26 anthracycline-pretreated patients, and of 69% in 23 previously untreated patients [12].

By employing continuous infusion of 5-FU, a high dose intensity can be achieved without significant myelosuppression, while the incidence of other toxicities such as mucositis depends on the duration of the infusion. Whereas the dose intensities of mitoxantrone were only slightly different in the four studies, the dose intensities of leucovorin/5-FU were different, respectively, 250/500 [12], 175/350 [10], 100/1000 [11] and approximately 88/1200 mg/m^2/week (median surface area 1.7 m^2, Table 1) in our trial. These figures suggest that a high dose intensity of 5-FU compensates for less dose intense leucovorin.

In conclusion, the MLF regimen, employing continuous infusion of high dose 5-FU, is a useful regimen for second-line palliative treatment of metastatic breast cancer. This regimen warrants assessment in first-line treatment, possibly with an even higher dose intensity of leucovorin/5-FU.

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Phase I/II Study of Low-dose Intravenous OKT3 and Subcutaneous Interleukin-2 in Metastatic Cancer

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In a phase I/II study the safety, immunostimulatory and antitumour effects of a combined OKT3/interleukin 2 (IL-2) treatment was studied in 15 cancer patients who failed IL-2 treatment. OKT3 was given as a 2-h intravenous infusion. Doses of 50, 100, 200 and 400 μg OKT3 were studied. Within 24 h, subcutaneous IL-2 was started 5 days/week for 4 weeks, at a dose of 9–18 × 10^6 U daily. Maximum tolerated dose was 400 μg OKT3 with neurotoxicity as dose-limiting toxicity. Toxicity of subcutaneous IL-2 was acceptable. At the maximum tolerated dose, 9 patients with renal cell carcinoma with measurable disease were treated in a phase II setting. 8 patients were evaluable for response. 4 patients had stable disease and 4 had progressive disease. An increase of activated lymphocyte subpopulations could not be found, although OKT3 was detectable on lymphocytes *in vivo*. Only if laboratory studies shed light on methods of improving immunostimulating effects of OKT3 will further clinical studies be warranted.
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INTRODUCTION

AFTER DISAPPOINTING results with non-specific stimulation of the immune system with Bacillus Calmette-Guérin or specific stimulation with tumour-derived vaccines [1], the use of recombinant cytokines has opened up a new approach in the immunotherapy of disseminated cancer. Objective responses in selected tumours have been observed using interferon or interleukin-2 (IL-2). Renal cell carcinoma (RCC) and malignant melanoma (MM) appear to be the most susceptible to this strategy. Interferons have shown an overall response rate of approximately

16% in RCC, but responses were usually of short duration [2]. IL-2 alone or in combination with lymphokine-activated killer (LAK) cells generated *in vitro* has resulted in objective tumour regressions with response rates of approximately 20–30% in RCC [3, 4]. Intravenous (i.v.) IL-2 treatment, however, is associated with severe toxicity for which hospitalisation and intensive care monitoring is necessary in a number of patients [3]. Recently, treatment regimens with intermittent subcutaneous (s.c.) injections have been developed with acceptable toxicity which also result in a response rate of approximately 20% in RCC patients [5–8]. Despite this obvious improvement in applicability by eliminating most of the toxicity of IL-2 treatment, the number of 80% non-responding patients remains a clinical challenge.

Resistance to IL-2 treatment could be due to lack of appropriately activated effector cells of the immune system. Besides the IL-2-induced non-specific natural killer (NK)/LAK activity, current efforts to improve immunotherapy have focused on the role of T cell-mediated antitumour activity. Previously, we found that during IL-2 therapy, the NK cells were the predominant stimulated populations, whereas T cells were only temporarily activated [9]. In this study, we aim to increase T cell immune reactivity in patients who failed with IL-2 treatment. For this purpose we used OKT3, a mouse monoclonal antibody (MAb) directed against the CD3 antigen of the human T cell receptor (CD3/TCR). Anti-CD3 MAb are both capable of activating and blocking the T cell receptor, depending on the dose used. Low doses of anti-CD3 MAb cause activation and proliferation of resting T-cells *in vitro* and in animal studies, resulting in induction of IL-2 receptor expression, cytokine production and both antigen-specific and non-specific cytotoxicity [10, 11]. Combinations of OKT3 and IL-2 in long-term lymphocyte cultures result in a 10-fold increase in the production of cytotoxic cells compared with cultures using IL-2 alone [12, 13]. A certain antitumour effect of anti-CD3 MAb has been reported [14, 15]. Bolus injections of 50–100 μg OKT3 caused neurotoxicity with symptoms of aseptic meningitis in 7 out of 13 patients [15].

