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Efficacy and toxicity of plasma-cell-reactive monoclonal antibodies B-B2 and B-B4 and their immunotoxins

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Abstract Immunotherapy based on the delivery of toxic agents to the tumor site using monoclonal antibodies (mAb) may be a promising modality in the treatment of hematological malignancies. In the selection of mAb, both for ex vivo but even more for in vivo therapy, not only their reactivity to the neoplastic cells should be considered, but also reactivity to other body constituents. Here we describe the screening of two human plasma-cell-reactive mAb B-B2 and B-B4, which may be used for immunotherapy of multiple myeloma. Cross-reactivity of B-B2 and B-B4 was determined by immunohistochemistry on a series of tissues. This revealed for both B-B2 and B-B4 a strong staining of epithelial cells in various organs, e.g. lung, liver, skin, kidney and gut, while only a weak and diffuse staining was seen with endothelial cells. In bone marrow reactivity was only found with plasma cells and not with hemopoietic precursors ($CD34^+$ cells). Immunotoxins from B-B2 and B-B4 were constructed by coupling them to the plant-derived ribosome-inactivating protein saporin. Both B-B2 and B-B4 immunotoxins appeared to be efficient in specific inhibition of protein synthesis in plasma cell lines (IC_{50} respectively 1 nM and 0.1 nm). The immunotoxins were also tested on epithelial cell line A431, on liver cell line HepG2 and on human umbilical vein endothelial cells. The epithelial cell line A431 was reactive with both B-B2 and B-B4, but was only inhibited by B-B4 immunotoxin. Cell line HepG2 was reactive with both mAb, but was not inhibited by either immunotoxin. The endothelial cells showed no reactivity with B-B2 and B-B4 and were not inhibited by either immunotoxin. Bone marrow treated with B-B2 and B-B4 immunotoxin did not show a decrease in colonies of hemopoietic precursor cells. Incubation of multiple-myeloma-derived bone marrow with these immunotoxin resulted in a clear decrease of the number of plasma cells.

From these data we conclude that B-B2 and B-B4 immunotoxin can be used for ex vivo bone marrow purging. Discrepancies were found between immunohistochemistry, binding assays and cytotoxicity assays with the mAb and the immunotoxin, which underlines the necessity for these various assays as a preclinical screening.

Key words Multiple myeloma · Plasma cell · Immunotoxin · B-B2 · B-B4

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Introduction

Multiple myeloma is a lymphoproliferative disease characterized by the presence of homogeneous immunoglobulins in blood, an increase in plasma cells in bone marrow and bone resorption due to increased osteoclast activity. Chemotherapy has only limited success in obtaining long-term disease-free survival and is considered palliative rather than curative [6, 17]. New approaches to anticancer therapy have been proposed on the basis of the delivery of therapeutically active agents to cancer sites by mAb [8, 18, 26, 39, 44, 47, 48]. For multiple myeloma, this approach is hampered by the elusiveness of the clonogenic cell in that disease, which may be either an early cell in the B cell series [10, 19, 25] a more mature B cell [38] or an early plasmacytoid cell [31]. Depletion of just one of these populations may not result in long-term disease-free survival.

There is considerable experience in immunotherapy of other B lymphoid malignancies, comprising either immunotoxins (IT) or radioimmunoconjugates using anti-CD19, anti-CD20, anti-CD21 or anti-CD22 [1, 15, 27, 30, 37, 46]. For a successful treatment it appears important to eliminate the plasma cells as well, as these cells are responsible for

the high morbidity of multiple myeloma, like bone pain and spontaneous bone fractures due to osteoclast activation. Anti-CD38 mAb are the most established mAb for flow-cytometric identification of plasma cells. Several other mAb restricted to the plasma cell have been described [2, 3, 23, 40, 42]. Some of these have been used as IT [16, 21, 22] or in combination with complement [4, 43] for ex vivo bone marrow purging. For this ex vivo treatment the possibility has to be excluded that the mAb react with and destroy stem cells in the bone marrow. For in vivo application, not only should reactivity to the neoplastic cells be considered, but also reactivity to other body constituents, further referred to as cross-reactivity. Serious problems can arise if cross-reacting mAb are used as there can be destruction of healthy tissues, as shown in recent trials [24, 33]. Immunohistochemistry is often used to evaluate this cross-reactivity, but data from this evaluation may not be the sole criterion, because they (a) demonstrate only binding and not subsequent capacities of destruction and (b) demonstrate both membrane and, even more clearly, cytoplasmic components, while membrane antigens are the targets for destruction. An alternative and additional way to study cross-reactivity is the screening of cell lines originating in different tissues [32].

We elaborate here the screening of two recently described non-competing plasma-cell-reactive mAb: B-B2 and B-B4 [13]. Different results were obtained when immunohistochemistry was compared to testing of cell panels including endothelial and epithelial cells. In the form of IT, both mAb proved not to be toxic for endothelial cells, nor was B-B2 toxic for epithelial cell lines. This was despite reactivity to these cell types in histochemistry or their capacity to bind to such cells in cell lines.

Materials and methods

Cell lines, mAb and saporin

Cells of the plasma cell line RPMI8226 (multiple myeloma bone-marrow-derived) were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin, in humidified air at 37 °C and 5% CO_2. Human umbilical vein endothelial cells were isolated from human umbilical vein and cultured as above, except for the FCS, which was replaced with 10% heat-inactivated human AB serum. The hepatocytic cell line HepG2 and the keratinocyte cell line A431 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin, in humidified air at 37 °C and 5% CO_2.

