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of the sucrose gradient, minor variations in degree of separation may be expected with samples of ascites fluid withdrawn at different times after inoculation of a host mouse.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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Method for Isolating Single Cells and Preparation of Clones from Human Bone Marrow Cultures.* (24235)

DOROTHY M. SCHENCK AND MERWIN MOSKOWITZ (Introduced by W. A. Hiestand)
Dept. of Biological Sciences, Purdue University, Lafayette, Ind.

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-

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† 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.

taining a single cell and placing them in culture vessels.

Materials. Glass squares. The glass squares were of pyrex glass 3 mm square and 0.13-0.16 mm thick. They were at first cut from #1 Corning brand coverslips after coverslips had been cleaned by tissue culture methods. They were later obtained cut to specifications by the Corning Glass Works. The latter squares were more uniform in size and had smoother edges, but were somewhat more difficult to clean because of their size. The observation apparatus consists of a dust guard, sterile moist cotton pledgets, sterile glass slide, and slide carrier (Fig. 1.) The dust guard was made by cementing 2 pieces of small glass tubing (3 mm o.d.) to edges of a glass slide. The slide carrier consists of plastic platform 15 mm high and is used merely to raise the remainder of the apparatus so that it may be more easily handled. *Vial cultures.* Flat bottomed shell vials 15 x 45 mm were used as culture vessels. To permit microscopic examination and photography with an inverted microscope, glass caps were used instead of rubber stoppers. The caps, 20 mm in height, were made by cutting off 19 x 65 mm vials. *CO_2 chamber.* Vial cultures were placed in desiccator for incubation. To facilitate handling, a long bolt was attached to the porcelain plate. Glass caps similar to those mentioned above were cemented to the plate to hold the culture vials. A small amount of water was placed in bottom of desiccator to minimize evaporation of fluid from the cultures. The pH of cultures was adjusted with CO_2. *Procedure.* One ml of nutrient medium was added to 60 mm Petri dish containing 30-36 of the 3 mm glass squares. The Petri dish was then shaken until all glass squares were wet and sank to bottom. Cells were washed from glass surface of culture vessel by forceful pipetting with a curved capillary pipette or trypsinized off with trypsin solution (0.05% Difco 1/250 in medium 1066). A cell count was taken with a hemocytometer and the cells diluted with nutrient medium to final concentration of 150 cells/4 ml. The cell suspension was added to the Petri dish and gently rotated to disperse the cells. The Petri dish was placed in a desiccator and the pH adjusted to 7.2 with CO_2. The culture was incubated at 37°C until cells settled and adhered to the glass. Time varied from 2 to 20 hours depending on cell type. The Petri dish was then rotated gently and the fluid discarded to remove unattached cells. Five cc of new nutrient fluid were added. The small glass squares were then examined for presence of single, healthy-appearing cells by removing them and observing them under microscope with the apparatus shown in Fig. 1. A sterile glass slide (B) was placed on slide carrier (C). Five or 6 of the glass squares were picked up with fine forceps and placed on glass slide. Care must be taken that the drop of fluid accompanying each square is spread on the glass so that no dark areas at edge of drop are present. A moistened, sterile cotton pledget was placed at each end of the slide to decrease evaporation of nutrient fluid and the slide was covered with a sterile dust guard (A). The glass squares were scanned at 75X magnification. If one cell was observed on a glass square the glass square and drop of fluid were reexamined at a higher magnification to verify presence of but a single cell. The desired glass squares were then transferred to vials containing 0.6 ml of medium preheated to 37°C. The vials were placed in the desiccator and

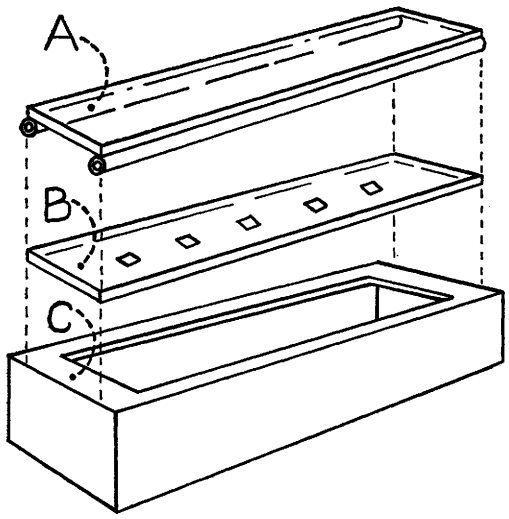

FIG. 1. Observation apparatus. A: Dust guard. B: Glass slide containing glass squares. C: Slide carrier.

pH adjusted with CO_2. They were incubated at 37°C until a relatively large colony developed. The colony was broken up and the cells dispersed over bottom of vial by forceful pipetting with capillary pipette. The cultures were reincubated until growth covered the bottom of vial. At that time the cells were loosened from the glass by forceful pipetting with a capillary pipette and the cell suspension transferred to a 13 x 100 mm pyrex tube. Fresh nutrient fluid was added to the vial and this culture was maintained until the clone was well established in tubes.

In some instances the cells were grown in solid medium. Three-tenths ml of medium containing 0.4% agar was placed in the vial and allowed to harden, the glass square was dropped onto the surface, and 0.3 ml of agar was then added on top. A colony developing in agar was allowed to grow until macroscopic in size. It was then dissected out, transferred to a vial containing 0.6 ml of liquid medium and thereafter treated as above.

