

Induction of Intercellular Adhesion Molecule 1 on Small Cell Lung Carcinoma Cell Lines by γ -Interferon Enhances Spontaneous and Bispecific Anti-CD3 \times Antitumor Antibody-directed Lymphokine-activated Killer Cell Cytotoxicity

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ABSTRACT

The interaction between LFA-1 and its natural ligand, ICAM-1, plays an important role in leukocyte adhesion and signal transduction. LFA-1-mediated T-cell adhesion is generally activated by CD3-mediated signal in association with T-cell receptor-mediated recognition of the antigen/major histocompatibility complex on antigen-presenting cells. In the present study, we compared spontaneous or bispecific antibody (BsAb)-directed LAK cell cytotoxicity against ICAM-1⁺ or ICAM-1⁻ small cell lung cancer (SCLC) cell lines. γ -Interferon (IFN- γ)-induced ICAM-1 expression on ICAM-1⁻ SCLC cell lines, and susceptibility to LAK cells was increased simultaneously. Increased cytotoxicity of the IFN- γ -treated SCLC was inhibited by an anti-ICAM-1 monoclonal antibody (mAb). Furthermore, LAK cell cytotoxicity directed by BsAb, which was composed of OKT3 and anti-SCLC mAb, was also increased by the IFN- γ treatment of SCLC, and this increase was inhibited by an anti-ICAM-1 mAb but not by anti-Class I or anti-CD2 mAb. These results suggest that a prior administration of IFN- γ would enhance the efficacy of the following specific targeting therapy utilizing BsAb and LAK cells by up-regulating the ICAM-1 expression on tumor target cells. The combinational use of IFN- γ and anti-CD3 \times anti-tumor BsAb might be a promising way of enhancing LAK cell-mediated adoptive immunotherapy in small cell lung cancer patients.

INTRODUCTION

The interaction between LFA-1 on killer cells and ICAM-1 on target cells plays an important role in various killer cell-mediated cytotoxicity. Anti-LFA-1 and anti-ICAM-1 mAbs² blocked CTL, NK, or LAK-mediated target cell lysis (1). LFA-1 not only acts as an adhesion molecule facilitating the TCR recognition of antigen/MHC on target cells but also delivers a costimulatory signal augmenting the TCR/CD3-mediated T-cell activation (2). In reverse, TCR/CD3-mediated activation signal up-regulates the LFA-1-mediated adhesion to ICAM-1 (2).

LAK cells exhibit MHC-unrestricted cytotoxicity (3). LAK cells induced from peripheral blood lymphocytes by culturing with high doses of IL-2 are mainly composed of NK cells until 1 week of the culture, but T-cells become dominant over 2 weeks (T-LAK cells). NK cells do not express TCR/CD3 and even the T-LAK cells expressing TCR/CD3 do not appear to use TCR in LAK cell recognition of target cells (1), and the

mechanism for MHC-unrestricted reactivity of LAK cells still remains unclear. Susceptibility to LAK cell-mediated cytotoxicity is valuable among various tumor cells. This is a major problem of adoptive immunotherapy with the use of LAK cells in treating various cancer patients, and is suspected to result from the differences in efficacy of recognition.

In order to bypass such an obscure step in LAK cell-mediated cytotoxicity and recruit all potentially cytolytic T-cells irrespective of their antigenic specificity, we established a STT by using BsAb composed of chemically conjugated anti-CD3 and anti-TAA mAbs (4). Such a BsAb greatly augmented the LAK cell cytotoxicity against various target cells expressing the TAA *in vitro* (4-6) and the clinical efficacy of LAK therapy in treatment of glioma patients (7). This augmentation of cytotoxicity is expected to result from the direct linkage of cytolytic T-cells and target cells by BsAb itself and triggering through CD3. Moreover, the up-regulation of LFA-1-mediated adhesion by CD3-mediated signal appears to be also involved, since anti-LFA-1 mAb inhibited the BsAb-directed LAK cell cytotoxicity.³

However, expression of the LFA-1 ligand, ICAM-1, is rather restricted in normal tissues to endothelium, germinal center, and macrophages (8); some tumor tissues also expressed ICAM-1 (9). If the target tumor tissue would not express ICAM-1, even the STT using BsAb would be less efficient.

