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LOCAL BUT NO SYSTEMIC IMMUNOMODULATION BY INTRAPERITONEAL TREATMENT OF ADVANCED OVARIAN CANCER WITH AUTOLOGOUS T LYMPHOCYTES RE-TARGETED BY A BI-SPECIFIC MONOCLONAL ANTIBODY

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We have reported a 27% overall anti-tumor response using i.p. immunotherapy of advanced ovarian carcinoma with autologous, *ex vivo* expanded, T lymphocytes re-targeted with bi-specific monoclonal antibody OC/TR, combined with soluble OC/TR and low-dose recombinant interleukin-2 (IL-2). This treatment had no effect on extraperitoneal disease. Therefore we studied in 13 patients whether this immunotherapeutic protocol resulted only in local or also in systemic immunomodulation. The phenotype of the *ex vivo* expanded lymphocytes was mainly $CD3^+, 4^-, 8^+, 16^-, 56^-$. Their OC/TR-re-targeted cytolytic activity against Igrov-1 ovarian-carcinoma cells was approximately as high in responders as in non-responders. Following most therapeutic cycles, the immunophenotype of lymphocytes recovered from the peritoneal fluid was similar to that of the infused T cells (*i.e.,* mainly $CD3^+, 4^-, 8^+$) and they were coated with OC/TR. However, cytolytic activity of the recovered lymphocytes against Igrov-1 cells was low in direct assays, and only slightly increased after additional *in vitro* re-targeting with OC/TR. Systemically, the i.p. immunotherapy resulted in a transient lymphopenia lasting for about 7 days, low (*i.e.,* 5 to 13 ng/ml) serum concentrations of free, functional OC/TR, and very weak coating of circulating T lymphocytes with OC/TR. These peripheral-blood T lymphocytes did not exert OC/TR-re-targeted cytolytic activity. Thus, locoregional OC/TR-re-targeted cellular immunotherapy resulted in substantial local immunomodulation and anti-tumor effects but virtually no systemic immunomodulation. *Int. J. Cancer* 73:211–219, 1997.
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The long-term results of chemotherapy of advanced ovarian carcinoma are disappointing because of the high frequency of relapse. In addition, immunotherapy of ovarian cancer using monoclonal antibodies (MAbs) or cytokines has met with only limited successes, *i.e.,* reduction of malignant ascites, or low response rates of short duration (Buckman *et al.,* 1992; Steis *et al.,* 1990). Adoptive immunotherapy with tumor-specific autologous cytotoxic T lymphocytes (CTL) has thus far not been feasible due to difficulties in generating sufficient numbers of such CTL (Ioannides *et al.,* 1991).

Re-targeting CTL with bi-specific monoclonal antibodies (bs-MAbs) directed to the CD3 molecule of the T-cell-receptor complex and a tumor-associated antigen on tumor cells combines the cytolytic potential of *in vitro*-expanded T lymphocytes with the tumor selectivity of MAbs (Van Dijk *et al.,* 1989). This approach has been effective in reducing or eliminating human tumors from xenotransplanted mice (Renner *et al.,* 1994), and has been clinically effective against malignant glioma (Nitta *et al.,* 1990), ovarian carcinoma (Canevari *et al.,* 1995) and malignant ascites of adenocarcinoma (Kroesen *et al.,* 1993).

The bs-MAb OC/TR is specific for CD3 on T lymphocytes and for a high-affinity folate-binding protein (MOv18) which is over-expressed by about 90% of ovarian carcinomas (Mezzanzanica *et al.,* 1988; Miotti *et al.,* 1987). OC/TR triggers the specific lysis of ovarian-carcinoma cells by *in vitro*-activated peripheral-blood T lymphocytes from healthy donors and ovarian-carcinoma patients (Lamers *et al.,* 1992; Pupa *et al.,* 1988). We have treated patients with advanced ovarian carcinoma i.p. with *ex vivo*-activated and -expanded autologous T lymphocytes re-targeted with OC/TR (Canevari *et al.,* 1995). The cellular therapy was combined with i.p. administration of IL-2 and soluble OC/TR to sustain proliferation and recycling of OC/TR-re-targeted cytotoxic T lymphocytes (Blank-Voorthuis *et al.,* 1993). We observed only local anti-tumor effects, and no effects on systemic, *i.e.,* extraperitoneal disease. The absence of systemic therapeutic effects might be related to the absence of systemic immunomodulating effects of therapy. Therefore, we studied (i) the correlation between immunological characteristics of the therapeutically used T lymphocytes and local immunological and anti-tumor effects, and (ii) whether or not the locoregional immunotherapy resulted in systemic immunomodulation.

MATERIAL AND METHODS

Patients and treatment schedules

Immunological studies were performed in 13 patients with advanced, non-mucinous epithelial, $MOv18^+$ ovarian carcinoma (FIGO stage III), and with residual tumor lesions less than 2 cm thick after debulking surgery. Eleven patients were treated with $F(ab')_2$ fragments of murine bs-MAb (OC/TR) and 2 with $F(ab')_2$ fragments of chimeric human-mouse bs-MAb (ch-OC/TR) (Warnaar *et al.,* 1994). The patients received i.p. infusions of *ex vivo* activated, expanded, bs-MAb-re-targeted autologous T lymphocytes, combined with soluble bs-MAb and low-dose IL-2 according to 3 protocols (Table I). The phase-I/II study (8 patients) is referred to as protocol 1, the phase-II study (3 patients) as protocol 2 and the ch-OC/TR pilot study (2 patients) as protocol 3 in this and subsequent sections.

Activation, expansion and re-targeting of T lymphocytes

Peripheral-blood mononuclear cells (PBMC) were obtained by a single leukapheresis 14 days before the start of the first series of infusions of bs-MAb-re-targeted T lymphocytes, and 10^8 PBMC were activated and expanded using PHA (HA16; Wellcome, Dartford, UK) and IL-2 (Proleukin; Eurocetus, Amsterdam, The Netherlands) as described (Canevari *et al.,* 1995; Lamers *et al.,* 1992). These expanded T lymphocytes were used for the first 2 series of infusions in all 3 protocols. The remainder of the apheresis product ($2 - 3 \times 10^8$ PBMC) was cryopreserved, thawed 2 weeks later, and served as a lymphocyte source for *ex vivo* expansion to enable a third cycle of infusions (protocol 1 only). Prior to i.p. administration, the *ex vivo*-activated and -expanded T lymphocytes were re-targeted with $F(ab')_2$ fragments of (ch-)OC/TR (Centocor Europe, Leiden, The Netherlands) at 1 mg per 10^9 viable cells by 30-min incubation at 37°C. The bs-MAb-re-targeted T lympho-

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TABLE I – TREATMENT SCHEDULES

