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Natural killer cytolytic activity is associated with the expression of killer cell immunoglobulin-like receptors on peripheral lymphocytes in human

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Although it has been shown that killer cell immunoglobulin-like receptors (KIRs) on peripheral lymphocytes are upregulated by interleukin-2 (IL-2), which activates natural killer (NK) activity, it has not been demonstrated whether the expression of KIRs is related to NK activity. Therefore, we investigated the association between the KIR expression on lymphocytes and NK activity. CD158a/b expression on lymphocytes obtained from 37 subjects was analyzed using flow cytometry. Simultaneously, NK activity was measured each sample using a ^{51}Cr-release assay. Additionally, lymphocytes were cultured in RPMI 1640 medium with or without IL-2 for 48 h, and then their CD158a/b expression and NK activity was analyzed. CD158a/b expression was significantly correlated with NK activity. Especially, the percentage of CD16+CD158a+ and CD8+ CD158a/b+ cells in lymphocytes showed a highly significant correlation with NK activity. However, analysis of CD8+ and CD16+ cells revealed that there was only a significant correlation between the percentage of CD8+CD158a+ cells among only CD8+ cells and NK activity. The upregulation of CD16+CD158a+/b+ cells in response to IL-2 tended to be related to the increase of NK activity, but the relationship was not significant. In conclusion, the level of KIR expression was correlated with NK activity, and IL-2 treatment resulted in an increase of NK activity as well as KIR expression, suggesting that upregulation of KIRs enhances the ability to sort target cells, such as virus-infected cells from uninfected cells, according to major histocompatibility complex class I expression.

Key words: Killer cell immunoglobulin-like receptors, Natural killing activity, Major histocompatibility complex class I, Inactivation

Introduction

Natural killer (NK) cells are a discrete population of lymphocytes characterized in humans by the expression of CD16 and CD56 cell surface antigens.[1] NK cells are most noted for their *in vitro* ability to spontaneously lyse transformed or virus-infected and some normal cells, and are also potent producers of lymphokines, predominantly interferon-γ and tumor necrosis factor-α. Given their rapid activation during an immune response and their dual role in cell-mediated cytotoxicity and lymphokine production, NK cells are considered an important component of natural resistance, active as a first line of defense against infectious agents.

Although the exact mechanisms that NK cells utilize to recognize and lyse target cells have remained poorly understood, the molecular cloning of novel NK receptors was achieved in 1995,[2–4] and it has subsequently been shown that these receptors, which are now called killer cell immunoglobulin-like receptors (KIRs), transmit positive and/or negative signals.[5,6] It is now widely accepted that the expression of KIRs is involved in the cytolytic function of NK cells, as demonstrated by a number of studies focusing on these receptors.[7]

We previously reported that the expression of both KIR2DL1 and KIR2DS1 (CD158a), and KIR2DL2, KIR2DL3 and KIR2DS2 (CD158b) on lymphocytes is upregulated by interleukin (IL)-2 but not by interferon-γ or IL-4, although IL-2 enhances the cytotoxicity of NK cells.[8] It has been speculated that the upregulation of KIRs by IL-2 results in enhanced ability to sort target cells such as virus-infected cells from uninfected cells according to major histocompatibility complex (MHC) class I expression. However, there has been no report of any investigation demonstrating an association between NK cytolytic activity and KIRs expression. In this study, we

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examined their relationship using peripheral blood lymphocytes in humans.

Methods

Reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-human CD8, FITC-anti-human CD16, phycoerythrin (PE)-conjugated anti-human CD158a (EB6) and PE-conjugated anti-human CD158b (GL183) were purchased from Immunotech (Marseille, France). Recombinant human IL-2 was obtained from Pharmabiotechnology (Hanover, Germany).

Cells

Peripheral blood mononuclear cells obtained from 37 volunteers (24 females and 13 males; age (mean ± standard deviation), 66.3 ± 25.4 years; range, 25–86 years) were separated from heparinized blood by Lymphoprep (Nyegaard, Oslo, Norway) gradient cenfugation.[9] Each peripheral blood mononuclear cell sample was a incubated in culture dish in a humidified 5% CO_2/95% air atmosphere at 37°C for 60 min. After the incubation, non-adherent cells were collected. These cell suspensions were washed twice in phosphate-buffered saline (PBS).

Cell culture

One million cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Biological Industries, Israel) in tissue culture dishes (Becton Dickinson, Flanklin, NJ, USA). The cell cultures were incubated in medium only or medium containing IL-2 at 100 U/ml in a humidified 5% CO_2/95% air atmosphere at 37°C for 48 h. After each incubation, cells were collected and their surface antigens were analyzed using flow cytometry (Epics XL; Beckman Coulter, Marseille, France). Simultaneously, the NK activity in each sample was measured using a ^{51}Cr-release assay. Each experiment was carried out in duplicate.

