

In vivo Targeting of Human Neutralizing Antibodies against CD55 and CD59 to Lymphoma Cells Increases the Antitumor Activity of Rituximab

Paolo Macor,¹ Claudio Tripodo,⁴ Sonia Zorzet,² Erich Piovan,⁵ Fleur Bossi,¹ Roberto Marzari,³ Alberto Amadori,⁵ and Francesco Tedesco¹

Departments of ¹Physiology and Pathology, ²Biological Sciences, and ³Biology, University of Trieste, Trieste, Italy; ⁴Department of Human Pathology, University of Palermo, Palermo, Italy; and ⁵Department of Oncology and Surgical Sciences, University of Padova, Padova, Italy

Abstract

An *in vivo* model of human CD20+ B-lymphoma was established in severe combined immunodeficiency mice to test the ability of human neutralizing miniantibodies to CD55 and CD59 (MB55 and MB59) to enhance the therapeutic effect of rituximab. The miniantibodies contained single-chain fragment variables and the hinge-CH2-CH3 domains of human IgG₁. LCL2 cells were selected for the *in vivo* study among six B-lymphoma cell lines for their high susceptibility to rituximab-dependent complement-mediated killing enhanced by MB55 and MB59. The cells injected *i.p.* primarily colonized the liver and spleen, leading to the death of the animals within 30 to 40 days. Thirty percent of mice receiving biotin-labeled rituximab (25 µg) *i.p.* on days 4 and 11 after cell injection survived to 120 days. Administration of biotin-labeled rituximab, followed by avidin (40 µg) and biotin-labeled MB55–MB59 (100 µg) at 4-h intervals after each injection resulted in the survival of 70% of mice. Surprisingly, 40% of mice survived after the sole injection of avidin and biotin-labeled MB55–MB59, an observation consistent with the *in vitro* data showing that the miniantibodies induced killing of ~25% cells through antibody-dependent cell cytotoxicity. In conclusion, MB55 and MB59 targeted to tumor cells represent a valuable tool to enhance the therapeutic effect of rituximab and other complement-fixing antitumor antibodies. [Cancer Res 2007;67(21):10556–63]

Introduction

Several chimeric and humanized monoclonal antibodies (mAbs) against different antigens expressed by neoplastic cells have been developed to obtain more specific and less toxic agents for cancer treatment. However, only relatively few molecules have been approved for human therapy and new approaches are needed to increase their efficacy *in vivo* (1–3). Satisfactory results in the immunotherapy of B-cell malignancies have been obtained using two mAbs recognizing CD20 (rituximab; refs. 4, 5) and CD52 (alemtuzumab, Campath1-H; ref. 6), expressed on the surface of tumor cells and approved by the Food and Drug Administration for the treatment of non-Hodgkin lymphoma and chronic lymphocytic leukemia, respectively (7).

Most of the information available on the mechanisms of action of these mAbs has been obtained from *in vitro* and *in vivo* studies of rituximab. This mAb has been reported to control tumor growth via different effector mechanisms that act either alone or, more often, in combination, and include complement-dependent cytotoxicity (CDC; refs. 8, 9), antibody-dependent cell cytotoxicity (ADCC; ref. 8), or apoptosis (10, 11). Xenograft models of lymphoma in mice lacking FcR-γ chain (12), or treated with neutralizing antibodies against mouse FcγR (13, 14), have provided evidence for the contribution of ADCC to the therapeutic activity of rituximab. Data from several groups are also available indicating that B-lymphoblastoid cell lines and fresh B-lymphoma cells expressing CD20 can be killed by complement activated by rituximab (9, 15–17). The susceptibility of these cells to CDC is partly related to the expression level of CD20 on the cell surface, but it is also dependent on the ability of rituximab to induce the translocation of CD20 into lipid rafts (18), which favors the clustering of antibodies and, in turn, promotes C1q binding. The important role played by complement as an effector system in the anticancer activity of rituximab is supported by the findings that this mAb is less effective in controlling tumor growth in C1q KO mice (9) and in mice treated with cobra venom factor (19) than in complement-sufficient mice.

The complement system has a definite advantage over cytotoxic cells as an *in vivo* defense system against cancer because it is made of soluble molecules that can easily reach the tumor site and diffuse inside the tumor mass. Moreover, complement components are readily available as a first line of defense against cancer cells because they are locally synthesized by many cell types, including macrophages (20), fibroblasts (21), and endothelial cells (22, 23). However, a major limitation of complement as an effector system against tumor cells is represented by the complement-regulatory proteins expressed on the cell surface (mCRPs; refs. 24, 25), which provide a means of evasion from complement attack. It has been previously shown that CD55 and CD59 mainly contribute to the protection of complement-mediated killing of CD20+ cells induced by rituximab (8). This observation led us to hypothesize that neutralizing these two CRPs would greatly improve the beneficial effect of rituximab on B lymphoma cells. To this end, we have recently produced two human mAbs neutralizing CD55 and CD59, named MB55 and MB59, respectively (26). They were initially isolated as single-chain fragment variables (scFv) from a human phage display library and then engineered to contain the hinge-CH2-CH3 region of human IgG₁ in order to produce a divalent molecule, with characteristics similar to those of a complete immunoglobulin. *In vitro* studies have shown that CDC of a lymphoma cell line induced by rituximab is significantly enhanced in the presence of MB55 and MB59 (26).

Requests for reprints: Francesco Tedesco, Department of Physiology and Pathology, University of Trieste, via Fleming 22, 34127 Trieste, Italy. E-mail: tedesco@units.it.

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The purpose of this investigation is to evaluate the *in vivo* therapeutic effect of MB55 and MB59 used in combination with rituximab to treat a human B-lymphoma established in severe combined immunodeficiency (SCID) mice. Evidence will be presented indicating that MB55 and MB59 targeted to tumor cells increase the ability of rituximab to eradicate established EBV+ B-lymphoma xenografts enhancing both CDC and ADCC.

Materials and Methods

Antibodies and sera. The anti-CD20 chimeric mAb rituximab was kindly provided by Dr. Josée Golay (Division of Haematology, Ospedale Riuniti, Bergamo, Italy). The anti-CD20, anti-CD55, anti-CD59 mAbs, and the isotype-matched control IgG were obtained from ImmunoTools. Rat mAb YTH53.1 to CD59 was a kind gift from Prof. Seppo Meri (Haartman Institute, University of Helsinki, Helsinki, Finland) and mAb BRIC216 to CD55 was purchased from the International Blood Group Reference Laboratory. All the secondary antibodies to human and mouse immunoglobulins were purchased from Sigma-Aldrich. MB55 and MB59 were purified from the supernatant of transfected HEK293T cells as previously described (26). Human sera from AB Rh+ blood donors were kindly provided by the Blood Transfusion Center (Trieste, Italy) and pooled as a source of complement (PNHS). For some experiments, the serum (NHS) and the erythrocytes obtained from a single blood donor were also used.

