Hum Genet (1985) 69:332–336

Human Genetics

Progressive muscular dystrophy (Duchenne): Biochemical studies by flow-cytometry

M. Hirsch-Kauffmann[1], G. Valet[2], J. Wieser[1], and M. Schweiger[1]

[1]Institut für Biochemie der Universität, A-6020 Innsbruck, Austria
[2]Max-Planck-Institut für Biochemie, D-8033 Martinsried, Federal Republic of Germany

Summary. The peanut lectin (PNL) receptor density of the cell membrane and several metabolic parameters of cultured fibroblasts of normal human individuals and of patients with muscular dystrophy were measured by simultaneous two and three parameter flow cytometry. The PNL-receptor density was significantly decreased on muscular dystrophy fibroblasts (between 20.7 and 33.6%) as compared to normal fibroblasts. The cell volume, the esterase activity, the intracellular pH, and the percentage of proliferating cells of both types of fibroblasts were not significantly altered. The mean cell volume of different fibroblast cultures varied between 2500 and 6000 μm^3. The concentration of the intracellular esterase activity of fibroblasts was low (0.169 relative units) as compared to lymphocytes and granulocytes of the peripheral blood (1.56 and 2.17 relative units). The fibroblasts had an acidic intracellular pH of 6.52 while lymphocytes and granulocytes had basic pH values of 7.30 and 7.17. Some of the fibroblasts were in the S+G2/M phase of the cell cycle (20%). The study shows that the measurement of biochemical parameters of vital and fixed single fibroblasts by flow-cytometry is of great interest for the recognition of differences between normal individuals and muscular dystrophy patients.

Introduction

Progressive muscular dystrophy (Duchenne; DMD) is a gonosomal hereditary disease (for reviews see Rowland 1977; Lunt and Marchbanks 1978; Witkowski and Jones 1981; Ebashi and Ozawa 1983; Moser 1984). The disease affects approximately 1 in 3000 male births; 30% of the cases being due to new mutations. A progressive degeneration of muscles leads to the death of the patient in late childhood or early adolescence. The biochemical defect is unknown: a specific myopathic defect as well as a basic metabolic defect are generally considered to be involved. For ethical reasons, biochemical studies on muscular dystrophy are restricted, and although several animal models are available there is uncertainty as to their comparability with human DMD.

The possibility of employing human fibroblast cell cultures for investigations into the molecular defect in DMD means the beginning of a new era in research on this hitherto baffling disease. Several attempts have been made to find differences between fibroblasts from DMD patients and from normal probands. Since the results are to some extent unequivocal (Witkowski and Jones 1981), a thorough study of the physiology of fibroblasts from DMD patients and normal probands was undertaken. The fact that fibroblasts can be grown under standardized cell culture conditions renders them eminently suitable for biochemical analysis.

A new approach to the analysis of such cultures is the use of flow-cytometric methods. Biochemical parameters of individual cells, such as lectin receptor densities (Malin-Berdel et al. 1984; Siegert et al. 1980), enzyme activities (Malin-Berdel and Valet 1980), intracellular pH (Valet et al. 1981), and cell volume can be simultaneously measured at high speed. The purpose of this study was to explore whether, using flow cytometric measurements, it is possible to distinguish between fibroblasts from normal individuals and fibroblasts from patients with Duchenne muscular dystrophy.

