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A correlation of NK cytolitic test and BLT esterase test in determining activity of NK cells, stimulated by tumor target cells

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We examined the granule exocytosis in natural killer (NK) cells by measuring N-benzyloxycarboxy-L-lysine esterase activity. As a source of NK cells we used buffy-coat isolated NK cells or peripheral blood lymphocytes (PBL). The exocytosis was triggered by incubating cells with ionomycin/PMA or by NK cell susceptible tumour target cells K562. When we stimulated purified NK cells with tumour target cells, a close correlation (Corr=0.84) of cytolitic NK test results and BLT test results was obtained. We may conclude that BLT test can provide a valuable tool to discriminate further NK cell deficiency in patients with low cytolitic NK test results.

Key words: killer cells, natural; exocytosis, cell degranulation

Introduction

Secretory granules of lymphocytes may be important organelles, serving to concentrate and store different enzymes, cytolitic proteins and biologically active molecules. It is possible that exocytosis of granules plays an important role in cytolitic activity of cytotoxic T lymphocytes (CTL). Trypsin-like serine esterase (BLT esterase) was described as an easily detectable biochemical marker of granules.[1] It was shown that secretion of this enzyme is triggered by incubating CTL with antigen-bearing target cells or with immobilised anti-TCR monoclonal antibodies - in both cases the crosslinking of T-cell receptors (TCR) is required. The exocytotic process can be also triggered by activation of protein kinase C (with phorbol esters) and by simultaneous translocation of calcium through CTL plasma membrane with Ca^{2+} ionophores.[2] It has been shown that external Ca^{2+} is obligatory for both the TCR-triggered and for the phorbol ester/ Ca^{2+} ionophore - triggered exocytosis, because Ca^{2+} chelators (EDTA, EGTA) and Ca^{2+} channel blockers (nifedipine, verapamil) inhibit exocytosis.[3]

Natural killer cells (NK cells) are a subpopulation of lymphocytes, whose function has been defined to date by their ability to mediate the *in vitro* destruction of certain

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neoplastic and virally infected cells in a rapid, non-MHC restricted fashion. Because NK cells do not express antigen receptors they must recognise their targets in a way, that differs from T cells antigen recognition.[4,5] Nevertheless, the killing mechanisms of both cell types may be similar and they include granule exocytosis that releases enzymes, cytolitic proteins and biologically active molecules into areas bounded by the close apposition of effector and target cell plasma membranes.[6,7]

In our experiments, we provided an evidence that the BLT esterase, besides the use for monitoring CTL exocytosis, can also be used as a biochemical marker of NK cell granule exocytosis. We used BLT-esterase assay for monitoring NK cell exocytosis. As a source of NK cells we used buffy-coat isolated NK cells or buffy-coat isolated lymphocytes (PBL) as well. The exocytosis was triggered by incubating cells with ionomycin/PMA or by NK cell susceptible tumour target cells K562.

Materials and methods

Separation of human peripheral blood lymphocytes (PBL)

Buffy coats from normal blood donors were obtained with the kind co-operation of Transfusion Centre in Ljubljana. Buffy coats were diluted with two parts of sterile PBS and overlayered on Ficoll-Isopaque. Gradients were spun at room temperature for 20 min at 250 g. Cells (PBL) were subsequently harvested from above with a Pasteur pipette and washed three times. An aliquot from each sample was then suspended in trypan-blue solution for counting in a counting chamber.

