Frequency and spontaneous cytotoxicity of natural killer cells in healthy children: preliminary results

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Abstract

Natural killer (NK) cells account for up to 15% of all peripheral blood lymphocytes and are characterized phenotypically by the presence of CD56/CD16 antigens and the lack of CD3 antigen. Natural killer cells exert cytotoxic functions against a variety of target cells including tumor cells, virus-infected cells and normal hematopoietic cells expressing allogeneic major histocompatibility complex (MHC) antigens. Human NK cells can be divided into five subsets with different biological functions: CD56bright CD16⁻, CD56bright CD16dim, CD56dim CD16⁻, CD56dim CD16bright, and CD56⁻ CD16bright.

The aim of the study was to analyze the frequency and activity of NK cells from the peripheral blood of healthy children aged 6-17 years (n = 18).

The tests were performed with the use of flow cytometer EPICS XL (Beckman Coulter).

We have shown that the cytotoxic activity of NK cells differed significantly from one another. The study proves that the overall cytotoxic activity of NK cells depends on the number of peripheral blood cells (p = 0.01) in a healthy children population.

Key words: NK cells, cytotoxicity, innate immunity, children.

Introduction

Normal function of the immune system depends on the proper number and activity of immunocompetent cells of different lineages. Natural killer (NK) cells are a subset of lymphocytes that contribute to innate immunity and can be defined as CD16+/CD56+/CD3– cells.

Natural killer cells exert spontaneous cell-mediated cytolysis against tumor cells, virus infected cells and other host cells with a decreased expression of allogeneic MHC (major histocompatibility complex) antigens. Natural killer cells account for up to 15% of circulating lymphocytes [1-6].

In humans five different subsets of NK cells may be distinguished on the basis of cell surface density of CD56 and expression of CD16: CD56bright CD16–, CD56bright CD16dim, CD56dim CD16–, CD56dim CD16bright, and CD56– CD16bright. There is some evidence that CD56dim cells show a higher cytotoxic activity in comparison with CD56bright cells [7, 8]. A high expression of CD16 molecule on NK cell surface enables more effective antibody-dependent cytotoxicity. On the other hand, CD56bright cells are most effective in cytokine production [interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), GM-CSF, interleukin (IL) 10, IL-13 [9].

Natural killer cells induce cytolysis through cytolytic granule-mediated apoptosis or antibody-dependent cell-mediated cytotoxicity (ADCC). Direct cytotoxic effect depends on the balance between stimulatory and inhibitory signals. Three major receptor families have been described on the surface of NK cell membrane: killer cell Ig-like receptors (KIR) which recognize HLA-A, -B and C; lectin-like receptors including CD94 and NKG2A/B receptors recognizing HLA-E, and natural cytotoxicity receptors including Nkp30, Nkp44 and NKP46 with unknown ligands [2, 10-17]. Nkp30 and NKP46 receptors are constitutively expressed on NK cells, whereas NKP44 appears only after...
stabilization [18]. CD16 molecule plays an essential role in biology and function of NK cells. This is a low affinity FcyRIII receptor which binds to antibody-opsonized target cells and signals through associated subunits containing an immunoreceptor tyrosine-based activation motif (ITAM) to direct antibody-dependent cell-mediated cytotoxicity (ADCC). Most of NK cells with a high CD56 expression do not express CD16 antigen or its expression is low, whereas over 95% of NK cells with a low CD56 expression are CD16 positive [7].

Cytolytic abilities of NK cells depend on the content and activity of intracellular granulosomes. A cortex of granulosome contains proteolytic enzyme – perforin, whereas a core contains granzymes. In the first stage, membrane of target cell is permeabilized by perforin, then proteolytic enzymes – granzymes – activate intracellular targets cell caspases leading to apoptosis. Several cytokines including: IL-2, IFN-α, IFN-β, IL-12, IL-18, IL-21, IL-15, as well as bacterial and viral proteins, can enhance spontaneous cytotoxicity of NK cells [19-22].

Immunosuppression and disorders affecting the function and/or number of NK cells may lead to instability of the immune system and uncontrolled proliferation of pathologically changed cells. In consequence, this may lead to development of tumors and/or autoimmunization [23, 24]. It is also suggested that some kinds of cancers may induce anergy in NK cells through secretion of inhibitory factors or elevated NFκB activity [25, 26].

