functioning of our own trials, but for every surgeon dealing with breast cancer. This standard should be adapted when new elements in cancer treatment are available. We should take the responsibility of promoting the implementation of these standards in non-specialised centres dealing with breast cancer. Furthermore, this quality assurance experience in breast conservative treatment could enhance similar projects in other aspects of oncological surgery.

Until quality assurance becomes an integral part of surgical treatment for cancer, there will be an unnecessary additional chaotic component within clinical trials. For improvements in systemic therapy to become manifest, it is important that local therapy is optimal, and the best method for improving surgery will be by means of quality assurance.

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Papers

Immunological Response to Intrathecal and Systemic Treatment with Ganglioside Antibody R-24 in Patients with Malignant Melanoma

Wolfgang Dippold, Helga Bernhard and Karl-Hermann Meyer zum Büschenfelde

Murine monoclonal antibody (MAb) R-24 reacts with the ganglioside GD3 that is highly expressed on malignant melanoma. 2 patients with melanosis of the meninges received MAb R-24 intrathecally. Regressive changes in tumour cells were observed in both patients after intrathecal application of MAb R-24 (1–10 mg, 8–10 h, over 5–6 weeks). The first patient suffered from brain metastases and died a few weeks later, whereas the second achieved a complete remission with no evidence of disease 6 years after intrathecal R-24 treatment. No R-24-related neurotoxicity has occurred to date. The administration of MAb R-24 caused an increase of inflammatory cells in the cerebrospinal fluid (CSF) of both patients. Cytotoxic lymphocytes, cultured from the CSF, showed high cytolytic activity against allogeneic melanoma cells *in vitro*. In addition, 15 patients with advanced melanoma, in which the brain was not affected, were treated with R-24 intravenously using high dose R-24 (5 or 10 mg/m^2) or low dose R-24 (1 mg/m^2). No remissions were registered in the high dose group, with only 1/6 patients experiencing a mixed response. In contrast, 2/9 patients treated with low dose R-24 achieved a partial remission, one achieving a minor response and the other a mixed response. Toxicity was related to the dose of R-24 administered. Urticaria, burning and pruritus were the prominent side-effects, mostly occurring at the high dose level. Immunological monitoring during and after intravenous treatment showed no significant changes in peripheral blood lymphocytes, natural killer cell activity or antibody-dependent cellular cytotoxicity, although transient changes were observed. There was no correlation between immunological parameters and clinical response.
Eur J Cancer, Vol. 30A, No. 2, pp. 137–144, 1994

INTRODUCTION

A LARGE NUMBER of antigens have been identified on human melanoma cells using monoclonal antibody (MAb) technology. Gangliosides in particular are strongly expressed on the cell surface of malignant melanoma. Several MAbs directed against the gangliosides (GD2, GD3, GM2 and GM3) have been developed [1–8]. The mouse MAb R-24 generated by Dippold and colleagues reacts with the trisaccharide structure NeuAcα2-8NeuAcα2-3Gal, which must be in a terminal position of the molecule [1,9,10]. This epitope is found in the disialoganglioside GD3 which is highly expressed on malignant melanoma and other tumours, and tissues of neuroectodermal origin [8,11–14].

Table 1. Clinical features and tumour responses of patients treated with MAb R-24 intravenously

Patient no.	Age/sex	Prior therapy	R-24 dose (mg/m²)	Sites of disease	Response/duration (weeks)
Group A: high-dose R-24					
1	53/M	Melphalan + actinomycin DTIC + vindesine + cisplatin	5	Skin	Mixed response/16
2	28/F	Cisplatin + ifosfamide + actinomycin	5	Lung	Progression
3	39/M	Cisplatin + vindesine	5	Skin, peritoneum, lymph nodes	Progression
4	36/F	Lamustine procarbazine + vincristine	5	Lung skin, lymph nodes	Progression
5	44/M	DTIC/TNF	10	Lung, liver, skin, lymph nodes	Progression
6	46/M	DTIC	10	Lymph nodes	Progression
Group B: low-dose R-24					
7	29/F	DTIC/IFN-α	1	Ovary, adrenal, lymph nodes	Partial remission/19
8	47/M	DTIC	1	Bone, lymph nodes	Partial remission/10
9	26/M	None	1	Lung	Minor response/17
10	69/F	Local XRT/IFN-β and IFN-γ	1	Lung, skin, liver, lymph nodes	Mixed response/4
11	46/M	DTIC/IFN-α	1	Skin, liver, lymph nodes	Progression
12	60/M	Local XRT	1	Bone, oropharynx, lymph nodes	Progression
13	64/F	DTIC/Local XRT	1	Skin, liver, bone, adrenal	Progression
14	25/M	None	1	Peritoneum	Progression
15	58/F	Local XRT	1	Skin	Progression

M, male; F, female; TNF, tumour necrosis factor; IFN, interferon; XRT, radiotherapy.

