ABSTRACT

A combination of recombinant human interleukin 2 (rhIL-2) and mouse monoclonal antibody R24 (recognition the ganglioside GD3) was evaluated in patients with metastatic melanoma in a phase I trial. rhIL-2 was given at a constant daily dose of 1 x 10^6 units/m^2 i.v. over 6 h on days 1-5 and 8-12. R24 was given on days 8-12 at four dose levels (1, 3, 8, and 12 mg/m^2 daily). Twenty patients were evaluable for toxicity and response, five at each dose level. The toxicity of the combination was not overlapping and generally mild. There was a rebound peripheral blood T-lymphocytosis at the end of treatment increasing with the dose of R24. The median lymphocyte count on day 12 of treatment was 3108 ± 554/m^3 in patients treated at R24 doses of 8 and 12 mg/m^2 versus 2299 ± 672/m^3 at doses of 1 and 3 mg/m^2. This evidence and other data suggested that R24 enhanced IL-2-mediated T-cell activation in vitro. Two patients demonstrated increases in R24-mediated antibody-dependent cellular cytotoxicity for GD3-expressing cells during treatment. rhIL-2 appeared to accelerate the development of human anti-mouse antibody; three patients developed human anti-mouse antibody by the fifth day of R24 treatment, earlier than observed in prior studies using R24 alone and one patient during the first week of rhIL-2 alone, prior to R24 treatment. One patient had a partial response in soft tissue sites lasting 6 months and two patients had minor responses. This clinical trial extends the previous observation that R24 enhances lymphocyte proliferation in vitro.

INTRODUCTION

Four mAbs have been reported to demonstrate antitumor activity in patients with metastatic melanoma in recent phase I clinical trials (1-8). All four mAbs bind to glycolipid antigens and three are potent mediators of ADCC. One of these mAbs, R24, reacts with GD3, a disialoganglioside abundantly expressed on human melanoma cells. The mechanism of tumor regressions observed after administration of R24 remains unknown. However, several preclinical and clinical observations have suggested a role for lymphocytes. First, ADCC with R24 and other mouse IgG3 mAbs is mediated by lymphocytes (9-12). Second, R24 and other anti-GD3 mAbs directly induce proliferation and activation of a GD3 subset of T-lymphocytes (13, 14). Third, treatment with R24 induces inflammatory responses at tumor sites, including infiltration with CD8^+/Ia^+ T-lymphocytes (2, 15). In related studies, cytotoxic CD8^+ T-cell clones that specifically lyse autologous tumor cells have been isolated from the peripheral blood of a responding patient after treatment with R24.

We have evaluated a combination of R24 and rhIL-2 in patients with metastatic melanoma in a phase I trial for the following reasons: (a) both mAb R24 and rhIL-2 individually have demonstrated antitumor activity in patients with metastatic melanoma (1-3, 16-19); (b) rhIL-2 has been shown to augment ADCC mediated by mouse IgG3 mAb (12, 20-22), presumably by expanding and activating lymphocyte subpopulations that mediate ADCC; (c) mAb R24 can induce T-cell activation in vitro which is augmented by rhIL-2 (13, 14); and, (d) both rhIL-2 and R24 treatment can induce infiltration of T-cells into metastatic melanoma sites (2, 15, 23). In this trial, rhIL-2 was administered initially as a single agent for 5 days by i.v. infusion to expand potential effector cell populations and then combined with R24 mAb. Toxicity and antitumor effects at four different dose levels of R24 were evaluated. In addition, we assessed the effects of treatment and R24 dose on peripheral blood and tumor lymphocyte populations, in vitro cytotoxicity, and the human immune response to mouse immunoglobulin.

