

Cytolytic response of human T cells against allogeneic small cell lung carcinoma treated with interferon gamma

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Summary. Human lymphocytes stimulated *in vitro* by allogeneic small cell lung carcinoma cell lines did not show any significant cytolytic activity against the stimulator tumor cells. However, a high level of lysis was observed when both stimulator and target small cell lung carcinoma cells were pretreated with interferon γ , which increased considerably the expression of major histocompatibility class I molecules by these cells. The demonstration that small cell lung carcinoma cells can be lysed by cytolytic T lymphocytes, suggests that it will be feasible to study the autologous T cell response of patients against this tumor.

Introduction

Many recent reports have described autologous cytolytic T lymphocyte (CTL) responses directed against human tumors [2]; CTL clones that lyse autologous tumor cells and do not lyse either autologous Epstein-Barr virus (EBV)-transformed B lymphocytes or natural killer (NK) target cell K562, have been described for several types of tumors including melanoma, colon carcinoma, and breast carcinoma [7, 8, 16, 20, 21]. Most of these CTL are CD8⁺ and their restriction elements are HLA class I molecules [1, 9, 14, 15, 18, 19, 21].

To our knowledge, no autologous CTL response directed against small cell lung carcinoma (SCLC) has been reported so far. We have recently described a procedure that ensures efficient production of CTL and CTL clones directed against allogeneic and autologous melanoma [8]. We wished to examine whether this procedure would also produce CTL against SCLC. As a first step, we examined whether lymphocytes stimulated by allogeneic SCLC cells could lyse these tumor cells. We report here that lysis was observed on three SCLC lines obtained in our laboratory [11], provided the level of class I major histocompatibility complex (MHC) molecules expressed on the SCLC cells was increased by prior treatment with interferon γ (IFN γ).

Materials and methods

1. *Cells and reagents.* The SCLC lines OC1, OC2, and OC3 were derived in our laboratory from three patients (LB-11,

LB-12, and LB-13), and were grown in RPMI 1640 (GIBCO) supplemented with 10 mM HEPES, L-arginine 116 mg/l, L-asparagine 36 mg/l, L-glutamine 216 mg/l, and 10% fetal bovine serum. These cell lines were identified as SCLC by their production of L-dopadecarboxylase and creatinine kinase brain isoenzyme, and by their binding to three anti-SCLC monoclonal antibodies developed in our laboratory [11, 13]. The B lymphoblastoid line, LG-2-EBV, was obtained by EBV transformation of lymphocytes from a melanoma patient. This cell line was cultured in the same medium as the SCLC lines.

Human recombinant interleukin 2 (IL2) was kindly provided by Dr. Fiers [5], and 1 unit of IL2 was defined as the concentration that gave 50% of the maximum proliferation of the CTLL-2 subclone 15H [3].

Human recombinant IFN γ was supplied by Ernst Boehringer laboratory (Ernst-Boehringer-Institut für Arzneimittelforschung, Bencor-Co Ges mbH Wien, Dr. Boehringer-Gasse 5–11, A-1121 Wien, Österreich). Its specific activity was 2×10^7 reference units/mg of protein.

Monoclonal anti-HLA-ABC (W6/32) and anti-HLA-DR (L243) antibodies were produced by hybridomas purchased from the American Type Culture Collection.

2. *Mixed lymphocyte tumor cell culture.* Peripheral blood mononuclear cells (PBMC) from melanoma patient LB-5 were isolated from heparinized blood by centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden). They were frozen at -80°C in RPMI 1640 supplemented with HEPES, 10% human serum, and 10% dimethyl sulfoxide, and thawed immediately before mixed lymphocyte tumor cell culture (MLTC) was started. These PBMC were used as responder cells in the allogeneic MLTC which was performed as described in detail elsewhere [8]. Briefly, 10^6 responder cells were mixed in 2 ml wells (nunc, Roskilde, Denmark) with 10^5 irradiated (10,000 rads from a $^{137}\text{cesium}$ source) stimulator SCLC cells pretreated or not with IFN γ in 2 ml RPMI 1640 medium supplemented with 10 mM HEPES buffer, 10% human serum, L-arginine (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), and 25 units/ml IL2 was added on day 3. On day 7, responder lymphocytes were collected, washed once, and 3×10^5 responder cells/well were restimulated with 10^5 irradiated SCLC (pretreated or not with IFN γ), in 2 ml of the same medium supplemented with 25 units/ml of IL2. This restimulation procedure was repeated every 7 days.

