Combined effect of recombinant CD19 × CD16 diabody and thalidomide in a preclinical model of human B cell lymphoma

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Combining different treatment strategies offers the possibility of improving treatment results for cancer patients. The aim of our study was therefore to investigate the combination of treatment of established s.c. human B non-Hodgkin's lymphoma in severe immune deficient mice using a recombinant bispecific CD19 × CD16 diabody (targeting natural killer cells to $CD19^+$ cells) and the angiogenesis inhibitor thalidomide. Monotherapy with either thalidomide or diabody caused an approximate 50% reduction in tumor growth rate. The combined treatment showed evidence for a synergistic effect resulting in a 74% reduction in median tumor size. In the combined treatment group, two of five animals had complete remissions of their s.c. tumor. These results suggest that a combination treatment with recombinant diabodies and angiogenesis inhibition represents a useful approach in cancer therapy. *Anti-Cancer Drugs* 15:915–919 © 2004 Lippincott Williams & Wilkins.

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Introduction

Bispecific antibodies (biAbs) have high therapeutic potential by directing immune effector cells to the tumor [1]. BiAbs derived from quadromas [1] or chemical cross-linking [2] display severe drawbacks that can be circumvented using recombinant biAbs [3]. Recently we reported the successful *in vivo* application of different recombinant biAbs targeting natural killer (NK) cells [4] or T cells [5,6] to human tumors. As neo-vascularization is a crucial process in tumor development, anti-angiogenic approaches have also been successfully used for several years [7]. Thalidomide (*N*-phthalimidoglutarimide), a potent angiogenesis inhibitor [8], exerted anti-tumor activity in some animal models [9]. It has also been shown to be effective as monotherapy or in combination with dexamethasone or dexamethasone plus chemotherapy in multiple myeloma [10,11]. A combination of these two strategies may represent a possible solution in treating 'therapy-resistant' tumors, such as relapsing or refractory B cell lymphomas. Thus, by combining tumor-specific antibody therapy with an anti-angiogenesis compound, both the tumor cell and the supporting blood vessels are attacked [12]. Here, we report the results of *in vivo* treatment with a recombinant CD19 × CD16 diabody for the recruitment of NK cells towards the tumor site followed by systemic treatment with thalidomide.

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Material and methods

Therapeutic reagents

Thalidomide solution was prepared according to the manufacturer's instructions (Grünenthal, Aachen, Germany).

Construction of V_H16–V_L19 and V_H19–V_L16 hybrid single-chain variable fragments (scFvs) genes was performed by exchange of anti-CD3 V_H and V_L genes in plasmids pHOG3-19 and pHOG19-3 [13] for their anti-human CD16 counterparts [4]. Expression vector pKID19x16 was constructed by ligation of the *Bgl*II–*Xba*I restriction fragment from pHOG16-19 comprising vector backbone and the *Bgl*II–*Xba*I fragment from pHOG19-16. CD19 × CD16 diabody was expressed in *Escherichia coli* XL1-Blue (Stratagene, Groningen, The Netherlands) and isolated from periplasma and culture medium by ammonium sulfate precipitation and immobilized metal affinity chromatography (IMAC) [13].

In previous *in vitro* experiments it was demonstrated that the CD19 × CD16 bispecific diabody was able to effectively recruit human NK cells for killing $CD19^+$ lymphoma cells [14].

Cytotoxicity assays

The influence of thalidomide on NK cell cytotoxicity was determined using a standard JAM test [15] and a ^{51}Cr-release assay.

