A Membrane Fluidizing Factor in Sera from Duchenne Muscular Dystrophy Patients: Effect on Lymphocyte Membranes of Incubation in Patient and Control Lipoproteins

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ABSTRACT. Sera from Duchenne muscular dystrophy (DMD) patients showed a membrane fluidizing effect on DMD and control lymphocytes. To look for the cause of this membrane fluidizing effect, human lymphocytes from healthy subjects were incubated in sera and in different serum fractions from DMD patients and healthy control subjects, and membrane fluidity was determined by steady state fluorescence polarization of the probe diphenyl hexatriene. DMD sera and total DMD lipoproteins showed a similar membrane fluidizing effect ($p < 0.02$) after incubation, whereas lipoprotein deficient serum did not show any effect on membrane fluidity. Control and DMD HDL showed a membrane fluidizing effect, the fluidizing effect of DMD HDL being slightly higher as compared to control HDL. Control LDL and DMD LDL showed a membrane rigidifying effect, the rigidifying effect of DMD LDL being significantly lower as compared to control LDL ($p < 0.02$). These data demonstrate that the membrane fluidizing effect of DMD serum is lipoprotein associated, LDL being the most important mediator of this effect. (*Pediatr Res* 25: 656-659, 1989)

Abbreviations

DMD, Duchenne muscular dystrophy
DPH, 1,6-diphenyl-1,3,5-hexatriene
LPDS, lipoprotein-deficient serum

The defective gene in DMD has been isolated (1), and the deficient gene product dystrophin has been characterized (2, 3). Dystrophin is expressed in all types of normal muscle cells (4, 5) whereas, except for central nervous system tissues, dystrophin expression in normal nonmuscle tissues correlates with smooth muscle myosin expression in these respective tissues (5). In DMD muscle biopsy specimens, dystrophin levels were less than 3% of normal levels (6). Dystrophin is localized at the cytoplasmic face of the plasma membrane of muscle fibers (7-10) and shows some chemical and structural analogies with spectrin and with α -actinin (3) probably indicating a cytoskeleton function (3). The localization of dystrophin in muscle cell plasma membranes (7) and the analogies of dystrophin to spectrin (3) may correlate to

other previous observations of "delta" lesions in DMD muscle membranes (11, 12). Erythrocyte spectrin is known to play an important role in membrane stability and fluidity (13, 14) and in maintenance of phospholipid asymmetry (15).

By contrast, indications for a defect in intracellular redox systems in DMD muscle cells are given by several studies. In avian muscular dystrophy, increased turnover of oxygen-derived free radicals is observed in the dystrophic muscle (16), and, in rat muscle cells, correlations between lipoxigenase activity and creatine kinase efflux are described (17). In DMD plasma lipid peroxidation products are elevated (18). Thus, it might be possible that dystrophin deficiency not only influences muscle membrane fluidity, but, as a secondary consequence lipid peroxidation in muscle cells and in serum fractions as well.

In a previous study, we showed that plasma membranes of DMD nonmuscle cells are not primarily affected, but that DMD sera contain a membrane-fluidizing factor (19). In the present study, we try to identify the membrane-fluidizing factor of DMD sera further, examining the influence of lipoproteins and of the different lipoprotein fractions on membrane fluidity. The results are discussed with respect to published alteration of DMD lipoproteins and to oxidation defects in DMD.

MATERIALS AND METHODS

Intact human lymphocytes from healthy subjects were isolated by Ficoll density gradient centrifugation according to Böyum (20). DPH fluorescence anisotropy was measured at 21°C in a Shimadzu spectrofluorophotometer RF 540. The preparation steps were done as described previously (19). Lymphocytes were incubated in the fluorescence marker DPH (21°C) for 10 min and for 20 min. Total fluorescence intensities (21, 22) were monitored to detect changes in fluorescence lifetime of DPH in lymphocytes after incubation in DMD *versus* control sera and serum fractions. In none of the measurements were such changes observed. Lipoproteins and LPDS were isolated by density gradient centrifugation using a modification of the method of Redgrave *et al.* (23). The density cuts were as follows: VLDL ($d < 1.019$ g/mL), LDL ($d = 1.045$ to 1.065 g/mL), HDL ($d = 1.095$ to 1.130 g/mL), and LPDS ($d > 1.150$ g/mL).

Incubations of lymphocytes in sera and in serum fractions were carried out at 4°C (19). For incubation experiments in total lipoproteins, DMD lipoproteins were added to control LPDS and vice versa. For incubation experiments in HDL, LDL, and VLDL, the respective DMD and control lipoprotein fractions were adjusted to an equal protein content with control LPDS. There were no differences in fluorescence anisotropy values between the incubation experiments performed with control

Received August 29, 1988; accepted February 8, 1989.

