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ORIGINAL ARTICLE

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# In vitro effects of R-verapamil on the cytokine environment and T-lymphocyte proliferation when human T-lymphocyte activation takes place in the presence of acute myelogenous leukemia blasts

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**Abstract** We investigated the effects of R-verapamil on the cytokine environment and T-lymphocyte proliferation when human T-lymphocytes were activated in the presence of accessory cells containing a large population of acute myelogenous leukemia (AML) blasts (nonirradiated blasts for cytokine studies, 50 Gy irradiated blasts in proliferation studies). In the presence of AML blasts, R-verapamil inhibited interleukin 4 (IL4) and interferon-$\gamma$ (IFN$\gamma$) release from polyclonal T-cell lines activated with the T-cell mitogen phytohemagglutinin (PHA). R-verapamil also inhibited both the proliferation and the release of IFN$\gamma$ and IL10 by normal T-cells stimulated with allogeneic peripheral blood mononuclear cells derived from AML patients. This antiproliferative effect of R-verapamil was seen in the presence of exogenous IL2 but was not observed in the presence of exogenous IL1$\beta$ or granulocyte/macrophage colony-stimulating factor GM–CSF). In addition R-verapamil inhibited the release of IL1$\beta$ and tumor necrosis factor $\alpha$ during allogeneic stimulation.

**Key words** T-lymphocytes · Acute myelogenous leukemia · R-verapamil

**Abbreviations** *AML* Acute myelogenous leukemia · *cpm* counts per minute · *ELISA* enzyme-linked immunosorbent assay · *G-CSF* granulocyte colony-stimulating factor · *GM-CSF* granulocyte/macrophage colony-stimulating factor · *IL* interleukin · *PBMC* peripheral blood mononuclear cells · *PHA* phytohemagglutinin · *TCL* T-cell line · *TNF*$\alpha$ tumor necrosis factor $\alpha$.

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## Introduction

Conventional chemotherapy in patients with acute myelogenous leukemia (AML) results in a long-term disease-free rate of survival of less than 50% [24]. To improve the prognosis for patients for whom allogeneic bone marrow transplantation is not available, the following therapeutic approaches have been suggested: (a) cytokine enhancement of antileukemic immune reactivity [18, 26, 39], (b) cytokine-induced increase of AML blast proliferation to render the blasts more susceptible to cell-cycle-specific cytotoxic drugs [3, 11], (c) inhibition of AML blast proliferation by cytokines [15, 28], and (d) decrease of cytotoxic drug extrusion from AML blasts by inhibition of the multidrug-resistance (MDR1) transport protein in the cell membrane [20, 32, 36, 37]. R-verapamil inhibits MDR1 in vitro [2, 30, 36, 37], but at the same time the drug may also interfere with the other three antileukemic mechanisms: (a) it may alter antileukemic reactivity by inhibiting T-cell activation [6, 12, 33], and it may alter AML blast proliferation either (b) by direct inhibition or (c) indirectly via effects exerted on T-cell secretion of hematopoietic growth factors [4, 5, 8].

To characterize further the effects of R-verapamil on interactions between human T-cells and AML blasts (native and 50-Gy-irradiated blasts), we characterized the effects of the drug on the cytokine environment and T-cell proliferation when human T-cells were activated in the presence of accessory cells containing a majority of AML blasts

## Materials and methods

### Cell donors

The clinical data on the AML patients included in the study are summarized in Table 1, and the serological HLA types of the patients and healthy individuals are shown in Table 2.