MATERIALS AND METHODS

Preparation and Schedule of R24 and rhIL-2. R24 was produced from hybridoma ascites of pristane-primed, irradiated (BALB/c × C57Bl/6)F1 mice by ammonium sulfate precipitation and purification over protein A as previously described (1). Safety testing and antibody activity was evaluated as previously reported (1). rhIL-2 was kindly provided by the Cetus Corporation (Emoryville, CA). All patients were treated on the general medical ward as inpatients. The treatment plan was as follows: rhIL-2 was administered i.v. at a dose of 1 x 10^6 units/m^2 over 6 h daily on days 1-5 and 8-12. R24 was given by i.v. infusion daily in 100-250 ml of 0.9% (w/v) sodium chloride in 5% (w/v) human serum albumin on days 8-12 at the following dose levels: 1, 3, 8, and 12 mg/m^2 and given at a rate of 5-8 mg/h starting 2 h after the start of rhIL-2. Five patients were treated at each dose level of R24 and doses were not escalated between dose levels.

Eligibility, Toxicity, and Response Evaluation. Patient eligibility requirements included: histologically confirmed stage 1V metastatic melanoma according to the classification of the American Joint Committee for Cancer Staging and End Results Reporting (24); Karnofsky performance status, ≥70; creatinine, ≤2.0 mg/dl; bilirubin, ≤1.5 mg/dl; prothrombin time, <1.3-fold control; granulocyte count, ≥1000/mm^3; platelet count, ≥100,000/mm^3; no evidence of tumor on computed tomographic scan of the brain; no chemotherapy, immunotherapy, or radiation therapy within 4 weeks of commencement of therapy; informed consent; and bidimensionally measurable disease which did not include effusions or bone metastases. Patients were excluded if they...
had clinically significant cardiac disease (New York Heart Association class III or IV), had active infections requiring antibiotic therapy, or were receiving treatment with corticosteroids or nonsteroidal anti-inflammatory drugs. All patients had a negative skin test (wheat, ≤2 cm in diameter) for immediate hypersensitivity with 0.1 µg R24 before the start of treatment. All patients had complete blood cell counts with WBC differential, platelets, prothrombin time, partial thromboplastin time, urinalysis, and complement measurements including C3 and CH50. Serum chemistry profiles (total bilirubin, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, calcium, phosphorus, albumin, total protein, uric acid, electrolytes, glucose, blood urea nitrogen, and creatinine) before treatment, days 1, 5, and 12 during treatment, and then every 2–4 weeks after treatment until progression of disease was evident. Urine was monitored for protein and glucose on days 5 and 12 and then every 2–4 weeks after treatment. R24 antibody levels and HAMA were measured before treatment, days 5, 8, 10, 12, and every 2–4 weeks after treatment. Flow cytometry of PBLs was done before treatment, days 5, 8, 10, and 12. Toxicity was graded according to the modified World Health Organization toxicity scale (25). Criteria for response were as follows:

A partial response was defined as a reduction of measurable tumors by at least 50% of the sum of the two greatest perpendicular diameters for a minimum of 30 days without the appearance of new lesions or the progression of any lesion; minor response was defined as a reduction of all measurable tumor by at least 25% of the sum of the product of greatest perpendicular diameters for a minimum of 30 days without appearance of new lesions or progression of any lesion; progression was defined as the appearance of any new lesions or an increase in the size of any measurable tumor by 50% of the product of two perpendicular diameters (25).

**Immunological Monitoring.** Cell-mediated cytotoxicity was evaluated using PBLs recovered by Ficoll-Hypaque fractionation, washed and cryopreserved at −180°C in 90% fetal calf serum and 10% dimethyl sulfoxide. Cells were subsequently thawed for use as effector cells against target tumor cells in an 18-h ¹ⁱ⁴C-release assay. Viability was usually >90% and always >80%. Cultured target cells were labeled for 60 min with 250 µCi of Na¹¹¹CrO₄, washed 3 times, and resuspended at a final concentration of 5 x 10⁶ cells/ml. Target cells were the human melanoma cell line SK-MEL-30 for ADCC and LAK assays, the erythroleukemia cell line K562 for natural killer assays, and autologous melanoma cell lines for ADCC and cell-mediated cytotoxicity. Target cells were mixed with effector cells in round-bottomed microtiter plates in 0.2 ml final volume at effector:target cell ratios of 6.25:1, 12.5:1, 25:1, and 50:1. R24 was used at a final concentration of 10 µg/ml for ADCC assays. After 18 h of incubation at 37°C, supernatants were collected using a supernatant collection system (Skatron, Lier, Norway) and the radioactivity was counted. The percentage of specific lysis was calculated from the average of triplicate values calculated as follows:

\[
\% \text{ of specific lysis} = \frac{\text{cpm test} - \text{cpm medium}}{\text{cpm max} - \text{cpm medium}} \times 100
\]