Cells. Three *in vitro*-derived EBV+ lymphoblastoid cell lines (LCL1–3) and three EBV+ cell lines obtained *ex vivo* from SCID mice injected with peripheral blood mononuclear cells (PBMC) from EBV+ donors (Hu-SCID1–3; ref. 27) were grown in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (Invitrogen). PBMC were isolated from buffy coats obtained from blood donors or from murine blood by Ficoll-Hypaque (Pharmacia Biotech) gradient centrifugation, and maintained in RPMI 1640 supplemented with 5% FCS, 2 mmol/L of glutamine and streptomycin (100 µg/mL). Human erythrocytes were stored in veronal-buffered saline at 4°C until use.

Animals. Female SCID mice (4–6 weeks of age) were purchased from Charles River and maintained under pathogen-free conditions. Blood as a source of PBMC and serum was obtained from female BALB/c mice kept as a colony at the Animal House of the University of Trieste.

All the experimental procedures were done in compliance with the guidelines of the European (86/609/EEC) and the Italian (D.L.116/92) laws and were approved by the Italian Ministry of University and Research as well as by the Administration of the University Animal House.

Immunofluorescence analysis. Lymphoma cells (5×10^5) were first incubated with the primary antibodies to CD20, CD55 or CD59, or isotype-matched control IgG (5 µg/mL) in PBS containing 0.1% bovine serum albumin for 1 h at 37°C and then with the appropriate FITC-conjugated secondary antibodies for 30 min at 37°C. After washing with PBS containing 0.1% bovine serum albumin, the cells were fixed with 1% paraformaldehyde (Sigma-Aldrich). Cell fluorescence was evaluated on a FACScalibur instrument (BD Biosciences) using CellQuest software.

Complement-mediated lysis. The CDC procedure described by Ziller et al. (26) to evaluate the effect of the neutralizing miniantibodies on the complement susceptibility of B-lymphoma cells was followed in detail. Briefly, $2 \times 10^5/50$ µL cells were incubated with rituximab (2 µg/mL) with or without MB55 and MB59 (10 µg/mL each) to a final volume of 100 µL for 10 min at room temperature prior to the addition of PNHS (25%). After further incubation at 37°C for 1 h, the number of residual viable cells was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (28) and the percentage of dead cells was calculated.

Biotin-labeling of rituximab, MB55, and MB59. The purified antibodies were biotin-labeled using biotin-aminocaproate *n*-hydroxysuccinimide ester (Sigma-Aldrich) as previously described (22). The procedure included overnight dialysis of the IgG fraction against 0.1 mol/L of sodium bicarbonate buffer (pH 8.8), incubation with biotin at a protein/biotin ratio of 8.25:1 (w/w) for 15 min at room temperature, addition of 1 mol/L of ammonium chloride at pH 6.5 (1:100 v/v) to block the reaction, and final dialysis against PBS to remove free biotin.

Mouse model of B-lymphoma. A SCID/LCL2 xenograft model described by Piovan and coworkers (29) was employed to investigate the *in vivo* effects of MB55 and MB59. Briefly, EBV+ lymphoblastoid cells were expanded *in vitro* prior to i.p. injection (2×10^6) into SCID mice. Lymphoma-bearing mice were treated with saline (as a control), rituximab, avidin, or miniantibodies. The mice were examined every 4 days for sign of sickness, and followed until they were sacrificed for serious illness, or up to 120 days.

Samples of peritoneal mass, spleen, liver, kidney, lung, and skeletal muscle from lymphoma-bearing mice either dead from the tumor or sacrificed at day +120 were obtained at necropsy. For morphologic evaluation, the specimens were fixed in 10% buffered-formalin solution and embedded in paraffin. Four-micrometer-thick sections were stained with H&E. Immunohistochemical analyses were done on snap-frozen samples embedded in optimum cutting temperature compound embedding medium (Diagnostic Division, Miles, Inc.). Four- to 6-µm sections were fixed in cold 100% methanol for 15 min. Immunohistochemical analysis was done using the avidin-biotin-peroxidase complex method according to standard procedures (30), and the slides were examined under a Leica DM2000 optical microscope.

ELISA. The presence of rituximab and avidin in the mouse peritoneal washes was evaluated by ELISA. Microtiter plate wells were coated with 100 µL of diluted (1:50) peritoneal washes by overnight incubation in 0.1 mol/L of sodium bicarbonate buffer (pH 9.6) at 4°C and the unbound sites were blocked by incubation with PBS containing 2% nonfat milk for 1 h at 37°C. The amount of chimeric antibodies was measured using 1:7,500 antihuman IgG conjugated with phosphatase and the enzymatic reaction was developed with paranitrophenylphosphate and read at 405 nm as previously reported (31). Avidin was revealed by the Vectastain Elite ABC kit (Vector Lab) according to the manufacturer's instructions. The percentage of residual protein was then calculated.

Apoptosis. To investigate the ability of MB55 and MB59 to induce apoptosis of lymphoma cells, $2 \times 10^5/50$ µL LCL2 cells were incubated with bio-rituximab (2 µg/mL) and/or bio-MB55 and bio-MB59 (10 µg/mL) for 48 h and apoptosis was assessed by terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay (Roche) according to the manufacturer's instructions.

ADCC. The procedure described by Spiridon et al. (32) to investigate the ability of MB55 and MB59 to induce cell cytotoxicity was followed with some modifications. Briefly, lymphoma cells were stained with the green fluorescence cytoplasmic dye 5- and (6)-carboxyfluorescein diacetate, succinimidylester (CFSE; Molecular Probes, Inc.), using 25 µL of a 10 µmol/L solution/ 10^6 cells for 7 min at 37°C. After washing, the labeled-LCL2 cells (2×10^5) were incubated first with bio-rituximab (2 µg/mL) for 30 min at 4°C and, when required, with the mixture of bio-MB55 and bio-MB59 (10 µg/mL each) for an additional 30 min at the same temperature. PBMC as effector (E) cells were mixed with target (T) cells at 50:1 E/T cell ratio and incubated for 4 h at 37°C in 5% CO₂. The cells were then centrifuged and assayed for dye release with Infinite F200 (Tecan). The percentage of specific lysis was determined as: [(test release – spontaneous release) / (total release – spontaneous release)] × 100. Dead cells were stained with propidium iodide (50 µg/mL; Sigma-Aldrich) and analyzed on a FACScalibur instrument to control the data obtained by measuring dye release.

