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ADHERENCE CHROMATOGRAPHY OF RAT BONE MARROW CELLS

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SUMMARY

Model experiments were carried out with rat bone marrow cells to investigate the possibility of fractionating complex cell populations into several fractions by gradient elution chromatography. Cells adhering with different strengths to glass beads in a column could be eluted stepwise by using an increasing concentration gradient of EDTA as the eluant. Standard chromatographic equipment was used and the fractions were evaluated by a turbidimetric method.

Typical elution curves have been obtained with marked peaks following after each increase in the concentration of EDTA, especially in the interval between 1.5–4 m*M*. Different types of cells were found in fractions corresponding to the individual peaks.

INTRODUCTION

The development of chromatography has led to its application to the separation of larger and larger molecules and particles and recently even of such complex and sensitive moieties as living animal cells. Reports have been published describing the separation of certain types of cells on columns filled with glass[1] or cotton wool[2,3], with glass beads[4] or siliconized glass[5–7], with glass or plastic beads carrying a fixed antigen or hapten[8–11], with ion exchangers[12], etc. Non-adhering lymphocytes and erythrocytes of human blood have been separated from other leucocytes that do adhere to siliconized glass[2,5]. Some of the adhering cells were eluted by using a standard 0.02 % solution of the disodium salt of ethylenediaminetetraacetic acid (EDTA)[5,6,13]. Splenic cells have been fractionated in a similar way into two sub-populations according to their adherence to glass beads and their elution with 10 m*M* EDTA solution[4]. The role of a Mg^{2+}- and Ca^{2+}-dependent serum factor that influences the adherence of cells has also been described[4,6,7]. The viability of cells was generally unaffected by such separation procedures[5–7].

In the present work we investigated in model experiments the possibility of fractionating the very complex suspension of rat bone marrow cells into several fractions by gradient elution chromatography. The work was based on the assumption that different living cells adhere with different strengths to the surface of glass

beads in the column and could be eluted stepwise by a discretely growing concentration gradient of EDTA solution. As the phenomenon of active cell adherence[6,14] seems to be fundamental to this technique, we have used the term "adherence chromatography" for such kinds of separations. We also ascertained the possibility of using for the chromatography of cells the standard equipment that is commonly used for column chromatography and for the photometric evaluation of effluents.

MATERIALS AND METHODS

Rat bone marrow cells were flushed from femurs and tibias of four freshly killed Wistar male rats. The crude cell suspension in the basal Eagle's medium (BEM, Sevac, Czechoslovakia) containing 10 or 20 % of calf serum was filtered through a fine-mesh polyamide sieve to remove tissue debris. The cells were then washed and suspended in the same medium to give a final concentration of $75 \cdot 10^6$ cells per ml. The cells were either chromatographed immediately or within a few hours of storage at 4°.

Chromatographic columns were 30–40 cm high and 1.3 cm in diameter, jacketed, connected with a Hoepler ultrathermostat and maintained at 37°. The bottom was covered with a fine polyamide net. Before use, glass beads of diameter 0.2 mm (Jablonecké sklárny n.p., Czechoslovakia) were washed either in boiling 65 % nitric acid for 90 min or in a hot (95°) mixture of concentrated sulphuric acid and potassium dichromate for 20 min. The beads were then rinsed with distilled water in a funnel until the pH of the eluate reached about 6. The wet slurry of the beads was poured into the columns up to a height of 10 cm. Immediately before chromatography, the columns were washed either with water or preferably with 200 ml of saline (0.9 % sodium chloride solution) followed by 30–50 ml of BEM, both adjusted with sodium hydrogen carbonate solution to pH 7.1–7.2.

