STUDIES ON CELL DEFORMABILITY

III. Some Effects of EDTA on Sarcoma 37 Cells

LEONARD WEISS

From the Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York

ABSTRACT

The nonlethal procedure of incubation in EDTA solution makes the peripheral regions of ascites sarcoma 37 cells more easily deformable, as reflected in measurements of the decreased amount of negative pressure required to suck out standard hemispherical bulges from the cells into micropipettes. The facilitation of deformability was abolished after reincubation of cells in calcium-containing saline, and this mechanical parameter was partially restored to normal after reincubation in magnesium-containing saline; the mechanical effect of EDTA treatment is, therefore, thought to be due mainly to the removal of calcium from the cell periphery. As EDTA treatment produces no detectable change in cellular electrophoretic mobility, it is concluded that peripheral calcium must be bound to anionic sites deeper than about 10 A from the cellular hydrodynamic slip plane. The data are discussed with emphasis on the view that they should not be extrapolated freely to other cell types.

INTRODUCTION

The role of calcium in the cell periphery in relation to cell contact phenomena has been the subject of a good deal of experimentation, speculation, and argument. The present communication is concerned with some effects on murine ascites sarcoma 37 cells of exposure to solutions of ethylenediaminetetra-acetic acid (disodium salt) (EDTA). This treatment, which is thought to remove calcium from the cell periphery, has been studied by its effect on the mechanical and electrokinetic parameters of this region, as well as by other techniques. The results are discussed in terms of the localization of calcium-binding sites in the periphery of sarcoma 37 cells.

MATERIAL AND METHODS

Sarcoma 37 cells were grown in ascitic form in an inbred strain of Swiss mice, and harvested 9–11 days after inoculation. Initial experiments showed that the measurements were not demonstrably different whether the ascitic fluid was collected in Hanks' balanced salt solution (HBSS) or calcium- and magnesium-free HBSS (CMF); consequently, 1 ml samples of ascitic fluid were routinely collected into 5 ml of CMF in order to prevent clotting, and washed twice in CMF by centrifugation at 400 g for 1 min. Aliquots corresponding to 0.33 ml of the original ascitic fluid, which had a packed cell volume of about 50% of which 30% was entrapped fluid, were resuspended in various test solutions and incubated with frequent agitation for 30 min at 37°C. After incubation, the cells were centrifuged and resuspended in CMF or HBSS as indicated.

The test solutions used were HBSS: CMF which consisted of standard HBSS with the calcium and magnesium salts omitted and 4.7 mg/liter NaCl added; disodium EDTA, 0.2% in CMF: CMF plus calcium chloride, 140 mg/liter; CMF plus magnesium chloride 200 mg/liter.

Cell deformability was assessed by determining 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 smooth square-ended pipettes of 5–6 μ internal diameter. The apparatus and techniques have been described in detail in a previous paper (1).

Cellular electrophoretic mobility was measured in a cylindrical cell apparatus of the type described by Bangham et al. (2). Measurements were made in CMF (pH 7.8), or HBSS (pH 7.2) of equal ionic strength, at 30°C. Cells were timed to travel 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.

Radioautographs were made from cells incubated with ^{14}C-labeled EDTA (0.2% in CMF) which had a specific activity of 2.1 mC/mmole. After being washed, the cells were spread on slides and either air fixed or alcohol fixed, treated with Wright's stain, coated with nuclear track emulsion by standard techniques, and exposed for 3 wk before development.

Cell viability was determined by means of standard trypan blue exclusion tests, and by animal inoculation. Cells incubated in CMF or EDTA-CMF, as in the other experiments, were suspended in CMF, in concentrations of 40,000 cells per ml. Injections of 0.25 ml of the suspensions were given subcutaneously
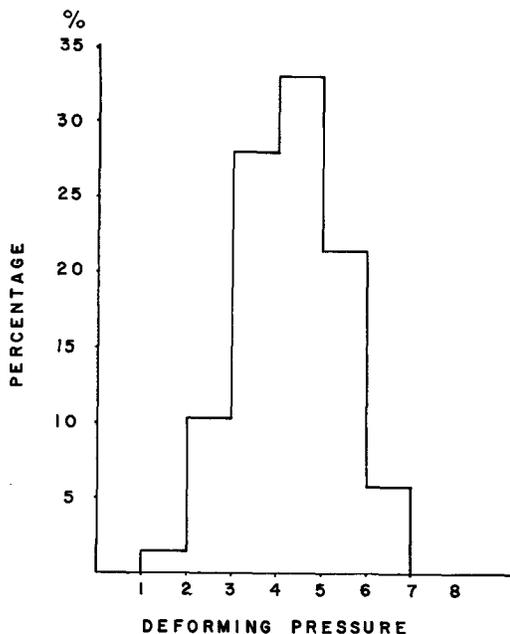

FIGURE 1 Histogram showing the percentage distribution of deforming pressures (in cm of Brodie's fluid) of sarcoma 37 cells incubated in calcium- and magnesium-free Hanks' balanced salt solution. (100% = 136 observations).

into the axillae and groins of Swiss mice, each mouse receiving two injections of cells from both treatment groups; and the appearance of tumors was noted.

