

## ORIGINAL ARTICLE

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## Role of perforin, granzymes and the proliferative state of the target cells in apoptosis and necrosis mediated by bispecific-antibody-activated cytotoxic T cells

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**Abstract** Bispecific monoclonal antibodies (bi-mAb), directed against a tumor-associated antigen and the CD3 or CD28 antigen on T lymphocytes, induce activation of resting T lymphocytes and target-specific tumor cell lysis. We now show that both necrosis and apoptosis contribute to T-cell-mediated tumor cell destruction. Even though T cells up-regulate FAS/APO-1 expression upon bi-mAb stimulation, FAS/APO-1-mediated apoptosis does not contribute to bi-mAb-mediated destruction of Hodgkin's cells. CD8<sup>+</sup> lymphocytes were the most potent effectors of bi-mAb-mediated cytotoxicity and had the highest levels of mRNA coding for perforin and granzyme A and B. Ca<sup>2+</sup>-complexing agents, which abrogate perforin activity, led to decreased levels of necrosis, while inhibition of granzyme activity in effector or target cells had a similar effect on apoptosis. Granzyme-mediated apoptosis critically dependent on the proliferative state of the target cells, while perforin-induced necrosis was not cell-cycle-dependent. Our results underline the importance of the expression levels of perforin and granzymes in the effector T cells and of the proliferative state of the target cells in bi-mAb-mediated apoptosis and necrosis of tumor cells.

**Key words** Hodgkin's lymphoma · Bispecific antibodies · T lymphocytes · Perforin · Granzymes

### Introduction

Since T cells have been recognized as the most effective mediators of antitumor cytotoxicity, many approaches of

experimental tumor therapy have aimed at the specific and effective activation of T cells against tumor cells [24]. One of the most successful strategies uses bispecific monoclonal antibodies (bi-mAb), which are able to target and activate a broad range of resting T cells against antigen-positive tumor cells, resulting in tumor destruction [5, 18]. Using Hodgkin's lymphoma as a model, we demonstrated that a combination of two bi-mAb, one arm of which recognizes the Hodgkin's-associated CD30 antigen, the other the CD3 and CD28 antigen on T cells, induce an efficient tumor cell lysis in vitro and in vivo [25]. Studies on the mechanisms involved in tumor cell lysis revealed that CD8<sup>+</sup> lymphocytes were the most potent effectors in this model [26]. However, the precise mechanism by which activated lymphocytes eliminate tumor cells still has to be elucidated. Early experiments that stressed the role of cytokines in tumor cell lysis [23] were recently challenged by experimental evidence [2] that the contribution of cytokines to tumor cell lysis is not by direct participation in processes involved in rapid cell destruction [3]; rather, lymphokines are now believed to function primarily by down-regulating the cell-cycle activity of tumor cells (e.g. interferon  $\gamma$ ) and by augmenting the cytolytic activity of effector lymphocytes, natural killer cells and macrophages [11, 21]. Much attention has been recently paid to two pathways used in T-cell-mediated lysis: first, the granule-exocytosis pathway, which is based on the directed cosecretion of granzymes and perforin, and second, the FAS/APO-1 pathway, where ligand-mediated triggering of target cell-surface receptors induces apoptosis [1]. According to the granule-exocytosis model of lymphocyte-mediated cytotoxicity, granules containing perforin and granzymes are directionally released from activated lymphocytes after specific recognition of and conjugate formation with a target cell. After entering the target cells, perforin monomers probably assemble to polymeric pore structures, which are inserted into the plasma membrane thus causing osmotic cytolysis and the typical picture of necrosis [8]. This functional similarity between perforin and complement C9 is also reflected in the considerable homology between their amino acid sequences [15, 30]. In contrast to perforin-mediated necrosis, the

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mechanisms underlying granzyme-induced target cell lysis remain largely unknown as their physiological functions and natural substrates have not been firmly established [29]. Recently published results provide experimental evidence that granzymes, secreted by cytotoxic lymphocytes in response to triggering by the target cell, enter the target cell and initiate an internal disintegration pathway that leads to DNA fragmentation (apoptosis) and lysis [19].

