Distinct and Non-Cross-Reactive Epitopes are Recognized on B16 Melanoma by LAK Cells and Anti-B16 Monoclonal Antibodies[1]

(43037)

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Abstract. **Two rat anti-B16 melanoma monoclonal antibodies (MoAb), designated IB16-6 and IB16-8, recognize an epitope expressed with high density on the surface of B16 parental cells and B16-F1, F10, F10FLR, and BL6 sublines. The purpose of this study was to define by means of cytolytic and clonogenic assays whether these MoAbs reacted with the same or distinct determinants as those recognized on B16 targets by lymphokine-activated killer (LAK) cells. Using ^{125}I-labeled antibody and Scatchard analysis, the affinity constant (K_A) of IB16-6 was determined to range from 5.6 to 9.4 × 10^8 liter/*M* and the number of receptor sites per B16 cell was 4.8 × 10^4 to 2.5 × 10^5. The effects of anti-B16 MoAb on LAK activity were determined by either preincubating ^{51}Cr-labeled B16 target cells with varying concentrations of MoAb, followed by the cytolytic assay, or exposing unlabeled B16 cells to MoAb, and then carrying out a 10-day clonogenic assay. Over a wide range of antibody concentrations, IB16-6 and IB16-8 had minimal effects on LAK activity, and even at MoAb concentrations up to 1 mg there were no changes in target cell sensitivity or colony-forming ability. Enzymatic treatment of B16 melanoma cells with either trypsin or pronase completely removed the epitope recognized by MoAb IB16-6 but did not alter B16 sensitivity to LAK cells. These observations indicate that the LAK recognition unit was distinct from the epitope reactive with MoAb IB16-6 and that the B16 determinant(s) recognized by LAK cells is resistant to proteolytic enzymes. The molecular structure of each of these remains to be determined.** [P.S.E.B.M. 1990, Vol 193]

The adoptive transfer of lymphokine-activated killer (LAK) cells into tumor-bearing hosts has produced regression of established metastases in both experimental animals (1–4) and humans (5, 6). The *in vivo* administration of LAK cells, generated *in vitro* by exposure to recombinant interleukin 2 (rIL-2), decreased pulmonary metastases following intravenous injection of B16 melanoma cells into C57BL/6 mice (1). Monoclonal antibodies (MoAb) reactive with tumor-associated antigens have been utilized for the immunotherapy of established cancers (7, 8). Several MoAb directed against melanoma-associated antigen have been produced for the treatment of melanomas (9, 10). The therapeutic effects of these MoAb were limited, in part, by the antigenic heterogeneity of tumor cells (11, 12). It has been postulated that antibody-dependent cell-mediated cytotoxicity (ADCC) is one of mechanisms by which MoAb exert their antitumor effects (13). ADCC mediated by interleukin 2-activated lymphocytes has been reported to be another possible mechanism for the *in vivo* therapeutic effects of LAK cells (14, 15). A panel of anti-B16 melanoma MoAb have been produced in our laboratory (16), two of which designated IB16-6 and IB16-8, showed reactivity to a surface antigen expressed on B16 parental cells and the B16-F1, F10, F10FLR, and F10-BL6 sublines. The purpose of the present study was to define whether these antibodies recognized the same or different determinants as those recognized by LAK cells on the B16 melanoma, as evidenced by their ability to alter target

Received July 18, 1989. [P.S.E.B.M. 1990, Vol 193]
Accepted December 11, 1989.

0037-9727/90/1934-0285$2.00/0

cell sensitivity. The cytolytic activity of LAK cells was demonstrated against B16 target cells using a ^{51}Cr release assay, and their antiadherent and antiproliferative activities were detected by means of a clonogenic assay. Over a broad range of concentrations anti-B16 melanoma MoAb IB16-6 and IB16-8 had no biologically significant effects on target cell sensitivity, suggesting that they recognized antigens that were distinct from the determinant(s) recognized by LAK cells.

Materials and Methods

Animals and Tumors. C57BL/6 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. B16 melanoma cells, kindly provided by Dr. I. J. Fidler, were passaged *in vivo* by injecting cultured tumor cells subcutaneously into C57BL/6 mice and explanted for tissue culture in supplemented Dulbecco's modified Eagle's medium (SDMEM) (Gibco, Grand Island, NY). The tumor cells used in the cytolytic assays were propagated *in vitro* for less than 10 passages.

