Cancer Immunol Immunother (1996) 43: 275–282

ORIGINAL ARTICLE

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Human T lymphocyte activation in the presence of acute myelogenous leukaemia blasts: studies of allostimulated interferon-γ secretion

Received: 4 June 1996 / Accepted: 15 October 1996

Abstract Normal peripheral blood mononuclear cells (PBMC responders) were cultured together with non-irradiated allogeneic PBMC (more than 95% leukaemia blasts) derived from patients with acute leukaemia (referred to as leukaemic PBMC stimulators). Cytokine secretion was determined as cytokine concentrations in supernatants. Both normal PBMC and enriched $CD4^+$ and $CD8^+$ T cells responded to allostimulation with interferon (IFNγ) secretion. Interleukin-1 (IL-1) receptor antagonist and IL-2-neutralizing antibodies decreased IFNγ secretion. Exogenous IL-1β, IL-2 and IL-7 increased allostimulated IFNγ secretion, whereas decreased levels were seen in the presence of IL-6, IL-10 and granulocyte-colony-stimulating factor (G-CSF). During allorecognition IFNγ -neutralizing antibodies decreased acute myelogenous leukaemia (AML) blast secretion of G-CSF. We conclude that (i) both $CD4^+$ and $CD8^+$ T cells show allostimulated cytokine secretion in response to allogeneic stimulator cells containing a dominating population of native, cytokine-secreting leukaemia blasts, and (ii) IFNγ released during this response can modulate the function of allogeneic AML blasts.

Key words T lymphocytes · Acute myelogenous leukaemia · Interferon γ

Introduction

Conventional chemotherapy of acute myelogenous leukaemia (AML) results in a long-term disease-free survival of less than 50% [27]. However, the prognosis can be improved by allogeneic bone marrow transplantation (BMT) [26]. Several factors contribute to this improved prognosis, including posttransplant antileukaemic effects mediated by graft versus host disease (GVHD) or by specific antileukaemic reactivity termed graft versus leukaemia (GVL) effects [22, 32, 33]. Graft T cells are probably important both for the GVL effects and for allorecognition during GVHD [22, 33]. The capability of human T cells to mediate antileukaemic effects is further demonstrated by the observation that T cell depletion can reduce the risk of GVHD after allo-BMT from an unrelated bone marrow donor, but without increasing the risk of leukaemia relapse as otherwise seen when using T cell depletion in HLA-identical sibling donors [2, 22]. Thus, decreased antileukaemic activity caused by T cell depletion is compensated for by increased allorecognition.

Interferon γ (IFNγ) has antileukaemic activity both in vitro and possibly also in vivo [15, 23, 30, 39, 40]. However, these effects of IFNγ on leukaemia cells are dependent on the cytokine network, and IFNγ may even have an enhancing effect on leukaemia blasts in the presence of certain cytokines [29]. It remains to be established whether IFNγ functions as an antileukaemic T cell effector mechanism during GVHD or GVL reactions, or whether the local cytokine network and the constitutive cytokine secretion by native AML blasts then will modulate IFNγ secretion or IFNγ effects [14]. In this context we used an in vitro model to characterize allostimulated IFNγ secretion in the presence of excess AML blasts.

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Materials and methods

Cell donors

The clinical data of the seven acute leukaemia patients are presented in Table 1, and the HLA types of patients and healthy blood donors are included in Table 2.

Table 1 Clinical characteristics of acute leukaemia patients included in the study. Patients were regarded as positive when more than 20% of blast cells stained positive, judged from flow-cytometric analysis. *PBMC* peripheral blood mononuclear cells, *NT* not tested

Patient	Sex	Age (years)	Cytogenetic analysis	FAB classification	Membrane molecule expression								Blasts among PBMC (%)	HLA-class-II-positive blasts (%)
					CD3	CD13	CD14	CD15	CD19	CD20	CD33	CD34		
1	M	21	Normal	ALL	–	–	–	–	+	+	–	+	97	97
2	M	30	Normal	AML-M2	–	–	–	NT	–	–	+	–	95	–
3	F	36	Normal	AML-M5	–	+	+	+	–	–	+	–	97	30
4	M	27	Normal	AML-M2	–	+	–	+	–	–	+	+	95	86
5	F	61	t(2;3), (q37; q21) (q13; q21; q21) der(11q)19q$^+$	AML-M5	–	–	+	+	–	–	+	+	98	94
6	M	47	Normal	AML-M4	–	+	+	NT	–	–	–	–	>99	43
7	M	49	Normal	AML-M4	–	+	–	+	–	–	+	–	96	69

