ABSTRACT

A Phase I trial of active specific immunotherapy for melanoma was performed to measure the toxicity and immunological effects of the therapy. A mixture of mechanical lysates (homogenates) of two melanoma cell lines was injected together with a novel adjuvant, DETOX, into 22 patients. Several types of cell-mediated and humoral immunity to melanoma-associated antigens were measured serially. In the 17 patients with measurable disease, the sizes of lesions were also noted serially. At least six patients per group were injected s.c. with either 100, 200, or 400 antigenic units (approximately 10, 20, and 40 million tumor cell-equivalents) of the lysates mixed with 0.25 ml of DETOX s.c. on weeks 1, 2, 3, 4, and 6. Three patients at each dose level also received 300 mg/m² of cyclophosphamide i.v. 4 days before the start of immunization.

Evidence for successful immunization was obtained in 13 of the 22 patients. An increase in the frequency of peripheral blood cytotolic lymphocyte precursors reacting against melanoma cells occurred in 12 patients, as measured by a limiting dilution assay involving in vitro re-exposure to irradiated melanoma cells for 9–10 days. Eight of the 12 patients had received cyclophosphamide. By cold-target competition assays, these cytolytic lymphocytes appeared to be atypical T-cells, which recognized melanoma-associated antigens on several allogeneic lines without apparent major histocompatibility complex restriction. An increase in antibody titers against melanoma-associated antigens, measured by enzyme immunoassay, was found in five of 22 patients, and an increase in delayed hypersensitivity against the melanoma lysate, in three patients. Responses were found at all three dosage levels of lysate, without an obvious dose optimum. No toxicity except minor local soreness was noted. Therefore, no maximum tolerable dose was defined.

Five of 17 patients with measurable lesions had a remission of their melanoma, two complete and three partial, with three additional minor responses. A patient whose complete remission lasted 5.5 months, has no evidence of disease 22+ months after entry onto the study, with the aid of surgical resection of small s.c. recurrences on two separate occasions. Sites of regression included s.c. nodules, lymph nodes, and pulmonary nodules, with no responses in liver, adrenal gland, or bone. The patients who had an increase in cytolytic lymphocyte precursors comprised all eight with a clinical remission (five major, three minor). In contrast, none of the seven patients lacking an increase in cytotoxic lymphocytes had a clinical response.

This version of active specific immunotherapy with allogeneic lysates elicited immunological reactivity to melanoma-associated antigens and may prove useful therapeutically.

INTRODUCTION

The incidence of malignant melanoma is increasing steadily, particularly in “sunbelt” states such as those in the American Southwest, and among Caucasians in Australia and Hawaii. This is attributable, at least in part, to an increase in intermittent, intense sun exposure among individuals with fair complexion and those who have predominantly indoor occupations. The current incidence among Caucasians in California and Australia is at least 15 per 100,000, with double that number in Hawaii (1). Approximately 28,000 new cases are expected in the U.S. during 1988. Surgery has been successful in the early treatment of the primary tumor, but in its disseminated form melanoma has been resistant to all conventional types of therapy, such as chemotherapy and radiation therapy (2). Recent immunological approaches to this disease have included the use of interferon-α (3) or interleukin-2, either preceded by cyclophosphamide (4) or together with lymphokine-activated killer cells elicited ex vivo (5). Deep invasion of melanoma at the time of diagnosis, or involvement of regional lymph nodes; portends a poor prognosis, indicating probable microscopic dissemination in the s.c. region of the skin or in the visera. Adjuvant (“adjuvant”) treatment at that time has seemed logical, in an attempt to forestall or perhaps eradicate sites of microscopic metastatic seeding, but none has proved effective thus far.

It would therefore be very useful to have an effective and preferably nontoxic immunological treatment that could be used after removal of the primary tumor in patients at high risk of recurrence, at a time when the disease itself was not causing symptoms. With this aim in mind, we have performed a Phase I trial of “active specific immunotherapy”, i.e., treatment designed to augment the specific immune response to autologous melanoma through the administration of melanoma-associated antigens. The work of Kahan, Pellis, LeGrue, and their colleagues in mice (6–8) suggested that active immunotherapy with butanol extracts of methylcholanthrene-induced sarcomas could prevent the “take” of tumor cells, block the development of metastases, and could even treat macroscopic 4-mm tumors when administered after chemotherapy. In humans, several groups have attempted active immunotherapy of melanoma with various preparations of whole melanoma cells or lysates, each demonstrating some degree of success in inducing durable clinical remissions (9–12).

We will describe our experience with a preparation consisting of a mixture of mechanical lysates (homogenates) of two allogeneic melanoma cell cultures we derived from biopsies of metastatic s.c. nodules. The mixture of lysates was injected together with a novel immunological adjuvant called DETOX (13). For ethical reasons, we first studied patients with disseminated melanoma, rather than the more favorable group with little or no disease. After we found that there was essentially no toxicity to the regimen the protocol was amended to allow inclusion of five patients referred to us with a high risk of recurrence but with no measurable metastases. All of the latter had previously had regional lymph nodes proved to contain melanoma cells. Our Phase I trial was designed to determine the toxicity and immunological consequences of several dose levels of melanoma-associated antigens, but we also carefully monitored any therapeutic effects of the regimen.

MATERIALS AND METHODS

Patients

Twenty-two patients were entered onto the study, selected by preestablished entry criteria. Seventeen of the 22 patients had measurable...
lesions. Eligibility criteria included melanoma histologically confirmed by our Pathology Department’s review of biopsy slides, a Karnofsky performance status of at least 70%, absence of central nervous system metastases and reactivity to at least one of a panel of five common microbial skin test antigens: candida, mumps, trichophyton, intermediate strength PPD and tetanus toxoid. Patients were required to be at least 18 years old, not pregnant, and at least 4 weeks past any previous therapy. Since antibiotics were used in the growth medium for the melanoma cells at the time of this trial, which is no longer our practice, patients with a verified allergy to penicillin or streptomycin were also excluded. All patients signed an informed consent form and the California Bill of Rights enabling them to resign from the study at any time. The characteristics of the patients, including the treatments they had received, are shown in Table 1.

