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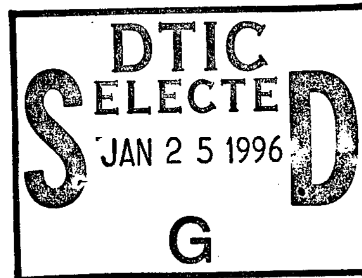

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Objective: To draw together in a common environment doctoral students from different basic science disciplines and provide them with a strong multidisciplinary background in breast cancer research.

Approach: 1) Provide future basic scientists (predoctoral students) in a given discipline (e.g., physiology, biochemistry, etc.) a stable multidisciplinary foundation from which to develop cutting-edge research in breast diseases, resulting in a dissertation dealing with a specific breast cancer research topic. 2) From these doctoral (Ph.D.) students, develop a cadre of postdoctoral fellows who will devote their entire professional careers to the field of breast cancer research and will be able to generate their own funding through competitive grants and contracts. It is expected that this group of researchers will swiftly and effectively provide translational (i.e., clinically relevant) bench research results into the clinical arena.

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T.K. Das Gupta — 10/26/95

PI - Signature — Date

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Two Reprints:
Waltz MR, Pandelidis SM, Pratt W, Barnes D, Das Gupta TK, Gendler SJ, Cohen EP. Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients.
Sun T, Kim TS, Waltz MR, Cohen EP. Interleukin-2-secreting mouse fibroblasts transfected with genomic DNA from murine neoplasms induce tumor-specific immune responses that prolong the lives of tumor-bearing mice. Cancer Gene Therapy 2:183-190, 1995.

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5. Introduction

The purpose of this grant is to draw together in a common environment doctoral students from different basic science disciplines and provide them with a strong multidisciplinary background in breast cancer research.

We have tried to provide the milieu of a multidisciplinary basic research forum for training the future generation of basic scientists so that they can develop an appropriate basis from which to pursue clinically relevant (i.e., translational) research in breast cancer. We have also tried to provide a stable multidisciplinary foundation from which to develop cutting-edge research in breast diseases, resulting in a dissertation dealing with a specific breast cancer research topic.

6. Body

The tasks listed in the grant proposal are:

Task 1: Provide to predoctoral fellows in various disciplines (e.g., Physiology, Biochemistry) a multidisciplinary basic foundation and emerging knowledge in breast cancer biology.

Task 2: Develop a well-focused dissertation theme related specifically to breast cancer.

Task 3: Generate a cadre of well-trained doctoral students (Ph.D.s) who will devote their professional careers to the field of breast cancer research and will be able to generate their own funding through competitive grants and contracts.

Task 4: Provide the milieu of a multidisciplinary basic research forum for training and educating clinical scientists, so that they can develop an appropriate basis from which to pursue clinical research.

To date (i.e., during the first year of this proposal), we have established a participating faculty committee for selection of the three graduate students funded by the grant.

The selection committee consists of Dr. Das Gupta (chairman) and Drs. E.P. Cohen (Department of Microbiology and Immunology), R.L. Davidson (Department of Genetics), R.R. Mehta (Department of Surgical Oncology), M.B. Mokyr (Department of Biochemistry), P. Raychaudhuri (Department of Biochemistry), and I.G. Roninson (Department of Genetics). This committee has selected the first three graduate students from the candidates nominated by each basic science department to be supported by this training grant. The candidates selected are Lisa Shamon, Margaret R. Waltz, and Lavanya Lall. These three were chosen from a pool of 22 candidates from all the basic science departments. The paramount reasons for selecting these three graduate students were their commitment to breast cancer research, their grades, and, finally, the disciplines in which they were pursuing their graduate studies. Ms. Shamon is a student in the Department of Medicinal Chemistry and Pharmacognosy in the College of Pharmacology; Ms. Waltz is a student in the Department of Microbiology and Immunology, College of Medicine; and Ms. Lall is in the Department of Genetics.

These graduate students are not only taking all the courses necessary to fulfill the requirements of the parent department, but also completing a course offered by the program faculty as a prerequisite for the Breast Cancer Research Fellowship. This is a specially designed course, titled "Basic Concepts in Cancer Biology." The objective of this course is to provide the predoctoral students with a larger vista in cancer biology, so that these students evolve into mature cancer biologists. Currently, this course is being offered in the form of a seminar once a month (2 hours); after the students complete all their required departmental courses, the seminar series will be more frequent.

Each of these trainees has also developed a research program dealing with some aspect of breast cancer, under the respective preceptorship of Drs. John M. Pezzuto (Department of Medicinal Chemistry & Pharmacognosy), Edward A. Cohen (Department of Microbiology and Immunology), and Richard L. Davidson (Department of Genetics). The trainees' dissertations will be written about the research performed in breast cancer. Dr. Das Gupta is a member of the respective thesis committees of these three graduate students. Descriptions and progress reports on their research activities are provided below.

<u>Research Projects of Lisa Shamon (Preceptor: Dr. J.M. Pezzuto)</u>

Summary

The proto-oncogene erbB-2 (also referred to as HER-2/neu) encodes a 185-kD ($p185^{neu}$) epidermal growth factor receptor-related transmembrane protein with tyrosine kinase activity. Amplification and overexpression of the erbB-2 proto-oncogene occur in as many as 30% of breast cancers and have been found to be associated with aggressive disease, increased probability of tumor recurrence, and poor patient survival rates. Furthermore, experimental evidence suggests that erbB-2 overexpression may play a critical role in the development of human tumors. Thus, an inhibitor of erbB-2 overexpression or tyrosine kinase activity may be able to repress cell transformation.

The overall goal of this project is to discover inhibitors of erbB-2 activity. In the first phase, approximately 290 compounds are being evaluated for cytotoxic potential against three cultured human breast cancer cell lines. Two of the cell lines, designated UISO-BCA-1 and MAXF-401, overexpress the erbB-2 (HER-2/neu) proto-oncogene, while the third cell line, UISO-BCA-2, does not. Of the compounds tested, 27 have so far demonstrated differential activity between at least one of the erbB-2-overexpressing and non-overexpressing cell lines. The ED_{50} values for the most active compounds to date are listed below.

			ED_{50}
Compound (unit)	UISO-BCA-1	MAX-F401	UISO-BCA-2
4-demethyldeoxypodophyllotoxin (mM)	<0.4	<0.4	49.0
genistein (mM)	12.2	8.8	57.2
robinetin (mg/ml)	39.0	0.9	ND
theonellisocyanide (mg/ml)	1.7	1.1	>20
vinblastine (mg/ml)	<0.5	<0.5	15.6

Some compounds evaluated in the present study have been shown previously to inhibit tyrosine kinases; many of the compounds are flavonoids. Therefore,

non-flavonoid compounds, including 4-demethyldeoxypodophyllotoxin and theonellisocyanide, will be given first priority for further investigation. These two compounds also demonstrated a greater difference in cytotoxicity than most other compounds tested in this study.

Methods

Cell culture conditions

MAXF-401, UISO-BCA-1, and UISO-BCA-2 human breast cancer cell lines were obtained from the Department of Surgical Oncology, University of Illinois at Chicago. MAXF-401 and UISO-BCA-2 were maintained in minimum essential media with Earle's salts (MEM-E) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B, 0.1 mM non-essential amino acids, 5 mg/ml insulin, and 15% fetal bovine serum. UISO-BCA-1 were maintained as above, but without insulin and with 5% bovine calf serum. All cells were plated in tissue culture flasks and incubated at 37°C in 95% air and 5% CO_2. The medium was changed twice weekly. Cells growing in a monolayer were passaged using trypsin-EDTA (0.5% trypsin, 0.2% EDTA in HBSS).

Evaluation of cytotoxic potential

The cytotoxic potential of each compound was determined according to a standard protocol. Cells in log-phase growth were harvested by trypsinization, quantified and diluted to 8-10 X 10^4 cells/ml using the same media preparations as described above. Test samples were initially dissolved in DMSO and diluted to a concentration of 10% DMSO. Further dilutions were made in 10% DMSO. 10 ml of test samples was added to wells of 96-well microtiter plates; 10% DMSO was used as a control. Cells (190 ml) were then added to the wells, and the plates were incubated at 37°C in 95% air with 5% CO_2 for 72 h. Zero-day controls were performed for each assay by plating cells into several wells of a 96-well plate and incubating for 30 min. At the end of the incubation period, cells were fixed with cold aqueous trichloroacetic acid. Plates were washed with tap H_2O, air-dried, and stained with 0.4% sulforhodamine B (SRB) in 1% HOAc. Free SRB was removed by washing with 1% HOAc. Plates were air-dried, then the bound dye was solubilized with 200 ml 10 mM Tris base (pH 10). Absorbance was measured at 515 nm with an ELISA plate reader. After correcting for zero-day controls, ED_{50} values were calculated relative to the solvent-treated controls.

Research Projects of Margaret R. Waltz (Preceptor: Dr. E.P. Cohen)

Margaret R. Waltz, a second-year graduate student in the Department of Microbiology and Immunology, is a trainee supported by the Army Training Grant. Ms. Waltz's current project is to investigate the immune response to polymorphic epithelial mucin (PEM), a previously described human breast cancer-associated antigen, in transgenic mice genetically modified to form PEM as a self-determinant. The cellular immunogen is a mouse breast cancer cell line modified to form PEM (E3 cells). The study is performed in collaboration with Dr. Joyce Taylor-Papadimitriou of the Imperial Cancer Research Fund Laboratories, London. Both the E3 cells and the transgenic mice modified to form PEM were prepared in Dr. Taylor-Papadimitriou's laboratory. In preparation for this project, Ms. Waltz spent two months training in Dr. Taylor-Papadimitriou's laboratory.

Thus far, Ms. Waltz has reconfirmed that E3 cells form progressively growing tumors in syngeneic mice. She is in the process of modifying the cells by retroviral transduction to form each of six different cytokines (IL-2, IL-4, TNF-A, GM-CSF, IL-12 and interferon-g) and will evaluate the cells' immunogenic properties in transgenic mice with established neoplasms.