Offprint requests to: M. Hirsch-Kauffmann

Materials and methods

Cell strains and culture methods

Cells were derived from skin biopsies from eight boys with clinically manifested DMD, ages ranging from 3–12 years. MDA and MDB are siblings. Corresponding control cells were taken from healthy persons, six males and one female. The cells were propagated in minimal essential medium (MEM) supplemented with 10% fetal calf serum at 37° C in air with 5% CO_2. Cells were taken for analysis either after having reached confluency or two days after trypsinisation and reseeding 5×10^5 cells into 100 ml polycarbonate culture flasks. The number of passages never exceeded 20 and all cultures were free of mycoplasma. The cells were mechanically scraped off from the culture flasks with a rubber spatula, washed twice in a 0.15 *M* NaCl solution buffered with 10 m*M* Tris/HCl to pH 7.35 (TBS), and resuspended at a concentration of 5×10^6/ml in TBS. The cells were either stained immediately or after fixation with formaldehyde. The cells were fixed by 1:1 (v/v) dilution of the cell suspension with a 3.5% formaldehyde solution in TBS. The formaldehyde solution was freshly diluted from a 35% stock solution (Merck, Darmstadt, Federal Republic of Germany, 1 part + 9 parts of TBS), cooled to 0° C, and adjusted to pH 7.35 before addition to the cell suspension. Cells were fixed at 0° C overnight, washed twice with 10 ml TBS to remove remaining formaldehyde, and resuspended in TBS at a concentration of 1.5×10^6/ml.

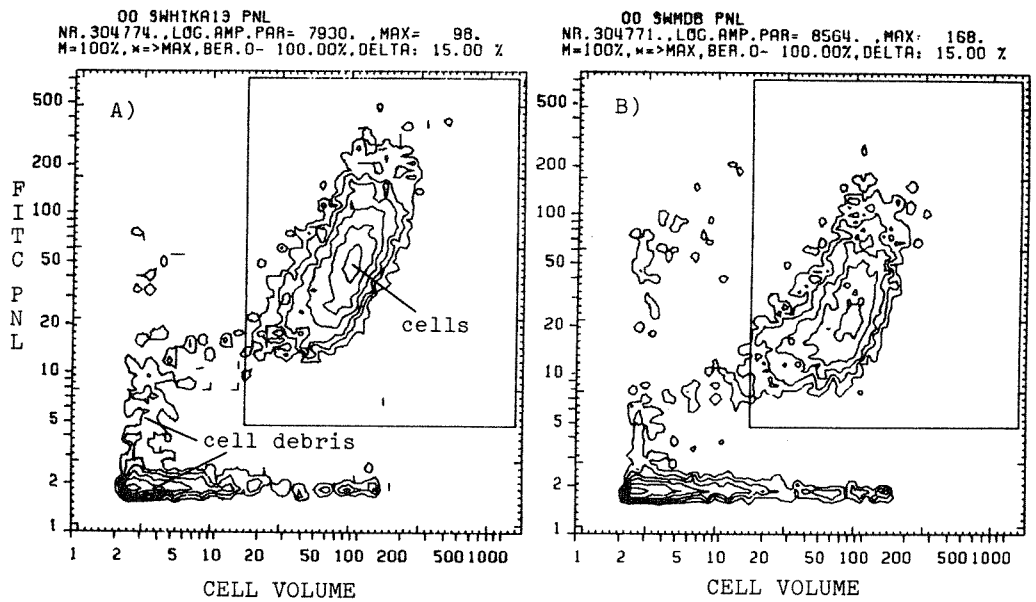

Fig. 1A, B. Cell volume versus cell-bound FITC-PNL of fibroblasts of a normal individual (**A**) and of a patient with muscular dystrophy (**B**). One volume unit on the logarithmic scale corresponds to 33.8 μm^3. The logarithmic fluorescence scale is graded in relative fluorescence units. The contour lines were calculated from the logarithmic channel contents of each histogram channel, starting at the maximum channel content as 100%. Contour lines were plotted in equal steps of 10% of the maximum logarithmic amplitude downwards. The mean FITC-PNL surface density of the cell cluster delimited by the straight lines was calculated in relative units as 1.089 for the normal fibroblasts (**A**) and as 0.735 for the fibroblasts of the muscular dystrophy patients (**B**). The mean cell volumes of the fibroblasts were 2556 μm^3 and 2857 μm^3, and 7930 and 8564 cells were measured per histogram, respectively

