Cancer Immunol Immunother (1994) 39: 391–396

Cancer Immunology Immunotherapy

Unprimed CD4+ and CD8+ T cells can be rapidly activated by a CD3×CD19 bispecific antibody to proliferate and become cytotoxic

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Received: 6 June 1994/Accepted: 11 August 1994

Abstract. We previously reported that a CD3×CD19 bispecific antibody (bsAb) can induce efficient killing of tumour cells by preactivated T cells isolated from patients with B cell malignancy. For future intravenous application we investigated whether resting T cells from peripheral blood can be stimulated to proliferate and become cytotoxic with the CD3×CD19 bsAb alone. Indeed peripheral blood mononuclear cells, isolated from healthy donors or patients with B cell malignancy, started to proliferate within 1 day in response to CD3×CD19 bsAb. Within the same time span cytotoxic activity against CD19-positive tumour cells was already detectable. Maintenance of cytotoxic activity was seen during 3 days of culture but optimal lysis of the target cells then required fresh CD3×CD19 bsAb in the cytotoxicity assay. Essentially the same results for proliferation and cytotoxicity were found when separated CD4-positive and CD8-positive T cells were activated by the bsAb in the presence of autologous monocytes. These results may be relevant for the in vivo application of the bsAb when used as immunotherapy in patients with B cell malignancy.

Key words: Immunotherapy – Bispecific monoclonal antibodies – T cell activation – Cytotoxicity

Introduction

Bispecific antibodies (bsAb) combining reactivity against the T cell receptor (TCR)/CD3 complex and a tumour cell antigen can induce T cells to lyse these tumour cells; this is known as targeted cytotoxicity. Most T cells from the peripheral blood (PBL-T) have little or no cytotoxic activity. Therefore, PBL-T have been activated ex vivo with mitogens or CD3 mAb in combination with interleukin-2 (IL-2), loaded with bsAb and used for loco-regional administration in patients with solid tumours (reviewed in [9, 10, 25]). For haematological tumours, including lymphoma, loco-regional administration is not sensible because these tumours are generally disseminated; intravenous administration of bsAb seems to be more logical. Apart from logistic problems, ex vivo activation and expansion of T cells results here in an unfavourable recirculation pattern of the activated T cells [23]. Naive T cells traffic preferentially through lymphoid tissues and low numbers are found in non-lymphoid tissues whereas memory T cells and activated T cells or T cell clones have a strong preference for non-lymphoid tissues, especially the lungs and the liver (reviewed in reference [17]). The problem of an altered recirculation pattern may be avoided, when the bsAb by itself can activate T cells in vivo in lymphoid tissues. Cross-linking of the CD3 complex by the bsAb may occur locally when the bsAb-coated T cell comes across target tumour cells expressing the second antigen recognized by the bsAb. Transduction signals through the TCR/CD3 complex may then lead to functional responses including cytotoxicity and production of cytokines [27].

In this study we describe the activation, with CD3×CD19 bsAb only, of peripheral blood mononuclear cells (PBMC) isolated from healthy donors and patients with non-Hodgkin's lymphoma (NHL) in remission. In all PBMC cultures the development of cytotoxic T cells in response to CD3×CD19 bsAb activation was seen within 2 days of stimulation. This held for both the CD4+ and the CD8+ T cell subset.

Materials and methods

Bispecific monoclonal antibody: SHR-1. The SHR-1 is a fusion product between the YTH12.5 and the MG1CD19 cell lines. YTH12.5 is a

This work was supported by grant IKMN 90-10 from the Dutch Cancer Society. M.C. was supported by a grant from the UK Medical Research Council

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rat IgG2b mAb and has a specificity for the human CD3 ε antigen [6]. MG1CD19 is a mouse IgG1 mAb, specific for human CD19 [7]. The production and purification of the SHR-1 has been described elsewhere [7]. Briefly, SHR-1 was purified by ion-exchange chromatography. Fractions were analysed using isotype-specific assays, and in addition by (reducing) sodium dodecyl sulphate and native polyacrylamide gel electrophoresis, as well as isoelectric focusing.

