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EFFECT OF SOLUBLE HLA CLASS I MOLECULE ON NK/LAK CELLS ACTIVATION INDUCED BY POLY I:C

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ABSTRACT

Background: Natural Killer cells express killer inhibitory receptors specific for HLA-class I molecules. These receptors could induce signals that determine NK cells ability to mediate cytotoxicity. Purified soluble form of HLA class I molecules (sHLA) could bind to NK cell receptors and down-regulate the NK killer function.

Objective: To evaluate the influence of sHLA and two monoclonal antibodies (mAbs) against killer inhibitory receptors on the poly I:C-treated freshly and IL-2 activated NK cells (LAK cells).

Methods: Isolation of $CD56^+$ NK cells and $CD56^-$ cells was performed using the magnetic cell separation technique. $CD56^+$ cells were activated by rIL-2 and anti-HLA-B7 specific cytotoxic T lymphocytes (CTLs) were established from $CD56^-$ cells. Flow cytometry analysis was performed to determine the percentage of CD3, CD16/CD56 and CD8 positive cells. LAK and specific CTLs were tested for cytotoxicity against M4 cells using the ^{51}Cr-release assay. Freshly isolated NK cells and LAK cells were pre-incubated with 0.7-11 μg/ml of sHLA-B7 fused to the Fc portion of IgG molecule and subsequently tested for killing activity. The influence of the sHLA molecule on the cytotoxicity of the cells after treatment with poly I:C as an inducer of interferon was also studied. LAK cells were pre-treated with 0.01 to 10 μg/ml of two mAbs against NK inhibitory receptors, NKB1 and NKAT2. The effect of these mAbs on the killing activity of poly I:C and non-poly I:C treated LAK cells against K562 target cell was determined.

Results: In flow cytometry analysis of LAK cells, 99.5% were found to be $CD56^+$. Analysis of generated CTLs showed 88.4 % positivity for CD8. Cytotoxicity of M4 stimulated CTLs was 12.3% at 1.5/1 E/T ratio to 97.1% at 25/1 E/T ratio. NK cells had no effect on M4 cells. Anti-NKB1 and Anti-NKAT2 mAbs decreased the cytotoxic activity of LAK cells. These mAbs showed no effect on the poly I:C activated LAK cells. Increasing concentrations of sHLA molecule decreased cytotoxic activity of LAK cells from 17.2% to 13.8% and poly I:C activated NK cells from 62.5% to 51.8%. Treatment of LAK cells with poly I:C and exposure of these cells with increasing concentrations of sHLA resulted in an increase in the target cell killing of LAK cells from 20.1% to 27.6%.

Conclusion: LAK cells were not able to kill M4 cells with high expression of HLA molecules, whereas CTLs were efficient in killing these cells. Recombinant sHLA fusion protein inhibited NK/LAK activity against K562 cells, whereas poly I:C activation of LAK cells reverted this effect indicating that poly I:C treatment of LAK cells could change the expression of NK inhibitory receptors.

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Key Words • NK cells • LAK cells• HLA

Introduction

Natural killer (NK) cells are a group of lymphocytes with a characteristic morphology and function.[1] These cells are distinct from both T and B lymphocytes in their circulation patterns, profile of surface markers, receptor repertoire and their ability to discriminate self from non-self.[1-3] NK cells are physiologically important in mediating immunity against viruses, intracellular bacteria and parasites, and in anti-tumor immune responses.[1-3] In fact, these cells were originally described on a

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functional basis according to their capacity of killing certain tumor cells of hematopoietic origin in the absence of previous stimulation.[3] The killing activity of NK cells has often been referred to as non-major histocompatibility complex (MHC) restricted.[4] NK cells selectively kill target cells that fail to express self-MHC class I molecules.[5] The molecular mechanisms involved in NK cells ability to identify and kill virally infected or tumor target cells but spare normal self-cells have recently been clarified. NK cells express a number of inhibitory receptors that recognize MHC class I molecules expressed on normal cells.[5-6] Two major types of these receptors specific for groups of human histocompatibility leukocyte antigens, HLA class I type A, B, C have been identified on human NK cells.[5-6]

