Effect of Trypsin and Ethylenediamine Tetra Acetic Acid on the Rate of Adhesion of KB Cells

Despite the wide utilization of trypsin and ethylenediamine tetra acetic acid (EDTA) to release cells in suspension, relatively little information is available about the effect of these two compounds on the adhesiveness of living cells[1-5]. In a preliminary work we developed a method to measure the kinetic of adhesion of living KB cells to the glass[6]. The phenomenon studied by this method being mainly related to active sticking on glass of single cells, we became interested to evaluate to what extent this process of adhesion was affected after pretreatment of the cell population with EDTA or Trypsin.

Materials and methods. KB cells (human carcinoma of the pharynx) were obtained from the American Type Culture Collection (Rockville, Md., USA) in 1970 (KB-364-CCL17), carried through 5 passages and stored in liquid nitrogen. Growth media were Eagle's Minimum Essential Medium on Hank's salt solution (MEM) and Eagle's Minimum Essential Medium Joklik modified for suspension cultures (MEMJ). Both media were purchased as powder media from Gibco (Grand Island N.Y., USA). Calf serum obtained from Sclavo (Siena, Italy) was always added to growth media at 10% concentration. Trypsin was used as 0.25% solution, in Ca- and Mg-free solution (CMFHS), of the 1:250 trypsin from Difco (Detroit, Mich.). EDTA was used as a 0.02% solution, in CMFHS, of the disodium salt of ethylene diaminotetracetic acid.

The experiment was performed as previously described[6]: 1 ml aliquots of cell suspension in MEM+10% serum were distributed in slanted test tubes in a slow rotating roller drum. After different lengths of time, groups of 5 test tubes were randomly chosen and the 2 populations of living adhering cells and non-adhering cells were evaluated by means of a Coulter Counter Mod. B.

Treatment was as follows: monolayers were shortly washed in CMFHS and then treated by trypsin or EDTA solutions for the time indicated under results.

The cell suspensions recovered at the end of treatment were checked for viability by erythrosine dye exclusion

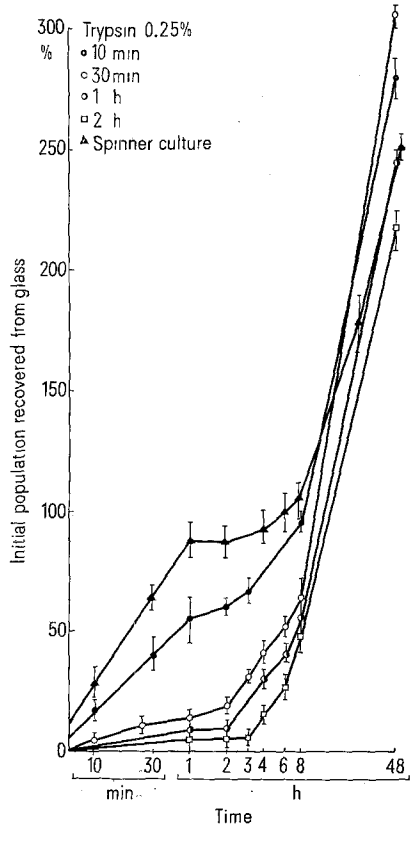

Fig. 1. Effect of trypsin. Percentage of the initial population as a function of time (log scale): Adhering cells recovered from glass by 10 min treatment with trypsin. Vertical bars represent the standard error of the means ($n = 5$).