The ICAM-1 expression could be induced in various tissues and cells by inflammatory cytokines such as IFN- γ , IL-1, and TNF (8, 10-14). These cytokines have been reported to induce the ICAM-1 expression in several tumor cell lines *in vitro* (10, 15).

In the present study, we examined the difference in susceptibility to the BsAb-directed cytotoxicity between ICAM-1⁺ and ICAM-1⁻ SCLC cell lines and whether IFN- γ induction of ICAM-1 expression results in a higher susceptibility to BsAb-directed cytotoxicity. We finally discuss the possible clinical application of IFN- γ pretreatment for enhancing the STT efficacy by up-regulating ICAM-1 expression in the tumor.

MATERIALS AND METHODS

Cell Lines. Human SCLC cell lines H69 and Lu135 (obtained from National Cancer Center in Japan) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 4 mM L-glutamine, 100 units/ml penicillin (Banyu, Tokyo, Japan), 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, and 25 mM 2-mercaptoethanol (Sigma) (complete medium).

Preparation of Effector Cells. Human peripheral blood mononuclear cells were isolated from buffy coats obtained from normal healthy donors by centrifugation at 1500 rpm for 30 min on Ficoll-Hypaque

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² The abbreviations used are: mAb, monoclonal antibody; NK, natural killer; TCR, T-cell receptor; MHC, major histocompatibility complex; IL-1, IL-2, interleukin 1 and 2; STT, specific targeting therapy; BsAb, bispecific antibody; TAA, tumor-associated antigen; IFN- γ , γ interferon; SCLC, small cell lung cancer.

³ Unpublished data.

(Pharmacia, Uppsala, Sweden) (16). Peripheral blood mononuclear cells were depleted of CD16⁺ NK cells by treatment with an anti-CD16 mAb (Leu-11b; IgM, Becton-Dickinson, Mountain View, CA) and rabbit complement (Low tox-H; Cedarlane, Hornby, Ontario, Canada). The resulting mononuclear cells contained CD3⁺ T-cells to more than 90%. This T-cell-enriched cell suspension was cultured with 100 units/ml recombinant interleukin-2 (Sionogi, Osaka, Japan) for 1 week (T-LAK).

Preparation of BsAb. The BsAb used in this study was prepared from anti-CD3 mAb (OKT3; American Type Culture Collection) and anti-SCLC mAb (LU246; kindly provided by Dr. Setuo Hirohashi, National Cancer Center), which specifically reacts with SCLC, glioma, and neuroblastomas (17), as described previously (4, 7, 18). Briefly, OKT3 and LU246 were digested by pepsin and papain, respectively, to prepare F(ab')₂ fragments. OKT3 F(ab')₂ was reduced with 0.5 mM dithiothreitol (Sigma) for 30 min at pH 7.5, and derivatized by addition of 5,5-dithiobis-2-nitrobenzoic acid (Sigma), at a final concentration of 5 mM. The nitrobenzoic derivative of OKT3 Fab' fragment (Fab'-S-NB) was separated by gel chromatography (TSK3000SWXL, TOSO, Japan). F(ab')₂ fragment of LU246 was also reduced to Fab'-SH with 0.5 mM dithiothreitol and excess reducing reagents were removed by gel filtration. OKT3 Fab'-S-NB and LU246 Fab'-SH were mixed at a 1:1 ratio and incubated for 4 h at 37°C to conjugate with each other. Heteroconjugated F(ab')₂ fragment was purified by gel filtration.

