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Repetitive intravenous application of the bispecific antibody BIS-1 in carcinoma patients receiving subcutaneous interleukin-2

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Submitted
Abstract

**Purpose:** The bispecific monoclonal antibody BIS-1 binds simultaneously to the CD3 complex present on T cells and to a pancarcinoma-associated antigen called epithelial glycoprotein-2 (EGP2, also known as EpCAM). We studied the feasibility, toxicity profile and optimal biological dose (OBD) of repetitive intravenous (IV) BIS-1 application.

**Methods:** Twelve patients with disseminated EGP2+ tumors from kidney, lung or colon were included. Cohorts of at least two patients were given daily doses of 3, 5, 8 and 13 µg/kg BIS-1 F(ab’)2 as a 2-hour IV infusion for five days while receiving subcutaneous (SC) IL-2. Peripheral vasoconstriction and chills were experienced by all patients at 60-90 minutes of infusion and appeared to be independent of BIS-1 dose. Fever was noted in 5/7 patients at the two highest dose levels and was preceded by temporary dyspnea in all but one, requiring adjustment of the infusion rate. A dose-dependent decline in mono- and lymphocyte numbers in the peripheral blood was observed in the course of each infusion; the decrease in lymphocyte numbers paralleled clinical toxicity. BIS-1 F(ab’)2 was found to saturate CD3 in a dose-dependent fashion, with a dose of 5 µg/kg/d inducing optimal binding degrees for in vitro tumor lysis. Plasma TNFα increased at the end of each infusion; IFNγ remained undetectable. No anti-tumor responses were observed.

**Conclusion:** Treatment incorporating repetitive IV BIS-1 F(ab’)2 infusions in concert with SC IL-2 is feasible, giving immunologically active CD3 saturations at well-tolerated doses. Based on these findings, an OBD of 5-8 µg/kg/d BIS-1 F(ab’)2 is suggested.
Introduction

Recombinant human interleukin-2 (IL-2) has clinical activity in the treatment of selected malignancies, with renal cell carcinoma (RCC) being one of the most susceptible ones. Until now, low-dose subcutaneous (SC) IL-2 regimens have proven to be equal in efficacy to the more toxic high-dose intravenous (IV) regimens, reaching objective response rates in the order of 20%, including 5% complete remissions [1-5]. Nevertheless, almost all other tumor types and the vast majority of RCC still remain refractory to IL-2, probably due to insufficient activation of cytotoxic effector cells, inadequate migration of these effector cells to the tumor site and (or) lack of specific recognition and subsequent killing of tumor cells by the cellular immune system.

To partially overcome IL-2 resistance and to extend the range of tumor targets, the killing capacities of IL-2-activated cytotoxic effector cells and the specific binding properties of monoclonal antibodies (MAb) may be joined in a concept of redirected T cell-mediated cytotoxicity using bispecific monoclonal antibodies (BsMAb). By virtue of a dual specificity for activation markers on cytotoxic effector cells and for tumor-associated antigens (TAA) on target cells, BsMAb are capable of redirecting effector cells to induce tumor lysis [6]. In combination with IL-2-induced effector cell activation, BsMAb might provide a specific stimulatory signal by cross-linking the triggering molecules of the effector cells at the tumor site [7, 8]. This approach has been extensively tested both in vitro and in vivo for a broad array of effector and tumor target cell populations [9-17]. Data indicate that BsMAb-retargeted cytotoxic effector cells can efficiently lyse tumor cell lines in vitro as well as eradicate small established tumors in vivo in animal models [18-20]. Clinical phase-I studies showed BsMAb-induced anti-tumor cytotoxicity after local application in patients with malignant glioma [21, 22], ovarian carcinoma [23-25], and malignant pleural or peritoneal effusions [16]. No anti-tumor responses have been reported yet in clinical studies using IV applied BsMAbs.