Normal cells like melanocytes, adrenal medulla and various parts of the brain exhibit the GD3 ganglioside. Functional *in vitro* studies revealed that R-24 mediates a variety of biological effector functions: activation of complement, mediation of antibody-dependent cellular cytotoxicity, growth inhibition of cultured melanoma cells, and induction of lymphocyte proliferation [15–19]. Preliminary clinical studies with intravenous (i.v.) application of R-24 have established anti-tumour activity in patients with advanced malignant melanoma [20–23]. This is the first report concerning the injection of ganglioside antibody R-24 into the cerebrospinal fluid of 2 patients suffering from melanosis of the meninges. In addition, 15 patients with no evidence of disease in the brain received R-24 i.v. The aim of the study was to monitor closely the immunological effects of R-24 with regard to different forms of application, various dose levels, toxicity and tumour response.

MATERIALS AND METHODS

Antibody preparation

Production and specificity analyses of murine MAb have been published previously [1,9,15]. Briefly, MAb R-24 (IgG3) is a murine ganglioside antibody that preferentially binds to disialoganglioside GD3. MAb R-24 was prepared from hybridoma cell cultures and purified over Protein A sepharose columns (Pharmacia, Freiburg, Germany). Safety testing was verified by standard procedures (Behring-Werke, Marburg, Germany).

Antibody administration

Intrathecal (i.th) application of MAb R-24 occurred after lumbar puncture. MAb R-24 was dissolved in 5 ml 0.9% (w/v) NaCl supplemented with 0.6% (w/v) human serum albumin. Before antibody application, 5 ml of cerebrospinal fluid (CSF) were withdrawn each time for cell and antibody monitoring. Patient A received 10 times MAb R-24 i.th. (dose 1: 2 mg, doses 2–10: 10 mg) over a period of 5 weeks, and additionally R-24 (27 mg) was given i.v. three times during the fourth week. Patient B received 1 mg R-24 i.th. 8 times over a period of 6 weeks.

MAb R-24 was administered by i.v. infusion in 5% human albumin/0.9% saline over 4 h. Group A patients received 5 mg/m² (nos. 1–4) or 10 mg/m² (nos. 5–6) daily, 10 to 21 times (Table 1). Patients of group B (nos. 7–15) were each given a reduced dosage of 1 mg/m² R-24 every other day, seven times.

Patient selection

Selection criteria for i.v. R-24 treatment permitted the inclusion of only those patients with GD3 antigen-positive metastatic melanoma (> 80% GD3-positive tumour cells). The presence of the target antigen GD3 was determined by immunoperoxidase technique on frozen sections of fresh biopsy specimens as described below. Eligibility requirements included adequate function of the bone marrow [white blood cells (WBC) ≥ 3.500/μl, platelet count ≥ 100.000/μl], the liver (serum bilirubin ≤ 5 mg%), and the kidney (serum creatinine ≤ 2.0 mg/dl). Furthermore, life expectancy had to be more than 12 weeks. Patients were excluded if they had significant heart disease, and had received anticancer therapy within 3 weeks. A formal consent was required from all patients. Patients with solid central nervous system metastases were excluded. However, patients with malignant melanosis having GD3-positive melanoma cells in the CSF were treated with i.th. applications of MAb R-24.

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Histochemical and cytochemical analyses

Tissue biopsies from patients with metastatic malignant melanoma were snap-frozen and stored in liquid nitrogen. Unfixed frozen sections, 5–7 μm thick, were air-dried for 1 h before staining. CSF was cytocentrifuged (Cytospin 2 Shandon) on to glass slides, and the cells were air-dried. Indirect immunoperoxidase staining of tissue sections or cytocentrifuged cells was performed as described previously [12].

