In Vitro *Activation of Murine Kupffer Cells by Lymphokines or Endotoxins to Lyse Syngeneic Tumor Cells*

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Murine Kupffer cells (KC) were isolated in sufficient number and purity to allow *in vitro* investigations of their tumoricidal capabilities. The identity of the adherent cells as KCs was established by morphologic, histochemical, and functional criteria. The yield of KCs varied from young (high) to old (low) mice but was not affected by the mouse strain. KCs activated *in vitro* by either endotoxins (lipopolysaccharide) or lymphokines were rendered highly cytotoxic against syngeneic melanoma or fibrosarcoma target cells. These studies indicate that KCs may indeed play a role in destruction of tumor cells *in vivo* and thus be important in host defense against developing hepatic cancer metastases. (Am J Pathol 1984, 117:372–379)

THE LIVER is an important component of the reticuloendothelial system.[1] The liver contains more fixed macrophages, i.e., Kupffer cells (KCs), than any other organ. The KCs constitute a major phagocytic barrier of the host. Together with other fixed macrophages, KCs can remove up to 90% of all blood-borne foreign and effete autologous particulate matter.[2] The ability of the KCs to accomplish this task has been widely studied and expressed both in terms of liver clearance[3–5] and production of degradative enzymes.[6–8] KCs can also participate in the initiation of specific immune responses.[9] Ia antigen is expressed on the surface of KCs,[10] and they can present antigens and induce the proliferation of antigen specific T-lymphocytes.[11–14]

We have been interested in determining the role that macrophages play in host defense against cancer metastasis.[15] The liver is a most common site for cancer metastasis; and under certain conditions, KCs are capable of phagocytosing tumor cells.[16–19] To date, most studies dealing with macrophage activation to the cytotoxic state have concentrated on peritoneal exudate cells (PEM), alveolar macrophages (AMs), or blood monocytes.[20–22] Few data are available, however, on the tumor cytotoxic properties of KCs.[23] This has probably been due to the tremendous difficulties associated with obtaining relatively pure and viable KCs of sufficient quantities to allow for such studies. The present report concerns the adaptation of a recovery procedure for KCs that allowed us to investigate in vitro the role of KCs in host defense. We also demonstrate that, like PEMs and AMs, KCs can be activated *in vitro* by either endotoxins or lymphokines to become cytotoxic against syngeneic target cells.

Materials and Methods

Animals

Specific pathogen-free mice of the inbred C57BL/6, C3H/HEN (mammary tumor virus-negative), and athymic nude mice were obtained from the NCI-Frederick Cancer Research Facility's Animal Production Area (Frederick, Md).

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Tumor Cell Cultures

The B16 melanoma, syngeneic to C57BL/6,[24] and the ultraviolet radiation-induced fibrosarcoma UV-2237 syngeneic to the C3H mouse[25] were used routinely. Both cell lines grow as monolayer cultures and were maintained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids, L-glutamine, and gentamycin (50 μg/ml). The components of this medium were obtained from Flow Laboratories (Rockville, Md). The cultures were incubated at 37 C in a humidified atmosphere of 5% CO_2. All cultures were free of mycoplasma.

Reagents

Collagenase Type II (292 U/mg, lot 22F-6802) and deoxyribonuclease (DNase) Type I (800 U/mg, lot 121F-9625) were purchased from Sigma Chemical Company (St. Louis, Mo). Collagenase (70 U/ml) and DNase (300 U/ml) were dissolved in Hanks' balanced salt solution (HBSS), pH 7.2. Metrizamide (30% wt/vol; Accurate Chemical and Scientific Corporation, Westbury, NY) was prepared in Gey's balanced solution without sodium chloride. All solutions were filtered through 0.45-μ Millipore units. Rat anti-sheep red blood cell antibody was a gift of Dr. Goro Hisano (NCI–Frederick Cancer Research Facility). Crude rat lymphokines containing macrophage-activating factor (MAF) were obtained as described previously.[26] Lipopolysaccharide (LPS) B (*Escherichia coli* 026:B6) was obtained from DIFCO Laboratories (Detroit, Mich).