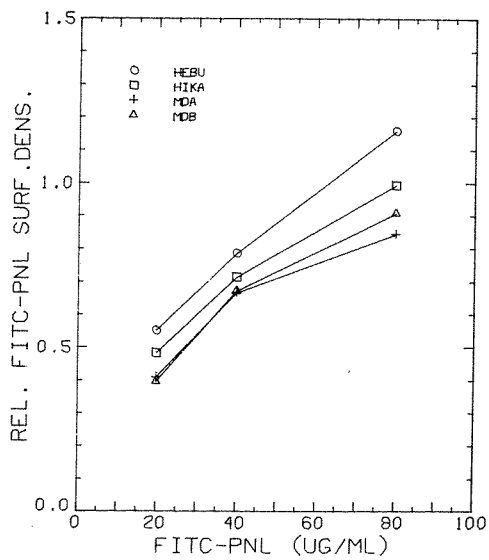

Fig. 2. Concentration dependence of FITC-PNL binding to fibroblasts of normal human individuals (HEBU, HIKA) and to fibroblasts of patients with muscular dystrophy (MDA, MDB). The binding curves have a similar shape indicating that the decreased FITC-PNL binding to the MDA and MDB cells is not due to a different affinity of PNL to the cell membrane receptor but to the decreased density of the receptor on the cell surface of fibroblasts from muscular dystrophy patients

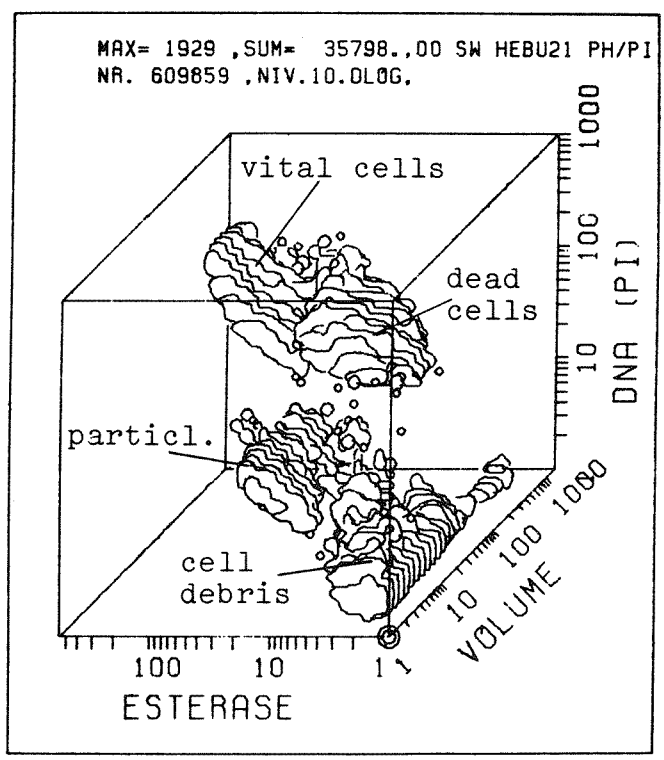

Fig. 3. Cloud display of a simultaneous three parameter measurement of ADB/PI-stained normal human fibroblasts. The vital and dead cells, the calibration particles, and the cell debris are visible. The contour lines surround the space where cells and particles are present. They are calculated from the logarithmic channel contents of the three parameter histogram by setting the maximum channel content as 100% and plotting contour lines at 10% of this level. The contour lines include histogram channels with two particles, which means that the locations of most of the 35798 cells of the histogram are displayed

FITC-PNL staining

Two hundred and fifty µl fresh or fixed cells were incubated with 10µl fluoresceinisothiocyanate coupled peanut lectin (PNL) solution (1mg/ml, FITC-PNL, F/P ratio 1.8 (mol/mol)), for 2h at 0°C in TBS. PNL (Boehringer, Mannheim) was fluorescinated by incubation with FITC, in a 50m*M*