Phase/treatment cycle	Day of therapy relative to leukapheresis	1st infusion[1,2]	2nd infusion[1,2]
Protocol 1 (Phase I/II: murine OC/TR)			
I—Cell dose escalation	14	10^6 Ly × OC/TR[3] + IL-2	IL-2
	15	10^7 Ly × OC/TR[3] + IL-2	IL-2
	16	10^8 Ly × OC/TR[3] + IL-2	IL-2
	17	10^9 Ly × OC/TR[3] + IL-2	IL-2
II—1st therapeutic cycle	20, 21, 22, 23, 24	10^9 Ly × OC/TR[4] + IL-2	OC/TR + IL-2
	25, 26	OC/TR + IL-2	OC/TR + IL-2
II—2nd therapeutic cycle	41, 42, 43, 44, 45	10^9 Ly × OC/TR[4] + IL-2	OC/TR + IL-2
	46, 47	OC/TR + IL-2	OC/TR + IL-2
Protocol 2 (Phase II: murine OC/TR)			
1st therapeutic cycle	14, 15, 16, 17, 18	10^9 Ly × OC/TR[4] + IL-2	OC/TR + IL-2
	19, 20	OC/TR + IL-2	OC/TR + IL-2
2nd therapeutic cycle	21, 22, 23, 24, 25	10^9 Ly × OC/TR[4] + IL-2	OC/TR + IL-2
	26, 27	OC/TR + IL-2	OC/TR + IL-2
Protocol 3 (chimeric human/murine OC/TR)			
1st therapeutic cycle	14, 15, 16, 17, 18	10^9 Ly × ch-OC/TR[4] + IL-2	ch-OC/TR + IL-2
2nd therapeutic cycle	21, 22, 23, 24, 25	10^9 Ly × ch-OC/TR[4] + IL-2	ch-OC/TR + IL-2

[1]The 1st infusion was administered at 12 a.m. and the 2nd infusion at 8 p.m.–[2]Ly × (ch-)OC/TR, autologous T lymphocytes loaded with (ch-)OCTR bs-MAb; IL-2, 0.6 × 10^6 IU IL-2; (ch-)OC/TR, 1 mg soluble (ch-)OC/TR bs-MAb. All infusions were given in 500 ml of Ringer's lactate solution supplemented with 1% human serum albumin.–[3]Unbound OC/TR bs-MAb was removed by washing.–[4]Unbound (ch-)OC/TR bs-MAb was not removed.

cytes were washed to remove unbound bs-MAb during the cell-dose-escalation cycle (protocol 1), but not during the treatment cycles (all 3 protocols).

Immunomonitoring schedule

The day of leukapheresis was designated day 0. EDTA-anti-coagulated venous blood samples were obtained for assessments of absolute lymphocyte counts, peritoneal fluid and heparinized blood samples for immunophenotyping assays and assessments of cytolytic activities, and serum samples for quantification of free functional bs-MAb at various time points before, during and after therapy, as indicated in ''Results''.

PBMC were isolated from blood samples by Ficoll-Isopaque density centrifugation. Serum and peritoneal-fluid samples were cleared from cells and debris by centrifugation (1500 *g* for 10 min) and stored at −80°C until further investigation. Immunophenotyping was performed on fresh PBMC and peritoneal cells at all time points, while cytotoxicity assays were performed on cryopreserved and thawed PBMC to allow simultaneous testing of all samples from each patient.

Flow-cytometric immunophenotyping and detection of cell-bound OC/TR

The following MAb mixtures were used for immunophenotyping: CD45 (anti-HLE-1/FITC) + CD14 (anti-Leu M3/PE), CD3 (anti-Leu 4/FITC) + CD56 (anti-Leu 19/PE), CD4 (anti-Leu 3ab/FITC) + CD8 (DAKO-CD8/PE), CD19 (anti-Leu12/FITC) + CD16 (anti-Leu 11c/FITC) and mouse (m) IgG1/FITC + mIgG1/PE as isotype controls. The CD8 MAb was obtained from DAKO (Glostrup, Denmark), all others from Becton Dickinson (San Jose, CA). Detection of OC/TR bound to PBMC or peritoneal-fluid lymphocytes was determined using goat anti-mouse (GAM) IgG1/PE, with GAM IgG2a/PE as conjugate control (both from Southern Biotechnology, Birmingham, AL). Absolute lymphocyte counts, immunostaining and flow cytometry were performed as described (Gratama *et al.,* 1996).

Cytotoxicity assays

Cytolytic activity was determined in a standard 3-hr ^{51}Cr-release assay (Lamers *et al.,* 1992). From the percentages of specific lysis derived from 4 effector- to -target ratios (*i.e.,* 50:1, 25:1, 12.5:1 and 6.3:1), the weighed mean of specific lysis (WMSL) was calculated as

$$\text{WMSL} = \tfrac{1}{4} \times \sum_{i=1}^{4} (\text{SLi/ETi}) \times 17.7$$

where SLi is the percentage of specific lysis at E:T ratio ETi, and ETi is E:T ratio i. The WMSL expresses the average cytolytic activity standardized at E:T ratio = 17.7, *i.e.,* the mean log E:T ratio. For the bs-MAb-re-targeted cytotoxicity assay, the MOv18-antigen-positive ovarian-carcinoma-derived cell line Igrov-1 (Bénard *et al.,* 1985) was used as target cell. Effector T lymphocytes were pre-incubated with 200 ng/ml OC/TR F(ab′)$_2$ at 37°C for 30 min, and OC/TR-re-targeted cytotoxicity was expressed as ΔIgrov, *i.e.,* the increase (Δ) of WMSL of Igrov-1 cells by OC/TR-re-targeted effector cells relative to effector cells that had not been re-targeted. The OKT3 hybridoma cell line, expressing membrane-bound CD3 MAb, served as a positive target-cell control for reversed-antibody (CD3)-dependent cellular cytotoxicity (ADCC). The K562 erythroleukemia and Daudi Burkitt's-lymphoma cell lines were used as target cells for the assessment of natural-killer(NK) and lymphokine-activated-killer(LAK) activity respectively.

For the detection of functional unbound bs-MAb in serum and peritoneal fluid, the CD3$^+$, 4$^-$, 8$^+$, TCRαβ$^+$ CTL clone D11 (Bolhuis and Van de Griend, 1985) was incubated in serial dilutions of bs-MAb (*i.e.,* 0, 0.01, 0.1, 1, 10, 100 and 1000 ng/ml in human AB serum), or in patient serum, for 60 min at 37°C. The D11 cells were then washed and processed in a 3-hr ^{51}Cr-release assay against Igrov-1 target cells at 8 E:T ratios ranging from 50:1 to 1.25:1. Per experiment, the serial dilutions of bs-MAb were plotted on a logarithmic scale against the corresponding WMSL value (at E:T ratio = 7.9:1, *i.e.,* the mean log E:T ratio), yielding a sigmoid standard curve. From the linear part of that curve, the concentration of free functional bs-MAb in patient serum was determined by interpolation. The lower limit of detection of this assay was 1 ng/ml.

RESULTS

Clinical anti-tumor response

The clinical anti-tumor response was investigated by laparotomy in 12 of the 13 patients and classified according to standard WHO criteria (Canevari *et al.,* 1995): 2 partial remissions (PR), 1 stable

disease (SD) and 5 progressive disease (PD) in protocol 1; 1 complete remission (CR), 1 PR and 1 PD in protocol 2 and 1 PR in protocol 3. Laparotomy was not performed in the second patient in protocol 3.

