

INHIBITION OF BISPECIFIC MONOCLONAL ANTIBODY (bsAb)-TARGETED CYTOLYSIS BY HUMAN ANTI-MOUSE ANTIBODIES IN OVARIAN CARCINOMA PATIENTS TREATED WITH bsAb-TARGETED ACTIVATED T-LYMPHOCYTES

Cor H.J. LAMERS¹, Jan W. GRATAMA¹, Sven O. WARNAAR³, Gerrit STOTER² and Reinder L.H. BOLHUIS^{1,4}

Departments of ¹Clinical and Tumor Immunology and ²Medical Oncology, Daniel den Hoed Cancer Center, Rotterdam; and ³Centocor Europe B.V., Leiden, The Netherlands.

T lymphocytes of 8 patients with ovarian cancer were targeted to the tumor cells using F(ab')₂ fragments of a bispecific monoclonal antibody (bsAb), specific for CD3 (a component of the T lymphocyte receptor for antigen) and for the folate receptor MOv18 (overexpressed by ovarian carcinoma cells) as part of a phase I/II study. Phase I (days 0 to 3) consisted of increasing intraperitoneal (i.p.) numbers (10⁶–10⁹) of bsAb-targeted T lymphocytes plus low-dose interleukin-2 (IL-2). Phase II (days 6 to 13, and 27 to 33) consisted of daily i.p. infusions of 10⁹ targeted T lymphocytes, 2 mg soluble bsAb, and low-dose IL-2. Using enzyme-linked immunosorbent assays (ELISA), human anti-mouse antibodies (HAMA) were detected in all patients: in the serum from day 13 onwards and in the peritoneal fluid from day 20 onwards. A significant proportion of the HAMA appeared to be directed against the idiotypes of the bsAb specific for CD3 and MOv18, as suggested by (1) the clearly higher ELISA titers against OC/TR bsAb as compared to those against a monoclonal antibody (MAb) with unrelated specificity, and (2) failure to abrogate the capacity of peritoneal fluid containing HAMA to block the binding of OC/TR bsAb to MOv18⁺ or CD3⁺ cells by absorption of human anti-mouse IgG-framework antibodies in peritoneal fluid to immobilized mouse IgG. The OC/TR-targeted cytotoxicity of the MOv18⁺ ovarian carcinoma cell line Igrov-1 by autologous T lymphocytes was inhibited by peritoneal fluid samples containing relatively high HAMA titers. Such inhibitory activity was never detected at the start of phase II, but coincided with the last series of i.p. infusions of targeted T lymphocytes in 2 patients.

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Mouse MAbs directed against tumor-associated antigens (TAA) and conjugated to radioisotopes, chemotherapeutic agents or toxins are increasingly being used for *in vivo* diagnosis and treatment of malignancies (Mach *et al.*, 1981; Schroff *et al.*, 1985). The infusion of murine immunoglobulins (Ig) elicits human anti-mouse Ig antibody (HAMA) responses. Such HAMA responses have been reported in clinical studies of tumor imaging (Courtenay-Luck *et al.*, 1986) and tumor therapy (Schroff *et al.*, 1985; Courtenay-Luck *et al.*, 1988; Saleh *et al.*, 1993). HAMA responses develop over a wide dose range (*i.e.*, from <1 mg to >1 g) (Khazali *et al.*, 1988), and occur more frequently after repeated administrations of MAbs (Lind *et al.*, 1991). Fab fragments of murine MAbs and genetically engineered mouse-human MAbs are less immunogenic than intact murine Ig molecules (reviewed by Khazali *et al.*, 1994). Initially, HAMA with specificities for the constant regions of murine Ig develop, followed by HAMA with anti-idiotypic specificities. These latter antibodies develop particularly well after multiple administrations of MAbs (Schroff *et al.*, 1985; Saleh *et al.*, 1993). Adverse clinical reactions upon administration of MAbs to patients with pre-existing HAMA are uncommon, but a highly increased clearance of the administered MAb is seen in such patients, which may prevent the MAbs from interacting with their target (Khazali *et al.*, 1994).

Targeting of the cytotoxic activity of T lymphocytes against tumor cells can be achieved using bispecific monoclonal antibodies (bsAbs) which are directed against a T-cell activation epitope on the one hand (*e.g.*, CD3) and a TAA on the other. Targeting of cytotoxic T lymphocytes (CTL) with bsAb

results in selective destruction of tumor cells following MHC-unrestricted binding of the CTL to tumor cells (Segal and Snider 1989; Van Dijk *et al.*, 1989). This approach has been effective in reducing or eliminating tumor in several mouse model systems (Segal and Snider, 1989), in human malignant glioma (Nitta *et al.*, 1990) and in ovarian carcinoma (Bolhuis *et al.*, 1992). The OC/TR bsAb is specific for CD3 and MOv18 (Mezzanzanica *et al.*, 1988). MOv18 has been identified as the glycosyl-phosphatidyl-inositol (GPI)-anchored, high-affinity folate-binding protein gp38 (Coney *et al.*, 1991), and is overexpressed on ovarian carcinoma cells as compared to normal ovarian cells. The OC/TR bsAb was used as part of the intraperitoneal (i.p.) treatment of ovarian carcinoma in a phase-I/II clinical trial (Bolhuis *et al.*, 1992). Patients entered into this trial received 10 therapeutic i.p. infusions with OC/TR and autologous OC/TR-targeted activated T lymphocytes. This report concerns the kinetics of HAMA responses in these patients and the interference of HAMA with bsAb-targeted cytotoxicity.

