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Killing of human leukaemia/lymphoma B cells by activated cytotoxic T lymphocytes in the presence of a bispecific monoclonal antibody (αCD3/αCD19)

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SUMMARY

Bispecific antibodies (BsAb) can be used to retarget T cells irrespective of their specificity to certain target cells inducing target cell lysis. We have tested the efficacy of the BsAb SHR-1, directed against the T cell antigen CD3 and the B cell antigen CD19 to induce (malignant) B cell kill by T cells as measured in a ^{51}Cr-release assay. Two cytotoxic T cell clones (CTL), expressing TCR$\alpha\beta$ or TCR$\gamma\delta$, were effective in killing CD19 expressing B cell lines at different stages of differentiation in the presence, but not in the absence, of the BsAb. CD19$^-$ target cells were not killed. Fresh CD19$^+$ leukaemia/lymphoma cells were also efficiently killed by SHR-1 preincubated CTL clones. In addition, phytohaemagglutinin (PHA) or CD3-activated IL-2 expanded peripheral blood mononuclear cells (PBMC) of normal donors did so after 2 weeks of stimulation. A concentration of 100 ng/ml of the BsAb was sufficient to obtain optimal lysis of all target cells tested. These results show that fresh human leukaemia/lymphoma cells, freshly derived from active lymphoblastic leukaemia (ALL) as well as non-Hodgkin's lymphoma (NHL) patients, can be effectively killed in the presence of this BsAb by activated T cells.

Keywords malignant B cells bispecific monoclonal antibodies activated T cells cytotoxicity

INTRODUCTION

In a large portion of non-Hodgkin's lymphoma (NHL) and acute lymphoblastic leukaemia (ALL), both haematologic malignancies, the malignant cell belongs to the B cell lineage. Although these cases respond to chemotherapy fairly well, relapse often occurs, even after intensive chemo-radiotherapy and autologous bone marrow transplantation [1]. New therapeutic approaches are being investigated, of which immunotherapy is a promising one [2]. MoAbs for the destruction of tumour cells may be used, utilizing an extrinsic effector mechanism (e.g. toxins) or host effector mechanisms; antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cellular cytotoxicity [2–4].

Immunotoxins, however, may suffer from serious aspecific (cyto)toxicity; ADCC against lymphoma cells is not easily demonstrated *in vitro*, and results obtained *in vivo* with antibodies such as αCD19 [5,6], αCD20 [7] and αId [8,9] whilst encouraging, are presently limited, showing only transient elimination of malignant B cells, and partial or short term remissions. A complete but transient remission has been achieved in a B-lymphoma patient using humanized CAMPATH-1H [10]. No durable, complete remission has been obtained so far. Other biological response modifiers in immunotherapy, e.g. IL-2 to obtain LAK cells or activation of natural killer (NK) and T cells *in vivo*, also have met variable results [4,11].

A novel approach involves the use of MoAbs with a dual specificity; these so-called bispecific antibodies (BsAb) may be used to retarget (activated) cytotoxic T lymphocytes (CTL) onto tumour cells [12–14]. BsAb directed to the TCR/CD3 complex can trigger the effector function of T cells and bridge the CTL to the target cell via the second arm of the BsAb recognizing an antigen on the surface of the tumour cell [15–19]. In this system BsAb activate and retarget CTL to lyse selected target cells, irrespective of their intrinsic antigenic reactivity. Results so far obtained *in vitro* [20–23] and in locoregional application *in vivo* of solid tumours are promising [24–26]. It may be expected that haematologic malignancies would be better targets than solid tumours for immunotherapy due to their better accessibility [27]. Moreover, with chemotherapy a state of minimal residual disease can often be obtained, in which immunotherapy may be more effective.

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In this study we have tested the efficacy of a BsAb against the CD3 and CD19 antigens to retarget CTLs as effectors towards human B cells. Three B cell lines, which together represent different stages of lymphoid differentiation, as well as fresh leukaemic/lymphoma cells were used as targets. Two CTL clones and activated peripheral blood mononuclear cells (PBMC) were used as effector cells. In all cases a specific cytotoxicity was found only when the BsAb, SHR-1, was present.

MATERIALS AND METHODS

Parental hybridoma cells

YTH12.5 is a rat IgG2b MoAb. The antibody has specificity for the human CD3 antigen [28] and was included with the CD3 panel in the Third and Fourth International Workshop on Human Leukocyte Differentiation Antigens [29,30]. MG1CD19 is a mouse IgG1 MoAb, having a specificity for the human CD19 [31].