Anti-B16 Melanoma Monoclonal Antibodies. MoAb were produced as described previously (16). Briefly, Fischer rats were immunized by repeated subcutaneous implantation of B16-BL6 melanoma. Following the demonstration of serum antibodies to B16 melanoma, rats were killed, and a suspension of splenocytes was prepared and fused with the rat myeloma cell line 210.RCY3Ag1.1.2.3.(Y3). Specific hybridomas were cloned and expanded in serum free SDMEM, and propagated in pristane-primed x-irradiated Fischer rats. Two of these MoAb, designated as IB16-6 and IB16-8, were selected for this study. MoAb were twice precipitated from ascites with 50% ammonium sulfate and dialyzed against phosphate-buffered saline (PBS). They were then dissolved in PBS, precipitated with 17% sodium sulfate, dialyzed extensively against PBS, and stored at −80°C. They were checked for purity on sodium dodecylsulfate-polyacrylamide gel electrophoresis, and under reducing conditions there were two bands corresponding to light and heavy chains, respectively.

Determination of Affinity Constant. The affinity constant (K_A) of MoAb IB16-6 was determined by adding varying concentrations of ^{125}I-labeled antibody ranging from 5 ng to 5 μg to duplicate samples of 5×10^6 viable B16 cells and incubated at 37°C for 1 hr. Following this, cells were washed three times with 1 ml of cold washing solution containing a mixture of 20 ml of McCoy's medium containing 30% fetal bovine serum and 100 ml of Hanks' balanced salt solution (Gibco). Cells were sedimented at 1800 rpm for 10 min, and radioactivity was quantified in a Tracor Analytic model 1185 gamma scintillation counter. From this, the amount of bound Ab could be assessed and the K_A and the number of receptor sites per cell (r) were determined by means of Scatchard analysis (17).

Generation of LAK Cells. Recombinant human interleukin 2 was generously supplied by Dr. J. Winkelhake, Cetus Corp., Emeryville, CA, in a powder form with 3×10^6 units/vial. The stock solution was aliquoted by diluting the powder in PBS and stored at −20°C. Spleens were removed aseptically, crushed with the hub end of a 5-ml syringe against a Cellector (Bellco Biotech, Vineland, NJ), and dispersed into a single-cell suspension by using a syringe fitted with a 26-gauge needle. Mononuclear cells were isolated after centrifugation on Histopaque-1077 gradients (Sigma, St. Louis, MO) at 300*g* for 20 min. Macrophages and B lymphocytes were removed by incubating mononuclear cells in 100-mm petri dishes at 37°C for 1 hr. The phenotypes of nonadherent cells, as determined by immunofluorescence, were 65.7% Thy-1.2 (+), 9.4% sIg (+), 0.6% Mac-1 (+), and 19.2% asialo-GM_1 (+). The nonadherent mononuclear cells (NMNC) prepared in this way were used as control effector cells. LAK activity was generated by incubating the NMNC with 250 units of rIL-2/ml of complete medium (CM). The CM consisted of RPMI 1640 (Gibco), 1 μm of sodium pyruvate, 0.1 m*M* nonessential amino acids, 0.03% glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum, and 5×10^{-5} *M* 2-mercaptoethanol.

Cytotoxicity Assay. Labeling of B16 melanoma cells was carried out by adding 5×10^3 cells to each well of a 96-well flat-bottomed microtiter plate (Corning, Corning, NY) in combination with 2 μCi of ^{51}Cr (Amersham Corp., Arlington Heights, IL) and incubating them at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hr. B16 target cells were all adherent at 24 hr and their viability was >95%. This method assured that all adherent target cells were maximally labeled with ^{51}Cr and would not be washed away during subsequent washing procedures (J. R. Wunderlich, personal communication). Various concentrations of IB16-6 and IB16-8, ranging from 0.1 to 1000 ng, were added to the wells and the plates were incubated at 4°C for 30 min. Normal rat IgG (Miles Lab., Naperville, IL) was used as control protein. Cell-free MoAb were washed away, and then LAK cells or control NMNC were added at effector to target (E:T) ratios ranging from 3:1 to 50:1. Spontaneous release was determined following incubation of the target cells in SDMEM alone. Maximum release was measured by lysis of the target cells with 0.1 *N* HCl. The ratio of spontaneous release to maximum release was <30% in the 18-hr assay. The plates then were incubated at 37°C for 18 hr. At the end of the incubation period, the plates were centrifuged at 300*g* for 30 sec and 100 μl of supernatant were removed from each well for counting in a Tracor Analytic model 1185 gamma scintillation counter. Percentage of specific release of ^{51}Cr was determined from the formula: % specific lysis = [(experimental cpm −

spontaneous cpm)/(maximal cpm − spontaneous cpm)] × 100%.

