Cancer Immunol Immunother (1996) 42: 369–375 

ORIGINAL ARTICLE

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# Further characterization of cytotoxic T cells generated by short-term culture of human peripheral blood lymphocytes with interleukin-2 and anti-CD3 mAb

Received: 6 December 1995 / Accepted: 4 June 1996

**Abstract** In this study we have specifically investigated the participation of T cells in the cytotoxic activity of peripheral blood lymphocytes (PBL) activated by interleukin-2 (IL-2, 50 U/ml) alone or in combination with an anti-CD3 mAb (BMA030, 10 ng/ml, IgG2a). Purified $CD3^+$ T cells, incubated in the presence of the anti-CD3 mAb for 4 days, mediated a cytotoxic activity against HL60 and U937 tumor cell lines. Several findings suggested the involvement of a redirected-cytotoxicity phenomenon, since the lytic process was restricted to target cell lines bearing the high-affinity Fcγ receptor (FcγRI) and T lymphocytes stimulated by IL-2 alone did not lyse these cell lines. Furthermore, anti-CD3 mAb $F(ab')_2$, anti-CD3 IgG1 (UCHT1), phytohemagglutinin or staphylococcal enterotoxin A did not induce a similar cytotoxic activity in T lymphocytes. The cytotoxic process occurred in the presence of a very low level of anti-CD3 antibodies (in the nanomolar range). The cytotoxic activity of T cells stimulated by IL-2 or by IL-2 + BMA030, against OVCAR-3 cells ($MOv18^+$ ovarian tumor cell line), was also compared in the presence of a bispecific antibody (OC/TR, anti-CD3 × MOv18). The stimulation by IL-2 + BMA030 induced approximately a twofold higher cytotoxic activity than IL-2-activated T cells. This could be related to the state of activation of effector cells stimulated by IL-2 + BMA030, since the phenotypic analysis showed an increased proportion of T cells expressing several activation/differentiation markers (CD25, HLA-DR, CD45R0, adhesion molecules). These findings could be applied to the design of therapeutic protocols using anti-CD3 ×antitumoral bispecific antibodies.

**Key words** Anti-CD3 mAb · Redirected cytotoxicity · Fcγ receptor (FcγR) · Bispecific antibody · Activation

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## Introduction

A large number of reports have addressed the cytotoxic properties of lymphokine-activated killer cells (LAK cells) generated by short-term culture of peripheral blood lymphocytes (PBL) with interleukin-2 (IL-2) [10, 24, 26, 28]. Despite interesting in vitro properties, the clinical efficacy of LAK cell therapy in cancer-bearing patients has not been satisfactory in most cases [55, 56]. Essentially these unsatisfactory results might be related to the fact that LAK cells belong to the natural killer (NK) subset [46, 50]. Parmiani [49] has even proposed that successful adoptive therapy with LAK cells could depend on the recruitment of activated host T lymphocytes. Therefore, it would be interesting to evaluate alternative protocols of immunotherapy based on T cells, rather than NK cells. Moreover, the use of bispecific antibodies that bind both the T cell receptor (TCR) complex and a target cell-surface molecule has renewed the interest in the development of T cell stimulation protocols [5, 6, 20, 37].

Soon after the production of the first murine monoclonal antibodies directed against the human TCR-CD3 complex, a large number of studies concentrated on the effects of these reagents on the activation, proliferation and cytotoxic differentiation of T cells [15, 45, 48]. In view of the important secretion of cytokines (including IL-2) induced by these antibodies [11, 17, 19, 51] it was proposed that anti-CD3 mAb could participate in the induction of a non-MHC-restricted LAK-like cytotoxic activity by T cells [12]. Recently, two groups [16, 43] have demonstrated the induction of antitumor activity using anti-CD3 mAb, in an in vivo murine model. In these studies, the injection of anti-CD3 [16] alone or in combination with IL-2 [43] in tumor-bearing mice induced the reduction [43] or the eradication [16] of tumor metastasis. However, very few studies carefully evaluated the antitumoral activity of T cells stimulated by anti-CD3 mAb with sorted lymphocyte subsets, most likely because classical sorting procedures are laborious and interfere with the function of cytotoxic effectors. We recently described an original protocol for

