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Gijsbert C. de Gast · Inez-Anne Haagen · Anja A. van Houten · Sigrid C. Klein
Ashley J. Duits · Roel A. de Weger · Thea M. Vroom · Mike R. Clark · Jenny Phillips
Anette J. G. van Dijk · Wim B. M. de Lau · Bert J. E. G. Bast

CD8 T cell activation after intravenous administration of CD3 × CD19 bispecific antibody in patients with non-Hodgkin lymphoma

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Abstract A bispecific antibody directed to T and B cells (CD3 × CD19 bsAb) was daily infused intravenously in escalating doses from 10 μg up to 5 mg in three patients with chemotherapy-resistant non-Hodgkin lymphoma; in this way we aimed to activate T cells to kill the malignant B cells. Only limited toxicity was observed, consisting of moderate fever preceded by chills or shivers and mild thrombocytopenia. No human anti-(mouse Ig) antibodies were found. Pharmacokinetics showed a $t_{1/2}$ of 10.5 h with peak levels of 200–300 ng/ml after infusion of 2.5 mg bsAb. bsAb in serum was functionally active in vitro. After bsAb infusion a rise in serum tumour necrosis factor α was observed, accompanied by an increase in soluble CD8 and to some extent in soluble interleukin-2 receptor (IL-2R), but not in interferon γ, IL-4 or soluble CD4. No evidence was found for monocyte activation (no increases in IL-6, IL-8 or IL-1β in serum). No gross changes in histology or number of IL-2R$^+$, CD4$^+$ or CD8$^+$ cells were found in the lymph nodes after therapy, but one patient showed activated CD8$^+$ T cells within the tumour nodules. In conclusion, after intravenously administered CD3 × CD19 bsAb only moderate toxicity was found, probably due to CD8$^+$ T cell activation and cytokine release, without CD4$^+$ T cell activation.

G. C. de Gast · I.-A. Haagen · A. J. Duits · A. J. G. van Dijk
W. B. M. de Lau · B. J. E. G. Bast
Department of Immunology, University Hospital Utrecht, The Netherlands

G. C. de Gast (✉) · A. A. van Houten
Department of Haematology, University Hospital Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands

S. C. Klein · R. A. de Weger · T. M. Vroom
Department of Pathology, University Hospital Utrecht, The Netherlands

M. R. Clark · J. Phillips
Department of Pathology, University of Cambridge, UK

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Introduction

Intensification of chemotherapy as a primary treatment for non-Hodgkin lymphoma (NHL) has not led to a better disease-free survival [1]. These results have stimulated the search for alternative strategies like immunotherapy. An interesting modality is the application of a bispecific antibody (bsAb) with one specificity for the CD3 antigen of T cells and the other for a tumour-associated antigen. Such bsAb can retarget T cells to the tumour, regardless of their original specificity, and locally activate effector T cells, resulting in cytokine release and cytotoxicity [2]. bsAb therapy in patients with solid tumours was recently reviewed [3]. In such patients bsAb treatment was mainly given locoregionally after ex vivo activation and loading of T cells with bsAb, sometimes with favourable results [4, 5]. Owing to the lack of real tumour-specific antibodies systemic application of bsAb seems to be hazardous in these patients [6].

In contrast to the situation in solid tumours, several monoclonal antibodies specific for B cells are available, making in vivo application of intravenous bsAb in patients with B cell tumours possible and less dangerous. In this respect the CD19 mAb is suitable. The CD19 antigen is expressed in the whole B lineage from the pro-B cell to the plasma cell, it is not shed, is uniformly expressed on all tumour cells and is absent from stem cells [7]. A potential disadvantage is that normal B cells may also be killed by CD3 × CD19 bsAb treatment. However, B cells will be rapidly replaced by differentiation from stem cells; moreover, antibody/immunoglobulin levels will not be seriously affected as plasma cells lack CD19.

In vitro, CD3 × CD19 bsAb has been shown to induce killing of malignant B cells by T cell clones or

activated peripheral blood T cells [8]. Even peripheral blood T cells from patients with final-stage leukaemia/lymphoma have shown the capacity to kill autologous tumour B cells in the presence of CD3 × CD19 bsAb [9]. Unprimed $CD4^+$ and $CD8^+$ T cells could be rapidly activated to proliferate and become cytotoxic by a CD3 × CD19 bsAb [10]. In vivo animal experiments with several mouse lymphoma models have shown that intraperitoneally seeded lymphoma B cells were killed after intravenous administration of CD3 × antitumour (anti-idiotype) bsAb [11–13].

