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Phase IB trial for malignant melanoma using R24 monoclonal antibody, interleukin-2/α-interferon

R Katherine Alpaugh[1], Margaret von Mehren[1], Irma Palazzo[2], Michael B Atkins[3], Joseph A Sparano[4], Lynn Schuchter[5], Louis M Weiner[1] and Janice P Dutcher[4]

[1]Fox Chase Cancer Center, Philadelphia; [2]Jeanes Hospital, Philadelphia; [3]Tufts University/New England Medical Center, Boston, MA; [4]Albert Einstein Cancer Center/Montefiore Medical Center, Bronx, New York; [5]University of Pennsylvania, Philadelphia, MA, USA

The inflammatory tumor lymphocytic infiltrates and spontaneous tumor regressions seen in patients with metastatic malignant melanomas suggest a cellular immune involvement. Enhancement of such responses has been the goal of R24 (GD3 ganglioside-specific) monoclonal antibody trials, alone and in combination with other agents. This study reports the results of 21 patients treated in a phase IB trial employing R24 (0, 5, 25, 50 mg/m^2) administered by continuous i.v. infusion on days 1–5 followed by 3 MU each of interleukin-2 (IL-2) and alpha interferon (α-IFN) given subcutaneously on days 8–12, 15–19 and 22–26. R24-related toxicities occurred pre-dominantly at the 25 and 50 mg/m^2 doses. One patient (50 mg/m^2 R24) exhibited a dose-limiting Grade 4 anaphylaxis. Cytokine-related toxicities required IL-2/α-IFN dose reduction in two patients and early termination of treatment in five additional patients. Nine of 20 baseline biopsies showed chronic inflammation; six with lymphocytic tumor infiltration and three where inflammation was confined to the perivascular/peritumoral spaces. No day 8 or 29 biopsies in the R24-treated groups demonstrated treatment-induced tumor lymphocytic infiltrates. However, one patient randomized to no R24 treatment, showed a significant inflammatory tumor lymphocytic infiltration at days 8 and 29. Eighteen of 21 treated patients were evaluable for response. One (5%) patient receiving IL-2/α-IFN alone had stable disease lasting 1.5 years. Five (28%) R24, IL-2/α-IFN-treated patients had stable disease ranging from 6 to 32 weeks, with one patient remaining alive 2.5 years post-treatment. Although this combined treatment program was generally well tolerated, no objective responses were seen and significant R24-induced tumor lymphocytic infiltrates were not demonstrated.

Keywords: monoclonal antibody; R24; IL-2; α-IFN; melanoma

Introduction

Multiple phase I trials have been conducted employing the monoclonal antibody R24 in patients with malignant melanoma. Partial remissions in up to 19% of some treated cohorts have been reported.[1–9] R24 is an immunoglobulin G3 (IgG3) murine monoclonal antibody that has specificity for the disialoganglioside GD3, a cell-surface ganglioside which may play a role in cell growth and differentiation. Overexpression of GD3 is seen in melanoma, astrocytoma and sarcoma.[10] *In vitro* R24 is capable of promoting complement-mediated

Correspondence: R Katherine Alpaugh PhD, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA. Tel: (215) 728 2629; Fax: (215) 728 2741
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lysis and antibody-dependent cell cytoxicity (ADCC). Normal tissue reactivity with R24 is restricted to melanocytes, neuronal and glial cells in the central nervous system, parotid gland, adrenal medullary cells, and rare cells in the connective tissue.[10]

GD3 gangliosides are also expressed on a subset of T-lymphocytes, (14% of unstimulated peripheral blood lymphocyte (PBL) and 44% of phytohemagglutinin (PHA) stimulated PBL).[11–15] R24 can potentiate the proliferative response of GD3+ lymphocytes to interleukin-2 (IL-2) and PHA stimulation. This proliferative response *in vitro* was found to be maximal following 5 to 7 days of stimulation, and was preceded by the expression of both membrane-bound and soluble IL-2 receptor.[12–15] Increased cytotoxicity and cytokine induction – gamma interferon (γ-IFN), TNFα and IL-6 – were detected within 72 hours following R24 stimulation.[14,15]

