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Monensin-induced inhibition of cell spreading in normal and dystrophic human fibroblasts

(adhesion/Duchenne dystrophy/secretion)

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ABSTRACT **Cultured skin fibroblasts from normal individuals and from patients with Duchenne muscular dystrophy spread equally rapidly when seeded on a glass substratum. Exposure to the ionophore monensin substantially suppresses normal and dystrophic fibroblast spreading in serum-free media for up to at least 100 min. Preincubation of normal fibroblasts with monensin causes a further reduction in cell spreading. Dystrophic fibroblasts fail to spread as well as normal cells after monensin preincubation. Such findings indicate that there is a delay in the secretion of functional adhesive surface proteins in monensin-preincubated normal fibroblasts and that this lag period is significantly longer in dystrophic fibroblasts. These data are consistent with findings of altered adhesive and secretory properties of fibroblasts from patients with Duchenne muscular dystrophy.**

Fibronectin is a pericellular multidomain glycoprotein that is involved in the active spreading of adherent fibroblasts (for review, see ref. 1). It is also a major component of the extracellular matrix (2, 3) and, together with collagen, is secreted from human fibroblasts via the Golgi complex–endoplasmic reticulum–lysosome (GERL) system (4). We have recently reported that, in the absence of serum, the monovalent ionophore monensin greatly reduces the ability of cultured human skin fibroblasts to spread on a glass substratum (5). This is probably a result of reduced matrix protein secretion (6) due to a monensin-induced blockade at, or near, the Golgi complex in human fibroblasts (7, 8) and other cell types (9–11). Furthermore, fibronectin that is secreted by long-term monensin-treated fibroblasts is abnormally glycosylated (12); this may also affect the biological properties of this glycoprotein in mediating cell spreading (5).

Matrix proteins, such as collagen (13, 14) and fibronectin (15), are abundant in muscles of patients suffering from Duchenne muscular dystrophy (DMD), an X chromosome-linked, progressive wasting disease of skeletal musculature (16). These observations, together with findings that collagen synthesis (17, 18) and secretion (19) are increased in cultured DMD fibroblasts, have led to suggestions that the connective tissue proliferation which is seen in DMD muscle may be closely associated with the etiology of the disorder (20, 21).

Although the nature of the primary defect in DMD is unknown, many reports have suggested an abnormality of the cell surface in the disorder (for review, see ref. 22). Cultured human skin fibroblasts provide a convenient system for investigating this possibility, since any membrane-mediated changes are unlikely to be a consequence of probable secondary degenerative events, as in the case of muscle cells *in vivo* (23, 24). Nor could the presence of putative circulating factors (25) account for any membrane abnormality. We (26, 27) and others (28) have found that DMD fibroblasts appear to be less adhesive than normal cells, and this may be related to altered cell surface properties in DMD (29). We have studied the spreading ability of normal and DMD fibroblasts under conditions in which protein secretion is altered by exposure to monensin and report here that there is no difference in the rate or extent of radial spreading between normal and DMD fibroblasts in serum-free media. However, although monensin suppresses the spreading of normal and DMD cells to a similar degree, we demonstrate that, after prolonged monensin preincubation, DMD fibroblasts fail to spread as efficiently as normal cells, especially when monensin is removed from the seeding media. These results are considered in relation to a possible defect in the secretory and adhesive properties of cultured DMD fibroblasts.

MATERIALS AND METHODS

Cell Cultures. Skin biopsy samples were obtained from the upper surface of the thigh from four boys (1–12 years old) who were investigated for muscle weakness but subsequently diagnosed as having no neuromuscular disease and from nine boys with DMD (Table 1) as described (30). Cultures were established from explants in 35-mm Petri dishes in Eagle's minimal essential medium supplemented with 10% fetal calf serum, penicillin at 50 international units/ml, and streptomycin at 50 μg/ml. When a sufficient number of cells had grown out from the explants, these cells were dissociated with 0.25% trypsin in Ca^{2+}/Mg^{2+}-free Hanks' basal salts solution (CMF) and transferred to 25-cm^2 plastic culture flasks. When these cultures were confluent, the cells were treated with trypsin once more and transferred to 80-cm^2 flasks. Cells were then subcultured weekly with 1:2 splits in minimal essential medium supplemented with 10% newborn calf serum and buffered with 10 mM Hepes. All media and plastics were purchased from GIBCO Europe.