NK cell isolation

Ficoll-isolated PBL cells were first incubated in nylon-wool columns in order to remove adherent cells (monocytes, B lymphocytes). Columns consisted of 10-ml plastic syringes, filled with 0.5 g of nylon wool. Each column was pre-washed with 30 ml of warm RPMI (37°C) and loaded with 1 ml of PBL suspension. Maximal cell load/column was 100 million cells. After 1 hr incubation at 37°C in a 5% CO_2 humidified atmosphere, non-adherent cells were gently washed out with 20 ml of warm RPMI (37°C). For further NK cell isolation, a commercially obtained preparation of polyvinylpyrrolidone-coated silica (Percoll, Pharmacia, Uppsala, Sweden) was first made isotonic for use with living lymphocytes. 1.5 M phosphate-buffered saline (PBS) was added to the stock solution of Percoll in a ratio of one part PBS to nine parts Percoll. For subsequent use, this stock (100%) was diluted with 0.15 M PBS or cell culture medium for cell separations. Discontinuous gradients were generated in 12 ml sterile plastic centrifuge tubes, which had been previously wetted with a small amount of serum to allow even flow of the Percoll. Non-adherent PBL cells, obtained from nylon-wool columns, were resuspended in 2 ml of 100% Percoll and successively less dense solutions layered carefully on top in 2 ml aliquots. Gradients for lymphocytes were run between 60% and 40%, with a 10% differential between layers. Once generated, gradients were spun for 10 min at 450 g. After centrifugation, distinct bands could be observed at the various interfaces. Gradients were harvested from above with a Pasteur pipette, and the cellular composition of each fraction was evaluated flow cytometrically. Cell preparations, containing more than 85% of NK cells (CD3-CD56+ cells) were considered NK cell homogenous and were used in subsequent experiments.

NK-enriched PBL cells

Ten ml of heparinised blood was diluted 1/2 with RPMI and layered on Ficoll-Isopaque.

Isolated PBL cells were further separated by Percoll centrifugation as described above. Low density cells were collected with a Pasteur pipette and used for NK test and BLT-E release assay.

Short-term cell culture with IL-2

Isolated cells were ($2x10^6$ cells/ml) cultured in a 24-well plate in the presence of 100 units/ml of recombinant human IL-2 (Sigma). After 18 hours of incubation (37°C in humidified 5% CO_2 atmosphere) the cells were recovered and used for subsequent studies.

NK test

Effector cells were tested against ^{51}Cr-labelled K562 target cells. K562 cells ($5x10^6$) in 0.5 ml medium were labelled by the addition of 100 (Ci sodium chromate ($Na_2{}^{51}CrO_4$, sp. act. 150-500 (Ci/mg) and incubated at 37°C for 1 hr. The cells were then washed three times in medium and resuspended to a concentration of 1×10^6 cells/ml in complete medium. Effector cells were prepared at a concentration of 5×10^6/ml and added to each well of the microtiter plate in a volume of 100 (l. Triplicate cultures were established in U-bottomed microtiter plates in a total volume of 100 (l, diluted in complete medium to obtain target/effector ratio (T/E) ranging from 1/4 to 1/64. Hundred µl of target cells were then added to effector cells. Plates were incubated for 4 h at 37°C in a 5% CO_2 humidified atmosphere, and released radioactivity was measured in a gamma counter. The percentage cytotoxicity was calculated by the equation: % cytotoxicity = (cpm experimental release - cpm spontaneous release) x 100 / (total radioactivity incorporated into cells). Spontaneous release was determined by incubating the target cells in medium alone. To compare the results at different E/T ratios, data are expressed in lytic units (LU). One LU represents the number of effector cells required for lysis of 20% of target cells. LU were determined from log-linear plots of the data points, and are reported at LU20/107 effector cells.[8]

