INNATE IMMUNE PROPERTIES OF THE IMMORTALIZED MACROPHAGE CELL LINE I-9.5

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SUMMARY

A colony stimulating factor-1-dependent macrophage cell line, I-9.5, originally derived from a BALB/c splenic macrophage colony, was maintained in culture and examined for the expression of certain properties key to its innate immune function. Chemotaxis, phagocytosis, and superoxide release were assessed in this cell line and compared to either freshly isolated elicited murine peritoneal or splenic macrophages from BALB/c mice. Three separate experiments indicated that I-9.5 displayed comparable phagocytosis of 1*C-radio-labeled Staphylococcus aureus and similar levels of superoxide release in response to opsonized zymosan. I-9.5, however, demonstrated impaired chemotaxis toward the chemotactic agent, N-formyl-methionyl-leucyl-phenylalanine, and displayed impaired random migration in response to a balanced salt solution. This observation suggests that I-9.5 may serve as an important model for elucidating the structural and molecular correlates of chemotaxis.

Key words: culture; cell lines; colony stimulating factor; macrophage.

INTRODUCTION

The elucidation of macrophage function is critical to a full understanding of the operation of both innate and acquired immunity. The development of immortalized prolific macrophage cell lines has contributed to better recognition of the molecular processes involved in macrophage function. Continuous macrophage lines have been isolated from a number of different species and tissue sources but particularly from murine and human tumors (7,15,20). In response to the need for permanent and prolific macrophage cell lines from nontumor sources, a number of investigators have turned to retroviral transformation in developing immortalized lines. Several investigators have used recombinant retroviruses containing the oncogenes, v-raf and v-myc, to transfect mouse bone marrow cells and generate a number of permanent macrophage cell lines capable of continuous growth in culture (5,6). Using similar transfection procedures, Roberson and Walker have employed the v-raf/mil, v-myc, and R-myc oncogenes to immortalize mouse splenic macrophages and bone marrow cells (16). Such cell lines, however, often continue to exhibit extraneous properties characteristic of transformed cells. They proliferate independent of macrophage growth factors, produce lethal tumors when injected into nude or syngeneic mice, and produce and release retroviruses (23). Additionally, certain transfected cells derived from bone marrow precursors often do not express macrophage receptors or display phagocytic properties (11,17). An alternative procedure was used by Wilson and co-workers, which exploited the effects of macrophage colony stimulating factor (CSF-1) to establish immortalized cell lines from splenic macrophage progenitors rather than traditional bone marrow precursors (23).

We have obtained an established cell line, I-9.5, from the Walker laboratory. It was originally derived 4 years ago from a murine BALB/c splenic macrophage colony that had been maintained on CSF-1-enriched media for over 4 mo. This cell line will neither form tumors when injected into nude or syngeneic mice nor will it release retroviruses. Our results describe and compare functional properties of I-9.5 with those of in vivo derived murine macrophages isolated from either the peritoneal cavity or spleen of BALB/c mice. The properties chosen for study—chemotaxis, phagocytosis, and oxygen-free radical release—are those that are critical to macrophage performance in the innate immune response.

MATERIALS AND METHODS

Growth and harvesting of I-9.5. The CSF-1-dependent, immortalized murine (BALB/c) splenic macrophage cell line, I-9.5 (mycoplasma negative), was obtained from the laboratory of Walker et al. (St. Jude Children's Research Hospital, Memphis, TN). The line was maintained in liquid culture using growth medium (GM) prepared with Dulbecco’s modified Eagle’s medium (DMEM) containing l-glutamine (Sigma Chemical Co., St. Louis, MO). GM was buffered with sodium bicarbonate and supplemented with 10% heat-inactivated fetal bovine serum (FBS, BioCell Laboratories, Rancho Dominguez, CA) and conditioned medium from a CSF-1-secreting mouse bone marrow cell line, LADMAC (17). When confluent approximately 15% of the cells were subcultured in GM. Complete medium (CM) used to harvest and wash I-9.5 cells was prepared with RPMI-1640 (Sigma) containing l-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS, BioCell Laboratories, Rancho Dominguez, CA) and conditioned medium from a CSF-1-secreting mouse bone marrow cell line, LADMAC (17). When confluent approximately 15% of the cells were subcultured in GM. Complete medium (CM) used to harvest and wash I-9.5 cells was prepared with RPMI-1640 (Sigma) containing l-glutamine supplemented with 10% heat-inactivated FBS and 2.5 × 10⁻⁵ M 2-mercaptoethanol (Sigma). Neither GM nor CM contained antibiotics.

The CSF-1-secreting murine bone marrow cell line, LADMAC (mycoplasma negative), was grown in 75 ml polystyrene flasks in Eagle’s minimum essential medium (EMEM, Sigma) containing l-glutamine. Cells were...
maintained in a water-jacketed CO₂ incubator at 37 °C and 5% CO₂. The cell line was split and its supernatant collected every 3 to 7 d from confluent flasks. To determine the appropriate concentration of LADMAC conditioned supernatant to use in preparing the GM for macrophage line I-9.5, a bone marrow proliferation assay was performed according to procedures outlined by Sklar et al. (17). Seven-day cultures of bone marrow cells were enumerated and that concentration of LADMAC supernatant supporting the greatest cell growth was used to prepare I-9.5 GM.