Varying concentrations of IB16-6 and IB16-8, ranging from 1 to 100 μg, were incubated with 1000 B16 target cells at 4°C for 30 min. The B16 targets then were seeded into 6-well tissue culture plates (Costar, Cambridge, MA) for the clonogenic assay. The effects of MoAb on the cytolytic activity of LAK cells against B16 targets were determined by using the 18-hr ^{51}Cr release assay.

Time Kinetics of LAK Activity. Nonadherent mononuclear cells (1.5×10^7) were incubated in T25 culture flasks (Corning) containing 250 units of rIL-2/ml of CM to generate LAK activity. Control NMNC were cultured in the absence of rIL-2. The flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Effector cells, harvested at daily intervals ranging from 2 to 11 days, were added at effector to target (E:T) ratios ranging from 6:1 to 100:1 in an 18-hr ^{51}Cr release assay.

Clonogenic Assay. LAK cells (5×10^4/well), generated after 4 days of incubation with rIL-2, were seeded into 6-well tissue culture plates in the presence of 250 units of rIL-2/ml of CM. Control NMNC were cultured in the absence of rIL-2. Following this, B16 target cells (1000/well) were added to wells containing either control NMNC or LAK cells and were incubated at 37°C in a humidified incubator containing 5% CO_2 for 10 days. The B16 colonies then were fixed for 3 min by adding 3 ml of 37% formaldehyde directly to each well, decanted, washed, and stained with 1% crystal violet. The numbers of colonies were enumerated by using an Artek 880 image analyzer (Artek System Co., Farmingdale, NY): plating efficiency = (number of B16 colonies enumerated/total number of B16 cells plated) × 100% and surviving fraction = [number of B16 colonies enumerated in the presence of effector cells/(total number of B16 plated × plating efficiency)] × 100%.

Effects of Enzymatic Treatment of B16 Melanoma Cells. B16 parental cells were treated with trypsin or pronase (1 mg/ml) for 30 min at 37°C, washed, exposed to 1–100 μg of IB16-6 for 30 min at 4°C, then exposed to fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (ICN, Lisle, IL) for 30 min at 4°C, and evaluated for binding of MoAb IB16-6 by indirect immunofluorescence using a Zeiss fluorescent microscope with epiillumination. Reactivity was scored from − (nonfluorescent) to 4+ (very strong fluorescent) as compared with negative and positive controls. ^{51}Cr-Labeled target cells also were treated with trypsin or pronase (1 mg/ml) for 30 min at 37°C, exposed to 10 ng to 100 μg of IB16-6 for 30 min at 4°C, and effector cells added at an E:T ratio of 50:1. The percentage of ^{51}Cr release was calculated as described previously.

Statistical Analysis. The statistical significance was determined by the one-way analysis of variance test for the differences between means obtained from the ^{51}Cr release assay.