Here we show the first clinical results of intravenous administration of CD3 × CD19 bsAb in patients with NHL.

Patients and methods

Patients

Eligible patients, 18–70 years old, had chemotherapy-resistant $CD19^+$ B lineage malignancy, WHO performance status 0, 1 or 2, no other severe disease and a serum creatinine, blood urea nitrogen and bilirubin below 2.5 × the upper limit of normal. Prior intravenous chemotherapy within 3 weeks of study was not allowed. The protocol was approved by the Local Institute's Ethical Committee. Three patients with negative skin tests to CD3 × CD19 bsAb fulfilled the entry criteria and gave informed consent. The characteristics of these patients are given in Table 1.

Bispecific antibody

The bispecific antibody used in this study is the complete CD3 × CD19 bsAb, SHR-1, produced by the somatic hybrid of the parental hybridomas, YTH12.5 and MG1CD19. YTH12.5 is a rat IgG2b-mAb-secreting hybridoma with a specificity for human CD3-ε, MG1CD19 is a mouse IgG1-mAb-secreting hybridoma, specific for human CD19 antigen [14]. The development of SHR-1 has previously been described [15]. After testing the bsAb for the absence of endotoxin, virus contamination and functional activity, SHR-1 was approved for a phase I study by the appropriate committee of the Dutch Government.

Design of clinical study

Pretreatment screening included history, physical examination, complete blood count and differential count, extensive chemistry, urinalysis, electrocardiography, chest X-ray, computed tomography scanning and marrow examination. The patient was hospitalized and a technetium colloid scan was performed as well as a lymph node biopsy. A peripheral catheter was inserted and escalating doses of bsAb were infused intravenously in 0.5 h: 10 μg on day 1, 100 μg day 2, 400 μg days 3 and 4, 1.0 mg days 5 and 6, 2.5 mg days 7 and 8 and 5.0 mg day 9, giving a total dose of 12.9 mg. During and for 3 h after the infusion, blood pressure, temperature and pulse were monitored. Daily blood samples were taken for blood counts, and a chemistry profile was obtained three times a week; other tests and investigations were carried out according to the judgement of the investigator. After the last dose of bsAb on day 9, a technetium colloid scan and a lymph node biopsy were again performed. On day 10 the patient was discharged and attended a weekly follow-up in the outpatient department, regular blood samples being taken until day 35.

Immunological monitoring

The monitoring was designed to document B cell elimination and T cell activation by surface markers and cytokine release, as well as to test for monocyte/macrophage activation or elimination.

Immunophenotyping

Surface antigens on peripheral blood mononuclear cells were detected by immunofluorescence staining and analysed on a FACScan cell sorter (Becton Dickinson, San Jose, Calif.). If non-conjugated antibodies were used, a second step was introduced with conjugated

Table 1 Patient characteristics. *LG* lowgrade, *IG* intermediate grade, *NHL* non-Hodgkin lymphoma, *CB-CC* centroblastic centrocytic, *COP* cyclophosphomide/vincristine/prednisone, *CHOP* cyclophosphamide/doxorubicin/vincristine/prednisone, *CNOP* cyclophosphamide/mitoxantrone/vincristine/prednisone, *ProMACE-MOPP* procarbazine/methotrexate/doxorubicin/cyclophosphamide/etoposide (*VP16*) chlormethine/vincristine/procarbazine/prednisone

	Patients		
Characteristic	1	2	3
Sex/age (years)	F/61	M/64	M/42
Performance status	2	1	1
Diagnosis	LG-NHL Follicular CB-CC	IG-NHL Diffuse CB-CC	LG-NHL Follicular CB-CC
Stage	IV-B	IV	IV-B
Leukaemic	+	−	−
BM involvement	+	+	+
Lymph node mass	Abdominal	Cervical, abdominal, axillary	Inguinal, iliac
Lymph oedema	+ + +	−	+ +
Splenomegaly	−	−	+ +
Hepatomegaly	−	−	+
Previous treatment	Chlorambucil COP/CHOP radiotherapy dexamethasone	CNOP ProMACE-MOPP VP16/prednisolone	COP, doxorubicin/VP16 fludarabine
Interval since diagnosis	16 years	35 months	30 months

antibodies of the goat anti-(mouse Ig) subclass (SBA, Birmingham Ala.). The following markers were used: CD3/DR, CD3/CD16 + CD56, CD4/DR, CD8/DR, CD14/CD45, CD5, CD7, CD10, CD20, CD22 (Becton Dickinson), CD15, CD19 (Dakopatts, Glostrup, Denmark).

