IMMUNOTHERAPY OF A MURINE TUMOR WITH INTERLEUKIN 2
Increased Sensitivity after MHC Class I Gene Transfection

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The lymphokine, IL-2, is a glycoprotein that mediates a variety of immunologic effects in animal models (1). When administered systemically to mice, IL-2 can mediate the regression of subcutaneous tumors as well as pulmonary and hepatic metastases from selected tumors (2, 3). The characteristics of tumor cells that determine sensitivity to IL-2 immunotherapy have not been identified. Recent data from our laboratory demonstrated that IL-2 is capable of mediating regression of 10-d pulmonary macrometastases from two weakly immunogenic 3-methylcholanthrene (MCA)1-induced sarcomas, yet has no impact on macrometastases derived from two nonimmunogenic sarcomas (4). An Lyt-2-bearing cell was implicated as a mediator of the regression of weakly immunogenic tumors. Analysis of clonally derived murine lymphocytes has shown a correlation between Lyt-2 phenotype and class I recognition (5). Tumor cell determinants may be recognized in an associative reaction with self MHC to generate an immune response, and a role for MHC antigens in controlling tumor immune responses is suggested by experiments involving alteration of class I expression on tumor cells by selection (6, 7) or gene transfection (8–10). Increased class I expression by transfection of several virally transformed or carcinogen-induced murine tumors was shown to change metastatic potential (8), tumorigenicity (9), or immunogenicity (10).

In this paper we demonstrate that class I MHC molecules are present on two weakly immunogenic tumors that respond to high-dose IL-2 therapy, whereas tumor macrometastases from two nonimmunogenic tumors that were not responsive to IL-2 immunotherapy express little or no class I MHC antigen. To test whether modulation of MHC expression could render tumor cells sensitive to IL-2 immunotherapy, cell lines were derived from a murine melanoma, B16BL6, by transfection of the class I gene encoding K0 and the class II gene encoding Iaβ (Tanaka, K., and G. Jay, unpublished data).

The original B16BL6 melanoma expressed little or no class I antigen and was not susceptible to therapy with IL-2. Class I expression induced sensitivity of macrometastases to therapy with high-dose IL-2 and sensitivity of micrometastases to high- and low-dose IL-2. Lyt-2-bearing cells were involved in regression
of micro- and macrometastases. High-dose IL-2 could also mediate regression of class I transfectant micrometastases without involving Lyt-2 cells. Class II expression did not increase the sensitivity of B16BL6 melanoma to IL-2 therapy.

Materials and Methods

Mice. Female C57BL/6 (C57) mice and (C3H × C57)F1 mice, 12–16 wk old, were obtained from the Small Animal Section, Veterinary Resources Branch, National Institutes of Health, Bethesda, MD.

Tumor Cells. MCA-101, -102, -105, and -106 are MCA-induced fibrosarcomas of C57 origin. They were maintained in vivo in syngeneic mice by serial subcutaneous transplantation of cryopreserved tumor samples as described previously (11). All tumors used were in the 3rd to 7th transplantation generation. The P815 mastocytoma, syngeneic to DBA/2 mice, was maintained in vivo as ascites tumor.

Interleukin 2. Human IL-2 was kindly supplied by the Cetus Corp., Emeryville, CA. The biological and biochemical activities of IL-2 have been described (12). Purified material had a specific activity of 3.6 × 10^6 U/mg. In our proliferation assays, ~1 U of Cetus Corp. rIL-2 is equivalent to 2–3 U of Biological Response Modifier Program IL-2 standard. The endotoxin level in the purified preparation was <0.1 ng/10^6 U IL-2, as measured by a standard limulus assay.

Preparation of Single Cell Suspensions. Single cell suspensions from solid tumor and from spleen were prepared as described previously (13). Single cell suspensions were prepared from monolayers by brief incubation with trypsin at 37°C, removal of cells by pipetting, centrifugation, and suspension of the pellet in DME containing 10% FCS for 30 min at 37°C. Cells were centrifuged, washed several times with HBSS, and injected into mice.

Protocol for Therapy of Pulmonary Metastases. 1–4.5 × 10^5 tumor cells were injected as a single cell suspension in 1 ml of HBSS into the tail veins of mice. After 3 d, micrometastases were present and invading pulmonary alveoli. By 10 d after injection, grossly visible metastases were apparent on the lung surface. At 3 or 10 d after tumor cell injection, therapy with intraperitoneal IL-2 began. Occasional doses of high-dose IL-2 were withheld from all mice if some appeared lethargic. At 17–18 d after injection, mice were ear-tagged, randomized, and killed. Lungs were removed by opening the thoracic cavity. 15% India ink was injected into the trachea and lungs were bleached in Fekete's solution as previously described (13, 14). White tumor nodules on black lung were counted without knowledge of the treatment given that mouse. Lungs with too many nodules to count were assigned a value of 300, the approximate upper limit for reliable counting. After all data were recorded, codes were broken and data analyzed.

Immunoperoxidase Staining. 6–7 µm frozen sections on gelatin-coated slides were used. The avidin-biotin complex technique was employed using a Vector ABC Kit (Vector Laboratories, Inc., Burlingame, CA). Sections were incubated overnight at 4°C with the primary antibody. Affinity-purified goat anti–mouse IgG2A was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). Endogenous peroxidase activity was quenched in all samples with methanol/peroxide. Counterstaining was done with a 1-min exposure to Mayer's hematoxylin. Normal C57BL/6 spleen sections served as positive controls for class I or II staining. Mouse mAb 211, which recognizes human T cells and lymphokine-activated killer (LAK) cells (Fox, B., and S. A. Rosenberg, manuscript in preparation), or 16-1-2, which detects class I K^b, served as irrelevant controls.

