STUDIES ON CELL DEFORMABILITY

II. Effects of Some Proteolytic Enzymes

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ABSTRACT

Murine sarcoma 37 ascites cells were treated with the proteolytic enzymes, trypsin and chymotrypsin, after which cellular deformability and electrophoretic mobility were measured. It was shown that incubation with trypsin increased the ease with which the cells could be deformed without changing electrophoretic mobility, and that diisopropylfluorophosphate (DFP)-trypsin was inactive, a fact which suggests that trypsin-sensitive peptide linkages help to maintain the "tension" at the cell periphery. On the other hand, chymotrypsin reduced cellular electrophoretic mobility without appreciably altering deformability. This suggests that, although chymotrypsin-sensitive bonds do not contribute to "tension," they are in some way associated with charged groups at the cell periphery.

Many present-day concepts of the molecular structure of the plasma membrane originated with the observation that the "tension" measured at the periphery of a variety of cells was lower than expected from a "bare" lipid surface. This led to the well known Danielli-Davson-Harvey model in which a bimolecular lipid leaf is depicted as being sandwiched between protein. In this communication, experiments are described in which the effects of exposure of murine sarcoma 37 ascites cells to proteolytic enzymes are correlated with "tension" at their peripheries as determined by measurements of cell deformability.

MATERIAL AND METHODS

Sarcoma 37 tumors were grown in ascitic form in young adult males of an inbred strain of Swiss mice, and harvested 5 to 7 days after inoculation. Unless otherwise stated, 0.5 ml samples of ascitic fluid were made up to a volume of 5 ml with Hanks' balanced saline (pH 7.2) at 4°C, and kept at this temperature for at least 20 min before washing twice in saline by centrifugation at approximately 350 *g* for 5 min. The original supernatant fluid was saved, and the washed cells were then resuspended in various test solutions to give a final volume of 10 ml containing some 50,000 cells per milliliter. These suspensions were incubated for 30 min at 37°C, washed once, and resuspended in the original supernatant fluid which contained 10% ascitic fluid in Hanks' solution, and in which all measurements were made.

The various "test" solutions in which the cells were incubated consisted of: trypsin (Difco, Laboratories, Detroit, Michigan, 0.2%) in trypsin diluent (NaCl, 8.0 g/liter; KCl, 0.4 g/liter; $Na_2HPO_4 \cdot 7H_2O$, 0.1134 g/liter; KH_2PO_4 (anhydrous), 0.060 g/liter; $NaHCO_3$, 0.50 g/liter), with diluent or Hanks' solution as controls; crystalline trypsin (Worthington X2 crystallized, salt-free, 0.2% in Hanks' solution), with both Hanks' solution and 0.2% diisopropylfluorophosphate (DFP)-treated trypsin (Worthington Corporation, Harrison, New Jersey) as controls; α-chymotrypsin (Worthington, X3 crystallized, 0.2% in Hanks' solution), with Hanks' solution as control; neuraminidase (Behringewerke, Marburg-Lahn, West Germany, 50 units per milliliter in Hanks' solution), with Hanks' solution as control.

The deformability of cells was expressed as the negative pressure (measured manometrically in

Brodie's fluid of 1.007 relative density) required to suck a hemispherical bulge of cell into fluid-filled micropipettes of 5 to 6 μ internal diameter (1). The cells were micromanipulated under a cover slip on a ridged slide while observed at a magnification of 430. The rate of application of negative pressure was controlled by means of a screw, which raised and lowered a platform holding a large fluid reservoir which was connected to a micropipette held in a de Fonbrune micromanipulator. The vertical movements of the fluid level in the reservoir were measured by connecting a second reservoir on the same platform to a manometer having a mirror-backed scale. The reservoirs were lowered at a rate of 5 cm per 23 to 25 sec. As described by Mitchison and Swann (2), the initial zero pressure position of the tip of the micropipette relative to the fluid levels in the reservoirs was determined by observation of the movements of small pieces of cell debris. The zero pressure position was taken when these markers stopped movement, in or out of the pipette.