Cell Spreading. All cultures used (Table 1) were between passages 8 and 20 and were grown to near confluence. Cultures were washed in CMF and incubated for 20 min at room temperature in 0.25% trypsin in CMF. Cells were then collected, washed in CMF, and resuspended at a final concentration of 2×10^5 cells per ml in serum-free minimal essential medium at 4°C, as described (5). In most cases (see Table 1), cell suspensions were divided into two aliquots which contained either 0.5 μM ethanolic monensin (from Calbiochem–Behring) or 0.1% ethanol alone (monensin control). Cells were allowed to attach and spread at 37°C onto clean glass multitest slides (Flow Laboratories) for 10, 30, 60, and 100 min. All cells were seeded within 30 min of dissociation. Spreading was stopped by washing the slides briefly in 0.1 M sodium cacodylate buffer, pH 7.2, and fixing for 30 min at

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Abbreviations: DMD, Duchenne muscular dystrophy; GERL, Golgi complex–endoplasmic reticulum–lysosome.

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Table 1. Identities of cultures used at each time point

Spreading medium	Normal				Dystrophic			
	10 min	30 min	60 min	100 min	10 min	30 min	60 min	100 min
No monensin preincubation								
Minimal essential medium	JM/1	JM/1	JM/2	JM/2	CR/1	CR/1	CR/1	CR/1 AV/1
	JM/2	JM/2	AP/1	JM/3	DS/1	DS/1	DS/1	DS/1 NS/1
		AP/1	AP/2	AP/1	SA/1	SA/1	SA/1	SA/1 BP/1
		AP/2		AP/2		BP/1	BP/1	MS/1 BP/2
		SB/2		AP/3		MS/1	MS/1	RS/1 BP/3
Minimal essential medium/monensin	SB/1	SB/1	SB/1	SB/1	CR/1	CR/1	CR/1	CR/1
	JM/2	JM/2	JM/1	JM/1	DS/1	DS/1	DS/1	DS/1
		AP/1	JM/2	JM/2	SA/1	SA/1	SA/1	SA/1
		AP/2	AP/1	AP/1		BP/1	BP/1	BP/1
			AP/2	AP/2		MS/1	MS/1	MS/1
Monensin preincubation*								
Minimal essential medium and	JM/4	JM/4	JM/4	JM/4 AP/3	CR/2	CR/2	CR/2	CR/2 DS/2
minimal essential medium/monensin	SB/2	SB/2	SB/2	SB/2	DS/2	DS/2	DS/2	CR/3
	AP/3	AP/3	AP/3	SB/3	SA/2	SA/2	SA/2	AC/1
				SB/4				ND/1
				VB/1				SA/2

Initials followed by a different number refer to a replicate culture. Initials followed by the same number indicate that cells from the same culture flask were used.
*Twenty hours with 0.5 μM monensin.

room temperature in 2.5% (wt/vol) glutaraldehyde in cacodylate buffer.

The effect of monensin preincubation and recovery was investigated by using normal and DMD fibroblast cultures that had been established as described above. These cultures, with a developed fibronectin matrix, were incubated in minimal essential medium supplemented with 10% newborn calf serum with 0.5 μM monensin for 20 hr at 37°C prior to the final trypsin treatment. Incubating cultures with 0.1% ethanol for 20 hr before dissociation had no effect on the attachment or spreading of normal or DMD cells, as judged by using the parameters described below. Preincubated cultures were washed in CMF at 4°C, treated with trypsin, and prepared for spreading as before. Prior to seeding of the cells, suspensions were again divided into two aliquots; either monensin was reintroduced (monensin block maintained) or ethanol was added (monensin block released).

Cell Area Measurements. Cells were examined by using differential interference contrast microscopy on a Leitz Orthoplan microscope. From each slide, 50 cells were photographed that fell on, or near, orthogonal diameters drawn randomly across the visual field of the microscope. Measurements of cell area were made from prints at a final magnification of ×1340 by using a MOP-1 digitizer (Kontron, Slough, U.K.) interfaced to a Commodore microcomputer (27).

In a population of spreading cells, the measured cell areas fit a negative binomial and not a Gaussian distribution (5), thus cell area frequency distributions were compared by using the Kolmogorov–Smirnov two-sample test (31), which we have found suitable for this type of analysis (5). Cell areas were also logarithmically transformed to allow a parametric test of significance (Student's t test) to be performed (Table 2). These tests were conducted on the means of ln(area) and were obtained from the individual cultures listed in Table 1. For cells allowed to spread for 100 min, significance tests were additionally performed on the basis of individual *subjects* (Table 3). In cases where replicate cultures from the same subject were employed, the grand mean of ln(area) for the subject was used.

