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Differences in Cytotoxic Effects of Activated Murine Peritoneal Macrophages and J774 Monocytic Cells on Metastatic Variants of B16 Melanoma^{1,2,3}

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ABSTRACT—The cytotoxic effects of activated peritoneal macrophages and the J774 reticulum cell sarcoma cell line on B16 melanoma cells of differing metastatic potential were investigated in vitro. The melanoma target cells were sublines of low (B16-F1) or high (B16-F10) lung colonization potential as well as a subline of high (B16-B14b) brain colonization ability. Thioglycolate-elicited peritoneal macrophages from syngeneic C57BL/6 mice and J774 cells were activated in vitro with polyinosinic-polycytidylic acid (poly I:C) and used as effector cells. Macrophage-mediated cytotoxicity was determined by means of 24- to 72-hour radioactivity release assays with [¹²⁵I]5-iodo-2'-deoxyuridine-labeled melanoma cells; the results indicated that the more metastatic sublines B16-F10 and B16-B14b were less susceptible to cytotoxicity by activated macrophages and J774 cells than was the poorly metastatic B16-F1 subline. The poly I:C-activated effector cells also released soluble cytotoxin(s), which resulted in melanoma cell lysis and growth inhibition. Cytotoxin-mediated melanoma cell cytotoxicity was determined by counting the number of viable mitomycin-treated target cells remaining after a 40-hour incubation period in the absence or presence of various concentrations of cell-released factors, and cytotoxin-mediated cytotoxicity was performed with the use of the same procedures without mitomycin pretreatment of targets. The factors released from both activated macrophages and J774 cells were more effective against the poorly metastatic B16-F1 cells than against the highly metastatic B16-F10 or B16-B14b cells. In addition, the activity of the factors from both activated effector cells was inhibited by fetal bovine serum. The J774 cells and the activated peritoneal macrophages demonstrated similar activities against B16 melanoma variants, indicating that the J774 cell line may be suitable as a model for the study of macrophage cytotoxicity. The results suggested that the potential of the highly metastatic melanoma cells to implant, survive, and grow at secondary sites may be due, in part, to their ability to circumvent host antitumor mechanisms.—JNCI 1983; 70:717-724.

Two fundamental characteristics of malignant tumors that distinguish them from benign tumors or normal tissue are their abilities to invade normal tissue and to metastasize to distant sites. These characteristics endow cancers with their life-threatening properties. The formation of metastases involves a wide range of complex cellular interactions that occur via sequential, highly selective steps (1-3). Only a small percentage of the malignant cells released by the primary tumor were able to survive each step of the metastatic process, suggesting that successful metastatic cells possess unique characteristics that allow for their survival and subsequent growth at secondary sites (4, 5).

For study of the unique properties of metastatic cells, it is important to have tumor models consisting of variant sublines with high and low capacities for metastasis derived from the same parental cell type so the variants can be directly compared at the cellular and molecular levels. A tumor cell system meeting this criterion was established by

Fidler (6) using murine B16 melanoma variant lines which were selected sequentially in vivo for enhanced lung colonization. Using this strategy, investigators have developed several other B16 sublines for their abilities to colonize liver (7), ovary (8), or brain (9-11).

Important properties of successful metastatic cells may be their abilities to resist host destruction or be stimulated by T-lymphocytes, natural killer cells (8), or activated macrophages (5, 12). Activated macrophages are well-known effectors in host resistance to tumor progression. For example, these cells have been observed infiltrating tumor allografts, chemically induced tumors, and tumor metastases in many experimental animal models (13-15). Treatments that activate macrophages have been shown to render mice more resistant to weakly immunogenic tumors (16-19), and agents that impair macrophage function in vitro, such as trypan blue, chlorine, carageenan, or silica, increase tumorigenicity (20, 21) and metastatic potential (22, 23).

Tumor cells are, in some cases, able to partially resist attack by macrophages. Several investigators have shown that tumor growths in mice and rats are capable of depressing the capacity of the host to mobilize and concentrate macrophages at sites of inflammation (24-28). Furthermore, in vitro studies on macrophage function indicate that macrophages obtained from progressing murine tumors are less

ABBREVIATIONS USED: DMEM=Dulbecco's modified Eagle medium; FBS=fetal bovine serum; [¹²⁵I]IdUrd=¹²⁵I-labeled 5-iodo-2'-deoxyuridine; LAH=lactalbumin hydrolysate; MCT=macrophage cytotoxin(s); PBS=phosphate-buffered saline; PEC=peritoneal exudate cells; poly I:C=polyinosinic-polycytidylic acid; TG-PM=thioglycolate-elicited peritoneal macrophages.