Hybrid hybridoma

SHR-1 is a fusion product between the YTH12 and the MG1CD19 cell lines. Cell fusion and antibody purification are described elsewhere [28,31]. Briefly, one parental cell line had been preselected for resistance to the drug 6-thioguanine and sensitivity to counter selection by the drug combination of hypoxanthine, aminopterin and thymidine (HAT). This cell line was fused using polyethylene glycol treatment with the second cell line pretreated with a supralethal dose of iodoacetamide. Hybrid-hybridoma cells were selected for in medium supplemented with HAT. Supernatants from growing cultures were screened for the different immunoglobulin isotypes in ELISA or erythrocyte-linked assays. Specificity for cell surface antigens (CD3 and CD19) was assessed by indirect immunofluorescence assays. Hybrids from positive cultures were recloned twice. The selected clone SHR-1.6 secreted BsAb with the first specificity for the human CD3 ε-chain and a second specificity for the human CD19. The antibody was purified by ion exchange chromatography and purified fractions were analysed by gel electrophoresis as well as isoelectric focusing. Pure bifunctional BsAb as determined by various biochemical methods [31] was used in this study.

SHL45.6 was produced by fusing the hybridoma cell YTH12.5 (ratIgG2b-αCD3) with a mouse myeloma cell line (J558L) which only produces a mouse lambda light chain [31]. During ion exchange chromatography the fraction containing the parental YTH12.5 MoAb was collected and was further indicated as SHL45.6. This antibody is used as a control as it was produced from a rat-mouse hybridoma cell line (i.e. is similar in this respect to SHR-1) and it has been grown, purified and stored in an identical manner to the bispecific CD3/CD19.

Target cells

Cytotoxicity was determined against a variety of (tumour) cells, including various human CD19$^+$ and CD19$^-$ cell lines, as well as fresh tumour cells isolated from patients.

Three CD19$^+$ hu-cell lines were used: Nalm-6, a pre-B-ALL; APD, an Epstein–Barr virus (EBV)-B transformed cell line [32,33] and CRL1484(HS-Sultan), a plasmacytoma cell line (ATCC, Rockville, MD). One CD19$^-$ cell line was used, the RPMI 8226 (CCL155), a human myeloma cell line (ATCC).

Target cell lines were all maintained in RPMI 1640 with 10 mM HEPES (GIBCO, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO); hereafter referred to as complete medium, supplemented with 10% fetal calf serum (FCS; GIBCO).

The tumour cell lines were regularly tested for mycoplasma.

Isolation of lymphocytes and tumour cells

Preparation of PBMC from the peripheral blood (PBL) and the bone marrow (BM). Heparinized cell samples were obtained from healthy individuals (PBL) or from patients with NHL or ALL (PBL/BM). Mononuclear cells were isolated on Ficoll–Paque (Pharmacia, Uppsala, Sweden). Cells were stored in liquid nitrogen until use.

Preparation of single cell suspensions from lymphnodes. Pieces of lymphnode were removed during operation. Parts of the biopsies were transported in sterile RPMI 1640 medium containing penicillin and streptomycin. A single cell suspension was obtained by mechanical disaggregation using scalpels and incubated in MEM (GIBCO) containing 15 U/ml collagenase (Sigma, St Louis, MO) for 1 h at 37°C, while gently kept in movement. After incubation, the free cells were washed twice with RPMI 1640 containing 10% FCS and stored in liquid nitrogen. Viability, tested by trypan blue exclusion, was >90% in all cases.

Those fractions of patient material containing CD19$^+$ cells are used in the cytotoxicity assays as target cells. The percentage of CD19$^+$ cells exceeded 60%. The night before the assay cells were thawed and cultured in about 7–10 ml RPMI 1640 complete medium plus 10% FCS in 25 cm^2 culture flasks (Nunc, Roskilde, Denmark): when the viability of the material permitted (>80%), the following morning cells were used as targets in the cytotoxicity assay.

Cloned effector cells

Two alloreactive cytotoxic T cell clones, CAK11-D11 (TCR$\alpha\beta$) and AK4 (TCR$\gamma\delta$) were used. Their generation, stimulation and culture maintenance have been described elsewhere [32,33].