**Table 1** Clinical characteristics of acute leukemia patients (*ALL* Acute lymphocytes leukemia *NT* not tested)

| Patient | Sex | Age (years) | Previous hematological disease | FAB classification | Membrane molecule expression[a] | | | | | | | Blasts among PBMC[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CD3 | CD13 | CD14 | CD15 | CD19 | CD33 | CD34 | |
| 1 | F | 55 | | AML-M4 | − | + | + | + | − | − | − | 97% |
| 2 | M | 73 | Chronic myelofibrosis | AML-M2 | − | + | − | NT | − | − | − | 98% |
| 3 | F | 71 | Second relapse | AML-M1 | − | + | − | + | − | − | + | 98% |
| 4 | F | 67 | | AML-M1 | − | + | − | NT | − | − | − | 98% |
| 5 | F | 83 | Primary myelo-dysplastic syndrome | AML-M2 | − | + | − | NT | − | + | − | 95% |
| 6 | M | 56 | | AML-M4 | − | + | − | + | − | + | − | 95% |
| 7 | F | 72 | Multiple myeloma | AML-M2 | − | − | + | NT | − | + | − | 97% |
| 8 | F | 67 | | AML-M2 | − | + | − | NT | − | + | − | 95% |
| 9 | M | 67 | Primary myelo-dysplastic syndrome | AML-M2 | − | + | − | NT | − | + | − | 97% |
| 10 | F | 54 | | AML-M2 | − | + | − | NT | − | + | − | 96% |
| 11 | F | 64 | | AML-M2 | − | + | − | NT | − | − | − | 94% |
| 12 | M | 75 | Non-Hodgkin's lymphoma, CHOP therapy | AML-M4 | − | + | − | NT | − | + | NT | 95% |
| 13 | F | 71 | | AML-M5 | − | + | + | + | − | + | − | 97% |
| 14 | M | 80 | | AML-M4 | − | − | + | NT | − | − | − | 97% |
| 15 | F | 56 | Primary myelo-dysplastic syndrome | AML-M2 | − | + | + | − | − | + | + | 99% |
| 16 | M | 72 | Primary myelo-dysplastic syndrome | AML-M1 | − | + | − | NT | − | − | + | 95% |
| 17 | M | 30 | | AML-M2 | − | − | − | NT | − | + | − | 95% |
| 18 | F | 46 | | AML-M5 | − | + | + | + | − | + | − | 96% |
| 19 | M | 27 | | AML-M2 | − | + | − | + | − | + | + | 95% |
| 20 | M | 47 | | AML-M4 | − | + | + | NT | − | − | − | 99% |
| 21 | M | 49 | | AML-M4 | − | + | − | + | − | + | − | 96% |
| 22 | F | 64 | | AML-M5 | − | − | + | + | − | + | − | 98% |
| 23 | M | 20 | | ALL | − | − | − | − | + | − | − | 98% |

[a] Patients were regarded as positive when more than 20% of blast cells stained positive as judged from flow cytometric analysis. All AML patients were negative for CD20 (Data not shown)

[b] Blast numbers are expressed as the percentage of blasts among PBMC as judged by light microscopy of May-Grünwald-Giemsa-stained preparations

**Table 2** HLA typing of healthy individuals and acute leukemia patients included in the allorecognition experiments (*NT* Not tested)

| Individual | Serological HLA types | | | |
|---|---|---|---|---|
| | A | B | DR | DQ |
| Control 1 | 2,3 | 7,15 | 2,4 | NT |
| Control 2 | 1,2 | 7,8 | 3,4 | NT |
| Control 3 | 2,9 | 12,27 | 2,4 | 1,3 |
| Patient 17 | 11,28 | 35,60 | 1,4 | 1,3 |
| Patient 18 | 2, − | 8,40 | 3,6 | 1,2 |
| Patient 19 | 11,19 | 5,27 | 1,8 | 1,4 |
| Patient 20 | 2,30 | 57,60 | 2,6(13) | 1, − |
| Patient 21 | 2,3 | 7,14 | 2,6 | 1, − |
| Patient 22 | 10,19 | 18, − | NT | NT |
| Patient 23 | 1,2 | 8,15 | 3,4 | 2,3 |

## Reagents

The culture medium was RPMI 1640 containing HEPES and glutamine (Gibco, UK), to which was added 10% inactivated fetal calf serum (HiClone, USA) and gentamicin at 100 μg/ml. R-verapamil (kindly donated by Knoll AG, Ludwigshafen, Germany) was dissolved in culture medium at a concentration of 25 mg/ml and stored frozen at −20 °C. Cytokines were used at the following concentrations [1, 4, 6, 21, 25]: GM-CSF, at 100 ng/ml (Sandoz, Switzerland); IL1$\beta$, at 50 ng/ml (R&D Systems): and IL2, at 50 ng/ml (R&D Systems)

## Cell preparation

### *Peripheral blood mononuclear cells*

PBMC were isolated by density-gradient separation (Ficoll-Hypaque, NyCoMed, Norway: specific density 1.077). Leukemic PBMC were prepared from patients with high blast counts in their blood (WBC $> 30 \times 10^9$/1, at least 75% blasts) and contained $\geq 95\%$ blasts (Table 1). Cells were stored in liquid nitrogen until used in the experiments as described previously [4].