Immunophenotype and cytolytic activities of ex vivo*-expanded lymphocytes*

During the 14 to 25 days of *ex vivo* expansion, the $CD3^+$, 4^-, 8^+, 16^-, 56^- immunophenotype became predominant in all protocols (Fig. 1, and data not shown). Patients treated as per protocols 2 and 3 received fewer $CD3^+$ lymphocytes than those treated as per protocol 1 ($p = 0.01$, Wilcoxon's test; Table II). That difference was partly due to the fact that patients in protocol 1 were scheduled to receive a total of 11.1×10^9 OC/TR-re-targeted lymphocytes in 3 cycles *vs.* 10.0×10^9 lymphocytes in 2 cycles according to protocols 2 and 3. In addition, the *ex vivo* expanded lymphocytes of 3 of the 5 patients in protocols 2 and 3 contained relatively many lymphocytes with a NK phenotype (*i.e.,* $CD3^-$, 16^+, 56^+) and, therefore, relatively low proportions of $CD3^+$ lymphocytes (Fig. 1, and data not shown). The reason for the preferential expansion of NK cells in these 3 patients was unknown; the frequencies of $CD16^+$, 19^- and $CD3^-$, 56^+ lymphocytes after *ex vivo* expansion did not correlate with the proportions of such cells in the apheresis products from which the cultures were initiated. The total numbers of $CD4^+$, $CD8^+$, $CD16^+$ and $CD56^+$ lymphocytes administered to the patients in protocols 1 *vs.* 2 and 3 were not significantly different (Table II).

The CD3 MAb-dependent and OC/TR-re-targeted cytolytic activity of the unstimulated PBMC in the apheresis products was, as expected, similar to that observed with PBMC from healthy donors (Fig. 1; Lamers *et al.,* 1992). Lymphocytes derived from fresh PBMC exerted significant levels of CD3-MAb-dependent cytolytic activity (median WMSL 79%, range 25 to 128%) and OC/TR-re-targeted cytolysis (ΔIgrov, 35%, range 5 to 86%) at the start of the first therapeutic cycle (following 14 or 20 days of *ex vivo* activation; Fig. 1). Lymphocytes derived from cryopreserved PBMC exerted lower levels of CD3 MAb-dependent and OC/TR-re-targeted (ΔIgrov) cytolytic activity at the start of the second therapeutic cycle of protocol 1 (following 17 days of *ex vivo* activation): median 52% (range 0 to 113%) and 27% (3 to 46%) respectively. The difference between cytolytic activity exerted by lymphocytes derived from fresh and from cryopreserved PBMC was not significant. The NK and LAK activity of the activated and expanded lymphocytes showed wide variation between patients (data not shown).

The immunophenotype (data not shown) and cytolytic activity of the *ex vivo* expanded lymphocytes at the first and last days of each therapeutic cycle did not differ significantly between the 5 patients with an anti-tumor response (CR or PR) and the 7 patients without anti-tumor response (SD or PD). Specifically, the median (range) WMSL of OC/TR-re-targeted cytolytic activity (ΔIgrov) of lymphocytes infused at the first and last days of each of the 2 therapeutic cycles was 38% (6 to 58%) in responding patients and 42% (11 to 51%) in non-responding patients.

Immunophenotype and cytolytic activities of peripheral-blood and peritoneal-fluid lymphocytes

Before treatment, most patients had lower absolute lymphocyte counts than healthy individuals (Fig. 2). In particular, absolute counts of $CD3^+$ (Fig. 2) and $CD4^+$ (not shown) T lymphocytes were lower, whereas those of $CD8^+$ (Fig. 2) T, $CD56^+$, 3^- and $CD16^+$ NK, and $CD19^+$ B lymphocytes (not shown) were normal. During the first therapeutic cycle, absolute lymphocyte counts decreased transiently (*i.e.,* for about 7 days). The magnitude of this transient drop in absolute lymphocyte counts was less during the second therapeutic cycle. Immunological assays had been scheduled only before and after each cycle. At these time points, absolute counts of lymphocytes and their sub-sets did not differ significantly from pre-treatment values (Fig. 2, and data not shown). The levels of OC/TR-re-targeted cytolytic activity (ΔIgrov) by pre-treatment PBMC were low (median 7%, range 1 to 48%), as was to be expected from unactivated T lymphocytes (Fig. 2; Lamers *et al.,* 1992). Post-treatment PBMC exerted only low levels of cytolytic activity against the Igrov-1 ovarian-carcinoma cell line in spite of the fact that they were coated with bs-MAb (see Figs. 2, 4, and below). In addition, the levels of OC/TR-re-targeted cytolytic activity of PBMC after *in vitro* pre-incubation with 200 ng/ml OC/TR were similarly low after each therapeutic cycle (Fig. 2).

Before immunotherapy, the proportions of peritoneal-fluid lymphocytes expressing T-cell markers were similar to those in blood, *i.e.,* approximately 75%, being $CD3^+$, with a ratio of about 1 between $CD4^+$ and $CD8^+$ sub-sets (Fig. 3, and data not shown). The lymphocytes recovered from the peritoneal fluid following the therapeutic cycles had an immunophenotype (*i.e.,* $CD3^+$, 4^-, 8^+, 16^-, 56^-) similar to the *ex vivo*-expanded lymphocytes (Fig. 3, and data not shown). The pre-treatment level of OC/TR-re-targeted cytolysis (ΔIgrov) of intraperitoneal lymphocytes was as low (median 6%, range 3 to 22%) as that of PBMC (Figs. 2, 3). The lymphocytes recovered from the peritoneal fluid following the dose escalation and therapeutic cycles exerted higher levels of OC/TR-re-targeted cytolytic activity than those isolated from the peritoneal fluid prior to therapy, but lower levels of OC/TR-re-targeted cytolytic activity than *ex vivo*-expanded T lymphocytes (Figs. 1, 3). Of note, post-treatment peritoneal-fluid lymphocytes exerted only low levels of cytolytic activity against the Igrov-1 ovarian-carcinoma cell line, in spite of the fact that they were still coated with bs-MAb (see Figs. 3, 4, and below).

In vivo *coating of peritoneal-fluid and peripheral-blood lymphocytes with bs-MAb*

The presence of bs-MAb on peritoneal-fluid lymphocytes was visualized by a rightward shift of the majority of the lymphocyte population in the fluorescence histogram after labeling with GAM IgG1/PE. Data from patient 9 (protocol 2) are shown in Figure 4. A comparison of the lower left 2 panels of Figure 4 reveals the presence of bs-MAb on the lymphocytes recovered from the peritoneal fluid 1 day after the first therapeutic cycle. Similarly, placement of the marker at the foot of the peak of the GAM IgG2a/PE conjugate control staining revealed a median of 61% (range 9 to 85%) positive lymphocytes at the end of the first therapeutic cycle in 10 patients (protocol 1, $n = 6$; protocol 2, $n = 2$; protocol 3, $n = 2$). Lymphocytes recovered from patient 9's peritoneal fluid at 12 hr after the last OC/TR infusion of the second therapeutic cycle showed 51% positivity (Fig. 4, lower right panel). She had not developed human anti-mouse antibodies (HAMA) at detectable levels by that time, *i.e.,* 14 days after the start of bs-MAb and lymphocyte infusions. However, OC/TR was only barely detectable (6 and 11%) on peritoneal-fluid lymphocytes of 2 patients in protocol 1 at the end of the second therapeutic cycle, 28 days after start of bs-MAb and lymphocyte infusions. Both patients had developed HAMA (Lamers *et al.,* 1995).