Lipids were extracted from the supernatant fluids of about 3 × 10^8 centrifuged EDTA-treated and control cells by means of chloroform: methanol (2:1) mixtures and examined by the technique of thin-layer chromatography (3). Supernatants were also tested for the presence of carbohydrates by the technique of DuBois (4) in which 5 ml of concentrated H_2SO_4 are layered onto 2 ml of test solution containing 0.5 ml of a 45% (WW) aqueous solution of phenol. An orange-yellow color at the interface, which indicates a positive reaction, was given by as little as 0.00001% glucose and sucrose, and 0.025% sialic acid.

RESULTS

The histogram shown in Fig. 1 indicates the distribution of deformation pressures in 136 cells washed and incubated in CMF.

Some of the results are summarized, together with statistical analyses in Table I. It is shown that the cells incubated in CMF revealed no change in deformability compared with the controls incubated in normal HBSS, which contains calcium and magnesium ions. Following treatment with EDTA, the cells became significantly more easily deformable. After EDTA treatment, normal deformability properties could be restored to the cells by incubating them in either HBSS or HBSS containing calcium but not magnesium ions. After EDTA treatment, incubation of the cells in HBSS containing magnesium but not calcium ions did *not* restore mechanical properties to those of the controls. However, comparison of the measurements of cells treated with EDTA and then calcium-free, magnesium-containing HBSS, with those of cells treated with EDTA for the same total period of time, shows for the former cells a significant increase in negative pressure (t = 3.97 with 68 df; $p < 0.001$), indicating a partial restoration of mechanical properties following incubation with magnesium ions in the absence of calcium.

In one series of experiments, after treatment with EDTA the cells were washed in CMF and then incubated for 30 min at 37°C with either CMF or EDTA in CMF. For 55 cells measured after reincubation in CMF, 4.42 ± 0.13 cm of Brodie's fluid were required to produce standard deformation, as compared to 3.78 ± 0.14 cm required for 30 cells reincubated in EDTA solution; these means were significantly different from each other (t = 3.11 with 83 df; $0.01 > p > 0.001$),

TABLE I

The Negative Pressures* Required to Pull Out a Hemispherical Bulge from Sarcoma 37 Cells into a Micropipette of 5 μ Internal Diameter, Following the Indicated Treatment

The mean pressures are compared with those of control cells washed and incubated in Hanks' saline (HBSS). Note that Ca^{++}-free HBSS contains magnesium ions.

Treatment	Deforming pressure ± se cm of Brodie's fluid	No. of measurements	t-Tests (against controls)
Controls (washed and incubated in HBSS)	4.42 ± 0.07	20	
CMF	4.31 ± 0.02	56	$t = 1.13$ with 74 df. $0.3 > p > 0.2$
EDTA-CMF	2.55 ± 0.01	30	$t = 7.39$ with 48 df. $p < 0.001$
EDTA-CMF; then HBSS	4.97 ± 0.08	20	$t = 1.40$ with 38 df. $p \approx 0.2$
EDTA-CMF; then Mg^{++}-free HBSS	4.59 ± 0.05	20	$t = 0.48$ with 38 df. $0.7 > p > 0.6$
EDTA-CMF; then Ca^{++} free HBSS	3.18 ± 0.01	40	$t = 5.03$ with 58 df. $p < 0.001$

* In cm of Brodie's fluid.

TABLE II

The Electrophoretic Mobilities of Sarcoma 37 Cells Treated as Indicated

The t-tests show no significant difference between the three treatment groups.