Serological tests

Concentrations of MAb R-24 in serum and CSF were measured by enzyme-linked immunosorbent assay (ELISA), as described previously [22]. Human anti-mouse antibodies (HAMA) were also measured by ELISA. Antibody titres were evaluated as positive if the corresponding ELISA values were greater than three times the negative control.

In vitro *culture of lymphocytes*

Peripheral blood lymphocytes were obtained by density gradient centrifugation of 10 ml heparinised blood in 25 ml RPMI medium and 15 ml Ficoll-Hypaque (Pharmacia) at 3000 *g* for 15 min. Lymphocytes obtained from the CSF were counted and cloned by limiting dilution (100, 10, 1 cell/well), in 96-well V-bottom plates (Nunc) in RPMI culture medium + 10% fetal calf serum (FCS). Growing cultures from the lowest dilution were cultured and cloned again. Lymphocyte growth was stimulated with 0.5 μg/ml phytohemagglutinine (PHA) (Wellcome, Burgwedel, Germany) for 3 days on feeder cells [1×10^6 peripheral blood lymphocytes (PBL)/ml, irradiated with 10000 rad]. Then PHA-containing medium was removed from the cultures, and replaced by 200 μl RPMI-1 containing 10% FCS, 2% L-glutamine and 10 ng/ml interleukin-2 (IL-2) (Biotest, Frankfurt, Germany). Cultures were fed twice/week, and every 10 days fresh feeder cells were added.

Cellular cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC)

Melanoma cell lines SK-MEL 29, leukaemia cell line K-562 and the Epstein–Barr virus (EBV)-transformed B cells SK-MEL 29B and LAZ 509 were used as targets. Standard ^{51}Cr release cytotoxicity was used, as described previously [15].

Flow cytometric analysis

Fluorescence-activated cell sorter (FACS) analyses of peripheral blood lymphocytes were performed according to standard procedures. Cells were incubated with MAbs at saturating concentrations for 30 min on ice, washed, and stained with polyclonal goat anti-mouse $F(ab')_2$ antibody fragments, coupled with fluorescein isothiocyanate (Coulter) for another 30 min on ice. Specimens were analysed in an Epics V cell sorter (Coulter).

RESULTS

Patients treated with R-24 intrathecally

Patients' characteristics. Patient A was a 34-year-old male, Karnofsky performance status 80%, with no solid tumour mass in computed tomography (CT) scans. He had already been on dexamethasone (9 mg daily) for the relief of cerebral symptoms, including visual disturbances, nausea, vomiting and headache 3 weeks before starting MAb treatment. Patient B, a 44-year-old male, Karnofsky performance status 60%, presented with a malignant melanoma located at the medulla oblongata, infiltrating the routes of the nervi XI and XII and the arteria vertebralis (Fig. 1a). The tumour was removed completely, but black spots of tumour cells on the meninges remained. The CSF of both patients proved to contain melanoma cells, which were GD3-ganglioside positive, as shown by immunocytochemical analyses using ganglioside antibody R-24.

Toxicity. The 2 patients who received MAb R-24 i.th. experienced no serious side-effects. Patient A appeared confused for several hours after each antibody treatment, but no continuous neurological toxicities were observed.

Clinical response. Regressive changes in tumour cells were observed in the CSF of patient A 6 weeks after starting antibody therapy. Treatment was stopped at the point when routine cytological examination showed no tumour cells at two successive lumbar punctures. Two weeks later, tumour cells reappeared.