A typical chromatographic run was as follows. Eagle's medium of pH 7.1–7.2 (30 ml) was passed through the column at a rate of about 1 ml cm^{-2} min^{-1}. 1 ml of the cell suspension containing $75 \cdot 10^6$ cells was applied and soaked into the column at the same rate. Eagle's medium containing 10 % of inactivated calf serum (Sevac, Czechoslovakia) was then applied in two 1-ml portions, the flow-rate was adjusted to 0.4–0.5 ml cm^{-2} min^{-1} and fractions of 2–3 ml were collected by using an automatic fraction collector. Then 17 ml of the same Eagle's medium were added and passed through the column at the lower flow-rate until a total of 10 ml of effluent were collected. The flow-rate was then increased again to 1 ml cm^{-2} min^{-1} and was maintained at this level for the remainder of the experiment. Successive 20-ml portions of Eagle's medium, containing 10 % of calf serum, and 1.5–10 mmoles of EDTA were then added, always at the moment when the previous solution had just soaked in. Each solution was adjusted to pH 7.1–7.2 with 7.5 % sodium hydrogen carbonate solution. The tip of the applicator (a 20-ml pipette) was bent upwards to avoid turbulence in the upper layer of the column.

The turbidity (which was proportional to the cell count) was measured with the TK turbidity attachment of the Spekol photometer (G.D.R.) at 480 nm in 0.495-cm cuvettes. Previously 1 drop (about 0.03 ml) of fresh 7.5 % sodium hydrogen carbonate solution was added to each test-tube to ensure that the same degree of phenol red coloration was present in BEM.

RESULTS AND DISCUSSION

Fig. 1 shows a typical elution pattern of rat bone marrow cells after column chromatography on glass beads treated with boiling nitric acid and pre-washed only with water. We used a randomly chosen discretely growing concentration gradient of EDTA solution over the range 3.0–10 m*M* in Eagle's medium containing 20 % of calf serum.

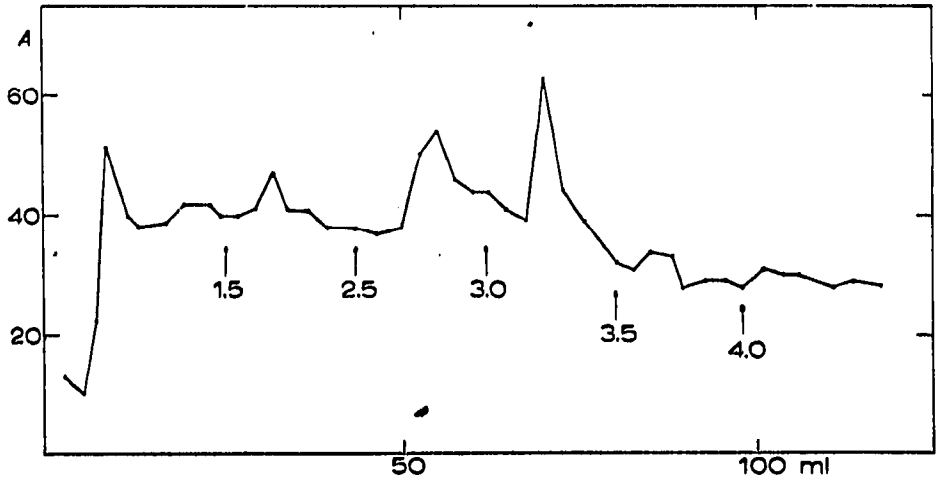

Fig. 1. Adherence chromatography of rat bone marrow cells. The 10 × 1.3 cm column of glass beads, 0.2 mm diameter, treated with nitric acid, then washed with water followed by BEM. $75 \cdot 10^6$ cells suspended in 1 ml of BEM containing 20% of calf serum were applied, pH 7.1–7.2, 37°. Elution with BEM +20% of calf serum containing an increasing concentration of EDTA. Applications and concentrations of EDTA in mmoles are indicated by arrows.

Fig. 2 shows the results of a similar experiment in which the glass beads were treated first with the solution of potassium dichromate in concentrated sulphuric acid, then washed with water followed by a rinse with saline adjusted to pH 7.1–7.2 with sodium hydrogen carbonate solution. BEM contained only 10 % of calf serum.

The elution curve in Fig. 3 was obtained on glass beads treated with boiling nitric acid (as in Fig. 1) but then washed with saline and sodium hydrogen carbonate solutions (as in Fig. 2).