Preparation and Purification of KCs

Mice killed by CO_2 suffocation were submerged in iodine and alcohol and placed into a laminar flow hood. All subsequent procedures were carried out under aseptic conditions. The liver was perfused *in situ* through the portal vein with 10 ml of cold HBSS containing 50 μg/ml gentamycin. The inferior vena cava was severed to allow free outflow. After the perfusion, the liver was excised, washed in cold Dulbecco's phosphate-buffered saline (PBS), Ca^{2+}- and Mg^{2+}-free. The liver was minced and passed through a 60-mesh stainless steel screen into enzyme solution (collagenase, 70 U/ml, DNase 300 U/ml). The suspension, consisting of 1 g of liver tissue per 15 ml of enzyme solution, was incubated in a 37 C water bath for 10 minutes and continuously stirred by a magnetic bar as described previously.[10] This procedure leads to the total dissociation of the liver aggregates and the destruction of many parenchymal cells. At this time, the suspension was diluted with an equal volume of cold HBSS (Ca^{2+}- and Mg^{2+}-free) and centrifuged at 50 *g* for 10 minutes. The pellets were washed three times with HBSS, then resuspended in 2.5 ml of HBSS, mixed with 3.5 ml of 30% (wt/vol) metrizamide solution, overlaid with 1 ml PBS, and centrifuged at 1400 × g for 15 minutes at 4 C.[27] Nonparenchymal cells (NPCs) were collected from the interface, washed twice in HBSS, and resuspended in medium containing 5% FBS. The preparation of NPCs was considered homogeneous, because little to no contamination with parenchymal cells or erythrocytes occurred.

KCs were isolated from the NPC population by adherence. Different numbers of NPCs in supplemented medium were seeded into 35-mm tissue-culture dishes and into the wells of microtest II plates (Costar, Cambridge, Mass). The cultures were incubated at 37 C for 1 hour. The supernatants were carefully removed, and fresh medium was added. KCs adhered to the bottom firmly and began to spread 3–4 hours after incubation. At this time, nonadherent cells were removed by vigorous washing (4–5 times) with warm HBSS before the addition of immunomodulators of tumor cells. For some studies, nonadherent NPCs were collected from the HBSS washes. These cells were incubated in culture dishes containing various activating agents for 24 hours. The cells were washed and counted prior to further studies.

Preparation of Peritoneal Macrophages

PEMs were harvested according to a method described previously.[30] Briefly, thioglycollate-induced PEMs were harvested and washed with HBSS (Ca^{2+}- and Mg^{2+}-free). The PEMs were resuspended in serum-free medium and plated into the 38 sq mm wells for 1 hour. Nonadherent cells were removed by vigorous washing. This procedure yields an adherent population with >95% viability of >95% phagocytic cells.[30]

Histochemical Identification of KCs

Similar to other macrophages, KCs demonstrate esterase activity. Naphthol-AS-acetate (N-AS-A) esterase activity was measured with the nonspecific esterase-activity agents (Technicon Instruments Corporation, Dallas, Tex) with 1% methyl green used as a counterstain. Under these conditions, the nucleus of positively reacting cells stains green, and the cytoplasm stains brownish red.[28]

To differentiate further between KCs and other cells in the NPC population, we measured peroxidase activity.[29] The reagents for the histochemical demonstration of leukocyte peroxidase were purchased from Sigma Chemical Company (Histozyme Kit 390-A).