Discussion. The percentage of glass squares found to contain but a single cell ranged from 12 to 27% of those examined. The percentage of single cells on glass squares that developed into cultures under specific conditions ranged from zero to 70%. Under a specific set of conditions some cells have proliferated well, whereas others have failed to proliferate or have done so very slowly, developing into transferable colonies only after 78 days as compared to 22 days for the former. All the different types of cells isolated from the Mox strain, at least those that can be divided into types on the basis of their morphology, are capable of developing into cultures from single cells but will do so only when growth conditions are appropriate. For instance, with one type of cell no colonies developed from single cells in the regular medium, but 60% of the cells proliferated well when the serum concentration was decreased.

This technic has certain advantages. Since the cell is attached to glass rather than suspended in fluid its morphology and condition may be more easily determined. Only cells which appear to be healthy need be selected for use. In instances where cells may be differentiated on morphological grounds, the selection of a particular type of cell is possible. The small glass squares are easily handled and may be transferred with a minimum of mechanical trauma to the cell to any desired culture vessel or to testing apparatus if individual cells are to be studied. When a particular type of cell does not grow under the regular cultural conditions it is possible to alter the conditions under which attempts are made to grow the single cells. By using glass vials with glass caps rather than tubes for culturing the cells, the depth of fluid may be varied easily without interfering with microscopic examination.

A disadvantage of this technic is that in the course of observing the cells on the glass squares they are exposed to light which may injure them. This, however, is an unavoidable evil with any procedure for isolating single cells in which microscopic examination is necessary. The change in oxidation-reduction potential in the course of microscopic examination may also affect the cells, but if the examination is done rapidly these effects can be minimized. There is the possibility of a second small cell being attached to the edge of the glass square where it cannot easily be detected. However, if this were the case, more than one colony would be detected growing and in no case has this been found to occur.

Although the technic has been utilized thus far only with bone marrow cells it would appear to be applicable for use with other types of cells. The conditions under which clone cultures may be developed from different types of cells may vary, but with the cells attached to the glass squares it is a simple matter to introduce the glass square into the necessary cultural conditions. Although a particular type of cell in a mixed culture may be maintained over many passages in association with other types of cells under a particular set of conditions, it may not be able to grow under such conditions by itself. With this technic it is possible to select cells that appear similar and attempt to grow them under a variety of cultural conditions to determine those which are optimum or necessary for the multiplication of the single cell. It

may be necessary to alter the nutrient medium, depth of fluid, gaseous environment, pH, or temperature of incubation in order to get a particular type of cell to grow. The nutrient medium may be pre-conditioned by previous growth of living cells or an irradiated feeder layer(2) may be used.

In attempting to establish clones from primary isolates of tissue, simply plating cells out involves an initial selection process in that only those types of cells that can grow under the conditions used will do so. However, with this technic, a partciular type of cell may be selected and placed under a variety of different cultural conditions in the hope of finding one that will permit the growth of the desired type of cell. Attempts are currently under way to isolate single cells directly from bone marrow and to develop from them clone cultures which can be maintained in serial passage.

Summary. A simple method for isolating single tissue culture cells has been developed. The cells are isolated on small glass squares which can be transferred to any desired culture vessel or testing apparatus. This procedure has been used for the preparation of clone cultures.

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Immediate Effects of Intravenous Injections of Tolbutamide and Insulin on Blood Glucose and Amino Acids.* (24236)

ROGER C. DEMEUTTER, AVEDIS K. KHACHADURIAN AND ALEXANDER MARBLE
Cancer Research Institute and Baker Clinic Research Laboratory, N. E. Deaconess Hospital, Boston, Mass.

After several years of study there is no general agreement regarding the details of the mechanism of action of the hypoglycemic sulfonylureas and more studies are needed to clarify this problem(1,2). The present study was undertaken with two purposes in mind: (1) To determine the time of onset of hypoglycemic action of tolbutamide following its intravenous administration and to compare, in normal and diabetic individuals, the blood glucose curve thus obtained with that produced by insulin; (2) To determine if, like insulin, tolbutamide causes a fall in the serum amino acids.

Materials and methods. Three groups of individuals were studied: (1) Five normal male volunteers, age 28 to 35, body weight 70 to 80 kg. (2) Seven patients with stable diabetes, age 42 to 73, weight 70 to 88 kg, classified as suitable for tolbutamide therapy according to commonly accepted criteria(3). (3) Two patients with unstable diabetes requiring more than 50 units of insulin daily. Prior to study, all subjects were fasted 12 hours. Patients on long-acting insulin were taken off this preparation for more than 48 hours and, when necessary, crystalline insulin was given for control of diabetes. However, no insulin was given later than 15 hours prior to test. The tests were performed on recumbent, rested subjects; no smoking was allowed. Blood samples were drawn through a needle fitted with stylet and kept in place in a brachial vein during the whole test. Stasis was avoided and no tourniquet used whenever possible. After a short control period of saline infusion, glucagon-free insulin† was injected intravenously at fixed dose of 0.1 unit/kg actual net body weight. One or several days later the same procedure was used and sodium

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