Ms. Waltz was also the primary investigator in a study designed to detect alteration of microsatellite DNA in the neoplastic cells of breast cancer patients. The project was designed to determine if genetic instability was present in breast cancer cells. The results of her studies are summarized in the Appendix, in the manuscript titled "Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients." The manuscript is in the final stages of preparation and will be submitted for publication.

Ms. Waltz also participated in a project designed to investigate the immunogenic properties of a mouse fibroblast cell line modified to form IL-2 that was transfected with genomic DNA from various mouse neoplasms. The results of these investigations were recently published; a reprint is enclosed in the Appendix.

Sun T, Kim TS, Waltz MR, Cohen EP. Interleukin-2-secreting mouse fibroblasts transfected with genomic DNA from murine neoplasms induce tumor-specific immune responses that prolong the lives of tumor-bearing mice. Cancer Gene Therapy 2:183-190, 1995.

Participation in national meetings

Ms. Waltz was either the primary or co-author of five papers presented in national meetings. The published abstracts are:

Waltz M, Pandelidis S, Pratt W, Barnes D, Hand R, Das Gupta TK, Gendler SI, Cohen EP. Alterations in Mucin by PCR and Southern Blotting. 3rd International Workshop on Carcinoma-Associated Mucins, Cambridge, England

Cohen EP, Kim TS, Sun T, Waltz MR. Interleukin-2-secreting mouse fibroblasts transfected with genomic DNA from different mouse neoplasms induce tumor-specific immune responses that prolong the survival of tumor-bearing mice. Cold Spring Harbor Symposia, Conference on Gene Therapy. New York, 1994.

Cohen EP, Das Gupta T, Kim TS, Sun T, Waltz MR. Interleukin-2-secreting fibroblasts transfected with genomic DNA from mouse neoplasms induce tumor-specific immune responses that prolong the lives of tumor-bearing mice. Gene Therapy of Cancer, San Diego, 1994.

Cohen EP, Sun T, Kim TS, and Waltz MR. Immunization with IL-2-secreting mouse fibroblasts transfected with genomic DNA from different mouse neoplasms induce tumor-specific immune responses. American Association for Cancer Research Annual Meeting, 1995.

Cohen EP, Sun T, Kim TS, Waltz MR. The survival of tumor-bearing mice treated with LM fibroblasts transfected with genomic DNA from different mouse neoplasms is prolonged--the anti tumor immune responses are specific for the

type of tumor from which the DNA is obtained. Third International Congress on Biological Response Modifiers. Cancun, Mexico, 1995

Additional Training

During the year, Ms. Waltz was selected to be a participant in a week-long course sponsored by the American Association for Cancer Research, titled "Histopathology of Neoplasia."

Research Projects of Lavanya Lall (Preceptor: Dr. R.L. Davidson)

High levels of thymidine (dT) are mutagenic to mammalian cells in culture, due to their ability to inhibit the enzyme ribonucleotide reductase, which catalyzes the reduction of CDP to DCDP. Such inhibition of ribonucleotide reductase decreases DCDP pools and, consequently, DCTP pools, leading to an increase in the intracellular DTTP/DCTP ratio. Subsequent mispairing of thymine (as DTTP) with template guanine (G) leads to a GC->AT transition at the next round of replication. Such dT-induced mutagenesis exhibits a high degree of sequence specificity, occurring preferentially at the 3'G of runs of two or more adjacent guanine residues. Oncogenic activation of the human ras proto-oncogenes is often associated with GC->AT transitions at the 3'G of one or the other of several GG doublets in their coding regions. Such GC->AT transitions have also been observed in many human breast tumors with mutant p53 proteins. The role of sequence-specific misincorporation of dT as a source of sequence-specific mutagenesis leading to oncogenic activation has been investigated for the GG doublet within codon 12 (the most frequently mutated ras codon in solid human tumors) of the human H, K, and N ras oncogenes, as well as for some GG doublets in the p53 coding sequence that appear to be hot spots in human breast tumors. We used an in vitro misincorporation assay which employs two primers for each gene of interest, one of which extends by incorporation of a base opposite the 3'G and the other opposite the 5'G. The primer pairs were annealed individually to DNA templates containing the GG doublet of interest, in the presence of dT and repair-deficient Klenow polymerase. Analysis of the reaction products on DNA sequencing gels revealed 5- to 20-fold more efficient misincorporation of dT opposite the 3'G than opposite the 5'G of the various ras and p53 GG doublets. Competition experiments, in which dT and dc (the correctly matched base) were provided, revealed a similar propensity for misincorporation of dT opposite the 3'G as compared to the 5'G of a given doublet. The different abilities of the 3'G and 5'G residues of these doublets to mispair with dT in a repair-free environment may explain the preferential mutation of the 3'G (over the 5'G) in the ras and p53 GG doublets analyzed, if such mutations (found in transformed cells and human tumors) were a consequence of dT-induced increases in the intracellular dTTP/dCTP ratios.

7. Conclusions

We are pleased with the projects developed by the trainees and the rewarding results and promising futures of each project. The results of this research have been presented at national scientific meetings and submitted to peer-reviewed journals for publication.

We have chosen trainees from widely varied fields (Medicinal Chemistry & Pharmacognosy, Microbiology and Immunology, and Genetics), to attack the

problem of breast cancer from many directions. All three trainees are women, affirming our commitment to recruiting more minorities and women into the translational research arena.

8. References

Waltz MR, Pandelidis SM, Pratt W, Barnes D, Das Gupta TK, Gendler SJ, Cohen EP. Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients. In preparation

Sun T, Kim TS, Waltz MR, Cohen EP. Interleukin-2-secreting mouse fibroblasts transfected with genomic DNA from murine neoplasms induce tumor-specific immune responses that prolong the lives of tumor-bearing mice. Cancer Gene Therapy 2:183-190, 1995.

Waltz M, Pandelidis S, Pratt W, Barnes D, Hand R, Das Gupta TK, Gendler SI, Cohen EP. Alterations in Mucin by PCR and Southern Blotting. 3rd International Workshop on Carcinoma-Associated Mucins, Cambridge, England

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Cohen EP, Sun T, Kim TS, and Waltz MR. Immunization with IL-2-secreting mouse fibroblasts transfected with genomic DNA from different mouse neoplasms induce tumor-specific immune responses. American Association for Cancer Research Annual Meeting, 1995.

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Lall L. Sequence specific mutagenesis in human oncogenes. 12th Annual University of Illinois Biological Retreat. Lake Geneva, Wisconsin, September 9th, 1995.

Lall L, Davidson RL. Sequence specific mutagenesis in human oncogenes. In Preparation.

9. Appendix

Waltz MR, Pandelidis SM, Pratt W, Barnes D, Das Gupta TK, Gendler SJ, Cohen EP. Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients. In preparation.

Sun T, Kim TS, Waltz MR, Cohen EP. Interleukin-2-secreting mouse fibroblasts transfected with genomic DNA from murine neoplasms induce tumor-specific immune responses that prolong the lives of tumor-bearing mice. Cancer Gene Therapy 2:183-190, 1995.

Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients.

Margaret R. Waltz[1], Steven M. Pandelidis[1], Wendy Pratt[2], Diana Barnes[3], Roger Hand[1], Tapas Das Gupta[1], Sandra J. Gendler[4] and Edward P. Cohen[1,5]

Running head: Alterations of microsatellite DNA in breast cancer

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Abstract

Paired DNA samples from the neoplastic and non neoplastic cells of 118 patients with the sporadic, non familial form of breast cancer were analyzed for evidence of genetic alteration at each of five microsatellite loci, and at the polymorphic epithelial mucin locus (MUC1) gene. The analysis of the microsatellite loci was carried out by the polymerase chain reaction (PCR), using oligonucleotide primers that flanked the regions of interest. The variable number tandem repeat (VNTR) region of the MUC1 gene was analyzed by Southern blotting. The microsatellite loci investigated were D17s579, at 17q21, D18s34 at 18q21, D1S104 at 1q21, APO-A2 at 1q21 and a microsatellite within the MUC1 gene at 1q21. At least 87% of patients were heterozygotic at one or more locus. The results revealed an alteration of at least one locus in the neoplastic, but not the non neoplastic cells of 30.0% of the patients. Instability of microsatellite DNA was present in at least one locus in 17.5% of patients. The VNTR region within the MUC1 gene was altered in 61.4% of informative cases. There was no significant association, however, between changes at the VNTR region and an alteration of microsatellite DNA at any of the loci investigated. In 16.5 % of cases, hMSH2, a replication error repair (RER) gene mapped to 2p16, was altered. A C to T transition at the -6 position of the splice acceptor site, a conserved region, was detected in each of the altered cases. The hMSH2 locus was altered in both the neoplastic and non neoplastic cells of the same patients. There was no association between an alteration of the hMSH2 locus and the presence of microsatellite instability in this group of patients.

Introduction

Recent evidence indicates that an accumulation of genetic changes at specific loci within an individual somatic cell are responsible for conversion to the malignant phenotype (Fearon and Vogelstein, 1990). Subsequent genetic changes in the malignant cells as the tumor progresses may lead to the appearance of cell-derivatives with metastatic capability and resistance to drugs commonly used for chemotherapy.

The discovery of mismatch repair genes in mammalian cells (Fishel *et al.*, 1993; Leach *et al.*, 1993; Papadopoulos *et al.*, 1994; Bronner *et al.*, 1994), homologous to mismatch repair genes described previously in E. coli (Levinson *et al.*, 1987) and in yeast (Strand *et al.*, 1993) provided insight into a mechanism that might be responsible for the genetic changes. The data indicate that mutations in genes responsible for error repair during DNA replication lead to the accumulation of numerous genetic alterations. The hMSH2 gene, a replication repair gene mapped to chromosome 2p16 (Fishel *et al.*, 1993; Leach *et al.*, 1993) is one of several recently described mismatch repair genes.