B-B2 and B-B4 are two recently described plasma-cell-reactive mAb [13]. B-B2 is of the IgG2b and B-B4 of the IgG1 class. The two mAb do not compete each other. Cell populations were identified using CD3, CD14, CD19, CD34 and CD38 (Becton and Dickinson, San José, Calif.).

Large-scale saporin purification was performed as described by Barbieri et al. [5].

Construction of IT

IT of B-B2 and B-B4 with the type-1 ribosome-inactivating protein saporin were prepared essentially according to the method described by Tazzari et al. [41]. Briefly, Sulfhydryl groups were introduced separately into the mAb and into the toxin by 2-iminothiolane treatment (0.6 mM and 1 mM, respectively added). To quantify the amount of toxin conjugated in the resulting IT, a trace of ^{125}I-labelled saporin was added to the toxin solution. The excess 2-iminothiolane was removed by G-25 gel filtration. The modified toxin was reduced with 20 mM 2-mercaptoethanol, and loaded onto a G-25 column. The derivatized moiety was separated from 2-mercaptoethanol and was collected directly from the column onto the unreduced derivatized mAb. After concentration, the conjugation was allowed to proceed for 16 h at room temperature. The immunotoxin was collected from this reaction mixture by gel filtration on Sephacryl S200. The mAb and toxin content of the IT was estimated by spectrophotometry at A280 and radioactivity. Binding activity of the IT was tested by means of competition experiments with biotin-labeled mAb and compared to the competition of free mAb. RPMI8226 cells were incubated with 5 μg/ml biotin-labeled B-B2 or B-B4 in the presence of increasing amounts of IT or free mAb for 20 min at 4 °C and subsequently stained with a phycoerythrin-conjugated streptavidin. Cells were measured using a FACScan flow cytometer (Becton Dickinson).

Protein synthesis inhibition assays

Ribosome-inactivation activity of free and conjugated saporin was tested in a reticulocyte lysate system, as described by Parente et al. [35].

The cytotoxic effect of the IT on cells was assessed by measuring their ability to inhibit protein synthesis in a concentration-dependent way. Cells were seeded in a 96-well round-bottom plate and incubated with either B-B2 or B-B4 IT for 72 h in concentrations ranging from 0.1 pM to 10 nM. [^{3}H]Leucine (1 μCi) was then added to each well and incubated overnight. Cells were harvested on glass-wool filters and counted on a beta plate scanner. Cell numbers used were chosen so that [^{3}H]leucine incorporation was a linear function of the number of cells. Results were expressed as the percentage [^{3}H]leucine incorporation with regard to mock-treated cells.

Clonogenic assay

A modification of the clonogenic assay according to Bast was performed [7, 28, 36]. Briefly, a series of 12 serial fivefold dilutions (six aliquots of 100 μl/dilution) were prepared from plasma cell line RPMI or epithelial cell line A431 (starting concentrations 10^6, 10^5, 10^4 and 10^3 cells/ml) in 96-well flat-bottom plates. Cells were incubated with medium, mAb and saporin separately or IT at a concentration of 10 nM in a total volume of 200 μl at 37 °C and 5% CO_2. After 14 days the plates were microscopically scored for colony outgrowth. The number of clonogenic units was calculated using a Spearman estimate as described by Johnson and Brown [29]. The logarithmic (log) cell killing brought about by the IT can be determined by comparing the numbers of clonogenic units from treated and untreated cells.

Bone marrow mononuclear cells

Normal bone marrow was obtained from patients undergoing cardiac surgery. Heparinized samples were diluted with an equal volume of phophate-buffered saline (PBS) and fractionated by gradient centrifugation on Ficoll-Hypaque. The interphase, containing the mononuclear cells, was collected. The cells were subjected to FACS analysis and hemopoietic precursor cell assays.

Immunohistochemistry

Specimens of human spleen, stomach, duodenum, esophagus, tonsil, thyroid gland, liver, lung, kidney, heart muscle, skin, thymus, brain and lymph node were presented to the Department of Pathology for histopathological diagnosis. Conventional histopathology on formalin-fixed paraffin-embedded tissue showed a normal "architecture". In addition a skin sample with immunocytoma and a nasopharynx containing a plasmacytoma were included in the analysis. Parts of the specimens were snap-frozen and stored at −20 °C. Immunohistochemistry was done on frozen tissue sections of 6–8 μm thickness, after

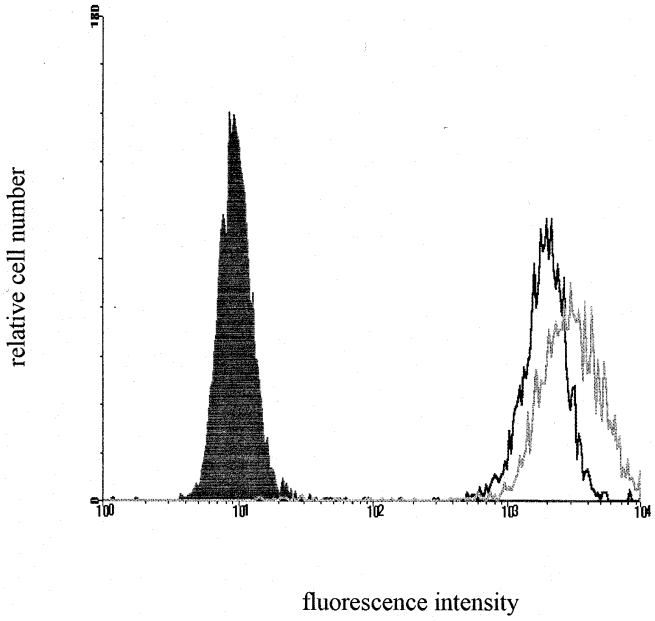

Fig. 1 Reactivity of B-B2 and B-B4 with plasma cell line RPMI8226. Cells were incubated with optimal concentrations of mAb for 30 min at 4 °C and, after two wash steps, stained with fluorescein-conjugated goat anti-(mouse Ig). Cells were analyzed with a flow cytometer. Isotype-matched mouse Ig (IgG1 and IgG2b) were used as a negative control. Incubation with control mAb (*solid peak*), B-B2 (*black outline*) and B-B4 (*grey outline*)