Flow Cytometric Analysis and Sorting. SCLC cell lines, H69 and Lu135, were analyzed for the expression of ICAM-1, LFA-3, and MHC Class I by immunofluorescence. Each cell (1 × 10⁶) was treated with mAb for 30 min at 4°C. After two washes with phosphate-buffered saline, fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin antibody (Caltag, San Francisco, CA) at 1:20 dilution was added and incubated for a further 30 min at 4°C. After two washes, the cells were analyzed on a FACScan (Becton Dickinson). The mAbs used were as follows: anti-ICAM-1 (RR1/1, IgG1, kindly provided from Dr. T. A. Springer) (19, 20), anti-LFA-3 (TS2/9, kindly provided by Dr. S. J. Burakoff) (21), and anti-MHC Class I (W6/32, IgG2a, Dakopatts). Normal mouse serum was used as a control.

Lu135 cell line partially expressed ICAM-1 molecule after treatment with 10 units/ml IFN- γ (Sionogi, Osaka, Japan) for 16 h. We isolated the ICAM-1⁺ subpopulation (Lu135PS) and the ICAM-1⁻ subpopulation (Lu135NS) by sorting on a FACStar Plus (Becton Dickinson). ICAM-1 molecule was not originally expressed on both subpopulations. After pretreatment with IFN- γ , ICAM-1 expression was induced on almost all Lu135PS, but not at all on Lu135NS. Although LFA-3 and MHC Class I were constitutionally expressed, MHC Class I expression was increased by IFN- γ treatment on both subpopulations.

Cytotoxicity Assay. Cytotoxic activity was measured in a standard 4-h ⁵¹Cr-release assay (22). Lu135PS, Lu135NS cells, with or without IFN- γ pretreatment, and H69 cells were labeled with 100 μ Ci of sodium [⁵¹Cr]chromate (DuPont/NEN) for 1 h. After washing twice, 1 × 10⁴ target cells in 100 μ l complete medium were seeded in triplicate in 96-well U-bottomed microtiter plates (Costar, Cambridge, MA). In order to estimate BsAb-directed cytotoxicity, radiolabeled target cells in microtiter plates were incubated with OKT3 × LU246 BsAb for 30 min at 20°C before assay. The enhancement of LAK cell cytotoxicity reached a plateau with BsAb at more than 1 μ g/ml. Similar results were obtained against malignant glioma cells (18). Thus, we used BsAb at a concentration of 1 μ g/ml in this study. Varying numbers of effector cells in 100 μ l complete medium were then added to each well. In antibody blocking study, anti-CD2 (OKT11, American Type Culture Collection) and anti-MHC Class I (W6/32) mAbs were added at 10 μ g/ml and anti-ICAM-1 (RR1/1) mAb was added at 1:100 dilution of the ascites. After a 4-h incubation at 37°C, 100 μ l of supernatants were harvested and counted in a gamma counter.

Percentage of cytolysis was calculated according to the following formula:

$$\frac{\text{Test cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100$$

The spontaneous cpm were measured from the well without addition of the effector cells. The maximum cpm were measured from the well with target cells supplemented with 1 μ l of 20% Triton-X.

RESULTS

Expression of ICAM-1 and LFA-3 on SCLC Cell Lines. We analyzed the expression of ICAM-1 and LFA-3 on SCLC cell lines, H69 and Lu135, by immunofluorescence and flow cytometry. In contrast to LFA-3, which was moderately expressed on both cell lines, ICAM-1 was expressed on H69 but not on Lu135 at all (Fig. 1).

Difference in Susceptibility to BsAb-directed LAK Cell Cytotoxicity between H69 and Lu135. To compare the susceptibility of H69 and Lu135 to LAK cell-mediated cytolysis, 4-h ⁵¹Cr-release assay was performed in the presence or absence of OKT3-LU246 BsAb, and the contribution of ICAM-1 was estimated by blocking with an anti-ICAM-1 mAb, RR1/1 (Table 1).

OKT3-LU246 BsAb increased cytolytic activity of LAK cells against both H69 and Lu135 cells. BsAb-directed cytotoxicity against H69 was always greater than that against Lu135 in three repeated experiments. Anti-ICAM-1 mAb partially inhibited the BsAb-directed cytotoxicity against H69 but not that against Lu135 at all.