The BsMAb used in the present study, BIS-1, is reactive with the CD3 complex on T cells on the one hand and a membrane-bound TAA (epithelial glycoprotein-2 (EGP2), also known as EpCAM) expressed by most carcinomas on the other. A recently conducted phase-I trial using single IV BIS-1 F(ab')2 infusions in RCC patients receiving SC IL-2 revealed a maximum tolerated dose (MTD) of 5 μg/kg BIS-1 F(ab')2 with little clinical efficacy [17]. To intensify BIS-1 F(ab')2 therapy, we performed a new phase-I trial in which multiple doses of IV BIS-1 F(ab')2 were applied in concert with SC IL-2. Patients with EGP2-expressing tumors from colon, lung or kidney, not responding to first-line therapy were included. In the present report, we describe the feasibility and immunological impact of this treatment.
Materials and methods

Patient selection
Patients entered into the study had a histological confirmation of a disseminated EGP2-expressing tumor from kidney, lung or colon, unresponsive to first-line therapy. They were required to have a performance status \(2\) (World Health Organization, WHO scale [26]), an age \(18\) years, an estimated life expectancy of more than \(3\) months, a rest period of at least \(4\) weeks after previous immunotherapy in the case of RCC, and adequate hematological (white blood cell (WBC) counts \(4 \times 10^9/L\) and platelet counts \(120 \times 10^9/L\)), renal (serum creatinine > \(120\) mmol/L) and hepatic (serum bilirubin > \(30\) mmol/L) functions. Patients with non-evaluable tumor lesions only, uncontrollable disease apart from the tumor, concurrent treatment with corticosteroids and (or) previous therapy with mouse-antibodies were excluded. The study protocol was approved by the Medical Ethical Committee of the University Hospital of Groningen, The Netherlands. Written informed consent was obtained from each participating patient.

IL-2
IL-2 (Proleukin®, EuroCetus b.v., Amsterdam, The Netherlands) with a biological activity of 18 million International Units (MIU) per milligram was used. Vials of \(1\) mg were reconstituted with \(1.2\) mL sterile water before SC administration.

BIS-1 F(ab')\(_2\)
The BIS-1 producing quadroma was made by fusion of the hybridomas RIV-9 (anti-CD3, IgG3) and MOC-31 (anti-EGP2, IgG1), as described previously [16]. Large-scale production and isolation of BIS-1 F(ab')\(_2\) was performed as described previously [17]. In short, the BIS-1 quadroma was grown in a hollow fiber culture system (Endotronics Inc., Minneapolis, MN, USA) under Good Manufacturing Practice (GMP) guidelines. Purification of the hybrid (IgG3/IgG1) from parental-type antibodies (IgG3 and IgG1) was performed by protein-A sepharose chromatography. BIS-1 was digested by pepsin (Worthington, Freehold, NJ, USA) using a final BIS-1 : pepsin weight ratio of 100 : 1, immediately followed by S200 superdex FPLC gel filtration to separate BIS-1 F(ab')\(_2\) from undigested IgG, fragmented Fc-portions, and pepsin. The purified BIS-1 F(ab')\(_2\) solution was adjusted to a concentration of 0.2 mg/mL with 0.9% NaCl. Human serum albumin (HSA; Institute Merieux, Lyon, France) was added to a concentration of 0.5% and the preparation was then passed through a \(0.22\) mm filter and stored sterile at 4°C. Sterility of the BIS-1 F(ab')\(_2\) preparation was confirmed by culturing in Clausur medium; the preparation was pyrogen-free as tested by a Limulus Amoebocyte Lysate Assay and by IV administration of the preparation to rabbits. Abnormal toxicity was tested by administration of the BIS-1 F(ab')\(_2\) preparation to mice and guinea pigs, both IV and intraperitoneally, according to the protocol of
the Dutch Pharmacopee IX (1980) [27], and was found to be absent. The ability of the BIS-1 F(ab')\textsubscript{2} preparation to redirect the lytic capacity of T cells towards EGP2\textsuperscript{+} tumor cells was assessed in a standard \textsuperscript{51}Cr-release assay, in which \textit{in vitro} activated T cells were used as effector cells and GLC-1M13 (EGP2\textsuperscript{+}), GLC-1 (EGP2\textsuperscript{-}) and P815 (Fc-receptor\textsuperscript{+}) were used as target cells. The target cell line P815 was used to check whether the BIS-1 F(ab')\textsubscript{2} preparation was devoid of undigested Fc-containing BIS-1 IgG or not. BIS-1 F(ab')\textsubscript{2} was shown to mediate excellent GLC-1-M13 toxicity, whereas GLC-1 and P815 were not killed.

