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Received May 22, 1958. P.S.E.B.M., 1958, v99.

Fractionation of Cell Types in Ehrlich Ascites Fluid by Sucrose Gradient Centrifugation.* (24234)

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For studies in our laboratories concerning the influence of X-irradiation on cell metabolism, it was necessary to obtain a supply of Ehrlich Mouse Ascites Tumor Cells free from erythrocytes, leucocytes and other cell types usually present in ascites fluid.

Erythrocytes may be removed by lysis with hypotonic saline which appears to leave ascites cells intact(1,2). Centrifugation of the mixed cells in Krebs-Ringer bicarbonate or isotonic saline affords a rough separation between erythrocytes and tumor cells whereby either layer may be scooped out with a glass spatula (3). The counter-streaming centrifugal procedure of Lindahl and Klein(4) yields considerably purified tumor cell preparations, but none in which contaminants are completely absent.

We have shown that centrifugation of the mixed cells through a sucrose gradient affords a separation into 4 distinct bands of different cell types. One of these appears to consist of pure ascites tumor cells which are viable.

Methods. Fractionation of cell types. A suitable sucrose gradient was prepared in a centrifuge tube (17 x 3 cm diameter). After first filling with 0.07 M phosphate buffer of pH 7.4, a layer of 50% w/v sucrose in phosphate buffer was introduced into the bottom 2 cm of the tube using a long Pasteur pipette with a small upturned tip. Successive 1 cm layers of sucrose solutions in phosphate buffer, each solution 5% w/v less in concentration than the previous one, were then introduced by upward displacement through the Pasteur pipette until a concentration gradient of 10-50% w/v of sucrose was obtained. After standing 15-30 minutes a glass rod was moved gently up and down through the solution to ensure an even gradient. Mouse ascites fluid (10 ml) was mixed with sodium citrate anticoagulant solution on withdrawal, then centrifuged. The cell mass (2-3 g wet wt) was twice washed in isotonic saline at 5°C and finally resuspended in 10 ml saline. A portion of the cell suspension (1.5-2.0 ml) was carefully layered on top of the sucrose gradient, and the tube centrifuged at 250-300 rpm for 30 minutes at 10-15°C.

Viability tests. Separate experiments were carried out to test the effect of strongly hypertonic (1.17 M) 40% sucrose solution on the viability of ascites tumor cells.

1. *Oxygen uptake of ascites cells treated with 40% sucrose solution in phosphate buffer.* A suspension of saline-washed mixed ascites cells was prepared in phosphate buffer containing 40% w/v sucrose. The suspension was divided into 2 portions, one of which was allowed to stand 40 minutes at 23°C,

* The work was conducted at the instigation of Professor J. van Rooijen. I am indebted to Dr. A. Polson for suggesting the use of a sucrose gradient, to Dr. G. de Kock for microscopical identification of the cells, to Dr. M. H. Silk and Mr. I. J. C. Macintosh for manometric measurements, and to Mr. H. F. Montauban van Swijndregt for valuable technical assistance. The Ehrlich ascites tumor was originally supplied to us by favour of Dr. Kanematsu Sugiura of the Sloan-Kettering Institute.

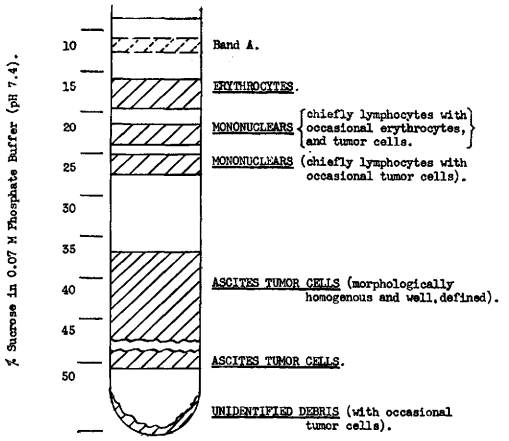

FIG. 1. Fractionation of cell types present in Ehrlich mouse ascites fluid by means of centrifugation in a sucrose gradient.

and the other for the same time at 0°C. Thereafter both portions were dialysed for 1½ hours at 4°C against 0.15 M sucrose (5% w/v) in phosphate buffer. The cells were removed from residual sucrose solution by centrifugation, after which they were resuspended in a medium consisting of mouse ascites serum previously neutralized with hydrochloric acid, buffered with 0.03 M phosphate to pH 7.4 and fortified with 0.25% w/v d-glucose. The final suspension contained 40 mg of sucrose-treated cells/1 ml of fluid and was used for measurement of oxygen uptake in Warburg manometers at 37°C.

2. *In vivo test of viability of ascites cells treated with 40% sucrose in phosphate buffer.* In addition to measurements of respiratory function, the viability of 40% sucrose treated cells was tested *in vivo*. Portions (0.1 ml) of the final suspension of treated cells in phosphate buffered and glucose fortified ascites serum were inoculated into mice under conditions used for routine transplantation of Ehrlich Ascites tumor.