On peripheral-blood lymphocytes, we also detected OC/TR during therapy. Labeling of patient 9's PBMC with GAM/IgG1/PE revealed a dim staining pattern after the first (day 21 relative to leukapheresis) and during the second therapeutic cycle (day 25) as compared with pretreatment (day 14). Lymphocyte-bound OC/TR became undetectable 12 hr after the last OC/TR infusion (day 28; Fig. 4, upper panels).

Serum concentrations of functional unbound bs-MAb

The bs-MAb-re-targeted CTX assay was also used to quantify the concentrations of functional unbound (ch-)OC/TR in patient sera. Lymphocytes derived from the $CD3^+$, 8^+ T-cell clone D11 were pre-incubated with patient sera before their use in a CTX assay against Igrov-1 cells. Functional unbound OC/TR became detectable (5 to 13 ng/ml) in sera of 3 of 4 patients in protocol 1 12 hr after the last i.p. OC/TR infusion of the first therapeutic cycle. However, functional unbound OC/TR was no longer detectable in

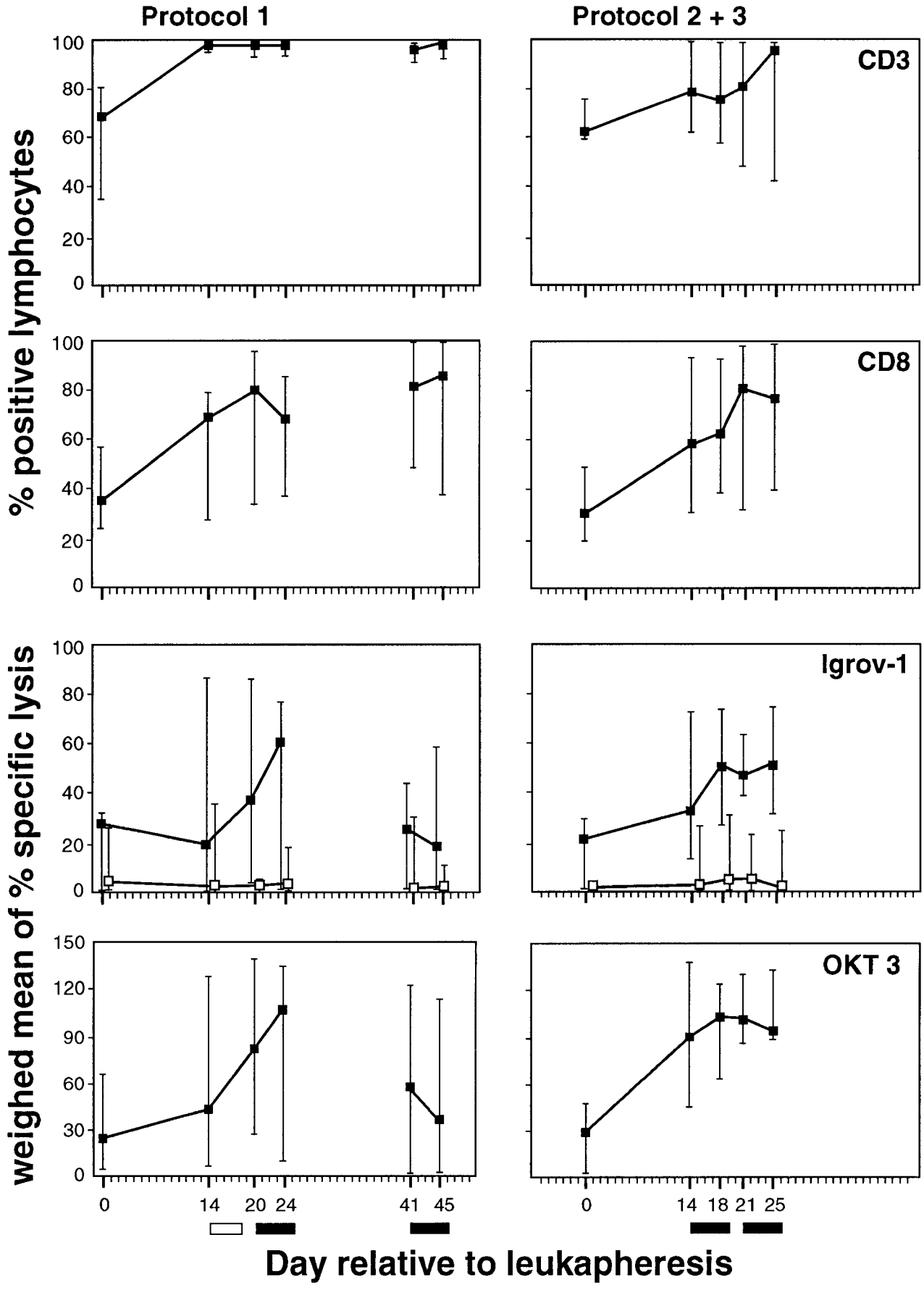
Protocol 1
Protocol 2 + 3
CD3
CD8
Igrov-1
OKT 3
% positive lymphocytes
weighed mean of % specific lysis
Day relative to leukapheresis
0
14
20
24
41
45
18
21
25

FIGURE 1

TABLE II – NUMBERS OF RE-INFUSED *EX VIVO*-EXPANDED LYMPHOCYTES: IMMUNOPHENOTYPICAL ANALYSIS

CD markers	Protocol 1 (n = 8)		Protocol 2 + 3 (n = 5)
	Treated as per protocol (n = 5)[1]	Incomplete treatment (n = 3)[2]	Treated as per protocol[1]
CD3	10.4 (10.0–10.7)[3]	6.9 (4.1–7.5)[3]	8.1 (5.2–9.9)[3]
CD4	3.7 (1.8–3.4)	3.1 (1.6–4.2)	1.8 (0.3–6.1)
CD8	8.7 (8.3–9.0)	3.6 (1.6–5.0)	7.0 (3.6–9.6)
CD16	0.2 (0.0–0.6)	0.2 (0.0–0.2)	1.9 (0.0–4.8)
CD56	0.1 (0.0–0.6)	0.2 (0.1–0.2)	1.7 (0.0–4.8)
Total lymphocytes	10.7 (10.1–11.1)	7.1 (4.3–7.8)	10.0 (10.0)

[1]Patients were considered as having been treated as per protocol if they had received >90% of the scheduled cell number (*i.e.,* 11.1×10^9).–[2]Two patients received only 70% and 39% of the planned cell numbers, respectively, due to slow *ex vivo* expansion of their T lymphocytes. The third patient received only 64% of the planned cell number because therapy had to be abrogated after the first day of the second therapeutic cycle, due to her poor clinical condition (unrelated to the immunotherapy).–[3]Median (range) number of cells infused ($\times 10^9$) during the entire treatment period (*i.e.,* cell-dose escalation, 1st and 2nd therapeutic cycle).

their sera 12 hr after the final i.p. OC/TR infusion of the second therapeutic cycle. All these 4 patients had developed HAMA at the end of the second therapeutic cycle (Lamers *et al.,* 1995). In contrast, functional unbound OC/TR was still detectable (8 to 9 ng/ml) in the sera of 2 patients in protocol 2 12 hr after the final i.p. OC/TR infusion of the second therapeutic cycle. These patients did not develop detectable HAMA until 15 to 25 days after completion of therapy (data not shown). Functional unbound ch-OC/TR was detectable (6 ng/ml) in 1 of the 2 patients in protocol 3 at 2½ days after the final i.p. infusion of ch-OC/TR of the first therapeutic cycle, but not at the corresponding time point after the second therapeutic cycle. None of the 2 patients in protocol 3 developed HAMA (data not shown). Thus, functional unbound bs-MAb was present in the circulation for at least 12 hr after i.p. infusion, unless a HAMA response had developed.