**Treatment program**

Patients were hospitalized during treatment and received daily SC IL-2 injections in two 5-day cycles with a 2-day rest period in between. Treatment consisted of 9 MIU IL-2 on the first two days of each cycle and 18 MIU IL-2 on subsequent days. During the second IL-2 cycle, 4 to 5 hours before the IL-2 injection, IV BIS-1 F(ab')\textsubscript{2} was given once daily for 5 consecutive days. For each infusion, BIS-1 F(ab')\textsubscript{2} was placed in 100 mL of 0.9% NaCl solution and administered over 2 hours. The initial BIS-1 F(ab')\textsubscript{2} dose of 3 µg/kg/d was chosen based on a previously conducted phase I trial by our group [17]. Subsequent dose levels were 5, 8 and 13 µg/kg/d BIS-1 F(ab')\textsubscript{2}, each given to at least two patients. A maximum oral dose of 3 g acetaminophen per day was prescribed concurrently to ameliorate pyretic reactions.

**Toxicity**

Toxicity was graded according to standard WHO criteria. Body weight and temperature were recorded daily throughout therapy. Vital signs were recorded every 30 minutes during BIS-1 F(ab')\textsubscript{2} infusion and every two hours thereafter. Complete blood cell counts, differential counts, renal and hepatic function, as well as serum levels of sodium, potassium, calcium, total protein and albumin were obtained biweekly during treatment and two weeks after completion of therapy.

**Cytokine analysis**

For cytokine analysis, EDTA-blood samples were obtained at days 1 and 5 of BIS-1 F(ab')\textsubscript{2} administration, i.e., before infusion (T=0), upon completion of the infusion (T=2), as well as one (T=3) and three (T=5) hours thereafter. Immediately after collection, plasma was separated by centrifugation at 2500 rpm at 4°C for 5 minutes and stored at -20°C until use. A quantitative sandwich immunoassay (R&D Systems Europe, Abingdon, Oxon, UK) was used for measuring plasma levels of tumor necrosis factor-α (TNFα) and interferon-γ (IFNγ; detection limit, 30 pg/mL).
Flow cytometry

For lymphocyte subset phenotyping, serial samples of 100 mL EDTA-blood were stained using the following MAbs: Leu-4 (anti-CD3); Leu-2α (anti-CD8); Leu-3 (anti-CD4); anti-IL2R (anti-CD25) and anti-HLA-DR (Becton Dickinson, Mountain View, CA, USA). All MAbs were directly conjugated with fluorescein-isothiocyanate or phycoerythrine. Analyses were carried out by three-color flow cytometry using a Coulter Elite Cytometer (Coulter Electronics, Hialeah, Fl, USA). Detection of BIS-1 F(ab’)2 on the surface of peripheral T cells was performed using an indirect immunofluorescence procedure with biotinylated goat-anti-mouse Ig (GαM-bio), unlabeled goat-anti-mouse Ig (GαM-unl) (Southern Biotechnologies Inc., Cambridge, MA, USA) and strepatavidin-phycoerythrin (SAPE) (Becton Dickinson, Mountain Views, CA, USA). 100 ml peripheral EDTA-blood was incubated with phosphate buffered saline (PBS) or a saturating amount of BIS-1 F(ab’)2 (2 µg/mL) at 4°C for 30 minutes. After one wash with 2 mL PBS, 50 mL GαM-bio (dilution 1:20 in PBS containing 1% pooled human serum) or, as a control for aspecific binding of the conjugate, unlabeled GαM (dilution 1:40) was added to the cell pellet and incubated at 4°C for 30 minutes. After one wash with 2 mL PBS, 10 mL SAPE was added to the cell pellet and the samples were incubated at 4°C for 30 minutes. Cells were resuspended in 2 mL FACS-lysing solution (Becton Dickinson, Mountain View, CA, USA), incubated for 10 minutes at room temperature, washed once with PBS and resuspended in a final volume of 150 mL PBS for flow cytometrical analysis. The CD3 occupancy was calculated according to the following formula:

\[
\frac{\text{MFI}_{t=x} (\text{PBS/GαM-bio/SAPE}) - \text{MFI}_{t=x} (\text{PBS/GαM-unl/SAPE})}{\text{MFI}_{t=x} (\text{BIS-1/GαM-bio/SAPE}) - \text{MFI}_{t=x} (\text{BIS-1/GαM-unl/SAPE})} \times 100% \]

in which the sequential incubation steps are given between brackets; MFI, mean fluorescence intensity; bio, biotinylated; unl, unlabeled; t=x represents time point of blood collection.