Cytokine assays

Cytokine levels were determined in plasma derived from EDTA-treated blood kept directly on ice after collection and promptly centrifuged and frozen at − 20 °C, according to the manufacturer's instructions. The following cytokines were assayed by enzyme-linked immunosorbent assay (ELISA): tumour necrosis factor α (TNFα), interferon γ (IFNγ), interleukin-1β (IL-1β) (Medgenix, Fleurus, Belgium), IL-4, IL-6, IL-8 (CLB, Amsterdam, the Netherlands), soluble interleukin-2 receptor (sIL-2R; Eurogenetics, Tessenderlo, Belgium), soluble CD8 (sCD8) and sCD4 (T cell Diagnostics, Cambridge, Mass.). The measurable threshold levels were respectively 10 pg/ml for TNFα, 10 U/ml for INFγ, 10 pg/ml for IL-1β, 10 pg/ml for IL-4, 1 pg/ml for IL-6, 10 pg/ml for IL-8, 100 U/ml for sIL-2R, 100 U/ml for sCD8 and 20 U/ml for sCD4. At least two samples were taken on different days before the start of the trial.

Ig levels

Serum IgM, IgG and IgA levels were determined by nephelometry in an array protein system (Beckman).

HAMA

Human anti-(mouse Ig) antibodies (HAMA) were measured by ELISA (Immunomedics, Warren, N.J.; the assay sensitivity is 40 ng/ml).

Pharmacokinetic study

This was performed on day 8 after infusion of 2.5 mg bsAb. Free serum bsAb was measured in regularly collected blood samples in an ELISA in microtitre plates coated with goat anti-(mouse IgG1) (GAM-IgG1) and, as a second step, serial dilutions of sera. Bound SHR-1 was then detected by adding GAM-Ig coupled to alkaline phosphatase. Results are given in nanograms per millilitre. Functional activity was measured in a conventional chromium-release assay in microtitre plates [8]; briefly, 100 µl effector cells (CTL-D11/TCRαβ) were mixed with 50 µl serial dilutions of purified bsAb or serum samples. After 30 min, chromium-labelled target cells were added to each well and the plates were incubated for 3 h at 37 °C. Subsequently supernatants were removed and assayed for release of chromium. Functional activity of the serum samples was deduced in comparison with the data obtained with purified bsAb.

Histochemistry

Cryostat sections (6 µm) of frozen lymph nodes were used for immunohistochemistry. Tissue sections were incubated with an appropriate dilution of the monoclonal antibodies directed to CD2, CD3, CD4, CD5, CD8, CD19, CD21, CD25, CD56, CD57 and CD68 (Becton Dickinson) and CD20, CD22 and CD45 (Dakopatts) and stained according to a three-step immuno-alkaline-phosphatase method [16]. The expression of perforin was tested with a rat anti-(mouse perforin) (clone AL27; a kind gift of Dr. Tschopp, Lausanne, Switzerland), detected by a biotinylated sheep anti-(rat Ig), followed by streptavidin-peroxidase and stained with diaminobenzidine and H_2O_2 in the presence of $Ni(NH_4)_2SO_4$.

Results

Three patients satisfied the entry criteria and gave informed consent. They completed the trial and were followed-up for at least 5 months.

Toxicity

Toxicity was limited; the only grade 2 toxicity consisted of chills or shivers after 1–2 h, followed by fever after 2–5 h in all three patients (Table 2). There was no relationship between the severity of the reaction and the dose administered: shivers/chills and fever did occur with 100 µg and with 5.0 mg bsAb i.v. to the same extent and nearly at the same time after infusion. Interestingly the patient with leukaemic lymphoma (patient 1) had shivers after 1 h, followed 1 h later by fever, whereas the other two patients, who were not leukaemic, had chills after 2 h and fever several hours later. Patient 1 showed a major urinary-tract infection on day 6, which reacted rapidly to antibiotics; it was probably not related to the bsAb treatment as a similar episode had occurred 2 months earlier due to hydronephrosis caused by enlarged abdominal lymph nodes obstructing the ureter.

None of the patients showed a decrease in uptake of technetium colloid by the liver or spleen, as measured after treatment. Patient 3 showed an unchanged uptake but decreased spleen size, probably due to a decrease in malignant infiltration. The consistent toxicity was mild (grade 1) thrombocytopenia without granulocytopenia. No organ toxicity was seen. No HAMA was found up to 35 days after the infusion.