Statistical analysis. The data were expressed as mean ± SD and analyzed for statistical significance by the two-tailed Student's *t* test to compare two paired groups of data. The Kaplan-Meier product-limit method was used to estimate survival curves and the log-rank test was adopted to compare different groups of mice.

Results

CDC of lymphoblastoid cells induced by rituximab is enhanced by MB55 and MB59. To evaluate the contribution of complement-mediated killing of lymphoma cells sensitized by anti-CD20 and the enhancing effect of neutralizing antibodies to CD55 and CD59 in an *in vivo* model, we first screened human

EBV-lymphoblastoid cell lines (LCL1-3 and Hu-SCID1-3) established by Piovan and coworkers (27) for their susceptibility to CDC. These cell lines were shown to induce lymphoma in SCID mice (29), and were therefore considered suitable to test the *in vivo* therapeutic effect of rituximab, MB55, and MB59.

The cells were incubated with murine mAbs to CD20, CD55, and CD59 and analyzed by flow cytometry using appropriate FITC-labeled secondary antibodies. The results obtained (data not shown) indicate that CD20 was variably expressed on the cell lines examined, with the highest value observed on LCL1 and the lowest on LCL3, whereas CD55 and CD59 were uniformly distributed on the surface of these cells. Similar results were obtained with rituximab, MB55, and MB59 extending previous data reported by Ziller et al. (26) for Hu-SCID1.

Next, we assessed the effect of rituximab (2 µg/mL) on complement-dependent killing of EBV+ lymphoblastoid cell lines and the ability of MB55 and MB59 (10 µg/mL each), neutralizing CD55 and CD59, respectively, to enhance the cytolytic activity of complement. To this end, the cells were exposed to the chimeric mAb and PNHS (25%) as a source of complement, and the number of cells killed was counted after 1 h of incubation at 37°C. As shown in Table 1, the CDC obtained with rituximab ranged between 13% for LCL2 and 31% for Hu-SCID2. Such low levels of cytotoxicity suggested that surface-expressed complement regulators might account for the resistance of these cells to complement attack. Because CD55 and CD59 were previously found to be mainly responsible for cell protection from CDC (8), the cells were incubated with rituximab and PNHS in the presence of MB55 and MB59. The results presented in Table 1 show that the percentage of cells killed under these experimental conditions increased 2- to 6-fold as compared with the values observed in the absence of the two miniantibodies. MB55 and MB59 were not able to induce complement-dependent lysis when incubated with cells and PNHS. Similar data were obtained using two well-known neutralizing mAbs, BRIC216 and YTH53.1, directed against CD55 and CD59, respectively (data not shown). For further experiments, we selected LCL2 because these cells were found to be the most resistant to rituximab-induced CDC in the absence of MB55–MB59 (13%) and the most susceptible to killing after neutralization of CD55 and CD59 (84%).

Mouse sera obtained from BALB/c and SCID mice were also tested as a source of complement in the cytotoxic assay of LCL2. As expected, mouse complement was less efficient than human complement causing only 6% of cell killing induced by rituximab, but this value increased to 36% in the presence of MB55 and MB59. No difference in CDC was observed between BALB/c and SCID mice sera.

Establishment of a human lymphoma model in SCID mice. To ascertain if the mixture of MB55 and MB59 and rituximab had a synergistic therapeutic effect, a lymphoma model was developed in

SCID mice by injecting i.p. LCL2 cells (2×10^6). This resulted in the formation of a tumor leading to the death of the animals within 30 to 40 days after cell inoculation (Fig. 1A). At necropsy, peritoneal tumor masses were seen in all these mice as a visible sign of lymphoproliferative disease. At histologic examination, the masses appeared as small-to-medium sized aggregates of irregularly-shaped lymphoid cells (Fig. 1B) positive for the human B-cell antigens CD20 and CD79a (Fig. 1C and D). Foci of lymphoid cells were seen in the spleen and liver of mice which died as a result of tumor growth, but we failed to detect lymphoma cells infiltrating lungs, kidneys, and skeletal muscles (data not shown). The bone marrow from these mice was not available for examination.

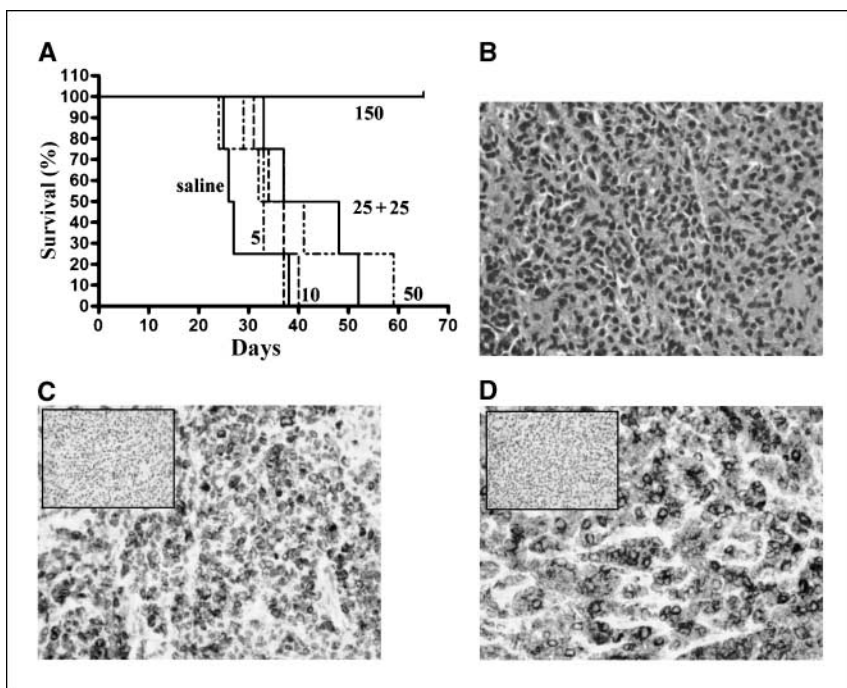
In the first series of experiments, we tested the *in vivo* therapeutic activity of increasing amounts of rituximab with the intent to find the minimal active dose that allowed us to evaluate the beneficial effect of MB55 and MB59, as combined therapy with the chimeric anti-CD20 mAb. To this end, the animals received LCL2 cells on day 0, followed 4 days later by a single injection of rituximab at doses of 5, 10, 50, or 150 µg. Four mice were used for each dose of mAb. One group of mice received 25 µg of rituximab twice on days 4 and 11 after injection of the cells on day 0. As shown in Fig. 1A, the therapeutic efficacy of rituximab was clearly dose-dependent, with the maximal effect observed at 150 µg, which corresponds to the standard dose of 375 mg/m² used in patients, and progressively decreased using lower doses of mAb. For further experiments, the protocol of double injections of rituximab (25 + 25 µg) on days 4 and 11 after tumor engraftment was adopted as the therapeutic scheme that was able to induce a slight, but statistically significant, increase in the number of surviving mice as compared with the control group of untreated mice.