As a measure of the uniformity of radial spreading in the seeded fibroblasts, a form factor was calculated for all cells measured. The form factor is a measure of the circularity of an object and was adapted for this study as a parameter to exclude cells that were either irregularly spread or had ceased radial spreading and had become polarized. The form factor is given by $4\pi A/P^2$, in which A and P are the object area and perimeter, respectively. Thus a perfect circle has a form factor of 1.0 and, in practice, cells with a form factor >0.60 can be regarded as unspread or showing radial spreading. All cells measured in this study were found to have a form factor >0.55.

RESULTS

Cell Spreading in the Presence or Absence of Monensin. Within 10 min of seeding, ≈90% of normal and DMD fibroblasts were attached. No morphological differences could be detected between the two genotypes. However, after 30 min, most cells in both groups had started to spread as indicated by the higher values for transformed means (Fig. 1*A*) of these cells and the skewness of area frequency distributions (data not shown).

Uniform radial spreading continued up to 100 min (Fig. 1*A*; Table 2) and by this stage nearly all normal and DMD cells have spread with a well-established skirt of cytoplasm extended around them (Fig. 2 *A* and *B*). At stages later than 100 min, the area of many normal and DMD cells was less than the values found at 60 or 100 min. This decrease in cell area is concomitant with the change in morphology from radial to polarized cells.

The addition of monensin to the seeding media did not appear to affect the attachment of normal or DMD cells. Similarly, initial cell morphology and mean cell area (Table 2) were unaffected by monensin treatment. By 30 min, however, spreading of monensin-treated cells appeared to be inhibited, although it did not reach significance. At later stages there is little further spreading of treated cells, and the difference from untreated cells is significant at both 60 min ($P < 0.02$) and 100 min ($P < 0.01$). Furthermore, the skirt of the cytoplasm was ragged in profile (Fig. 2 *C* and *D*) compared with the more regular outline of untreated cells (Fig. 2 *A* and *B*). The difference in area distribution between treated and untreated cells was greatest at 100 min. This can be seen by

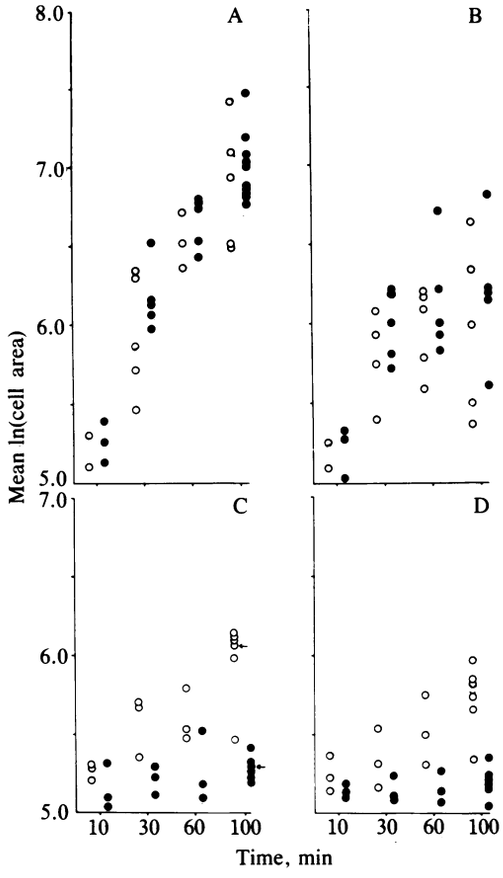

FIG. 1. Distribution of mean cell areas (μm^2) after natural-logarithmic transformations. Values are shown for normal (○) and DMD (●) cells seeded in the absence (*A*) or presence (*B*) of monensin. The effect of 20-hr monensin preincubation is shown in *C* and *D*: monensin-preincubated cells seeded in the absence (*C*) or presence (*D*) of monensin. The arrows in *C* indicate the cultures used for the distributions shown in Fig. 3.

the back-transformed data of Table 2 and by the distribution of the (transformed) means (Fig. 1).

