Cancer Immunol Immunother (2000) 49:173–180 © Springer-Verlag 2000

ORIGINAL ARTICLE

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Initiation of humoral and cellular immune responses in patients with refractory Hodgkin's disease by treatment with an anti-CD16/CD30 bispecific antibody

Received: 16 September 1999 / Accepted: 6 January 2000

Abstract Fifteen patients with refractory Hodgkin's disease were treated in a dose-escalation trial with the bispecific monoclonal antibody (bi-mAb) HRS-3/A9, which is directed against the Fcγ receptor III (CD16 antigen) and the Hodgkin's-associated CD30 antigen. Treatment consisted of four cycles of four bi-mAb infusions given over 1 h every 3–4 days at different dose levels ranging from 1 mg/m^2 to 64 mg/m^2. Measurable serum levels (above 0.1 μg/ml) of circulating bi-mAb could be detected in patients treated with doses above 4 mg/m^2, reaching peak levels of 9.5 μg/ml immediately after the end of antibody infusion on the highest dose level. Bi-mAb elimination corresponded to second-order kinetics with a terminal half-life time ($t_{1/2,\beta}$) of 28–32 h. Bi-mAb treatment induced the occurrence of human anti-(mouse Ig) antibodies (HAMA) in 6 out of 13 patients initially testing negative. All 6 patients not only developed anti-isotypic anti-(mouse Ig) but also anti-idiotypic and anti-anti-idiotypic antibodies. While no consistent changes of peripheral blood cell counts, or of any lymphocyte subpopulation including natural killer (NK) cells, has been observed, 4 out of 6 evaluable patients treated with doses of at least 4 mg/m^2 showed an increase of NK cell activity within 2 weeks after treatment, which lasted for a maximum of 12 weeks. Circulating amounts of soluble CD30 antigen could be detected in the serum of 6 patients. However, like the results and time courses of all the other immunological parameters evaluated, this was not predictive for treatment outcome.

Key words Immunotherapy · Monoclonal antibodies · Hodgkin's lymphoma

Introduction

Among many immunotherapeutic approaches to malignant diseases, monoclonal antibodies (mAb) were one of the first to have their efficacy confirmed in clinical trials. The success of treating colorectal cancers [26] in an adjuvant setting, or relapsed low-grade lymphoma [17] with antibodies, demonstrates the clinical potential of therapeutic strategies that redirect humoral and cellular effector functions to tumor cells. The major handicap of native monoclonal antibodies is their variable and usually low cytotoxic potential. Therefore, concepts have been developed that take advantage of the high tumor specificity of mAb for the specific delivery of cytotoxic drugs, enzymes or radioisotopes [7, 27]. In addition, antibodies have been generated that are able to redirect and activate potential cytotoxic effector cells to the tumor site [31]. This concept of bispecific antibodies (bi-mAb) has proven its efficacy in multiple tumor systems in vitro and in preclinical studies with animal models. Immunological effector cells that can be activated by such bi-mAb include granulocytes, macrophages, natural killer (NK), and T cells [9, 11, 29]. In contrast to T cells, NK cells are genuine killer cells with pre-stored cytoplasmic granula that contain cytotoxic molecules such as perforin and granzymes [10]. These molecules are rapidly released when appropriate trigger receptors, expressed on the membrane of NK cells (e.g. CD16), are crosslinked by an antibody [21]. The advantage of bi-mAb is their ability to bridge tumor and effector cells together and induce local cell destruction. Although this concept has been known for many years and the number of papers on this topic is quite high, there are only two reports of clinical trials with

Supported by grants PF135/3-1 of the Deutsche Forschungsgemeinschaft and W5/93/PF-3 of the Deutsche Krebshilfe/Dr. Mildred-Scheel-Stiftung to M.P.C.R. and F.H. contributed equally to this work

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NK-cell-activating bi-mAb [13, 34]. This is mostly because of problems inherent to the production of sufficient amounts of antibody of clinical-grade quality.