R24 monoclonal antibody therapy has been reported to induce T-cell infiltration of melanoma lesions.[1,2] Maintenance and expansion of these lymphocytes would be expected to enhance the clinical anti-tumor properties of R24. Both IL-2 and alpha interferon α-IFN have been shown to enhance T-cell responses, particularly when used in combination.[16–21] IL-2 is a potent T-cell activator and growth factor with well documented anti-tumor activity in patients with metastatic melanoma.[16,18–21] IL-2 therapy leads to the induction of tumor-infiltrating lymphocytes, some of which have specificity for putative melanoma antigens such as MAGE-1, MAGE-3, tyrosinase, MART-1/Melan-A, and gp100.[17] A decreased recurrence rate in high-risk melanoma patients has been observed following α-IFN therapy.[19,21] Whilst the mechanisms responsible for this adjuvant effect have not been identified, α-IFN has numerous immunomodulatory effects that may contribute to the resulting clinical benefit.[19,21] Combined IL-2 and interferon therapy has been widely employed in the therapy of both metastatic renal cell carcinoma and metastatic malignant melanoma and has been shown to have efficacy in these diseases.[18,19,21]

The current trial used a continuous i.v. administration of R24 for one 5-day cycle which is known to produce sustained plasma levels of this antibody.[2] Cytokine therapy in the current trial was delayed to allow R24 to exert maximally its proliferative effects on GD3-expressing T lymphocytes, to upregulate IL-2 receptor expression,[12,14,15] to target tumor[1–3] and to mobilize effector cell infiltrates, promoting both complement-mediated lysis and ADCC. The IL-2/α-IFN therapy was given on days 8–12, 15–19 and 22–26 and was designed to expand further the effector cells at tumor sites, and to activate both the peripheral blood and tumor-infiltrating effector cell populations. The primary goal of this trial was to determine the dose of R24 in conjunction with a defined IL-2/α-IFN schedule which produces an optimal expansion of antibody-activated tumoricidal infiltrates compared with IL-2/α-IFN or R24 alone. In addition, toxicity of therapy where R24 is given prior to IL-2/α-IFN and the effects of the therapy on the peripheral blood T cell populations were determined.

Materials and methods

Patient eligibility

Patients were required to have histologically proven malignant melanoma, incurable by conventional therapy and have a minimum of three 1 × 1 cm biopsy-accessible lesions. Patients were excluded for an Eastern Cooperative Oncology Group performance status (ECOG) greater than 1; prior exposure to murine antibody, alpha-interferon (α-IFN) or interleukin-2 (IL-2), chemotherapy or radiation therapy within 4 weeks (6 weeks if nitrosoureas or mitomycin C); and WBC < 3000/mm^3, platelets < 100 000/mm^3, BUN > 30, creatinine > 2.0, total bilirubin > 1.8, SGOT and SGPT > 2 × upper limit of normal. Initially, prior IL-2 and α-IFN therapy was not permitted but the study was amended to permit such prior therapy after the sixth patient had been enrolled. Patients were required to have normal cardiac function, no existing brain metastases or active peptic ulcer disease. Anti-coagulants, corticosteroids and additional cancer therapies were excluded while the patient was on study. All patients gave written informed consent.

Clinical monitoring and laboratory studies

Patients were monitored by clinical laboratory tests including complete blood counts and serum chemistries (electrolytes, BUN, creatinine, SMA-12, LDH, ALT) at baseline and periodically during the 29-day cycle. Clinical responses and toxicity were graded using standard ECOG criteria. CT scans and X-rays were used to document measurable or evaluable disease at baseline and day 29. When accessible, serial tumor biopsies were performed at baseline, before treatment day 8 and day 29. Immunophenotyping of blood

peripheral lymphocyte samples was performed at baseline, day 8 and day 29.

