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SEVEN MURINE CELL LINES WITH PROPERTIES OF MACROPHAES,(U)
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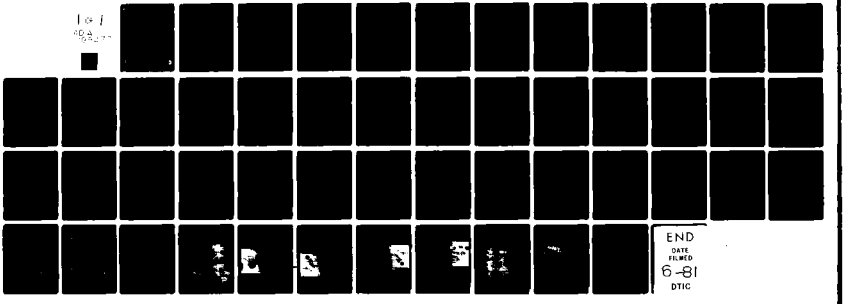

Seven Murine Cell Lines with Properties of Macrophages

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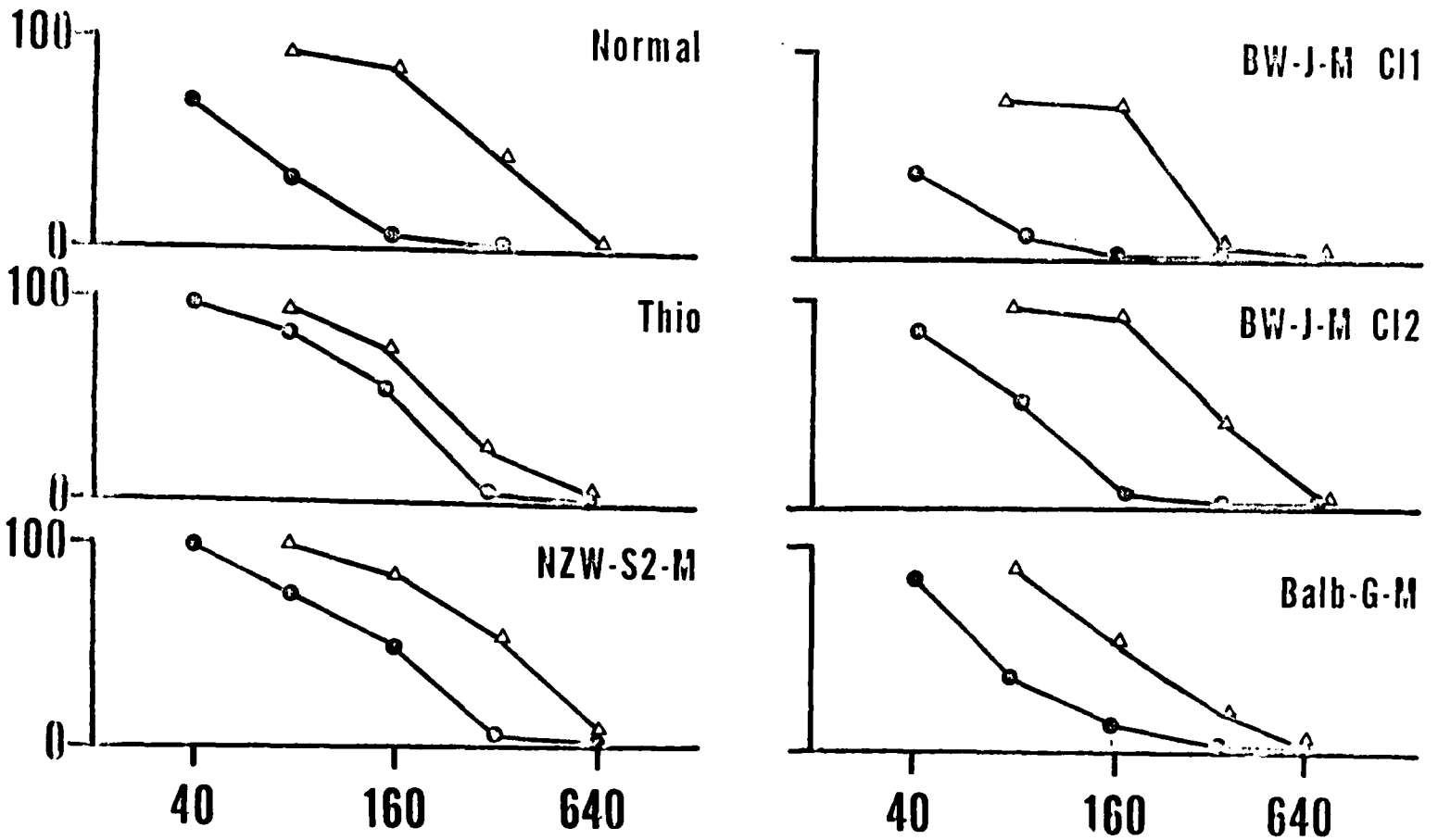
Normal
BW-J-M Cl1
Thio
BW-J-M Cl2
NZW-S2-M
Balb-G-M
100
0
Percent Rossettes
40
160
640
Reciprocal Dilution IgG anti-SRBC

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Seven phagocytic murine cell lines established from cultures of thymic lymphomas closely resembled authentic mouse peritoneal macrophages in their morphology and phagocytic properties. They secreted lysozyme and contained large quantities of nonspecific esterase, β-glucuronidase, acid phosphatase, and lysozyme. They lacked the surface antigens of thymic lymphocytes (Thy-1,2 antigen) or bursa-equivalent lymphocytes (immunoglobulin), but they expressed receptors for immunoglobulin and complement. Complement-mediated rosettes did not occur in the absence of divalent cations. Efficient phagocytosis of sheep →

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erythrocytes required opsonization with rabbit IgG antibodies. Ia8 antigen was resent on all four H2D cell lines. Two of the cell lines can be readily cloned; we used these to demonstrate that variation in receptor expression and morphology was not due to the presence of multiple cell types. None of the cell lines was tumorigenic in nude mice or normal syngeneic mice. These macrophage-like cell lines provide well-characterized models which can be used to examine certain aspects of macrophage function under defined conditions without lymphoid cell admixture.

ABSTRACT

Seven phagocytic murine cell lines established from cultures of thymic lymphomas closely resembled authentic mouse peritoneal macrophages in their morphology and phagocytic properties. They secreted lysozyme and contained large quantitites of nonspecific esterase, β-glucuronidase, acid phosphatase, and lysozyme. They lacked the surface antigens of thymic lymphocytes (Thy-1.2 antigen) or bursa-equivalent lymphocytes (immunoglobulin), but they expressed receptors for immunoglobulin and complement. Complement-mediated rosettes did not occur in the absence of divalent cations. Efficient phagocytosis of sheep erythrocytes required opsonization with rabbit IgG antibodies. Ia8 antigen was present on all four H2D cell lines. Two of the cell lines can be readily cloned; we used these to demonstrate that variation in receptor expression and morphology was not due to the presence of multiple cell types. None of the cell lines was tumorigenic in nude mice or normal syngeneic mice. These macrophage-like cell lines provide well-characterized models which can be used to examine certain aspects of macrophage function under defined conditions without lymphoid cell admixture.

Macrophages have provided a widely exploited model in cell research. In addition to their classical functions of phagocytosis and motility these cells are active in pinocytosis (7), secretion of factors regulating growth and function of other cells (37), synthesis of extracellular proteases (11) including plasminogen activator (40) and collagenase (43), and production of complement (C) components (9). They bear cell surface receptors for C (18, 34) and immunoglobulin (Ig) (39); there is a complex interaction between the physiological state of the macrophage and the ability of these receptors to initiate binding and phagocytosis of particles coated with Ig or C (4). Macrophages also have an important place in the immune response and participate in precise genetic interactions with thymus-processed lymphocytes (27). Potent soluble mediators from thymic lymphocytes or other substances can control the physiological state of the macrophage with profound alterations in enzyme content and secretion, receptor function, phagocytosis, spreading and motility (reviewed in 42). These so-called "activated" macrophages are also capable of recognizing malignant cells and inhibiting their growth or killing them (1, 15). Other stimuli such as conditioned medium can induce the macrophage to leave its usual G_o state and initiate DNA synthesis followed by cell division (13, 35, 41).