DISCUSSION

I.p. immunotherapy of advanced ovarian carcinoma using autologous *ex vivo*-activated and -expanded T lymphocytes re-targeted with the bs-MAb OC/TR specific for CD3 and the folate-binding protein over-expressed by ovarian-carcinoma cells, can induce local tumor regressions as evidenced by a 27% overall response rate (Canevari *et al.,* 1995). However, this locoregional treatment had no effect on extraperitoneal disease. In view of these results, we studied (i) the correlation between immunological characteristics of the therapeutically used T lymphocytes, and local immunological and anti-tumor effects, and (ii) whether or not the locoregional immunotherapy resulted in systemic immunomodulation.

Although our activation and expansion protocol based on PHA + IL-2 resulted in the preferential expansion of $CD3^+$, 4^-, 8^+, 16^-, 56^- T lymphocytes, confirming our pre-clinical studies (Lamers *et al.,* 1992), there was considerable variation in the numbers of NK lymphocytes ($CD16^+$, 56^+, 3^-) administered to the patients (Table II). In addition, cytolytic activity of the expanded lymphocytes varied over a wide range (Fig. 2). However, neither immunophenotype (CD3, CD4, CD8, CD16, CD56) nor levels of cytolytic activity (OC/TR-re-targeted cytotoxicity, reversed ADCC, NK and LAK) of the infused lymphocytes correlated with an anti-tumor response (CR or PR *vs.* SD or PD).

Because the growth rate of T lymphocytes levels off to zero after 4 weeks of IL-2-supported culture (Lamers *et al.,* 1992), fresh PBMC cultures could not produce enough lymphocytes between days 35 and 39 of culture for the second therapeutic cycle of protocol 1. Hence, we used cryopreserved and thawed PBMC to generate lymphocytes for the second therapeutic cycle. The elimination of the 2-week rest period between the 2 therapeutic cycles in protocols 2 and 3 not only enabled the generation of all lymphocytes required for both therapeutic cycles from a single fresh PBMC culture, but also reduced the risk that HAMA development would interfere with therapeutic efficacy (Lamers *et al.,* 1995).

We studied local immunological effects of therapy by comparing the immunophenotype and cytolytic activities of peritoneal-fluid lymphocytes obtained before and 2 days after each series of lymphocyte infusions. The major immunophenotype in the lymphocyte cultures ($CD3^+$, 4^-, 8^+) was also found among peritoneal-fluid lymphocytes following most therapeutic cycles, but the latter T lymphocytes did not exert levels of OC/TR-re-targeted cytolytic activity as high as those exerted by the *ex vivo*-expanded T lymphocytes prior to infusion. Moreover, post-treatment peritoneal-fluid lymphocytes still coated with bs-MAb exerted only low levels of cytolytic activity against ovarian-carcinoma cells. As we reported earlier, OC/TR-re-targeted CTL cannot enter multiple lytic cycles *in vitro,* unless newly synthesized CD3 molecules on their surface have interacted with freshly added OC/TR bs-MAb (Blank-Voorthuis *et al.,* 1993). Therefore, infusions of soluble bs-MAb were administered which resulted in i.p. bs-MAb concentrations of approximately 1,500 ng/ml 12 hr post-infusion (data not shown). These infusions were combined with low-dose IL-2 (0.6×10^6 IU IL-2 per infusion) to maintain cytolytic activity of the infused T lymphocytes and to recruit such activity from resident T lymphocytes. Thus, the low cytolytic activity of bs-MAb-coated peritoneal-fluid T lymphocytes may be explained by induction of transient anergy by their interactions with $MOv18^+$ cells (Blank-Voorthuis *et al.,* 1993).

One of the systemic effects of the i.p. therapy was the transient (about 7 days) lymphopenia, especially during the first therapeutic cycle. That effect was due to the i.p. infusions of soluble bs-MAb and not to the infused bs-MAb-re-targeted lymphocytes and/or IL-2, since it was not observed during the dose-escalation phase when no soluble OC/TR was administered. A transient disappear-

FIGURE 1 – Immunophenotype and cytolytic activity of lymphocytes during *ex vivo* activation and expansion. Upper 4 panels: Proportions of lymphocytes (% of viable lymphocytes as defined by flow-cytometric light-scatter gating) expressing CD3 or CD8 during *ex vivo* activation and expansion using PHA and IL-2 (see ''Material and Methods''), relative to treatment protocol. Lower 4 panels: Cytolytic activity of *ex vivo* PHA-activated and IL-2-expanded lymphocytes against Igrov-1 ovarian-carcinoma cells without (open symbols) or with (closed symbols) OC/TR-re-targeting of the effector cells, and against OKT3 cells (*i.e.,* CD3-mediated or reversed ADCC) at various time points during culture relative to treatment protocol. Results are expressed as weighed mean of percent specific lysis at E/T ratio 17.7:1 (see ''Material and Methods''). Median values (indicated by symbols) and ranges (indicated by bars) of 8 patients in protocol 1 (left panels) and 5 patients in protocols 2 and 3 (right panels) are shown. The symbols below the X-axis represent the treatment cycles: open box, cell-dose escalation (phase I); closed boxes, therapeutic cycles (phase II).