Treatment

R24 (provided by NCI, Bethesda, MD, USA: MoAb R24, NSC 608918) was administered by continuous i.v. infusion on days 1–5, followed on days 8–12, 15–19 and 22–26 by subcutaneous injections of 3 MU/day each of IL-2 and α-IFN (provided by NCI, Bethesda, MD, USA: recombinant human interleukin-2–rIL-2, IL-II–NSC 600664, BB-IND 2186; interferon alpha-2a CTEP/DCT/NCI–Roferon-A®–alpha-interferon–rIFN-A, rLeuA–NSC 367982, BB-IND 1913, supplied by Hoffman-LaRoche, Nutley, NJ, USA to the NCI). Patients were not premedicated with antihistamine or steroids. Patients received only one course of a randomly assigned R24 dose. The maximal R24 dose was previously determined in the single agent trials.[1,2] The IL-2/α-IFN regimen was piloted at the Biologic Response Modifiers Program of the National Cancer Institute (personal communication 1991, M Sznol, NCI). Patients with stable or responding disease were eligible for a second cycle of four weeks of IL-2/α-IFN therapy following a two-week break. Thereafter, only patients with responding disease could continue receiving cycles of cytokine therapy.

Flow cytometric analysis

Heparinized or EDTA peripheral blood was collected by venipuncture. 100–300 μl of whole blood was mixed and incubated at 37°C with the appropriate fluoroscein (FITC) and phycoerythrin (PE) coupled antibodies. After incubation, erythrocytes were lysed with FACS Lysing Solution, washed and fixed with 1% paraformaldehyde. Flow cytometry analysis to obtain the percentage of the total population expressing a specific surface marker used a FACScan (Becton-Dickinson, San Jose, CA, USA), gating on the CD45 positive population. Antibodies (Becton-Dickinson, San Jose, CA, USA) anti-Leu4 (CD3), Leu2a (CD8), Leu3a (CD4), Leu12 (CD19), Leu M3 (CD14), Leu19 (CD56), CD11a, Leu11c (CD16), CD25, Leu23 (CD69), HLADR, and the R24 monoclonal antibody were used for analyses.

Immunohistochemistry

Serial biopsies collected at baseline, days 8 and 29 were snap frozen and stored at –70°C until reviewed. Two micron sections were stained with R24 (40 μg/ml), S-100, HMB45, LN5, LCA (leukocyte common antigen), CD57 and developed using a BioGenex (San Ramon, CA, USA) biotin-streptavidin detection system and AEC chromogen as per manufacturers' directions. Tissue staining with R24 monoclonal antibody (mAb) was graded for intensity (+1 to +5), and the percentage of tumor cells expressing the reactive GD3 epitope. Immunohistochemical analyses were performed to evaluate for evidence of necrosis and perivascular or intra-tumoral inflammation, and to describe the cellular composition. Serial sections were stained to define and estimate the number of infiltrating cells per high power field (400 × magnification) positive for LCA, LN5 and CD57. Any observed perivascular or peritumoral lymphocytes were documented but not otherwise scored.

Results

Patient characteristics

Twenty-one patients were treated in this Phase IB trial, as outlined in Table 1. The median age was 49 years. There were 13 men and 8 women. ECOG performance was 0 in 10 and 1 in 11. Eleven patients were previously treated with chemotherapy and all had undergone prior surgery. No patient had prior IL-2 treatment, one patient had received granulocyte colony-stimulating factor (G-CSF) and another had previously received a chemotherapy regimen and tamoxifen combined with α-IFN. All patients had metastatic disease (Table 2). Twenty of 21 patients were evaluable for toxicity and 18 for tumor response.

Toxicity

Non-hematological effects Skin rashes and urticaria of grade 2 or higher were observed at the 25 and 50 mg/m^2 doses (Table 3). R24-related toxicities

Table 1 Dose schema

Level	*R24 dose (mg/m^2/day)*[a,b]	*No. of patients*
I	0	5
II	5	6
III	25[c]	4
IV	50	6

[a]R24 dose was randomized and administered by continuous intravenous infusion daily on days 1–5 with 3.0 MU/day each of IL-2 and α-IFN given subcutaneously on days 8–12, 15–19, and 22–26.
[b]A second four week cycle of IL-2 and α-IFN following a two week break was given to patients with stable or responding disease; if second evaluation showed response, patients were eligible for additional cytokine cycles.
[c]The 25 mg/m^2/day dose level was eliminated from the randomized assignment following the treatment of patient 17.