In our study [13, 25], we evaluated an NK-cell-activating CD16/CD30 bi-mAb (HRS-3/A9) in patients with Hodgkin's disease refractory to (high-dose) chemotherapy and radiotherapy. In vitro and in vivo experiments using Hodgkin's-derived tumor cell lines heterotransplanted into SCID (severe combined immunodeficiency) mice demonstrated that the CD16/CD30 bi-mAb was very effective in redirecting human NK-cell-mediated lysis to $CD30^+$ Hodgkin's cells [15]. Despite the fact that the patients who were entered into the subsequent phase I/II study presented with absolute lymphocyte numbers characteristic for end-stage Hodgkin's patients, 30% of the heavily pretreated patients responded to therapy, and remissions lasting up to 15 months were observed [13]. Repeated treatment of responding patients was not possible as all patients showed allergic reactions when a second bi-mAb application was tried 4 weeks after the initial treatment.

To gain further insights into possible immunological effects induced by the treatment with CD16/CD30 bi-mAb, we analyzed in more detail the pharmakokinetics of the antibody and the generation of human anti-(mouse Ig) antibodies (HAMA). In addition, we performed NK cell assays with frozen peripheral blood lymphocytes to test NK cell activity at different times during and after treatment. As nothing is known about the evolution of NK cell activity during and after treatment with NK-cell-activating bi-mAb, a careful characterization of these parameters could suggest important improvements of the infusion regimen, which should ultimately lead to an increased treatment efficacy with higher and longer-lasting remissions.

Patients and methods

Patient and treatment schedule

The patient characteristics have been described extensively before [13]. Altogether, 15 patients with progressive Hodgkin's disease, who were refractory to standard treatment procedures, entered the phase I/II clinical trial and were eligible for analysis. All patients gave written informed consent. Patients with negative delayed-type hypersensitivity (DTH) after bi-mAb exposure were treated with four HRS-3/A9 infusions given intravenously in 250 ml 5% human albumin solution over 1 h every 3–4 h. The starting dose was 1 mg/m^2 for each infusion. Two patients were treated at each dose level, and the dose was doubled for the next 2 patients if no side-effects of CTC grade 3 or 4 were observed until the maximum dose (because of the limited amount of bi-mAb available) of 64 mg/m^2 was reached.

Cell lines and fusion proteins

The $CD30^+$ human Hodgkin's-derived cell lines (L540CY and HDLM2), lymphoblastoid cell lines (Daudi, Raji) and the erythroleukemic K562 cell line have been described elsewhere [5, 22]. All cell lines were cultured in RPMI-1640 medium (Gibco, Karlsruhe, Germany), supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, streptomycin (100 mg/ml) and penicillin (100 IU/ml). A fusion protein (CD30-Ig), consisting of the extracellular CD30 domain and a murine IgG1 Fc fragment, was generated in our laboratory [22]. The cell lysate from CD30-positive (L540CY) and negative cell lines (HPB-ALL) was extracted as previously described [12].

Antibodies

The generation, purification and characterization of the parental anti-CD30 (HRS-3) and anti-CD16 (A9) antibodies and of the bispecific anti-CD16/CD30 antibody have been described previously [15]. For the clinical study, the bispecific antibody had been produced under conditions of good manufacturing practice by Biotest Pharma GmbH (Dreieich, Germany) and contained more than 95% intact murine IgG1 bi-mAb. The mAb used in this study for fluorescence-activated cell sorting (FACScan) analysis were phycoerythrin(PE)-labeled anti-CD3, fluorescence-isothiocyanate(FITC)-labeled anti-CD14, anti-HLA-DR-PE, anti-CD16-FITC, anti-CD19-PE and FITC-labeled goat anti-(mouse Ig) serum (Becton Dickinson, Heidelberg, Germany). Polyclonical goat anti-(mouse Ig) serum (unconjugated and peroxidase (POX) conjugated) and polyclonal biotinylated goat anti-[human $F(ab)_2$] were all purchased from Dianova (Hamburg, Germany).

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated by Ficoll-Hypaque (Pharmacia, Sweden) density gradient centrifugation as described before [22]. Macrophages were removed by plastic adherence (37 °C, 1 h)

Blood count analysis and FACScan analysis

Blood counts, with differentiation (Coulter STKS) and analysis of circulating lymphocyte subsets (FACScan, Becton Dickinson), were performed before and after the end of the bi-mAb infusion as well as 1, 3, 6, 24, 48, 72 h thereafter. FACScan analysis with directly labeled antibodies or FITC-conjugated goat anti-(mouse Ig) serum was performed as previously described [13, 22].