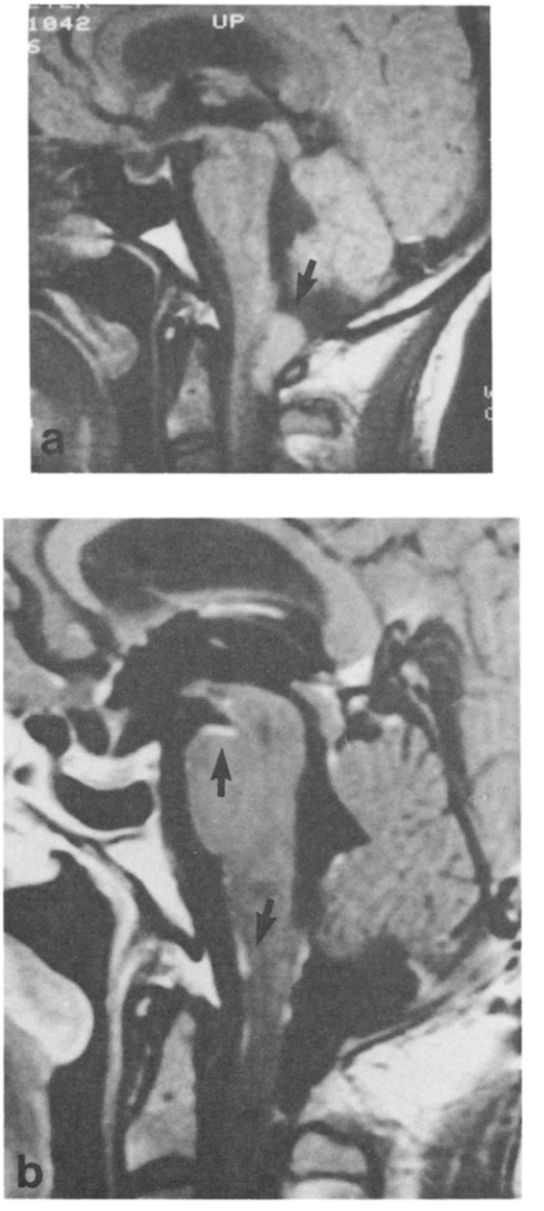

Fig. 1. Brain NMR scan of patient A. Before resection of a solitary melanoma metastasis at the medulla oblongata (a), arrow points to sites of disease. No evidence of disease 6 years after intrathecal MAb R-24 treatment following resection of the metastasis (b), arrows point to the reactive gliosis of the meninges.

At this time, CT scans of the brain again did not show any solid tumour, but a nuclear magnetic resonance (NMR) image performed for the first time, revealed three large (up to 1.5 cm) tumour nodules. The patient died 6 weeks later.

In contrast, a depletion of tumour cells was attained in the CSF of patient B after antibody therapy, and 6 years have passed with no evidence of tumour obtained either by cytological studies or by CT or NMR scans of this patient. Interestingly, the repeated NMR images now show an increasing gliosis of the meninges (Fig. 1b). Furthermore, the protein levels of the CSF are raised.

Immunological monitoring. Tumour cells and inflammatory cells in the CSF were monitored during and after treatment. Antibodies applied i.th. were detectable in the CSF and serum of both patients. Patient A developed HAMA in CSF and serum. In the case of patient A, HAMA developed 15 days after the first application in the serum, but not until the 25th day in the CSF. HAMA titres rose up to 1:25 000 in the serum and 1:1600 in the CSF of patient A (Fig. 2). The reactivity of the HAMA response was not anti-idiotypic, because they reacted with two unrelated mouse MAbs equally well. Patient B did not develop HAMA in the CSF or in the serum.

The administration of MAb R-24 caused an increase in the number of inflammatory cells in the CSF of both patients. The CSF of patient A was monitored over a period of 3 months, as shown in Table 2. The percentage of some cell types changed significantly. For example, the number of granulocytes increased at the beginning of therapy, and the number of plasma cells at the end. At high R-24 concentrations in the CSF, melanoma cells become GD3-ganglioside-negative on days 5, 7 and 9. One month after the beginning of R-24 therapy, tumour cells in the CSF of patient A became necrotic, but vital tumour cells that were GD3-positive reappeared (Table 2). However, there was no evidence of tumour cells in the CSF of patient B after R-24 treatment.

Lymphocytes of the CSF were cultured from patient B 6 weeks after the end of treatment, because at that time point necrotic tumour cells had been observed in patient A. Lymphocytes were cultured by limiting dilution, and tested for tumour cell cytotoxicity. Lymphocytes grew in 13 out of 96 wells plated. All 13 lymphocyte cultures showed a high degree of cytotoxicity for allogeneic malignant melanoma cell line SK-MEL 29 and the natural killer cell target K-562 (Fig. 3). The cytolysis was less for the allogeneic EBV-transformed B cells SK-MEL 29B and LAZ 509. One lymphocyte culture grew well to enable examination of the marker profile. These lymphocytes expressed the antigens CD2 (99%), CD3 (54%), CD8 (68%) and CD56 (54%), but not CD4. A parallel approach of culturing peripheral blood lymphocytes of the same patient was performed, but no melanoma cytotoxic lymphocyte cultures were obtained.