Isolation of ICAM-1⁻ and ICAM-1⁺ Lu135 Subpopulations after Treatment with IFN- γ . In order to estimate the contribution of LFA-1/ICAM-1 interaction to the enhancement of LAK cell cytotoxicity by BsAb, we prepared ICAM-1⁺ and ICAM-1⁻ subpopulations from the same cell line. A small portion of

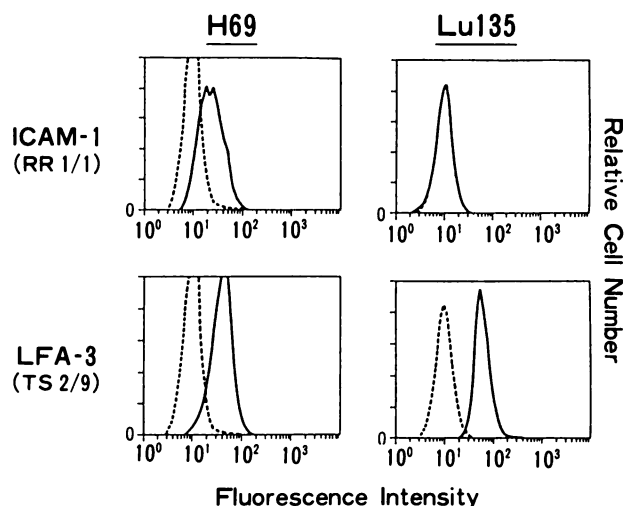


Fig. 1. FACS analysis of ICAM-1 and LFA-3 expression on SCLC cell lines (—). Antibodies used were RR1/1 (anti-ICAM-1) and TS2/9 (anti-LFA-3). ---, staining with normal mouse serum.

Table 1 Comparison of BsAb-directed LAK cell cytotoxicity against H69 and Lu135

LAK cells supplemented with OKT3 × Lu246 BsAb were tested for their cytotoxicity in a 4-h ⁵¹Cr-release assay. Effector/target ratio was 5. Values indicate mean ± SD of triplicated wells.

LAK cells with	% of cytotoxicity against	
	H69	Lu135
Control	8.0 ± 1.4	5.4 ± 1.6
OKT3 × Lu246	29.0 ± 1.7	17.8 ± 2.9
OKT3 × Lu246 + anti-ICAM-1	20.9 ± 2.0	18.6 ± 1.5

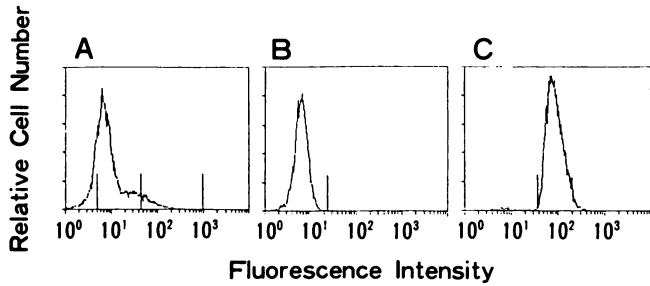


Fig. 2. Fractionation of Lu135 pretreated with IFN- γ based on ICAM-1 inducibility. *A*, staining pattern of ICAM-1 on Lu135 cell line after treatment with 10 units/ml IFN- γ for 16 h. *B*, sorted ICAM-1⁻ fraction (Lu135NS). *C*, sorted ICAM-1⁺ fraction (Lu135PS).

Lu135, on which no ICAM-1 expression was detected, expressed ICAM-1 after the treatment with 10 units/ml IFN- γ for 16 h (Fig. 2*A*). Then, the ICAM-1⁻ and ICAM-1⁺ subpopulations were isolated by fluorescence-activated cell sorting, and were named Lu135NS (Fig. 2*B*) and Lu135PS (Fig. 2*C*), respectively.