In Vivo Depletion of Lymphocytes by mAb. A hybridoma (2.43), producing a rat IgG2b mAb directed against the Lyt-2 T cell antigen was obtained from American Type Culture Collection, Rockville, MD. mAb was harvested as ascites from sublethally irradiated (500 rad) DBA/2 mice and kindly provided by Dr. James Yang, NIH. For in vivo lymphocyte depletion (15), C57 mice received one intravenous injection of 100 µl of monoclonal ascites fluid diluted to 0.5 ml with HBSS, from 6–12 h before the first IL-2 injection. This procedure has been shown to induce depletion of T cell subpopulations in vivo for up to 14 d (Yang, J., personal communication). Injection of purified rat IgG (Miles...
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Laboratories Inc., Naperville, IL) served as a control. It was mixed as 2 mg/ml in 0.5 ml HBSS. Hybridoma 2.43 ascites was diluted to 2 mg/ml or less in 0.5 ml HBSS.

Fluorescence-activated Cell Sorter Analysis. Flow cytometry analysis of lymphocyte or tumor cell surface phenotypes was carried out by indirect fluorescence using a FACScan flow microfluorometer (Becton Dickinson & Co., Mountain View, CA), interfaced with a PDP 11/24 computer (Digital Equipment Corp., Maynard, MA). Pelleted single cell suspensions of 10^6 cells were incubated for 45 min at 4°C with 10-30 µg of mAbs diluted in PBS containing 3% FCS and 0.1% NaN3. Bound antibodies were detected by incubation with an appropriately titered FITC-labeled mouse mAb to rat κ light chains (MAR 18.5, Becton Dickinson & Co.) or a goat FITC-labeled mAb to mouse IgG heavy and light chains (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 45 min at 4°C. Anti-Kb mAbs 20-8-4 and 28-8-6, anti-Ia^b mAb 34-5-3 and anti-Ia^x mAb 10.2-16 were gifts of Dr. David Sachs, National Institutes of Health. Anti-Lyt-2 and anti-L3T4 FITC-labeled mAbs were obtained from Becton Dickinson & Co.

B16 Clones. BL6-13, CL8-1, and CL8-2 are cell lines derived from murine melanoma B16BL6 subclone 8 by calcium phosphate cotransfection with a pRSVneo plasmid containing the selectable marker gene encoding resistance to neomycin (BL6-13) or both the neomycin resistance plasmid and another pRSV plasmid containing the DNA encoding Kb, a class I antigen (CL8-1, CL8-2). Line BL22 was derived from B16BL6 Clone 8 by calcium phosphate cotransfection with pRSVneo and another plasmid containing the genes encoding the α and β chains of Ia^x, a class II antigen (Tanaka, K., and G. Jay, unpublished data). All lines were maintained in continuous culture in DMEM with 10% FCS and passaged twice weekly. Before each experiment, expression of class I antigen on the cell surface was verified with indirect immunofluorescence on the FACScan, using mAb 28-8-6.

Generation of LAK Cells. C57 spleen cells in complete medium (CM) were prepared as previously described (11). 1 ml of CM containing 4 x 10^6 viable splenocytes was added to each well of a 24-well culture plate (no. 3524, Costar, Cambridge, MA). 1 ml of CM containing 2,000 U of IL-2 was added to each well. After 3 d of incubation at 37°C with 5% CO2, cells were harvested and tested for lytic activity in a 4-h chromium-release assay (11, 16).

In Vitro Sensitization to Allogeneic Cells. Conditions for allogeneic in vitro sensitization have been described in detail (11). Briefly, 4 x 10^6 responder C57 splenocytes and 2 x 10^6 irradiated (3,000 rad) DBA/2 spleen stimulator cells were cultured in 2 ml of CM in individual wells of a 24-well tissue culture plate (Costar), for 4 d at 37°C with 5% CO2. The cells were harvested and tested for cytotoxic activity against tumor or P815 targets in a 4-h chromium-release assay as described (11, 16). EL-4 targets were specificity controls.

Statistics. Statistical analyses were performed by the Wilcoxon rank sum test (13). Two-sided p values are presented in all experiments. No mice were excluded from the statistical evaluations.

Results

Correlation of MCA Sarcoma Class I MHC Expression In Vivo with IL-2 Sensitivity. Previous work from our laboratory (4) using four murine sarcomas, all induced by the carcinogen 3-methylcholanganthrene, established that high-dose IL-2 immunotherapy mediated 80-90% reduction of 10-d pulmonary macrometastases of weakly immunogenic tumors MCA-105 and -106 but had no effect on macrometastases of nonimmunogenic tumors MCA-101 and -102.

Expression in vivo of class I and II MHC molecules by macrometastases from these four sarcomas was tested by immunoperoxidase staining of frozen sections of lung from mice 10 d after injection with tumor cells. The results in the first panel of Table I show that tumors 105 and 106 stain positively for class I MHC using a murine mAb that identifies Kb. Tumors MCA-101 and -102 show no
TABLE I
Correlation Between Class I MHC Expression on Murine Sarcomas and Susceptibility to IL-2 Therapy

| Tumor | Immunohistochemical staining of lung tumor* | Immunofluorescence of tumor cells in vitro‡ | Susceptibility of macrometastases to therapy with high-dose IL-2 |
|-------|------------------------------------------|-------------------------------------------|---------------------------------------------------------------|
|       | Class I | Class II | Class I | Class II | % |
| 101   | -       | -        | - (0)   | -        | 0 |
| 102   | -       | -        | + (50)  | -        | 0 |
| 105   | +       | -        | ++ (95) | -        | 99|
| 106   | +       | -        | ++ (80) | -        | 83|

* Mice were injected with 1-3 × 10⁵ tumor cells intravenously. Lungs were removed from animals killed on day 18 after injection, imbedded in OCT media, and quick-frozen. 6-μm sections were stained with an immunoperoxidase technique using a vector ABC kit (Vector Laboratories Inc.). Murine mAb 28-8-6 was used for K<sup>b</sup>, a class I determinant. Murine mAb 34-5-6 is specific for IA<sup>a</sup>, a class II determinant.