JAM test

The NK cells were negatively enriched from human peripheral blood mononuclear cells [isolated from the blood of healthy donors by Ficoll (Sigma-Aldrich, Taufkirchen, Germany) density gradient centrifugation] by immunomagnetic depletion of human T cells, B cells and myeloid cells using the NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). As the target cell, the CD19-expressing Raji cell line was used. Raji cells were cultured in RPMI 1640 (Invitrogen/GIBCO, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin (both PAA Laboratories, Cölbe, Germany) and 2 mmol/l L-glutamine (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2. For the cytotoxicity assay, thalidomide was used in a concentration of 5 µg/ml. Target cells labeled with [^{3}H]thymidine were incubated with effector cells (E:T ratio = 25:1) for 4 h at 37°C in a 5% CO_2 atmosphere in round-bottom microtiter plates. The cells were harvested and radioactivity was measured with a liquid scintillation β counter (LKB, Wallach, Germany). Cytotoxicity related to the apoptosis-induced DNA fragmentation was calculated as percent specific lysis = $(S-E)/S \times 100$, where E is experimentally retained labeled DNA in the presence of NK cells (in c.p.m.) and S is retained DNA in the absence of NK cells (spontaneous). Each measurement was performed in triplicate.

^{51}Cr-release assay

Additionally, tumor cell lysis by effector cells was quantitated in a 4-h ^{51}Cr-release assay. NK cells (isolated as described above) were incubated for 5 days in complete RPMI and Aldesleukin (300 U/ml, Proleukin; Chiron, München, Germany). Thalidomide (1 µg/ml cell culture medium) dissolved in DMSO was added daily. As control, DMSO alone was added to the cell preparations. Target cells (1×10^6) were labeled with 200 µCi ^{51}Cr (Hartmann Analytic, Braunschweig, Germany) and plated at 1×10^4 cells/well. Effector cells were added at various E:T cell ratios and after 4 h of incubation, the supernatant was removed and radioactivity measured in a γ counter. The percent cytotoxicity was determined as follows: percent specific lysis = [(sample release − spontaneous release)/(maximum release − spontaneous release)] × 100. Maximum release of target cells was measured following treatment with 2% detergent (Triton X-100) (Merck, Darmstadt, Germany).

Cell viability assay

Raji cells were seeded in six-well plates at a density of 5×10^5 cells/well. The cells were treated with thalidomide in various concentrations. Triplicate wells of each treatment group were counted at day 7. The viability of the cells was determined by Trypan blue exclusion.

Preparation of human peripheral blood lymphocytes (PBLs) for animal experiments

Human PBLs were isolated from buffy coats of healthy donors by Ficoll density gradient centrifugation.

Animal experiment

SCID mice (BALB/c background) aged 6–8 weeks old (Charles-River, Sulzfeld, Germany), kept under specific pathogen-free conditions, were irradiated (300 rad) and received i.p. injections of 10 µl anti-asialo-GM_1 antibody (Wako, Neuss, Germany). One day thereafter, 10^7 Raji tumor cells were injected s.c. dorsolaterally. Treatment was started when tumors reached a size of 5 mm in diameter (day 0). At days 0, 3 and 6, mice received i.v. injections of either PBS (control) or 5×10^6 human PBLs. Four hours after PBL injections, 50 µg CD19 × CD16 diabody were injected i.v. Thalidomide was administered i.p. at 120 mg/kg body weight at days 0–6. Animals from the combination group received at days 0, 3 and 6 diabody (plus 5×10^6 PBL) and at days 13–19 thalidomide. As we were preferentially interested to investigate the NK cell-independent mechanism of therapeutic effect of combined treatment we decided to perform sequential administration of thalidomide (as opposed to simultaneous treatment).

Tumor size was measured at least twice weekly and animals were sacrificed when tumors reached diameters greater than 15 mm.