Mononuclear cell cultures. For isolation, expansion and activation of PBMC, samples from patients were taken after informed consent. PBMC samples from normal donors and patients with NHL in remission were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation. PBMC were cultured at a concentration of 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10 mM HEPES (Gibco, Grand Island, N.Y.), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), hereafter referred to as complete medium, and 10% fetal calf serum (FCS; Gibco). Cells were activated with CD3×CD19 bsAb or CD3 mAb rIgG2b (50 ng/ml), or without additives. Cultures were maintained at 37 °C, in 5% CO_2 for 3–4 days.

Simultaneously proliferation assays were started: PBMC were resuspended in RPMI-1640 complete medium with 10% FCS. Cells (1×10^5/well) were added to round-bottom 96-well plates (Nunc, Roskilde, Denmark) and stimulated for 3 days with 1, 10, or 100 ng/ml Ab prepared in complete medium in a final volume of 200 µl/well. During the last 18 h of culture cells were pulsed with 1 µCi/well [^{3}H]thymidine. Each combination was performed in triplicate. Incorporated radioactivity was quantified by liquid scintillation counting. The labelling index of the cells was measured with bromodeoxyuridine (BrdUrd) as described by the manufacturer (Boehringer Mannheim, Germany). Briefly, cells were incubated for 30 min, 5% CO_2 at 37 °C with BrdUrd. After slides had been prepared, cells were fixed in 70% ethanol (in 50 mmol/l glycine buffer, pH 2.0). Slides were subsequently exposed to anti-BdrUrd and fluorescein-isothiocyanate(FITC)-labelled goat anti-(mouse Ig) Ab and examined in a phase-contrast immunofluorescence microscope. The surface phenotype of the cells was determined on day 0 and repeated when cells were used in the cytotoxicity assay.

Isolation of PBMC subpopulations. PBMC were first depleted of monocytes by adherence to plastic for 2 h. Monocytes were recovered from the plastic and stored on melting ice. Non-adherent cells were used for T cell isolation by rosette formation with 2-aminoethyl-isothiouronium (AET)-treated SRBC followed by Ficoll-Paque density centrifugation [26]. For separation in CD4$^+$ or CD8$^+$ subpopulations, T cells were divided and incubated with either two antibodies to CD4, i.e. RIV7 (Dr. F. Uitdehaag, RIVM, The Netherlands) and Leu3A (Becton Dickinson, San Jose, Calif.), or with two antibodies to CD8, i.e. WT83 and Leu2A (Becton Dickinson). After rosetting with ox erythrocytes coated with goat anti-(mouse IgG) (Tago, Burlingame, Calif.) [13, 22], rosettes were separated from non-rosetting cells by Ficoll-Paque density centrifugation. Only negatively selected cells were used in the proliferation assays. The purity of the cell fractions was determined by immunofluorescence staining using CD3, CD4, CD8, CD16, CD19, CD14, CD45 mAb (Simultest; Becton Dickinson). T cell preparations, and T cell subpopulations were found to be more than 98% pure in all cases, and CD14$^+$ cells were not detectable. The percentages of CD4 and CD8 in the isolated subpopulations did not change during the 3 days in culture.

T cell cultures. Purified 1×10^5 CD4$^+$ or CD8$^+$ T cells were cultured in 200 µl/well in round-bottomed 96-well plates with autologous monocytes (2×10^4 cells/well) for 3 days. T cells were stimulated with the CD3×CD19 bsAb or CD3 mAb (100 ng/ml). Proliferation was measured by [^{3}H]thymidine incorporation as described. Cytotoxic potential was tested in a ^{51}Cr-release assay on day 5.