‡ Fresh tumor digested with hyaluronidase, collagenase, and DNAase was passed once in DMEM with 10% FCS until a homogeneous monolayer grew; cells were trypsinized and washed, then analyzed using a FACS II analyzer. Murine mAb 28-8-6 was used to detect K<sup>b</sup>; 34-5-6 was used for IA<sup>a</sup>. The numbers in parentheses represent percent fluorescence in the control subtracted from the experimental sample.

§ See reference 4. The number represents percent reduction of 10-d pulmonary metastases treated with 150,000 U of IL-2 intraperitoneally, three times per day for 4 d versus treatment with no IL-2.

staining when tested with the same mAb. A different mAb that identifies both K<sup>b</sup> and D<sup>b</sup> fails to bind to tumors 101 and 102 (data not shown). No staining of any of the four sarcomas is seen when a mAb identifying the class II molecule, IA<sup>a</sup>, is used. The staining data suggest that tumors 101 and 102 do not express class I or II MHC molecules. Similar staining data (not shown) were obtained using subcutaneous tumors generated by the four MCA sarcomas. Tumor cells derived from the four sarcomas by enzymatic digestion and one passage in vitro were analyzed by microfluorometry for the presence of class I or II MHC molecules using the same murine mAbs used for immunoperoxidase staining. The flow microfluorometry profiles are shown in Fig. 1. Strongly positive class I staining is seen for tumors 105 and 106, intermediate staining for 102 and none for 101. None of the MCA sarcomas stain with an antibody that identifies the class II molecule IA<sup>a</sup>, whereas normal C57BL/6 splenocytes stain strongly using the same mAb, 34-5-3, in the same experiment. The anti-class I H-2<sup>b</sup> mAb did not bind to H-2<sup>b</sup> C3H splenocytes, and anti H-2<sup>k</sup> mAb 16-1-2 did not bind to the MCA sarcomas. Class I MHC is expressed by 105 and 106, both of which are weakly immunogenic and susceptible to IL-2 immunotherapy. Little or no class I expression is seen on MCA tumors 101 and 102, both nonimmunogenic and resistant to IL-2 immunotherapy. None of the tumors expressed class II MHC molecules.

Characterization of B16 Cell Lines Transfected with Class I and II MHC Genes. To test whether class I MHC expression could increase sensitivity to IL-
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ANTIBODY USED FOR STAINING

CONTROL  ANTI-CLASS I  ANTI-CLASS II

TUMOR CELL

CELL NUMBER

LOG FLUORESCENCE

FIGURE 1. Flow microfluorometric analysis of sarcoma tumor cells. The control antibody was goat anti-mouse FITC-conjugated Ig; the anti-class I mAb was 28-8-6, which recognizes K\textsuperscript{b} + D\textsuperscript{b}; the anti-class II mAb was 34-5-3, which recognizes I\textalpha\textbeta. Cell number was measured on the ordinate, and fluorescence intensity was measured on the abscissa in arbitrary logarithmic units. A total of 10\textsuperscript{6} cells were analyzed in each panel.

In this study, we used cell lines derived from a subcloned murine melanoma, B16BL6, that were transfected with either: (a) a plasmid encoding the selectable marker for Neomycin resistance, neo'; (b) class I MHC gene encoding K\textsuperscript{b} and the neo' plasmid; (c) class II genes I\textalpha\textsuperscript{a} and I\textalpha\textsuperscript{b} and the neo' plasmid (Tanaka, K., and G. Jay, unpublished data). These cell lines were designated BL6-13, CL8-2, and BL-22, respectively. A northern blot of RNA from these cell lines is shown in Fig. 2. Cytoplasmic polyadenylated RNA from the transfected cell lines and control cells was fractionated on agarose-formaldehyde gels. A synthetic oligonucleotide probe specific to the K\textsuperscript{b} gene was used for hybridization in the left lanes A1–A4 of Fig. 2; a strong signal in lanes A3 and A4 at 1.8 kb indicates the presence of class I mRNA in the cell lines CL8-1 and CL8-2, two cell lines transfected with the neo' plasmid and the K\textsuperscript{b} class I gene. In lanes A1 and A2, B16BL6 clone 8 and BL6-13, the parental cell line and the line transfected with the neo' plasmid, do not express detectable class I mRNA. Right lanes B1–B4 show that B16 clone 8, BL6-13, CL8-1, and CL8-2, express normal amounts of actin mRNA as a positive control. A northern blot of cytoplasmic polyadenylated RNA has been performed (Tanaka, K., and G. Jay, unpublished data), showing that BL22, a cell line transfected with neo' plasmid and the genes encoding \( \alpha \) and \( \beta \) chains of I\textalpha\textsubscript{a}, expresses mRNA for both chains.