Table 2 Patient characteristics

Total patients			21
Age	mean	49	
	range	(29–71)	
Sex	male	13	
	female	8	
Performance status	0-10		
	1-11		
Prior therapy			
Chemotherapy			
none		10	
single		6	
combination		7	
Radiation		5	
Immunotherapy		5	
Surgery		20	
Current disease status			
local disease			1
regional disease, in transit			3
regional node disease			4
distant nonvisceral disease			4
distant visceral disease			9
Metastatic sites			
lung			9
liver			5
bone marrow/bone			4
subcutaneous/skin			15
node			11
Other (gastrointestinal, adrenal, groin, brain)			6

occurred generally on days 1–3 of administration and subsided on days 4 and 5. R24 was discontinued on day 2 in one patient due to grade 4 anaphylaxis and grade 3 hypotension. One hour after R24 infusion started, this patient noted a rash and was treated with diphenhydramine. Twelve hours after starting therapy, the R24 infusion was discontinued for signs and symptoms of anaphylaxis. The patient improved with fluids and volume expanders, but required dexamethasone and epinephrine for complete resolution of symptoms.

All patients experienced grade 2 or higher IL-2 and α-IFN-induced toxicities. These toxicities included diarrhea, nausea/vomiting, fever, fatigue, depression, neuromotor (depression, hallucinations/confusion), or pulmonary (shortness of breath) (Table 3). Four patients were removed from study due to such toxicity. In two patients the IL-2/α-IFN doses were reduced by 50%. One patient was removed from study on day 9 due to a pulmonary embolus, associated with a lower extremity deep venous thrombosis. Patients were noted to have increases in their liver transaminases, alkaline phosphatase and LDH with IL-2/α-IFN treatment. Increases in transaminases were in the range of 2–4 times baseline values, usually peaking after the first four days of cytokine therapy. Alkaline phosphatase and LDH values tended to continue rising throughout the cytokine therapy. Only one patient required discontinuation of cytokines because of increases in liver function tests on day 18 of cycle 1. This patient subsequently received a second cycle of IL-2/α-IFN without a further dose reduction and did not develop any hepatic toxicities. Two other patients received two cycles of cytokine therapy.

Hematological effects Although significant fluctuations in total white counts were not evident, an IL-2/α-IFN-related transient grade 3–4 lymphopenia was noted at day 12 in 7/9 patients in whom white blood cell differentials were performed at each time point. However, lymphocyte counts returned to normal and showed a subsequent rise on days 15 and 22, with a 2–3 fold increase in the absolute lymphocyte count compared to baseline values. Minor changes in red blood cell and platelet counts were noted but without correlation with the R24 dose.

Flow cytometric analysis

Patient samples were analyzed at baseline (day 1), prior to the initiation of IL-2/α-IFN (day 8), and upon the completion of the cycle (day 29). Complete flow cytometric data was available for analysis on only nine patients. In those nine patients (3 at 0 mg/m^2, 2 at 5 mg/m^2, 2 at 25 mg/m^2, 2 at 50 mg/m^2 R24), preferential expansion of the $CD8^+$ lymphocyte population was not detected at day 8 in the R24-treated cohort and no change in the percentage expression of $GD3^+$ or $CD3^+$ lymphocyte populations correlated to R24 treatment. An R24-induced 1.3 to 2-fold increase in the percentage of CD25-expressing cells noted on day 8 in 6 of 16 patients was non dose-related. Flow cytometric analyses at day 29 showed no variation from baseline (or day 8) which could be associated with the IL-2/α-IFN treatment (data not shown).

Immunohistochemistry

Twenty of 21 patients were biopsied at baseline and 16 of 20 were serially biopsied on days 8 and/or 29. All samples had a histological diagnosis of metastatic malignant melanoma. Baseline inflammation was noted in 9/20 samples (45%), with six demonstrating leukocyte infiltration within the tumor bed; in the remaining three samples, leukocyte infiltration was confined to the perivascular spaces within the fibrocollagenous tissue surrounding the tumors and in the peritumoral stroma. Immunohistochemistry for GD3-expression and the characterization of the lymphocytic infiltrates was performed on the serial biopsies of seven of 16 selected patients, six of whom had shown baseline inflammation