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Supported by Deutsche Forschungsgemeinschaft (Hu 408/1-4). A portion of this study is thesis work for U.W.

versus DMD LPDS-adjusted lipoprotein fractions nor with autologous versus heterologous LPDS-adjusted lipoprotein fractions.

The age of the DMD patients was 10 ± 4 y, the age of the controls was 20 ± 9 y. The results correlated neither with the age of the control subjects nor with the age of the DMD patients nor with the general health conditions of the DMD patients.

Total cholesterol, free cholesterol, triglycerides, and phospholipids in sera and serum fractions were determined by standard enzymatic colorimetric methods (Diagnostica, Boehringer Mannheim GmbH, West Germany). Cholesteryl esters were estimated by comparing total cholesterol and free cholesterol contents. Extraction of lipids and determination of fatty acid methyl esters of lipoprotein fractions were done as described previously (24). Apoproteins were determined by single radial immunodiffusion (combi R.I.D., Immuno AG, Vienna, Austria).

Statistical analysis was carried out using the nonparametric Mann-Whitney test. The results are expressed as means $\pm$ SD.

RESULTS

After incubation in DMD sera, DPH fluorescence anisotropy in lymphocyte plasma membranes was decreased as compared to the values after incubation in control sera (Fig. 1). After incubation in DMD total lipoproteins, fluorescence anisotropy in lymphocytes was decreased as compared to the values after incubation in control lipoproteins (Fig. 1). These data indicate a membrane fluidizing effect of DMD sera and lipoproteins. After incubation in DMD and control LPDS, no effect on DPH fluorescence anisotropy in lymphocytes was observed, indicating no change in membrane fluidity (Fig. 1).

After incubation in control HDL, fluorescence anisotropy values decreased as compared to initial values ($p < 0.02$ for DPH 10 min and for DPH 20 min anisotropy values), indicating a membrane fluidizing effect of HDL (Fig. 1). When comparing the incubation effects of DMD versus control HDL, anisotropy values of DMD HDL were slightly lower versus controls (Fig. 1).

After incubation in control LDL, fluorescence anisotropy values increased as compared to initial values ($p < 0.0002$) indicating a membrane rigidifying effect of LDL (Fig. 1). When comparing the incubation effects of DMD versus control LDL, anisotropy values of DMD LDL were significantly lower versus controls (Fig. 1).

After incubation in control VLDL, fluorescence anisotropy values decreased as compared with initial values ($p < 0.02$),

Fig. 1. Fluorescence anisotropy (21°C) of DPH 10 min (marker incubation time, ○) and of DPH 20 min (●) in plasma membranes of intact human lymphocytes from healthy subjects after incubation in control (—) vs. DMD (---) sera (controls $n = 9$, DMD $n = 10$), lipoprotein deficient sera (LPDS; controls $n = 7$, DMD $n = 9$), lipoproteins (controls $n = 9$, DMD $n = 10$), and lipoprotein fractions (controls $n = 7$, DMD $n = 9$), respectively; p values for DMD vs. control data after a 4 h of incubation.

indicating a membrane fluidizing effect of VLDL (Fig. 1). The incubation effects of DMD versus control VLDL on fluorescence anisotropy showed nearly no difference (Fig. 1).

Contents of cholesterol, triglycerides, phospholipids, and proteins in DMD lipoprotein fractions were not different from controls (Table 1). Fatty acid composition of DMD HDL was normal. In DMD, content of LDL C18:1 was increased, and in DMD VLDL, the contents of several fatty acids were different as compared to control VLDL (Table 2). In none of the DMD lipoprotein fractions were the wt ratios of saturated to cis-

Table 1. Composition of lipoprotein fractions used for the lymphocyte incubation experiments (mg/mL serum)*