Briefly, cells were cultured using RPMI 1640 supplemented with 10% non-heat-inactivated pooled human AB serum, 2 mM glutamine, 1 μg/ml indomethacin (Sigma), 1 μg/ml leucoagglutinin (Pharmacia) and penicillin-streptomycin.

Expansion of responder cells. One thousand cells/well were seeded in 96-well round-bottomed microtitre plates (Nunc) with 2×10^4 irradiated (2000 rad) allogeneic PBL feeder cells and 1×10^4 allogenic irradiated EBV-B-LCL (APD, BSM) cells per well. After 5 days four wells were harvested and expansion of the cells was measured; 1 week after restimulation cells were used in the cytotoxicity assay. Cells were replated and restimulated once a week. The phenotype of CTLs was checked every 3 weeks.

Peripheral blood-derived effector cells

PBMC from healthy donors were isolated as described and incubated at 1×10^6 cells/ml in 80 cm^2 cell culture flasks (Nunc) in RPMI 1640 complete medium with 5% FCS. Aiming to generate efficient cytotoxic effector cells [20,34], cells were activated using either 1 μg/ml phytohaemagglutinin (PHA; HA16, Wellcome Diagnostics, Dartford, UK) plus 25 U/ml IL-2 (EuroCetus BV, Amsterdam, The Netherlands), or with 10 ng/

ml WT32 (αCD3-mIgG2a MoAb, kindly provided by Dr W. Tax, Nijmegen, The Netherlands) plus 25 U/ml IL-2, or with 10 ng/ml SHL45.6 plus 25 U/ml IL-2. Three days after the culture had been started cells were counted, viability was determined and if necessary diluted to $0{\cdot}5 \times 10^6$ cells/ml using fresh complete medium plus 5% FCS supplemented with 100 U/ml IL-2. This was repeated every 2 or 3 days.

Surface-phenotype of the cells was determined at day 0 and repeated when cells were used in a cytotoxicity assay which was once a week for 3 consecutive weeks. Activated recovered lymphocytes were used as effector cells in a ^{51}Cr-release cytotoxicity assay against the CD19$^+$ cell line and malignant B cells.

Phenotype determination

Surface antigens on CTLs, freshly isolated cells, PBL-derived effector cells and patient material were detected by immunofluorescence staining. Characterization of patient material (PBL, BM, lymph node) was done on cryopreserved cells.

MoAbs used were: CD3-FITC/DR-PE (Leu-4/HLA-DR, Becton Dickinson, Mountain View, CA), CD4-FITC/CD8-PE (Leu-3a/Leu-2a, Becton Dickinson), anti-human TCRγδ-FITC and TCRαβ-FITC (Becton Dickinson), CD16 (Leu-11a-FITC, Becton Dickinson; or CLB-FcR granI, kindly provided by Dr T. Huizinga, Amsterdam, The Netherlands), CD19 (CLB, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). When indicated FITC-conjugated goat anti-mouse (GAM) subclass specific antibodies against IgG1 and IgG2a were used (SBA, Southern Biotechnology Associates Inc., Birmingham, AL), or GAM-IgM + IgG-FITC (Tago, Burlingame, CA).

For cell-staining, $0{\cdot}5 \times 10^6$ to 1×10^6 pelleted cells were incubated with the relevant MoAbs. If non-conjugated antibodies were used (the CLB-CD19, CLB-FcR granI), a second incubation step was introduced with a GAM-FITC. One- and two-colour immunofluorescence stained cells were analysed using a FACScan (Becton Dickinson).

Cytotoxicity assay for effector cell targeting

Cytotoxicity was measured in a standard chromium release assay in a U-bottomed 96-well plate using complete medium plus 10% FCS. Briefly, varying numbers of effector cells in 100 μl were mixed with 50 μl of diluted antibodies or for control values 50 μl medium in the wells of the plate. After about 30 min, a fixed number of ^{51}Cr-labelled target cells (2×10^3 targets/well if cell lines were used or 3×10^3 targets/well when using fresh patient material) in 50 μl was added. Plates were centrifuged for 3 min at 100 ***g*** and incubated for 3·5 h at 37°C, 5% CO_2. Plates were again centrifuged for 4 min to spin down the cells and assayed for released ^{51}Cr by collecting 100 μl supernatant and determined in a gammacounter (Minaxi, Auto-gamma Counter, Packard Instrument Co., Meriden, CT).

