Purification without Loss of Viability of Neoplastic Plasma-Cells from the Ascites Passaged Mouse Myeloma, X5563

By BENJAMIN L. GORDON, II, JACOB FONG AND BEN W. PAPERMASTER

PLASMA-CELL NEOPLASMS of the mouse have been widely studied as models for immunoglobulin synthesis. The transplantable tumor X5563 was one of the earlier murine plasma-cell tumors to be described.[1] Arising as a spontaneous tumor of the ileocecal soft tissue of a female adult C3H/He mouse, it was found to be transplantable to other C3H/He mice, and was subsequently adapted to an ascites form. However, when X5563 is grown as an ascites tumor, the ascites fluid harvested from the peritoneal cavity is laden with many types of cells: erythrocytes, lymphocytes, polymorphonuclear neutrophils, and macrophages are seen in large numbers in addition to the neoplastic cells.

For conducting biologic and biochemical studies on these neoplastic plasma-cells, we wanted to obtain them in as highly purified a state as possible, without significant loss of viability. The technic described below enabled us to obtain highly purified, viable preparations of tumor cells.

MATERIALS AND METHODS

The mice used in these experiments were C3H/HeJ males, between 6 and 10 weeks of age. They were obtained from Jackson Laboratories. The tumor strain was supplied to us by Dr. Myron Silverman of the United States Naval Radiological Defense Laboratory, San Francisco, California, and has been serially propagated in the ascites form in C3H/He mice in our laboratory for over one year. The ascites tumors were passaged at a dose of 10^7 viable cells, injected intraperitoneally, at intervals of 10–14 days. Tumor cells were harvested by opening the abdominal skin with a scissors and forceps without disturbing the underlying musculature forming the wall of the peritoneum, and the ascites fluid was withdrawn by means of a hypodermic needle and syringe. The fluid from several animals was pooled, and one unit of heparin per ml. of ascites fluid was added to prevent clotting.

The purification of myeloma cells was accomplished by differential centrifugation of the ascitic fluid followed by passage through a glass bead column. Myeloma cells sediment in light centrifugal fields more rapidly than do red cells, lymphocytes, mast cells, and polymorphonuclear neutrophils (see Discussion). Differential centrifugation was accomplished at room temperature in a model UV International Centrifuge, equipped with a

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Supported in part by U. S. Public Health Service Research Grant No. CA-08126 from the National Cancer Institute to Jacob Fong and by Research Grant No. E-338 from the American Cancer Society and USPHS Research Grant No. AI-06501 to B. W. Papermaster.

First submitted July 21, 1966; accepted for publication Sept. 20, 1966.

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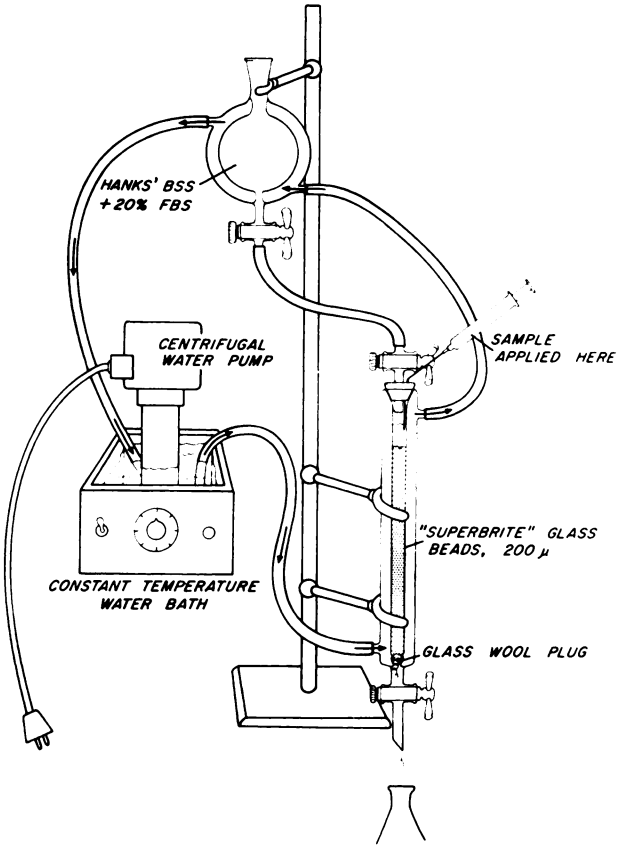

Fig. 1.—Glass bead column for retention of histocytes.