Cell phenotype

Surface phenotyping was carried out using a two-color immunofluorescence staining technique, with isotype-specific mouse anti-human antibody conjugated with either FITC or PE.[10] Each sample of stained cells was suspended in 0.5 ml of PBS and analyzed by flow cytometry. Lymphocyte subsets were identified by gating analysis and fluorescence profiles were obtained for 10,000 cells of each sample. Negative controls for each experiment were performed with FITC-labeled and PE-labeled mouse immunoglobulin G.

NK cytolytic activity

NK activity was assessed against K562, a myelogeneous leukemia cell line, in a 3 h ^{51}Cr-release assay as described previously. The effecter cells were mixed with labeled target cells at various cell concentrations to give effecter to target ratios of 20:1 or 10:1. Duplicate samples were prepared for each ratio. The supernatants were withdrawn after a 4 h incubation period, and the ^{51}Cr released from lysed targets was quantitated in a gamma counter (Packard Instrument Co., Boston, MA, USA). Incubation of targets with medium or saponin (7 mg/ml) and ethylenediamine tetraacetic acid (0.1 mg/ml) was used to determine spontaneous and maximal release, respectively. The percent cytotoxicity was calculated by the formula: (cpm of effecter cells − cpm of spontaneous release)/(cpm of maximal release − cpm of spontaneous release) × 100.

Statistical analysis

Data are expressed as mean (standard deviation) values. All data were collected in a computer database and analyzed using the StatView-J 4.02 program (Abacus Concept, Berkeley, CA, USA). Spearman rank correlation analysis was performed between each part of NK activity and KIR expression. For all statistical tests, correlations were regarded as statistically significant if $p < 0.05$.

Results

The relationship between the percentage of CD158a+ or CD158b+ cells in lymphocytes and NK cytolytic activity

There was no relationship between the expression of CD16 and NK cytotoxicity (Table 1). In contrast, the expression of CD158a or CD158b correlated with the NK cytolytic activity significantly (Table 1). The correlation of CD158a expression with NK cytotoxicity is shown in Fig. 1.

The relationship between the percentage of CD8+CD158a+/b+ or CD16+CD158a+/b+ cells in lymphocytes and NK cytolytic activity

There was a significant relationship between the percentage of CD8+CD158a+/b+ cells and NK cytolytic activity (Table 1). Similarly, the percentage of CD16+CD158b+ cells tended to be correlated with NK cytolytic activity, although the correlation was not significant (Table 1).

Table 1. Correlation of NK cytolytic activity with several cell surface antigens

NK cytolytic activity V.S.**	Probability*	Linear regression coefficient
CD16	0.086	0.295
CD158a	0.010	0.424
CD158b	0.032	0.362
CD8CD158a	0.008	0.432
CD8CD158b	0.006	0.458
CD16CD158a	0.018	0.398
CD16 CD158b	0.130	0.257
CD8+CD158a+ cell/CD8+ cell	0.026	0.375
CD8+CD158b+ cell/CD8+ cell	0.117	0.270
CD16+CD158a+ cell/CD16+ cell	0.163	0.241
CD16+CD158b+ cell/CD16+ cell	0.790	0.047

*If probability < 0.05, correlations are regarded as ststistically significant.
**V.S. = versus.

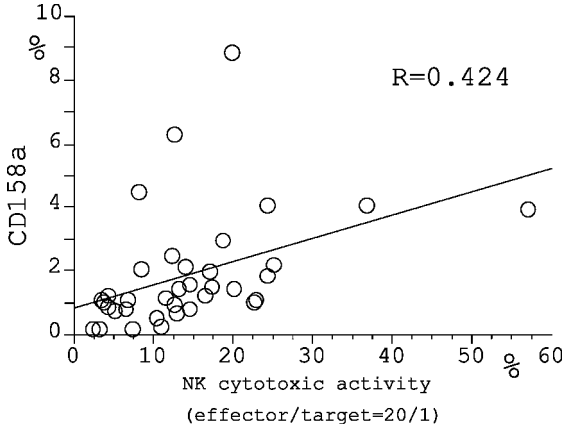

FIG. 1. The correlation of CD158a+ cells with natural killing activity is shown. $P = 0.0099$, $r = 0.424$.