Enriched macrophage populations are easily obtained from the rodent peritoneal cells commonly used for these studies, but there is always some admixture of other cell types (23). The purification exploits the adherence of macrophages which are then difficult to obtain in suspension without cell damage.

Thus, when we encountered growth of macrophage-like cells in murine thymic lymphoma cultures, we attempted to isolate these as pure strains.

We report here seven such macrophage-like cell lines and compare them to authentic murine macrophages and selected control cell lines.

MATERIALS AND METHODS

Establishing macrophage-like cell lines. A spontaneous or murine leukemia virus (MuLV) (Scripps)-induced lymphoma (26) in its first to sixth transplant generation was minced and stirred for 15 min with 0.25% trypsin at 37 C. After washing, 20 ml of cells at a concentration of 5×10^6 to 5×10^7/ml were transferred to a 75-cm^2 plastic flask. The medium used, unless otherwise noted, was Eagle's minimum essential medium (EMEM, Autopow, Flow Laboratories, Rockville, MD) with supplemental 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 1% nonessential amino acids (#6-244D, Flow Laboratories) 2 mM glutamine, 50 µg/ml streptomycin and 50 U/ml penicillin ("complete medium"). During the early part of the study 20 mM Hepes (Gibco, Grand Island, NY) was added. Cultures were fed weekly by removing and centrifuging the supernatant fluids and returning unattached cells to the flask in fresh medium. When obvious signs of growth occurred, attempts were made to establish subcultures by transferring floating cells or by treating attached cells with trypsin-EDTA. Cell lines were designated according to mouse strain of origin: NZB = NZB/Scr, NZW = NZW/Scr, BW = NZB x NZW F1, BALB = BALB/cSt followed by a letter denoting which transplanted thymic lymphoma was cultured. The last letter denotes whether the cell line resembles a macrophage (M), a thymic lymphoma (T) or a fibroblast (F). For example, Balb-G-M was derived from culturing a thymic lymphoma (G) induced in a BALB/cSt mouse and was macrophage-like.

Maintenance of macrophage-like cell lines. The seven macrophage lines were fed at 5-7-day intervals and subcultured at ratios of 1:3 to 1:5 using vigorous agitation, trypsin-EDTA (#16-891-49, Flow Laboratories)

or xylocaine (30) to remove adherent cells (See Results). Suspension cultures were seeded twice weekly at 2 x 10^5 cells/ml of complete medium and incubated stationary or on a rotary shaker at 100 gyrations/min. Roller bottle cultures were established from the contents of one or two confluent 75-cm^2 flasks in 75 to 100 ml of complete medium and rotated at 0.5 rpm.

Additional cell lines. Cell lines included in some tests for comparison with the macrophage cell lines were BW-J-T, NZW-D-T, and BALB-G-T, MuLV (Scripps)-induced Thy-1.2 positive lymphoma cell lines established during the course of this study; BALB-G-F, a fibroblast-like line derived from the same culture as BALB-G-M by cloning; L929 cells, a gift from Dr. Rolf Zinkernagel, Scripps Clinic and Research Foundation; and TE-1, a cloned cell line of epithelial appearance established from a spontaneously transformed BALB/c thymus culture. The NZB cell line 60a (19) was used as a target for complement-mediated cytotoxicity to Thy-1.2. The MuLV (Rauscher)-induced BALB/c tumor cell line T5 was obtained from Dr. Bruce Chesebro, Rocky Mountain Laboratory, Hamilton, MT, for use as a target in complement-mediated cytotoxicity tests for Ia8 (6). All cells were grown in complete medium except T5 which was propagated in RPMI 1640 supplemented with 5 x 10^{-5} M 2-mercaptoethanol, 10% FCS, and antibiotics.

Macrophages. The peritoneal cavities of BALB/c mice untreated or injected 3 to 4 days previously with thioglycollate (1 ml Brewer's thioglycollate medium, Difco Laboratories, Detroit, MI) were washed with EMEM, without Ca^{++} or Mg^{++}, containing 50 µg/ml streptomycin plus 50 U/ml penicillin. The cells thus removed were centrifuged, washed in complete medium, and seeded 4 to 24 hr before use. Macrophages were assumed to constitute 30% of resident peritoneal cells and 70% of

thioglycollate-induced cells. Four hours after being plated, the cells were vigorously washed in medium to remove nonadherent lymphoid cells. Morphology viewed with Giemsa staining performed 2 hr after adding 100:1 zymosan particles indicated the cells to be greater than 95% macrophages.

Histochemical stains. Acid phosphatase (Naphthol AS-MX phosphate substrate, pH 5.2) intracellular granules were demonstrated using a kit from Sigma Chemical Co., DeKalb, MO. Nonspecific esterase stains were done by Dr. M. Tavassoli (Scripps Clinic and Research Foundation) by the method of Li et al. (20) using α-naphthylacetate substrate at pH 6.3.

Enzyme assays. Cells were seeded in duplicate at 5×10^5 to 10^6 cells/ 25-cm^2 flask and incubated 3 days. Supernatants were clarified by centrifugation for 15 min; cells were harvested by scraping with a rubber policeman in 1 ml of water. The cells were then subjected to three freeze-thaw cycles (8). Lysozyme was assayed by the lysis of Micrococcus lysodeikticus as described by Parry et al. (25) and compared to purified egg white lysozyme (Sigma Chemical Co.). Acid phosphatase (p-nitrophenyl phosphate substrate, pH 4.8) and β-glucuronidase (phenolphthalein glucuronic acid subst rate, pH 4.5) were determined using commercially available reagents (Sigma Chemical Co.). All assays were performed under conditions where enzyme was limiting.

Cell surface receptors for Ig and C3. Receptors characteristic of macrophages were sought on the macrophage-like cell lines. Sheep erythrocytes (E) (Colorado Serum Co., Denver, CO) were washed twice in Dulbecco's PBS and suspended at 10% v/v in PBS. An equal volume of antibody was added with mixing, the cells were incubated at 37 C for 30 min, washed three times in PBS, and resuspended to 5×10^8/ml in the appropriate diluent. Unless otherwise noted, IgG or IgM rabbit antisera to E (Cordis Laboratories, Miami, FL) were used at a 1:40 dilution which

represented one-half the agglutinating concentration. Immune adherence negative EAC were prepared by centrifuging 1 ml EA (IgM) and resuspending in 0.8 ml veronal buffered saline (Complement fixation test diluent tablets (Oxoid Ltd., London, England). Two hundred microliters C5-deficient mouse serum (B 10.D2 old/J) were added before incubating 30 min at 37 C, washing twice and resuspending in 1 ml of the appropriate diluent (36).

Four to 6 hr before use, adherent cells were added to 8-well slides (#4808, Lab-Tex Products, Naperville, IL) in 200 µl of diluent or to 24-well tissue culture dishes (#3008, Falcon, Oxnard, CA) containing 12-mm cover slips in 500 µl of diluent resulting in a cell density of $7.5 \times 10^5/cm^2$. Strongly adherent cells detached with trypsin-EDTA or xylocaine were seeded at $5 \times 10^5/cm^2$ and left overnight in complete medium before use. EMEM with antibiotics was the diluent for complement studies and EMEM with 2% heat-inactivated FCS and antibiotics for all other studies. Indicator E were added at a 25:1 ratio in 50 µl of diluent, mixed, and incubated at 37 C for 30 min in 95% air-5% CO_2 atmosphere. The excess E were removed by gentle washing with a pipette; the slides were fixed in 2% glutaraldehyde in PBS, and the rosettes were scored after staining with Giemsa. Cells with three or more E adherent or ingested were considered positive. Tests were performed in duplicate and 200-300 cells were counted.

Cultured thymic lymphomas, which were not adherent, were tested by adding 25:1 indicator E to 10^6 cells with 200 µl of diluent in a conical centrifuge tube. After incubation at 37 C for 30 min, the cells were resuspended and counted in a Neubauer chamber (36).

Phagocytosis. Phagocytic cells, E, and EA were prepared as for determination of surface receptors. Zymosan (Sigma Chemical Co.) was

suspended at 20% v/v in PBS and then autoclaved. The particles were counted in a hemocytometer and just before use, diluted in EMEM with 2% heated FCS and antibiotics to yield a 25:1 multiplicity. One hour after addition of the substance to be tested, the cells were washed in PBS; those exposed to E were treated with distilled water for approximately 5 sec before fixation in 2% glutaraldehyde-PBS. Slides were stained with Giemsa and scored at 960X magnification. Cells scored as phagocytic contained three or more of the test particles. At least 200 cells were counted for each value shown and duplicate determinations were performed.