Maximum release was measured by incubating the target cells in 1% Triton-X100 and spontaneous release was determined by incubating the cells in medium alone. Tests were carried out in triplicate determinations. The percentage of specific ^{51}Cr-release was determined as: % specific release = (experimental release − spontaneous release)/(total release − spontaneous release) × 100.

RESULTS

SHR-1-dependent lysis mediated by CTL clones on different target cell lines: specificity and concentration of bispecific antibody

Two CTL clones, CAK11-D11 (TCRαβ) and AK4 (TCRγδ) were used to determine the optimal concentration for the induction of cytotoxicity. As shown in Fig. 1, titration of SHR-1 bispecific MoAb in an effector cell targeting cytotoxicity assay shows the killing of the CD19$^+$ target cells of the B cell lineage with the SHR-1 titrated in a range of 10 pg/ml to 1 μg/ml. No lysis was induced by the CTL alone. Induction of lysis was observed at 1 ng/ml and higher. Lysis was dose-dependent and reached a plateau at about 100 ng/ml, used in further studies.

The SHR-1 efficiently promoted target cell lysis by CTL clones at the E:T ratios of 27:1; similar results were obtained with an E:T ratio of 9:1 (data not shown). APD (EBV-LCL) was most sensitive and Nalm-6 (pre-B ALL) the least sensitive target cell in this assay. When the CTLs were incubated with a CD19 MoAb, MG1CD19 or CLB-CD19, or a CD3 MoAb, YTH12.5 or SHL45.6, up to 1 μg/ml, or a mixture of CD3 and CD19 MoAbs, essentially no induction of cytotoxicity was seen (Fig. 1).

No killing of bystander cells: specificity on target cell level

In contrast to CD19$^+$ targets CD19$^-$ target cells (RPMI8226) were not killed in the presence of CTL-D11 and BsAb (Table 1). When CD19$^-$ targets (RPMI8226) were mixed with CD19$^+$ targets (APD) in a 1:1 ratio and added to SHR-1-incubated effector cells, only the CD19$^+$ were killed. These data suggest a

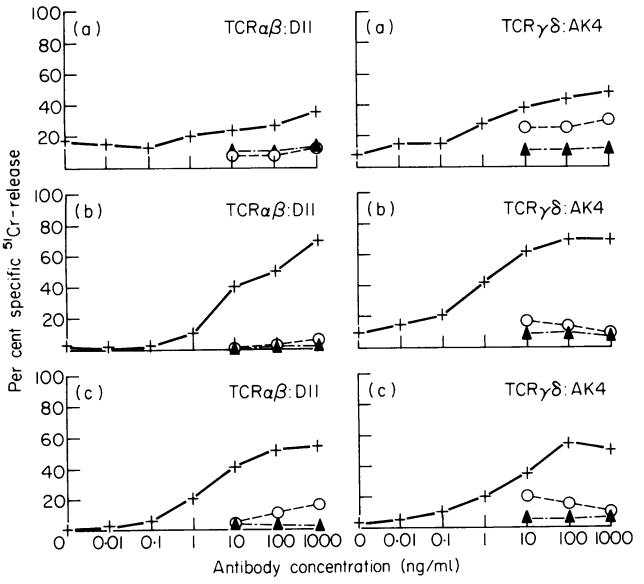

Fig. 1. CTL-mediated killing of three target cell lines induced by the bispecific antibody (BsAb) SHR-1. The tests were performed in the absence of antibody, in the presence of different concentrations of SHR-1 (+), parental CD3 (○) or parental CD19 (▲) at two E:T ratios: 27:1 and 9:1. Only the results of experiments using the E:T ratio 27:1 are shown. Effector CTL clones used are: TCRαβ-D11 and TCRγδ-AK4. The following target cells were used: pre-B cell line, Nalm-6 (a); EBV-B line, APD (b) and a plasmacytoma line, CRL1484 (c). Cytotoxic activity was measured in a 3·5 h ^{51}Cr-release assay.

Table 1. No killing of bystander cells

Target labelled	Cold	No MoAb 27:1*	No MoAb 9:1	SHR-1 27:1	SHR-1 9:1
RPMI8226	RPMI8226	6†	1	5	5
RPMI8226	APD	12	2	9	9
APD	APD	3	3	56	39
APD	RPMI8226	5	2	61	43

$CD19^-$ RPMI8226 cells were mixed with $CD19^+$ APD cells.
E:T ratio is given for labelled targets.
* Effector to labelled target ratio.
† Per cent specific ^{51}Cr-release.