Patients treated with R-24 intravenously

Patients' characteristics. The clinical characteristics of the patients treated with R-24 intravenously are summarised in Table 1. All patients suffered from stage IV melanoma. They had all undergone previously resection of their primary lesion, and many of them had undergone further surgical procedures for resection of metastatic recurrences. Furthermore, 10 patients were given prior chemotherapy and 2 had received prior local irradiation. 2 patients had no systemic therapy before R-24 treatment. All patients had progressive metastatic disease at the time they entered the study. Sites of measurable disease included skin (8 patients), oropharynx (1), lymph nodes (9), lung (5), peritoneum (2), ovary (1), liver (4), adrenal (2) and bone (3).

Toxicity. All patients entered were evaluated for toxicity. No haematological, renal, cardiac or neurological toxicities was observed during or after treatment. Following treatment with R-24, local pain in the tumour sites occurred in 4 patients. Reddening around tumour nodules was observed in 8 out of 12 patients having cutaneous metastases or peripheral lymph nodes.

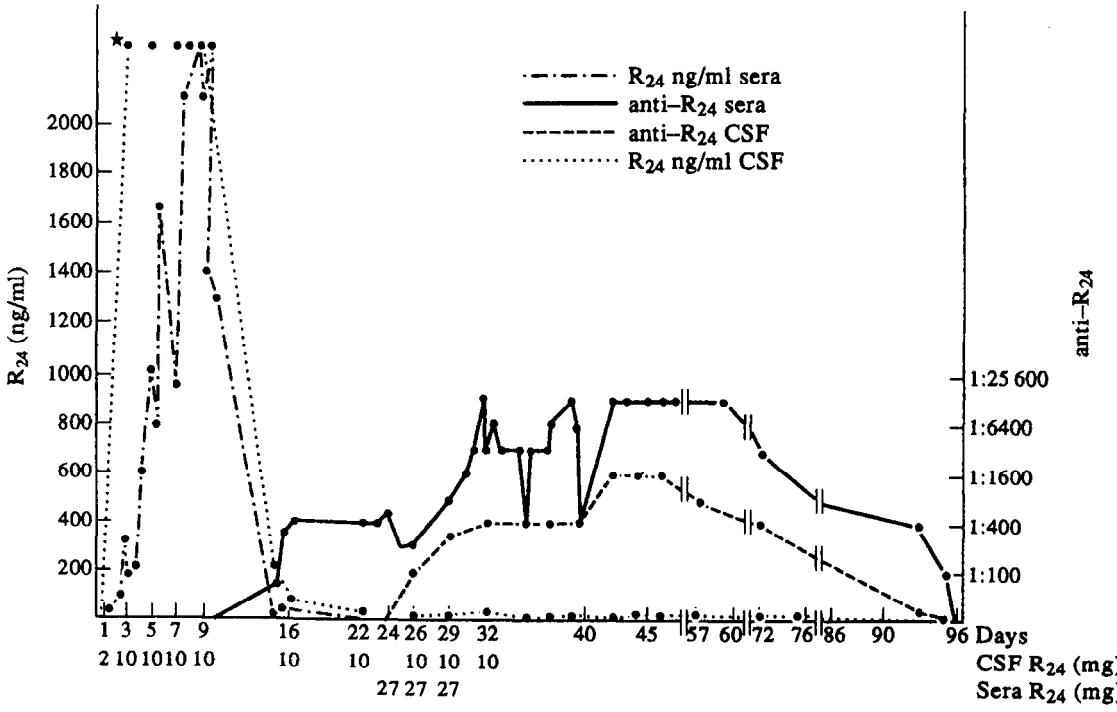

Fig. 2. Serological studies in serum and CSF of patient A treated with MAb R-24. Treatment was applied intrathecally on day 1 (2 mg), on days 3, 5, 7, 9, 16, 22, 26, 29 and 32 (10 mg) and intravenously on days 24, 26 and 29 (27 mg).