### *T-cell lines*

The TCL were prepared by a method corresponding to long-term expansion of human T-cell clones [7]. This culture method is based on weekly T-cell stimulation using accessory cells plus mitogen plus IL2, and IL2 is also added one additional time each week [6, 7]. In brief, normal PBMC at $1 \times 10^6$/ml were cultured with phytohemagglutinin at 1 μg/ml (PHA HA16, Wellcome, UK) plus IL2 at 10 ng/ml using 24-well tissue-culture plates (Costar, UK) containing 2 ml medium/well. The cells were restimulated on days 7 and 14 by

exchange of 1 ml medium/well and simultaneous addition of $2 \times 10^5$ pooled allogeneic, irradiated (30 Gy), normal PBMC plus PHA at 2 μg/well plus IL2 at 20 ng/well. IL2 was also added on days 4, 11, and 18 by changing of 1 ml medium/well. Cultures were divided when wells contained a monolayer of cells. The cells were frozen on day 21 and are hereafter referred to as T-cell lines (TCL). Three TCL were prepared: TCL-GH, containing 60% $CD4^+$ and 41% $CD8^+$ cells; TCL-RME, containing 69% $CD4^+$ and 32% $CD8^+$ cells; and TCL-AS, containing 81% $CD4^+$ and 21% $CD8^+$ cells as determined by flow cytometric analysis. All TCL showed minimal proliferation (<10% of controls cultured with irradiated PBMC plus PHA plus IL2) and no detectable secretion of the T-cell cytokine IL4 when stimulated with PHA plus IL2. The TCL-GH line showed the lowest proliferation in response to PHA plus IL2 and was selected for the major proportion of these experiments.

### Proliferation assays

#### *PHA-stimulated cultures*

TCL at $5 \times 10^4$/well were cultured in U-bottomed microtiter plates (Costar, USA) in 200 μl medium with 50-Gy-irradiated leukemic PBMC at $5 \times 10^4$/well and PHA at 1 μg/ml. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% $CO_2$. [$^3$H]-Thymidine at 37 kBq/well (TRA 310, 1 mCi/nmol; Amersham, UK) was added after 2 days, and nuclear radioactivity was determined 18 h later by liquid scintillation counting.

#### *Allorecognition*

Irradiated (50 Gy) leukemic PBMC at $5 \times 10^4$/well were cultured in 200 μl medium in U-bottomed microtiter plates together with $5 \times 10^4$ normal PBMC as responder cells. [$^3$H]-Thymidine incorporation was assayed after 6 days.

### Cytokine secretion

#### *PHA stimulation*

Nonirradiated leukemic PBMC at $2.5 \times 10^5$ were cultured with $2.5 \times 10^5$ TCL and PHA at 1 μg/ml in 1 ml medium in each well of 24-well tissue-culture plates. Supernatants were collected after 2 days.

#### *Allorecognition*

Nonirradiated leukemic PBMC at $2 \times 10^6$ were cultured with $1 \times 10^6$ normal allogeneic PBMC in 2 ml medium in each well of 24-well tissue-culture plates. Supernatants were then collected after 6 days.

#### *Cytokine analysis*

Supernatants were stored frozen until cytokine concentrations were determined by specific ELISA analysis (Quantikine ELISA kits, R&D systems, UK) performed strictly according to the manufacturer's instructions [8–10].

### Presentation of the data

Proliferation assays were performed in triplicate, and median responses were used for calculations and statistical analysis. Proliferation was regarded as significant when it (a) exceeded the negative control by at least 1000 counts per minute (cpm) and (b) exceeded the mean cpm of negative controls by at least 3 SD. The incremental response was defined as the response observed in stimulated cultures minus the responses seen in unstimulated controls.

For cytokine secretion, all concentrations were transformed to logarithmic values and the difference between cultures prepared in the the absence and presence of R-verapamil were then ranged. Wilcoxon's test for paired samples was used for statistical analysis, and differences were regarded as statistically significant when $P < 0.05$.

