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Cytokine regulation of cell-to-cell interactions in lymphokine-activated killer cell cytotoxicity in vitro

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Abstract. The permanent pancreas carcinoma cell line, PCI-24, was developed in order to analyse cytokine regulation on pancreas carcinoma and lymphokine-activated killer (LAK) cell interaction. PCI cells expressed ICAM-1 and HLA-ABC, but not HLA-DR antigens. PCI cells showed augmented ICAM-1 and HLA-ABC expression when incubated with interferon γ (IFNγ) and tumour necrosis factor α. A similar but weak augmentary effect on the HLA-ABC and ICAM-1 surface expression was seen with interleukin-1β treatment. Natural attachment of LAK to PCI cells was augmented by recombinant IFNγ in close association with ICAM-1 up-regulation on PCI cells. In addition, natural attachment was significantly inhibited by anti-LFA-1 and anti-ICAM-1 antibody treatments. Cytotoxicity of the LAK cells against PCI cells was also significantly inhibited with the same treatment. Thus, the attachment of LAK cells to PCI cells through LFA-1/ICAM-1 molecules appeared to be essential for the cytotoxicity for PCI cells. Pretreatment of PCI cells, but not of LAK cells, with IFNγ or other cytokines resulted in a decrease of susceptibility for LAK cell cytotoxicity. The decreased susceptibility inversely correlated with HLA-ABC expression on the PCI cells. The collective evidence indicates that, although LAK cell attachment to pancreas carcinoma cells through the LFA-1/ICAM-1 molecule is augmented by IFNγ, IFNγ treatment of pancreas carcinoma cells reduces LAK cell cytotoxicity possibly through an increase in HLA-ABC or a regulation of molecules closely associated to HLA-ABC expression.

Key words: Pancreas cancer – LAK – IFNγ

Introduction

Lymphokine-activated killer (LAK) cells have cytotoxic activity against numerous carcinoma and sarcoma cell lines [14, 21, 35]. LAK cells, in combination with interleukin-2 administration, have an in vivo effect as well [20, 32]. The cytotoxic activity of LAK cells appears to be through a direct contact with the target cells [11]. Recent investigations revealed that divergent families of surface molecules, such as adhesion and major histocompatibility complex (MHC) molecules play critical roles on LAK cell/target cell interaction [28]. Thus, regulation of expression or of affinity to ligands is occurring on these surface molecules [6], and cytokines are clearly one of the most important regulatory elements [26]. We report here that attachment of LAK cells to PCI cells through an LFA-1/ICAM-1 interaction is a prerequisite for LAK cell cytotoxicity, and that this attachment is enhanced by interferon γ treatment. However, the overall effect of IFNγ on LAK and PCI interaction is an acquisition of resistence of PCI cells for LAK cytotoxicity, which appears to be inversely correlated with the up-regulated expression of HLA-ABC molecules on the PCI cells.

Materials and methods

Cells. A pancreas carcinoma cell line, PCI-24, was established from a primary carcinoma site of a 67-year-old man with a moderately differentiated adenocarcinoma. In brief, 1-mm cubes of the primary-site tissue were cut with scissors, and gently stirred into a medium supplemented with 1 mg/ml collagenase type I (Wako, Japan) and 1 turbidity-reducing unit/ml hyaluronidase (Amano, Japan) for 2 h at room temperature. The dispersed cells were washed three times in RPMI-1640 medium and cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS). Fresh medium was added daily in the first week, then every 3 days. In 2 months, a continuously growing cell line with a pavement-like arrangement was obtained. These cells were passaged by trypsinization and gentle scraping. The cell line generated subcutaneous tumour nodules with a moderately differentiated tubular adenocarcinoma histology, when innoculated into BALB/c *nu/nu* mice. A colon carcinoma cell line, DLD-1, was purchased from the American Type Culture Collection, Maryland. LAK cells were induced from peripheral blood lymphocytes of healthy volunteers by incubation with 1000 U/ml recombinant IL-2 for 7–8 days. The resultant blastic cells were used as LAK cells.