the positive selection of T cells using a magnetic cell sorter (MACS), magnetic beads and F(ab′)$_2$ anti-CD3 mAb. When performed at the effector stage this procedure was shown not to modify the function of T cells [29]. Therefore, we used this technique to characterize TCR-dependent and TCR-independent (LAK-type) cytotoxic activity of T cells after short-term culture of PBL with IL-2 compared with that with IL-2 + anti-CD3 mAb.

In this study, we have attempted to define the type of effector cells generated by the stimulation of human PBL with anti-CD3 mAb and to characterize their antitumoral activity. The cultured cell populations were sorted into T cells and non-T cells or NK cells and non-NK cells by using MACS or flow-cytometry sorting (FACS) procedures.

Several aspects of the cytotoxic phenomenon were considered, including the generation of serine esterase (granzyme) activity [57] and the direct lysis of tumor cell lines detected by a $^{51}$Cr-release assay. On the basis of our data, we suspected that residual anti-CD3 mAb at the surface of activated T cells were involved in the lysis of Fc-receptor-positive (FcγR$^+$) tumor cell lines. Similarly, the cytotoxic potential of activated T cells was also tested in the presence of bispecific anti-CD3 × anti-MOv18 mAb, which can bridge T cells to FcγR$^-$ tumor cell lines bearing the MOv18 epitope.

Overall the results suggest that cells cultured with anti-CD3 mAb provide an optimal activation of T cells compared with culture with IL-2 alone. Nevertheless, the short-term cytotoxic process strictly requires binding to the tumor cell either by a bispecific mAb or by residual membrane mAb.

## Material and methods

### Preparation and culture of PBL

Healthy donors were used as a source of PBL (Centre de transfusion de Liège, Belgium). The lymphocytes were prepared by centrifugation on Lymphoprep (Nycomed, Oslo, Norway), washed three times and counted. The culture medium consisted of RPMI-1640 medium (Gibco BRL, Gent, Belgium) supplemented with 1% non-essential amino acids (Gibco), sodium pyruvate (1 mM Gibco), 30 U/ml penicillin/streptomycin (Gibco) and 5% pooled heat-inactivated human AB serum. Cells were cultured ($1.25\times10^6$ cells/ml) in the presence of 10 ng/ml anti-CD3 mAb (BMA030, IgG2a; Behringwerke, Marburg, Germany) and 50 U/ml human recombinant (r)IL-2 kindly provided by Glaxo Institute for Molecular Biology (AG, Genève, Switzerland). For some experiments, the cell were cultured with other anti-CD3 mAb (UCHT1, IgG1, Immunotech, Marseille, France; SPVT3 F(ab′)$_2$, Zymed, San-Francisco, Calif.; BMA033, IgG3, Behringwerke) or in the presence of staphylococcal enterotoxin A (10 ng/ml, Sigma, Bornem, Belgium) or in the presence of phytohemagglutinin (PHA; 0.1%, Difco, Mich).

### Cell separation

The magnetic cell separation was previously described [29]. Briefly, the cells were incubated in phosphate-buffered saline (Gibco) with a biotinylated anti-CD3 mAb F(ab′)$_2$ (Zymed) for 20 min. The cells were then incubated with fluorescein-isothiocyanate(FITC)-labelled streptavidin (Boehringer Mannheim, Brussels, Belgium). Finally, the cells were incubated in the presence of 100 µl 1/100 diluted biotinylated magnetic beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) for 5 min. The cell sorting was done with the MACS instrument (Becton Dickinson, Erembodegem, Belgium) [38]. The purity of the positive and negative cell fractions were respectively verified by flow cytometry (FACStar$^+$, Becton Dickinson). When needed, further purification was performed by a flow-cytometry cell sorter (FACStar$^+$). For some experiments NK cells (CD56$^+$ and CD16$^+$ cells) were depleted by flow-cytometry cell sorting.