Response

Prior to treatment, patients were staged by physical examinations; complete blood counts; serum tests of liver, kidney and bone marrow functions; and echographic or radiological recording of disease extension. Response was monitored at the end of BIS-1 F(ab’)2 treatment and 2 weeks later. A complete response was defined as the disappearance of all evidence of tumor for a minimum of 4 weeks. A partial response was defined as a 50% or greater reduction of the sum of the products of all diameters of evaluable lesions. Patients with a response less than partial or an increase of less than 25% were classified as stable disease, whereas progressive disease was defined as an increase of more than 25% or the development of new lesions.
Statistics
The two-tailed Student's \( t \)-test, paired Student's \( t \)-test, and Wilcoxon test were used for statistical analysis. \( p \) values < 0.05 were considered significant. Unless otherwise stated, the two-tailed Student's \( t \)-test was used.

Results

Toxicity
Twelve patients with EGP2-expressing tumors from colon (2), lung (6) or kidney (4) were enrolled in this trial. Patient's characteristics are outlined in Table 1. All patients were assessable for clinical, hematological and biochemical toxicity.

<table>
<thead>
<tr>
<th>Table 1. Patients' characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>Male : Female</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>WHO performance</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

Clinical side effects arising from SC IL-2 administration were similar to previously reported findings by our group [28]. In short, all patients experienced mild to moderate constitutional symptoms, grade I-III fever and chills, as well as transient inflammation and induration at the IL-2 injection sites. Nausea and vomiting did not occur, nor did diarrhea or hypotension. All side effects abated immediately after cessation of IL-2 therapy, except for the inflammatory skin nodules, which resolved slowly during several months after IL-2 discontinuation. Elevations in serum levels of urea and creatinine were observed in 8% and 17% of patients, respectively. Hepatic toxicity, as reflected by increases in liver enzymes, was seen in all patients. Specifically, elevations in serum levels of aspartate aminotransferase and alanine aminotransferase were noted in 58% and 92% of patients, respectively, whereas elevations in serum levels of alkaline phosphatase and \( \gamma \)-glutamyl transferase were observed in all. Levels immediately returned to baseline upon completion of treatment. In one lung cancer patient, hepatic dysfunction persisted after IL-2 discontinuation due to the presence of multiple liver metastases.
Toxicities associated with IV BIS-1 F(ab')<sub>2</sub> infusion are summarized in Table 2. The majority of patients at all dose levels experienced peripheral vasoconstriction and chills, occurring suddenly at the end of the infusion (90-120 min.) and persisting for approximately 30 minutes.

Table 2. Toxicities associated with IV BIS-1 F(ab')<sub>2</sub> infusion

<table>
<thead>
<tr>
<th>No.</th>
<th>Tumor</th>
<th>Dose µg/kg/d</th>
<th>Toxicity Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>colon</td>
<td>3</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>2.</td>
<td>colon</td>
<td>3</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>3.</td>
<td>lung</td>
<td>3</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>4.</td>
<td>lung</td>
<td>5</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>lung</td>
<td>5</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>lung</td>
<td>8</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>8.</td>
<td>kidney</td>
<td>8</td>
<td>C, F, D, V</td>
<td>C, D</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>9.</td>
<td>kidney</td>
<td>13</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10.</td>
<td>kidney</td>
<td>13</td>
<td>C, F, D</td>
<td>C, F, D</td>
<td>C, F, D</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11.</td>
<td>kidney</td>
<td>13</td>
<td>C, F, D, V</td>
<td>C, V</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12.</td>
<td>lung</td>
<td>13</td>
<td>C, F, D</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
</tbody>
</table>

C, chills ± peripheral vasoconstriction; F, fever; D, dyspnea; N, nausea; V, vomiting.

