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Mouse monoclonal IgG3 antibody detecting G_{D3} ganglioside: A phase I trial in patients with malignant melanoma

(complement activation/inflammation/immunotherapy)

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ABSTRACT **R_{24} is an IgG3 mouse monoclonal antibody that identifies G_{D3}, a prominent ganglioside on the surface of melanoma cells and other cells of neuroectodermal origin. Twelve patients with metastatic melanoma were treated with R_{24} at three dose levels, 8, 80, or 240 mg/m^2, over a period of 2 weeks. Peak antibody levels in the serum were dose related and ranged from <0.1 to 62 μg/ml. Inflammatory reactions (urticaria, pruritus, erythema, subcutaneous ecchymoses) were observed around tumor sites in patients treated at doses ≥80 mg/m^2. Tumor biopsies during and after treatment showed lymphocyte and mast cell infiltration, mast cell degranulation, and complement deposition. Side effects were mild and were readily controlled by antihistamines. Major tumor regression has been observed in three patients.**

Mouse monoclonal antibodies have identified a large number of antigens on the surface of melanoma cells (1, 2). Althougn none of the antigens are melanoma specific, some antigens have characteristics of differentiation antigens that mark melanocytes, melanoma, and other cells of neuroectodermal origin (3). An IgG3 mouse monoclonal antibody, designated R_{24}, identifies one of the most restricted of these neuroectodermal markers. R_{24} was generated by Dippold *et al.* (4) during a study of surface antigens of cultured melanoma cells and Pukel *et al.* (5) have shown that R_{24} identifies the disialoganglioside G_{D3}. Analysis of cultured cells (4) and normal and malignant tissues (6, 7) showed that R_{24} reacts with melanocytes, astrocytes, melanomas, astrocytomas, and a subset of sarcomas. R_{24} also mediates a variety of biological effector functions, including tumor cell aggregation, human complement-mediated cytotoxicity, and antibody-dependent cell-mediated cytotoxicity with human effector cells (refs. 8–10; unpublished work).

In the present study, we have investigated the response of melanoma patients to R_{24} with regard to different dose levels, toxicity, serological parameters, and tumor response.

MATERIALS AND METHODS

Patients. Patients' tumors were shown to express G_{D3} by indirect immunofluorescence tests on frozen sections prior to treatment. All patients had objectively measurable disease, a performance status (Karnofsky scale) of at least 60, and had been off anticancer therapy for at least 4 weeks. No concurrent anticancer therapy was given during evaluation. Patients were considered evaluable 6 weeks after initiation of therapy; 10 patients are evaluable and 2 have not yet reached the 6-week mark.

Preparation and Administration of R_{24}. R_{24} was prepared from ascites of (BALB/c × C57BL/6)F_1 mice and purified by ammonium sulfate precipitation, chromatography over protein A-Sepharose and Sephadex G-25 columns, and filtration. Each R_{24} batch was tested for antibody reactivity and assayed for nucleic acids, 16 mouse viruses, bacteria, fungi, and mycoplasma. Preparations underwent standard safety testing in mice and guinea pigs and pyrogenicity testing in rabbits.

R_{24} was administered by i.v. infusion in 100–200 ml of 0.9% saline/5% human serum albumin. Skin tests with 0.1 μg of R_{24} were done before the first treatment. The schedule of treatment was 1 or 10 mg/m^2 every other day for eight treatments or 30 mg/m^2 per day by continuous infusion on days 1–5 and 8–12.

Serological Tests. R_{24} antibody titers were determined by testing serum samples in protein A mixed hemadsorption assays (11) against the melanoma target cell line SK-MEL-28. R_{24} concentrations were measured by an enzyme-linked immunoassay. Falcon 3034 plates were precoated with purified R_{24} at 125 μg/ml. Rabbit anti-mouse IgG3 (Bionetics, Kensington, MD) diluted 1:100 was mixed (1:1, vol/vol) with patients' serum samples diluted 1:4 and incubated for 120 min. The mixture was transferred to the precoated wells and incubated for 60 min, and wells were washed with phosphate-buffered saline. Wells were incubated with goat anti-rabbit IgG linked to alkaline phosphatase (Sigma) for 60 min. Alkaline phosphatase activity was determined using *p*-nitrophenyl disodium phosphate substrate (12). R_{24} concentrations were determined by comparison with standards using different concentrations of purified R_{24} diluted in a pretreatment serum sample from the patient.