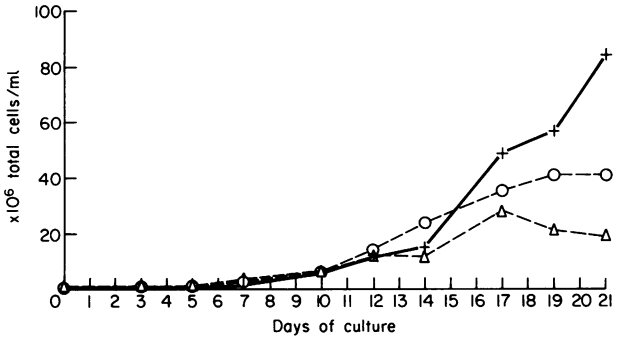

Fig. 2. Expansion of peripheral blood mononuclear cells (PBMC) cultured for 3 weeks in the presence of stimulating agents, i.e. phytohaemagglutinin (PHA)+IL-2 (+), WT32-CD3+IL-2 (△) or SHL45.6-CD3+IL-2 (○) as indicated in Materials and Methods. Cells were counted and multiplication factor was determined. Data represent the total cell number, assuming that all cells were maintained in culture.

Table 2. SHR-1-dependent cytolytic activity of CTL-clones against malignant B cells

Diagnosis	Source	SHR-1*	CTL-clones TCRαβ:D11 27:1†	TCRαβ:D11 9:1	TCRγδ:AK4 27:1	TCRγδ:AK4 9:1
NHL	PBL	−	−9‡	−7	0·6	0·2
		+	46	39	47	44
	LN	−	−4	−3	5	6
		+	33	29	50	42
	Pericard.	−	−2	3	0·2	ND
		+	48	39	45	42
ALL	PBL	−	−4	−5	−1	0
		+	25	17	40	28
	BM	−	9	8	2	1
		+	33	31	36	31

* SHR-1 (100 ng/ml) induced killing of tumour-cell-targets by the two CTL clones tested at E:T ratios (†) of 27:1 and 9:1. Targets: 3×10^3 cells/well.
‡ Results expressed as percentage specific ^{51}Cr-release in a 3·5 h assay. ND, not determined.
PBL, Peripheral blood lymphocytes; LN, lymphnode; BM, bone marrow; NHL, non-Hodgkin's lymphoma; ALL, acute lymphoblastic leukaemia.

cytolytic activity towards $CD19^+$ cells and no 'bystander' lysis of $CD19^-$ cells.

CTL clones mediated BsAb-dependent lysis of $CD19^+$ fresh leukaemia/lymphoma B cells

To test a possible clinical application, fresh isolated lymphoma/leukaemia B cells were then used as target cells in the cytotoxicity assay (Table 2). The cells from NHL or ALL patients were isolated from the lymphnodes (three samples), pericardial fluid (PF; one sample) and PBL (two samples), or PBL (two samples) and BM (one sample) respectively. The total post-purification/Ficoll fractions were labelled with chromium and used as target cells. The percentage of $CD19^+$ cells in the cell preparations shown in Table 2 was > 70%. In general, all types of malignant lymphoma or leukaemia B cells were susceptible in this type of cytotoxicity. The spontaneous release of labelled tumour cells never exceeded 15–20% of the maximum release. Some quantitative differences in killing efficiency of the targeted CTLs were found.

Table 3. Phenotypic analysis of activated peripheral blood mononuclear cells (PBMC)

Week of stimulation	Monoclonal antibodies CD3	CD3/DR	CD4	CD8	TCRγδ	CD16	CD19
0	63*	0	45	33	2	8	15
1	85	19	42	33	1	5	11
2	96	30	40	60	2	2	2
3	98	49	25	75	1	1	1

Fluorescence-activated cell sorter analysis of activated-PBMC (data represent five donors) during a 3-week culture period. PBMC were cultured in the presence of PHA+IL-2 or a CD3 MoAb+IL-2 as described in Materials and Methods.
* Percentage of positive cells determined by gated lymphocytes.