### Cell lines

The tumor cell lines used in this study are K562 [35], U937 [59], Daudi [30], HL60 [14], CESS [42], LY, Nalm-6 [27], Molt-3, Molt-4 [39], Jurkat [62], A431 [22], OVCAR-3 [25] and CK2 [23]. The cell lines were cultured in RPMI-1640 medium with the exception of CK2, which was maintained in Dulbecco's modified Eagle's medium (Gibco). Both media were supplemented with 10% fetal calf serum (Gibco).

### Cytotoxicity assays

The $^{51}$Cr-release cytotoxic assays were performed in 96-well V-bottom plates using 5000 $^{51}$Cr-labelled (Amersham, Belgium) tumor targets/well. Six serial dilutions of effector cells were prepared (effector cell:target ratios, E:T, 20:1–0.75:1). Plates were centrifuged for 6 min at 400 *g* to ensure cell contact and incubated for 4 h at 37 °C. Samples of 100 µl supernatant were then recovered from each well and the radioactivity was measured in a γ counter (Cobra auto-gamma Packard, Downers Grove, Ill.). Maximum chromium release was obtained by adding 100 µl detergent (RBS 10%, Chemical Products, Belgium) to 100 µl of target cell suspension and spontaneous release was given by incubation of target cells without effector cells. The percentage of target lysis was calculated as previously described [29]. For some experiments, OC/TR F(ab′)$_2$ bispecific antibody (kindly provided by Centocor, Leiden, The Netherlands) (100 ng/ml) was added to the target cells 30 min before the cytotoxic assay. The bispecific antibody OC/TR recognizes CD3 and a folate-binding protein, a molecule overexpressed in about 90% of ovarian carcinomas [36]. Serine esterase activity was measured using *N*-benzyloxycarbonyl-L-lysine-thiobenzyl-ester esterase (BLT assay), as previously described [58].

### Immunofluorescence and flow cytometry

Double and triple staining were performed with antibodies directed against CD2 (T11 FITC), CD3 (IOT3 FITC), CD4 (IOT4 FITC), CD45R0 [phycoerythrin(PE)-labelled] and CD25 (PE) from Dako (Golstrup, Denmark). The anti-CD3 (PerCP), anti-CD28 (PE), anti-CD45RA (FITC), anti-CD11a (LFA-1 FITC), anti-CD16 (Leu11c PE), anti-CD56 (PE), anti-CD57 (FITC) and anti-HLA-DR (FITC) were purchased from Becton Dickinson. The anti-CD8 (PE) and anti-CD19 (PE) were obtained from Immunotech. The antibodies directed against the FcγRI (CD32, 32.2 FITC) and the FcγRII (CD64, IV.3 FITC) were purchased from Medarex (West Lebanon, N.H.). A goat anti-mouse Ig, FITC-labelled (Immunotech), was used to detect on the cell surface the presence of BMA030 used for the stimulation. The cells were analyzed for fluorescence intensity on a FACStar$^+$ (Becton Dickinson).

### Statistical analysis

The data were analyzed using Student's *t*-test (Instat Mac 2.01 software, GraphPad, San Diego, Calif.).

## Results

### Lymphocyte phenotype obtained after activation with IL-2 or with IL-2 + BMA030

To determine the cell populations and the state of cell activation obtained after stimulation by IL-2 or by IL-2 +

BMA030, the cell phenotype was characterized by a panel of antibodies. A higher percentage of T lymphocytes ($CD2^+$ $CD3^+$) was obtained in the IL-2 + BMA030 culture conditions, compared to what was observed with IL-2 alone (Table 1). The presence of BMA030 in the culture medium also induced a higher percentage of $CD25^+$ (68% versus 13%), HLA-$DR^+$ (38% versus 14%), $CD45R0^+$ (63% versus 41%) and $CD28^+$ (86% versus 74%) cells compared to that observed with IL-2 alone (Table 1). Moreover, an increased frequency of T cells expressing a high density of LFA1 ($CD11a^{high}$, 61% versus 30%) and CD2 ($CD2^{high}$, 55% versus 12%) was obtained when lymphocytes were stimulated with IL-2 + BMA030.