Human IgG antibody against mouse Ig was detected by enzyme-linked immunoassays. Falcon 3034 plates precoated with R_{24} at 50 μg/ml were incubated with patients' serum samples diluted 1:50 for 60 min and washed with phosphate-buffered saline. Anti-human IgG linked to alkaline phosphatase (Sigma) was incubated in wells for 60 min, and reactions were measured by spectrophotometry (12).

Indirect immunofluorescence and immunoperoxidase procedures were carried out as described (13). The following reagents (Ortho Diagnostics) were used for testing tissue sections: OKT3, OKT4, OKT8 antibodies to T-cell markers; OKB2 and OKB7 antibodies to B-cell markers; OKM1 and OKM5 antibodies to macrophage markers; and OKIa1 to human Ia antigens. Goat antisera to the human complement components C3, C5, and C9 were provided by Carl-Wilhelm Vogel. R_{24} was used at a concentration of 40 μg/ml. Mouse IgG in tumor tissues was detected by incubating sections directly with biotinylated goat anti-mouse IgG, then with avidin-peroxidase conjugates and substrate. The toluidine blue staining method was used to detect tissue mast cells.

RESULTS

Patient Characteristics. Table 1 lists the clinical features of the 12 patients included in the study. The patients received total doses of 8 mg/m^2 (three patients), 80 mg/m^2 (six pa-

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Table 1. Clinical features and tumor responses of patients treated with R_{24}

Patient/age, yr/sex	Primary site	Performance status*	Prior therapy	Dose: Level, mg/m^2	Dose: Total, mg	Site(s) of disease	Response and duration (by site)
1/55/♀	Foot	90	Intralesional therapy	8	16.8	Skin†	Progression after 4 weeks
2/57/♀	Leg	70	Radiation therapy	8	13.6	Skin†	Partial response‡, 10 weeks
						Brain	Stable, 19 weeks
3/36/♀	Trunk	90	BCG, *cis*-retinoic acid	8	13.6	Axilla and paratracheal lymph node†	Partial response‡, 38+ weeks
						Lung	Progression after 24 weeks
4/30/♂	Leg	70	Interferon, intralesional therapy	80	168	Skin and lymph nodes†	Partial response‡, 28+ weeks
5/52/♂	Unknown	70	Intralesional therapy	80	90	Skin and lymph nodes†	Progression after 6 weeks§
6/25/♀	Neck	70	Autologous tumor cell vaccine	80	144	Skin†	Progression after 10 weeks
						Brain	
7/44/♀	Leg	70	Intralesional therapy	80	136	Skin and lymph nodes (axilla, retroperitoneum)†	Progression after 5 weeks§
8/30/♂	Choroid	60	Methotrexate, BCNU, dacarbazine, hydroxyurea	80	144	Skin†	Progression, died at 6 weeks
						Liver†	
9/67/♂	Trunk	90	Dacarbazine, interferon	80	152	Skin†	Progression after 6 weeks
						Lung†	Partial response‡, 10 weeks
10/58/♂	Trunk	70	Dacarbazine, CCNU, dibromodulcitol, pimozide	240	528	Skin†	Partial response‡, 6 weeks
11/34/♀	Leg	90	Dacarbazine, actinomycin D	240	400	Retroperitoneal lymph nodes†	Stable, 4+ weeks
12/26/♀	Leg	90	Cytoxan	240	400	Skin, mediastinum, and lung†	Stable, 4+ weeks

BCG, bacillus Calmette–Guerin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.
*Karnofsky scale.
†Site of measurable disease.
‡Partial response: ≥50% reduction in the sum of the products of the maximum and perpendicular diameters of all measurable lesions for at least 4 weeks.
§Patients 5 and 7 subsequently achieved partial responses with dacarbazine treatment.

tients), or 240 mg/m^2 (three patients). The median age was 40 years (range 25–67) and the median performance status was 70 (range 60–90). Seven patients had received prior chemotherapy, radiation therapy, or interferon treatment. All patients had skin or soft tissue disease. In addition, visceral metastases were present in six patients, including lung (three patients), brain (two patients), and liver (one patient).

