Cure of Xenografted Human Tumors by Bispecific Monoclonal Antibodies and Human T Cells

Christoph Renner, Wolfram Jung, Ugur Sahin, Ralf Denfeld, Christoph Pohl, Lorenz Trümper, Frank Hartmann, Volker Diehl, René van Lier, Michael Pfreundschuh*

Tumor immunotherapy should increase both the number of T cells that kill the tumor and the likelihood that those cells are activated at the tumor site. Bispecific monoclonal antibodies (Bi-mAbs) were designed that bound to a Hodgkin's tumor–associated antigen (CD30) on the tumor and to either CD3 or CD28 on the T cell. Immunodeficient mice were cured of established human tumors when mice were treated with both the CD3-CD30 and the CD28-CD30 Bi-mAbs and then given human peripheral blood lymphocytes that had been incubated with the CD3-CD30 Bi-mAb and cells that expressed CD30. The enrichment of human T cells within the tumor and the fact that established tumors can be cured may indicate in situ activation of both the T cell receptor and the costimulatory pathway.

According to the concept of the two-signal T cell activation system, an engagement of the T cell receptor (TCR) complex must be supplemented with a costimulatory signal in order to induce the complete array of T cell effector functions, including proliferation, cytokine secretion, and cytotoxicity. The best characterized costimulatory molecule expressed on resting T cells, CD28, acts as a receptor for ligands expressed on antigen-presenting cells, the well-known B7 (B7-1), BB-1 (B7-3), and the recently described B7-2 antigen (*1*). Cytotoxic T cells can be specifically stimulated and recruited for tumor cell–specific lysis in vitro by the use of bispecific antibodies to the TCR-CD3 complex and tumor-associated antigens (*2*) if the T cells have been previously activated by lymphokines, antigen-presenting cells, or the additional cross-linking of the CD28 antigen (*3*). Combinations of either chemically linked or hybrid-hybridoma (tetradoma)–derived Bi-mAbs with specificities for tumor antigen and CD3 or tumor antigen and CD28 have been used successfully for the induction of tumor cell lysis in vitro (*4*).

Previous work in a xenografted mouse model has shown that Bi-mAbs are able to prevent tumor growth if given in vivo in combination with T cells that had been preactivated by antibody to CD3 (anti-CD3) and interleukin-2 (*5*). To determine the in vivo efficacy of an antitumor approach that uses only Bi-mAbs for activation and targeting of the effector cells, we used severe combined immunodeficiency disease (SCID) mice, which have no functional T and B cells. These mice do not reject xenografts of human lymphocytes or human tumors and can be repopulated with human lymphocytes (*6*). We therefore used the SCID mouse as a model for immunomodulatory therapies of xenografted human tumors with human effector cells (*7*). Hodgkin's lymphoma was chosen as a model human tumor because of the availability of mAbs to the Hodgkin's-associated CD30 antigen (*8*). To take advantage of the possibility of stimulating resting T cells by combined triggering of the TCR complex and CD28, we concentrated on Bi-mAbs to CD3-CD30 and CD28-CD30. These reagents could theoretically activate T cells of any specificity to proliferate and kill CD30$^+$ tumor cells, thus creating a pool of cytotoxic cells at the tumor site. Tetradomas producing Bi-mAbs with reactivity to CD3 and CD28 and the CD30 antigen were obtained by somatic fusion of the hybridoma cell lines producing the parental antibodies (*9*). To determine the efficacy of the Bi-mAbs in inducing proliferation, we tested them in vitro. A combination of purified CD3-CD30 and CD28-CD30 Bi-mAbs induced a significantly stronger proliferation of CD3$^+$ cells in monocyte and natural killer (NK) cell–depleted human peripheral blood lymphocytes (PBLs) in the presence of CD30$^+$ Hodgkin's-derived L540 cells than did a combination of the parental monospecific mAbs to CD3, CD28, and CD30 or either of the Bi-mAbs alone (*10*) (Fig. 1). Despite this strong proliferation-inducing capacity, the combination of CD3-CD30 and CD28-CD30 did not induce significant in vitro cytotoxicity of resting human PBLs against the Hodgkin's-derived cell line L540CY (*11*). The combination of CD3-CD30 and CD28-CD30 was cytotoxic only when PBLs were used that had been previously stimulated with the Bi-mAb CD3-CD30 in the presence of CD30$^+$ cells. No stimulation was achieved in the presence of CD30$^-$ cells (Fig. 2). Prestimulation with only the CD28-CD30 Bi-mAb was not effective (*9*).