Perforin and granzymes are key components in the lytic machinery of T cells and represent potent cytotoxic molecules that can suppress tumor growth [31, 33]. Experiments using perforin-deficient mice have shown that this peptide is crucially involved in antitumor immune responses, in the elimination of certain viruses, in graft rejection and in some autoimmune diseases [12, 32]. To clarify the mechanisms responsible for target cell lysis induced by bi-mAb-activated T cells, we analyzed the role of the FAS/APO-1 system and the molecules of the granule exocytosis pathway in T-cell-induced cytotoxicity with special emphasis on T cell subpopulations, the kinetics of tumor cytolysis and target-cell-dependent factors.

## Materials and methods

### Antibodies and cell lines

The generation, purification and characterization of the bi-mAb (anti-CD3/CD30 and anti-CD28/CD30) have been described previously [25, 26]. The established human cell lines (HDLM2, L540CY) and the generation of a CD30-transfected cell line and a CD30 fusion protein (CD30-FP) have been described elsewhere [26].

### T cell preparations

Peripheral blood mononuclear cells (PBMC) were isolated and T cells were negatively enriched by the magnetic activated cell sorting (MACS) technique as described [26]. The remaining lymphocytes were more than 95% CD3<sup>+</sup>. Contaminating cell fractions were always less than 0.5%; no proliferation after phorbol 12-myristate 13-acetate (10 ng/ml) or phytohemagglutinin (100 ng/ml) stimulation for 2–5 days was observed. Isolation of CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes was performed accordingly, yielding a more than 97% pure cell fraction.

### Activation of T lymphocytes

Purified T lymphocytes were activated by the combination of both bi-mAb (anti-CD3/CD30 and anti-CD28/CD30) in the presence of CD30-FP for 3–5 days as described [26].

### Determination of necrotic cells

bi-mAb-mediated necrosis was measured using the technique of time-resolved fluorimetry [26]. Samples of  $1 \times 10^4$  Eu-labeled tumor cells were used as targets and preactivated lymphocytes as effector cells. Fresh bi-mAb was added at the onset of the assay at a final concentration of 100 ng/ml. After 4 h of culture at 37 °C, 10  $\mu$ l supernatant was collected from each well, mixed with 100  $\mu$ l enhancement solution (Pharmacia, Freiburg, Germany) and counted in a time-resolved fluorimeter (LKB Wallac, Turku, Finland). Maximum release was determined by adding 0.5% Triton X-100 (Sigma, Munich, Germany) to labeled cells. The percentage of specific lysis was calculated as described [26]. Assays were performed in triplicate; standard deviations did not exceed 8%.

### Determination of apoptotic cells

For measuring necrosis and apoptosis simultaneously, target cells were labeled with a fluorescent dye (PKH-26, Sigma, Munich, Germany) according to the manufacturer's recommendations prior to EuCl electroporation. The cytotoxicity assays were performed as described above, the supernatants were collected after the indicated time for the determination of the Eu release and the cell pellets were harvested. Apoptosis was measured using (7-AAD; 20  $\mu$ g/ml) as an indicator for apoptotic cells as described by others [28]. Flow-cytometric analysis was performed with a FACScan cell cytometer applying a gate for PKH-26-labeled target cells and measuring 7-amino-actinomycin D (7-AAD) uptake in these cells. Samples comprising  $1 \times 10^5$  marked cells were analyzed in each experiment and the percentage of apoptotic cells was calculated as described [28]. To confirm results obtained by 7-AAD uptake, two control assays measuring apoptosis by *in situ* nick translation or DNA fragmentation were performed. For *in situ* nick translation, PKH-26-labeled target cells were treated as described [7] and the intensity of incorporated fluorescein-12-UTP measured by FACScan analysis. DNA fragmentation was performed using standard techniques for DNA extraction and DNA fragments were visualized on a 2% agarose gel stained with ethidium bromide [17].

### Depletion of extracellular Ca<sup>2+</sup>

Ca<sup>2+</sup> depletion with chelating agents was performed as described [16] to reduce perforin activity. Indicated amounts of EGTA were added to each well prior to the seeding of the lymphocytes and cytolysis was determined.

### Inhibition of granzyme activity in effector cells

As described by Hayes et al. [10], bi-mAb-activated lymphocytes were treated with medium alone or 3  $\mu$ g/ml monensin at 37 °C for 30 min. Then, phenylmethylsulfonyl fluoride (PMSF; 1 mM) was added for an additional 30 min at 37 °C and cells were washed and used as effector cells for cytotoxicity assays.