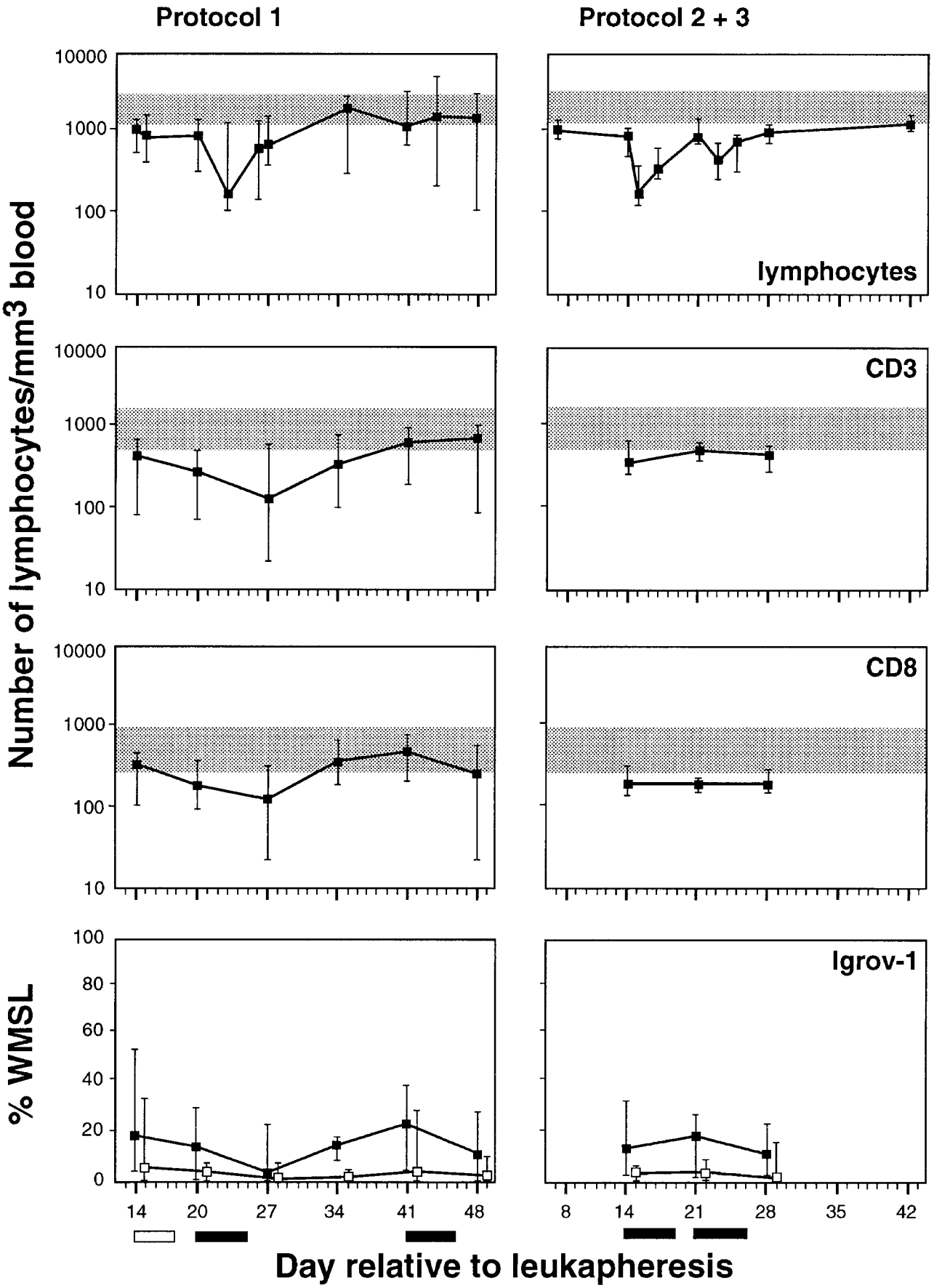
Protocol 1
Protocol 2 + 3
Number of lymphocytes/mm3 blood
% WMSL
lymphocytes
CD3
CD8
Igrov-1
10000
1000
100
10
100
80
60
40
20
0
14
20
27
34
41
48
8
14
21
28
35
42
Day relative to leukapheresis

FIGURE 2

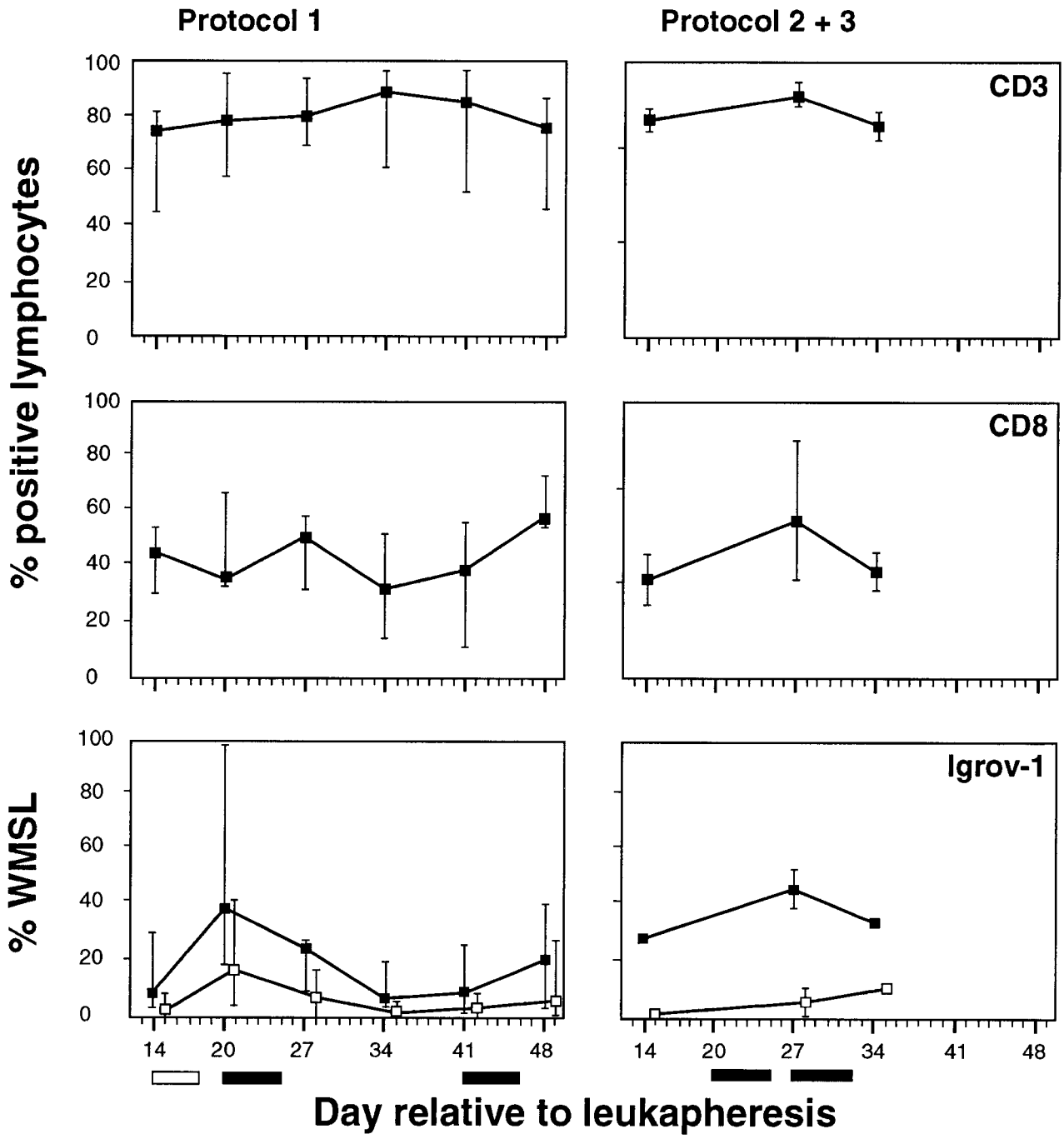

FIGURE 3 – Immunophenotype and cytolytic activity of lymphocytes in peritoneal fluid. Proportions of $CD3^+$ (upper 2 panels) and $CD8^+$ (middle 2 panels) lymphocyte sub-sets (expressed as % of total lymphocytes), and cytolytic activities against Igrov-1 ovarian-carcinoma cells without (open symbols) or with (closed symbols) OC/TR-re-targeting of the effector cells, by lymphocytes in peritoneal-fluid samples obtained before, during and after immunotherapy (lower 2 panels). Symbols represent median values; bars indicate ranges of observations per time point. See further the legend to Figure 1.