3. *Cytotoxic assay.* The SCLC cells pretreated or not with IFN γ and K562 cells were used as targets in a conventional chromium (Cr) release assay [8]. The cells were incubated for 45 min at 37°C in RPMI medium containing 10% fetal calf serum (FCS) and 200 μ Ci/ml of Na⁵¹CrO₄, washed, and distributed into 96-well microplates (conical wells: Linbro, Hamden, Conn.) in aliquots of 100 μ l containing 10³ cells. The CTL were added in 100 μ l amounts and assays were run in duplicate. The plates were incubated at 37°C in a 5.5% CO₂ atmosphere. After 4 h the plates were centrifuged and the supernatant collected and counted. The percentage of specific ⁵¹Cr release was calculated as follows: (ER-SR) \times 100/(MR-SR), where ER was the observed experimental ⁵¹Cr release, SR the spontaneous release, and MR the maximum release obtained by adding 100 μ l 0.3% (v/v) Triton X-100 (Sigma, St. Louis, Mo.) to the target cells.

4. *Cytometric analysis of HLA expression induced by IFN γ .* After 3 days of incubation in culture medium containing IFN γ , SCLC cells were washed and labeled with biotinylated anti-class I or anti-class II monoclonal antibodies for 45 min at 4°C in medium containing 137 mM NaCl, 5 mM KCl, 0.4 mM MgSO₄, 0.3 mM MgCl₂, 5 mM glucose, 4 mM NaHCO₃, 1 mM EDTA, penicillin (500 units/ml), streptomycin (1 μ g/ml), and 5% FCS, and buffered with phosphate (1 mM, pH 7.4). The cells were then washed, incubated further with fluoresceinated streptavidin (Amersham International Bucks, England) for 45 min at 4°C, fixed with 0.6% paraformaldehyde, and analyzed on an ATC 3000 flow cytometer (ODAM, Wissembourg, France). Dead cells were excluded on the basis of forward angle light scatter values. For fluorescence intensity measurements the cytometer was calibrated with fluoresceinated microbeads to ensure uniform sensitivity for all experiments. For sorting, PBMC were labeled in the same medium for 45 min at 4°C with fluoresceinated monoclonal antibody (anti-leu-2a, Becton Dickinson, Mountain View, Calif.) and immediately sorted using the ATC 3000 cytometer.

Results

In the course of preliminary experiments performed with human SCLC lines OC1 and OC2, we observed that allogeneic PBMC stimulated with these cells for 3 weekly cycles displayed an insignificant level of lysis on these SCLC lines. The level of lysis observed was not significantly higher than that observed on NK target K562 cells. This was not unexpected since it has been clearly demonstrated by Doyle et al. [6] that fresh tumor samples and culture lines of human SCLC express a very low amount of MHC class I molecules. Since the same authors showed that treatment of SCLC with IFN γ increased the level of surface class I molecules, we examined whether this lymphokine could also increase the lysis of SCLC by allogeneic effectors.