Determination of antibody levels and the induction of anti-idiotypic antibodies

Levels of circulating bispecific antibody were assessed by a sandwich enzyme-linked immunosorbent assay (ELISA). In brief, ELISA plates were pre-coated with goat anti-(mouse Ig) serum (dilution 1:2000, 4 °C, overnight) blocked with 1.5% gelatin in TRIS-buffered saline and the patient's serum was added in serial dilutions. A purified murine antibody of the IgG1κ subclass was used as standard. Binding of bispecific antibodies was detected by a peroxidase-labeled goat anti-(mouse Ig) serum (dilution 1:5000, room temperature, 1 h). Human anti-(mouse Ig) antibody (HAMA) response to HRS-3/A9 was assayed as described previously [13] with minor modifications. Briefly, ELISA plates were coated with bi-mAb HRS-3/A9 (2 μg/ml, 50 μl/well) overnight at 4 °C; non-specific binding was blocked by 1.5% gelatin (w/v) in phosphate-buffered saline at room temperature; dilutions of patients' sera were incubated for 1 h at room temperature; after washing, 50 μl/well of a 1:1000 diluted biotinylated goat anti-[human-$F(ab')_2$] was added as the secondary antibody for 1 h at room temperature. After extensive washing, a 1:50 000 dilution of alkaline-phosphatase-conjugated streptavidin (Boehringer Mannheim, Germany) was incubated for 15 min at room temperature (100 μl/well) and, after additional washing, the reaction product was developed with nitrophenylphosphate (Sigma, Munich, Germany) as substrate, stopped with HCl, and read at 405 nm on an ELISA reader (Dynatech MR 4000). All patients' sera were pre-incubated (1 h, 37 °C) with 5 μg/ml irrelevant IgG1 control antibody [28] to absorb anti-isotypic antibodies.

To distinguish between the different variants of anti-idiotypic antibodies (Ab2), standard ELISA experiments were performed as described [19]. Ab2γ and Ab2β were detected by measuring the ability of a patient's serum to block the binding of the CD30-antigen-specific, biotinylated HRS-3 antibody (HRS-3b, 0.5 μg/ml) to CD30-positive cell lysate (diluted 1:50). Serum and HRS-3b were pre-incubated (1 h, 37 °C) before being added to the cell lysate. Binding was detected as described above. An HRS-3-specific mAb2β (9G10) was used as positive control. The anti-idiotypic antibody (16H5) with specificity for the IRAC antibody [28] served as negative control in all experiments. Human Ab3 with specificity for the CD30 antigen were determined as described [20]. CD30-positive cell lysate (diluted 1:50) was co-incubated with serial dilutions of patient serum (1 h, 37 °C) and washed; HRS-3b was added in a concentration (0.25 μg/ml) known to be submaximal. Binding was detected as mentioned before and the percentage of specific inhibition calculated [20]. Lysate from the HPB-ALL cell line, which is known to be CD30-antigen-negative, served as the negative control in all experiments [19].

sCD30 levels

Soluble CD30 (sCD30) was determined as previously described [23] by a sandwich ELISA based on two CD30-specific antibodies recognizing different epitopes. To block unspecific binding by HAMA, serum was preabsorbed with an unrelated isotype-matched murine antibody (5 μg/ml, room temperature, 1 h). Recombinantly expressed CD30 antigen was used as standard [22].

Assay for cytolytic activity

NK-cell-mediated cytotoxicity was measured, using the previously described modifications of time-resolved fluorometry [23]. The NK-sensitive K562 and the NK-resistant Daudi or Raji cell lines were used as targets. Effector:target cell (E:T) ratios ranged from 40:1 to 5:1. Samples comprising 1×10^4 europium-labeled tumor cells were used as targets and PBMC as effector cells. After 4 h of culture at 37 °C, 10 μl supernatant was collected from each well, mixed with 100 μl enhancement solution (Pharmacia, Freiburg, Germany) and counted in a time-resolved fluorometer (LKB Wallac, Turku, Finland). The percentage specific lysis was calculated as described [22]. Maximum release was determined by adding 0.5% Triton X-100 (Sigma, Munich, Germany) to labeled cells. Each blood sample was analyzed in triplicate.