**Table 1** Cell-surface phenotype. Results are means % ± SD of positive cells from 5–36 independent experiments. *P* values were calculated by comparing the results of interleukin-2 (IL-2) and IL-2 + anti-CD3 mAb stimulation. *$CD11a^{high}$*, *$CD2^{high}$* cells expressing a high density of cell-surface marker

| Antigen | Control | Cells stimulated by IL-2 | Cells stimulated by IL-2 + anti-CD3 mAb | $P^a$ |
|---|---|---|---|---|
| CD2 | 83 ± 5 | 84 ± 6 | 93 ± 5 | 0.0015 |
| CD3 | 78 ± 7 | 79 ± 7 | 85 ± 5 | 0.0004 |
| CD25 | 5 ± 3 | 13 ± 5 | 68 ± 10 | 0.0001 |
| CD28 | 76 ± 4 | 74 ± 4 | 86 ± 5 | 0.0315 |
| $CD3^+$ $DR^+$ | 6 ± 3 | 14 ± 7 | 38 ± 13 | 0.0007 |
| CD45R0 | 40 ± 11 | 41 ± 10 | 63 ± 11 | 0.0001 |
| CD45RA | 63 ± 11 | 67 ± 10 | 49 ± 17 | 0.0001 |
| $CD11a^{high}$ | 22 ± 12 | 30 ± 13 | 61 ± 13 | 0.0001 |
| $CD2^{high}$ | 4 ± 4 | 12 ± 6 | 55 ± 17 | 0.0060 |

## Comparison of cytotoxic activity of T cells stimulated with IL-2 with that of cells treated with IL-2 and anti-CD3 mAb

The lysis of K562 and Daudi cells, which are classical target cells for NK and LAK cell activities, was similar with lymphocytes cultured for 4 days with either IL-2 or IL-2 + BMA030, but the stimulation by IL-2 + BMA030 induced a higher cytotoxic activity against HL60 and U937 than did IL-2 alone ($P < 0.005$ for HL60 and $P < 0.01$ for U937) (data not shown). In parallel, a higher increase of serine-esterase activity was observed in the presence of both IL-2 and BMA030 compared to the value for IL-2 stimulation ($P \geq 0.05$) or for unstimulated cells ($P \geq 0.01$) (data not shown). To determine the respective participation of NK cells and T lymphocytes in this lytic activity, the mixed population was sorted for CD3 expression before the cytotoxicity assay. According to a previously published protocol [29], we used MACS alone or MACS + FACS to achieve a purity superior to 95% in both $CD3^+$ and $CD3^-$ cell fractions. The majority of $CD3^-$ cells were NK cells as assessed by the presence of cell-surface CD16 and/or CD56 (data not shown).

To determine more accurately the spectrum of cytotoxic activity displayed by PBL activated by IL-2 + anti-CD3, a panel of ten hematopoietic tumor cell lines was used (Table 2). The $CD3^-$ cells killed all cell lines, whereas the $CD3^+$ T cells were poorly cytotoxic to most of the cell lines, except for HL60 and U937. By contrast, after stimulation with IL-2 alone, the $CD3^+$ T cells did not show any significant cytotoxic activity against these target cell lines (data not shown). Similar results were obtained when the T cell fraction was purified by negative selection, showing that the sorting procedure did not interfere with the cytotoxic properties of the effectors (data not shown).