HLA class I molecules are expressed on the most nucleated cells and are known to serve as restriction elements for the lysis of target cells by cytotoxic T-lymphocytes (CTLs).[7] Soluble forms of these molecules (sHLA) have been detected in serum of normal individuals.[8] It is suggested that sHLA molecules are of biological significance. sHLA could down-regulate the CTL cytotoxicity.[9] In a recent study the ability of sHLA to induce apoptosis in alloreactive T cells mediated by CD95-L up-regulation has been shown.[10]

A possible immunoregulatory function for sHLA has also been suggested in NK cell-target recognition. Webb et al. have shown the ability of sHLA to inhibit NK cytotoxicity.[11] The mechanism of sHLA modulatory activity on NK cells is not clearly understood. It is postulated that these molecules can bind to specific receptors expressed on the NK cell surface and deliver a negative signal to the NK cells.[12] Whether this inhibitory signal is altered by up-regulation of NK cell activity has not been fully investigated. In a normal immune response, activation of NK cells to higher levels of lytic activity is more important than basal NK cell lytic function.[2-4] In the present study, freshly isolated and also IL-2 activated NK cells (LAK cells) were treated with polyinosinic-polycytidilic acid (poly I:C) and the effect of sHLA exposure on the lytic function of these cells against tumor cell lines was investigated. The influence of poly I:C as an inducer of interferon (IFN) production and activator of NK cells[13] on the expression of two specific receptors of MHC class I molecules, NKB1 and NKAT2 involved in the negative regulation of NK cell-mediated cytotoxicity was also investigated.

Materials and Methods

Antibodies, cell lines and culture condition:

The Epstein Barr virus-transformed cell line M4, kindly provided by Dr. N. Zavazava from the Institute of Immunology, Kiel, Germany, and K562, an MHC class I negative erythroleukemia cell line were used in this study. These cell lines were cultured in RPMI 1640 medium supplemented with 2mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Germany). FITC conjugated anti-CD16/CD56 and anti-CD3, and PE conjugated anti-CD8 were obtained from Immunotech, Germany. Purified mouse monoclonal anti-human NKAT2 and NKB1 were purchased from Pharmingen, Sweden. Recombinant truncated soluble HLA-B7 fusion protein, HLA-B7-IgG (Fc) was kindly provided by Dr. N. Zavazava.

NK cell separation and LAK cell production:

Peripheral blood mononuclear cells (PBMCs) from healthy normal individuals were isolated by density gradient centrifugation using Ficoll-hypaque. After washing, cells were re-suspended in PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA. Twenty μl of MACs colloidal super-paramagnetic micobeads (Miltenyi Biotec, Germany) conjugated to monoclonal mouse anti-CD56

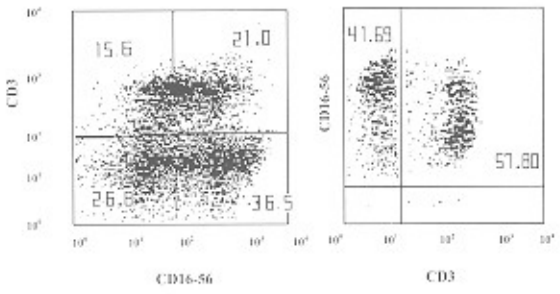

Figure 1: Flow cytometry analysis of CD56$^+$ lymphocytes isolated by magnetic microbeads conjugated with anti-CD56 antibody. Cells were stained after one **(Left)** and three weeks **(Right)**.

antibody suspension was added. After washing, cells were applied onto the magnetic separator with positive selection column in the magnetic field. After passing the negative cells through, the positive cells were flushed out using the plunger. The CD56 negative cells were collected for generation of cytotoxic T cells and the CD56 positive cells were washed and re-suspended in culture medium with 100 IU/ml rIL-2 (Glaxo, Switzerland). Cells were also stimulated with irradiated M4 cells (50 Gy) at responder/stimulator ratio (R/S) of 50/1 and irradiated allogeneic PBMCs (20 Gy) of 20/1.