Results

Antibody distribution and elimination

No data exist to date for the optimal treatment regimen with bispecific antibodies. Therefore, we decided to use a dose-escalating scheme as shown in Table 1. The first patients entered on the 1-mg/m^2 level received three bolus infusions every 3–4 days and were treated with a double dose on the fourth infusion. When no relevant toxicity was observed, the next 2 patients were started at the latter level. As stated in the previous publication, we reached the maximum intended dose of 64 mg/m^2 without any clinically relevant side-effects [13]. Significant levels of circulating bispecific antibodies could be detected by the standard ELISA technique in patients treated with 4 mg/m^2 or higher doses (Fig. 1A). Peak levels, measured directly at the end of the infusion, reached values of 9.5 μg/ml for the highest dose level. Saturation with bi-mAb of circulating NK cells in the peripheral blood was not reached with that dose, suggesting that higher bi-mAb doses might be even more efficient (data not shown). The serum half-life ($t_{1/2,\beta}$) of the bispecific antibody was measured by the ELISA technique and showed consistent results for all groups

Table 1 Characteristics of the treatment schedule. Two patients were entered on each dose level and antibody was administered on the days indicated. The first patients entered on level 1. Progression to the next higher dose level was allowed if 2 patients had completed the treatment without the occurrence of severe side-effects (WHO grade III or IV)

Treatment level	Dose (mg/ml) for stages I–IV in the cycle			
	I (day 1)	II (day 4)	III (day 8)	IV (day 11)
1	1	1	1	2
2	2	2	2	4
3	4	4	4	8
4	8	8	8	16
5	16	16	16	32
6	32	32	32	64
7	64	64	64	64

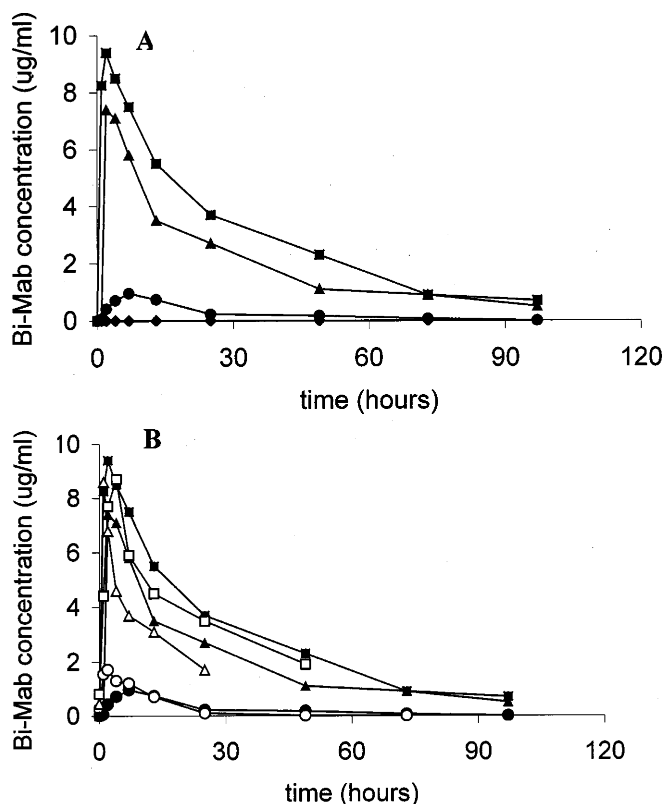

Fig. 1 A Serum levels of circulating antibodies. Antibody levels were determined by sandwich enzyme-linked immunosorbent assay as described. Significant levels of circulating murine antibodies were detectable in patients treated with 4 mg/m^2 or higher doses. The mean antibody profile of the first treatment cycle is shown for levels 1 (◆), 3 (●), 5 (▲) and 7 (■). **B** Variation of serum antibody concentration correlated to cycle number. Compared are the concentrations of circulating antibodies in treatment cycles I (● ▲ ■) and IV (○ △ □). Data are presented for level 3 (● ○), 5 (▲ △) and 7 (■ □)

treated with doses higher than 4 mg/m^2. Bi-mAb elimination followed second-order kinetics and ranged between 28 h and 32 h, being more rapid in the last treatment cycle than in the first. The average antibody half-life time dropped from 28 h to 17 h (level 6, 32 mg/m^2) or from 32 h to 28 h (level 7, 64 mg/m^2), respectively (Fig. 1B).