10 min fixation in acetone at room temperature. The first incubation was done with mAb at a predetermined optimal dilution (30 min, room temperature). The second and third incubations were performed with rabbit anti-(mouse immunoglobulin) and sheep anti-(rabbit immunoglobulin), respectively, both conjugated to horseradish peroxidase (Dakopatts, Glostrup, Danmark). Color development was done with 3′3-diaminobenzidine tetrahydrochloride and hydrogen peroxide as substrates. Sections were then counterstained with hematoxylin. Controls included replacement by an irrelevant antibody and resulted in the only occasional peroxidase labeling of polymorphonuclear leukocytes, when present.

Flow-cytometric analysis

Cells growing in monolayer were detached in PBS with 20 mM EDTA, incubated for 30 min at 4 °C and subsequently washed once in culture medium. Cells growing in suspension were drawn from the culture and washed once in culture medium. Cells ($0.1-0.2\times10^6$/sample) were incubated for 15 min at 4 °C with the mAb (10 μg/ml). After two washes in RPMI-1640 medium supplemented with 10% FCS, the cells were incubated for another 15 min at 4 °C with goat antimouse Ig antibodies conjugated to fluorescein isothiocyanate or phycoerythrin. The cells were again washed twice in RPMI-1640 medium supplemented with 10% FCS and finally suspended in PBS supplemented with 1% bovine serum albumin and 0.1% NaN_3 and analyzed with a FACScan flow cytometer (Becton Dickinson).

Fluorescent cell staining

Bone marrow cells from myeloma patients were membrane-stained with B-B2 or B-B4, cytocentrifuged and incubated for cytoplasmic staining with either goat anti-(human κ chain) or goat anti-(human λ chain) conjugated to rhodamine tetramethyl isocyanate (Southern Biotechnology Associates). Preparations were analyzed by fluorescence microscopy.

Table 1 Biochemical characterization and activity of B-B2- and B-B4-saporin. The activity shown is the inhibition of cell-free protein synthesis, after reduction of the disulfide bond, tested in a reticulocyte lysate system as described by Parente et al. [35]. The concentration is that at which 50% of the protein synthesis is inhibited (ng saporin/ml)

Toxin or immunotoxin	SH groups introduced		Ratio toxin/mAb	Activity (ng/ml)
	Saporin	mAb		
Saporin	–	–	–	1.80
B-B2-saporin	1.00	2.46	2.00	3.02
B-B4-saporin	1.23	1.74	2.55	4.17

Colony assays of hemopoietic progenitor cells

Bone marrow mononuclear cells were resuspended in RPMI-1640 medium containing 10% AB serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin with or without 10 nM IT or mAb + saporin separately. For the enumeration of granulocyte/macrophage-colony-forming units (CFU-GM), 100 units/ml granulocyte/macrophage-colony-stimulating factor and 10 units/ml interleukin-3 (IL-3) were added, for erythroid-burst-forming units (BFU-E) 3 units/ml erythropoietin, and for granulocyte/erythroid/macrophage/megakaryocyte-colony-forming units (CFU-GEMM) 10 units/ml IL-3 and 3 units/ml erythropoietin. Methylcellulose was added to a final concentration of 0.9%. Finally the cells (200 000) were plated out in 3-cm petri dishes and incubated at 37 °C and 5% CO_2. After 14 days colonies of more than 20 cells were counted.

Results

Binding of the mAb and biochemical characterization of the IT

Expression of B-B2 and B-B4 on plasma cell line RPMI8226 was determined. Cells were stained with optimal concentrations of B-B2 and B-B4. Results are shown in Fig. 1. Expression of B-B4 is twofold higher than that of B-B2.

The biochemical characterization of both IT is shown in Table 1. Saporin activity is retained sufficiently as compared to free saporin and other immunotoxins (data not shown). Binding activity of the mAb part of the IT to the target cell was compared to binding of the native mAb. The plasma cell line RPMI8226 was stained with directly conjugated B-B2 or B-B4-FITC in the presence of increasing concentrations of native mAb or IT. Results are shown in Fig. 2. B-B2-saporin showed an approximately fourfold decrease in binding compared to B-B2. Binding of B-B4-saporin was similar to binding of B-B4.

Immunohistochemistry

Immunohistochemistry was carried out on samples of lymphoid (spleen, tonsil, and thymus) as well as non-lymphoid tissues (stomach, duodenum, esophagus, thyroid, liver, lung, kidney, heart, brain, and skin). For B-B2 and B-B4 similar staining patterns were observed. B lymphocytes in lymphoid follicles and plasma cells in various organs were reactive. Staining was also seen for other tissue