The first indication of the replication error repair (RER^{+}) phenotype in cancer patients was the discovery of widespread alterations in microsatellite DNAs in malignant cells (Aaltonen *et al.*, 1993). Microsatellites are di-, tri, or tetranucleotide repeats present thoughout the genome (Weber and May, 1989). Their highly polymorphic nature, precise chromosomal localization, and relative ease of detection by the PCR render them ideal for the detection of genetic instabilities in neoplastic cells. Alterations in microsatellite DNAs have been found in colorectal carcinoma cells of patients with hereditary non-polyposis colon cancer (HNPCC) (Thibodeau *et al.*, 1993; Aaltonen *et al.*, 1993) and in the neoplastic cells of a small proportion of patients with bladder (Gonzalez-Zuluetta *et al.*,1993), endometrial (Risinger *et al.*, 1993) or gastric cancer (Peltomaki *et al.*., 1993). Whether or not alterations in microsatellite DNAs are present in the malignant cells of breast cancer

patients, and their possible relationship to mutations in the hMSH2 gene, is uncertain.

Here, we compared each of five polymorphic microsatellite loci in the neoplastic cells of breast cancer patients with the analogous loci in the non neoplastic cells of the same individuals. In total, 118 patients with the sporadic form of the disease were investigated. The analysis was performed by the PCR, using oligonucleotide primer DNAs that flanked the regions of interest. Three of the five microsatellite loci investigated were chosen because of their known association with disease. The locus at D17S579 is closely linked to the BRCA1 locus, involved in hereditary early-onset breast cancer (Hall *et al.*, 1992). Also, LOH at D17S579 has been demonstrated in sporadic breast cancers (Futreal *et al.*, 1992). D18s34 is linked to the DCC gene (Weber and May, 1990), a suspected tumor suppressor gene. Previously, Thompson *et al.*, (1993) detected LOH at the DCC locus in 31% of patients with non familial breast cancer. The third microsatellite investigated lies within intron 6 of the MUC1 gene, a site of frequent alteration in sporadic breast cancer. (Gendler et al., 1990). Two other loci at 1q21, APO A2 and D1s104, are not known to be primarily involved in breast cancer. locus (Pratt *et al*, unpublished) at 1q21. The results indicated that one or more of the microsatellite loci was altered in the neoplastic, but not the non neoplastic cells of 30.0% of the patients. The variable number tandem repeat region (VNTR) within exon 2 of the MUC1 gene was also investigated. Finally, the hMSH2 gene, a mismatch repair gene, was analyzed for alteration in the paired DNA specimens.

Materials and Methods.

Human tissue samples.

DNA was extracted from the neoplastic and non neoplastic cells of 118 patients with the sporadic, non familial form of breast cancer. Fresh solid tumor tissues, taken during the usual course of the patient's treatment, were dissected free of fat and stored in liquid nitrogen. DNA from the tumor was prepared from the tissue pellets used for estrogen and progesterone receptor analysis. This method conserved a portion of the neoplasm for subsequent use and ensured that the DNA was isolated from non-necrotic portions of the specimen. Peripheral blood buffy coat cells obtained at the time of surgery were used as the source of DNA from non neoplastic cells from the same individuals. The histological type and grade of the tumor were determined in stained paraffin-embedded sections, prepared according to conventional techniques.

Preparation of high molecular weight DNAs from paired neoplastic and non neoplastic cells of the same individuals with breast cancer.

High molecular weight DNAs were obtained from the neoplastic and non neoplastic cells of the same breast cancer patients. Frozen tumor tissues (approximately 0.3 gms) were disaggregated in a Braun (Melsuugen, Germany) Mikro-disembrator II, and the DNA was isolated from the cells in an Applied Biosystems (Foster City, CA) 340A DNA extractor. Non neoplastic cells from the patient's peripheral blood were lysed with water before addition to the extractor. After dialysis, the DNA concentrations were measured spectrophotometrically. Before the various analyses were performed, aliquots of undigested DNA from the paired samples were subjected to electrophoresis through 0.7% agarose gels, to ensure that they were undegraded.

Analysis of microsatellite DNAs from paired neoplastic and non neoplastic specimens of the same patients.

Analyses of each of five microsatellite loci were performed by the PCR, using oligonucleotide primers that flanked the region of interest. The loci investigated and the primer sequences are presented in Table 1. A 50 ul reaction mixture contained approximately 300 ng of genomic DNA from neoplastic or non neoplastic cells, 10X reaction buffer (consisted of 500 mM KCl, 100 mM Tris-HCl, pH 9.0 and 1% Triton X-100), 2mM $MgCl_2$, 1.25 mM of each dNTP, 50 pMol of each primer, 3 uCi α-^{32}P-dCTP and 0.5 units Taq polymerase (Promega, Madison,WI). The samples were overlaid with oil, placed in a thermocycler (Perkin-Elmer, Norwalk, CT) and subjected to 27 cycles of 94^0, 45 sec; 55^0, 45 sec; 72^0, 45 sec. The PCR products were separated in 8% polyacrylamide denaturing gels and then exposed to XAR-5 film (Kodak, Rochester, NY) at -70^0 for 1 to 3 days before the films were developed and analyzed.

In some instances, the studies were performed by the "Booster" PCR method. In the initial reaction, a 50 μl reaction mixture consisting of 300 ng genomic DNA in reaction buffer (0.55 mM $MgCl_2$, 50 mM each dNTP, 2.5% formamide, 50 pMol each primer and 0.5 units Taq polymerase) were overlaid with oil and cycled 14 times at: 94^0, 1 min.; 45^0, 1 min.; 72^0, 1 min. For the second phase, 50 ul of a mixture containing the reaction buffer, 2.35 mM $MgCl_2$, 150 uM each dNTP, 2.5% formamide, 50 pMol each primer,10 μCi α-^{32}P dCTP and 0.5 units Taq polymerase were added and the reaction was continued for another 20 cycles.

The autoradiographs were examined to determine if deletions, partial deletions, or instability was evident at each microsatellite locus in tumor DNA. .Instability of microsatellite DNA was indicated by a gain or loss of base pairs in one or both alleles, or the presence of additional bands in DNA from the neoplastic but not non neoplastic cells of the same individual.

Analysis of the VNTR region of the MUC1 gene at 1q21 in the neoplastic and non neoplastic cells of breast cancer patients.

Southern blotting was used to detect possible alterations in the VNTR region of the MUC1 gene in the neoplastic cells of the breast cancer patients. Approximately 10 μg of paired tumor and normal DNAs were digested to completion with Hinf1 (Gibco BRL, Gathersburg, MD), according to the directions of the supplier. Afterward, the digested DNAs were fractionated in 0.7% agarose gels. Hind III digested lambda DNA (Gibco BRL, Gathersburg, MD) was used as a fragment size reference. After fractionation, the DNAs were denatured in alkali and transferred to nylon membranes (Biodyne, Pall, Glen Cove, NY), according to methods described previously (Gendler*et al.*, 1990). After transfer, the membranes were baked for 1 hr at 80^{0} under vacuum, and then hybridized with a probe homologous to the VNTR region at exon 2 of the MUC1 gene (Gendler *et al.*, 1987). The probe was labeled to high specific activity with [α-^{32}P]-dCTP by the random priming method (Feinberg and Vogelstein, 1984). To reduce non specific binding, the hybridization reactions were performed at 42^{0} in the presence of non specific DNA (herring testis, Promega, Madison, WI) in a buffer containing 5X SSC (1X SSC is 150 mM NaCl, 15 mM trisodium citrate, (pH 7.0) and 50% formamide. After incubation, the filters were washed extensively, first at 55^{0} with 2 X SSC/0.1% $NaDoSO_4$, and then at 55^{0} with 0.1 X SSC/0.1% $NaDoSo_4$. The washed filters were exposed to XAR-5 film (Kodak, Rochester, NY) at -70^{0} for 1 to 3 days before the films were developed and analyzed.

Analysis by SSCP of the hMSH2 locus in the paired neoplastic and non neoplastic specimens of the breast cancer patients.

The analysis of the hMSH2 locus in the paired specimens was performed according to the method described by Orita *et al* (1989), using oliogonucleotide primers specific for codons 668-736 of the hMSH2 locus (Leach*et al.*, 1993). The primers were:

PF CGC GAT TAA TCA TCA GTG,
PR GGA CAG AGA CAT ACA TTT CTA T (Fishel*et al.*, 1993).

The reaction mixture consisted of 200 ng genomic DNA, 5 μl of 10X reaction buffer, 1.5 mM $MgCl_2$, 50 pMol of each primer, 25 nMol of each nucleotide, 10 μCi [α-^{32}P]-dCTP and 2.5 Units Taq polymerase in a total volume of 50 ul. The reaction mixtures were cycled 27 times, at 94°, 45 sec; 57°, 45 sec; and 72°, 45 sec for each cycle. Afterward, 6 ul of the mixture was heated to 95^0 for 3 minutes before it was placed in a neutral 7% polyacryamide gel containing 10% glycerol. The gels were subjected to electrophoresis at room temperature for 6 hrs at 50 watts. Afterward, they were dried and then exposed to XAR-5 film (Kodak, Rochester, NY) at -70^0 for 1 to 3 days. Sequencing of the PCR products was performed using a double strand DNA Cycle Sequencing System (Gibco/BRL, Gaithersburg, MD), according to the directions of the supplier.

Results

Alteration of microsatellite DNAs in breast cancer cells.

Paired DNA specimens from the neoplastic and non neoplastic cells of 118 patients with breast cancer were analyzed for alterations at each of five microsatellite loci. The loci and their chromosomal localizations were D17s579, at 17q21, D18s34 at 18q21, D1s104 at 1q21, APO-A2 at 1q21 and a microsatellite within the MUC1 gene at 1q21. Primer sequences are given in Table 1. Each of the patients was heterozygotic at one or more of the loci, and was therefore suitable for analysis.

A deletion, partial deletion or a change in size of one allele in the neoplastic, but not non neoplastic cells of the same patient, or the presence of additional allelic bands in the neoplastic cells was taken as an indication of an alteration. Representative examples are shown in Figure 1. At least one of the five loci examined was altered in 30.0% of informative cases. Six percent of the patients revealed an alteration at more than one locus. The incidence of an alteration at D17s579 and the microsatellite within the MUC1 gene was significantly ($p < .001$) higher than that of the other loci (Table 2).

Microsatellite instability was found in at least one microsatellite locus in 17.5% of patients. Instability at more than one locus was present in the tumor DNA of one patient. Complete or partial deletions of one allele was found in the neoplastic cells of 14.3% of cases at D17S579 and 12.1% of cases at the microsatellite within the MUC1 locus.