Treatment	Mobilities ± se (No. observations) μsec^{-1}. $volts^{-1}$. cm	t-Tests between treatment groups
1. Controls (washed, incubated, and measured in HBSS at pH 7.2)	−1.468 ± 0.034 (100)	1 v 3 $t = 0.89$ with 198 df. $0.4 > p > 0.3$
2. CMF (measured in CMF at pH 7.8)	−1.451 ± 0.038 (100)	1 v 2 $t = 0.33$ with 198 df. $0.8 > p > 0.7$
3. EDTA (measured in CMF at pH 7.8)	−1.513 ± 0.038 (100)	2 v 3 $t = 0.3 > p > 0.2$

although the deformability of cells reincubated was not significantly different from that of the controls (4.42 ± 0.07) shown in Table I, which indicated a restoration of mechanical properties following incubation in CMF after previous EDTA treatment. On the other hand, the cells reincubated in the EDTA solution showed incomplete restitution of deformability compared with the controls ($t = 2.37$ with 48 df; $p \approx 0.02$).

Electrophoretic Mobility

The mobilities of the cells under the specified conditions, together with a comparison of their means by the t-test, are shown in Table II. It is shown that the cells incubated in CMF demonstrate no significant change in mobility compared with "control" cells incubated in HBSS. Furthermore, no significant change is demonstrable between the cells incubated in HBSS and those treated with EDTA, nor between the cells treated with EDTA and those incubated in CMF.

Radioautography

The results are summarized in Fig. 2 (*a–f*) which shows progressive optical cuts through three cells in the same microscope field. It can be seen that with progressive cuts a peripheral distribution of exposed grains is revealed, indicating that at least some of the EDTA is located at or near the peripheral zone of the sarcoma cells. Optical cuts on radioautographs of cells labeled with tritiated uridine did not reveal a similar peripheral distribution of grains.

Thin Layer Chromatography

No differences were detected between the two groups of supernatant fluids from centrifuged cells, previously treated with either EDTA or
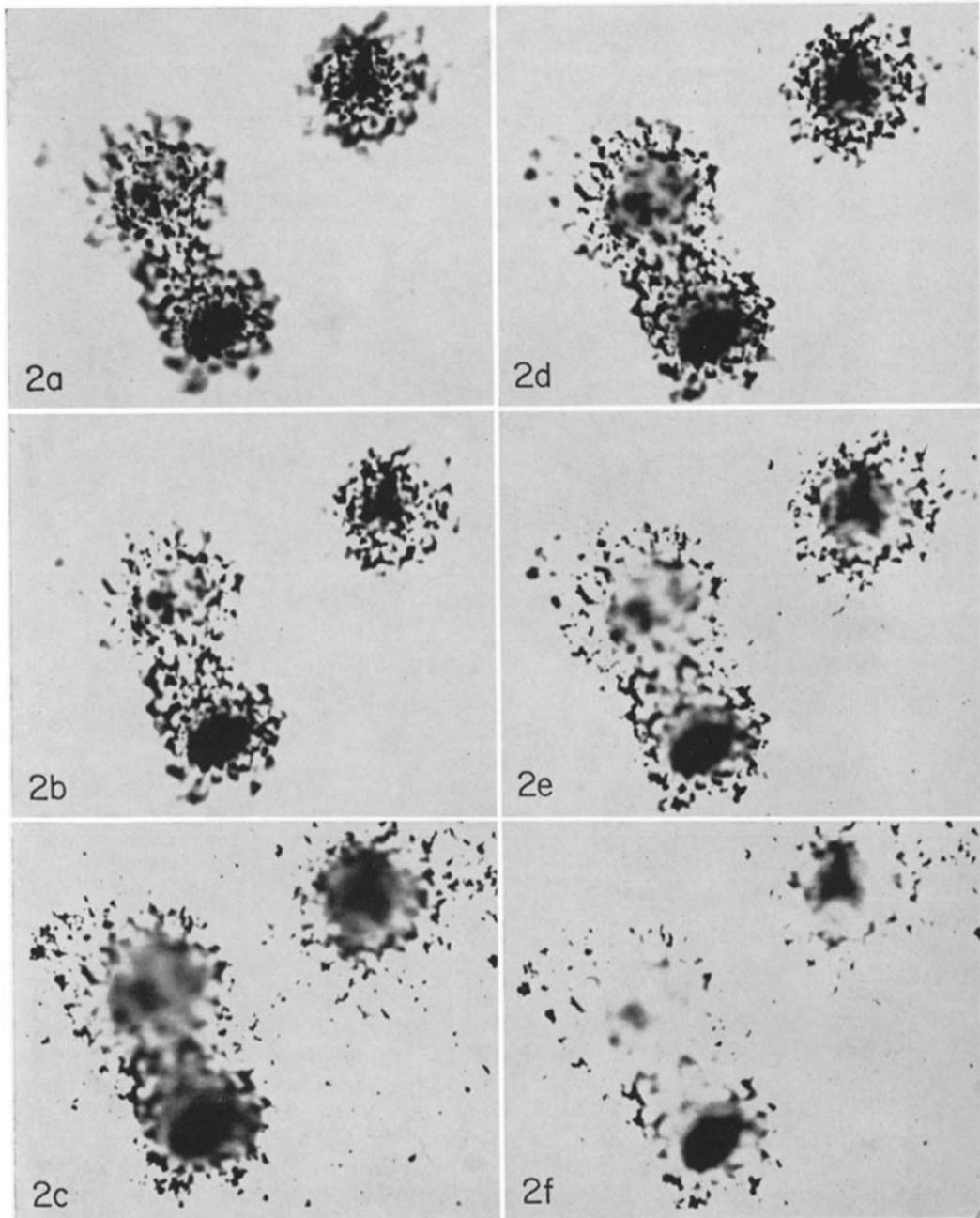
FIGURE 2   Radioautographs of cells incubated with EDTA-^{14}C. The figures a to f show progressive optical "cuts," in steps of approximately 1 μ, and indicate that much, if not all, of the EDTA is located in the peripheral zones of the cells.