Targeting of MB55–MB59 to lymphoma cells via the avidin-biotin system. Because CD55 and CD59 are widely expressed on the surface of circulating and tissue cells, we devised a strategy to target MB55 and MB59 to rituximab-coated tumor cells, thus avoiding the risk that these antibodies might bind to host cells and cause undesired complement attack following neutralization of the two CRPs. A three-step biotin-avidin system, currently employed in patients to target radionuclides to cancer cells, was used (33–35). First, using fluorescence-activated cell sorting analysis, we checked if biotin-labeled rituximab (bio-rituximab), MB55 (bio-MB55), and MB59 (bio-MB59) were still able to recognize CD20, CD55, and CD59 using FITC-streptavidin (data not shown). Next, bio-rituximab was tested for its ability to cause CDC of lymphoma cells in the presence or in the absence of bio-MB55 and bio-MB59. As shown in Fig. 2, bio-rituximab caused the death of 12% of LCL2 in the absence of bio-MB55 and bio-MB59, and >80% in their presence. These results did not differ from those obtained with unlabeled rituximab and the two neutralizing miniantibodies (Table 1).

Table 1. Complement-mediated killing of lymphoblastoid cell lines

	LCL1	LCL2	LCL3	HuSCID1	HuSCID2	HuSCID3
Rituximab	28 ± 7	13 ± 3	21 ± 4	25 ± 5	31 ± 11	14 ± 5
MB55–MB59	4 ± 2	4 ± 2	5 ± 2	4 ± 3	7 ± 2	7 ± 5
Rituximab + MB55	55 ± 7	49 ± 5	55 ± 7	32 ± 6	41 ± 9	35 ± 9
Rituximab + MB59	64 ± 9	55 ± 6	71 ± 4	53 ± 8	42 ± 12	41 ± 9
Rituximab + MB55–MB59	73 ± 5	84 ± 7	83 ± 11	61 ± 8	63 ± 7	45 ± 7

Figure 1. Effect of increasing doses of rituximab on survival of SCID mice challenged with LCL2 cells. *A*, survival curve. Mice were injected i.p. with 2×10^6 LCL2 cells and treated on day 4 with saline or rituximab at the indicated doses (5–150 μ g). One group of mice received 25 μ g of rituximab twice on days 4 and 11 after challenge. *B–D*, representative sections from peritoneal masses stained with H&E (*B*); anti-CD20 mAb, AEC chromogen (*C*); anti-CD79a mAb, and 3,3'-diaminobenzidine chromogen (*D*). *Insets*, controls incubated with isotype-matched IgG and peroxidase-labeled secondary antibody (*C* and *D*). Original magnification, $\times 400$.



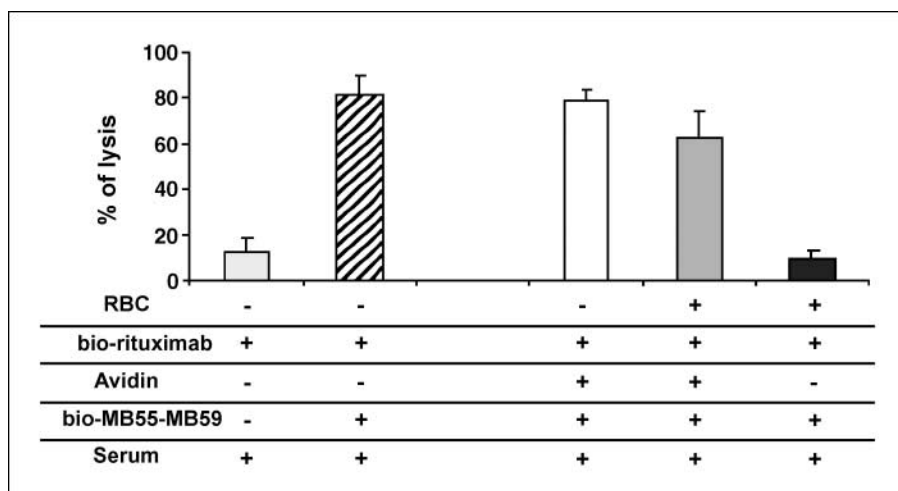
Having ascertained that the biotin labeling procedure did not alter the functional activity of rituximab, MB55, and MB59, we sought to test *in vitro* the three-step biotin-avidin system prior to its use in *in vivo* experiments. To this end, LCL2 cells (2×10^5) were incubated with bio-rituximab (2 μ g/mL) for 10 min at 37°C, followed by 30 min incubation first with avidin (5 μ g/mL) and then with bio-MB55–MB59 (10 μ g/mL each) at 37°C to a final volume of 150 μ L with washes between each step. NHS (50 μ L) obtained from a single donor was added as a source of complement and the number of residual viable cells was evaluated after 1 h of incubation at 37°C. The data presented in Fig. 2 indicate that the CDC of lymphoma cells observed under these experimental conditions was not significantly different from that obtained with unlabeled rituximab, MB55, and MB59.

To confirm the efficacy of the three-step biotin-avidin system in targeting MB55 and MB59 to EBV+ lymphoblastoid cells, LCL2 cells were incubated with biotin-labeled antibodies, avidin, and serum

as indicated above, but this time in the presence of 40% human erythrocytes expressing CD55 and CD59 obtained from the same donor of NHS. Despite the high number of red cells, the complement-dependent killing of LCL2 exceeded 60%, a value not significantly different from that obtained in the absence of erythrocytes (Fig. 2). Conversely, only 9% of LCL2 cells were killed in the absence of avidin, suggesting that bystander erythrocytes bound most of the bio-MB55 and bio-MB59, preventing their targeting to LCL2 which was coated by bio-rituximab (Fig. 2). However, binding of bio-MB55 and bio-MB59 to red cells did not result in cell lysis (data not shown), confirming our previous finding that the two miniantibodies are unable to activate complement (26).