Lytic units, defined as the number of effector cells required to lyse 20% of 5000 target cells, were calculated according to the equation

\[ y = A(1 - e^{-kx}) \]

where \( y \) = fractional chromium release, \( x \) = lymphocyte:target ratio, \( k \) = slope of the target cell survival curve obtained by plotting ln(A − y) versus x, and \( A \) = the maximum cell-mediated lysis (26).

Values of percentage of specific lysis for ADCC were determined by subtracting the LAK activity observed at each effector:target ratio. R24 titers of established autologous cell lines were determined by protein A mixed hemadsorption assay as previously described (1, 2). Briefly, sera from patients were incubated with the G09 human melanoma cell line SK-MEL-28 for 1 h, washed with PBS, and incubated for 45 min with human erythrocytes coated with protein A (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Target melanoma cells were scored positive when >50% of the cell circumference was covered by erythrocytes: titers were determined by the last serum dilution giving ≥20% positive target cells.

Proliferation assays were performed using PBLs isolated by Ficoll-Hypaque (10⁶ cells/ml) incubated in triplicate flat-bottomed microwell cultures in the presence or absence of 100 µg/ml mAb R24 or 1% (w/v) PHA. At 4 days, triplicate cultures were pulsed for 4 h with [³¹P]orthophosphate (0.5 µCi/ml; New England Nuclear, Boston, MA), and wells were harvested using a semiautomated cell harvester (Skatron) onto filters and counted by liquid scintillation.

The following mAbs were used for cell surface antigen determination: OKT-3, -4, -8, -9, -10, -11, OKM-5, (Ortho Diagnostic Systems, Raritan, NJ); Leu-7, -11a, anti-IL-2r, anti-HLA-DR (Becton-Dickinson, Mountainview, CA), and B1 and B2 (Coulter Immunology, Hialeah, FL). Direct immunofluorescence staining was performed using 100-µl aliquots of whole blood stained with 5 µl of the appropriate mAb conjugated with FITC. Tubes were incubated on ice for 30 min, lysing solution (lysing reagent, Ortho Diagnostic Systems) was added, and the mixture was incubated for 10 min at room temperature until lyss was complete. Leukocytes were washed with cold PBS and analyzed for T-lymphocyte subsets, percentage of lymphocyte subpopulations, and total WBC counts using the SPECTRUM III laser flow cytometer (Ortho Diagnostic Systems). For indirect immunofluorescence, cells were stained with unconjugated primary mAbs followed by staining with FITC-conjugated secondary antibody. PBLs (100 µl) at a concentration of 1 x 10⁶ cells/ml in cold PBS containing 2% BSA was mixed with 100 µl of an appropriate dilution of unconjugated primary mAb and incubated on ice for 30 min. Cells were washed three times with cold PBS and 2% BSA, and 100 µl of 1:40 dilution of FITC-conjugated goat anti-mouse IgG (Ortho Diagnostic Systems) was added to the cell pellet. Cells were incubated on ice for 30 min. Cells were washed again three times with cold PBS and 2% BSA and adjusted to a concentration of 1 x 10⁶ cells/ml and analyzed by flow cytometry using an H-50 Cytometer (Ortho Diagnostic Systems).

