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Original Paper

Reduction of Metastatic Carcinoma Cells in Bone Marrow by Intravenously Administered Monoclonal Antibody: Towards a Novel Surrogate Test to Monitor Adjuvant Therapies of Solid Tumours

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In a pilot, prospective randomised study, 40 patients with breast and colorectal cancer presenting with metastatic cytokeratin (CK)-positive tumour cells in bone marrow were treated either with six doses of 100 mg of a monoclonal Lewis Y antibody during 2 weeks or with a placebo regimen, consisting of six infusions of human serum albumin (HSA). CK-positive cells in marrow were monitored prior to and on days 15 and 60 after commencement of treatment. In 30 patients presenting with relatively low tumour cell numbers (1–11 per 4 × 10^5 bone marrow cells), a therapy-induced reduction of CK-positive cells could not be conclusively determined. More meaningful quantitative data were obtained in 10 breast cancer patients presenting with more than 20 tumour cells per 4 × 10^5 nucleated bone marrow cells. 7 of these patients had been randomised to the antibody arm, and 5 showed an eradication or a distinct reduction of CK-positive/Lewis Y-positive cells of at least one log unit, while 2 patients, presenting with Lewis Y-negative tumour cells, showed no corresponding decrease. Similarly, in all 3 patients randomised to the placebo arm, tumour cells were not reduced. Because the antibody exerted a marked cytotoxicity on tumour cell lines when tested *ex vivo* in serum taken from these patients after antibody infusion, we postulate that the observed, prompt reduction of individual tumour cells in bone marrow was due to the cytotoxic action of the injected antibody. Although monitoring micrometastatic cells in bone marrow of patients with high tumour cell counts appears to be feasible, the immunocytochemical assay needs to be improved for patients with lower cell numbers before it can be applied as a surrogate test for adjuvant therapies.

Key words: breast cancer, colorectal cancer, monoclonal antibody therapy, Lewis Y antigen, micrometastases, bone marrow, surrogate marker
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INTRODUCTION

ADJUVANT CHEMOTHERAPY is increasingly used in patients, following successful surgical resection of primary cancers. Its main goal is to pre-empt a later relapse from occult metastatic cells in approximately a third of those patients. Because of the occultness of disseminated tumour cells, the efficacy of adjuvant therapy can thus far be assessed only indirectly by comparative trials on large numbers of patients observed for a minimum of 5 years. Consequently, progress in this form of therapy has been extremely slow and, in addition, it has been difficult to tailor therapy to the special needs of the individual patient or to the particular biology of the tumour.

Thus, the importance of an assay that would permit the immediate assessment of therapy-induced cytotoxic effects on residual cancer cells cannot be overemphasised. In the present, prospective, randomised study, we attempted to use an immunocytochemical test to monitor disseminated epithelial tumour cells in bone marrow during passive antibody therapy. Several groups have demonstrated that dispersed epithelial tumour cells can be reliably identified in bone marrow aspirates with monoclonal antibodies against cytokeratins [1–10]. As shown by the present placebo-controlled trial, infusion of a cytotoxic antibody specific for Lewis Y blood group antigen [11–13] leads to a reduction or eradication of micrometastatic epithelial cells

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from bone marrow without apparently affecting overt metastases. Because of the consistency of the findings, it is suggested that monitoring of micrometastatic cells in bone marrow is feasible and might be developed into a novel surrogate assay for adjuvant therapies in minimal residual cancer.

PATIENTS AND METHODS

Patients

40 patients (19 breast and 21 colorectal cancer patients), presenting cytokeratin (CK)-positive epithelial tumour cells in their bone marrow, were consecutively recruited into this study. In order to find these 40 patients, 172 patients had to be screened.

In all patients, the diagnosis of carcinoma was confirmed histologically. Staging of the tumour was performed according to the primary tumour/regional nodes/distant metastasis (TNM) classification of the International Union against Cancer (UICC). All patients had either failed or refused current treatment modalities. Patients receiving any treatment with biological response modifier substances within 3 months or any kind of conventional therapy within 3 weeks of the expected inclusion date were excluded. During the study period, patients received no further antitumoral therapy.

Of the 40 patients, 20 were randomised to a placebo group and 20 to the monoclonal antibody (MAb) treatment arm. The treatment consisted of six infusions of 100 mg of MAb ABL364, administered over 2 weeks on days, 1, 3, 5, 8, 10 and 12. The control patients, receiving human serum albumin according to the same regimen, were offered a cross-over to the antibody treatment at day 60.