Generation of CTLs:

Anti-HLA-B7 cytotoxic T cells were generated by co-culturing previously HLA-typed responder PBMCs lacking HLA-B7 allotype and irradiated allogenic PBMCs that were different only in HLA-B7. M4 cell line (A1/2 B7/37 Cw6/7 DR2/-) was also used as stimulator at a 20/1 R/S ratio. Cells were re-stimulated every 7-8 days. On the third and subsequent re-stimulations, culture medium was supplemented with 30 IU/ml IL-2.

Flow Cytometry:

Flow cytometery was performed using a FACStar PLUS (Becton Dickinson, USA). Cells were incubated with FITC and PE conjugated monoclonal antibodies (mAbs) for 30 minutes at 4°C and then analysed for fluorescence intensity.

Cytotoxicity and inhibition assays:

Cytotoxic activity was tested by incubating ^{51}Cr-labeled target cells with LAK cells, PBMCs or CTLs as effector cells (E), at 37°C and 5% CO2 for 4 hours. Target cells were M4 and K562 tumor cell lines. Specific release was calculated as (x-y) 100/z-y, where x is experimental release and y is spontaneous release. Z is total release measured in the presence of 10% Triton X-100. LAK cells, PBMCs or CTLs were incubated with various concentrations of sHLA molecules (0.7 to 11 μg/ml) at 37°C for 30 minutes. After that, target cells (T) at E/T ratios of 25/1, 12.5/1, 6.25/1 and 3.12/1 were added and cytotoxicity was determined. In the case of anti-NKB1 and anti-NKAT2, LAK cells were pre-treated with 0.01 to 10 μg/ml of mAbs 30 minutes before cytotoxicity assay.

Poly I:C activation of NK/LAK cells:

Freshly isolated PBMCs and LAK cells were incubated with 100 μg/ml of poly I:C (Sigma, USA) at 37°C in the CO2 incubator (Pharmacia, Sweden) overnight. After that cells were washed and used for cytotoxicity assay.

Results

Generation of LAK cells and CTLs and flowcytometry analysis:

In this study two groups of CD56$^+$ Natural Killer cells and CD56$^-$ cells were separated from PBMCs. In order to establish LAK cells, isolated CD56$^+$ cells were cultured with rIL-2 and irradiated M4 cell line. The purity of CD56$^+$ cell population was analyzed with mAbs for different subsets of mononuclear cells. As shown in Figure 1, after one week,

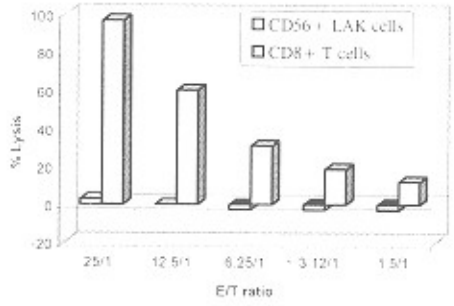

Figure 2: Cytotoxicity of HLA-B7 specific $CD8^+$ T cells and $CD56^+$ LAK cells against M4 cell line measured by 4-hours ^{51}Cr-release assay. LAK cells showed no cytotoxic activity compared to high activity of specific $CD8^+$ T cells.

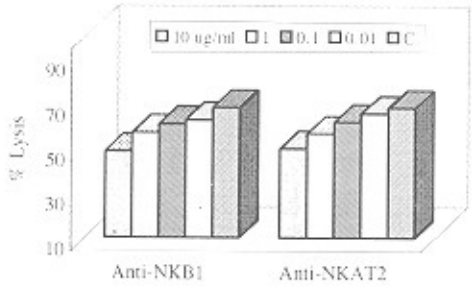

Figure 3: Activity of LAK cells after exposure with different concentrations of anti-NKB1 and anti-NKAT2 mAbs measured by ^{51}Cr-release assay. Control is without antibody. The E/T ratio in cytotoxicity assay was 12.5/1

15.6% of the cells were $CD3^+$, 36.5% $CD16^+CD56^+$ and 21% CD3/CD56 positive. Three weeks later $CD3^+$ cells were markedly decreased and the percentage of $CD16^+CD56^+$ and CD3/CD56 positive lymphocytes increased to 57.8% and 41.69%, respectively, showing that the isolation procedure would give a population consisting of 99.5% $CD56^+$ cells.