The micropipettes were made from standard Pyrex glass tubing of 3 mm external diameter, which was first drawn out by hand into pipettes with tips of approximately 200 μ diameter; and their ends were bent into small hooks. Pipettes were fitted into the de Fonbrune microforge, a 4 g weight was hung on the hook, and the forge heating filament was activated in a carefully regulated manner so that the pipette tip was drawn out smoothly and slowly, until the weight caused it to break cleanly at a predetermined internal diameter, close to 5 μ. Measurements of the micropipette diameters were later made on a microscope with an eye-piece micrometer at a magnification of 430, and pipettes which had diameters discernibly different from 5 μ were rejected, as were those not having smooth square tips.

It was found that, if cells were kept at room temperature (24°C) for more than about 30 min, they became progressively more easily deformable. Consequently, in any particular experiment, measurements were made with the same micropipette on only ten "experimental" cells followed by ten "control" cells. A common pool of cells was kept on ice until the beginning of incubation with either the enzymes or their controls, and these treatments were staggered. After twenty such measurements, a fresh specimen of cells was obtained and the order of treatment reversed, so that measurements were first carried out on "control" cells. It has not been practicable, in our experience, to run more than two groups of cells at one time in any one experiment, owing to both eye fatigue in the observer and changes in the cells themselves.

Electrophoretic mobility was measured in the type of apparatus described by Bangham et al. (3), with the cells suspended in Hanks' solution of pH 7.2, at 30°C. Cells were timed over 25 μ in both directions with a potential difference of 50 v applied over 16.5 cm, when 0.9 ma of current was flowing.

RESULTS

Deformability

The results shown in Table I indicate that incubation with Difco trypsin produces a highly significant increase in cellular deformability. This facilitation of deformability was not due to the trypsin diluent per se, since no difference was detected between cells incubated in trypsin diluent and Hanks' balanced saline. The effects of Difco trypsin and crystallized trypsin were numerically the same, and trypsin inactivated with DFP produced no change. Incubation with crystalline trypsin produced effects numerically similar to those of neuraminidase, and chymotrypsin produced no observable effect.

It is noted that the control series of cells required about 4 to 5 cm of negative pressure to deform them, as compared to the 15 cm (approximately) previously described (1) for similar cells with similar pipettes. In the previous experiments, the negative pressure was applied over 5 sec, that is, at a rate of -3 cm per second, compared with the present experiments where the rate of application was -0.2 cm per second. This rate dependency is taken to indicate the dilatancy of the deformed cell, and will be discussed later in terms of so called "anomalous" rheology. In the previous experiments, neuraminidase-treated cells were deformed by some 30% less pressure than their controls; in the present experiments, approximately 30% less pressure is also required.

Electrophoretic mobility

Mobilities measured after cells had been incubated under conditions similar to those in the deformability experiments are shown in Table II. It is seen that incubation with trypsin produced no significant change in mobility; incubation with chymotrypsin produced an 11% decrease in mobility which is significant at the 1 to 2% level, and neuraminidase produced a 45% decrease which is significant beyond the 0.1% level.

DISCUSSION

In the previous paper in this series (1), it was pointed out, with reference to much previous work of others, that, although measurement of an arbi-