MATERIAL AND METHODS

Clinical study

Eight ovarian-carcinoma patients were treated with i.p. infusions of targeted T lymphocytes and the bsAb OC/TR in a phase-I/II clinical trial. Patients with advanced epithelial ovarian carcinoma (FIGO stage III, MOv18-positive tumor, and thickness of residual tumor lesions of less than 2 cm after debulking surgery), were eligible. T lymphocytes were activated *in vitro* and expanded using phytohemagglutinin (PHA; Wellcome, Dartford, UK) and interleukin-2 (IL-2; Proleukin, Eurocetus, Amsterdam, The Netherlands) as described previously (Lamers *et al.*, 1992). The bsAb OC/TR F(ab')₂ fragments were produced by Centocor Europe (Leiden, The Netherlands). Prior to i.p. administration, the cultured T lymphocytes were incubated for 30 min at 37°C with OC/TR bsAb (1 mg per 10⁶ to 10⁹ viable cells; Table I). Thereafter, unbound bsAb was washed away in phase I but was not removed in phase II, and 0.6 × 10⁶ IU IL-2 were added prior to administration in all cases. According to the schedule (Table I) the patients were to receive 3 cycles of i.p. therapy of autologous OC/TR-labeled T lymphocytes, soluble OC/TR and IL-2. Thus, they should have received a total of 10 × 10⁹ OC/TR-labeled T lymphocytes, 28 mg OC/TR bsAb and 16.8 × 10⁶ IU IL-2 during phase II. The actual treatment differed from schedule in 3 of the 8 patients. Patient 2 received a third therapeutic cycle from days 48 to 52 because her first therapeutic cycle was considered inadequate due to insufficient fluid distribution in the peritoneal cavity. For patient 6,

⁴To whom correspondence and reprint requests should be sent, at the Department of Medical and Tumor Immunology, Daniel den Hoed Cancer Center, P.O. Box 5201, 3008 AE Rotterdam, The Netherlands. Fax: 31 10 4 841 885.

the interval between phase I and the first therapeutic cycle of phase II was extended to 14 days because of slow *in vitro* expansion of the autologous T lymphocytes. Treatment of patient 8 was abrogated on day 28 (*i.e.*, after the first day of the second therapeutic cycle) due to her poor clinical condition. Serum and peritoneal fluid samples were taken prior to the first infusion and on days 6, 13, 27, 34, 49, and around days 70 and 100. Serum and peritoneal fluid samples were cleared from cells and debris by centrifugation and stored at -80°C until further investigation.

Detection of human anti-mouse antibodies (HAMA)

The presence of HAMA in serum and peritoneal fluid samples was assessed using a 2-step sandwich enzyme-linked immunosorbent assay (ELISA). As solid phase, F(ab')_2 fragments of the OC/TR bsAb ($\text{IgG}_1/\text{IgG}_1$), of the 323/A3 MAb (an IgG_1 , κ MAb with pan-carcinoma specificity; Edwards *et al.*, 1986) and of the MOv18 MAb (IgG_1 , κ) were used (all from Centocor Europe, Leiden). The tracer antibody was goat anti-human Fc(IgG) conjugated with alkaline phosphatase (Affipure; Jackson Immunotech, West Grove, PA) and the substrate was p-nitrophenylphosphate disodium dissolved in 2-amino-2-methyl-1-propanol (Sigma, St. Louis, MO) at pH 10.3. The wells of 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with $2\ \mu\text{g}$ of antibody in $50\ \mu\text{l}$ of 0.1 M glycine, 0.5 mM EDTA, pH 8.5, for 16 hr at 4°C . After washing with PBS (pH 7.4), free binding sites on the matrix were blocked with $150\ \mu\text{l}$ PBS containing 1% BSA, for 1 hr at 37°C . The assay samples ($80\ \mu\text{l}/\text{well}$), diluted in PBS + 1% BSA, were incubated for 2 hr at 37°C . The initial dilution was 1:20. A HAMA-positive serum and a pool of normal human sera were run with each assay plate as positive and negative controls, respectively. The plates were washed 5 times with PBS containing 0.1% Tween-20 and incubated with $75\ \mu\text{l}/\text{well}$ of tracer antibody (diluted 1:2,000 in PBS + 1% BSA) for 1 hr at 37°C . After washing with PBS + 0.1% Tween-20, the plates were incubated with $100\ \mu\text{l}/\text{well}$ substrate for 30 min at 37°C , and the reaction was abrogated by adding $50\ \mu\text{l}$ 3N NaOH. The optical density (OD) was read at 405 nm. The reciprocal dilution of the serum sample with $\text{OD}_{405} = 1.0$ was arbitrarily referred to as the HAMA titer.

Removal of human antibodies to murine IgG from peritoneal fluid

Human anti-murine IgG antibodies were removed from sera and peritoneal fluid by affinity absorption using mouse IgG (Sigma) coupled to Affi-gel 10 (BioRad, Richmond, CA). For coupling, 10 mg mouse IgG in 0.5 ml 1 M NaCl, 80 mM CaCl_2 and 0.1 M HEPES (pH 7.2), was incubated with 1 ml Affi-gel 10 for 4 hr at 4°C on a roller bank. Residual reactive Affi-gel binding sites were blocked by incubation with 0.1 M ethanolamine-HCl for 1 hr. For absorption of human antibodies to murine IgG, 1 ml peritoneal fluid was mixed with 0.25 ml of coupled Affi-gel 10 and incubated for 16 hr at 4°C on a roller bank. Thereafter, the peritoneal fluid was separated from the Affi-gel 10 by centrifugation. This absorption procedure was repeated once with freshly coupled Affi-gel 10. The processed peritoneal fluid samples were tested for residual HAMA using the ELISA described above.

Flow cytometric assay of HAMA-mediated inhibition of OC/TR bsAb binding to Igrov-1 and D11 cells

The MOv18⁺ ovarian carcinoma-derived cell line Igrov-1 (Bénard *et al.*, 1985) and the $\text{CD}3^+$, 4^+ , 8^+ , $\text{TCR}\alpha\beta^+$ CTL clone D11 (Van de Griend *et al.*, 1984) were used to analyze HAMA-mediated inhibition of binding of OC/TR bsAb to these cells. From each cell line, $50\ \mu\text{l}$ containing 0.50×10^6 cells were incubated for 30 min at 0°C with $50\ \mu\text{l}$ of (1) PBS + 1% BSA; (2) $1\ \mu\text{g}/\text{ml}$ OC/TR bsAb; (3) W6/32 (anti-HLA-A,B,C MAb obtained from SeraLab, Crawley Down, UK) diluted in PBS + 1% BSA; (4) HAMA containing peritoneal

fluid (diluted 1:2 in PBS + 1% BSA); (5) same as (4), with OC/TR bsAb; and (6) same as (4), with W6/32 MAb added 30 min prior to the assay. Thereafter, the cells were washed once and incubated with goat-anti-mouse Ig conjugated with phycoerythrin (PE; Southern Biotechnology, Burlington, VA) for another 30 min at 0°C . After washing once, the cells were fixed in PBS containing 10 mg/ml paraformaldehyde and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). For data acquisition, list mode data of 10,000 cells per sample were obtained. In the analyses of those data, dead cells and cell fragments were excluded on the basis of their low forward light scatter signals, after which PE fluorescence characteristics were studied using histogram statistics.