From 10 to 100 min after seeding, no differences were detected between normal and DMD cells in response to monensin treatment by either morphological appearance or analysis of cell area distributions. It is important to note here that all of these experiments (and those described below) were conducted in serum-free media. When serum was present during cell spreading (see *Discussion*) monensin failed to suppress this process in DMD cells, as we have already shown for normal skin fibroblasts (5).

Effect of Preincubation with Monensin. The effect of prolonged exposure to monensin, and any recovery from this sustained blockade to secretion, was investigated by using cells that had been preincubated with the ionophore for 20 hr prior to trypsin treatment. This extended exposure to monensin did not affect cell size (Table 2), morphology, or attachment in either normal or DMD fibroblasts, and it was not initially affected by the addition or omission of monensin in the seeding medium. However, within 30 min, many normal cells in which monensin was removed from the medium had spread to a greater extent than those in which monensin was maintained. The greater inhibition of normal cell spreading (if monensin is reintroduced to the seeding medium) was maintained for at least 100 min (Fig. 1*D*, Tables 2 and 3). Spreading in these cells was more reduced than in any of the other experiments on *normal* cells described here (Table 4). Normal cells from which monensin was removed continued to spread (although to a limited extent), and by 100 min (Fig. 1*C*) they had spread to a similar extent ($P > 0.05$) as those normal or DMD cells that were exposed to monensin only at the time of seeding (Fig. 1*B*). The majority of the former cells had a well-defined rim of cytoplasm spread around them, but, as in the short-term monensin-treated cells, the cell perimeter lacked the smoother profile of normal and DMD untreated cells (Fig. 2*E*).

DMD cells responded to the challenge of prolonged monensin preincubation and recovery in a manner different from that of normal cells, and this was noticeable within 30 min of seeding (Fig. 1*D*). The removal of monensin had no significant effect on the spreading ability of DMD cells; even after 100 min, spreading of these cells was as inhibited as spreading of those cells in which monensin was reintroduced (Fig. 1*C*; Tables 2 and 3). Both groups of DMD cells had spread less than the equivalent normal cells by 100 min, but the greatest difference was between normal and DMD monensin-released cells at this time point (Figs. 1*C* and 2 *E* and *F*). This difference was found whether genotypes were compared on the basis of individual cultures (Table 2) or subjects (Table 3). The latter classification was used to test whether an unfairly weighted contribution was being made to the data by, e.g., a single spurious subject from which replicate cultures were analyzed.

Table 2. Area analysis of untreated and monensin-treated normal (N) and DMD cells

		Area, μm^2							
		10 min		30 min		60 min		100 min	
Spreading medium		N	DMD	N	DMD	N	DMD	N	DMD
No monensin preincubation									
Minimal essential medium	MCA	184	194	380	480	682	775	973	1085
	CL	51–657	140–268	239–605	373–619	444–1050	633–948	594–1591	929–1266
Minimal essential medium/monensin	MCA	178	183	315	398	391	462	391	491
	CL	63–502	121–276	193–516	303–524	281–543	300–713	200–766	290–833
Monensin preincubation*									
Minimal essential medium	MCA	195	173	265	184†	273	195	413	198‡
	CL	169–224	119–251	164–427	146–232	179–417	111–342	296–577	183–215
Minimal essential medium/monensin	MCA	183	171	209	171	249	174	307	183‡
	CL	139–240	152–191	132–331	140–209	142–434	134–225	245–385	165–203

All values represent back-transformed data, thus the MCA values are not the *observed* mean cell areas. These data are generated from the values shown in Fig. 1. MCA, mean cell area; CL, 95% confidence limits. Since the transformation was nonlinear, these are asymmetric about the mean.

*Twenty hours with 0.5 μM monensin.

†Significantly different from normal at $P < 0.02$, by Student's *t* test.

‡Significantly different from normal at $P < 0.001$, by Student's *t* test.