### Inhibition of granzyme activity in target cells.

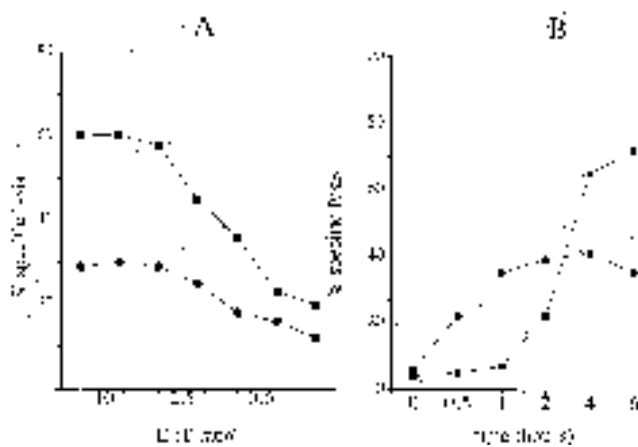
Aprotinin, actinomycin D and bovine serum albumin (BSA; as control) were used at a concentration of 2 mg/ml and electroporated into the target cell concomitantly with EuCl under the conditions described above. The efficiency of protein loading was evaluated using fluorescein-isothiocyanate (FITC)-conjugated BSA and FACScan analysis.

### Cell-cycle analysis

To discriminate between proliferating and resting target cells, a triple fluorescent analysis was performed using a FITC-conjugated Ki-67 antibody (Dako, Hamburg, Germany). Target cells were stained as described [6] and actinomycin D (20  $\mu$ g/ml) was added to the fixation solution in order to prevent the loss of specific 7-AAD staining. In some experiments, cell-cycle analysis was performed with the cell cycle analysis kit (Becton Dickinson, Heidelberg, Germany) on a FACScan using CELL FIT software for analysis.

### Northern blot analysis

Total RNA was isolated with guanidinium isothiocyanate [4]. Samples (20  $\mu$ g) from MACS-sorted lymphocytes were fractionated on 1% formaldehyde agarose gels, then transferred and crosslinked to nitrocellulose. Blots were hybridized with <sup>32</sup>P-labeled probes in a formamide buffer system (50% formamide, 5 $\times$  standard saline citrate, 5 $\times$  Denhardt's, 1% sodium dodecyl sulfate and 200  $\mu$ g/ml heat-denatured salmon sperm) at 42 °C for 12–16 h. After hybridization, membranes were washed twice at 42 °C for 15 min and then exposed to X-ray films for 24–48 h. cDNA specific for perforin, granzyme A and granzyme B was generated by the reverse transcriptase/polymerase chain reaction



**Fig. 1A, B** Bispecific-monoclonal-antibody (bi-mAb)-mediated cytotoxicity of target cells. Target cells were labeled with PKH-26 and EuCl to measure simultaneously apoptosis (●) and necrosis (■). **A** The E:T ratio ranged from 20:1 to 0.3:1; the bi-mAb concentration was kept at a constant level (100 ng/ml). Cytotoxicity was determined after 4 h and the percentage of cytolysis calculated as described. **B** The cytotoxicity assay was repeated at a constant E:T ratio (20:1) and the percentage of necrosis or apoptosis measured at the indicated time

and cDNA fragments 300–500 bp long, labeled by random priming (Pharmacia, Freiburg, Germany). A  $\beta$ -actin probe was used as control for equal RNA loading per lane.

#### Determination of FAS/APO-1 ligand expression and crosslinking of FAS/APO-1

FAS/APO-1 antigen expression on Hodgkin's derived tumor cell lines was measured by FACSscan analysis using a monoclonal antibody (DX2, 5  $\mu$ g/ml, Dianova, Hamburg, Germany). This antibody induces apoptosis in FAS-antigen-positive tumor cells when crosslinked by polyclonal goat anti-(mouse Ig) sera (20  $\mu$ g/ml, Z259, Dako, Hamburg, Germany). Up-regulation of FAS/APO-1-ligand-specific mRNA in bi-mAb-activated T cells was controlled by Northern blot analysis with a cDNA probe covering a 580-bp fragment of the coding region.