1. Increased MHC class I expression by SCLC lines OC1, OC2, and OC3 after IFN γ treatment

The OC1, OC2, and OC3 lines were incubated for 72 h with IFN γ at doses ranging from 5 to 500 units/ml. The expression of class I MHC molecules was then measured by flow cytometry with monomorphic anti-class I monoclonal

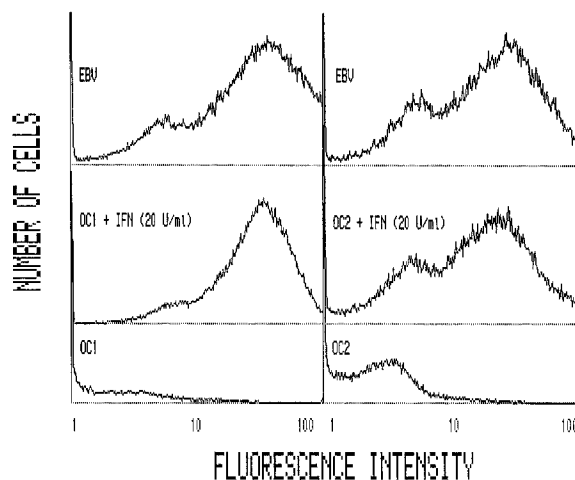


Fig. 1. Flow cytometry analysis of major histocompatibility complex (MHC) class I expression on small cell lung carcinoma (SCLC) lines OC1 and OC2 pretreated or not with 20 units/ml interferon γ (IFN γ) for 72 h. The cells were labeled with biotinylated anti-class I monoclonal antibody W6/32. The lymphoblastoid cell line LG-2-EBV (EBV) was used as a positive control (not treated with IFN γ)

al antibody W6/32. As shown in Figs. 1 and 3, untreated OC1, OC2, and OC3 cells expressed very low amounts of class I molecules. The level of expression was more than 30-fold lower than that observed on EBV-transformed B cells. We confirmed that IFN γ induced a large increase in the expression of class I molecules on SCLC cells. For OC1 and OC2, treatment with 5 units/ml of IFN γ raised the level of surface class I expression up to the level observed on EBV-transformed lymphoblastoid cells. For OC3 the same increase was obtained with 20 units/ml. For OC1, a significant increase in the level of class I expression was observed with doses as low as 0.05 units/ml (Fig. 2). The effect of the IFN γ was quite persistent: 4 days after the end of the treatment, SCLC cells still expressed half the amount of class I molecules that was observed immediately after the treatment. Similar results were obtained by Carrel et al. with melanoma cells [4].

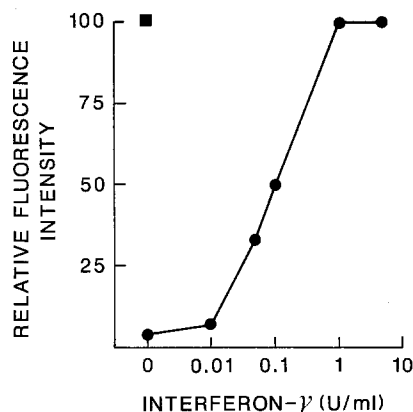


Fig. 2. MHC class I expression on OC1 (●) treated for 72 h with IFN γ at concentrations ranging from 0 to 10 units/ml. The level of class I expression is expressed as the mean fluorescent intensity of the cells, relative to the mean fluorescence observed on lymphoblastoid cells LG-2-EBV (■), which was arbitrarily set to a value of 100

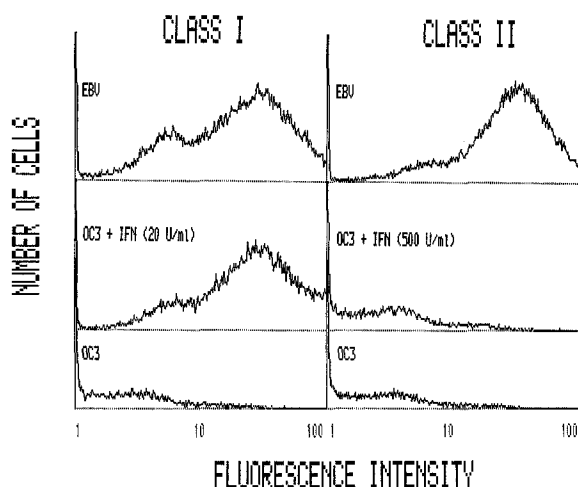


Fig. 3. Flow cytometry analysis of the level of MHC class I and class II expression on OC3 pretreated or not with IFN γ for 72 h (with a saturating dose of 20 units/ml for the expression of class I and of 500 units/ml for the expression of class II). The cells were labeled with biotinylated monoclonal antibody W6/32 (class I) or L243 (class II). The lymphoblastoid cell line LG-2-EBV was used as a positive control