**Preparation of Immunizing Materials**

Cultures of melanoma cells were started in our laboratory from biopsies of s.c. nodules from two different female patients. One culture was begun in 1980 from biopsies of two lesions on the calf of a female patient who had a remarkably protracted course of metastatic s.c. melanoma, encompassing more than 15 years. The second was begun in 1981 from a lesion on the back of a female patient who died within 12 months of that time. These tissue cultures were designated MSM-M-1 (M-1) and MSM-M-2 (M-2). We phenotyped each of the cultures on several occasions, at first with monoclonal antibodies W6/32 and Q5/13, and then with monoclonal antibodies more specific for HLA\(^{1}\) antigens. M-1 was amelanotic, grew rather slowly, and expressed both HLA-A,B,C and HLA-DR antigens. M-2 was highly pigmented, smaller, grew more rapidly, and expressed HLA-A,B,C antigens but not HLA-DR.

Both cultures were permitted to grow to near confluence in roller bottles (more recently, in NUNC “Cell Factories,” NUNC, Roskilde, Denmark), in RPMI 1640 medium with 10% fetal bovine serum containing penicillin and streptomycin. Before harvesting, the cells were grown in RPMI medium without fetal bovine serum for 3–4 days to remove most of the fetal bovine serum from the cell surface. The two cultures of melanoma cells were combined and mechanically disrupted in the cold with a Polytorn stainless steel high speed tissue homogenizer (Tekmar Co., Cincinnati, OH), followed by three cycles of freeze thawing. No enzymes were used either to remove the cells from the culture flasks or for their lysis.

The immunogens used for active specific immunotherapy comprised a mixture of M-1 and M-2 lysates. In the first batch, “MAC-1” (melanoma antigens from cultured cells) the ratio of M-1 to M-2 was 1:1. Because of slower growth of M-1 for the second preparation, “MAC-3”, used for 17 of the 22 patients, the ratio was 1:6. The number of tumor cell-equivalents (t.c.e) was noted, and the number of antigenic units (a.u.) per ml was determined by a binding inhibition assay to be described. The lysates were then aliquoted into 2-ml sterile, nonglycine glass vials, at a concentration of 200 a.u./ml. Vials chosen at random were sent to licensed commercial laboratories for microbiological testing and the remainder stored at −70°C.

The presence exclusively of disrupted melanoma cells was confirmed by microscopic examination of random samples of the lyse mixture. The absence of viable melanoma cells in our preparations was proved by inoculation of 0.2 ml (2–3 × 10⁶ t.c.e) into each of five nude mice and into RPMI medium with 10% fetal bovine serum, which showed no growth of tumor cells over the course of the next 4 weeks.

Tissue cultures were monitored periodically for the presence of mycoplasma and were discarded if they were contaminated. We now prepare the melanoma cells in antibiotic-free medium, which makes any contamination more apparent and rapidly detectable. The lyse mixture preparations were proved sterile, mycoplasma-free, nonpyrogenic, endotoxin-free, and devoid of both hepatitis virus and AIDS virus, the latter by direct hybridization and culture with a standard susceptible T-cell line. The investigations for sterility and general safety in test animals were performed in licensed commercial laboratories, in accordance with regulations of the Food and Drug Administration under an Investigational New Drug license.

**Assay for Antigenic Content by Binding Inhibition**

In addition to determining the number of tumor cell-equivalents, we quantitated the content of a melanoma-associated antigen: p250, the target of mouse monoclonal antibody 9.2.27. This antibody was kindly given to us by Dr. Ralph Reisfeld of Scripps Clinic and Research Foundation, La Jolla, CA. We used a binding-inhibition assay based on the enzyme immunoassay of Harper et al. (14). One hundred fifty to 300 ng of monoclonal antibody 9.2.27 labeled with horseradish peroxidase were preincubated with 30 μl of melanoma lyse in a medium of PBS, 1% bovine serum albumin, and 0.05% Tween-80 for 1 h at 37°C. Fifty μl of the mixture were added in triplicate to target melanoma cells, fixed to polyvinyl Falcon microtiter wells, and incubated for 1 h at 37°C. After washing, the peroxidase substrate solution, containing o-phenylenediamine, was added, and after 30 min the reaction was stopped and the absorbance read at 490 nm. A standard curve of binding, repeated each time an assay was performed, was constructed with a melanoma lysate derived from a patient with a large abdominal metastasis. One a.u. was defined as that amount of antigen present in 10 μl of undiluted standard melanoma extract, which bound maximally (80% inhibition) to antibody 9.2.27. A computerized program (“Curvefit”) devised by Jeffrey S. Mitchell (Epstein, Mitchell and Blackford, Computer Consultants, New Haven, CT) was used to calculate the number of antigenic units in each new lot of melanoma lysate directly from the equation describing the standard curve.

**Immunological Adjuvant: DETOX**

DETOX (Ribi ImmunoChem Research, Inc., Hamilton, MT) is a novel immunological adjuvant containing detoxified endotoxin (monophosphoryl lipid A) from *Salmonella minnesota* (13), cell wall skeletons of *Mycobacterium phlei*, squalene, and emulsifier. This material has been tested in patients by intradermal and s.c. injection (15–16) and is known to stimulate both antibody- and cell-mediated immunity in animals. An Investigational New Drug is held by Ribi ImmunoChem Research Inc., who granted permission to us to cross-file for its use in this study.