Written informed consent was obtained from all patients. The study had been approved by the review board of the Ethics Committee of the University of Munich and the Zentralklinikum Augsburg.

Antibodies

Murine MAb ABL364, specific for the Lewis Y carbohydrate antigen, was secreted as an IgG_3 globulin by the BR55-2 hybridoma [11] and was exclusively manufactured *in vitro* for human use by Sandoz (Switzerland). Purification of the IgG_3 antibody was achieved by a sequence of ion-exchange chromatographies. After reconstitution of lyophilised material, the antibody was >99% pure according to HPLC and SDS–PAGE analysis.

For immunocytochemical detection of epithelial tumour cells in bone marrow, the MAb CK2, specific for cytokeratin 18 (Boehringer Mannheim, Germany), was applied according to the previously described technique [1]. Additional slides of the same cell suspension were prepared from the 10 patients presenting with more than 20 CK-positive cells/4×10^5 bone marrow cells. These were stained with MAb ABL364 specific for the Lewis Y antigen. Normal bone marrow cells do not react with antibody CK2 or antibody ABL364 [1, 3, 11]. Appropriate dilutions of mouse myeloma immunoglobulin served as isotype controls.

Immunocytochemistry

Bone marrow samples were aspirated from left and right iliac crest (spina iliaca posterior superior) at entry. Follow-up aspirates were obtained 3 days after the last antibody or placebo administration (day 15), at the site where the highest count of CK-positive cells per 4×10^5 bone marrow cells had been found, and 8 weeks after treatment (day 60). A mean volume of 4.5 ml of bone marrow was obtained per aspiration. After density centrifugation through Ficoll–Hypaque, interface cells were harvested and cytocentrifuged on glass slides. The average number of cells per slide was 8×10^4. Following overnight air drying and subsequent fixation with acetone for 10 min, slides were either stained immediately or stored at −70°C. Routinely, five slides per aspiration site, comprising a total of 4×10^5 nucleated cells, were examined, and additional slides were prepared for immunoglobulin isotype control. Following incubation with primary antibody at pretested optimal concentrations in the range 4–10 μg/ml, the reaction was developed with alkaline phosphatase using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique. All slides were examined blind by two independent observers.

Determination of mouse IgG in serum

Serum concentration of MAb ABL364 was determined by incubating serum in dilutions of up to1:2000 in microtitre wells coated with rabbit anti-mouse IgG (Nordic, The Netherlands). After washing, bound ABL364 was determined by incubation with rabbit anti-mouse IgG/peroxidase conjugate (Dianova, Germany) and developed with o-phenylenediamine/hydrogen peroxide. Measurements were made using an ELISA reader at wavelengths of 492 nm/620 nm.

Determination of complement-dependent cytotoxicity (CDC)

The lytic activity of fresh or thawed serum, obtained from patients treated with antibody, was tested in a ^{51}Cr release assay using the Lewis Y antigen-positive SKBR5 breast cancer cell line as target cells. ^{51}Cr-labelled SKBR5 cells were incubated for 4 h with fresh or heat-inactivated serum (dilution1:2.5) as a source of complement and isotope release was determined according to standard procedures.

RESULTS

In a previous phase I trial, a single 100 mg dose of MAb ABL364 was well tolerated [13]. In order to obtain high transvascular gradients of cytotoxic antibody, elevated blood concentrations were maintained for prolonged periods of time by a multiple dose regimen consisting of six doses of 100 mg MAb ABL364 administered on alternate days over 2 weeks. The time course of actual serum antibody concentrations and the cytolytic activity as obtained with fresh non-inactivated serum of one patient are depicted in Figure 1. Very similar data were obtained from other antibody-treated patients. Heating the sera for 30 min at 56°C abolished the cytotoxic activity completely.

3 patients, 1 antibody-treated and 2 placebo-treated patients, did not consent to follow-up examinations. The numbers of tumour cells in bone marrow for all 37 evaluable patients are shown in Tables 1–4. Due to sampling error, monitoring of CK-positive cells was difficult to evaluate in the majority of patients (27/37), as these cells occur only at low frequencies, in the order of 1–11/4×10^5 mononuclear bone marrow cells (Tables 1, 2). Nevertheless, on day 60, CK-positive cells were detected in only 2 of the 12 antibody-treated patients (17%; Table 1) compared with 6 of the 15 placebo-treated patients (40%; Table 2).