	DMD-HDL	Control-HDL
Free cholesterol	0.10 ± 0.04 (6%)	0.10 ± 0.04 (4%)
Cholesteryl esters	0.35 ± 0.06 (19%)	0.52 ± 0.09 (20%)
Triglycerides†	0.11 (6%)	0.07 ± 0.03 (3%)
Phospholipids	0.37 ± 0.11 (20%)	0.56 ± 0.18 (22%)
Apoprotein A-I	0.7 ± 0.2 (38%)	1.0 ± 0.4 (39%)
Apoprotein A-II	0.2 ± 0.1 (11%)	0.3 ± 0.1 (12%)
	DMD-LDL	Control-LDL
Free cholesterol	0.14 ± 0.05 (6%)	0.17 ± 0.04 (7%)
Cholesteryl esters	1.03 ± 0.26 (47%)	1.19 ± 0.24 (47%)
Triglycerides†	0.13 (6%)	0.13 ± 0.02 (5%)
Phospholipids	0.30 ± 0.07 (14%)	0.33 ± 0.11 (13%)
Proteins	0.59 ± 0.12 (27%)	0.72 ± 0.21 (28%)
	DMD-VLDL	Control-VLDL
Free cholesterol	0.04 ± 0.02 (3%)	0.03 ± 0.02 (4%)
Cholesteryl esters	0.26 ± 0.17 (17%)	0.16 ± 0.06 (19%)
Triglycerides†	1.03 (67%)	0.48 ± 0.21 (58%)
Phospholipids	0.08 ± 0.06 (5%)	0.08 ± 0.05 (10%)
Proteins	0.12 ± 0.06 (8%)	0.08 ± 0.04 (10%)

* $n = 9$ for DMD and $n = 7$ for control samples except for determinations of † triglycerides ($n = 2$ for DMD and $n = 3$ for controls).

Table 2. Relative contents of fatty acids of lipoprotein fractions used for the lymphocyte incubation experiments*

Subject	Fatty acids	Wt % (HDL)	Wt % (LDL)	Wt % (VLDL)
DMD	16:0	23.8 ± 4.0	18.9 ± 3.3	27.5 ± 2.1 †
Controls		19.9 ± 3.0	24.1 ± 5.3	19.7 ± 5.7
DMD	18:0	11.6 ± 1.8	8.6 ± 1.8	9.3 ± 1.9
Controls		10.9 ± 1.7	11.5 ± 2.5	9.1 ± 1.4
DMD	18:1‡	17.3 ± 3.1	19.7 ± 1.2 †	29.7 ± 3.3 §
Controls		14.8 ± 1.6	15.1 ± 2.9	20.7 ± 7.9
DMD	18:2	18.1 ± 5.0	21.7 ± 7.2	9.8 ± 3.7 §
Controls		21.9 ± 6.0	15.3 ± 5.1	14.8 ± 2.6
DMD	20:3 (n-6)¶	1.0 ± 0.5	0.7 ± 0.2	0.4 ± 0.3
Controls		1.1 ± 0.5	0.8 ± 0.4	0.7 ± 0.2
DMD	20:4 (n-6)**	3.6 ± 1.8	1.9 ± 1.3	0.9 ± 0.6 †
Controls		3.4 ± 1.4	1.7 ± 0.6	1.7 ± 0.5
DMD	22:0	0.3 ± 0.1	0.6 ± 0.3	0.3 ± 0.2
Controls		0.5 ± 0.3	1.0 ± 0.9	0.6 ± 0.6
DMD	22:6 (n-3)††	0.9 ± 0.5	0.3 ± 0.2	0.3 ± 0.2
Controls		0.9 ± 0.5	0.6 ± 0.8	0.6 ± 0.8
DMD	24:0	0.3 ± 0.1	0.5 ± 0.4	0.3 ± 0.2
Controls		0.4 ± 0.3	0.6 ± 0.4	0.5 ± 0.3

* $n = 7$ for DMD and for control HDL; $n = 4$ for DMD and for control LDL; $n = 7$ for DMD and $n = 5$ for control VLDL.

† $p < 0.05$ vs. controls.

‡ 18:1 (cis-9).

§ $p < 0.02$ vs. controls.

|| 18:2 (cis,cis-9,12).

¶ 20:3 (all cis-8,11,14).

** 20:4 (all cis-5,8,11,14).

†† 22:6 (all cis-4,7,10,13,16,19).

unsaturated fatty acids different from controls (data not shown). Protein content of total DMD lipoproteins (31.9 ± 4.4 mg/mL; $n = 5$) was not different from control lipoproteins (32.3 ± 5.7 mg/mL; $n = 7$).

DISCUSSION

After incubation in DMD sera, lymphocytes from DMD patients and from healthy controls showed an increased membrane fluidity due to a membrane fluidizing factor in DMD sera (19). Our present data on DMD serum incubation (Fig. 1) are in accordance with our previous results (19). The present incubation experiments demonstrate that DMD lipoproteins (Fig. 1), but not DMD LPDS, contain the membrane fluidizing factor which we previously proposed (19).