HAMA was detected by enzyme-linked immunoassays. Ninety-six-well microtiter plates were precoated with R24 or 3F8 (anti-Gro5 IgG3) at 2 µg/ml. After blocking with 2% BSA, serial dilutions of serum samples were added for 60 min. HAMA that reacted with 3F8 was considered to have specificity against shared, nonidiotype determinants. After extensive washing, bound human IgG was detected using goat anti-human IgG conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) as a second antibody. The reaction was developed using a p-nitrophenyl phosphate substrate and the absorbance at 410 nm was monitored. A panel of sera from 28 healthy donors, 15 untreated melanoma patients, and pretreatment sera of 44 patients was used to establish negative values.

Immunopathological studies were conducted using tumor tissues obtained prior to and subsequent to treatment in eight patients. Histological sections were cut and stained with hematoxylin and eosin. Lymphocytic infiltration in tumor biopsies was evaluated with immunohistochemical stains and will be reported elsewhere.

**Biostatistical Considerations.** Assessment of a significant change of a parameter for an individual patient was accomplished by establishing a pretreatment baseline for that parameter. The pretreatment baseline was defined as the 95% prediction interval for a random observation of the parameter drawn from a balanced or unbalanced one-way random effect model with normal errors. All the available pretreatment replicated patient values were used to establish both the intrapatient and the interpatient variances by restricted maximum likelihood method. Power transformations were performed on some parameters to promote normality so that the usual inference procedures based on normality assumption could be applied to the data: To relate change of a parameter at two different time points to dose, linear regression with replicated observations at the selected dose levels was performed, adjusting for regression toward the mean if necessary. If there was a significant dose-change relationship, further calculation was performed to determine a threshold dose above which either significant elevation or suppression took place. If a systematic regression relationship could
not be established, then either Student's t test or the Wilcoxon test was applied. When pretreatment baseline values were available for a parameter and comparisons were with the established baseline, the generalized form of the paired t test was used. If a systematic dose-change relationship could not be established and the data suggested comparing the low dose groups (dose levels 1 and 3 mg/m²) with the high dose groups (8 and 12 mg/m²), then the two-sample t test was used to compare the population means or the Mann-Whitney test was used to compare the two population medians.

RESULTS

Toxicity. Twenty-one patients were entered in the trial, and 20 were evaluable for toxicity and response. One patient was not evaluable due to the development of hematuria, ureteral obstruction, and urosepsis on day 3 of treatment. A subsequent cystoscopy revealed melanoma diffusely involving the urothelium, and the patient was removed from the trial. Characteristics of the 20 evaluable patients are given in Table 1. Observed toxicities in all patients are given in Table 2. Two patients had grade 4 hypotension, one each at dose level I and III. Both toxicities in all patients are given in Table 2. Two patients had urticaria, one each at dose level I and III. Both toxicities in all patients are given in Table 2. Two patients had urticaria, one each at dose level I and III. Both toxicities in all patients are given in Table 2.

Adverse effects related to R24 included urticaria, dyspnea, and substernal chest pressure. Urticaria was related to the dose of R24. It was minimal in patients at 1 and 3 mg/m² of R24 but more prominent and con fluent in patients treated at the 8- and 12-mg/m² dose levels. Symptoms of urticaria were controlled in all patients with diphenhydramine. Pulmonary toxicity consisted of dyspnea during R24 infusion and was readily controlled by decreasing the rate of infusion of R24 or by brief interruption and recommencement at a lower rate of infusion. No patient required the use of epinephrine. Substernal pressure was present in 8 patients during the time of R24 infusion but no electrocardiographic changes were noted. Seven of the eight patients experiencing substernal chest pressure were treated at the two highest dose levels of R24. We conclude that the combination of R24 and IL-2 at these doses and schedule does not result in overlapping toxicity.

Analysis of PBLs. There was a significant increase in the total lymphocyte count on day 12 of treatment compared to paired baseline pretreatment values (P = 0.001). Day 12 lymphocyte counts were obtained 18 h after the last dose of rhIL-2 and thus represent a rebound lymphocytosis which has been previously observed after rhIL-2 as a single agent (16-19, 27-30). The median lymphocyte count (+ SE) observed on day 12 in patients treated with R24 doses of 8 and 12 mg/m² was 3108 ± 554/m(12) versus 2239 ± 672/ml, respectively, in patients treated with doses 1 and 3 mg/m². One explanation for this enhanced response is that R24 augmented the T-cell mitogenic response to rhIL-2 in vivo (13). Based on in vitro data, R24 serum concentrations attained by treatment at 8 and 12 mg/m², but not 1 or 3 mg/m², could induce T-cell activation and proliferation.