## Results

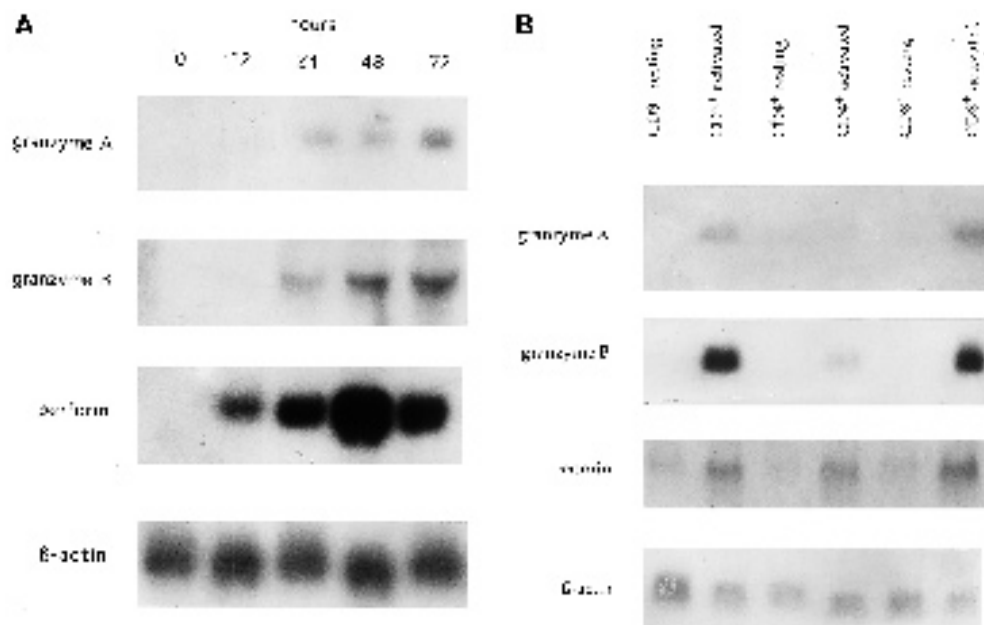
### T-cell-mediated apoptosis and necrosis of tumor cells

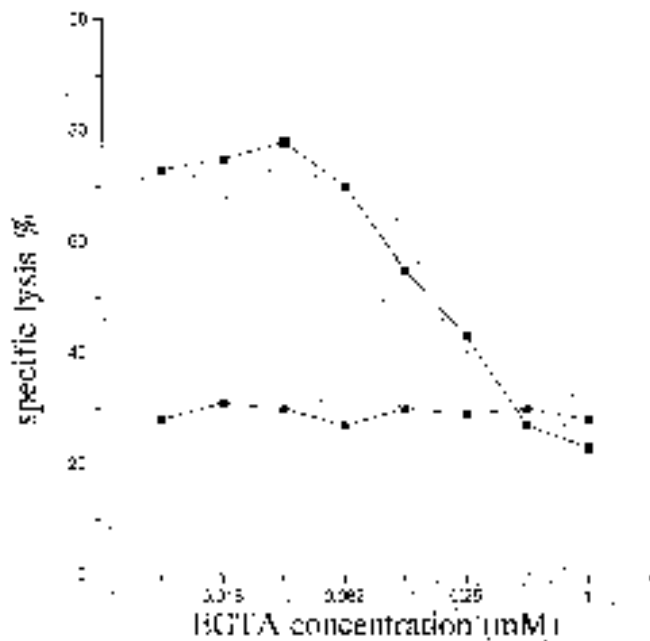
The lysis of tumor cells by bi-mAb-preactivated T lymphocytes was mediated both by apoptosis and necrosis, as determined by the Eu release and 7-AAD uptake of PKH-26-labeled cells, respectively. Both apoptosis and necrosis showed a good correlation with the effector:target cell ratio (Fig. 1A) and the bi-mAb concentration (data not shown). In addition to 7-AAD uptake, apoptosis of target cells was also demonstrated by DNA fragmentations assays and nick translation, with all assays showing a good correlation (data not shown). During the time course of bi-mAb-mediated T cell cytotoxicity, apoptosis of the target cells was significant within 30 min, while necrosis became prominent only after 2 h (Fig. 1B).

