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ORIGINAL ARTICLE

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CD3 × CD19 bispecific antibodies and CD28 costimulation for locoregional treatment of low-malignancy non-Hodgkin's lymphoma

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Abstract In advance of using bispecific antibodies for the treatment of B cell lymphoma in humans, we analysed CD3 × CD19 bispecific antibodies for their capacity to induce T cell activiation in cell suspensions from follicular lymphoma lymph nodes. Here, we demostrate that the lack of costimulatory molecules, such as members of the B7 family, on the tumour cells resulted in insufficient activation of autologous T lymphocytes. However, stimulation and proliferation of T cells could be induced by addition of monospecific CD28 antibodies. Moreover, we show that bispecific CD3 × CD19 antibodies can protect severe combined immunodeficiency (SCID) mice from human Epstein-Barr-virus (EBV)-induced B cell lymphoma growth. In these *in vivo* studies, CD28 costimulation did not show a significant benefit, possibly because of the high-level expression of CD80 and CD86 on the surface of the lymphoma cells. Furthermore, the treatment of SCID mice with bispecific antibodies, with or without CD28 antibodies, induced tumour-protective effects, as determined by a rechallenging experiment in long-term-surviving animals with the autologous EBV-transformed tumour B cell line. Treatment of a follicular lymphoma patient by intratumoural injection of both antibodies resulted in immunological responses with increases in the T/B ratio of peripheral blood as well as enhanced NK cell activity without toxic systemic side-effects.

Key words CD3 × CD19 bispecific antibodies · CD28 · Locoregional treatment · Non-Hodgkin's lymphoma

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Introduction

Bispecific anti-CD3 × antitumour antibodies are capable of redirecting cytotoxic T lymphocytes towards malignant cells that express tumour-associated antigens (TAA) [1, 4,7, 8, 11, 15, 22, 23]. This treatment modality has been intensively examined in preclinical models, as well as in initial clinical studies, including solid and non-solid tumours [12, 20]. Since many tumours do not express accessory molecules for costimulatory signalling, attempts have been made to evade the need for costimulation. This was achieved by either using subcutaneous interleukin-2 (IL-2) administration [17] or injection of effector cells preactivated *in vitro* [16]. However, clinical feasibility was usually limited because of the toxic effects of exogenous IL-2 or use of lymphokine-activated killer cells.

Here, we describe an approach using CD3 × CD19 bispecific antibodies in combination with monospecific CD28 antibodies. The use of costimulating CD28 antibodies may replace the natural ligand-receptor interaction between CD28 and the members of the B7 family, namely B7-1 (CD80) and B7-2 (CD86), which are expressed on antigen-presenting cells such as macrophages and dendritic cells [9, 14]. CD28-B7 interaction usually occurs during antigen presentation, simultaneously with binding of the CD3/TcR complex to MHC-bound antigens. A lack of costimulation via this pathway is thought to be of importance for elimination of autoreactive T lymphocytes, since CD3/TcR signalling alone results in anergic T cells [10, 13]. In addition, insufficient costimulation of effector cells may be one possible mechanism for malignant cells to escape immune surveillance. Activation of tumour-infiltrating lymphocytes (TIL) and redirection of T cell-mediated cytotoxicity towards the malignant cells, using CD3 × anti-TAA bispecific antibodies, may result in recruitment of tumour-specific memory T cells present in the TIL population.

Here we show that CD3 × CD19 bispecific antibodies in combination with CD28 monospecific antibodies can be used for activation of autologous T cells in lymph nodes of

patients with low malignancy B cell non-Hodgkin's lymphoma. In addition, *in vivo* studies in severe combined immunodeficiency (SCID) mice with Epstein-Barr virus (EBV)-induced human B cell lymphomas showed a tumour-protective effect of CD3 × CD19 bispecific antibodies that is also demonstrable after rechallenge with autologous lymphoma cells. Based on these experimental findings, a compassionate treatment in patients with relapsed follicular lymphoma, by locoregional application of CD3 × CD19 bispecific antibodies together with CD28 antibodies, was initiated. Analysis of immunological responses upon non-systemic immunotherapy revealed temporary increases in the peripheral T/B ratio as well as enhanced natural killer (NK) cell cytotoxicity, while neither toxic effects nor systemic increases in cytokine levels could be demonstrated.