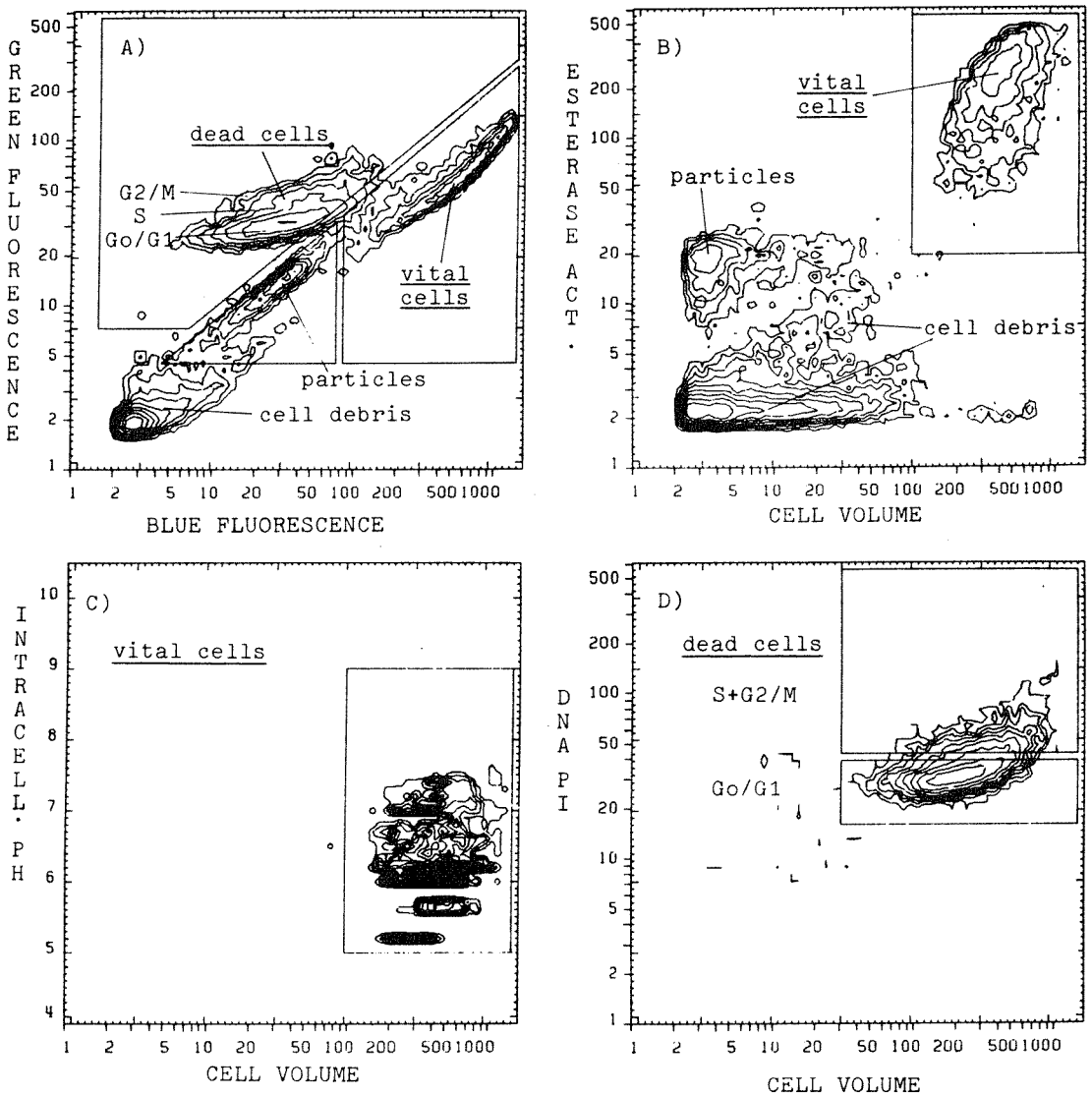

Fig. 4A–D. Esterase versus DNA histogram (**A**) of the cloud display of Fig. 3. The two parameter histogram was obtained by orthogonal projection of Fig. 3 onto the esterase-DNA-plane (blue versus green fluorescence). The areas limited by *straight lines* were used for the calculation of the absolute cell concentration (1.24×10^6/ml) and the percent vital (22.8%) and dead cells (77.2%) in the cell suspension. The cell volume versus esterase histogram (**B**) of the vital cells was calculated from Fig. 3 by projecting only the vital cells and the calibration particles of Fig. 4A onto the cell-volume–esterase-plane. The mean esterase concentration of the vital cells was calculated within the delimited area (0.214 relative units). The cell volume versus intracellular pH histogram of the vital cells (**C**) was calculated from the cloud plot by calculating the ratio between blue and green fluorescences for the vital cells only. The cell volume versus DNA histogram of the dead cells (**D**) was calculated by projecting the dead cell of the cloud plot onto the cell-volume–DNA-plane. The number of cells in the G0/Gl (85.2%) and the S+G2/M phase (14.8%) of the cell cycle was determined from the cells in the two delimited areas. One logarithmic cell volume class corresponds to 13 μm^3. The fluorescence scales are in relative logarithmic units

$NaHCO_3$ buffer pH 9.5 for 3h at 0°C, followed by dialysis against TBS with 0.01% sodium azide to remove free FITC and to preserve the FITC-PNL against microbial degradation.

ADB/PI stainings

Two hundred and fifty µl fresh cells were incubated for 5 min at room temperature with 5 µl of a stain cocktail containing 1,4-diacetoxy-2,3-dicyano-benzene (ADB, Paesel, Frankfurt, Federal Republic of Germany) 1 mg/ml dissolved in dimethylformamide (DMF), and propidium iodide (PI, Sigma Chemicals, St. Louis, USA) 2 mg/ml. ADB permeates the cell membrane and is cleaved by intracellular esterases into the