Reagents

The culture medium was RPMI-1640 with HEPES and glutamine (Gibco, UK) to which was added 10% inactivated fetal calf serum (HiClone, USA) and 100 μg/ml gentamicin. Recombinant human cytokines were used at the following concentrations [1, 9–11, 24, 34]: 50 ng/ml interleukin-1β (IL-1β ; R&D Systems Europe, UK), 20 ng/ml IL-2 (R&D Systems), 20 ng/ml IL-3 (R&D Systems), 40 ng/ml IL-4 (R&D Systems), 40 ng/ml IL-6 (R&D Systems), 40 ng/ml IL-7 (R&D Systems), 20 ng/ml IL-10 (kind gift from Schering Plough, USA), 50 ng/ml IL-13 (R&D Systems), 50 ng/ml granulocyte-colony-stimulating factor (G-CSF; Roche, Switzerland), 50 ng/ml granulocyte/macrophage-colony-stimulating factor (GM-CSF; Sandoz, Switzerland). For cytokine blocking the following reagents were used (R&D Systems Europe, UK): 100 ng/ml interleukin-1 receptor antagonist (IL-1RA) [34], anti-IL-2 monoclonal antibody 5 μg/ml (0.03 μg/ml neutralizing 50% of the biological effect of 2 ng/ml IL-2), 25 μg/ml anti-IL-4 polyclonal antiserum (3 μg/ml neutralizing 50% of the biological effect of 4.5 ng/ml IL-4), 25 μg/ml-anti-IL-6 polyclonal antiserum (0.5 μg/ml neutralizing 50% of the biological effect of 7.5 ng/ml IL-6), 5 μg/ml anti-IFNγ monoclonal antibody (0.2–0.6 μg/ml neutralizing 50% of the biological effect of 5 ng/ml IFNγ). For control cultures the corresponding control antibodies were added.

Cell preparation

Peripheral blood mononuclear cells (PBMC)

PBMC were isolated by density-gradient separating (Ficoll-Hypaque, NyCoMed, Norway: specific density 1.077 g/cm^3) and stored in liquid nitrogen. Leukaemic PBMC were prepared from patients with high white blood cell counts (above 30×10^9/l; more than 85% blasts) and contained more than 95% blasts (Table 1).

Enriched CD4$^+$ and CD8$^+$ T cells

Immunomagnetic beads coated with CD4- or CD8-specific monoclonal antibodies (Dynabeads; Dynal, Norway; final concentration 0.5×10^7 beads/ml) were incubated with PBMC (three beads per cell) for 30 min at 4 °C under constant rotation. The beads plus attached cells were removed by a magnetic field, and the beads were then detached and removed after incubation with Detachabeads 15 μl (Dynal, Norway) at 18 °C for 1 h under constant shaking. Light microscopy of isolated cells after incubation with CD4- or CD8-specific beads confirmed the purity of the CD4/CD8 cell populations.

Depletion of CD2$^+$ cells

Depletion was performed using CD2-specific immunomagnetic beads (Dynal, Norway) and a similar separation procedure to that described above for CD4/CD8 enrichment.

Tissue culture

Proliferation assays

Irradiated (50 Gy), leukaemic PBMC 5×10^4/well were cultured in 200 μl medium in U-bottomed microtitre plates together with 5×10^4 normal PBMC responder cells. [^{3}H]thymidine incorporation (Amersham, UK; TRA 310, 37 kBq/well) was assayed after 6 days.

Cytokine secretion

Non-irradiated leukaemic PBMC were cultured in 2 ml medium/well with 2.5×10^5/ml normal allogeneic PBMC. Unless otherwise stated, leukaemic and normal PBMC were cultured at a ratio of 1:1. Supernatants were collected after 6 days.

Analysis of cytokine concentrations

Enzyme-linked immunosorbent assay (ELISA) analysis (R&D Systems Europe, UK) was used to determine concentrations of IL-1β , IL-1RA, IL-4, IL-6, IL-10, IL-12, IL-13, G-CSF, tumour necrosis factor α (TNFα) and IFNγ (CD4/CD8 experiments, cytokine blocking experiments). In the other experiments IFNγ concentrations were determined by another ELISA method (IFNγ screening line; Medgenix, Belgium) performed as recommended by the manufacturer: (i) microtitre wells were coated with 2.5 μg/ml anti-IFNγ capture monoclonal antibody (200 μl/well; 18 h incubation at 4 °C) and aspirated; (ii) wells were incubated with blocking solution (2 h at 18 °C) and washed four times; (iii) 200 μl/well standards (Medgenix IFNγ calibrator; twofold dilution between 250 pg/ml and 3.7 pg/ml) or samples were incubated with 50 μl/well biotinylated anti-cytokine monoclonal antibody (2 h at 18 °C, constant shaking) and thereafter washed four times; (iv) 200 μl/well streptavidin/peroxidase conjugate in phosphate-buffered saline (Boehringer Mannheim; Streptavidin POD Konjugat 5000, final dilution 1:20 000) was added (30 min incubation at 18 °C, continuous shaking) and wells were then washed four times; (v) substrate solution 200 μl/well (Medgenix TMB solution) was added (30 min incubation at 18 °C, continuous shaking) and (vi) stop solution was added and the absorbance determined at 450 nm.