Cell-targeted MB55 and MB59 increase the therapeutic effect of rituximab. The *in vitro* data showed that LCL2 could efficiently be killed even in the presence of erythrocytes provided that the antibodies are targeted to tumor cells. To prove that the

Figure 2. Complement-mediated killing of lymphoblastoid cell lines using the three-step avidin-biotin system. LCL2 were incubated with bio-rituximab followed by avidin and the mixture of bio-MB55 and bio-MB59 (bio-MB55–MB59). NHS was added and residual viable cells were measured. When required, RBC and LCL2 were incubated together with biotin-labeled antibodies, avidin, and serum. Experimental details are given in the Results. *Columns*, means of three different experiments; *bars*, SD.



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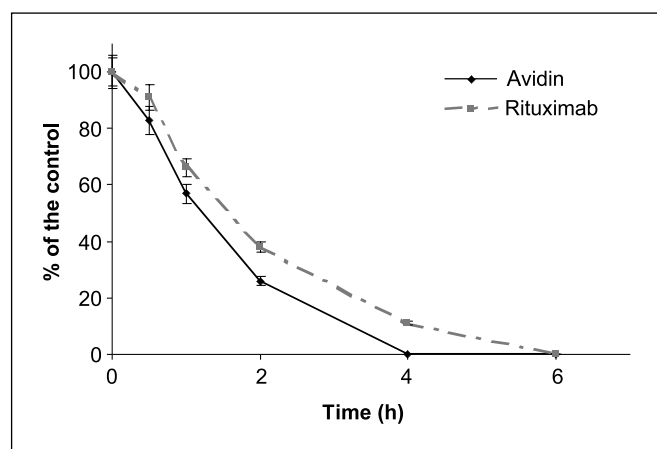


Figure 3. Clearance of rituximab and avidin from mouse peritoneal cavity. Rituximab (25 μ g) and avidin (11 μ g) were injected i.p. and their levels were measured by ELISA at various time intervals with reference to the value obtained 1 min after injection (100%). Points, means from three different mice; bars, SD.

model system was also effective *in vivo*, bio-rituximab and avidin were injected i.p. at an equal molar ratio (corresponding to 25 and 11 μ g, respectively) and the miniantibodies at thrice the molar ratio (30 μ g each), on days 4 and 11. The time interval between the injections was chosen by evaluating the time of disappearance of the individual reagents from the peritoneal cavity as tested by ELISA in the peritoneal washes (1 mL of saline). The results presented in Fig. 3 show that 4 h after i.p. injection, only 10% of rituximab was detected in the peritoneal cavity whereas avidin was undetectable, suggesting that by this time, a substantial proportion of these reagents had left the peritoneum. Based on these results, we selected 4 h as the time interval between the injections of the reagents in our *in vivo* model.

To test the effect of bio-rituximab, bio-MB55, and bio-MB59, SCID mice received an i.p. injection of LCL2 and were then divided into four groups. Group 1 received saline, group 2 received bio-rituximab, whereas group 3 was treated with bio-rituximab, avidin, bio-MB55, and bio-MB59. Group 4 received avidin, bio-MB55, and bio-MB59 and served as a control. These treatments were repeated twice on days 4 and 11, and the animals were followed up to 120 days. The combined results from two independent experiments ($n = 10$ per group) presented in Fig. 4 show that only 30% of the mice treated with bio-rituximab survived, whereas the long-term survival of animals receiving a combination of bio-rituximab, bio-MB55, and bio-MB59 increased to 70%, indicating the synergistic effect of the three antibodies ($P < 0.01$ in a log-rank test). Neither of the two miniantibodies used individually in combination with bio-rituximab and avidin had an increased therapeutic effect compared with bio-rituximab alone. Surprisingly, bio-MB55 and bio-MB59 administered alone had a therapeutic effect similar to that of bio-rituximab, suggesting the direct antitumor activity of the two miniantibodies. Tumor development was unaffected by treating the mice with avidin alone (data not shown).

MB55 and MB59 control tumor growth via ADCC. The *in vivo* effect of bio-MB55 and bio-MB59 on tumor growth was unexpected because we have previously found that the miniantibodies are unable to activate complement (26). To explore the possibility that the two anti-CRP antibodies might cause cell killing via apoptosis, LCL2 cells were incubated with the miniantibodies for 48 h and

analyzed by TUNEL assay. We failed to detect a direct apoptotic effect of MB55 and MB59 used both as single antibody or in combination, and no synergistic effect of the two miniantibodies with rituximab was observed (Fig. 5A). Next, we evaluated the contribution of ADCC to MB55 and MB59-mediated killing of lymphoblastoid cells because ADCC is believed to be an important mechanism of cell killing induced by the mAbs used in cancer therapy (19). The results obtained with human PBMC as effector cells show that MB55 and MB59 are able to cause cell cytotoxicity only when used together (Fig. 5B). Mouse PBMC proved as effective as human PBMC in inducing cell cytotoxicity mediated by the two miniantibodies (data not shown). These antibodies were found to increase rituximab-dependent ADCC of LCL2 from 20% to 35%. Neither apoptosis nor ADCC induced by bio-rituximab were enhanced by the addition of avidin and either bio-MB55 or bio-MB59 (data not shown).

Discussion

The complement system is one of the effector systems involved in the antitumor activity of rituximab (9, 36, 37), the mAb currently used to treat patients with non-Hodgkin lymphoma with satisfactory results and, more recently, approved by the Food and Drug Administration for the treatment of patients with rheumatoid arthritis and other autoimmune diseases (38, 39). Unfortunately, overexpression of CRPs by tumor cells restricts the destructive effect of complement activated by rituximab, limiting its therapeutic efficacy (8, 40). We now provide data indicating that administration of neutralizing antibodies to CD55 and CD59, in combination with rituximab, enhances the therapeutic effect of the chimeric anti-CD20 mAb in an *in vivo* model of B-cell lymphoma.

The present finding that B-lymphoma cells are protected from CDC mainly by CD55 and CD59 with a negligible contribution from CD46, despite the good expression of this CRP on the cell surface, extends previous observations published by Golay et al. (8) These data clearly indicate that the expression level of the CRPs does not necessarily correlate with their functional activity and that the *in vivo* neutralization of the CRPs should be tailored according to their protective role.