Toxicity. No side effects were observed in the three patients treated with the lowest dose of R_{24} (8 mg/m^2). All patients receiving a total dose of 80 mg/m^2 or greater had skin reactions, manifested by urticaria and pruritus usually developing 2–4 hr after starting treatment. The intensity of skin reactions was related to the dose level and rate of antibody infusion. Urticaria characteristically appeared over tumor sites in the skin and subcutaneous soft tissue and around surgical scars where tumor had been removed. One patient (patient 6) developed urticarial lesions at sites where she had received melanoma cell vaccines 8 months previously. No reactions developed at R_{24} skin test sites or around surgical scars unrelated to tumor treatment. Six patients went on to develop more generalized urticarial lesions over the face, trunk, or limbs (patients 5, 7, 8, 10, 11, 12). Patient 5 experienced mild wheezing after rapid infusion of antibody (10 mg/hr); in this case the dose of R_{24} was reduced to 66% of the total intended dose. Diphenhydramine was effective in controlling side effects but was only used for systemic symptoms.

At a dose level of 80 mg/m^2, the severity of skin reactions, particularly pruritus, was found to be related to the rate of infusion of R_{24}. Treatment was tolerated well when the infusion rate was maintained at <5 mg/hr. At this rate, skin reactions usually occurred only after the first, second, and third infusions and not after subsequent treatments. At 240 mg/m^2, R_{24} was administered by continuous infusion to maintain an infusion rate of <5 mg/hr. All three patients treated at this dose level developed urticaria initially restricted to tumor sites, which later became generalized. Patients 10 and 11 experienced mild nausea and vomiting between 4 and 8 hr after the start of treatment. Temperature elevation (up to 37.8°C) was seen in patients 11 and 12 near the end of treatment. No hepatic, renal, hematopoietic, or neurological toxicity was observed and no changes were noted in vision or skin pigmentation over a period of up to 9 months follow-up.

Antitumor Effects. Table 1 summarizes tumor responses in patients treated with R_{24}. Major tumor regression was observed in the three patients presented below.

Patient 3 is a 36-year-old woman with primary malignant melanoma of the back, Clark's level IV, 5-mm depth of invasion diagnosed Sept. 1982. In Aug. 1983, the patients was found to have recurrent tumor and on Nov. 30, 1983, treatment with R_{24} was started, 1 mg/m^2 (1.7 mg) every other day for eight doses. No toxicity or reactions at tumor sites were noticed during treatment. Sites of measurable disease included a firm 7 × 9 cm right axillary mass, a 4 × 2.5 cm subcutaneous nodule over the right hip, and a 3 × 4.5 cm right paratracheal mass (Fig. 1). A poorly defined density was present in the right upper lobe of the lung. Regression of tumor in the axilla and paratracheal region was first observed 5 weeks after starting treatment. The paratracheal mass has been undetectable since March 1984 (Fig. 1). The right axillary mass measured 1.2 × 0.8 cm in May 1984 and has continued to

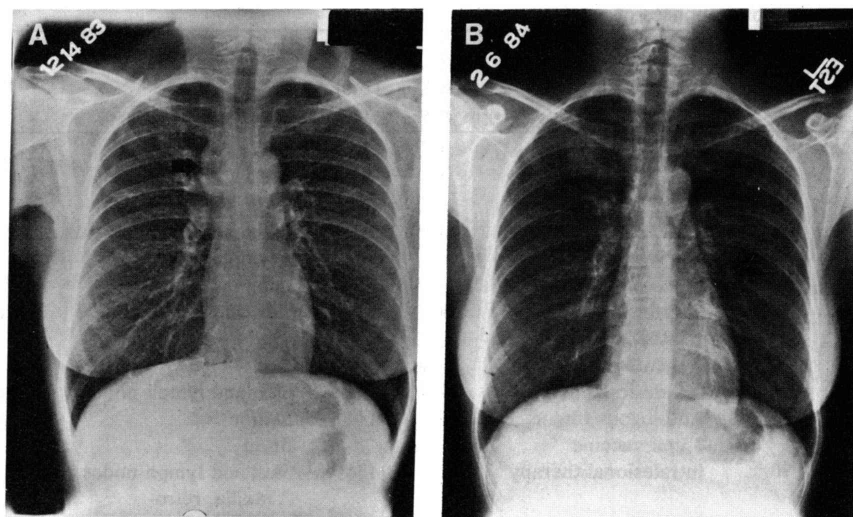

FIG. 1. Chest x-rays of patient 3. (*A*) At end of R_{24} treatment (no change compared with pretreatment x-ray). (*B*) Eight weeks later. The arrow points to the paratracheal mass.

regress. The subcutaneous nodule over the right hip did not change in size but became very tender and inflamed by Feb. 2, 1984. Excisional biopsy of this lesion revealed hemorrhagic necrosis and inflammatory cell infiltrates with small nests of melanoma cells that stained weakly or not at all with R_{24}. By May 1984, the density in the right upper lung field had become better defined and a needle biopsy revealed melanoma cells that reacted strongly with R_{24}.