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cytotoxic than those from regressing tumors (29, 30), and factors extracted from murine tumors inhibit the cytolytic capacity of lipopolysaccharide-activated peritoneal macrophages (31).

The actual mechanism(s) by which activated macrophages lyse neoplastic targets is unknown. Many laboratories studying this process have found direct contact of macrophages with the target cell necessary for lysis (32–35), whereas other reports indicate that supernatants derived from cultures of activated macrophages contain cytotoxins that selectively lyse neoplastic target cells (36–39). We have found that alloimmune peritoneal macrophages can be activated to induce allogeneic or syngeneic tumor cell lysis by contact with tumor target cells or by treatment with poly I:C *in vitro* (40). Both activation processes also result in the release of a cell-lytic molecule(s), termed “MCT”, which can bind to and lyse allogeneic or syngeneic tumor cells (39).

Several murine macrophage-like cell lines have been established that release cytotoxin(s) (41–45). Because of the need to study well-defined systems, we are investigating the effects of these cell lines and their toxins on metastatic cells *in vitro*.

Few studies have related the metastatic ability of tumor cells with their capacity to resist macrophage killing *in vitro* (46–48). We have demonstrated that highly metastatic sublines and clones derived from murine RAW 117 lymphosarcoma are more resistant to cytolysis and cytostasis by poly I:C-activated, syngeneic peritoneal macrophages than a poorly metastatic subline and clones derived from the same tumor (Miner KM, Nicolson GL: Submitted for publication). Here we report that poly I:C-activated peritoneal macrophages from C57BL/6 mice and poly I:C-activated J774 reticulum cell sarcoma cells differentially lyse metastatic variants of B16 melanoma. The B16 variants of high metastatic potential are more resistant to cytolysis and cytostasis than poorly metastatic B16 lines. These results suggest that the high metastatic potential of certain tumor cells may be due, in part, to their abilities to circumvent destruction and/or growth inhibition by activated macrophages. Furthermore, we show that poly I:C-activated J774 monocytic cells appear to be a suitable model in which to study the differential cytolytic effects observed among metastatic variants in that their behavior reflects that of poly I:C-activated macrophages.

MATERIALS AND METHODS

Animals.—Female C57BL/6 mice 8–10 weeks old were obtained from The Jackson Laboratory, Bar Harbor, Maine. They were fed a normal diet and received tap water with a chlorine content of 0.25–0.80 ppm.

Tumor cell lines and culture conditions.—The murine B16 melanoma variant subline B16-F1 (poor lung colonization potential) and B16-F10 (high lung colonization potential) were obtained from Dr. I. J. Fidler (NCI-Frederick Cancer Research Facility, Frederick, Md.). Variant subline B16-B14b, selected 14 times for brain colonization, has high lung-, ovary-, and brain-colonization potentials and was developed in our laboratory from B16-F1 (10, 11).

The B16 sublines were maintained *in vitro* in plastic flasks and grown in DMEM containing 10% FBS (Flow Laboratories, Inglewood, Calif.) and 1% nonessential amino acids without antibiotics. All cultures were kept at 37°C in a humidified incubator containing 5% CO₂ and 95% air.

The BALB/c reticulum cell sarcoma line J774 was obtained from Dr. R. Berens (St. Louis University, St. Louis, Mo.). The J774 cell line was maintained as a monolayer culture in glass bottles and grown in RPMI-1640 medium supplemented with 10% newborn calf serum, 2 mM glutamine, and the antibiotics penicillin (100 U/ml) and streptomycin (20 µg/ml). The cells were subcultured by nonadherent cells released from confluent monolayers being passed into bottles with the same medium.

All tumor cell lines were examined for and found to be free of mycoplasma contamination (Mycoplasma Testing Facility, The University of California, Irvine Medical Center, Irvine, Calif.). All lines were used within 10 passages from frozen stocks.