A microfluorometric flow analysis of the control, class I, and class II transfectant lines was performed using mAbs recognizing K\textsuperscript{b}, I\textalpha\textsuperscript{b}, and I\textalpha\textsuperscript{a}. The immu-
Fluorescence profiles are shown in Fig. 3. Control cell line BL6-13 does not bind antibodies 28-8-6 or 34-5-3, indicating that it expresses no class I or II MHC molecules at its surface. CL8-2 binds mAb 28-8-6 but not 34-5-3, indicating that it expresses class I but not class II MHC molecules. BL22 binds mAb 10.2-16 but not 28-8-6, indicating the presence of class II Ia but no syngeneic class I molecules. Immunoperoxidase staining of pulmonary metastases or subcutaneous tumors generated by BL6-13 or class I transfectant CL8-2 shows positive staining by a class I mAb only for CL8-2 (data not shown). Therefore, the class I transfectant line CL8-2 transcribes mRNA for Kb and expresses the Kb antigen molecule at its surface in vitro and in pulmonary or subcutaneous tumor in vivo. The class II transfectant line BL22 similarly transcribes class II mRNA and expresses the class II molecule at its surface. In contrast, control line BL6-13 neither transcribes mRNA for, nor expresses class I or II MHC molecules.

**LAK and Allogeneic CTL Lysis of Transfected Cell Lines.** Class I transfectant line CL8-2 and control line BL6-13 were tested for sensitivity to lysis in vitro by syngeneic LAK cells, and by allogeneic CTL. DBA/2 splenocytes sensitized to irradiated C57BL6 splenocytes should lyse allogeneic b haplotype class I–expressing cells, but not cells bearing either syngeneic d haplotype or no class I molecules. LAK cells are generated by 3-d incubation of normal C57BL6 splenocytes in IL-2, and show MHC-unrestricted lysis of many fresh and cultured tumor cell lines in vitro (17). The top panel of Fig. 4 shows that control line BL6-13 and transfected line CL8-2 are lysed equally by LAK cells in a 4-h chromium-release assay. However, in the bottom panel, allogeneic CTLs lyse class I transfected CL8-2 cells, but not control line BL6-13. Therefore, the class I molecules expressed on line CL8-2 are functional in an MHC-restricted allogeneic CTL reaction in vitro. LAK lysis in vitro is not an MHC-restricted phenomenon (17), and LAK cells lyse CL8-2 and BL6-13 equally well.
Expression of MHC Class I Renders B16 Transfectant Micro- and Macrometastases Sensitive to IL-2 Immunotherapy.} Micrometastases were generated by intravenous injection of control line BL6-13 and class I transfectant CL8-2. 3 d after injection, IL-2 therapy at varying doses began and continued for 5 d. Lung metastases were counted at day 17. The results of two identical experiments are shown in Table II, and one experiment is plotted in Fig. 5. Control BL6-13 micrometastases are sensitive to IL-2 immunotherapy in one of the experiments only at the highest dose of IL-2, 182 vs. 82 metastases, \( p < 0.05 \). In contrast, class I transfected CL8-2 micrometastases showed statistically significant and considerable regression at all IL-2 doses, 283 vs. 15 metastases at the highest IL-2 dose in Exp. 1, 201 vs. 17 metastases in Exp. 2. Expression of class I MHC increased crometastatic sensitivity to IL-2 at doses from 10,000 to 100,000 U i.p. every 8 h given for 5 d, beginning 3 d after injection of tumor cells.

Pulmonary macrometastases were generated by intravenous injection of tumor cells from parental line B16BL6, control line BL6-13, and class I transfectant line CL8-2. Table III shows the results of two experiments in which mice received IL-2 therapy from day 10 to day 14 after tumor injection, and lung metastases were counted on day 18. In neither experiment was there a demonstrable impact of IL-2 therapy on the number of pulmonary macrometastases generated by
FIGURE 4. LAK or allogeneic CTL lysis of control clone BL6-13 or class I transfectant clone CL8-2. Splenocytes from C57BL6 mice were incubated in complete medium containing 1,000 U of IL-2 per milliliter for 3 d to generate LAK cells. Splenocytes from DBA/2 mice were incubated with irradiated C57BL6 splenocytes in complete medium for 5 d at a 2:1 ratio to generate allogeneic CTL. BL6-13 or CL8-2 were labeled with ^51Cr and either LAK or allogeneic CTL cells were used as effectors in a 4-h ^51Cr-release assay. Allogeneic CTLs lysed control target EL4 but cytotoxic activity was <2% for syngeneic target P815 (not shown).

TABLE II

Expression of Class I MHC Antigen Renders B16 Transfectant 3-d Pulmonary Metastasis Sensitive to Low- and High-dose IL-2

| Exp. | Tumor* | Number of metastases (± SEM) after treatment with the following units of IL-2 |
|------|--------|-----------------------------------------------------------------------------|
|      |        | 0                         | 10,000                          | 30,000                          | 100,000                         |
| 1    | BL613  | 118 (17)                  | 120 (17)                        | 137 (28)                        | 102 (14)                        |
|      | CL8-2  | 283 (16)                  | 108± (12)                       | 174± (6)                        | 15± (5)                         |
| 2    | BL613  | 187 (13)                  | 180 (20)                        | 162 (17)                        | 82± (12)                        |
|      | CL8-2  | 201 (24)                  | 34± (13)                        | 44± (35)                        | 17± (9)                         |

* 2-4.5 × 10^6 tumor cells were injected intravenously. IL-2 therapy started day 3 after tumor injection, every 8 h intraperitoneally for 5 d. Mice were killed on day 17 for lung metastases counting. Each group contains 6-10 mice.