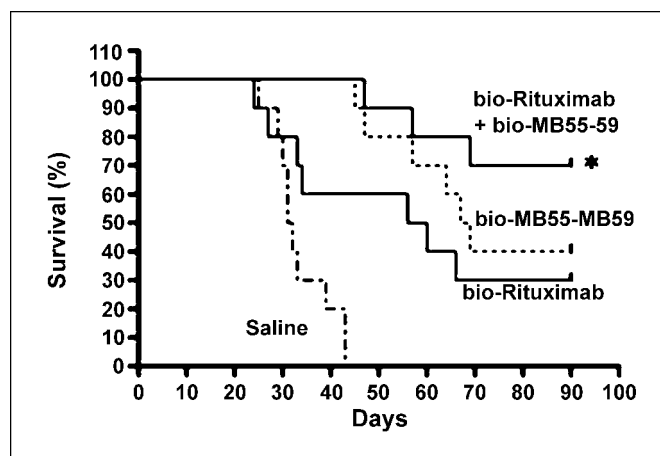


Figure 4. Synergistic effect of bio-rituximab and the mixture of bio-MB55 and bio-MB59. Survival curve. SCID mice ($n = 10$ per group) received 2×10^6 LCL2 cells i.p. and one of the following treatments on days 4 and 11 after challenge: saline, bio-rituximab (25 μ g), bio-MB55 and bio-MB59 (30 μ g each), or bio-rituximab (25 μ g), avidin (11 mg), and the mixture of bio-MB55 and bio-MB59 (bio-MB55-MB59). *, $P < 0.01$ versus rituximab.

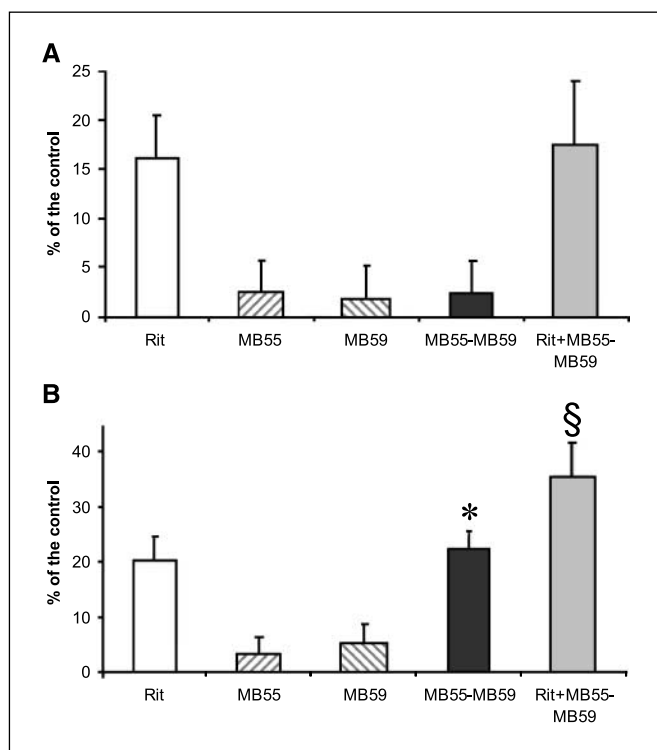


Figure 5. Apoptosis and ADCC of lymphoblastoid cell lines induced by the mixture of MB55–MB59. *A*, for apoptosis analysis, LCL2 were incubated with bio-rituximab (2 $\mu\text{g}/\text{mL}$), with bio-MB55 (10 $\mu\text{g}/\text{mL}$) or bio-MB59 (10 $\mu\text{g}/\text{mL}$), or the mixture of bio-MB55 and bio-MB59 (each at 10 $\mu\text{g}/\text{mL}$) for 48 h and apoptosis was evaluated by TUNEL assay. *B*, for ADCC, CFSE-labeled LCL2 were coated with bio-rituximab (2 $\mu\text{g}/\text{mL}$), with bio-MB55 (10 $\mu\text{g}/\text{mL}$) or bio-MB59 (10 $\mu\text{g}/\text{mL}$), or the mixture of bio-MB55 and bio-MB59 (each at 10 $\mu\text{g}/\text{mL}$) and mixed with human PBMC at a 50:1 cell ratio for 4 h. Specific lysis was evaluated as detailed in Materials and Methods. Columns, means of three different experiments; bars, SD. *, $P < 0.01$ versus MB55 or MB59; §, $P < 0.01$ versus rituximab.

To increase complement-mediated killing by neutralizing the inhibitory activity of CRPs, we have isolated scFv to CD55 and CD59 from a human phage display library to be used in combination with complement-fixing rituximab and other complement-activating mAbs (26). The therapeutic use of scFv has been limited by problems encountered in their large-scale production as well as by difficulties in achieving effective and sustained levels at tumor sites (41). To overcome these problems, the anti-CD55 and anti-CD59 scFvs were genetically fused to the hinge-CH2-CH3 domains of human IgG₁ in order to produce miniantibodies which exhibit increased antigen binding and *in vivo* stability (42). The neutralizing MB55 and MB59 were effective in increasing the complement-mediated killing of lymphoblastoid cell lines induced by rituximab to a value of 80%. It should be pointed out, however, that the susceptibility of the cell lines to CDC depends not only on the expression of mCRPs, which restrict complement activation, but also on the cell surface level of CD20 and on the ability of the anti-CD20 mAb to induce antigen translocation into the lipid raft (18).

The combined effect of rituximab, MB55, and MB59 was tested in a xenograft model of B-lymphoma established in SCID mice using EBV+ CD20+ lymphoblastoid cell lines (29), and was shown by Mosier et al. (43) to induce the formation of multiple masses made of lymphoblastoid and plasmacytoid human B-lymphocytes in the peritoneum, liver, and lymph nodes. These findings were

confirmed in the present study. The cells used to test the therapeutic activity of MB55 and MB59 in the *in vivo* experiments were selected for their susceptibility to killing by human complement following neutralization of CD55 and CD59. Our major concern was that the contribution of complement to the *in vivo* control of tumor growth in mice could not be properly evaluated because murine complement is not as efficient as human complement in lysing tumor cells. The results of the *in vitro* cytotoxic assay show that SCID mouse serum could kill ~40% of LCL2 cells sensitized by rituximab in the presence of MB55 and MB59. Although this value is approximately half of that obtained with human complement, we believe that it was sufficient to control tumor growth in mice receiving complement-activating rituximab. This conclusion is also supported by the finding that the therapeutic activity of rituximab is abolished or greatly reduced in syngeneic and xenograft models of lymphoma established in C1q-deficient mice or in animals treated with cobra venom factor to deplete complement (9, 19, 44).

The strategy adopted in this study to evaluate the beneficial effect of MB55 and MB59 in combination with rituximab in lymphoma-bearing mice was to treat the animals with the minimal dose of mAb that prolonged their survival slightly beyond that of the control group of mice. The selected dose was administered twice with an interval of 1 week based on the observation made by Di Gaetano et al. (9) that cancer cells were still detected in the spleen and bone marrow of tumor-bearing mice 2 to 3 weeks after i.p. injection of rituximab. The complement system is an ideal effector system to eradicate surviving tumor cells in the animal model and, likewise, to control minimal residual disease in cancer patients undergoing chemotherapy that impairs ADCC by reducing the number of effector cells (44).