Having defined the conditions for the induction of Bi-mAb–mediated T cell cytotoxicity in vitro, we treated SCID mice bearing established human L540CY Hodgkin's-derived tumors of 6 to 8 mm in diameter by injection of Bi-mAbs CD3-CD30 and CD28-CD30 or the respective parental

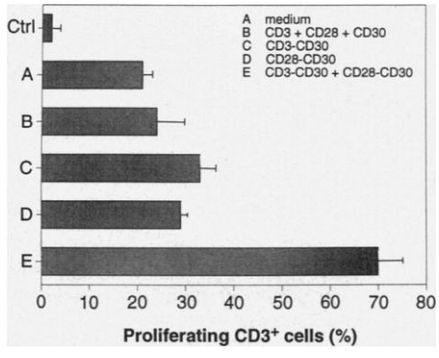

Fig. 1. Proliferation of CD3$^+$ human PBLs in response to Hodgkin's-derived L540CY cells and mABs after incubation of NK cell– and monocyte-depleted mononuclear cells (48 hours, 37°C) with medium (control), with L540CY cells preincubated with medium (A), or with a combination of parental mAbs CD3 + CD28 + CD30 (B), with Bi-mAb CD3-CD30 (C), with Bi-mAb CD28-CD30 (D), or the combination of Bi-mAbs CD3-CD30 + CD28-CD30 (E). The percentage of proliferating CD3$^+$ cells (mean ± SD) was determined as the percentage of CD3$^+$ cells in S, G_2, and M phase.

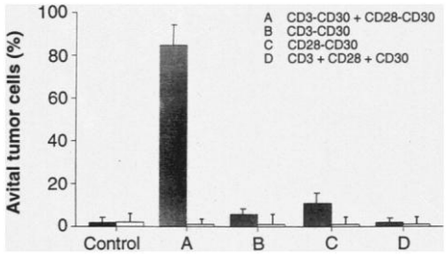

Fig. 2. Cytotoxicity of human PBLs against the Hodgkin's-derived cell line L540CY. For the induction of cytotoxicity, NK cell– and monocyte-depleted mononuclear cells were first stimulated by incubation (72 hours, 37°C) with CD30$^+$ Hodgkin's-derived L540CY cells (hatched columns) or CD30$^-$ HPB-ALL cells (open columns) in the presence of Bi-mAb CD3-CD30. Prestimulated cells were incubated with CD30$^+$ L540CY cells that had been preincubated with an irrelevant CD30–anti-alkaline phosphatase Bi-MAb (control), a combination of Bi-mAbs CD3-CD30 + CD28-CD30 (A), CD3-CD30 alone (B), CD28-CD30 alone (C), or a mixture of the parental mAbs CD3 + CD28 + CD30 (D). Columns show the percentage (mean ± SD) of avital tumor cells.

C. Renner, W. Jung, U. Sahin, R. Denfeld, L. Trümper, F. Hartmann, M. Pfreundschuh, Medizinische Klinik und Poliklinik, Innere Medizin I, Universität des Saarlandes, D-66421 Homburg, Germany.
C. Pohl and V. Diehl, Medizinische Klinik I, Universität zu Köln, D-50921 Cologne, Germany.
R. van Lier, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, 1006 Amsterdam, Netherlands.

*To whom correspondence should be addressed.

antibodies through the tail vein. Tumors were excised and subjected to immunohistological analysis at 12, 24, 36, 48, 72, and 96 hours after injection of the antibodies (*12*). Specific localization of the Bi-mAbs and the mAb to CD30, but not of the parental mAbs to CD3 and CD28, was demonstrated during the entire observation period. Therefore, tumor-bearing animals received 1×10^7 previously stimulated human PBLs intravenously 24 hours after administration of the antibodies. In accordance with the in vitro cytotoxicity data, only human T cells that had been previously stimulated in vitro with $CD30^+$ L540CY cells in the presence of CD3-CD30 Bi-mAb induced a regression of the established tumors: All animals (two series of 10 animals in each treatment group) that had received the mixture of Bi-mAbs CD3-CD30 and CD28-CD30 were cured of the xenografted human tumor. All other animals had progressing tumors and were killed by day 80 (*13*) (Fig. 3).