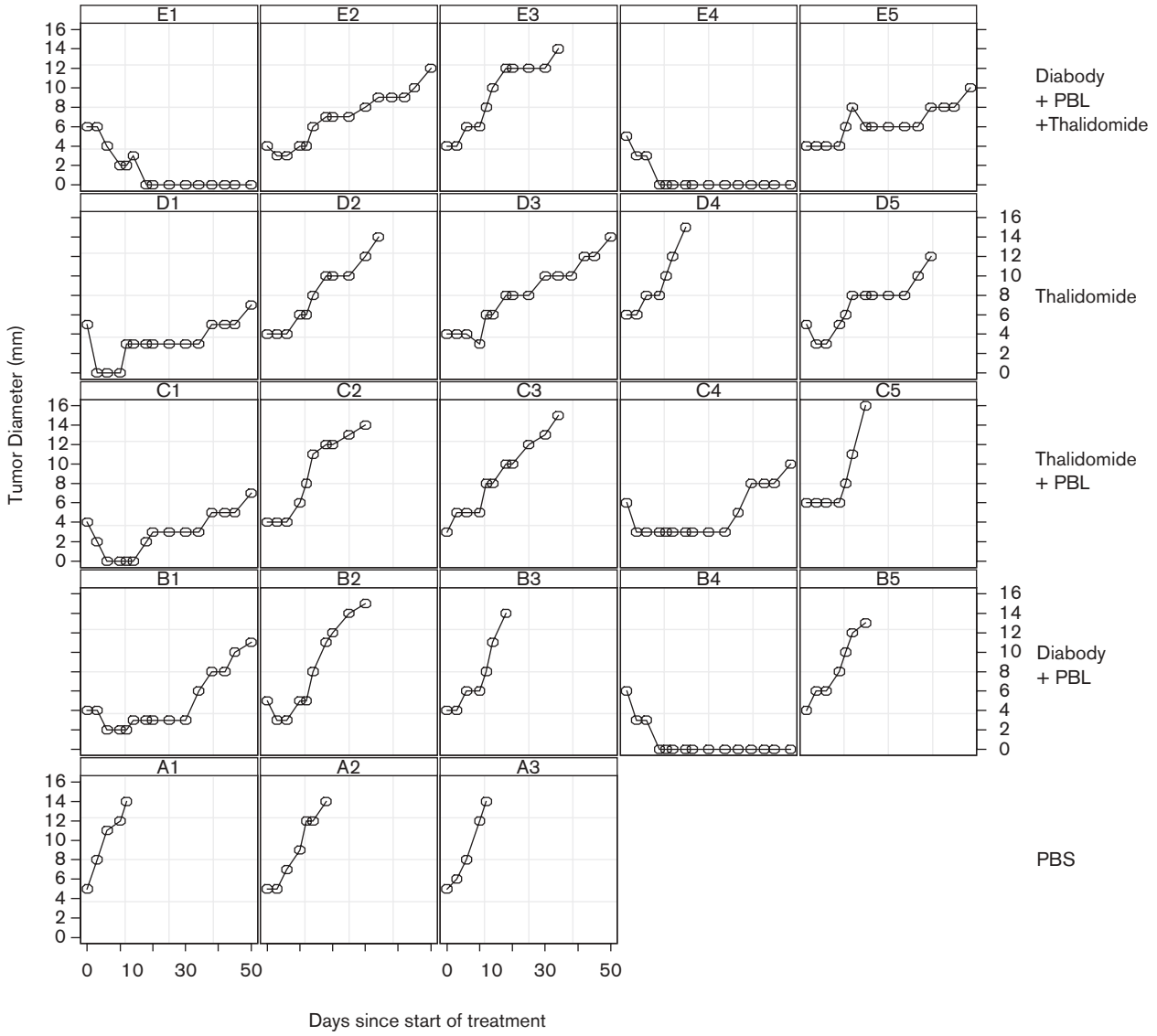
Statistical analysis

Survival time distribution was estimated following Kaplan and Meier [16]. Statistical analysis of tumor progression and treatment effects was done by analysis and comparison of tumor growth rate over time (Fig. 1), and by calculation of predicted median tumor size at days 5, 10, 15, 20 and 25 (Table 1). To compare tumor regression and tumor growth over time, a linear mixed-effects model for time-dependent data was applied [17]. Two-sided tests were performed (Software: S-Plus 3.4; SAS 8.1).