Patient 4 had a malignant melanoma diagnosed Feb. 1982, 7 mm thick, Clark's level IV, with tumor in 10 of 12 regional lymph nodes. By Jan. 1984, the patient had extensive bulky skin and soft tissue metastases (>100 lesions) on the left thigh, in the left and right inguinal areas, and on the lower abdomen, scrotum, and penis (Fig. 2). Treatment with R_{24}, 10 mg/m^2 every other day for eight doses, was started on Jan. 18, 1984. Two hours after the start of the first infusion, the patient developed severe pruritus and urticarial lesions around all tumor sites. Urticaria progressed to confluent erythema over tumor sites and adjacent areas of the thigh, inguinal areas, and lower abdominal wall. These reactions disappeared 18 hr later. A milder reaction was seen after the second and third doses and no reactions were seen during subsequent treatments. Eight weeks after the end of R_{24} treatment, there was enlargement of lesions in the right groin and left thigh and new lesions had appeared over the abdominal wall. However, 4 weeks later, all measurable lesions had decreased in size by >50%. There has been continued tumor regression over the past 6 months and most sites are now tumor-free (Fig. 2).

Patient 10 is a 61-year-old man who developed a melanoma, 2.8 mm thick, Clark's level III, over the right scapular area in 1977. A solitary lesion of the left frontal lobe of the brain was detected in Jan. 1982 and treated by left frontal lobe craniotomy followed by whole brain radiation therapy.

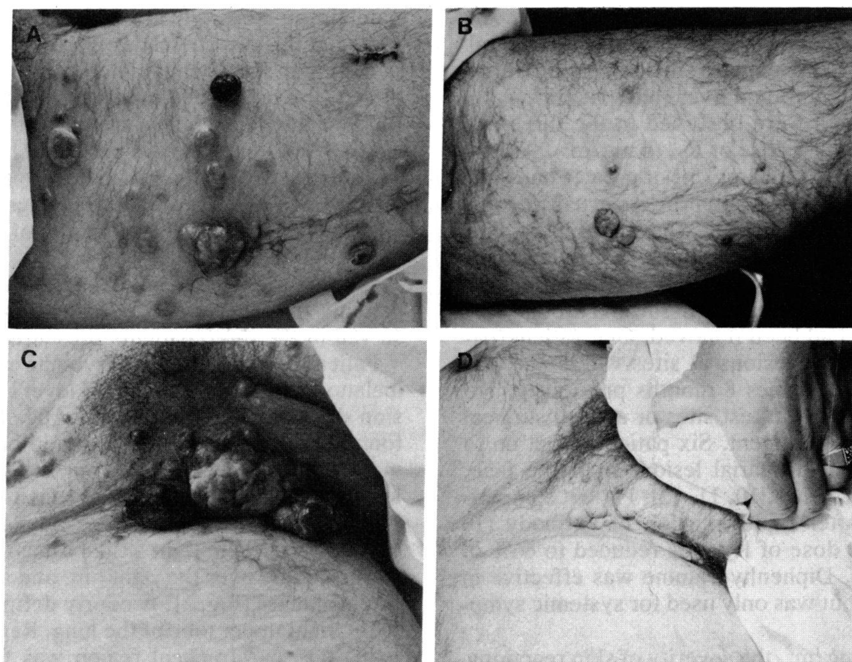

FIG. 2. Response of patient 4 to R_{24} treatment. (*A* and *B*) Left thigh. (*C* and *D*) Right inguinal area. (*A* and *C*) At start of treatment. (*B* and *D*) Twenty-two weeks later.