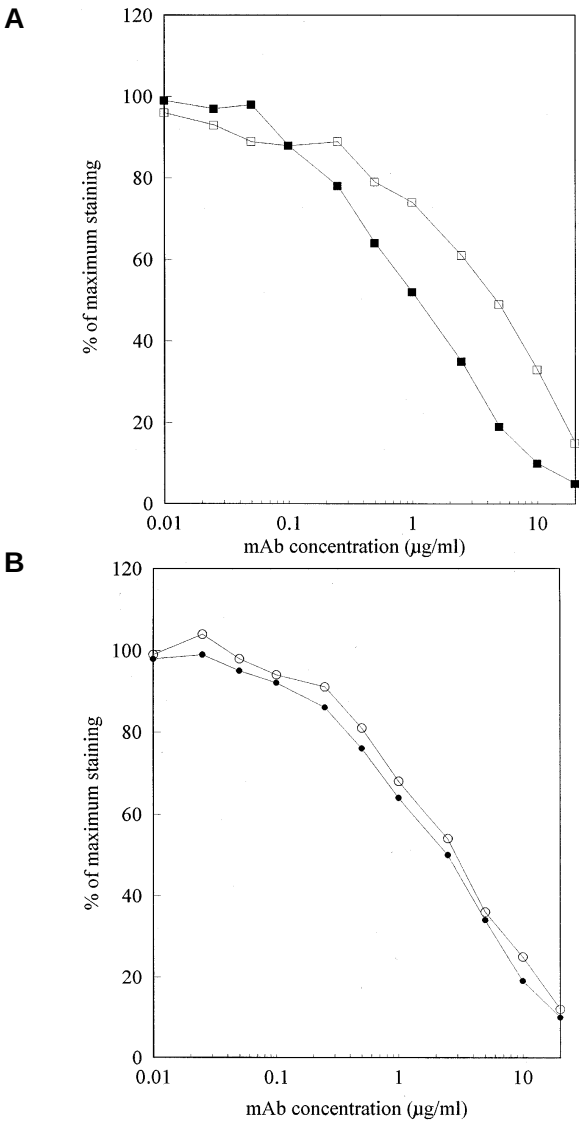

Fig. 2A, B RPMI8226 cells were stained with biotin-labeled B-B2 (**A**) or B-B4 (**B**) in the presence of increasing amounts of free mAb or immunotoxin to determine the amount of competition as a measure of binding activity. Cells were incubated for 20 min at 4 °C and stained with phycoerythrin-conjugated streptavidin. Cells were analyzed with a flow cytometer. Data are expressed as a percentage of the staining with the biotin conjugate alone. Symbols represent B-B2 (■), B-B2-saporin (❑), B-B4 (●) and B-B4-saporin (❍)

components. This included a strong labeling of epithelium in various organs tested, and a weak intensity labeling of endothelium, in almost all organs tested. In liver, hepatocytes showed reactivity. In spleen tissue sections a diffuse staining of the red pulp was seen. There was no reactivity of connective tissue, muscle and nervous tissue. In the samples of skin and nasopharynx manifesting a plasmacytoma, immunolabeling of the plasma cells was observed. A typical staining pattern B-B4 is shown on a duodenum tissue section in Fig. 3, which is similar to that of B-B2.

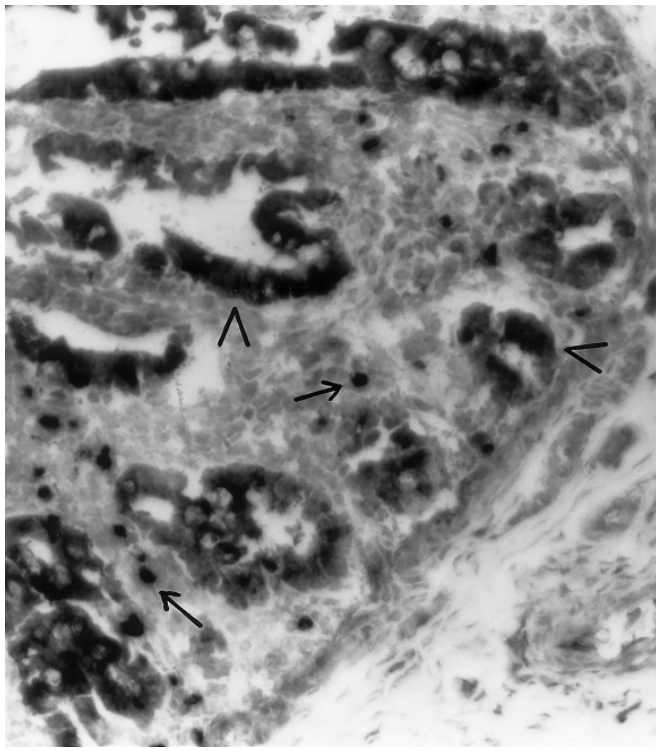

Fig. 3 Immunohistochemistry with B-B4 on duodenum tissue. A frozen specimen of duodenum was stained with B-B4 and a second step peroxidase conjugate. Color development was done with 3′3-diaminobenzidine. The staining pattern of B-B2 was similar to that of B-B4: there is strong labeling of epithelium (*arrowhead*). Also strong staining of plasma cells can be seen (*arrow*)

Reactivity with healthy bone marrow and peripheral blood

Double-staining cytofluorography was performed on bone marrow from cardiac surgery patients with B-B2 and B-B4 and various markers. B-B2 and B-B4 labeled less than 1% of the cells, and no double staining was seen with CD34, CD33, CD19, CD10, CD21, CD3 or CD14. When tested for CD38, only cells with high CD38 expression were reactive with both B-B2 and B-B4. Conversely all B-B2- and B-B4-reactive cells were $CD38^+$. The various cell populations in blood, e.g. monocytes, lymphocytes ($CD3^+$, $CD19^+$) and polymorphonuclear granulocytes showed no reactivity with B-B2 or B-B4.

Reactivity with epithelial, hepatocytic and endothelial cell line cells

Epithelial cell lines A431 (keratinocyte-derived) and HepG2 (hepatocyte-derived) and human umbilical vein endothelial cells were stained with B-B2 and B-B4 to evaluate the results found in immunohistochemistry. In addition the T cell line CEM was included as a negative control. Cells were stained for cytofluorography as well as immunocytochemistry. Results are shown in Fig. 4. A431 and HepG2 were reactive with both B-B2 and B-B4, but not as strongly as plasma cell line RPMI8226. The endothelial cells were negative.