The use of the primers outside the microsatellite within the MUC1 locus resulted in the presence of secondary bands. Alteration of the secondary bands was found in the neoplastic but not non neoplastic cells of seven patients.

Alterations of the VNTR region of the MUC1 locus in the neoplastic but not non neoplastic cells of breast cancer patients.

The VNTR region within the coding region of the MUC1 gene (Figure 2), was analyzed by Southern blotting to determine if an alteration in this region was accompanied by changes at the microsatellite within the locus. Conceivably, the same molecular defect responsible for alterations in the microsatellites could affect the coding region of the MUC1 gene as well.

Paired DNA samples from the neoplastic and non neoplastic cells of 70 breast cancer patients were investigated. Fifty seven patients (81.4%) were heterozygous and were therefore informative. There was LOH in three cases (5.1%). In 32 patients, (56.1%) one of the two alleles was amplified in the neoplastic, but not the non neoplastic cells, as indicated by a hybridization signal of relatively greater intensity (Figure 1). The larger of the two alleles was amplified in 23 (40.3%) of the informative cases. In one specimen, a recombination event that included the MUC1 gene had taken place in the malignant, but not in the non malignant cells of the same individual (Figure 1).

DNAs from the neoplastic cells of patients with an alteration at the VNTR region were investigated for alterations at each of the five microsatellite loci, including the microsatellite within intron 6 of the MUC1 gene. The results failed to indicate a significant association between changes at the VNTR region within the coding region and alteration in microsatellites either within or without the gene ($p \geq .067$).

Alterations of the hMSH2 locus in the neoplastic and non neoplastic cells of breast cancer patients.

The hMSH2 locus is a site of alteration in patients with HNPCC (Fishel *et al.*,1993). To determine if an analogous alteration was present in the hMSH2 locus in the neoplastic or non neoplastic cells of breast cancer

patients, the paired DNA-extracts were investigated by SSCP, using primers for codons 668-736 of the hMSH2 locus (Leach *et al.*, 1993). This portion of the gene is conserved across species and is believed to code for the DNA binding region of a mismatch binding protein. The results indicated that an alteration of the hMSH2 locus was present in 16 of 97 cases examined (16.4 %). In each instance, however, an alteration in the neoplastic cells was accompanied by the same alteration in the non neoplastic cells of the same individual. It is likely that the alterations represented a polymorphism or a germ line mutation. A typical result is indicated in Figure 3.

The region (codon numbers 668 to 736 and flanking sequences; corresponding to bps 2072 to 2208) was sequenced in patients revealing an alteration by SSCP. As indicated (Figure 4), a T to C substitution at the -6 position of the splice acceptor site was found. It was present in both the neoplastic and non neoplastic cells of the same individuals. Eight paired specimens which failed to indicate an alteration at the hMSH2 locus by SSCP were sequenced as well. No alterations were detected in these cases.

We made an attempt to associate changes at the hMSH2 locus with alterations in the microsatellite DNAs in neoplastic cells from the same individuals. There was no significant association between a change in the hMSH2 gene and a change in any of the microsatellite loci investigated. The one patient whose tumor demonstrated instability of microsatellite DNA at two of the five loci investigated did not have an alteration at the hMSH2 locus.

Discussion.

Microsatellite DNA was first described in humans and has been found in almost all other eukaryotes investigated. In humans, 76 percent of microsatellites are repeats of A, CA, AAAN, AAN or AG, in decreasing order of frequency. The repeated bps are found in 5' and 3' untranslated regions, and introns (Tautz and Renz, 1984). The repetitive regions are

normally stable and the number of base pairs in the repeat is highly polymorphic. The segregation of alleles of heterozygous parents is considered to be unambiguous. These highly polymorphic microsatellite loci have proved useful in the development of genetic maps and in the detection of chromosomal aberrations in disease.

Here, we described the results of a comparative analysis of five microsatellite loci along with the VNTR region of the MUC1 gene in malignant and non malignant cells of 118 breast cancer patients. The investigation was an extension of prior studies in which we found that the MUC1 gene was frequently altered in patients with primary breast carcinomas (Gendler *et al.*, 1990).

As reported by others, we too found LOH in the region of the BRCA1 locus. In this instance, the neoplastic cells of 14.3% of patients with sporadic breast cancer demonstrated LOH. Thus, an alteration at this locus is not limited to patients with familial neoplasia.

Analysis of the microsatellite within the MUC1 gene revealed alterations in the secondary bands in 7 of 99 informative cases. Weber and May (1990) stated that the formation of secondary bands is a consequence of PCR, and not secondary to genetic mosaicism. We included changes in secondary bands in our overall analysis of alterations at the CA repeat within the MUC1 gene.

We were unable to detect an association between alterations in the VNTR portion of the MUC1 gene with mutations in the microsatellite within the locus. Sixty one percent of patients revealed a deletion or amplification of the MUC1 gene, and 23.3% of patients had an alteration of the microsatellite within the gene. However, in those instances in which one portion of the gene was altered, the other portion had a no greater than random likelihood of exhibiting a change. Most changes within the VNTR region of the MUC1 gene were amplifications. Given the proximity of the CA repeat at intron 6 of the gene, one would also predict a similar change there. Amplification of a microsatellite by the PCR would be difficult to detect given

that the PCR is not generally considered to be a quantitative technique. Also, amplifications of the MUC1 gene may be limited to the VNTR region. Deletions of the CA repeat at intron 6 of MUC1 in a tumor were not accompanied by deletions at the VNTR region. Perhaps what appeared to be amplifications of the VNTR region in one allele of a tumor, could have actually represented a deletion of the opposite allele with background contamination by non neoplastic DNA.

Investigators have described specific genetic changes in HNPCC and other cancers in the HNPCC syndrome.(Leach et al., 1993; Fishel et al., 1993; Peltomaki et al.,1993; Papadopoulos et al.1994; Bronner et al. 1994) The RER^{+} phenotype has not only been found in HNPCC colon cancers, but also in sporadic tumors which are part of the HNPCC syndrome. (Peltomaki et al, 1993).

Whether or not breast cancer is part of the HNPCC syndrome is a uncertain. Lynch and others (Lynch et al 1991) report no excess of breast cancer cases in HNPCC kindreds as compared to the normal population, while Itoh and others (Itoh et al.,1990) report a five-fold incidence of breast cancer in HNPCC families.

Peltomaki and others (Peltomaki et al., 1993) found no sporadic breast cancers which demonstrated the RER+ phenotype. They did not find a single incident of microsatellite instability among 7 microsatellite loci examined in 84 breast cancers.

In our investigation of sporadic breast cancer, microsatellite instability was occasionally detected; however most of the alterations detected were losses of heterozygocity Only one of our tumors would fit the RER+ phenotype as defined by Aaltonen and others. (Aaltonen et al., 1993) These investigators defined the RER+ phenotype as microsatellite instability in at least 2 of 7 microsatellites.

The microsatellite instability we detected appears different than that demonstrated in tumors of the HNPCC syndrome.(Aaltonen et al., 1993; Gonzalez-Zuluetta et al., 1993; Petolmaki et al., 1993; Risinger et al., 1993) In those tumors, one sees expansions from the two original alleles of a microsatellite to multiple alleles which represent inadequately repaired DNA replication errors resulting from multiplications of the original error-prone clone. The microsatellite instability we detected only on rare occasions showed expansions. Usually what we saw were changes in size of one of the alleles, or just on or two additional bands.(Figure 1) Wooster and others (Wooster et al., 1994) also found similar sorts of changes when they examined microsatellites in breast cancer.

Fishel *et al.* (1993) and Leach *et al.* (1993) found that abpproximately 60% of patients with colon cancer with the RER+ phenotype had mutations of the hMSH2 gene. In an analogous manner, we attempted to detect changes at the hMSH2 locus in breast cancer patients. No significant association between an alteration at the hMSH2 locus and alterations of the microsatellite DNAs were detected. The mutation we detected, a T to C substitution at the -6 position of the splice acceptor site, was present in 16 of 97 cases. Since it was present in both the neoplastic as well as non neoplastic cells of the same individual, it was likely a polymorphism unrelated to the disease. Leach *et al.* (1993) found the same mutation in 2 of 20 tumor-free individuals. The one patient in our series whose tumor fit Aaltonen's definition of the RER+ phenotype did not have an alteration of the portion of the hMSH2 gene we studied.

It is likely that the rare microsatellite instability we detected results from a different mechanism than seems to be responsible for the widespread instability seen in cancers of HNPCC and the HNPCC syndrome. Our results confirm the high frequency of alteration in the region of the BRCA1 locus and in the region of the MUC1 gene in sporadic breast cancer.

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TABLE 1

SEQUENCE OF PRIMERS USED FOR ANALYSIS OF MICROSATELLITES

MICROSATELLITE	PRIMER SEQUENCE	REFERENCE
D17S579	5'-AGT CCT GTA GAC AAA ACC TG 5'-CAG TTT CAT ACC AAG TTC CTA	Hall et al.,1992
D18S34	5'-CAG AAA ATT CTC TCT GGC TA 5'-CTC ATG TTC CTG GCA AGA AT	Weber and May,1990
APO-A2	5'-GGT CTG GAA GTA CTG AGA AA 5'-GAT TCA CTG CYG TGG ACC CA	Weber and May,1989
D1S104	5'-ATC CTG CCC TTA TGG AGT GC 5'-CCC ACT CCT CTG TCA TTG TA	Weber et al.,1990
MS within MUC1	5'-AGG AGA GAG TTT AGT TTT CTT GCT CC 5'-TTC TTG GCT CTA ATC AGC CC	Pratt, unpublished

TABLE 2

FREQUENCY OF ALTERATION IN BREAST CANCER CELLS OF MICROSATELLITE DNAs AT MUC1 AND FOUR OTHER LOCI

	MUC1	D17S579	D18S34	D1S104	APOA2
# of cases	104	101	94	74	70
# of informative cases	100 (96.1%)	90 (89.1%)	81 (87.2%)	65 (87.8%)	70 (100%)
Total # of changes	24 (23.3%)	17 (18.7%)	5 (6.2%)	3 (4.7%)	2 (2.8%)
Deletion	5	9	3	0	0
Partial Deletion	7	4	1	0	0
Change in size	4	4	1	2	1
2o band alteration	7	0	0	0	0
Additional bands	1	0	0	1	1

LEGENDS

Figure 1: Representative examples of alterations of microsatellite DNAs and the MUC1 locus
B represents blood (non neoplastic) DNA, and T represents tumor DNA.