PEC and culture conditions.—Three milliliters sterile thioglycollate (Baltimore Biological Laboratories, Cockeysville, Md.) was injected ip into each C57BL/6 mouse to obtain PEC. Approximately 15–20 × 10⁶ PEC per mouse were harvested aseptically 3 days later by peritoneal lavage with 7–8 ml cold PBS (pH 7.2). The exudate was pooled and centrifuged for 5 minutes at 400 × *g* at 4°C. The pellet was resuspended in RPMI-1640 medium supplemented with 10% newborn calf serum (KC Biologicals, Lenexa, Kans.), 2 mM glutamine, and 50 µg gentamicin/ml.

Experimental metastasis assays.—B16 melanoma variant lines were grown to subconfluence and were harvested by the cells being overlaid with 0.25% trypsin–2 mM EDTA in calcium-, magnesium-free PBS (detaching buffer) for less than 2 minutes. The cells were washed twice in serum-free DMEM and injected iv (2.0 × 10⁴ cells in 0.2-ml aliquots) into groups of C57BL/6 mice. Tumor cell viability was assessed in parallel cell samples by trypan blue exclusion, and all experiments were performed with single cell suspensions of viabilities greater than 95%. All animals were killed at a designated period of time ranging from 5 to 8 weeks after injection depending on the experiment. Tumor foci were determined visually with the aid of a dissecting microscope.

Macrophage-mediated direct-cytolysis assays.—Direct cytotoxicity was determined by a modification of the microcytotoxicity radioactive release assay described by Raz et al. (49). PEC (obtained as described above) or J774 cells in growth medium were plated into wells of 96-well plates (Micro-Test II; Falcon Plastics, Oxnard, Calif.) at densities of 5, 10, and 25 × 10³ cells/well. After 2 hours of incubation at 37°C, macrophage monolayers were washed once with growth medium to remove nonadherent cells. The subconfluent target cells (B16-F1, B16-F10, or B16-B14b) were prelabeled for 24 hours with 4.0 µCi [¹²⁵I]IdUrd per 10⁵ cells in 25-cm² tissue culture flasks containing 5 ml growth medium. After 24 hours the target cells were washed extensively with medium and removed from the flasks with detaching buffer, 1 × 10³ cells were added to the macrophage monolayers, and the melanoma cells were allowed to adhere overnight. Target cells were also added to wells without macrophages.

After the overnight incubation, the media were removed from all wells; one-half of the wells in each plate received 40 μ g poly I:C/ml (P-L Biochemicals, Inc., Milwaukee, Wis.) in DMEM, and the remaining one-half received DMEM alone. After 24, 48, or 72 hours, culture supernatants were absorbed with a Titertek Supernatant Collection System (Flow Laboratories) and counted in a gamma counter. The percent macrophage-mediated cytotoxicity was calculated as follows: Percent direct cytotoxicity = $100 (A-S)/T$, where A = cpm released from target cells in the presence of activated macrophages, S = spontaneous cpm released from target cells in the absence of macrophages, and T = total cpm in target cells plated in each well.

Production of cytotoxin(s).—Subconfluent monolayers ($1 \times 10^6/5 \text{ cm}^2/\text{ml}$) of either the J774 cell line or thioglycollate-elicited PEC were maintained as described above. These cells were induced to release MCT by treatment with 40 μ g poly I:C/ml in medium plus 0.01% LAH for 1 hour at 37°C . The induced cells were washed five times with fresh medium and were incubated in medium plus 0.01% LAH for 2 hours in the absence of poly I:C. The cell supernatants were then collected and concentrated 10- to 15-fold by means of an Amicon PM-10 membrane (Amicon Corporation, Danvers, Mass.).

MCT-mediated cytotoxicity and cytostasis assays.—For cytostasis assays, singly suspended B16 melanoma cells (>95% viable) were added in 1.0-ml aliquots to glass tubes containing growth medium plus gentamicin (50 μ g/ml) at a density of 5×10^4 cells/ml. For cytotoxicity assays, melanoma cells (1×10^5 cells/ml) were incubated in medium containing mitomycin C (5.0 μ g/ 10^6 cells; Sigma Chemical Co., St. Louis, Mo.) to inhibit cell division. Target cells were allowed to adhere to the glass tubes for 12 hours before MCT preparations were added. In some experiments treatment with MCT was done with the use of 0.01% LAH as a serum substitute. In these assays, medium was removed from the glass adherent cells. The cells were then washed with serum-free medium, and fresh DMEM supplemented with either FBS or LAH was added before the addition of MCT. The target cells were incubated for 36–48 hours at 37°C in stoppered glass tubes purged with 5% CO_2 –95% air. Target cells were then washed with 5 ml of 10 mM PBS (pH 7.2) and treated with 0.1% trypsin in PBS for 5 minutes at 37°C . The trypsinized cells were suspended by being whirled in PBS containing 0.01% Formalin, and one-tenth of the cells were enumerated in a Coulter counter. All experiments were performed in quadruplicate, and controls consisted of target cells treated by the same procedure, except that cytotoxin was absent.