One of the most severe disorders characterized by NK cell cytotoxic function impairment is hemophagocytic lymphohistiocytosis (HLH). It may lead to uncontrolled hyper activation of macrophages which phagocyte erythrocytes, leukocytes, platelets and their precursors within bone marrow, liver and lymph nodes. As a consequence, patients suffer from fever, advanced pancytopenia and hepatosplenomegaly. Hemophagocytic lymphohistiocytosis is a life-threatening disease. The major aim of HLH treatment is to suppress severe hyper inflammation by a combined action of dexamethasone, cyclosporine and etoposide. Steroids are cytotoxic for lymphocytes and inhibit secretion of cytokines. Cyclosporine prevents T lymphocytes proliferation. Etoposide is highly effective in treatment of monocyte and histiocytic diseases. Patients with a low risk (without genetic background of HLH) may be treated only with steroids and cyclosporine. In the most severe congenital cases, only stem cell transplantation and replacement of defective immune cells by normal functioning cells is a successful treatment [27].

Currently, methods evaluating the phenotype, cytokine production and proliferation capacity of NK cells are well described, but cytotoxic activity is still rarely investigated. Moreover, the range of cytotoxic activity in normal subjects of different age and sex is not completely known. To date the reference cytotoxicity test based on radioactive chromium as an indicator of target cells was the only diagnostic method of spontaneous cytotoxicity. Nowadays, the technique brings several technical problems for numerous laboratories. The use of radioactive chemical elements requires specialized, highly qualified and certified isotopic laboratory. The best alternative is a new flow cytometric method applied and described by many authors [28-32]. In this method, staining with radioactive chromium is replaced with various fluorescence stains. Flow cytometry gives a broad operating range with diverse modifications and applications [33]. However, the flow cytometry method is still rarely used in routine diagnostic procedures and demands additional operation research.

The aim of this study was to find a correlation between spontaneous cytotoxic activity, measured by the flow cytometric method, and the number of circulating NK cells in a group of healthy children.

Material and methods

Eighteen healthy children and adolescents aged from 6 to 17 years (10 girls and 8 boys) were enrolled to the study. Parents of the investigated children gave informed consent during the enrollment visit, having been fully informed of the nature, risk and potential benefits of the study. For elder children (> 15 years) their permission was also obtained. The Ethical Committee of the Medical University of Warsaw approved the study protocol. Each individual was assessed clinically by a pediatrician or by a research physician for eligibility to take part in the study. Children were free from any allergic diseases, immune and hematological disorders. Healthy children referred to the Children Hospital of Medical University of Warsaw were qualified for routine health screen or minor surgical procedures. Blood collection was performed at least three months after the last episode of infection, vaccination and any medication, and at least 3 months after the last episode of viral diseases such as mononucleosis or chickenpox. For all analyses, 3.2 ml of venous blood was drawn to the tubes containing heparin.

Flow cytometric analysis of the phenotype and frequency of natural killer cells

Leukocyte concentration in the whole blood sample was adjusted to 4 × 10⁹/ml with NaCl. Next, 100 μl of blood was incubated with 10 μl of ready-to-use monoclonal antibodies solutions: anti-CD3 FITC, anti-CD16/CD56 PE, anti-CD45PC5 (Becton Dickinson) according to the manufacturer’s protocol, protected from light, 20 min at room temperature. After staining, the erythrocytes were lysed with 500 μl of OptiLyse C reagent (Beckman Coulter). The samples were analyzed by flow cytometry using appropriate isotype controls. Lymphocytes were recognized and gated according to the side scatter (lymphocytes present side scatter lower than monocytes and
polymorphonuclears) and CD45 expression (lymphocytes present the highest expression of CD45 antigen among whole blood leukocytes). Nonviable cells and debris were removed from analysis based on very low forward scatter and side scatter and lack of CD45 antigen expression. Acquisition was run to the total number of 10,000 events in lymphocyte gate. Flow cytometric analyses were performed using Cytomix FC 500 Beckman Coulter. Instrument setup and calibration was performed with the use of Flow-Check Pro Fluorospheres (Beckman Coulter) according to manufacturer’s protocol.

**Flow cytometric cytotoxicity test**

Peripheral blood mononuclear cells (PBMC) from 3 µl of anticoagulated, heparinized blood were separated by density gradient centrifugation (Histopaque 1077, Sigma-Aldrich). After separation, the cells were suspended in RPMI media at a final concentration of 4 × 10^6/µl.

Target cells were harvested from the culture, washed and suspended in RPMI media at the final concentration of 1 × 10^6/ml. Natural killer-mediated cytotoxicity was analyzed as described previously [30]. Target cells (K562) were labeled with fluorescent dye DiO (3,3-dioctadecyloxacarbocyanine perchlorate, Sigma-Aldrich). 10 µl of the stock solution (3 mM in DMSO) was added to 1 × 10^6 of K562 cells suspended in 1 ml of RPMI media and incubated at 37°C, 5% CO₂ for 30 min. After incubation, the cells were washed 3 times in PBS and once in RPMI (to wash out the excess of unbound DIO dye) and suspended in RPMI media to the final concentration of 1 × 10^6/ml.