CD3 or PHA activated/IL-2 expanded cytotoxic effector cells derived from PBMC

Growth kinetics. PBMC from five healthy donors were stimulated on day 0 with PHA plus IL-2 or with a CD3 MoAb (WT32 or SHL45.6) plus IL-2, and the cultures were maintained subsequently with IL-2 only. Generally, cells began to expand around day 4. Figure 2 shows the growth curve of PBMC from one representative donor. Although the initial percentage may differ, phenotypic analysis during the culture period showed that a population of $CD3/DR^+$ cells and the subpopulation of $CD8^+$ cells increased up to 95–99% and 50–80% respectively. As shown in Table 3, the $CD16^+$ and $CD19^+$ populations gradually disappeared during the time of culturing. Data obtained for the three different activation protocols described

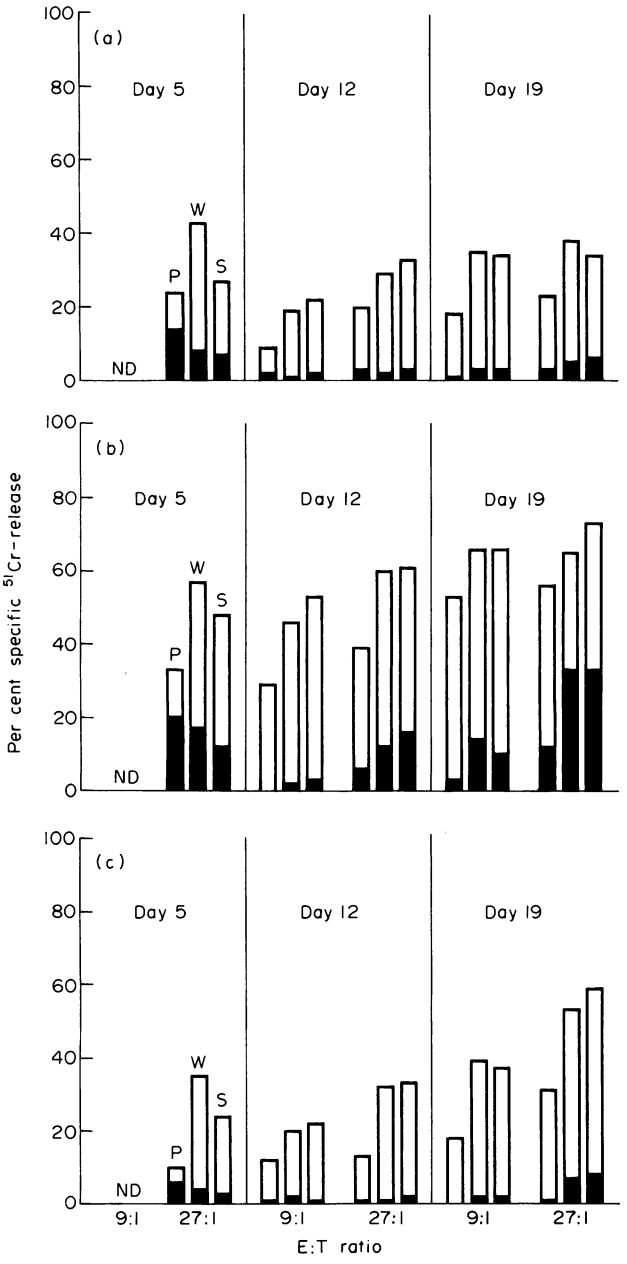

Fig. 3. Activation kinetics of peripheral blood mononuclear cells (PBMC) of one representative donor. Cells were activated as indicated for 3 weeks. The cytotoxic activity against the three CD19$^+$ cell lines, (a) Nalm-6, (b) APD, (c) CRL1484, was determined in a 3·5 h ^{51}Cr-release assay at weekly intervals, with (□) or without (■) incubation with 100 ng/ml SHR-1. E:T ratios shown are 27:1 and 9:1 (not on day 5). The three bars shown at each E:T ratio always represent the following: first bar P, phytohaemagglutinin (PHA) + IL-2 stimulation; second bar W, WT32 + IL-2 stimulation; third bar S, SHL45.6 + IL-2 stimulation. ND, Not determined.

were similar, indicating that both CD3 MoAbs as well as PHA (HA16) are suitable inducers of T cell expansion and cytotoxic activity (see below).

SHR-1-mediated lysis of CD19$^+$ cell lines mediated by in vitro *activated PBMC.* The lytic activity of the *in vitro* activated PBMC was determined 1, 2 and 3 weeks after starting the cultures (Fig. 3). As found using the target cell lines and CTL clones, also here the APD, EBV-LCL, was most susceptible to killing. Against all targets some spontaneous activity was apparent. On the other hand SHR-1-mediated cytotoxicity by PBMC gradually increased during this culture period, resulting in a much higher level of cytotoxicity in all cases compared with the spontaneous cytotoxic activity. Although CD16$^+$ cells may partly explain the spontaneous activity, this phenomenon is found also after a percentual decrease of these cells during culture.