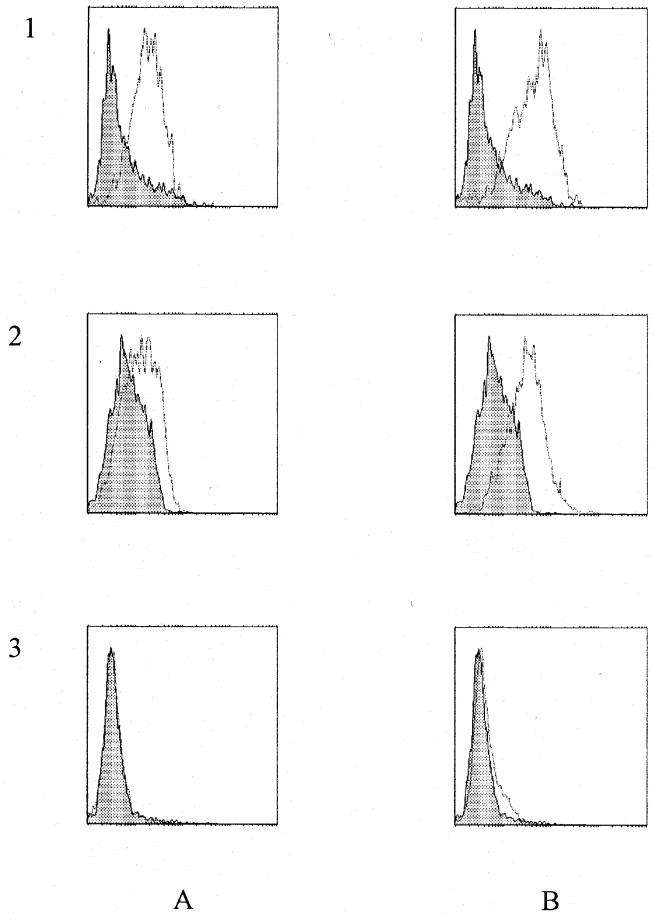

Fig. 4A, B Flow-cytometric analysis of cells of different origin stained with B-B2 (**A**) and B-B4 (**B**). Cells were stained with optimal concentrations of the first mAb, followed by a second-step fluorescein-isothiocyanate-labeled goat antimouse IgG. Two isotype-matched irrelevant mouse Ig (IgG1 and IgG2b) were used as a negative control. Each histogram depicts the logarithmic green fluorescent intensity of a gated cell population specific for the cell type tested. *1* A431; *2* HepG2; *3* human umbilical vein endothelial cells (HUVEC). *Shaded peak* negative control

Cytotoxicity of B-B2 and B-B4 IT for target cells

The specific activity of both IT was studied by treatment of the plasma cell line RPMI8226. Results are shown in Fig. 5a. Cells were incubated with IT in varying concentrations from 10 nM to 0.1 pM. After 72 hours protein synthesis was measured by [^{3}H]leucine incorporation. B-B2-saporin and B-B4-saporin are highly cytotoxic for RPMI8226, inhibiting 50% of the protein synthesis (IC_{50}) at respectively 1 nM and 0.1 nM. This means an increase in cytotoxicity by factors of 10^2 and 10^3 compared to free saporin, which has an IC_{50} of 0.1 μM (data not shown). So B-B4-saporin has a tenfold stronger effect than B-B2-saporin. Both IT, however, completely block protein synthesis at 10 nM, at which concentration there is no cytotoxic effect of free saporin or mAb. No additive effect was seen on the cytotoxic potency when both IT were combined.

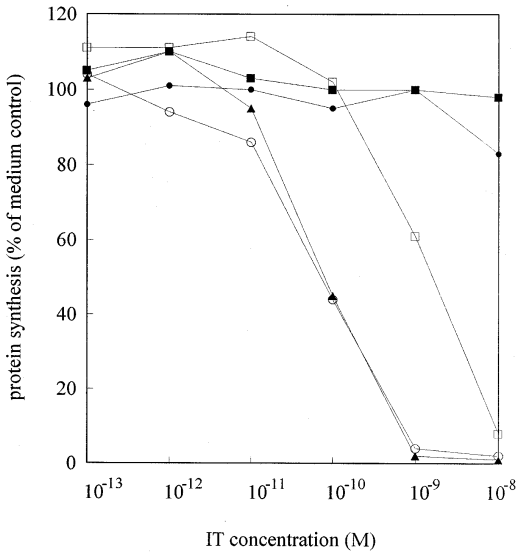

Fig. 5 Cytotoxicity of B-B2-saporin and B-B4-saporin to plasma cell line RPMI8226. Cells were incubated with different concentrations of immunotoxin (IT) or mAb + saporin as control for 72 h and pulse-labeled with [^{3}H]-leucine. Inhibition of protein synthesis is expressed as percentage of [^{3}H]-leucine incorporation of untreated cells. Concentrations refer to the amount of saporin in the IT used. ■ B-B2 + saporin, ❑ B-B2-saporin, ● B-B4 + saporin, ❍ B-B4-saporin and ▲ combination of both IT. Data represent means of three independent experiments. Standard deviations were less than 10%

Since immunohistochemistry revealed binding of both B-B2 and B-B4 to epithelial and endothelial cells, the effect of the IT on protein synthesis by these cells was investigated. Figure 6 shows the effect of an increasing concentration of IT on endothelial and epithelial cell lines. Cells of the A431 keratinocyte cell line were clearly sensitive to B-B4-saporin, but not to B-B2-saporin. No cytotoxicity was seen on hepatocytic cell line HepG2 with either IT, although there seemed to be some influence of B-B4-saporin at 10 nM. This is, however, not significant. Endothelial cells were also not inhibited by the two IT, since the same rate of inhibition was observed in the presence of saporin alone in combination with the native mAb. So endothelial cells seem to be more sensitive to saporin alone than do other cells.

