Differential Sensitivity of Tumor Targets to Liver Macrophage-mediated Cytotoxicity

Carol R. Gardner, Arthur J. Wasserman, and Debra L. Laskin

Department of Pharmacology and Toxicology, Rutgers University [C. R. G., D. L. L.], and Department of Pathology, University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School [A. J. W.], Piscataway, New Jersey 08854

ABSTRACT

Liver macrophages activated in vivo with bacterially derived lipopolysaccharide (LPS) display enhanced chemotaxis, phagocytosis, and oxidative metabolism. To determine if LPS also activates these mononuclear phagocytes for tumor cell killing, we compared the cytotoxic activity of macrophages from livers of rats treated with LPS (5 mg/kg, i.v.) with resident Kupffer cells. We found that both macrophage cell types displayed cytotoxicity towards rat NISI hepatoma and RBL-1 basophilic leukemia cells. Cytotoxicity of resident and LPS-activated liver macrophages towards these targets increased with incubation time, was dependent on the effector/target cell ratio, and appeared to involve extracellular lysis. No direct correlation between macrophage activation and cytotoxicity was observed towards these targets. While liver macrophages from LPS treated rats were more cytotoxic towards NISI cells, resident Kupffer cells were more cytotoxic towards RBL-1 cells. In further studies, resident Kupffer cells were also found to display extracellular cytolytic activity towards mouse P815 mastocytoma cells. In contrast, LPS-activated liver macrophage-mediated killing of these targets involved phagocytosis of intact tumor cells, as evidenced by light and electron microscopy and by uptake of 51Cr-labeled cells. These results suggest that cytotoxicity mediated by liver macrophages depends on the type of macrophage and the nature of the tumor cell target. In addition, cytotoxicity towards tumor targets appears to involve at least two different mechanisms including extracellular cytolysis and phagocytosis.

INTRODUCTION

The liver contains the largest number of macrophages in the body (1) and thus would be expected to provide a formidable defense against tumor invasion (2). In the rat, it has been reported that tumor cells injected directly into the portal vein are temporarily sequestered in the liver, and then, over a 4-h period, slowly released as dead or damaged cells (3). Similarly, in mice, the number of lymphocytic leukemia cells present in the liver decreases sharply within 1–5 days following tumor cell inoculation (4). The mechanism underlying tumor cell destruction in the liver is unknown. Nonparenchymal cells, a heterogeneous mixture of Kupffer, endothelial, and NK3 cells have been temporarily sequestered in the liver, and then, over a 4-h period, slowly released as dead or damaged cells (3). Similarly, in mice, the number of lymphocytic leukemia cells present in the liver decreases sharply within 1–5 days following tumor cell inoculation (4). The mechanism underlying tumor cell destruction in the liver is unknown. Nonparenchymal cells, a heterogeneous mixture of Kupffer, endothelial, and NK3 cells have been reported to be cytotoxic towards a number of different tumor targets in vitro (5–8). Although it has been suggested that NK cells are the major effectors responsible for cytotoxicity (6, 7, 9, 10), Kupffer cells, in fact, possess considerable tumoricidal activity (7, 8, 11–13). Light and electron microscopy has revealed that certain tumor cells, when infused directly into livers, are rapidly and preferentially engulfed by Kupffer cells (14–16). Furthermore, depletion of Kupffer cells appears to be related to the development of chemically induced hepatic tumors (2, 17). Based on these observations, it has been hypothesized that a population of macrophages (Kupffer cells) resides in the liver that protects against locally arising hepatocellular carcinoma (2) and other metastatic tumors (8).

Destruction of tumor cells by macrophages typically requires the macrophages to become activated (18). In previous studies we demonstrated that treatment of rats with bacterially derived LPS leads to the accumulation of activated macrophages in the liver (19). These cells display altered morphology, enhanced chemotaxis and phagocytosis, and release reactive oxygen mediators. In addition, treatment of murine Kupffer cells with LPS in vitro stimulates cytotoxicity towards syngeneic tumor targets (20). To determine if LPS also activates liver macrophages for tumor cell killing in vivo, we compared the cytotoxic activity of macrophages isolated from livers of rats treated with LPS with resident Kupffer cells towards several different tumor targets. We found that cytotoxicity by liver macrophages depends on both the type of macrophage and nature of the tumor target.