## Results

### Effect of R-verapamil on PHA-stimulated TCL proliferation

The three TCL were cultured with 50-Gy-irradiated leukemic PBMC (patients 18, 22) in the presence either of PHA at 1 μg/ml or of PHA at 1 μg/ml plus IL2 at 10 ng/ml. R-verapamil (0.025–50 μg/ml) significantly inhibited TCL proliferation only at high concentrations in tests of both PHA alone ($n = 6$; $P = 0.032$ for R-verapamil at 25 and 50 μg/ml) and PHA plus IL2 ($n = 6$; $P = 0.032$ for R-verapamil at 50 μg/ml).

### Effect of R-verapamil on PHA-stimulated IL4 and IFN$\gamma$ release

Normal PBMC ($n = 5$) were cultured with PHA at 1 μg/ml and IFN$\gamma$ concentrations were determined after 3 days. R-verapamil at 5 μg/ml caused only weak inhibition of IFN$\gamma$ release, whereas inhibition exceeding 50% was seen for R-verapamil at 25 μg/ml (data not shown).

High concentrations of IFN$\gamma$ were detected in cultures containing TCL-GH plus PHA plus nonirradiated leukemic PBMC (patients 1–16), and R-verapamil at 5 μg/ml decreased the IFN$\gamma$ concentrations (Fig. 1; $n = 16$, $P = 0.003$). Control cultures containing leukemic PBMC alone, TCL-GH, or TCL-GH plus leukemic PBMC showed either undetectable or low IFN$\gamma$ levels (less than 10% of cultures containing TCL plus PHA plus leukemic PBMC). Detectable IL4 levels were also seen for all patients (patients 1–15) whose cultures contained TCL–GH plus PHA plus leukemic PBMC, and R-verapamil at 5 μg/ml decreased the IL4 concentrations (Fig. 1; $n = 15$, $P < 0.\ 0.001$). IL4 was not detected in control cultures containing TCL-GH, PHA plus TCL, or PHA plus leukemic PBMC.

### Effect of R-verapamil on IL1$\beta$ and G-CSF release in cultures containing TCL plus PHA plus leukemic PBMC

Concentrations of IL1$\beta$ (patients 1, 3, 4, 7–12, 16) and G-CSF (patients 1, 3–7, 9, 11–14) were determined in

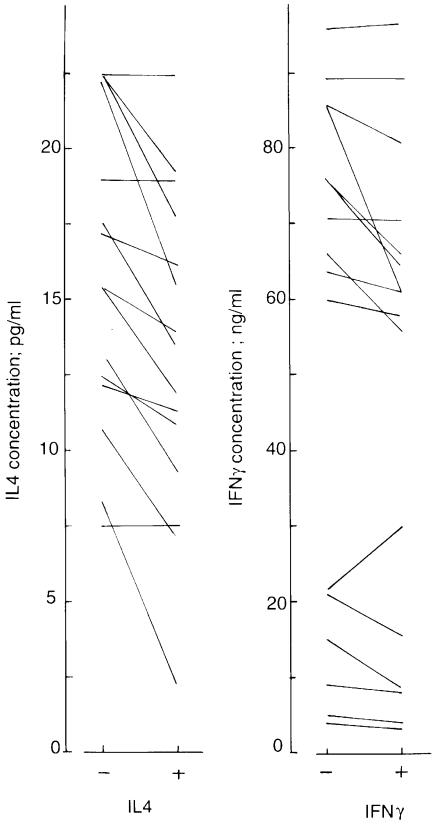


**Fig. 1** Effect of R-verapamil at 5 μg/ml on IL4 (patients 1–15) and IFN$\gamma$ (patients 1–16) secretion in cultures containing TCL-GH + PHA + nonirradiated leukemic PBMC. The results are presented as cytokine concentrations measured in 48-h supernatants for cultures prepared in the presence (+) and absence (−) of R-verapamil at 5 μg/ml