FIGURE 2 – Immunophenotype and cytolytic activities of peripheral-blood lymphocytes. Absolute numbers of total lymphocytes (upper 2 panels), $CD3^+$ and $CD8^+$ lymphocyte sub-sets (middle 4 panels), and cytolytic activities of PBMC against Igrov-1 ovarian-carcinoma cells without (open symbols) or with (closed symbols) OC/TR-re-targeting of effector cells (lower 2 panels) before, during and after immunotherapy. The shaded areas represent the normal ranges of absolute numbers of lymphocytes and lymphocyte sub-sets as defined by the 95% confidence intervals obtained in 72 apparently healthy individuals. Symbols represent median values; bars indicate ranges of observations per time point. See further the legend to Figure 1.

ance of bs-MAb-coated lymphocytes from the circulation also occurred between 4 and 48 hr following i.v. bolus administration of (CD3 × anti-tumor) bs-MAb (Kroesen *et al.*, 1994; Tibben *et al.*, 1996). The low serum bs-MAb levels in our study, which ranged between 5 and 13 ng/ml at 12 hr following the last i.p. bs-MAb infusion, were nevertheless sufficient for *in vivo* coating of peripheral-blood T lymphocytes and to cause their transient disappearance from the circulation, probably due to their interactions with $MOv18^+$ endothelial cells, but not the clinical syndrome of massive cytokine release by activation of T lymphocytes

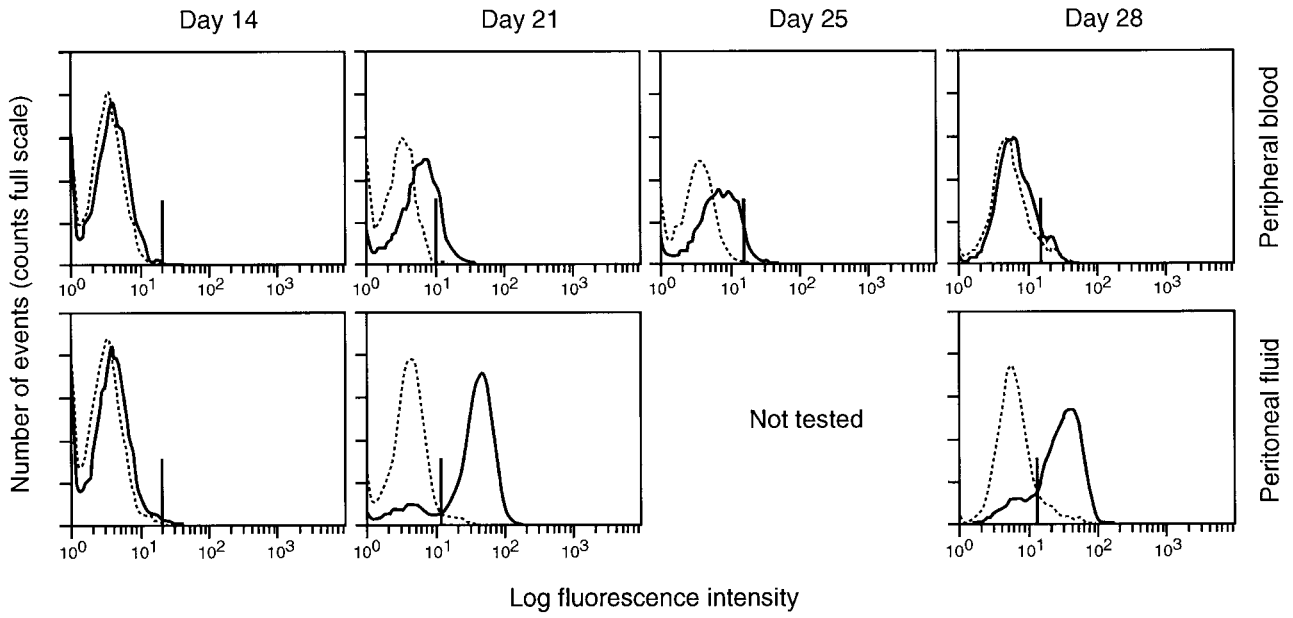

FIGURE 4 – Coating with OC/TR of peripheral-blood lymphocytes (upper 4 panels) and peritoneal-fluid lymphocytes (lower 3 panels) of patient 9 (protocol 2) before immunotherapy (day 14 after leukapheresis), 12 hr after the last OC/TR infusion of the first therapeutic cycle (day 21), during the second therapeutic cycle (day 25) and 12 hr after the last OC/TR infusion of the second therapeutic cycle (day 28). OC/TR coating was visualized by staining using GAM IgG1/PE (closed lines). Labeling with GAM IgG2a/PE (broken lines) served as conjugate control. The marker settings to discriminate between negative and positive fluorescence signals, set at the foot of the peak of the conjugate control, are shown.

(Kroesen *et al.,* 1994; Tibben *et al.,* 1996). These low serum bs-MAb concentrations only induce bs-MAb-re-targeted cytolysis by activated T lymphocytes such as cloned CTL (Van Ravenswaay Claasen *et al.,* 1993; this study) and *in vitro*-activated PBMC (Pupa *et al.,* 1988). The fact that post-treatment PBMC did not lyse Igrov-1 ovarian-carcinoma cells in spite of their coating with bs-MAb may be explained by anergy of their bs-MAb-retargeted T-cell receptors (Blank-Voorthuis *et al.,* 1993). That contention is supported by the observation that the PBMC after each treatment cycle did not exert significant levels of OC/TR-re-targeted cytolysis even after *in vitro* pre-incubation with 200 ng/ml OC/TR.

Taken together, i.p. infusions of bs-MAb-targeted T lymphocytes, soluble bs-MAb and IL-2 resulted in significant local immunomodulation and anti-tumor effects but virtually no systemic immunomodulation. The release of soluble bs-MAb from the peritoneal cavity resulted in serum bs-MAb concentrations that were sufficient for *in vivo* coating of peripheral-blood T lymphocytes and their transient disappearance from the circulation, but insufficient for their activation and induction of significant levels of bs-MAb-re-targeted cytotoxicity. The absence of systemic immunomodulation, in particular bs-mAb-retargeted cytolytic activity, may explain the lack of systemic antitumor effects of this locoregional immunotherapeutic protocol.

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REFERENCES

BÉNARD, J., DA SILVA, J., DE BLOIS, M.C., BOYER, P., DUVILLAND, P., CHIRIC, E. and RIOU, G., Characterization of a human ovarian adenocarcinoma line, IGROV1, in tissue culture and in nude mice. *Cancer Res.,* **45,** 4970–4979 (1985).