Expression of ICAM-1, LFA-3, and MHC Class I on Lu135PS and Lu135NS after IFN- γ Treatment. Both Lu135NS and Lu135PS did not express ICAM-1 before treatment with IFN- γ (Fig. 3). ICAM-1 expression was detected after a 16-h treatment with IFN- γ on Lu135PS but not on Lu135NS (Fig. 3). LFA-3 was expressed on both Lu135NS and Lu135PS cells to a similar level before and after IFN- γ treatment. MHC Class I was expressed on both Lu135NS and Lu135PS cells to a similar level, and the expression was increased by IFN- γ treatment on both cell lines to a similar extent.

Inhibitory Effect on Anti-ICAM-1 mAb on BsAb-directed T-LAK Cells against IFN- γ -treated Lu135PS. Next we examined the BsAb-directed T-LAK cell cytotoxicity against Lu135NS and Lu135PS with or without IFN- γ pretreatment. And then we examined whether the enhanced cytotoxicity

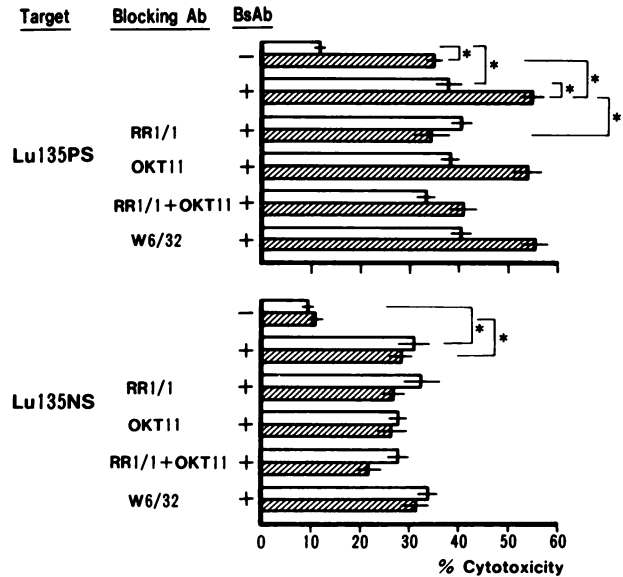


Fig. 4. Inhibitory effect of anti-ICAM-1 mAb on BsAb-directed LAK cell cytotoxicity against IFN- γ -treated Lu135PS and Lu135NS. OKT3 \times LU246 BsAb was used at 1 μ g/ml. E:T ratio was 10. \square , untreated cell lines; \blacksquare , IFN- γ -treated cell lines. Values indicate mean \pm SD of triplicated wells. *, $P < 0.001$.

would be inhibited by anti-ICAM-1 (RR1/1), anti-CD2 (OKT11), and/or anti-MHC Class I (W6/32) mAbs.

Spontaneous cytotoxicity of LAK cells was increased by the IFN- γ pretreatment against Lu135PS but not against Lu135NS (Fig. 4). This result is consistent with that with neuroblastoma cells recently reported by others (15). Addition of OKT3-LU246 BsAb enhanced the cytotoxicity against both target cells without IFN- γ pretreatment to a similar extent, but to a higher extent against Lu135PS when pretreated with IFN- γ . This enhanced cytotoxicity of BsAb-directed LAK cells by IFN- γ pretreatment of Lu135PS was specifically inhibited