Complement-mediated cytotoxic tests for Ia8 and Thy-1.2. Cells labeled with ^{51}Cr were reacted with heat-inactivated antisera and rabbit complement in microplates as previously described (26). Unknown cells were typed by absorbing 75 µl of antiserum diluted to plateau titer with varying quantities of cells. After 1 hr incubation at 4 C, the mixture was centrifuged for 15 min; duplicate 25-µl portions of supernatant were tested for their ability to lyse 2×10^4 known positive target cells. Fifty percent decrease in cytotoxicity was considered a positive reaction. Cells were considered to be negative if 20 times the effective number of target cells failed to decrease toxicity. Thy-1.2 was measured by blocking the lysis of 60a cells by AKR anti-C3H thymocyte serum and Ia8 by blocking lysis of T5 by (B10.AxA/WySn) F_1 anti-C57BL/10 serum. BALB/c spleen cells blocked the Ia8 reaction about three times as well as T5 and thymocytes about 0.4 times as well as T5.

Fluorescent antibody tests. To locate intracellular immunoglobulin, cell smears were fixed for 10 min in cold absolute ethanol and stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse globulin (36). Cell surface Ig was determined by staining viable cells (36).

Antisera. AKR anti-C3H Thy-1.2 was obtained from Litton Bionetics, Kensington, MD. Antiserum to mouse immunoglobulins was prepared by hyperimmunizing goats with 50% saturated ammonium sulfate-precipitated mouse globulins. B10.AxA/WySn anti-C57BL/10 serum specific for Ia8 antigen on BALB/c cells was a gift of Dr. Bruce Chesebro (6).

Tumorigenicity. To assess the ability of the macrophage-like cell lines to cause tumors in adult syngeneic mice and nude mice we injected 6-12 week old mice s.c. or i.p. with 1 to 5 x 10^7 cells and observed them for 3 to 5 months. Nu/nu mice on BALB/c or CBA backgrounds were obtained from the Scripps vivarium.

Other manipulations. Gamma irradiation was administered at 109 R/min by a ^{137}Cs source (Gamma cell 40, Atomic Energy of Canada Ltd., Ottawa, Canada). Mitomycin C (Sigma Chemical Co.), treatment was performed by the method of Bach and Voynow (3) using 25 μg/ml to block 6-20 x 10^5 cells/ml. Macrophage spreading was assessed as described by Rabinovitch and DeStefano (28, 29) using 0.5, 1.0, and 5.0 mM $MgCl_2$ in 0.15 M NaCl, 10 mM Tris (pH 7.3) or 10-fold dilutions of 0.25% trypsin (Gibco) in EMEM as inducers. The cells were fixed in glutaraldehyde-PBS after 30 min and scored at 320X with phase-contrast optics. Spreading after 48 hr cocultivation with thymocytes was performed by adding 5 x 10^6 thymocytes (separated with wire mesh screens) to 5 x 10^5 cells from one of the lines in complete medium in a 25-cm^2 plastic flask (Falcon #2033).

Statistical analysis. Correlations were assessed by Kendall's Tau statistic (10). Values obtained with resident and thioglycollate-elicited peritoneal macrophages were included with those from the seven macrophage-like cell lines.

RESULTS

Establishment of cell lines. Four (BW-J-M, BW-L-M, NZW-D-M, BALB-G-M) of the seven marcophage-like cell lines were established during attempts to propagate transplanted MuLV-induced thymic lymphomas in vitro. The tumors, which were trypsinized for culture, contained undefined stomal elements and immune effector cells. Within several days of preparing the cultures, most of the lymphocytes forming the bulk of the tumor disintegrated leaving scattered small round cells loosely adherent and floating in the supernatant, cells resembling cultured fibroblasts, and variable numbers of macrophage-like cells. The latter were further identified in some cultures by their ability to phagocytose zymosan particles rapidly.

In two of these cultures small cells were found increasingly in the supernatant beginning at about 4 months, but could not be cultured independently. At 6 months, larger round cells were noted both in the supernatants and attached to the containers. When placed in new flasks, the large round cells adhered to the plastic, had macrophage-like morphology, and ingested zymosan. By 7 months, transfer of supernatants to new flasks resulted in a pure culture of large cells (BW-L-M) in one case and a mixed culture (BW-J-M and BW-J-T) in the other case. Fractionation of the BW-J culture on discontinuous albumin density gradients (31) yielded relatively pure populations of large cells (BW-J-M) and small denser thymic lymphoma cells (BW-J-T). These were subsequently cloned. In a third culture, a thymic lymphoma (NZW-D-T) was established by 3 months. Supernatants were discarded over the next 3 months and adherent cells were passed with trypsin-EDTA at approximately 2-week intervals. Larger rounded cells appeared in the supernatant; again we cultured the floating cells. Over the succeeding 4 months the

large phagocytic cells were established as a continuous culture (NZW-D-M). The fourth line (BALB-G-M, Fig. 1c) began to grow progressively 7 months after the culture was initiated. Small cells resembling thymic lymphoma cells were evident during the first month of culture only. The Thy-1.2-positive lymphoblastoid cell line (BALB-G-T, Fig. 1a), which we used for comparison, was established from another culture of the same tumor. BALB-G-M propagated well at first but became overgrown by rapidly growing fibroblastic cells (Fig. 2). The fibroblasts were readily isolated by cloning (BALB-G-F, Fig. 1b), but the macrophages could be cloned only by using a lethally irradiated BALB-G-F feeder layer.

The remaining three of our seven phagocytic cell cultures were established from an NZW (NZW-S1-M, NZW-S2-M) and an NZB (NZB-S-M) spontaneously occurring, poorly differentiated lymphocytic lymphoma in transplantation. The original NZW tumor cells expressed Thy-1.2, but did not phagocytize zymosan or form EA or EAC rosettes. The surface characteristics of the NZB tumor passed in vivo were not studied. The cultures were composed predominantly of adherent cells that resembled macrophages from the first week. Within 4 to 6 months these cells were more rounded and began to proliferate. When confluent, moderate numbers of cells could be removed by vigorous shaking and further passages were carried out in this manner or by using trypsin-EDTA.

Growth characteristics and morphology. (Table 1). In general, the cell lines from MuLV-induced thymomas resembled one another and differed somewhat from those of the spontaneous tumors. The former were less adherent, more easily dislodged by vigorous shaking and spread less on glass or plastic surfaces. Most of these cells were rounded, although

many had small processes when examined by phase microscopy (Fig. 3). There were occasional cells with the typical appearance of macrophages. Early cultures of BALB-G-M resembled the other three lines from MuLV-induced lymphoma cultures but after cloning the cells were flattened and strongly adherent (Fig. 1c). The three cell lines arising from cultures of spontaneous tumors contained cells which were more strongly adherent and required trypsin-EDTA or xylocaine for complete detachment. Many of these cells closely resembled macrophages by phase-contrast microscopy, often with pseudopods and ruffled membranes (Fig. 4). Both scanning and transmission electron micrographs of selected cell lines confirmed their macrophage characteristics (Figs. 5-7).

Cells from actively growing cultures passed at weekly intervals were relatively uniform in size; however, as cultures aged they often became more heterogeneous with some larger cells reaching 25-50 μ in diameter. Confluent cultures contained increased numbers of well-spread cells and rounded cells began to detach into the supernatant (Figs. 3, 4). These floating cells sometimes reached 1-5 x 10^5/ml and would attach and spread if transferred to a new flask.

Since the cultures were morphologically heterogeneous even after cloning (see below), the possibility of differentiation was considered. Gamma radiation (Table 2) or mitomycin C was used to arrest mitosis completely, and then cultures were assessed by phase microscopy. By one week, about half the original number of BW-J-M, BW-L-M, NZW-S1-M and NZW-S2-M cells was still present, and 10 to 20% persisted until 3 weeks. Many of the remaining cells were larger than those usually found in unmanipulated cultures, but it was impossible to be sure whether they were the result of growth and phagocytosis, abortive mitosis, or selective death of smaller cells. There was a higher proportion of

well-spread, adherent cells with pseudopods, although a few viable floating cells also remained.