The survival of prestimulated human effector cells labeled with [^{3}H]uridine was examined in the SCID mice for up to 7 days after intravenous injection, when necrosis of the appropriately treated tumors made continuing measurements difficult. An uptake of the effector cells in the tumors specifically mediated by the Bi-mAbs was demonstrated during the whole observation period, with quantitatively similar enrichment of human effector cells in tumors of animals that had been treated with either one of the Bi-mAbs (CD3-CD30) or the combination of CD3-CD30 and CD28-CD30 Bi-mAbs (Table 1). In contrast to the increasing uptake in the tumors of Bi-mAb–treated animals, the activity in other organs (blood, spleen, liver, and lung) was highest at the first time points (3, 6, and 24 hours) and declined thereafter. It is unlikely that quantitative differences in effector cell binding are responsible for the success of the treatment, because the pattern of enrichment of the human effector cells within the tumor is similar for the group treated with one Bi-mAb alone or the combination of the two Bi-mAbs. Whether this indicates in situ activation of prestimulated human T cells by the combination of both Bi-mAbs remains to be shown. Immunohistology of tumor biopsies taken at 96 hours after administration of the prestimulated human PBLs demonstrated human $CD3^+$-$CD4^+$ and $CD3^+$-$CD8^+$ T cells in the regressing tumors of appropriately treated animals. No human NK cells (as indicated by staining with anti-CD16), B cells (anti-CD19), monocytes (anti-CD14), or granulocytes (anti-CD15) were detected in these tumors (*12*).

Our data show that Bi-mAbs with reactivity to T cells and human tumor-associated antigens localize specifically to human tumor xenografts in SCID mice. Both CD3-CD30 and CD28-CD30 Bi-mAbs can target human T cells to the tumor cells in vivo, but only the combination of both Bi-mAbs is able to induce sufficient tumoricidal activity to cure the tumors. It remains to be shown whether molecules other than CD28, for example, CD2 or CD45, are as effective costimulants as CD28 in this model or might even act alone or in combination to CD28 synergistically. This immunotherapeutic approach is highly effective against established human Hodgkin's tumors that grow as solid tumors in SCID mice. The CD30 molecule is a member of the nerve growth factor receptor family (*14*). Its ligand, CD30L, is a member of the tumor necrosis factor family of cytokines and induces proliferation of Hodgkin's-derived tumor cell lines in vitro (*15*). Because the mAb to CD30 used for the production of the Bi-mAbs, HRS-3, has no receptor activating or inhibiting capacity (*16*), it is unlikely that the efficacy of our approach is due to interference of the CD3-CD30 or CD28-CD30 Bi-mAbs with CD30 receptor–mediated signaling. Rather, the immunotherapeutic approach presented here should be applicable to all other human tumors for which mAbs to tumor-associated antigens are available. In contrast to genetherapeutic approaches, which also try to use the T cell stimulating activity of the CD28 antigen or its ligands (*17*), the Bi-mAb approach is simple and readily applicable to the clinical situation, especially because the parental HRS-3 antibody has been shown to bind specifically to Hodgkin and Reed-Sternberg cells in patients with Hodgkin's disease (*18*). The efficacy of this model seems to be comparable to approaches using immunoconjugates with cytotoxic drugs (*19*) and supports the clinical evaluation of this approach in human malignancies that cannot be cured by standard radiotherapy and chemotherapy.

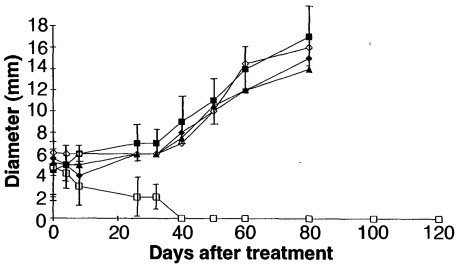

Fig. 3. Bi-mAb–mediated regression of human Hodgkin's-derived tumor xenografts in SCID mice. Tumors regressed only in mice that received the combination of Bi-mAbs CD3-CD30 + CD28-CD30 followed by intravenous injection of human PBLs that had been prestimulated in vitro in the presence of $CD30^+$ L540CY cells and CD3-CD30 Bi-mAbs (□). No significant effect, as compared to medium-treated controls, was seen in animals that received either Bi-mAbs CD3-CD30 (◆) or CD28-CD30 (▲), or a mixture of the parental mAbs CD3, CD28, and CD30 (■), or were treated with a combination of Bi-mAbs CD3-CD30 + CD28-CD30 without human lymphocytes (◇). The tumor diameters represent the mean tumor diameter (± SD), for the group treated with both Bi-mAbs or parental mAbs, of 10 animals in each treatment group. The data shown are from one out of two independent experiments.