Table 2 Toxicity profile

Toxicity	Presence	Grade
Fever, chills/shivers	3/3	2
Haematological		
Thrombocytopenia	3/3	1
Granulocytopenia	0/3	
Macrophage elimination	0/3	
Major (urinary) infection	1/3	3[a]
Renal function impairment	1/3	1[a]
Liver function impairment	1/3	1
Hypotension	1/3	1
Erythema, eosinophilia	1/3	1
Other organ toxicity	0/3	
Human anti-(mouse Ig) antibodies	0/3	

[a]Probably not related to bispecific antibody therapy

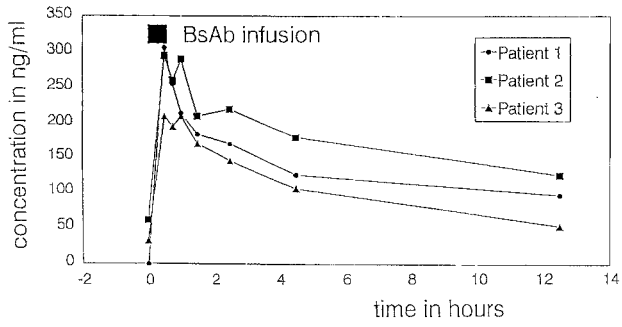

Fig. 1 Results of pharmacokinetic studies on day 8 after infusion of 2.5 mg bispecific antibody (*BsAb*). Peak levels of bispecific antibody of 200–300 ng/ml were measured in the serum directly after infusion. Levels of 50–100 ng/ml were measured for 12 h after an initial rapid decrease

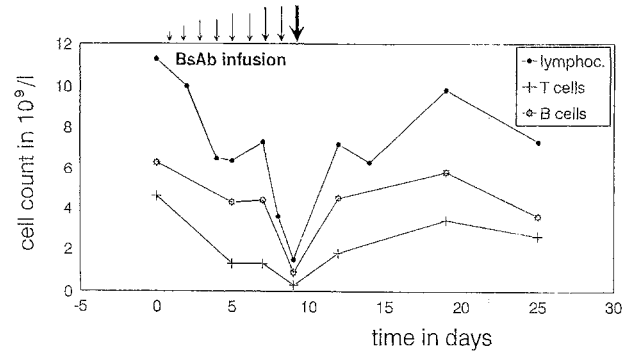

Fig. 2 Effect of bispecific antibody (*BsAb*) infusion on T and leukaemic B cells in blood of patient 1 with leukaemic lymphoma. T and B cells disappeared during the therapy, but returned rapidly after it stopped

Pharmacokinetics

Peak levels of 200–300 ng/ml were noted directly after infusion of 2.5 mg bsAb (Fig. 1). bsAb levels of 50–100 ng/ml were registered for a period of at least 12 h. The calculated $t_{1/2}$ was 10.5 h (6.5, 11.5 and 13 h). bsAb isolated from the blood appeared to be functional in the cytotoxicity assay (data not shown).

Immunological effects

Profound lymphocytopenia did occur in all three patients. Values before daily administration of bsAb in the leukaemic patient are shown in Fig. 2; patient 1 had about 60% (malignant) B cells and 40% normal T cells in the circulation, which both disappeared gradually. This was noted for all T cell markers (CD3, CD4, CD5, CD7, CD8) as well as B cell markers (CD19, CD20, CD22), thus excluding a masking of these markers by the CD3 × CD19 bsAb. Every day, directly after administration of bsAb, T and B lymphocytes and monocytes disappeared from the blood but reappeared, to some extent, later on the same day (data not shown). Cytokine release, as measured in the serum, is shown in Table 3. Moderate TNFα increases were present in all three patients on day 8 (Fig. 3). It was accompanied by elevations of sCD8, but not of IFNγ, IL-4 or sCD4 (Table 3). High levels of sIL-2R and sCD8 were found in pretreatment samples from patients 1 and 3 with low-grade lymphoma, which increased further during treatment. Levels of sCD4 were low or absent before treatment and increased only marginally. An increase in IL-6 and IL-8 was only found in patient 1 on the day of a major urinary tract infection (day 6). Pretreatment cytokine levels in each patient were constant.

Immunoglobulin levels showed minor decreases during bsAb treatment with a rise to pretreatment levels after the treatment was stopped (data not shown).