### Expression of perforin and granzymes in effector cells

Northern-blot analyses were performed for the expression of granzymes and perforin by effector cell subpopulations. Studies on the kinetics of perforin and granzyme induction revealed that their expression was up-regulated after 24 h and reached a maximum 72 h after the initiation of prestimulation with CD30-FP in the presence of both bi-

**Fig. 2A, B** mRNA expression of activated lymphocyte subsets. **A** mRNA analysis of induced perforin and granzyme message was performed at the indicated time using bi-mAb-activated lymphocytes. Blots were hybridized with radiolabeled perforin-, granzyme-A- or granzyme-B-specific cDNA and exposed to X-ray films overnight.  $\beta$ -Actin cDNA was used as control to ensure equal loading of RNA in each lane. **B** Resting or activated (48 h) lymphocytes of indicated subsets were activated for 48 h and mRNA subjected to Northern blot analysis





**Fig. 3** Impact of  $\text{Ca}^{2+}$  depletion on bi-mAb-mediated cytotoxicity. The experiment was performed with a constant E:T ratio of 20:1 using bi-mAb-activated lymphocytes and constant amounts of bi-mAb (100 ng/ml). The indicated concentrations of EGTA were added and cytotoxicity including necrosis (■) and apoptosis (●) measured after 4 h. Levels of EGTA higher than 1.5  $\mu\text{M}$  were toxic for target cells and enhanced spontaneous release significantly

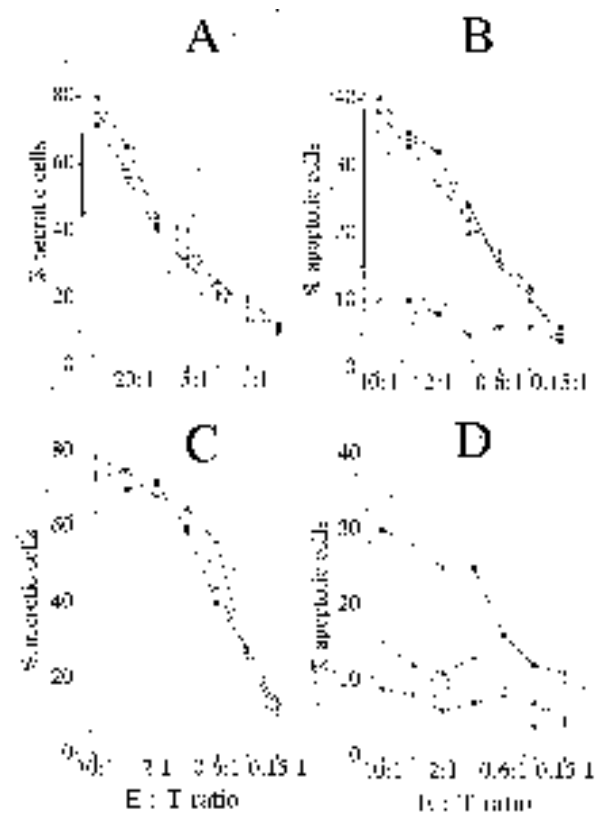
mAb (Fig. 2A). The maximal expression of granzymes and perforin coincided with the empirically determined optimum length of in vitro prestimulation of unstimulated T cells in our previous studies.  $\text{CD8}^+$  lymphocytes expressed significantly higher levels of mRNA for granzymes and perforin than did  $\text{CD4}^+$  cells, unsorted T cells and resting lymphocytes (Fig. 2B).

#### Inhibition of perforin activity

To delineate further the role of perforin in bi-mAb-mediated cytotoxicity, inhibition experiments were performed. As the pore-forming process is strictly  $\text{Ca}^{2+}$ -dependent, it can be inhibited by  $\text{Ca}^{2+}$ -chelating agents, e.g. EGTA. As shown in Fig. 3, T-cell-mediated necrosis was reduced in correlation with the EGTA concentration, while apoptosis was virtually unaffected by  $\text{Ca}^{2+}$  depletion.