Induction of human anti-(mouse Ig) antibodies

As the bispecific antibody HRS-3/A9 is of murine origin, the induction of HAMA during treatment was expected and measured by the standard ELISA technique. Two patients already had HAMA before treatment but did not show any signs of allergic side-effects under treatment. The level of HAMA in these patients did not increase during treatment and stayed very low over the entire observation period (data not shown). Of the remaining 13 patients, 6 developed HAMA (Fig. 2A). The absolute concentration of anti-idiotypic antibodies ranged between 153 ng/ml (patient 7) and 1002 ng/ml (patient 2) with a median of 232 ng/ml. Patient 3, treated with 2 mg/m^2 bi-mAb tested positive for HAMA after the third infusion. All other patients presented with a positive HAMA titer 4 weeks after the last infusion.

To determine the quality of induced HAMA, we analyzed the percentage of anti-idiotypic and anti-anti-idiotypic antibodies. Anti-idiotypic antibodies (AB2) were detected by their ability to block the binding of the parental HRS-3 antibody with the CD30 target antigen (Fig. 2B). All HAMA-positive patients presented with blocking antibodies, leading to a reduction of CD30-specific binding ranging between 94% and 14% (Fig. 2B) at a serum dilution of 1:1. As the observed inhibition could be caused, to some extent, by anti-anti-idiotypic antibodies (AB3), we performed an additional test to measure the amount of human CD30-specific antibodies. Again, all patients positive for HAMA and anti-idiotypic antibodies presented with detectable levels of these AB3 antibodies. These treatment-induced human antibodies provided a specificity for the CD30 antigen and did not cross-react with CD30-antigen-negative tumor cells (Fig. 3).

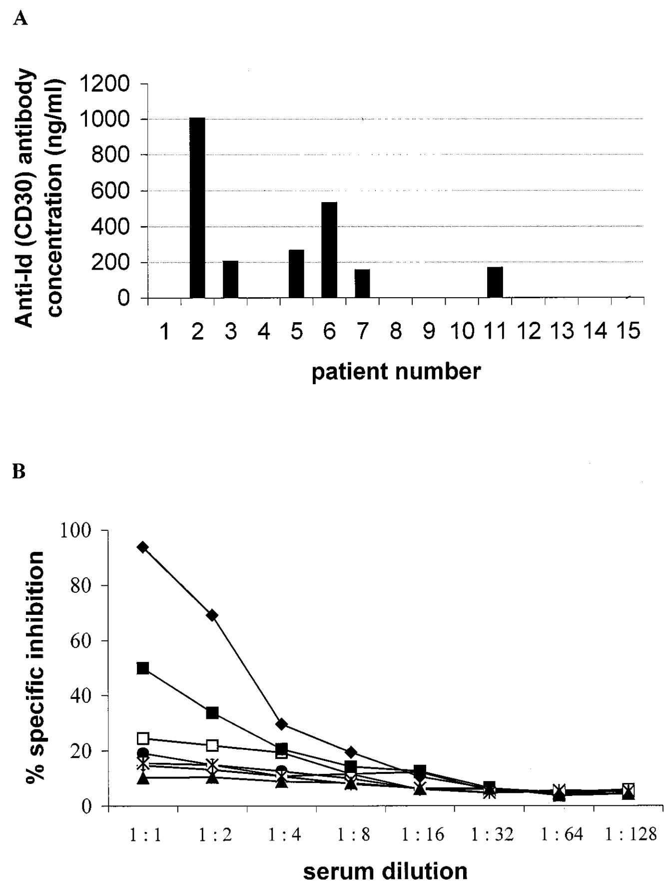

Fig. 2A, B Induction of anti-idiotypic antibodies. The existence of anti-idiotypic antibodies Ab2γ and 2β was determined 4 weeks after treatment on the basis of a competition assay. The test analyzed the ability of the patient's serum to block the binding of submaximal amounts of biotinylated anti-CD30 mAb (0.5 μg/ml) with its antigen. **A** Serial serum dilutions were compared to a standard curve and translated into antibody concentrations (ng/ml). Standard data were obtained as described by using an anti-idiotypic Ab2β (9G10) at various concentrations. **B** Specific inhibition of binding of tumor-antigen-specific anti-CD30 mAb to its target antigen by patient serum. The indicated dilutions of serum from human anti-(mouse Ig)-positive patients [patients 2 (◆), 3 (●), 5 (□), 6 (■), 7 (■) and 11 (■)] were analyzed and compared to control serum from healthy donors (▲). Pre-treatment serum of all patients was tested and did not induce any specific inhibition (data not shown)

sCD30 antigen level under treatment

In Hodgkin's patients, the circulating levels of sCD30 correlate with the activity of the disease or can be used as an early detection marker for relapse. Out of the 15 patients included in the studies, 6 presented with measurable sCD30 levels before or under treatment (Fig. 4). There was no correlation between the presence of detectable sCD30 and the clinical efficacy of CD16/CD30 bi-mAb treatment. However, rapidly rising sCD30 levels indicated emerging relapse or progression, as shown for patient 3, who showed stable disease under treatment, but had a fast-developing progression after the last treatment cycle.