Table 3 Cytokine patterns in serum. Results show values before/maximal values during bispecific antibody therapy. *ND* not detectable, *ELISA* enzyme-linked immunosorbent assay, *IFN* interferon, *IL* interleukin, *TNF* tumour necrosis factor, *sCD4* soluble CD4

Cytokine (ELISA)	Threshold levels	Patient		
		1	2	3
IFNγ (U/ml)	10	ND/ND	ND/ND	ND/ND
IL-4 (pg/ml)	10	ND/ND	ND/ND	ND/ND
sIL-2R (U/ml)	100	1450/2750	ND/ND	1122/1750
TNFα (pg/ml)	10	65/250[a], 210	ND/580	145/310
sCD4 (U/ml)	20	ND/31	ND/32	38/40
sCD8 (U/ml)	100	1840/3663[b]	190/2400	944/1934
IL-6 (pg/ml)	1	2/249[a], 5	ND/16	ND/ND
IL-8 (pg/ml)	10	ND/248[a], 14	ND/ND	ND/ND
IL-1β (pg/ml)	10	ND/ND	ND/ND	ND/ND

[a]Measured on day 6 during major urinary-tract infection
[b]Measured on day 8 because of the urinary-tract infection

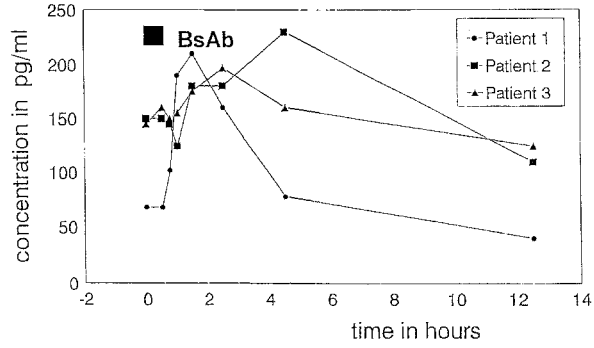

Fig. 3 Tumour necrosis factor α (INFα) levels in serum on day 8 of pharmacokinetics. TNFα levels increased rapidly after bispecific antibody infusion (patient 1) or slowly (patients 2 and 3) to levels of 200–250 pg/ml

Lymph nodes before and after bsAb treatment contained $CD19^+$, $CD20^+$, $CD22^+$ and monoclonal sIg^+ malignant cells in nodular or follicular structures. In between these nodular structures $CD3^+$, $CD4^+$ or $CD8^+$ T cells were found. This distribution did not

change after treatment, nor did the expression of $CD25^+$ and $CD68^+$ cells. In none of the nodes after treatment mouse Ig could be detected by immunohistochemistry. In patient 1 a lymph node decreased in size during treatment; here the tumour nodules showed a less dense structure and $CD8^+$, $CD25^+$ and $CD68^+$ cells were present within those nodules, whereas they were absent in the lymph node before therapy. Similar changes were not observed in lymph nodes of the two other patients. Perforin expression was only weakly positive in patient 1 after therapy.

Clinical effects were limited. In the two patients with low-grade NHL (patients 1 and 3) a transient decrease in lymph oedema was found and a transient decrease of splenomegaly in patient 3. A progression-free survival of 6 months was noted in patient 1 until a cerebral hemorrhage occurred, from which she died. Patient 3 was progression-free for 2 months and then received palliative radiotherapy. In patient 2 with intermediate-grade NHL progression did occur during the bsAb treatment necessitating additional oral chemotherapy. He died 3 months later from his NHL.

Discussion

In this study with intrapatient escalation of intravenously administered bispecific antibodies (CD3 × CD19 bsAb), only limited toxicity was observed, consisting of moderate (grade 2 WHO) fever and chills or shivers. The toxicity was not related to the dose administered. The only other consistent toxicity observed was mild thrombocytopenia. No HAMA response was found, as seen in a previous study of escalating doses of CD19 mAb in NHL patients [17]. On the basis of the high toxicity seen in an earlier study using CD3 × Mov18 bsAb [6], the low toxicity found in our study was not anticipated. In our planned dose range (highest dose 5 mg, total dose 12.9 mg) a maximum tolerated dose was not reached and dose-limiting toxicity could not be determined. The optimal biological dose, therefore, has to be determined by pharmaco-kinetics and immunological activity of the bsAb.