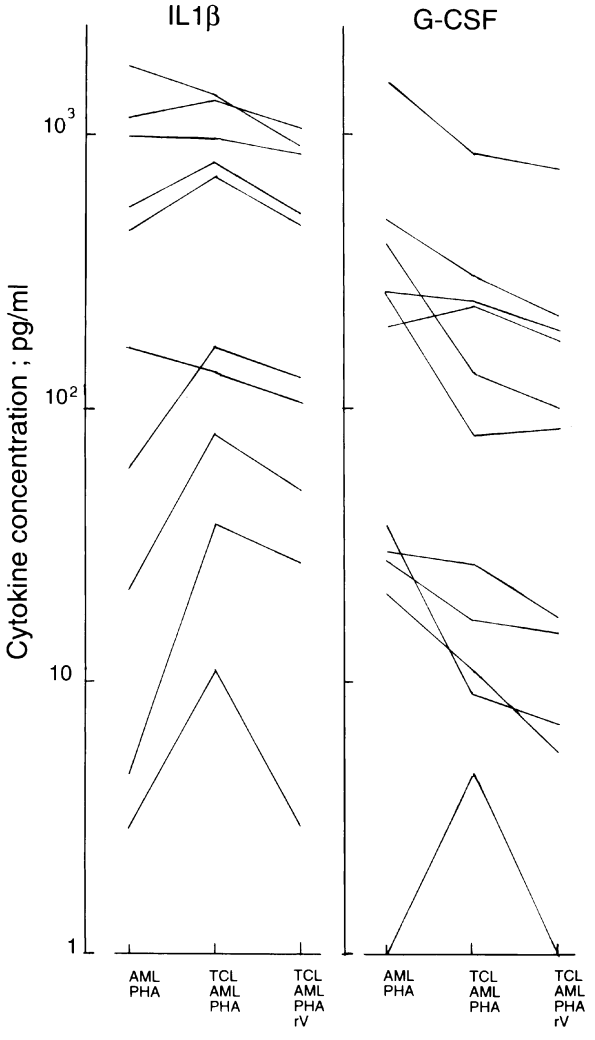


**Fig. 2** Concentrations (pg/ml) of IL1$\beta$ (10 AML patients) and G-CSF (11 AML patients) measured in supernatants derived from cultures containing PHA + leukemic PBMC (*AML.PHA*). TCL-GH + PHA + leukemic PBMC (*TCL.AML.PHA*), and TCL-GH + PHA + leukemic PBMC + R-verapamil at 5 μg/ml (*TCL.AML.PHA.rV*)

cultures containing TCL-GH plus PHA plus nonirradiated leukemic PBMC, and for a majority of patients, increased IL1$\beta$ levels were seen as compared with leukemic PBMC plus PHA (Fig. 2). However, subsequent addition of R-verapamil at 5 μg/ml decreased the IL1$\beta$ levels ($n = 10$, $P = 0.002$). For a majority of patients, decreased G-CSF concentrations were seen in cultures containing PHA plus TCL plus leukemic PBMC as compared with leukemic PBMC alone, and addition of R-verapamil at 5 μg/ml further decreased the G-CSF levels (Fig. 2; $n = 11$, $P = 0.002$).

## Effect of R-verapamil on allostimulated proliferation of normal PBMC

PBMC derived from two healthy individuals (controls 2, 3) were cultured with 50-Gy-irradiated allogeneic leukemic PBMC (patients 17–23). Detectable proliferation was seen for 12 of the 14 stimulator-responder combinations, and R-verapamil at 5 μg/ml significantly inhibited the allostimulated proliferation (Fig. 3; $n = 12$, $P = 0.01$). The allostimulated cultures were also prepared with IL1$\beta$ at 50 ng/ml, IL2 at 10 ng/ml, or GM-CSF at 100 ng/ml, and for each cytokine, cultures were prepared in the presence and absence of R-verapamil at 5 μg/ml. Although IL2 increased allostimulated proliferation, R-verapamil inhibited these responses even in the presence of excess IL2 (Fig. 3; $n = 13$, $P = 0.032$). In contrast, R-verapamil at 5 μg/ml did not significantly inhibit allostimulated proliferation in the presence of IL1$\beta$ or GM-CSF (Fig. 3.).