Figure 2: MUC1 gene
The variable number tandem repeat region (VNTR) is located within exon 2 of the gene. The CA repeat is located within intron 6 of the gene.

Figure 3: Detection of hMSH2 alteration by SSCP
Lanes 1 and 2 are the blood and tumor DNA (respectively) of a patient with unmodified hMSH2, as indicated by analysis by SSCP. Lanes 3 and 4 represent blood and tumor DNA (respectively) of a patient with an alteration of hHMSH2.

Figure 4: Sequencing of a portion of the hMSH2 gene
Arrow denotes change of T (left gel) to C (right gel) at the -6 position of splice acceptor site in hMSH2.

Interleukin-2–secreting mouse fibroblasts transfected with genomic DNA from murine neoplasms induce tumor-specific immune responses that prolong the lives of tumor-bearing mice

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Genetic alterations are a common feature of the malignant phenotype. Among other properties, altered genes may be responsible for invasion and metastasis, as well as for resistance to chemotherapeutic agents. Under appropriate circumstances, the products of other altered genes expressed by malignant cells may act as tumor-associated T-cell epitopes, capable of provoking antitumor immune responses. As a novel means of augmenting the immunogenicity of the gene products, unfractionated, sheared genomic DNA from various tumor cell lines (B16F1 melanoma, B16F10 melanoma, MOPC-315 plasmacytoma, C1498 lymphoma, or J558 myeloma), or from non-neoplastic liver cells of tumor-free mice, was transfected into LM cells, a mouse fibroblast cell-line ($H-2^k$) that had been modified previously by retroviral gene transfer to secrete interleukin-2 (IL-2). The IL-2–secreting transfected cell populations were then tested for their immunogenic properties toward B16F1 ($H-2^b$) or C1498 ($H-2^b$) cells in syngeneic C57BL/6 mice. The antitumor responses were specific for the type of tumor from which the DNA was obtained. The survival of C57BL/6 mice injected with a mixture of viable B16F1 cells and IL-2–secreting LM cells transfected with DNA from B16F1 cells was significantly prolonged. In a similar manner, the survival of C57BL/6 mice injected with a mixture of C1498 cells and IL-2–secreting LM cells transfected with DNA from C1498 cells was prolonged as well. The immunity was mediated predominantly by $CD8^+$ and natural killer/lymphokine-activated killer (NK/LAK) cells. These data raise the possibility that a cell line altered previously for cytokine secretion may be readily modified to provide immunologic specificity for the neoplasms of individual cancer patients.

Key words: Interleukin-2; immunity; melanoma; lymphoma.

Numerous reports[1–3] indicate that the secretion of various cytokines by tumor cells augments their immunogenic properties. The cytokine-secreting cells are rejected by experimental animals that are susceptible to the growth of the unmodified cells. The injection of inbred mice with syngeneic neoplastic cells genetically modified to secrete interleukin-2 (IL-2),[4–6] IL-4,[7,8] IL-6,[9,10] IL-7,[11,12] interferon-gamma (IFN-γ),[13,14] tumor necrosis factor-alpha (TNF-α),[12] and granulocyte-macrophage colony stimulating factor (GM-CSF),[3] among others, resulted in antitumor cellular immune responses that in some instances were capable of prolonging the lives of mice with established neoplasms. Because the antitumor responses were directed toward the neoplastic cells alone and nonmalignant cells were unaffected, it is likely that the immunity was directed toward unique T-cell determinants expressed by the malignant cells.

The identity of the products of genes specifying tumor-associated T-cell epitopes has not been completely established. In some instances, the T-cell epitopes may result from the modification of genes specifying various products of non-neoplastic cells. The products of mutated or rearranged oncogenes, for example, were found to act as "targets" of immune-mediated attack.[15,16] Alterations of genes in neoplastic cells that controlled the expression of genes such as MAGE[17] or tyrosinase,[18] or of genes specifying proteins designated gp100 and MART-1,[19] have also been described. These examples may be only several representatives of a larger number of analogous genetic events in tumor cells resulting in an array of tumor-associated determinants. Under appropriate circumstances, the gene products may become antigenic.

Here, we describe the immunogenic properties of an IL-2–secreting mouse fibroblast cell line that was transfected with sheared, unfractionated genomic DNA from different mouse neoplasms. We reasoned that certain of the undefined genetic events in the neoplastic cells that resulted in the formation of tumor-associated T-cell epitopes might be represented as gene products in the population of cytokine-secreting, transfected cells. We reasoned further that the number of cells in the subpopulation of transfected cells that expressed the prod-

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ucts of such genes, represented as tumor-associated T-cell epitopes, would be sufficient to induce antitumor immune responses in tumor-bearing mice.

The results indicated that immunization with the population of transfected cells resulted in NK/LAK and $CD8^+$ cell-mediated antitumor immune responses that were specific for the type of tumor from which the DNA was obtained.

MATERIALS AND METHODS

Cell lines and experimental animals

B16F1 ($H\text{-}2^b$) is a highly malignant melanoma cell-line derived from a tumor that occurred spontaneously in a C57BL/6 mouse ($H\text{-}2^b$). B16F10 is a derivative selected for its metastatic properties. Both cell-lines, obtained originally from I. Fidler (M.D. Anderson Cancer Center, Houston, Tex), were maintained by serial passage in histocompatible C57BL/6 mice (Jackson Laboratories, Bar Harbor, Me) or at 37°C in a humidified 7% CO_2/air mixture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (growth medium) (GIBCO BRL, Grand Island, NY). MOPC-315 cells ($H\text{-}2^d$), a mineral oil–induced plasmacytoma of BALB/c mouse origin ($H\text{-}2^d$) (from M. Mokyr, University of Illinois, Chicago, Ill), were maintained by serial passage in BALB/c mice ($H\text{-}2^d$). C1498 cells ($H\text{-}2^b$), a spontaneously occurring mouse lymphoma cell line, J558 cells ($H\text{-}2^d$), a mineral oil–induced mouse myeloma cell line, and LM cells ($H\text{-}2^k$), a mouse fibroblast cell line, were obtained from the American Type Culture Collection (Rockville, Md). Each cell line was maintained at 37°C in a humidified 7% CO_2/air atmosphere in growth medium. IL-2–dependent CTLL-2 cells (from A. Finnegan, Rush Medical College, Chicago, Ill) were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L glutamine, 0.5 mmol/L sodium pyruvate, 15 mmol/L HEPES, 5×10^{-2} mmol/L 2-β-mercaptoethanol, and 100 U/mL IL-2 (Genzyme, Cambridge, Mass).

Eight- to 12-week-old pathogen-free C57BL/6 or BALB/c mice were maintained and treated according to National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. The mice were obtained from the Jackson Laboratories, Bar Harbor, Me.

Preparation of IL-2–secreting LM cells

LM cells were modified for IL-2 secretion by transduction of the retroviral vector pZipNeoSVIL-2[20] (from M.K.L. Collins, Institute of Cancer Research, London, England). The vector specified the human IL-2 gene and the *neo^r* gene, both under control of the Moloney leukemia virus long terminal repeat (LTR). The *neo^r* gene conferred resistance to the aminoglycoside antibiotic, G418.[21] For use as controls, aliquots of the suspension of LM cells were transduced with pZipNeoSV(X) (from R. Mulligan, Whitehead Institute for Biomedical Research, Cambridge, Mass). Like pZipNeoSVIL-2, pZipNeoSV(X) specified the *neo^r* gene but lacked the gene for IL-2. To select transduced cells, the cell population was maintained for 14 days in growth medium containing 400 μg/mL G418. One hundred percent of nontransduced LM cells maintained in the selection medium died within this period. Colonies of cells proliferating in the medium were pooled for later, additional modification.

Secretion of IL-2 by the G418-resistant cells was assayed with the IL-2–dependent cell line, CTLL-2,[22] as described previously.[23] One unit of IL-2 resulted in half-maximal proliferation of CTLL-2 cells. Every third transfer, the IL-2–secreting LM cells (LM–IL-2 cells) were passaged in growth medium containing 300 μg/mL G418 to assure that IL-2 secretion was maintained.

Preparation of B16F1 antibodies

Antibodies reactive with B16 cells were raised in C57BL/6 mice by repeated intraperitoneal and subcutaneous injections of irradiated (5000 rad) B16 cells in complete Freund's adjuvant (Spex Industries, Metuchen, NJ), as described previously.[28] The ascitic fluids and sera reacted with B16 cells, but not with a variety of cells from various organs and tissues of tumor-free C57BL/6 mice.[24]

Immunofluorescent staining and cytofluorometric measurements

Quantitative immunofluorescence measurements were performed in an Epic V flow cytofluorograph (Coulter Electronics, Hialeah, Fla) equipped with a multiparameter data-acquisition and display system (MDADS), as described previously.[20] One-parameter fluorescence histograms were generated by analyzing at least 1×10^4 cells.

Transfection of LM–IL-2 cells with genomic DNA from B16F1 melanoma, B16F10 melanoma, MOPC-315 plasmacytoma, J558 lymphoma, or C1498 lymphoma cells, or with DNA from the livers of tumor-free C57BL/6 mice

High molecular DNA was isolated from each of the tumor cell lines, or from liver cells of tumor-free naive C57BL/6 mice, using the method described previously.[25] The DNAs were sheared by passage through a 25-gauge needle before they were used to transfect the IL-2–secreting LM cells. Ten milligrams of DNA from one of the tumor cell types, or from the liver cells, was mixed with pHyg (1 μg) (from L. Lau, University of Illinois, Chicago, Ill) and added to approximately 1×10^6 LM–IL-2 cells. The cationic liposome-mediated transfection method[26] (Lipofectin, GIBCO BRL) was applied to facilitate DNA uptake. The plasmid pHyg carries an *Escherichia coli* gene encoding a hygromycin B phosphotransferase.[27] As an additional control, other LM–IL-2 cells were transfected with pHyg (1 μg) alone. Afterward, the cells were maintained for 14 days in growth medium containing 400 μg/mL hygromycin (ICN Biochem, Cleveland, Ohio). One hundred percent of non-

transfected LM–IL-2 cells maintained in the hygromycin-growth medium died within this period. The surviving colonies (more than 6×10^3 in each instance) were pooled and used in the experiments described below.