Results

Reactivity of Anti-B16 Melanoma MoAb. IB16-6 and IB16-8, two of a panel of anti-B16 MoAb against B16 melanoma targets, were selected for the present study (Table I). Both MoAb were IgG_2 as determined by immunodiffusion. Neither of them bound to normal thymocytes, renal adherent cells, or F98 glioma cells, as determined by an immunofluorescent binding assay. IB16-6 recognized a surface antigen expressed on >93% of parental B16 cells and the B16-F1, F10, F10FLR, and F10-BL6 sublines, as determined by flow cytometry. IB16-8 reacted with a surface and cytoplasmic antigen expressed on 32–59% of B16 parental and sublines. IB16-6 was not rapidly internalized and as determined by flow cytometry >70% remained on the surface of B16 cells after 3 hr of incubation (16). MoAb IB16-8, but not IB16-6, was cytolytic in the presence of rabbit complement. Neither IB16-6 nor IB16-8 supported antibody-dependent cell-mediated cytotoxicity. Using ^{125}I-labeled MoAb and Scatchard analysis, the affinity constant ranged from 5.6 to 9.4×10^8 M^{-1} and the number of receptor sites per B16 tumor cell was 4.8×10^4 to 2.5×10^5 (Fig. 1). In order to control for nonspecific binding, ^{125}I-IB16-6 was tested against F98 glioma cells and showed only 0.3% and 1% binding compared with >90% binding on B16 melanoma cells (unpublished data). The antigen recognized by IB16-6 could not be chemically isolated despite repeated attempts. Since IB16-6 was a rat IgG_2, it was not possible to purify it by means of affinity chromatography on either a staphylococcal protein A or an antigen-coupled agarose column. Because of this the K_A, as determined by using ^{125}I-labeled MoAb and Scatchard analysis, should be interpreted with some caution. Nevertheless, this should not affect our conclusions concerning the reactivity of this antibody with B16 tumor cells.

Time Kinetics of LAK Activity. The cytolytic activity of LAK cells against B16 targets was determined by means of an 18-hr ^{51}Cr release assay from Day 2 to Day 11 following the culture of murine NMNC (Figs. 2 and 3). Over a range of effector to target cell ratios from 6:1 to 100:1, the cytolytic activity of LAK cells progressively increased and reached a peak at Day 5. The percentage of specific lysis mediated by LAK cells was ≈ 100% in an 18-hr assay on Day 5. Control NMNC showed 35% cytolytic activity at an E:T ratio of 100:1 on Day 5 in an 18-hr assay. After 7 days of incubation, LAK cells lost their cytolytic activity and the percentage of lysis decreased to <20% in an 18-hr assay. LAK activity could be restored by adding rIL-2 on Day 11, following which effector cells regained their high cytolytic activity.

Effects of MoAb on LAK Activity. The interaction

Table I. Reactivity of Anti-B16 Melanoma Monoclonal Antibodies IB16-6 and IB16-8 against Melanoma and Nonmelanoma Target Cells

Antibody	Subclass	% Positive reactivity against target cells[a]							Complement-mediated reactivity	
		Thymocytes	RAC	B16	B16-F1	B16-F10	B16-F10FLR	B16-BL6	Fixation	% Lysis[b]
IB16-6	IgG_{2a}	0	0	93.6	92.5	96.8	96.0	97.7	−	0
IB16-8	IgG_{2b}	0	0	32.0	36.0	36.0	59.2	52.3	+	74

[a] Target cells were exposed to saturating quantities of IB16-6 or IB16-8 and then to fluorescein isothiocyanate-conjugated rabbit anti-rat IgG and binding was quantified by either fluorescence microscopy for thymocytes and renal adherent cell or by flow cytometry for B16 melanoma and its sublines. Saturating quantities of the MoAb were defined as the concentration of antibody that gave maximum fluorescence.
[b] Determined by trypan blue exclusion following exposure of B16 parental cells to saturating quantities of MoAb and rabbit complement.

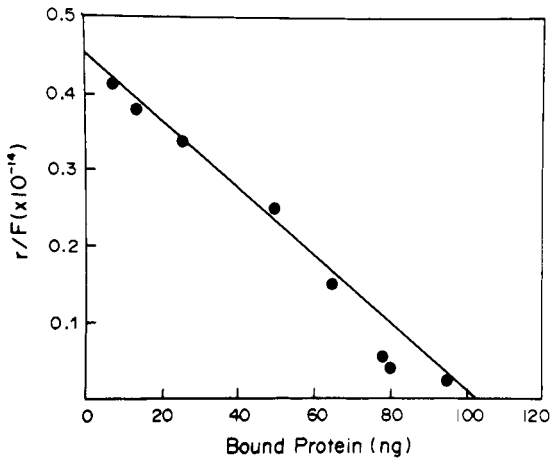

Figure 1. Binding affinity of MoAb IB16-6. Binding affinity was determined by adding varying concentrations of MoAb ^{125}I-IB16-6 to duplicate samples of 5×10^6 viable B16 cells and incubated at 37°C for 1 hr. After subtracting background and nonspecific binding, the data in this figure were obtained and subjected to Scatchard analysis. r, number of binding or receptor sites; F, molar concentrations of free ^{125}I-labeled antibody.