Monoclonal antibodies. CD3-FITC/DR-phycoerythrin(PE) (Leu4/HLA-DR), CD4-FITC/CD8-PE (Leu3a/Leu2a), antibodies to human TCRγδ-FITC and TCRαβ-FITC, CD3-FITC/CD16-CD56-PE (Leu4/Leu11c-Leu19), and CD45-FITC/CD14-PE (Hle-1/LeuM3) were from Becton Dickinson (San Jose, Calif.). CD19 (CLB, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), CD11a/αLFA-1 (F8.8) and CD54/ICAM-1 (F10.2) were obtained from Dr. A. Bloem (Utrecht, The Netherlands). CD44/NKI-P1 was from Dr. C. Figdor (Amsterdam, The Netherlands), CD32/AT10 from Dr. M. Glennie (Southampton, UK), B7-24 from Dr. M. de Boer (Zwijnaarde, Belgium [3]), MHC-1 [W6/32, anti-(HLA class I common epitope)] from Seralab (Crawley Down, Sussex, UK). Mouse IgG1 and IgG2a CD3 mAb (CLB-T3/4) were provided by Dr. L. Aarden (CLB), and rat IgG2b CD3 mAb (SHL45.6) by Dr. M. Clark. When indicated, FITC-conjugated goat (anti-mouse Ig subclass)-specific Ab against mIgG1 and mIgG2a were used (SBA, Southern Biotechnology Associates Inc., Birmingham, Ala.).

Phenotype determination. Cell fractions were determined for purity by immunofluorescence staining [16]. Samples containing 0.5×10^6–1×10^6 pelleted cells were incubated with the relevant mAb. If non-conjugated Ab were used, a second incubation step was introduced with a FITC-labelled goat anti-(mouse Ig). Cells stained for one- and two-colour immunofluorescence were analysed with a FACScan cell sorter (Becton Dickinson).

Cytotoxicity assay for effector cell targeting. Cytotoxicity was measured in a standard chromium-release assay in round-bottomed 96-well plates (Nunc) using complete medium with 10% FCS [16]. Briefly, 100 µl effector cells was mixed with 50 µl diluted antibodies. After 30 min, 50 µl of a fixed number of ^{51}Cr-labelled target cells (2×10^3 targets/well when cell lines were used or 3×10^3 targets/well when using fresh patient material) was added. A CD19-positive Epstein-Barr-virus(EBV)-transformed B cell line (APD), and (in the case of NHL patients) malignant autologous B cells were used as targets. Plates were centrifuged for 3 min at 100 *g* and incubated for 3.5 h at 37 °C, 5% CO_2. Plates were then centrifuged for another 4 min, and 100 µl supernatant was assayed for released ^{51}Cr in a gamma counter (Minaxi, Autogamma counter, Packard Instrument Co., Meriden, Conn.). Maximum release was measured by incubating the target cells in 1% Triton X-100, and spontaneous release was determined by incubating the cells in medium alone. All tests were carried out in triplicate. The percentage specific ^{51}Cr release was determined as specific release (%) = 100× (experimental release – spontaneous release)/(total release – spontaneous release).

Cell lines. The CD19$^+$ B cell line APD, and EBV-transformed B cell line [14, 15], was used as target cells in the cytotoxicity assays with an alloreactive cytotoxic T cell clone; CAK11-D11 (TCRαβ) as effector cells [14–16]. Cells were regularly tested for *Mycoplasma* infection (Gen-Probe, San Diego, Calif.).

Results

Proliferative response of PBMC after stimulation with CD3×CD19 bsAb

The ability of the monovalent CD3×CD19 bsAb to activate PBMC cultures was compared to activation of these cells by bivalent parental CD3 mAb. PBMC were stimulated with increasing amounts of CD3×CD19 bsAb or CD3 mAb for up to 3 days. Included in the proliferation assay were cells isolated from eight patients with NHL in remission, and results were compared to data obtained from ten healthy donors. In the CD3×CD19 bsAb stimulation a plateau was reached at 10 ng/ml, similar to that following rat IgG2b CD3 mAb stimulation. There were no differences