The rebound lymphocytosis was comprised of cells expressing CD2, CD3, CD4, CD8, CD16, CD25, and HLA-DR cell surface antigens. During treatment, there was a statistically significant increase in the absolute number/ml (but not the percentage) of PBLs expressing these markers on day 12 compared to pretreatment or day 1 values (Table 3). No detectable differences were observed in individual PBL populations relative to R24 dose. Although there were increases in the absolute number/ml of T10+ and GoT+ lymphocytes in individual patients, no statistically significant changes occurred in T10+ or GoT+ PBLs in the overall patient population during treatment. The inability to detect significant change in GoT+ lymphocytes is of interest. On the one hand, R24 lyases and depletes GoT+ T-cells (≤ grade 2) in all patients. Grade 3 temperature elevation (>40°C) occurring during the rhIL-2 infusion was seen in one patient at each dose level.

Table 1  Patient characteristics

<table>
<thead>
<tr>
<th>Median age (yr) (range)</th>
<th>47 (21–65)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median performance status (range)</td>
<td>90 (80–90)</td>
</tr>
<tr>
<td>Male/female</td>
<td>14/6</td>
</tr>
</tbody>
</table>

Table 2  Clinical toxicity

Five patients were evaluable for toxicity at each dose level. Toxicity was graded as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>R24 dose level and grade*</th>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
<th>Level 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urticaria</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
<tr>
<td>Subspleural pressure</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
<tr>
<td>Chills/fever</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
<tr>
<td>Hypotension</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
<tr>
<td>Myalgias</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1 2 2</td>
<td>1 4 2</td>
<td>1 4 1</td>
<td>1 5 1</td>
</tr>
</tbody>
</table>

* Level 1, 1 mg/m²/day; level 2, 3 mg/m²/day; level 3, 8 mg/m²/day; level 4, 12 mg/m²/day.

* No observed toxicity.

Antigen*  Day 1  Day 12

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Day 1</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>1074 (103)</td>
<td>1625 (246)</td>
</tr>
<tr>
<td>CD4</td>
<td>644 (57)</td>
<td>1089 (160)</td>
</tr>
<tr>
<td>CD8</td>
<td>422 (76)</td>
<td>630 (110)</td>
</tr>
<tr>
<td>CD2</td>
<td>1210 (126)</td>
<td>2130 (247)</td>
</tr>
<tr>
<td>CD16</td>
<td>142 (35)</td>
<td>254 (56)</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>259 (24)</td>
<td>768 (93)</td>
</tr>
<tr>
<td>CD25</td>
<td>72 (18)</td>
<td>294 (41)</td>
</tr>
<tr>
<td>T10</td>
<td>152 (36)</td>
<td>560 (196)</td>
</tr>
<tr>
<td>Leu-7</td>
<td>283 (79)</td>
<td>303 (98)</td>
</tr>
<tr>
<td>GD3</td>
<td>189 (85)</td>
<td>324 (145)</td>
</tr>
</tbody>
</table>

* Antibodies used for cell surface antigen are listed in “Materials and Methods.”

* Values observed on day 12 were compared to values obtained pretreatment using the paired t test.

* Mean number cells/ml (SE).

* NS, no statistically significant differences.
in vitro in the presence of human serum by complement-mediated cytotoxicity, particularly at higher concentrations of R24 (14). Competing with this effect, R24 stimulation of PBLs can lead to expansion of the GD3+ population. It is not possible from our data to determine whether either or both of these competing effects was occurring in vivo.