Results. After centrifugation the sucrose gradient was examined against the light. Four distinct bands of cells were revealed, together with some coarser material deposited at bottom of the tube. Microscopic examination of the bands was carried out, following their careful removal with a sampling pipette. The composition of the various layers is illustrated in Fig. 1.

In some runs a thin white band (Band A—Fig. 1) of unidentified material was seen above the erythrocyte layer. The main bulk of ascites cells was found to sediment at the 40% sucrose level. In certain cases continued centrifugation (15-30 minutes) resulted in separation of a further narrow band of ascites cells from below the main bulk, indicated by a broken line at the 45% sucrose level in Fig. 1.

Ascites cells removed from the sucrose gradient appeared to be intact. The cell membrane was somewhat crinated and ratio of cytoplasmic to nuclear volume reduced owing to a certain amount of dehydration. The cells appeared to be more intensely stained by haematoxylin-eosin than fresh untreated ascites cells.

Although large mononuclears were probably present in the main band of ascites cells, these were not directly sought by means of the special technics required to distinguish them from ascites cells.

The respiratory function of ascites cells was apparently not impaired after contact with strongly hypertonic 40% w/v sucrose. The Q_{O_2}† of the cells maintained for 40 minutes in 40% w/v sucrose at 23°C was −5.1 while that of cells maintained for the same time at 0°C was −5.4. Both values are within the limits of −5.0 to −10.0 quoted by Warburg(5) for ascites cells metabolizing in ascites serum, and would indicate that viability of the cells is not affected by contact with 40% w/v sucrose for the time required during centrifugation.

Tumors were produced in 100% of mice inoculated with the 40% sucrose treated ascites cells thus providing additional evidence of viability.

Application of Schreck's staining test(6) to a sample of 40% sucrose-treated cells showed that at least 50% were definitely viable.

Discussion. Centrifugation of mixed ascites cells through a sucrose gradient appears to be a satisfactory method of obtaining pure

† Q_{O_2} values expressed as ul O_2/mg dry wt/hour. Respiration remained undiminished in measurements of Q_{O_2} over 60 min.

ascites tumor cells which are viable and morphologically intact though somewhat dehydrated. Since the fractionation achieved depends upon density of the various cells present as well as viscosity and temperature of the sucrose gradient, minor variations in degree of separation may be expected with samples of ascites fluid withdrawn at different times after inoculation of a host mouse. The method affords a rough fractionation between small and large ascites tumor cells, as well as between these and the erythrocytes and lymphocytes usually present in ascites fluid. The technic forms a useful basis for study of tumor cell-lymphocyte inter-relationships(7). For example, the influence of the lymphocytic cells on *in vitro* growth of ascites tumor cells is being studied in these laboratories.

Summary. Mixed cell types present in Ehrlich ascites tumor fluid have been separated by centrifugation through a sucrose gradient. The method affords a fractionation into 4 distinct bands of cell types. One of these appears to consist of pure ascites tumor cells, although no special technics were applied to determine whether any large mononuclears are also present in this band. Ascites cells removed from the gradient appear to be intact. The cell membrane is somewhat crinated and ratio of cytoplasmic to nuclear volume reduced owing to a certain amount of shrinkage through dehydration. The cells have been shown to be viable after contact with hypertonic sucrose in the gradient. Their oxygen uptake is equivalent to that of fresh untreated ascites cells, and they are capable of producing tumors when inoculated into mice. The separation technic forms a useful basis for study of tumor cells—lymphocyte inter-relationships in ascites fluid.

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Received May 23, 1958. P.S.E.B.M., 1958, v99.

Method for Isolating Single Cells and Preparation of Clones from Human Bone Marrow Cultures.* (24235)

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In our studies with normal human bone marrow cells† attempts were made to obtain cultures derived from single cells. The technic of Sanford(1) using capillary tubes proved inefficient and the plating technic of Puck (2) did not appear reliable insofar as certainty that the clones were derived from a single cell. When these cells are plated in a Petri dish they grow in apparent clones on the glass; however, inasmuch as they migrate on surface of glass and are occasionally released from the glass and settle at other locations we could not be certain that a culture obtained from such a colony had indeed been derived from a single cell. To obtain cultures which would unquestionably be derived from single cells the technic which will be described was developed. This technic involves placing small glass squares in a Petri dish, adding a suspension of cells to the dish, removing the individual glass squares and observing them microscopically, selecting glass squares con-

* These studies were supported in part by research grant from Nat. Science Fn.

† The cells were the Mox strain obtained from Dr. McCulloch of Connaught Med. Res. Labs., Univ. of Toronto, Can. This is an established strain normally maintained on medium 1066 plus 20% horse serum. The 1066 is obtained from Connaught Med. Res. Labs.