Materials and methods

Cells and antibodies

The generation and isolation of bispecific antibodies from tetradoma supernatant has been described elsewhere [5, 19]. Briefly, one of the parental cell lines (OKT3, IgG2a-κ, anti-CD3; American Type Culture Collection, USA) was selected for hypoxanthine-guanine phosphoribosyltransferase deficiency and then fused with the second fusion partner (6A4, IgG1-κ, anti-CD19; R. Levy, Stanford, Calif., USA) pretreated with a lethal dose of iodoacetamide using polyethylene glycol (Sigma, Germany), whereupon hybrid hybridomas were cultured in hypoxanthine/aminopterin/thymidine-supplemented medium. The tetradoma cell line 6A4 × OKT3 and the hybridoma cell line 15E8 (anti-CD28; Dr. R. van Lier, NCB, Amsterdam, The Netherlands) were grown in hollow-fibre bioreactors. The supernatants were purified by single-step hydrophobic interaction chromatography on phenyl-superose columns [18a]. Antibody preparations were tested for bi-isotypic antibodies by double-isotype enzyme-linked immunosorbent assay, isoelectric focusing and sodium dodecyl sulphate/polyacrylamide gel electrophoresis. The bispecific binding of the antibody preparations was confirmed by their ability to stimulate T lymphocytes and to redirect cytotoxicity of cytotoxic T cells towards CD19-positive B cell lines as described previously [5].

T cell stimulation assays

Lymph node specimens from patients with follicular lymphomas were minced and then homogenised to a single-cell suspension. After the cells had been filtered through 70 µm nylon strainers to remove aggregates, the mononuclear cells were enriched by Ficoll density centrifugation. Cells were resuspended to a concentration of 5×10^6/ml in RPMI-1640 medium supplemented with Glutamax-I and 5% fetal calf serum (cell culture media and supplements by Gibco, Germany) and incubated with antibodies (1 µ g/ml) in 24-well plates at 37 °C and 7% CO_2 in a humidified atmosphere. After the incubation period of 6 days, cells were harvested, washed in phosphate-buffered saline/1% bovine serum albumin and analysed by flow cytometry after immunofluorescent surface staining (CD2-FITC, CD4-PerCP, CD8-FITC, CD20-PE, CD25-PE, anti-HLA-DR-PerCP, all reagents by Becton Dickinson, Germany). The cell culture supernatants were checked for secreted cytokines [human IL-2 (hIL-2), human interferon γ (hIFN-γ), hIL-4, hIL-12].

SCID mouse assays

Human peripheral blood mononuclear cells (PBMC) were obtained from healthy, EBV-seropositive donors. For SCID mouse engraftment,

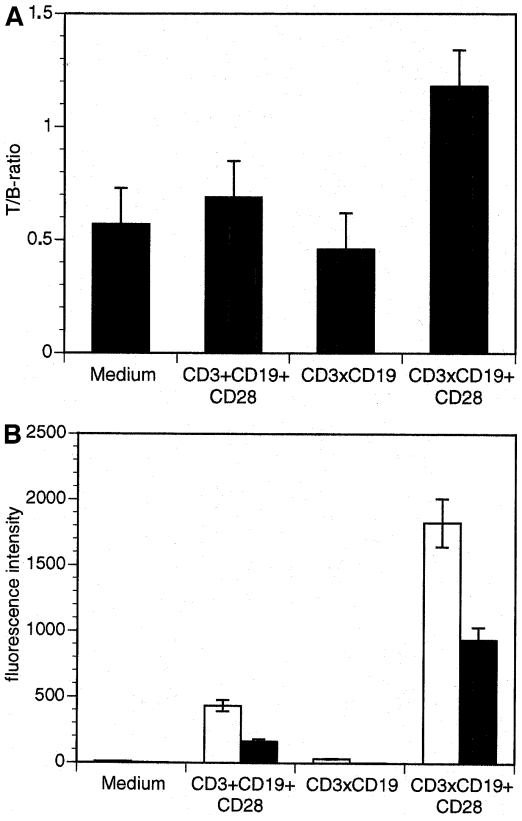

Fig. 1A, B T cell stimulation in follicular lymphoma. Lymph node cell suspensions were incubated for 6 days with the indicated antibody combinations (1 µg/ml), harvested and phenotypically analysed by flow cytometry. Increases in T/B ratio (**A**) and up-regulation of activation molecules such as CD25 (**B**) on CD4-positive (*open bars*) and CD8-positive T cells (*closed bars*) could be demonstrated upon stimulation with CD3 × CD19 and CD28 antibodies. The data show a representative experiment. *Error bars* indicate standard error of the mean