When used at high doses, anti-CD3 MAb cause immunosuppression *in vivo*, a property that is exploited extensively in renal transplant patients for the suppression of graft rejection [16, 17].

The purpose of this study is to explore the safety, immunomodulatory and antitumour properties of slow infusion with escalating, low doses of OKT3, followed by a fixed regimen of intermittent s.c. injections of IL-2 in a phase I/II setting.

MATERIALS AND METHODS

Patient selection

Eligible patients had a confirmed histological diagnosis of disseminated RCC or MM for which no effective therapy was available after monotherapy with IL-2 had failed or the tumour had relapsed. Patients pretreated with other therapies were eligible as long as a minimum of 3 weeks had elapsed since the completion of previous treatments. Additional qualifications for eligibility included a performance status of WHO grade II or less. Patients who required steroids for control of central nervous system (CNS) localisations and patients with uncontrollable disease apart from the tumour were excluded from the study. For the phase II study, measurable or evaluable disease was required. The study protocol was approved by the Medical Ethical Committee of the University Hospital Groningen, The Netherlands. All patients gave informed consent before entry in the study.

OKT3

The mouse monoclonal antibody OKT3 was obtained from the Orthoclone Pharmaceutical Corporation (Raritan, New Jersey, U.S.A.). It is available in vials containing 5 mg in 5 ml. A one hundred-fold dilution was available for this study.

IL-2

IL-2 was obtained from EuroCetus (Amsterdam, The Netherlands). It is available as Proleukin, with a biological activity of 18 million U/mg. Vials of 1 mg were reconstituted with either 1 or 2 ml sterile water and administered s.c. within 24 h after preparation, as has been described previously [7, 8].

MAbs

The MAbs Leu4 (anti-CD3); Leu3 (anti-CD4); Leu2 (anti-CD8); Leu19, (anti-CD56 + 16); anti-HLA-Dr and anti-IL-2 receptor alfa (CD25) were obtained from Becton Dickinson (Mountain View, California, U.S.A.) and were directly conjugated with phycoerythrin (PE) or fluorescein-isothiocyanate (FITC).

Immunostaining of cells and flow cytometry

Whole ethylenediaminetetraacetic acid (EDTA) blood was stained within 4 h after collection. To 100 μl of whole blood, 20 μl of MAb preparation (containing 0.5 μg of assessed MAb) was added and the sample was incubated at room temperature for 15 min. Fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson) (2 ml) was added and the cells were incubated for an additional 10 min. Subsequently, the solution was centrifuged and the cells were washed in phosphate-buffered saline supplied with 0.092 mg/ml heparin and resuspended in 150 μl. To analyse the binding of OKT3 on T cells, whole blood aliquots of 100 μl were incubated with 10 μl of anti-Leu4-FITC plus 50 μl of goat antimouse (GAM)-IgG2a-PE (Southern Biotechnology Associates, Alabama, U.S.A.; 20-fold diluted) or with 10 μl of anti-Leu16-FITC plus 50 μl of GAM-IgG2a-PE. The samples were immediately analysed on a FACSTAR (Becton Dickinson), with the laser tuned at 488 nm. Lymphocytes were gated using standard FSC/SSC settings, which excluded the monocytes and granulocytes from analysis.

Soluble IL-2 receptor and soluble CD8

Soluble IL-2 receptor (IL-2R) and soluble CD8 levels in serum were measured using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) (T-cell Sciences, Cambridge, Massachusetts, U.S.A.).