A problem to be solved for the therapeutic use of MB55 and MB59 in cancer patients was to devise a strategy to target the two antibodies to rituximab-coated tumor cells. The widespread distribution of mCRPs on circulating and tissue cells would prevent the binding of the two mAbs to tumor cells in a sufficient amount to be clinically effective and may also cause undesired side effects. We addressed this issue using the three-step biotin-avidin system employed by Paganelli and coworkers in the clinic to target radio-nuclides to breast cancer (33–35). One drawback of this system is that the procedure of biotin-labeling may impair the functional activity of antitumor mAbs, as shown for biotin-labeled anti-GD3-ganglioside mAb by Jokiranta and Meri (45). Fortunately, this was not the case for the mAbs used in this study because they were still capable of recognizing their antigens after biotin-labeling. In addition, bio-rituximab retained the ability to activate the complement system and to induce ADCC, and both bio-MB55 and bio-MB59 kept their neutralizing activity for CD55 and CD59, respectively. The target specificity of the labeled miniantibodies for lymphoma cells was supported by the finding that >60% of LCL2 cells coated by bio-rituximab were killed in the presence of human erythrocytes used as bystander cells. The presence of avidin in this system was critical because the percentage of killing of lymphoma cells dropped to 9% in the absence of this protein, suggesting that bystander red cells bound most of the blocking antibodies. We failed to detect the release of hemoglobin under these conditions confirming the inability of the two MBs to activate complement (26).

Bio-MB55 and bio-MB59 synergized with bio-rituximab to improve the survival of the lymphoma-bearing mice at 120 days from 30% in the group of animals treated only with rituximab to 70% in

mice receiving both anti-CD20 antibodies and the mixture of MB55 and MB59. It is interesting to note that the increased survival was obtained using one third of the dose of rituximab that eradicated the tumor. The finding that 40% of mice treated with MB55 and MB59 in the absence of rituximab survived 4 months after the inoculum of tumor cells was unexpected. It is important to emphasize that this effect was seen only because the miniantibodies were specific for human CD55 or CD59 and did not recognize murine CRPs (26). Failure of MB55 and MB59 to activate complement or to cause DNA fragmentation of lymphoblastoid cells revealed in this study by the negative TUNEL staining rules out the possibility that CDC and apoptosis are responsible for their antitumor activity, although their *in vivo* contribution cannot be excluded. Conversely, the miniantibody-mediated killing of ~20% of lymphoblastoid cells induced by human or mouse PBMC acting as effector cells rather suggests that ADCC contributes, at least in part, to the increased survival of mice treated with bio-MB55 and bio-MB59.

The definition of the relative contribution of CDC and ADCC to tumor cell destruction *in vivo* was outside the scope of the present investigation because it would require the use of animals that selectively lack either effector cells or complement. However, the finding that >80% of lymphoblastoid cells were killed via CDC when exposed to rituximab, MB55, and MB59, as opposed to 35% of cells killed through ADCC, is in favor of a major role played by complement in the control of tumor growth under this experimental condition, although the *in vivo* contribution of ADCC should not be underscored.

In conclusion, we have shown that the neutralizing miniantibodies, MB55 and MB59, could be targeted to tumor cells providing a novel therapeutic approach to improve the antitumor activity of rituximab. These miniantibodies have the advantage of being entirely human and could therefore be used in human therapy in association with rituximab, ofatumumab, a newly developed anti-CD20 mAb (Humax-CD20; ref. 46) and other antitumor antibodies. There are now ways to modify hinge or Fc protein sequences to improve the ability of these antibodies to activate complement or to induce ADCC (47). Repeated injections of avidin may cause an antibody response in the recipient. Avidins purified from different sources or produced as a recombinant protein are now being screened in several laboratories to find the least immunogenic for human use (48–50). Efforts are also being made in our laboratory to produce a bispecific antibody (51) carrying the specificity of the anti-CD20 mAb and of one of the miniantibodies, MB55 and MB59, as a more suitable and efficient treatment for malignant B cells.