At the two lowest dose levels, no other side effects were observed. Two out of three patients at the 8 µg/kg dose level and three out of four patients at the 13 µg/kg dose level developed fever up to WHO grade III, generally starting about 30-60 minutes after the end of the infusion and lasting for 1-2 hours. Peak temperatures did not appear to be dose-related and fever was confined to the first two or three days of BIS-1 F(ab')<sub>2</sub> infusion. In four patients, fever was preceded by moderate to severe dyspnea at rest, occurring at the end of the infusion and persisting for approximately 30 minutes. Symptoms gradually resolved thereafter. Just like fever, dyspnea was limited to the first two or three days of BIS-1 F(ab')<sub>2</sub> treatment. At the 8 µg/kg dose level, the infusion was protracted to 3 hours in order to control pulmonary toxicity. At the 13 µg/kg dose level, dyspnea required interruption of the infusion in one patient. No hypotension occurred during these episodes. Nausea and vomiting were observed in two patients at the highest dose levels, but did not require anti-emetics. No short- or long-term BIS-1 F(ab')<sub>2</sub>-associated toxicities were noted. With dyspnea as dose limiting toxicity, a BIS-1 F(ab')<sub>2</sub> dose between 8 and 13 µg/kg/d was considered to be the maximum tolerated dose (MTD). No correlation between sex, age, performance status and toxicity profile could be identified.

**Immunobiological effects**

During SC IL-2 administration, the majority of patients developed peripheral blood eosinophilia, with eosinophils increasing from (mean ± SD) < 0.6 ± 0.4 x 10<sup>9</sup>/L at baseline to 1.4 ± 1.4 x 10<sup>9</sup>/L at the end of the first treatment cycle. Eosinophil numbers further rose during the second
treatment cycle up to a maximum of $7.6 \pm 2 \times 10^9/L$. Lymphocyte counts increased from (mean ± SD) $1.1 \pm 1.2 \times 10^9/L$ to $3.3 \pm 1.8 \times 10^9/L$ within one week of IL-2 therapy. No sustained effects on the numbers of monocytes and basophils were noted.

Infusion of BIS-1 F(ab')$_2$ had definite but transient effects on the number of lympho- and monocytes, as shown in Figures 1 and 2, respectively. These effects appeared to be dose-related and transiently, and had completely resolved at follow-up.

Figure 1. Mean changes in lymphocyte numbers during BIS-1 F(ab')$_2$ infusions with 3 (E), 5 (G), 8 (C) and 13 (H) µg/kg/d.

At the 3 and 5 µg/kg dose levels, lymphocyte numbers tended to decrease during each BIS-1 F(ab')$_2$ infusion (not significantly, [NS]) and almost returned to baseline within 24 hours. At the 8 µg/kg dose level, lymphocyte counts significantly decreased during the first BIS-1 F(ab')$_2$ infusion. During subsequent infusions, the lymphocyte counts tended to drop, but the difference did not reach statistical significance. Because lymphocyte counts did not fully return to baseline within 24 hours, a cumulative decrease was noted during BIS-1 F(ab')$_2$ treatment (mean ± SD; day 1, T=0, $6.4 \pm 0.4 \times 10^9/L$; day 5, T=0, $3.1 \pm 0.5 \times 10^9/L$; p < 0.02). At the 13 µg/kg dose level, lymphocyte counts significantly declined during the first three BIS-1 F(ab')$_2$ infusions only, with a trend towards a decrease during subsequent infusions (NS). Again, lymphocyte counts cumulatively decreased throughout BIS-1 F(ab')$_2$ treatment (mean ± SD; day 1, T=0, $3.1 \pm 0.2 \times 10^9/L$; day 5, T=0, $2.0 \pm 0.7x \times 10^9/L$; p < 0.04).
Figure 2. Mean changes in monocyte numbers during BIS-1 F(ab')_2 infusions with 3 (É), 5 (G), 8 (C) and 13 (H) µg/kg/d.