Treatment and study design

Prior to entry, patients were staged by full clinical examination, measurement of blood cell counts, blood chemistry, thyroid function tests, chest radiograph and appropriate computed tomographic scanning or ultrasound sonography. OKT3 dissolved in 100 ml NaCl 0.9%, was given as a 2-h i.v. infusion. The starting dose of OKT3 was established as 50 μg since this

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dose showed a certain toxicity as a bolus injection in a previous study [15]. Doses of 50, 100, 200 and 400 μg were tested. The dose was escalated after at least 2 patients had shown no toxicity exceeding WHO grade II at that dose. Within 24 h of the infusion, subcutaneous IL-2 was started 5 days/week for 4 weeks at a dose of 18×10^6 U/day in the first week. The dose in the first 2 days of the following weeks was reduced to 9×10^6 U/day. While on the study, patients were not allowed treatment with non-steroidal anti-inflammatory drugs (NSAID). After a short stay in hospital, patients were treated at home and IL-2 injections were self-administered. Acetaminophen 250–500 mg orally every 4–6 h was given to prevent pyretic reactions. Metocloperamide 20 mg suppository was given for nausea and vomiting. Toxicity was scored according to WHO criteria during and after the OKT3 infusion and before each weekly IL-2 cycle. For toxicity greater than WHO grade II, IL-2 therapy was withheld until toxicity was resolved. When a delay of more than 7 days was necessary, the patient was removed from the study.

Blood samples were taken on the mornings before and 18 h after the OKT3 infusion, and on days 7, 14 and 28 during IL-2 treatment. Blood counts, electrolytes, renal and liver function tests, lymphocyte subsets and lymphocyte activation markers were determined for evaluation of side-effects and immunological changes. In 3 patients, soluble IL-2 receptors and soluble CD8 were measured. To be evaluable for response, patients with measurable disease had to be followed for at least 4 weeks after the start of treatment. Radiological evaluation for determination of tumour measurements was repeated after 4 weeks of treatment, or when there was evidence of disease progression (PD) during a course. Complete response (CR) indicated the documented disappearance of all signs and symptoms of detectable tumour and no development of new lesions. A partial response (PR) was defined as a decrease of at least 50% in the sum of the product of the two largest perpendicular diameters of all measurable lesions and no concomitant occurrence of new lesions. The situation in which no change or a decrease of less than 50% of the sum of the products of the two largest perpendicular diameters of measurable lesions occurred was defined as stable disease (SD). PD was defined as a 25% increase in the aforementioned lesions.

RESULTS

10 patients were entered in the phase I protocol. Patients' characteristics are shown in Table 1. All patients were evaluable for toxicity. Maximum tolerated dose (MTD) was 400 μg OKT3 with neurotoxicity in 2 out of 4 patients (Table 2). Patient 2 (50 μg) developed neurological symptoms with vertigo during treatment. Computed tomography (CT) of the brain showed cerebellar metastases, whereupon IL-2 treatment was discontinued. Patient 7 (400 μg) had a psychotic reaction with confusion and disorientation 36 h after the OKT3 infusion. No lateralisation of symptoms was found and a CT scan of the brain showed no abnormalities. All symptoms were transient and IL-2 treatment was restarted after 5 days without complications. Patient 9 (400 μg) was somnolent 4–36 h after the infusion. Patients 4 (100 μg) and 6 (200 μg) had a mild headache responding to acetaminophen.

Subcutaneous IL-2 treatment was well tolerated, but transient inflammation and local induration at the injection sites occurred in all patients. Fever and chills were common and in most cases, not controllable with antipyretic treatment, leading to grade I and II toxicities in 3/33 and 13/33 treatment weeks, respectively. A grade III toxicity was seen in 4/33 weeks. Nausea and vomiting grades I and II occurred in 8/33 and 14/33 weeks, respectively, and was in most cases not controllable with anti-emetic therapy. Diarrhoea grade I was observed in 3/33 weeks. Hypotension, defined as systolic tension below 100 mmHg, occurred only once in 33 treatment weeks. Hepatic and renal toxicity was mild. An increase in serum bilirubin levels was not found. Serum creatinine levels remained stable during treatment. Increases in

Table 1. Patients' characteristics

	Phase I	Phase II
No. of patients	10	5
Age (years)		
Median	57	56
Range	29–73	45–64
Male/female	8/2	3/2
Performance (WHO)		
0	5	1
1	3	4
2	2	
Diagnosis		
Renal cell carcinoma	9	5
Metastatic carcinoma	1	
Prior chemotherapy	1	3
Prior surgery	9	1
Prior radiotherapy	2	5
Prior immunotherapy	10	
Metastatic sites		
Lung	9	5
Liver		1
Bone	2	1
Local recurrence	2	1
Primary		2
Others	3	1