Determination of Fc Receptors and Phagocytosis

Sheep red blood cells (SRBCs) were maintained in Alsever's solution (GIBCO, Grand Island, NY) at 4 C. The SRBCs were washed carefully, suspended in HBSS, and incubated at 37 C with anti-SRBC antibody for 30 minutes. The opsonized SRBCs were washed and diluted to a 1% suspension, and 0.1-ml aliquots were added to dishes containing adherent KCs. Incubation was carried out at 4 C for 1 hour. At this time the cultures were thoroughly rinsed, fixed, and stained with Giemsa for examination.

For phagocytosis studies, 0.1 ml of 1% opsonized SRBCs was added into the KC culture. The KC–SRBC mixture was incubated at 37 C for 1 hour, then rinsed gently. A hypotonic shock with distilled water for 30 seconds was used for lysis of uningested erythrocytes. The KCs were then fixed and stained with Giemsa. Phagocytosis was also measured by the uptake of carbon particles. India ink (Gunter Wagner, Hannover, West Germany) diluted 1:100 in HBSS was added into the culture dishes. After 1-hour incubation at 37 C, the KCs were thoroughly rinsed, fixed, and stained with Giemsa.

In Vitro Activation of KCs

Four hours after the initial plating of the KCs, the cultures were washed and refed with either medium alone, medium containing LPS (10 μg/ml), or lymphokines containing MAF activity. Twenty-four hours later, the KC cultures were washed before the addition of target cells.

Assay of KC-Mediated Cytotoxicity *in Vitro*

KC-mediated cytotoxicity was assessed by a radioactive release assay as detailed previously.[30] Briefly, target cells were incubated for 24 hours in medium containing 0.2 μCi/ml ^{125}I-iododeoxyuridine (^{125}I-IUdR; specific activity 200 mCi/mmol; New England Nuclear, Boston, Mass). The target cells were washed to remove unbound radiolabel, harvested by a short trypsinization, and resuspended in supplemented medium. Target cells (1×10^4) were plated into the wells containing KCs. The effector target cell ratio was calculated from the number of adherent KCs, compared with the number of plated tumor cells. At this population density, untreated KCs exhibited low to no spontaneous cytotoxicity against neoplastic cells, whereas activated KCs did. Radiolabeled target cells were also plated alone as an additional control group. All cultures were refed with fresh medium containing 10 ng/ml LPS, 24 hours after the plating of tumor cells. The interaction of KCs and target cells was terminated after 72 hours of cocultivation. At this time the cultures were washed twice with PBS, and the remaining adherent cells were lysed with 0.1 ml of 0.1 N NaOH. The lysate was monitored for radioactivity in a gamma counter.

The cytotoxicity activity of the KCs was calculated as follows:

$$\%\ \text{cytotoxicity} = 100 \times \frac{\begin{array}{c}\text{CPM in target cells}\\ \text{cultured with}\\ \text{control effector cells}\end{array} - \begin{array}{c}\text{CPM in target cells}\\ \text{cultured with}\\ \text{test effector cells}\end{array}}{\begin{array}{c}\text{CPM in target cells cultured}\\ \text{with control effector cells}\end{array}}$$

(CPM = counts per minute)

The results of the *in vitro* KC-mediated cytotoxicity experiments were analyzed for their statistical significance with the Student two-tailed *t* test.

Ultrastructural Studies of KCs

KCs cultured on 35-mm plastic culture dishes were fixed with a fixative containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer for 1 hour. The samples were washed with buffer and then fixed with 1% buffered osmium tetroxide for 30 minutes. Samples for scanning electron microscopy were removed by cutting out the bottom of the culture dishes, dehydrated in a graded series of ethanol, and substituted with Freon 113. The samples were critical-point-dried in Freon 13 in a Bomar critical-point dryer SPC-900/EX (Bomar Co., Tacoma, WA). They were then coated with platinum–palladium alloy (100–150 Å of 80:20 alloy) by evaporation and examined in a Hitachi HFS-2 field emission scanning electron microscope. Samples for transmission electron microscopy were fixed with the glutaraldehyde–paraformaldehyde fixative and postfixed with the same fixative containing 1% tannic acid. The samples were washed thoroughly with buffer, postfixed with 1% buffered osmium tetroxide for 30 minutes, and washed twice with distilled water. The samples were stained *en bloc* with 1% aqueous uranyl acetate for 30 minutes, dehydrated with a graded series of ethanol, embedded in Epon,[31] and polymerized for 3 days at 56 C. The blocks were sectioned on an LKB Ultratome III. The sections were stained with lead citrate and examined in a Hitachi HU-12A transmission electron microscope at an accelerating voltage of 75 kv.