Therefore, to detect at least a one log decrease after antibody therapy, we chose to analyse separately those patients who presented with more than 20 tumour cells per 4×10^5 bone marrow cells at one aspiration site. The clinical characteristics of these 10 patients and the actual numbers of disseminated tumour cells present prior to therapy are given in Table 3. All 10 patients had breast cancer, 3 of whom had no clinically visible metastatic lesions as defined by conventional diagnostic

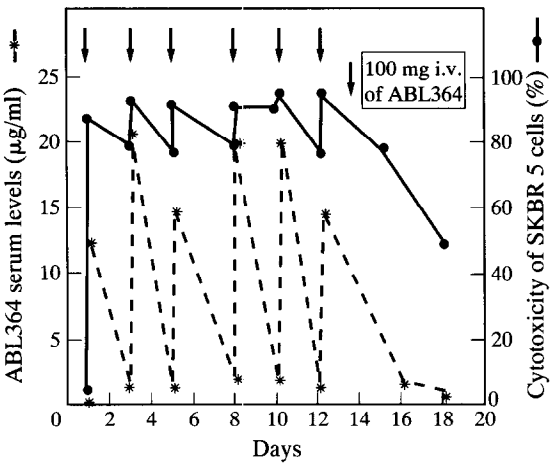

Figure 1. Antibody concentrations (-*-) and cytotoxicity (-●-) in the serum of a representative patient treated with MAb ABL364. Cytotoxicity is given as percentage cytotoxicity in a 4 h ^{51}Cr release assay of the patient's fresh frozen serum (see Patients and Methods). The serum half-life of MAb ABL364 in blood had been previously extensively studied at three dose levels of 50, 100 and 300 mg. The median half-life is 50 h (range 20–105).

Table 1. Evaluation of epithelial tumour cells in the bone marrow of antibody-treated patients who presented with less than 20 CK-positive cells per 4 × 10^5 bone marrow cells

Patient	Stage	Tumour	Number of tumour cells/ 4 × 10^5 bone marrow cells Day 0	Day 15	Day 60
1	$T_2N_1M_0$	CRC	2	1	1
2	$T_4N_0M_0$	CRC	2	3	1
3	$T_1N_0M_0$	BR	1	0	0
4	$T_2N_0M_1$	BR	1	0	0
5	$T_2N_0M_0$	BR	1	2	0
6	$T_3N_0M_0$	CRC	2	0	0
7	$T_4N_0M_0$	CRC	2	3	0
8	$T_3N_2M_1$	CRC	1	1	0
9	$T_3N_0M_0$	CRC	1	0	0
10	$T_2N_0M_0$	CRC	1	0	0
11	$T_3N_0M_0$	CRC	1	3	0
12	$T_2N_0M_0$	CRC	11	0	0

BR, breast cancer; CRC, colorectal cancer.

techniques (X-ray, computed tomography (CT) scan). Prior to antibody therapy, no Lewis Y-positive cells could be detected in 4 patients, whereas in the other 6 patients 80–100% of the CK-positive cells stained with MAb ABL364 (Table 4). After treatment with six doses of 100 mg antibody, 5 of the 7 patients showed a distinct decrease or disappearance of CK-positive, Lewis Y-positive cells. Interestingly, at day 60 after treatment, the CK-positive tumour cells appeared to be negative for the Lewis Y antigen, suggesting that tumour cells lacking the target antigen had been selectively expanded. Interestingly, 2 of the 7 antibody-treated patients (cases 33 and 34), presenting exclusively Lewis Y-negative metastatic tumour cells, did not show a notable decrease of CK-positive cells. Similarly, no decrease or eradication of those cells was observed in patients receiving human serum albumin only, regardless of their Lewis Y phenotype.