Results and discussion

To reflect the clinical situation treatment was started (day 0) when s.c. human lymphoma tumors (Raji) were established and had reached a diameter of 5 mm. Figure 1 displays growth curves of individual tumors. Table 1

Fig. 1



Treatment of SCID mice bearing human Burkitt's lymphoma xenografts. The following treatment was performed: group A received PBS (day 0, 3 and 6), group B human PBLs followed by the administration of CD19 × CD16 diabody 4 h later (day 0, 3 and 6), group C human PBLs (day 0, 3 and 6) and thalidomide (day 0–6), group D thalidomide (day 0–6), and group E CD19 × CD16 diabody 4 h after PBL injection (day 0, 3 and 6) and thalidomide at days 13–19. Tumor sizes were measured twice weekly. Tumor growth curves of individual animals of each experiment are presented.

shows the estimated median tumor size at days 5, 10, 15, 20 and 25 of all groups.

Tumors of control animals displayed aggressive tumor growth kinetics, reaching a mean diameter of 15 mm within 2 weeks after begin of treatment. Tumor progression (tumor growth curve) in mice treated with thalidomide in the presence or absence of human PBL was significantly delayed ($p = 0.04$). Correspondingly, the reduction in the predicted mean tumor size at day 25 (Table 1) in the thalidomide groups was 53.1% (with PBL) and 54.1% (without PBL).

As expected from previous experience, CD19 × CD16 diabodies caused tumor growth delay with growth kinetics similar to the thalidomide-group. One of five animals receiving CD19 × CD16 diabody displayed tumor regression with complete eradication of tumor. The predicted reduction in mean tumor size (day 25) by the diabody was 53%.

Table 1 Estimated tumor size profiles

Group (*n*)	Estimated median tumor size (mm)				
	Day 5	Day 10	Day 15	Day 20	Day 25
Control (3)	8.0 (6.1, 10.0)	11.4 (8.6, 14.2)	14.8 (10.9, 18.6)	18.1 (13.2, 23.0)	21.5 (15.5, 27.5)
CD19 × CD16 + PBL (5)	4.1 (2.6, 5.5)	5.6 (3.4, 7.7)	7.1 (4.2, 9.9)	8.6 (5.0, 12.2)	10.1 (5.8, 14.4)
Thalidomide + PBL (5)	4.0 (2.6, 5.5)	5.5 (3.4, 7.6)	7.0 (4.1, 9.8)	8.5 (4.9, 12.0)	10.0 (5.7, 14.3)
Thalidomide, no PBL (5)	4.4 (3.0, 5.9)	5.8 (3.6, 7.9)	7.1 (4.2, 9.9)	8.4 (4.8, 12.0)	9.7 (5.4, 14.0)
CD19 × CD16 + PBL + thalidomide (5)	3.9 (2.5, 5.4)	4.3 (2.2, 6.4)	4.7 (1.9, 7.5)	5.1 (1.5, 8.6)	5.5 (1.2, 9.6)

The predicted median tumor size (diameter in mm) at days 5, 10, 15, 20 and 25 after start of therapy for the control group and each treatment is shown using a linear mixed-effects model fit by maximum likelihood [17]. According 95% confidence intervals are specified in brackets.

Analysis of tumor growth rate revealed statistically significant time-dependent (i.e. $p < 0.05$) differences between the combination group and each of the other groups (diabody: $p = 0.04$; thalidomide plus PBL: $p = 0.042$; thalidomide, no PBL: $p = 0.08$; PBS: $p < 0.0001$).

The predicted median tumor size reduction of the combined therapy at day 25 was 74.4% compared to the control group. Remarkably, two of five mice treated with diabody plus thalidomide showed complete tumor regression for the whole period of observation (50 days). The statistical comparison of the tumor growth curves of individual groups based on the predicted tumor size at days 5, 10, 15, 20 and 25 (Table 1) indicates a multiplicative, i.e. synergistic, effect of the CD19 × CD16 diabody plus thalidomide treatment from day 20 on (thalidomide was administered at days 13–19).