Standards and sample dilutions were prepared in medium. Standard curves, fitting straight lines, were constructed using logarithmic plots of the mean of duplicate determinations, and samples were analysed at a dilution resulting in a measured value within the range of the curve.

Table 2 Interferon γ (IFNγ) concentrations in allostimulated cultures using normal PBMC responder cells and non-irradiated leukemic PBMC as stimulator cells. Allostimulated cultures were prepared using 1×10^6 non-irradiated leukaemic PBMC as stimulator cells and 5×10^5 normal PBMC as responder cells. The cells were cultured in 2 ml culture medium for 6 days before supernatants were harvested and concentrations of IFNγ determined. All concentrations are presented as the mean ± standard deviation of duplicate determinations

	IFNγ concentration (pg/ml)				
Patients/stimulator cells	Healthy responder I A1,9;B12,27;DR2,4; DQ1,3	Healthy responder II A2,11;B40;DR6,8; DQ1,4	Healthy responder III A1,2;B8,15;DR2,4; DQ1,3	Healthy responder IV A2,3;B8,15;DR2,4	Healthy responder V A1,3;B7,8;DR3,4
1. A1,2;B8,15; DR3,4;DQ2,3	426 ± 7.8	1860 ± 32	51 ± 0.7	116 ± 6.4	103 ± 0.7
2. A11,28;B35,60; DR1,4;DQ1,3	7.9 ± 1.1	7.0 ± 0.3	14.9 ± 0.5	9.2 ± 0	1.7 ± 0.1
3. A2;B8,40; DR3,6;DQ1,2	25.5 ± 1.2	23 ± 0	316 ± 6.4	42.5 ± 4.8	405 ± 7.4
4. A11,19;B5,27; DR1,8;DQ,1,4	1360 ± 110	460 ± 27	1680 ± 115	550 ± 76	1560 ± 285
5. A10,19;B18	3.1 ± 0.2	not tested	not tested	3.3 ± 0.5	9.5 ± 0.2
6. A2,30;B57,60; DR2,13;DQ1	1140 ± 191	1090 ± 110	1360 ± 284	1280 ± 346	1305 ± 7.8
7. A2,3;B7,14; DR2,6;DQ1	30.5 ± 1.1	36.5 ± 0.8	56 ± 6.4	27 ± 0.7	194 ± 3.2

The minimal detectable concentrations were IL-1β 0.3 pg/ml, IL-1RA 6.5 pg/m, IL-4 4 pg/ml, IL-6 0.7 pg/ml, IL-10 1.5 pg/ml, IL-12 5.0 pg/ml, IL-13 32 pg/ml, G-CSF 7.2 pg/ml; TNFα 4.4 pg/ml, IFNγ (R&D Systems) 3.0 pg/ml and (Medgenix) 3.7 pg/ml.

Presentation of the data

Proliferation assays were performed in triplicate and median responses used for calculations. Proliferation was regarded as significant when exceeding the negative control by (i) at least 1000 cpm and (ii) at least three standard deviations (SD). The incremental response was defined as the response in stimulated cultures minus the response in corresponding unstimulated controls. For the cytokine studies all concentrations were transformed to logarithmic values before differences were calculated and ranked. The Willcoxon test for paired samples was used for statistical comparisons and the Kendall test for correlation analysis. Differences were regarded as statistically significant when P was less than 0.05 (corrected for the number of comparisons).

Results

Allostimulated cytokine secretion using normal PBMC as responder cells and leukaemic PBMC as stimulator cells

Normal PBMC (5×10^5 cells/well) derived from five healthy individuals were cultured in 2 ml medium together with unirradiated leukaemic PBMC (1×10^6 cells/well) derived from 1 acute lymphocytic leukaemia patient and six AML patients (Table 1). IFNγ concentrations were determined in the supernatants after 6 days (Table 2). Control cultures containing responder or stimulator cells alone showed no detectable IFNγ (less than 3.7 ng/ml). Although IFNγ concentrations showed a wide variation, increased IFNγ levels were detected for all responder/stimulator combinations. IFNγ concentrations were also determined for cultures prepared using responder/stimulator ratios of 1:1 and 10:1 (responder cells 5×10^5/well). IFNγ levels did not differ when responder/stimulator ratios of 1:2 and 1:1 were compared. When a ratio of 10:1 was used IFNγ was detected for 14 out of the 33 combinations tested, but concentrations were decreased compared with cultures with a ratio of 1:1 or 1:2 (data not shown).