Percent cytostasis was determined from data obtained with dividing target cells and was calculated according to the formula: Percent cytostasis = $[1 - (E - T)/(C - T)] \times 100$, where T = number of target cells added per tube, C = number of target cells in control tubes at the conclusion of the experiment, and E = number of target cells remaining after cytotoxin treatment.

Percent cytotoxicity was determined from data obtained with nondividing target cells (mitomycin C treated) and was calculated according to the formula: Percent cytotoxicity = $[(C - E)/C] \times 100$, where E = number of target cells after incubation in the presence of cytotoxin and C = number of

target cells incubated alone.

The mean numbers of cells and standard deviations were determined for each quadruplicate sample.

Statistical analysis.—Differences among control and experimental populations were analyzed for statistical significance by a one-way analysis of variance. The statistical significance of differences between sublines was determined by the Mann-Whitney U test.

RESULTS

Metastatic potential of B16 melanoma variant sublines.—The metastatic potentials of B16 melanoma lines were determined by iv injection of viable, singly suspended cells into C57BL/6 mice. Pigmented metastatic foci were determined visually 5–8 weeks later. Often, the animals died before the assay date, especially those given injections of B16-B14b or B16-F10 cells. Therefore, the animals were killed just prior to death to assess accurately brain tumor colonization. Lung colonization characteristics of sublines B16-F10 and B16-B14b differed significantly. B16-B14b lung tumor nodules were larger and were generally clear or white (amelanotic) and nearly replaced the lung tissue at the time of death compared to B16-F10 cells, which yielded smaller but more numerous lesions. B16-F10 cells were generally black, and more than 200 of these colonies could be counted at the time of death. Lung, ovary, and brain tumor colonizations were routinely confirmed by either histologic examination or explanation of tumor cells into culture (11). As indicated in table 1, B16-B14b and B16-F10 cells were more highly metastatic than B16-F1 cells. Animals given iv injections of B16-B14b cells routinely had brain and ovary tumors, as well as massive lung and thoracic involvement at the time of autopsy. B16-F10 cells colonized the lung and, in some experiments, the ovary, but these cells only occasionally colonized the brain. Animals given injections of B16-F1 cells developed fewer tumor colonies in lung, ovary, or brain than if given injections of either B16-F10 or B16-B14b cells (table 1).

In vitro cytotoxicity of B16 melanoma variants by poly I:C-activated peritoneal macrophages or J774 cells.—In vitro cytotoxicity of B16 variants was determined by means of a radioactive release assay with [^{125}I]IdUrd-labeled target cells as described in

TABLE 1.—Experimental metastasis produced by B16 melanoma variant sublines in syngeneic hosts

Cell line	Average day of sacrifice	Death prior to assay date ^a	Colonization of organ sites ^b		
			Lung and thoracic cavity	Ovary	Brain ^c
B16-F1	33	2/10	4/10	0/10	2/10
B16-B14b	29	6/10	10/10	3/10	9/10
B16-F10	28	8/10	10/10	6/10	3/10

^aThe number of animals that died before the assay date/total number of animals.

^bData are numbers of animals with tumors/total number of animals given injections.

^cMeningeal cell colony pigmentation indicative of melanoma.

"Materials and Methods." Cytolysis was mediated either by TG-PM from C57BL/6 mice or by J774 cells. As shown in text-figures 1 and 2, all three B16 variants were lysed by poly I:C-activated J774 cells (text-fig. 1A) and poly I:C-activated TG-PM (text-fig. 1B) during 72-hour assays; however, the more metastatic sublines B16-B14b and B16-F10 were more resistant to cytolysis. Cytolysis was dependent on the number of macrophages present in the assay whether mediated by activated J774 cells (text-fig. 2A) or activated TG-PM (text-fig. 2B). The spontaneous release of radioactivity from control target cells plated in the absence of effectors was approximately 35–40% for all metastatic variants. There was no apparent reutilization of radioactive material by effector cells, because the radioactivity released from target cells exposed to unactivated J774 cells was similar to that released from target cells plated in the absence of effectors.