MATERIALS AND METHODS

Tumor Targets. Sprague-Dawley rat-derived NISI hepatoma, Wistar rat-derived RBL-1 basophilic leukemia, and DBA/2 mouse-derived P815 mastocytoma cells were purchased from ATCC (Rockville, MD). The cells were cultured in DMEM containing 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 0.05 units/ml bovine insulin, and 1% penicillin/streptomycin (10,000 IU/10,000 mg/ml).

Isolation of Liver Macrophages. Macrophages were isolated from livers of outbred female Sprague-Dawley rats (200–225 g; Taconic Breeders, Germantown, NY) using a modification of previously described methods (19). Rats were anesthetized with 50 mg/kg sodium pentobarbital and the livers perfused through the portal vein with Ca2+/ Mg2+ free Hanks' balanced salt solution containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 0.5 mM ethyleneglycol bis(2-aminoethoxy) ether)-N,N,N',N'-tetraacetic acid. This was followed by perfusion for 15 min with Liebovitz's L-15 medium containing 0.05% protease type XIV (Sigma) and 100 units/ml collagenase type IV. The liver was then excised, combed, and digested for an additional 40 min at 37°C with 0.2% protease type XIV and 0.001 % Dnase I. The cells were then washed (50 × g, 1 min) to remove contaminating hepatocytes, and the nonparenchymal cells recovered by centrifugation (300 × g, 4°C, 5 min). Macrophages were purified from the nonparenchymal cells by differential centrifugation on a metrizamide gradient as described by Pilaro and Laskin (19, 21). Liver macrophages were identified morphologically and histochemically using nonspecific esterase as previously reported (19). Macrophages were washed twice in buffered L-15 medium, resuspended (5 × 10⁶ cells/ml) in DMEM culture medium, and inoculated into 96-well dishes (1 × 10⁴, 5 × 10⁴, 1 × 10⁵, or 2 × 10⁵ cells/well). After 4 h, the macrophages were washed with warm DMEM to remove nonadherent cells and debris and refed with 0.1 ml of fresh culture medium. Resident Kupffer cells were obtained from livers of untreated rats, while LPS-activated macrophages were from livers of rats injected i.v. with 5 mg/kg of Escherichia coli LPS 0128:B12 48 h prior to cell isolation.

Cytotoxicity Assay. Cytotoxicity was measured using a modification of methods described by Meltzer (22) and Russell (23). Tumor targets (1 × 10⁶ cells) were prelabeled for 24 h with 1HdThd (specific activity, 44 Ci/mmol; New England Nuclear, Boston, MA). NISI and RBL-1 cells were labeled with 0.5 μCi/ml and P815 with 2 μCi/ml. Four h prior to use, the labeled targets were washed (300 × g, 5 min) and then...
incubated with 5 ml of DMEM culture medium. Separate cultures of P815 mastocytoma cells \((1 \times 10^6)\) cells were labeled for 1 h with 200 
\(\mu\text{Ci}^{51}\text{Cr}(\text{sodium salt}; \text{specific activity,} \, 275–400 \text{mCi/mg}; \text{New England Nuclear}),\) followed by 1 h incubation with 10 ml of culture medium. Target cells were then washed and resuspended \((1 \times 10^6)\) cells/ml in culture medium. One hundred \(\mu\)l of tumor targets were then added to the plates containing liver macrophages to give E:T ratios of 1:1, 5:1, 10:1, and 20:1. Control wells containing only target cells were used to monitor spontaneous release of the radiolabel. Following incubation for 24–72 h, the plates containing the cells were centrifuged for 2 min at 800 \(\times g\). Quadruplicate 0.1-ml samples of the culture supernatant were removed and counted for radioactivity. Each experiment was repeated 3–4 times with similar results. The data were analyzed using the Mann-Whitney 2-Sample Rank Test.