Allostimulated proliferation of normal PBMC when leukaemic PBMC stimulator cells were used

Allostimulated PBMC proliferation (responder/stimulator ratio 1:1) was tested for 33 responder/stimulator combinations using 50 Gy irradiated leukaemic PBMC stimulator cells. Allostimulated proliferation and IFNγ secretion showed a significant correlation (n = 33; range of proliferative responses 1.680–29.284 cpm; Kendall's test $P<0.001$). Low but significant proliferation was detected when normal and leukaemic PBMC stimulator cells were used at concentrations as low as 400 cells/well (data not shown).

Allostimulated IFNγ secretion by $CD4^+$ and $CD8^+$ T cell subsets with leukaemic PBMC stimulator cells

Purified $CD4^+$ and $CD8^+$ T lymphocytes were prepared from healthy individuals I–IV and cultured with allogeneic leukaemic PBMC from patients 3, 6 and 7. After 6 days, detectable IFNγ levels were seen both for $CD4^+$ and $CD8^+$ T cells for 11 out of the 12 responder/stimulator combinations investigated, and IFNγ concentrations were significantly correlated for the two subsets when the same responder/stimulator combinations were tested (Kendall's test, $P<0.001$). $CD4^+$ cells (mean concentration 3075 pg/ml) showed significantly higher IFNγ concentrations than the corresponding $CD8^+$ cells (mean 1.509 pg/ml, n = 11; Willcoxon's test for paired samples, $P<0.001$). IFNγ was not detected in control cultures containing $CD4^+/CD8^+$ cells

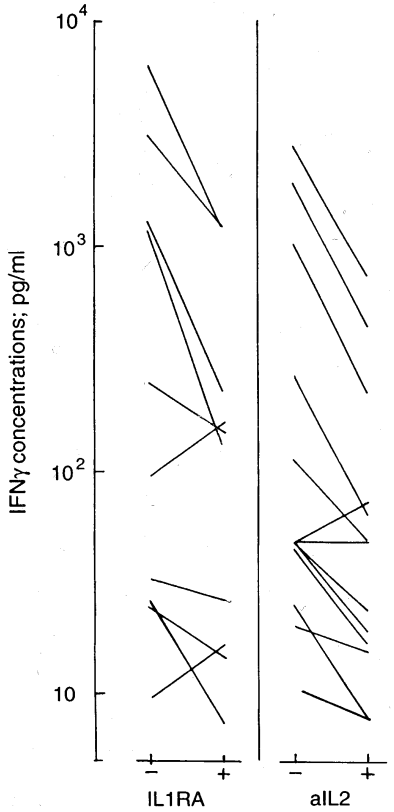

Fig. 1 The effect of cytokine blocking on allostimulated interferon γ (IFNγ) secretion. IFNγ concentrations were analysed after 6 days in supernatants derived from cultures containing different combinations of normal peripheral blood mononuclear cells (PBMC; healthy individuals I–IV) and native leukaemic PBMC (patients 3, 4, 6, 7). Cultures were prepared without (–) and with interleukin-1 receptor antagonist (IL1RA; +) and, when testing anti-IL-2 antibodies (aIL-2), cultures were prepared with either control antiserum (–) or with anti-IL-2 antibodies (+)

alone. Cultures containing $CD4^+/CD8^+$ cells plus phytohaemagglutinin (PHA) and leukaemic PBMC+PHA showed IFNγ concentrations less than 5% of allostimulated levels.

The effect of cytokine blocking on allostimulated IFNγ secretion when unirradiated leukaemic PBMC were used as stimulator cells

Normal PBMC (individuals I–IV) were cultured with leukaemic PBMC (patients 3, 4, 6, 7), cultures being prepared with IL-1RA, anti-IL2, anti-IL4 or anti-IL6 antibodies. IL-1RA significantly decreased IFNγ concentrations determined on day 6 (Fig. 1; $n = 10$, $P = 0.032$) without having an effect on allostimulated proliferation (50 Gy-irradiated leukaemic PBMC stimulator cells; data not shown). Anti-IL2 antibodies (Fig. 1; $n = 13$, $P = 0.004$) also decreased IFNγ levels, whereas anti-IL4 ($n = 13$) and anti-IL6 antibodies ($n = 10$) did not alter IFNγ concentrations significantly (data not shown).