CMF. By this technique, classes of neutral lipids (sterol esters, triglycerides, fatty acids, and sterols) and phosphatides (phosphatidylserine, phosphatidylinositol, sphingomyelins, lecithins, sulphatides, phosphatidylethanolamines, and ceramides) can be identified. All four classes of neutral lipids and ceramides were detected in trace amounts in both groups.

Viability Tests

Tumors developed in 9/16 of sites injected with EDTA-treated cells, and in 11/16 of sites injected with CMF-treated controls; these results are not significantly different ($\chi^2 = 0.133$, $0.8 > p > 0.7$). Trypan blue exclusion tests revealed 96.4% viable cells in the EDTA-treated group, compared with 97.6% in the CMF-treated controls.

DuBois' Test

No carbohydrates were detectable in the supernates from either the EDTA-treated cells or their CMF-treated controls.

DISCUSSION

It must be emphasized that the present discussion is based on experiments done with murine sarcoma 37 ascites tumor cells and should not be extrapolated to other types of cell in the absence of experimental data.

As discussed in the previous papers in this series, (1, 5), measurements of cell deformability reflect mechanical changes taking place in the cell periphery but, owing to the physical complexity of this region, the changes cannot be analyzed mathematically. The experimental results indicate that cells became more easily deformable following treatment with EDTA, and that this change was reversed on incubation in calcium-containing saline. These data suggest that the changes in cellular mechanical properties are directly related to the loss of calcium ions from the peripheral regions of cells. The fact that the restoration of mechanical properties is only partial following EDTA treatment and incubation in saline containing magnesium ions but no calcium, is in accord with other observations (6, 7) that calcium ions cannot be replaced by magnesium ions in a variety of cell contact phenomena. The observation that after EDTA treatment cells regained normal deformability properties upon incubation in calcium- and

magnesium-free saline suggests that calcium ions were leaving an "inner" cellular compartment to replace those removed by the EDTA. This suggestion was substantiated by the observation that EDTA-treated cells did not regain their measured mechanical properties when reincubated in the presence of more EDTA, which presumably complexed the calcium entering the peripheral regions from the "inner" compartment. The failure of cells to regain their normal deformability after EDTA treatment and on reincubation in saline containing magnesium but not calcium ions may possibly be explained in terms of the presence of magnesium retarding or inhibiting the centrifugal diffusion of calcium ions.

The increased deformability on incubation of cells with EDTA, together with the absence of demonstrable change when they were incubated with calcium- and magnesium-free saline, suggests that the calcium ions are not bound to readily dissociable groups in the cell periphery, but are strongly bound to the extent that they can only be removed to form the higher energy coordinates with EDTA. It is not possible to comment further on the energetics of calcium binding, as no data are presently available for the dissociation constants of calcium salts or coordinates at the cell periphery.

Wherever the calcium ions are bound in the cells, the lipid and sugar analyses militate against the deformability changes being due to loss of membrane lipids or lipopolysaccharides, as observed in EDTA-treated *E. coli* (8); and the changes are unlikely to be associated with cell death, since only intact cells were measured and the animal inoculation tests indicated no demonstrable changes in viability of cell populations as a whole.