No. 240 head. The fluid was centrifuged repeatedly at 600 rpm (80 g) for 3 minutes. After each centrifugation, the supernatant fluid was discarded and the pellet was resuspended in chilled Hanks' solution supplemented with 20 per cent fetal bovine serum (Microbiological Associates) and 1 unit of heparin per ml. of medium. Ten such centrifugations were performed and the pellet was resuspended in the above medium. The differential centrifugation procedure described above yields myeloma cells free of red cells but still contaminated with peritoneal macrophages.

Macrophages were removed by passage of the cell suspension through a glass bead column. This procedure depends on the property of macrophages to rapidly adhere to glass surfaces. A glass bead column 32 × 2 cm. was constructed according to the model described by Rabinowitz[2] and was modified according to Figure 1. Both the column and the reservoirs were jacketed and water-heated to 37 C. in a thermostatic-controlled water bath was circulated through the column jacket by means of a centrifugal pump. The glass beads employed were No. 100–15 Superbrite (Minnesota Mining and Manufacturing Company). These were washed by boiling in several changes of 12 Molar HCl, and rinsed exhaustively in 30–40 changes of distilled water. The beads, column, reservoirs, and all components in contact with either cells or wash solutions were treated with Siliclad.* Introduction of the sample onto the top of the column was performed by means of a syringe needle fitted with a catheter. After layering the cell suspension above

*Clay-Adams Co.

Table 1.—*Cell Composition by Hemocytometer Count*

Sample No.	Total Tumor Cells per ml. of Sample	Total Histiocytes per ml. of Sample	Total Red Cells per ml. of Sample
A (Original)	1.5×10^8	3.5×10^6	5.0×10^8
B (Centrifuged)	1.5×10^8	3.8×10^6	Less than 1×10^5*
C (Column Passaged)	1.2×10^8	Less than 1×10^5*	Less than 1×10^5*

*Smallest detectable number using a hemocytometer and white blood cell pipette.

the beads and allowing all components to reach 37 C., the cell suspension was drawn onto the glass beads and washout solution added above the beads from the reservoir. The elution solution consisted of the same medium in which the cells were suspended—namely, Hanks' BSS supplemented with 20 per cent fetal bovine serum and 1 unit of heparin per ml. of medium. After drawing the cells onto the beads, the column was allowed to equilibrate for 30 minutes to allow adsorption of the macrophages. The neoplastic plasma-cells were then eluted off the column by running the medium through the column until the eluate appeared clear of cells.

Smears were prepared on glass slides, air-dried, and fixed in absolute methanol for 5 minutes. They were transferred directly to a saturated solution of May-Grunwald Stain (National Aniline Co.) in absolute methanol and immersed in this solution for 10 minutes, then they were transferred directly to a freshly prepared solution of Giemsa Stain (10 ml. Giemsa Stock Solution* to 200 ml. of pH 7.00 phosphate buffer prepared by diluting a standard concentrated buffer solution† 25-fold with distilled water) where they were allowed to remain for 30 minutes. The slides were washed in cold running tap water until all color stopped coming off, passed rapidly through acetone, and immersed in toluene for five minutes. They were then mounted in Permount.

An enzymatic method of determining the viability of cells has been described by Rotman and Papermaster.[3] Cells are exposed to 10^{-6} Molar fluorescein diacetate (FDA); living (FDA +) cells fluoresce green after enzymatic hydrolysis of the substrate while dead (FDA −) cells do not. This property, termed fluorochromasia, provides a simple, precise test of the intact cell membrane of living cells.[3] The counting of (FDA +) and (FDA −) cells per field enables one to determine the percentage of viable cells in a mixture.