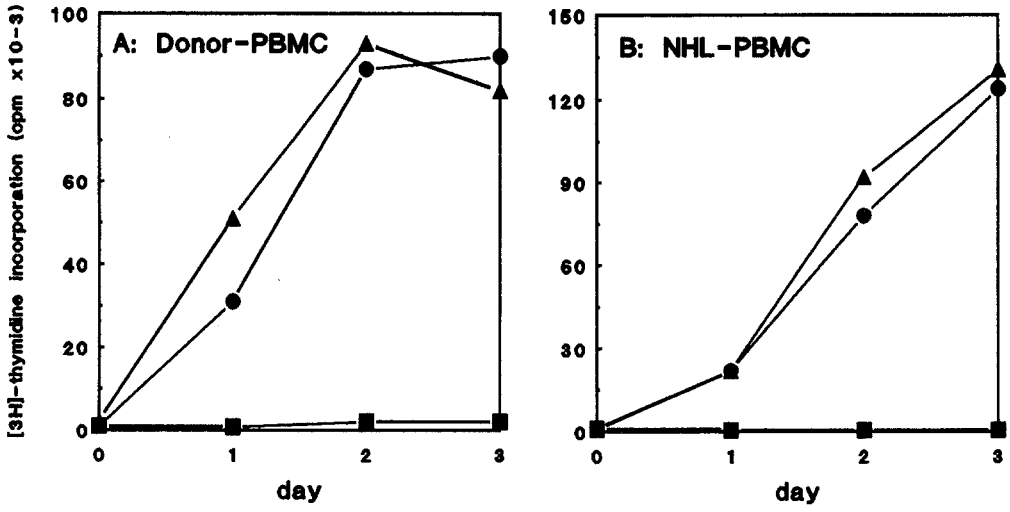

Fig. 1A, B. Activation rate of antibody-induced proliferation of freshly isolated peripheral blood mononuclear cells (*PBMC*). PBMC from a healthy donor (**A**) and a non-Hodgkin's lymphoma (*NHL*) patient (**B**) were stimulated with 10 ng/ml soluble CD3×CD19 bsAb (●), and parental CD3 mAb rIgG2b (▲), and CD19 mAb mIgG1 (■) for 3 days. Results are representative of those from four donors. Proliferation was daily quantified by [^{3}H]thymidine uptake and is expressed as the mean of triplicate measurements

in the dose responses between PBMC isolated from patients or those from healthy donors (data not shown). The time for PBMC from both donors and patients to become activated was next examined. In Fig. 1 data are given for stimulation with 10 ng/ml Ab during 3 days of culture. Proliferation was observed after 1 day of culture. The slight difference in kinetics between donor PBMC (Fig. 1A) and patient PBMC (Fig. 1B) may be related to the initial percentage of T cells, 70% compared to 30%, present in the PBMC fractions. Similar results were obtained when 1 ng/ml CD3×CD19 bsAb or CD3 mAb was used (data not shown).

Development of cytolytic activity in freshly isolated PBMC

For immunotherapy with bsAb, T cells need to be activated to exert lysis on tumour target cells after cross-linking to the target cells by the bsAb. Since proliferation of PBMC was observed after 1 day when cultured with the bsAb, we next examined whether these cells also employed cytolytic activities. Freshly isolated PBMC were cultured for up to 3 days with CD3×CD19 bsAb or CD3 mAb and were followed for their ability to lyse CD19$^+$ target B cells. Figure 2 shows that donor PBMC stimulated with CD3×CD19 bsAb