Results

Morphologic Identification of KCs

To identify phagocytic KCs in the first experiment positively, we gave mice intravenous injections of colloidal carbon 1 hour prior to liver perfusion. The ad-

herent cells recovered from such livers contained carbon particles in their cytoplasm. Under phase-contrast microscopy, these cells exhibited a morphology similar to that of PEMs. Ultrastructural examination of KCs revealed that the cells were elongated, measured 12–14 μ, and contained numerous osmiophilic particles resembling residual bodies, lysosomes, or both. Lipid vacuoles were also prominent in the cytoplasm. Abundant cytoplasmic processes were common at the periphery of the cells (Figure 1).

Yield of Recovered KCs

All *in vitro* assays of KCs depend on the recovery of sufficient cells with high viability. We first investigated whether the yield of recoverable KC could be influenced by the age of the mouse. The combined results of our experiments (188 independent liver samples) are shown in Table 1 and demonstrate that up to 2×10^6 KCs/g liver could be obtained from mice 4–8 weeks old. In mice 11 weeks or older, this yield decreased to as few as 1×10^6 KCs/g liver. The reduced yield of KCs in older mice could not be attributed simply to increased liver size, and thus to overloading of the metrizamide gradient (Table 1). We also compared the yield of recovered KCs from C57BL/6, C3H, and nude mice. The data of 60 independent liver analyses do not demonstrate strain differences in the yield of recoverable KCs. Specifically, the yield of KCs from C57BL/6 mice was $2 \pm 0.4 \times 10^6$, from C3H it was $2.7 \pm 0.9 \times 10^6$, and from nude mice it was $2.35 \pm 0.3 \times 10^6$ cells.

Identification and Functional Characterization of KCs

The combined data of thirty observations are shown in Table 2. Greater than 95% of the adherent cells were mononuclear and exhibited typical macrophage morphology (Figure 2A). Greater than 95% of the adherent cells were positive for nonspecific esterase stain (Figure 2B; Table 3). Previous reports[32] suggested that peroxidase activity could distinguish positive KCs from negative endothelial cells in the NPC fraction of the liver. Indeed, greater than 75% of the adherent KCs were intensely positive for peroxidase staining and exhibited dark brown-black intracellular granules. The other cells exhibited a lower intensity of peroxidase staining.

The presence of Fc receptors was determined subsequent to incubation of the KCs with opsonized SRBCs. Greater than 70% of the cells formed rosettes with opsonized SRBCs (Figure 3). The binding was firm: the SRBCs could not be detached from the KCs by vigorous washing. Phagocytosis of the opsonized SRBCs was determined by light microscopic examination. At least 80% of the adherent cells internalized SRBCs in their cytoplasm. A similar level of phagocytosis was observed after incubation with colloidal carbon.

Table 1 – Age of Donor Mice Influences the Yield of Kupffer Cells

Age (weeks)	Number of mice	Liver weight (g) (mean ± SD)	Number of recovered KCs (10^6/g liver)
4	40	0.9 ± 0.02	2.33 ± 0.18
6	54	1.09 ± 0.14	2.19 ± 0.42
8	55	1.20 ± 0.21	2.18 ± 0.62
11	18	1.38 ± 0.15	1.21 ± 0.66
12	15	1.43 ± 0.12	1.15 ± 0.66*
17	15	1.63 ± 0.06	1.03 ± 0.53*

* Significantly different from 4-week-old mice ($P < 0.001$).