The effect of exogenous cytokines on alloresponsiveness when leukaemic PBMC were used as stimulator cells

IFNγ concentrations were determined when unirradiated leukaemic PBMC (patients 1–3, 5–7) and normal PBMC (individuals IV, V) were cultured at a ratio of 1:1 and cultures prepared with and without exogenous cytokines. Increased IFNγ levels were seen in the presence of exogenous IL-1β (Fig. 2; $n = 12$, $P < 0.005$), IL-2 ($n = 12$, $P < 0.005$) and IL-7 ($n = 12$, $P < 0.02$), whereas decreased levels were seen when IL-6 ($n = 11$, $P < 0.005$), IL-10 ($n = 11$, $P < 0.005$) and G-CSF ($n = 11$, $P = 0.03$) were added to the cultures. IL-β , IL-4, IL-13 and GM-CSF caused no significant alteration of IFNγ release (data not shown).

The effect of the same exogenous cytokines on allostimulated proliferation was tested using responder PBMC from individuals I, IV and V (21 responder/stimulator combinations tested). Only IL-10 caused a significant alteration of allostimulated proliferation ($n = 21$, range of cytokine-free controls 2941–34734 cpm; mean inhibition 37%, Willcoxon's test for paired samples, $P = 0.04$).

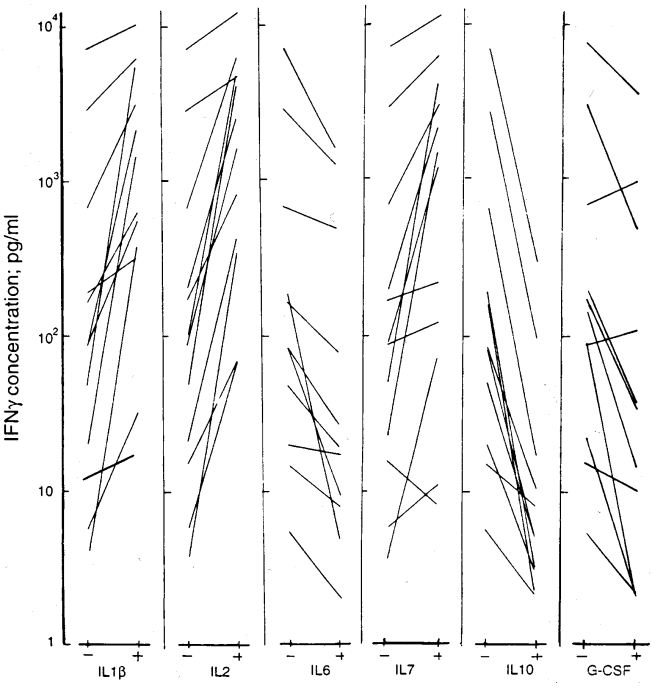

Fig. 2 IFNγ concentrations (median of duplicate determinations) in supernatants derived from cultures containing normal responder PBMC (healthy individuals IV, V) and native allogeneic leukaemic PBMC as stimulator cells (patients 1–3, 5–7). Cultures were prepared either in medium alone (–) or with 50 ng/ml exogenous IL-1β, 20 ng/ml IL-2, 40 ng/ml IL-6, 40 ng/ml IL-7. 20 ng/ml IL-10 or 50 ng/ml granulocyte-colony-stimulating factor (G-CSF; +) and supernatants were collected after 6 days. The figure only includes the results for responder/stimulator combinations giving significant IFNγ secretion

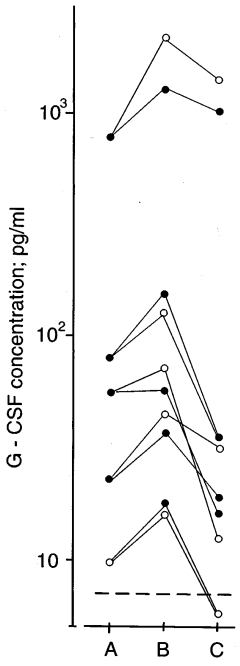

Fig. 3 The figure shows G-CSF concentrations (pg/ml) in culture supernatants derived from cultures containing acute myelogenous leukaemia (AML) blasts alone + control antibody (A), AML blasts + CD4+ responder cells + control antibody (B) and AML blasts + CD4+ responder cells + neutralizing IFNγ antibodies (C). Responder cells were derived from healthy individuals I (○) and V (●) and stimulator cells were leukaemic PBMC from patients 3–7 (––; not detectable)