The total amount of radiolabel capable of being released from \[^{3}H\]dThd-labeled tumor cells (total release) was determined by counting 0.1-ml samples of solubilized extracts of labeled tumor cells lysed in 1% sodium dodecyl sulfate. Total release of \(^{51}\text{Cr}\) from P815 cells was measured by adding an equal volume of distilled water to the tumor cells and then freezing and thawing the cells 4 times. Lysates of P815 cells were then centrifuged at 850 \(\times g\) for 5 min to remove debris and triplicate 0.1-ml aliquots of supernatants removed for counting. Cytoxicity is presented as a percentage of specific release of radiolabel from target cells and was calculated as experimental release divided by total release times 100. Spontaneous release was subtracted from experimental and total release prior to calculation of the percentage of specific release. In the absence of macrophages, \(^{51}\text{Cr}\)-labeled P815 cells were found to spontaneously release significant amounts of radiolabel into the culture medium. At 48 h, spontaneous release was about 35–40% of the total \(^{51}\text{Cr}\) incorporated into the cells and by 72 h, about 75–80%. In contrast, the maximum spontaneous release of \[^{3}H\]dThd from labeled targets was approximately 30–40% even after 72 h. Therefore, experiments with \(^{51}\text{Cr}\)-labeled P815 cells were terminated after 48 h cocultivation.

Phagocytosis Assay. Liver macrophages were inoculated into 24-well culture plates \((6 \times 10^4)\) cells/well and allowed to adhere for 4 h. Cells were then washed 2 times with warm DMEM and \(^{51}\text{Cr}\)-labeled P815 cells were added to each well to give E:T ratios of 10:1 and 20:1. Seventy-two h later, the cells were washed 3 times with phosphate buffered saline (pH 7.3), solubilized with 1 ml of 0.1 N NaOH, and counted for radioactivity as previously described (24, 25). The data were analyzed using 1-way analysis of variance and Duncan’s Multiple Range Test.

Electron Microscopy. Cultures of macrophages and tumor cells were fixed with cacodylate buffered 2% glutaraldehyde and postfixed in cacodylate buffered 1% OsO\(_4\). For transmission electron microscopy, samples were block stained in acetone buffered 1% uranyl acetate, dehydrated, and embedded in Polybed 812 (Polysciences, Warrington, PA). Thin sections were cut, double stained with uranyl acetate and Reynolds lead citrate (26) and photographed on a Philips 420 transmission electron microscope. For scanning electron microscopy, post-osmicated samples were dehydrated and subjected to critical point drying followed by gold sputter coating. Samples were photographed in an AMRAY 1400 scanning electron microscope equipped with a titanium sublimation pump.

**RESULTS**

Cytotoxicity of Liver Macrophages towards NISI Hepatoma Cells. Initially, we compared the cytotoxic activity of liver macrophages obtained from untreated and LPS-treated rats towards the rat hepatoma cell line NISI1. We found that both macrophage cell types displayed cytotoxicity towards \[^{3}H\]dThd-labeled hepatoma cells in a time-dependent manner reaching a maximum after 72 h coincubation (Fig. 1). Although there were no significant differences in cytotoxicity between the 2 liver macrophage types at early time points, after 72 h LPS-activated liver macrophages were approximately 2–3 times more cytotoxic towards the NISI1 hepatoma cells than were resident Kupffer cells. Cytotoxicity of liver macrophages towards NISI1 hepatoma cells was also found to be dependent on the E:T ratio. For both resident Kupffer cells and LPS-recruited liver macrophages, as the number of effector cells was increased, cytotoxicity also increased, reaching a maximum at the 20:1 E:T ratio (Table 1).

Cytotoxicity of Liver Macrophages towards RBL-1 Basophilic Leukemia Cells. To determine if liver macrophage-mediated cytotoxicity varied with the nature of the tumor target, we next compared the ability of resident Kupffer cells and LPS-recruited liver macrophages to kill RBL-1 rat basophilic leukemia cells. As observed with the NISI1 hepatoma, both liver macrophage populations exhibited cytotoxicity towards the leukemia cells in a time-dependent manner reaching a maximum after 72 h coincubation (Fig. 2). In contrast to the results obtained using the hepatoma cells, by 72 h resident Kupffer cells were significantly more cytotoxic towards RBL-1 cells than were lige macrophages from LPS-treated rats.