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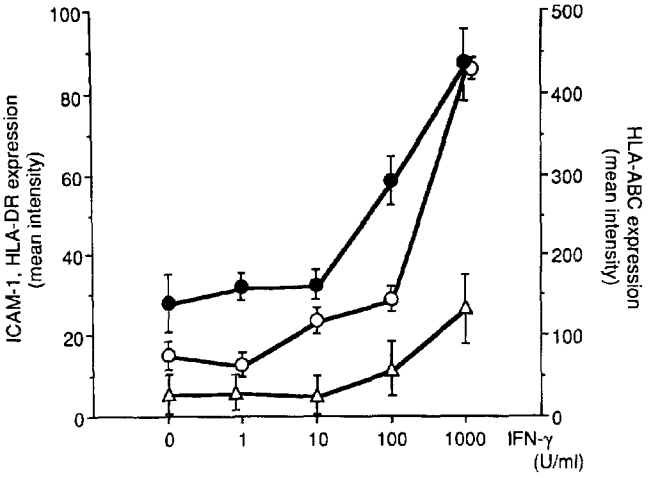

Fig. 1. Surface antigen expression on PCI cells. PCI-24 cells were incubated with interferon γ (*IFNγ*) for 4 days, at several concentrations, and HLA-ABC, DR, and ICAM-1 antigens were detected by flow cytometry. ○——○, HLA-ABC; ●——●, ICAM-1; △——△, HLA-DR

Flow-cytometric detection of surface molecules. Surface MHC, HLA-ABC, DR, and DQ, and ICAM-1 molecules on PCI cells were detected by indirect-flow cytometry, using a FACScan (Becton-Dickinson, Calif.). Monoclonal antibodies, W6/32, IgG2a, [1], HOK7, rat IgG2b, [10], SK10, IgG1, and HA58, IgG1, [30], were used as the primary antibodies for HLA-ABC, DR, DQ, and ICAM-1 respectively. Anti-LFA-1 antibody, 25.3.1 (IgG1), was purchased from Funakoshi, Japan. The HA58 antibody was a generous gift from Dr. K. Imai, Sapporo Medical College. The fluorescein-isothiocyanate-labelled goat anti-mouse immunoglobulin was used as the secondary antibody. Similarly, surface expression of the T cell receptor, CD3, DC56, and DC57 on the LAK cells was detected by flow cytometry. These antibodies were purchased from Becton Dickinson, Calif.

Natural attachment and LAK cell cytotoxicity assay. Natural attachment between LAK and PCI cells was measured by the method described by Dustin et al. [4]. In brief, 0.1 ml 1×10^7/ml LAK cells was labelled with 0.1 mCi ^{51}Cr for 2 h at 37° C. Samples containing 3×10^4 cells/well PCI cells were cultivated in a 24-well microtiter plate in an attached, monolayered form at the bottom; 2×10^5 LAK cells were then added to the PCI-24 cells in a well and incubated for periods of 15–240 min. Following the incubation, wells were extensively washed four times in phosphate-buffered saline (PBS). The attached LAK cells were all lysed by addition of NaOH (0.1M). The ^{51}Cr-containing medium was collected and the radioactivity was measured by gamma counter.

LAK cell cytotoxicity against PCI cells was measured by the 4-h ^{51}Cr-release assay. In brief, 0.1 ml 1×10^7/ml PCI cells was incubated with 0.1 mCi ^{51}Cr for 1 h at 37° C and, after a wash in PBS, 1×10^4 cells/well PCI cells and $(0.5-100) \times 10^4$ cells/well LAK cells were co-cultivated in a round-bottom 98-well microtiter plate for 4 h. Cetrimide was used to lyse all the target cells (maximal release). Supernatants from various effector : target (E : T) ratios were collected, and radioactivity in each well was measured with gamma counter. The percentage cytotoxicity was calculated as follows:

$$\text{cytotoxicity (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Blocking experiments were performed using W6/32, HA58, and 25.3.1 monoclonal antibody. Samples of 20 μg/ml of each antibody were incubated with 1×10^6 PCI cells/ml for 30 min at room temperature, and antibody-treated cells were extensively washed in RPMI-1640 medium supplemented with 10% fetal bovine serum. As controls, 20 μg/ml either SK10 (anti-DQ, IgG1) or mouse myeloma proteins (IgG1 and IgG2a, Cappel, N.C.) were used. The cells were then used as effector or target cells in either natural attachment or LAK cell cytotoxicity assays. In other experiments, 20 μg/ml antibodies was added to the medium where natural attachment or LAK cell cytotoxicity was determined.