Unactivated TG-PM have little or no ability to lyse any of the B16 variants (table 2). The amount of killing with unactivated TG-PM was always low and variable (0–7%) even at 72 hours. Macrophages activated with poly I:C for 1 hour prior to the addition of target cells demonstrated an increased ability to lyse B16 cells, and the differential effect between various B16 variants was evident. When poly I:C was present throughout the assay, the greatest lytic effect as well as the greatest differential capacity to lyse poorly metastatic B16 variants was observed (table 2). These results

TEXT-FIGURE 1.—B16 melanoma variants treated for different times in the presence of poly I:C-activated effectors at an effector-to-target ratio of 25:1. A) J774 cells were the effectors. B) TG-PM were the effectors. Data are presented as percent cytotoxicity as determined by a radioactive release assay with the use of [125 I]IdUrd-prelabeled targets. Poly I:C was present throughout the assays. The differences in percent cytolysis between poorly and highly metastatic sublines was statistically significant by the Mann-Whitney *U* test at the $P=0.0043$ level by means of six independent observations when the assay was done for 72 hr at an effector-to-target ratio of 25:1.

TABLE 2.—Effect of poly I:C activation on in vitro cytolysis mediated by peritoneal macrophages or the J774 cell line against B16 melanoma variants^a

Target cell	Effector cell ^b	E:T ^c	No poly I:C		Poly I:C, 1-hr pulse		Poly I:C, continuous	
			cpm $\pm$ SD ^d	% cytolysis ^e	cpm $\pm$ SD ^d	% cytolysis ^e	cpm $\pm$ SD ^d	% cytolysis ^e
B16-F1	TG-PM	0	206 $\pm$ 54		257 $\pm$ 63		825 $\pm$ 110	
		5	244 $\pm$ 89	7	384 $\pm$ 43	6	1,129 $\pm$ 87	16
		10	245 $\pm$ 79	7	366 $\pm$ 25	6	1,229 $\pm$ 182	21
		25	223 $\pm$ 54	6	457 $\pm$ 69	10	1,298 $\pm$ 254	24
B16-B14b	TG-PM	0	122 $\pm$ 15		157 $\pm$ 45		440 $\pm$ 18	
		5	128 $\pm$ 35	0	172 $\pm$ 44	1	517 $\pm$ 79	5
		10	138 $\pm$ 17	1	188 $\pm$ 27	2	587 $\pm$ 67	11
		25	135 $\pm$ 35	1	195 $\pm$ 51	3	673 $\pm$ 85	17
B16-F10	TG-PM	0	292 $\pm$ 44		ND		580 $\pm$ 29	
		5	243 $\pm$ 56	0			664 $\pm$ 3	7
		10	260 $\pm$ 56	0			731 $\pm$ 32	13
		25	228 $\pm$ 26	0			766 $\pm$ 30	16
B16-F1	J774	0	118 $\pm$ 8		126 $\pm$ 40		236 $\pm$ 85	
		5	76 $\pm$ 16	0	297 $\pm$ 76	13	431 $\pm$ 59	15
		10	72 $\pm$ 21	0	501 $\pm$ 164	28	576 $\pm$ 57	26
		25	102 $\pm$ 47	0	529 $\pm$ 78	30	587 $\pm$ 56	26
B16-14b	J774	0	30 $\pm$ 11		54 $\pm$ 15		125 $\pm$ 23	
		5	30 $\pm$ 5	0	96 $\pm$ 26	8	147 $\pm$ 12	4
		10	70 $\pm$ 48	8	126 $\pm$ 36	14	180 $\pm$ 24	11
		25	30 $\pm$ 15	0	150 $\pm$ 50	19	201 $\pm$ 27	15
B16-F10	J774	0	216 $\pm$ 49		ND		514 $\pm$ 75	
		5	208 $\pm$ 55	0			606 $\pm$ 19	8
		10	198 $\pm$ 35	0			643 $\pm$ 82	11
		25	187 $\pm$ 19	0			660 $\pm$ 94	12

^aCytotoxicity was determined by a 72-hr radioactive release assay with the use of [125 I]IdUrd-prelabeled targets. Macrophages were either not activated with poly I:C (none) or preactivated with poly I:C for 1 hr and then washed once before the addition of target cells (1-hr pulse), or poly I:C was added to macrophages at the time target cells were added and it remained in the supernatant throughout the assay (continuous). See "Materials and Methods" for details.