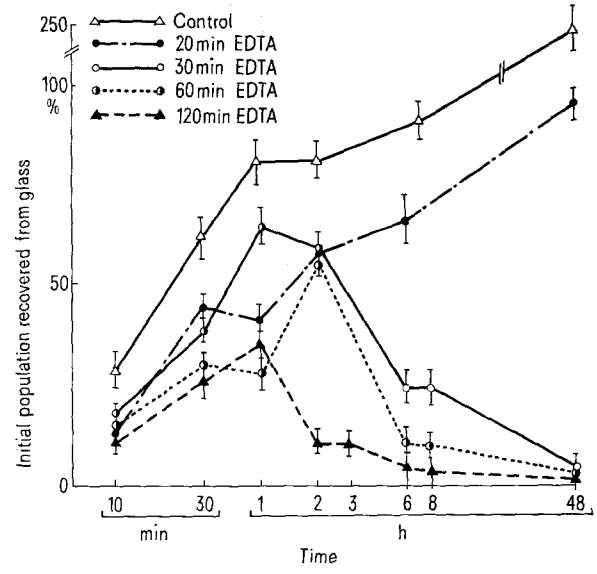

Fig. 2. Effect of EDTA. Percentage of the initial population as a function of time (log scale): Adhering cells recovered from glass by 10 min treatment with trypsin. Controls are the same spinner cultures of Figure 1. Vertical bars represent the standard error of the means ($n = 5$).

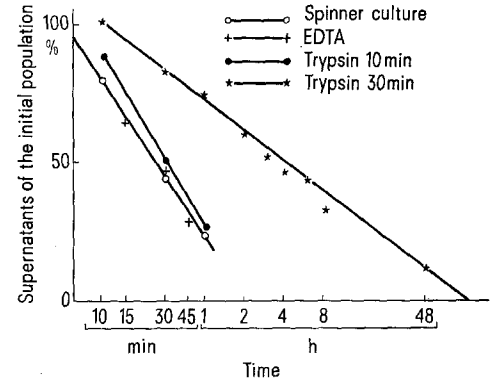

Fig. 3. Percentage of the initial population as a function of time (log scale): Non-adhering cells recovered from supernatants. Regression lines obtained after variance analysis.

[1] A. C. Taylor, Expl Cell Res., suppl. *8*, 154 (1961).
[2] S. Nordling, Acta path. microbiol. scand., suppl. *192* (1967).
[3] A. A. Moscona and M. H. Moscona, Expl Cell Res. *45*, 239 (1967).
[4] G. Poste, Expl Cell Res. *65*, 359 (1971).
[5] L. Weiss and D. L. Kapes, Expl Cell Res. *41*, 601 (1966).
[6] L. Morasca, E. Dolfini and A. M. Frache, Experientia *28*, 1263 (1972).

test after washing and resuspending in MEM+10% serum. Cells grown in spinner flasks as cell suspensions were washed from MEMJ+10% serum and resuspended in MEM+10% serum to reconstitute the normal concentration of Ca and Mg ions able to support adhesion.

Results. The rate of adhesion of a viable cell population treated for 10 min with trypsin is shown in Figure 1, compared with the rate of adhesion of untreated cells grown in suspension (spinner culture). The 2 functions follow an exponential pattern during the first hour followed by a plateau and a secondary exponential phase of growth. Trypsin-treated cells have a slower rate of adhesion than untreated ones showing a less pronounced plateau before the start of the cellular growth.

Cells treated for longer periods of time by trypsin (Figure 1) show proportionally slower rates of adhesion in which no plateau is present. This may be due to the fact that after cell growth is started, the adhesion of part of the population is still taking place and therefore the function obtained is the resultant of the simultaneous presence of these 2 phenomena.

The same experiment performed on EDTA-treated cells is shown in Figure 2. The rate of adhesion follows an irregular pattern during the first hour and later falls to zero, showing that these cells were not viable despite their capacity to adhere during the first few hours. This late toxicity of EDTA is also evident in the 20 min sample that reaches the value of the initial population only after 48 h. This sample, however, was released in suspension by scraping because its time of contact with EDTA was too short to release in suspension the cells.

Figure 3 reports the 3 functions obtained for nonadhering cells. It appears that 10 min trypsin-treated cells, EDTA-treated cells, and spinner cultured cells, all disappear from culture medium at the same rate while 30 min trypsin-treated cells disappear at a lower rate. This conclusion was reached by performing analysis of variance on the data obtained.

Discussion. It may first be noted that by the approach of measuring both the rate of adhesion of living cells and the rate of disappearance of non-adhering cells, it becomes much easier to separate the true process of cell adhesion from the cell growth and death, normally present in every cell population. In this way it can be seen that EDTA and trypsin have quite a different effect on adhesion and on the subsequent growth of KB cells.