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\(^1\) The abbreviations used are: HLA, human leukocyte antigenation; a.u., antigenic units; PBS, phosphate buffered saline; IL-2, interleukin-2; EIA, enzyme immunoassay; MHC, major histocompatibility complex; CT, computerized tomography; CR, complete remission; PR, partial remission.

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Table 1 Characteristics of patients on study

<table>
<thead>
<tr>
<th>Sex</th>
<th>Male: 16</th>
<th>Female: 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Median, 42.5 years</td>
<td>Range, 26–68 years</td>
</tr>
<tr>
<td>Prior treatment</td>
<td>Surgery only: 12</td>
<td>Chemotherapy: 1</td>
</tr>
<tr>
<td>Sites of disease</td>
<td>Subcutaneous: 13</td>
<td>Lymph node: 6</td>
</tr>
</tbody>
</table>

Chemotherapy + radiation + immunotherapy: 3
Chemotherapy + immunotherapy: 1
Chemotherapy: 1
Immunotherapy: 1
Surgery only: 12
Female: 6
Male: 16
Liver: 5
Lung: 7
Median, 42.5 years
Range, 26–68 years
Liver: 5
Lung: 7
Male: 16
Female: 6
Other: Adrenal: 1
Small bowel: 2
Pelvis: 1
No measurable or evaluable disease: 5

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\(^4\) C. M. Pinsky et al., data to be published.

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**Immunization**

In the hospital pharmacy just before administration, 0.25 ml of DETOX (containing 250 μg of cell wall skeletons and 25 μg of endotoxin) was mixed thoroughly, added to the appropriate amount of melanoma lysate in the vial, and again mixed thoroughly. The appropriate dose was administered s.c. into two sites, in the deltoid or triceps regions or the buttocks. No injection was given into regions of previous lymphadenectomy or extensive involvement with tumor nodules.

Patients were injected on weeks 1, 2, 3, 4, and 6. At least two baseline determinations of immunological measurements were made, after which immunological tests were performed weekly during and for 2 weeks after the period of immunization. We treated at least six patients per group with 100, 200, or 400 a.u. per dose of lysate mixture.

Because of our concern *a priori* that we might not achieve any immunization with our preparation due to the influence of suppressor T-cells, we decided in this exploratory trial to administer a low dose of cyclophosphamide to the last three patients in each group before moving to a higher dose level. A dose of 300 mg/m² of cyclophosphamide was given by a 15-min i.v. infusion 4 days before the course of immunization was begun. Although this design did not allow us to compare the influence of cyclophosphamide on immunization within each dose level in a statistically rigorous way, comparisons between cyclophosphamide-treated and -untreated patients were nonetheless possible.

A skin test for preexistent delayed-type hypersensitivity to melanoma-associated antigens was performed with 16.6 a.u. of the melanoma lysate after we determined that a patient was not anergic. Patients were to be stratified to receive one-half the above doses if they were skin-test positive, but only the first patient on study (C. C.) had a positive skin test before vaccination, receiving 50 a.u. as a consequence.

**Immunological Studies**

Delayed-type Hypersensitivity to Melanoma Lysate. A skin test with 16.6 a.u. of lysate mixture without DETOX was administered before and 1 week after the course of vaccination. A positive skin test reaction was defined as one in which 5 mm or more diameter of induration was observed at 48 h. Conversion of a negative skin test to positive or an increase in more than 25% of the product of the diameters was considered indicative of a change in delayed hypersensitivity to the components of the lysate mixture.

Limiting Dilution Analysis of Cytolytic Lymphocyte Precursors. As one serial measurement of changes in cell-mediated reactivity to melanoma, we chose to quantitate the frequency of cytolytic lymphocytes in the peripheral blood, by our adaptation of the method of Vose (17). More precisely, because the method involves restimulation with melanoma cells *in vitro*, it principally measures *precursors* of cytolytic lymphocyte precursors as well as mature effector cells. We will therefore refer to the lymphocytes whose activity we measured in this assay as "cytolytic lymphocyte precursors." Heparinized whole blood was passed through a nylon wool column to remove adherent cells and platelets. Peripheral blood lymphocytes were prepared from the column-treated blood by Ficoll-Hypaque gradient centrifugation, and were resuspended in RPMI 1640 medium with 10% human AB serum (“complete” RPMI). The lymphocytes were washed and resuspended in complete RPMI. A portion of the peripheral blood mononuclear cells irradiated with 2000 rads was used as autologous feeder cells (2.0 x 10⁴/well).

Recombinant IL-2 (Cetus) was added as a growth factor. We used 100 Cetus units/ml in these experiments, but we have since found that only 5 to 20 units/ml are required, and are in fact optimal. For the assay itself, 2 x 10⁴ to 1.25 x 10⁵ responder cells were plated repetitively into 96-well round-bottomed microplates in 0.1 ml of complete RPMI. Thirty-two replicates at each responder cell number were routinely used for statistical accuracy. Five thousand irradiated tumor cells were added to wells in which a micro-mixed lymphocyte-tumor cell reaction was set up. IL-2-induced cytotoxicity was assessed in wells containing lymphocytes, IL-2 and 2.0 x 10⁴ irradiated mononuclear feeder cells alone. The cultures were fed on Day 6 with 50 μl/well of fresh medium containing IL-2. Plates were cultured for 9–10 days to determine the frequency of cytotoxic cells. Microcultures of feeder cells alone and feeders with tumor cells and IL-2, served as indicators of background.