$CD56^+$ group of cells were used to generate alloreactive CTLs. Flow cytometry analysis showed that 88.4% of generated CTLs were CD8 positive (data not shown).

Cytotoxicity of NK/LAK cells and CTLs against M4 cells:

Cytotoxicity of LAK cells against M4 cells was measured using ^{51}Cr-release assay and compared to the $CD8^+$ T lymphocytes. As shown in Figure 2, the NK cell killing against M4 cells in all E/T ratios was less than 3%. Whereas cytotoxicity of M4 stimulated $CD8^+$ T cells increased from 12.3% in 1.5/1 E/T ratio to 97.1% in 25/1 E/T ratio, indicating the strong cytotoxicity of these cells against M4 cell line.

Effect of sHLA, NKB1, NKAT2 and poly I:C on NK/LAK killing activity:

Two mAbs were used to bind with two receptors of KIR family, NKB1 and NKAT2. As shown in Figure 3, LAK cytotoxicity against K562 target cell decreased in both cases. Percentage of lysis of K562 cells in the presence of anti-NKB1 (ranging from 49% to 62.9%) was generally less than the control (68.5%). LAK activity in the presence of anti-NKAT2 decreased from 65.9% at 0.01 μg/ml antibody concentration to 50.2% at 10 μg/ml. These results show the inhibitory effect of both mAbs on the LAK cytotoxic function. Poly

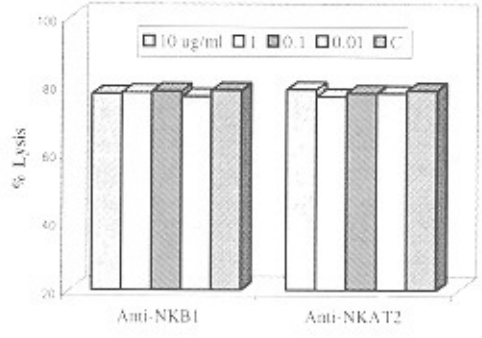

Figure 4: LAK cells treated with poly I:C and then exposed to anti-NKB1 and anti-NKAT2 mAbs. The E/T ratio for ^{51}Cr-release assay was 12.5/1. Control is without antibody.

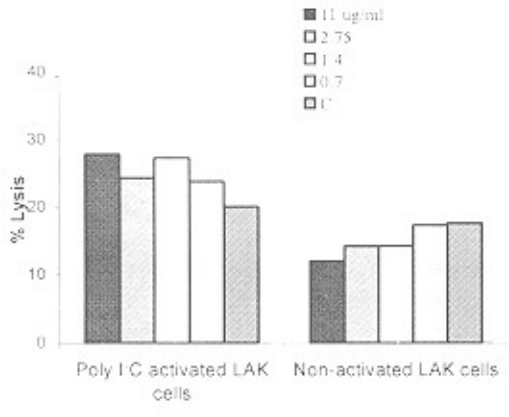

Figure 5: Effect of different concentrations of soluble HLA-B7-Fc(IgG) on the cytotoxic activity of LAK cells before and after treatment with poly I:C. Control is without sHLA. The E/T ratio in cytotoxicity assay was 1.5/1.