The chromatographed cells for which the results are given in Figs. 2–4 were pooled from one group of animals.

The results shown in the figures illustrate that the hypothesis of adherence chromatography and gradient elution of cells is valid and that the technique is easy to perform with the use of standard chromatographic equipment.

Each addition of a higher concentration of EDTA solution (indicated by arrows) was followed by a markedly increased elution of cells from the column. The first peak, however, corresponded to cells that did not adhere to the glass beads. In Figs. 2 and 3 the first peaks show two marked maxima.

The most effective concentrations of the EDTA solutions used in the eluant were between 1.5 and 4.0 m*M*. The different elution profiles in Figs. 1, 2 and 3 indicate that the separation was very sensitive to variations in the experimental conditions, *e.g.*, pre-washing of the glass beads, concentration of serum and contingent variability of experimental animals.

To demonstrate the fractionation of rat bone marrow cells, which consist of a very complex mixture of cells in different stages of maturation, we simplified their classification by a gross differentiation of six main morphological types. The results in Fig. 4 show that the fractions corresponding to the maxima of indi-

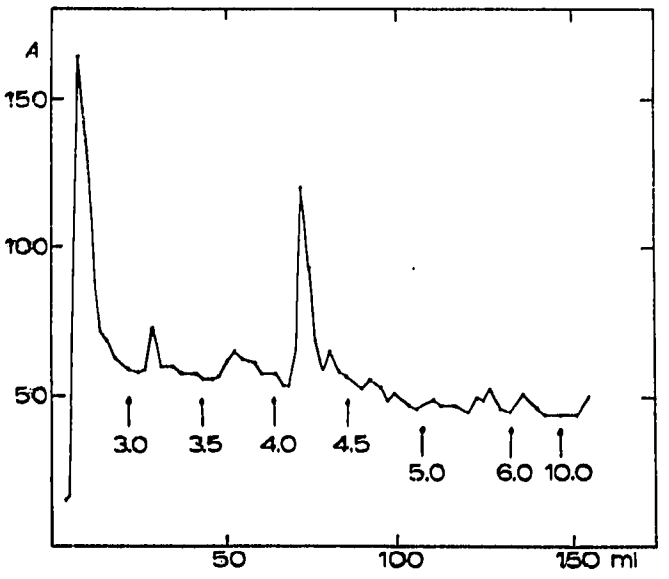

Fig. 2. Adherence chromatography of rat bone marrow cells. 10 × 1.3 cm column of glass beads, 0.2 mm diameter, treated with potassium dichromate in concentrated sulphuric acid, then washed with 0.9% sodium chloride solution adjusted to pH 7.1–7.2 with sodium hydrogen carbonate solution followed by BEM. Other details as in Fig. 1. The eluant contained only 10% of calf serum.

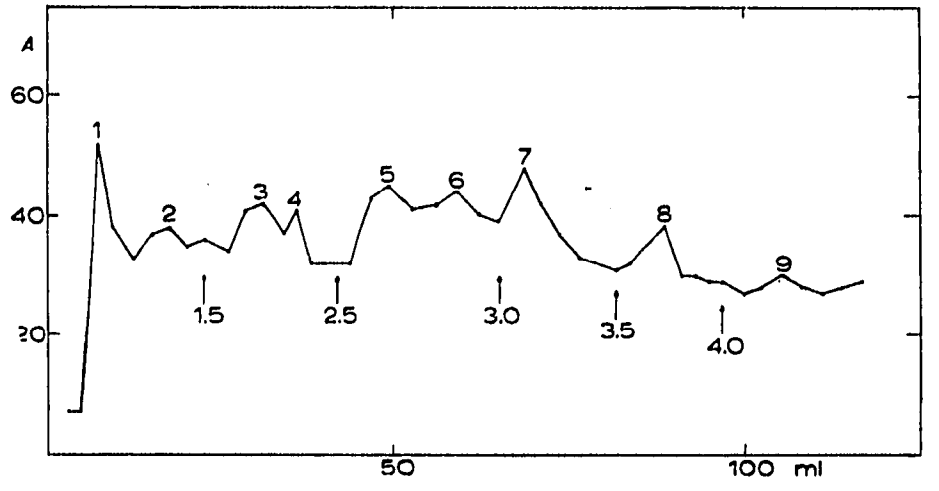

Fig. 3. Adherence chromatography of rat bone marrow cells. 10 × 1.3 cm column of glass beads, 0.2 mm diameter, treated with nitric acid. Washing and further details as in Fig. 2.