Effect on clonogenic growth of cell lines

The desired effect of IT treatment is to stop cell proliferation. Therefore, cells were treated with IT and outgrowth of these cells was monitored with a clonogenic assay (Table 2). This way a log(cell kill) value could be determined for the IT both on a plasma cell line and on an epithelial cell line. Clearly B-B4-saporin showed the best inhibition of cell growth on RPMI8226 [log(cell kill) = 6]. A431, which is also reactive with B-B4, is only marginally inhibited by B-B4-saporin. B-B2-IT has a substantial effect on the plasma cell line, whereas the epithelial cell line is not inhibited in its cell growth

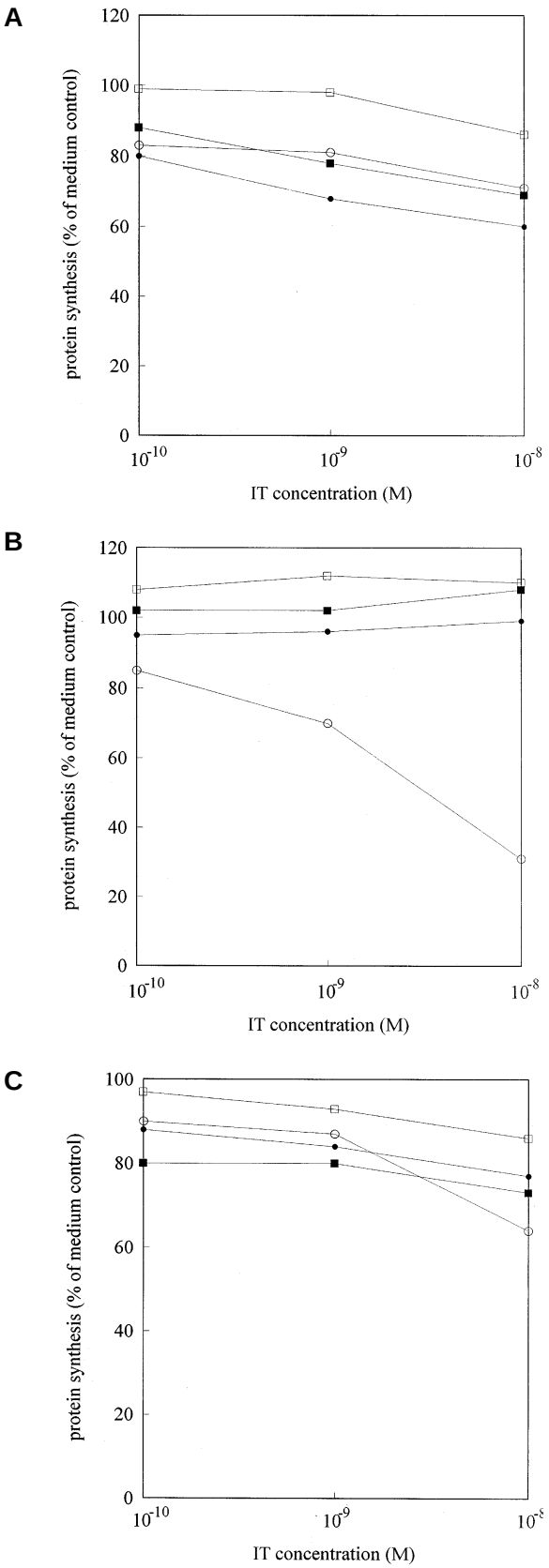

Table 2 Effect of immunotoxin treatment on the clonogenic growth of a plasma cell line and an epithelial cell line. Clonogenic assay was performed as described in Materials and methods. Cells were cultured in the presence of only culture medium or with a single dose of 10 nM (as saporin) irrelevant mAb-saporin conjugate (control), B-B2-saporin or B-B4-saporin. Clonogenic units were determined after 2 weeks of culture and calculated using a Spearman estimate

	RPMI8226		A431	
Culture conditions	Clonogenic units	log (cell kill)	Clonogenic units	log (cell kill)
Medium	0.8×10^6	–	0.8×10^6	–
Control	1×10^6	0	0.8×10^6	0
B-B2-saporin	1.1×10^4	2	1×10^6	0
B-B4-saporin	0	6	0.7×10^5	1

Effect on hemopoietic precursor cells

The effect of treatment of bone marrow with one of the two IT was studied by incubating bone marrow mononuclear cell preparations with both IT (10 nM) and subsequent determination of the CFU-GM, BFU-E and CFU-GEMM. Neither IT inhibited colony growth of normal hemopoietic precursor cells, whereas an anti-CD71 IT completely abrogated colony formation (Table 3).

Effect on freshly isolated multiple myeloma bone marrow mononuclear cells

Myeloma-cell-containing cell suspensions isolated from bone marrow from patients with multiple myeloma were incubated with both IT to determine efficacy on the eventual target cell (Fig. 7). As a measure for cytotoxicity the number of plasma cells was determined at different times by cytospin preparations. In this way only the cytotoxicity on plasma cells is established. The efficacy on the clonogenic cells may be different, but since a reliable clonogenic assay for multiple myeloma is not available at present this can not be established. Treatments with B-B2-saporin and B-B4-saporin resulted in a similar decrease in the number of plasma cells. There is difference in the relative effect of treatment between the different patient samples. However, in each sample the plasma cells are sensitive to the IT.