The pharmacokinetic study with 2.5 mg bsAb showed blood levels of 50–100 ng/ml for about 10–12 h after the initial peak levels and a $t_{1/2}$ of 10.5 h with individual variation (6.5–13 h). Levels of 10–100 ng/ml had been shown in vitro to induce cytotoxicity effectively [8, 9]. On the basis of these data, a dose of 1 mg bsAb given once or twice a day should be effective. bsAb in serum obtained from the patients after i.v. administration appeared to be functional in vitro, as measured in the cytotoxicity assay. No bsAb could be demonstrated by immunohistology in the lymph node, probably due to the low amount of bsAb infused and the large tumour load. In another study with high doses of CD19 mAb (up to 1000 mg total dose), CD19 mAb could indeed be demonstrated in the nodes [17].

An important goal of the study was to document any sign of T cell activation by the CD3 × CD19 bsAb. A first indication was the rapid, although transient, disappearance of T cells from the blood after each bsAb infusion. Better evidence was the occurrence of shivers or chills and fever, accompanied by TNFα release and increases in sCD8, indicating T cell activation [18]. In the two patients with low-grade NHL (patients 1 and 3) high levels of sIL-2R and sCD8 were found even before the study, probably because of pre-existing $CD8^+$ T cell activation. Both were increased further by bsAb therapy. Remarkably no increases in IL-4 and IFNγ and a minimal increase in sCD4 were found in two patients, suggesting that only $CD8^+$ T cells already present in the lymph nodes were further activated and $CD4^+$ T cells were not triggered. Soluble forms of lymphoid cell surface molecules have been shown to exist and release may occur by different mechanisms. These include (a) alternative splicing of mRNA resulting in a secretory protein lacking a transmembrane domain, as demonstrated for sCD8 [19]; (b) proteolytic cleavage at the cell surface as suspected for sIL-2R [20]; (c) cleavage of phosphatidylinositol linkages, as found for sCD16 [21]. The mechanism of sCD4 release is not known. Increases in sCD4 and sIL-2R have been reported in patients with viral infections and autoimmunity [22–24], but not in patients with tumours. In the last-mentioned category, including patients with haematological malignancies [25], increases in sCD8 have been reported, as were found in our patients. The presence of weakly perforin-positive T cells in the lymph node of only one patient suggests that the $CD8^+$ T cell activation did not result in adequate differentiation into cytotoxic cells. It was unclear whether circulating T cells were targeted to the tumour. If this is true only B cell malignancies that are naturally infiltrated with T cells may respond to CD3 × CD19 bsAb therapy alone.

TNFα increases were seen in all three patients and were probably due to T cell activation, as no simultaneous increase in IL-6, IL-8 and IL-1 was found, which would be the case if it were due to monocyte activation [26 27]. This point is illustrated in the first patient, where an increase in TNFα, IL-6 and IL-8 was found during the major urinary-tract infection, accompanied by chills and fever. Production of TNFα by activated NK cells [28] seems less likely, as NK cells lack the CD3 antigen. According to the technetium colloid scans no complete elimination of macrophages occurred.

Alterations in the target organ, the lymph node, are of special interest. In patient 1, CD8, CD25 and weakly perforin-positive T cells, as detected by immunohistochemistry, appeared in the tumour nodules. In the other patients we found no clear increase in activated ($CD25^+$) T cells or altered CD4/CD8 ratio. The malignant B cells showed a normal expression of CD19 next

to the other B cell markers CD20 and CD22, so no CD19-negative population was selected.

Despite the phase I character of the study, the possible efficacy was determined. It was limited in our study to a transient decrease in lymph oedema and in splenomegaly. Both an in vitro clonogenic assay [29] and animal experiments indicate that T cell activation by CD3 × antitumor bsAb alone is rather limited. This may be due to the fact that CD3 cross-linking alone induces anergy, and consistent T cell activation requires a second signal [30]. The major costimulation pathway seems to be given by the B7 system [31], which may be lacking on the tumour cell. In order to deliver such a second signal, CD28 mAb [32] CD28 × antitumour bsAb [33, 34], or IL-2 may be added to the bsAb. The addition of IL-2 did make bsAb therapy 100–1000 times more effective in a clonogenic assay [29], as well as in a mouse lymphoma model [35]. Moreover, in a mouse model with liver metastases using low-dose CD3 mAb and IL-2, it was found that only the combined administration resulted in perforin-positive T cells in the liver [36]. Indeed the second signal is also important for the generation of effector cells [31]. In a recent study in renal carcinoma patients intravenously applied bispecific antibodies combined with subcutaneous IL-2 resulted in TNFα and IFNγ release, showing good T cell activation [37]. Based on these data a phase I/II study of CD3 × CD19 bsAb, combined with low-dose subcutaneous IL-2, is now planned in NHL patients.

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