Expression of Class II MHC Does Not Increase Sensitivity of B16 Transfectant Micrometastases to IL-2. Line BL22 is derived from B16BL6 by cotransfection with neo and the genomic DNA encoding the α and β chains of Iaβ. The parental B16BL6 (250 vs. 266 metastases), or control line BL6-13 (166 vs. 150 metastases) in Exp. 1. Neither cell expresses class I MHC antigens. A statistically significant regression of 10-d micrometastases generated by class I transfected line CL8-2 is seen in both experiments at the higher dose of IL-2 (152 vs. 27 metastases in Exp. 1, 126 vs. 71 metastases in Exp. 2). Micrometastases generated by a second class I transfected cell line, CL8-1, are also sensitive to high-dose IL-2 (data not shown).
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TABLE III
Exposure of Class I MHC Renders B16 Transfectant 10-d Pulmonary Metastases Sensitive to High-dose IL-2

| Exp. | Tumor* | Number of metastases (± SEM) after treatment with the following units of IL-2 |
|------|--------|-------------------------------------------------|
|      |        | 0 | 20,000 | 100,000 |
| 1 B16 | 271 (18) | 281 (18) | 284 (14) |
| BL6-13 | 166 (15) | 142 (23) | 150 (27) |
| CL8-2 | 152 (19) | 83 (29) | 271 (12) |
| 2 B16 | 238 (10) | 239 (10) | 218 (14) |
| BL6-13 | 144 (23) | 157 (17) | 144 (21) |
| CL8-2 | 126 (18) | 92 (11) | 711 (11) |

* 1-4.5 × 10⁷ tumor cells were injected intravenously. IL-2 therapy started day 10 after tumor injection, every 8 h intraperitoneally for 5 d. Mice were killed on day 18 for counting of lung metastases. Each group contains 6-10 mice.

† Wilcoxon rank sum test of treated groups compared with groups receiving HBSS alone. p < 0.05. All others were not significant.

transfection of a H-2k haplotype class II gene necessitates the use of F1 C3H × C57 animals for generation of pulmonary metastases. 3 d after intravenous injection of class II transfectant BL22 or control BL6-13 cells, IL-2 therapy began and continued for 5 d. Lung metastases were counted at day 17. The results of two identical experiments are shown in Table IV. As a positive control to ensure that IL-2 could mediate regression of micrometastases in F1 animals, two groups of mice were injected with class I transfectant CL8-2 and treated in parallel with BL22 or BL6-13 injected mice. In each of the two experiments, high-dose IL-2 can mediate impressive regression of CL8-2 micrometastases in F1 mice. Similar to our findings in C57BL6 mice, high-dose IL-2 can mediate a small but statistically significant regression of BL6-13 control micrometastases in F1 mice, 210 vs. 74 and 51 vs. 21 in two experiments. Class II transfectant micrometastases show no increased sensitivity to low- or high-doses of IL-2 in either experiment. No significant regression is seen for BL22 in Exp. 1, 172 vs. 115, and moderate regression in Exp. 2, 109 vs. 57, p < 0.05. There is no increase in micrometastatic regression of BL22 versus BL6-13, but there is
between BL6-13 and CL8-2. This result suggests that expression of a class II MHC gene by B16BL6 melanoma cannot induce micrometastatic sensitivity to IL-2 in semisyngeneic F1 mice.

**Lyt-2 Cells Mediate Reduction of CL8-2 Macrometastases by High-dose IL-2.** To establish whether an Lyt-2 cell was involved in mediating regression of 10-d pulmonary macrometastases induced by class I transfected line CL8-2, rat anti-Lyt-2 mAb 2.43 was injected intravenously before the onset of IL-2 immunotherapy to deplete the Lyt-2 lymphocyte subset. After 5 d of IL-2 therapy, the lymphocyte populations were examined to verify Lyt-2 depletion. Fig. 6 shows microfluorometric analyses of splenocytes 5 d after antibody treatment. There is >85% depletion of Lyt-2+ cells in mice treated with mAb 2.43. As a control, fluorescein-conjugated anti-L3T4 mAb was used to measure the L3T4+ lymphocyte subset in mice treated with anti-Lyt-2 mAb or rat Ig–treated control mice. The bottom two panels of Fig. 6 show no change in the L3T4+ subset after treatment with mAb 2.43. The splenocytes from anti-Lyt-2-treated mice are also unable to generate an allogeneic CTL response in vitro. Splenocytes from C57BL6 mice were removed on day 5 after mAb 2.43 or rat Ig injection, on the last day of IL-2 therapy of CL8-2 macrometastases, and cocultured with irradiated DBA/2 splenocytes for 5 d to generate allogeneic CTLs. The allogeneic CTLs were used as effectors in a 4-h chromium-release assay with tumor line P815 as the target. The results are shown in Fig. 7. Mice treated with mAb 2.43 fail to generate allogeneic CTL lysis of P815, whereas control mice treated with rat Ig generate good allogeneic CTL killing of P815 in vitro. The results of these microfluorometric and allogeneic CTL assays of in vivo cell depletion are similar at 14 d after mAb injection (Yang, J., personal communication).

The results of two identical anti-Lyt-2 depletion experiments on the generation of CL8-2 macrometastases treated with high-dose IL-2 are shown in Table V. High-dose IL-2 is capable of mediating significant regression of CL8-2 macrometastases in both experiments in rat Ig–treated control animals (292 for HBSS

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### Table IV

**Expression of Class II MHC Does Not Render B16 Transfectant 3-d Pulmonary Metastases More Sensitive to IL-2 Therapy**

| Exp. | Tumor* | Number of metastases (± SEM) after treatment with the following units of IL-2 |
|------|--------|--------------------------------------------------------------------------------|
|      |        | 0      | 10,000 | 20,000 | 100,000 |
| 1    | BL6-13 | 210 (21) | 177 (18) | 152 (15) | 75† (18) |
|      | BL22   | 172 (23) | 156 (20) | 164 (22) | 115 (16) |
|      | CL8-2  | 140 (27) | —      | —      | 2† (1)   |
| 2    | BL6-13 | 51 (12)  | 75 (20)  | 26 (4)  | 21† (9)  |
|      | BL22   | 109 (12) | 83 (16)  | 66 (20) | 57† (16) |
|      | CL8-2  | 24 (7)   | —      | —      | 2† (1)   |

*2–4.5 × 10⁵ tumor cells were injected intravenously. IL-2 therapy started day 5 after tumor injection, every 8 h intraperitoneally for 5 d. Mice were killed on day 17 for lung metastases counting. Each group contains 6–10 mice.