All the lines tested, BW-J-M, BW-L-M, NZW-S1-M, and NZW-S2-M, grew well in roller bottles after a few days lag period. They often detached from the glass and floated in small clumps, reaching concentrations of 5×10^5 to 1.5×10^6/ml. NZW-S1-M adapted poorly to growth on the rotary shaker, but the other three multiplied fairly well, although they were sometimes difficult to detach.

BW-J-M and BW-L-M proliferated slowly in complete medium containing only 5% FCS and persisted for several days in 2% FCS. The other lines were more sensitive to low FCS concentrations (Table 1). Cells showed increased adherence, more spreading and a more macrophage-like morphology in low FCS concentrations.

Increased spreading was also seen after passaging the cells with trypsin-EDTA. Use of 0.25% trypsin alone augmented spreading, but failed to detach the macrophage cell lines. These observations prompted us to quantitate the effects of Mn^{++} and trypsin, two known potent inducers of macrophage spreading (28, 29). In control preparations of Tris-buffered saline 5% of the BW-J-M cells had begun to spread by 30 min (surrounding halo 1/3 or more of cell diameter) and the addition of only 0.5 mM Mn^{++} increased this to 59% with a maximum of 70% at 5 mM Mn^{++} (Fig. 8). BW-L-M had 20% spontaneous spreading rising to 72% at 1.0 mM and declining to 52% at 5 mM. Trypsin augmented the spreading of BW-J-M from 4% in EMEM alone to 86% in the presence of 0.25 mg/ml trypsin (Fig. 9) with some increase evident even in 0.025 mg/ml trypsin (23% spreading). BW-J-M, BW-L-M, and NZW-D-M also evidenced increased spreading after 48 hr of cocultivation with BALB/c or NZB thymocytes (Fig. 10).

Enzymes. (Table 3). The nonspecific esterase stain clearly separated macrophages and macrophage-like cell lines from controls. The activity was slightly inhibited by fluoride (data not shown), but not to the extent seen with human monocytes (20). The histochemical reaction for acid phosphatase was strongly positive in most of the macrophage-like cell lines, but was also positive in two control cell lines. Thymic lymphoma cell lines were negative. These results were paralleled by those of quantitative assays of cell homogenates. The presence of relatively large quantities of lysozyme in cell lysates and supernatants and of β-glucuronidase in cell lysates also distinguished macrophages and their analogues from controls. The degree of enzyme expression varied considerably among the cell groups, with the NZW-D-M line consistently low in all categories. Lysozyme concentration in cells and supernatants correlated well ($P < 0.01$) but there were no other statistically significant relations among enzyme activities.

Surface markers. (Table 4). From 45 to 90% of each of the putative macrophage cell lines formed rosettes with EAC compared with 70 to 80% of normal peritoneal macrophage controls. None of the seven cell lines formed greater than 2% rosettes with E alone or E sensitized with rabbit IgM antibody; none of the three thymic lymphoma cell lines, BALB-G-F, or TE-1 bound EAC. When EAC were tested in Ca^{++}- and Mg^{++}-free EMEM containing 10 mM EDTA, less than 2% rosettes were found. EA prepared with a rabbit IgG antibody formed rosettes with 90 to 100% of cells from the seven phagocytic cell lines. The three thymic lymphoma cell lines, BALB-G-F and TE-1 failed to rosette with EA.

To define the affinity of these cells for lower concentrations of erythrocyte-bound antibody, we studied the responses of three cell lines to E sensitized with decreasing amounts of IgG (Fig. 11). Two clones of

BW-J-M required E sensitized by 1.2 to 2.7% antiserum to give 50% rosetting and resembled unstimulated peritoneal cells (1.9%). NZW-S2-M gave 50% rosettes with E sensitized by only 0.7% antibody, similar to thioglycollate-induced peritoneal macrophages (0.6%). When these same EA were coated with complement by treating them with C5-deficient mouse serum, rosetting was markedly enhanced, and 50% rosettes were obtained using E treated with 0.3 to 0.5% antibody.

Ia8 antigen was sought and detected on cells derived from H2D (BALB/c x NZB) mice or their hybrids (NZB x NZW) (Table 4). The quantities detected were 0.5 to 1.8 times those present on T-5 cells. Thy-1.2 was not detected on any of the seven macrophage-like cell lines by absorption of cytotoxic antibodies or by indirect fluorescent antibody tests using AKR anti-C3H Thy-1.2 followed by FITC-labeled goat anti-mouse globulin. Thymic lymphoma cell lines (BW-J-T, BALB-G-T and NZW-D-T) tested as controls lacked Ia8 but were positive for Thy-1.2 by absorption of cytotoxicity and fluorescence. Neither the phagocytic nor the thymic cell lines had surface or intracellular Ig.

Phagocytosis. All seven cell lines proposed here as macrophage models were actively phagocytic for particulate matter such as latex particles or India ink. When quantitative studies were performed with zymosan particles and IgG-coated E, 55 to 98% of the cells had ingested zymosan and 80 to 100% contained EA within 1 hr. The ingestion of EA was mediated by the Fc receptor, since less than 3% of cells ingested E under the same conditions. BW-J-T, NZW-D-T, BALB-G-T, BALB-G-F, L929 and TE-1 control cells were all nonphagocytic under these conditions.

Evidence from clones. I attempted to clone the cultures in microplates several times, but only succeeded with BW-J-M and BW-L-M. With an input of 0.3 cell/well, 58 to 66% of the predicted number of

wells grew colonies. Twenty BW-J-M and 24 BW-L-M clones were studied in the microtiter wells in which they were initially isolated. Although observation conditions were not optimal, there were no significant morphological differences. BW-J-M clones formed 35 to 70% EAC rosettes compared to 60 to 75% values obtained for the parent cultures on different occasions. BW-L-M (30 to 40% EAC rosettes) yielded clones with 18 to 61% rosettes. Four clones of BW-J-M were studied further (Table 5), but there were no clear-cut correlations among the properties measured. Although the clones were initially homogeneous in size, older cultures became as heterogeneous in appearance as the parent culture. However, clones 1 and 2 were passed for 6 weeks without loss of their differential adherence properties.

Lack of tumorigenicity. Subcutaneous inoculation of 5 to 10 nu/nu and i.p. injection of 10-15 syngeneic adult mice with 1 to 5 x 10^7 BW-J-M, BW-L-M, NZW-D-M, NZW-S1-M and NZW-S2-M cells did not produce tumors over a 3- to 5-month observation period.

DISCUSSION

The seven cell lines described here share many properties with murine macrophages: morphology, enzymes, surface antigenicity, surface receptors, and phagocytic capacity. Why they began to grow and eventually became established as permanent lines is unclear. Macrophage populations contain cells which can undergo mitosis when cultured with conditioned media (5, 35, 41) or with supernatants from immunological reactions (13). We and others (41) have consistently observed the slow growth of macrophages in cultures of mixed cell types derived from the peritoneum of thioglycollate-injected mice and from spleen or thymus explants. Some of these underwent several population doublings over periods of up to 6 months, but they never acquired the ability to proliferate rapidly or exclusively of other cell types. We have studied more lymphoma cultures than normal organ explants, and the promising cultures were maintained with more persistence, so it may be coincidental that the cell lines we established were derived from tumor cultures. Possibly macrophages, like other murine cell types, transform spontaneously if kept in culture for a sufficient time under proper conditions. However, we cannot disregard the presence of MuLV in the virus-induced thymomas used to establish some of the cultures and possible stimulation of macrophage multiplication by tumor or immune lymphocytes (13) in the culture flasks. In other situations, virally transformed cells have produced soluble factors which in turn promote transformation (17).

It is clear that six of the seven cell lines do not represent the tumor which contributed the bulk of cells used to seed the initial cultures. The Thy-1.2 antigen marker was present on the original tumor; lymphoblastoid cell lines bearing Thy-1.2 are available from three of the tumors. Indeed, one "pair" of cell lines (BW-J-M and BW-J-T) was

derived from the same flask and NZW-D-M came from a subsequent in vitro passage of the culture which had given rise to NZW-D-T. BALB-G-M also yielded a fibroblast-like cell line, and nonphagocytic cells resembling fibroblasts were present in the other cultures during the early phases of cultivation. The macrophage cell lines presumably originated from host macrophages infiltrating the tumor. The situation is less certain with the NZB-S-M cell line arising from culture of an spontaneous NZB lymphoblastic lymphoma, since markers of the original tumor were not studied.