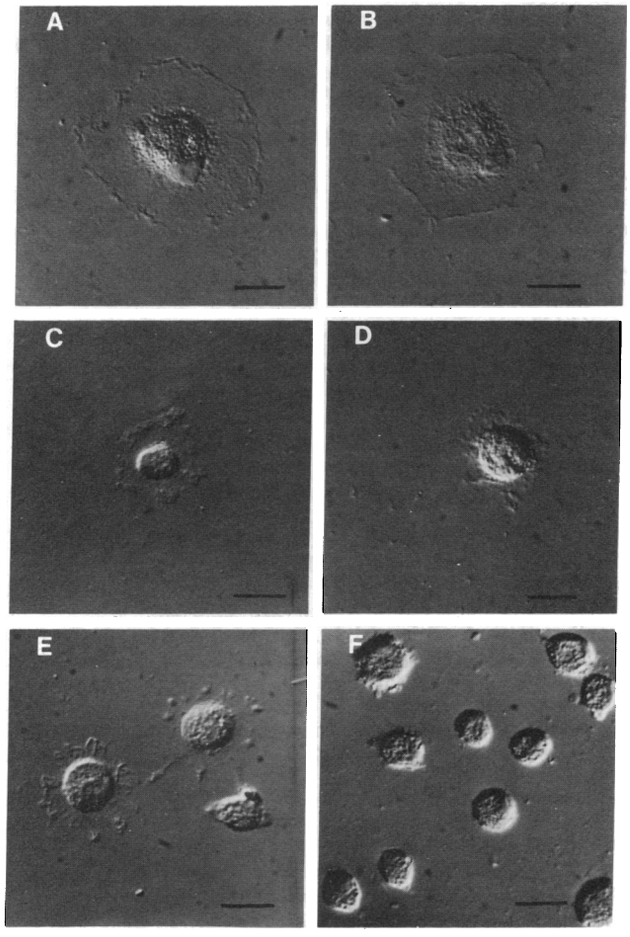

FIG. 2. Normal (*A*, *C*, and *E*) and DMD (*B*, *D*, and *F*) fibroblasts allowed to attach and spread for 100 min on glass substrata. (*A* and *B*) Untreated cells. (*C* and *D*) Cells seeded in the presence of 0.5 μM monensin. (*E* and *F*) Cells preincubated with monensin for 20 hr and then allowed to spread in monensin-free medium. All micrographs are shown for illustration only; measurements were made from prints at much greater magnification. (Bars = 20 μm.)

Most (>60%) of the DMD cells were completely unspread by 100 min, and the remainder often exhibited only short filopodia which contributed very little to any increase in cell area. Cell area frequency distributions of normal and DMD monensin-released cells show the inhibition of spreading in the latter by the much-reduced right-hand tail of the graph (Fig. 3). Analysis of the pooled values for normal and DMD monensin-released cells by the Kolmogorov–Smirnov two-sample test also shows a significant ($P < 0.01$) difference between these two populations. This behavior of normal and DMD cells was seen consistently in six normal and six DMD cultures (Fig. 1*C*).

Table 3. Mean cell area analysis of 100-min samples on the basis of subjects

Spreading medium	Area, μm^2 Normal	DMD	P*
No monensin preincubation			
Minimal essential medium	911	1034	NS
Minimal essential medium/ monensin	354	491	NS
Monensin preincubation			
Minimal essential medium	453	200	<0.001†
Minimal essential medium/ monensin	309‡	186	<0.001†

All values represent back-transformed data. Number of subjects is given in Table 1.

*Probability values derived from Student's t test. NS, not significant.

†It should be noted that these differences could be due to a gene independent of DMD. In this case, the probability of four normal and five DMD subjects forming two, discrete distributions is given by $1:2^8$—i.e., $P < 0.004$.

‡Significantly different from monensin-preincubated normal cells seeded in minimal essential medium alone ($P < 0.02$).

Table 4. Summary of cell spreading in control and monensin-treated skin fibroblasts

Spreading medium	Spreading Normal	DMD
No monensin preincubation		
Minimal essential medium	++++	++++
Minimal essential medium/ monensin	+++	+++
Monensin preincubation*		
Minimal essential medium	+++	+
Minimal essential medium/ monensin	++	0/+

Spreading ability is represented by estimates of increasing cell area on an arbitrary scale from 0 to ++++. All values represent the extent of spreading 100 min after cell seeding.

*Twenty hours with 0.5 μM monensin.