I-9.5 cells were grown on 100 mm polyethylene terephthalate plates (VWR Scientific, San Francisco, CA) and maintained at 37 °C and 5% CO₂. The cells were split and transferred into fresh medium every 7 to 10 d, when plates became confluent. For use, I-9.5 were separated from the plates by gently scraping the cell monolayers with a rubber policeman. The resulting cells were pelleted by centrifugation at 800 Xg and washed twice in CM. Cells were counted and cell viability determined by trypan blue dye exclusion.

Peritoneal exudate cells (PEC). Four days prior to cell harvesting BALB/c mice were injected intraperitoneally with 1 ml 10% Brewer’s thiglycolate broth (Scientific Products, Irvine, CA). Animals were sacrificed via CO₂ asphyxiation and cold HBSS was introduced into the peritoneal cavity. Macrophages that migrated into the cavity in response to the irritant were harvested in HBSS by syringe. After the cavity was washed several times the resulting cell suspension was pelleted by centrifugation (1000 Xg) and transferred to CM. Cell and viability counts were performed and all subsequent treatment of the PEC was in parallel with I-9.5.

Splenic macrophages. Spleens were removed from BALB/c mice and cells were teased from the capsule using forceps. A single cell suspension was prepared by gently aspirating these cells 20–30 times with a Pasteur pipette and then filtering them through Nitrex mesh (3).

Chemotaxis. A modification of previously described procedures was used to ascertain the extent of chemotaxis displayed by I-9.5, PEC, or splenic macrophages (9,18). The synthetic tripeptide, formyl-methionyl-leucyl-phenylalanine (f-MLP, Sigma), served as the chemotactant and was used at a concentration of 10⁻⁶ M in HBSS. The f-MLP was added to the lower well of a Neuro Probe 48 well micro chemotaxis chamber (Neuro Probe, Cabin John, MD). Certain lower wells designated as non-chemotactic controls contained only HBSS without chemotactant to allow for assessment of random migration. The upper wells of the chamber were separated from the lower ones by an 8 #m pore gelatin-coated polycarbonate filter (Nucleopore Corp., Pleasanton, CA). The chambers were inoculated with 3 X 10⁵ I-9.5 cells, PEC, or splenic macrophages in HBSS and incubated for 1 h at 37 °C, after which time the filter was removed, fixed, and stained using a Diff-Quick Staining kit (Scientific Products). Migration was assessed by counting the number of macrophages traversing the filter in 15 randomly selected fields under an oil immersion lens (X1000).

Phagocytosis. To assess phagocytic activity, 3 X 10⁶ I-9.5 and PEC in CM were incubated in 35 mm polystyrene plates in the presence of ¹⁴C-labeled Staphylococcus aureus (strain CA24) (Nuclopro Corp., Pleasanton, CA). The monolayers were incubated at 37 °C for 30 min and the supernatants were removed and measured by a modification of previously described methods (10,14). Briefly, macrophage monolayers were formed in 96-well flat-bottom tissue culture plates. Reaction mixtures containing cytochrome c and where appropriate, opsonized zymosan, were added to the wells in HBSS. Certain wells also received superoxide dismutase and served as controls to ensure that the reduction of cytochrome c was indeed due to superoxide release. The monolayers were incubated at 37 °C for 30 min and the supernatants were read in a microplate reader (Dynatech Laboratories, Alexandria, VA) at 550 nm. Protein determinations were performed and final results were expressed as a moles of superoxide released per milligram monolayer protein using a molar extinction coefficient of 21 X 10⁶ M⁻¹ cm⁻¹. All comparisons between I-9.5 and in vivo macrophages were made using a two-tailed Student’s t test.

Results
Bone marrow cells cultured in medium lacking LADMAC conditioned supernatant exhibited no macrophage proliferation or differentiation. These cells were not adherent to the surface of the culture vessels and showed none of the cytoplasmic extensions characteristic of macrophages. The most pronounced CSF-1 stimulatory activity was demonstrated by cells cultured in 30% LADMAC supernatant. Proliferation was dramatically increased, resulting in confluent monolayers with cells assuming the stellate shape of vigorous macrophages extending pseudopodia onto the surface of the culture well. This concentration was used to supplement GM for all further experiments and I-9.5 remained dependent on CSF-1 for growth throughout the study. Prior to the start of individual functional assays, all macrophages were transferred from LADMAC-containing media to either HBSS or CM and processed as described below.

In three separate experiments, the chemotactic responsiveness of I-9.5 was assessed and compared to BALB/c PEC. Results shown in Table 1 are expressed as the number of cells traversing the chemotaxis filter per oil immersion field. As shown, I-9.5 macrophages demonstrated chemotaxis in response to f-MLP. Approximately twice as many cells crossed the filter when the chemotactant was used than when HBSS was present in the lower well. This salt solution was used to assess random migration. Surprisingly, however, the level of both random migration and chemotaxis was considerably below that seen with PEC. Three times as many PEC responded to the chemotactant than I-9.5 cells and nearly twice as many PEC randomly migrated to the underside of the filter. In order to assess whether the decreased level of migration observed in I-9.5 was inherent to the cell line or a common feature of splenic macrophages from BALB/c mice, splenic macrophages were isolated and migration was compared to PEC. The mean results obtained for two separate experiments indicated that there were no significant differences between the two macrophage populations. Splenic and peritoneal macrophages demonstrated a chemotactic responsiveness of approximately 45 and 13 migrated cells per oil immersion field in the presence of f-MLP and HBSS, respectively.

In contrast to the dramatic disparity in f-MLP-induced chemotaxis, I-9.5 cells and BALB/c PEC exhibited comparable levels of