The statistical analysis of the interpretation of the above assays was by Student's *t*-test.

Cytokine and anti-IFNγ antibody. Recombinant human cytokines, IFNγ, tumour necrosis factor α (TNFα), and IL-1β, were purchased from Genzyme, Cambridge, Mass. PCI cells were preincubated with cytokines, and the natural attachment and LAK cell cytotoxicity assays were performed. Anti-IFNγ for neutralizing IFNγ was purchased from Genzyme.

Results

MHC and ICAM-1 expression and regulation on PCI cells by cytokines

PCI-24 cells expressed HLA-ABC and ICAM-1, but not HLA-DR antigens. PCI cells expressed HLA-DR antigens when incubated with 100 U/ml or more IFNγ for 4 days. Similarly, HLA-ABC expression was augmented by the addition of 10 U/ml or more IFNγ for 4 days (Fig. 1). ICAM-1 expression augmentation was seen at 100 U/ml or more IFNγ pretreatment (Fig. 1). HLA-DQ antigen was not expressed even after incubation with IFNγ. Surface HLA-ABC expression on PCI-24 cells was much higher than that of ICAM-1 (Fig. 1). HLA-ABC and DR expression on PCI cells was gradually increased by IFNγ treatment for up to 7 days. ICAM-1 up-regulation was seen from day 1, and remained almost unchanged up to day 7. TNFα, 500 U/ml, and IL-1β, 100 U/ml, augmented ICAM-1 expression on the PCI cells (Fig. 5b). PCI cells did not express LFA-1 antigen.

Natural attachment between PCI and LAK cells

LAK cells used in this study expressed CD56 (20.9%), CD57 (25.3%), as well as CD3 (69.0%) and T cell receptor, therefore these cells were a mixture of natural killer and T lymphocytes. They intensely expressed HLA-ABC (97.9%, mean intensity, 196.8) and, weakly, ICAM-1 (26.0%, mean intensity, 18.9). LAK cells also expressed LFA-1 antigen (54.7%, mean intensity, 40.0).

Natural attachment between PCI and LAK cells occurred after 15 min, reached a maximum at 60 min then gradually decreased. The attachment of 2×10^5 LAK cells to PCI-24 cells depended on the number of PCI cells as long as the number did not exceed 6.4×10^4. When the number of PCI cells exceeded 6.4×10^4, natural attachment between these cells appeared to be saturated and there was no further increase.

Effect of IFNγ on natural attachment of LAK cells to PCI cells

Natural attachment between PCI-24 and LAK cells was augmented by preincubation of PCI cells with IFNγ for 4 days. A significant augmentation of attachment occurred

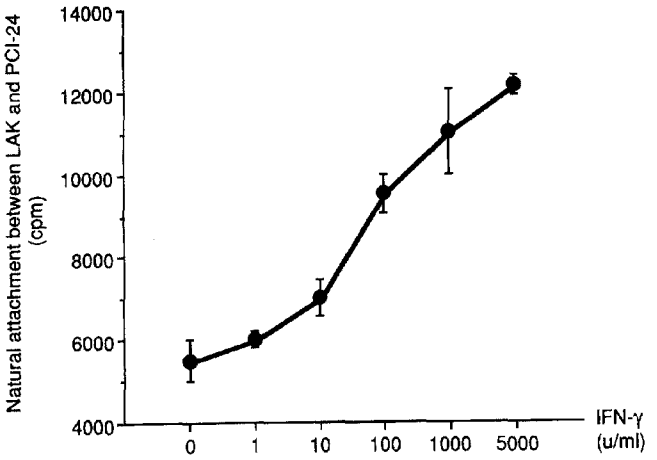

Fig. 2. Dose-dependent augmentation of natural attachment by IFNγ pretreatment of PCI cells for 4 days