When using the different lipoprotein fractions, we found that after incubation in HDL, fluorescence anisotropy in lymphocytes decreased (Fig. 1); *i.e.* membrane fluidity increased, probably because cholesterol efflux from the cell is mediated by HDL (25). Our finding of an HDL-mediated fluidizing effect on lymphocyte membranes is in accordance with the finding that HDL-cholesterol has a fluidizing effect on erythrocyte membranes (26). The fluidizing effect of DMD HDL was slightly higher than that of control HDL (Fig. 1).

After incubation in the cholesterol-rich LDL (Table 1), fluorescence anisotropy increased (Fig. 1); *i.e.* membrane fluidity decreased, probably because cholesterol influx into cells is mediated by LDL (25). Our finding of a LDL-mediated rigidifying effect on lymphocyte membranes corresponds to the finding of a LDL-mediated increase of cholesterol content in lymphocyte (27) plasma membranes. An increased cholesterol content corresponds to increased DPH fluorescence anisotropy; *i.e.* decreased membrane fluidity (24). The rigidifying effect of DMD LDL was significantly lower than that of control LDL (Fig. 1).

Our findings demonstrate that the membrane fluidizing effect of DMD sera is lipoprotein-associated (Fig. 1). It is mainly caused by a less rigidifying effect of DMD LDL as compared to control LDL, but probably also by a more fluidizing effect of DMD HDL as compared to control HDL.

Regarding the incubation temperature of 4°C, our findings are not likely to be due to enzymatic reactions. A major difference of lipid (Table 1) or of the fatty acid composition (Table 2) of DMD lipoproteins is also not likely to be the cause of the membrane fluidizing effect. In DMD HDL, neither fatty acid composition nor the saturated to cis-unsaturated fatty acid ratio were different *versus* controls, and, though there was an elevated content of C18:1 in DMD LDL (Table 2), the saturated to cis-unsaturated fatty acid ratio was normal. Thus, a hitherto undetected difference in lipid composition of DMD LDL and HDL or an abnormal apoprotein could be responsible for the fluidizing effect of DMD lipoprotein fractions. Arthur *et al.* (28) found an increased electrophoretic mobility of DMD LDL. Increased electrophoretic LDL mobility can be due to an elevated content of the cis-unsaturated fatty acid C18:1 (29), to a structural aberration in the protein moiety (28), or to an increased lipid oxidation (30). We assume that the finding of Arthur *et al.* (28) of an abnormal electrophoretic mobility of DMD LDL and our finding of a membrane fluidizing effect of DMD LDL have the same biochemical cause. An increased C18:1 content (Table 2), therefore, is not likely to be the cause of the abnormal electrophoretic mobility (28), for, if it were the cause of the membrane fluidizing effect of DMD LDL, one would expect a decrease of the saturated to cis-unsaturated fatty acid ratio (24). An abnormal apoprotein also is not likely to be the cause of the abnormal electrophoretic mobility (28) nor of the fluidizing effect, as all the genes of the human apolipoproteins so far cloned are located on several autosomes, but not on chromosome X (31). Thus, increased lipid oxidation is most likely the cause of the abnormal electrophoretic mobility (28) and the fluidizing effect of DMD LDL. This hypothesis is in accordance with the findings of elevated products of lipid peroxidation in DMD sera (18).

Steinbrecher *et al.* (30) demonstrated that cultured endothelial cells modify LDL in a way that leads to an increase of its electrophoretic mobility. They also showed that this modification of LDL involved free radical-initiated LDL peroxidation (30). In analogy to the findings of Steinbrecher *et al.* (30), it might be possible that DMD muscle tissue modifies LDL in a similar way. According to Morel *et al.* (32), oxidized LDL might be an important link between oxidative free radical generation and tissue damage. Although our results of the fluidizing effect of DMD LDL on lymphocytes do not correlate with the clinical stage of the patients, tissue damage by oxidized LDL nevertheless might add to the progradient clinical course of DMD.

Although we so far cannot further identify the membrane-fluidizing component in DMD LDL or HDL, it seems worthwhile to study in detail lipids and lipid oxidation in LDL and HDL from DMD patients. The pathogenesis of the clinical progression of DMD might be related to an interaction between dystrophin deficiency and lipoproteins.

Acknowledgments. The authors thank Dr. R. Beckmann, Department of Pediatrics, University of Freiburg, West Germany; Dr. E. Boltshauser, Department of Pediatrics, University of Zurich, Switzerland; Dr. F. Hanefeld, Department of Pediatrics, University of Göttingen, West Germany; Dr. H. G. Lenard, Department of Pediatrics, University of Düsseldorf, West Germany; Dr. W. Mortier, Children's Hospital, Wuppertal, West Germany for supplying biological material from DMD patients. The excellent technical assistance of M. Knorre and I. Wernicke is gratefully acknowledged.

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