Cytotoxicity (CTX) assay

Cytolytic activities were assessed in a standard 3-hr ^{51}Cr -release assay, using T lymphocytes from ovarian cancer patients, activated and expanded using PHA + IL-2, and the CTL clone D11, specific for HLA-Cw3, as effector cells (Bolhuis and Van de Griend, 1985). The ovarian carcinoma cell line Igrov-1 and the Epstein-Barr virus-transformed, HLA-Cw3⁺ lymphoblastoid cell line BSM served as the source of target cells. The CTX assay medium was RPMI-1640 supplemented with 10% newborn bovine calf serum (GIBCO, Grand Island, NY). The effector cells were pre-incubated with OC/TR for 30 min at 37°C ; $100\ \mu\text{g}/\text{ml}$ of OC/TR were sufficient for optimal targeting of cloned and bulk-cultured CTL (Mezzanzanica *et al.*, 1988). In one experiment, chimeric F(ab')_2 OC/TR (chOC/TR; Centocor Europe), in which the murine constant part of the antibody was replaced by the corresponding human constant part, was used to target D11 cells. Varying numbers of effector lymphocytes were seeded in triplicate in 96-well, round-bottomed microtiter plates ($100\ \mu\text{l}/\text{well}$), followed by 2,500 target cells in $100\ \mu\text{l}$ per well. The target cells were labeled with $100\ \mu\text{Ci}$ ^{51}Cr per 0.5×10^6 cells for 2 hr at 37°C . The effector to target (E:T) ratios used in most cases were 50, 25, 12 and 6:1. At the end of the 3-hr incubation period (37°C and 5% CO_2), supernatants were collected and ^{51}Cr release was measured in a gamma-counter. The percentage of specific lysis was calculated according to standard formulas. The data were expressed as percentage specific lysis for each of the 4 E:T ratios tested.

For analysis of the interference of HAMA in peritoneal fluid with *in vitro* OC/TR-mediated cytolytic activity, autologous T lymphocytes that had been activated and expanded using PHA and IL-2 were targeted with OC/TR F(ab')_2 as described above, and subsequently washed twice to remove unbound OC/TR. OC/TR-targeted or untargeted T lymphocytes were resuspended in undiluted, heat-inactivated (30 min at 56°C) peritoneal fluid samples. The target cells were resuspended in the corresponding peritoneal fluid samples. A similar CTX assay performed in CTX assay medium instead of peritoneal fluid served as positive control in each experiment, whilst CTX assays using untargeted T lymphocytes served as negative controls.

RESULTS

HAMA in serum and peritoneal fluid become detectable during therapy and contain anti-idiotypic antibodies

HAMA became detectable in the serum and peritoneal fluid of all 8 patients during treatment (Fig. 1). Serum IgG antibodies reactive with OC/TR bsAb were first detectable after a median of 20 days (range 13 to 27) and reached their peak after a median of 49 days (range 27 to 70) after the start of phase I. The median peak serum titer was 107,000 (range 511 to 1.4×10^6). Serum IgG antibodies reactive with 323/A3 showed similar kinetics but reached lower titers (median peak serum titer, 12,000 [range 50 to 45,000]). Thus, it appeared