pH-indicator dye DCH (1,4-dihydroxy-2,3-dicyano-hydrochinon) and acetate. DCH accumulates in vital cells. The intensity of its fluorescent light is a measure of the intracellular esterase activity, and the fluorescence color is a measure of the intracellular pH (Malin-Berdel and Valet 1980). PI stains the DNA of dead cells. Five µl of DCH-stained, porous latex particles bearing NH_2 groups were also added to the cell suspension as an internal standard for cell concentration, cell volume, and cell fluorescence immediately before the flow-cytometric measurement. The DCH-particles were prepared by admixing 10 µl unstained particles (5×10^8/ml) and 10 µl DCH (1 mg/ml in DMF) to 500 µl TBS-buffer, followed by incubation for 2h at 0°C for complete equilibration with the DCH.

Flow-cytometry

The volume and one or two fluorescences of each cell were simultaneously determined at a speed between 500 and 1000 cells/s in a FLUVO-METRICELL flow cytometer (Kachel et al. 1977) in combination with a CYTOMIC data analyzer (Kachel et al. 1984) (HEKA, D-6071 Forst, FRG). The cell volume was measured electrically in a cylindrical orifice of 85 µm diameter and 100 µm length with a current of 0.088 mA or 0.229 mA in TBS-buffer, after hydrodynamically focusing the cells through the center of the orifice. The fluorescence of the cells was excited by epi-illumination with light from a HBO-100 high pressure mercury arc lamp at the outlet of the orifice.

Fluorescence excitation was between 300 and 400 nm for ADB/PI. The amount of blue fluorescence light emitted by intracellular DCH was measured between 420 to 440 nm by the first photomultiplier tube, the green fluorescence of DCH, and the red fluorescence of PI between 500 and 700 nm by the second photomultiplier tube. FITC-fluorescence was excited between 400 to 500 nm and the fluorescence light emitted was collected between 500 and 600 nm by the first photomultiplier tube. The second photomultiplier tube was not used in the FITC-measurement.