Induction of cytolysis was slightly higher upon activation with αCD3 as compared with PHA. No difference was observed between activation with WT32 or SHL45.6.

In vitro *activated PBMC incubated with SHR-1 do kill fresh leukaemia/lymphoma B cells.* In view of possible therapeutic use of the BsAb, we tested the lytic potential of activated T cells towards fresh tumour cells. Malignant B cells, i.e. lymphoma cells isolated from the PF of a NHL-patient and BM-cell suspension of an ALL patient, already used as targets for the CTL clones (Table 1), were now used as targets for SHR-1-mediated killing by activated donor PMBC. As is seen in Table 4, PF-NHL cells and BM-ALL cells were readily lysed in the presence of the SHR-1 (100 ng/ml), while virtually no killing was seen in the absence of BsAb. Therefore, these data confirm the results obtained with CTL clones. The data presented in Fig. 3 and Table 4 are obtained from different representative donors. In Table 4 cytotoxic activity against the EBV-B (APD) cell line is included for comparison.

Collectively, these data demonstrate that suitable cytolytic activity could be induced against all target cells, i.e. cell lines and fresh leukaemia/lymphoma cells, by cloned CTL (TCRαβ/TCRγδ) as well as PHA- or CD3-activated IL-2-expanded PBMC as effector cells. Activation with the parental CD3 MoAb, the SHL45.6 does not interfere with the SHR-1-mediated cytotoxicity and is suitable for activation of PBMC *in vitro.*

DISCUSSION

In this study a BsAb, SHR-1, αCD3/αCD19 was tested *in vitro* on its efficacy to retarget human T cells to malignant human B cells.

The surface molecule chosen as a target on the effector cells is critical for the activation and anti-tumour effects of this cell. T cells are present in the peripheral blood in a high percentage and these cells are readily activated and/or expanded. An obvious choice is the use of αCD3 MoAb which is known to activate all T cells. It has become clear already that targeting of activated CTLs, by using BsAbs via the CD3 can effectively trigger the lytic machinery of these cells, despite the monovalent binding of BsAbs [17,18,35].

The surface molecule chosen to target to malignant B cells is the CD19 antigen. About 80–85% of NHL are B cell malignancies and most ALL are malignant proliferations of pre-B cells (common ALL) all expressing the CD19 antigen [1,36]. These tumour cells are considered to represent lymphoid cells frozen at a certain stage of differentiation and they can be related phenotypically to their normal counterparts in the immune system. CD19 is expressed during almost the complete spectrum of B cell development: during early stages of lymphoid differentiation (pre-pre-B, pre-B) as well as on immature B cells and mature B cells; and is gradually lost upon terminal differen-

Table 4. Activated peripheral blood mononuclear cells (PBMC) targeted by SHR-1 bispecific antibodies (BsAb) specifically lyse CD19⁺ leukaemia/lymphoma B cells

Days of activation	Target cells	BsAb SHR-1	Activation protocol: PHA+IL-2 27:1*	PHA+IL-2 9:1	WT32+IL-2 27:1	WT32+IL-2 9:1	SHL45.6+IL-2 27:1	SHL45.6+IL-2 9:1
7	APD	−	14†	ND	15	ND	14	ND
		+	30	ND	46	ND	42	ND
	NHL-PF	−	0	ND	4	ND	3	ND
		+	6	ND	22	ND	19	ND
	ALL-BM	−	2	ND	7	ND	5	ND
		+	24	ND	34	ND	33	ND
14	APD	−	13	5	14	5	10	1
		+	54	38	56	45	54	37
	NHL-PF	−	1	−1	2	0	0	−1
		+	23	18	25	24	22	18
	ALL-BM	−	0	−1	0	0	1	−2
		+	32	30	34	33	31	30
21	APD	−	13	4	13	5	18	5
		+	93	69	90	57	98	72
	NHL-PF	−	6	4	5	2	6	4
		+	43	40	47	42	50	42
	ALL-BM	−	3	1	2	0	3	2
		+	61	49	56	40	57	47

Activation kinetics of PBMC of one donor, experiments presented in Table 3 and Fig. 3 were done with PBL from different donors. Cells were activated as indicated for 3 weeks. The cytotoxic activity was determined against CD19⁺ cells isolated from the pericardial fluid (PF) of a non-Hodgkin's lymphoma (NHL) patient and leukaemia cells from the bone marrow (BM) of an active lymphoblastic leukaemia (ALL) patient. As a control on cytotoxic activity the APD cell line was included. Effector cells were stimulated and added to the assay as indicated. Targets: 3×10^3 cells/well.