Table 2. Cytochemical studies in the CSF of patient A treated with MAb R-24 intrathecally

ADCC (%)	17	20				24	20						0				0			34							28
Total cell counts/ml	0,3	1,1		7,1	7,2	4,5	1,1	3,3	28	42	14	5,3	3,8	4,3	5,6	3	2,6	7,2	1,5		0,2		$\times 10^5$			
Lymphocytes (%)	59				41		57	38				64		24		33	44	11	56		37					
Reticulum cells (%)	29				8		18	16				5		8		4	7	6	13		13					
Granulocytes (%)	5				46		22	40				26		54		28	21	43	24		40					
Macrophages (%)					1		1	1				1														
Plasma cells (%)							1	3				4		14		35	27	40	7							
Tumour cells/ml		⊢						5	−60		⊢						⊢		0–30*	⊢			50			
Tc. MAb-R24 %	100	50	0	0	0	100	100	90	80	100	100	0	80	100	0				0		100					
day	1	3	5	7	9	16	22		26		32		37	40	42	44	46	57	60	72	76					

*Necrotic tumour cells. The days of treatment correspond to the ones shown in Fig. 2. Tumour cells were GD3 ganglioside negative on days 5, 7 and 9, when MAb levels in the CSF were highest. On days 35, 42 and 57, no tumour cells were observed in the CSF.

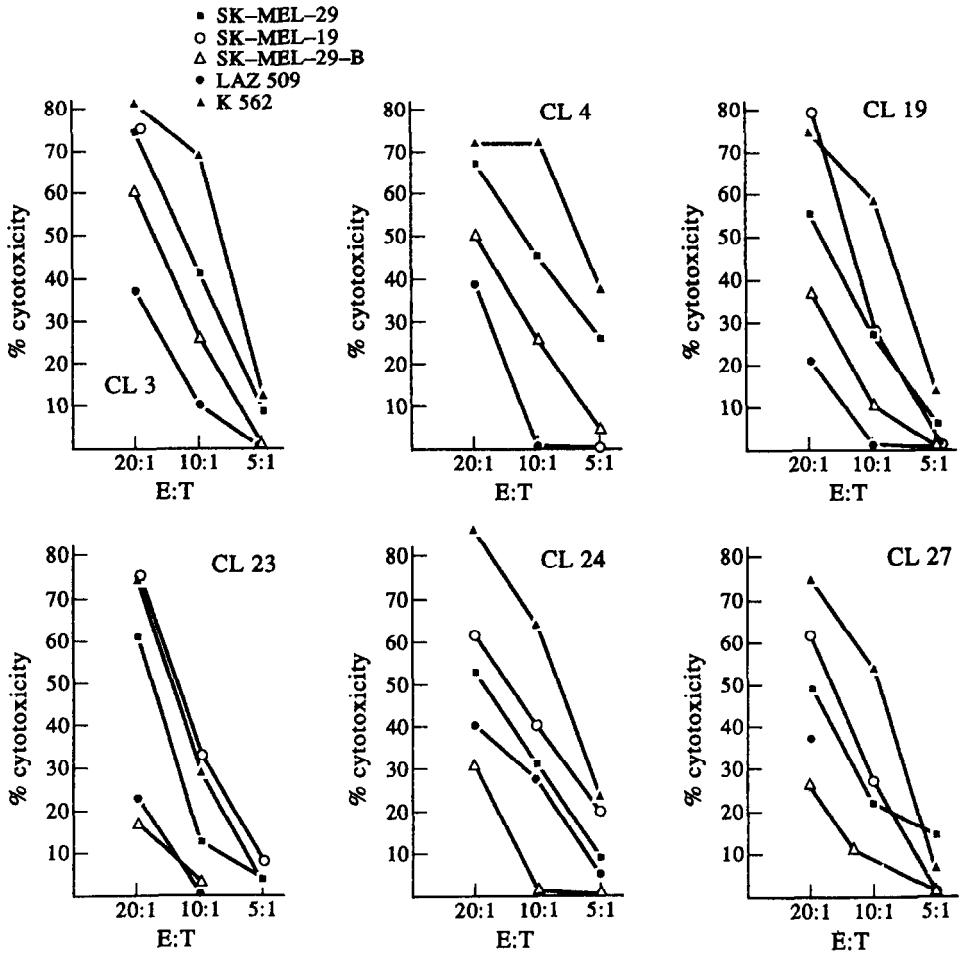

Fig. 3. Cytolytic activity of cytotoxic lymphocytes generated from the CSF of patient B after intrathecal MAb R-24 treatment. % CYTO, % cytotoxicity; CL, culture; E:T, effector: target ratio.