Table 1. Tumor uptake of human effector cells mediated by Bi-mAbs. Prestimulated human PBLs were labeled with [^{3}H]uridine (*19*) and injected intravenously into SCID mice (three per experiment) bearing established L540CY human Hodgkin's tumors. Tumor uptake was determined by measurement of radioactivity in tumors and was expressed as the percentage of injected dose per gram of tumor tissue. Measurements were done at the time indicated (days after intravenous injection of [^{3}H]uridine-labeled effector cells). The differences between CD3-CD30 and the combination CD3-CD30 + CD28-CD30 were not significant ($P > 0.05$; two-sided Student's unpaired t test), whereas the differences with the control were significant (day 1, $P < 0.05$; day 2, $P < 0.01$; day 3, $P < 0.001$; day 4, $P < 0.05$).

Treatment	Percentage of injected dose per gram of tissue at:			
	1 day	2 days	4 days	7 days
CD3-CD30	8.3 ± 3.0	10.0 ± 4.2	15.1 ± 2.5	12.0 ± 5.0
CD3-CD30 + CD28-CD30	7.3 ± 1.5	8.2 ± 2.5	13.7 ± 3.5	13.0 ± 6.2
Control (no Bi-mAb)	2.7 ± 0.5	2.5 ± 1.0	2.0 ± 1.0	1.8 ± 2.0

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10. Peripheral blood lymphocytes were obtained from healthy donors by density centrifugation and were depleted of monocytes and NK cells by adherence to plastic and lysis with leucine methyl ester. For the determination of proliferation, 1×10^6 cells were incubated (48 hours, 37°C) with 1×10^5 L540CY cells, which had been preincubated (1 hour, 4°C) with medium, Bi-mAb CD3-CD30 plus CD28-CD30 (1 μg/ml), CD28-CD30 alone, CD3-CD30 alone, or a mixture of the three parental mAbs CD3 (OKT-3, obtained from ATCC), CD28 (15E8), and CD30 (HRS-3). The percentage of proliferating fluorescein isothyocyanate–conjugated anti-CD3$^+$ cells was determined by flow cytofluorometry after treatment with ribonuclease and DNA staining with propidium iodide as the percentage of CD3$^+$ cells in S, G_2, and M phase.
11. For the induction of cytotoxicity, human PBLs were depleted of NK cells and monocytes (to exclude nonspecific cytotoxicity), adjusted to 1×10^6 cells per well, and prestimulated by incubation in RPMI 1640 plus 20% human serum (72 hours, 37°C) with 1×10^5 CD30$^+$ Hodgkin's-derived L540CY cells or CD30$^-$HPB-ALL cells and Bi-mAb CD3-CD30 (1 μg/ml). After prestimulation, 2.5×10^4 fluorochrome PKH2-labeled vital CD30$^+$ L540CY cells that had been preincubated (1 hour, 4°C) with Bi-mAb or parental mAb (1 μg/ml) were added and cocultured (4 hours, 37°C). The uptake of propidium iodide (2 μg/ml) by avital PKH2$^+$ cells was determined by flow cytofluorometry. The percentage of avital L540CY cells was calculated as the 100-fold ratio of (experimental uptake − spontaneous uptake) to (maximal cell number − spontaneous uptake).
12. Binding of Bi-mAb to tumor cells was demonstrated by immunohistological staining of frozen tissue sections with the APAAP technique (*8*). Human PBL subpopulations in the tumors were identified by staining with biotinylated mAbs and streptavidin-alkaline phosphatase (2 μg/ml).
13. Pathogen-free SCID mice were obtained from the Institut fur Versuchstierzucht, Hannover, Germany. The animal experiments had been approved by the Animal Welfare Committee of the State of North Rhine Westfalia and were performed in accordance with German federal guidelines. Solid L540CY Hodgkin's-derived tumors were established in SCID mice by injection of 1.5×10^7 L540CY cells subcutaneously in the ventral thoracic wall of 4- to 6-week-old female mice. After 10 to 14 days, tumors had grown to a minimal diameter of 0.6 to 0.8 cm. Groups of 10 animals received 50 μg of the Bi-mAbs CD3-CD30 and CD28-CD30 or a mixture of the respective parental antibodies on day 0, followed by intravenous injection of 1×10^7 monocyte- and NK cell–depleted human PBLs after 24 hours (day 1). Tumor diameter was measured with a caliper.
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Control of Cell Behavior During Vertebrate Development by *Slug*, a Zinc Finger Gene