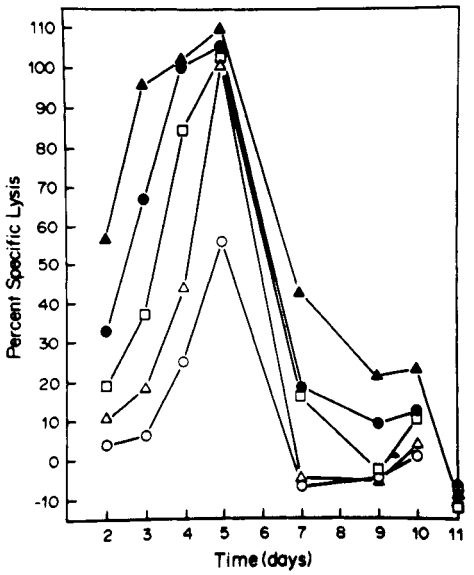

Figure 2. Cytolytic activity of LAK cells against B16 target cells determined by an 18-hr ^{51}Cr release assay. LAK cells were generated in the presence of 250 units of IL-2/ml of CM, harvested at daily intervals, and then added to each well containing 5×10^3 ^{51}Cr-labeled B16 targets at E:T ratios ranging from 6:1 to 100:1 (○, E:T = 6:1; △, E:T = 12:1; □, E:T = 25:1; ●, E:T = 50:1, ▲, E:T = 100:1). The plates were incubated at 37°C for 18 hr. At the end of the incubation period, 100-μl aliquots of supernatant were harvested from each well for counting in a gamma scintillation counter. Cytotoxicity was determined by the formula: % specific lysis = [(experimental cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm)] × 100%.

between MoAb and LAK cells was determined by preincubating ^{51}Cr-labeled target cells with MoAb at 4°C, followed by an 18-hr ^{51}Cr release assay (Figs. 4 and 5). Normal rat IgG was used as a negative control protein. Over a wide range of concentrations of IB16-6 and IB16-8, there were no significant differences, as determined by one-way analysis of variance test, in the cytolytic activity of control NMNC and LAK cells at E:T ratios ranging from 3:1 to 50:1 in the presence of MoAb compared with control rat IgG. Even at concentrations up to 1 mg of MoAb, there were no effects on target cell lysis. The effects of MoAb IB16-6 on LAK activity in the clonogenic assay were studied by pretreating B16 melanoma cells with 1–100 μg of IB16-6 at 4°C for 30 min and co-cultivating with LAK cells for 10 days. MoAb IB16-6 did not affect the surviving fraction of B16 colonies in this assay.

Clonogenic Assay. The *in vitro* antiadherent and antiproliferative activities of MoAb and LAK cells were defined by means of a clonogenic assay (Table II). The plating efficiency of B16 colonies was 30% in 6-well tissue culture plates. B16 parental cells were exposed to 1–100 μg of MoAb IB16-6 at 4°C for 30 min and seeded into tissue culture plates. MoAb IB16-6 did not affect the plating efficiency of B16 melanoma cells. The surviving fraction of B16 targets co-cultivated with control NMNC was 100%. In contrast to this, the number of B16 colonies, enumerated with an Artek Counter, was reduced to 0 after 10 days incubation of LAK cells supplemented with rIL-2. Representative plates, shown in Figure 6, demonstrate the striking antiadherent effects of LAK cells.

Effects of Enzymatic Treatment of B16 Melanoma Cells. The binding of IB16-6 to trypsin- or pronase-treated B16 melanoma cells was evaluated by an indirect immunofluorescence assay. Control cells,

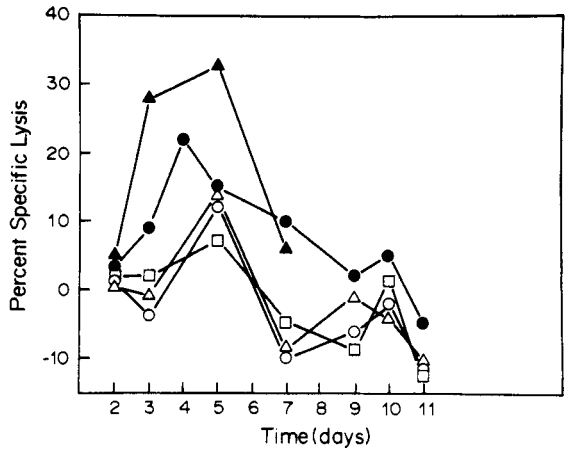

Figure 3. Cytolytic activity of control NMNC against B16 target cells determined by an 18-hr ^{51}Cr release assay. ○, E:T = 61; △, E:T = 12:1; □, E:T = 25:1, ●, E:T = 50:1; ▲, E:T = 100:1.