Cytotoxicity towards RBL-1 cells was also dependent on the
LIVER MACROPHAGE-MEDIATED CYTOTOXICITY

Fig. 2. Cytotoxicity of liver macrophages towards rat RBL-1 basophilic leukemia cells. Resident Kupffer cells (A) or liver macrophages from LPS-treated rats (B) were coincubated with [3H]dThd-labeled RBL-1 cells at an E:T ratio of 20:1 for increasing periods of time. Cytotoxicity was measured by release of radiolabel into the culture medium and is presented as a percentage of specific release of radiolabel. Points, mean ± SE (bars) of 12 samples; *, statistically significant (P < 0.05) differences between resident Kupffer cells and LPS-activated liver macrophages.

Fig. 3. Time-dependent decrease in spontaneous release of radiolabel from RBL-1 cells cocultured with resident Kupffer cells (A) or LPS-recruited liver macrophages (B). Macrophages were cocultivated with [3H]dThd-labeled RBL-1 cells at an E:T ratio of 1:1 for increasing periods of time. Data are the percentage of spontaneous release of radiolabel. Points, mean ± SE (bars) of 16 samples.

Fig. 4. Cytotoxicity of liver macrophages towards mouse P815 mastocytoma cells. Resident Kupffer cells (A) or liver macrophages from LPS-treated rats (B) were coincubated with [3H]dThd-labeled P815 cells at an E:T ratio of 10:1 for increasing periods of time. Cytotoxicity was measured by release of radiolabel into the culture medium and is presented as a percentage of specific release of radiolabel. Points, mean ± SE (bars) of 12 samples; *, statistically significant (P < 0.05) differences between resident Kupffer cells and LPS-activated liver macrophages.

LIVER MACROPHAGE-MEDIATED CYTOTOXICITY

number of macrophages and target cells present in the cultures. Maximum killing of targets was observed with an E:T ratio of 20:1 (Table 1). Cytotoxicity towards RBL-1 cells was not detected at the lower E:T ratios by either macrophage cell type. In fact, at the 1:1 E:T ratio, we observed a time-dependent decrease in the release of [3H]dThd from the leukemia cells (Fig. 3). This decrease correlated with an increase in the number of target cells observed in the cultures, suggesting that the RBL-1 cells were proliferating and reincorporating the radiolabel. Similar stimulation of tumor target growth by macrophages at low E:T ratios has been reported previously (27, 28).

Cytotoxicity of Liver Macrophages towards P815 Mouse Mastocytoma Cells. We next determined if liver macrophages were cytotoxic towards the xenogeneic mouse tumor cell line, P815 mastocytoma. Both liver macrophage populations were found to exhibit cytotoxicity towards [3H]-labeled P815 cells in a time-dependent manner as evidenced by the specific release of [3H]-dThd into the culture medium (Fig. 4). As observed with the RBL-1 cells, resident Kupffer cells were significantly more cytotoxic towards these tumor cells than were LPS-recruited liver macrophages after 72 h cocultivation. The cytotoxic activity of resident Kupffer cells towards P815 cells was dependent on E:T ratio, with the maximum release of radiolabel occurring at an E:T ratio of 10:1 (Table 1). In contrast, release of radiolabel from [3H]-labeled P815 cells coincubated with liver macrophages from LPS-treated rats did not appear to be dependent on E:T ratio (Table 1). Approximately 50% specific release of radiolabel was observed at all E:T ratios.