† Wilcoxon rank sum test of treated groups compared with groups receiving HBSS alone, two-sided, showing p < 0.05.
Figure 6. Flow microfluorometric analysis of splenocytes from mice treated with anti-Lyt-2 mAb 2.43 (left panels) or control animals treated with rat Ig (right panels). Antibody was administered intravenously 5 d before analysis, as 100 µl of crude ascites suspended in 0.5 ml HBSS. Spleens were removed and single cell suspensions were generated as described previously (13). The two left panels show staining profiles of control splenocytes using anti-Lyt-2 and anti-L3T4 FITC antibodies (Becton Dickinson & Co.). In the right panels, the staining profiles of anti-Lyt-2-depleted splenocytes are shown. Cell number is measured on the ordinate, and fluorescence intensity is measured on the abscissa in arbitrary logarithmic units. A total of 10^6 cells is analyzed in each panel.

Figure 7. Ability of splenocytes from animals treated with anti-Lyt-2 mAb to generate allogeneic CTL in vitro. 5 d after C57BL/6 mice received an intravenous injection of either mAb 2.43 or rat Ig, spleen cells were removed and cultured with irradiated DBA/2 stimulator cells at a ratio of 2:1 for 4 d in RPMI 1640 complete medium. The C57 splenocytes were used as effectors in a 4-h chromium-release assay using 51Cr-labeled P815 cells grown in ascites as the allogeneic target. Lysis of control syngeneic target EL-4 was <5% for all effectors (not shown).

vs. 169 for 100 ×10^3 U IL-2, and 55 vs. 28, both significant at p < 0.05). As seen previously, lower doses of IL-2 do not impact on CL8-2 macrometastases. The effect of high-dose IL-2 on CL8-2 macrometastases in both experiments is completely abrogated in animals treated with anti-Lyt-2 mAb 2.43 (232 for
TABLE V
Lyt-2\(^+\) Cells Mediate Reduction of CL8-2 10-d Pulmonary Metastases byHigh-dose IL-2

| Exp. | Treatment* | Number of metastases (± SEM) after treatment with the following units of IL-2 |
|------|------------|--------------------------------------------------------------------------------|
|      |            | 0 | 10,000 | 30,000 | 100,000 |
| 1    | Rat Ig     | 292 (5) | 260 (13) | 248 (32) | 169\(^\text{a}\) (25) |
|      | Anti-Lyt-2 | 232 (31) | 287 (22) | 246 (24) | 247 (19) |
| 2    | Rat Ig     | 55 (9)  | 55 (17)  | 65 (14)  | 28\(^\text{a}\) (5) |
|      | Anti-Lyt-2 | 61 (7)  | 56 (14)  | 77 (11)  | 53 (6)   |

* 4.5 \times 10^5 tumor cells were injected intravenously. Antibody was injected intravenously 12 h before starting IL-2 therapy. HBSS or IL-2 was injected intraperitoneally for 5 d every 8 h starting on day 10 after tumor injection. Each group contains 6–10 mice.

\(^\text{a}\) Wilcoxon rank sum test of treated groups compared with groups receiving HBSS alone, two-sided; \(p < 0.05\).

TABLE VI
Lyt-2\(^+\) Cells Mediate Reduction of CL8-2 3-d Pulmonary Metastases by Low-dose, but not High-dose IL-2

| Exp. | Treatment* | Number of metastases (± SEM) after treatment with the following units of IL-2 |
|------|------------|--------------------------------------------------------------------------------|
|      |            | 0 | 10,000 | 30,000 | 100,000 |
| 1    | Rat Ig     | 81 (21) | 16\(^\text{a}\) (10) | — | 9\(^\text{a}\) (5) |
|      | Anti-Lyt-2 | 93 (12) | 77 (23) | — | 12\(^\text{a}\) (4) |
| 2    | Rat Ig     | 262 (23) | 186 (71) | 51\(^\text{a}\) (20) | 15\(^\text{a}\) (6) |
|      | Anti-Lyt-2 | 268 (31) | 254 (23) | 233 (53) | 32\(^\text{a}\) (6) |

* 4.5 \times 10^5 tumor cells were injected intravenously. Antibody was injected intravenously 6 h before starting IL-2 therapy. HBSS or IL-2 was injected intraperitoneally for 5 d every 8 h starting on day 3 after tumor injection. Each group contains 6–10 mice.

\(^\text{a}\) Wilcoxon rank sum test of treated groups compared with groups receiving HBSS alone, two-sided. \(^\text{a}\) \(p < 0.05\). \(^\text{a}\) \(p < 0.005\). All others were not significant.

HBSS vs. 247 for 100 kD IL-2, and 61 vs. 55, both not significant). This result suggests that an Lyt-2-bearing cell is involved in the high-dose IL-2-mediated regression of CL8-2 10-d pulmonary metastases.