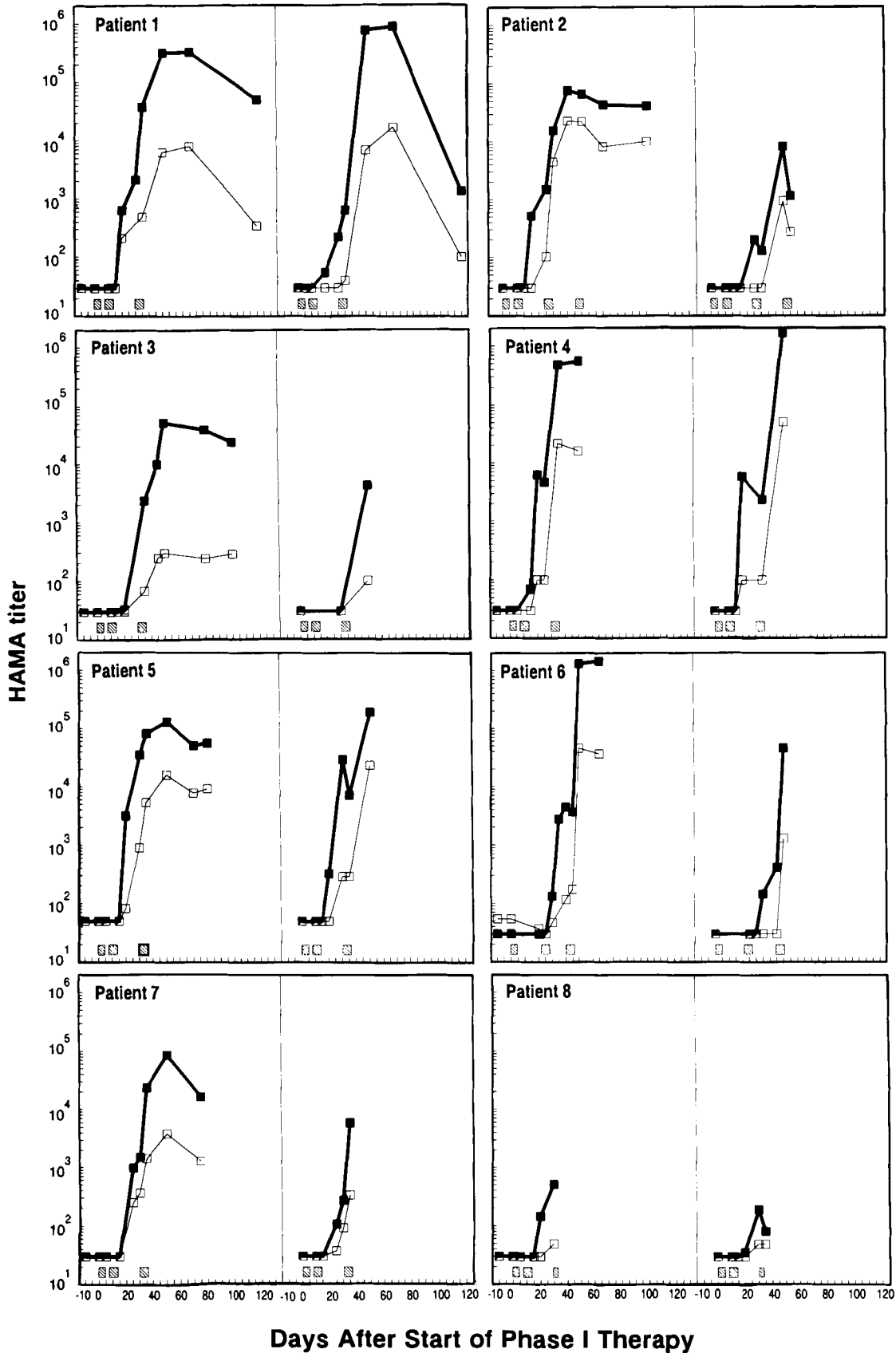


FIGURE 1 – HAMA titers in sequential serum and peritoneal fluid samples from 8 patients. For each patient, serum titers are set out in the left panel and peritoneal fluid titers in the right panel. HAMA titers against OC/TR bsAb are represented by closed symbols connected by bold lines; those against 323/A3 MAb (with specificity unrelated to that of OC/TR) by open symbols connected by thin lines. Hatched boxes represent time periods during which immunotherapy (1 phase-I cycle, followed by 2 to 3 phase-II cycles) was administered. Logarithmic scales were used for the Y axes in order to compress the figures.

that a significant component of HAMA was directed against the idiotypes of OC/TR bsAb.

The kinetics of IgG antibody titers reactive with OC/TR bsAb or 323/A3 MAb in peritoneal fluid paralleled those in the serum. HAMA reactive with OC/TR bsAb were first detectable after a median of 22 days (range 20 to 34) and reached their peak after a median of 48 days (range 27 to 70) after the start of phase I. The median peak titer was 27,000 (range 200 to 1.6×10^6). As in the serum, the titers of HAMA reactive with 323/A3 were lower than those reactive with OC/TR bsAb (median peak titer, 1,075 [range 45 to 50,000]). The fluctuations of HAMA titers in the peritoneal fluid observed in patients 2, 4 and 5 may have been caused in part by dilution by the i.p. infusions of targeted T lymphocytes (2×500 ml daily) and by the rinsing of the peritoneal cavity with saline between the 2 therapeutic cycles.

Further evidence that HAMA by and large comprised anti-idiotypic antibodies reactive with OC/TR bsAb was obtained by the observation that removal of human anti-mouse IgG antibodies by absorption to murine IgG coupled to Affi-gel 10 from the day-48 peritoneal fluid sample of patient 2 did not abrogate its capacity to block the binding of OC/TR bsAb to Igrov-1 ovarian carcinoma cells or to the CTL clone D11. The 2-fold absorption procedure of this specimen with mouse IgG coupled to Affi-gel 10 led to a 98% reduction in titer of antibodies reactive with 323/A3 MAb (*i.e.*, from 4,200 to 70), whilst only a 43% reduction in titer of antibodies reactive with OC/TR bsAb was seen (*i.e.*, from 11,300 to 6,400). Figure 2 shows that addition of OC/TR bsAb to both unabsorbed (upper panels) and absorbed (lower panels) peritoneal fluid, 30 min prior to incubation with D11 or Igrov-1 cells, led to clear reductions in the levels of reactivity with these cell types (*i.e.*, approaching those of the respective peritoneal fluid samples in the absence of OC/TR bsAb) as compared to the levels of reactivity obtained with OC/TR

added to PBS + 1% BSA. In contrast, addition of W6/32 MAb with specificity different from that of OC/TR (*i.e.*, anti-HLA-A,B,C) to unabsorbed peritoneal fluid led only to partial reductions in the levels of reactivity with D11 and Igrov-1 cells as compared to the levels of reactivity obtained with W6/32 added to PBS + 1% BSA (Fig. 2, upper panels). Moreover, the reactivity of W6/32 MAb, following addition to absorbed peritoneal fluid, with D11 and Igrov-1 cells was only slightly lower than that of W6/32 added to PBS + 1% BSA (Fig. 2, lower panels). These results confirmed that HAMA comprised both anti-murine IgG-framework and anti-idiotypic antibodies reactive with OC/TR bsAb.

Interaction between HAMA and OC/TR-targeted cytotoxicity of Igrov-1 ovarian carcinoma cells

Since MOv18 is shed, although to a limited extent, from the surface of fresh ovarian tumor cells and cell lines (Miotti *et al.*, 1992), ascitic or peritoneal fluid samples from such patients may contain free MOv18 glycoproteins which may interfere with *in vitro* assays of OC/TR-targeted cytotoxicity. However, we observed that the levels of bsAb-targeted cytotoxicity of Igrov-1 cells in the presence of peritoneal fluid obtained prior to treatment (day 0) were similar to those in the presence of standard assay medium in all of the 7 patients investigated in this respect (Fig. 3).

Next, we studied whether or not peritoneal fluid samples containing HAMA could inhibit OC/TR-targeted cytotoxicity of Igrov-1 cells. Inhibition was detected in peritoneal fluid samples of 6 of the 8 patients, and particularly in samples with relatively high HAMA titers (Fig. 3). Seven of 8 peritoneal fluid samples with HAMA titers of 5,778 or more inhibited OC/TR-targeted cytotoxicity of Igrov-1 cells *vs.* only 1 of 20 samples with HAMA titers $< 5,778$ (Fisher's exact test, $p < 0.001$). Inhibition was only detected after completion of phase II in 4 patients (1, 4, 6 and 7), but coincided with therapy