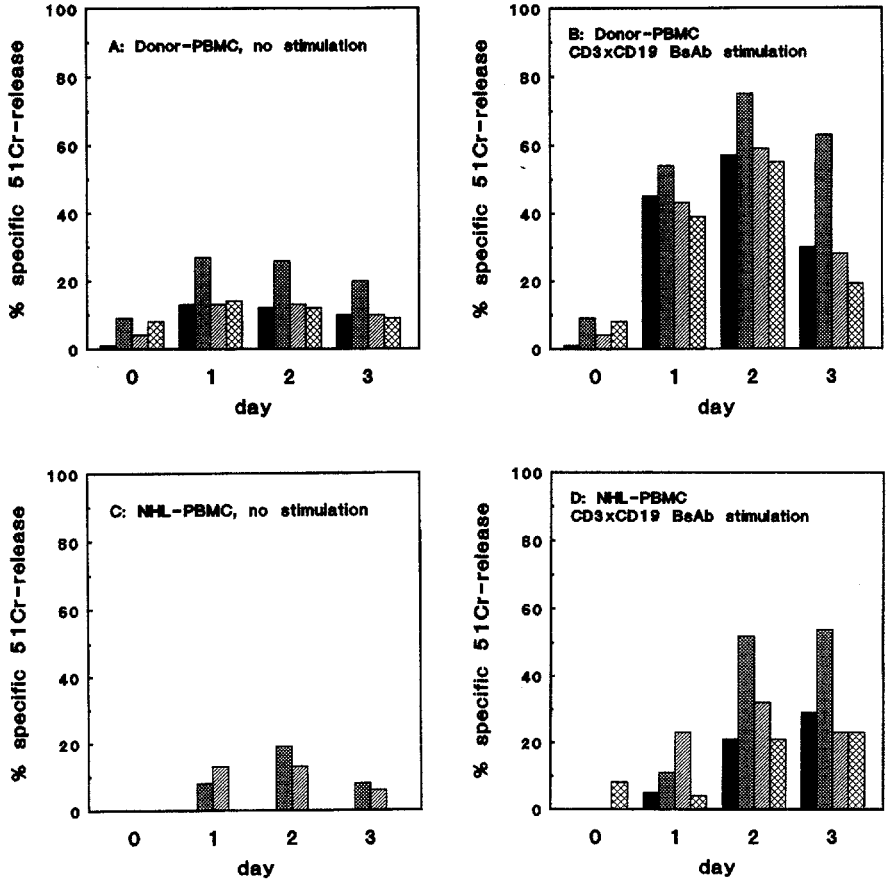

Fig. 2A–D. Cytotoxic potential of donor PBMC (**A, B**) and NHL PBMC (**C, D**) to CD19$^+$ Epstein-Barr-virus-transformed (EBV-B) or autologous tumour target cells. PBMC were cultured with 50 ng/ml CD3×CD19 bsAb for 3 days (**B, D**) or without activation stimulants (**A, C**). Development of cytotoxic potential was measured daily in a ^{51}Cr-release assay, at an effector-to-target ratio of 27 : 1, in the presence of the indicated antibodies (100 ng/ml): CD3×CD19 bsAb (▩), CD3 mAb rIgG2b (▨), or CD19 mAb mIgG1 (▩), or with no antibody (■). Data are expressed as the means of triplicate samples and are representative for three separate experiments, using PBMC from four donors and two NHL patients

Table 1. Labelling index of CD4+ and CD8+ cells in peripheral blood mononuclear cell cultures

	BrdUrd-positive cells (%) in T cell subpopulation:	
Ab (50 ng/ml)	CD4+	CD8+
No mAb	ND	1
CD3×CD19 bsAb	30	35
CD3 mAb	22	25

Table 2. Cytolytic activity of CD3+CD4+ and CD3+CD8+ T cell subpopulations against a CD19+ target cell

	Lysis (%) by T cell subpopulation:			
	CD4+		CD8+	
Stimulus in 5-day culture	No mAb	bsAb	No mAb	bsAb
No mAb	1	21	0	27
CD3×CD19 bsAb	21	38	17	42
CD3 mAb	15	30	16	40

T cell subpopulations were cultured for 5 days in the presence of autologous monocytes. Bispecific (bs) Ab were added to the cytotoxicity assay (100 ng/ml), and the percentage lysis was measured in a ^{51}Cr-release assay at an effector to target cell ratio of 27:1

became cytotoxic after 1 day of culture (Fig. 2B). Even on day 0 PBMC cultured with bsAb showed some activity during the 4-h cytotoxicity assay. The cytolytic activity of donor PBMC without stimulation, is shown in Fig. 2A. After activation of donor PBMC with the bsAb (Fig. 2B) during 1 or 2 days of culture, no extra addition of bsAb to the cytotoxicity assay was needed. At day 3, bsAb-mediated cytotoxic activity was still present but the addition of bsAb during the cytotoxicity assay increased it substantially. Staining of the PBMC with goat anti-(mouse Ig) Ab showed that, until day 2, bsAb was detectable on the surface of the cells. On day 3 bsAb could not be detected by immunofluorescence analysis although cytotoxic activity was still found, indicating the presence of the bsAb on the cells in low amounts.