^bTG-PM were from C57BL/6 mice; J774=monocytic cell line of BALB/c origin.

^cRatio of number of effector cells (macrophages or J774) to number of target B16 cells.

^dPercent cytotoxicity as compared with control target cells cultured without macrophages. ND=not determined.

^ePercent cytotoxicity as compared with control target cells cultured without macrophages.

were consistent with those of Murray and Cohn (50), who recently reported that activated murine peritoneal macrophages were more efficient in cytotoxicity assays if the activating agent was presented to the macrophages periodically throughout the cultivation period. Unactivated J774 cells demonstrated little or no capacity to lyse B16 cells (table 2). However, J774 cells activated with poly I:C for 1 hour or activated throughout the assay lysed the B16 variants, and the differential lytic effect on B16-F1 cells was evident (table 2). Differences in percent cytotoxicity of highly and poorly metastatic B16 variants mediated by TG-PM or J774 effector cells were statistically significant at the $P=0.0005$ level by Mann-Whitney analysis with the use of eight independent observations.

In vitro cytotoxicity and cytostasis by factor(s) released from poly I:C-activated peritoneal macrophages or J774 cells.—In vitro cytotoxicity and cytostasis were determined in the presence or absence of effector cell supernatants (table 3). The highly metastatic sublines B16-B14b and B16-F10 were more resistant to lysis by MCT generated from either poly I:C-activated TG-PM or J774 cells than was the poorly metastatic subline B16-F1. Differential cytostatic effects on poorly and highly metastatic variants by MCT generated from poly I:C-activated TG-PM or J774 cells were also observed routinely (table 3). The reduction in number of cells observed in cytotoxicity assays was due to lysis and not merely to cell detachment, because cells released from the substrate were not viable (<5%) by dye exclusion. The difference in percent cytotoxicity between the poorly and highly metastatic sublines was statistically significant by the Mann-Whitney U test at the $P=0.0022$ level with the use of six independent observations. By the same criteria, differences in percent cytotoxicity between poorly and highly metastatic B16 sublines were statistically significant at the $P=0.0130$ level.

TEXT-FIGURE 2.—B16 melanoma variants treated with poly I:C macrophages for 72 hr at different effector-to-target ratios. A) J774 cells were the effectors. B) TG-PM were the effectors. Data are presented as percent cytotoxicity as determined by a radioactive release assay with the use of [125 I]IdUrd-prelabeled targets. Poly I:C was present throughout the assays.

icant at the $P=0.0130$ level.

Effect of serum on in vitro cytotoxicity mediated by poly I:C-activated TG-PM and J774 cells.—Adams et al. (38) reported that cytotoxin(s) released from BCG-activated macrophages were inhibited by FBS. We also found that FBS inhibited cytotoxicity by MCT generated from poly I:C-activated TG-PM (table 4). This effect was found to be more dramatic with

TABLE 3.—In vitro cytotoxicity and cytostasis mediated by MCT induced by activated J774 cells and TG-PM against metastatic variants of B16 melanoma