Between June 1983 and Oct. 1983, multiple subcutaneous tumors developed over the trunk, and the patient continued to progress during sequential treatment with dacarbazine, pimozide, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and dibromodulcitol. Treatment with R_{24}, 240 mg/m^2, by intravenous infusion over 2 weeks was started on June 11, 1984. The patient had more than 30 skin and soft tissue lesions on the trunk, extremities, scalp, face, and neck measuring between 1 and 5 cm in diameter. Four hours after starting treatment the patient developed urticaria, first around tumor lesions and then becoming generalized by 6 hr. The skin reactions abated over the next 12 hr and were gone by the 4th day of treatment. Regression (>90%) of several pigmented tumors was seen at the end of R_{24} treatment, and generalized regression of lesions was observed by 4 weeks after starting therapy. During tumor regression, subcutaneous ecchymoses were noted over six responding tumor sites. The patient achieved a partial remission (>50% regression of all measurable lesions) and regression of lesions has continued. In the face of regression of skin and soft tissue lesions, the patient developed left mild hemiplegia 8 weeks after the start of therapy. The computerized tomography image of the brain showed an enlarging lesion in the putamen with a necrotic center.

Mixed responses were observed in two other patients (patients 2 and 9) (Table 1). Patient 7 had rapid progression of bulky skin and soft tissue disease with deterioration of performance status and received dacarbazine 5 weeks after the start of R_{24} treatment; she achieved a partial response 3 weeks after dacarbazine treatment and has remained in remission for more than 20 weeks. Patient 5 also showed progression of disease with development of new skin nodules at 6 weeks. Treatment with dacarbazine resulted in a partial response lasting now more than 20 weeks.

Serology. Table 2 presents the results of serological studies performed on patients treated with R_{24}. Several points are apparent from these studies. (*i*) There was heterogeneity in the expression of G_{D3} in the melanoma specimens obtained before treatment, ranging from 40% positive cells to 100% positive cells. (*ii*) Peak R_{24} levels were related to the amount of antibody received. Median peak R_{24} levels were 0.8 μg/ml at 8 mg/m^2, 7 μg/ml at 80 mg/m^2, and 58 μg/ml at 240 mg/m^2. R_{24} levels fell off rapidly after the last R_{24} treatment and were usually <5% of peak levels by 18 hr after the end of therapy. (*iii*) Elevated levels of human IgG against mouse Ig were detected in all evaluable patients between 15 and 40 days after the start of therapy. (*iv*) There was no evidence of antigenic modulation during therapy. Tumor cells biopsied or aspirated during therapy in patients 4 and 10 showed continued expression of G_{D3} ganglioside in the face of substantial levels of circulating and tumor-bound R_{24}. (*v*) Progression of tumor after R_{24} treatment was not related to out-

Table 2. Serology and immunopathology of patients treated with R_{24}

	R_{24} reactivity with melanoma biopsy sample*		R_{24} serum level: Peak			R_{24} serum level: 18 hr after end of treatment			
Patient	Intensity	% + cells	Day	Titer†	Mouse IgG3,‡ μg/ml	Titer†	Mouse IgG3,‡ μg/ml	Human IgG anti-mouse Ig§	Immunopathology
1	3+	100	4	1/64	0.8	1/8	<0.1	4.1 (164)	Not examined
2	3+	80	7	1/32	<0.1	0	<0.1	5.6 (70)	Increased infiltration with lymphocytes and mast cells; frequent mast cell degranulation; no mouse IgG detected (13)
3	2+	70	9	1/128	2	0	<0.1	3.9 (100)	Increased infiltration with lymphocytes and mast cells (64)
4	3+	100	7	1/256	13	1/32	0.5	3.8 (80)	Increased infiltration with $T8^+/Ia^+/T3^+/T4^-/B2^-/B7^-/M1^-/M5^-$ lymphocytes; increase in number of mast cells with frequent degranulation; deposition of complement (C3, C5, C9); mouse IgG in tumor biopsy sample primarily around vessels (13)
5	2+	100	7	1/256	3	1/16	<0.1	2.1 (40)	Increased infiltration with lymphocytes and mast cells; frequent mast cell degranulation (32)
6	2+	70	7	1/128	7	1/32	<0.1	2.0 (40)	No inflammatory infiltrate (64)
7	2+	80	14	1/512	5	1/128	1	2.3 (40)	Not examined
8	2+	80	4	1/64	6	0	<0.1	1.3 (35)	Not examined
9	2+	70	9	1/256	8	0	<0.1	3.3 (40)	Not examined
10	3+	100	12	1/1024	58	1/128	NT	1.8 (30)	Infiltration with $T8^+/Ia^+/T3^+/T4^-/B2^-/B7^-/M1^-/M5^-$ lymphocytes; increase in number of mast cells with frequent degranulation; deposition of complement (C3, C5, C9); mouse IgG in tumor biopsy sample around vessels and in tumor (11)
11	3+	90	5	1/256	18	1/128	1	1.2 (20)	Not examined
12	2+	40	5	1/800	62	1/200	2	1.3 (14)	Not examined

NT, not tested. Numbers in parentheses represent days after start of treatment.