EDTA does not affect adhesion but, in agreement with data on riaggregation[3], shows quite a toxic effect on living cells. EDTA being unable to modify the rate of disappearance of non-adhering cells, it appears that a non-viable population of cells may adhere to glass at the same rate as a living one.

Trypsin is able to decrease the rate of adhesion and apparently reduces the lag, period before growth resumption. Since any trypsin possibly adsorbed on the plasma membrane during the treatment[4] is readily inactivated by the serum added to the culture medium[7], the hypothesis that membrane adsorbed trypsin could interfere with the process of adhesion can be ruled out. At the contrary, the decrease of the rate of adhesion being proportional to the time of contact of cells with trypsin, the effect on adhesion may be interpreted as a removal of membrane proteins, as was suggested by other authors[8] investigating the process of cell aggregation.

In conclusion, the adhesion of KB cells to a glass surface is a process not necessarily related to the viability of the cell population. EDTA and trypsin, in the experimental conditions here utilized, may well serve the purpose to demonstrate this dissociation and to help to understand the mechanisms involved in cell adhesiveness[9].

Riassunto. La velocità di adesione al vetro di cellule pretrattate con Tripsina ed EDTA è stata comparata a quella di cellule cresciute in sospensione, utilizzando un metodo che permette di valutare la popolazine di cellule adese vitali. I risultati mostrano che il pretrattamento con Tripsina riduce la velocità di adesione senza ridurre la vitalità delle cellule, mentre il trattamento con EDTA pur essendo citotossico non altera la velocità di adesione.

L. MORASCA, G. BALCONI, E. DOLFINI and C. OLDANI

Istituto di Ricerche Farmacologiche, 'Mario Negri', Via Eritrea 62, I-20157 Milano (Italy), 21 February 1973.

[7] M. D. ROSENBERG, Biohpys. J. *1*, 137 (1960).
[8] B. PESSAC and F. ALLIOT, C.r. hebd. Séanc. Acad. Sci. (D), Paris *269*, 755 (1969).
[9] This work has been supported by CNR Contract No. 115/1134/05033.

The Effect of Glibenclamide on Non-Esterified Fatty Acid Levels in the Plasma of the Severely Alloxan Diabetic Rat

In addition to their ability to stimulate insulin release from the pancreatic β-cell, it has also been postulated that the hypoglycaemic sulphonylureas exert an effect at certain extrapancreatic sites[1,2]. BEWSHER and ASHMORE[3] observed a direct inhibition of hepatic lipase activity by tolbutamide, and WEISS et al.[4] showed glycodiazin would inhibit triglyceride lipase bound to the lysosomal structure. These results explain the often observed inhibition of ketogenesis in vitro by the hypoglycaemic sulphonylureas.

SCHMIDT et al.[5] showed a reduction in the plasma concentration of non-esterified fatty acid (NEFA) and β-hydroxybutyrate in alloxan diabetic rats 1 h after the i.p. injection of glibenclamide 0.5 mg/kg. The effect on plasma β-hydroxybutyrate has been confirmed by other workers[6]. SCHMIDT[5] observed no change in plasma insulin levels at sacrifice both histologically and immunologically and suggested that an effect of glibenclamide on adipose tissue lipase was responsible for the fall in NEFA observed[7]. The animals used in the latter investigation were given alloxan i.p. (75 mg/kg), and left for 10 days to develop stable diabetes. Animals were then selected for the experimental group on the basis of blood sugars in excess of 300 mg/100 ml. In our laboratory rats given alloxan 75 mg/kg i.v. developed diabetes after 48 h. of a severity which closely paralleled that expected after pancreatectomy. Indeed, treatment of these rats with 100 mg/kg glibenclamide orally every 5 h for 15 h (4 doses in all), produced no detectable elevation of the plasma insulin level (FOY and STANDING, unpublished observation). The experiment of SCHMIDT was, therefore, repeated and then a similar investigation was carried out using our own animal model, comparing the response to glibenclamide with tolbutamide and chlorpropamide.

Materials and methods. Male C.S.E. (Bradford Strain) rats weighing 200–220 g, were used in all experiments. Diabetes was induced by slow i.v. injection of 75 mg/kg