It is of interest that the five cell lines tested failed to transplant in syngeneic normal mice or nu/nu mice at a dose of 1 to 5 x 10^7 cells, even though they were obviously "transformed" in the sense of continuous in vitro growth after an initial crisis.

Once established, the cultures presented a complex pattern of growth. Recently passed cultures tended to be predominantly adherent cells. Some resembled macrophages with prominent spreading pseudopodia and ruffled membranes, but the majority were rounded and attached by multiple tiny processes. As the cultures became increasingly confluent, larger numbers of cells were found in the supernatant. The adherent cells then evidenced more spreading and a number of larger cells appeared. Adherence and spreading were usually more prominent in the lines derived from cultures of spontaneous tumors. Older cultures of the latter lines became virtually confluent with cells of typical macrophage morphology.

We can suggest three explanations for this morphological heterogeneity. a) genetic: the cultures may contain two or more distinct populations which vary in their relative proportions depending on the conditions of cultivation; b) differentiation: the round cells

could be proliferating blast cells and under appropriate conditions, some fraction irreversibly differentiates to mature macrophage-like cells; and c) stimulation: certain cells may be differentially responsive to external stimuli, for example, because of their position in the cell cycle or ingestion of debris.

The presence of genetically different cell populations is unlikely to be the sole explanation. Tests of phagocytic function, histochemical demonstration of granules, and rosette formation by surface receptors often showed 80 to 100% of the cells to be positive and there was no obvious correlation with morphology. We examined the most variable property, EAC-rosettes, in more detail in the two cell lines which could be cloned efficiently. Twenty clones of BW-J-M and 24 clones of BW-L-M each contained 18 to 80% rosetting cells. Furthermore, even though recently isolated clones were more uniform in appearance they eventually became heterogeneous. I attempted to demonstrate differentiation within the cultures by blocking replication with gamma irradiation, mitomycin C, or low serum concentration. These manipulations led to a gradual increase of adherent, well-spread cells over a period of several days, but the changes did not suggest a uniform progressive maturation and were never complete. Thus, the third explanation seems most likely. Differences in morphology and receptor expression within a culture presumably depend on the physiological state of the cell, although in uncloned cultures differing genetic composition may play a role as well.

Spreading of macrophages may be seen as the result of membrane-substrate adhesive forces and the cytoskeletal organization. These transformed cell lines have different cell surface properties than normal macrophages, as can be seen from the rounded-up cells so prominent in BW-J-M, BW-L-M, and NZW-D-M and by our ability to detach the cell lines

with trypsin-EDTA, unlike authentic macrophages. It was thus of interest that the cell lines tested spread in response to Mn^{++} and trypsin as do normal macrophages (28, 29). An incidental observation which we have not investigated further was the marked spreading induced in some of the lines cocultivated for 48 hr with allogeneic or semi-syngeneic thymocytes.

Histochemical stains for nonspecific esterase were positive only in macrophages and the macrophage cell lines. This enzyme is considered specific for the monocyte-macrophage lineage of normal or malignant human blood cells (20). Acid phosphatase-positive granules were present both in macrophages and in some control cells, as has been reported for other cultured cells (5). Quantitative determinations of β-glucuronidase further supported the analogy to authentic macrophages. Most importantly, all seven cell lines were found to contain and secrete large quantities of lysozyme. Although myeloid cells may contain lysozyme in their granules, macrophages or monocytes are the only leukocytes known to secrete this enzyme (24) and it is secreted by other cell lines thought to be of this type (32).

The antigenic composition of the cell lines was also consistent with a macrophage origin. Two important lymphoid cell surface markers were absent, Ig and Thy-1.2. Intracellular Ig was not detected. Ia8 was detected on all four H2D lines tested. This antigen may be a differentiation marker for macrophages (21).

The receptors which these cells bear also resemble those of authentic macrophages. Immune adherence-negative EAC prepared with C5-deficient mouse serum bound to all the macrophage-like cell lines, suggesting the presence of C3d receptors (33, 34). Binding of these EAC did not occur in the absence of divalent cations, a property of murine macrophage

receptors (18). The cell lines formed rosettes with rabbit IgG-coated E, a measure of Fc receptor function. The dependence of these rosettes on IgG concentration and enhancement by complement was studied for selected cell lines. The results fell between those for normal and thioglycollate-induced peritoneal macrophages.

Since these macrophage-like cell lines were first identified by their ability to phagocytose zymosan, it was not surprising that virtually all cells from every culture ingested particles such as India ink, latex, or zymosan when offered in excess for several hours. However, since many cultured cell types are capable of extensive endocytic activity (44), it was important to establish that they were actively phagocytic for zymosan and EA under conditions where control cells had no activity. Furthermore, phagocytosis of EA presumably required Fc receptor interaction since unsensitized E were scarcely ingested.

Two of the macrophage-like lines, BW-J-M and BW-L-M, have the additional advantage of cloning with high efficiency. This allowed us to prove that the low frequency of EAC-rosettes in these cultures was due to heterogeneity of expression rather than the presence of receptor-positive and receptor-negative sublines. If adequate selection procedures can be devised, these cell lines could be the source of powerful mutants of use in the study of receptors, cell adherence and other aspects of cell biology. For example, Muschel et al. (22) reported the isolation of phagocytosis-negative clones from mutagenized cultures of the phagocytic reticulum cell sarcoma J774, and Unkeless (38) isolated variants of the macrophage-like cell line $P388D_1$ with unusual Fc receptor properties.

Several reports have described murine cell lines which simulate macrophage function (reviewed in 21). At least two other macrophage-like

cell lines (2, 16) may represent other examples of proliferation of macrophages from a tumor culture. Several of the reported cell lines have been tested for phagocytosis, Fc receptors, C3 receptors, lysozyme secretion, lysozymal enzyme content, etc., and share these properties with authentic macrophages and the cell lines reported here. The seven cell lines in this report are unique in representing "new" macrophage-like lines quantitatively compared by several parameters in a single laboratory to resident and thioglycollate-induced peritoneal macrophages. Variations in the properties of these cell lines make certain members uniquely suitable for specialized studies. Furthermore, we are completing quantitative studies of C3 and Ig receptor function, phagocytosis, tumor cell killing, and antibody-dependent cellular cytotoxicity which extend the similarities of the cell lines reported here to authentic murine macrophages (C. J. Peters, Phagocytosis and cytotoxicity by murine macrophage cell lines. Manuscript in preparation).

Preliminary studies with our cell lines have already given promising results in the study of membrane function and the influence of pharmacological agents (12) and the analysis of neutral protease secretion and production of plasminogen activator (D. Loskutoff and C. J. Peters, unpublished data). However, none of the seven cell lines migrated out of agar droplets (J. T. Harrington and C. J. Peters, unpublished data), and so are not useful as substrates for migration-inhibition factor studies (14).

The question of whether these are "real" macrophages is largely unanswerable. The fertilized zygote gave rise to the entire mouse, so we know that any structural or functional analysis of the cell lines can be attacked by arguing that the genetic substrate was available. There

is as yet no clear knowledge of the possible transmutations of "differentiated" cells in culture. We favor the interpretation that these cells are derived from host effector macrophages present in cultured lymphomas, but the validity of this assertion is really irrelevant to the practical usefulness of these cell lines in the laboratory.

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TABLE 1

Characteristics of phagocytic murine cell lines

Cell Line	Derivation[a]	Adherence[b]	Spreading[c]	Diameter[d] ± S.D.
BW-J-M	MT	++	+	16.2 ± 2.5
BW-L-M	MT	+	+	16.6 ± 2.9
NZW-D-M	MT	++	++	16.0 ± 3.0
BALB-G-M	MT	++++	+++	14.8 ± 1.9
NZW-S1-M	ST	+++	++	17.7 ± 2.7
NZW-S2-M	ST	+++	+++	15.3 ± 2.6
NZB-S-M	S	+++	+++	16.2 ± 2.7

[a]Macrophage-like cell line derived from culturing a MuLV-induced Thy-1.2 positive lymphoma (MT), a spontaneous Thy-1.2 positive lymphoma (ST), or a spontaneous lymphoma which was not further characterized (S).

[b]+, Detach with brief shaking; usually have 20 to 50% cells in suspension. ++, Detach with vigorous shaking; most cells adherent until culture near confluency. +++, Many cells detach with shaking, but trypsin EDTA is required to remove all; cells in suspension only when confluent. ++++, Few cells detach with shaking; no cells released in suspension even when confluent.