We investigated the effect of thalidomide on NK cell activity as a possible mechanism of action in our *in vivo* setting using cytotoxicity assays. Neither in a JAM test nor in a ^{51}Cr-release assay did we observe an increase in the specific NK cell-mediated tumor cell lysis in the presence of thalidomide [JAM test percent specific lysis: 6.87 without thalidomide versus 9.97 with thalidomide 5 μg/ml; ^{51}Cr-release assay percent specific lysis: 44 without thalidomide versus 45 with thalidomide (results from different NK cell preparations)].

This is to our knowledge the first report on the combination of bispecific antibody-based therapy with angiogenesis inhibition in a preclinical model of a human hematologic malignancy. As thalidomide was found to induce significant anti-tumor activity against multiple myeloma, several mechanisms of action of thalidomide *in vivo* are currently under considerations [18]. Interestingly, thalidomide increases activation of NK cells [19]. As NK cell-mediated tumor cell lysis is considered the major mechanism of action for the diabody used in this study, enhancement of diabody pre-activated NK cells deserves attention. However, two observations argue against a direct activation of immune-effector cells by thalidomide as the main mechanism in our model. First, in our model thalidomide exerted significant anti-tumor activity in the absence of any immune cells since we used mouse NK cell-depleted SCID mice without reconstitution with human PBLs in one group of animals. Second, a corresponding group of animals reconstituted with human PBLs did not show significant differences in tumor growth characteristics or survival as compared to the group without human PBLs. In addition, we did not observe an NK cell enhancing activity using two different *in vitro* systems.

Table 2 Cell viability assay

Concentration (μg/ml)	Viability (%)	
	Mean	SD
Thalidomide		
5	79.33	2.05
20	80.33	2.36
DMSO		
5	75.0	3.74
20	71.67	2.49
Medium control	70.0	0.82

Direct anti-tumor cell activity of thalidomide against multiple myeloma cells *in vitro* has previously been shown [20]. This effect was demonstrated only at high concentrations, which are not achievable in patient plasma. At the concentrations of 5 and 20 μg/ml used in the *in vitro* cell viability assay (Table 2), thalidomide failed to inhibit tumor cell proliferation. The therapeutic efficacy of thalidomide in these groups is therefore most likely mediated by a direct effect on the stromal microenvironment, e.g. anti-angiogenic effects. As in other tumor models of immunoincompetent mice [21,22] we found by immunohistochemical analysis active angiogenesis in Raji tumors (data not shown). The anti-angiogenic activity of thalidomide was initially demonstrated in rabbit models, whereas in murine models it is a controversial matter. Anti-angiogenic activity of thalidomide was shown in murine embryoid bodies [23]. D'Amato *et al.* also explored thalidomide in mouse models of corneal angiogenesis: the effect was reproducibly obtained by i.p. application [24] which was also the route of administration in our experiment. Recently, thalidomide has been demonstrated to have anti-angiogenic properties in experimental neuroblastoma [21].

Synergistic therapeutic activity of anti-angiogenic agents with tumor cell-directed antibodies has been shown [12] and is also suggested in our experiment.

Investigation of microvessel density (MVD) in this model is of potential interest, but Folkman and others have pointed out that MVD measurement is not a reliable technique to assess anti-angiogenic effect of therapeutic compounds [25].

Conclusion

We found that thalidomide is therapeutically active in human B-NHL xenografts in mice. Combination therapy using thalidomide and a recombinant bispecific CD19 × CD16 diabody resulted in a 74% reduction in median tumor size.

Our model raises several possibilities for further investigation of the *in vivo* effect of thalidomide on B cell lymphomas, and for the study of the interactions between the immune system and angiogenesis mechanisms in tumors [26].

Clinical trials using thalidomide to enhance therapeutic efficacy of antibody-based therapies in lymphoma patients are encouraged.

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