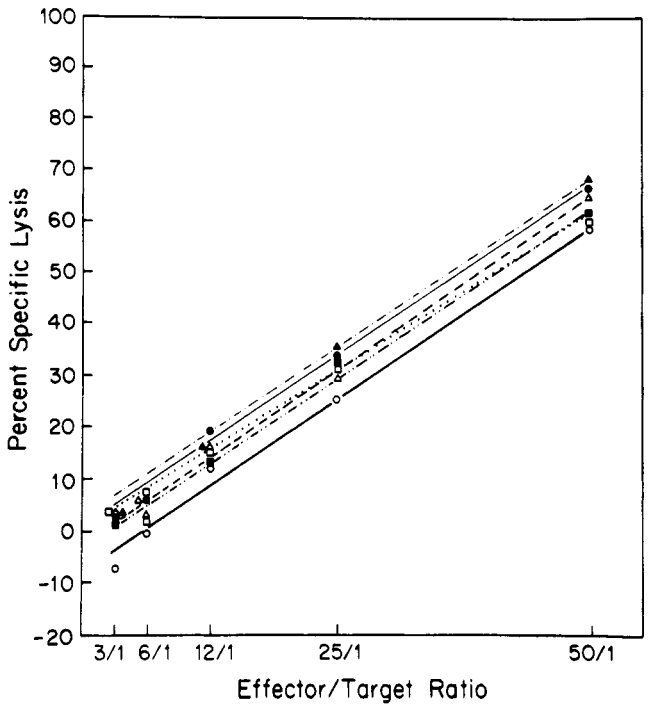

Figure 4. Effects of MoAb IB16-6 (○——, 0.1 ng; △– –, 1 ng; □·····, 10 ng; ●——, 100 ng; ▲–·–, 1000 ng; ■–···–, none) on LAK activity against B16 target cells. Various concentrations of IB16 6 were added to the wells containing 5×10^3 ^{51}Cr-labeled B16 targets and the plates were incubated at 4°C for 30 min. Following this, LAK cells or control NMNC were added at an effector to target ratios ranging from 3:1 to 50:1. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 18 hr, after which percentage of specific lysis was determined.

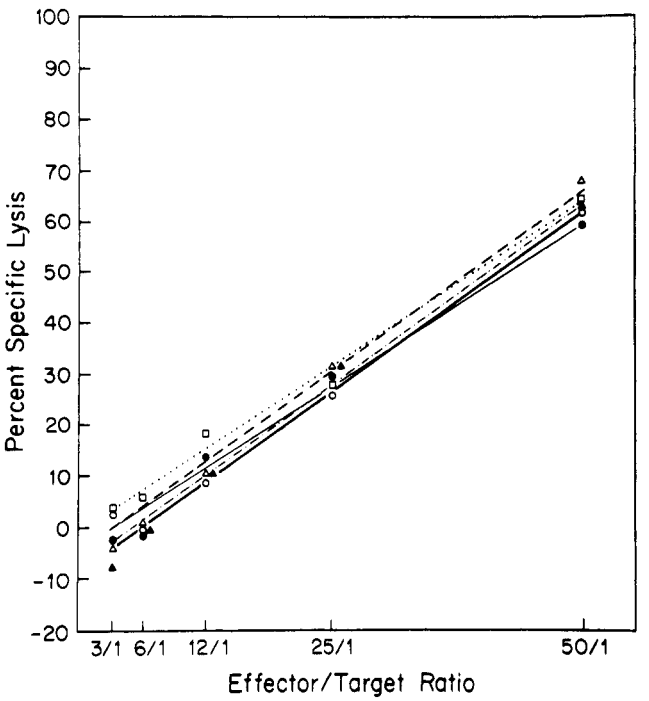

Figure 5. Effects of MoAb IB16-8 on the LAK activity against B16 targets. The procedure employed was the same as previously described. The concentrations of IB16-8 were: ○——, 0.1 ng; △– –, 1 ng; □·····, 10 ng; ●——, 100 ng; ▲–·–, 1000 ng.