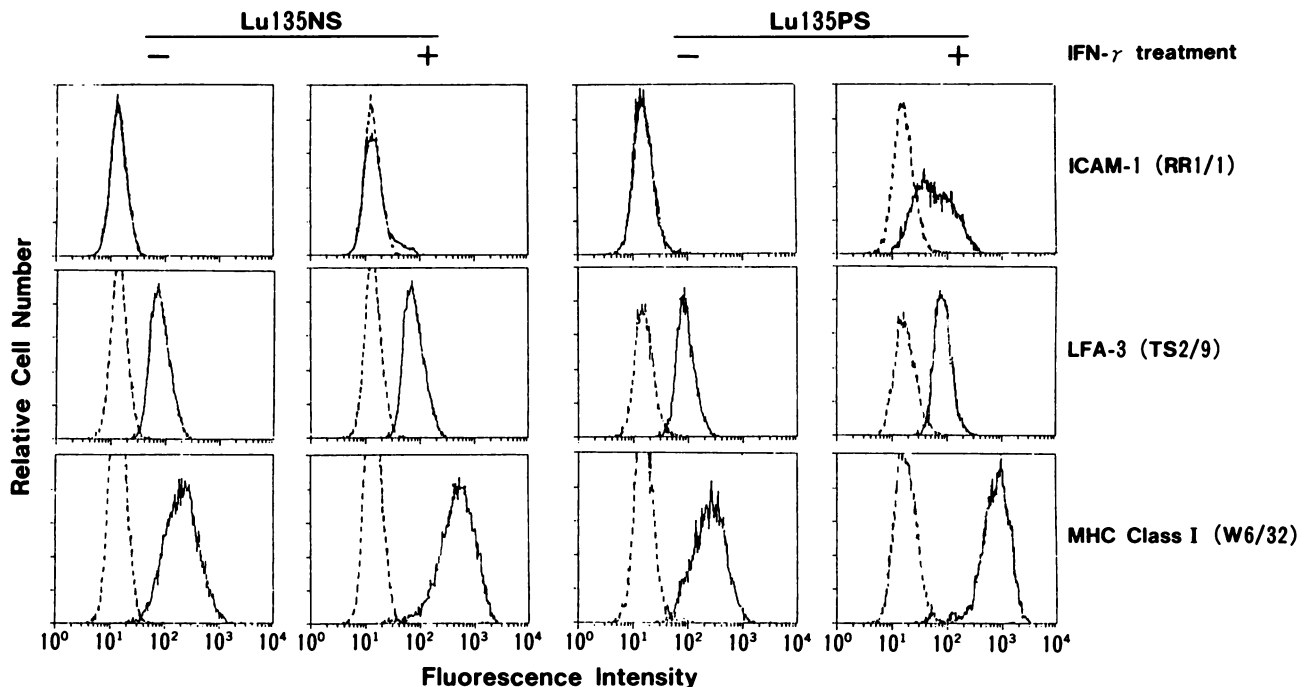


Fig. 3. Comparison of cell surface expression of ICAM-1, LFA-3, and MHC Class I on Lu135PS and Lu135NS before and after IFN- γ treatment. Both cell lines were treated or not treated with 10 units/ml IFN- γ for 16 h and stained with the mAbs (—), or normal mouse serum as a control (---).

by anti-ICAM-1 mAb (RR1/1) but not by anti-CD2 (OKT11) or anti-Class I (W6/32) mAb. A combination of anti-ICAM-1 and anti-CD2 mAbs did not result in a stronger inhibition as compared with anti-ICAM-1 mAb alone. In contrast, the BsAb-directed cytotoxicity against Lu135NS was not inhibited by all these mAbs even after the IFN- γ pretreatment (Fig. 4).

DISCUSSION

In this study, we initially examined the effect of IFN- γ on the expression of adhesion molecules on SCLC cell lines. Recently, it was reported that several cytokines play important roles in up- or down-regulating the expression of adhesion molecules on various cells and tissues (8, 10, 12–14). It is also known that IFN- γ , IL-1, or TNF- α induces or enhances the expression of ICAM-1 on certain tumor cell lines (1, 15). In the case of human SCLC, we revealed that the treatment with 10 units/ml IFN- γ induced or increased the expression of ICAM-1 within 16 h. In contrast, IL-1 and TNF- α could not induce or enhance ICAM-1 expression on SCLC cell lines (data not shown).

H69 cell line that originally expressed ICAM-1 (Fig. 1) was more susceptible to both spontaneous and BsAb-directed LAK cell cytotoxicity than Lu135 cell line that did not express ICAM-1 (Table 1). In order to make more clear the influence of ICAM-1 expression on target cells on their susceptibility to the BsAb-directed cytotoxicity, comparison was performed between two sublines which was originated from the same cell line and become ICAM-1⁺ and ICAM-1⁻ after IFN- γ pretreatment.