Separated PBMC were cultured with target K562 cells in 3 sterile tubes: tube 1 – effector (E) to target (T) cells ratio (E : T) 13 : 1, tube 2 – control with effector PBMC without target K562 cells, tube 3 – control with target K562 without PBMC. The culture was incubated for 4 h in humidified at 37°C, 5% CO₂ atmosphere. After 4 h the tubes were stained with 5 µl of propidium iodide (PI) (50 µg/ml stock) and left for another 30 min incubation. Afterwards, the samples were acquired to flow cytometer to the final number of 5000 events presenting DiO fluorescence.

Propidium iodide as well as DiO are excited with an argon-ion laser tuned to 100 mW at 488 nm. Red fluorescence from propidium iodide-stained cells was deflected by 610 nm short-pass dichroic, passed through a 630 band-pass filter and detected on photomultiplier FL3. Green fluorescence from DiO stained cells was collected through a 560 nm short-pass dichroic, passed through a 530 nm band-pass filter and detected on photomultiplier FL1. A two-parameter cytogram, log (FL1) versus log (FL3) was plotted to discriminate the different cell types in the experiment. Live target (K562) cells showed only DiO fluorescence (D4), dead target cells showed both DiO and PI fluorescence (D2) (Fig. 1B). The percentage of dead target cells was calculated according to the following formula: dead cells × 100%/total number of target cells. A specific lysis index (% of cytotoxic activity) was calculated as follows: [the percentage of dead target cells (including effector cells)] – [dead target cells (without effector cells)] (Fig. 1A).

![Fig. 1. Cytotoxicity test using flow cytometry (A) target cells without effector cells, (B) target cells with effector cells](image-url)
Statistical calculations were performed using Statistica 10 software. Shapiro-Wilk test was used to analyze distribution of the studied variables. Differences between two groups were assessed using the Mann-Whitney U test. Correlation strength between the number of NK cells and cytotoxicity was assessed using Spearman correlation test. Statistical significance was considered at \( p < 0.05 \).

This work was approved by the Ethical Committee of the Medical University of Warsaw. The study was conducted according to the Helsinki Declaration.

### Results

The percentage of NK cells in a population of lymphocytes was assessed by flow cytometry using anti CD16+56, and CD3 monoclonal antibodies. Only cells expressing CD56 and/or CD16 antigens without the expression of CD3 were considered NK cells.

The detailed results of cytotoxicity tests and the percentage of NK cells in whole blood are shown in Table 1.

Although the mean value of spontaneous NK activity in the group of girls was higher than in the group of boys, statistical significance was not achieved (\( p = 0.7 \)) (Fig. 2). No difference was found in the percentage of circulating NK cells between girls and boys (\( p = 0.8 \)) (Fig. 3).

There was a high correlation between spontaneous NK cell cytotoxicity and the frequency of NK cells in the bloodstream (\( p = 0.01; \ r = 0.55 \)). A positive correlation was observed in the whole analyzed population (Fig. 4), as well as in the groups of boys and girls when analyzed separately.

### Discussion

The phenomenon of natural cytotoxicity was discovered at the beginning of the 1970s as the ability of a certain population of lymphocytes to exert a cytotoxic reaction against tumor cells. In the group of patients suffering from malignant diseases, the activity of lymphocytes against autologous and/or allogeneic neoplastic cells was lower when compared to the activity of lymphocytes isolated from healthy subjects. The function of NK cells is currently understood to be controlled by a dynamic signal balance between activating and inhibiting receptors engaged upon interaction with ligands presented on the surface of the target cell [7, 9, 34].

The range of NK cells number in the whole blood of healthy subjects as well as spontaneous cytotoxic activity may vary. It was found that individual NK cells activity depends on the genetic background and environmental factors [18, 35, 36]. On the basis of repeated analyses of the same individuals, the activity of NK cells turned out to be quite stable. Probably it is settled during the first year of life and is only slightly modified by environmental factors [37]. It was also confirmed that relative and absolute numbers of NK cells remain almost invariable during early childhood. In further life, the percentage of circulating NK cells seems to increase, but absolute counts remain almost invariable [38]. All children enrolled to our study (except one) had NK cell numbers within age-related normal values.

Certain differences in NK cell number and their activity in blood were observed between men and women. At the beginning of the 1980s, Penschow and Mackay had investigated cytotoxic activity of NK cells in a group of healthy subjects (26 women and 17 men). They found that spontaneous NK activity was significantly higher in men than in women [39]. Yovel with collaborators had analyzed the correlation between gender, menstrual cycle, or