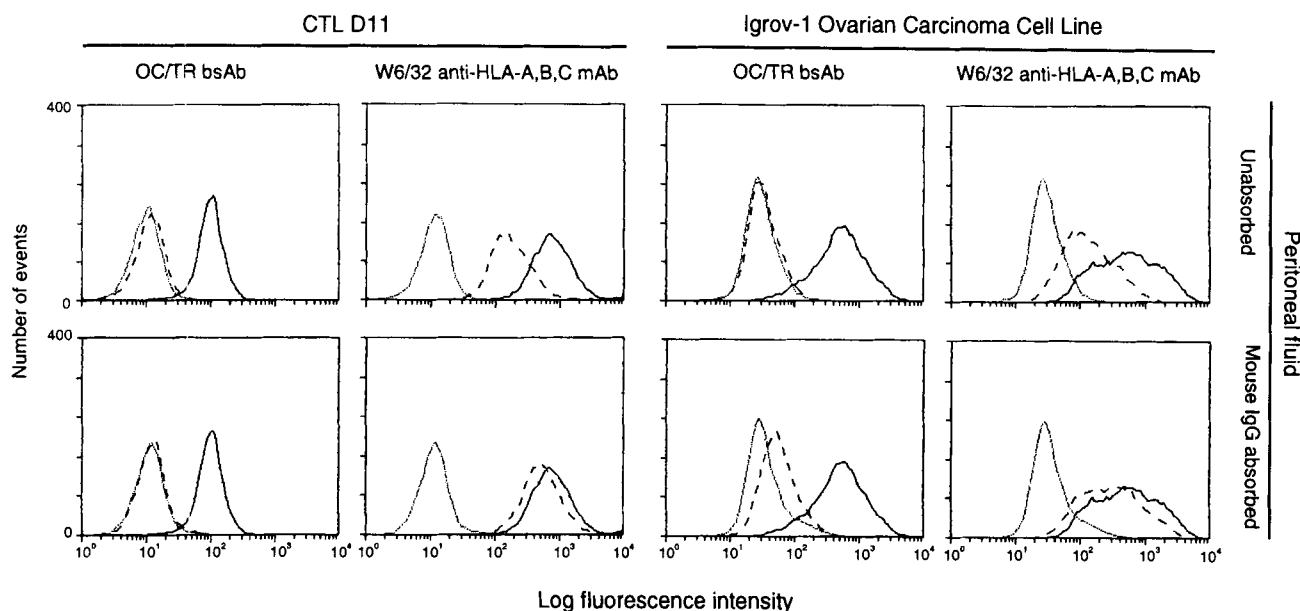
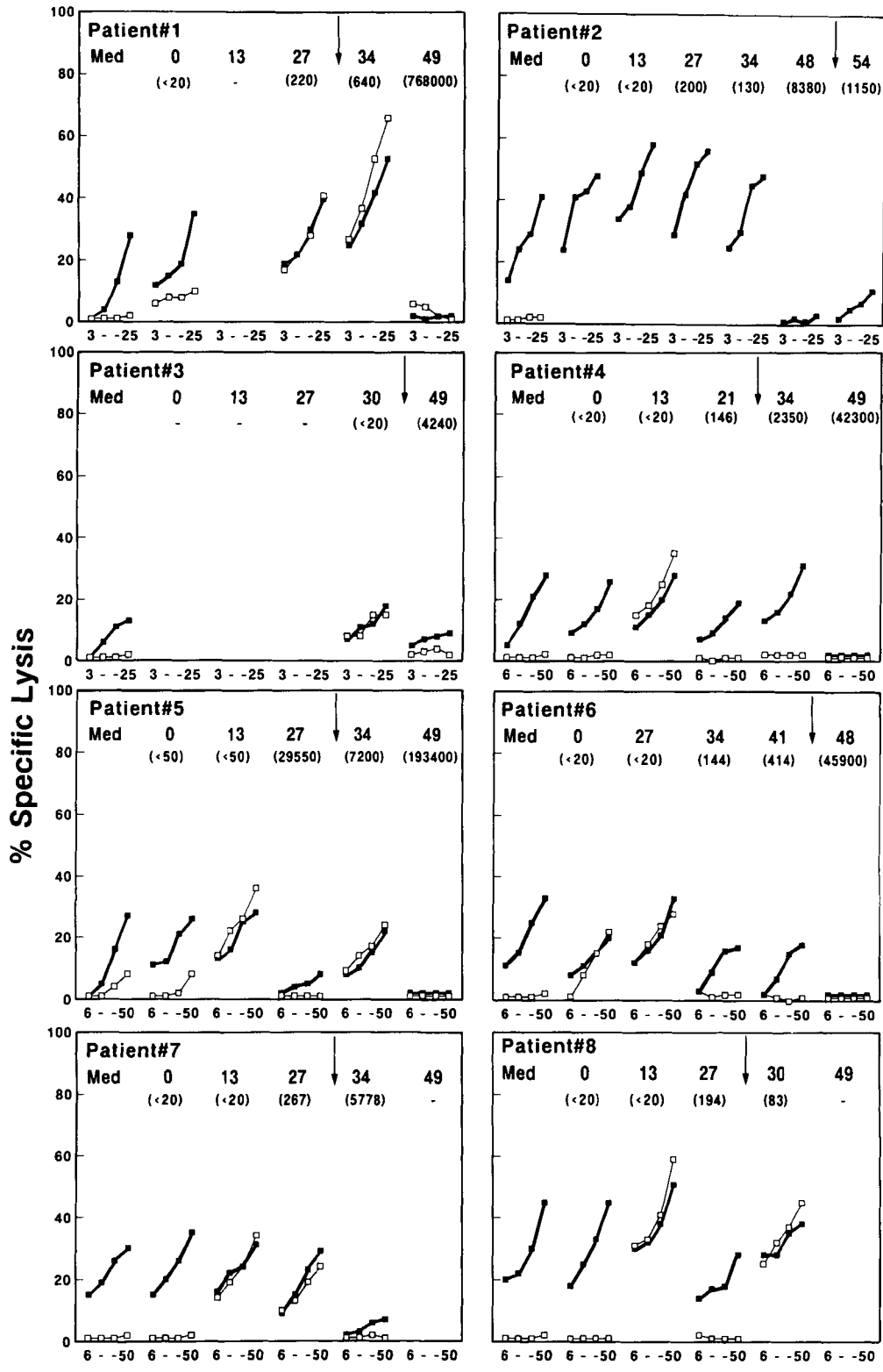