At the 3 µg/kg dose level, monocyte numbers tended to drop simultaneously with lymphocyte numbers during each BIS-1 F(ab')_2 infusion (NS) and returned to baseline levels within 24 hours. At both the 5 and 8 µg/kg dose level, a significant decline in the number of monocytes was noted during the first BIS-1 F(ab')_2 infusion only. During subsequent infusions, monocyte numbers tended to decrease (NS). Numbers had completely returned to baseline before the next infusion. At the 13 µg/kg dose level, the number of monocytes significantly decreased during each BIS-1 F(ab')_2 infusion, returning to baseline within 24 hours. No clear effects on other WBC types were observed during BIS-1 F(ab')_2 treatment.

**BIS-1 (Fab')_2 binding to CD3 on circulating T cells**

Before and after IV BIS-1 F(ab')_2 administration, the degree to which available CD3 molecules on circulating T cells were loaded with BIS-1 F(ab')_2 was determined by flow cytometry. Figure 3 summarizes the results obtained from patients treated with increasing doses of BIS-1 F(ab')_2. Since mean CD3 occupancy was found to be highest directly after each BIS-1 F(ab')_2 infusion, only data on T=2 are shown. Except for the 5 µg/kg BIS-1 F(ab')_2 dose level, mean CD3 occupancy appeared to be dose-related and maximal between days 3 and 5 of treatment. At 3, 5, 8 and 13 µg/kg BIS-1 F(ab')_2, CD3 occupancy increased over the course of BIS-1 F(ab')_2 treatment up to (mean ± SD) 22 ± 1%, 15 ± 11%, 57% and 61 ± 21%, respectively. At the two highest dose steps, BIS-1 F(ab')_2 was still detectable on peripheral T cells after 24 hours [data not shown].
Figure 3. Mean CD3 occupancy of circulating T cells, measured directly after infusion with 3 (E), 5 (G), 8 (C) and 13 (H) \( \mu g/kg \) BIS-1 F(ab')\(_2\) per day. Dotted lines represent optimal range for \textit{in vitro} target cell lysis, as reported previously [17].

**Cytokine release**

Serum levels of TNF\(\alpha\) were assessed in one patient receiving 3 \( \mu g/kg \) BIS-1 F(ab')\(_2\) and in two patients per each subsequent dose level. Data on TNF\(\alpha\) are listed in Table 3.

<table>
<thead>
<tr>
<th>No.</th>
<th>Dose</th>
<th>Day 1</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu g/kg/d )</td>
<td>T=0</td>
<td>T=2</td>
</tr>
<tr>
<td>3.</td>
<td>3</td>
<td>50</td>
<td>&lt;30</td>
</tr>
<tr>
<td>4.</td>
<td>5</td>
<td>78</td>
<td>146</td>
</tr>
<tr>
<td>5.</td>
<td>5</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>6.</td>
<td>8</td>
<td>92</td>
<td>nd</td>
</tr>
<tr>
<td>8.</td>
<td>8</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>9.</td>
<td>13</td>
<td>95</td>
<td>72</td>
</tr>
<tr>
<td>10.</td>
<td>13</td>
<td>42</td>
<td>&lt;30</td>
</tr>
</tbody>
</table>

nd, not done

At baseline, several patients showed TNF\(\alpha\) production, probably due to prior IL-2 therapy. In one patient at the 5 \( \mu g/kg \) dose level, TNF\(\alpha\) levels remained below the detection limit throughout BIS-1 F(ab')\(_2\) treatment. This patient showed no signs of clinical toxicity at all. At increasing dose steps of BIS-1 F(ab')\(_2\), peak levels of TNF\(\alpha\) were found 2-3 hours after the start of each infusion, with concentrations up to 1415 pg/mL at the highest dose level. Serum levels of IFN\(\gamma\) were assessed in one patient receiving 5, in one patient receiving 8 and in two patients
receiving 13 μg/kg BIS-1 F(ab')₂. In all patients, levels of IFNγ remained below detectable limits during BIS-1 F(ab')₂ treatment.

Response
All patients were assessable for tumor response. No complete or partial responses were seen. Stable disease was observed in nine of twelve patients (75%), whereas progressive disease was noted in three patients (no. 2, 6 and 8; 25%). One month after cessation of therapy, five patients who had stable disease showed tumor progression.