From day 1, CD3/HLA-DR double-positive cells appeared in donor PBMC cultures with bsAb. The ratio between CD4+ and CD8+ cells in the culture did not change in the 3-day period. On day 3, cultures were separated by fluorescence-activated cell sorting in CD4+ and CD8+ cell fractions: in the cultures stimulated with CD3×CD19 bsAb or CD3 mAb both T cell subpopulations incorporated BrdUrd, used as a marker for cell proliferation. The labelling indices of both CD4+ and CD8+ fractions in the bsAb- or CD3-mAb-stimulated cultures were high (22%–35%), while a labelling index of 1% was found in the unstimulated PBMC culture (Table 1).

Similar results were obtained with PBMC from two NHL patients in remission, using autologous tumour cells as targets (Fig. 2C, D). The PBMC fraction contained 47% CD19+ cells on day 0. After stimulation with CD3×CD19 bsAb, cells became cytotoxic from day 2. Addition of fresh

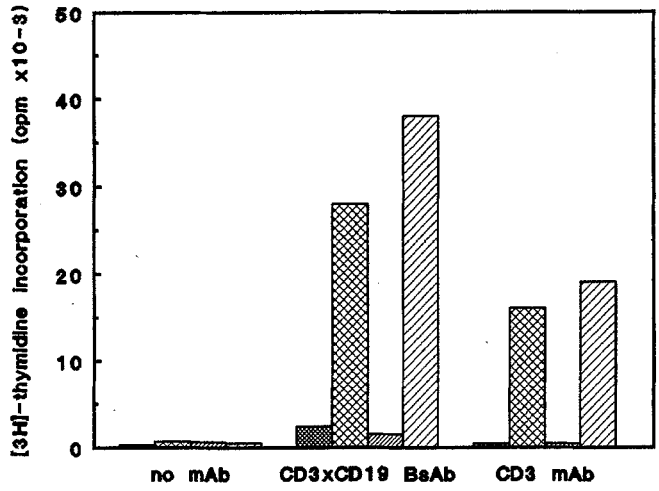

Fig. 3. Proliferation of freshly isolated and purified CD4+ and CD8+ T cell subpopulations. T cells (T) were cultured with CD3×CD19 bsAb and CD3 mAb rIgG2b (100 ng/ml) in the presence of autologous monocytes (M) (more than 99% CD14+) at a T:M ratio of 1:0.4. ▩, CD4+ T cells; ▨, CD4+ T cells plus monocytes; ▨, CD8+ T cells; ▨, CD8+ T cells plus monocytes. Each point represents the mean of triplicate determinations. One representative experiment of three is shown

bsAb during the assay increased cytotoxicity up to 55% at an effector:autologous-tumour-cell ratio of 27:1, while CD3 mAb and CD19 mAb had no effect. Spontaneous lytic activity was minimal (less than 15%). Staining with goat anti-(mouse Ig) Ab during the culture period showed that both the CD19+ and the CD3+ population were coated with the bsAb. Control cultures were performed by culturing PBMC with a CD3 rIgG2b mAb. CD3×CD19-bsAb-stimulated PBMC were found to be as cytotoxic as CD3-mAb-stimulated PBMC (data not shown).

Analysis of CD3×CD19-bsAb-mediated lysis by CD4+ and CD8+ T cell subpopulations

Stimulation by CD3×CD19 bsAb induced proliferation of both CD4+ and CD8+ T cells, as confirmed by BrdUrd incorporation in short-term cultures. Also, expansion of the lymphocytes for 14 days with the bsAb showed that both CD4+ and CD8+ T cells remained in culture (data not shown). We therefore examined the requirements for activation of highly purified CD3+CD4+ or CD3+CD8+ T cell fractions, obtained by negative selection, using accessory cells and Ab. Results are presented in Fig. 3. After 3 days of stimulation both the CD4+ and CD8+ T cell subpopulation were activated by the CD3×CD19 bsAb in the presence of autologous monocytes without further additives. Stimulation with CD3 rIgG2b mAb in combination with monocytes was nearly as high as in the same culture stimulated with CD3×CD19 bsAb. This was found for both the CD4+ and the CD8+ T cell fraction.