<p>| Table 1. Results of cytotoxicity test and the percentage of NK cells |
|-----------------|----------|---------|-------|-------|</p>
<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
<th>% NK Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>16</td>
<td>17.7 22.5</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>10</td>
<td>6.5   4.0</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>11</td>
<td>18.5 14.3</td>
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<tr>
<td>4</td>
<td>F</td>
<td>12</td>
<td>12.0 10.6</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>11</td>
<td>10.8 11.3</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>17</td>
<td>9.8   11.4</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>10</td>
<td>9.3   4.8</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>12</td>
<td>9.4   19.8</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>13</td>
<td>6.3   5.1</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>12</td>
<td>7.2   14.5</td>
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<tr>
<td>11</td>
<td>M</td>
<td>10</td>
<td>1.2   1.2</td>
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<tr>
<td>12</td>
<td>F</td>
<td>11</td>
<td>7.0   13.2</td>
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<tr>
<td>13</td>
<td>M</td>
<td>13</td>
<td>13.0  13.2</td>
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<tr>
<td>14</td>
<td>F</td>
<td>10</td>
<td>12.6  15.8</td>
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<tr>
<td>15</td>
<td>F</td>
<td>11</td>
<td>10.7  21.6</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>10</td>
<td>8.11  4.5</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>6</td>
<td>5     10.8</td>
</tr>
<tr>
<td>18</td>
<td>F</td>
<td>6</td>
<td>11.8  9.7</td>
</tr>
<tr>
<td>median</td>
<td></td>
<td></td>
<td>10.1  11.3</td>
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<tr>
<td>25 percentile</td>
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<td></td>
<td>7.0   5.1</td>
</tr>
<tr>
<td>75 percentile</td>
<td></td>
<td></td>
<td>12.0  15.8</td>
</tr>
</tbody>
</table>

M – male, F – female
oral contraceptives and NK cells number and their cytotoxic activity. They revealed that NK activity was higher in men than in women with regular menstrual cycles. The lowest values were found in a group of women taking oral contraceptives [40].

In our study, the number of NK cells and their cytotoxic activity were higher in the group of girls, but most girls were in the pre-pubertal age.

Gender-related differences in the number and activity of NK cells highlight the link between gonadotropic and sex hormones and NK cell development. The concentration of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol and progesterone increases in girls between 8 and 10 years of age. Approximately at the age of 16 the concentration of all sex hormones reaches the level observed in mature women [40]. Seaman et al. had demonstrated in an animal study that prolonged exposure to estrogen significantly decreased cytotoxic activity [41]. Other authors had observed the correlation between NK activity and gonadal hormones level in the course of the menstrual cycle. Sulke et al. had found a significant drop of NK cytotoxic activity at the moment of ovulation. Natural killer cells activity was higher in the follicular phase than in the luteal phase [42].

Physical activity also has an important influence on NK cells number and efficiency. This influence depends on the intensity of the exercise. It was revealed that a serious and rapid increase in NK activity after physical effort was connected with an increased number of circulating CD56+ cells. Moreover, it was observed that moderate, long-lasting effort may cause a persistent increase in resting activity of NK cells [37, 43, 44]. Psychological stress and regular blood donation are also considered as factors decreasing cytotoxic activity of NK cells [45, 46].

The presence of several disturbances of NK cells activity may lead to the development of pathological conditions, such as cancer, viral infections or autoimmune diseases.

Fig. 2. The difference in the value of spontaneous cytotoxicity between girls (G) and boys (B) (Mann-Whitney U test p = 0.7)

Fig. 3. The difference in the number of circulating NK cells between girls (G) and boys (B) (Mann-Whitney U test p = 0.8).

Fig. 4. The Spearman correlation between the number of NK cells and spontaneous cytotoxicity (p = 0.01; r = 0.55)
It is well known that patients suffering from autoimmune disorders are highly predisposed to development of severe complications, like macrophage activation syndrome (MAS) and hemophagocytic syndrome (HLH). Macrophage activation syndrome is caused by hyperactivation of immune response as a consequence of an impaired NK cells function. Patients with such autoimmune disorders as systemic juvenile idiopathic arthritis (SJIA) and type 1 diabetes are predisposed to this syndrome. Hemophagocytic syndromes were also described as a complication of several other autoimmune disorders, e.g. systemic lupus erythematosus (SLE), juvenile dermatomyositis or Kawasaki disease [47-50]. Unfortunately, numerous cases of MAS and HLH remain undiagnosed and appropriate treatment is not applied. Lack of the right diagnosis may result in severe complications including patients’ death [51]. It is therefore essential to recognize NK cell defects and predict or even prevent the progression of the underlying disease.

In our study, we found a positive correlation between the number of circulating NK cells and the activity of spontaneous cytotoxicity. Such correlation might not always be true for NK cells. The presence of disturbances in NK cell function may affect this correlation. So that it might be an oversimplification to assume normal NK cell function when the cell count falls within normal range.

The results of the presented study indicate that the level of individual cytotoxicity in children without defects in NK cells function or severe immune diseases depends on the frequency of NK cells. However, the influence of the other factors like age, gender and state of health should also be considered.

The authors declare no conflict of interest.

References