To test for cytotoxicity, 5 x 10⁵ M-1 melanoma cells labeled with ⁵¹Cr-sodium chromate were added to each well in complete RPMI. The reaction mixture was incubated for 6 h at 37°C, after which 100-μl aliquots of supernate were harvested and their radioactivity measured in a gamma counter. Test wells were considered "positive" when values of cpm released exceeded the mean control values, in wells containing autologous feeder and stimulator cells with IL-2, by more than three standard deviations. The number of cells plated that corresponds with 37% negative wells on a plate provides an estimate of frequency, indicating one cytotoxic cell in that population according to the Poisson distribution.

Originally we constructed regression lines of the number of responders plated versus the log percentage of negative wells in order to calculate our minimum estimate of the frequency of cytotoxic cells. We further measured the significance of differences between lines for different responders within the same experiment by the method of Taswell (18), which uses the minimization of chi-square differences as its basis. In most of our work we used the analysis described by Fazekas de St. Groth (19), an iterative calculated based on minimization of variance. The latter method also permits calculation of the 95% confidence intervals for the frequency and the chi square value of differences between two groups. In most circumstances the frequencies calculated by both methods agreed closely, but particularly where only 3 to 4 effector:target cell levels could be tested, a more accurate estimate was given by the second method. We have developed a computer program based upon Lotus 1-2-3 which directly calculates these values from raw data of cpm of ⁵¹Cr released. This program is available to interested investigators upon request.

Specificity of the Cytolytic Lymphocytes. In order to analyze the specificity of the cytotoxic lymphocytes we tested the ability of various unlabeled ("cold") target cells to inhibit the lysis of ⁵¹Cr-labeled M-1 melanoma cells by the effector lymphocytes. The cytotoxic cells were generated in mixed lymphocyte-tumor cells cultures containing 2 x 10⁶ peripheral blood mononuclear cells and 10⁶ irradiated M-1 melanoma cells/ml in RPMI medium supplemented with 10% human AB serum and 20 or 100 Cetus IL-2 units/ml. After 9–10 days in culture the effector cells were harvested and cold target competition assays were set up in round-bottomed microculture wells in a total volume of 200 μl/well. Each well contained 5 x 10⁶ ⁵¹Cr-labeled M-1 target cells, 2 x 10⁶ effector cells, and various numbers of unlabeled cells as competitors. The ratio of inhibitors to target cells ranged from 1.25 to 10. The microplates were spun at 1000 rpm for 5 min and incubated for 6 h at 37°C. To estimate the amount of released ⁵¹Cr, 100 μl of supernate was harvested and counted in an automated gamma counter. Percent cytotoxicity was calculated as 100 x [cpm in test well - spontaneous release / maximal release - spontaneous release]. Percentage of inhibition was calculated as 100 x [1 - (a/b)], where a = cytotoxicity in the presence of "cold" targets and b = cytotoxicity against labeled target cells alone.

The cells used as putative inhibitors in the various experiments included six melanoma cell lines (M-1 to M-5 and UCLA M21), a squamous cell carcinoma of the lung (Lu-1), two B-cell lymphomas (Daudi and Raji), erythroleukemia K-562, and concanavalin-A-induced lymphoblasts from normal donors or two patients with melanoma.

**Serum Antibodies.** An IFA procedure was adapted from Harel and Nelsen (20). M-1 or A375 melanoma cells were washed three times in serum-free medium and seeded at a concentration of 2 x 10⁴ cells per well in flat-bottomed 96-well plates for 24 h in serum-free RPMI medium. The cells were fixed to the wells for 5 min with 0.05% glutaraldehyde followed by three washes with PBS + 0.05% Tween-20. A further overnight incubation was performed at 4°C with 1% bovine serum albumin to saturate the vacant protein binding sites in the wells. Fifty μl/ml of serial dilutions of the test sera were added to each well and incubated 60 min at room temperature, followed by three washes with PBS-Tween 20. The concentration of IgG antibodies bound to the melanoma cells was detected by peroxidase-labeled Staphylococcal protein A (1,100 in 1% bovine serum albumin, 45-min incubation, three washes), and the substrate 3-phenylenediamine (50 μl/well, 20–30 min). A dose-dependent color intensity developed in the wells containing specific antibodies to the melanoma cells. The specificity of
the antibodies was determined in selected patients by absorptions with various types of tumor and normal cells, including melanoma cultures of various deviations, squamous lung cancer, B-lymphoma, and allogeneic normal lymphoblasts. The ratio of serum to packed cells was 1:1 v/v, with an incubation at 30 min at room temperature, then 30 min at 4°C. The sera were then collected by centrifugation at 1000 × g and tested against the melanoma target cells.

**Toxicity**

Careful records were kept of the subjective and objective toxicity of the treatment, aided by weekly laboratory analyses of peripheral blood cells and serum. Standard WHO criteria were used to classify the degree of toxicity.