NK cell activity under treatment

As already stated in our previous report, the absolute counts of lymphocytes, thrombocytes and erythrocytes did not change during treatment (data not shown). To determine the impact of antibody treatment on NK cell activity, peripheral blood lymphocytes (PBL) were collected at different times, stored and analyzed all at the same time at the end of the observation period. Only 9 out of the total 15 patients could be included in this analysis as their PBL met the criteria of 90% viability after thawing. For these patients, NK-cell-mediated cytolysis was measured by using NK-sensitive and -resistant target cell lines (Fig. 5). All patients, with the

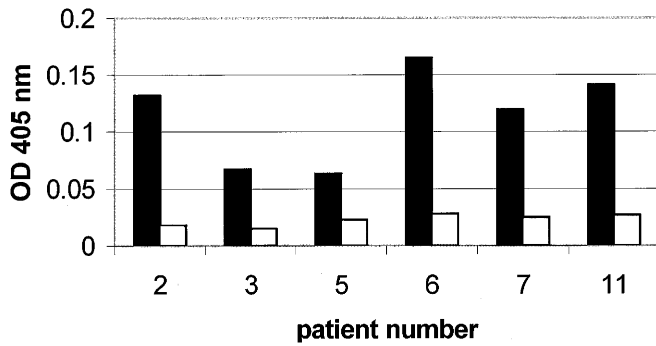

Fig. 3 Generation of anti-anti-idiotypic antibodies (Ab3). Serum (1/10 dilution) from patients positive for anti-idiotypic antibodies was tested 4 weeks after treatment for the presence of Ab3 on $CD30^+$ (*black columns*) and $CD30^-$ (*shaded columns*) cell lysate. Serum samples obtained prior to treatment were used as controls and revealed absorbance values below 0.01 for all patients (data not shown). As no human anti-CD30 mAb exists, no standardization could be performed and data are given as absorbances

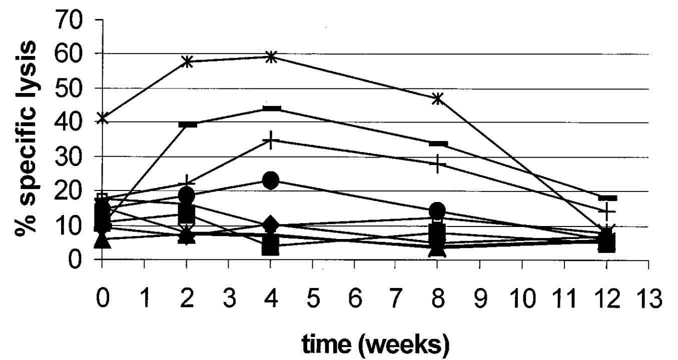

Fig. 5 Cytotoxic activity of patients' natural killer (NK) cells during and after bispecific mAb treatment. At each assay time, NK cell activity was tested in triplicate on the NK-sensitive K562 and NK-resistant Daudi tumor cell line. Lysis of Daudi cells was below 12% in all experiments (data not shown). Specific lysis is defined as the average value obtained for K562 lysis at the indicated time at the given E:T ratio. NK cell activity was monitored up to 10 weeks after treatment. The following patients could be tested: patients 1 (▲), 2 (■), 3 (▲), 5 (r), 6 (–), 10 (●), 11 (■), 14 (□), 15 (■). Data are shown for the E:T ratio of 20

exception of patient 6, had low levels of NK cell activity at the beginning of treatment. A rise of NK cell activity under treatment was observed in 4 out of 6 patients who received 4 mg/m^2 bi-mAb or more. NK cell activity peaked 2 weeks after treatment and declined from this point, reaching pre-therapeutic or even lower levels 12 weeks after treatment. There was no clear correlation between the rise of NK cell activity and clinical outcome, as 2/4 patients with increasing NK cell activity had progressive disease as determined by staging 4 weeks after treatment.