Lu135 cells did not originally express ICAM-1, but a small subpopulation became ICAM-1⁺ after treatment with 10 units/ml IFN- γ for 16 h (Fig. 2). Then, the Lu135 cells treated with IFN- γ was fluorescence-activated cell sorted into ICAM-1⁺ (Lu135PS) and ICAM-1⁻ (Lu135NS) subpopulations, and we examined the sensitivities of these two sublines to spontaneous and BsAb-directed LAK cell cytotoxicity. The sensitivity of Lu135PS, on which ICAM-1 expression was induced, but not that of Lu135NS, on which ICAM-1 expression was not induced, was significantly increased by IFN- γ pretreatment. This increased susceptibility by IFN- γ pretreatment was induced due to ICAM-1 induction, since anti-ICAM-1 but not anti-CD2 nor anti-Class I mAb could inhibit it (Fig. 4).

In addition, a simple cross-linking of CD3 on LAK cells by the addition of OKT3 plus anti-mouse immunoglobulin did not enhance the LAK cell cytotoxicity against untreated or IFN- γ -treated Lu135PS and Lu135NS cells (data not shown). This indicates that the IFN- γ enhancement of the BsAb-directed cytotoxicity requires not only T-cell activation but also a direct linkage of LAK and target cells by BsAb, and that it is not mediated by an increased susceptibility of the target cells to a bystander-type killing mediated by secretion of cytotoxic lymphokines. This further stresses the critical contribution of ICAM-1 as an adhesion molecule in BsAb-directed cytotoxicity.

It was recently reported that the T-cell stimulation through CD3 complex could strengthen the interaction between LFA-1 molecule on T-cells and ICAM-1 molecule on target cells(2). Such a mechanism would also work in the LAK cell cytotoxicity directed by BsAb containing OKT3 and stimulating T-cells through CD3.

In our present results, LAK cell cytotoxicity was enhanced by OKT3 \times LU246 BsAb against both Lu135PS and Lu135NS to a similar extent before IFN- γ treatment, but to a higher extent

against ICAM-1⁺ Lu135PS after IFN- γ treatment. This indicates that the ICAM-1 expressed on target cells did contribute to the enhanced cytotoxicity by BsAb but also suggests that the direct linkage by BsAb itself might be efficient in inducing a substantial level of BsAb-directed cytotoxicity. Alternatively, some other cell-cell adhesion system than the LFA-1/ICAM-1 interaction plays a dominant role in the BsAb-directed LAK cell cytotoxicity against Lu135. However, the CD2/LFA-3 interaction, which is another well-known pathway (23–25), also appeared not to be involved, based on the lack of inhibition by anti-CD2 mAb. The adhesion molecules involved in this system and whether their interaction is up-regulated by the BsAb, like the LFA-1/ICAM-1 interaction, remain to be determined.

The cytotoxicity of LAK cells prepared from three healthy individuals were similarly enhanced by the BsAb, not only against SCLC cell lines, but also against LU246-reactive glioma and neuroblastoma cell lines, to a higher extent against ICAM-1⁺ cells than against ICAM-1⁻ cells after IFN- γ treatment (data not shown). Therefore, the IFN- γ enhancement of BsAb-directed cytotoxicity by ICAM-1 induction appears to be generally applicable to various tumors.

In conclusion, IFN- γ induction of the ICAM-1 expression on the target cells clearly enhanced OKT3-LU246 BsAb-directed LAK cell cytotoxicity. BsAb composed of anti-CD3 and anti-TAA mAbs is a powerful tool for enhancing and specifically directing T-cell cytotoxicity against tumor target cells expressing the TAA (4, 5), and has been successfully used for clinical treatment of glioma patients (7). However, its efficacy was further enhanced by pretreatment of target cells with IFN- γ *in vitro*, which resulted in ICAM-1 induction. In our preliminary examination, glioma tissues as well as SCLC tissues frequently lacked the ICAM-1 expression. Therefore, a combinational use of IFN- γ (and possibly TNF- α and IL-1, which could also up-regulate ICAM-1 expression on certain tumor cells) will be a promising way for enhancing the clinical efficacy of the STT with LAK cells and BsAb.

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