Lyt-2 Cells Mediate Reduction of CL8-2 Micrometastases by Low-dose, Not High-dose IL-2.
To establish whether a Lyt-2-bearing cell mediates regression of 3-d pulmonary micrometastases generated by class I transfected line CL8-2, rat mAb 2.43 was injected intravenously 3 d after tumor cell administration. IL-2 therapy was begun 6 h later and continued every 8 h for 5 d. Lymphocyte populations were examined to verify Lyt-2 depletion as in the prior experiments. Virtually complete depletion was obtained with data similar to that in Figs. 6 and 7 (data not shown). The results of two similar anti-Lyt-2 depletion experiments on the generation of CL8-2 micrometastases treated with varying doses of IL-2 are shown in Table VI. IL-2 at doses ranging from 1–10 \times 10^4 U i.p. every 8 h for 5 d was capable of mediating significant and substantial regression of CL8-2 micrometastases in rat Ig–treated control animals. The regression is invariably
greatest at the highest dose of IL-2 (262 vs. 15, and 81 vs. 9, \( p < 0.005 \)). In anti-Lyt-2-treated animals, the effect of low-dose IL-2 (1–3 \( \times 10^3 \) U) is abrogated, whereas IL-2 at 10 \( \times 10^4 \) U still mediates significant regression of CL8-2 micrometastases (268 vs. 37, and 93 vs. 12, \( p < 0.005 \)). A Lyt-2-bearing cell is involved in low-dose IL-2-mediated regression of CL8-2 micrometastases. In contrast, high-dose IL-2 can mediate significant tumor elimination independent of a Lyt-2-bearing cell. This suggests that CL8-2 micrometastases may be eliminated by at least two mechanisms mediated by IL-2; one involving Lyt-2 cells, and one that does not.

Discussion

Observations made in our laboratory and others suggest that regression of tumor in animals treated with IL-2 is mediated via the host immune system. The effect of IL-2 can be eliminated in animals treated with doses of radiation that suppress cell-mediated immunity (14). Similarly, lethally irradiated mice reconstituted with T cell–depleted bone marrow have complete abrogation of the IL-2 antitumor effect (11). Mule et al. (4) have demonstrated that treatment with high-dose IL-2 can mediate regression of 3-d pulmonary micrometastases from weakly immunogenic and nonimmunogenic sarcomas. Lyt-2+, as well as asialo GM1+ cells, were involved in regression of immunogenic tumors, though only asialo GM1+ cells were involved in IL-2-mediated regression of nonimmunogenic 3-d tumors. 10-d pulmonary macrometastases from immunogenic tumors were susceptible to therapy with high-dose IL-2, but macrometastases from nonimmunogenic tumors were not. An Lyt-2-bearing cell was involved in the regression of macrometastases from these weakly immunogenic sarcomas. L3T4-bearing cells were not involved in the regression of micro- or macrometastases from either non- or weakly immunogenic sarcomas treated with IL-2. These data indicated that Lyt-2+ cells are intermediaries in IL-2 immunotherapy of weakly immunogenic sarcomas.

Since the presence of Lyt-2, a murine T cell marker, determines both cytolytic function and class I MHC associated recognition of antigen (5), IL-2 may stimulate specific cytolytic T cells to recognize tumor antigens in association with self MHC. Class I MHC molecules are surface glycoproteins that serve as recognition structures in the interaction of cytolytic T cells and virus-infected cells (18), but little is known about their role in tumor cell recognition by cytolytic cells. Murine tumors induced by viral or chemical carcinogens can have attenuated surface expression of class I antigens (8–10). Similarly, many human tumors appear to be class I deficient, such as neuroblastomas (19), basal cell carcinomas (20), small cell lung tumors (21), or choriocarcinomas (22).

Modulation of class I expression on murine tumor cells by gene transfection has been shown to reverse oncogenesis (9), decrease metastatic capability (8), and potentiate immunogenicity (7, 10). Oncogenic strains of adenoviruses and retroviruses have evolved mechanisms to selectively downregulate MHC class I expression (23, 24). Reduced class I expression may represent a mechanism for tumor cells to evade destruction by cellular immunity, and may be an integral feature of oncogene-mediated cellular transformation (25).

As we have shown, IL-2 mediates regression of advanced weakly immunogenic
sarcoma metastases via a Lyt-2$^+$ T cell, and cannot impact on advanced metastases from nonimmunogenic sarcomas (4). Since interaction of Lyt-2$^+$ cells with tumor cells seemed necessary for the immunotherapeutic effect of IL-2 on advanced sarcoma metastases, we hypothesized that decreased sensitivity to IL-2 therapy would correlate with attenuated expression of class I MHC. Upregulation of class I should promote T cell-mediated tumor recognition and result in an increased sensitivity to IL-2 immunotherapy.

In accord with this hypothesis, the experiments presented herein show that weakly immunogenic tumors MCA-105 and -106 express class I MHC molecules in vivo and in vitro and are susceptible to therapy with high-dose IL-2; nonimmunogenic tumors 101 and 102 express little or no class I MHC in vivo or in vitro and advanced metastases are not sensitive to IL-2 therapy. None of the four tumors express class II MHC antigens. A murine melanoma that expressed minimal class I MHC was subcloned and transfected with genes encoding class I molecule K$^b$ or class II molecule Ia$^b$ (Tanaka, K., and G. Jay, unpublished data). Expression of class I MHC significantly increased the sensitivity of advanced 10-d metastases from the B16 transfectant tumor to IL-2 therapy. A more impressive increase in IL-2 susceptibility was seen when treating 3-d pulmonary micrometastases. Lower doses of IL-2, which did not cause regression of B16 transfectant 10-d macrometastases, strongly impacted on micrometastases.