## Role of membrane-bound mAb in the cytotoxic activity induced in culture with IL-2 and anti-CD3 mAb

We then checked for the presence of FcγR on the target cells. Interestingly, HL60 and U937 were both positive for FcγRI, a receptor with a high affinity for murine IgG2a antibodies (Table 1). This observation suggested that residual BMA030, an IgG2a antibody, on the cultured lymphocytes may have acted as a bridge between activated T cells and FcγRI-bearing target cells, thus being responsible for redirection of the cytotoxic activity against the targets. Accordingly, when PBL were cultured with an anti-CD3

**Table 2** Cytotoxic activity of lymphocytes (unsorted and sorted cells) stimulated by IL-2 + anti-CD3. Cells were cultured for 4 days in the presence of IL-2 + anti-CD3 mAb (BMA030) and sorted into $CD3^+$ and $CD3^-$ cell populations. The cytotoxic activity was determined in a 4-h $^{51}Cr$-release assay against ten hematopoietic tumor cell lines with different patterns of Fcγ receptors, their pattern being determined by fluorescein-isothiocyanate-labelled antibodies (*FcγRI* and *FcγRII*) or phycoerythrin-labelled antibody (*FcγRIII*). The data are shown for an E : T of 20 : 1. The results represent means ± SD of 3–6 independent experiments. *P* values were calculated by comparing the cytotoxic activity of $CD3^+$ and $CD3^-$ cells

| | FcγR | | | Cytotoxicity (%) | | | |
|---|---|---|---|---|---|---|---|
| Cell lines | I | II | III | Unsorted cells | $CD3^-$ cells | $CD3^+$ cells | *P* |
| K562 | – | + | – | 47 ± 16 | 91 ± 12 | 9 ± 2 | 0.0003 |
| Daudi | – | + | – | 65 ± 16 | 75 ± 11 | 8 ± 6 | 0.0001 |
| Ly | – | – | + | 19 ± 9 | 30 ± 11 | 5 ± 2 | 0.0260 |
| Cess | – | – | + | 17 ± 12 | 32 ± 22 | 3 ± 3 | 0.0800 |
| Nalm-6 | – | + | – | 15 ± 1 | 54 ± 8 | 13 ± 6 | 0.0290 |
| Jurkat | – | – | – | 67 ± 30 | 76 ± 1 | 13 ± 3 | 0.0250 |
| Molt-3 | – | – | – | 35 ± 6 | 53 ± 9 | 3 ± 1 | 0.0160 |
| Molt-4 | – | – | – | 27 ± 17 | 59 ± 1 | 2 ± 1 | 0.0001 |
| U937 | + | + | – | 57 ± 13 | 62 ± 1 | 56 ± 1 | 0.2500 |
| HL60 | + | + | – | 55 ± 3 | 56 ± 17 | 45 ± 4 | 0.3000 |