Table 3 Toxicity/outcome

Patient No.	*R24 dose (mg/m²)*	*Toxicity (grade) R24*	*IL-2/α-IFN*	*Best response (weeks duration)*
3[a]	0		+3nausea; +3 neuromotor (weakness)	P
6			+2 fever; +3 fatigue (myalgia)	P
10			+2 fatigue	S (80)
15			+2 nausea; +3 fatigue/depression	P
16			+3 nausea	P
2[a]	5			NE
8[a]			+3 nausea/vomiting	S (16)
11[a]			+2 fever/liver function; +3 neuromotor/stomatitis	NE
17			+3 diarrhoea; nausea/vomiting; neurological (psych)	S (6)
20[a]		hives	+3 myalgias	S (8)
22		ND		NE
1[a]	25	+2 hives		P
7[a]			+2 nausea/vomiting	P
12		+2 skin rash	+2 fatigue; nausea/vomiting	P
13			+2 fatigue; alopecia	P
4	50		+2 nausea/vomiting; diarrhoea anorexia; +3 fatigue	P
5		NT		
9[a]		+3 hypotension		S (24)
		+4 anaphylaxis	+3 diarrhoea; vomiting; +4 fatigue	P
14			+3 paresthesia	
18				P
19		+2 nausea +3 skin rash; shortness of breath (↑ LFTs)		P
21[a]		+2 skin rash; +4 hives; ↑ LFTs		S (32)[b]

ND - no data available; NT - not treated; NE - not evaluable; P - progression; S - stable.
[a]inflammation at tumor site noted in pre-treatment biopsy; [b]alive at 2.5 years post-treatment.

(see above). A non tumour-infiltrating perivascular-peritumoral inflammatory lymphocytic expansion was noted in five of the six reviewed patients following R24 treatment (2 at 5 mg/m^2, 2 at 25 mg/m^2, 1 at 50 mg/m^2 of R24 mAb). One patient treated with no R24 showed an IL-2/α-IFN-induced inflammatory response, but no R24-induced inflammatory responses were documented (data not shown).

The presence of therapy-targeted murine immunoglobulin could not be detected in any biopsies obtained at days 8 or 29 in those patients showing baseline staining for GD3-expression (Table 4). In the six evaluated patient samples baseline GD3-expression ranged from a low of 30% of tumor cells giving a +2 intensity to 95–100% of tumor cells with a +2–3 intensity. Days 8 and 29 biopsies showed evidence of GD3 down-modulation with GD3-expression decreasing 30–80%.

The composition and biological features of tumor infiltrating effector cells did not vary according to the R24-treatment permutations received by these patients. No induction of an antibody-activated tumoricidal infiltrate was documented. Although four out of six biopsies from patients with stable disease showed some degree of baseline inflammation (Table 3 footnote a), treatment-related augmentation of this baseline inflammation was noted only in one patient who received IL-2/α-IFN therapy alone (data not shown).

Clinical response

Eighteen of 21 patients were evaluable for clinical response (Table 3). Sixteen patients received the assigned dose of R24. No objective tumor responses

Table 4 Tumor cell GD3 modulation[a]

Patient No.	*R24 dose (mg/m²)*	*R24-reactivity (intensity/%)*[b] *Day 1*	*Day 8*	*Day 29*
3	0	+2–3/100	+2–3/100	+2–3/100
8	5	+1–2/95	+1–2/15	NA
11	5	+2–3/95	NA	NA
20	5	+2/100	+1–2/70	NA
22	5	+2–3/100	+2–3/70	NE
9	50	+2/30	NA	NA
21	50	+2–3/95	+1/95	+1/40(focal)

[a]Performed on the selected patients indicated;
[b]Intensity of R24 staining +1–+5; % GD3 + tumor cells;
NA - not available; NE - staining results not evaluable.

were observed. Stable disease was documented in six of 18 (33%) patients, with one (6%) patient remaining alive 2.5 years post treatment, and subsequently lost to follow-up. Survival time for the 19 patients who succumbed to disease following treatment ranged from 1 to 24 months (mean/median of 1 year).