Discussion
BsMAb reactive with TAA on the one hand and cytotoxic T-lymphocytes (CTL) on the other have been demonstrated to efficiently mediate tumor regression in a variety of experimental models [20, 29, 30]. Based on in vitro data, the murine BsMAb BIS-1 F(ab')₂ has been implicated to specifically arm circulating CTL for lysis of EGP2-expressing tumor targets. However, a clinical phase I trial using single IV BIS-1 F(ab')₂ infusions in metastatic RCC patients receiving standard SC IL-2 therapy had shown systemic toxicities at relatively low doses with undetectable clinical effectiveness [17]. To obtain better anti-tumor responses, we conducted a novel trial utilizing repetitive BIS-1 F(ab')₂ infusions in carcinoma patients receiving SC IL-2.

Acute BIS-1 F(ab')₂-associated toxicity consisted of peripheral vasoconstriction, chills, fever and temporary dyspnea. While peripheral vasoconstriction and chills occurred at all dose levels, fever and dyspnea were observed at the two highest dose levels only. Remarkably, these symptoms were predominantly confined to the first two days of BIS-1 F(ab')₂ administration. Much of the toxicity observed is thought to result from T cell activation with subsequent release of cytokines such as TNFα and IFNγ. Theoretically, T cells loaded with BIS-1 F(ab')₂ might leave the circulation to be crosslinked to EGP2 via BIS-1 F(ab')₂ resulting in their activation [31]. In our patients, no plasma IFNγ could be detected during BIS-1 F(ab')₂ therapy. Peak levels of plasma TNFα were found 2-3 hours after the start of BIS-1 F(ab')₂ infusion, at the time flu-like symptoms also appeared. This, and the fact that no TNFα was detected in the toxicity-free patient (no. 5), undoubtedly hints at a causal role of TNFα in acute clinical toxicity. Larger studies are needed to determine whether BIS-1 F(ab')₂-related TNFα-release is dose-dependent or not.

However, the addition of dyspnea to the pattern of flu-like toxicity at the two highest dose levels cannot be explained by the release of TNFα and suggests a dose-dependent toxicity of BIS-1 F(ab')₂. Indeed, at the highest dose level, dyspnea was more discomforting, requiring interruption of the infusion in one of the patients. In all cases, the pattern of dyspnea was
identical: severe dyspnea occurred at the end of the infusion, especially on the first day, and required adjustment of the infusion rate to approximately 3 $\mu$g/kg/h. This confirmed our experience in a prior phase-I trial with the same BIS-1 F(ab')$_2$ regimen limited to one day, where among six patients at the 5-6 $\mu$g/kg dose, three patients developed the same pattern of dyspnea [17]. Data on the 13 patients treated with BIS-1 F(ab')$_2$ at doses 5 $\mu$g/kg/d in these two phase-I studies allow the conclusion that a dose of 5-8 $\mu$g/kg/d, with infusion rate adjustments if necessary, is feasible. Although the discomfort of dyspnea to the patient can be considerable, even higher doses can be contemplated as far as toxicity is concerned by mitigating the infusion rate. Nevertheless, data on potential effectiveness in vitro would not support this approach, as a linear dose-dependency of effectiveness is unlikely. In particular, $^{51}$Cr-release studies, performed to assess BIS-1 F(ab')$_2$-redirected T cell cytotoxicity, revealed maximum target cell lysis at a CD3 occupancy of 20 to 30%, with a substantial reduction at higher degrees [17]. Considering the CD3 occupancies attained in our patients, with maximum degrees up to approximately 60% at the two highest dose levels, the optimal biological dose (OBD) of BIS-1 F(ab')$_2$ would appear to be approximately 5 $\mu$g/kg/d. Although cumulative CD3 occupancies with repeated BIS-1 F(ab')$_2$ infusions were shown, this effect did not translate into improved clinical benefit.

BIS-1 F(ab')$_2$ has been shown to induce significant lympho- and monocytopenia at the two highest dose levels. An explanation for the observed reduction in cell numbers might be the extravasation of both lympho- and monocytes, probably due to the release of secondary cytokines like TNF$\alpha$. These cells might also be sequestrated in lungs, liver or spleen or might even stick to the endothelium for a certain period of time, mimicking extravasation and migration, after which they return to the blood stream.