Effects of allostimulated IFNγ release on AML blast function

Enriched CD4+ T lymphocytes (healthy controls I and V, 0.33×10^6 cells/ml) were cultured together with unirradiated allogeneic AML blasts (0.66×10^6 cells/ml). Cultures were prepared with anti-IFNγ antibodies or corresponding control antibodies. After 5 days of culture, detectable G-CSF levels were seen for five patients when blasts were cultured alone (patients 3–7). The addition of alloreactive CD4+ cells increased G-CSF levels, but further addition of neutralizing IFNγ antibodies decreased G-CSF levels (Fig. 3; Willcoxon's test for paired samples $P = 0.001$). The results were confirmed in repeated experiments, and the effect of anti-IFNγ was similar when tested on day 6. Neither alloreactive cells nor anti-IFNγ induced detectable G-CSF levels for AML blasts showing undetectable levels when cultured alone (data not shown).

Allostimulated cultures with and without anti-IFNγ were prepared using native AML blast stimulator cells (patients 3, 4, 6, 7; 5×10^5 cells/ml) and normal PBMC responder cells (2.5×10^5 cells/ml). [3H]thymidine at 185 kBq/ml was added on day 6; 24 h later adherent cells and CD2+ cells were removed and [3H]thymidine incorporation of the CD2– subset was assayed. IFNγ neutralization decreased proliferation of CD2– cells only for two of the four patients examined, and this heterogeneity between AML patients was confirmed in repeated experiments and when testing responder cells from both healthy individuals I (data not shown) and V (Table 3).

The cytokine environment during alloactivation was characterized by determining IL-4, IL-6, IL-10 and IL-12 levels in culture supernatants. IFNγ concentrations (see Table 2) showed a significant correlation to the supernatant levels of IL-6 ($n = 19$; range of IL-6 levels 12–2050 pg/ml; Kendall's test, $P = 0.045$) and IL-10 (Fig. 4; $n = 33$, $P = 0.006$). IL-4 and IL-12 could not be detected in allostimulated cultures.

Certain AML blasts caused relatively high allostimulated IFNγ release for all responder cells examined (Table 1). To investigate whether these differences are caused by differences in constitutive AML blast cytokine secretion, cytokine concentrations were determined in supernatants for cultures containing native AML blasts alone (AML blasts 1×10^6/ml cultured for 48 h; see Table 4). Allostimulated IFNγ levels showed no clear correlation with constitutive cytokine secretion or IL-1RA release. Only one of the two patients causing high allostimulated IFNγ release (patient 6 but not patient 4) showed high constitutive cytokine secretion, and high constitutive secretion was also seen in a patient (patient 3) who caused only low/intermediate allostimulated IFNγ release.

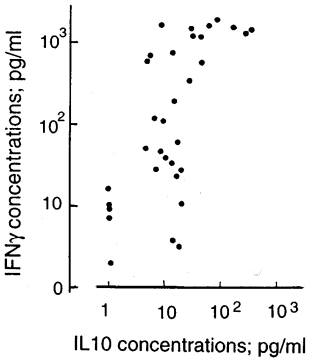

Fig. 4 The correlation between IFNγ concentrations and IL-10 concentrations in cultures containing normal responder PBMC and allogeneic leukaemic PBMC as stimulator cells. Cytokine concentrations are presented as the mean of duplicate determinations

Discussion

T lymphocytes are possible targets for immunotherapy in acute leukaemia, and this approach is based on two major observations: (i) clinical data support a role of graft T cells in the GVHD and GVL effects after allo-BMT, and (ii) the normal T cell repertoir may encompass leukaemia-specific T lymphocytes [2, 4, 6, 12, 17, 19, 20, 22, 32, 33, 35, 43]. In general, T lymphocyte activation depends on (i) antigen-presenting accessory cells that also deliver costimulatory signals and (ii) the cytokine environment [37]. The constitutive cytokine secretion by leukaemia blasts may then modulate T cell activation and T cell effector functions

Table 3 Proliferation of the CD2-negative subset during allostimulation of normal responder PBMC cultured with native allogeneic leukaemic PBMC. Normal responder PBMC (2.5×10^5/ml) were cultured together with native leukaemic stimulator cells (5×10^5/ml [^{3}H]thymidine was added after 6 days and, 24 h later, cells were depleted of adherent cells and CD2$^+$ cells. Incorporation of [^{3}H]thymidine in the CD2$^-$ subset is expressed as the mean ± standard deviation of triplicate cultures. Cultures were prepared with and without anti-IFNγ-neutralizing antibodies. Negative controls and responder PBMC incubated in medium + anti-IFNγ or control antibody corresponded to less than 500 cpm. The results for patients showing decreased proliferation in the presence of anti-IFNγ are in bold type