The test was performed as follows: 2 µl. of an acetone solution containing 5 mg. per ml. of FDA was added to 5 ml. of protein-supplemented or plain Hanks' BSS containing the cells to be tested for fluorochromasia. Where light suspensions of cells are used—e.g., 10^4 cells per ml., the reaction is instantaneous. Where heavier suspensions of cells are used—e.g., 10^7 cells per ml.—a few moments wait may be required to allow all of the viable cells to accumulate enough intracellular fluorescein to fluoresce brightly.

Results

Bloody ascitic fluid was harvested from tumor-bearing mice and pooled. Sample A represents the original whole ascitic fluid, sample B represents the centrifugally purified fluid, and sample C represents the final purified tumor cell suspension. Samples A, B, and C were counted by means of a hemocytometer with a phase-contrast microscope. The composition of each sample is expressed in Table 1.

Smears were made from each sample and these were stained by the modified May-Grunwald-Giemsa method described above. Differential counts

*National Aniline Co.

†Van Waters and Rogers, Inc. Buffer #3725.

Table 2.—*Differential Count from Stained Smears*

Sample Number	Total Cells Counted	Per Cent Cell Types Expressed in Smallest Significant Decimal				
		Lymphs.	PMN's	RBC's	Hists.	Tumor Cells
A	2061	0.5	0.5	67.2	0.8	30.5
B	2032	0.0	0.2	0.0	0.6	99.2
C	2030	0.0	0.0	0.1	0.0	99.9

were performed on each sample with these smears, and these differential counts reflect the stepwise purifications achieved.

The differential count, expressed in per cent cell types to the least significant decimal place, is given in Table 2.

In many experiments a 99 per cent level of purity was achieved. The viability of the purified cell suspensions as determined by fluorochromatic assay is generally about 99 per cent. The lowest viability we have obtained by these methods was 77 per cent, but the tumor used for the preparation was 14 days old, and it is probable that the initial viability was less in this case then usually obtained with 10–12 day old tumors.

The fluorochromatic assay for viability used in these experiments appears to be a reliable parameter of cell viability. Rotman and Papermaster have correlated this property closely with cloning efficiency of a mouse lymphoma (ML-388) in vitro.[3] We have compared viability of X5563 cell suspension by fluorochromasia with viability by Trypan Blue exclusion and have found these to correlate well.

Discussion

It is believed that the method of purifying tumor cells described above offers the advantages of rapidity, simplicity, and ease of manipulation without damage to the cells. The differential centrifugation employed as the first step of purification causes only a negligible loss of myeloma cells but results in a purified cell suspension consisting almost entirely of myeloma cells, with less than 0.5 per cent macrophages as the sole detectable contaminating cell. This property of rapid sedimentation in light centrifugal fields is primarily a function of cell size, rather than actual buoyant density. Our experiments involving density-gradient centrifugations of myeloma ascites in bovine serum albumin gradients have demonstrated that the myeloma cell buoyant density is, in fact, less than that of red cells. Therefore, the method is probably applicable for purifying many other ascites tumor cells, provided that the neoplastic cells are significantly larger or greater in mass than other types of cells found in the ascites fluid.

This property of macrophages to adhere to glass beads is well-known and has been discussed by Rabinowitz.[1] The column described above has been adapted from his description of columns used to separate monocytes from peripheral blood.

Summary

A simple scheme for purification of murine myeloma cells from ascites fluid without loss of viability is presented. This is accomplished by repeated

differential centrifugations followed by passage through a glass bead column. The cells so obtained are 99.9 per cent pure and 99 per cent viable in a large number of repetitions of this technic.

Summario in Interlingua

Es presentate un simple systema pro le purification de cellulas de myeloma murin ab liquido de ascites sin perdita de viabilitate. Le methodo consiste in repetite centrifugationes differentiation sequite per le passage a transverso un columna de granos de vitro. Le cellulas assi obtenite ha un puritate de 99,9 pro cento e un viabilitate de 99 pro cento a judicar per numerose repetitiones del procedimento.

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