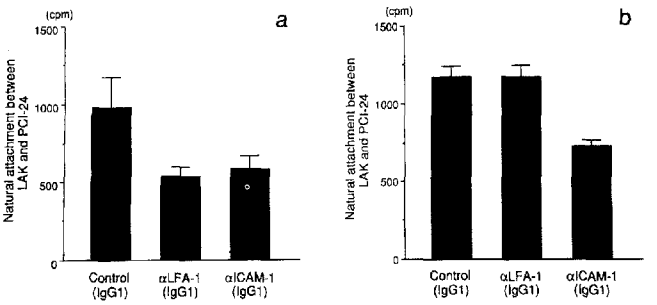

Fig. 3 a, b. Inhibitory effect of monoclonal antibodies (IgG1) on natural attachment between lymphokine-activated killer (*LAK*) and PCI cells. Anti-LFA-1, ICAM-1, and HLA-DQ (control) monoclonals (20 μg/ml) were added to either a mixture of LAK/PCI (**a**) or PCI (**b**) cells

at 100 U/ml or more IFNγ (P <0.01) (Fig. 2). Kinetic studies revealed that the augmentation of attachment reached a peak on the first day of 100 U/ml IFNγ incubation, and remained almost unchanged.

Blocking effect of surface molecules by monoclonal antibodies on attachment and cytotoxicity of LAK cells to PCI cells

Mixtures of LAK and PCI-24 cells were incubated with monoclonal antibodies against HLA-DQ, ICAM-1, and LFA-1 for 30 min at room temperature, and natural attachment was determined. Anti-DQ was used as an isotype-matched control antibody. Anti-LFA-1 and ICAM-1 treatment of LAK cells led to a significant reduction of attachment as compared with control antibody (P <0.05) (Fig. 3a). Similar antibody treatments were then performed on target PCI cells, but not on LAK cells. Anti-ICAM-1 treatment of PCI cells yielded a significant decrease of attachment (P <0.01), but anti-LFA-1 treatment of PCI cells did not block attachment (Fig. 3b).

Effects of anti-HLA-ABC, ICAM-1, and LFA-1 monoclonals on LAK cell cytotoxicity against PCI-24 cells were also examined. LAK cell cytotoxicity for 1×10^4 PCI cells

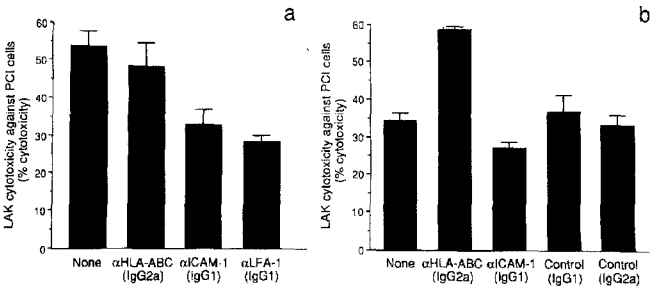

Fig. 4 a, b. Inhibitory effect of monoclonal antibodies on LAK cell cytotoxicity for PCI cells. Anti-HLA-ABC, ICAM-1, LFA-1, or control mouse myeloma proteins (IgG1 and G2a) were added to either a mixture of LAK/PCI (**a**) or PCI (**b**) cells

depended on the target PCI cells at an enzyme:target (E:T) ratio of 25 or less, and was not augmented at an E:T ratio of 50 or more. First, mixtures of LAK and PCI cells were incubated with monoclonal antibodies, and LAK cell cytotoxicity against PCI cells was determined at E:T = 25. Anti-LFA-1, and anti-ICAM-1 treatment of the LAK/PCI cell mixture diminished the cytotoxicity of LAK cells against PCI cells (P <0.05) (Fig. 4a). Similar antibody treatments were next performed on PCI, but not on LAK cells, using control myeloma proteins, IgG1, and IgG2a, and LAK cell cytotoxicity was determined at E:T = 25. Anti-HLA-ABC treatment of PCI cells significantly augmented the cytotoxicity of LAK cells against PCI (P <0.01) (Fig. 4b). A significant attenuation of cytotoxicity was observed by anti-ICAM-1 treatment of PCI cells (P <0.05) (Fig. 4b).