### Table 2 Summary of Phase I trial

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dosage in a.u.</th>
<th>Cytolytic lymphocytes</th>
<th>Skin test</th>
<th>Antibodies by EIA</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. C.</td>
<td>50 (MAC-1)</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>PR</td>
</tr>
<tr>
<td>L. S.</td>
<td>100 (MAC-1)</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>N. C.</td>
<td>100 (MAC-1)</td>
<td>0; 15 15</td>
<td>0</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>S. O.</td>
<td>100 + Cy (MAC-1)</td>
<td>0; 15 22</td>
<td>0</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>T. B.</td>
<td>100 + Cy (MAC-1)</td>
<td>0; 7 7</td>
<td>0</td>
<td>+</td>
<td>MR</td>
</tr>
<tr>
<td>E. E.</td>
<td>100 + Cy</td>
<td>0; 43 43</td>
<td>0</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>T. W.</td>
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<td>0; 43 43</td>
<td>0</td>
<td>+</td>
<td>MR</td>
</tr>
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<td>0</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>L. A.</td>
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<td>+</td>
<td>CR</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
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<td>0</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>M. R.</td>
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<td>0</td>
<td>+</td>
<td>PR</td>
</tr>
<tr>
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<td>0</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>T. S.</td>
<td>200 + Cy</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>J. B.</td>
<td>200 + Cy</td>
<td>0; 22 22</td>
<td>0</td>
<td>+</td>
<td>CR</td>
</tr>
<tr>
<td>D. C.</td>
<td>200 + Cy</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>J. S.</td>
<td>400</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>B. M.</td>
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<td>0</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>W. M.</td>
<td>400</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>G. M.</td>
<td>400 + Cy</td>
<td>0; 8 15</td>
<td>0</td>
<td>+</td>
<td>PR</td>
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<tr>
<td>G. G.</td>
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<td>0; 8 15</td>
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<td>+</td>
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<td>G. F.</td>
<td>400 + Cy</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>None</td>
</tr>
</tbody>
</table>

* ++ = Elevation noted; n1 = day rise first noted; n2 = day peak noted.
* 0—+ = conversion to positive; ++ + = increase >25% over original area.
* Antibody present before treatment but not significantly increased afterwards.
* Abbreviations: a.u., antigenic units; EIA, enzyme immunoassay; CR, complete remission; PR, partial remission; MR, minor response; NE, not evaluable (no measurable disease).

**Evaluation of Clinical Responses**

By physical examination, plain X-rays, or CT scans we measured the longest perpendicular diameters of tumor masses that were chosen as sentinel lesions before, at weekly intervals during and for 2 weeks after treatment. Strict standard definitions involving both degree and duration of response were used to categorize a clinical response as a complete remission, partial remission, minor response, or no response. Thus, a complete remission was a disappearance of all measured lesions for at least 4 weeks; a partial remission was a decrease of 50% in the sum of the products of greatest perpendicular diameters of all measured lesions, lasting at least 4 weeks; a minor response was a decrease of 25–50% in the sum of the products of greatest perpendicular diameters of all measured lesions, or 50% decrease for less than 4 weeks; and no response was any response less than a minor response.

**RESULTS**

**Toxicity**

There was no toxicity of the treatment, except for slight soreness at the sites of injections 1 day later in 40% of the patients. Palpable lumps, proved by biopsy in one instance to be a granuloma, formed at several sites of injection in two patients (9%) whose injections were perhaps given more superficially than they should have been. No systemic side-effects were observed, and a variety of standard hematological and chemical blood tests that were performed weekly showed no evidence of toxicity to vital organs. Likewise, no adverse immunological effects such as rash or anaphylaxis were seen.

**Immunological Effects**

**Cytolytic Lymphocyte Precursors in Peripheral Blood.** The frequency of circulating cytolytic lymphocyte precursors was increased in 12 of the 22 patients (Table 2). In eight of the 12 there was a rise 1 to 3 weeks after beginning the vaccination, with the peak value at week 4. Four patients had a significant elevation only on the 6th week. The rise in cytolytic lymphocyte precursors accompanied or antedated the onset of a clinical
remission in the eight patients with an early increase in frequency. Fig. 1 indicates the kinetics of the augmentation of circulating cytolytic lymphocyte precursors in the blood of six patients who represent the range of responses we observed, from complete remission to a minor response. The cytolytic lymphocyte precursors remained at their zenith for a relatively brief time, and may have represented a transient overflow of immunologically committed lymphocytes from the lymphoid organs.

It is of interest that while eight of 11 patients treated with cyclophosphamide developed increases in circulating cytolytic lymphocyte precursors, only four of 11 who did not receive cyclophosphamide had such an increase.

A frequency of approximately 1 cytolytic lymphocyte per 5,000 peripheral blood lymphocytes was obtained as a baseline in these patients. The peak increase ranged from 2- to 10-fold among the 12 responders, with a mean peak frequency of 1:1000. The highest level achieved was 1:350. At increased dose levels, no increased mean peak frequency or increased percentage of patients responding were noted.

Specificity of Cytolytic Lymphocytes. In order to determine whether the cytolytic lymphocytes generated in our limiting dilution assays after a 10-day incubation with M-1 melanoma cells were in fact T-lymphocytes directed against melanoma-associated antigens, we performed cold-target competition assays on 11 patients, seven of whom had a cytotoxicity of 30% or more at an effectortarget cell ratio of 40:1. Fig. 2A summarizes the percentage of inhibition achieved by each inhibitor cell line, while Fig. 2, B and C, show the actual values for cytotoxicity in a single patient. At an effectortarget ratio of 40:1, the ratio we examined in cold-target competition assays, a mean cytotoxicity of 42% was observed.

In all instances, several different unlabeled allogeneic melanoma cell lines inhibited the cytotoxicity of the patient's lymphocytes against the target M-1. Other types of tumor cell, including targets sensitive to lymphokine-activated killer cells, did not inhibit nor did the allogeneic lymphoblasts from two patients with melanoma, in two separate experiments. Only K-562 showed any consistent competition, in approximately 30% of the tests, and was equal to M-2 in its degree of inhibition for reasons that have not yet been explained. That discrepancy notwithstanding, the data have suggested that "atypical" T-lymphocytes directed against melanoma-associated antigens, but not recognizing either self or allogeneic HLA antigens on the target cells, were elicited by the specific immunotherapy with allogeneic lysates.
Serum Antibodies. Table 2 and Fig. 3 depict those patients who have had antibody titers measurable by the EIA. All five patients given MAC-1, whose values are shown in Fig. 1, left, had a significant rise in serum antibodies after immunization. High titers, exceeding 1:500 in all five patients were noted, but this included not only the one patient, C. C., who had a partial remission, but two other patients who failed to have any response, and a third who had only a transient regression of his nodules. Three of the 10 patients in Group 2 (T. W., L. A., and J. B.) also had serum antibodies, but their antibodies antedated the vaccination and their titer did not rise significantly after treatment.