The OC1, OC2, and OC3 lines also expressed very low amounts of class II MHC molecules. In marked contrast with the results observed with melanoma and colon carcinoma [4, 17], we did not observe any increase in the level of class II MHC molecules after IFN γ treatment of our SCLC lines (Fig. 3).

2. Allogeneic cytolytic T cell response directed against SCLC treated with IFN γ

We initiated MLTC using irradiated OC1, OC2, and OC3 cells as stimulators and allogeneic PBMC from a melanoma patient as responder lymphocytes. The stimulator SCLC cells had been previously incubated for 72 h in the presence of 20 units/ml of IFN γ and extensively washed. The responder cells were restimulated at weekly intervals. After 3 to 6 weeks of stimulation, the lytic activity of the

responder cells was assayed against OC1, OC2, and OC3 target cells. As shown in Table 1, very little lysis was observed on the SCLC cells when these target cells had not been treated with IFN γ . By contrast high lytic activity of the effector cells on SCLC target cells treated with IFN γ was observed. This lysis was considerably higher than that observed on K562 cells and showed specificity for the stimulator tumor cells (Table 2). The cytolytic activity observed against SCLC cells appeared to be largely due to CD8 $^+$ T cells. Responder cells obtained from an allogeneic MLTC stimulated with OC1 were found to contain more than 90% CD8 $^+$ cells. Upon sorting, these CD8 $^+$ cells showed strong lytic activity directed against the stimulator SCLC line (Table 3).

The treatment of SCLC cells with IFN γ before MLTC also increased the proliferation of lymphocytes induced by these stimulator cells. The cumulative responder cell proliferation observed after 3 weeks was approximately 10-fold higher when the stimulator SCLC cells had been pretreated with IFN γ (Fig. 4).

Discussion

We have confirmed that SCLC cell lines have very low expression of class I MHC molecules, but that this expression can be increased considerably by treatment with IFN γ . Our results indicate that cytolytic T cells can be obtained upon allogeneic stimulation with IFN γ -treated SCLC cells. The cytolytic T cells specifically lysed the stimulator SCLC cells, but only when these target cells had been previously treated with IFN γ . It is not surprising that most of these cytolytic T cells were CD8 $^+$, since this subset of CTL is usually restricted by MHC class I products [1, 9, 14, 15, 18, 19, 21].

The observation that SCLC cells can be lysed by CTL will be used as a starting point for study of the autologous cytolytic T cell response against SCLC. Considering the MHC restriction of CTL, it is very likely that in order to observe an autologous CTL response directed against a SCLC tumor-associated antigen after MLTC stimulation, we will have to increase the level of class I expression of the tumor cells with IFN γ . Possibly, tumor-associated SCLC antigens that do not elicit any response in vivo

Table 1. Cytolytic activity of lymphocytes stimulated with allogeneic SCLC

Stimulator cells ^a	Weeks	E/T ^b	Percent specific ⁵¹ Cr release from target cells ^c						
			OC1	OC1 + IFN γ	OC2	OC2 + IFN γ	OC3	OC3 + IFN γ	K562
OC1 + IFN γ	6	10/1	9	66					11
		3/1	3	47					6
		1/1	4	27					2
OC2 + IFN γ	4	10/1			0	57			6
		3/1			0	51			3
		1/1			1	18			1
OC3 + IFN γ	3	10/1					0	75	25
		3/1					0	50	11
		1/1					0	25	4

^a Allogeneic PBMC were stimulated in MLTC with irradiated OC1, OC2, OC3 cell lines previously treated with IFN γ (20 units/ml, 72 h). The cytolytic activity of the MLTC cultures was tested on weeks 3–6 as indicated