Preparation of spleen cell suspensions for cytotoxicity determinations

Mononuclear cells from the spleens of C57BL/6 mice immunized with the population of IL-2–secreting LM cells transfected with DNA from one of tumor cell lines were used as sources of effector cells for the cytotoxicity determinations. Spleen cell suspensions were prepared from the immunized mice by forcing the spleens through a 40-gauge stainless steel screen using a small quantity of growth medium to aid dispersion. Afterward, the cells were transferred to 15 mL conical centrifuge tubes (Sarstedt, Newton, NC). Large clumps of cells and cell debris were allowed to settle for 1 minute. The cells remaining in the supernatant were collected, overlaid onto a Ficoll/Hypaque gradient (Pharmacia, Piscataway, NJ), and then centrifuged (2000 rpm) for 30 minutes at room temperature. The viability of the mononuclear cells collected from the gradient was greater than 98%, as determined by the exclusion of trypan blue dye (0.4%). Before the cytotoxicity determinations were performed, aliquots of the cell suspensions were coincubated in growth medium at 37°C for 5 days with mitomycin C–treated (Sigma Chemical, St Louis, Mo) (50 μg/mL for 45 minutes at 37°C) cells of the same type as used to immunize the mice. After incubation, the population that failed to adhere to the plastic cell culture flasks was collected and used as the source of effector cells for the cytotoxicity determinations against the tumor cells.

Spleen cell–mediated cytotoxicity toward the tumor cells

Spleen cells from mice immunized with one of the transfected cell lines were tested for their cytotoxic effects toward the tumor cells, using a standard ^{51}Cr-release assay. Briefly, 5×10^6 tumor cells as indicated in the relevant tables were labeled with ^{51}Cr during a 1-hour incubation at 37°C in growth medium containing 100 μCi of ^{51}Cr (Amersham, Arlington Heights, Ill). After three washes with Dulbecco's modified Eagle's medium, 1×10^4 ^{51}Cr-labeled B16 cells were incubated for 4 hours at 37°C with the non–plastic-adherent population of spleen cells from the immunized mice at varying effector:target (E:T) ratios. The percentage of specific cytolysis was calculated as follows:

$$\frac{\text{Experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{Maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100$$

In some instances, asialo GM1 monoclonal antibodies (MAbs) (Wako Chemical, Dallas, Tex) or CD8 MAbs (Pharmingen, San Diego, Calif) were added to the cultures before the ^{51}Cr-release assays were performed.

Statistical analyses

Life table methods and log rank analyses were used to determine the statistical differences between the proportion of mice surviving after the different treatments. Student's t test was used to determine the statistical differences between cytotoxic activities in mice in various experimental and control groups. A P value of less than .05 was considered significant.

RESULTS

Modification of LM mouse fibroblasts to secrete IL-2

LM cells, a mouse fibroblast cell line of C3H mouse origin (H-2^k), were modified for the secretion of IL-2 by transduction of the retroviral vector pZipNeoSVIL-2.[23] The transduced cells formed approximately 10,000 U IL-2 in 48 hours (LM–IL-2 cells). Nontransduced LM cells or LM cells transduced with the IL-2–negative vector pZipNeoSV(X) did not form detectable amounts of IL-2. Every third passage, the LM–IL-2 cells were placed in medium containing G418. Under these circumstances, the G418-resistant cell population formed equivalent quantities of IL-2 for more than 6 months of continuous culture. An analysis by Southern blotting indicated that two copies of the IL-2 gene had been integrated into genomic DNA of the transduced LM cells (these data are not presented).

The survival of tumor-bearing mice treated with IL-2–secreting fibroblasts transfected with genomic DNA from one of several mouse neoplasms

To test the hypothesis that genetic alterations in the neoplastic cells included changes that resulted in the formation of (undefined) gene products that might act as tumor-associated antigens, LM–IL-2 cells were transfected with sheared, unfractionated genomic DNA from B16F1, B16F10, C1498, J558, or MOPC-315 cells. DNA from pHyg, a plasmid-carrying hygromycin resistance gene, was included for selection. In parallel, LM–IL-2 cells were cotransfected with pHyg and DNA from non-neoplastic (liver) cells of tumor-free, naive C57BL/6 mice. The size of the sheared genomic DNAs was greater than 23 kb, as determined by standard agarose gel electrophoresis techniques. After selection in growth medium containing a sufficient concentration (400 μg/mL) of hygromycin to kill 100% of nontransfected cells, the surviving colonies were pooled and used in the experiments described below. In each instance, a pool of at least 6000 colonies of cells proliferating in the selection medium was used. After transfection, the hygromycin-resistant cells were reassayed for IL-2 secretion. LM–IL-2 cells transfected with DNA from the tumor cells, or from the liver cells, formed the same amounts of IL-2 as LM–IL-2 cells.

To detect antitumor immunity, C57BL/6 mice were injected subcutaneously with a mixture of viable C1498 lymphoma cells and LM–IL-2 cells transfected with DNA from C1498 cells (LM–IL-2/C1498). The mice received two additional subcutaneous injections at

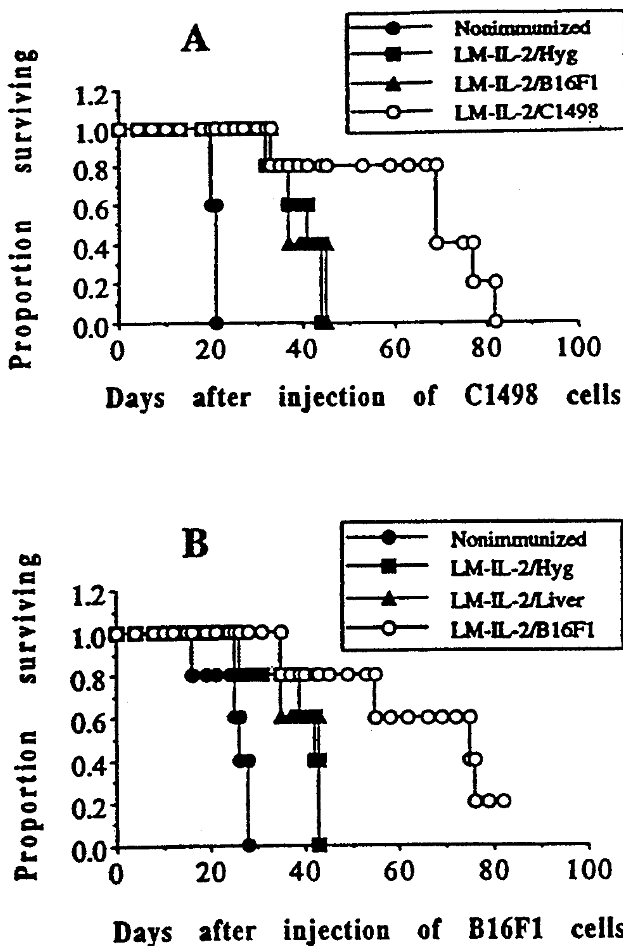

Figure 1. The survival of mice injected with a mixture of B16F1 melanoma or C1498 lymphoma and LM–IL-2 cells transfected with genomic DNA from B16F1 or C1498 cells. C57BL/6 mice (five per group) were injected with 4×10^6 cells of one of the cell types and 1×10^5 C1498 cells **(A)** or 5×10^3 B16F1 cells (B). The mice received two weekly additional injections with 4×10^6 cells of the same IL-2–secreting cell types used for the first injection, but without additional tumor cells. **(A)** Median survival time (days) after the injection of C1498 cells: Nonimmunized, 21.6 ± 3.7; LM–IL-2/Hyg, 39.6 ± 5.1; LM–IL-2/B16F1, 39.4 ± 5.4; LM–IL-2/C1498, 66.0 ± 19.3. $P < .03$, LM–IL-2/C1498 versus LM–IL-2/B16F1. **(B)** Median survival time (days) after the injection of B16F1 cells: Nonimmunized, 24.6 ± 5.0; LM–IL-2/Hyg, 38.4 ± 7.7; LM–IL-2/Liver, 38.0 ± 7.6; LM–IL-2/B16F1, 64.6 ± 19.4. $P < .03$, LM–IL-2/B16F1 versus LM–IL-2/Liver.

weekly intervals of viable LM–IL-2/C1498 cells alone. The survival of these mice was compared with the survival of mice injected with viable C1498 cells and LM–IL-2 cells transfected with genomic DNA from B16F1 cells (LM–IL-2/B16F1), followed by two additional injections of LM–IL-2/B16F1 cells alone. Other C57BL/6 mice were injected with C1498 cells and LM–IL-2 cells transfected with pHyg alone (LM–IL-2/Hyg cells), or the mice were injected only with C1498 cells.

The results (Fig 1A) indicate that mice injected with C1498 cells and LM–IL-2/C1498 cells survived significantly ($P < .03$) longer than mice in any of the control groups, including mice injected with C1498 cells and LM–IL-2/B16F1 cells. The survival of mice injected with C1498 cells and LM–IL-2/B16F1 cells was not significantly different from that of mice injected with C1498 cells and LM–IL-2/Hyg cells. Mice injected with C1498 cells alone died in significantly shorter intervals than mice injected with C1498 cells and LM–IL-2/C1498 cells ($P < .01$).