Mitogenic Response of PBLs. Both rhIL-2 and R24 induce proliferation of PBLs. During the first week of treatment (rhIL-2 alone), there was no consistent change in in vitro mitogenic responses of PBLs to R24 or PHA. However, at the end of the second week of treatment (R24 plus rhIL-2), there was a decrease in proliferative response induced by R24 (Table 4). The ratio of proliferation induced by R24 versus PHA (R24:PHA ratio) decreased with a median change of −71% (range, −238 to +40%) compared to values obtained prior to treatment with R24. This decrease in the mitogenic response was only apparent at higher doses of R24. The median R24:PHA ratio for all patients on day 8 was 0.16. This ratio was unchanged (0.17) in patients treated with 1 and 3 mg/m2 but declined to 0.08 in patients treated with 8 and 12 mg/m2 (P < 0.005). There are several possible explanations for a dose-dependent decrease in R24-induced proliferation of PBLs. Depletion of a GD3+ subpopulation of T-cells by complement-mediated lysis has been demonstrated in vitro to abrogate R24-induced proliferation (14). However, no consistent decrease in GD3+ lymphocytes was detected. More likely, maximum R24 proliferation had been attained in vivo, prior to stimulation of PBLs in in vitro assays. PBLs that were activated by R24 in vivo were no longer responsive in vitro. Consistent with this explanation, we have shown that R24-induced T-cell proliferation in vitro peaks on days 5–7 and declines thereafter (14).

Cytotoxicity Assays against Allogeneic and Autologous Melanoma Cells. There was a statistically significant increase in LAK activity against SK-MEL-30 target cells (P = 0.002) and NK activity against K562 target cells (P = 0.009) during treatment that declined to baseline values after completion of therapy (Fig. 1). These effects have been demonstrated during treatment with rhIL-2 alone (16–19, 28, 30). We could not detect augmentation of NK or LAK induced by R24 during the second week of treatment over that observed in during the first week. Two patients demonstrated increases in ADCC mediated by R24 against the GD3+ melanoma cell line SK-MEL-30 during treatment compared to pretreatment values (Fig. 2).

We were able to establish autologous melanoma cell lines in six patients. There was no detectable cytotoxicity against autologous tumor cells in 4-h chromium release assays by unstimulated PBLs taken before, during, or after treatment in any of the six patients. This finding suggests that detectable “arming” of PBLs by R24 mAbs in vivo had not occurred. In ADCC assays with R24, PBLs from three of six patients lysed autologous tumor cells in the presence of R24 compared to no cytotoxicity with PBLs alone. The lack of any ADCC for the remaining three cell lines of the patients was presumably related to relatively low GD3 expression since early passage R24 titers on these cell lines was ≤1:12,500. An increase in ADCC against autologous tumor occurred in one patient (patient 9) during treatment; no detectable changes were observed in the remaining five patients during treatment.

Levels of Serum Complement during Treatment. There was no change in levels of the complement component C3 or total hemolytic complement during treatment at any dose level. This is consistent with our previous results with R24 as a single agent in which significant decreases in serum complement were observed only at doses of 30 mg/m2 and higher.

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**Table 4** Mitogenic response of peripheral blood lymphocytes to phytohemagglutinin

<table>
<thead>
<tr>
<th>Day of treatment</th>
<th>Dose level*</th>
<th>R24 proliferation*</th>
<th>PHA proliferation*</th>
<th>Ratio R24:PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1, 3, 8, 12</td>
<td>4,281 ± 1,438</td>
<td>31,298 ± 6,055</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>8</td>
<td>1, 3, 8, 12</td>
<td>3,853 ± 1,442</td>
<td>18,705 ± 5,111</td>
<td>0.17 ± 0.17</td>
</tr>
<tr>
<td>12</td>
<td>1, 3, 8, 12</td>
<td>3,224 ± 1,347</td>
<td>24,265 ± 7,268</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>12</td>
<td>1, 3</td>
<td>3,990 ± 2,132</td>
<td>35,946 ± 10,739</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>12</td>
<td>8, 12</td>
<td>1,873 ± 1,079</td>
<td>24,614 ± 9,143</td>
<td>0.08 ± 0.04</td>
</tr>
</tbody>
</table>