M. Angela Nieto, Michael G. Sargent, David G. Wilkinson, Jonathan Cooke*

***Slug*, a vertebrate gene encoding a zinc finger protein of the Snail family, is expressed in the neural crest and in mesodermal cells emigrating from the primitive streak. Early chick embryos were incubated with antisense oligonucleotides to chick *Slug*. These oligonucleotides specifically inhibit the normal change in cell behavior that occurs at the two sites in the emerging body plan in which the gene is expressed. This change, which is the transition from epithelial to mesenchymal character, occurs at the formation of mesoderm during gastrulation and on emigration of the neural crest from the neural tube.**

The events leading to emergence of the body plan in birds closely resemble those of mammalian development but occur in a flat sheet of tissue, the blastoderm, where normal embryo formation can be continuously observed in a simple culture system. We have found that preincubation of blastoderms with antisense oligonucleotides (oligos) can cause specific, transient failure of function of certain early-acting genes. We have screened a complementary DNA (cDNA) library from a 2-day-old chick embryo with a probe from *Xsna* (*1*), the *Xenopus* homolog of the *Drosophila snail* gene (*2*), and isolated clones belonging to genes encoding related zinc finger proteins (*3*). In addition to the chick homolog of *Xsna* and of the mouse gene *Sna* (*4*), we have isolated a gene similar to *Xsna* that we have named *Slug* (Fig. 1). *Slug* displays a dynamic pattern of transcription during early development, which we have analyzed in detail by whole-mount in situ hybridization in embryos of stages 4 to 19 (*5*). This period, from about 18 hours to 3.5 days of development, extends from gastrulation to the onset of differentiation in many organs. Treatment with antisense oligos to the *Slug* gene causes specific developmental failures at the sites of *Slug* transcription. These failures implicate *Slug* in the control of a particular program of cell activity that normally occurs at these sites. The amenability of the early chick embryo to in vitro culture, combined with the use of antisense oligos, may thus provide a powerful tool for analyzing early stages in the genetic control of vertebrate embryogenesis.

The predicted amino acid sequence for the Slug protein (Fig. 1) shows that it contains five zinc fingers in the 3′ half of the protein. The finger region has 89% amino acid identity relative to *Xsna*, and the NH_2-terminal region (residues 1 to 49) has 76% identity. The intervening portion shows only low conservation (30%). In the finger region, Slug is more similar to Escargot (*6*), another member of the *Drosophila* Snail family, than it is to Snail (80% and 69% conservation, respectively). There are apparently no substantial similarities between *Drosophila* and vertebrates in the nonfinger regions in genes with this distinctive 3′ position and type of zinc finger region.

Slug is transcribed in the flask-shaped epiblast cells that form the flanks of the primitive streak (Fig. 2A). Examination of transversely sectioned material after in situ hybridization suggests that the gene turns on in these cells shortly before they emerge through the disrupted basement membrane at the streak to migrate as mesenchyme in the process of gastrulation. However, *Slug* is never expressed in the strip of tissue that is left in front of the regressing Hensen's node and forms the notochord in the future dorsal midline (Fig. 2B). Unlike other tissue leaving the streak, notochord never enters a freely migrating mesenchymal phase. Even when many anterior somites have formed, *Slug* expression continues in the flanks of the streak-like region that provides a source of tissue for progressive construction of the body posteriorly (Figs. 2F and 3D). *Slug* RNA disappears rather rapidly from cells that have emerged from the front half of the streak, and that are assigned to the somite-forming tissue. Somite formation involves reacquisition of epithelial character. More lateral mesoderm, which retains mesenchymal character, maintains detectable expression of *Slug* for a longer period after its generation at gastrulation, so that this lateral *Slug* signal is visible more anteriorly as the body forms.

M. A. Nieto, Instituto Cajal, Doctor Arce, 37, 28002 Madrid, Spain.
M. G. Sargent, D. G. Wilkinson, J. Cooke, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.
*To whom correspondence should be addressed.