Discussion

Treatment of refractory Hodgkin's patients with an anti-CD16/anti-CD30 bispecific antibody induced objective responses in 30% of all patients [13]. The distribution and elimination of bispecific antibodies could be determined in patients receiving 4 mg/m^2 or higher antibody doses. The serum antibody half-life in patients treated on the same dose levels was fairly homogeneous without significant inter-individual differences. When the first infusion cycle was compared with the last one, a more rapid antibody elimination from the circulation was observed. This increased elimination profile is a typical sign of the induction of immune complexes indicating the emergence of HAMA, which can escape detection at this stage because of technical limitations. The fact that 50% of all patients developed HAMA antibodies was not surprising since the bispecific antibody used is entirely of murine origin. Moreover, the fact that the last injection of a treatment cycle was given with an increased dose might have resulted in an immunogenic boost favoring HAMA generation. No clear correlation could be established between the antibody dose administered and the degree of HAMA response, which is in accordance with observations made by others [4]. All but 1 HAMA-positive patient developed detectable antibody titers 4 weeks after treatment. The time of measurable HAMA induction after antibody treatment seems to be extremely variable and dependent on the

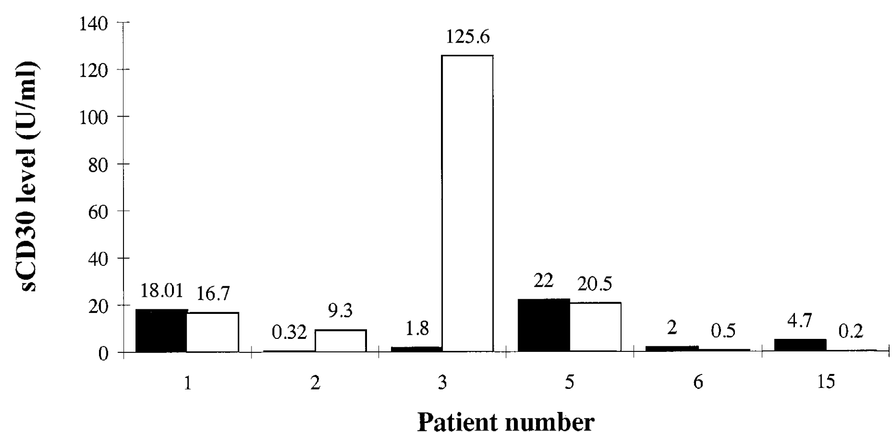

Fig. 4 Soluble CD30 (sCD30) levels under treatment. sCD30 was determined before (*black columns*) and 4 weeks after treatment (*shaded columns*) as described. Absolute values for sCD30 are presented on the top of the columns (U/ml). All other patients were negative for sCD30

individual murine antibody. Dillman and coworkers [6] observed HAMA induction in 41% of their cancer patients within 14 days after a single antibody injection. According to the literature, the proportion of HAMA-positive patients varies among different cancer types, with 27% of all patients with colorectal tumors developing HAMA compared to 55% and 56% in the group of melanoma and prostate cancers respectively. Although almost all murine antibodies used for clinical trials belong to the IgG1 subclass, they show substantial differences with regard to HAMA induction, ranging from very low values of 4% of all patients treated to 100% [2, 8].

Two patients in our study presented with low-level HAMA that were detectable in sera taken prior to murine bi-mAb therapy. They did not develop allergic side-effects in the mandatory DTH test, which had to be performed before the first antibody injection was given. This observation is in accordance with other publications, indicating that 9%–11% of the population of the Western hemisphere have low-level antibodies reacting with murine immunoglobulins [1, 33]. The origin of these antibodies remains unknown so far. Surprisingly, the HAMA titer in our 2 patients did not increase and was stable under treatment. The explanation for this phenomenon is still unclear.

The anti-idiotypic response was characterized in more detail 4 weeks after treatment, as a previous report on bispecific antibodies had suggested that the amount of anti-idiotypic antibodies peaked 3–5 weeks after treatment [4]. The absolute concentration of anti-idiotypic antibodies in our study was significantly lower when compared to the bispecific antibody trial from Weiner's group [4], where 10/17 patients presented with anti-idiotypic antibody concentrations of more than 1000 ng/ml 4 weeks after treatment. This difference in immune response might be explained by the difference in antibodies used. Moreover, the patients entering our trial were heavily pretreated and had absolute T and B cell counts well below the normal range, suggesting that this group had an impaired humoral immune response at the time of treatment.