PBMC were resuspended to a concentration of 2.5×10^8 cells/ml in the supernatant of the EBV-secreting cell line B95/8. SCID mice (age 5–7 weeks, non-leaky, ten animals per group) were engrafted with human PBMC by intraperitoneal injection of 5×10^7 cells in 200 µl EBV-containing medium (B95/8 supernatant). On day 3 after PBMC engraftment, the mice were treated with antibody solutions (150 µl i.p., 150 µl i.v., 50 µg each indicated antibody). EBV-transformed B cell lines (LCL, large cellular lymphoma cell lines) were generated by culturing 5×10^7 cells/ml in B95/8 supernatant supplemented with 5 µg/ml ciclosporin A. The cells were used for rechallenging experiments when the cultures were free from contaminating T cells, as detected by flow cytometry (fewer than 0.1% CD3-positive cells). Control SCID mice used in the rechallenging experiment were chosen to be age-matched (100 days).

Locoregional treatment of a B cell non-Hodgkin's lymphoma patient

A 57-year-old woman with low-malignancy non-Hodgkin's lymphoma (follicular lymphoma, stage IV) was chosen for a compassionate treatment with CD3 × CD19 bispecific antibodies and CD28 monospecific antibodies. After the primary chemotherapy with eight cycles COP (cyclophosphamide, vincristine, prednisone) and adjuvant abdominal radiation therapy (40 Gy) the lymphoma relapsed after a 13-month partial remission, and was then treated with a second chemotherapy regimen with six cycles CHOEP (cyclophosphamide, Adriamycin, vincristine, etoposide, predisone) with limited clinical reponse.

Table 1 Treatment of human B cell lymphoma in severe combined immunodeficiency (SCID) mice. *PBMC* peripheral blood mononuclear cells, *LCL* large cellular lymphoma cell line, *PBS* phosphate-buffered saline

Theraupeutic group	Primary survival(%) (day 100 after PBMC engraftment)	Survival after rechallenge (%) (day 70 after LCL challenge)
PBS	10	–
CD3+CD19+CD28	0	–
CD3 × CD19	50	25
CD3 × CD19+CD28	70	50
Control (rechallenge)	–	0

Fifteen months after the second chemotherapy regimen, bispecific antibody treatment was started after written informed consent had been given. For locoregional treatment, 270 μg each antibody (CD3 × CD19+CD28) was injected under ultrasonographic control into a cervical lymph node. Vital signs and toxic effects as well as blood chemistry and immunological parameters of peripheral blood, like flow cytometric phenotyping of peripheral blood lymphocytes, natural killer cell cytotoxicity, and cytokine levels, were monitored before and after the antibody injection. On day 4 after antibody therapy the injected lymph node as well as a contralateral involved lymph node were excised for comparative phenotypic analysis.

Results

T cell stimulation in cell suspensions of follicular lymphoma lymph nodes

Incubation of lymph node cell suspensions from patients with low-malignancy non-Hodgkin's lymphomas with CD3 × CD19 bispecific antibodies together with monospecific CD28 antibodies revealed up-regulation of activation markers such as CD25 (α-chain of IL-2 receptor) on both CD4- and CD8-positive T cells (Fig. 1b). This resulted not only in a proliferative response of T lymphocytes but also in a decrease of malignant B cells (Fig. 1a). This was due to increased cytotoxic activity of the activated T cells redirected to the CD19-positive target cells by the bispecific antibodies as demonstrated by cytotoxicity assays using malignant B cells as target cells and autologous T cells activated by CD3 × CD19 and CD28 antibodies as effector cells (data not shown). Costimulation via the CD28-B7 pathway seems to be crucial in these stimulation assays since incubation with bispecific antibodies alone did not result in sufficient activation of T cells or significant changes in numbers of T cells and B cells. This may be explained by the low expression level of B7 molecules on the surface of the malignant B lymphocytes in the lymph nodes analysed (data not shown).