For comparison purposes, cytotoxicity of liver macrophages towards [51Cr]-labeled P815 cells was also analyzed. We found that when resident Kupffer cells were used as the effector cells in the cytotoxicity assays, the results obtained were similar to those observed with [3H]dThd labeled P815 cells. Cytotoxicity, measured by specific release of [51Cr] into the culture medium, increased with cocultivation time (not shown) and was dependent on the E:T ratio (Fig. 5A). In contrast, when liver macrophages from LPS-treated rats were used as effectors, we observed markedly different results. After 48 h cocultivation with [3H]dThd labeled targets, LPS-recruited liver macrophages induced a 30 and 40% increase in specific release of [3H]dThd at all E:T ratios tested. In contrast, no specific release of radiolabel was observed from [51Cr]-labeled P815 cells after the same incubation time (Fig. 5B). In fact, there was a macrophage and time-dependent decrease in [51Cr] released from the targets (not shown). The maximum retention of radiolabel was observed after 72 h with E:T ratios of 10:1 and 20:1. Light microscopic examination of cocultures of P815 cells and LPS-activated liver presence of apparently intact P815 cells inside the macrophages (Fig. 6). This finding suggested that the target cells were being phagocytized by the macrophages. No intact target cells were observed inside resident Kupffer cells (not shown). Additionally, neither N151 hepatoma or RBL-1 leukemia cells appeared to be phagocytized, intact, by liver macrophages (not shown). Phagocytosis of P815 targets by LPS-recruited liver macrophages was confirmed by transmission and scanning electron microscopy. Liver macrophages appeared to capture and envelope P815 cells by means of a highly plastic crop of lamellipodia (Fig. 7A). Focal contact was observed...
Fig. 5. Cytotoxicity of resident Kupffer cells (A) or liver macrophages from LPS-treated rats (B) towards P815 cells. Macrophages were cocultured with [H]TdR- (A) or [Cr] (B) labeled P815 cells for 48 h at increasing E:T ratios. Cytotoxicity was measured by release of radiolabel into the culture medium as described in "Materials and Methods" and is presented as a percentage of specific release of radiolabel. Points, mean ± SE (bars) of 16 samples.

Fig. 6. Light photomicrograph of LPS-recruited liver macrophage phagocytosis of P815 cells. Macrophages were cocultured with P815 cells for 72 h. Note the presence of intact P815 cells inside the macrophage. × 1000.

between the 2 cell types where the macrophages involuted and pulled target cells inward. Eventually P815 cells were entirely internalized by the macrophages (Fig. 7, B and C).

To quantify phagocytosis of P815 cells, liver macrophages from LPS-treated rats were coincubated with [Cr] labeled tumor targets for 24–72 h. Phagocytosis was measured by uptake of radiolabeled targets by the macrophages. We found that LPS-activated liver macrophages phagocytized P815 cells in a time-dependent manner, reaching a maximum after 72 h incubation (Fig. 8). Phagocytosis of P815 targets also varied with E:T ratio. The maximum response was observed with a 20:1 E:T ratio (not shown).
LIVER MACROPHAGE-MEDIATED CYTOTOXICITY

Fig. 8. Phagocytosis of P815 mastocytoma cells by liver macrophages from LPS-treated rats. Macrophages were cocultivated with 51Cr-labeled P815 cells at an E:T ratio of 20:1 for increasing periods of time. Macrophages were then solubilized in 0.1 N NaOH and counted for radioactivity. *, mean±SE (bars) of 16 samples; * statistically significant (P ≤ 0.01) differences between time points.

DISCUSSION

The results reported in these studies demonstrate that resident Kupffer cells and liver macrophages from LPS-treated rats are cytotoxic towards different tumor targets. However, cytotoxicity varies with the type of macrophage and the nature of the tumor cell. We found that treatment of rats with LPS results in the accumulation of macrophages in the liver that display enhanced extracellular cytolytic activity towards liver-derived tumor cells when compared to resident Kupffer cells. Cytotoxicity towards NISI hepatoma cells was maximal at 72 h and was dependent on the E:T ratio. Enhanced cytotoxicity by activated liver macrophages has been reported previously using cells treated in vitro with liposome-encapsulated myeloid peptide (29), interferon (6), macrophage activating factor (6), LPS (20), and activated in vivo with Corynebacterium parvum (12), glucan (8) or Mycobacterium bovis (30). In contrast, we found that resident Kupffer cells were more cytotoxic towards RBL-1 basophilic leukemia and P815 mastocytoma cells than were the LPS-activated liver macrophages. These data suggest that enhanced cytotoxicity does not always correlate with the state of macrophage activation but may instead depend on the nature of the tumor target. Furthermore, the results indicate that resident macrophages may participate in the natural antitumor defense system of the liver. The variations in sensitivity of the different tumor targets to killing by liver macrophages may be related, at least in part, to the tissue origin of the tumors. The N1S1 cell line was established from the Novikoff hepatoma, a liver-derived tumor (31), while the RBL-1 and P815 cells are blood-derived tumors (32, 33). It may be that resident Kupffer cells are already maximally primed in vivo to kill foreign blood-derived tumor cells. However, at this time we cannot exclude the possibility that differences in cytotoxicity towards the N1S1 and RBL-1 cells are due to the fact that these tumor targets are derived from distinct rat strains. In addition, P815 cells are of mouse origin and may be particularly sensitive to extracellular killing by resident Kupffer cells. It is also possible that the differences in sensitivity of the targets may be due to the presence of specific cell surface receptors and recognition molecules for the tumor cells on the different macrophage populations (34). Further studies comparing the mechanism of killing of different tumor targets by resident and activated liver macrophages are required to understand the specificity of this macrophage-mediated response.