Absorption analyses were performed to determine the specificity of the antibodies, of which one performed with the high-performance liquid chromatography-purified IgG fraction of the high-tiered serum of patient T. B. is shown in Fig. 4. By these methods, patient T. B. appeared to have antibodies directed against antigens associated with melanoma cells, rather than against components of the medium or similarly nonspecific determinants.

Clinical Responses

Shrinkage of measurable lesions was noted in several patients. As shown in Table 2, and illustrated in Fig. 5, the active immunotherapy induced complete or partial remissions of melanoma in five of the 17 patients with measurable disease. Two patients had complete regression of their disease and three others, a partial remission. Minor responses were also noted in three other patients, including more than 50% regression of large nodules in one patient, but which did not last 4 weeks. Nine patients had no response, i.e., progressive disease despite treatment.

Of most interest were the two complete responses. Patient L. A., a 30-year-old woman, had received 2 trials of immunotherapy at other universities without obvious benefit. The second treatment, intralesional injections of buffy coat lymphokines, extended from November, 1985, through May, 1986, ending 1 month before her entry onto our study. She was given 200 a.u. of lyysate mixture and DETOX weekly. She began to have shrinkage of the numerous s.c. nodules on her right thigh and buttock within 1 week after her first dose on 6/11/86 (Fig. 5A). This rapid onset of resolution was common to all patients who responded to the 200-a.u. dosage, whereas at the 50 or 100-a.u. level regression began 3 to 5 weeks after the beginning of treatment. Many of her nodules were impalpable within 3 weeks and all were gone within seven. The patient had a 4-cm necrotic nodule, which was slowly diminishing, excised during the sixth week because it was causing discomfort when she was seated. This lesion was entirely necrotic by histopathology of several tissue sections. When the resolution began her skin felt indurated, but over several months it gradually regained its normal texture. She had a single new 5-mm lesion in the skin of the right lower quadrant, just above the inguinal ligament, which was immediately resected. 5.5 months after the remission began, and another of the same size on the thigh at 10 months. Patient L. A. also had a 1.5-cm diameter pulmonary nodule that disappeared completely on x-ray and CT scan by the seventh week (Fig. 6). Two further "maintenance" courses of immunization were given in September–October, 1986, and May–June, 1987, with monthly "boosters" from September, 1987, to the present. She remains fully active with no evidence of disease 23+ months from the date of complete remission (as of June, 1988), by physical examination and CT scans of the chest, abdomen, and head.

Fig. 7, A and B, shows pre- and post-treatment biopsies of one of the s.c. nodules. After treatment there were macrophages with ingested melanin (i.e., melanophages) (arrows), but no viable tumor cells were seen in the specimen by the dermatopathologist. A modest perivascular lymphocytic infiltrate was also noted, but few if any lymphocytes were adjacent to the dead tumor cells.

Patient J. B. had had resections of large lymph nodes involved with melanoma from his right axilla for the 3 months prior to his entering the study, having had ostensibly radical resection of axillary disease before that. He had measurable shrinkage of his only lesion, a 2.5-cm diameter lymph node, 1 week after the first injection, which continued weekly until it disappeared entirely on physical examination and CT scan by the 6th week (Fig. 5B). During the 8 weeks of study, and for at least 5.5 months thereafter no new lymphadenopathy appeared. Retro-
Fig. 5. Kinetics of response of measured lesions in patients with a complete or partial remission (A–E) and one with a minor response (F) (>50% regression for <4 weeks). In D: △, adrenal metastases; X, lung nodules that did not change significantly as a result of the treatment. Responses were seen within 6 weeks in all, many of which began after the first injection. A, complete remission (L. A.); B, complete remission (J. B.); C, partial remission (C. C.); D, partial remission (M. R.); E, partial remission (G. M.); F, < partial remission (T. B.).

Fig. 6. Closeup views of a chest X-ray of the left upper lobe of patient L. A. A, a 1.5-cm diameter nodule was present at the onset of treatment; B, the nodule disappeared within 7 weeks, and has not reappeared.

peritoneal lymphadenopathy was found on a CT scan at that time, and he was considered off-study.

The partial remissions were also interesting, because the patients in question had bulky or numerous s.c. or lymph nodal tumor masses. Patient C. C. had lesions scattered over a region on her right lower extremity from calf to groin, measuring from 0.8 to 2.2 cm in diameter. She had had progression of the nodules despite radiation therapy from May to July, 1985, entering our study in November. All lesions shrank during the 1-week hiatus after the fourth injection, and most remained small until the middle of a second course of lysate mixture, 5 or 6 weeks later. Two nodules became impalpable within 3 weeks. Biopsy of a remaining nodule showed considerable necrosis of tumor cells, with some peripheral lymphocytic infiltration. During both courses, especially the second, the nodules fluctuated in size, with a peculiar and thus far unexplained 3- or 4-day periodicity confirmed by our own measurements. (Note also the irregular terminal phase of regression of one lesion in Fig. 5C). This phenomenon was also reported by patient T. B. Patient C. C. was given the lowest dose on the study, 50 a.u. weekly, because she was the only one to have a positive melanoma skin test before therapy.