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References

- Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. *Nat Biotechnol* 2005;23:1147–57.
- White CA, Weaver RL, Grillo-Lopez AJ. Antibody-targeted immunotherapy for treatment of malignancy. *Annu Rev Med* 2001;52:125–45.
- Carter P. Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer* 2001;1:118–29.
- McLaughlin P, White CA, Grillo-Lopez AJ, Maloney DG. Clinical status and optimal use of rituximab for B-cell lymphomas [discussion 9-70, 75-7]. *Oncology (Williston Park)* 1998;12:1763–9.
- Reff ME, Carner K, Chambers KS, et al. Depletion of B cells *in vivo* by a chimeric mouse human monoclonal antibody to CD20. *Blood* 1994;83:435–45.
- Dyer MJ, Hale G, Hayhoe FG, Waldmann H. Effects of CAMPATH-1 antibodies *in vivo* in patients with lymphoid malignancies: influence of antibody isotype. *Blood* 1989;73:1431–9.
- Cheson BD. Monoclonal antibody therapy for B-cell malignancies. *Semin Oncol* 2006;33:S2–14.
- Golay J, Zaffaroni L, Vaccari T, et al. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab *in vitro*: CD55 and CD59 regulate complement-mediated cell lysis. *Blood* 2000;95:3900–8.
- Di Gaetano N, Cittera E, Nota R, et al. Complement activation determines the therapeutic activity of rituximab *in vivo*. *J Immunol* 2003;171:1581–7.
- Byrd JC, Kitada S, Flinn IW, et al. The mechanism of tumor cell clearance by rituximab *in vivo* in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. *Blood* 2002;99:1038–43.
- Bannerji R, Kitada S, Flinn IW, et al. Apoptotic-regulatory and complement-protecting protein expression in chronic lymphocytic leukemia: relationship to *in vivo* rituximab resistance. *J Clin Oncol* 2003;21:1466–71.
- Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate *in vivo* cytotoxicity against tumor targets. *Nat Med* 2000;6:443–6.
- Funakoshi S, Longo DL, Murphy WJ. Differential *in vitro* and *in vivo* antitumor effects mediated by anti-CD40 and anti-CD20 monoclonal antibodies against human B-cell lymphomas. *J Immunother Emphasis Tumor Immunol* 1996;19:93–101.
- Uchida J, Hamaguchi Y, Oliver JA, et al. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* 2004;199:1659–69.
- Golay J, Lazzari M, Facchinetti V, et al. CD20 levels determine the *in vitro* susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* 2001;98:3383–9.
- Reff ME, Hariharan K, Braslawsky G. Future of monoclonal antibodies in the treatment of hematologic malignancies. *Cancer Control* 2002;9:152–66.
- Bellosillo B, Villamor N, Lopez-Guillermo A, et al. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated *in vitro* by a caspase-independent mechanism involving the generation of reactive oxygen species. *Blood* 2001;98:2771–7.
- Cragg MS, Morgan SM, Chan HT, et al. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. *Blood* 2003;101:1045–52.
- Cragg MS, Glennie MJ. Antibody specificity controls *in vivo* effector mechanisms of anti-CD20 reagents. *Blood* 2004;103:2738–43.
- Tedesco F, Bulla R, Fischetti F. Terminal complement complex: regulation of formation and pathophysiological function. In: Szebeni J, editor. *The complement system: novel rules in health and disease*; 2004. pp. 97–127.
- Garred P, Hetland G, Mollnes TE, Stoerovold G. Synthesis of C3, C5, C6, C7, C8, and C9 by human fibroblasts. *Scand J Immunol* 1990;32:555–60.
- Langelegen H, Pausa M, Johnson E, Casarsa C, Tedesco F. The endothelium is an extrahepatic site of synthesis of the seventh component of the complement system. *Clin Exp Immunol* 2000;121:69–76.
- Langelegen H, Berge KE, Macor P, et al. Detection of mRNA for the terminal complement components C5, C6, C8 and C9 in human umbilical vein endothelial cells *in vitro*. *APMIS* 2001;109:73–8.
- Gelderman KA, Tomlinson S, Ross GD, Gorter A. Complement function in mAb-mediated cancer immunotherapy. *Trends Immunol* 2004;25:158–64.
- Fishelson Z, Donin N, Zell S, Schultz S, Kirschfink M. Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. *Mol Immunol* 2003;40:109–23.
- Ziller F, Macor P, Bulla R, et al. Controlling complement resistance in cancer by using human monoclonal antibodies that neutralize complement-regulatory proteins CD55 and CD59. *Eur J Immunol* 2005;35:2175–83.
- Piovan E, Bonaldi L, Indraccolo S, et al. Tumor outgrowth in peripheral blood mononuclear cell-injected SCID mice is not associated with early Epstein-Barr virus reactivation. *Leukemia* 2003;17:1643–9.
- Monks A, Scudiero D, Skehan P, et al. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 1991;83:757–66.
- Piovan E, Tosello V, Indraccolo S, et al. Chemokine receptor expression in EBV-associated lymphoproliferation in hu/SCID mice: implications for CXCL12/CXCR4 axis in lymphoma generation. *Blood* 2005;105:931–9.
- Florena AM, Tripodo C, Iannitto E, et al. Value of bone marrow biopsy in the diagnosis of essential thrombocythemia. *Haematologica* 2004;89:911–9.
- Tedesco F, Pausa M, Nardon E, et al. The cytolytically inactive terminal complement complex activates endothelial cells to express adhesion molecules and tissue factor procoagulant activity. *J Exp Med* 1997;185:1619–27.
- Spiridon CI, Ghetie MA, Uhr J, et al. Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antitumor activity of a human breast cancer cell line *in vitro* and *in vivo*. *Clin Cancer Res* 2002;8:1720–30.

33. Paganelli G, Magnani P, Zito F, et al. Three-step monoclonal antibody tumor targeting in carcinoembryonic antigen-positive patients. *Cancer Res* 1991;51:5960-6.
34. Paganelli G, Bartolomei M, Grana C, et al. Radioimmunotherapy of brain tumor. *Neurol Res* 2006;28:518-22.
35. Goldenberg DM, Sharkey RM, Paganelli G, Barbet J, Chatal JF. Antibody pretargeting advances cancer radioimmunodetection and radioimmunotherapy. *J Clin Oncol* 2006;24:823-34.
36. van der Kolk LE, Grillo-Lopez AJ, Baars JW, Hack CE, van Oers MH. Complement activation plays a key role in the side-effects of rituximab treatment. *Br J Haematol* 2001;115:807-11.
37. Kennedy AD, Beum PV, Solga MD, et al. Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. *J Immunol* 2004;172:3280-8.
38. Cohen Y, Nagler A. Treatment of refractory autoimmune diseases with ablative immunotherapy. *Autoimmun Rev* 2004;3:21-9.
39. Cambridge G, Leandro MJ, Edwards JC, et al. Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis. *Arthritis Rheum* 2003;48:2146-54.
40. Manches O, Lui G, Chaperot L, et al. *In vitro* mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* 2003;101:949-54.
41. Adams GP, Schier R. Generating improved single-chain Fv molecules for tumor targeting. *J Immunol Methods* 1999;231:249-60.
42. Powers DB, Amersdorfer P, Poul M, et al. Expression of single-chain Fv-Fc fusions in *Pichia pastoris*. *J Immunol Methods* 2001;251:123-35.
43. Mosier DE, Gulizia RJ, Baird SM, Wilson DB. Transfer of a functional human immune system to mice with severe combined immunodeficiency. *Nature* 1988;335:256-9.
44. Golay J, Cittera E, Di Gaetano N, et al. The role of complement in the therapeutic activity of rituximab in a murine B lymphoma model homing in lymph nodes. *Haematologica* 2006;91:176-83.
45. Jokiranta TS, Meri S. Biotinylation of monoclonal antibodies prevents their ability to activate the classical pathway of complement. *J Immunol* 1993;151:2124-31.
46. Bayes M, Rabasseda X, Prous JR. Gateways to clinical trials. *Methods Find Exp Clin Pharmacol* 2005;27:49-77.
47. Lazar GA, Dang W, Karki S, et al. Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci U S A* 2006;103:4005-10.
48. Hytonen VP, Laitinen OH, Grapputo A, et al. Characterization of poultry egg-white avidins and their potential as a tool in pretargeting cancer treatment. *Biochem J* 2003;372:219-25.
49. Hytonen VP, Nyholm TK, Pentikainen OT, et al. Chicken avidin-related protein 4/5 shows superior thermal stability when compared with avidin while retaining high affinity to biotin. *J Biol Chem* 2004;279:9337-43.
50. Nordlund HR, Hytonen VP, Laitinen OH, Kulomaa MS. Novel avidin-like protein from a root nodule symbiotic bacterium, *Bradyrhizobium japonicum*. *J Biol Chem* 2005;280:13250-5.
51. Ridgway JB, Presta LG, Carter P. 'Knobs-into-holes' engineering of antibody CH3 domains for heavy chain heterodimerization. *Protein Eng* 1996;9:617-21.