Table 2. Evaluation of epithelial tumour cells in the bone marrow of placebo-treated patients who presented with less than 20 CK-positive cells per 4 × 10^5 bone marrow cells

Patient	Stage	Tumour	Number of tumour cells/ 4 × 10^5 bone marrow cells Day 0	Day 15	Day 60
13	$T_3N_2M_1$	CRC	8	1	1
14	$T_3N_0M_0$	CRC	1	0	2
15	$T_3N_0M_0$	CRC	1	0	1
16	$T_2N_0M_0$	CRC	1	3	4
17	$T_3N_0M_0$	CRC	1	1	1
18	$T_2N_1M_0$	BR	5	1	2
19	$T_2N_0M_0$	BR	2	1	0
20	$T_3N_2M_1$	BR	4	0	0
21	$T_1N_0M_0$	BR	1	0	0
22	$T_3N_0M_0$	CRC	1	0	0
23	$T_2N_0M_0$	CRC	1	0	0
24	$T_2N_0M_0$	CRC	4	0	0
25	$T_2N_0M_0$	CRC	1	0	0
26	$T_4N_0M_0$	CRC	1	0	0
27	$T_3N_0M_0$	CRC	1	1	0

BR, breast cancer; CRC, colorectal cancer.

3 placebo patients were crossed over to the treatment arm as permitted by the protocol. While the first patient (case 35) exhibited a relatively stable course of CK-positive/Lewis Y-positive cells over a period of 60 days, cells decreased by more than 95% on day 75, i.e. approximately 48 h after the last antibody infusion. The other placebo patients (cases 36 and 37) who also crossed over to the antibody treatment arm, presented initially with metastatic cells lacking the Lewis Y antigen altogether. Here, after antibody treatment at day 75, the number of tumour cells did not only not decrease, but rather increased.

As to the clinical manifest metastases (Table 3), no objective regression of the metastatic lesions could be ascertained by conventional diagnostic techniques, such as X-ray or CT analysis. In the remaining 3 patients with no evidence of clinical metastasis (M0), no relapse of the disease has yet occurred.

Except for nausea (WHO grade 3–4) and cutaneous allergy (WHO grade 2) seen in all antibody-treated patients, no major adverse effects were observed. In particular, no anaphylactic reactions were noted during the 2 week period of treatment, although low titres of human anti-mouse immunoglobulin were found in all treated patients.

DISCUSSION

The main purpose of this study was to determine whether the pronounced *in vitro* cytotoxicity of this particular IgG_3 antibody could be verified *in vivo* by assessing CK-positive tumour cells in bone marrow before and after antibody infusion. In patients with more than 20 epithelial tumour cells per 4 × 10^5 bone marrow cells, the notion that the observed reduction or eradication of CK-positive cells is an effect of the infused antibody was supported by the absence of a similar decrease in patients with Lewis Y-negative tumour cells and in the placebo-treated group of patients. Thus, the question may arise whether treatment with Lewis Y antibody leads to a preferential selection of Lewis Y-negative tumour cell clones. Masking of Lewis Y antigen by the administered antibody is an unlikely event since control preparations of bone marrow, stained with secondary

Table 3. Characteristics and clinical courses for patients who presented with more than 20 CK-positive cells per 4 × 10^5 bone marrow cells

Patient	Primary tumour	Number of initial CK+ cells per 4 × 10^5 bone marrow cells	Stage*	Treatment allocation	Clinical course
28	Breast	860	M_1 bone	Verum	Progressive bone and brain metastases; death 12 months after MAb therapy
29	Breast	27	$T_2N_0M_0$	Verum	Stable disease; no locoregional or distant metastases for 18 months
30	Breast	22	M_1 bone, liver, lung	Verum	Progressive lung metastases; death by pulmonary emboli 10 weeks after MAb therapy
31	Breast	114	$T_2N_0M_0$	Verum	Stable disease; no distant metastases for 12 months
32	Breast	37	$T_1N_1M_0$	Verum	Stable disease; no distant metastases for 14 months
33	Breast	349	M_1 bone, liver peritoneum	Verum	Progressive peritoneal metastases; death by ileus 4 months after MAb therapy
34	Breast	62	M_1 bone	Verum	Stable disease for 3 months
35	Breast	320	M_1 bone, liver	Verum after placebo	Progressive disease, death 3 months after MAb therapy
36	Breast	1051	M_1 bone, skin	Verum after placebo	Progressive disease
37	Breast	22	M_1 bone, skin	Verum after placebo	Progressive disease

*Patients presenting only CK-positive cells in bone marrow, but were found to have no visible metastatic lesions by conventional diagnostic techniques (X-ray, CT scan), were attached to stage M_0.