* mg/m2/day of R24.
* Counts/min.
* Number in parentheses, number of values available to determine the median.
Analysis of HAMA. HAMA developed in all patients between 5 and 37 days after starting R24 treatment. rhIL-2 treatment may have accelerated the development of HAMA in three patients, all of whom developed HAMA by the fifth day of R24 treatment (protocol day 12). HAMA on day 5 is earlier than was observed in the phase I trial of R24 alone. One patient developed HAMA at the end of the first week of rhIL-2 treatment and prior to i.v. injection of R24. This may have been either a response to the intradermal skin test performed on day 1 or enhancement of previously existing, nondetectable HAMA. In either case, it is possible that rhIL-2 helped elicit or facilitate a HAMA response in this patient. HAMA titers were similar to those observed in the phase I trial of R24 given as a single agent, and, thus, it appears that rhIL-2 did not generally augment the amplitude HAMA response. HAMA was directed against shared mouse immunoglobulin determinants and not idiotypic determinants.

Antitumor Activity. Patient 16 achieved a partial response lasting 6 months. This patient was a 22-year-old woman previously treated systemically with cyclohexylaminosorosourea and with palliative radiation therapy to osseous metastases. The patient was treated at the 12-mg/m² dose level of R24. Response occurred in s.c. lesions measuring 4.8 x 3.8 and 3.8 x 3.9 cm. Two patients had minor responses. Patient 14, a 52-year-old male treated at the 8-mg/m² dose level, had approximately a 30% reduction in two s.c. masses measuring 2.0 x 3 and 1.4 x 0.7 cm lasting 5 months. Patient 5, a 46-year-old female treated at the 1-mg/m² level, demonstrated a minor response in s.c. lesions (1.8 x 1.0 and 6.0 x 5.8 cm) and a lymph node (4.5 x 4.5 cm) lasting 3 months. These results suggest that on this schedule and these dose levels, treatment with a combination of R24 and rhIL-2 does not result in enhanced antitumor effects over each agent alone.

DISCUSSION

rhIL-2 has been shown to augment ADCC by mouse IgG3 mAbs and enhance stimulation of T-cells by R24 in vitro assays. In this trial, we explored whether treatment with rhIL-2 in vivo could enhance R24-mediated ADCC, augment proliferative responses of T-lymphocytes to R24, and increase infiltration of lymphocytes in tumor sites. We measured an increase in R24-mediated ADCC by PBLs during treatment in 2 patients. One of these patients had a minor response and the second patient had stable disease for 8 months. We could not unambiguously evaluate an additional responding patient for ADCC (patient 16; partial response) due to a lack of pretreatment values. However, even in this patient there was a substantial increase in ADCC against both autologous and allogeneic target melanoma cells between days 5 and 40 of treatment compared to values observed 107 days after treatment (data not shown). The number of patients in this trial is too small to determine correlations between increased ADCC and antitumor activity but this observation warrants further evaluation in trials using multiple biological agents.

Fifteen of 20 patients had observed increases in LAK activity. No correlation between increases in LAK activity and antitumor response was observed; 3 of 3 responding patients and 12 of 17 nonresponding patients had increased LAK cytotoxicity.

Recently, it has been shown in experimental animals that treatment with mAbs can lead to tumor rejection through direct activation of T-cells. This approach presumably does not require targeting of antibody to tumor but rather to circulating immune cells. T-cells can be activated by mAbs against the CD3 cell surface antigen, a molecular complex closely associated with the T-cell receptor, leading to activation of peripheral blood T-cells and prevention of tumor outgrowth in mice (31, 32). mAbs against another cell surface antigen, Ly6, expressed on lymphocytes and other immune cells can also inhibit outgrowth of murine sarcomas, leukemias, and melanomas in both immunocompetent and athymic nu/nu mice (33). There was evidence that T-lymphocytes were stimulated by R24 in vivo, demonstrated by an increase in lymphocytosis related to R24 dose. Lymphocytes rebounding after rhIL-2 and R24 were comprised of T-cell populations. The effects of R24 on T-cells in vivo are complex because R24 can lyse Gp100+ target T-cells, effectively abrogating T-cell activation (14). If activation of T-cells is important in the antitumor effects of R24, then a potential strategy is to use F(ab′)2 fragments that retain the ability to stimulate T-cells but do not activate complement (14). Finally, there was evidence of increased infiltration of lymphocytes, predominantly T-cells, in tumor sites induced by treatment (data not shown). It was our impression that T-cell infiltration induced by the combination of R24 and rhIL-2 was not substantially different from that observed with either R24 or rhIL-2 alone (2, 23). More reproducible methods to quantitate cellular infiltrates will be important to evaluate this crucial end point.