Table II. Clonogenic Assay for LAK Activity

Effector cells	B16 colonies[a]	Surviving fraction[b] (%)
None	300 ± 14	—
Control NMNC	300 ± 12	100
LAK cells	0	0

[a] Values are mean ± SD for triplicate experiments.
[b] Surviving fraction = [number of B16 colonies enumerated in the presence of effector cells/(total number of B16 plated X plating efficiency)] × 100%. Plating efficiency = (number of B16 colonies enumerated/total number of B16 cells plated) × 100%.

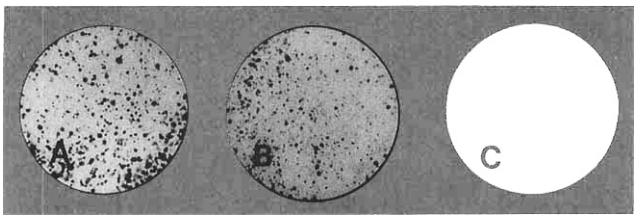

Figure 6. The representative photographs of the results of a clonogenic assay. One thousand B16 melanoma cells were incubated with SDMEM medium only (A) or with control NMNC (B) or LAK cells (C). Ten days later, B16 colonies were fixed with 37% formaldehyde and stained with 1% crystal violet.

treated with PBS, showed strong fluorescence (4+) whereas cells treated with either trypsin or pronase were nonfluorescent (−). These results also have been confirmed by flow cytometry. In contrast to this, trypsin- or pronase-treated target cells retained their sensitivity to LAK cells at an E:T ratio of 50:1 (Table III). There were no significant differences in their sensitivity to LAK cells at E:T ratios ranging from 3:1 to 100:1, indicating that the epitope recognized by MoAb IB-16-6 was distinct from the determinant(s) recognized by LAK cells.

Table III. Effects of Enzymatic Treatment of B16 Melanoma Cells on the Cytolytic Activity of LAK Cells

Effector cells	IB16-6	Cytolytic activity of effector cells[a]		
		PBS	Trypsin	Pronase
Control NMNC	0	−5.3 ± 4.0	−1.7 ± 3.6	−1.4 ± 2.9
LAK cells	0	65.2 ± 1.6	65.5 ± 1.6	63.0 ± 2.2
LAK cells	10 ng	63.6 ± 3.6	65.1 ± 0.7	63.3 ± 4.0
LAK cells	100 ng	66.8 ± 3.2	63.6 ± 2.6	64.9 ± 3.6
LAK cells	1 μg	65.5 ± 3.6	66.6 ± 1.5	62.9 ± 1.0
LAK cells	10 μg	63.0 ± 2.3	64.2 ± 1.4	64.5 ± 1.1
LAK cells	100 μg	63.8 ± 3.0	63.4 ± 1.7	63.6 ± 3.7

[a] Values are expressed as percentage of specific lysis (mean ± SD) at an E:T ratio of 50:1 for triplicate experiments determined by means of an 18 hr ^{51}Cr release assay.

Discussion

The culture conditions that we have defined for the *in vitro* generation of maximum murine LAK activity correlate well with previously reported data (18, 19). With murine LAK cells we were able to maintain cell viability for >10 days at a density of 1.5×10^6 cells/ml with 250 units of rIL-2/ml of medium, following which LAK activity could be restored by adding rIL-2. These observations confirm the importance of supplementation with rIL-2 for the maintenance of both viability and cytolytic activity of LAK cells in long-term cultures (20, 21). The culture of murine NMNC in the presence of 250 units of rIL-2/ml of complete medium generated LAK cells with peak cytolytic activity at 4–5 days. At this time, the percentage of specific lysis reached 100% at an E:T ratio of 100:1 in an 18-hr ^{51}Cr release assay.

The high specific release (35%) mediated by unstimulated splenocytes against B16 targets may have been due to the higher E:T ratio (100:1) that was used and/or the longer duration of the assay (18 hr vs 4 hr usual). In addition, B16 melanoma cells with low H-2K^b Ag expression have been shown to be more sensitive to lysis by natural killer cells than those that express high amounts of H-2K^b (22). We have observed that B16 parental cells, transplanted into BALB/c mice, formed progressively growing tumors, suggesting that they expressed low levels of H-2K^b (unpublished observations).