Treatment of human B cell-lymphoma-transplanted SCID mice

CD3 × CD19 bispecific antibodies were analysed in a human B cell lymphoma model in SCID mice (Table 1) as previously described [6]. After intraperitoneal engraftment of human PBMC together with EBV-containing B95/8 supernatant, SCID mice usually die within 6–7 weeks from anaplastic EBV-positive lymphoma. While 90% (9/10) of the untreated control mice (PBS) and 100% (10/10) of the mice injected with a combination of monospecific antibodies (CD3+CD19+CD28) died of tumour development within 50 days, the groups treated with bispecific antibodies alone (CD3 × CD19) or in combination with CD28 antibodies (CD3 × CD19+CD28) revealed 50% and 70% long-term survival respectively. The differences between these groups were not significant, suggesting that costimulation via the CD28 molecule was not indispensable to the generation of sufficient T cell responses towards the tumour cells in the chosen tumour model. Flow cytometric analysis of costimulatory molecules on the surface of the EBV-transformed B cells showed high expression levels of the

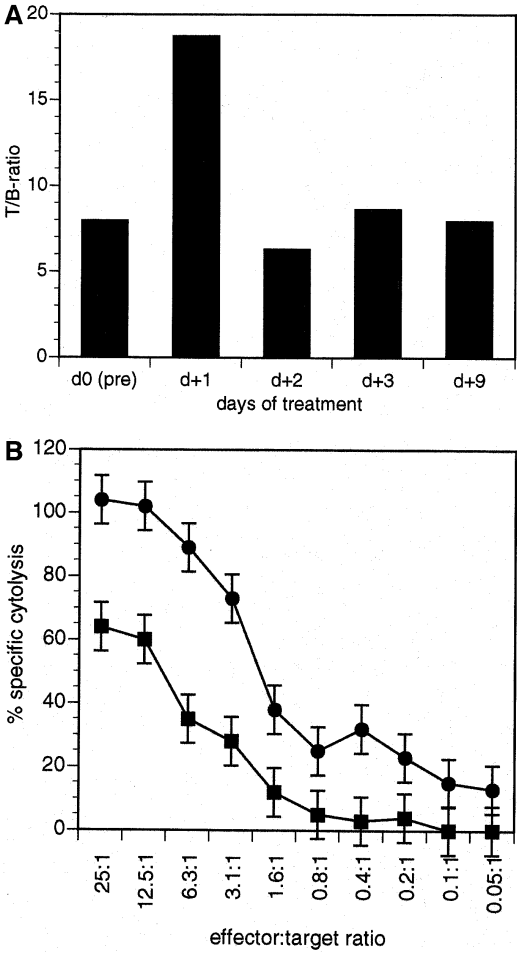

Fig. 2 A, B Monitoring of immunological parameters in peripheral blood during locoregional treatment with bispecific antibodies. Peripheral blood samples were obtained before and after injection of CD3 × CD19 and CD28 antibodies into a cervical lymph node. Peripheral blood lymphocytes were phenotypically analysed by flow cytometry (**A**) for changes in T/B ratio. Natural killer cell activity was determined 1 day before (■) and 5 days after antibody treatment (●) by a standard cytotoxicity assay (4 hours europium release) using the natural-killer-sensitive cell line K562 as target cells (**B**). For standardization the effector:target ratio that resulted in 50% specific cytolysis of K562 cells was calculated

B7 molecules CD80 (B7-1) and CD86 (B7–2) respectively (data not shown). These findings strengthen the hypothesis that costimulatory signalling by CD28 antibodies is necessary in tumours with weak expression of accessory molecules, although it is not required if the tumour cells show sufficient surface expression of B7 molecules.

In a rechallenging experiment, long-term-surviving mice from the first SCID mice assay were intraperitoneally reinjected with the autologous B cell line transformed *in vitro* by EBV. As shown, long-term-surviving animals were capable of rejecting the tumour cells without additional antibody treatment, as determined by survival analysis compared to age-matched, non-leaky control mice (Table 1). However, since no human cells could be recovered from long-term-suriving animals, the effector cell compartment responsible for the tumour-protective effect remains to be proved.