Table 4. Monitoring breast cancer patients who presented with more than 20 CK-positive cells per 4 × 10^5 bone marrow cells

	Treatment		Number of CK+ cells per 4 × 10^5 bone marrow cells			
Patient	Day 0 Placebo	Day 60 ABL364	Day 0	Day 15	Day 60	Day 75†
35*	+	+	320	300	380	22
36*	+	+	1051‡	900‡	259‡	924‡
37*	+	+	22‡	55‡	192‡	247‡
	ABL364					
28	+		860	28‡	47‡	
29	+		27	0	0	
30	+		22	10	0	
31	+		114	2‡	12‡	
32	+		37	12	0	
33	+		349‡	437‡	n.d.	
34	+		62‡	353‡	950‡	

*Crossing over to MAb on day 60; † On day 75, an analysis was carried out in cross-over patients only, i.e. on day 15 after MAb infusion; ‡All cells were Lewis Y antigen-negative in parallel staining. n.d., not done.

anti-murine Ig antibodies alone, remained negative. Unfortunately, less convincing data were obtained in patients presenting with lower numbers of CK-positive cells in the bilateral aspirates. Due to the greater sampling error at such low cell frequencies, an interpretation of the longitudinal analysis in individual patients proved to be difficult. Mansi and associates [14] found that disseminated tumour cells in bone marrow became undetectable after surgery in untreated breast cancer patients, who remained disease-free. However, this study had a long-term follow-up with a median interval of 18 months (range 4–56), whereas we used a fixed interval of 60 days between the first and last aspiration.

The reduction of individual dispersed tumour cells stands in sharp contrast to the absence of any notable effect of the antibody therapy on macroscopic tumour nodes. This is congruent with the general experience of inefficacy of antibodies in advanced solid tumours. With the exception of a few anecdotal and transient remissions, no consistent survival benefit occurred for treated patients. The explanations for this failure are still contentious [15]. One hypothesis suggests that unmodified murine antibodies lack the capacity to kill epithelial tumour cells *in vivo*, while another simpler explanation holds that malignant epithelial cells, due to their location in solid tumour parenchyma are largely inaccessible for intravenously administered antibodies [16]. Indeed, very recent evidence demonstrates that unconjugated murine MAbs may be life saving when used in patients with minimal residual disease, as demonstrated in colorectal cancer patients after surgical resection of the tumour [17].

It is noteworthy that the antibody used in the present study belongs to a group of MAbs, all of IgG_3 isotype, that, more frequently than others, induced clinical remissions in individual patients [18, 19]. ABL364 is distinct from several antibodies by exhibiting a marked cytotoxicity (Figure 1) when added *ex vivo* in the serum, freshly drawn from an infused patient, to various tumour cell lines. The IgG_3 isotype, exhibiting strong C1q activation via a specific Fc–Fc interaction, may account for the potent mobilisation of the lytic complement cascade [20]. Whether this particular IgG_3 activity or the Lewis Y specificity incapacitates the inhibitory function of complement control proteins, such as decay accelerating factor (DAF) or CD59, is not yet known [21].

The Lewis Y antigen belongs to a family of difucosylated oligosaccharides that are expressed during embryogenesis and

become re-expressed during malignant transformation [22]. In primary lung cancer, it has been hypothesised that sialyated Lewis antigen functions as an adhesion ligand for various selectins. In this tumour, its presence appears to be closely correlated with tumour cell motility, invasiveness and lastly with survival [23]. Thus, a blockade of these structures might contribute to a therapeutic effect.

Although the assay in its present form is far from being perfect, it is evident that the qualitative and quantitative information obtained by double-staining of single individual cells cannot be provided by current polymerase chain reaction techniques [24, 25]. Recently, the test has been further improved, so that it routinely allows the counting of 1×10^6 nucleated cells; furthermore, by use of a pan-CK antibody, the sensitivity of the test can be increased considerably [26]. Thus, the critical limit of a low tumour cell count may soon be overcome.

Therefore, characterisation and monitoring of individual epithelial cancer cells, as demonstrated here, for patients with higher tumour load, may also become possible in patients with minimal residual disease, where the frequency of isolated tumour cells is relatively low. As stated above, it is for this vulnerable stage of tumour development that a surrogate test for guiding and developing adjuvant therapies is most needed. Currently, another trial is being initiated in which a cut-off point of 20 tumour cells per 2×10^6 nucleated bone marrow cells will be used prospectively as an entry criterion to assess the effect of a human/mouse chimeric antibody.

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