Cytotoxic Effector Cell Function at Different Stages of Human Monocyte-Macrophage Maturation

Reinhard Andreesen, Jürgen Osterholz, Klaus J. Bross, Annegret Schulz, G. Albrecht Luckenbach, and Georg W. Löhrl


ABSTRACT

Human blood-borne monocytes were cultured for up to 22 days on disposable Teflon foils. Within 8 days, these monocytes developed into mature macrophages. At various stages of differentiation, the cells were recovered from the hydrophobic membrane and were assayed for typical monocyte-macrophage enzymes and morphology, binding of monoclonal antibodies (OKM1, OKIal),Fc and transferrin receptors, phagocytic activity, lysozyme production, and ability to inhibit the growth of an allogeneic tumor target cell line (U873). A significant antitumor activity of mature macrophages was found, which developed along with the differentiation of the monocyte precursor cells. In addition, cytotoxic effector macrophages could be activated by lymphokine-rich medium and synthetic alkyl-lyosphospholipids. After density gradient separation, light cells (<1.05 and <1.06 g/ml) showed enhanced cytotoxicity, whereas cells from the dense fraction (>1.06 g/ml) with low base-line activity could be best activated for cytotoxicity by lymphokines. If monocyte-macrophages are involved in a natural surveillance mechanism, our results may indicate the importance of unimpaired macrophage maturation to generate effective host defense against tumor development.

INTRODUCTION

The role of the immune system in defense against tumor growth has been under debate for more than 30 years (12).

Besides the humoral and cell-mediated immune responses to tumor-specific or tumor-associated antigens, the unspecific surveillance function of monocyte-Mφ and natural killer cells has been attracting increasing attention (5, 11, 14, 21). The antitumor activity of murine Mφ has been documented in many in vitro and in vivo studies (13), but reports on the activity of human cells are try, and phagocytic activity. At any stage of differentiation, the cells had matured to M0 that were heterogeneous in terms of function in an uncommitted population and at different stages of maturation, we have cultured human monocytes on Teflon foils (1, 7, 27). To study the cytotoxic effector cell function in an uncommitted population and at different stages of differentiation, the cells were recovered from the hydrophobic membrane and tested. It is shown that these monocyte-derived Mφ are effective antitumor cells in vitro which respond to activation by lymphokines as well as by synthetic alkyl-lyosphospholipids (23). Tumor cytotoxicity greatly increases with maturation along the monocyte-macrophage lineage.

MATERIALS AND METHODS

Human Monocyte-Mφ Cultures. The method of growing human Mφ from blood-borne monocytes on hydrophobic Teflon is based on a report by Munder et al. (24) and has recently been published (2, 3). Briefly, 12 × 10^6 monocytes separated from lymphocytes by adherence to plastic were cultured in 30-ml Teflon bags (Bioloflex 25; W. C. Heraeus, D-6450 Hanau, Germany) in RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5 × 10^-5 M 2-mercaptopethanol, antibiotics, and 15% human AB-group serum pooled from selected donors (2). In some experiments, the cultures were fed with 5 ml of supplemented RPMI 1640 plus 20% FCS. Except for the studies on cytotoxicity at different stages of Mφ maturation, cells from nonfed cultures were assayed as effectors. At any stage of differentiation, the cells could be easily recovered from the hydrophobic membrane by exposure to 4° and by gentle agitation of the pliable bags. Viability of the recovered cells ranged from 70 to 90%. Functional and phenotypic analysis of mature Mφ and differentiating monocytes was performed and is described in Table 1.

Tumor Growth Inhibition Assay. Effector Mφ, 5 × 10^5 or 1 × 10^6, were seeded in supplemented RPMI 1640 plus 20% FCS into microtiter plates (0.2 ml/well) in triplicate with and without activating compounds. Twenty-four hr later, the medium was removed, and the adherent Mφ layer was rinsed twice with warm medium; then 1 × 10^5 target cells [U873-clone 4 (31)] from a human histiocytic lymphoma cell line, kindly provided by Kenneth Nilsson, Wallenberg Laboratory, Uppsala, Sweden, were added to the microwells. After a 48-hr incubation, tumor cells were brought into suspension by vigorously pipetting the cultures. Tumor cell suspension (0.1 ml) was transferred to a new microplate, washed once, and resuspended in fresh medium. Microscopy revealed no tumor targets left attached to the Mφ layer. 0.2 μCi [3H]dThd (Amersham and Buchler, D-33 Braunschweig, Germany) was then added to each well. After another 6-hr incubation, cells were harvested, and incorporated radioactivity was measured by liquid scintillation. In some control experiments, we counted viable nonadherent tumor cells after coculture with the Mφ, seeded 1 × 10^5 cells/well in microtiter plates, and cultured them in fresh medium for 3 days. After 24 and 72 hr, the incorporation of [3H]dThd was measured as described above.