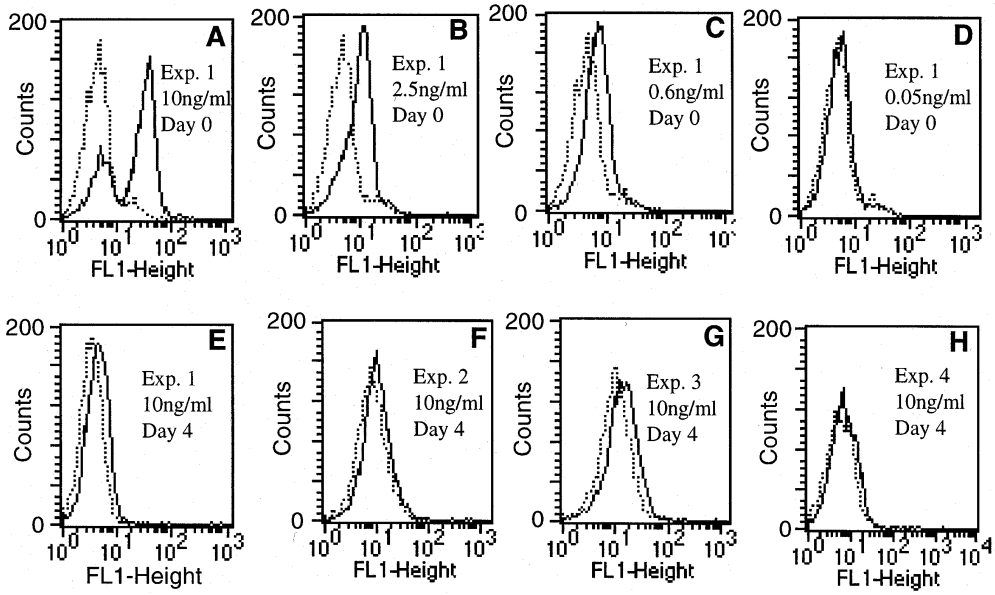


**Fig. 1A–H** Detection of BMA030 after 4 days of culture. An incubation of peripheral blood lymphocytes with different concentrations of BMA030: **A** 10 ng, **B** 2.5 ng/ml, **C** 0.6 ng/ml, **D** 0.05 ng/ml, was performed and the cells were run through fluorescence-activated cell sorting to determine the minimum amount of antibody detectable by this technique using a fluorescein-isothiocyanate (FITC)-labelled goat anti-mouse Ig. - - - The control fluorescence. **E** The same cells were cultured for 4 days in the presence of interleukin-2 + BMA030 and loaded with FITC-labelled goat anti-mouse Ig. **F**, **G** and **H** Three other independent experiments on anti-CD3 mAb detection after 4 days of culture

F(ab′)$_2$ fragment or with a non-mAb TCR ligand such as staphylococcal enterotoxin A or PHA, the sorted T cells did not display any significant cytotoxic activity against HL60 and U937 (less than 10% lysis). Similarly, the use of an anti-CD3 mAb (UCHT1, IgG1) that has a poor affinity for FcγRI did not induce a strong cytotoxic activity against these targets (data not shown). By contrast, stimulation with an anti-CD3 IgG3 mAb, which has the same affinity as IgG2a mAb for FcγRI, induced similar cytotoxicity to BMA030 (IgG2a). Despite this evidence for a participation of the anti-CD3 mAb in the effector stage of the lytic process, only a small amount of residual anti-CD3 mAb (in the nanomolar range) could be detected on activated T cells by a goat anti-mouse Ig mAb (Fig. 1). In fact, we observed that, in most experiments, the amount of membrane mAb molecules on our effectors corresponded to a standard staining assay performed with a working concentration of 0.6 ng/ml mAb (Fig. 1).

### Comparison of OC/TR bispecific mAb retargeting of T cells stimulated with IL-2 with that of cells stimulated with IL-2 + BMA030

The hypothesis that anti-CD3-stimulated PBL could kill their target through a “redirected” pathway, even in the presence of low amounts of mAb, led us to investigate the activity of these cells in the presence of bispecific antibodies directed against both CD3 and a tumor-specific antigen. OC/TR is a monoclonal bispecific antibody, which recognizes both CD3 and a folate-binding protein (MOv18), a cell-surface molecule overexpressed on ovarian tumors. We determined whether the short-term culture with anti-CD3 mAb plus IL-2 could be more efficient than IL-2 alone in generating effector cells against the MOv18$^+$ OVCAR-3 tumor cell line. The MOv18$^-$ A431 tumor cells were used as negative controls.

After 4 days of culture in the presence of IL-2 or IL-2 + BMA030, the CD16$^+$ and CD56$^+$ cells were depleted from the cell population to eliminate NK activity. In three independent experiments, a specific cytotoxic activity could be detected against the MOv18$^+$ OVCAR-3 tumor cell line after addition of F(ab′)$_2$ bispecific antibody OC/TR in the cytotoxic assay (Fig. 2). Retargeted cytotoxicity was more pronounced after stimulation with IL-2 + BMA030 ($P < 0.05$) (Fig. 2). The stimulation of T cells by PHA + IL-2 was not as effective in generating retargeted cytotoxicity, as was the stimulation by IL-2 + BMA030 (data not shown).