Effect of cytokines on cytotoxicity of LAK cells to PCI cells

PCI-24 cells were preincubated with either 100 U/ml IFNγ, 500 U/ml TNFα, or 100 U/ml IL-1β for 4 days, and LAK cell cytotoxicity for cytokine-treated PCI cells was examined. As shown in Fig. 5a, treatments with IFNγ and TNFα decreased the susceptibility to LAK cell cytotoxicity (P <0.01), in that order. IL-1β treatment yielded a marginal decrease in LAK cell susceptibility. Simultaneous flow-cytometry assay for surface molecules revealed that the increase of HLA-ABC but not ICAM-1 or HLA-DR expression on PCI cells correlated with this decrease (Fig. 5b).

Dose dependence of IFNγ modulation of LAK cytotoxicity

Effects of preincubation of PCI-24 cells with 1, 10, 100 and 1000 U/ml IFNγ on LAK cell cytotoxicity were examined at several E:T ratios. A decrease in the susceptibility for LAK cytotoxicity was evident with 10 U/ml or over of IFNγ (Fig. 6). Since significant augmentation of HLA-ABC surface expression was evident with 10 U/ml or more of IFNγ (Fig. 1), the increased HLA-ABC expression on PCI cells was also shown here to correlate with a decrease in susceptibility for LAK cytotoxicity. In contrast, the sur-

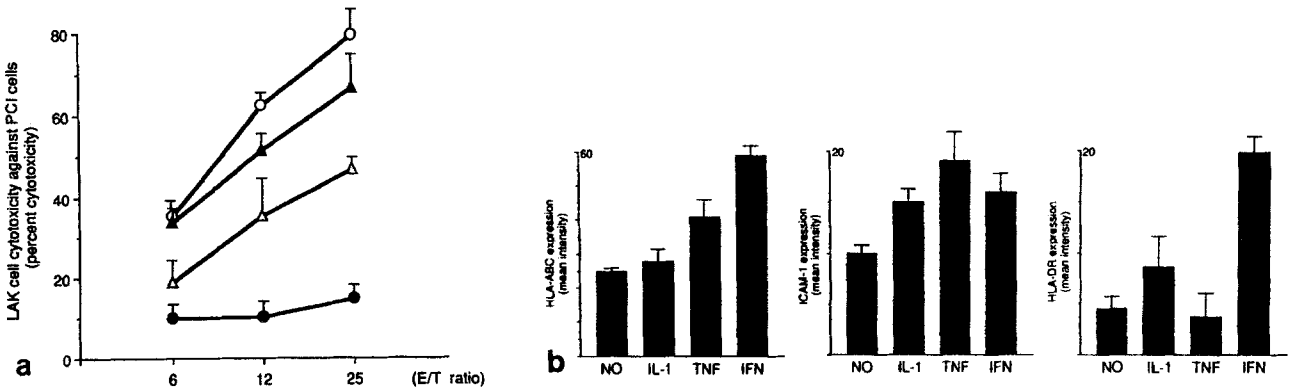

Fig. 5 a, b. Cytokine regulation of LAK cytotoxicity for PCI cells. **a** Interleukin-1β (IL-1β; 100 U/ml), tumour necrosis factor α (TNFα; 500 U/ml), and interferon γ (IFNγ; 100 U/ml) treatments of PCI cells led to a resistance for LAK cell cytotoxicity. O——O, No cytokine added; ▲——▲, IL-1β, △——△, TNFα; ●——●, IFNγ. **b** Change of susceptibility for LAK cell cytotoxicity correlated with HLA-ABC, but not with HLA-DR or ICAM-1 expression on PCI cells