It was noted that about 5 to 10% of the adherent Mφ had detached after the 48-hr cocultivation with the tumor cells. Data are expressed either as cpm, as a percentage of [3H]dThd incorporation of the control, or as a percentage of inhibition of [3H]dThd incorporation in tumor cell control cultures.

Mφ Activation. In some experiments, Mφ were activated by lymphokine-rich supernatants of PHA-stimulated peripheral blood leukocytes. This conditioned medium (kindly provided by Dr. H. Neumann, Medizinische Universitätsklinik, D-78 Freiburg, West Germany) was prepared...
as described previously (4) and used at a dilution of 1:10. Rac-1-O-octadecyl-2-methoxy-sn-glycero-3-phosphocholine (ET18-OCH3, NSC 324368) has been described elsewhere (23) and was purchased from Medmark Chemicals, D-8032 Grünwald b. München, Germany. Activation is expressed as activation index, which is defined as $[^{3}H]$dThd uptake by tumor cells cultured in the presence of normal $\text{M}$0 divided by $[^{3}H]$dThd uptake by tumor cells cultured with activated $\text{M}$0.

**Separation of Mature $\text{M}$0 on Density Gradients.** Colloidal silica coated with polyvinylpyrrolidone (Percoll; Pharmacia Fine Chemicals, S-75104 Uppsala 1, Sweden) was adjusted to various densities by dilution with phosphate-buffered saline (1.6 mM phosphate-0.9% NaCl, 0.155 M). Three-ml samples at densities of 1.04 g/ml, 1.05 g/ml, and 1.06 g/ml were sequentially overlayered in 12-ml Falcon tubes. Three ml of $\text{M}$0 suspension (2 x 10^9/ml) were placed on top of the last gradient aliquot, and gradients were centrifuged at 400 g for 30 min at 4°C. Following centrifugation, the interface cells and the cell pellet were removed with a Pasteur pipet, washed 3 times with phosphate-buffered saline, and resuspended in appropriate medium for assay.

**RESULTS**

Human blood-borne monocytes were separated from lymphocytes by adherence to plastic and were cultured on hydrophobic Teflon membranes (2, 24). These adherent monocytes transformed into $\text{M}$p in 8 to 12 days, with some cells already appearing as mature $\text{M}$p after 4 days. This probably reflects the presence of different stages of differentiation in the blood monocyte population (35). $\text{M}$p also varied in their behavior in culture; i.e., some adhered firmly to the Teflon membrane and others stayed non-adherent at all times and formed clusters of 30 to 50 cells. The percentage of cells adherent to the hydrophobic Teflon substrate differed among the individual cultures. Fig. 1 shows a $\text{M}$p population harvested from the Teflon bag after 12 days and cultured on plastic Petri dishes. They are heterogeneous in size, cell shape, content of nuclei, and intensity of cytoplasmic staining. The majority of the cells readily adhered to plastic surfaces, whereas 10 to 15% remained in suspension. A full phenotypic and functional analysis of an in vitro-generated $\text{M}$p population is given in Table 1.

To assess the antitumor activity of the monocyte-$\text{M}$p, we used a postlabeling assay in which the target cells were separated from the effector cells after cocultivation and resuspended in fresh medium before the incorporation of $[^{3}H]$dThd was measured. This excluded the influence of unspecific factors released by $\text{M}$p. The U937 cells were chosen from among other cell lines tested (MOLT4, HL60, K562, Daudi, Raji) for several reasons: (a) they were found to be the most sensitive target to untreated and activated $\text{M}$p; (b) the results obtained with the cells from individual donors were reproducible; and (c) neither enhancement nor inhibition of growth was observed in $\text{M}$p supernatants.