FIGURE 2 – Interaction between OC/TR bsAb and unabsorbed (upper panels) or mouse IgG-absorbed (lower panels) peritoneal fluid containing HAMA led to clear reductions in the levels of OC/TR reactivities with CTL D11 and the Igrov-1 ovarian carcinoma cell line. In contrast, reactivity of W6/32 MAb (anti-HLA-A,B,C) with those cell types was only partially or minimally blocked by interaction with unabsorbed or absorbed peritoneal fluid, respectively. The day-48 peritoneal fluid sample of patient 2 was used. Prior to absorption, the HAMA titer against OC/TR bsAb was 11,300 and the titer against 323/A3 MAb was 4,200. Following absorption, these titers were 6,400 and 70, respectively. Uninterrupted lines: reactivity of D11 or Igrov-1 cells with OC/TR or W6/32, visualized using GAM/IgG/PE; interrupted lines: reactivity of D11 or Igrov-1 cells with OC/TR or W6/32 that had been pre-incubated with unabsorbed or absorbed peritoneal fluid; dotted lines; reactivity of D11 or Igrov-1 cells with GAM/IgG/PE after incubation with unabsorbed or absorbed peritoneal fluid.



E:T Ratio for Each Assay in Medium or Peritoneal fluid

FIGURE 3 – Interaction between HAMA in sequential peritoneal fluid samples and OC/TR-targeted cytotoxicity of Igrov-1 ovarian carcinoma cells by autologous T lymphocytes activated and expanded using PHA + IL-2. The sampling time point of each peritoneal fluid sample is indicated as day relative to start of phase I. Arrows indicate the end of phase II. The HAMA titer against OC/TR bsAb (ELISA assay) is indicated between brackets for each peritoneal fluid sample. CTX assays using OC/TR-targeted T lymphocytes as effector cells are represented by closed symbols connected by bold lines; the corresponding assays using untargeted T lymphocytes are represented by open symbols connected by thin lines. CTX assays performed in CTX assay medium (indicated by "Med") instead of peritoneal fluid served as positive controls for OC/TR-targeted cytotoxicity of Igrov-1 cells in each experiment. The E:T ratios in assays designated 3–25 were 3, 6, 12 and 25:1, respectively; those in assays designated 6–50 were 6, 12, 25 and 50, respectively. All peritoneal fluid samples were heat-inactivated (30 min at 56°C) prior to the CTX assays.

TABLE I – TREATMENT SCHEDULE

Phase	Day of therapy	1 st Infusion ^{1,2}	2 nd Infusion ^{1,2}
I	0	10 ⁶ Ly × OC/TR ³ + IL2	IL2
	1	10 ⁷ Ly × OC/TR ³ + IL2	IL2
	2	10 ⁸ Ly × OC/TR ³ + IL2	IL2
	3	10 ⁹ Ly × OC/TR ³ + IL2	IL2
II (1 st therapeutic cycle)	6, 7, 8, 9, 10	10 ⁹ Ly × OC/TR ⁴ + IL2	IL2 + OC/TR
	11, 12	IL2 + OC/TR	IL2 + OC/TR
II (2 nd therapeutic cycle)	27, 28, 29, 30, 31	10 ⁹ Ly × OC/TR ⁴ + IL2	IL2 + OC/TR
	32, 33	IL2 + OC/TR	IL2 + OC/TR

¹The 1st infusion was administered at 12 A.M. and the 2nd infusion at 8 P.M. – ²Ly × OC/TR, autologous T lymphocytes targeted with OC/TR bsAb; IL-2, 0.6 × 10⁶ IU IL-2; OC/TR, 1 mg soluble OC/TR bsAb. All infusions were given in 500 ml of Ringer's lactate solution supplemented with 1% human serum albumin. – ³Unbound OC/TR bsAb was washed away. – ⁴Unbound OC/TR bsAb was not removed.

in 2 patients (2 and 5). Patient 2 received a third therapeutic cycle (days 48–52). Peritoneal fluid obtained at days 48 and 54 completely inhibited OC/TR-targeted cytotoxicity of Igrov-1 cells. The day-27 sample of patient 5, obtained immediately before the start of the second phase-II therapeutic cycle (Table I), also inhibited OC/TR-targeted cytotoxicity of Igrov-1 cells. This sample had an exceptionally high HAMA titer (*i.e.*, 29,550) in comparison to the day 27 samples of other patients (*i.e.*, ranging between <20 and 267; patients 1, 2, 6, 7 and 8). The increased lysis of Igrov-1 target cells by untargeted T lymphocytes in patient 1 (days 27 and 34), patient 4 (days 13), patient 5 (days 13 and 34), patient 6 (day 27), patient 7 (days 13 and 27) and patient 8 (days 13 and 30) was caused by the presence of free OC/TR bsAb in those peritoneal fluid samples. This presence was detected by incubation of D11 and Igrov-1 cells in peritoneal fluid followed by indirect immunofluorescence (data not shown).

Finally, we analyzed the interaction between HAMA in peritoneal fluid samples, and (1) OC/TR-targeted cytotoxicity of Igrov-1 cells; (2) chOC/TR-targeted cytotoxicity of Igrov-1 cells, and (3) untargeted, HLA-Cw3-specific cytotoxicity of BSM cells, by D11 effector cells (Fig. 4). Three peritoneal fluid specimens were selected: one from patient 1, obtained on day 69 (HAMA titers [ELISA assay] against OC/TR bsAb, 896,000; against 323/A3 MAb, 17,000); one from patient 2, obtained on day 48 and diluted 1:4 in CTX medium (final HAMA titers 2,093 and 236, respectively); and one from patient 5, obtained on day 48 (HAMA titers 193,400 and 23,500, respectively). First, all 3 samples completely inhibited OC/TR-targeted cytotoxicity of Igrov-1 cells by the CTL clone D11 (Fig. 4, upper panel). A combination of human anti-idiotypic and anti-murine Ig-framework antibodies may be responsible for this inhibition. Second, the possible inhibitory effect of HAMA against the murine C_H and C_L domains was excluded in the subsequent assay in which the D11 cells were targeted using chOC/TR (Fig. 4, middle panel). The cytotoxicity of Igrov-1 cells by chOC/TR-targeted D11 cells was still completely inhibited in the assays with the highest HAMA concentrations, *i.e.*, those carried out using the peritoneal fluid samples of patients 1 and 5, whilst partial inhibition was seen in the assay with the lowest HAMA concentration, *i.e.*, the one carried out in the 1:4 diluted peritoneal fluid from patient 2. This observation indicates that the inhibition of OC/TR-targeted cytotoxic activity by patient 2's peritoneal fluid was partly due to anti-murine C_H and/or C_L antibodies. Third, HAMA had no effect on untargeted, HLA-Cw3-specific cytotoxicity of BSM cells by the D11 effector cells (Fig. 4, lower panel).