## Effect of R-verapamil on cytokine secretion during allostimulation of normal PBMC

Normal PBMC (controls 1, 2) were stimulated with allogeneic nonirradiated leukemic PBMC (patients 17–22). Cultures were prepared in the presence and

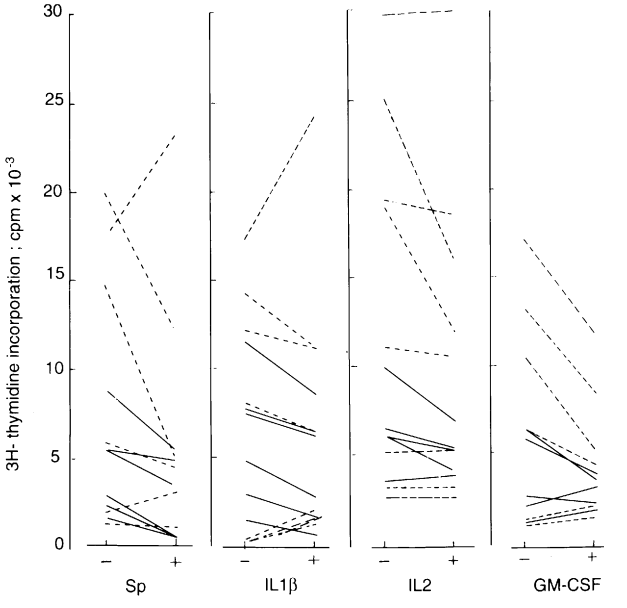


**Fig. 3** Allostimulated proliferation resulting from the use of normal PBMC responder cells (*solid lines* donor 2, *broken lines* donor 3) and 30-Gy-irradiated leukemic PBMC stimulator cells (patients 17–23). Cultures were prepared in medium alone (*Sp*) and in medium containing IL1$\beta$ at 20 ng/ml. IL2 at 10 ng/ml, or GM-CSF at 100 ng/ml; the results are expressed as median cpm of triplicate cultures prepared in the absence (−) or presence (+) of R-verapamil at 5 μg/ml. A total of 14 responder-stimulator combinations were investigated, and for each cytokine tested the results are included only for combinations showing significant proliferation

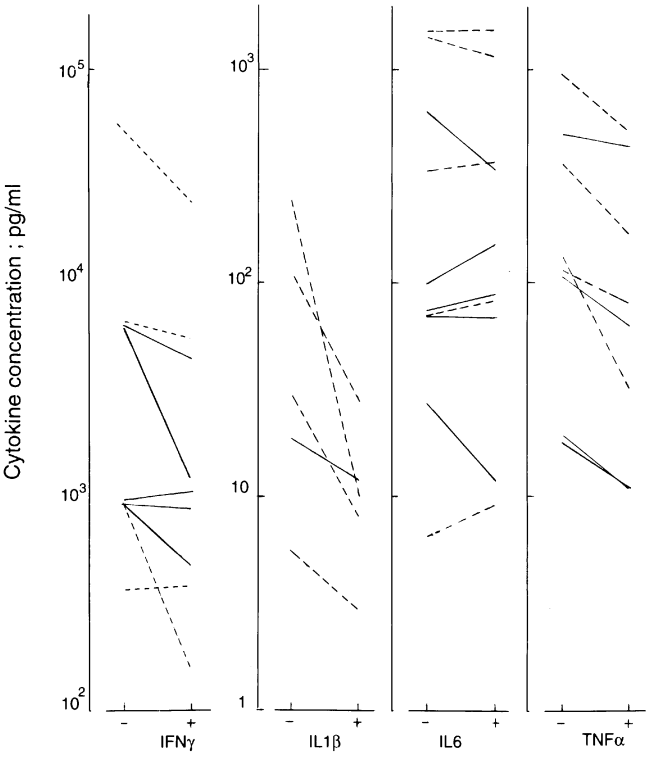


**Fig. 4** Concentrations (pg/ml) of IFN$\gamma$, IL1$\beta$, IL6, and TNF$\alpha$ measured in allostimulated cultures containing normal PBMC responder cells (*solid lines* donor 1, *broken lines* donor 2) together with nonirradiated leukemic PBMC stimulator cells (patients 17–23). Cultures were prepared in the absence (−) and presence (+) of R-verapamil at 5 μg/ml. For each cytokine tested the results are included only for responder-stimulator combinations where detectable cytokine levels were seen