BLT test

The amount of secreted BLT esterase was measured using 1 x 105 cells (PBL or NK cells) in 0.1 ml of cell culture medium in the 96-well microtiter plate in the presence of tumour target cells (K-562) or pharmacological stimuli - PMA (10 ng/ml) / A23187 (0.5 ug/ml). After 4-hr incubation at 37°C in a 5% CO_2 humidified atmosphere, cells were resuspended by gentle pipetting and centrifuged at 200 g for 5 min. Fifty µl aliquots of supernatant were used to assay BLT esterase activity. Total cellular content of the enzyme was determined using 0.1% Triton X-100 solubilized cells. Determinations were carried out in triplicate. Data are presented as specific percentage of enzymatic activity released which was calculated from the equation: % release = 100 x (E-S) / (T-S), where E represents the number of enzyme units in the supernatant of the experiment well, S the number of enzyme units in the supernatants of the well containing no stimuli, and T the total number of units of BLT esterase per well. The total BLT esterase content in target cells K-562 was always less than 5% of that in PBL or NK cells. For measuring BLT esterase activity, 0.05 ml of culture medium was mixed with 0.95 ml of BLT solution (0.2 mM BLT, 0.22 mM DTNB in PBS, pH 7.2). The mixture was incubated at 37°C for 20 min and the reaction was stopped by adding 5µl of 0.1 M PMSF, which was dissolved in dimethylsulfoxide. The solution was diluted by 1 ml of PBS and absorbency at 412 nm was measured in comparison to a blank solution (cell culture medium) that was treated exactly as experimental.

Flow cytometry

Flow cytometric analysis was performed by a fluorescence-activated cell sorter (FACSort, Becton Dickinson, Mountain View, CA, USA). A two-parameter analysis was performed to determine the proportion of CD3-CD56+ cells in samples.[9] Monoclonal antibodies (CD3-FITC, CD56-PE) were purchased from Becton Dickinson (Mountain View, CA, USA).

Statistical analysis

Correlation analysis was calculated by using a standard analysis package Quattro pro (Borland International, USA).

Results

For determining the time course of BLT-E secretion by NK cells, a secretion of Percoll-isolated NK cells was triggered by synergistic action of PMA and ionophore A23187 or NK cell susceptible tumour cells K562. Treatment of NK cells with PMA and A23187 results in faster release of granule enzyme than during incubation with K562 cells (Figure 1). When NK cells were incubated with K562 cells, BLT-E measurement reached stable values in 3-4 hours. Therefore a 4-hr incubation time was chosen for performing further experiments.

As a granule enzyme release is thought to be involved in NK cell cytotoxicity, we compared the influence of K562-induced NK cell triggering on BLT-E secretion and NK-cytotoxicity in healthy blood donors. In Figure 2 we present the data of experiments done on peripheral blood mononuclear cells. A poor correlation (Corr=0.54, SE =3.2) results most probably from low BLT secretion levels that are very close to a background release levels. Because NK cells represent only a minor part in PBL, BLT-E secretion results can be improved by using purified NK cells. Our results (Figure 3) demonstrate that detectable BLT-E secretion results can be achieved in experiments done on pure NK cells. Also a correlation (Corr = 0.84, SE=1.28) of NK cell cytotoxicity and BLT-E secretion results was improved substantially.

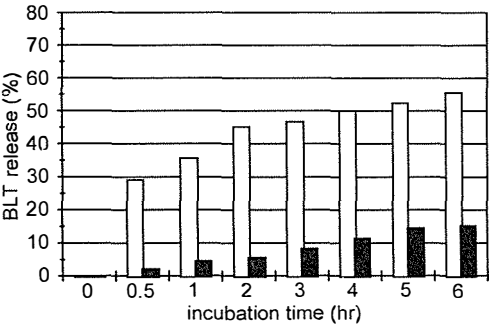

Figure 1. The time course of BLT esterase secretion by purified NK cells stimulated with PMA/A23187 (empty columns) or tumour target cells K562 (black columns). 1 x 105 purified NK cells were incubated in the presence of PMA/A2387 or tumour target cells K562. After incubation periods indicated on the abscissa, BLT esterase activity in the supernatant was measured. BLT esterase activity is presented as a percentage of maximal BLT release. Each point represent an average of at least four experiments, made in triplicates.