DISCUSSION

We have studied the kinetics of HAMA responses and the interference of HAMA with OC/TR bsAb-targeted cytotoxicity

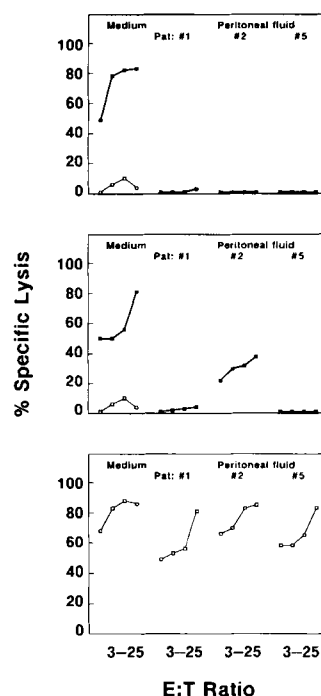


FIGURE 4 – Interaction between HAMA in peritoneal fluid and OC/TR-targeted (upper panel), chOC/TR-targeted (central panel) and HLA-Cw3-specific (lower panel) lysis by D11 CTL. The following peritoneal fluid samples were used: patient 1, day 69 (HAMA titers [ELISA assay] against OC/TR bsAb, 896,000; against 323/A3 MAb, 17,000); patient 2, day 48, 1:4 diluted (HAMA titers after 1:4 dilution, 2,093 and 236, respectively); and patient 5, day 48 (HAMA titers 193,400 and 23,500, respectively). CTX assays using OC/TR-targeted or chOC/TR-targeted D11 CTL as effector cells are represented by closed symbols connected by bold lines; assays using untargeted D11 CTL are represented by open symbols connected by thin lines. CTX assays performed in CTX assay medium (indicated by “Medium”) instead of peritoneal fluid served as positive controls in each experiment. The E:T ratios were 3, 6, 12 and 25:1, respectively. All peritoneal fluid samples were heat-inactivated (30 min at 56°C) prior to the CTX assays.

in 8 ovarian carcinoma patients receiving phase-I/II *i.p.* immunotherapy using OC/TR-targeted lymphocytes, soluble OC/TR bsAb and low-dose IL-2. HAMA were detected using ELISA in serum and peritoneal fluid samples of all 8 patients from 2 and 3 weeks following start of therapy onwards, respectively, and reached peak levels at 7 weeks. These results are consistent with published data on the serum kinetics of

primary IgG HAMA responses following intravenous (Courtenay-Luck *et al.*, 1986) or intraperitoneal (Maher *et al.*, 1992) administrations. A significant part of the HAMA appeared to be directed against the idiotypes of OC/TR bsAb specific for CD3 and MOv18, as suggested by (1) the clearly higher ELISA titers against OC/TR bsAb compared to those against a MAb with unrelated specificity, and (2) the failure to abrogate the capacity of peritoneal fluid to block the binding of OC/TR bsAb to MOv18⁺ or CD3⁺ cells by absorption of human anti-mouse IgG-framework antibodies in peritoneal fluid to immobilized mouse IgG. Indeed, anti-idiotypic HAMA responses have been observed in particular after repeated MAb administrations (Schroff *et al.*, 1985; Courtenay-Luck *et al.*, 1988; Saleh *et al.*, 1993).

The specificity of HAMA for the complementarity-determining region (CDR) of MAb has been demonstrated previously using inhibition of antigen binding (Traub *et al.*, 1988) or inhibition of anti-idiotypic antibody binding to that MAb (Chattopadhyay *et al.*, 1991). We have followed the first approach (Fig. 2). An important observation was that peritoneal fluid samples with relatively high HAMA titers inhibit *in vitro* OC/TR-targeted cytolysis of ovarian carcinoma cells (Fig. 3). This inhibitory activity was never detected at the start of the therapeutic infusions of OC/TR-targeted T lymphocytes (*i.e.*, phase II), but became detectable during phase II in 2 patients. This result suggests that HAMA may interfere with the goal of therapy, *i.e.*, the *in vivo* destruction of tumor cells by bsAb-targeted T lymphocytes.

Strategies for overcoming the immunogenicity problem include the use of murine-human antibody constructs: chimeric MAb, composed of murine V_H and V_L regions combined with human C_H and C_L regions, or CDR-grafted MAb, which consist not only of human constant regions but also of the framework of human V_H and V_L regions (reviewed by Khazaeli *et al.*, 1994). Although such molecules are probably less immunogenic than their entirely murine counterparts, they still elicit anti-idiotypic HAMA responses and, hence, antibodies with different anti-tumor idiotypes need to be used in the case of prolonged or repeated therapeutic interventions using bsAb-targeted T lymphocytes. An alternative to the use of autologous bsAb-targeted T lymphocytes is offered by genetically engineered T lymphocytes endowed with receptors specific for tumor-associated antigens (Hwu *et al.*, 1993), which may be less immunogenic.