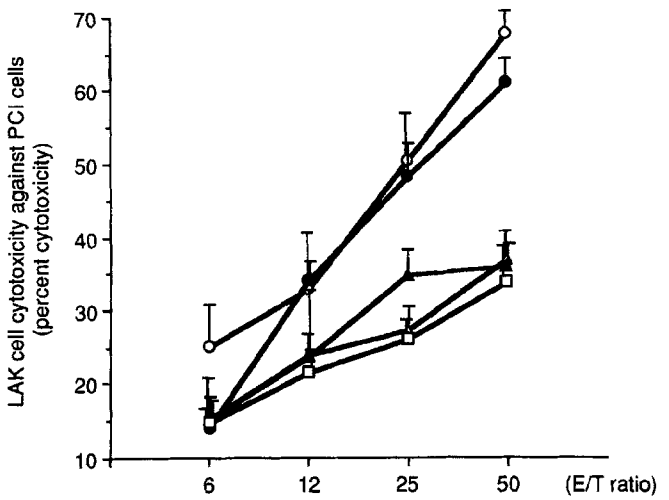

Fig. 6. Dose-dependent action of IFNγ on regulation of LAK cell cytotoxicity for PCI cells. Concentrations higher than 10 U/ml IFNγ yielded a significantly protective effect on LAK cell cytotoxicity at almost all E:T ratios examined. O——O, No IFNγ; ●——●, 1 U/ml IFNγ; △——△, 10 U/ml IFNγ; ▲——▲, 100 U/ml IFNγ; □——□, 1000 U/ml IFNγ

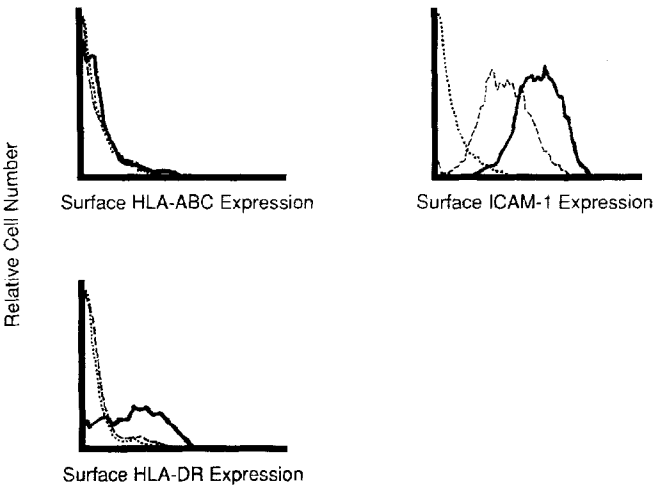

Fig. 7. Surface HLA-ABC, ICAM-1, and HLA-DR expression on DLD-1 cells. · · ·, background fluorescence without using the primary antibody; -----, surface HLA-ABC, ICAM-1, and HLA-DR expression without IFNγ preincubation; ———, surface HLA-ABC, ICAM-1, and HLA-DR expression with IFNγ (100 U/ml, 4 days)

face expression of ICAM-1 and HLA-DR was not augmented at 10 U/ml IFNγ; ICAM-1 and HLA-DR expression was augmented at 100 U/ml or more IFNγ (Fig. 1).

Effect of cytokines on LAK cell cytotoxicity for an HLA-ABC-negative cell line

To determine whether IFNγ exerts its effect through surface HLA-ABC antigen expression on PCI cells, an HLA-ABC-negative, and -non-inducible colon adenocarcinoma cell line, DLD-1, was used; indirect flow-cytometry failed to detect HLA-ABC antigen or its induction by IFNγ on the cell surface of DLD-1. ICAM-1 was positive and inducible by IFNγ. HLA-DR was negative, but inducible by IFNγ (Fig. 7). As shown in Fig. 8, treatment of DLD-1 cells with 100 U/ml IFNγ for 4 days did not alter the susceptibility for LAK cell cytotoxicity at any E:T ratio examined, thereby

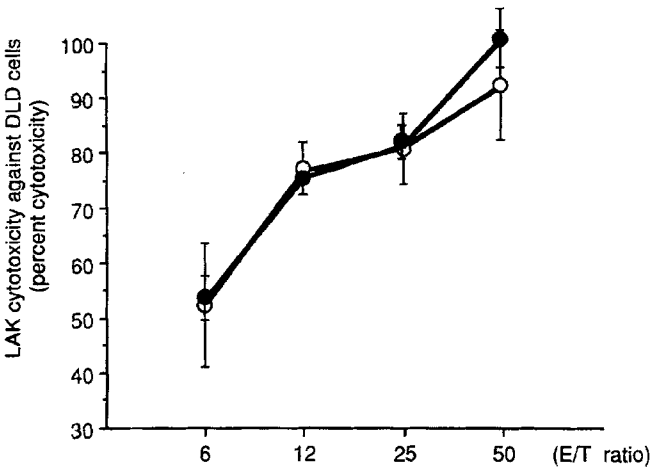

Fig. 8. IFNγ treatment of DLD-1, a colon adenocarcinoma cell line, did not alter susceptibility of the adenocarcinoma cells for LAK cell cytolysis. O——O, No IFNγ; ●——●, 100 U/ml IFNγ