Target cell	Effector cell	Relative cytotoxin concentration ^a	Average No. of target cells, $\times 10^{-2}$ ^b	Percent cytotoxicity ^{c,d}	Significance ^e	Average No. of target cells, $\times 10^{-2}$ ^f	Percent cytostasis ^g	Significance ^e
B16-F1	TG-PM	0	707 $\pm$ 74			4,267 $\pm$ 456		
		1 $\times$	575 $\pm$ 72	19	0.001	2,070 $\pm$ 158	58	0.001
		5 $\times$	525 $\pm$ 43	26		1,269 $\pm$ 125	80	
B16-B14b	TG-PM	0	493 $\pm$ 84			1,736 $\pm$ 403		
		1 $\times$	515 $\pm$ 53	0	0.025	1,079 $\pm$ 148	53	0.001
		5 $\times$	437 $\pm$ 23	11		882 $\pm$ 93	70	
B16-F10	TG-PM	0	1,136 $\pm$ 57			4,984 $\pm$ 606		
		1 $\times$	1,081 $\pm$ 32	5	0.01	2,911 $\pm$ 243	43	0.001
		5 $\times$	928 $\pm$ 28	18		1,762 $\pm$ 145	67	
B16-F1	J774	0	1,058 $\pm$ 37			3,998 $\pm$ 115		
		1 $\times$	894 $\pm$ 92	16	0.001	2,777 $\pm$ 204	35	0.001
		5 $\times$	701 $\pm$ 75	34		1,442 $\pm$ 54	74	
B16-B14b	J774	0	1,169 $\pm$ 72			1,653 $\pm$ 113		
		1 $\times$	1,091 $\pm$ 51	7	0.01	1,530 $\pm$ 204	11	0.001
		5 $\times$	1,048 $\pm$ 80	10		1,204 $\pm$ 184	39	
B16-F10	J774	0	1,455 $\pm$ 48			4,982 $\pm$ 606		
		1 $\times$	1,294 $\pm$ 152	11	0.001	4,662 $\pm$ 420	7	0.001
		5 $\times$	1,124 $\pm$ 66	23		3,312 $\pm$ 331	37	

^a1 $\times$ cytotoxin concentration=50 μ l of the concentrate (see "Materials and Methods") added to target cells; 5 $\times$ cytotoxin concentration=250 μ l of the concentrate added to target cells.

^bTarget cells were plated with mitomycin C in the presence and absence of MCT. Values are mean numbers of cells $\pm$ SD of quadruplicate samples.

^cPercent cytotoxicity as compared with control target cells cultures without MCT.

^dThe cytotoxicity assays were done in 0.01% LAH as a serum substitute.

^eDifferences between samples were significant at the indicated level by one-way analysis of variance.

^f5 $\times 10^4$ dividing target cells were plated in the presence and absence of MCT. Values are mean numbers of cells $\pm$ SD of quadruplicate samples.

^gPercent cytostasis as compared with control target cells cultured without MCT.

TABLE 4.—In vitro cytotoxicity mediated by MCT against B16 melanoma variants in the presence and absence of serum

Target cell ^b	Effector cell ^c	Cytotoxin concentration ^d	With FBS			Without FBS ^a		
			Average No. of target cells, $\times 10^{-2e}$	Percent cytotoxicity ^f	Significance ^g	Average No. of target cells, $\times 10^{-2e}$	Percent cytotoxicity ^f	Significance ^g
B16-F1	TG-PM	0	1,256±65			707±72		
		1×	1,221±104	3	NS	575±72	19	0.001
		5×	1,162±58	7		525±43	26	
B16-B14b	TG-PM	0	840±102			493±84		
		1×	826±85	2	NS	515±53	0	0.025
		5×	809±67	4		437±28	11	
B16-F10	TG-PM	0	1,649±105			1,136±57		
		1×	1,657±20	0	NS	1,081±32	5	0.01
		5×	1,454±80	12		928±28	18	
B16-F1	J774	0	1,605±27			1,058±37		
		1×	1,437±47	10	0.001	894±92	16	0.001
		5×	1,216±58	24		701±75	34	
B16-B14b	J774	0	1,425±38			1,169±72		
		1×	1,459±197	0	NS	1,091±57	7	0.01
		5×	1,323±63	7		1,048±80	10	
B16-F10	J774	0	1,934±30			1,455±48		
		1×	1,808±61	6	0.025	1,294±152	11	0.001
		5×	1,675±33	13		1,124±66	23	

^aIn these experiments targets were plated in growth medium containing 10% FBS for 8 hr. Just before the addition of MCT, the medium was removed and replaced with either medium plus 10% FBS or medium plus 0.01% LAH.

^bTarget cells were treated with mitomycin-C and plated in the presence and absence of cytotoxin for 40 hr as described in "Materials and Methods."

^cTG-PM were from C56BL/6 mice; J774=monocytic cell line of BALB/c origin. Designates the effector cell used to generate MCT.

^d1× cytotoxin concentration=50 μ l concentrate (see "Materials and Methods") added to target cells; 5× cytotoxin concentration=250 μ l concentrate added to target cells.

^eValues are mean numbers of cells $\pm$ SD of quadruplicate samples.

^fPercent cytotoxicity as compared with control target cells cultured without MCT.

^gDifferences between samples significant at the indicated level by one-way analysis of variance. NS=not significant.