Patient M. R. with three large (4–5 cm) s.c. nodules, and masses in the left adrenal gland and the lung, received a course of 200 a.u. per week, preceded by low-dose cyclophosphamide. His previous therapy at another institution had included chemotherapy with hydroxyurea, the BOLD regimen, and one dose of a combination of velban and platinum. He noted a rapid softening of all skin nodules within a week after receiving active immunotherapy, and by our measurements on Day 8 the lesions were indeed smaller and flatter. All reached their nadir within a month, and one became impalpable by the end of therapy. An adrenal and a lung nodule remained stable. The overall response was classified as a partial remission because the aggregate sum of the perpendicular diameters of all measured lesions was less than 50% of their original value (Fig. 5D).

A third patient, G. M., with no previous treatment for metastatic disease, had a flattening of his many, approximately 1-cm, s.c. nodules at the sixth week, on 400 a.u. per week preceded by cyclophosphamide. On the seventh week he had a clear
shrinking of all five sentinel lesions to an aggregate of less than 50% their original size (Fig. 5E). For various nonmedical reasons, he did not return for follow-up on schedule. When next seen, the nodules had become greater than their original size, and he was treated with our regimen of low-dose cyclophosphamide + IL-2 (4) on which he rapidly sustained another partial remission.

Fig. 8 shows pre- and posttreatment biopsies of a s.c. nodule from patient G. M. Note the degeneracy of the tumor cells in Fig. 8B, with vacuolated cytoplasm and a pyknotic nucleus. A moderate perivascular lymphocytic infiltrate (not shown in the photograph) was also present, as with patient L. A.

Patient T. B. (Fig. 5F) had more than 50% shrinkage of s.c. and lymph node masses that began 3 weeks after the onset of therapy with low dose cyclophosphamide and 100 a.u. of lysate mixture weekly, but which lasted only 3 weeks. However, one of this patient’s s.c. nodules became and has remained palpable. He then received cyclophosphamide + IL-2, had a persistent PR, and remained on maintenance therapy until August, 1987, when he relapsed. Patient T. W. had four large s.c. metastases, one of which began to shrink within 1 week, diminishing from 1.8 cm in diameter to 0.75 cm, and remained at 5-mm size thereafter. Since the other three s.c. masses and lesions in the liver did not shrink, his aggregate response was less than a PR. Patient G. G. had approximately 30% regression of his s.c. nodules lasting only 3 weeks.

Our starting dose level of 10 to 15 million tumor cell-equivalents (100 a.u.) was based on the work of others with melanoma or renal cell carcinoma, and we did not attempt to explore a broad spectrum of doses. The two CRs and one PR occurred at a dose of 200 a.u. per week, but the first patient on study given only 50 a.u. had a PR, as did one from the 400 a.u. group. Thus, there was no strict dose-response relationship demonstrable within this range.

In contrast to our findings on the increase in cytolytic lymphocyte precursors in the peripheral blood, administration of low-dose cyclophosphamide did not appear to influence the clinical response, which occurred with equal frequency regardless of whether the patients had also received the drug.

Association of a Clinical Response with an Increase in Cytolytic Lymphocytes

Two of the patients with a rise in cytolytic lymphocyte precursors had no measurable disease. Of the remaining 10 patients with measurable tumor masses who showed an increase in cytolytic lymphocyte precursors, five had a remission, three a minor response, and two no response. In contrast, none of seven patients who failed to show an increase in cytolytic lymphocyte precursors had a remission. Statistical analysis of the relationship between an increase in cytolytic lymphocyte...
precursors during immunization and a clinical response (complete or partial remissions), showed that the proportion of responders was significantly greater among those with an increase (five of ten) than among those without an increase (zero of seven) \( (P = 0.044, \text{Fisher's exact test, two tailed}) \) (Table 4).

If minor responders were also included, the differences were even more striking. Thus, the probability of attaining some degree of response for those with an increase in cytolytic lymphocytes (eight of ten) was significantly higher than for those without (zero of seven) \( (P = 0.002, \text{Fisher's exact test, two tailed}) \).

**DISCUSSION**

This Phase I trial was designed to determine the toxicity and immunological effects of allogeneic melanoma lysates mixed with the novel adjuvant DETOX. We have shown that the material at various dose levels was immunogenic in patients with advanced melanoma, increasing the frequency of cytolytic lymphocyte precursors directed against melanoma-associated antigens in 12 of 22 subjects, and increasing the titer of antibodies against melanoma in five patients. In addition, regression of autochthonous tumor masses in five of 17 patients with measurable lesions provided further evidence that the allogeneic lysates caused reactivity against the patient's own tumor-associated antigens.

The ability of a preparation of allogeneic melanoma lysates to increase the immune response against autologous melanomas is of interest theoretically and practically. The results reemphasize what work with mouse and human monoclonal antibodies has already demonstrated: that there are antigens common to many and perhaps all melanomas regardless of individual differences. A practical consequence is that it is feasible to treat large groups of patients with a standardized material, at something approximating a "dosage" in tumor cell-equivalents or antigenic units. The patients who might benefit most from immunotherapy are those with a small or microscopic tumor burden, who might not have sufficient numbers of tumor cells from which to make an autologous lysate mixture. In addition, patients with inaccessible lesions can also be treated. Furthermore, the number of injections in a course of treatment can be predetermined, rather than being limited by the amount of tumor obtained from an individual.

Even one remission in a Phase I trial of a cytotoxic chemical, which is designed solely to determine the maximum tolerable dose, is considered suggestive of its effectiveness. A response rate of five of 17 (29%) in advanced melanoma in this Phase I trial of active immunotherapy, while obviously requiring confirmation in a larger trial, is all the more encouraging. In advanced melanoma, the response to chemotherapy is usually less than 20%, and complete remissions are rare (2). Although biomodulators (biological response modifiers) will almost certainly be most useful where the tumor burden is relatively low, we have found that at least two of them are helpful even with metastatic neoplasms. Besides the active immunotherapy regimen we have described here, a regimen of low-dose cyclophosphamide and interleukin-2 has caused approximately a 25% remission rate in patients with disseminated melanoma (4), inducing partial remissions in three patients (N. C., T. B., and G. M.) who had been treated in this Phase I trial.