Table 2. Toxicity and response to OKT3/IL-2 treatment

Patient no.	OKT3 (μg)	Toxicity	Onset of symptoms (h)	Days of IL-2 treatment	Response
1	50	None		5	PD
2	50	Vertigo	32	2	NE
3	100	None		20	SD
4	100	Headache	12	20	PD
5	200	None		20	SD
6	200	Headache	12	20	PD
7	400	Confusion	36	20	SD
8	400	None		20	PD
9	400	Somnolence	4	20	SD
10	400	None		20	PD
11	400	None		20	SD
12	400	None		20	PD
13	400	None		20	SD
14	400	Headache	12	20	PD
15	400	Hypotension	48	5	NE

NE, not evaluable; SD, stable disease; PD, progressive disease.

serum alkaline phosphatase and lactic dehydrogenase were found frequently, but were transient in most cases. Patient 3 developed a clinical and biochemical hypothyroidism for which substitution therapy was indicated. Two and a half months after discontinuation of IL-2 treatment, thyroid function had normalised spontaneously. No objective responses were observed (Table 2). Because 4 patients with metastatic RCC with measurable disease were treated at the 400 μg OKT3 step, this number was extended with 5 other patients to study the efficacy at the MTD dose. Patients' characteristics are shown in Table 1. Toxicity consisted of hypotension with renal dysfunction in 1 patient and headache in another (Table 2). After 4 weeks treatment, 8 patients were evaluable for response. 4 patients had SD for 10, 7, 4 and 1 months and 4 patients showed PD. Toxicity of subcutaneous IL-2 was comparable to the toxicity seen in the other patients.

Immunological changes measured before and 18 h after the OKT3 infusion are shown in Table 3. Little increase was found in any lymphocyte subset. At the higher doses, peripheral blood lymphocytes tended to decrease after OKT3 infusion. No immunological activation as determined by an increase in the percentage of cells expressing the CD4/CD25, CD4/HLA-Dr, CD8/HLA-Dr or CD56 + 16/HLA-Dr phenotypes was found at any dose step. Before OKT3 administration no binding of the GAM-IgG2a-PE antibody was found. After OKT3 administration, the antibody was bound on Leu4-FITC-positive cells but not on Leu16-FITC-positive cells, indicating a specific staining of T cells by GAM-IgG2a-PE, caused by the binding of OKT3 on the T cell surface (Fig. 1). Soluble IL-2R and soluble CD8 levels, measured before and after OKT3 administration in 3 patients at the 400 μg dose level are shown in Table 4. Almost no increase in soluble factors was observed. During IL-2 treatment, peripheral blood lymphocytes (× 10^9/l) rose from 1.5 (range 0.5–2.4) to 3.4 (range 0.8–6.5). Eosinophils (× 10^9/l)

Table 3. Cell surface phenotype (% positive cells)

Patient	OKT3 dose (μg)		Ly	CD3	CD56 + 16	CD4	CD8
1	50	Before	44	70	21	53	18
		After	33	68	24	50	18
2	50	Before	18	75	8	45	32
		After	23	73	10	43	34
3	100	Before	28	76	14	56	19
		After	37	81	10	62	17
4	100	Before	28	69	20	37	34
		After	45	70	20	37	32
5	200	Before	24	71	17	52	16
		After	21	57	28	40	16
6	200	Before	17	67	18	34	30
		After	17	50	30	30	16
8	400	Before	26	61	22	37	25
		After	11	ND	ND	ND	ND
9	400	Before	20	60	31	30	28
		After	12	41	37	20	17
10	400	Before	28	55	32	41	13
		After	14	42	37	35	9
11	400	Before	28	55	40	35	16
		After	34	58	36	40	14
12	400	Before	13	52	36	12	33
		After	4	21	13	8	45
13	400	Before	27	65	19	46	15
		After	ND	32	27	29	7
14	400	Before	20	64	40	19	25
		After	19	66	40	21	23
15	400	Before	19	49	46	24	26
		After	13	41	48	21	21

Ly, lymphocytes; ND, not determined.