Several MoAb have been produced against B16 melanoma-associated antigens (23), and some of these have been reported to prevent the adherence of B16 melanoma cells *in vitro* and to reduce the frequency of metastases *in vivo* (9, 10). The therapeutic effects of using a combination of effector cells and MoAb directed against surface antigens expressed on melanoma cells have been reported by Schulz *et al.* (24, 25). One goal of the present study was to determine whether anti-B16 MoAb IB16-6 and IB16-8 would alter target cell sensitivity to LAK cells. The cytolytic activity of LAK cells against antibody-coated B16 target cells was similar to that observed with normal rat IgG-coated cells in the ^{51}Cr release assay. Over a wide range of concentrations from 0.1 to 1000 ng, similar results were obtained with IB16-8. We have previously shown that MoAb IB16-6 and IB16-8 did not mediate antibody-dependent cell-mediated cytotoxicity in the presence of either lymphocytes or thioglycollate-induced peritoneal macrophages (16, 26). These observations correlate with other data showing that murine lymphocytes had low ADCC activity (27–29). Shiloni *et al.* (14) have suggested that ADCC might be one mechanism by which LAK cells kill tumor cells. Although Fc receptor-bearing effector cells are thought to be responsible for the generation of ADCC (30, 31), we have not observed increased lysis of antibody-coated B16 cells by LAK cells, indicating that IB16-6 and IB16-8 did not mediate ADCC. Although MoAb IB16-6 and IB16-8 recognized an epitope expressed with high density on B16 melanoma cells, this was not the recognition unit for LAK cells, since even saturating quantities of either of these antibodies had only minimal effects on their cytolytic activity. Furthermore, it appears unlikely that either of these antibodies reacted with adhesion or triggering molecules, which play a major role in the cytotoxic activity of natural killer cells or cytolytic T lymphocytes against their targets (32–36).

Jacobs *et al.* (37) have reported that the cell surface determinant recognized on glioma targets by LAK cells was sensitive to trypsin and chymotrypsin. We have observed that enzymatic treatment of B16 melanoma cells with either trypsin or pronase completely removed the epitope recognized by MoAb IB16-6, but did not affect their sensitivity to LAK cells. These observations indicate that the recognition unit for LAK cells on different tumors is not uniformly sensitive to proteolytic enzymes and that it is distinct from the epitope that MoAb IB16-6 and IB16-8 recognize. This raises the possibility that MoAb IB16-6 and IB16-8 could be used for the selective targeting of tumoricidal agents without altering target cell sensitivity to LAK cells.

A final comment should be made about the *in vitro* clonogenic assay that also was used to demonstrate LAK activity against B16 target cells. The number of B16 colonies enumerated was reduced to 0 by cocultivation with LAK cells for 7 to 10 days. Similar results were observed with B16-F1, F10, F10FLR, and BL6 sublines at an E:T ratio of 400:1. LAK cells appeared to prevent the attachment of B16 cells as well as to cause already adhered cells to detach. Although the activity of cytotoxic effector cells, including cytolytic T lymphocytes, NK cells, and LAK cells are usually assessed by means of short term ^{51}Cr release assays, these may not provide a full explanation for their tumoricidal and antimetastatic activity. Data from the clonogenic assay suggest that besides being cytolytic, LAK cells may have antiadherent and antiproliferative

effects on tumor cells and this might provide an additional explanation for their *in vivo* antimetastatic activity. The clonogenic assay may prove to be a useful tool for delineating the ability of LAK cells to alter tumor cell adherence from their cytolytic activity, and for evaluating the interactions of other cytokines and effector cells with tumor target cells.

Supported in part by Grants DE-AC02-82ERG0040 from the Department of Energy, Grants R01 CA-41288 and P-30-CA-16058-14 from the National Cancer Institute, and a Research Challenge Grant from The Ohio State University.

We thank Dr. I. J. Fidler for providing us with B16 melanoma cells and sublines, Dr. J. Winkelhake for recombinant IL-2, useful discussions with Dr. J. Wunderlich, and Dr. R. B. Herberman for his advice and encouragement.

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