Locoregional treatment of human B cell non-Hodgkin's lymphoma

A female patient with relapsed follicular lymphoma, after initital chemotherapy and radiotherapy, which resulted in a partial remission, was treated by ultrasonographically controlled injection of 270 μg CD3 × CD19 bispecific antibodies and 270 μg CD28 monospecific antibodies into an involved cervical lymph node. Neither allergic or toxic reactions after the application, nor significant changes in clinical laboratory parameters occurred. Although no adverse systemic effects of the locoregionally administered antibodies could be detected, there were influences on immunological parameters. As shown in Fig. 2A, the T/B ratio in the peripheral blood showed a temporary increase from 8.0 before therapy to 18.7 on day 1 after antibody injection. Two days after therapy the T/B ratio dropped back to baseline values. In addition, there was a twofold increase in natural killer cell cytotoxicity against NK-sensitive K562 target cell line, when cytotoxicity assays of day 1 before therapy and day 5 after therapy were compared (Fig. 2B). The concentrations of cytokines measured in peripheral blood serum samples (IL-2, IL-6, IL-10, IFNγ) never exceeded 10 pg/ml during the whole observation period (data not shown).

Discussion

Although CD3 × antitumour bispecific antibodies have been intensively tested in preclinical as well as clinical studies, this concept of immunotherapy still has to prove its clinical value in the management of malignant disorders [24]. A major issue to be addressed in CD3-based bispecific antibody therapy is the requirement for costimulatory signalling towards the effector cells [2]. Since systemic T cell activiation prior to or during the application of bispecific antibodies has to involve toxic side-effects, it is desirable to deliver costimulatory signals towards the effector T cells in a more physiological way.

The results presented in this study clearly demonstrate that CD3 × CD19 bispecific antibodies can be used for *in vitro* stimulation of autologous T cells in lymph nodes of patients with follicular lymphoma if costimulatory signalling is provided by monospecific CD28 antibodies. Artificial costimulation, for example by using CD28 antibodies, seems to be indispensable in tumours that lack accessory molecules able to deliver the required second stimulus [3]. On the other hand, in the human B cell lymphoma model described in this study, CD28 costimulation shows only marginal additional effects upon the capability of bispecific antibodies to prevent tumour growth in immunocompromised SCID mice, possibly because of the high membrane expression of B7 molecules on the EBV-transformed tumour cells.

Since immunological memory is usually ascribed to the T cell and B cell repertoire [21], CD3-based immunotherapy, recruiting T lymphocytes as effector cells, is potentially advantageous compared to recruitment of effector cells of the myeloid lineage. In addition , there is evidence that tumour-specific T cells exist in malignant diseases [18, 25]. In B cell malignancies, T cell clones specific for the idiotype of the tumour cells have been isolated from the peripheral blood [26]. These findings strengthen the hypothesis that activation of tumour-specific T cells that may be present in many malignant diseases, but are ineffective in the control of tumour growth, can reconsitute tumour-specific immune surveillance.

In a rechallenging experiment, long-term-surviving SCID mice that had been pretreated with bispecific antibodies were intraperitoneally injected with the *in vitro* derived autologous EBV-transformed B cell line. No antibodies were injected during the rechallenging experiments. Survival data from these *in vivo* studies suggested a tumour-specific memory, since the rejection of the B cell lymphoma in animals treated with bispecific antibodies proved to be advantageous for the control of lymphoma growth after retransplantation of the autologous tumour cells. However, experiments on the recovery of the putative memory T cells as well, as their functional characterization, have to be performed to prove this hypothesis.

Analysis of T cell stimulation experiments in B cell malignancies with high expression of B7 molecules and with weak or absent expression of costimulatory molecules revealed inefficent B7 signalling in approximately 30% of all samples tested. Thus, we decided to include monospecific CD28 antibodies in the treatment schedule for locoregional application of CD3 × CD19 bispecific antibodies in human low-malignancy non-Hodgkin's lymphoma. A patient with follicular lymphoma underwent a compassionate treatment with a single-dose injection of CD3 × CD19 bispecific antibodies (270 μg) and monospecific CD28 antibodies (270 μg) into a cervical lymph node. Monitoring of clinical and immunological parameters before and during 4 weeks after the antibody injection showed increases in the T/B ratio of the peripheral blood and enhanced natural killer cell activity, while toxic side-effects could not be

demonstrated. On the basis of these experiments, the combination of CD3 × CD19 and CD28 antibodies is currently being tested in a "proof of concept" clinical analysis.

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