vidual peaks of the elution curve (Fig. 3) differed markedly in the type of cells that they contained. We did not find significant morphological damage of the cells that had passed through the column. Also, their viability (tested with neutral red) remained without greater changes.

These findings are encouraging for further experimentation on adherence

chromatography of cells and are also in general agreement with previous results[4-7]. However, there was considerable variability from experiment to experiment both in the total recovery of the cells and in the cell counts in individual peaks. This problem is being further investigated.

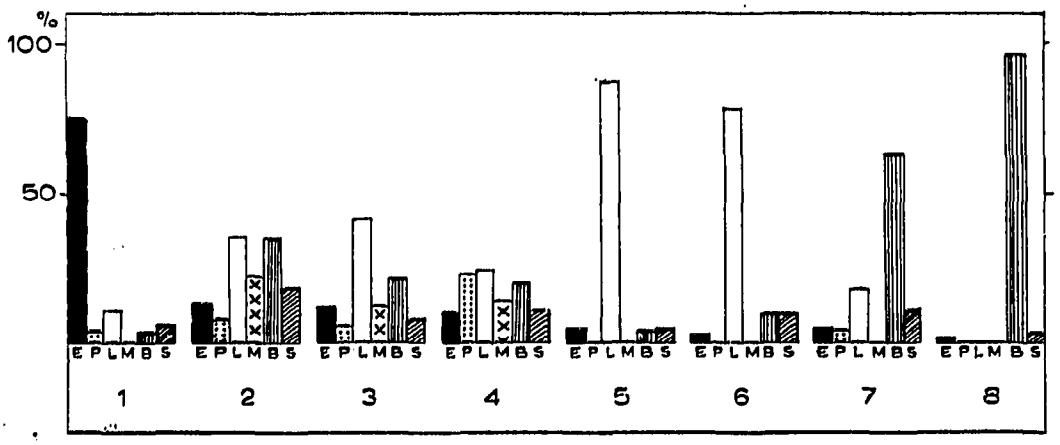

Fig. 4. Differences between the cell populations present in the individual peaks. 2–3-ml fractions corresponding to the maxima of the numbered peaks in Fig. 3 were used for differential cell counts. For simplification, all cells were classified into only six morphological groups: erythrocytes (E), polymorphonuclear leucocytes (P), lymphocytes (L), monocytes (M), large immature cells (B) and small immature cells (S). 100 cells were counted at random in each smear stained by the May–Grünwald and Giemsa–Romanowski techniques.

Generally, the cells in each peak have one characteristic in common: they have lost their ability to adhere to the glass beads at a given concentration of EDTA solution. It is still difficult and premature to interpret this elution phenomenon in detail, because many extremely complex factors are involved[4,6,7,14]. The forces involved may be roughly divided into those causing "passive adsorption" between various chemical groups on the glass and cell surfaces as well as electrostatic and hydrophobic interactions, and those causing "active adherence" due to phagocytic and similar active abilities typical for some types of living cells.

It is evident also that the complex composition of the incubation and elution medium (BEM + serum) that is necessary to ensure the viability of the cells, makes an exact interpretation still more difficult. Nevertheless, we assume that the results of adherence chromatography under standard conditions depend on the type, age and the general state and viability of the cells and on their specific resistance towards the given eluting agent (*e.g.*, EDTA).

From this aspect, adherence chromatography in a concentration gradient of EDTA solution or of another suitable eluant, seems to open new possibilities for a fine cell separation which is based not on the size or density but rather on the functional properties of living cells.

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