The relationship between the percentage of CD158a/b+ cells in CD8+ or CD16+ cells and NK activity

To investigate the significance of the KIR expression on CD8+ or CD16+ cells, we further analyzed the association between the percentage of CD158a/b+ cells in CD8+ or CD16+ cells and NK activity. There was a tendency toward a correlation between the percentage of CD8+CD158a+ cells in CD8+ cells and NK cytolytic activity. However, we could not find a significant correlation between the percentage of CD8+CD158b+ cells/CD8+ cells, CD16+ CD158a+ cells/CD16+ cells or CD16+CD158b+ cells/CD16+ cells, and NK cytotoxicity (Table 1).

The change of CD158a/b expression and NK activity when lymphocytes are treated with IL-2

We assessed the CD158a/b expression and NK cytolytic activity after treatment of eight donors with IL-2. NK cytotoxicity increased by treatment of IL-2 as previously shown[8,11] (Fig. 2A). Simultaneously, the percentage of CD158a+ and CD158b+ cells increased (Fig. 2B). In addition, the percentage of CD8+CD158a+/b+ cells and CD16+CD158a+/b+ cells also increased in parallel with the increase of NK cytotoxicity (Fig. 2B). The increases of the CD158a+/b+ cell population and NK cytolytic activity by treatment with IL-2 were dose dependent from 10 to 200 U/ml (data not shown).

Discussion

In this study, we first showed that NK cytolytic activity correlates with the expression of KIRs recognized by CD158a/b in humans. Second, we showed that upregulation of the KIR expression was accompanied by an increase of NK cytotoxicity when lymphocytes were incubated with medium plus IL-2. The present findings support the speculation described earlier. Namely, when the cytolytic activity increases, the expression of KIRs also increased. Alternatively, the percentage of CD16+CD158b+ cells did not correlate with NK cytolytic activity, although the percentage of CD16+CD158a+ and CD8+CD158a+/b+ cells correlated with it in our study. NK cytolytic activity is dependent on the signaling balance of KIRs, activating receptors and the expression of their ligands. Therefore, there is a not always a relationship between the NK cytolytic activity and the proportion of KIR-positive cells in the cross-sectional analysis. In contrast, the percentage of each cell population is related to the NK cytolytic activity after treatment with IL-2, indicating that the expression of KIRs is upregulated when NK activity increases. These findings are in accord with recently published data showing that downregulation of KIRs is accompanied by a decrease of natural killing activity.[12] However, it is unclear whether the upregulation of KIRs occurs before or after enhancement of NK activity.

Analysis of the percentage of KIR-expressing cells among CD8+ or CD16+ cells revealed that the percentage of CD158a expression on CD8+ cells, but not on CD16+ cells, correlated with NK activity. We cannot yet explain these phenomena from the