MCT generated from poly I:C-activated TG-PM than from J774 cells (table 4). TG-PM-generated MCT cytotoxicity of target cells was not generally observed in the presence of FBS.

DISCUSSION

Macrophages are thought to be an important component in host surveillance against tumor progression. The evidence in vitro and in vivo is overwhelming that "tumoricidal" or "activated" peritoneal exudate macrophages can discriminate between neoplastic and nonneoplastic cells, irrespective of the animal species or tumor origin (7). Because tumors such as B16 melanoma are known to be heterogeneous in their metastatic and other properties (3-11, 12, 46), it is possible that these cells differ in their abilities to resist destruction by activated macrophages in vitro. We have reported that highly metastatic cell sublines and clones derived from murine RAW 117 lymphoma-lymphosarcoma were more resistant to cytotoxicity and cytostasis by poly I:C-activated TG-PM from BALB/c mice than poorly metastatic sublines and clones derived from the same tumor (Miner KM, Nicolson GL: Submitted for publication). Here we have demonstrated that peritoneal macrophages from C57BL/6 mice activated in vitro with poly I:C also possess differential abilities to lyse variants of murine B16 melanoma. Furthermore, the J774 reticulum cell sarcoma, a cell line that behaves in a number of respects as peritoneal macrophages (41-45), was also shown to exhibit differential cytotoxicity. While both effector cell types possessed the ability

to lyse highly and poorly metastatic variants, the highly metastatic cell lines were always more resistant to effector cell destruction. We generally found that activated J774 cells were slightly more efficient in lysing B16 melanoma cells than activated TG-PM. This may be due to the fact that PEC are a heterogeneous population of macrophages containing only a certain percentage of macrophages that is actually cell-lytic. The J774 cell line may be a more homogeneous population representing cell-lytic macrophages. Another possibility is that the J774 cell line, derived from a BALB/c mouse and therefore allogeneic to the B16 melanoma cells, is better able to recognize the melanoma variants than are the syngeneic TG-PM.

The actual lytic and/or growth inhibitory mechanism(s) of tumoricidal macrophages has not been clearly defined, although a number of mechanisms has been suggested (38, 39, 51-57). Most of these mechanisms involve the secretion of toxic components from the activated macrophages. Activated macrophages from the rat, mouse, guinea pig, and human, in addition to four of five continuous murine macrophage-like cell lines tested, have been shown to release cell-lytic and growth inhibitory factors in vitro (36-38, 40, 41, 58-60). The factors have not been characterized; however, several groups have reported that supernatants from activated rodent peritoneal macrophages preferentially lysed neoplastic as compared to normal cells in vitro (36-39). We have reported that inflammatory or alloimmune C57BL/6 murine peritoneal macrophages can be activated by treatment with poly I:C to induce the release of the cell-

lytic neutral protease MCT, which can bind to and lyse allogeneic or syngeneic tumor cells but cannot bind to or lyse normal cells (39). Poly I:C-activated J774 cells also release a cell-lytic molecule(s), and preliminary evidence suggests that it may be similar to that released by peritoneal macrophages (Klostergaard J, Armstrong C, Granger JA: Manuscript in preparation).

We have demonstrated that the sublines B16-B14b and B16-F10 are more resistant to both cytolysis and cytostasis mediated by MCT released from either activated TG-PM or activated J774 cells than is the poorly metastatic B16-F1 line. This suggests that the cytotoxins can distinguish among the different B16 cell types and that recognition is not due only to discrimination by cell surface constituents of the macrophage.

Fidler (61) has not detected differences in macrophage cytolysis between highly and poorly metastatic variants of B16 melanoma. The reason for the discrepancy between this work and ours is unclear. It could be due to different methods of macrophage activation or to the different densities of macrophages and tumor cells used in our assays. In fact, we found that the greatest differential effect was observed when the macrophage-to-target ratio was 25:1; higher effector-to-target ratios tended to abrogate the difference.

It is apparent that certain tumor cells differ in their abilities to resist macrophage destruction. One of the unique properties of highly metastatic cells may be their ability to resist this destructive component of the host's surveillance system. The similarity observed between TG-PM and J774 cells in their capacities to lyse target cells suggests that this monocytic J774 cell line may be an appropriate model to study the mechanisms of tumor cell cytolysis and cytostasis.

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