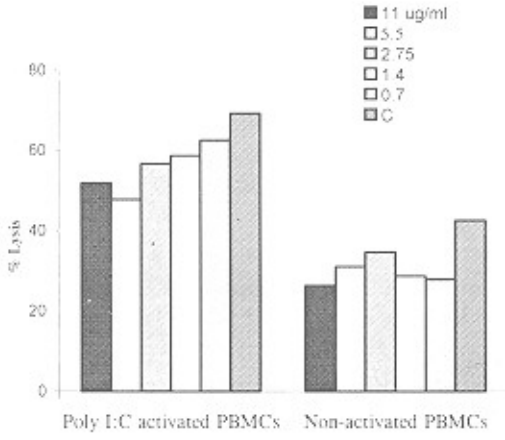

Figure 6: Effect of different concentrations of soluble HLA-B7-Fc (IgG) on the cytotoxic activity of PBMCs before and after treatment with poly I:C. Control is without sHLA. The E/T ratio in cytotoxicity assay was 25/1.

I:C activation of LAK cells decreased the inhibitory action of these mAbs on LAK cytotoxicity against k562 target. The mean ± SD percent killing of K562 cells in the presence of different concentrations of anti-NKB1 and anti-NKAT2 were 77.8 ± 0.7 and 78.0 ± 0.9, respectively, compared to that of the control of 78.8 ± 0.2 (Fig. 4).

As observed in Figure 5, the addition of increasing concentrations of sHLA to the LAK cells prior to culturing with K562 resulted in a decrease of LAK cell cytotoxicity from 17.2% to 13.8%. A similar effect was observed for NK activity of PBMCs (Fig. 6). The mean ± SD percent killing in the presence of different concentrations of sHLA was 29.8 ± 3.2 compared to the 42.6 ± 1.5 for the control, showing the inhibitory effect of sHLA on both non-poly I:C activated NK and LAK cells. The effect of sHLA on NK/LAK cells after exposure with poly I:C is shown in Figures 5 and 6. Poly I:C caused 26.7% increase in PBMCs cytotoxic activity. This effect on IL-2 activated LAK cells was lower (2.5%). Poly I:C activated PBMCs showed a cytotoxicity of 69.3% for K562 target cells. Addition of increasing concentrations of sHLA from 0.7 to 11μg/ml decreased NK activity from 62.5% to 51.8%. In the case of poly-I:C activated LAK cells, in contrast to non-poly-I:C-activated cells, target cell lysis was increased with increase in the sHLA concentration, as percentage of lysis at 0.7 μg/ml of sHLA, which was 23.8%, reached 27.6% at 11 μg/ml of sHLA.

Discussion

In the present study, the cytotoxic activity of $CD56^+$ LAK cells was first compared with the cytotoxicity of alloreactive cytotoxic T-cells generated from CD56 PBMCs. Both cells were pre-stimulated with M4 cells. M4 is a tumor cell line with a high surface expression of HLA class I molecules.[9] The results indicated that $CD8^+$ specific T cells were strongly efficient in killing this target cell whereas NK cells had no effect. This result is in agreement with data in the literature indicating the major difference between target

cell recognition and lysis in T and NK cells.[4-7] T cells need pre-stimulation to effect target cell killing and recognize antigen in the context of MHC molecules[7] In contrast, expression of MHC molecules protects target cells from LAK/NK lysis.[14] For the first time, Karre and co-workers proposed the missing-self model predicting that NK cells survey the body for MHC class I expression and kill cells in which it is abnormally down-regulated.[15] Consistent with this model has been the discovery of receptors on NK cells that bind to polymorphic determinants of MHC class I molecules and signal inhibition or activation of cytolysis.[16] Two types of these receptors including type I glycoproteins belonging to the immunoglobulin super-family, termed as killer cell immunoglobulin-like receptors (KIRs) and type II glycoproteins (CD94-NKG2) have been identified.[17-19] Both of these receptor families contain two types of inhibitory and activating receptors. Interaction of the inhibitory receptor with MHC class I molecules results in blockage of the cytotoxic activity of NK cells.[19-21] The function of activating receptors is not clear. Different isoforms of KIR receptors have been identified.[19] NKB1 and NKAT2 are included in KIR3DL and KIR2DL isoforms, respectively, capable of inhibiting NK cell activity.[20] NKB1 recognizes HLA-B allotypes and NKAT2 recognizes HLA-C allotypes.[22-24] MAbs against KIR receptors are useful as triggering or blocking agents. In this study, two mAbs used against NKB1 and NKAT2 have the property to mimic the effect of HLA class I molecules leading to the inhibition of LAK cell activity. This inhibitory activity was changed after exposure of LAK cells to poly I:C.