TABLE I

Deformability Measurements

Treatment	Deforming pressure ± SE (Hanks' fluid)	No. of measurements	*t* Tests
	cm		
Trypsin (Difco)	3.23 ± 0.16	30	t = 7.4 with 58 df
Trypsin diluent	5.36 ± 0.24	30	$P \ll 0.001$
Trypsin diluent	4.96 ± 0.27	30	t = 0.20 with 48 df
Hanks' solution	4.57 ± 0.37	20	$0.9 > P > 0.8$
Crystalline trypsin	3.57 ± 0.17	20	t = 5.3 with 48 df
Hanks' solution	5.21 ± 0.23	30	$P < 0.001$
DFP-trypsin	4.68 ± 0.46	20	t = 0.98 with 38 df
Hanks' solution	4.11 ± 0.37	20	$0.4 > P > 0.3$
Crystalline trypsin	3.02 ± 0.30	10	t = 0.43 with 18 df
Neuraminidase	3.20 ± 0.30	10	$0.7 > P > 0.6$
Chymotrypsin	4.19 ± 0.20	30	t = 0.77 with 58 df
Hanks' solution	4.46 ± 0.28	30	$0.5 > P > 0.4$

The negative pressures (± standard error) required to produce a 5 μ diameter, hemispherical bulge in sarcoma 37 ascites cells, treated as indicated. Pressures are expressed in centimeters of Brodie's fluid.

TABLE II

Measurements of Electrophoretic Mobility

Treatment	Mobilities ± SE	*t* Tests (vs. Hanks' control)
	$\mu \cdot sec^{-1} \cdot v^{-1} \cdot cm$	
Hanks' solution	−1.249 ± 0.031 (102)	
Trypsin	−1.183 ± 0.032 (91)	t = 1.375 with 191 df $0.2 > P > 0.1$
Chymotrypsin	−1.110 ± 0.055 (65)	t = 2.574 with 165 df $0.02 > P > 0.01$
Neuraminidase	−0.688 ± 0.085 (31)	t = 8.710 with 131 df $P < 0.001$

The mobilities of sarcoma 37 cells (± standard error) measured in Hanks' balanced salt solution + 10% ascitic fluid (pH 7.2) at 30°C, after incubation with the various enzymes indicated. The number of observations is given in parentheses.

trarily defined deforming pressure is not too difficult technically, the mathematical analysis of the results is difficult, if not impossible.

In a fluid droplet, where the true surface tension is γ_0 dynes per centimeter, S is the entropy of the system, T the absolute temperature, P the pressure, V its volume, A its surface area, n the number of molecules, and μ the chemical potential; then, where F is the total free (Helmholtz) energy:

$$dF = -SdT - PdV + \gamma_0 dA + \mu dn \quad \text{(ref. 4)}.$$

At constant temperature and volume, when the number of molecules in the system remains the same:

$$\gamma_0 = \frac{dF}{dA}.$$

If γ_0 is positive, then the Helmholtz free energy F will decrease when there is a diminution of surface area, i.e., $-dA$. When fluid drops are deformed, the deforming pressure must obviously exceed the true surface tension. However, in the case of cells deformed by sucking them up a pipette, as described and/or discussed by Harvey and Danielli (5), Mitchison and Swann (2), Rand and Burton (6), and others, then the measurements no longer

reflect only true surface tension, but also include the very complex rheological properties of the cell periphery. These are expected to deviate from "ideal" from the general considerations of "anomalous" rheology discussed by Weissenberg (7); by extrapolation of Mitchison and Swann's (2) data suggesting that the deformation of sea urchin eggs into micropipettes reveals both viscous and elastic elements; by extrapolation from the experimental data of Katchalsky et al. (8) on the erythrocyte periphery, which may be represented as a Kelvin unit which consists of elastic and viscous elements arranged in parallel; and from studies showing that the detachment of cultured mammalian cells from various substrata by shearing pressure is rate dependent (9).

The present data show that when ascites tumor cells are incubated with crystalline trypsin they become more easily deformable, and that this activity is due to the tryptic activity of the trypsin (DFP-trypsin is ineffective), and is not due to its solvent (cells suspended in Hanks' saline and trypsin diluent have the same deformability). The conformational and charge changes produced in trypsin, by phosphorylation of its active site, are most unlikely to affect either its adsorption to cells, or the effects of such adsorption on cellular deformability. It is well known that trypsin hydrolyzes the peptide bonds between the carboxyl groups of the basic amino acids, arginine or lysine, and other amino acids. It was previously shown (1) that cells could be more easily deformed after incubation with neuraminidase, and it was suggested that this was owing to the removal of terminal sialic acid carboxyl groups, which, by their mutual electrostatic repulsion, impose rigidity on the underlying cell periphery. The fact that incubation of these cells with trypsin produces no significant change in their electrophoretic mobility (Table II) may be interpreted as showing that the trypsin is not increasing cellular deformability simply by cleaving off ionized sialic acids, but is producing its effect by direct action on cellular proteins.

