al.* (34) and Ben-Bassat *et al.* (35) (linear regression analysis of the reported data in Ben-Bassat's report was carried out by us, $r = 0.81$, $p < 0.05$) revealed that capping capacity and fluorescence depolarization of DPH showed a positive linear correlation, indicating that with decreasing DPH fluorescence anisotropy (*i.e.* increasing membrane fluidity) capping capacity decreases in murine (34) and human leukemic lymphocytes (35). Our results showing decreased DPH fluorescence anisotropy (*i.e.* increased membrane fluidity) are in accordance with the findings of a diminished capping capacity in DMD lymphocytes (11–14).

We found a negative correlation between CK in sera and DPH fluorescence anisotropy in DMD patients, which suggests that there is an increasing membrane fluidity in DMD lymphocytes with increasing CK levels in sera (Fig. 1). Sensi *et al.* (13) found a negative correlation between CK activity in sera and capping capacity in DMD lymphocytes. The results of Ho *et al.* (12) also showed a negative correlation between CK activity in sera and capping capacity in DMD lymphocytes (linear regression analysis of the reported "Bcap" data was carried out by us, $r = -0.80$, $p < 0.01$). Considering the above mentioned correlation between DPH anisotropy and capping in lymphocytes (34, 35), our negative correlation between CK level and fluorescence anisotropy fits in with the reported findings of a negative correlation between CK activity and capping capacity (12, 13).

When incubated in DMD sera, DMD lymphocytes and control lymphocytes decreased to the same level of fluorescence anisotropy (Fig. 2). When incubated in control sera, DMD lymphocytes increased their membrane fluidity, while control lymphocytes showed decreased fluorescence anisotropy values (Fig. 2). Other experiments using non-DMD sera with elevated CK activity levels showed no influence of CK on membrane fluidity. The fact that CK level and lymphocyte membrane fluidity in DMD are significantly correlated (Fig. 1) and the fact that the elevated CK (and other elevated enzymes) originate from the dystrophic muscle (1), suggesting a "leak" in the muscle membrane, is compatible with the hypothesis that a toxic or defective serum factor attacks the lymphocyte membrane. This serum factor may originate from the dystrophic muscle. On the other hand, this serum factor, which possibly is a product of lipoxygenase activity (36), may simultaneously attack lymphocyte membranes and muscle membranes. Due to the fact that we found normal membrane fluidity in DMD fibroblasts and erythrocyte ghosts, we do not agree with the hypothesis that there is a general membrane abnormality in DMD.

Hunter and Mohamed (37) found elevated lipid peroxidation products in plasma from DMD patients. They also discuss a circulating toxic factor and suggest "that circulating lipid peroxides or their breakdown products might well be candidates for such factors" (37). We are now looking for experimental data that link chemical changes to our biophysical findings.

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Announcements

Abstract Deadline

The American Pediatric Society and the Society for Pediatric Research announce the abstract deadline for the 1988 Annual Meeting (May 2–6, Washington Sheraton Hotel, Washington, D.C.) has been set as *December 10, 1987. For further information contact:* **SPR**—Debbie L. Wogenrich, The Society for Pediatric Research, 2350 Alamo S.E., Suite 106, Albuquerque, NM 87106 (505)764-9099. **APS**—Dr. Audrey K. Brown, Secretary-Treasurer, Department of Pediatrics, Box 49, SUNY, Health Sciences Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, NY 11203, (718)270-1692.

Mechanisms and Management of Pediatric Hepatobiliary Disease

The conference will be held on February 28–March 1, 1988 at the Hyatt Regency Crystal City at Washington National Airport, 2799 Jefferson Davis Highway, Arlington, VA. The Conference is sponsored by the National Digestive Diseases Advisory Board, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Child Health and Human Development, and the American Liver Foundation.

The program is disigned for basic and clinical investigators and physicians in practice. The registration fee of $50.00 includes participation in all plenary and workshop sessions and three working lunches during the conference. To receive registration materials, please send name, title, affiliation, mailing address, and telephone number to Ms. Marti Bernstein, Prospect Associates, Suite 500, 1801 Rockville Pike, Rockville, MD 20852 or call the Conference Registrar at (301) 468-MEET. Limited travel funds may become available for young investigators. Those wishing to be considered should submit a letter of application to Ms. Bernstein, including reasons for wanting to attend the conference, its relevance to your work, a brief CV, and a letter from your preceptor or a senior associate. Applications for travel assistance must be received by December 1, 1987.