BLANK-VOORTHUIS, C.J.A.C., BRAAKMAN, E., RONTELTAP, C.P.M., TILLY, B.C., STURM, E., WARNAAR, S.O. and BOLHUIS, R.L.H., Clustered CD3/TCR complexes do not transduce activation signals after bi-specific-monoclonal-antibody-triggered lysis by cytotoxic T lymphocytes via CD3. *J. Immunol.,* **151,** 441–444 (1993).

BOLHUIS, R.L.H. and VAN DE GRIEND, R.J., PHA-induced proliferation and cytolytic activity in $T3^+$ but not in $T3^-$ cloned T lymphocytes requires the involvement of the T3 antigen for signal transduction. *Cell. Immunol.,* **93,** 46–57 (1985).

BUCKMAN, R. and 13 OTHERS, Intraperitoneal therapy of malignant ascites associated with carcinoma of ovary and breast, using radioiodinated monoclonal antibody 2G3. *Gynecol. Oncol.,* **47,** 102–109 (1992).

CANEVARI, S. and 17 OTHERS, Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T-lymphocytes re-targeted by a bi-specific monoclonal antibody: clinical proof of concept. *J. nat. Cancer Inst.,* **87,** 1463–1469 (1995).

GRATAMA, J.W., SCHMITZ, P.I.M., GOEY, S.H., LAMERS, C.H.J., STOTER, G. and BOLHUIS, R.L.H., Modulation of immune parameters in patients with metastatic renal-cell cancer receiving combination immunotherapy (IL-2, INFα and autologous IL-2 activated lymphocytes). *Int. J. Cancer,* **65,** 152–160 (1996).

IOANNIDES, C.G., PLATSOUCAS, C.D., RASHED, S., WHARTON, J.T., EDWARDS, C.L. and FREEDMAN, R.S., Tumor cytolysis by lymphocytes infiltrating ovarian malignant ascites. *Cancer Res.,* **51,** 4257–4265 (1991).

KROESEN, B.J., BUTER, J., SLEIJFER, D.T., JANSSEN, R.A.J., VAN DER GRAAF, W.T.A., THE, T.H., DE LEIJ, L. and MULDER, N.H., Phase-I study of intravenously applied bi-specific antibody in renal-cell-cancer patients receiving subcutaneous interleukin-2. *Brit. J. Cancer,* **70,** 652–661 (1994).

KROESEN, B.J., TER HAAR, A., SPAKMAN, H., WILLEMSE, P., SLEIJFER, D.T., DE VRIES, E.G.E., MULDER, N.H., BERENDSEN, H.H., LIMBURG, P.C., THE, T.H. and DE LEIJ, L., Local anti-tumor treatment in carcinoma patients with bi-specific-monoclonal-antibody-redirected T cells. *Cancer Immunol. Immunother.,* **37,** 400–407 (1993).

LAMERS, C.H.J., GRATAMA, J.W., WARNAAR, S.O., STOTER, G. and BOLHUIS, R.L.H., Inhibition of bi-specific-monoclonal antibody (bs-Ab)-targeted cytolysis by human anti-mouse antibodies in ovarian-carcinoma patients treated with bs-Ab-targeted activated T lymphocytes. *Int. J. Cancer,* **60,** 450–457 (1995).

LAMERS, C.H.J., VAN DE GRIEND, R.J., BRAAKMAN, E., RONTELTAP, C.P.M., BÉNARD, J., STOTER, G., GRATAMA, J.W. and BOLHUIS, R.L.H., Optimization of culture conditions for activation and large-scale expansion of human T lymphocytes for bi-specific-antibody-directed cellular immunotherapy. *Int. J. Cancer,* **51,** 973–979 (1992).

MEZZANZANICA, D., CANEVARI, S., MÉNARD, S., PUPA, S.M., TAGLIABUE, E., LANZAVECCIA, A. and COLNAGHI, M.I., Human ovarian-carcinoma lysis by cytotoxic T cells targeted by bi-specific monoclonal antibodies: analysis of the antibody components. *Int. J. Cancer,* **41,** 609–615 (1988).

MIOTTI, S., CANEVARI, S., MÉNARD, S., MEZZANZANICA, D., PORRO, G., PUPA, S.M., REGAZZONI, M., TAGLIABUE, E. and COLNAGHI, M.I., Characterization of human ovarian-carcinoma-associated antigens defined by novel monoclonal antibodies with tumor-restricted specificity. *Int. J. Cancer,* **39,** 297–303 (1987).

NITTA, T., SATO, K., YAGITA, H., OKUMURA, K. and ISHII, S., Preliminary trial of specific targeting therapy against malignant glioma. *Lancet,* **335,** 368–371 (1990).

PUPA, S.M., CANEVARI, S., FONTANELLI, R., MÉNARD, S., MEZZANZANICA, D., LANZAVECCHIA, A. and COLNAGHI, M.I. Activation of mononuclear cells to be used for hybrid-monoclonal-antibody-induced lysis of human ovarian-carcinoma cells. *Int. J. Cancer,* **42,** 455–459 (1988).

RENNER, C., JUNG, W., SAHIN, U., DENFELD, R., POHL, C., TRÜMPER, L., HARTMANN, F., DIEHL, V., VAN LIER, R. and PFREUNDSCHUH, M., Cure of xenografted human tumors by bi-specific monoclonal antibodies and human T cells. *Science,* **264,** 833–835 (1994).

STEIS, R.G. and 13 OTHERS, Intraperitoneal-lymphokine-activated killer-cell and interleukin-2 therapy for malignancies limited to the peritoneal cavity. *J. clin. Oncol.,* **8,** 1618–1629 (1990).

TIBBEN, J.G., BOERMAN, O.C., MASSUGER, L.F.A.G., SCHIJF, C.P.T., CLAESSENS, R.A.M.J. and CORSTENS, F.H.M., Pharmacokinetics, biodistribution and biological effects of intravenously administered bi-specific monoclonal antibody OC/TR F(ab′)$_2$ in ovarian-carcinoma patients. *Int. J. Cancer,* **66,** 477–483 (1996).

VAN DIJK, J., WARNAAR, S.O., VAN EENDENBURG, J.D.H., THIENPONT, M., BRAAKMAN, E., BOOT, J.H.A., FLEUREN, G.J. and BOLHUIS, R.L.H., Induction of tumor-cell lysis by bi-specific monoclonal antibodies recognizing renal-cell carcinoma and CD3 antigen. *Int. J. Cancer,* **43,** 344–349 (1989).

VAN RAVENSWAAY CLAASEN, H.H., VAN DE GRIEND, R.J., MEZZANZANICA, D., BOLHUIS, R.L.H., WARNAAR, S.O. and FLEUREN, G.J., Analysis of production, purification, and cytolytic potential of bi-specific antibodies reactive with ovarian-carcinoma-associated antigens and the T-cell antigen CD3. *Int. J. Cancer,* **55,** 128–136 (1993).

WARNAAR, S.O., DE PAUS, V., LARDENOIJE, R., MACHIELSE, B.N.M., DE GRAAF, J., BREGONJE, M. and VAN HAARLEM, H., Purification of bi-specific F(ab′)$_2$ from murine trioma OC/TR with specificity for CD3 and ovarian cancer. *Hybridoma,* **13,** 519–526 (1994).