* Effector:target ratio; only the data of 27:1 and 9:1 are shown.

† Results expressed as percentage specific ^{51}Cr release.

ND, Not determined.

tiation to plasma cells [37–39]. Recently it was shown that the CD19 antigen is not shed or secreted from the cell surface while modulation of the CD19 antigen with the bivalent CLB-CD19 MoAb *in vivo* did not necessarily occur [5,6]. In order to obtain immunoreactivity against all tumour cells it is important to know whether early clonogenic cells are CD19⁺ and are eliminated as well. A rather specific chromosomal breakpoint in follicular NHL (t14:18) takes place during gene rearrangement when the chromosome is exposed to many alterations [40,41]. Those events take place early in the development of the B cell at the same time as the CD19 antigen is expressed. Possible precursor cells of NHL and ALL will thus be expected to be CD19⁺ and therefore should be eliminated [38].

In the present study two CTL clones [32], one expressing the TCR$\alpha\beta$ and one expressing the TCR$\gamma\delta$, were equally effective in their lytic activity against the CD19⁺ B cell lines when preincubated with SHR-1. In all cases 100 ng/ml appeared sufficient to generate optimal induction of lysis of the CD19⁺ cell lines used.

The pre-B ALL cell line, Nalm-6, was less sensitive for SHR-1-mediated cytotoxicity compared to the EBV-LCL (APD) and the plasmacytoma line (CRL1484). Using fresh patient-derived material (ALL cells *versus* NHL cells) these differences were not seen. Nalm-6 cells differ phenotypically from APD and CRL1484 in exhibiting low LFA-1 and ICAM-1 expression. A recent report has demonstrated that adhesion molecules contribute to the susceptibility of the target cells to CD3/TCR-BsAb-mediated lysis [42]. This is currently tested further using fresh human LFA-1⁻ lymphoma B cells.

The lytic activity of the preincubated CTL clones on fresh leukaemia/lymphoma cell preparations was also examined. The target cells used, independent of the diagnosis and the source of the target cells, were efficiently killed by the CTLs even at low E:T ratios (9:1).

Important for the clinical use of the BsAb is whether activated PBMC efficiently kill CD19⁺ cell lines and even more important: are patient-derived malignant B cells susceptible to this BsAb-mediated cytotoxicity. Using activated PBMC at an E:T ratio of 9:1, efficient lysis is still observed (up to 49%); lower E:T ratios are also suitable, which offers good prospects for *in vivo* therapy.

Bispecific MoAb therapy should fulfill a number of criteria. The BsAb should be protected from damage or degradation. The desired effector cell, here the BsAb-coated CTL, might bind to and/or be scavenged by Fc-receptor bearing cells, interfering with their CTL function or indeed destroying them. However, the Fc-combination of SHR-1, mixed ratIgG2b/mouseIgG1, was previously found not to induce complement-mediated lysis

of human T blasts [31]. SHR-1 has monovalent specificities for both the antigens CD3 and CD19; in similar complement-mediated experiments this bispecific antibody was also found to be non-lytic for a $CD19^+$ B lymphoma cell line [31]. It has also previously been reported that a hybrid Fc with one ratIgG2b in an antibody did not mediate ADCC [28]. We also found, using NK ($CD3^-/CD16^+$) cells preincubated with SHR-1 as effector cells and the CTL clone D11 as target, that no killing of the CTL could be mediated (data not shown). Therefore undesired reactivity with Fc receptor-bearing cells apparently may not be a major problem with the SHR-1 MoAb.

Although freshly isolated human T cells show little cytolytic activity, T cells become cytotoxic already after 1 day of activation in the presence of SHR-1 and B cells or monocytes (unpublished results). We anticipate that a similar activation of T cells will occur *in vivo*, especially in lymph nodes where they are close together. We therefore plan to test the application of this BsAb *in vivo* without such pretreatment of T cells *in vitro*.

Prior to clinical application in a phase I study, activation of PBMC obtained from patients in different stages of their disease will be tested to determine whether they are still suitable for activation.

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