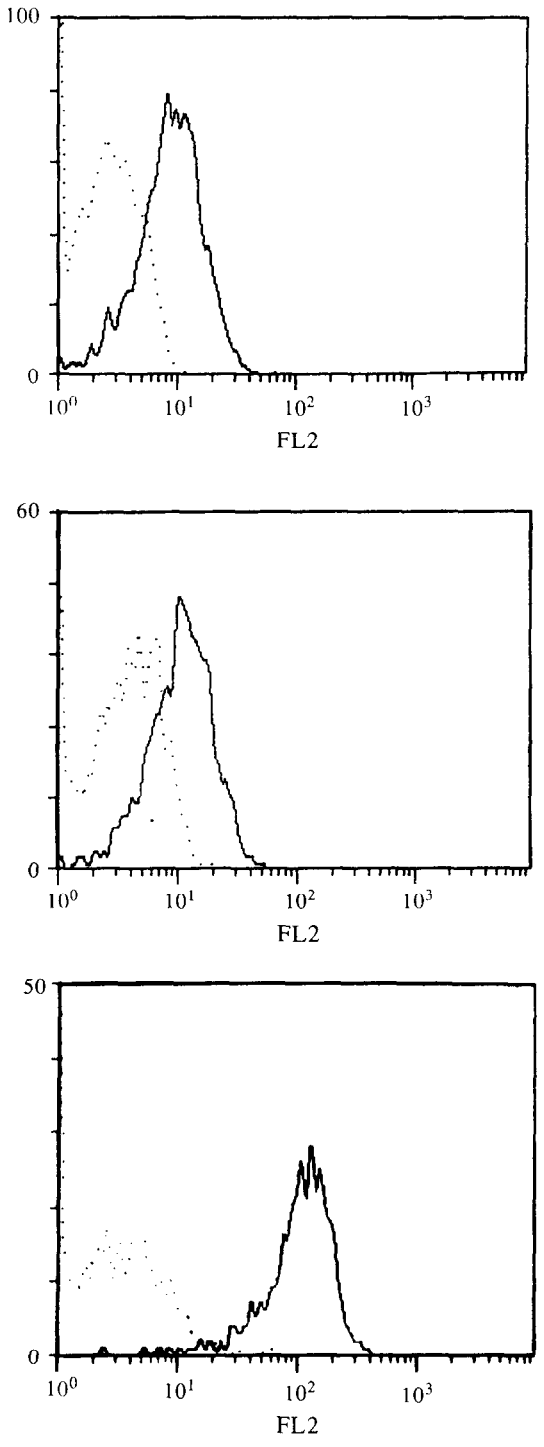

Fig. 1. OKT3 on CD3+ cells, before (dotted line) and after OKT3 administration (solid line) of 50, 100, 400 μg OKT3. Horizontal axis: log fluorescence intensity (arbitrary units). Vertical axis: relative cell number.

Table 4. Soluble IL-2R and soluble CD8

Patient	OKT3 dose (μg)		Soluble IL-2R (U/ml)	Soluble CD8 (U/ml)
7	400	Before:	873	561
		After:	901	536
11	400	Before:	1659	420
		After:	1440	415
14	400	Before:	3775	467
		After:	2523	411

rose from 0.3 (range 0.1–0.6) to 2.3 (range 0.2–7.3). The lymphocytosis and increases of eosinophils were not different from the changes observed during treatment with s.c. IL-2 alone [8].

DISCUSSION

We studied the *in vivo* safety, immunostimulatory and antitumour effects of a combined OKT3/IL-2 treatment in 15 cancer patients. MTD was 400 μg OKT3 with neutoxicity as the dose-limiting factor. Objective responses were not observed. Furthermore, no signs of enhanced T cell activation as indicated by the induction of CD25 or HLA-Dr expression were found after administration of the OKT3, although the antibody could be detected on the T lymphocyte surface. No enhanced rebound lymphocytosis during successive IL-2 treatment was observed, compared with treatment with s.c. IL-2 alone. A rise in soluble IL-2R or soluble CD8 indicating possible immune activation in other compartments than the peripheral blood was not observed at the MTD.