	[^{3}H]Thymidine incorporation (cpm)		
	Normal responder PBMC + leukaemic stimulator PBMC		
Stimulating leukaemic PBMC	Anti-IFNγ	Control antibody	Leukaemic PBMC +control antibody
3	**15672 ± 385**	**29151 ± 3089**	309 ± 150
4	15712 ± 343	14297 ± 1993	14996 ± 556
6	12002 ± 229	14644 ± 1670	8772 ± 326
7	**8896 ± 203**	**25892 ± 1768**	15518 ± 195

(including IFNγ secretion), as recently described for chronic lymphatic leukaemia [14]. In this context we used an in vitro model to characterize allostimulated proliferation and IFNγ secretion by normal T lymphocytes in the presence of excess acute leukaemia blasts. However, when this model is used, the results must be interpreted with caution: (i) our results may be representative only for the patient subset showing high blast counts in peripheral blood; (ii) the in vitro manipulation may induce AML blast cytokine expression [38]; (iii) in vitro culture may cause a relative increase in the clonogenic AML blast subset [31].

T cell alloreactivity depends on (i) direct recognition of antigens on foreign cells and (ii) indirect recognition of alloantigens presented by self-antigen-presenting cells [18, 25]. Low or undetectable IFNγ levels were seen for cultures containing PHA$^+$CD4$^+$/CD8$^+$ T cells, and these results, together with the microscopy controls, confirm that CD4$^+$/CD8$^+$ cells did not contain any functionally important accessory cell contamination [37, 41]. Thus, when the enriched CD4$^+$ and CD8$^+$ T cells are used, only the direct pathway is operative and both subsets are then capable of direct allorecognition in the presence of leukaemic PBMC. Both the direct and indirect pathways must be regarded as operative when normal PBMC responder cells are used.

Allorecognition of leukaemic PBMC can be caused by recognition of either leukaemia blasts or contaminating allogeneic dendritic cells. However, in our stimulator cell titration experiments a detectable alloresponse was seen even at very low stimulator:responder ratios corresponding to 1 stimulating dentritic cell (more than 95% enriched AML blasts)/2000 responder cells. Thus, stimulation by dendritic cells cannot be excluded but, on the basis of comparisons with previous studies, direct recognition of leukaemia cells seems more likely [41].

Activated T lymphocytes can secrete IL-2, IL-4, IL-10 and IL-13 (for references see [9–11]). The importance of IL2 as a regulatory signal during allorecognition was demonstrated by the decreased IFNγ levels during IL-2 neutralization and the increased levels when exogenous IL-2 was added. It is conflicting whether free IL-10 can be detected in ordinary mixed lymphocyte cultures [3, 42], but our results demonstrate that increased IL-10 levels are seen during allorecognition of leukaemic PBMC. Thus, IL-10 is a part of the T cell cytokine response during allorecognition (Table 4, Fig. 4), and the decreased allostimulated proliferation and IFNγ secretion caused by exogenous IL-10 further indicate its regulatory function during allorecognition. In contrast, a similar regulatory function could not be demonstrated for IL-4 and IL-13.

IL-1 is secreted by many different cells, including T cells and AML blasts [8, 17]. IL-1 neutralization decreased allostimulated IFNγ release, these results demonstrating an enhancing effect of IL-1 in the presence of leukaemia blasts. In contrast, other cytokines that have a growth-factor function for leukaemia blasts similar to that of IL-1 [13, 16, 17, 34, 36], either had no effect (IL-3, GM-CSF) or caused decreased IFNγ levels (G-CSF).

Our results indicate an enhancing effect of IL-7 on IFNγ secretion in response to leukaemic PBMC, and this is similar to the enhancing effect of IL-7 on IFNγ mRNA expression when anti-CD3 antibodies are used for T cell activation [5].

IL-6 is produced by both T lymphocytes and AML blasts [9–11]. IL-6 neutralization had no effect whereas exogenous IL-6 decreased IFNγ secretion. A possible explanation for this discrepancy could be dose-dependent differences in the effects of IL-6.

The wide variation in allostimulated IFNγ secretion cannot be explained by differences in blast expression of HLA-class II molecules or differences in HLA mismatch between various responder/stimulator combinations (Tables 1, 2). However, in normal mixed-lymphocyte cultures, T cell alloresponses are highest when responder/stimulator combinations are tested with a limited rather than a maximal HLA mismatch [42] , and similar mechanisms may also contribute to the variation seen in our experiments (Table 2).