Table 2 – Characterization of Adherent Kupffer Cells

Criteria	% Positive cells*
Nonspecific esterase staining	95
Peroxidase staining	76 ± 11
Fc Receptors	71 ± 13
Phagocytosis of opsonized SRBCs	80 ± 9
Phagocytosis of colloidal carbon	67 ± 8

* Mean ± SD of 30 measurements for each parameter.

In Vitro Activation of KCs by MAF and LPS to Become Cytotoxic

Previously published reports suggest that only 50% of recoverable KCs can adhere to plastic or glass.[27,33] In our experiments, we routinely obtained a monolayer of KCs in 7-mm culture wells by seeding 2.0×10^5 KCs

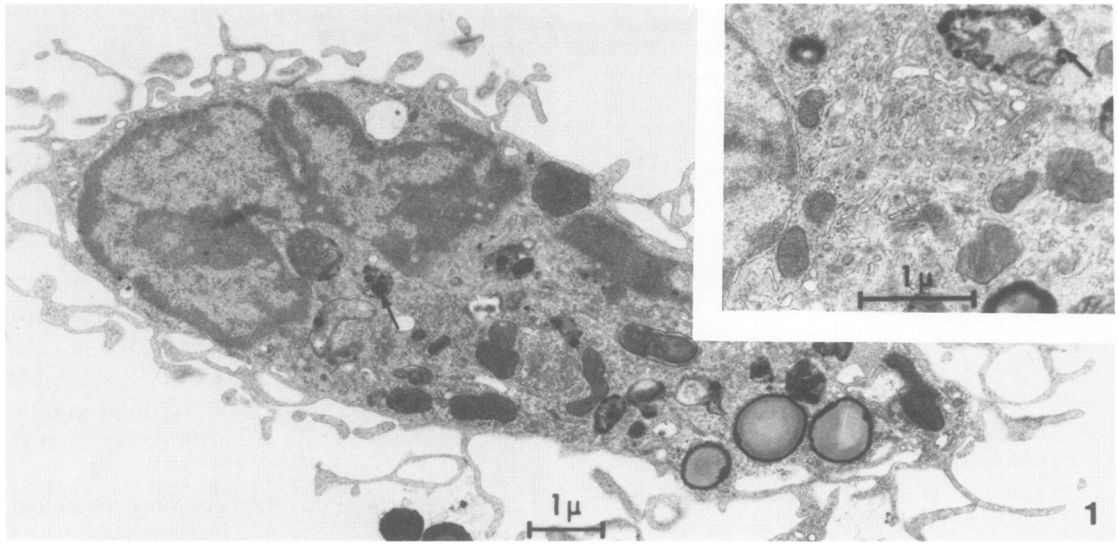
Table 3 – *In Vitro* Activation of Kupffer Cells and Peritoneal Exudate Cells by Endotoxins or Lymphokines to Become Tumor-Cytotoxic

Treatment of macrophages*	Radioactivity remaining in adherent target cells on day 3†	
	KCs	PEMs
No macrophages, tumors cells alone	977 ± 78	1036 ± 44
Medium	1080 ± 29	975 ± 125
MAF	560 ± 81 (48%)†	359 ± 12 (65%)†
LPS (10 μg/ml)	619 ± 94 (42%)	635 ± 124 (39%)†

* The KCs or PEMs were incubated with the indicated agents for 24 hours prior to the addition of target cells.

† Ten thousand ^{125}I-IUdR labeled B16 melanoma cells were added to 1.0×10^5 KC or 1.0×10^5 PEM cultures. The assays were terminated 72 hours later. Data are shown as mean counts per minute of triplicate cultures ± SD (per test group). This is a representative experiment of three.