Using in vivo antibody depletion to eliminate Lyt-2-bearing cells from treated mice, we have shown that high-dose IL-2-mediated regression of B16 transfectant macrometastatic tumor involves a Lyt-2$^+$ cell, possibly a tumor-specific cytolytic T cell. Two cell types appear to be involved in the regression of B16 transfectant 3-d micrometastases. At low doses of IL-2 the predominant cell appears to be Lyt-2$^+$. However, at higher doses of IL-2, regression of micrometastases can also be mediated by Lyt-2$^-$ cells, presumably LAK cells. These findings are in accordance with the work of Mulé et al. (4), in which weakly immunogenic sarcoma macrometastases, shown in this paper to express class I MHC were susceptible to IL-2 therapy, whereas nonimmunogenic sarcoma macrometastases shown herein to be devoid of class I MHC, were not susceptible to IL-2 therapy.

We thus postulate a model for IL-2-mediated antitumor effects in which Lyt-2$^+$ cells and LAK cells both play a role in regression of 3-d micrometastases, whereas only Lyt-2$^+$ cells are involved in elimination of established 10-d macrometastases. The expression of a single K$^b$ gene was sufficient to render a tumor sensitive to IL-2 immunotherapy. Class I MHC expression may be necessary for Lyt-2$^+$ cells to mediate the effects of high-dose IL-2 on established tumor since IL-2 immunotherapy had no impact on non-class I expressing macrometastases in the paper by Mulé et al. (4) or in our experiments. Regression of advanced tumor occurred only at the highest dose of IL-2 that was shown by Mulé et al. (4) to induce endogenous LAK activity in lung and splenic lymphocytes. However, antibody depletion experiments with Lyt-2 antibody established the necessary involvement of Lyt-2$^+$ cells in regression of macrometastases. These data suggest that tumor-specific cytolytic T cells may be the effectors of macrometastatic regression, and that tumor class I MHC antigens may be important for in vivo CTL recognition.
IL-2 mediates regression of 3-d micrometastases in a dose-dependent manner. At high doses of IL-2, both Lyt-2+ cells and endogenous LAK cells are involved in elimination of tumor in studies by Mule et al. (4). This antitumor effect was independent of the presence of class I MHC. Our work suggests that Lyt-2+ cells are not necessary for regression of B16 transfected micrometastases at high doses of IL-2 and tumor regression occurs in the presence or absence of class I MHC. At lower doses of IL-2, Lyt-2+ cells appear to be the major effectors of antitumor activity, and they do not impact on B16 micrometastases devoid of class I MHC. We conclude that IL-2 mediates its antitumor effect on 3-d micrometastases by at least two different mechanisms. Low-dose IL-2 stimulates antitumor activity of Lyt-2+ cells against class I-expressing micrometastases. Higher doses of IL-2 stimulate a broader population of effectors to show MHC-unrestricted antitumor activity, including Lyt-2+ and LAK cells. LAK cells are the predominant intermediary in regression of class I-negative micrometastases, whereas Lyt-2+ cells are more important in elimination of class I-expressing 3-d tumor.

The effects of IL-2 on advanced murine macrometastases are analogous with the immunotherapy of advanced cancer in humans. High-dose IL-2 alone or with the concurrent administration of LAK cells has been shown to be capable of causing significant objective tumor responses and a modest number of complete responses in a variety of human malignancies (26). Many tumors, however, remain resistant to IL-2 immunotherapy. Since advanced murine tumors that express class I MHC are more sensitive to IL-2 therapy, the patients whose tumors show no objective response to IL-2 may be deficient in class I expression at one or more of the HLA-A, -B, or -C loci. Ample evidence exists for downregulation of HLA class I loci in human tumors (22–24). We plan to assay levels of HLA-A, -B, or -C expression in tumors before IL-2 therapy to ask whether the level of class I expression predicts a response to IL-2. If this is true then patient tumor cell MHC modulation in vitro can be attempted using IFN-α or -γ or TNF-α, molecules known for their ability to upregulate HLA expression in human cells (27–29). Lymphocytes sensitized in vitro to tumor-expressing class I MHC may show increased antitumor activity after adoptive transfer.

Summary

We have shown that two weakly immunogenic MCA sarcomas developed in our laboratory that are sensitive to high-dose IL-2 immunotherapy express class I MHC in vivo and in vitro. Two nonimmunogenic MCA sarcomas are relatively insensitive to IL-2 therapy and express minimal or no class I MHC molecules in vivo and in vitro. To study the role of MHC in the therapy of tumors with IL-2, a class I-deficient murine melanoma, B16BL6, was transfected with the K\(^{b}\) class I gene. Expression of class I MHC rendered B16BL6 advanced pulmonary macrometastases sensitive to IL-2 immunotherapy. 3-d micrometastases of CL8-2, a class I transfected clone of B16BL6, were significantly more sensitive to IL-2 therapy than a control nontransfected line. Expression of I\(^{a}\), a class II MHC molecule, had no effect on IL-2 therapy of transfected pulmonary micrometastases in F\(_1\) mice. By using lymphocyte subset depletion with mAbs directed against Lyt-2, therapy of class I transfected macrometastases with high-dose IL-
2 was shown to involve an Lyt-2 cell. In contrast, regression of micrometastases treated with low-dose IL-2 involved Lyt-2+ cells, but regression mediated by high doses of IL-2 did not. We hypothesize that both LAK and Lyt-2+ T cells effect IL-2-mediated elimination of micrometastases, but only Lyt-2+ T cells are involved in macrometastatic regression. Low doses of IL-2 stimulate Lyt-2+ cells to eliminate class I-expressing micrometastases, but high doses of IL-2 can recruit LAK cells to mediate regression of micrometastases independent of class I expression. Only high-dose IL-2, mediating its effect predominantly via Lyt-2+ cells, is capable of impacting on MHC class I-expressing macrometastases. Macrometastases devoid of class I MHC antigens appear to be resistant to IL-2 therapy.

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