Of 6 patients who received 5 mg/m^2 R-24, 4 experienced urticaria and pruritus, whereas patients treated with 1 mg/m^2 R-24 showed none of these symptoms. No anaphylactic reactions were seen.

Response to therapy. Table 1 summarises the tumour responses and the response durations in patients treated with R-24 i.v. 1 of 6 patients treated with 'high-dose' R-24 (5 or 10 mg/m^2) had regressive skin metastases. However, other skin nodules were progressive during the same time. Therefore, the clinical response must be described as a 'mixed response'. Because of the ineffectiveness of MAb R-24 treatment in the 'high-dose' group, the following patients were given a reduced antibody dose (1 mg/m^2). In this 'low-dose' group, 2 patients received a partial response. Patient no. 7 was a 29-year-old woman with metastases in the right ovary, both adrenals and retroperitoneal lymph nodes. She failed conventional chemotherapy. Eight weeks after antibody treatment, a partial remission of the ovary metastasis was documented with the CT scan (Fig. 4). The response duration was 19 weeks, the survival time 3 years. Patient no. 8 had multiple bone metastases. He experienced a sclerosis of the osteolytic metastases. However, gastrointestinal metastases occurred after 10 weeks and he died of progressive disease. Patient no. 9 achieved tumour regression of his lung nodules for 17 weeks. Patient no. 10 had regressive skin nodules, but suffered from a progression of the other organ metastases at the same time. She died of brain metases a few weeks later.

Immunological monitoring. Prior to therapy, none of the patients had antibodies against mouse immunglobulins. Elevated levels of human IgG against mouse Ig were detected in 6 of 12 patients tested between 2 and 4 weeks after the start of therapy. The HAMA response did not depend on the i.v. dosage of R-24. Peripheral blood lymphocytes of the patients were monitored. FACS analyses using antibodies against CD3, CD4, CD8, CD56 or ganglioside GD3 showed no significant change after starting i.v. R-24 therapy. Prior to therapy, 8 out of 12 tested patients showed a natural killer (NK) cell cytotoxicity up to 45% as tested in the ^{51}Cr release assay, using K-562 as target cell line. Repeated tests during and after R-24 treatment showed no significant improvement of NK cell activity. ADCC using melanoma cell line SK-MEL 29 as a target and MAb R-24 as connecting antibody was examined before, during and after i.v. R-24 treatment. Peripheral blood lymphocytes from 6/13 tested patients were able to lyse SK-MEL 29 melanoma cells in the presence of MAb R-24 *in vitro*. Cytolyses before starting R-24 treatment ranged between 2 and 60%. A transient elevation of ADCC up to 80% was induced in several patients (5/13). However, improvement of ADCC *in vitro* did not correspond with tumour response *in vivo*. For example, patient no. 1 showed an elevation of ADDC from 30% before treatment to 80% after treatment, but suffered from disease progression. However, patient no. 7 who had a partial response, showed no ADCC before and after i.v. treatment with R-24.