‡ The number in parentheses is the specific cytotoxicity. This value is derived from comparison with tumor cells cultured with media-treated macrophages ($P < 0.005$).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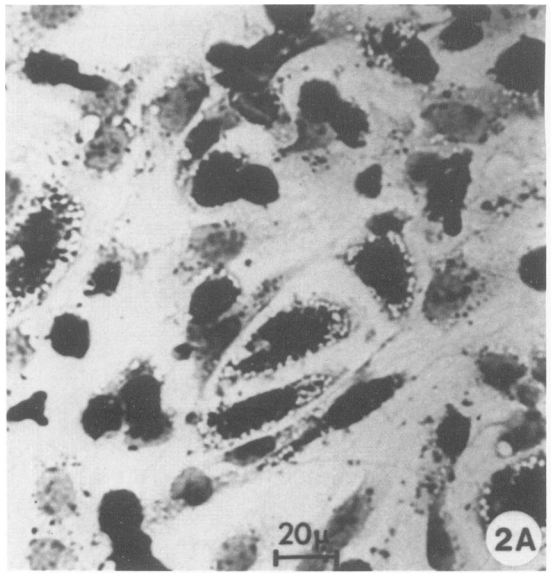
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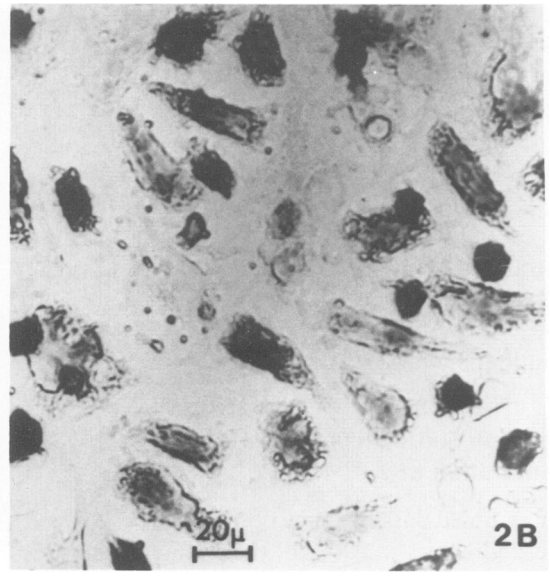
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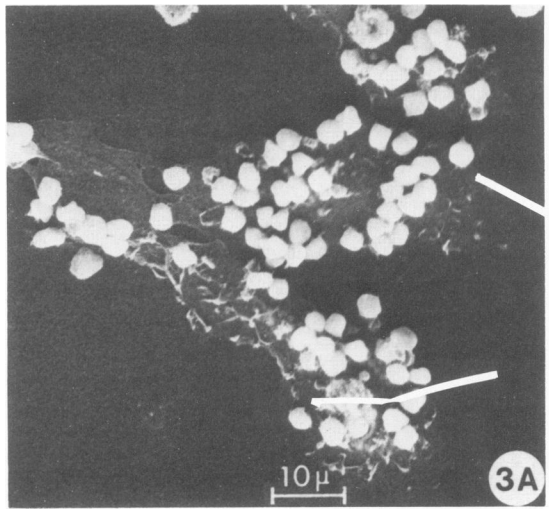
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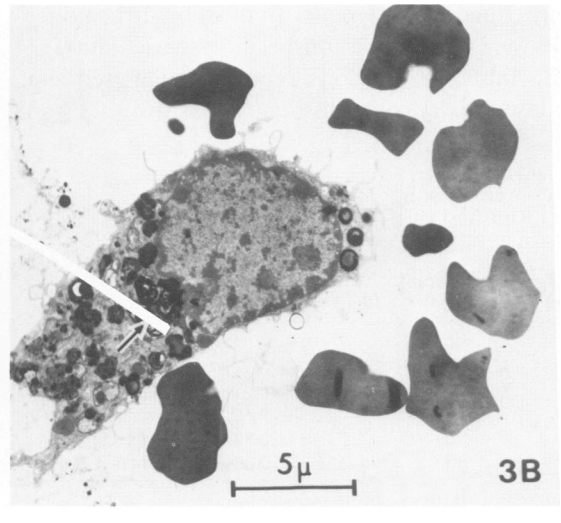
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(in 0.2 ml of medium). After washing, about 1×10^5 KCs adhered to the plastic. The addition of 1×10^4 targets therefore produced a 10:1 effector/target ratio (see below).