Discussion

This phase IB trial of R24, a GD3-specific murine IgG3 monoclonal antibody, and IL-2/α-IFN was a therapeutic permutation aimed at inducing enhanced lymphocytic inflammatory tumor infiltrates and improving response compared with their use as single agents in patients with metastatic malignant melanoma (Table 5). Using R24 as a single agent Houghton *et al*[1,2] observed a 19% response. In another R24 trial in patients with pulmonary metastases from soft tissue sarcomas,[5] no patient had subsequent development of metastases. Occasional responses have been observed in trials combining R24 with IL-2,[4] TNFα,[6] cisplatin and WR-2721[7] or GM-CSF.[8] In these studies, tumor responses were initially observed 3–20 weeks following therapy.[1,2,7]

Toxicities to the murine immunoglobulin administration were related to dose and rate of infusion. In general, when infusion was maintained at < 5 mg per hour, toxicities were mild, most prominent during the initial treatments and controlled by antihistamines. Similar R24-related side effects have been seen in previous studies. The more severe toxicities were all associated with the agent which was combined with the R24 therapy. In no trials, including this study, were synergistic toxicities encountered.[3,4,7–9] The majority of patients receiving R24, even at 1 mg/m^2 doses developed human anti-mouse antibody (HAMA) within eight days of treatment initiation, and when IL-2 was given prior to R24, an acceleration of HAMA induction was suggested.[4] R24–HAMA-related clearance has been documented following HAMA induction.[2,3,7]

In vitro stimulation of peripheral blood mononuclear populations with R24 has been shown by flow cytometric analysis to induce IL-2 receptor and HLADR expression on lymphocytes after 3–6 days.[14] In clinical trials of IL-2/R24, Bajorin *et al*[4] did not see variation in the percentage of cells expressing CD3, CD4, CD8, CD16, CD25 and HLADR, although R24 dose-related peripheral blood lymphocytosis was observed at the end of treatment supporting the *in vitro* data that R24 can potentiate lymphocyte proliferative responses to other mitogenic stimuli. Caulfield *et al*[3] reported decreases in both CD4$^+$ and CD8$^+$ populations following combined R24 and α-IFN treatment. It was not determined in that study whether the effect was due to R4, α-IFN, or a combined effect. Minor increases in CD25 expression were noted in this current study.

Biopsies taken on days 8 and 29 of treatment showed a varying degree of GD3 antigen modulation (Table 4). In previous studies of R24 therapy, the detection of murine IgG has been dose-dependent and weakly observed in perivascular spaces in biopsies obtained from patients treated with 10 mg/m^2. Penetration of the murine IgG into tumor, accompanied by increased perivascular deposits were seen following treatment with 50 mg/m^2 R24.[1,2,3,7] In the current trial, biopsies were performed on day 8 (3 days post antibody). No murine IgG was detected in the limited samples examined from patients who received greater than 5 mg/m^2 R24. Inflammatory reactions previously have been noted around skin lesions in patients treated with R24 doses ≥ 10 mg/m^2.[1,2] Houghton *et al*[1,2] saw evidence in two patients of an increase over baseline of infiltrating CD8$^+$ lymphocytes, suggesting treatment with R24 could produce a localized inflammation. Marked infiltration confined mainly to the peritumoral or perivascular spaces was found in day 5 biopsies in

Table 5 R24 clinical trials

R24 doses (mg/m^2)	*No. of R24 treatments*	*Other agents*	*Responses/ total*	*Date and reference*
1[e], 10[e], 30/day[f], 50/day[f]	8		4/21	1985[1,2]
8[g]	10	IFNα-2a[a] 10	0/15	1990[3]
0, 1, 3, 8, 12[j]	5	IL-2[g,b]	3/20	1990[4]
5[e]	6		[k]/10	1994[5]
10[e]	2	TNF[c]	1/8	1994[6]
0, 8, 16, 20, 40[g]	10	cisplatin/WR-2721[d]	2 (1)[m]/23	1994[7]
0, 10, 50[n]	8	GM-CSF[a]	3/20	1994[8]
1, 3, 10, 30, 50[p]	5	M-CSF[b]	0/19	1995[9]