absence of R-verapamil at 5 μg/ml, and concentrations of the T-cell cytokines IL4, IL10, and IFN$\gamma$ were then determined in the supernatants. IL4 was not detected for any combination tested. R-verapamil decreased IL10 concentrations in four of the five combinations tested (data not shown). IFN$\gamma$ was detected for nine combinations, and R-verapamil significantly decreased the IFN$\gamma$ levels (Fig. 4; $n = 9$, $P = 0.016$). Concentrations of IL1$\beta$, IL6, TNF$\alpha$, and G-CSF were also determined for cultures prepared in the presence and absence of R-verapamil at 5 μg/ml. R-verapamil caused no significant alteration in IL6 levels (Fig. 4; $n = 10$), whereas it significantly decreased concentrations of TNF$\alpha$ (Fig. 4; $n = 8$, $P = 0.008$) and IL1$\beta$ (Fig. 4; $n = 5$, $P = 0.031$). R-verapamil decreased G-CSF levels for all three combinations where G-CSF could be detected (data not shown).

## Discussion

T-lymphocyte activation depends on (a) accessory cells that present antigens/mitogens and deliver costimulatory signals and (b) the cytokine environment [34]. R-verapamil inhibits both T-lymphocyte and AML blast proliferation in vitro [5, 6, 8], and on the basis of this background we characterized the effects of R-verapamil when accessory cells included a majority of AML blasts showing spontaneous cytokine secretion [9, 13, 14, 28, 31, 35].

A possible mechanism for chemotherapy resistance in AML is extrusion of drugs by the MDR1 protein, and one therapeutic approach at trying to overcome drug resistance is MDR1 inhibition [20]. However, other therapeutic approaches are also being investigated. First, T-lymphocytes seem to mediate antileukemic reactivity [18, 26], and cytokine therapy to enhance T-cell activation is therefore tried in AML [39]. Second, certain cytokines may be used to increase AML blast proliferation [1, 5, 13, 14, 21] and, thereby, render the cells more susceptible to cell-cycle-specific cytotoxic drugs [3, 11]. Third, other cytokines may be used for direct inhibition of AML blasts [9]. With regard to these other three approaches, R-verapamil may interfere with cellular functions other than MDR inhibition and, thereby, modulate functions that are important as additional antileukemic mechanisms: (a) R-verapamil inhibition of T-cell functions may modulate antileukemic immune effects [6, 18, 26], and inhibition of AML blast proliferation by R-verapamil may (b) decrease susceptibility to cycle-specific cytotoxic drugs

or (c) have a beneficial additional effect in combination with antileukemic cytokines [5, 8, 9]. Thus, the final effect of combining chemotherapy and R-verapamil depends not only on MDR1 inhibition but also on interactions between R-verapamil and cellular functions regarded as important for other antileukemic mechanisms. Such additional effects may explain the importance of the treatment sequence of R-verapamil and cytotoxic drugs for the achievement of maximal enhancement of cytotoxicity [30].

Because of its lower cardiotoxicity, higher in vivo levels can be reached for R-verapamil than for racemic verapamil [16, 29, 38]. Previous investigations have shown that R-verapamil tested at concentrations of $> 1$ μg/ml has dose-dependent, nontoxic inhibitory effects on T-lymphocytes and AML blasts [5, 6, 8], and it has been suggested that this effect may be caused by interactions with potassium channels, calcium metabolism, or the utilization of essential nutritients (see [4–6, 8]). The serum levels of R-verapamil and its active metabolite nor-verapamil reached after intravenous therapy are up to 2 μg/ml (3–4 μ*M*) [2, 19, 27, 32]. The antiproliferative effects of R-verapamil are seen at concentrations corresponding to the higher in vivo levels of R-verapamil/non-verapamil [2, 32]. In tests of the sensitivity of lymphoma cell lines to doxorubicin in vitro, a plateau of maximal enhancement of doxorubicin cytotoxicity has been reached at R-verapamil concentrations of 3 μg/ml (6.6 μ*M*) and with 50% of the maximal effect seen at 0.25 μg/ml (0.5 μ*M*) [19, 27]. With the culture medium used in our models the free fraction of R-verapamil is 0.5 [19]. Several of our experiments were performed using R-verapamil at 5 μg/ml, a free drug concentration that is slightly below the level of maximal enhancement of cytotoxicity and representing a free in vitro drug fraction corresponding to the total levels of R-verapamil/nor-verapamil reached in vivo.