[c]+, 2 to 10% of cells spread under normal growth conditions. ++, 11 to 50% of cells spread under normal growth conditions. +++, 50 to 90% of cells spread under normal growth conditions.

[d]Resident peritoneal macrophages were 11.7 ± 2.0 and thioglycollate-induced were 15.1 ± 2.7 μm in diameter.

TABLF 2

Growth properties of macrophage-like cell lines

Cell Line	Cloning Efficiency[a]	Growth in Fetal Calf Serum[b]			Response to Radiation	
		5%	2%	None		
BW-J-M	66	G	P 5	P 5	2000[c]	3000[d]
BW-LM	58	G	P 5	P 1-2	2000	3000
NZW-D-M	< 0.1	G	P 3-5	P 1-2	-	-
BALB-G-M	< 0.1[e]	P 5	P 3-5	P 1/2-1	-	-
NZW-S1-M	< 0.1	P 5	P 2-3	P 1/2-1	1000	2000
NZW-S2-M	< 0.1[f]	P 5	P 2-3	P 1/2-1	2000	3000
NZB-S-M	< 0.1	P 5	P 2-3	P 1/2-1	-	-

[a]Percent of expected clones, assuming Poisson distribution when 200 µl of complete medium containing 1.5 cells/ml was seeded in 96-well microculture plates (Falcon #3040).

[b]Response of cell line to reduction of FCS in complete medium: G, slow growth; P, persistence for number of days indicated.

[c]Maximum rads permitting recovery and growth; -, not tested.

[d]Minimum rads to stop culture growth; -, not tested.

[e]Cloned on feeder layer of lethally irradiated BALB-G-F fibroblasts.

[f]Cloned on mitomycin C-treated feeder layer of NZW-S2-M.

TABLE 3

Enzymatic properties of macrophage-like cell lines

Cell	Histochemical				Lysozyme[d]	
	Nonspecific Esterase % Positive[a]	Acid Phosphatase[b]	Acid Phosphatase[b]	β-Glucuronidase	Supernatant	Cells
Macrophage-Like Lines						
BW-J-M	96	2+	14	1.38	190	15
BW-L-M	97	2+	15	0.74	38	4
NZW-D-M	92	2+	6	0.12	47	7
BALB-G-M	97	3+	28	0.74	350	57
NZW-S1-M	99	4+	26	1.21	235	47
NZW-S2-M	100	4+	29	0.94	330	68
NZB-S-M	99	3+	39	0.13	140	8
Macrophages						
Unstimulated	97	3+	7	0.34	203	37
Thioglycollate	92	2+	11	0.44	325	46
Control Lines						
BW-J-T	2	0	2	0.01	< 3	< 1
NZW-D-T	0	0	3	0.01	< 3	< 1
BALB-G-T	0	0	2	0.01	< 3	< 1

BALB-G-F	0	-[e]	-	-	< 3	< 1
TE-1	0	2+	15	0.01	< 3	< 3
L929	0	2+	-	-	< 3	< 3

[a]Intensity of staining graded: 0, 2% or fewer cells with scattered granules, to ++++, 90% or more cells having cytoplasm filled with granules.

[b]nmoles/min/10^6 cells p-nitrophenol released from p-nitrophenyl phosphate at 37 C by cell lysate.

[c]nmoles/min/10^6 cells phenophthalein released from phenolphthalein glucuronic acid at 37 C by cell lysate.

[d]ng egg white lysozyme-equivalent/10^6 cells present in 3-day culture supernatant or cell lysate.

[e]-, not tested.

TABLE 4

Surface markers of macrophage-like cell lines

Cell Line	EAC[a]	EA[a]	Ia8[b]	Thy-1.2[c]	SIg[d]
Macrophage-Like					
BW-J-M	+	+	1.0	0	0
BW-L-M	+	+	0.8	0	0
NZW-D-M	+	+	-	0	0
BALB-G-M	+	+	0.5	0	0
NZW-S1-M	+	+	-	0	0
NZW-S2-M	+	+	-	0	0
NZB-S-M	+	+	1.8	0	0
Control					
BW-J-T	0	0	0	+	0
NZW-D-T	0	0	-	+	0
BALB-G-T	0	0	0	+	0
BALB-G-F	0	0	-	-	-
TE-1	0	0	-	-	-

[a] +, > 25% of cells bound 3 or more EA or EAC; 0, < 2% of cells bound 3 or more EA or EAC.

[b] Ia8 antigen sought on H2D cell lines. Relative concentration to T5 indicated; 0, less than 50% blocking when 20 times the effective number of T5 cells used; -, not tested.

[c] Thy-1.2 antigen sought by blocking as in [b].

[d] Surface Ig detected by staining with fluoresceinated goat anti-mouse Ig.

TABLE 5

Properties of clones of BW-J-M

Clone	Adherence[a]	Lysozyme[b]		Receptors[c]		Zymosan Phagocytosis (% ingesting by min)		
		Supernatant	Cells	EA	EAC	15	30	60
1	+	330	16	94	51	5	18	61
2	+++	510	60	96	80	3	14	46
6	++	200	15	97	55	1	22	40
8	++	360	65	98	52	14	53	71
Uncloned	++	190	15	96	71	5	52	70

[a]See Table 1.

[b]Lysozyme concentration in cell lysates or supernatants from 3-day cultures expressed as ng/10^6 cells egg white lysozyme equivalents.

[c]Percent of cells binding 3 or more EA or EAC.

FIGURE LEGENDS

Fig. 1. Contrasting morphology of cell lines established from BALB-G tumor. (a) BALB-G-T, malignant thymocyte cell line. (b) BALB-G-F, so-called fibroblastic cell line. (c) BALB-G-M, macrophage cell line. Phase contrast, 320X.

Fig. 2. Culture of BALB-G tumor. Characteristic fibroblastic cells co-exist with macrophage-like cells (identified by rosetting and phagocytosis of EA). Giemsa stain, 160X.

Fig. 3. Growth of BW-J-M cell line, clone 1. (a) Recently passed culture with many rounded cells. (b) Older culture showing increased heterogeneity in size and more prominent spreading. Phase contrast, 320X.

Fig. 4. Growth of NZW-S2-M culture. (a) Recently passed. (b) Mature culture. Phase contrast, 320X.

Fig. 5. Electron micrographs of BW-J-M cells. (a) Scanning micrograph of representative cell with extended apron of cytoplasm and ruffling, 3200X. (b) Transmission micrograph of comparable cell, 3200X.

Fig. 6. Electron micrographs of NZW-S2-M cells. (a) Scanning micrograph showing typical flattened, elongated cells and less numerous rounded cell type, 1750X. (b) Transmission study illustrating polarized ruffling of an elongated cell, 3500X.