[a]Prior to R24 (GM-CSF days 1-21); [b]overlapping with R24 (M-CSF continuous i.v. days 1–14); [c]1 h i.v. 3–4 h following R24; [d]Day 1; immediately prior to R24; [e]every other day i.v.; [f]continuous i.v. days 1–5, 8–12; [g]6 h i.v. days 1–5, 8–12; [j]2 h after start of IL-2; days 8–12; [k]no patient had sequent development of pulmonary metastases; [m]2 pts receiving all agents; 1 pt received no R24; [n]continuous i.v. days 8–15; [p]several hours i.v. days 6–10.

trials of the concurrent administration of R24 and α-IFN. However, it was concluded there was no consistent change compared with pretreatment samples.[3] Casper *et al*[5] also found mild to moderate infiltrates around and within pulmonary metastases, but biopsies of primary tumor (pretreatment) from most of these patients had equivalent infiltrates. Biopsies from patients treated with cisplatin, WR-2721 and R24[7] showed variable infiltration of $CD3^+$, $CD4^+$ and $CD8^+$ cells but the results of pre-treatment biopsies were not discussed.

Several trials have assessed *in vitro* the cytotoxic capabilities of peripheral blood lymphocytes,[3,4,7] monocytes,[8] and/or granulocytes[8] pre- and post-treatment with R24, alone or in combination with other agents. Cells obtained from patients treated with R24 together with either α-IFN, cisplatin/WR-2721, or IL-2 all showed no enhancement of antitumor NK or ADCC activity following treatment. Two patients treated with IL-2/R24 did have evidence of some ADCC for $GD3^+$ tumor cells.[4] There was some upregulation of both monocyte and granulocyte ADCC with GM-CSF/R24 therapy[8] but GM-CSF-enhanced R24 responses could not be proven. These seemingly treatment-induced cytotoxic capabilities are pertinent only if this population can be sequestered at tumor.

Current therapies with murine monoclonal antibodies are hampered by toxicities at high doses and by the inability to deliver adequate amounts of antibody to tumor sites. Tumor targeting of R24 has only been detected at doses $> 10\,mg/m^2$. Moreover, repeat dosing with murine immunoglobulins is complicated by HAMA induction and HAMA antibody-complex clearance.[2,3] Of interest was the observation by Vadham-Raj *et al*[2] that patients with partial tumor responses had delayed HAMA induction. We have made similar observations in other immunotherapy protocols (unpublished data). Investigation into the mechanism of this delayed response could shed light on immunomodulating factors governing TH1 versus TH2 responses.

The goal of many immunotherapy trials is to increase potential effector cell infiltration at tumor site. Inflammatory infiltrates are most likely related to Fc-associated complement deposition and by-products such as C5a[1] and C3a components which are chemotactic for granulocytes and monocytes. However, antibody treatment-related toxicities are amplified by Fc domain-induced functions and cellular interactions.[22] Humanized or human chimeric recombinant antibody motifs lacking Fc-domains can minimize the adverse Fc-domain toxicity.[23] In addition, genetic engineering of antibody molecules to include chemotactic factors or cytokines could promote the desired inflammation at tumor sites,[1] decreasing intravascular systemic cellular activation. Thus, pre-targeting strategies using biologically active agents (M-CSF, GM-CSF) to induce proliferation of the desired cellular infiltrates, followed by administration of tumor-targeting proteins consisting of a tumor-specific antibody fragment recombinantly fused to chemotactic factors (or cytokines) and then the introduction of an activating biological (IL-2, α-IFN/γ-IFN) could create the desired cellular compositions at tumor sites.

In the current R24 trial the composition and biological features of the effector cells infiltrating the melanoma deposits were not treatment augmented. We could not confirm the hypothesis that a combined therapy with R24 and IL-2/α-IFN results in the induction of antibody-activated tumoricidal infiltrates. An IL-2/α-IFN *in situ* expansion of infiltrating lymphocytes was seen in one patient receiving IL-2/α-IFN alone and the expansion of peritumoral/perivascular lymphocytic populations was observed in five R24, IL-2/α-IFN-treated patients. However, no R24 dose-associated effects on tumor-associated effector cell populations were observed. No synergistic toxicities were encountered. The absence of objective clinical responses, and of effector cell infiltration at tumor sites, shows that this biological combination therapy does not provide a platform for incremental strategies employing these agents.

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