The electrical cell volume signal and the fluorescence signals from the photomultiplier tubes were logarithmically amplified (3 decade amplifiers). The maximum amplitude of each of the three pulses of a cell was digitized by 128-step analogue digital converters and stored sequentially on magnetic tape (list-mode). Channel two parameter histograms (64 × 64) (Figs. 1 and 4) or 32 × 32 × 32 channel three parameter histograms (Fig. 3) were calculated and quantitatively evaluated (INTERDATA 7/32 computer, Perkin Elmer, Oceanport, NJ, USA, 768Kbyte core memory, 200Mbyte disc, 800bpi magnetic tape) after completion of the measurement from the list-mode data of each cell sample with FORTRAN IV computer programs developed earlier (Valet 1980). The relative packing density of membrane bound FITC-PNL was calculated for each cell as the ratio of its total fluorescence divided by the cell surface. The cell surface (S) was calculated from the cell volume (V) as: $S = 4.84 \times V^{0.67}$ assuming a spheroid shape of the cells. The esterase concentration of each cell was obtained as the ratio of the DCH blue fluorescence divided by the cell volume. The intracellular pH of each cell was calculated as the ratio of the blue fluorescence divided by the green fluorescence of the DCH-stained vital cells using a calibration chart (Valet and Raffael 1984).

Results

The fibroblasts of normal and muscular dystrophy patients stained well with FITC-PNL (Fig. 1A, B). The volume distribution curves were broad and the mean volume of both cell types (Tables 1 and 4) was variable when the cells were collected from different passages or on different days. The cells shrunk between 5 and 30% on fixation with formaldehyde (Table 1). The variation of the mean fibroblast volume in different cell culture emphasizes the difficulty of comparing the results if the lectin binding data are expressed as lectin bound per cell. The results obtained from different cell cultures are comparable if relative lectin packing densities on the cell surface are calculated. This is accomplished by dividing the total cell-bound FITC-PNL fluorescence by the cell surface, which is derived from the cell volume. The results show (Tables 2 and 3) that the PNL receptor density on the cell surface of DMD fibroblasts is significantly reduced to between 68.9 and 79.3% of the values of fibroblasts from normal human individuals. This reduction could be due to a different binding affinity of PNL to the cell membrane receptors or to a reduction of receptor site density. The dose dependence of PNL binding (Fig. 2) was determined to clarify this point. It is apparent that saturation of PNL binding is not reached within the range 20 to 80 µg/ml PNL concentration. Forty µg/ml was the normal PNL concentration in the assay. This concentration was chosen because higher PNL concentrations which were closer to the saturation level (e.g., 0.4 mg/ml) aggregated the cells. It is seen from Fig. 2 that the PNL binding curves of all four cell lines show a similar inclination, indicating that the different PNL binding to normal and to DMD fibroblasts is due to a reduction of PNL receptor sites on the cell membrane and not to a change of receptor affinity.

It was now investigated whether the lowered PNL receptor density on the DMD fibroblasts was accompanied by changes of intracellular metabolic parameters. The esterase activity, the intracellular pH, the proportion of cells in S and G2/M phase of the cell cycle, and the vitality of the fibroblasts were investigated. The data were compared to the data of normal peripheral blood lymphocytes and granulocytes. Most parameters investigated were similar in normal and DMD fibro-

Table 1. Effect of formalin fixation on fibroblast mean cell volume

	Fresh cells (μm^3)	Fixed cells (μm^3)
Normal ($n = 6$)	4771 ± 948[a]	3425 ± 553
Dystrophy ($n = 9$)	3942 ± 889	3875 ± 266
$2P$ (Student)	N.S.	N.S.