Fig. 6A–C Cytotoxicity of B-B2-saporin and B-B4-saporin to cells of endothelial or epithelial origin. HUVEC (**A**), A431 (**B**) and HepG2 (**C**) cells were incubated with different concentrations of IT or mAb + saporin as control for 72 h and pulse-labeled with [^{3}H]leucine. Inhibition of protein synthe sis is expressed as the percentage of [^{3}H]leucine incorporation of untreated cells. Concentrations refer to the amount of saporin in the IT used. ■ B-B2 + saporin, ❑ B-B2-saporin, ● B-B4 + saporin and ❍ B-B4-saporin. Data represent means of three independent experiments. Standard deviations were less than 10%

Table 3 Influence of immunotoxin (*IT*) treatment of healthy bone marrow on hemopoietic precursor cells. Bone marrow mononuclear cells (n = 5) were incubated with either culture medium, IT or mAb with saporin. Anti-(transferrin receptor) mAb conjugated to saporin was used as positive control IT (aCD71-saporin). Granulocyte/erythroid/macrophage/megakaryocyte-colony-forming units (*CFU-GEMM*), granulocyte/macrophage-colony-forming units (*CFU-GM*) and erythroid-burst-forming units (*BFU-E*) were determined as described in Materials and methods. Because of the wide range of colonies found in the different samples, colony numbers are expressed as percentage of the number of colonies found with culture medium ± SD.

Culture conditions	CFU-GEMM (%)	CFU-GM (%)	BFU-E (%)
Medium	100	100	100
B-B2 + saporin	87 ± 17	105 ± 9	102 ± 20
B-B2-saporin	101 ± 11	103 ± 19	93 ± 12
B-B4 + saporin	90 ± 18	100 ± 10	97 ± 16
B-B4-saporin	112 ± 18	101 ± 5	96 ± 20
aCD71-saporin	0	0	0

Discussion

Two as yet unclustered plasma-cell-reactive mAb, B-B2 and B-B4, were screened to evaluate their potential use for immunotherapy in patients with multiple myeloma. Both mAb were coupled to the ribosome-inactivating protein saporin to form immunotoxins (IT). In another phase I trial with anti-CD30-saporin IT only very limited toxicity was seen with saporin as the toxin moiety [20].

These IT appeared to be very efficient in the specific inactivation of protein synthesis in plasma cell line RPMI8226 and also in the inhibition of clonogenic cell growth of this cell line. B-B4-saporin showed a higher cytotoxicity than B-B2-saporin, propably because of a lower reactivity of native B-B2 and a decreased binding of the IT compared to the native mAb, caused by the conjugation. Apart from efficacy to the target cells, the potential therapeutic application depends on other criteria, including undesired toxicity. For ex vivo use this requires the absence of reactivity with hemopoietic precursor cells to avoid interference with blood cell repopulation of the