## Discussion

This report illustrates several findings regarding T cell activation and antitumor cytotoxicity. (a) Short-term culture of PBL with BMA030, in the presence of a concentration of IL-2 sufficient to induce LAK cells [1, 4, 21, 60, 63], enhanced the differentiation of cytotoxic T cells devoid of any LAK-like activity (as shown by the absence of lytic activity against Daudi and K562). (b) The cytotoxic activity of T cells observed in a 4-h chromium-release assay, was strictly dependent on the presence of FcγRI on the target cells; interestingly the effect was obtained with a very low amount of anti-CD3 mAb, supposedly in the nanomolar range, in contrast to previously published models of redir-

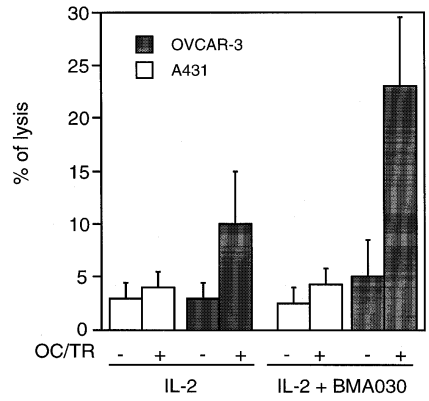


**Fig. 2** Cytotoxic activity in the presence of bispecific antibody (*OC/TR*). After 4 days of culture with IL-2 or IL-2 + BMA030, natural killer cells were depleted and a cytotoxic assay was performed against A431 an OVCAR-3 tumor cell line in the presence or absence of the bispecific antibodies (OC/TR). The means ± SD of three independent experiments are represented. E:T = 20:1

ected cytotoxicity [8, 33, 44, 53, 64]. (c) Accordingly, T cells from cultures activated with IL-2 + BMA030 mediated a higher bispecific mAb-dependent cytotoxicity than their counterparts cultured with IL-2 alone.

We formally demonstrated that, in the presence of a "LAK-inducing" concentration of IL-2, the cytotoxic activity induced in cultures of T lymphocytes with an anti-CD3 mAb, during a 4-hour $^{51}$Cr-release assay, was strictly of the "antibody-redirected" type since only target cell lines bearing FcγRI (high-affinity receptor) (HL60 and U937) [2] (and personal results) were lysed. As very few BMA030 molecules were detected on the cell surface at the end of the culture, it is likely that the cytotoxic process did not require a large number of anti-CD3 mAb molecules, unlike results previously reported with other models of redirected cytotoxicity [13, 33, 44, 53]. Furthermore, there was no correlation between the level of the cytotoxicity and the level of detection of BMA030. One could argue that the sensitivity of the detection assay, based on FITC-conjugated goat anti-mouse-Ig mAb was not sufficient to estimate the amount of antibody required to induce the lysis. We observed that, in most experiments, the amount of membrane mAb molecules on our effectors was in the nanomolar range, which is far below the dose usually recommended to obtain redirected cytotoxicity. Furthermore, with a more sensitive assay that has been reported to detect as few as 400 molecules/cell [65], no additional BMA030 was detected in any of the experiments (results not illustrated).

The cytotoxicity mediated by the bispecific antibody is very similar to the anti-CD3-redirected cytotoxicity, except that the lysis is not limited to FcγR$^+$ target cells. This type of cytotoxicity increases when activated effector cells are used [40]. We have demonstrated that stimulation of PBL with IL-2 + BMA030 increases the cytotoxicity against folate-binding protein FBP$^+$ target cells following retargeting via OC/TR bispecific antibody, in contrast to the cytotoxicity observed with IL-2-activated lymphocytes. Other authors have also demonstrated the advantages of anti-CD3 + IL-2 stimulation compared to stimulation with IL-2 alone for the enhancement of cytotoxic activity mediated by other bispecific antibodies [3]. However, in contrast to our study, the effectors T cells were not sorted, thus the contribution of NK cells (LAK activity) to the cytotoxic activity was not known. Lamers *et al.* [31] observed that the induction of OC/TR-antibody-directed cytotoxicity was more rapid after anti-CD3 mAb activation, than after PHA activation of lymphocytes.