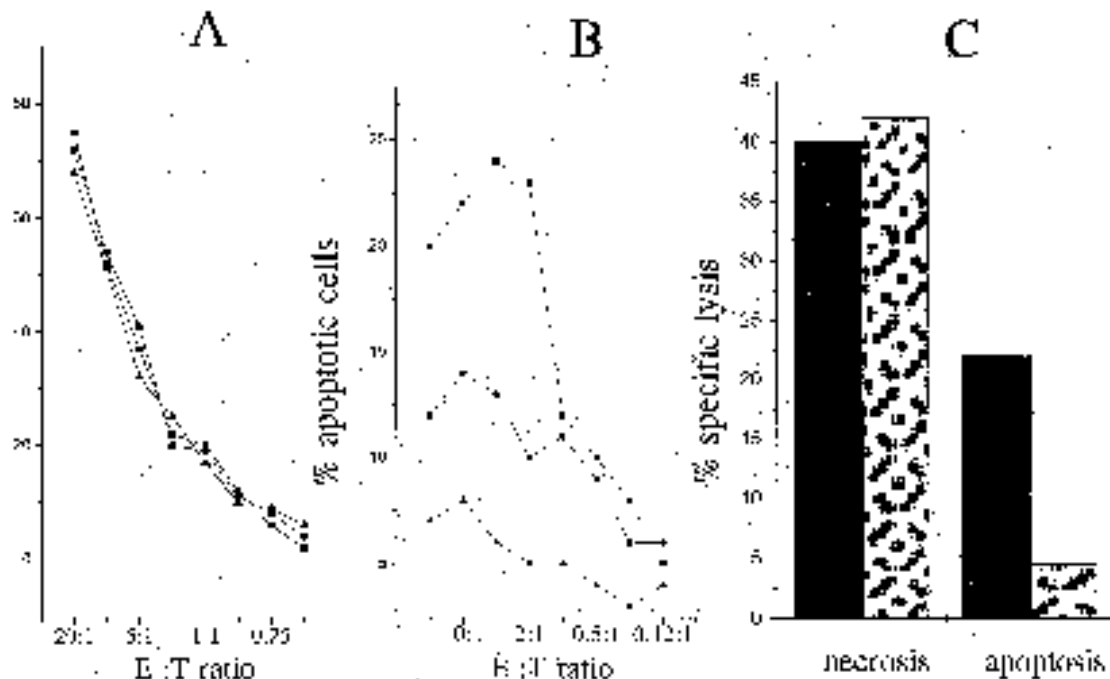
#### Inhibition of granzyme activity

To inhibit granzyme activity in effector and target cells respectively, the effects of two procedures on T-cell-mediated cytotoxicity were determined. In one set of experiments we used a combination of monensin and PMSF to inhibit protease activity in effector cells. In a second set of experiments, target cells were loaded with a



**Fig. 4A–D** Effect of granzyme inhibition of bi-mAb-mediated apoptosis. **A, B** Protein synthesis inhibitor phenylmethylsulfonyl fluoride (PMSF) was coincubated with bi-mAb-activated, monensin-pretreated T cells for 30 min. Cells were then washed and used as effector cells for cytotoxicity assays measuring apoptosis and necrosis. The experiment was conducted as previously described and cytotoxicity determined after 4 h. ■ Untreated, activated lymphocytes; ● monensin-treated; ▲ PMSF-treated; ▼ monensin + PMSF-treated. **C, D** In contrast to **A**, target cells were labeled by electroporation with a protease inhibitor (2 mg/ml) such as aprotinin (●) and an RNA synthesis inhibitor such as actinomycin D (▲). Bovine-serum-albumin-loaded target cells were used as control (■). Bi-mAb-activated lymphocytes and 100 ng/ml bi-mAb were added and cytotoxicity measured after 4 h. Again, apoptosis and necrosis were determined. The experiment was conducted three times and results are given as mean values

macromolecular protease inhibitor (aprotinin) or an RNA-synthesis inhibitor (actinomycin D), in order to inhibit granzyme-mediated DNA fragmentation in these cells. Pretreatment of effector lymphocytes with a combination of monensin and PMSF led to a diminished target-cell lysis, which was due to a reduction in apoptosis (Fig. 4B), while necrosis was unaffected (Fig. 4A). Similarly, pretreatment of target cells loaded with aprotinin or actinomycin D resulted in a significant reduction of apoptosis (Fig. 4D), again leaving necrosis unaffected (Fig. 4C).