It is important to discuss some of the issues concerning evaluation of biological end points in this trial. First, there was substantial interpatient variability in the ability of PBLs to mediate ADCC, as has been reported by others (11), and in R24 activation of PBLs. Given the relatively small number of patients studied, particularly at each dose level of R24, this variability makes it more difficult to detect statistical trends in the total patient population and between groups of patients treated at different dose levels. Second, there was a lack of evidence for increases in ADCC against autologous tumors in 5 of 6 patients. Only 1 of the 3 responding patients had an established autologous cell line. This patient did not have baseline ADCC values to determine unequivocally an increase in ADCC. However, ADCC on days 5 and 40 was substantially greater than that observed on day 107. The results with autologous tumors are harder to interpret, however, because of the heterogeneity of antigen expression and selection of subpopulations of cells during outgrowth in vitro. Although virtually all metastatic melanoma lesions express Gp100, there is substantial variability in levels of Gp100 in different lesions and within single lesions (2, 34). In addition, a decrease in expression of Gp100 frequently occurs during outgrowth of melanoma cells in culture (35) and, therefore, Gp100 expression in cell lines does not necessarily reflect Gp100 expression in vivo. Since density of antigen is a major factor in determining sensitivity to ADCC, assays against the cell line SK-MEL-30 that expresses high levels of Gp100 may more accurately reflect optimal activation of ADCC and may be more relevant to tumor response in the patient. Several future strategies can be considered when using the combination of rhIL-2 and mAb therapy. First, higher doses of IL-2 can be explored. The dose and schedule of IL-2 were based on a maximum tolerated dose, based on an earlier trial by Kolitz et al. (29), which could be administered in a non-intensive care unit setting. Results from clinical trials suggest that response to rhIL-2 is related to dose. In vitro data show that the composition of lymphocyte populations that are expanded in vitro depends on the concentration of rhIL-2. Cytotoxic T-cells are more effectively grown at low concentrations of rhIL-2, but
LAK cells are primarily expanded at high rhIL-2 concentrations. In animal models, treatment with mAbs and LAK cells activated by high doses of rhIL-2 have been shown to be effective against established B16 melanoma tumors (21, 35). Augmentation of ADCC by mouse IgG3 mAbs can be observed in vitro at relatively low concentrations of rhIL-2 (9, 12). However, at higher concentrations of rhIL-2, cytotoxicity by LAK cells predominates over ADCC (12). It is important to note that the effector cell population mediating ADCC and LAK activity are both comprised largely of CD16+ (natural killer) cells.

Higher doses of R24 mAb can be considered. The doses of R24 in this trial do not result in saturation of antigen sites on tumor (2), and ADCC in sensitive human melanoma cell lines is related to the concentration of R24 (36). Finally, other effector cell populations such as monocytes, which mediate ADCC, can be explored in combination with mAbs (37). One approach could be the use of monocytes either alone or stimulated by recombinant human macrophage colony-stimulating factor (38). Monocytes cultured in the presence of recombinant human macrophage colony-stimulating factor are particularly effective in ADCC against cultured human melanoma and neuroblastoma cells (39). Clinical investigation of this effector cell population with mAbs capable of mediating ADCC is warranted.

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Phase I Evaluation of a Combination of Monoclonal Antibody R24 and Interleukin 2 in Patients with Metastatic Melanoma

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