Active specific immunotherapy has been effective to some degree in the treatment of widespread neoplasia in several trials. Berd and colleagues treated nine patients with low-dose cyclophosphamide and then with irradiated autologous tumor cells (21) for several months, and noted two clinical responses in addition to eliciting an immune response in a proportion of the patients. A recent update of this work (22) indicates three complete and one partial remission in 33 patients (12%) treated in this way. Bystryn et al. (9) have been among the few to use allogeneic material (melanoma culture supernates without an adjuvant), and produced a clinical response in one of 13 patients. At least two other tumors have been affected by active immunotherapy. In renal cell carcinoma, Neidhart et al. (23) obtained objective responses in 7-13% of 57 patients, while McCune and colleagues (24, 25) found a 26% response (nine of 34) in their patient's. It is particularly interesting in regard to our own results that the two complete remissions reported by Neidhart et al. (23) were achieved when an allogeneic preparation was submitted for autologous material. In colon carcinoma, Hanna and Hoover (26, 27) have prevented the recurrence of disease and prolonged the survival of Dukes' B2 and C patients, with irradiated autologous tumor cells + *Bacillus Calmette-Guérin*, but they had no success in treating disseminated disease.

The ability of our lysate mixture to induce rejections of metastatic melanoma may be due in large part to the adjuvanticity of DETOX, the novel material with which our melanoma lysates were mixed. This adjuvant, containing detoxified endotoxin and mycobacterial cell wall skeletons in a degradable oil base, was chosen because it resembled Freund's adjuvant and was capable of augmenting not only antibody synthesis but cell-mediated immunity as well. Evidence in many animal tumor models has indicated that the latter property is more important than the former in the rejection of solid tumors. However, it is unlikely that the DETOX was solely responsible for the effects we observed. DETOX alone has never affected distant metastases in melanoma even when injected intralesionally (15-16). Furthermore, nonspecific active immunotherapy, such as *Bacillus Calmette-Guérin*, and *Corynebacterium parvum*, has been used extensively in the past in melanoma without any consistent effects on systemic disease. Our immunological evidence of antibody- and cell-mediated reactivity to antigens on melanoma cells also point to a more specific antitumor response than might be generated by nonspecific immunotherapy alone.

Indeed, the strongest immunological correlate with a clinical response in our series was an increase in the frequency of circulating precursors of cytotoxic lymphocytes reactive against M-1 melanoma, one of the immunogens in the lysate mixture. This was demonstrated by a limiting dilution assay involving
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reexposure of peripheral blood mononuclear cells to irradiated melanoma M-1 cells for 10 days (17). The cytotoxicity of these lymphocytes can be blocked by monoclonal antibodies to CD3, indicating that they are in fact T-cells. The lymphocytes may be the same type of unusual CD3+, (CD4-, CD8-) Leu 19+ cytotoxic T-cells described by Lanier and Phillips (28, 29), which do not show the same MHC restriction that limits the reactivity of so-called “classical” T-cells (30). Many of the nonclassical T-cells recently found in humans may have a γ-δ chain T-idiotypic (Ti) portion of the CD3-Ti receptor for antigen, rather than the α and β chains that characterize the more classical variety (31). Further support for our view that the cytolytic cells are T-cells comes from recent work by Finn and her colleagues, who found atypical CD3+ T cells in the regional lymph nodes of patients with pancreatic carcinoma, which were reactive only against several different pancreatic carcinoma cell cultures and not other types of tumor cell.

It is possible that the cytolytic T-cells recognize melanoma antigens in the context of public HLA determinants, rather than that they ignore the MHC entirely. Even if they are not recognized in their native, unaltered form by cytolytic T-lymphocytes, HLA antigens may be influential in the elicitation of reactivity against the tumor. Far from preempting the immune response, or causing it to be directed against the tumor antigens only in context of the allo-MHC, which were theoretical objections to an allogeneic lysate preparation, it is equally plausible that allo-MHC may somehow function in the presentation phase of tumor-associated antigens, causing them to be viewed in the sufficiently “foreign” context that their immunogenicity is increased. Expression of MHC may in fact be essential for the immunogenicity of autochthonous tumors (32), and may also influence the sensitivity of tumors to treatment with interleukin-2 (33).

While five of 10 of the patients whose cytolytic lymphocyte precursors were increased had a major clinical response, none of seven who lacked an increase had a response. In other words, the increase was necessary but not sufficient for a response. This raises the obvious but difficult questions of why these remainders of the patients did not have an elevation in their effector cells, and why clinical remissions did not ensue in everyone who was able to generate an increase in cytolytic lymphocyte precursors. Among the many possibilities is that suppressor influences were more prominent in the nonresponders, but our current evidence does not specifically support that. Those patients who received cyclophosphamide here had no better frequency of clinical response than those given the lysate mixture alone. Moreover, weekly measurements of concanavalin A-inducible suppressor T-cells in 17 of the 22 patients (data not presented here) has failed to indicate a consistently lesser degree of suppression at the outset, or a more consistent decrease to low levels during vaccination in those patients who responded versus those who did not. Berd et al. (10, 21) concluded from their data with autologous immunization that cyclophosphamide was required to permit the development of delayed-type hypersensitivity in their melanoma patients, and thus may have contributed to the response. Our data with cytolytic lymphocyte precursors, but not skin hypersensitivity, tend to confirm their immunological findings, but whether cyclophosphamide causes a superior number and duration of clinical responses should be the subject of a controlled trial.

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