^b Effector to target ratio

^c Specific release observed after 4 h incubation of the MLTC responder cells on Cr-labeled SCLC targets previously treated or not with IFN γ (20 units/ml, 72 h)

Table 2. Specificity of cytolytic activity of lymphocytes stimulated with allogeneic SCLC cells

Stimulator cells ^a	Weeks	E/T	Percent specific ⁵¹ Cr release from target cells			
			OC1 + IFN γ	OC2 + IFN γ	OC3 + IFN γ	K562
OC1 + IFN γ	5	1/1	91		12	8
		0.3/1	83		3	3
		0.1/1	55		3	2
	3	3/1	92	7		24
		1/1	58	4		5
		0.3/1	25	4		0
OC2 + IFN γ	3	10/1	21	81		25
		3/1	17	66		4
		1/1	6	20		3

^a Allogeneic PBMC were stimulated in MLTC either with OC1 or OC2 treated with IFN γ (20 units/ml, 72 h). The cytolytic activity was tested after 3–5 weeks of MLTC on K562 or SCLC lines pretreated with IFN γ (20 units/ml, 72 h)

Table 3. Cytolytic activity of sorted CD8⁺ lymphocytes

Effector cells ^a	E/T	Percent specific ⁵¹ Cr release from target cells ^b		
		OC1	OC1 + IFN γ	K562
Total	3	17	72	14
	1	19	70	2
	0.3	15	57	4
	0.1	2	46	0
CD8 ⁺	3	10	66	3
	1	5	64	0
	0.3	5	46	0
	0.1	1	26	0

^a Allogeneic PBMC were stimulated with IFN γ -treated OC1. After 4 weeks of MLTC the total responder cells and the sorted CD8⁺ responders were tested for their lytic activity on K562 or OC1 cells treated with IFN γ (20 units/ml, 72 h)

^b 4 h incubation

could be detected in vitro on IFN γ -treated cells. Indeed, results obtained with several mouse tumors have shown that tumor-specific antigens often fail to elicit any rejection response in vivo because of the lack of class I molecules [10, 12]. By raising the level of class I expression, it was possible to elicit immune responses against tumor-associated antigens [22]. For colon carcinoma, Pfizenmaier et al. [17] observed an increase in the autologous antitumor CTL response when the tumor cells were treated with IFN γ . It appears also that the expression of some tumor-associated antigens can be directly increased by IFN γ treatment. Carrel et al. [4] demonstrated that the expression of some melanoma and colon tumor-associated antigens recognized by antibodies increases markedly upon IFN γ treatment. On the basis of these results, it appears worthwhile to attempt a study of the autologous CTL response against SCLC cell lines treated with IFN γ .

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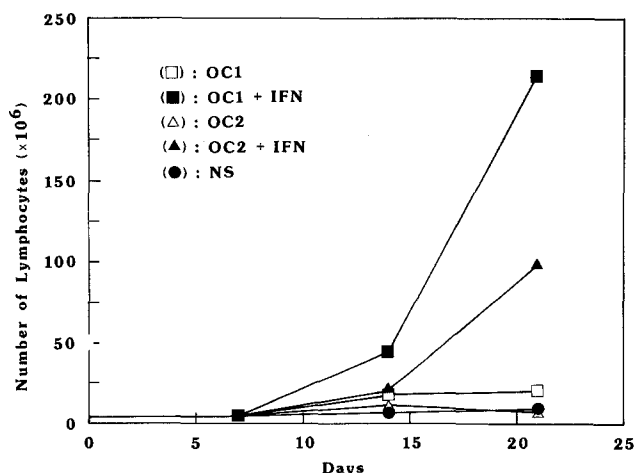


Fig. 4. Cumulative proliferation of allogeneic mixed lymphocyte tumor cell culture (MLTC): peripheral blood mononuclear cells (PBMC) from patient LB-5 were stimulated with OC1 or OC2 pretreated or not with 20 units/ml of IFN γ for 72 h. NS: no stimulator cells added

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