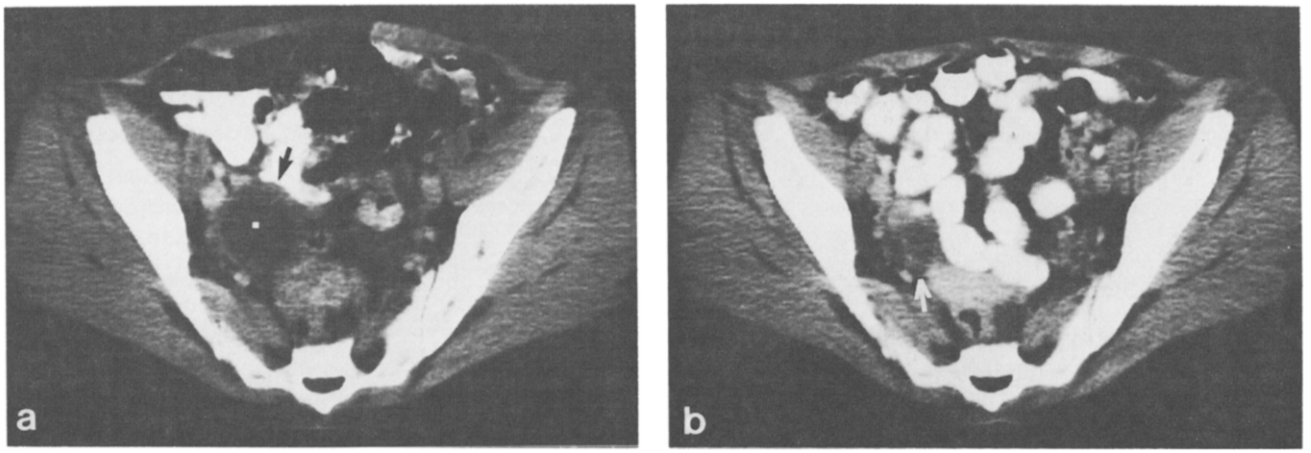

Fig. 4. Computed tomography scan of patient no. 7 shows a melanoma metastasis of the right ovary: (a) before MAb R-24 treatment; (b) after MAb R-24 treatment; arrows point to sites of disease.

DISCUSSION

In the present study, we have described the immunological and clinical effects of i.th. or i.v. application of ganglioside antibody R-24. 2 patients with melanosis of malignant melanoma received MAb R-24 i.th. MAb R-24 can be safely administered i.th. without major side-effects. It should be noted that no R-24-related neurotoxicity occurred, even though immunohistochemical examinations revealed a positive staining in the surrounding neuropils of many neuronal groups and in ependymal and glial cells [11]. Tumour cells in the CSF were no longer present in both patients after i.th. R-24 treatment. 1 patient had a relapse after a short period of time, but the second achieved complete remission, and there has been no evidence of disease since i.th. R-24 application 6 years ago. In order to evaluate the mechanisms causing tumour regression, the CSF of the patients treated were monitored during and after R-24 administration. MAb R-24 induced an increase in the number of inflammatory cells in the CSF. The generation of 13 highly cytotoxic lymphocyte cultures obtained from the CSF of patient B, 6 weeks after the conclusion of treatment, is striking. Similar cytotoxic cells could not be established from his peripheral blood lymphocytes. The CSF lymphocytes efficiently lysed melanoma cells and K-562 targets. The functional properties, as well as the antigenic phenotype, characterised these cells as cytotoxic T cells with NK cell activity. This phenotype is not characteristic of the major human NK effector cells which are non-T/non-B CD3-negative lymphocytes. However, human cells with NK activity are heterogeneous in their antigenic phenotype, and NK cells that display the CD8 and CD3 antigen have been described [24]. These data indicate the potential for immune response by the central nervous system. List and colleagues came to a similar conclusion after intraventricular injection of IL-2 into patients with leptomeningeal carcinomatosis. Intraventricular IL-2 application changed the cell composition of the CSF, and induced a complex cytokine response in the CSF [25]. Furthermore, cytotoxic lymphocytes could be induced in the CSF of patients suffering from glioma after i.th. administration of bispecific MAb-targeted lymphocytes [26].

15 patients were treated i.v. with high dose or low dose R-24. There was a trend towards more clinical effectiveness when lower doses of R-24 were administered. Similar observations were made by other investigators [22, 27]. To evaluate the effector functions responsible for tumour regression, the PBL of the patients treated were monitored. The phenotype of these did not change during or after R-24 treatment. The functions of the peripheral lymphocytes, such as NK cell activity and ADCC, showed either no change or a transient change caused by R-24 application. In contrast to these observations based on PBL, Kirkwood and colleagues reported that CD8- and CD4-positive tumour infiltrating lymphocytes changed after R-24 treatment, and correlated with tumour response [27].

The challenge now is to establish methods of augmenting the antitumour effects of ganglioside antibodies *in vivo*. The first step is to search for ganglioside antibodies with superior properties. Nine ganglioside antibodies with different epitope recognition, affinity and cytotoxicity have been established, as described previously [8, 15]. In addition, the development of humanised MAbs is underway to circumvent the problem of a HAMA induction [28]. An addition method of augmenting the effectiveness of ganglioside antibodies *in vivo* will be to administer them together with biological response modifiers such as interferon-α, granulocyte–macrophage colony-stimulating factor or IL-2 [29–31]. Recently, Creekmore and colleagues reported that IL-2 enhances the antitumour effects of MAb R-24, since 10 partial remissions out of 23 patients treated were achieved [30]. In conclusion, there are a number of possibilities to optimise treatment with ganglioside antibodies, and further clinical trials, in particular with patients of low tumour load and high risk of tumour recurrences, are warranted.

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