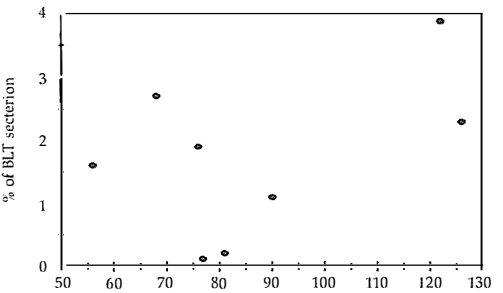

Figure 2. A correlation of NK cytolitic test and BLT-E test results in peripheral blood lymphocytes (PBL), stimulated with tumour targets. Peripheral blood lymphocytes of normal blood donors were stimulated with tumour target cells K562 at different effector/target ratios (E/T = 1/4 - 1/64). After 4 hour stimulation, supernatants were collected for cytolitic NK cell test and BLT-E release test as well. NK tests results are expressed in lytic units (LU) and BLT-E test results are expressed as percentages of maximal BLT-E release from effector cells. Each point represent an average of at least four experiments, made in triplicates.

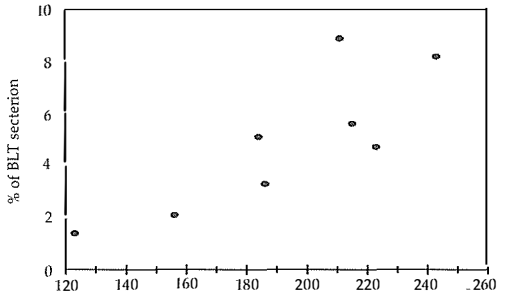

Figure 3. A correlation of NK cytolitic test and BLT-E test results in isolated NK cells, stimulated with tumour targets. Purified NK cells of normal blood donors were stimulated with tumour target cells K562 at different effector/target ratios (E/T = 1/4 - 1/64). After 4 hour stimulation, supernatants were collected for cytolitic NK cell test and BLT-E release test as well. NK test results are expressed in lytic units (LU) and BLT-E test results are expressed as percentages of maximal BLT-E release from effector cells. Each point represents an average of at least four experiments, made in triplicates.

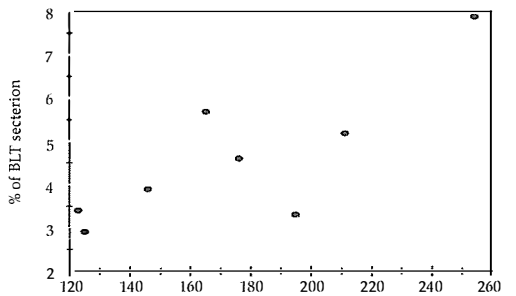

Figure 4. A correlation of NK cytolitic test and BLT-E test results in NK-enriched peripheral blood lymphocytes, stimulated with tumour targets. NK-enriched of normal blood donors were stimulated with tumour target cells K562 at different effector/target ratios (E/T = 1/4 - 1/64). After 4 hour stimulation supernatants were collected for cytolitic NK cell test and BLT-E release test as well. NK tests results are expressed in lytic units (LU) and BLT-E test results are expressed as percentages of maximal BLT-E release from effector cells. Each point represents an average of at least four experiments, made in triplicates.

Flow cytometrical evaluation of NK-enriched PBL revealed that 3-4 x enrichment of NK cell concentration was usually obtained, with NK cell content ranging from 40 to 65%. As shown in Figure 4, maximal NK lysis reached 250 LU while maximal BLT-E release was 10% of total BLT-E content of peripheral blood lymphocytes. The correlation of NK cell cytotoxicity and BLT-E secretion results(Corr = 0.79, SE=0.97) was close to correlation in experiments performed on purified NK cells.

Discussion

A vast majority of studies on granule exocytosis has been performed on cytotoxic T clones, while NK cell clones have been studied less frequently.[10-13] For studying exocytosis we used purified NK cells instead of cloned cells. An advantage of purified cells is that they better represent an *in vivo* physiological situation while cloned cells may have altered many of their phenotypic characteristics during long term *in vitro* cultivation. A disadvantage of studies on purified NK cells may be, besides laborious isolation procedure, a weak exocytotic activity of purified NK cells. Because previous reports [11] indicated that IL-2 can enhance, in a cytokine concentration-dependant manner, NK cell-mediated cytotoxicity, we established a short-term (18 hours) culture of purified cells to obtain exocytotic activities well distinct from background values.