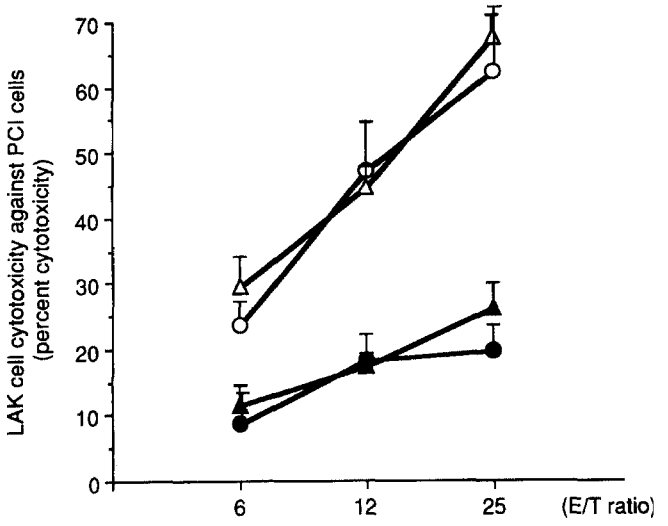

Fig. 9. IFNγ (100 U/ml) treatment on LAK cells did not alter their cytotoxic activity against PCI cells. ○——○, No IFN treatment; ●——●, only PCI cells were treated with IFNγ; △——△, only LAK cells were treated with IFNγ; ▲——▲, both PCI and LAK cells were treated with IFNγ

providing further evidence for the correlation between HLA-ABC expression and LAK cell cytotoxicity.

IFNγ treatment of LAK cells

In previous experiments, IFNγ treatment was exclusively performed using PCI cells. In this experiment, LAK cells were preincubated with 100 U/ml IFNγ for 4 days and the subsequent alteration of cytotoxicity was examined. IFNγ treatment of LAK cells did not modulate the cytotoxic activity of these cells on the PCI cells (Fig. 9) and did not alter surface HLA-ABC, DR, ICAM-1, or LFA-1 on the LAK cells (data not shown). A significant decrease in susceptibility was reproduced only when the target PCI cells were treated with IFNγ.

Discussion

The established pancreas carcinoma cell line, PCI-24, expressed HLA and ICAM-1 antigens on the surface, and surface expression of these molecules was up-regulated by recombinant IFNγ. Regulation of ICAM-1 antigen by cytokines has been investigated using normal and neoplastic cell lines [5, 8, 16, 31]. Regulation of this adhesion molecule has also been investigated in a variety of tissue sections [9, 27, 29, 33]. Most of the available data show that ICAM-1 up-regulation occurs in the presence of exogenous cytokines, especially IFNγ. The up-regulation of ICAM-1 expression on the PCI cells, as shown in this study, clearly revealed that these pancreas carcinoma cells are also ICAM-1-inducible. This implies that IFN treatment may alter adhesiveness or cytotoxic activity by LFA-1-positive effector cells such as LAK cells.

We obtained evidence for the attachment between PCI and LAK cells, which phenomenon was originally noted in phytohaemaggultinin blasts and fibroblasts [4]. Since this natural attachment was augmented by preincubation of PCI cells with IFNγ, and was inhibited by anti-ICAM-1 antibody treatment of PCI cells, ICAM-1 molecules on the PCI surface appear to play a critical role in attachment [23]. Anti-LFA-1 treatment of the LAK-PCI cell mixture also significantly diminished attachment, clearly demonstrating the critical importance of LFA-1 molecules on LAK cells: although another ligand for LFA-1 antigen, ICAM-2 [24], may participate in the natural attachment between LAK and PCI cells, the surface expression of ICAM-2 molecules in PCI cells has not been determined. Surface ICAM-2, however, does not appear to participate in the cytokine regulation of PCI/LAK attachment and cytotoxicity, since ICAM-2 expression is not regulated by cytokines [24].