We then investigated whether these activated T cell subpopulations acquired cytolytic activity when cultured with the bsAb. Cytotoxic activity was measured against a CD19+ EBV-tranformed B cell line in the presence of

CD3×CD19 bsAb. Both CD4+ and CD8+ T cells, when separately activated with monocytes in combination with the bsAb but without further additives, efficiently killed the target cells (Table 2). Control experiments with freshly isolated monocytes or monocytes activated with interferon γ (100 U/ml) showed no cytolytic activity on the part of the monocytes to the tumour target cells (EBV-B, APD) in the presence of bsAb (data not shown).

The level of cytolytic activity varied among the three donors tested, where bsAb-mediated lysis by CD8+ T cells (range from 33% to 84%) exceeded lysis by CD4+ T cells (range from 20% to 62%).

Discussion

Effective immunotherapy using intravenously administered bsAb, having recognition sites for CD3 and a tumour antigen, is dependent on induction of T cell activation and maturation towards a cytotoxic phenotype (reviewed in [1]). Under physiological conditions antigen-specific T cell activation occurs as a consequence of TCR/CD3 cross-linking in combination with costimulatory signals transferred via recognition of membrane molecules between T cells and antigen-presenting cells. If both activation of the T cell as well as its targeting to the tumour cell has to occur in vivo, the TCR cross-linking task can be taken over by the bsAb through Fab-mediated binding to CD3 on the T cell and by binding with their Fc to a Fcγ receptor on an accessory cell [19, 21, 32]. Fab-mediated binding to CD3 as well as to an antigen on the tumour target cell may replace the role of the FcγR+ cell, or just be supplementary [11, 18, 28, 31, 32].

On the basis of theoretical and practical considerations (see Introduction), we intend to use our biologically produced CD3×CD19 bsAb without prior ex vivo T cell activation. Monitoring the kinetics whereby cytotoxic T cells are generated by the action of the bsAb in in vitro cultures of PBMC revealed that within 1 day substantial target-cell-specific cytotoxicity could be detected. Cells retained this capacity during a 3-day culture period. Only at day 3 did they need repeated addition of bsAb to obtain optimal lysis. This may be ascribed to the inability of individual TCR/CD3 complexes to continue signalling once engaged [2]. Induction of proliferation was as good as with CD3 mAb, which is often used for ex vivo activation of T cells [20]. These results hold true both for normal donors and for NHL patients. Interestingly, our bsAb not only induced proliferation and cytotoxic activity in the CD8+ subset, but also in the CD4+ T cells. Stimulation of these CD4+ and CD8+ T cells subsets (obtained by negative selection), occurred in the presence of autologous monocytes and CD3×CD19 bsAb. CD4-executed cytotoxicity has previously been reported for mitogen- but not for bsAb-activated T cells [11, 12, 24, 29, 30]. The proliferation observed in vitro of CD4+ and CD8+ cells by CD3×CD19 bsAb may have a favourable effect on the effector-to-target-cell ratio in vivo as well as on the secretion of a number of cytokines [4, 27].

T cell activation in the PBMC cultures, as well as in the experiments with isolated CD4+ and CD8+ cells, was dependent on the presence of monocytes. We found that the accessory function provided by malignant B cells may be of minor importance (manuscript in preparation). Monocytes are well known for their supportive action in CD3-mAb-mediated T cell activation [19, 21], and can be held responsible for the bsAb-mediated T cell responses seen in our experiments. In mouse models of B cell tumours, the efficacy of intravenous application of CD3×anti-Id bsAb to kill malignant B cells has been shown [5, 8, 31]. The role of Fc-Fcγ receptor interactions contributed to the effectiveness of the therapy [8, 32].

In conclusion, we have demonstrated that unprimed T cells are in vitro rapidly induced to a cytotoxic state in the presence of CD3×CD19 bsAb and accessory cells. Both CD8+ and CD4+ T cells exert this potential. In vivo testing of this bsAb will reveal whether the activation of T cells will result in sufficient cytotoxicity.

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