We found that the cytotoxicity of PBMCs and LAK cells against K562 target cells were decreased in the presence of sHLA, indicating the inhibitory action of sHLA on LAK/NK activity. These results are in concordance with Carbone et al. who showed the inhibitory effect of a truncated HLA-B7 and several membrane-derived purified sHLA class I molecules on NK/LAK function.[14] The membrane-derived molecules were intact heterodimers consisting of a ß2-microglobulin and a heavy chain releasing to the supernatant of different HLA class I positive cell lines. Carbone et al. have indicated that sHLA molecules can down-regulate NK cell killing at the effector level. Moreover, they showed that different NK clones are able to specifically recognize sHLA antigen. sHLA used in this study was a chimeric protein consisting of recombinant truncated HLA-B7 bound to the Fc portion of an IgG molecule. The efficacy of recombinant truncated sHLA-B7 (lacking the transmembrane and intracellular domains) to modulate T cell function has been reported by Hansen et al.[25] They showed that this molecule is less effective than membrane-derived sHLA to modulate T cell function. To improve the molecular size of the class I molecules, fusion proteins linked to the Fc region of immunoglobulins have been proposed.[25] Aggregation of these molecules could result in the formation of dimeric proteins that are able to cross-link HLA receptors on the LAK and NK cells and facilitate the possible signaling.[25]

NK cells, in contrary to T cells, do not require sensitization by antigen and differentiation to become efficient killer cells.[3] Instead they are always ready to kill and their activity can be increased by exposure to cytokines such as IL-2, IL-12 and IFNs.[26] In situations of malignancy or viral infections, natural killer cells are usually in an activated state. We mimicked this state *in vitro* by treatment of PBMCs and LAK cells with poly I:C, a synthetic analog of viral double-stranded RNA. The effect of sHLA on these cells showed that stimulation of NK cells with poly I:C has no effect on the inhibitory action of sHLA whereas stimulation of LAK cells with poly I:C reduced this inhibitory effect. Comparing the effect of sHLA on poly I:C activated PBMCs cells and IL-2 activated cells (non-poly I:C activated LAK cells) showed that in both cases, sHLA has an inhibitory

effect. It could be postulated that up-regulation of NK cells activity due to IL-2 and poly I:C alone could not change the inhibitory action of sHLA. Conversely, poly I:C activation of LAK cells (IL-2 and poly I:C in combination) reduced the inhibitory action of sHLA indicating that synergistic effects of IL-2 and poly I:C as an inducer of IFN might create some changes in HLA receptors upon which the inhibitory action of sHLA decreased. These changes may occur in the density expression of either inhibitory or activating receptors. As has been shown, selective killing of NK cells results from a fine balance between inhibitory NK receptors for MHC class I molecules and activating receptors that are still largely unknown.[18] Although it is now accepted that KIRs deliver negative signals to natural killer (NK) cells regarding the recognition of target cells, it is still unclear as to how the expression of these receptors on lymphocytes is regulated. In another study, the regulation of expression of representative KIRs, CD158a and CD158b by cytokines such as IL-2, IL-4 and IFN-γ has been investigated.[27] Neither IL-4 nor IFN-γ affected the expression of CD158a/b, but incubation with IL-2, up-regulated the expression of the KIRs.[27] Results of our study in changing the inhibitory effects of two mAbs against NKB1 and NKAT2 after poly I:C activation of LAK cells is further evidence that the combination of cytokines such as IL-2 and IFNs may play an important role in regulating the expression of NK inhibitory receptors or changing their balance versus the stimulatory ones. Further studies are needed to demonstrate the precise impact of different factors particularly cytokines in alterations of the expression of NK receptors. In this regard, sHLA molecules seem to be a useful tool.

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Vank Church, blind arcade, mural painting, Julfa, Isfahan (1655)