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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 ovarium adenocarcinoma line, IGROV-1, in tissue culture and in nude mice. *Cancer Res.*, **45**, 4970-4979 (1985).
- BOLHUIS, R.L.H., LAMERS, C.H.J., GOEY, S.H., EGGERMONT, A.M.M., TRIMBOS, J.B.M.Z., STOTER, G., LANZAVECCHIA, A., DI RE, E., MIOTTI, S., RASPAGLIESI, F., RIVOLTINI, L. and COLNAGHI, M.I., Adoptive immunotherapy of ovarian carcinoma with bs-MAB-targeted lymphocytes: a multicenter study. *Int. J. Cancer, Suppl.* **7**, 78-81 (1992).
- 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 transmission. *Cell. Immunol.*, **93**, 46-57 (1985).
- CHATTOPADHYAY, P., SNEED, D., ROSENBERG, J., STARKEY, J., ROBERTSON, N., LEONARD, J. and RAYCHAUDHURI, S., Monoclonal anti-idiotypic antibodies to human melanoma-associated proteoglycan antigen: generation and characterization of anti-idiotypic antibodies. *Cancer Res.*, **51**, 3183-3192 (1991).
- CONEY, L.R., TOMASSETTI, A., CARAYANNOPOULOS, L., FRASCA, V., KAMEN, B.A., COLNAGHI, M.I. and ZURAWSKI, V.R., JR., Cloning of a tumor-associated antigen: MOv18 and MOv19 antibodies recognize a folate-binding protein. *Cancer Res.*, **51**, 6125-6132 (1991).
- COURTENAY-LUCK, N.S., EPENETOS, A.A., MOORE, R., LARCHE, M., PECTASIDES, D., DHOKIA, B. and RITTER, M.A., Development of primary and secondary immune responses to mouse monoclonal antibodies used in the diagnosis and therapy of malignant neoplasms. *Cancer Res.*, **46**, 6489-6493 (1986).
- COURTENAY-LUCK, N.S., EPENETOS, A.A., SIVOLAPENKO, G.B., LARCHE, M., BARKANS, J.R. and RITTER, M.A., Development of anti-idiotypic antibodies against tumour antigens and autoantigens in ovarian cancer patients treated intraperitoneally with mouse monoclonal antibodies. *Lancet*, **2**, 894-897 (1988).
- EDWARDS, D.P., GRZYB, K.T., DRESSLER, L.G., MANSER, R.E., ZAVA, D.T., SLEDGE, G.W., JR. and MCGUIRE, W.L., Monoclonal antibody identification and characterization of a M, 43,000 membrane glycoprotein associated with human breast cancer. *Cancer Res.*, **46**, 1306-1317 (1986).
- HWU, P., SHAFER, G.E., TREISMAN, J., SCHINDLER, D.G., GROSS, G., COWHERD, R., ROSENBERG, S.A. and ESHHAR, Z., Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ chain. *J. exp. Med.*, **173**, 361-366 (1993).
- KHAZAELI, M.B., CONRY, R.M. and LOBUGLIO, A.F., Human immune response to monoclonal antibodies. *J. Immunother.*, **15**, 42-52 (1994).
- KHAZAELI, M.B., SALEH, M.N., WHEELER, R.H., HUSTER, W.J., HOLDEN, H., CARRANDO, R. and LOBUGLIO, A.F., Phase I trial of multiple large doses of murine monoclonal antibody CO17-1A. II. Pharmacokinetics and immune response. *J. nat. Cancer Inst.*, **80**, 937-942 (1988).
- 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 bispecific antibody-triggered cellular immunotherapy. *Int. J. Cancer*, **51**, 973-979 (1992).
- LIND, P., LECHNER, P., HAUSMANN, B., SMOLA, M.G., KOELTRINGEE, P., STEINDORFER, P., CESNIK, H., PASSL, R. and EBER, O., Development of human antimouse antibodies (HAMA) after single and repeated diagnostic application of intact murine monoclonal antibodies. *Antibody Immunoconj. Radiopharmaceut.*, **4**, 811-818 (1991).
- MACH, J.P., BUCHEGGER, F., FORNI, M., RITSCHARD, J., BERCHE, C., LUMBROSO, J.D., SCHREYER, M., GIRARDET, C., ACCOLLA, R.S. and CARREL, S., Use of radiolabelled monoclonal anti-CEA antibodies for the detection of human carcinomas by external photoscanning and tomoscintigraphy. *Immunol. Today*, **2**, 239-249 (1981).
- MAHER, V.E., DRUKMAN, S.J., KINDERS, R.J., HUNTER, R.E., JENNINGS, J., BRIGHAM, C., STEVENS, S. and GRIFFIN, T.W., Human antibody response to the intravenous and intraperitoneal administration of the F(ab')₂ fragment of the OC125 murine monoclonal antibody. *J. Immunother.*, **11**, 56-66 (1992).
- MEZZANZANICA, D., CANEVARI, S., MÉNARD, S., PUPA, S.M., TAGLIABUE, E., LANZAVECCHIA, A. and COLNAGHI, M.I., Human ovarian carcinoma lysis by cytotoxic T cells targeted by bispecific monoclonal antibodies: analysis of the antibody components. *Int. J. Cancer*, **41**, 609-615 (1988).
- MIOTTI, S., ALBERTI, S., FACHERIS, P., MANTOVANI, L., FORNARO, M., STELLA, M., MÉNARD, S., CANEVARI, S. and COLNAGHI, M.I., Membrane association and shedding of the GPI-anchored Ca-MOv18 antigen in human ovary carcinoma cells. *Int. J. Cancer*, **51**, 499-505 (1992).

- 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).
- SALEH, M.N., KHAZAELI, M.B., GRIZZLE, W.E., WHEELER, R.H., LAWSON, S., LIU, T., RUSSELL, C., MEREDITH, R., SCHLOM, J. and LOBUGLIO, A.F., A phase I clinical trial of murine monoclonal antibody D612 in patients with metastatic gastrointestinal cancer. *Cancer Res.*, **53**, 4555–4562 (1993).
- SCHROFF, R.W., FOON, K.A., BEATTY, S.M., OLDHAM, R.K. and MORGAN, A.C., JR., Human anti-murine immunoglobulin responses in patients receiving monoclonal antibody therapy. *Cancer Res.*, **45**, 879–885 (1985).
- SEGAL, D.M. and SNIDER, D.P., Targeting and activation of cytotoxic lymphocytes. *Chem. Immunol.*, **47**, 179–213 (1989).
- TRAUB, U.C., DE JAGER, R.L., PRIMUS, F.J., LOSMAN, M. and GOLDENBERG, D., Anti-idiotypic antibodies in cancer patients receiving monoclonal antibody to carcinoembryonic antigen. *Cancer Res.*, **48**, 4002–4006 (1988).
- VAN DE GRIEND, R.J., GIPHART, M.J., VAN KRIMPEN, B.A. and BOLHUIS, R.L.H., Human T cell clones exerting multiple cytolytic activities show heterogeneity in susceptibility to inhibition by monoclonal antibodies. *J. Immunol.*, **133**, 1222–1229 (1984).
- 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).