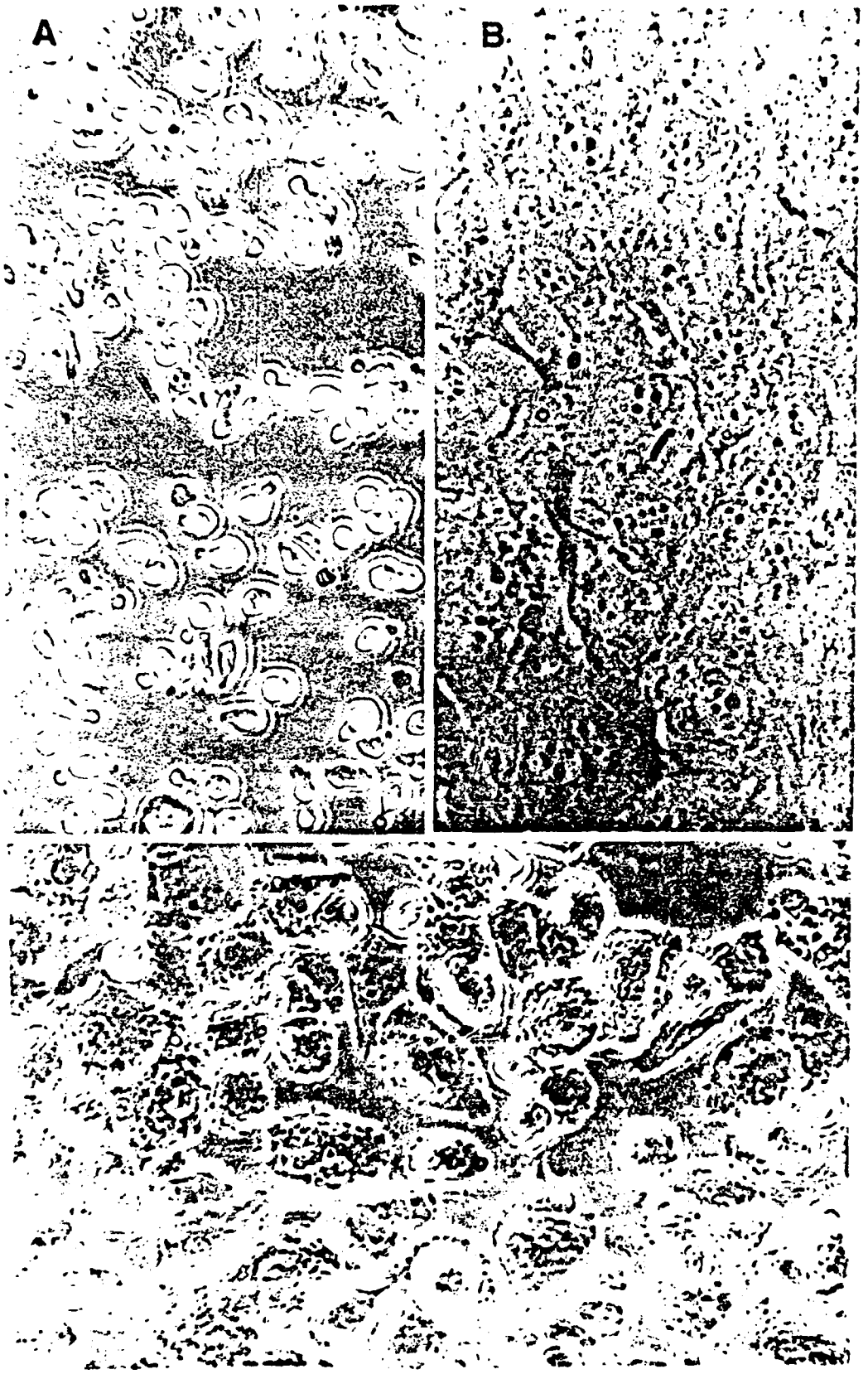
Fig. 7. Electron micrographs of BALB-G-M. (a) Scanning study demonstrates marked heterogeneity in spite of previous cloning, 1500X. (b) Transmission micrograph, 1750X.

Fig. 8. Spreading of BW-J-M cultures in the presence of Mn^{++}, 30 min after seeding cultures. (a) Control is Tris-saline. (b) Tris-saline plus 1 mM Mn^{++}. Phase contrast, 320X.

Fig. 9. Spreading of BW-J-M cultures in the presence of trypsin, 30 min after seeding. (a) Control is EMEM. (b) EMEM plus 0.25 mg/ml trypsin. Phase contrast, 320X.

Fig. 10. Spreading of BW-J-M after co-cultivation with NZB/Scr thymocytes for 48 hr. (a) Control. (b) With thymocytes. Phase contrast, 320X.

Fig. 11. Dependence of EA rosette formation on dilution of sensitizing rabbit IgG antibody (●——●) and enhancement after addition of C3d by treatment of EA with C5-deficient mouse serum (Δ——Δ).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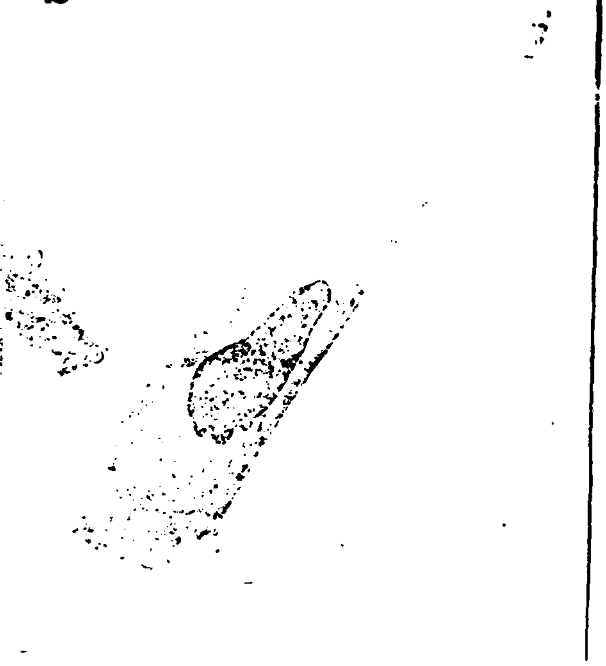

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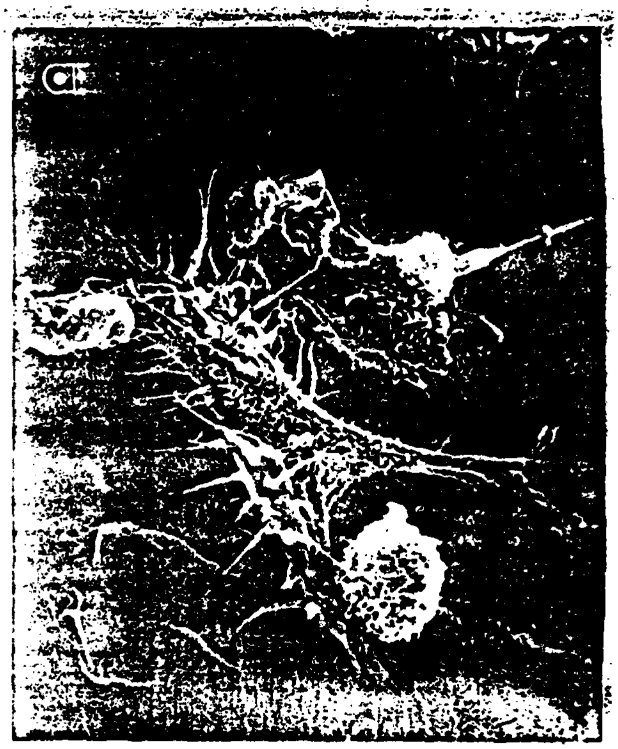
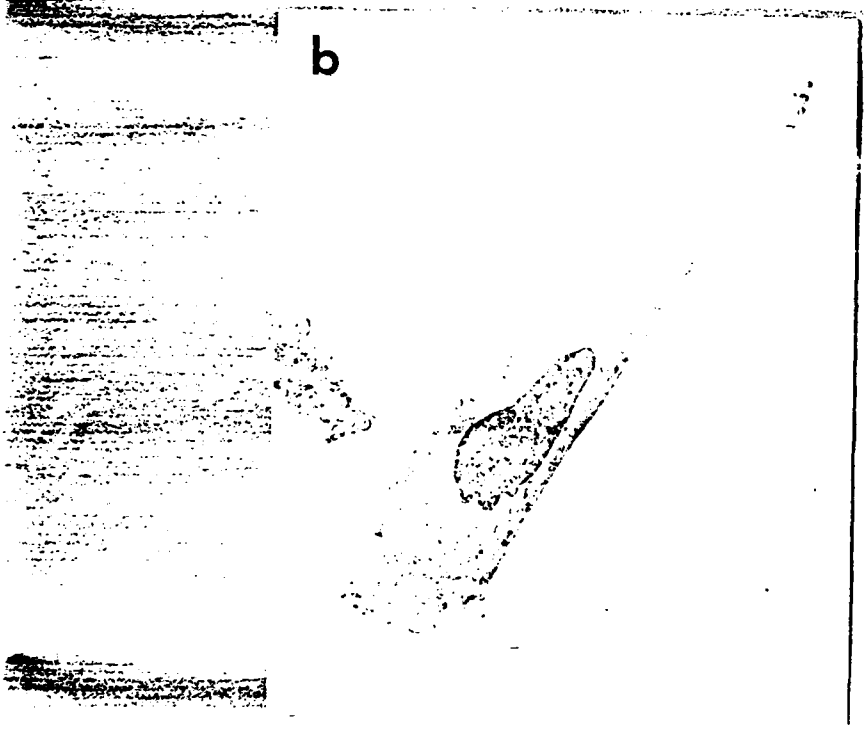