Both anti-LFA-1 and anti-ICAM-1 antibody treatment also significantly inhibited cytotoxicity of LAK cells against PCI cells. Therefore, attachment of PCI and LAK cells mediated by ICAM-1/LFA-1 molecules has a critical importance also in LAK cell cytotoxicity.

Anti HLA-ABC treatment of PCI cells yielded augmented cytotoxicity. The antibody treatment may invoke antibody-dependent cellular cytotoxicity (ADCC) [22]. Indeed, one of the major cytotoxic functions for antibody-coated target cells exerted by LAK cells has been attributed to ADCC [17, 34]. Surface expression of HLA-ABC antigens on PCI cells is much higher than that of ICAM-1, which may explain the appearance of an ADCC mechanism in anti-HLA-ABC treatment.

Preincubation of PCI cells, but not of LAK cells, with IFNγ did not augment LAK cell cytotoxicity against PCI cells, irrespective of ICAM-1 up-regulation and the augmentation of natural attachment. On the contrary, PCI cells obtained a resistance to LAK cytotoxicity. Previous studies have also shown that IFNγ treatment of a neoplastic cell line does not necessarily increase susceptibility of the cell line for LAK cell cytotoxicity [3, 15]; several malignant cell lines became resistant to LAK cell cytotoxicity [3, 15], whereas a neuroblastoma cell line became more susceptible to LAK cell killing when target neoplastic cells were treated with IFNγ [18]. Augmentation of surface HLA-ABC antigen expression or of the expression of antigen(s) closely associated with HLA-ABC may be attributable to the relative resistance of PCI-24 cells for LAK cell killing, since natural killer cytotoxicity was seen to be inhibited by an increase in surface HLA-ABC antigens on the surface of target cells [13, 19, 25], although this notion evokes controversy [2, 7]. Our study also suggests a correlation of surface HLA-ABC antigen expression on target PCI cells with a decreased susceptibility of these target cells for LAK cytotoxicity. This idea was further supported by the lack of alteration of LAK cell susceptibility in DLD-1, and HLA-ABC negative human colon adenocarcinoma cell line; DLD-1 is ICAM-1-positive, and a cell line where HLA-DR is inducible by IFNγ, but HLA-ABC antigen is neither expressed nor induced by IFNγ on these cells. Although the surface expression of ICAM-1 molecules on DLD-1 cells was augmented by IFNγ, the cytotoxicity induced by LAK cells did not increase. The DLD-1 may have already expressed ICAM-1 molecules (without IFNγ) sufficient to saturate LAK cell LFA-1, or

DLD-1 may express excessive amounts of adhesion molecules other than the ICAM-1/LFA-1 adhesion set, such as LFA-3/CD2 and extracellular matrix proteins/VLA, so that an increase in ICAM-1 expression on this cell line may not alter its adhesion status.

A slight cytostatic effect of 100 U/ml IFNγ on PCI-24 cells has been observed by a growth curve (data not shown). Since rapidly growing cells are killed to a greater degree by LAK cells, the cytostatic effect of the cytokine might be one of the reasons for the decreased susceptibility of IFNγ-treated PCI cells.

Cytokine production by LAK cells was noted by Lewis et al. [12]. IFNγ produced by LAK cells may give PCI cells some resistance to LAK cell cytotoxicity. When anti-IFNγ antibody was added to medium in the standard ^{51}Cr-release assay, we found no significant effect of the antibody treatment (data not shown). Thus it is unlikely that target PCI cells are rendered resistant to LAK cell cytotoxicity by IFNγ produced and secreted by LAK cells, in the 4-h ^{51}Cr-release assay.

In summary, we noted that attachment of LAK cells mediated by surface LFA-1 molecules has critical importance for LAK cell cytotoxicity against PCI cells. Although IFNγ treatment augmented surface ICAM-1 molecules on PCI cells, thus increasing the attachment between LAK and PCI cells, the efficiency of cytotoxicity of LAK cells declined with IFNγ treatment. The acquisition of resistence to LAK cell cytotoxicity appears to correlate with the up-regulated surface HLA-ABC expression on PCI cells. Modulation of surface HLA-ABC (or unknown molecules closely associated with HLA-ABC expression) and ICAM-1 antigens on PCI cells is presumably one of the most critical factors in cytokine regulation of LAK cell pancreas carcinoma interactions.

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