[a] Mean ± standard error of mean (SEM)

Table 2. Relative PNL-surface receptor density on fresh fibroblasts

	Expt. 1	Expt. 2	Expt. 3
Normal ($n = 4$)	1.092 ± 0.012[a]	0.645 ± 0.011	1.040 ± 0.064
Dystrophy ($n = 4$)	0.752 ± 0.045	0.512 ± 0.009	0.821 ± 0.063
Percent of normal	68.9	79.3	78.9
$2P$ (Student)	<0.001	<0.001	<0.10

[a] Mean ± SEM

Table 3. Relative PNL-surface receptor density on formalin-fixed fibroblasts

	Expt. 1
Normal ($n = 4$)	1.153 ± 0.062[a]
Dystrophy ($n = 4$)	0.761 ± 0.072
Percent of normal	66.4
$2P$ (Student)	<0.05

[a] Mean ± SEM

Table 4. Metabolic parameters of fibroblasts from normals and patients with muscular dystrophy

	Volume (μm^3)	Rel. est. conc.	Intracellular pH	S + G2/M (%)	Vital (%)
Normal ($n = 8$)	5012 ± 608[a]	0.169 ± 0.020	6.52 ± 0.09	22.2 ± 3.5	26.9 ± 4.1
Dystrophy ($n = 6$)	5000 ± 1147	0.166 ± 0.036	6.58 ± 0.06	17.7 ± 0.3	18.0 ± 3.9
$2P$ (Student)	N.S.	N.S.	N.S.	N.S.	N.S.
Blood lymphocytes ($n = 3$)	227 ± 17	1.564 ± 0.028	7.30 ± 0.02	< 1.0	98.9 ± 0.2
Granulocytes ($n = 3$)	434 ± 31	2.167 ± 0.044	7.17 ± 0.02	< 1.0	98.3 ± 0.5

[a] Mean ± SEM

blasts (Table 4). The number of vital cells in both cases was relatively low (26.9 and 18.0%). This was due to the mechanical harvesting of the cells which was of importance for the maintenance of intact cell surfaces for the PNL-binding studies. Incubation of the cells with trypsin for easier cell detachment from the culture dishes stripped many of the PNL receptors from the cell surfaces. The esterase concentration of the fibroblasts was only between 7 and 11% of the esterase concentration in the lymphocytes and granulocytes (Table 4) and the intracellular pH of the fibroblasts was significantly lower than the pH in the peripheral blood lymphocytes and granulocytes. A significant number of cells in normal and DMD fibroblasts were in the S + G2/M phase of the cell cycle (22.2, 17.7%) (Table 4).

Discussion

The experiments reported here indicate that the general physiologic state of the DMD fibroblasts is not pathologic. The cells grow and divide normally, and the intracellular pH and esterase activity are unaltered. There is no consistent difference in cell size as compared with fibroblasts from control subjects, and the distribution of cell cycles within the various cultures is similar. It can therefore be concluded that the major pathways in intermediary metabolism function normally in DMD fibroblasts. The finding of a reduced capacity to bind peanut lectin indicates that the cell surface of the DMD fibroblasts differs in some way from that of control cells. That each of our DMD cell lines exhibited significant differences as compared to control lines suggests a connection between this feature and the genetic defect. The consistent manifestation of such a defect in fibroblasts is of tremendous value for the future of DMD research.

That the predominant feature of DMD is the degenerative alteration of muscle tissue is probably attributable to the specific properties of this type of tissue. Evidence already exists that, in addition to the degenerative changes in muscle tissue, DMD is accompanied by pathologic alterations in other cell types including erythrocytes (Matheson and Howland 1974), lymphocytes (Hauser et al. 1979; Stern et al. 1979), and fibroblasts (Witkowski and Jones 1981; Moser 1984). Our finding of an abnormality at the cell surface of DMD fibroblasts is in agreement with earlier reports (for critical reviews Moser 1984; Jones and Witkowski 1983a, b) indicating membrane abnormalities in cultured skin fibroblasts from DMD patients. The evidence provided by our results places the use of cultured fibroblasts in DMD research on an unequivocal basis, with all the advantages that this implies.

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Received August 31, 1984 / Revised December 11, 1984