Using the assay system described, it is possible to study enzyme granule exocytosis of NK cells. Because BLT esterase was described by many authors as an easily detectable biochemical marker of cytolitic granules, we used BLT-E release assay to detect NK cell exocytosis. It was shown that BLT-E is released by immune cells, capable of cell-mediated killing - including CTL and NK cells as well. *In vivo*, CTL release cytotoxic granules after they recognise surface antigens presented by MHC I molecules on the surface of target cells (virus infected or tumour cells). *In vitro* a similar process can be triggered by incubating CTL with antigen-bearing target cells or with immobilised anti-TCR (CD3) monoclonal antibodies - in both

cases the crosslinking of T-cell receptors (TCR) is required. On the other hand, NK cell utilise, *in vivo* two distinct mechanisms to trigger exocytosis. One, termed antibody-dependent cellular cytotoxicity (ADCC), utilises NK cell surface Fc-receptors and antibodies directed against the target-cell antigens. The other involves direct interaction between NK cells and their target cells and utilises NK cell receptors that have not yet been fully characterised.[14,15] *In vitro*, a process of NK killing can be demonstrated by incubating NK cells with antibody coated (ADCC killing) or some tumour target cells (K562 cells).

The exocytotic process in both cell types (CTL and NK) can be also triggered *in vitro* by the activation of protein kinase C (with phorbol esters) and by simultaneous translocation of calcium through CTL plasma membrane with Ca^{2+} ionophores.[2] Our results demonstrate that PMA/A23187 very efficiently trigger BLT-E secretion of purified NK cells. In less than 2 hour incubation time almost half of the total BLT-E content is released from NK cells. On the other hand, when NK cells were incubated with K562 cells, BLT-E measurement reached stable values in 3-4 hours and, in that time less than one third of total BLT-E content was released.

Because PMA/A23187 triggering acts on CTL and NK cells, experiments should be done only with homogenous cell populations - CTL or NK cell clones or purified cells. On the other hand, K562 cells trigger NK cell exocytosis without influencing CTL exocytosis.[3] That would theoretically enable studies of NK cell exocytosis in mixed cell populations (e.g. patients PBL). Our results, when using PBL in BLT-release test, demonstrate very low BLT-E release. A possible reason lies in calculation of BLT-E release: an enzyme release is expressed as a percentage of specific release versus total enzyme cell content. Because a total cell enzyme content in PBL is a sum of total enzyme content in CTL and NK cells, NK cell release makes usually very low proportion of the sum. Furthermore, a calculation of BLT release can be greatly influenced by the proportions of CTL and NK cells in PBL.

Obviously the experiments are best to be performed by using isolated NK cells to obtain high BLT-E release/effector cell. On the other hand, to perform extensive clinical studies, NK cell isolation may not be appropriate method because considerable amounts of blood are required. As a compromise, we performed a simple NK-enrichment procedure by using Percoll gradient centrifugation. By using NK-enrichment procedure we were able to obtain PBL containing more than 45% of NK cells. Furthermore, to perform BLT-E release assay and NK-cytotoxicity test as well in most instances only 10 ml of blood was required i.e. the quantity that enables to perform more extensive clinical studies.

Our results confirm a close association between granule enzyme exocytosis and NK lytic process in healthy persons. A correlation between NK test and BLT-E release results increases with the purity of NK cells, used in the experiment. Because a granule enzyme exocytosis is supposed to be a part of a lytic process in NK cells, a BLT-E release assay could be used to further analyse the lytic process in NK cells. A described assay procedure is simple and requires only moderate blood volumes, so, it is possible to perform it in clinical studies. Its use might be important especially in patients with impaired NK cytotoxicity (cancer patients, immunodeficient patients) because a defect of NK cell cytotoxicity can be influenced also by the factors that do not include granule enzyme exocytosis (NK cell binding and recognition of target cells, cytokine stimulation, activation of NK lytic machinery).

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