*Before treatment; intensity is on a scale of 0–3+.

†Measured by protein A mixed hemadsorption assay against SK-MEL-28 melanoma target cells.

‡Determined by inhibition of enzyme-linked immunoassay.

§Determined by enzyme-linked immunoassay. Results are presented as the after treatment/before treatment ratio.

growth of G_{D3}^- cells. Biopsies done after tumor persistence or progression showed strongly G_{D3}^+ melanoma cells in the lung lesion of patient 3 and skin lesions of patients 5, 6, and 9. (*vi*) The amount of R_{24} reaching tumor cells appeared to correspond to the dose level of antibody given. Mouse IgG was not detected in lesions from a patient treated at 8 mg/m^2 (patient 2) but was detected weakly around vessels in patient 4 (80 mg/m^2) and strongly around vessels and on a proportion of tumor cells in patient 10 (240 mg/m^2). (*vii*) Inflammation at tumor sites involved several components associated with immune reactions. Tumors from patients 4 and 10 had increased numbers of mast cells with mast cell degranulation, evidence of complement deposition including C3, C5, and C9, and infiltration with $T8^+/Ia^+$ lymphocytes. Tumor tissue taken immediately before treatment did not show these characteristics.

DISCUSSION

A variety of mechanisms may be involved in the antitumor activity of R_{24}, ranging from antibody-directed complement-mediated cytotoxicity and cell-mediated cytotoxicity to tumor cell injury secondary to the inflammatory reaction elicited in the tumor bed by R_{24}. Tumor biopsy samples showed a range of inflammatory changes in R_{24}-treated patients, with infiltration of T cells and mast cells, mast cell degranulation, and deposition of complement components C3, C5, and C9 being among the most prominent. Dippold *et al.* (14) have also observed inflammatory reactions over tumor sites in two patients treated with R_{24}.

In our studies of a large number of mouse monoclonal antibodies to surface antigens of human cancer, R_{24} has been found to be unique in its ability to activate human complement to such a high degree and to mediate extremely strong cell-mediated cytotoxicity. Whether these are general characteristics of antibodies (such as R_{24}) that belong to the IgG3 subclass or are related to the nature of the G_{D3} antigenic determinant is not known. These questions can be addressed by studying other classes and subclasses of anti-G_{D3} monoclonal antibodies and IgG3 antibodies to other surface antigens. Complement components, such as C5a, that are generated during complement activation are known to have inflammatogenic activity (15), and it is likely that these are involved in R_{24}-directed inflammation at the tumor site. If complement activation plays a key role in R_{24}-induced inflammation and antitumor effects, attention needs to be directed at complement levels, both systemic and intratumoral, during R_{24} therapy to determine whether complement availability might limit the therapeutic activity of R_{24}.

Another aspect of R_{24} that needs clinical evaluation is the possibility that responses to chemotherapy may be enhanced by R_{24}. Studies in *nu/nu* rats and mice have shown that the antitumor effect of R_{24} is potentiated by drugs such as adriamycin. Increased tumor blood flow and altered susceptibility of tumor cells to the action of drugs as a consequence of antibody treatment are two possibilities that could account for increased drug sensitivity of R_{24}-treated animals. This observation may have its counterpart in the clear responses of two R_{24}-treated patients to dacarbazine.

It remains to be determined what dose and treatment schedule of R_{24} are necessary to achieve optimal therapeutic results. Because toxicity at 240 mg/m^2 is still mild and easily controlled, assessment of higher doses of R_{24} is desirable. However, a dose–response relation to R_{24} may not be straightforward, because patients' responses will be influenced by a number of other parameters—e.g., strength and heterogeneity of G_{D3} antigen expression, tumor cell accessibility, and availability of accessory factors such as complement, histamine, and inflammatory cells. The response of patient 3 to R_{24} at 8 mg/m^2 illustrates the difficulty of making predictions with regard to optimal R_{24} dose levels. Further studies in melanoma patients and in patients with other types of G_{D3}-expressing tumors (astrocytomas, sarcomas) will be necessary to evaluate the potential of R_{24} in the treatment of human cancer.

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