We used gradient separation to isolate enriched blast populations from the blood of patients with high blast counts [5, 9], as more extensive cell separation can induce AML blast cytokine expression [35]. However, some investigations indicate that the blast number in peripheral blood may be a prognostic factor in AML and may thus reflect intrinsic properties of the leukemia cells [23]. Our results may therefore be representative only for this subset of patients.

To investigate proliferative T-cell responses we used irradiated leukemic PBMC to avoid AML blast proliferation. In the cytokine studies we used native AML blasts, and cellular functions were then evaluated by cytokine analysis. R-verapamil modulated T-cell functions as well as AML blast cytokine secretion in tests of both irradiated and nonirradiated leukemic PBMC.

The TCL represent enriched T-cell populations. However, in tests of TCL this is an in vitro restimulation and should therefore be regarded as functionally different from the primary allostimulated responses of normal unstimulated PBMC [6, 7, 34]. In tests of PHA-stimulated TCL proliferation in the presence of AML blasts, R-verapamil inhibited proliferation only at high concentrations (25 and 50 μg/ml). In contrast, significant inhibition of IL4 and IFN$\gamma$ secretion was seen for all patients when R-verapamil was tested at 5 μg/ml.

T-cell alloreactivity depends on direct recognition of alloantigens on foreign cells and indirect recognition of alloantigens presented by self-antigen-presenting cells [17, 22]. To include both of these pathways in our in vitro models we used normal PBMC as responder cells. R-verapamil at 5 μg/ml inhibited both allostimulated proliferation and IL10 and IFN$\gamma$ release. A slight antiproliferative effect was also detected in the presence of exogeneous IL2 but was not seen in the presence of exogenous IL1$\beta$ or GM-CSF. Both IL1 and GM-CSF can modulate the constitutive AML blast secretion of cytokines acting as T-cell growth factors [10, 15, 28], and the reversal of R-verapamil effects on T-cell cytokine secretion by IL1$\beta$ and GM-CSF may therefore be indirectly mediated via alterations in this constitutive cytokine secretion. We conclude that (a) this antiproliferative effect of R-verapamil is mediated at least partly by mechanisms other than IL2 inhibition and (b) the antiproliferative effect of R-verapamil on alloreactive cells can be reversed by cytokines (IL1$\beta$, GM-CSF) capable of modulating AML blast functions.

IL$\beta$ and G-CSF detected in culture supernatants are regarded as AML blast-derived cytokines because (a) AML blasts produce high levels of IL1$\beta$ [9, 13], and IL1$\beta$ was not detected in our cultures containing TCL plus PHA; and (b) G-CSF is not produced by T-lymphocytes [14]. R-verapamil at 5 μg/ml decreased the concentrations of these cytokines during both mitogenic stimulation and allorecognition. This inhibition of AML-blast cytokine secretion is probably an indirect effect mediated via the activated T-cells, as R-verapamil shows no significant effect on spontaneous cytokine secretion in tests of leukemic PBMC alone [8].

IL6 and TNF$\alpha$ are secreted by both AML blasts and activated T-cells [9, 10, 26, 31, 35]. During allorecognition, TNF$\alpha$ levels showed a decrease similar to that displayed by IL1$\beta$ in the presence of R-verapamil, whereas R-verapamil did not alter IL6 levels.

From our overall results we conclude that R-verapamil not only may inhibit MDR1 in AML blasts but may also interfer with other antileukemic mechanisms by modulation of the cytokine environment or inhibition of T-cell activation. Generally, MDR-inhibitory drugs should be tested in vitro for antiproliferative and immunomodulatory effects prior to clinical trials. Assays using [$^3$H]-thymidine incorporation by immunocompetent and malignant cells may then be used [5, 6], but in the case of myeloid malignancies, colony-forming assays should also be included [8, 9]. Such additional effects should then be

considered when future clinical trials of MDR1 inhibition are designed both for AML patients and for patients with other malignant diseases.

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