Like PEMs, KCs were rendered tumor-cytotoxic *in vitro* subsequent to incubation with either MAF or LPS. The data shown in Table 3 were very reproducible. Neither medium-treated KCs nor medium-treated PEMs exhibited any cytotoxicity against the B16 melanoma cells. Incubation of KCs or PEMs with medium for 24 hours did not bring about a change in cytotoxic properties. In several preliminary experiments, KCs or PEMs freshly harvested were not cytotoxic against the tumor target (data not shown). Activation of KCs by MAF or LPS to the tumoricidal state was reproducible. In the different assays, the activity ranged from 40% to 52% and 37% to 46% for MAF or LPS, respectively.

To rule out the possibility that the extensive harvesting procedure used to obtain KCs was responsible for their interaction with LPS or MAF, PEMs were treated with the collagenase-DNase enzyme solution for 10 minutes at 37 C, washed, separated on metrizamide solution, and centrifuged at 1400*g* for 15 minutes at 4 C. The treatment did not influence the capacity of PEMs from either C57BL/6 or C3H mice to respond to activation stimuli and lyse B16 melanoma or UV-2237 fibrosarcoma targets. Specifically, control PEMs activated with LPS or MAF lysed 65% and 40% of B16 melanoma cells, respectively, whereas enzyme-pretreated PEMs activated with LPS or MAF lysed 63% and 42% of the B16 melanoma cells. Similar reactivities were observed with PEMs obtained from C3H mice cultured with syngeneic UV-2237 fibrosarcoma cells.

Adherent KCs, but not nonadherent NPCs, could be rendered tumor-cytotoxic *in vitro*. We base this conclusion on the data shown in Table 4. Nonadherent NPCs were collected as described in Materials and Methods and cultured with medium, LPS, or MAF for 24 hours. The cells were washed and mixed with radiolabeled target cells at up to a 50:1 ratio. No evidence of target cell lysis could be obtained after 72 hours of cocultivation. In contrast, adherent KCs (at a 10:1 effector/target ratio) were rendered tumor-cytotoxic subsequent to incubation with LPS or MAF (34% and 21%; $P < 0.01$). The possibility that nonadherent cells in NPCs could contain natural killer cells has not been ruled out.

Table 4—Tumoricidal Properties of Adherent Kupffer Cells and Nonadherent Nonparenchymal Liver Cells

Treatment of effector cells	Radioactivity remaining in viable target cells after 3 days*	
	Adherent—KCs†	Nonadherent—NPCs‡
Media	1449 ± 112	1315 ± 130 (3%)
MAF	1138 ± 94 (21%)§	1377 ± 128 (3%)
LPS (10 μg/ml)	961 ± 132 (34%)§	1394 ± 141 (2%)

* Mean counts per minute ± SD of triplicate cultures. The number in parentheses is the percent cytotoxicity calculated by a comparison with media treated effector cells.

† The KCs were incubated with the indicated agents for 24 hours prior to the addition of target cells. 10^4 ^{125}I-IUdR labeled B16 melanoma cells were added to 1.0×10^5 KCs (1:10 ratio). The assays were terminated 72 hours later. The data are shown as mean counts per minute of triplicate cultures ± SD (per test group). This is a representative experiment of three.

‡ Nonadherent nonparenchymal cells (NPCs) were collected from the supernates of NPC cultures and cultured for 24 hours with the indicated activators. At this time, the cells were washed, counted, and admixed with 1×10^4 target cells to produce an effector to target ratio of 50:1. The cultures were terminated 3 days later.