#### Cell-cycle-dependent sensitivity to T-cell-mediated cytotoxicity

To evaluate the influence of the proliferative state of the target cells on T-cell-mediated cytotoxicity, target cells were grown for 5 days at different concentrations of fetal calf serum (FCS) in order to obtain cell populations with different rates of proliferating cells. Staining with Ki-67 showed that cells grown in 20% FCS had a proliferating population of 57%, those grown in 5% had 34%, while those grown in 2% had only 17% proliferating cells. As shown in Fig. 5, the percentage of proliferating cells had no influence on T-cell-mediated necrosis (Fig. 5A), while the cell populations with the highest proliferative activity were the most sensitive to T-cell-mediated apoptosis (Fig. 5B). The influence of the proliferative state of the target cells on apoptosis was also confirmed by the observation that T-cell-mediated apoptosis against Ki-67<sup>-</sup> target cells was significantly reduced compared to Ki-67<sup>+</sup> target cells, while there was no significant difference in T-cell-mediated necrosis between the two cell fractions (Fig. 5C). Again, target-cell populations with high Ki-67 values were the most sensitive to effector-cell-induced apoptosis, while necrosis was not affected by the proliferative state, as demonstrated by the similar levels of necrosis for Ki-67<sup>+</sup> and Ki-67<sup>-</sup> target cells.

#### FAS/APO-1-receptor-mediated cytolysis

T cells up-regulate FAS/APO-1 ligand mRNA, starting 4 h after bi-mAb stimulation and reaching a maximum expression after 12 h, as demonstrated by Northern blot analysis. As, on the other hand, Hodgkin's-derived cell lines L540CY and HDLM2 strongly express FAS/APO-1 antigen on their surface, we expected the FAS/APO-1 pathway to contribute

**Fig. 5A–C** Impact of tumor cell proliferation on bi-mAb-mediated cytotoxicity. **A, B** CD30<sup>+</sup> Hodgkin tumor cells (L540CY) were cultured for 5 days in medium containing the indicated amount of fetal calf serum (FCS) and then checked by cell cycle analysis for the percentage of proliferating cells (■ 20% FCS = 57%, ● 5% FCS = 34%, ▲ 2% = 17%). Tumor cells were labeled as described, with PKH-26 and EuCl, to measure apoptosis and necrosis simultaneously. The cytotoxicity experiment was performed for 4 h with different E:T ratios and a constant amount of bi-mAb (100 ng/ml). After this period, necrosis and apoptosis were determined. **C** Role of Ki-67<sup>+</sup> tumor cell subpopulation on bi-mAb-mediated cytotoxicity. The experiment was performed as described above with the modification that necrosis and apoptosis were determined by flow cytometry, dividing targets into Ki-67<sup>+</sup> (■) and negative (□) cells. The analysis was repeated three times and data obtained are presented as mean values

to bi-mAb-mediated cytotoxicity. However, crosslinking of the FAS/APO-1 antigen by the mAb DX2, which induces death in cells expressing FAS/APO-1 on their surface, did not induce apoptotic DNA fragmentation in these Hodgkin's-derived cell lines (data not shown). As the FAS/APO-1 pathway is obviously not functional in these Hodgkin's-derived cell lines, it can be concluded that it does not contribute to target cell killing by bi-mAb-activated T cells in this system.

#### Discussion

Recent studies on the cytotoxic mechanisms involved in the immunological control of infections and malignant diseases have confirmed the importance of the FAS/APO-1-receptor-mediated and granule exocytosis pathway [12, 32]. However, there was no FAS/APO-1-mediated cytotoxicity in Hodgkin's cells, even though these cells strongly express the FAS/APO-1 antigen on their surface and bi-mAb-

activated T lymphocytes up-regulate the expression of the FAS ligand. The absence of FAS/APO-1-mediated cytotoxicity in Hodgkin's cells is not due to mutations of the FAS/APO-1 receptor in these cells, as determined by sequence analyses (unpublished data). Similar observations have been made in B cell lymphomas where tumor cells express the FAS/APO-1 antigen but can not undergo apoptosis after receptor crosslinking (R. van Lier, personal communication). The mechanism for this resistance remains unclear today.