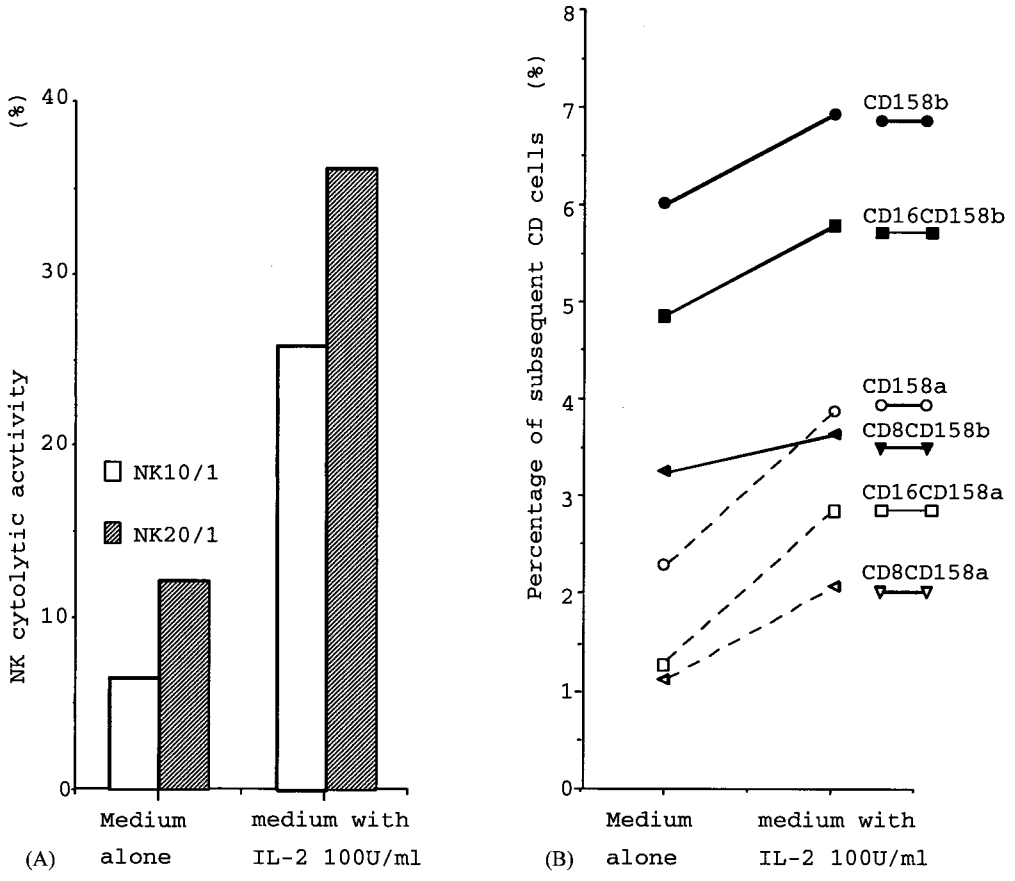

FIG. 2. The change of CD158a/b expression and NK activity when lymphocytes are treated with medium alone or medium containing IL-2 at 100 U/ml. Eight of 35 donors were assessed for CD158a/b expression and NK cytolytic activity. (A) NK cytolytic activity in bars representing medium alone and medium with IL-2. (B) Six lines presenting the percentage increase of subsequent CD cells. The percentage of each CD cell was increased significantly by the treatment of IL-2. The change of each CD cell was in parallel to the increase of NK activity.

immunological viewpoint. It is possible that the expression of KIRs on CD8+ cells is more important functionally than inhibitory receptors on CD16+ cells. Although the function of KIRs in CD8+ T cells is still obscure, these KIRs probably prevent autoaggression by cytotoxic T cells specific for self-antigens.[13,14] In T cells, the expression of the activating receptors such as TCR and CD2, and the MHC-restricted killing activity, are not affected by KIR downregulation.[15] Thus, the only known fail-safe system to escape attack by cytotoxic T cells is the inactivation by negative signals from KIRs on T cells. Indeed, in NK cells, activating receptors were found to be decreased by KIR–MHC class I interactions, and this receptor downregulation was correlated with a deficiency in the NK activity as well as CD16-mediated killing activity.[12,16] These phenomena appeared to be specific to NK cells.

KIRs recognized by CD158a/b consist of two kinds of receptors.[17,18] CD158a reacts with KIR2DL1 (inhibitory receptor (IR)) and KIR2DS1 (activating receptor (AR)), and CD158b reacts with KIR2DL2, KIR2DL3 (IR) and KIR2DS2 (AR). CD158a/b monoclonal antibodies (mAb) cannot distinguish between these receptors. Furthermore, the ligands that react with AR have been identified as ligands that react with IR and, importantly, the CD158b mAb we used bound equally to the AR and IR,[19] making it difficult to explain NK cell regulation. To date, it has been considered that the activating signal can be overridden by a dominant-negative signal from IR when IR interacts with its ligand on the target cell, although AR, when occupied, triggers lysis of the target cell being recognized.[20,21] These insights led us to speculate that KIRs increase as part of the defense system of non-infected cells when NK cells are activated by viral infection, although other activating receptors such as CD69[22] and 2B4[23] might be upregulated. However, several critical questions remain. The importance of AR upregulation parallel to IR upregulation is still not clear in NK cell activation. Recently, a biphasic response of NK cells expressing both AR and IR has been demonstrated.[24] High concentrations of CD158b mAb transmitted inhibitory signals to NK cells, whereas low concentrations transmitted activating signals.[24] Warren *et al*. speculated that there is a fail-safe mechanism for activating NK cells if other NK cell-activating receptors or target

cell ligands to activate those receptors are not present when histocompatibility antigen (HLA) concentrations on target cells are below that capable of inhibiting NK cell function. In our study, NK cytolytic activity correlated with the expression of CD158a/b-recognizing receptors. A possible rationale for the upregulation of both IR and AR during the course of viral infection or neoplastic disease is that NK cells are capable of lysing cells expressing low levels of HLA in early stages of diseases independently of other activating receptors, and NK cells are also inactivated by negative signals from IR if activating receptor-expressing NK cells encounter normal cells on which HLA molecules are highly expressed. Therefore, we speculate that it is beneficial for the bio-defense system to upregulate the expression of KIRs when NK cells acquire enhanced cytotoxic activity. Recently, Huard et al. reported that KIR downregulation on NK cells is associated with the downregulation of activating receptors such as 2B4 as well as with a lack of cell responsiveness12. Thereafter, several safety mechanisms may function to protect normal HLA-expressing cells.

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