In addition to the antibody/antigen interaction induced by the bispecific mAb, adhesion molecules could be involved in the binding of the effector cell to the tumor cell. In IL-2 + BMA030 cultures, the cells had an increased expression of adhesion molecules such as LFA1 (CD11a) and CD2 previously described to be involved in redirected cytotoxicity. For example, an interaction between LFA-1 and ICAM was found to be required for cytotoxic activity induced by an anti-(epidermal growth factor, EGF, receptor)/anti-CD3 bispecific mAb [18]. In fact, pretreatment of effector cells by anti-LFA1 mAb significantly inhibited cytolysis of all types of EGF-receptor-positive tumor cells. The cell-surface phenotype was also studied and a larger proportion of cells stimulated by IL-2 + BMA030 had an activated phenotype and a higher expression of CD28. All these properties (expression of adhesion molecules, activated/memory phenotype) could be associated with a lowered threshold for triggering of lysis after the interaction with the tumor cell. Moreover the culture condition IL-2 + BMA030 generated more effector T cells with a memory phenotype that has been shown to be associated with redirected cytotoxicity in a previous report [54]. Several investigations have already shown an increase of CD3$^+$ [40], CD25$^+$ [48], CD3$^+$HLA-DR$^+$ [47], and CD2$^{+high}$ [9] cells in the presence of IL-2 + anti-CD3. Percentages of positive cells obtained in these studies were similar to or smaller than ours. But, their culture conditions were different from ours since purified T cells were cultured or the anti-CD3 was removed from the culture medium by washing the cells at least 24 h before the cytotoxic assay.

The cytotoxic potential of effector cells can also be accessed by performing the serine esterase assay (BLT assay) or the tumor growth inhibition assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT assay). Preliminary results obtained using a BLT assay on unpurified lymphocytes stimulated by IL-2 or IL-2 + BMA030 showed that higher serine esterase activity was observed after culture in the presence of IL-2 + BMA030. Though the production of serine esterase after culture in the presence of IL-2 or anti-CD3 is well documented [7, 13, 32, 34], up to now comparison of the two culture conditions using the BLT assay has only been reported in a murine model [61]. In that model, the production of serine esterase was similar for the two culture conditions. Concerning the MTT assay, we also observed a higher tumor growth inhibition with lymphocytes stimulated by IL-2 + anti-CD3. This higher cytotoxic potential could be related to an increased production of cytokines interferon γ (IFNγ) and tumor necrosis factor α (TNFα) [52], as suggested by our analysis of PBL culture super-

natants. This hypothesis is correlated with a previous study where stimulation by IL-2 + BMA030 induced a higher production of TNFα and IFNγ than did stimulation with IL-2 [41].

In conclusion, these experiments have demonstrated the advantages of the combined use of anti-CD3 and IL-2 to induce an optimal differentiation of cytotoxic T cells capable of killing tumor cells in the presence of bispecific antibodies. Our results have a potentially interesting application in the design of immunotherapy protocols based on anti-CD3 × antitumor bispecific antibodies.

**Acknowledgements** This work was supported in part by the Belgian Fund for Medical Scientific Research, the Research Fund of the Faculty of Medicine of Liège, the Centre anticancéreux près l'Université de Liège (CAC) and the AIRC-FIRC. N. Jacobs is supported by grant from the Léon Fredericq Foundation. M. Trebak was supported by the CAC. M.P. Moutschen is a Senior Research Assistant of the National Fund for Scientific Research. The authors wish to acknowledge Dr. S. Canevari and Dr. S. Giannini for the critical reading of the manuscript and E. Franzen-Detrooz and G. Zeimers for the excellent technical assistance.

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