Differences in constitutive AML blast cytokine secretion are a possible explanation for the observation that certain stimulator cells caused relatively high IFNγ release for all responder cells (Table 1). The detailed characterization of constitutive cytokine secretion from the AML stimulators makes this explanation less likely, because (i) high IFNγ levels were seen for patients showing both high (patient 4) and low (patient 6) constitutive cytokine secretion and (ii)

Table 4 Constitutive cytokine secretion by native acute myelogenous leukaemia (AML) blasts. AML blasts (1×10^6/ml) were cultured for 48 h and cytokine concentrations (mean ± SD of duplicates) then determined in the culture supernatants. IFNγ (<3.7 pg/ml), interleukin (IL) 4 (<4.0 pg/ml), IL-10 (<1.5 pg/ml) and IL-12 (<5.0 pg/ml) could not be detected in the supernatants. *IL-1RA* interleukin-1 receptor antagonist, *TNF*α tumour necrosis factor α, *G-CSF* granulocyte-colony-stimulating factor

	Cytokine concentration (pg/ml) in patient:					
Cytokine	2	3	4	5	6	7
IL-1α	2.2 ± 0.9	128 ± 4.6	–	–	59 ± 2.4	–
IL-1β	49 ± 1.5	1.120 ± 32	<3.0	–	380 ± 43	5.0 ± 0
IL-1RA	<5.6	1.480 ± 31	6.100 ± 141	3700 ± 849	7900 ± 580	16500 ± 424
IL-6	5.6 ± 1.8	23;500 ± 240	7.0 ± 2.1	450 ± 23	14500 ± 630	120 ± 13
TNFα	19 ± 4.6	82.5 ± 16..4	<3.5	–	245 ± 33	67 ± 23
G-CSF	<7.2	1919 ± 12	<7.2	<7.2	99 ± 1.1	14 ± 5.4

only intermediate/low IFNγ levels were seen for another patient who showed high constitutive secretion (patient 3). This constitutive secretion by AML blasts cultured alone reflects their secretion during allostimulation because, even in the presence of exogenous cytokines, intrinsic properties of the blast are still the major determinant for their levels of cytokine secretion [8, 9, 11].

The final effect of IFNγ on AML blasts depends on the presence of other cytokines together with IFNγ [29], and the cytokine environment in our allostimulated cultures was therefore characterized in detail. Cytokines detected in the culture supernatants can be derived from either AML blasts or normal PBMC. IFNγ and IL-10 should be regarded as T-cell-derived cytokines (Table 3; for references see also [7, 9–11]. Although IL-6 is derived from both normal PBMC and AML blasts [9–11, 36], the correlation between IL-6 and IFNγ levels indicates that IL-6 release in regulated by the alloresponding T cells. In contrast, G-CSF is not released by T lymphocytes [16]. Thus, when enriched $CD4^+$ responder T cells are used G-CSF should be regarded as being derived from the enriched AML blasts [10, 36]. During allorecognition, increased levels of G-CSF were detected, but these levels could be reduced by IFNγ neutralization.

Proliferation of AML blasts during allorecognition was investigated after depletion of adherent cells and $CD2^+$ cells from the cultures. AML blasts are the major population among $CD2^-$ cells. The blasts are also the major determinant of the proliferative response of $CD2^-$ cells because the contaminating B lymphocytes show minimal proliferation during in vitro allostimulation [28]. In contrast to the effect on G-CSF release, anti-IFNγ decreased blast proliferation only for certain patients. This difference is probably caused by differences between patients in the cytokine requirement for blast proliferation. Similar effects are also seen when the effects of exogenous cytokines (II-4, IL-10, IL-13) on AML blasts are investigated. Exogenous cytokines then decreased cytokine secretion for all patients, whereas effects on proliferation show individual differences [8, 9, 11].

On the basis of our anti-IFNγ studies we conclude: (i) alloactivated T cells can modulate AML blast functions; (ii) IFNγ seems to enhance certain AML blast functions (G-CSF secretion, for some patients also blast proliferation) when acting in the cytokine environment determined by native AML blasts and alloreactive T cells.

Several forms of immunotherapy, such as IL-2 therapy, would be expected to influence IFNγ secretion as well as secretion of other cytokines and cytotoxic cell functions [4, 21, 43, 44]. The dependence of the effects of IFNγ on the cytokine environment must then be taken into account when T cell targeting in future therapeutic approaches is considered.

Acknowledgements The study received financial support from The Norwegian Cancer Society, Blix Medical Fund and Meltzers Medical Fund, University of Bergen, Norway. Serological HLA-typing was performed by The Institute of Transplantation Immunology, The National Hospital, Oslo, Norway. Flow-cytometric analysis was performed at the Broegelmann Research Institute, University of Bergen, Norway.

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