To determine if LM–IL-2 cells transfected with DNA from nonneoplastic (liver) cells of tumor-free C57BL/6 mice (LM–IL-2/liver) exhibited analogous antitumor effects, C57BL/6 mice were subcutaneously injected with a mixture of viable B16F1 cells and LM–IL-2/liver cells or with a mixture of B16F1 cells and LM–IL-2/B16F1 cells. As indicated (Fig 1B), the survival rate of mice injected with B16F1 cells and LM–IL-2/liver cells was significantly ($P < .03$) less than the survival rate of mice injected with B16F1 cells and LM–IL-2/B16F1 cells. The survival of mice injected with B16F1 cells and LM–IL-2/liver cells was not significantly different from that of mice injected with B16F1 cells and LM–IL-2/Hyg cells. Mice injected with B16F1 cells alone died in shorter intervals than mice in any of the other groups.

Thus, the most significant antitumor effects were in mice injected with a mixture of C1498 cells or B16F1 cells and LM–IL-2 cells transfected with genomic DNA from the same tumor cell type.

Antitumor cytotoxic immune responses generated in C57BL/6 mice injected with LM–IL-2/B16F1 or LM–IL-2/C1498 cells were specific for the type of tumor from which the DNA was obtained

To further investigate the immunologic specificity of the antitumor immune responses in mice injected with the transfected cells, C57BL/6 mice were subcutaneously injected with viable LM–IL-2/B16F1 cells or LM–IL-2/C1498 cells. The mice received three injections of viable LM–IL-2/B16F1 cells or LM–IL-2/C1498 cells, respectively, at weekly intervals. One week after the last injection, mononuclear cells from the spleens were tested for reactivity toward ^{51}Cr-labeled B16F1 cells or ^{51}Cr-labeled C1498 cells. As controls, other C57BL/6 mice were injected according to the same schedule with LM–IL-2 cells transfected with DNA from J558 cells (LM–IL-2/J558), with LM–IL-2/liver cells, or with LM–IL-2/Hyg cells. One group of mice was not injected. The results (Table 1) indicate that the cytotoxic activity (percent cytolysis) toward B16F1 cells in mice injected with LM–IL-2/B16F1 cells was significantly ($P < 0.001$) higher than that present in mice injected with LM–IL-2/C1498 cells or with any of the other control groups.

At the same time, other naive C57BL/6 mice were injected with LM–IL-2/C1498 cells, or, as controls, with LM–IL-2/B16F1, LM–IL-2/J558, LM–IL-2/liver, or LM–IL-2/Hyg cells, or the mice were not injected. Analogous to the previous finding, the cytotoxic activity of greatest magnitude toward C1498 cells was found in the population of spleen cells from mice injected with LM–IL-2/C1498 cells; it was significantly ($P < .001$) higher than

Table 1. Antitumor Cytotoxic Immune Responses in C57BL/6 Mice Immunized With IL-2–Secreting Cells Transfected With Genomic DNA From Various Mouse Neoplasms

		Percent Cytolysis (E:T = 100:1)	
Effector Cells*	Target Cells	GM alone	Anti-Asialo GM1
Immunized with			
LM-IL-2/B16F1	B16F1	41.14 ± 1.90†	21.53 ± 1.27‡
LM-IL-2/C1498	B16F1	16.15 ± 1.32	−0.29 ± 1.62
LM-IL-2/J558	B16F1	11.65 ± 2.44	0.46 ± 1.13
LM-IL-2/Liver	B16F1	5.96 ± 4.77	−3.83 ± 0.35
LM-IL-2/Hyg	B16F1	14.92 ± 1.15	0.91 ± 1.07
Nonimmunized	B16F1	0.12 ± 1.32	−0.32 ± 1.43
Immunized with			
LM-IL-2/B16F1	C1498	15.41 ± 2.51	3.37 ± 0.41
LM-IL-2/C1498	C1498	50.44 ± 2.65†	34.27 ± 6.55‡
LM-IL-2/J558	C1498	12.83 ± 3.47	2.79 ± 0.01
LM-IL-2/Liver	C1498	20.06 ± 2.45	7.07 ± 1.84
LM-IL-2/Hyg	C1498	15.62 ± 1.13	1.72 ± 0.62
Nonimmunized	C1498	5.08 ± 1.18	6.53 ± 0.28

*C57BL/6 mice (three per group) were injected with 4×10^6 of one of the cell types (LM IL-2/B16F1, LM IL-2/C1498, LM IL-2/J558, LM-IL-2/liver, or LM IL-2/Hyg), or the mice were not injected. The mice received two additional injections at weekly intervals of the same cell type injected initially. Seven days after the last injection, mononuclear cells recovered from the spleens of the mice were incubated for 5 days with mitomycin C–treated cells of the same type used for the initial injections (the ratio of spleen cells to the stimulator cells = 30:1). Excess quantities of anti-Asialo GM1 MAbs (five times the amount required to saturate the binding sites) or growth medium (GM) was added to the spleen cell suspensions 1 hour before a standad 4-hour ^{51}Cr-release assay toward B16F1 or C1498 cells was performed. The values represent the mean ± SD from triplicate determinations.

†$P < .001$, relative to any other group.

‡$P < .001$, relative to any other group treated with anti-asialo GM1 MAbs.

that found in mice injected with LM–IL-2/B16F1 cells or any of the other modified cell types.

To determine the relative contributions of NK/LAK cells to the antitumor response observed in mice injected with LM–IL-2 cells transfected with the tumor DNAs, excess quantities (five times the amount required to saturate the relevant binding sites) of asialo GM1 MAbs were added to aliquots of the spleen cell suspensions before the cytotoxicity reactions toward B16F1 or C1498 cells were performed. As indicated (Table 1), treatment of the cell population with asialo GM1 MAbs reduced but did not eliminate the antitumor responses in the spleen cell populations prepared from mice immunized with either LM–IL-2/B16F1 or LM–IL-2/C1498 cells, suggesting that other antitumor effector cell types were involved. The addition of asialo GM1 MAbs to spleen cell suspensions from mice injected with LM–IL-2/J558, LM–IL-2/liver, or LM–IL-2/Hyg cells reduced the antitumor cytotoxicity responses toward B16F1 or C1498 cells to "background," that is, to a level equivalent to that of spleen cells from nonimmunized mice. In these instances, NK/LAK cells were the predominant effector cell types.

To determine if $CD8^+$ (Lyt-2.2) T cells were involved in the antitumor responses, CD8 MAbs were added to spleen cell suspensions from mice immunized with LM–IL-2/B16F1 or LM–IL-2/C1498 cells before the cytotoxic effects toward ^{51}Cr-labeled B16F1 or C1498 cells were determined. As indicated (Fig 2), treatment with CD8 MAbs reduced ($P < .001$) the cytotoxic activity toward B16F1 cells in mice injected with LM–IL-2/B16F1 cells, and treatment with CD8 MAbs reduced the cytotoxic activity toward C1498 cells in mice injected with LM–IL-2/C1498 cells.

Thus, the injection of mice with LM–IL-2 cells transfected with genomic DNA from either of two mouse neoplasms resulted in the induction of specific cytotoxic immune responses toward the type of tumor from which DNA was obtained. In the case of mice injected with LM–IL-2/B16F1 or LM–IL-2/C1498 cells, both NK/LAK and $CD8^+$ cells were involved in mediating the antitumor responses.

Antitumor cytotoxic responses generated in tumor-bearing C57BL/6 mice injected with LM–IL-2/B16F1 or LM–IL-2/C1498 cells were specific for the tumor from which the DNA was obtained

The experiments described in the previous section were performed in tumor-free C57BL/6 mice. To determine if analogous effects were observed in tumor-bearing mice, C57BL/6 mice were subcutaneously injected with a mixture of viable B16F1 cells and LM–IL-2/B16F1 cells, or, as controls, with B16F1 cells and LM–IL-2/C1498 cells, LM–IL-2/J558 cells, or LM–IL-2/Hyg cells. One group received B16F1 cells alone. The animals received two further subcutaneous injections at weekly intervals of LM–IL-2/B16F1, LM–IL-2/C1498, LM–IL-2/J558, or LM–IL-2/Hyg cells, respectively, but without additional tumor cells. The group injected with B16F1 cells alone received no further injections. Seven days after the last injection, the spleens were removed and tested for cytotoxicity toward B16F1 cells.

As indicated in Table 2, the antitumor cytotoxic responses in mice injected with B16F1 melanoma and LM–IL-2/B16F1 cells were significantly ($P < .001$) higher than the antimelanoma responses in any of the control groups.

To determine if analogous responses were present in mice injected with C1498 and LM–IL-2/C1498 cells, naive C57BL/6 mice were injected according to the same protocol with C1498 cells and LM–IL-2/C1498 cells, or as a control, with C1498 cells and LM–IL-2/B16F1 cells. One group was injected with C1498 cells alone. As previously, the cytotoxicity response toward C1498 lymphoma in mice injected with C1498 cells and LM–IL-2/C1498 cells was significantly ($P < .001$) higher than cytotoxicity responses toward C1498 cells in mice from either of the control groups.