In contrast to the FAS/APO-1 system, the molecules of the granule-exocytosis pathway do play a critical role in the bi-mAb-mediated T cell cytotoxicity against Hodgkin's cells. As this secretory model does not depend on receptor/ligand interactions and, in contrast to the FAS/APO-1, is not limited to cells with strong expression of the FAS/APO-1 system, i.e. primarily cells of the hematopoietic system, the findings described here with bi-mAb-mediated T cells cytotoxicity against Hodgkin's cells should be applicable to the immune-mediated destruction of a wide range of human tumors.

The importance of perforin and granzymes for immune-mediated cytotoxicity has been demonstrated in experiments with perforin- or granzyme-deficient animals. In addition, up-regulation of granzyme and perforin expression in tumor-infiltrating lymphocytes has been shown [13]. Tumor-infiltrating lymphocytes, activated *ex vivo* by a combination of two independent signals (CD3 and interleukin-2), expressed high levels of perforin, granzyme and cytokine message [20]. Moreover, up-regulation of the respective molecules by the tumor-infiltrating lymphocytes after *ex vivo* stimulation correlated directly with the survival rate of tumor-bearing animals after adoptive transfer of the lymphocytes. Another hint about the role of perforin in the immune response against tumors stems from the observation that, in patients with follicular lymphoma, tumor-infiltrating lymphocytes displayed high levels of perforin, which was interpreted as a marker of cytotoxic activity and was speculated to be due to the endogenous local secretion of interleukin-2 (IL-2) by the tumor cells [14]. Finally, cytotoxic T cell activity *in vitro*, achieved by the administration of therapeutic doses of IL-2, correlated strongly with perforin and granzyme expression in patients suffering from metastatic melanoma [13]. In these patients, subcutaneous administration of IL-2 enhanced the pool of circulating perforin or granzyme-positive lymphocytes in a dose-dependent fashion and modulated tumor-specific cytotoxicity. The clinical significance of perforin and granzymes is also suggested by the observation that lymphocytes in endomyocardial biopsies, taken from human heart-transplant patients with a rejection episode, expressed enhanced levels of perforin and granzymes [9] and that these molecules might serve as an early predictive marker for graft rejection.

Our observation that CD8<sup>+</sup> cells are the most efficient cytotoxic cells suggests that the cytolytic capacity of an effector cell after bi-mAb stimulation may be largely determined by its up-regulation of expression of the major members of the granule exocytosis pathway, i.e. perforin and granzymes A and B. These molecules are

the most relevant contributors to tumor cell lysis by apoptosis and necrosis in the system of bi-mAb-mediated specific lysis of Hodgkin tumor cells. The specific inhibition of perforin and granzyme activity prove that the up-regulation of perforin and granzyme message is not an epiphenomenon of T cell activation, but rather a prerequisite for efficient target-cell lysis, with perforin being the major protein responsible for target-cell necrosis and granzymes being critically involved in target-cell apoptosis. Adding Ca<sup>2+</sup>-complexing agents (like EGTA) represents an accepted tool to inhibit perforin activity, as its lytic function is inextricably dependent on the presence of calcium ions [31]. One of the most elegant approaches, loading the target cells with macromolecular protease inhibitors to block granzyme activity, has been described recently by Nakajima [19] and was used in this report with slight modifications. In accordance with Nakajima, we could show that inhibition of granzyme activity in the tumor cell reduced effector-cell-mediated apoptosis significantly, leaving perforin-induced necrosis unaffected. Our observation that granzyme activity correlates directly with the proliferative state of the target-cell populations suggests that the proliferative state of a tumor cell is critical for its susceptibility against granzymes. These results confirm and extend a report by Nishioka and Welsh [22], who showed that target cells that enter into G1 or a G1-like state after transfection with *c-myc* or treatment with growth factors are rendered competent to die by apoptosis.

In summary, our results underline the importance of perforin and granzyme activity during the effector phase of bi-mAb-activated cytotoxic T cells. They suggest the members of the granule exocytosis pathway to be the prime molecules in bi-mAb-induced T-cell-mediated tumor destruction *in vitro*, which may explain the impressive results obtained in animal models where the combined use of these two T-cell-activating bi-mAb could even induce the cure of advanced disease [27]. Ongoing studies will have to clarify whether the role of perforin and granzymes that we have now demonstrated in bi-mAb-mediated T cell cytotoxicity *in vitro* is also operative in immune destruction of tumor cells *in vivo*.

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