§ $P < 0.01$.

Discussion

The difficulties associated with routine isolation of KCs in sufficient numbers, purity, and viability have prevented extensive investigations of this important cell. We have been interested in the role that free and fixed macrophages play in host defense. For this reason, we needed to find a procedure that could be used routinely for the isolation of KCs. Although a high yield of KCs can be obtained by the digestion of liver aggregates with pronase,[29,34] this treatment could well affect the function of the recovered KCs.[14] For this reason, we have chosen to concentrate on the use of collagenase-DNase dissolved in HBSS containing Ca^{2+} and Mg^{2+} for the dissociation procedure. With this mixture of enzymes, we obtained a preparation of single cells that did not contain erythrocytes or whole parenchymal cells. Because endothelial cells require exogenous fibronectin for attachment and spreading to glass or plastic, whereas KCs do not,[33] we used adhesion to separate the KCs from the NPCs. By 3 hours of incubation in supplemented medium, KCs firmly adhered to the dishes. Subsequent to vigorous washings practically all adherent cells exhibited typical macrophage morphology.

Figure 1—Transmission electron micrograph of adherent Kupffer cell showing indented nucleus, numerous cytoplasmic processes, lipid vacuoles, large mitochondria, many residual bodies (*arrow*). Prominent Golgi apparatus is shown in the **inset**. (×10,000; **inset**, ×16,000) **Figure 2**—Adherent Kupffer cells stained with Giemsa stain (**A**) and stained for nonspecific esterase (**B**). (×400) **Figure 3**—Rosette of KCs and SRBCs examined by scanning electron micrography (**A**) and by transmission electron micrograph (**B**). Notice the clustering of SRBCs in the perinuclear area in both figures. The transmission electron micrograph of the rosette also shows KCs with myelin figures plus residual bodies and lipid in the perinuclear area. Residual bodies are scattered in the cytoplasm. (**A**, ×1000; **B**, ×3250)

The exact number of KCs in the liver is unknown. A review of the literature revealed that the number of KCs recovered from the liver varied among different species and even among the same species, depending on the methods used for isolation.[10,27,32] We examined the question of whether the strain or age of mice could influence the yield of recoverable KCs. All three strains of mice we examined yielded similar levels of recoverable KCs. The number of recoverable KCs varied, however, between young (4–8 weeks) and old (>11 weeks) mice. Our combined analysis of 188 livers is indeed extensive, but it serves to demonstrate that differences in yield do exist among animals that are young or old. Livers of older rodents contain a high number of fat-storing cells or fibroblasts, or both,[35] and this may account for the fact that the number of recoverable KCs per gram of tissue is lower in the older mice. The adherent cells exhibited morphologic features compatible with those of fixed macrophages. The percentage of KCs exhibiting Fc receptors (70%) agreed closely with other published data.[27] This may be due to heterogeneity of the KC[36] or to surface damage mediated by enzymes released from parenchymal cells.[34]

Macrophages are an important component of host defense. The *in vitro* or *in vivo* activation of macrophages can render these cells bactericidal and tumoricidal.[20,26,37] The liver is a common site for cancer metastasis, and the role that activated KCs may play in the destruction of metastatic cells is of obvious importance. In this report, we demonstrate that similar to PEMs and AMs, KCs can be rendered tumor cytotoxic *in vitro* by treatment with endotoxins or lymphokines. The kinetics of the cytotoxicity mediated against the melanoma target were identical to those exhibited by other macrophages (data not shown). At the same time, the nonadherent cells exhibited no cytotoxic activity against the melanoma targets.

In conclusion, like other murine macrophages, the fixed macrophages of the liver can be rendered tumor-cytotoxic *in vitro*. The ability to activate the antitumor activity in KCs *in situ*, and thereby enhance host resistance to hepatic metastases, is now being investigated.

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