Calcium ions could conceivably be bound in the cell periphery to anionic groups which are deep or superficial with respect to the electrical double-layer surrounding the cell. As a cell moves through the electrophoresis apparatus in physiologically isotonic suspending fluids, only those charged groups located no farther than about 10 A from the hydrodynamic slip plane will reflect in cellular electrophoretic mobility (9). The fact that no change in mobility was detected following incubation in EDTA raises the possibility that, within the limits of the technique which is sensitive to mobility changes of the order of 5%, either the chelated calcium is bound to anionic groups in

the cell peripheral zone which lie deeper than 10 A from the hydrodynamic slip plane, or alternatively superficial anionic sites revealed by calcium removal are almost immediately masked by binding univalent cations. Rothstein and Meier (10) have studied the binding of anionic groups at the peripheries of yeast cells with a variety of cations. Their data on uranyl ion binding suggest that hydroxyl, carboxyl, and/or phosphatic groups are involved and that, of these, high polymeric polyphosphates are the most likely. They observed that, although the anionic groups at the yeast cell periphery showed considerable affinity for divalent cations including Ca^{++}, Mg^{++}, Zn^{++}, and Be^{++}, no affinity for Na^{+} or K^{+} was observed on the basis of displacement of bound uranyl ions. As the dissociation constants of univalent ions from anionic sites is expected to be different from that of divalent ions, it seems unlikely that anionic groups exposed by removal of calcium would be completely concealed electrically by newly bound univalent ions from the medium. Additional data revealing the poor binding capacity of superficial anionic groups at the cell periphery come from the work of Bangham and Pethica (11, 12) who showed that very high concentrations of calcium ions are required to produce charge reversal in murine Ehrlich ascites tumor cells. In other words, calcium is not bound at the cell surface in the sense that a surface is a two-dimensional planar structure, but is bound within the three-dimensional peripheral zone of these cells. This difference between the cell surface and its peripheral zone has been stressed in the context of cell contact phenomena (13).

In 1900, Herbst (14) demonstrated that blastomeres of *Echinus microtuberculatus* could be dispersed in calcium-free sea water. On the basis of this and other work (reviewed in reference 6), Coman (15, 16) suggested that the decreased mutual adhesion between some neoplastic cells might be related to their decreased calcium content; and, in support of this, Zeidman (17) observed that calcium removal facilitated the separation of cells from each other. It has been suggested that in a general way much of the metastatic process can be accounted for in terms of the low calcium content of tumors. In a critical review of the field (18), it has been pointed out that generalizations of this type should not be made because of the shortage of data.

It has been known for many years that mammalian cells carry a net negative surface charge, and, consequently, the role of calcium in cell adhesion has been postulated as a "bridge" linking anionic sites on adjacent cells (19). Other roles of calcium have been postulated in terms of its effect on the electrical double-layer (20), desolvation (21), and in stabilizing coacervates of intercellular mucosubstances (22). Experimental evidence from a number of different systems (23) indicates that these reactions of calcium are considerably more complex than the simple interactions of a divalent cation with any acidic group. The present results would suggest that, even if intercellular calcium "bridges" do exist between cells in general, they are unlikely to exist between sarcoma 37 ascites tumor cells, since the anionic groups are too deep within the peripheral zones to permit a 5-A calcium "bridge" to link them together. In view of the effect of EDTA treatment on cell deformability, calcium might well be visualized as forming tangentially oriented "bridges" within the cell peripheral zone, which would affect its mechanical strength but which would not affect cell adhesion. On the other hand, such deep, tangential bonds, by affecting the cohesive strength of the cells, would be expected to affect their ability to separate from each other, since it has been argued that cell separation is dependent on the cohesive strength of this region, as distinct from interfacial or intercellular adhesive strength (13).

It might well be argued that sarcoma 37 ascites cells exist only in a free state because they cannot adhere to each other by forming radially oriented calcium "bridges." However, these cells do adhere, apparently strongly, to the peritoneum. If radial calcium "bridges" are involved in the adhesion of ascites cells to peritoneal cells, it is difficult to understand why they are not involved in promoting mutual adhesions between the tumor cells. In assessing the role of calcium-binding in tumor cells, it must not be forgotten that the data of Bangham et al. (11, 12) also indicate that in isolated liver cells this binding is as poor as it is in Ehrlich ascites tumor cells.

As stated at the beginning of this discussion, it is not proposed to generalize about calcium in relation to the cell periphery on the basis of experiments done on one cell type. However, the data do suggest that in the cell type studied the calcium binding at the cell periphery may be considered in terms of deep anionic sites, as well as the "conventional," superficial, true surface sites.

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