In some control experiments, we compared the results obtained with our postlabeling assay with the regrowth capacity of viable tumor cells after coculture with the effector $\text{M}$p. As can be seen from Chart 1, untreated $\text{M}$p mainly induce tumor growth inhibition, whereas the impaired regrowth capacity of tumor cells after coculture with lymphokine-activated $\text{M}$p indicates more serious damage to the cells (Chart 1B). It should be noted that, in using the term "cytotoxicity," we refer to the effects measured in the postlabeling assay, not necessarily equating cytotoxicity with cell lysis.

In Chart 2, the results of a number of experiments evaluating $\text{M}$p cytotoxicity against the U937 cell line at an effector:target ratio of 5:1 are summarized. The activity of untreated $\text{M}$p and those activated by lymphokines or synthetic alkyl-lyso phospholipids is shown.

The heterogeneity of the mature $\text{M}$p in regard to morphology was also found to be evident in terms of density. Separation of the cells on density gradients revealed at least 2 populations which showed different spontaneous cytotoxicity. In Chart 3, the results of one of the preliminary experiments are shown. Here, 3 different populations could be separated. The untreated light cells (<1.05 and <1.06 g/ml) are the more active effector cells, whereas cells from the higher density fraction (>1.06 g/ml) could be best activated for cytotoxicity by lymphokines.

Because monocytes are only slightly cytostatic even at higher effector:target cell ratios, we followed the development of $\text{M}$p antitumor activity at different stages along the maturation pathway of monocytes to macrophages. In these experiments, we measured cytotoxicity at an effector: target ratio of 10:1. As parameters for differentiation, we recorded changes in morphological characteristics.

### Table 1

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<th>Non-specific esterase</th>
<th>Acid phosphatase</th>
<th>Peroxidase</th>
<th>OKM1</th>
<th>OKM1</th>
<th>OKT9</th>
<th>EA^b</th>
<th>EA^c</th>
<th>24 hr secretion colony-stimulating activity</th>
<th>Angelotensin-converting enzyme (per 10^6 M0)</th>
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<td>III</td>
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<td>&gt;95</td>
<td>63 ± 4</td>
<td>9229 ± 847</td>
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^a Monoclonal antibody binding was detected by the peroxidase-antiperoxidase method on polylysine-coated slides (5).

^b EA, sheep erythrocyte coated with rabbit anti-sheep IgG; ND, not determined; CFU-c, colony-forming cells in culture.

^c EA were used, M0 rosetting 5 or more EA were counted as positive.

^d EA rosetted M0 were incubated for 30 min at 37°C. M0 with 2 or more intracellular EA were counted as positive.

^e Ratio of stimulator cells to responder cells, 1:10.

^f Mean ± S.D.

^g Two x 10^6 nonadherent human bone marrow cells were cultured with 10% supernatant from 2 x 10^6 M0 in methylcellulose as described (6); data are the number of mixed colonies counted on Day 14.

^h Angiotensin-converting enzyme was kindly measured in culture supernatants by Dr. Burmeister (Medizinische Universitätsklinik, Freiburg, Germany).

^i One x 10^6 T-cells from a healthy individual, partially purified by rosetting with sheep erythrocytes, were cultured with 1 x 10^6 irradiated M0 for 6 days; data are cpm after 16-hr pulse with $[^{3}H]$dThd.
Activity and development of tartrate-resistant acid phosphatase (35) and nucleation (Chart 4A), loss of endogenous peroxidase staining (Table 1), expression of the transferrin receptor expressed as cpm; the mean of 3 cultures is given. Bars, S.D.

There is a significant and rapid increase in cytotoxicity against tumor cells by the monocyte Mφ populations recovered from the Teflon cultures within the first 7 days (Chart 5). In those cultures which were fed with fresh medium plus 20% FCS every 5 days, cytotoxicity declined, whereas cells from cultures in which the medium composition remained unchanged exerted constant high cytotoxicity. There was no evidence that Mφ from the fed and nonfed cultures differed in any other parameter tested.