OKT3, a mouse IgG2 MAb directed against the human CD3/TCR complex, is known for its immunosuppressive activity associated with rapid depletion of peripheral blood lymphocytes, CD3/TCR modulation and prolonged graft survival when used repeatedly in high doses (> 1 mg) in renal transplant recipients [17]. Besides immunosuppressive effects, anti-CD3 MAb have strong mitogenic activity in lymphocytes when used at low doses (μg) *in vitro* and *in vivo*. For its mitogenic activity at least three signals seem to be necessary: CD3/TCR receptor occupation, cross linking of CD3 molecules and additional accessory cell signals from monocytes. Mitogenic activity can be induced in the absence of cross linking and accessory cell signals when exogenous IL-2 is provided [18]. The most likely mechanism for anti-CD3 antibody-mediated triggering *in vitro* is the induction of IL-2 receptors [19]. Tumour regression after administration of low-dose anti-murine CD3 was found in a murine model [14]. In clinical use, signs of immune activation and cytokine release have been observed after first dose administration of OKT3 to renal transplant patients [20, 21]. OKT3-coated T cells, found in lymph node biopsies from renal transplant recipients within 2 h after bolus injection of the antibody, showed enhanced proliferation in the presence of IL-2 *in vitro* [22].

Possible reasons for the lack of enhanced T cell activation with the OKT3/IL-2 combination in our study could be that the OKT3 doses used are too low to activate T cell function. Another explanation could be the previous treatment with IL-2 in all patients entered in this study. T cell lytic function of patients treated with IL-2 has been found to be inhibited with only partial reversibility by subsequent T cell receptor activation using antiCD3 MAb [23]. Recent clinical phase I studies in cancer patients with OKT3 alone or combined with low-dose continuous infusion of IL-2 failed to enhance T cell phenotype or function at doses from 10 to 600 μg OKT3 [24, 25]. Further investigation is needed to explain the difference between the *in vitro* and *in vivo* findings of the effects of low dose OKT3 in combination with IL-2 on the induction of T cell activation. Only if such studies shed light on methods to improve stimulating effects of OKT3 will further clinical studies be warranted.

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Prognostic Significance of the CaMBr1 Antigen on Breast Carcinoma: Relevance of the Type of Recognised Glycoconjugate

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An extensive study of the expression of the blood group-related antigen CaMBr1 has been performed by immunohistochemistry, immunoblotting and high performance thin layer chromatography both on frozen and paraffin-embedded (paraffin) samples from normal and neoplastic breast tissues. The glycolipid antigenic fraction (from frozen samples) was preferentially expressed on functioning breast epithelium. In a prospective series of 143 breast cancer cases CaMBr1 expression was associated, on frozen sections, with the transferrin receptor ($P = 0.01$), the positivity with oestrogen receptor immunochemical assay ($P = 0.06$), premenopausal status ($P = 0.06$) and node negativity ($P = 0.07$). Non-significant correlation with longer disease-free survival (DFS) was observed. In a retrospective series of 862 cases on paraffin sections the glycoproteic antigenic fraction was significantly associated with premenopausal status ($P < 0.05$) and lobular histotype ($P < 0.01$), but failed to predict survival, although a trend for longer DFS was observed for positive cases.
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INTRODUCTION

MBr1 IS AN IgM monoclonal antibody (MAb) produced in our laboratory in 1982 [1]. It was raised against a crude membrane preparation of the human breast cancer cell line MCF-7. The recognised epitope (CaMBr1) is present both on lipid and protein carriers and is expressed on a variety of non-neoplastic epithelial cells and epithelial tumours [2]; moreover, its expression is modulated on normal breast during the ovarian cycle and decreases in infiltrating breast carcinomas [3].

In breast cancer, MBr1 has been demonstrated to be a useful tool for diagnostic purposes, whether employed alone or in combination with other antibodies, in the differential diagnosis of effusions [4], or in the search for breast cancer cells in bone marrow biopsies [5–7]. By relying on the living cells' ability to internalise this antibody [10], therapeutic approaches have also been attempted by conjugating MBr1 with restrictocin [8] or the ricin A chain [9]. Furthermore, the prognostic usefulness of MBr1 has been proposed both in breast cancer [11, 12] and in small cell lung cancer [13].

In the first part of this paper we report a parallel analysis of the expression of glycolipid and glycoprotein antigenic fractions, as studied by immunoblotting, high performance thin layer chromatography (HPTLC) and immunohistochemistry, both on frozen and paraffin-embedded breast samples. In the second part, the prognostic relevance of MBr1 in breast cancer is studied by analysing the relationships with well-known prognostic factors and the survival outcome in two series of patients.

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MATERIALS AND METHODS

Materials

Production and characterisation of the murine MAb MBr1 have been described [1, 14]. Radiolabelling was performed using the ^{125}I-Bolton Hunter reagent (Amersham, Bucks, U.K.). The