The increase in cell deformability following trypsin treatment is reminiscent of the decrease in "rigidity" observed by Mitchison (10) in trypsin-treated unfertilized sea urchin eggs. The question which is of immediate relevance to the present discussion is the site of action of the trypsin. Rosenberg (11) demonstrated that trypsin could be taken up by cultured cells and then slowly released. It is known that microsomal material is responsible for the basophilia of the ground substance of many cells (12) and that, in isolated fractions in vitro, trypsin removes ribosomes from the rough microsomal vesicles (13). It is therefore conceivable that the effect of trypsin could be due to its intracellular activity, as distinct from its action on the cell peripheral zone. Although Mitchison (10) concluded that the effects of trypsin observed by him were ". . almost certainly due to a decrease in the elastic modulus of the surface, since the interior cytoplasm of an unfertilized egg is so fluid that a decrease in its "elasticity" would be most unlikely to affect the elastimeter readings," the same argument cannot necessarily be used in the case of sarcoma cells. It is indisputable that trypsin affects the peripheries of many different types of cell; however, it is not known whether ingested trypsin affects the mechanical properties of the cytoplasm of viable cells. It would appear that trypsin does not cause widespread changes in cytoplasmic integrity which are reflected in cell death (14), and that trypsin-treated cells begin to regenerate factors affecting their contact reactions almost immediately (15). It therefore seems likely that the nonlethal effects of trypsin described here are attributable to its enzymatic activity at the cell periphery, and, although a secondary effect on the cytoplasm cannot be ruled out, there is no evidence to support it. The experimental evidence suggests, therefore, that proteins in the cell periphery are to some extent responsible for its mechanical properties, and would appear to contribute in a positive way to increasing "tension" rather than decreasing it, as previously suggested (5).

An attempt was made to show the expected additive effects of incubation of cells with trypsin and then neuraminidase, and vice versa. This was unsuccessful, as the cells were rendered so sticky that it was technically impossible to manipulate them with micropipettes without the occurrence of blockage.

Additional proof that the increases in deformability described here are not merely due to removal of ionized carboxyl or other acidic groups comes from the observations that, whereas incubation with chymotrypsin causes a significant (11%) reduction in electrophoretic mobility, it does not produce a detectable and significant increase in cell deformability. In addition, the fact that chymotrypsin produces an effect on mobility

at all suggests that L-tyrosine and/or L-phenylalanine (16) are present in the cell periphery, and that some charged groups in this region are in some way associated with these residues.

In advancing their now well known membrane model of a bimolecular lipid leaflet sandwiched between protein, Harvey and Danielli (5) in part used the evidence that the "tension at the surface" of various cells was lower than the tension expected from bare lipids, and that the tensions observed were compatible with protein-coated lipids. However, in their review, these authors made it quite clear that "tension at the cell surface," which is measured, is a quantity different from cell "surface tension," which is not.

Regardless of the precise interpretation of the "mechanical" data, it is known from chemical and immunological studies that protein is present in the cell periphery, although recent studies of erythrocyte ghosts with infra-red spectroscopy by Maddy and Malcolm (17) provide no evidence to support the hypothesis that a monolayer of protein in the extended β-conformation is associated with the membrane lipids. There have been many suggestions that proteins are in some way responsible for some of the mechanical properties of the cell periphery, one of the more recent being that of Haydon and Taylor (18), and the present data are possibly of some interest, since they provide experimental evidence to suggest this.

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