DISCUSSION

Monocytopoiesis is initiated from early multipotential stem cells in the bone marrow, and a path of differentiation is set up ending in fixed and mobile tissue Mφ (for review, see Ref. 35). Blood monocytes are intermediate precursor cells within the monocyte-Mφ lineage. In vitro, these cells undergo transformation to typical Mφ as they do in vivo once they have left the blood stream to migrate into tissues and body cavities. Our culture system provides the possibility to experiment with suspended Mφ in that the hydrophobicity of the Teflon membrane prevents irreversible adherence of the cells to their culture substrate (2, 3, 33, 34).

The results of the present investigation document the antitumor activity of monocyte-derived Mφ. They respond well to activation by physiological mediator molecules or synthetic compounds of the alkyl-lysophospholipid class (23). Despite the uniformity in common surface antigens, cytochemistry, and the capacity for phagocytosis, these in vitro-generated Mφ seem to represent a heterogeneous population, as is evident from morphology, cell size, nucleation per cell, adherence to plastic, and buoyant density. In addition, in the present study, we have shown that heterogeneity exists also in terms of tumor cytotoxicity and response to activating molecules. Data are too preliminary to

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**Chart 4.** Cell nucleation (A) and lysozyme secretion (B) at different stages of monocyte-M₄ maturation in vitro. For morphology, cells were put on polylysine coated slides and stained with Pappenheim, and nuclei were counted. Lysozyme was measured in the supernatants of 1 x 10⁵ M₄ cultured for 24 hr in supplemented RPMI 1640 plus 10% PCS according to the method of Gordon et al. (8). Hen egg lysozyme was used as a reference; values are not corrected for human standards. Each symbol represents one experiment.

It is evident from our data that monocytes acquire effective tumor cytotoxicity during their differentiation into M₄, although a substantial base-line activity may be present in the monocytes, as shown by others (15, 20, 29) using different targets and different experimental systems. The observed differences between fed and nonfed M₄ at late culture stages cannot yet be readily explained. Optimal nutrient supply in cultures fed 5 times daily may favor an antagonizing M₄ subpopulation, the presence of which is indicated in our experiments with cells of different density. In addition, M₄ seem to self-condition their growth medium as it accelerates and optimizes differentiation of freshly isolated monocytes (our own preliminary data). However, all cultures show the strong increase in cytotoxicity during M₄ maturation (Chart 5). Unimpaired M₄ maturation from immature precursor cells may thus be an important factor for the efficient activity of natural host defense systems against nascent tumor development. Although various types of effector mechanisms may be involved in this immune surveillance, defective monocyte differentiation might play a role in the increased incidence of cancers associated with immunosuppression (14, 16). Limited recruitment of mature cytotoxic effector cells from the M₄ type, together with the lack of M₄-activating lymphocyte products, may completely block monocyte-M₄ activity. For example, patients who received alkylating drugs subsequently developed primary malignancies (16, 28). The same is true for allograft recipients receiving immunosuppressive agents (10, 26). Furthermore, the rate of tumor development induced by chemical carcinogens is increased with suppression of immunity (9). It will be the aim of future studies in our system to investigate directly the effect of various drugs on monocyte maturation in vitro.

Effective surveillance by M₄ may be especially needed to stabilize disease states in which the mass tumor burden is removed using aggressive chemotherapy, radiation, and surgery. Relapse in patients with acute leukemia, for example, is sometimes accompanied by viral infection. It is of interest in this context that Lee and Epstein (18) have described inhibition of monocyte-M₄ maturation by interferon at physiological concentrations. Furthermore, impaired monocyte differentiation has been detected in patients with cancer (17), a phenomenon which may be linked to the causative process of tumor development rather than being induced by the malignant disease.

Nevertheless, our demonstration of the generation of effective tumor cytotoxicity during monocyte differentiation to M₄ should stress the need for a careful balance of beneficial and deleterious effects associated with aggressive tumor therapy.
ACKNOWLEDGMENTS

We are indebted to A. Kelly for excellent technical assistance.

REFERENCES


Fig. 1. Morphology of monocyte-derived human Mu. Cells were harvested from the hydrophobic Teflon bag after 12 days in culture, washed once, and plated on conventional plastic Petri dishes. Pappenheim stain, × 320.
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