Fig 6



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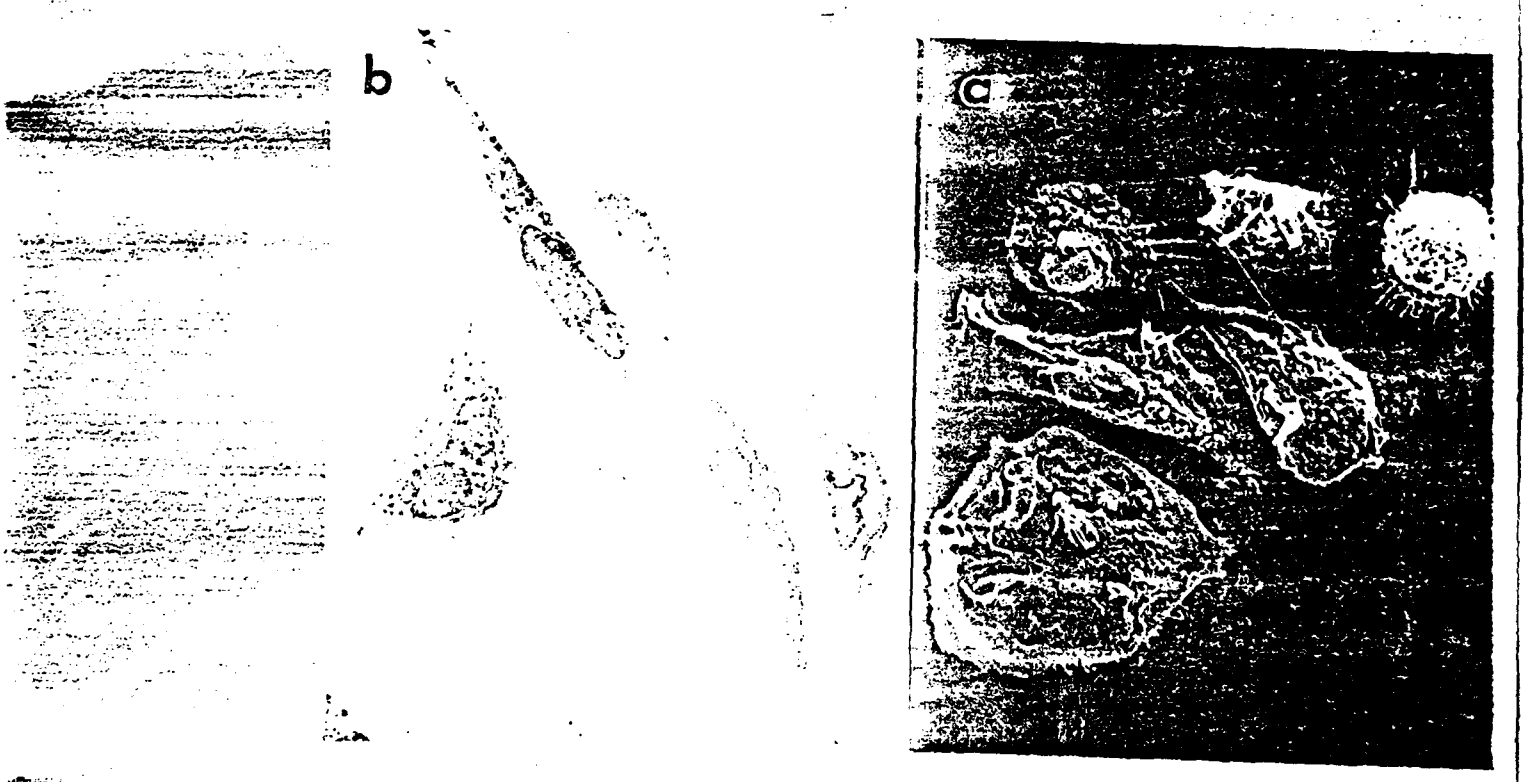

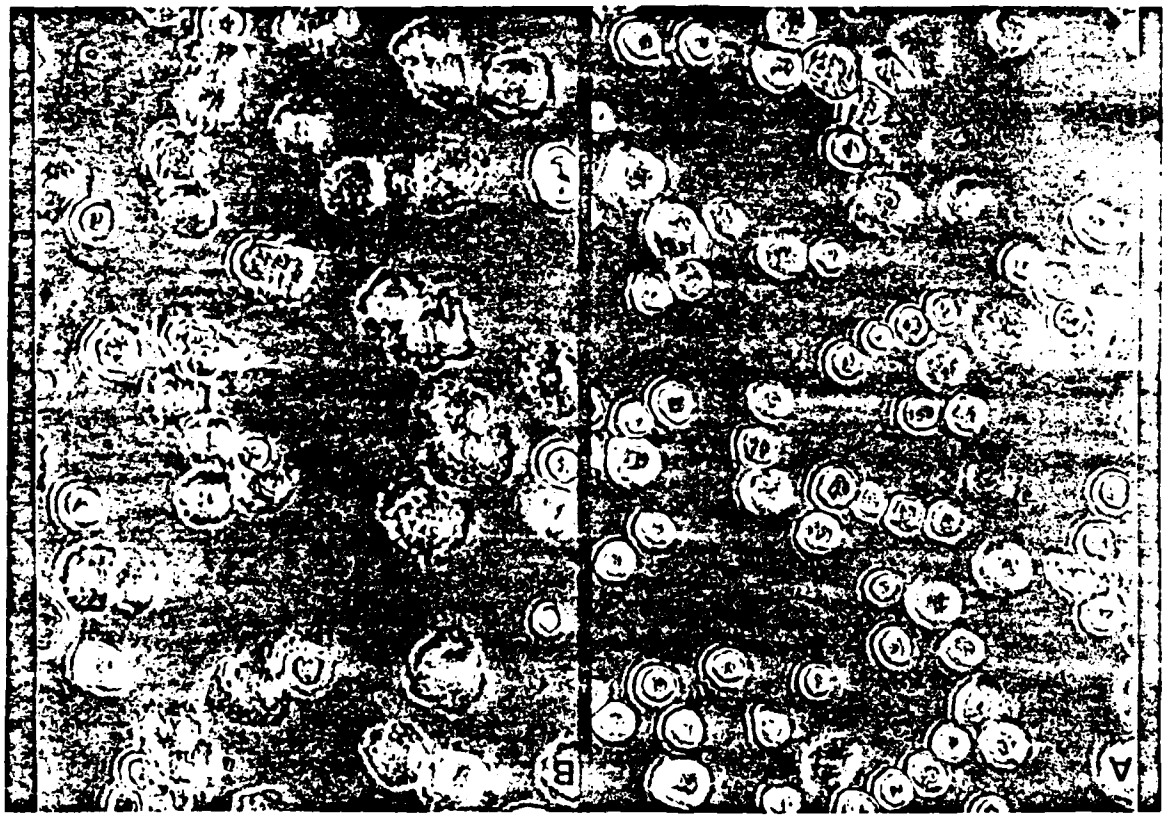
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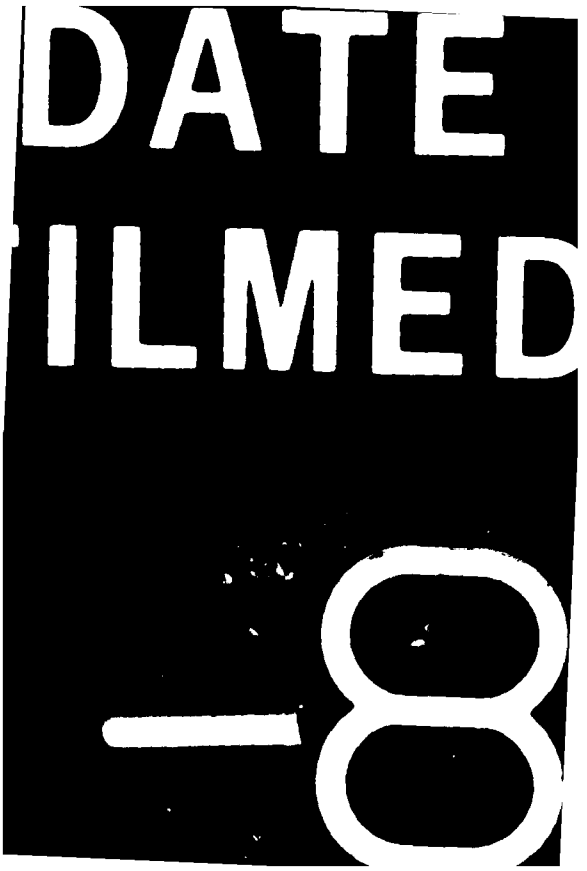
Fig 10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