The clinical importance of anti-idiotypic antibodies is still under debate. Anti-idiotypic antibodies can block binding of the bispecific antibodies with their target cells (as shown for patients 2 and 6), resulting in a reduced rate of bi-mAb-mediated tumor cell destruction. However, the induction of an anti-idiotypic immune response might have favorable effects, as shown in other antibody trials [30]. All 6 patients with an anti-idiotypic immune response had a measurable level of anti-anti-idiotypic antibodies as well. These so-called AB3 antibodies recognized the CD30 antigen and might have contributed to the observed tumor responses [18, 32]. The number of patients treated in our phase I/II trial was too small to allow a correlation between anti-idiotypic response and clinical outcome.

No correlation could be observed between clinical response and drop in sCD30 levels. This might be explained by a large tumor burden and inaccessible tumor sites as antibody penetration into tissue infiltrated by Hodgkin's lymphoma is crucial for effective cell killing. However, we could demonstrate that the rise of sCD30 is an early marker for relapse. This observation is in accordance with chemotherapy trials that proved increasing levels of sCD30 to be an independent marker for relapse [12].

The absolute counts for erythrocytes, thrombocytes, granulocytes and lymphocytes did not alter significantly during treatment nor were they significantly different between treatment groups. No dose-limiting toxicity was observed. This is surprising, because Weiner and coworkers [34], who had used a similar NK-cell-activating bi-mAb for the treatment of Her-2/neu-positive cancers, had reported the occurrence of dose-dependent and dose-limiting thrombocytopenia at bi-mAb doses as low as 5 mg/m^2. The mechanism underlying this thrombocytopenia remains unclear so far and future analysis has to focus on possible cross-reactions of the antibodies with thrombocytes. However, the CD16-antigen-specific antibodies that were used by Weiner's group [34] and ours recognize different non-overlapping CD16 epitopes [15]. If indeed the lack of thrombocytopenia in our study, even at ten times higher antibody doses, is due to the different antibodies used in the respective studies, this would favor the A9 parental monoclonal antibody to be used for future bispecific constructs that aim to redirect NK cell activity towards tumor cells.

Although absolute and relative NK cell counts remained largely unaffected by the CD16/CD30 bi-mAb treatment, a rise in NK cell activity was observed in 4 out of 6 patients who were treated with 4 mg/m^2 or higher doses. This treatment-associated, temporary rise in NK cell activity lasted for a maximum of 8 weeks. Several reports have stressed the importance of NK cell activity in terms of tumor response and control [32]. In neuroblastoma patients, relapse-free survival of patients in remission with NK activity above the median value was significantly longer than that of the patients with NK activity below the median. NK cell activity in tumor patients does not seem to correlate directly with T cell effector activity. We could show, in a recent publication, that Hodgkin's patients have a T cell defect that can be linked to a reduced expression of the T-cell-receptor-associated ξ chain [24]. However, the expression of the ξ chain in NK cells was normal, suggesting that they are regulated independently. Therefore, the significant reduction of absolute T cell counts that we observed in the patients enrolled in the present study does not imply an immunodeficiency that involves other arms of the immune system [13]. NK cell counts for our patients were lower than in healthy patients, but still within the normal range. This might be important, as Lode and co-workers [16] could prove for their anti-GD2/interleukin-2 fusion protein that the tumor response was dependent on intact NK cell function and not on the presence of T cells.

Recently we started a new clinical trial to compare the efficacy and the effects on immunological parameters of a regimen involving the continuous infusion of an antibody with those of a bolus injection. The continuous antibody infusion might have the advantage of reduced HAMA induction allowing multiple treatment cycles. While different application schemes might reduce murine bi-mAb immunogenicity in patients to a certain degree, for a definite solution of this problem, antibody constructs have to be designed with reduced immunogenicity [3, 14], e.g. so-called diabodies or bispecific $F(ab')_2$ fragments, which are derived from chimaeric or fully humanized antibody gene sequences. Such constructs should be suitable for long-term treatment. It is obvious that only concepts using repetitive cycles of sufficiently dosed antibody applications allow the full exploitation of this immunotherapeutic approach, which might ultimately lead to long-lasting remissions.

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