The ability of both rat and mouse macrophages to lyse P815 mastocytoma cells is well established (5, 35–37). In fact, these targets are typically used to confirm macrophage-mediated cytotoxicity since they are resistant to NK cell killing (37, 38, 39). We found that resident Kupffer cells, like other tissue macrophages, displayed extracellular cytolytic activity towards labeled P815 cells. In contrast, killing by liver macrophages from LPS-treated rats appeared to involve phagocytosis of intact P815 cells. This observation was supported by our light and electron microscopic observations of apparently intact tumor cells inside the macrophages and by our phagocytosis experiments. Phagocytosis of tumor cells by LPS-activated liver macrophages may also explain the results of our cytotoxicity studies demonstrating that P815 cells release 3HdThd but retain the 51Cr label. Following tumor cell killing, 3HdThd-labeled nucleic acids are hydrolyzed resulting in the release of free radiolabel. Since liver macrophages do not actively proliferate, they would not be expected to salvage large amounts of released 3HdThd or its nucleotides, and thus the label appears in the cell culture medium. In contrast, 51Cr released from phagocytosed P815 cells may readily bind to cytoplasmic and membrane components of the macrophages and therefore would not be released into the culture medium. Kupffer cells are active macrophages whose major function in the liver is phagocytosis and clearance of foreign antigens (40). These macrophages have been reported to phagocytize cellular fragments (41) as well as whole allogeneic and syngeneic tumor cells in vivo (15, 16). We have previously demonstrated that liver macrophages from LPS-treated rats display increased phagocytosis of sheep RBC when compared to resident Kupffer cells (19). It may be that LPS treatment induces upregulation of phagocytic receptors on these activated macrophages (34).

NK cells have been proposed as the major nonparenchymal cell type responsible for tumor killing in the liver, with Kupffer cells possessing only minimal activity (9). We found that both resident Kupffer cells and LPS-recruited liver macrophages effectively kill tumor targets. It is unlikely that NK cells contribute to tumor cell killing in our assays, since NK cell-mediated cytotoxicity is very rapid, typically detectable within 4 h and usually requires E:T ratios greater than 50:1 (7). We observed that the cytotoxic activity of liver macrophages was maximal after 72 h with no killing apparent at cocultivation periods of less than 24 h. In addition, we noted significant tumor cell killing using E:T ratios as low as 1:1. These findings, together with the fact that NK cells are not cytotoxic towards P815 mastocytoma cells (35), provides evidence for macrophage-mediated killing of tumor targets and suggests that these cells may be important immune elements in preventing metastasis. Furthermore, our data indicate that killing of tumor cells by liver macrophages occurs by at least 2 different mechanisms: extracellular lysis and phagocytosis. The mechanism is dependent on the type of liver macrophage and apparently, on the nature of the tumor target. The contribution of each of these processes to the antitumor defense system in the liver remains to be determined.

REFERENCES

LIVER MACROPHAGE-MEDIATED CYTOTOXICITY


Differential Sensitivity of Tumor Targets to Liver Macrophage-mediated Cytotoxicity

Carol R. Gardner, Arthur J. Wasserman and Debra L. Laskin


Updated version
Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/47/24_Part_1/6686

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.