At the lowest dose levels, the lymphocyte counts tended to decline after each BIS-1 F(ab')$_2$ infusion, while at the highest dose levels, lymphocyte counts significantly decreased during the first three infusions. Because lymphocyte behavior nearly paralleled the pattern of dyspnea, one could postulate that pulmonary toxicity might be caused by the induction of micro-aggregates of T cells coated with BIS-1 F(ab')$_2$. At the individual level, however, no clear correlation between lymphocyte counts and pulmonary toxicity was found. Specifically, not every patient with a substantial decrease in circulating lymphocytes developed dyspnea, suggesting that the acute BIS-1 F(ab')$_2$-related toxicity is not solely associated with removal of effector cells from the circulation.

In our patients, the repetitive application of SC IL-2 and IV BIS-1 F(ab')$_2$ did not induce objective anti-tumor responses. Basically, this lack of efficacy might be due to shortcomings at the level of effector cell activation, lack of migration of effector cells to the tumor site and (or) insufficient specific recognition and subsequent killing of tumor cells.

Appropriate T cell activation requires both an antigen-specific signal delivered through its antigen receptor and a second, antigen-nonspecific signal delivered by accessory receptors [7,
In this trial, IV BIS-1 F(ab')$_2$ was used to provide the first signal by cross-linking the CD3 complex present on T cells, while SC IL-2 was used to provide the co-stimulatory signal and, in addition, this might also promote the migration of T cells across the endothelium. The administration of SC IL-2 has previously been shown to induce the upregulation of HLA-DR on circulating CTL in vivo [33]. Because BIS-1 F(ab')$_2$ exclusively exerts its stimulatory effect in the context of its target antigen EGP2 and not in the peripheral blood, only in vitro data on BIS-1 F(ab')$_2$-related T cell activation are available. Experimental studies showed optimal tumor lysis at a concentration of 0.1 µg/mL BIS-1 F(ab')$_2$, implying a daily in vivo dose of approximately 500 µg BIS-1 F(ab')$_2$, which is comparable to the 5-8 µg/kg BIS-1 F(ab')$_2$ doses used in our trial. However, in vitro data are based on high effector : target cell (E:T) ratios (10-100). Since E:T ratios are much lower in the clinical setting, T cells loaded with BIS-1 F(ab')$_2$ may not be effective for treating bulky metastatic tumors as evaluated in the present study. Hence, to attain a clinically effective treatment, one either has to increase the T cell pool at the tumor site using T cell attracting substances or to substantially reduce the tumor burden before starting BIS-1 F(ab')$_2$ treatment. The application of BsMAb in the case of micrometastases or minimal residual disease seems to be quite promising [30, 34].

Another obstacle in BsMAb-based immunotherapy might be the migration of activated CTL towards tumor tissue. Firstly, it is not all clear that arming of T cells with BIS-1 F(ab')$_2$ does not influence T cell kinetics in vivo. It could be possible that higher doses of BIS-1 F(ab')$_2$ are less effective because they 'overload' T cells, resulting in impaired T cell mobility and extravasation. An alternative approach might be the intra- or peritumoral application of BIS-1 F(ab')$_2$ with or without IL-2. Secondly, it remains obscure whether in the tumor itself BIS-1 F(ab')$_2$-armed T cells efficiently home towards tumor cells or not. Additional studies to visualize in vivo T cell behavior during cancer immunotherapy are needed to resolve this problem.

The lack of therapeutic efficacy might be due to the fact that only massive tumor destruction can be evaluated as a tumor response. Alternatively, it is also possible that in this clinical setting this can be attributed to insufficient tumor cell recognition and subsequent lysis by activated T cells because of, e.g., local production of tumor-derived T cell inactivating substances such as transforming growth factor-β.

It can be concluded that cancer immunotherapy incorporating low-dose SC IL-2 in concert with repetitive IV BIS-1 F(ab')$_2$ infusions is feasible and immunologically active. Based on the presumed OBD and the toxicity profile, a BIS-1 F(ab')$_2$ dose of 5-8 µg/kg/d is recommended. Since the OBD is based on in vitro experiments, clinical phase II trials, with modifications to enhance T cell homing and tumor accessibility, are needed to determine whether the recommended dose is therapeutically active.
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References