DISCUSSION

In this report we have demonstrated that DMD cells settle onto glass substrata and spread to the same extent as normal cells and, further, that the spreading process is equally inhibited in both genotypes by monensin added at the time of plating. Uchida *et al.* (6) have demonstrated that 7-hr exposure to 50 nM monensin can reduce fibronectin and collagen secretion in human fibroblasts by ≈60%. Exposure to monensin (6, 32) produces a delay of up to 6 hr after incubation in monensin-free medium before secretion approaches control values. Under the conditions we have used, the efficiency of monensin appears to be sufficient to reduce glycoprotein se-

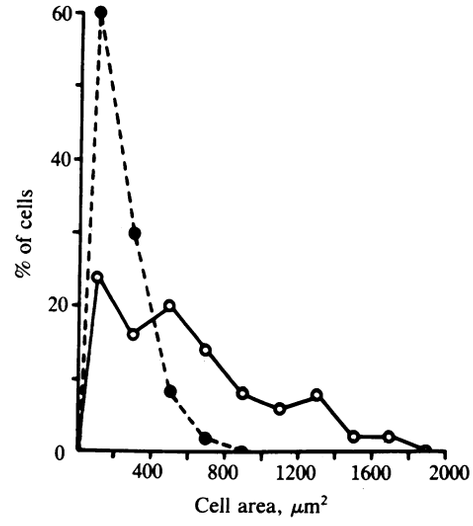

FIG. 3. Representative cell area frequency distributions of normal (○) and DMD (●) cells seeded for 100 min in monensin-free medium after 20-hr monensin preincubation. The transformed means of these distributions are shown arrowed in Fig. 1. The distributions of normal (AP/3) and DMD (CR/3) are those closest to the grand means for either genotype and are shown for illustration of true cell size. However, such relative inhibition of spreading in DMD cells under these conditions was seen in all cultures examined.

cretion below the threshold level (33) required for successful spreading: this applies to both normal and DMD fibroblasts.

It should be mentioned that monensin-induced spreading inhibition is masked by the presence of serum in the seeding medium (5). Since serum-derived fibronectin is known to be incorporated into cell layers (34) and substrata (35), it seems probable that both increased cell spreading in serum-supplemented media and the failure of monensin to suppress cell spreading under these conditions are due to the provision of sufficient serum-derived elements—e.g., fibronectin—to support rapid cell spreading.

Although short-term exposure to monensin has similar effects on normal and DMD cell spreading, preincubation with monensin for 20 hr causes a much more pronounced reduction in DMD cells, even when the drug is removed before seeding. In particular, it is most noticeable in such cells that have been allowed to spread in monensin-free medium for 100 min. This observation may be correlated with the renewed secretion of biologically active fibronectin after release from long-term monensin exposure (12). This is consistent with a series of studies (32, 36–38) confirming that the transit time for newly synthesized fibronectin to reach the cell surface is ≈30 min. The inability of DMD cells to recover spreading ability to the same extent as normal cells may thus be a consequence of a greater delay in the reconstitution of the GERL secretory pathway compared with normal fibroblasts. The pleiotropic action of monensin could distinguish between normal and DMD cells in other ways [for example, ionic substitution in model and cell membranes (39) and microtubule organization (40)], but this does not seem probable. However, our observations on seeding efficiency after prolonged exposure to the ionophore suggest no damage to the biochemical mechanisms required for cell–substrate attachment. Cell morphology, trypan blue exclusion, and the partial recovery of normal monensin-released cells within 30–100 min all demonstrate that long-term monensin exposure *per se* is neither lethal nor irreversibly disabling to the complex biochemical and ultrastructural systems required for cell–substrate spreading.

Finally, a defect in the recovery of cell spreading in DMD monensin-released fibroblasts may be correlated with previous reports of abnormal cell–cell adhesiveness (26, 27) and cell–substratum detachment (28) in DMD fibroblasts. Kent (28) has demonstrated that the detachment rate of DMD fibroblasts from plastic substrata by trypsin was greater than that of normal cells and also that DMD fibroblasts were more sensitive to lower concentrations of trypsin. We have found (unpublished data) that the reduction in intercellular adhesiveness of DMD cells is lost when the cells are given a 2-hr recovery period after trypsin treatment. This recovery period, prior to aggregation, allows for the replacement of surface material damaged or removed by trypsin (41). Such observations, taken with those of Kent (28), suggest that the failure of DMD cells to recover normally from a monensin blockade may be related to the adhesive surface properties of DMD cells and to the replacement of surface or secretory adhesive proteins. The 30-min transit time [in the case of fibronectin (36)] involves the *de novo* synthesis of the glycoprotein, post-translational modification, and transport through the GERL system. A defect in such secretion-related mechanisms could account for abnormal DMD cell adhesiveness (26, 27), the behavior of DMD cells in their response to monensin, and previous reports (17–19, 29) of abnormalities in the synthesis of GERL-associated proteins in DMD.

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