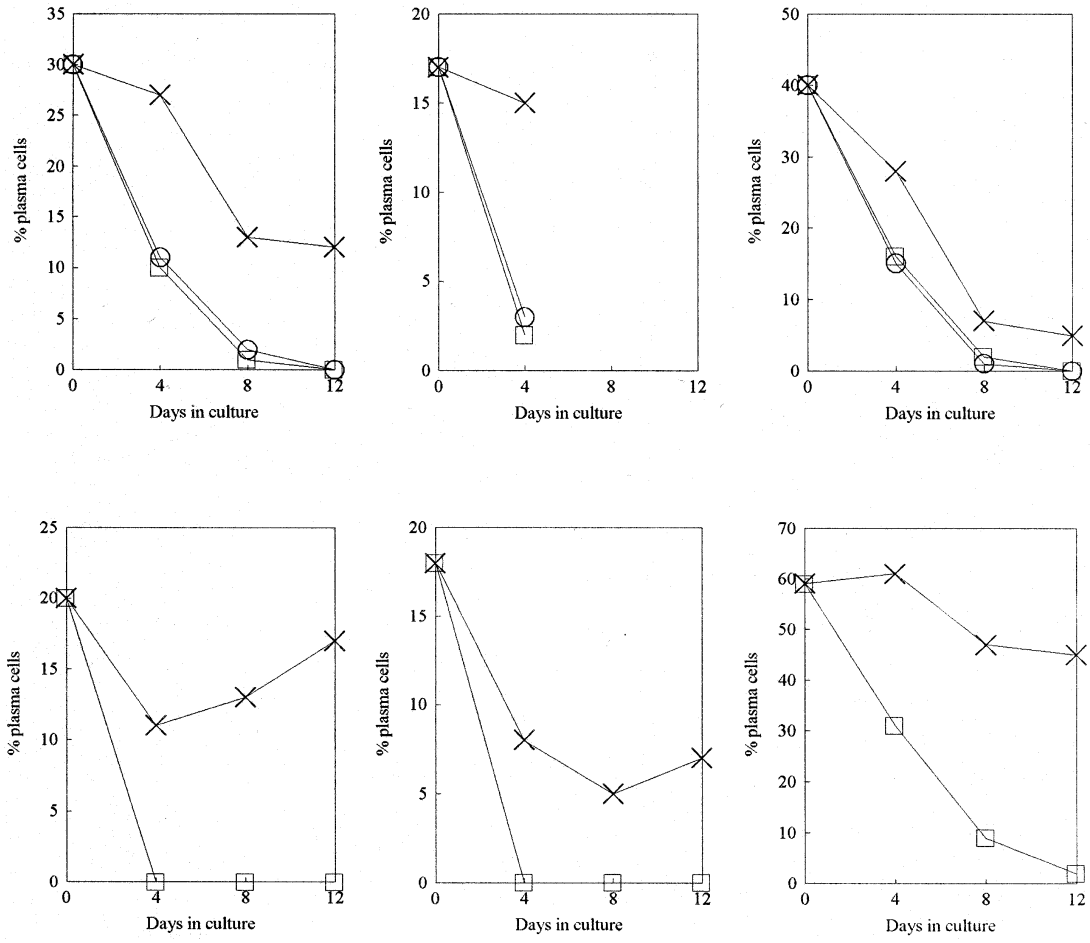

Fig. 7 Influence of IT treatment on myeloma-derived bone marrow mononuclear cells. Suspensions of these cells from patients with multiple myeloma were cultured in the presence of 10 nM B-B2-saporin (❑), 10 nM B-B4-saporin (❍), 10 nM unconjugated B-B2, B-B4 and saporin (*upper panel*, ×) or 10 nM unconjugated B-B4 and saporin (*lower panel*, ×). At various assay time cells were taken from the culture and the numbers of plasma cells were determined on cytospin preparations stained for cytoplasmic immunoglobulin light chain

treated bone marrow. For in vivo use an additional criterion is the absence of cross-reactivity with other tissues.

To test the applicability of these IT for ex vivo marrow purging, cytofluorography of bone marrow with both mAb was carried out followed by experiments with the IT in colony assays of hemopoietic precursor cells. Less than 1% of cells in normal adult bone marrow were labeled. B-B2 and B-B4 do react with normal plasma cells as described before [13]. The absence of reactivity with $CD34^+$ HPC was confirmed by the HPC colony assays. Malignant plasma cells in bone marrow samples of patients with multiple myeloma were susceptible to both IT, resulting in the disappearance of these cells after incubation with the IT. So both IT can be used for ex vivo marrow purging; B-B4 IT seems to be the more effective.

The undesired reactivity of B-B2 and B-B4 with tissue components was first evaluated by immunohistochemistry. Both antibodies showed strong reactivity with epithelial and weak reactivity with endothelial cells. The relevance of these data was evaluated in more detail, to discriminate between binding to intracellular (not accessible) and extracellular antigen. Cell membrane reactivity was determined by cytofluorography on epithelial cell lines and human umbilical vein endothelial cells (HUVEC). The endothelial cells did not show reactivity with both mAb. The epithelial cells, represented by keratinocyte cell line A431 and hepatocytic cell line HepG2, were stained in accordance with the tissue staining in immunohistochemistry. To discriminate between sole binding and toxic effect (inhibition of cell function) the cells were further tested with both IT in a protein synthesis inhibition assay. Results are summarized in Table 4. B-B4 IT showed toxicity to cell line A431, but not to HepG2 and HUVEC, while B-B2 IT, in spite of binding epithelial cells in cytofluorography, did not show any toxicity either to epithelial or to endothelial cells. Some toxicity on HUVEC was seen with saporin alone. This is in agreement with the occurrence of mild vascular leak symptoms reported in a phase I study with an anti-CD30-saporin conjugate [20]. The difference in toxicity to A431 of B-B2 and B-B4 could be due to a distinct internalisation of the two IT by A431 cells. The different susceptibility of A431 and HepG2 to B-B4-saporin may be caused by a lower reactivity of the latter with B-B4. Alternatively the B-B4-reacting antigen may have a different internalization on these cells. For CD30, differential behavior despite similar expression has been reported on cells of hemopoietic origin (Reed-Sternberg cell lines and erythroleukemic cell line K562) [9].

According to the results with the IT on different cell lines, B-B2-saporin seems to be suitable for in vivo use. The in vivo application of B-B4-saporin may not be recommended because of its toxicity for epithelial cell lines, although in a clonogenic assay B-B4 IT showed no inhibitory effect on outgrowth of A431 cells, whereas outgrowth of plasma cell line RPMI8226 was substantially inhibited by B-B2 IT and completely by B-B4 IT. Also, the accessibility for mAb in vivo differs greatly between different types of tissue; e.g. the accessibility of endothelium is much greater than that of epithelium [14]. In fact, epithelial-cell-reactive mAb have been used in vivo for the targeting of carcinomas, resulting in a much higher tumor uptake than uptake by normal epithelium [11, 12, 34, 45]. As the accessibility of epithelial cells to mAb (and IT) in tissue can only be tested in vivo, the next step would be to study tissue reactivity with radiolabeled B-B2 and B-B4 in radioimmunoscintigraphy. If these studies show an absence of reactivity with organs of epithelial origin, phase I studies with B-B2/B-B4 IT could be considered.

Table 4 Summary of results

Cells	Immuno-histochemistry B-B2/B-B4	Cyto-fluorography B-B2/B-B4	Immunotoxin assay B-B2/B-B4
Plasma cells/lines	+++/+++	++/++	++/+++
$CD34^+$/CFU-GM	ND	–/–	–/–
Endothelial cells	+/+	–/–	–/–
Epithelial cells	++/++	+/+	–/+
Hepatocytes	+/+	+/+	–/–

In conclusion, the in vitro efficacy and toxicity profile of two plasma-cell-reactive mAb, B-B2 and B-B4, indicates that, for toxicity testing, immunohistochemistry has to be combined with testing of cell lines of different origin. Even then testing with IT can give results that differ from those with the mAb alone. Both plasma-cell IT can probably be used for ex vivo marrow purging. However, the conditions for this kind of treatment still have to be determined in more detail. The in vivo application still depends on the accessibility of epithelia. The answer to this question may be given by radioimmunoscintigraphic studies with these mAb.

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