Thus, like the specificity of the antitumor responses in mice injected with LM–IL-2 cells transfected with genomic DNAs from different mouse tumors, the antineoplastic cell responses in mice with B16F1 melanoma or C1498 lymphoma injected with the transfected LM–

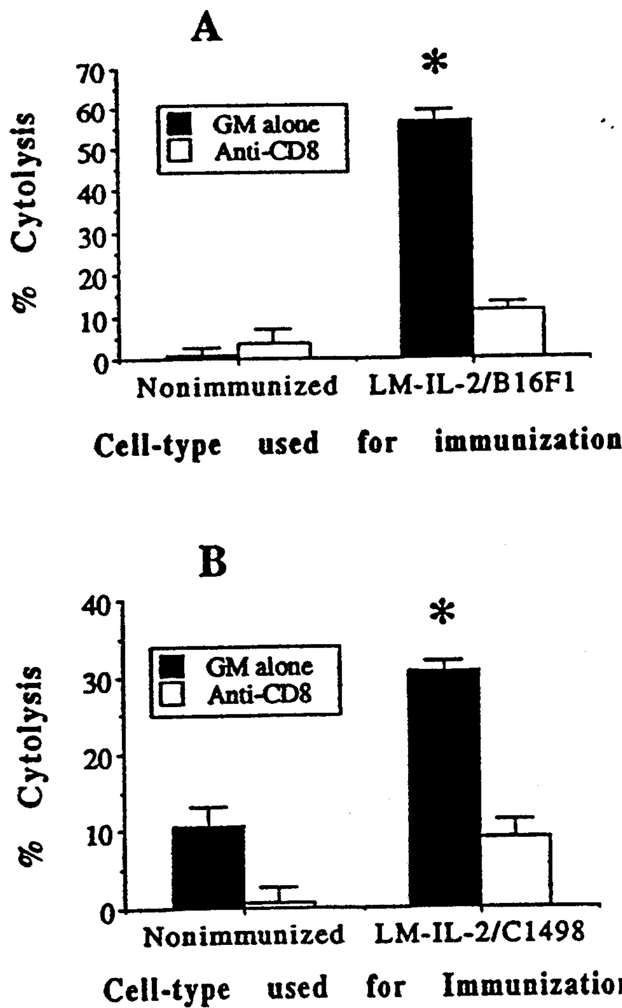

Figure 2. The effect of CD8 MAbs on spleen cell-mediated cytotoxic immune responses in C57BL/6 mice immunized with LM-IL-2 cells transfected with genomic DNA from B16F1 or C1498 cells. C57BL/6 mice (three per group) were injected with 4×10^6 LM-IL-2/B16F1 **(A)** or LM IL-2/C1498 cells **(B)**, or the mice were not injected. The mice received two additional injections at weekly intervals of the same cell type injected initially. Seven days after the last injection, mononuclear cells recovered from the spleens of mice were incubated for 5 days with mitomycin C-treated cells of the same type used for the initial injections (the ratio of spleen cells to the stimulator cells = 30:1). Excess amounts of anti-Lyt 2.2 (CD8) MAbs or growth medium were added to the spleen cell suspensions before a standard 4-hour ^{51}Cr-release assay toward ^{51}Cr-labeled B16F1 **(A)** or C1498 cells **(B)** was performed. The effector to target ratio was 100:1. The values represent the means ± SD of triplicate determinations. *$P < .001$, relative to the group treated with anti-CD8 MAbs.

IL-2 cells were directed toward the type of tumor from which the DNA was obtained.

The expression of B16F1 melanoma-associated antigens by LM-IL-2 cells transfected with genomic DNA from B16 melanoma cells

Quantitative immunocytofluorography was used to detect the possible expression of melanoma-associated antigens by LM-IL-2 cells transfected with genomic DNA from B16F1 melanoma cells. An antiserum prepared in C57BL/6 mice injected with (X-irradiated) B16F1 cells[21] was used to stain LM-IL-2/B16F1 cells or LM-IL-2/B16F10 cells, or, as controls, B16F1, B16F10, LM-IL-2/Hyg, or LM-IL-2/MOPC-315 cells.

The results (Fig 3) indicated that 3.75% of LM-IL-2/B16F1 cells and 3.26% of LM-IL-2/B16F10 cells stained positively. Under similar conditions, more than 97% of B16F1 cells and more than 95% of B16F10 cells stained. LM-IL-2/Hyg cells, nontransduced LM cells, or LM-IL-2 cells transfected with genomic DNA from MOPC-315 cells failed to stain.

Table 2. Spleen Cell-Mediated Cytotoxicity Toward Neoplastic Cells in Tumor-Bearing C57BL/6 Mice Immunized With IL-2-Secreting Cells Transfected With Genomic DNA From Different Tumor Cell Types

Effector Cells*	Target Cells	% Cytolysis (E:T = 100:1)
Immunized with		
LM-IL-2/B16F1	B16F1	36.2 ± 5.2†
LM-IL-2/C1498	B16F1	12.6 ± 1.6
LM-IL-2/J558	B16F1	17.4 ± 0.6
LM-IL-2/Hyg	B16F1	18.4 ± 4.1
Nonimmunized	B16F1	0.5 ± 1.1
Immunized with:		
LM IL-2/B16F1	C1498	11.5 ± 3.3
LM IL-2/C1498	C1498	31.2 ± 3.2†
Nonimmunized	C1498	−10.4 ± 3.2

*C57BL/6 mice were injected subcutaneously with 4×10^6 of one of the IL-2-secreting cell types as indicated and 5×10^3 B16F1 cells, or with 4×10^6 of one of the cell-types and 1×10^5 C1498 cells. The mice received two weekly additional injections with 4×10^6 of the same cell type used for the first injection, but without B16F1 or C1498 cells. Seven days after the last injection, a pool of mononuclear cells from the spleens of two mice in each group were incubated for 5 days with mitomycin C-treated cells of the same IL-2-secreting type as first injected. At the end of the incubation, ^{51}Cr-labeled B16F1 cells or ^{51}Cr-labeled C1498 cells were added and the mixed cell cultures were incubated for 4 additional hours, after which the specific release of isotope (% cytolysis) was determined. The values represent the mean ± SD of triplicate determinations.

†$P < 0.001$, relative to any other group.

DISCUSSION

Using two different tumor model systems, B16F1 melanoma and C1498 lymphoma, antitumor immune responses were generated in C57BL/6 mice injected with mixtures of tumor cells and IL-2-secreting LM mouse fibroblasts transfected with unfractionated, genomic DNA from either of the two neoplastic cell lines. The immune responses were of sufficient magnitude to prolong survival of the tumor-bearing mice. Immunizations with LM-IL-2 cells transfected with DNA from nonneoplastic cells or with LM cells transfected with plasmid DNA failed to inhibit tumor progression. This report is

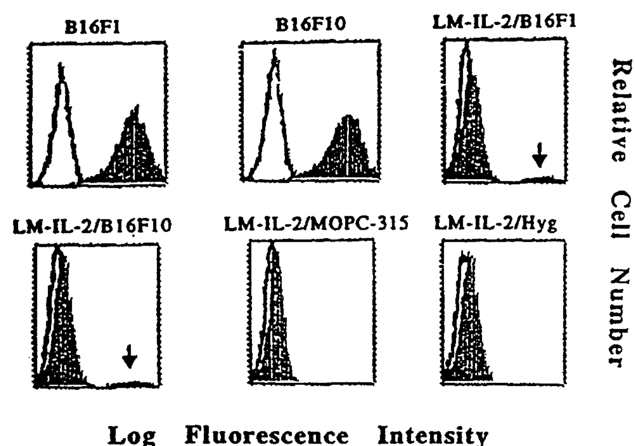

Figure 3. Immunocytofluorometric staining of LM–IL-2/B16F1 or LM–IL-2/B16F10 cells with polyclonal B16F1 antibodies. A quantity of 1×10^6 cells in each group were incubated with polyclonal B16F1 antibodies, followed by FITC-conjugated antimouse IgG. Unshaded area: cells incubated with FITC-conjugated antimouse IgG alone. Shaded area: cells stained with anti-B16F1 antibodies, followed by FITC-conjugated antimouse IgG.

an extension of an earlier study[23] in which C57BL/6 mice injected with B16F1 cells and LM–IL-2/B16F1 cells were found to survive significantly longer than C57BL/6 mice injected with B16F1 cells and LM–IL-2/MOPC-315 cells. In this report, we describe additional parameters of the immunologic specificity of the transfected IL-2–secreting cells and determine certain of the immune effector cell types activated in the immunized mice.

The antitumor immune responses were mediated predominantly by Lyt-2.2$^+$ (CD8$^+$) T cells and NK/LAK cells and were specific for the tumors from which the DNAs were obtained. The immunity generated in C57BL/6 mice injected with LM–IL-2/C1498 lymphoma cells was directed toward C1498 lymphoma and not B16F1 melanoma. At the same time, the immunity in mice injected with LM–IL-2/B16F1 cells was directed toward B16F1 melanoma and not C1498 lymphoma.

We hypothesize that the antitumor immune responses were the consequence of undefined genetic events in the neoplastic cells that included alterations of genes whose products, in a high local concentration of IL-2, became immunogenic, tumor-associated determinants. Whether or not the gene products were specified by mutated genes or resulted from an alteration in the expression of nonmutated genes was not determined. Conceivably, an array of altered genes was responsible for the antitumor responses. Genetic instability in mouse neoplasms is well known.

The number of LM–IL-2 cells cotransfected with pHyg and the tumor-derived DNAs was chosen so as to yield in excess of 6000 hygromycin-resistant colonies, in the expectation that the greatest array of potential tumor-associated T-cell epitopes would be expressed by cells in the population. No systematic investigation was performed to determine if immunizations with a pool derived from lesser numbers of transfected cells would yield equivalent immunotherapeutic results. The proportion of cells transfected with melanoma-derived DNA that stained with a polyclonal melanoma-specific antiserum was approximately 3%, consistent with the presence of multiple melanoma-associated antigens expressed by the transfected cells.

These data raise the possibility that a cytokine-secreting cell line can be prepared for subsequent modification to provide specificity for tumor-associated antigens of an individual neoplasm. Although it is of obvious theoretical importance, the number of genes specifying such determinants, or their precise characterization, is not critical if the cells are to be used for immunotherapy.

Here, the cell line chosen for modification expressed allogeneic major histocompatibility complex (MHC) determinants, for two complementary reasons. We found previously[28] that immunization with cells that coexpressed MHC determinants along with melanoma-associated antigens led to antimelanoma responses in allogeneic but not syngeneic mice. The immunity was persistent for at least 5 months after a single immunizing injection of the genetically modified cells. In addition, tumor growth was observed[20] in syngeneic C57BL/6 mice injected with IL-2–secreting B16 cells but not in C57BL/6 mice injected with allogeneic cells modified for IL-2 secretion. The cells were rejected as an allograft. Thus, the coexpression of allogeneic antigens augmented the cells' immunogenic properties as it protected the recipients against the growth of the modified cells.

Like other immunotherapeutic strategies, tumor growth eventually recurred in mice treated with the genetically modified cells. Because the tumor cell population is known to be heterogeneous in terms of its resistance to various cellular immune mechanisms, it is likely that a subpopulation of malignant cells, resistant to host immune mechanisms, survived. For further control, a combination of immunotherapeutic strategies will be required. Conceivably, immunization with a population of multiple cytokine-secreting cells transfected with genomic tumor-derived DNA would result in a cellular immunogen of greater efficacy than a population of modified cells secreting a single cytokine alone.

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